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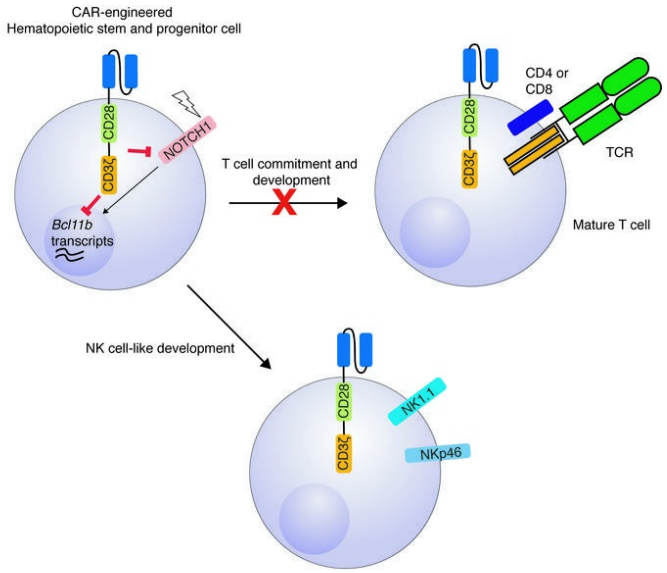
Graphical abstract



Research Article

Chimeric antigen receptor-induced BCL11B suppression propagates NK-like cell development

Marcel Maluski[1], Arnab Ghosh[2], Jessica Herbst[1], Vanessa Scholl[1], Rolf Baumann[3], Jochen Huehn[4], Robert Geffers[5], Johann Meyer[6], Holger Maul[7], Britta Eiz-Vesper[8], Andreas Krueger[9], Axel Schambach[6,10], Marcel R. M. van den Brink[2], and Martin G. Sauer[1]

[1]Department of Pediatric Hematology/Oncology and Blood Stem Cell Transplantation, Hannover Medical School, Hannover, Germany; [2]Department of Medicine and Immunology, Memorial Sloan Kettering Cancer Center, New York, New York, USA. [3]Clinic for Radiation Oncology, Hannover, Germany. [4]Experimental Immunology, [5]Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany. [6]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany. [7]Asklepios Clinic Hamburg, Germany. [8]Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany. [9]Institute of Molecular Medicine, Goethe University Frankfurt, Frankfurt a. M., Germany. [10]Division of Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, USA.

Corresponding author: Martin G. Sauer, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany; Phone: +49 (0)511 532 6716, Fax: +49 (0)511 532 9120

e-mail: sauer.martin@mh-hannover.de

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ABSTRACT

The transcription factor B Cell CLL/Lymphoma 11B (BCL11B) is indispensable for T lineage development of lymphoid progenitors. Here we show that chimeric antigen receptor (CAR) expression early in ex vivo generated lymphoid progenitors suppressed BCL11B, leading to suppression of T cell-associated gene expression and acquisition of natural killer (NK) cell-like properties. Upon adoptive transfer into hematopoietic stem cell transplant recipients they differentiated into CAR-induced killer cells (CARiK) that mediated potent antigen-directed antileukemic activity even across MHC barriers. A CD28 and active immune-receptor-tyrosine-based-activation-motifs were critical for a functional CARiK phenotype. These results give important insights into differentiation of murine and human lymphoid progenitors driven by synthetic CAR transgene-expression and encourage further evaluation of ex vivo generated CARiK cells for targeted immunotherapy.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) currently represents the best curative treatment option for very high-risk leukemias. However, relapse after HSCT remains the major cause for treatment failure. Redirecting T cell responses more specifically to tumor-associated antigens by T cell receptor (TCR) (1-3) or more recently chimeric antigen receptor (CAR) engineering has produced impressive clinical results (4-7). Nevertheless, important problems remain or have been underestimated using engineered T cells. This includes i) that the majority of successful clinical trials using genetic receptor transfer technologies have been conducted with autologous T cells, which requires the collection of a T cell product of sufficient quantity and quality from heavily pretreated patients, ii) the need to manufacture the product on a highly individualized basis resulting in treatment delays due to the scarcity of production slots and iii) on target/off tumor effects confining receptor engineering to a very narrow choice of suitable target antigens. Thus, a prefabricated T cell product, allowing for a wider choice of effectively targetable antigens, being applicable to a wider range of patients, and minimizing the risk of long-term sequalae from on target/off tumor effects would be highly desirable. In vitro pre-differentiated lymphoid progenitors from hematopoietic stem and progenitor cells (HSPCs) that undergo final maturation upon adoptive transfer (AT) in the recipient can be transplanted across major MHC barriers without triggering graft versus host disease. They give rise to a functional T cell population being both, tolerant and MHC-restricted to the host even in MHC-mismatched recipients (8-10). TCR-engineered lymphoid precursors with a host-restricted TCR lead to rapid thymic repopulation and their progeny can mediate potent and long-lasting anti-leukemia effects (11).

Here, in order to circumvent the limitation of TCR restriction, we used CAR-engineered hematopoietic progenitor cells to generate lymphoid precursors in vitro using the Notch-based OP9-DL1 culture system. Elegant proof-of-principle studies suggested the relevance of this concept for humans, however, exploration in vivo remains complex (12-14). Using a murine

and human CD19 CAR (15), we targeted a clinically relevant antigen and were able to assess its immunological impact on lymphoid progenitor development and demonstrate strong evidence for its translational relevance for humans

We show that CAR expression during early lymphoid differentiation can impact lymphoid progenitor fate profoundly via suppression of BCL11B. *Bcl11b* and *Notch1* transcripts are both indispensable for T cell development, both in mouse and man. As a result, T cell development was blocked in favor of a cell population acquiring NK cell-like properties. We termed this cell type CAR-induced killer (CARiK) cells. CARiK cells mediated strong anti-leukemic effects even across MHC barriers without evoking graft versus host disease (GVHD). We further demonstrate that this differentiation shift depends on the co-stimulatory domain and the activity of immune receptor-based activation motifs (ITAMs) used within the CAR-construct. Using CAR-engineered hematopoietic stem cells that had been isolated from human umbilical cord blood (UCB) we further show CAR-induced suppression of T cell differentiation in favor of CARiK cell development. These findings encourage efforts to further address the potential of CARiK cells as a cellular product of broader applicability for anticancer immunotherapy.

RESULTS

im1928z1-CAR expression in HSPCs cells prevents T cell but rather favors NK-like cell development of lymphoid progenitors in vitro and in vivo

HSPCs transduced with a host HLA-restricted TCR and differentiated into lymphoid progenitors of the T cells lineage have shown to mediate potent anti-leukemic upon co-transplantation with T cell depleted bone marrow (TCD-BM) (11). To evaluate the biological consequences of CAR expression in differentiating lymphoid progenitors both in vitro and in vivo, we cloned a previously published murine second-generation CAR directed against mouse CD19 containing a CD28 costimulatory domain and 1 functional ITAM within the CD3ζ signaling domain, termed im1928z1 (**Figure 1A and Supplemental Figure 1A**) (15). CAR expression was set under the control of a tetracycline-inducible (Tet-On) T11 promoter to study the impact of time-dependent CAR expression (11, 16). For inducible transgene expression, murine bone marrow-derived Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSKs) cells with an rtTA-M2 transactivator knock in were used. The Tet-on system was induced continuously for transgene expression during in vitro and in vivo experiments from the very early beginning unless noted otherwise. Lymphoid progenitors were generated from transduced LSKs using the OP9-DL1 co-culture system (**Supplemental Figure 1B**) (17). In contrast to previously published TCR-engineered lymphoid progenitors, the im1928z1 CAR was highly expressed on generated lymphoid progenitors in vitro (**Figure 1B**). Cells for adoptive transfer (AT) studies were at least 90% transgene positive and 50-60% were at the double negative (DN) 2 stage (CD25$^+$CD44$^+$/CD4$^-$CD8$^-$) (**Figure 1C and Supplemental Figure 1C**). Although the OP9-DL1 co-culture system is known to allow for limited NK cell development (17), we identified increased frequencies of NK1.1$^+$ cells (mean= 7.4%) with a CD25^{mid}CD44$^+$ phenotype within the im1928z1 group. This compared to around 0.6% NK1.1$^+$ cells for controls (ctrls) (**Figure 1C**).

To track the development of CAR-expressing lymphoid progenitors in vivo, irradiated syngeneic C57BL/6 (B6) recipients were transplanted with 3×10^6 T cell-depleted bone marrow (TCD-BM) cells and adoptively transferred with 8×10^6 im1928z1-engineered lymphoid progenitors (**Figure 1D and Supplemental Figure 1D**). Co-transplanted lymphoid progenitors have been shown to foster early repopulation of the thymus (8, 11). im1928z1-lymphoid progenitors did, however, completely fail to repopulate the thymus (**Figure 1E**). Importantly, adoptive transfer of im1928z1-expressing lymphoid progenitors resulted later in greater numbers of $NK1.1^+$ progeny in BM and spleen suggesting accentuated NK cell-like development (**Figure 1, F and G**). In contrast to the iTom ctrls, im1928z1 lymphoid progenitors sparsely matured into $CD4^+$ or $CD8^+$ single positive (SP) T cells or expressed the CD3/TCR complex (**Figure 1H**). Together, im1928z1-expression suggests T lineage development from early hematopoietic precursors in favor of a population with NK cell-resembling phenotype.

CAR-induced killer (CARiK) cells derived from im1928z1-engineered lymphoid progenitors demonstrate potent antileukemic activity across MHC barriers in vivo

In contrast to TCR expression initiated later during physiologic thymic T cell development, CARs started and continued to be expressed immediately after LSK transduction (14). Therefore, it was intriguing to assess the impact of CAR-triggering during the generation process of im1928z1-lymphoid progenitors. Early antigen exposure of CAR-transduced LSKs during in vitro differentiation slowed cell expansion and resulted in an increased $NK1.1^+CD25^{mid}CD44^+$ population (**Figure 2A** and **Figure 1C**). In vitro stimulation of im1928z1-lymphoid progenitors with CD19-expressing target cells caused prompt degranulation as seen by expression of CD107a and an IFN-γ response (**Figure 2B**) suggesting antigen specificity. Next, we assessed recovery of $CD19^+$ B cells after HSCT in co-transplanted recipients to evaluate the activity of matured im1928z1-lymphoid progenitors in vivo. We could

document induction of profound B cell aplasia following co-transplantation (**Figure 2C**). CAR-expressing progeny could be recovered from the mouse and further maintained in ex vivo cultures which required NK cell-like high interleukin (IL)-2 doses (1000 U/ml) in contrast to the standard T cell culture conditions (20 U/ml IL-2) (**Figure 2D**). Upon stimulation with CD19, CD107a expression and copious IFN-γ secretion again demonstrated specific reactivity of this im1928z1-expressing progeny (**Figure 2E**).

We next studied the anti-leukemia potential of "off-the-shelf" CAR lymphoid progenitors in a mouse model of post-HSCT relapse (**Figure 2F**). Recipients of syngeneic B6 TCD-BM, co-transplanted with either syngeneic (B6) or completely MHC mismatched (B10.A-mimicking "off-the-shelf") im1928z1-lymphoid progenitors, were challenged with a lethal dose of $mCD19^+$ leukemic cells (C1498-mCD19-GFP). Both groups showed a significant survival advantage over controls (**Figure 2G**). Consistent with prior reports GVHD was never observed after co-transplantation with MHC-mismatched lymphoid progenitors (data not shown) (9). A second leukemia challenge of 100-day survivors resulted in 100% lethality rate of the recipients suggesting the absence of long-term activity or functionally relevant memory cell formation (**Figure 2H**). Depletion of $NK1.1^+$ cells in transplant recipients using a NK1.1-depleting antibody led to a complete loss of anti-leukemia effects (**Figure 2, I and J**), demonstrating that the CAR-induced $NK1.1^+$ cells mediate the observed potent anti-leukemic effects. Due to NK cell-resembling phenotype and functionality of im1928z1-induced $NK1.1^+$ cells we will further refer to this cell population as CAR-induced killer cells, or CARiK cells.

im1928z1 expression on HSPCs leads to BCL11B suppression allowing for CARiK cell development and concomitantly decreases T cell-associated gene expression

To further substantiate CAR-induced differentiation of early lymphoid progenitors we asked whether the observed phenotypic and functional data would find an equivalent on the transcriptional level (18, 19). Therefore, we analyzed the gene expression profiles of engineered

lymphoid progenitors at the end of in vitro culture immediately prior to co-transplantation and of their progeny after in vivo differentiation 28 days after co-transplantation. (**Figure 3A**). Principal component analysis (PCA) showed a distinct transcriptional profile of im1928z1-lymphoid progenitors and CARiK cells when compared to respective ctrls (**Figure 3B**). These differences became more prominent during further in vivo development. Altogether, 449 differently expressed genes were identified in im1928z1-lymphoid progenitors. Genes related to the TCR/CD3 complex and enzymes involved in TCR rearrangement (*Rag2*, *Dntt*) did show decreased transcriptional activity in im1928z1-lymphoid progenitors (**Figure 3C**). This was accompanied by a complete lack of detectable D (diversity)-J (joining) recombination segments within the *Tcrb* locus (**Figure 3D)** suggesting the absence of TCR rearrangement in im1928z1-engineered CARiK cells. Complementing the observed phenotype transcripts known to be essential for T cell identity (e.g. *Cd8a, Cd8b1, and Cd4*) were markedly decreased in lymphoid progenitor-derived im1928z1-CARiK cells **(Supplemental Figure 2)**.

We assessed the biological consequences of CAR expression in lymphoid progenitor cells and studied transcription factors (TFs) which are known to be involved in T and NK cell development. Our whole transcriptomic data showed that transcripts of *Notch1* and *Bcl11b*, which are indispensable for T cell development and commitment (20-23), are decreased in im1928z1-engineered cells (indicated by arrows) (**Figure 3, C and E**). This was reflected in flow cytometric analysis which showed that the surface NOTCH1 expression was decreased on im1928z1-engineered lymphoid progenitors when compared to transgene-negative controls (**Figure 3F**). Concomitantly, BCL11B protein expression was substantially suppressed when compared to lysates obtained from iTom controls (**Figure 3G**) indicating that the CAR expression on early lymphoid progenitors fundamentally intervenes at the transcription checkpoint of NK cell versus T cell development.

Concurrently, transcripts associated with NK cell-like development and functionality could be documented for both, im1928z1-expressing cells in vitro and in vivo (**Figure 3H and**

Supplemental Figure 2). This included a pronounced expression of NK cell-related developmental factors (e.g. *Nfil3* and *Id2*) (24, 25) and the identification of transcripts such as *Car5b, Dapk2, Adamts14, Spry2, Klri2, Ncr1, Aoah, Serpinb9b, and Gzma* that have been reported to be related to NK cells (19). Our results indicate that CAR expression in differentiating lymphoid progenitors enforces expression of NK cell-like transcripts generating CARiK cells which is paralleled by decreased activity of relevant genes and TFs being closely associated with T cell development.

Transcriptional profile analysis locates CARiK cells at the interface of T lymphocytes and NK cells

To more distinctively describe the developmental relation of CARiK cells to T- and NK cells, we compared transcriptional profiles of CARiK cells, that were isolated 28 days after co-transplantation, to those of sorted T cells, NKT cells, γδT cells, and NK cells (**Figure 4A**). PCA analysis revealed a distinct localization from all other lymphoid cell populations (**Figure 4B**). Hierarchical clustering of the 500 genes with the most variable mRNA expression (adjusted p-value < 0.05) confirmed that CARiK cells were distinct from both, NK cells and the other T-lymphoid subsets (**Figure 4C**).

To further elucidate the lineage of CARiK cells we grouped key transcripts according to function and association with distinct lymphoid cell types (26) (**Figure 4D**). CARiK cells demonstrated decreased expression of transcripts associated with T cell identity such as for *Cd4* and *Cd8*. Of note, mRNA transcript expression for *Bcl11b* was further decreased in CARiK cells when compared to other T lymphoid subsets and NK cell-associated transcripts for *Ncr1* and *Nfil3* were significantly overexpressed. Nevertheless, CARiK cells expressed transcripts such as *Zbtb16*, *Rorc*, and *Cxcr6* that are known to be associated with γδT cells, NKT cells or innate lymphocytes. Expression strength of transcripts important for cytotoxicity mediators such as *Ifng*, *Gzmb*, and *Gzma* was comparable between CARiK cells, NK cells or NKT cells.

High transcriptional activity for inhibitory receptors (*Pdcd1, Ctla4, Lag3, Havcr2*) suggests a high activation status. Together these data suggest a CARiK identity at the interface between NK- and T cell development.

CAR expression early during lymphoid progenitor cell differentiation is required for CARiK cell generation at the expense of T cell development

Under standard experimental conditions CAR expression was induced early on following transduction and maintained thereafter. To assess the role of antigen-exposure for further NK cell differentiation in vivo, engineered im1928z1-CARiK cells were co-transplanted into *Cd19*-knockout mice. For these experiments BM of *Cd19*-knockout donors was used and co-transplanted with im1928z1-lymphoid progenitors into *Cd19*-knockout mice to create a complete CD19-antigen free environment in vivo (**Supplemental Figure 3A**) (27). Compared to *Cd19* wildtype (WT) recipients, similar numbers of NK1.1-expressing progeny arose in BM and spleens suggesting that decisive signals for killer cell differentiation had been initiated by CAR expression at an early time point (**Supplemental Figure 3B**).

In order to evaluate the timing aspect of CAR expression in lymphoid progenitors, we compared early (day 0, standard experimental set up in **Figure 1D**) versus delayed (day 21) im1928z1-expression in vivo (**Figure 5A and Supplemental Figure 4**). In contrast to early im1928z1 expression, delayed im1928z1 induction allowed for limited T cell development as indicated by CD3 and TCRβ expression of the respective progeny (**Figure 5B**). This was paralleled by reduced frequencies of $NK1.1^{+}$ CARiK cells (**Figure 5C**). B cell suppression occurred to a similar extend in both groups, however with delayed onset when the CAR was "switched on" late (**Figure 5D**). In vivo persistence was akin of early im1928z1-CARiK cells versus late induced CAR T cells, and reconstitution of the B cell compartment was paralleled by both, progressive extinction of the lymphoid progenitor-derived CAR T cells and CARiK cells (**Figure 5D**). Collectively, the developmental shift of CAR-expressing lymphoid

progenitors occurred early during development leading to functional CARiK cells whereas delayed CAR expression allows for the generation of functional CAR T cells.

CARs containing a CD28 costimulatory domain induce killer cells with superior functionality

To study the impact of CAR design on lymphoid progenitor development, we generated and compared a more diverse panel of lentiviral CAR vector constructs: im19delta without an immunoreceptor tyrosine-based activation motif (ITAM); im19z1 and im19z3 with one or three ITAMs active, both without the costimulatory domain; and im1928z1 and im1928z3 both containing a CD28 moiety and one or three active ITAMs within the CD3ζ chain (**Figure 6A and Supplemental Figure 5A**). CAR constructs were comparatively expressed on resulting lymphoid progenitors (**Supplemental Figure 5B**). Both, im19delta- and iTom-transduced LSKs generated very few $NK1.1^+$ lymphoid progenitors (**Supplemental Figure 5C**). Number of ITAMs and the presence of the costimulatory moiety CD28 correlated with the occurrence of a $CD25^{mid}CD44^+$ population in vitro containing the $NK1.1^+$ CARiK cell subset (**Figure 6, B and C and Figure 1C**). The size of this population differed between CAR constructs, was more accentuated when the CAR contained the CD28 costimulatory domain, and seemed independent of 1 versus 3 ITAMs being active. This was paralleled by a significant decrease of the DN2 population and a more pronounced reduction of DN3 cells demonstrating a block of T cell development at this stage (**Figure 6, B and C**). In contrast, a comparable increase of the $CD122^+NK1.1^+$ fraction was observed with the exception of the 19z1 construct, which produced a less pronounced CARiK-shift (**Figure 6D**). While all signaling competent CAR variants promoted the generation of $CD122^+$ lymphoid precursors and finally a $NK1.1^+NKp46^+$ CARiK cell population in the bone marrow after transplantation, higher numbers were derived from CARs with CD28 co-stimulation (**Figure 6E**). In addition, the use of CARiK cells expressing a CAR with CD28 co-stimulatory domain resulted in prolonged B cell suppression

(**Figure 6, F and G**). This was closely mirrored by strong anti-leukemic activity upon co-transplantation (**Figure 6 H**) suggesting that a CD28 co-stimulatory domain allows for enhanced functionality of lymphoid progenitor-derived CARiK cells.

CAR-induced developmental shifting from T cell to NK cell-like differentiation translates to humans.

We next sought to evaluate if the impact of CAR expression on developing human lymphoid progenitors was comparable to that seen in mice by studying human UCB-derived $CD34^+$ HSPCs. We cloned a previously published and clinically tested second generation human CD19 CAR containing the CD28 costimulatory domain (h1928z3) (28) into a lentiviral backbone. The respective human signaling-incompetent h19delta CAR served as control (**Figure 7A**). CAR-HSPCs were co-cultured onto OP9-DL1 monolayers supplemented with hIL-7, hSCF, hTPO, and hFLT3L to perform differentiation experiments (14). Equivalent to the murine system, CAR expression could already be detected on early hematopoietic progenitor cells (**Figure 7B**). In human T cell development, the expression of CD1a during early lymphoid development marks an important step for lineage choice (29, 30). We show that CAR-expression on human lymphoid progenitors resulted in decreased frequencies of $CD1a^+$ cells in the $CD7^+CD5^+$ subpopulation (**Figure 7C**). This was associated with decreased NOTCH1 cell surface expression **(Figure 7D**) and enhanced frequencies of $CD56^+CD161^+$ cells (**Figure 7E**). This phenotype was strongly enhanced by stimulation with the hCD19 antigen during the differentiation process (**Figure 7, C-E**), demonstrating the importance of signaling strength for the developmental shift. Importantly, rearrangement at the *TCRB* locus was blocked in h1928z3-expressing lymphoid progenitors **(Figure 7F)**.

To further substantiate these findings, we performed microarray analysis experiments of the engineered human lymphoid progenitors. Unlike that seen in mouse, h1928z3 expression on lymphoid progenitors resulted in a transcriptional profile which was comparable to the h19delta

control **(Figure 7G)**. However, stimulation of the h1928z3 CAR led to decreased transcript expression of the regulators of T cell development such as *PTCRA*, *GATA3*, *NOTCH3*, and most pronounced *IL7R* **(Figure 7H)**. In contrast, NK cell-related genes including *ID2* and transcripts for functional molecules (*GZMB*, *GZMH*) were overexpressed. Similar to the data obtained from mice, *BCL11B* expression was significantly decreased in the human CAR-stimulated lymphoid progenitors as shown by quantitative reverse transcription PCR **(Figure 7I)** supporting our findings that signaling strength mediated by the CAR profoundly impacts lymphoid differentiation from early hematopoietic progenitors.

DISCUSSION

In general, low cost production methods of approved agents have to compensate for investments during early phases of drug development. This paradigm has been profoundly challenged by the appearance of clinically highly effective cellular biologicals such as CAR-engineered T cells for the treatment of B cell malignancies. Since the drug needs to be produced on demand on an individualized basis, production remains expensive resulting in very high costs for a single treatment. Thus, there is a burning economical and ethical need to develop cell products that can be used for a broader range of patients, independently of their HLA-type. Using a syngeneic and an MHC class I/II mismatched murine hematopoietic progenitor cell transplantation model for CD19 expressing leukemia we intended to study the impact of a T cell depleted bone marrow graft that had been enriched with prefabricated CAR-expressing lymphoid progenitor cells.

For proof of principle we initiated the studies using a murine CAR against the clinically relevant antigen CD19 to transduce murine hematopoietic stem cells that we had originally planned to further differentiate into precursor T cells in vitro. The expression of the CAR-encoding gene was additionally set under the control of an inducible promoter system (16) in order to evaluate its impact on T cell fate in a time-dependent manner.

We found that forced CAR expression early in hematopoietic progenitors can profoundly interfere with T cell development in favor of a population with NK cell-like properties. This developmental shift is initiated early during in vitro lymphoid differentiation, which then persists as what we termed -closely reflecting a nomenclature introduced earlier by others (23)- CARiK cells upon co-transfer in vivo. These CARiK cells mediate strong anti-CD19-directed activity as shown by profound B cell aplasia and leukemia eradication even across a complete MHC class I/ II mismatch. Both, the degree of this developmental shift as well as the in vivo persistence of CARiK cells depend heavily on the presence of a CD28 co-

stimulatory moiety in the CAR construct and seems to be influenced to a lesser extent by the number of active ITAMs within its ζ -chain.

We explored the mechanism of these findings by conducting microarray technology-supported whole transcription analysis both, on in vitro generated CAR-engineered lymphoid precursors previous to co-transplantation and on their respective progeny after in vivo maturation. We show that CAR expression resulted in decreased transcriptional activity for *Bcl11b* and *Notch1*, transcription factors that have shown to exert gate keeper function for T cell development. Deletion of *Bcl11b* has been shown to result in reprogramming of T cells to natural killer-like cells and was associated with a reduction of Notch transcripts (22, 23, 31). This was paralleled by a distinct NK cell-associated transcriptional profile that becomes further pronounced after in vivo maturation (18, 19, 24, 32). In order to substantialize these data, we investigated whether these findings would translate to the protein level. Indeed, western blotting experiments showed a marked reduction of BCL11B in CAR-engineered lymphoid precursors at the end of the in vitro differentiation process already. In addition, NOTCH1 expression on the cell surface was also reduced in concordance with the transcriptional data.

The distinct transcriptional profile on Microarray analysis distinguished CARiK cells from other lymphoid subsets and located their identity at the interface of T lymphocytes and NK cells. Similar to T progenitor cells that had been generated from induced pluripotent stem cells, CARiK cells had overlapping expression profiles with γδT cells, NKT cells, and NK cells (26).

A growing body of evidence suggests that the introduction of either a TCR or a CAR into human hematopoietic progenitor cells can prevent endogenous rearrangement of the TCRα and TCRβ locus thereby giving rise to mono-specific T cells, that express the transgenic antigen receptor only (12, 33, 34). We could demonstrate that like in the murine model, early signals mediated by a CAR, that had been transduced into CD34+ hematopoietic human stem cells, result in decreased expression of NOTCH1 which leads to a suppression or regular progenitor

T cell development in favor of NK-like cell differentiation. As in murine lymphoid progenitor cell development this was associated with a profound block of TCR-gene rearrangement. Although downregulation of *BCL11B* did not reach significance in the performed microarrays, quantitate reverse transcription PCR did reveal significant *BCL11B* suppression. Further assessment of its impact in vivo has been frequently hampered by limitations of currently available humanized mouse models. Using in vitro generated murine precursor T cells that had been engineered with a CAR against human CD19, Zakrzewski and colleagues did find CAR-expressing $CD3^+CD4^+$ and $CD3^+CD8^+$ peripheral T cells in murine recipients hinting normal positive and negative selection processes of transduced progenitor T cells (9). However, the use of a first-generation human CAR in an otherwise murine environment might not have fully met the signaling strength required for a shift in differentiation. We observed that the murine CAR (im1928z1) used in our studies exhibits significant tonic signaling activity as assessed in a NFAT reporter cell line (data not shown) inducing a decisive NK cell-like differentiation shift in vitro already. Although Notch culture systems might allow for some B cell differentiation, the early developmental shift in the absence of measurable antigen during in vitro culture is further indicative of functional relevant tonic signaling already known for CARs (35-37).

In contrast, the human CAR that was used required additional stimulation with CD19 to fully reproduce the differentiation characteristics seen in the murine system.

The observation that in the murine system CAR-engineered lymphoid progenitor cells continued further differentiation into CARiK cells both, in a CD19 competent (WT) and a CD19 deficient host environment suggests that the differentiation program was already initiated early in the in vitro culture. This is supported by the fact that *Bcl11b* levels were reduced at this point. Since control experiments with a signaling-deficient CAR variant, both, in the murine and human system, did again allow for T cell development to the level of CAR-negative controls, the observed shift in differentiation is suggestive of being dependent on CAR-mediated signaling. In contrast to effects caused by early CAR expression, delayed CAR expression

largely fails to induce the CARiK cell phenotype. Delayed induction of CAR expression however permits normal T cell development to some extent as indicated by the occurrence of CAR transgene-positive mature $CD3^+TCR\beta^+$ T cells. This was paralleled by decreased numbers of CARiK cells.

We further show that the CAR-expressing NK cell-like progeny of co-transplanted lymphoid progenitors mediated strong anti-leukemia activity after hematopoietic stem cell transplantation. Early in vivo depletion after HSCT using an NK-depleting antibody led to a complete abrogation of anti-leukemia effects. In contrast to lymphoid progenitors, that had been engineered to express an anti-leukemic TCR matching the recipient's MHC type, CAR-induced $NK1.1^+$ progeny did not build a cellular long-term memory (11). In vivo persistence and as a consequence CARiK cell mediated effects were limited, and in its extent associated with the use of a CD28 co-stimulatory domain. The anti CD19-directed activity lasted for about 60 days. This has important implications from a safety perspective, since we found a single injection of the stem cell-derived product to produce durable activity. After that, transgene-positive cells became undetectable in vivo. Leukemia survivors that were re-challenged with a lethal dose of CD19-expressing leukemia cells succumbed to the disease supporting the notion that no memory cell population had been developed. Although the cell product did not persist, the ready availability of a prefabricated and aliquotted product would_permit repeated infusions as needed.

Umbilical cord blood (UCB)-derived lymphoid progenitor cells (termed proT2) being $CD34^+CD7^+CD5^+$ have been identified to be a potential equivalent of the in vitro generated murine DN2 precursor T cell population (13, 38). We and others have shown that UCB-derived lymphoid progenitors can be genetically engineered (12, 14). Our data on TCR rearrangement and gene expression in both, murine and human systems support earlier studies that human and murine lymphoid development are much more similar than originally reported (30) and encourage its further exploitation for clinical use.

The use of genetically engineered HPSCs for adoptive transfer leads to concerns of safety when it persists in the body for an unduly long duration. Although long term persistence has demonstrated to be important in targeting B cell malignancies (5, 7, 39, 40), it has, however, remained unclear, why complete eradication of a target cell population would make further persistence of engineered T cells necessary. Of interest, recently cures were achieved using third party gene-edited T cells with limited persistence (41). Targeting antigens beyond CD19/20 might obviate the need for long term persistence of CAR-engineered immune cells. Our generated CARiK cells with limited in vivo persistence might be of interest when antigen structures are considered for which ”off tumor/on target” effects are of concern. In this scenario tumor cell eradication might be followed by the recovery of a physiologic cell population sharing the targeted antigen such as normal myelopoiesis after the treatment of a myeloid malignancy. Several other approaches have been taken to develop CAR cell products for a more universal use. The emergence of potent genome editing technologies has shown to transform a mature post-thymic T cell into a universally applicable cellular carrier of CARs by disruption of both, the endogenous TCR and HLA class I expression (41-43). As a promising alternative, induced pluripotent stem cells (iPSCs)-derived lymphoid cells (26) offer an attractive platform for immune engineering providing both the generation of T and NK cells (10, 44). Despite of impressive progress being achieved major challenges remain. This includes the necessity to control T- and NK lineage specifications, identification of the optimal maturational stage and preservation of an optimal functional and proliferative potential. Our data predict that the choice of the antigen receptor including its structural composition as well as the progenitor cell population going to be engineered can fundamentally direct final cell differentiation thereby determining biologic properties, both, in mouse and man.

MATERIALS AND METHODS

Mice

C57BL/6 (B6, $H2^b$) mice were purchased from Charles River. B10.A ($H2^a$) mice were purchased from Taconic laboratories. R26-M2rtTA knock-in mice (B6.rtTA, $H2^b$) express a reverse tetracycline-controlled transactivator for doxycycline inducible transgene expression and were used for transgene expression studies. To create B10.A-R26-M2rtTA (B10.A.rtTA) mice, B6.rtTA mice were backcrossed onto B10.A. *Cd19* knockout mice on B6 background (B6 CD19 KO) were a kind gift from K.-H. Lee (Hannover Medical School, Hannover, Germany).

Primary human UCB samples and generation of human engineered lymphoid progenitors

UCB samples were processed as described earlier (14). Briefly, purified $CD34^+$ HSPCs were transduced with lentiviral supernatant and transferred onto OP9-DL1 stromal cells in the presence of human (h)IL-7, hSCF, hTPO and hFLT3L. Human engineered lymphoid progenitors were harvested every 3-4 days and put onto new OP9-DL1 monolayers supplemented with the respective cytokines. h1928z3-lymphoid progenitors were stimulated by adding irradiated Daudi cells from day 4 of culture.

Cell lines

The C1498 myeloid leukemia cell line ($H2^b$, B6 origin) was transduced with retroviral supernatant of murine CD19 alone in the pAlpha.SIN.MPSV.wPRE vector (45) or linked with an IRES GFP cassette to generate the murine $CD19^+$ cell lines C1498-mCD19 or C1498-mCD19-GFP. For isolation of stable mCD19 expression, cells were sorted twice for CD19 or CD19 and GFP (FACSAria Ilu, BD Bioscience). C1498, C1498-mCD19, C1498-mCD19-GFP, Daudi and $58\alpha^-\beta^-$ cells were cultured in RPMI-1640 and 293T cells were cultured in DMEM (Capricorn). Both media were supplemented with 10% heat-inactivated fetal calf serum

(Capricorn), L-glutamine, Hepes buffer and penicillin/streptomycin (all Gibco). OP9-DL1 cells were cultured in complete αMEM (life technologies) supplemented with 20% FCS, L-glutamine, Hepes buffer and penicillin/streptomycin. The hybridoma cell line HB-191 (ATCC) expressing an anti-NK1.1 antibody (clone: PK136) was cultured in HybridomMed Dif 1000 (Biochrom). All cell lines were tested for mycoplasm-negativity by PCR.

Bone marrow and lymphoid progenitor co-transplantation, leukemia challenge and NK cell in vivo depletion

Total body irradiation of 8-week old B6 recipients was performed with 10,5 Gy from a linear accelerator. After 24h, mice were co-transplanted with 3×10^6 T-cell-depleted B6 bone marrow (TCD-BM) and 8×10^6 lymphoid progenitors as previously described.(11) All adoptive transfer studies were performed under permanent administration of Doxycycline (Dox)-containing water or food for transgene expression unless otherwise noted. For leukemia studies, 1.2×10^6 C1498-mCD19-GFP leukemia cells were injected via the lateral tail vein on day 20 after transplantation. $NK1.1^+$ cell depletion in vivo was performed in CAR lymphoid progenitor-transplanted mice challenged with leukemia cells by i.p. injection of 200µg anti-NK1.1 antibody weekly and PBS as control. All mice have been randomly assigned to experimental groups and no blinding of investigators was performed.

Lentiviral CAR constructs and production of supernatant

The murine CD19 CAR construct (im1928z1) and sequence were described previously (15). im1928z1 contains an anti-CD19 scFv, the transmembrane domain and co-stimulatory domain from mouse CD28 and the CD3ζ signaling domain with 1 functional ITAM. The im19BBz1 construct has the same ITAM configuration but a 4-1BB costimulatory domain instead of CD28. Im1928z3 has the same configuration like im1928z1 but 3 functional ITAMs. im19z1 and im19z3 were designed without CD28 co-stimulatory domain. The im19delta construct

contains only the scFv and the CD28 transmembrane domain and lacks the co-stimulatory and signaling. All CAR sequences were cloned under the control of the Tetracycline inducible T11 promotor in combination with an IRES dTomato (Tom) cassette in a shortened version of an all-in-one lentiviral backbone (11, 16). A dTomato only construct was used as control vector (iTom). A functional human CD19 CAR (h1928z3) (28) and the signaling-deficient h19delta construct for control were cloned in a lentiviral backbone under a SFFV promotor that was linked to a dTomato reporter cassette. Lentiviral supernatants were produced via transient transfection of 293T cells with the viral plasmids pMD2.G, pRSV.Rev, pcDNA.GP.4×CTE (plasmids produced by PlasmidFactory) and the respective CAR plasmid by using the calcium phosphate transfection method. The RD114/TR envelope was used for transduction of $CD34^+$ HSPCs. Harvested supernatant was filtered and concentrated via ultracentrifugation. The $58\alpha^-\beta^-$ hybridoma cell line transduced with the M2 transactivator was used for viral titer determination of murine constructs.

Generation of engineered murine lymphoid progenitors

Murine hematopoietic stem and progenitor cells were isolated from bone marrow with antibodies against lineage markers and sorted for c-kit^+ and Sca-1^+ (LSK) cells. LSKs were then transduced with lentiviral supernatant and cultured as published (11). Briefly, transduced LSK cells were transferred to OP9-DL1 monolayer cells in complete αMEM medium (life technologies) supplemented with 10% heat-inactivated FCS, FLT3-L (5 ng/mL), IL-7 (5 ng/mL) (Peprotech) and Dox (2 mg/mL) (Sigma). Lymphoid progenitors were transferred to new OP9-DL1 monolayer every three to four days. Transduced lymphoid progenitors were sorted on day 10 of OP9-DL1 co-culture for dTomato expression (Tom^+) and frozen on day 13 to 17. For adoptive transfer studies, engineered lymphoid progenitors were thawed and cultured for further 7 days on OP9-DL1 with cytokines and Dox. Engineered lymphoid progenitors between days 20-24 of co-culture were used for co-transplantation. Cultures were supplemented

with Dox for permanent transgene expression unless otherwise noted. For in vitro antigen stimulation of im1928z1-lymphoid progenitors, irradiated C1498-mCD19 or C1498 cells were added in a 1:10 ratio to lymphoid progenitor culture.

Flow cytometry

Single cell suspensions of murine origin were stained with the following Fluorochrome-conjugated antibodies that were purchased from eBiosciences, BD Pharmingen or Biolegend: CD3ε (Brilliant Violet (BV) 421/ PE-Cy7; clone:145-2c11), CD4 (PE-Cy7/ BV 421; GK1.5), CD8α (FITC/APC; 53-6.7), TCRβ-(PE-Cy7/ APC; H57-597), CD25 (PE-Cy7; PC61), CD44 (APC/FITC; IM7), CD122 (FITC/ PE-Cy7; TM-β1), CD19 (FITC/ APC/ BV 421; 6D5), NK1.1 (APC/ FITC; PK136), NKp46 (BV 421; 29A1.4), IFNγ (APC/PE-Cy7; XMG1.2), CD107a (APC/PE-Cy7; 1D4B), NOTCH1 (APC; HMN-1-12), anti-rat IgG2a isotype (APC; eBR2a or PE-Cy7; MOPC-21). For lineage$^-$c-kit$^+$Sca-1$^+$ sort, antibodies against lineage markers CD3, CD4, CD8, CD19, NK1.1, Gr-1 (clone RB6-8C5), and CD11b (M1/70) in FITC and c-kit (APC; ACK2) and Sca-1 (PE; D7) were used. For CAR expression detection, cells were stained with goat-anti-rat Fab Fragment conjugated with Alexa Fluor 647 (Jackson ImmunoResearch). Human cells were stained with the following antibodies after blocking with Human TruStain FcX (catal. no. 422302) purchased from Biolegend: CD34 (PE-Cy7; 581), CD7 (FITC; CD7-6B7), CD5 (BV421; UCHT2), CD1a (APC; HI49), CD56 (APC; HCD56), NOTCH1 (APC; MHN1-519), and CD161 (FITC; HP-3G10). Flow cytometry was performed using a FACSCanto or LSR-II (BD Biosciences) and the data were analyzed with FlowJo software (TreeStar). FACS analysis was based on fluorescence minus one (FMO) controls. Relative numbers from Thymi, BM and splenocytes were calculated from Tom$^+$ gate.

Western Blotting

Cell lysates of transgene-positive sorted lymphoid progenitors or wild-type B6 thymocytes were prepared in radioimmunoprecipitation assay (RIPA) buffer as described (46). Equal masses of protein lysates were separated by SDS-PAGE and transferred onto PVDF membranes (Amersham). Membranes were blocked with 5% skim milk and BCL11B was stained with rat anti-mouse primary antibody (clone 25B6; Biolegend) and detected with HRP-coupled goat anti-rat secondary antibody (Poly4054; Biolegend).

Ex vivo short-term culture

Splenocytes from co-transplanted mice were harvested 28 days after transplantation and brought into short-term culture under T cell conditions with ConA (5mg/mL) (Sigma), IL-7 (5ng/mL) and IL-2 (20 U/ml) (Peprotech) or cultured with high IL-2 concentrations (1000 U/ml). Cells were split every 2 days and used in functionality assays after 4-6 days of culture.

Intracellular cytokine staining and degranulation assay

Ex vivo cultured splenocytes were co-incubated with C1498-mCD19 cells and intracellular IFNγ staining performed as previously described.(47) For detection of degranulation, ex vivo cultured splenocytes were co-incubated on 96-well plates with C1498-mCD19 cells in a 10:1 ratio in the presence of an anti-CD107a antibody. After 1h, GolgiStop (BD Bioscience) was added. Cells were harvested after 4 hours and treated with the CytoFix/CytoPerm Kit (BD Bioscience) for FACS analysis.

PCR for rearrangement on *TCRβ* locus

Murine and human engineered lymphoid progenitors were harvested from in vitro culture and sorted for Tom^+ cells. Genomic DNA was isolated (Qiagen) and D-J rearrangement at the *Tcrb* locus assessed via PCR using TCRB_Jβ2-Rev TGAGAGCTGTCTCCTACTATCGATT and

TCRB_Dβ2-Fwd GTAGGCACCTGTGGGGAAGAAACT as primers (5`-3`) as described (23) for mouse TCR rearrangement. Human *TCRB* rearrangement was assessed using Primers as described (48).

Microarray and data analysis

Murine Tom^+ cells were sorted from iTom or im1928z1-lymphoid progenitors' in vitro culture on day 20 or from harvested splenocytes for their progeny on day 28 after transplantation. For the comparison of different lymphocyte subsets and CARiK cells, T cells ($CD3^+\gamma\delta TCR^-NK1.1^-$), NKT cells ($CD3^+NK1.1^+$), γδ T cells ($CD3^+\gamma\delta TCR^+$), and NK cells ($CD3^-NK1.1^+$) were sorted from wildtype B6-derved splenocytes. Tom^+ CARiK cells were sorted on day 28 after co-transplantation. RNA was extracted using Qiagen RNAeasy (Qiagen) according to manufacturer´s manual. For human samples, RNA of Tom^+ sorted cells was extracted with the Single cell lysis kit (Invitrogen). Microarray analysis was performed with Affymetrix Clariom™ S (400 Format) Pico chips. Raw data were analyzed by R/Bioconductor packages oligo and Biobase. Summarized probeset data was log2 transformed followed by RMA normalization procedure. Normalized data sets were filtered for informative genes (showing at least expression values >log2(10) in more than two samples). Datasets were tested across all groups (ANOVA) or pairwise using linear models to assess differential expression in context of the multifactorial designed experiment. For statistical analysis and assessing differential expression the R/BioConductor package "limma" was used that utilizes an empirical Bayes method to moderate the standard errors of the estimated log-fold changes (49). Functional analysis was performed by R package "clusterProfiler" (50).

Microscopy

Thymi of mice co-transplanted with engineered lymphoid progenitors were harvested on day 14 after transplantation. Sections from Tissue-Tec O.C.T. (Sakura) embedded thymi were

analyzed with a Zeiss Axio Imager 2 microscope (x20 magnification) and acquired with Zen pro software (Zeiss). Images were equally processed with ZEN lite software.

RNA extraction and quantitative reverse transcription PCR

RNA from sorted samples on day 18 of OP9-DL1 co-culture was extracted using the RNAeasy Mini Kit (Qiagen) and converted into cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR reactions were performed using the QuantiTec SYBR Green PCR kit (Qiagen) on a Applied Biosystems 7300 Real-Time PCR System (Thermo). The QuantiTec Primer assay for *BCL11B* (QT00080983) and *ACTB* (QT00095431) were purchased from Qiagen. Relative expression of *BCL11B* was normalized to *ACTB*. Non-transduced lymphoid progenitors were used for controls.

Statistics

Statistics were performed and graphed with GraphPad Prism 5 software for Mac (GraphPad Software). Survival curves were compared using Mantel-Cox (log-rank) test. Student´s *t* test (two-tailed) was applied for two group comparisons and one-way ANOVA with Tukey´s post-test for comparing more than two groups. Data were represented with means ± s.e.m. P values < 0.05 were considered to be significant and indicated by *; **P < 0.01, ***P < 0.001, ****P < 0.0001.

Data availability

Raw data of microarray experiments are deposited in the Gene Expression Omnibus (GEO) under the accession number GSE104512 and GSE135015.

Study approval

All animal experiments were approved by the State Government of Lower Saxony, Germany (Approval code: 33.14-42502-04-15/1781) and performed in accordance with institutional animal care and use guidelines. Human UCB-derived samples were collected after written, informed consent by the child´s mother. Procedures for the use of UCB for this study were reviewed and approved by the medical ethics committee of Hannover Medical School and handled in accordance with the Declaration of Helsinki.

AUTHORSHIP CONTRIBUTIONS

Contribution: M.M. designed research, performed experiments, analyzed and interpreted data, and drafted and edited the manuscript; J.H. and V.S. performed experiments; R.G., J.M., H.M. and B.E.-V. contributed vital new technology and reagents; A.G., M.R.M.v.d.B., J.H., R.B., A.K., and A.S. analyzed and interpreted data and edited the manuscript; and M.G.S. designed the general concept and research, analyzed, and interpreted data, drafted and edited the manuscript.

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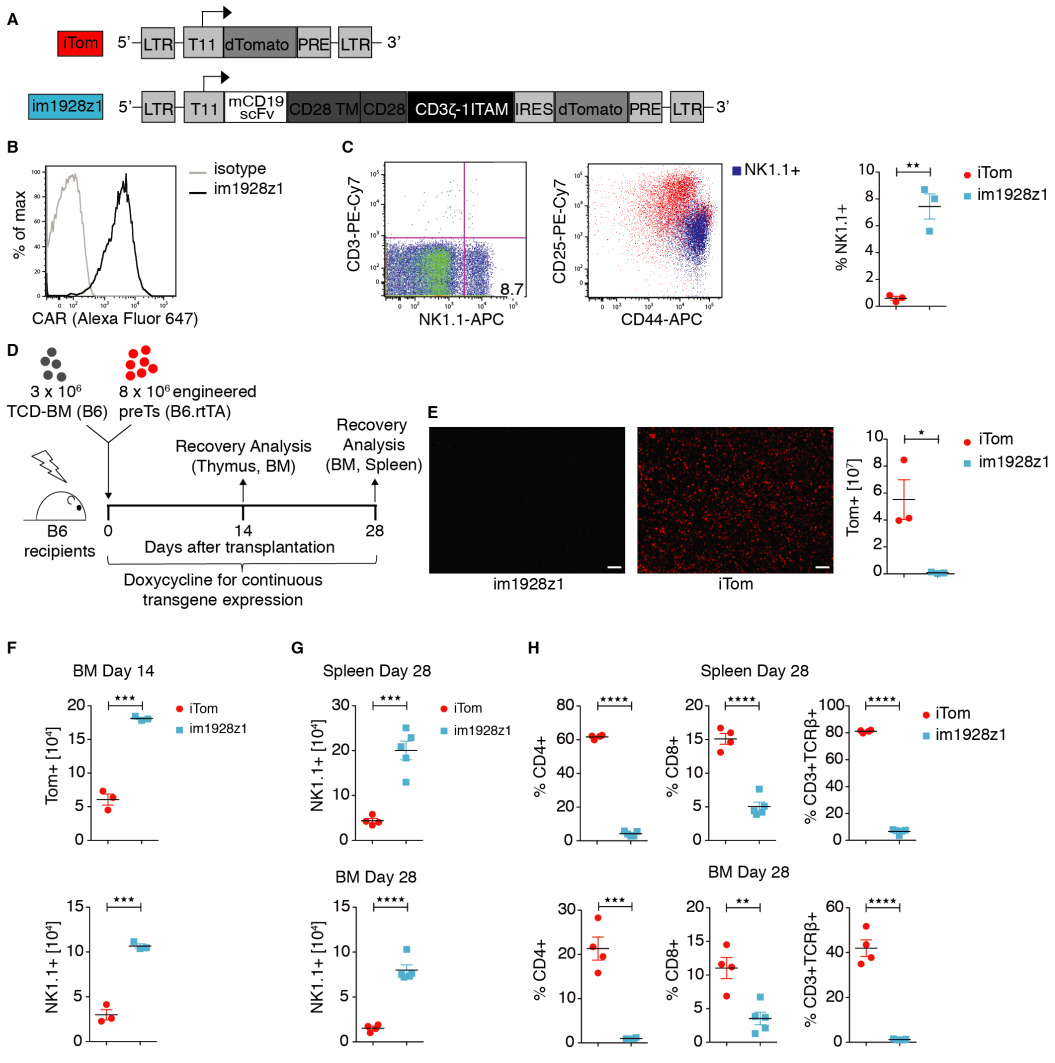

Figure 1. im1928z1-CAR expression in HSPCs cells prevents T cell but rather favors NK-like cell development of lymphoid progenitors *in vitro* and *in vivo*. (**A**) The lentiviral control and the murine CD19 CAR construct: iTom (inducible dTomato reporter gene only) and im1928z1 (inducible murine CD19 CAR, CD28 co-stimulation, 1 functional ITAM containing CD3ζ domain) linked to an IRES dTomato cassette. LTR, long terminal repeats; T11, doxycycline-inducible promotor; scFv, single chain variable fragment; TM, transmembrane domain; IRES, internal ribosome entry site; PRE, Woodchuck hepatitis virus posttranscriptional regulatory element. (**B**) Representative data of im1928z1 expression on in vitro generated lymphoid progenitors. (**C**) Representative FACS plots of NK1.1 and CD3 expression on in vitro generated im1928z1-engineered lymphoid progenitors (left); $NK1.1^+$ population within $CD25^+CD44^+$ lymphoid progenitors (middle) and $NK1.1^+$ expression on

771 iTom and im1928z1-transduced lymphoid progenitors previous to co-transplantation (right) (n=
772 3 independent cultures were pooled). (**D**) Irradiated B6 recipients were reconstituted with 3 x
773 10^6 B6 T cell depleted BM (TCD-BM) and co-transplanted with either 8 x 10^6 im1928z1- or
774 iTom-engineered lymphoid progenitors. (**E**) Thymic sections were imaged for Tom^+ cells (scale
775 bar: 50µm; x 20 magnification). Single cells from harvested thymi were analyzed by FACS for
776 Tom^+ progeny of co-transplanted lymphoid progenitors (n= 3 mice, respectively). (**F**)
777 Lymphoid progenitor-derived progeny in the BM on day 14 (top). Numbers of $NK1.1^+$ cells
778 within the Tom^+ population are depicted (bottom) (n= 3 mice per group). (**G**) Numbers of
779 $NK1.1^+$ and (**H**) frequencies of $CD4^+$, $CD8^+$, and $CD3^+TCR\beta^+$ progeny within the Tom^+ gate
780 in BM and spleens on day 28 (im1928z1, n= 5; iTom, n= 4). Results from one of two
781 independent experiments are shown. Statistics was performed by using Students t-test (two-
782 tailed). Data represent means ± s.e.m. Significant differences are indicated by *P < 0.05, **P <
783 0.01, ***P < 0.001, ****P < 0.0001.
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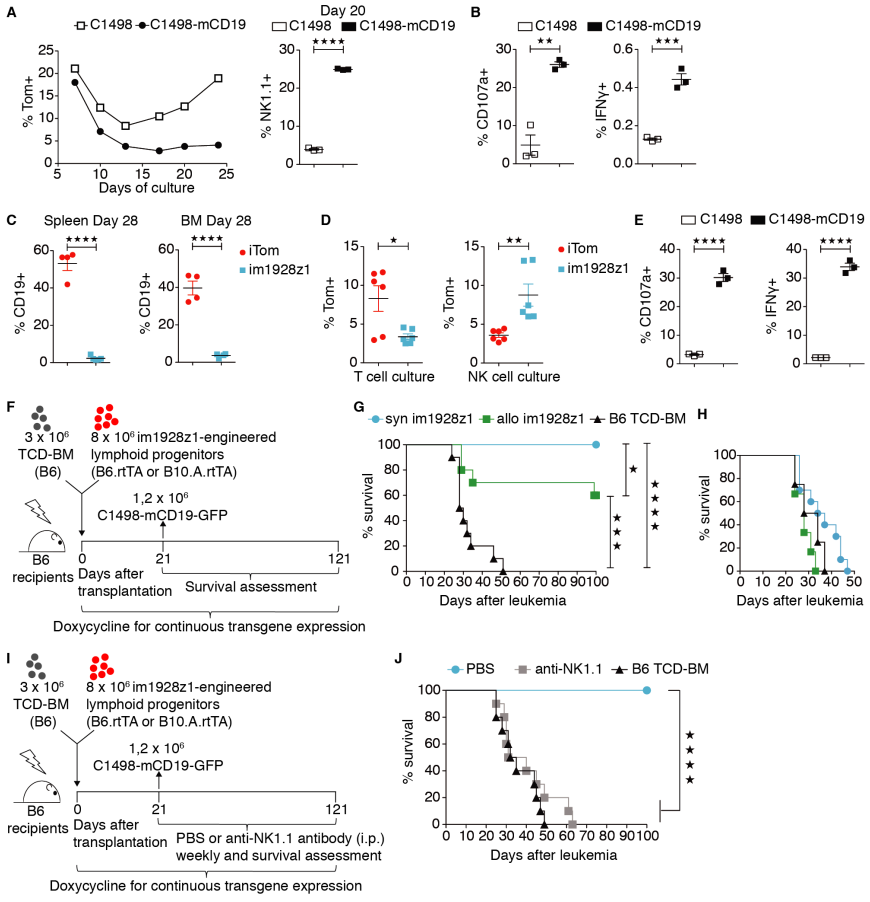


Figure 2. CAR-induced killer (CARiK) cells derived from im1928z1-engineered lymphoid progenitors demonstrate potent antileukemic activity across MHC barriers in vivo. (**A**) Generation of either stimulated or non-stimulated im1928z1-lymphoid progenitors. Frequencies of Tom^+ progenitors (left) and $NK1.1^+$ im1928z1-CARiK cells on day 20 of culture (right). (**B**) Responses of im1928z1-lymphoid progenitors upon stimulation were quantified via CD107a degranulation (left) or IFNγ production (right). Data from one of two experiments are shown. **(C)** $CD19^+$ B cell recovery of irradiated B6 recipients of B6 TCD-BM and either im1928z1-engineered progenitors or iTom ctrls (n= 4 mice, respectively). (**D**) Splenocytes were harvested on day 28 and re-cultured *ex vivo* under T cell- or NK cell culture conditions (n= 6, respectively; left). (**E**) $CD107a^+$ degranulation (middle) and IFNγ (right) responses to antigen were assessed (n= 3, respectively). (**A-E**) Students t-test was used for analysis. Data represent means ± s.e.m. (**F**, **G**) B6 recipients of 3 x 10^6 B6 TCD-BM (n=10/group) with or without 8 x 10^6 syngeneic (syn) or MHC class I and II mismatched (allo) im1928z1-expressing progenitors

799 received 1.2 x 10^6 C1498-mCD19 cells on day 21 after transplantation and monitored for
800 survival. Results from one of two independent experiments are graphed. (**H**) Survivors were re-
801 challenged with 1.2 x 10^6 C1498-mCD19 cells on day 100 and re-assessed for survival. TCD-
802 BM only recipients (n= 4) were added for control. (**I, J**) B6 recipients of 3 x 10^6 B6 TCD-BM
803 with or without 8 x 10^6 syngeneic im1928z1-progenitors were treated with weekly i.p.
804 injections of an anti-NK1.1 antibody (clone: PK136; 200µg/dose). PBS was used for control
805 (n= 10 per group). All mice were challenged with 1.2 x 10^6 C1498-mCD19 cells on day 21 after
806 transplantation (**J**). Survival curves were compared using Mantel-Cox (log-rank) test.
807 Significant differences are indicated by *P < 0.05, ***P < 0.001, ****P < 0.0001.
808

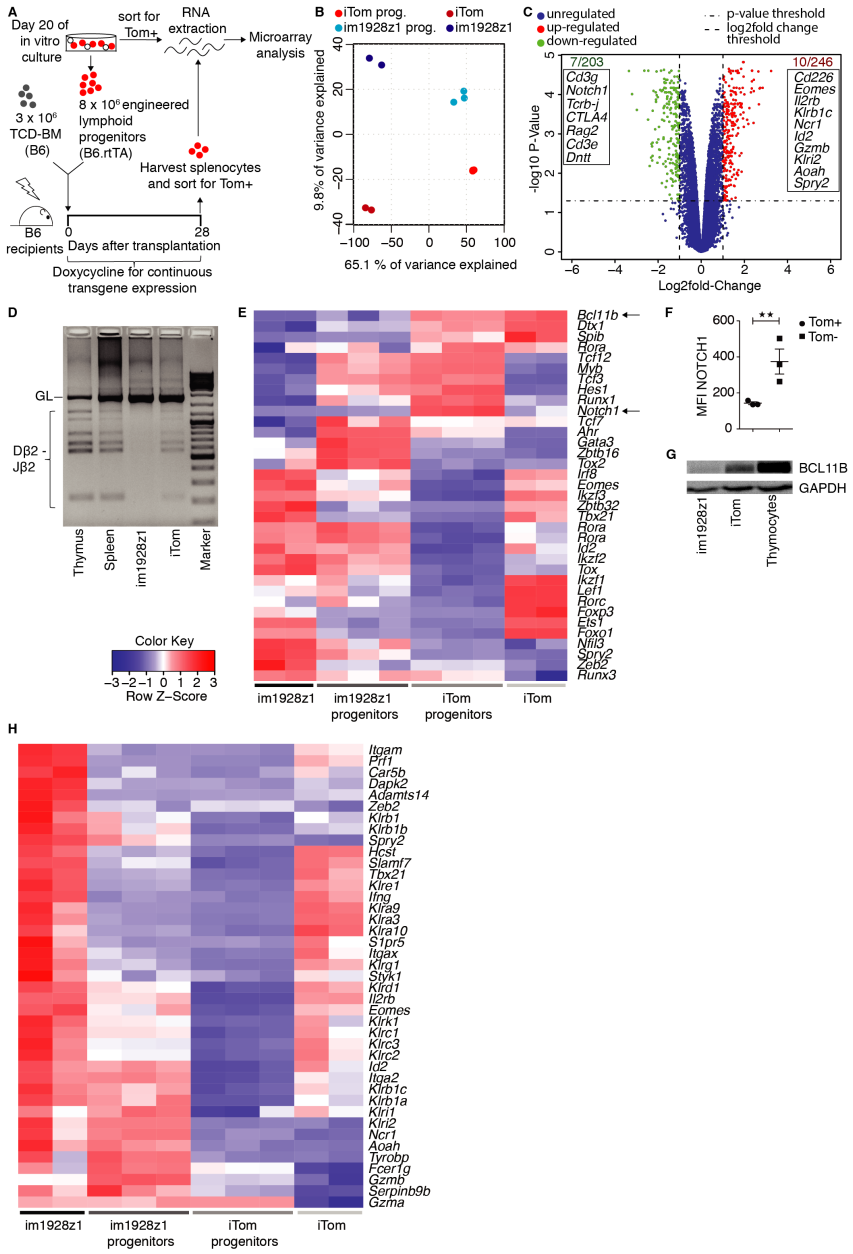
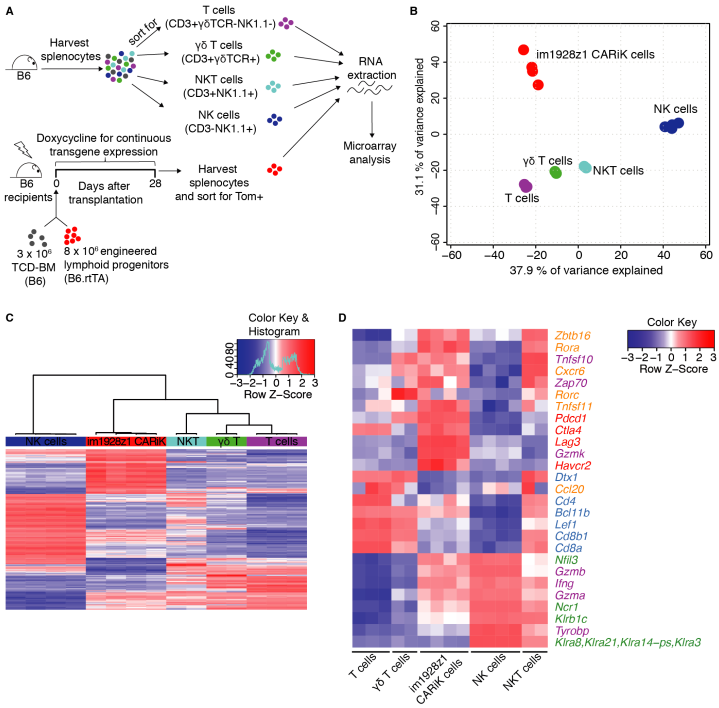


Figure 3. im1928z1 expression on HSPCs leads to BCL11B suppression allowing for CARiK cell development and concomitantly decreases T cell-associated gene expression. (**A**) For Microarray data analysis, RNA from Tom^{+}-sorted im1928z1- (n= 3) or iTom-lymphoid progenitors (n= 3) immediately previous to co-transplantation or from spleen-derived progeny (n=2, respectively) was isolated on day 28 after transplantation. (**B**) Principal component analysis (PCA) of total transcriptome profiles from either engineered lymphoid progenitors or their respective progeny is graphed. (**C**) Volcano plot for comparison of differently regulated transcripts in im1928z1-lymphoid progenitors and iTom ctrls. Gene symbols in the boxes

818 indicate selected transcripts found to be down-regulated (green) or up-regulated (red) at least
819 twofold (P < 0,05) in im1928z1-lymphoid progenitors as compared to ctrls. (**D**) Recombination
820 of D and J regions of the *TCRβ* locus in engineered lymphoid progenitors. Genomic DNA of
821 engineered progenitors was isolated on day 20 of culture, and rearrangements were detected by
822 PCR. Splenocytes and thymocytes from wildtype B6 mice were used as controls. Results from
823 one of two independent experiments are shown. GL, germ line band. (**E**) Heat map showing the
824 relative expression of transcripts for selected transcription factors. Data are normalized
825 according to expression in each row. (**F**) NOTCH1 expression on transgene-positive (Tom+)
826 or transgene-negative (Tom-) lymphoid progenitors engineered with im1928z1. Statistics were
827 performed by using Students t-test. Data represent means ± s.e.m. Significant differences are
828 indicated by **P < 0.01. (**G**) Western blot analysis for BCL11B in lysates from iTom lymphoid
829 progenitors, im1928z1-lymphoid progenitors or B6 WT thymocytes. Representative data from
830 one of two independent experiments are shown. (**H**) Relative expression of selected transcripts
831 for NK cell receptors, integrins, adaptors, effector molecules and transcription factors in
832 engineered lymphoid progenitors and their progeny. Data are normalized according to
833 expression in each row.
834



835

836 **Figure 4: Transcriptional profile analysis locates CARiK cells at the interface of T**
837 **lymphocytes and NK cells**

838 **(A)** Schematic representation of the experimental setup for transcriptional comparison of
839 CARiK cells and different lymphoid cell populations. Splenocytes of 12-week old wildtype B6
840 mice were harvested and sorted for T cells ($CD3^+\gamma\delta TCR^-NK1.1^-$; n= 3), NKT cells
841 ($CD3^+NK1.1^+$; n= 2), γδ T cells ($CD3^+\gamma\delta TCR^+$; n= 2), and NK cells ($CD3^-NK1.1^+$; n= 4). Tom^+
842 CARiK cells (n= 4) were harvested from recipients on day 28 and consecutively sorted.
843 Extracted RNA samples from all lymphoid subsets were compared by microarray analysis.
844 Experiment was performed once. **(B)** PCA analysis of transcriptional profiles derived from the
845 sorted lymphoid cell populations. **(C)** Hierarchical clustering of the 500 most differentially
846 expressed (adjusted p-value < 0.05) transcripts across CARiK cells and respective lymphoid
847 lineages. **(D)** Selected transcripts expressed by lymphoid subsets were color-coded according
848 to function or lymphoid cell type. Orange: γδ T cells, NKT cells, and innate lymphocytes;
849 Purple: Cytotoxicity mediators; Red: Inhibitory receptors; Blue: T lymphocytes; Green: NK
850 cells.

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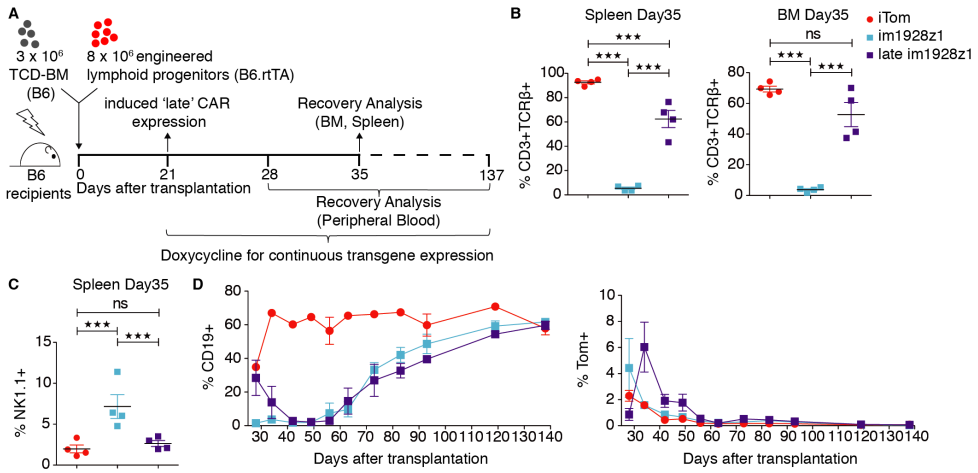

852

853 **Figure 5. CAR expression early during lymphoid progenitor cell differentiation is**
854 **required for CARiK cell generation at the expense of T cell development.** (**A-D**) Irradiated
855 B6 recipients received 3 x 10^6 B6 TCD-BM and either 8 x 10^6 im1928z1- or iTom-lymphoid
856 progenitors. CAR expression was either induced early (day 0) or late (day 21) after HSCT.
857 Indicated time points refer to the day after transplantation. (**B**) Frequencies of $CD3^+TCR\beta^+$ cells
858 were analyzed within the transgene-positive gate on day 35, both in the BM and spleens. (**C**)
859 Comparative analysis of $NK1.1^+$ cells in spleens of early versus late im1928z1-lymphoid
860 progenitor recipients on day 35 after AT. (**B, C**) Each analysis was done with n= 4 mice. Gating
861 was done on the Tom^+ population. Statistics were performed by using one-way ANOVA with
862 Tukey´s post-test. Data represent means ± s.e.m. Significant differences are indicated by **P <
863 0.01, ***P < 0.001. ns, not significant. (**D**) $CD19^+$ B cell recovery (left) and frequencies of
864 Tom^+ cells (right) in the PB of transplant recipients after early or late im1928z1 induction (n=
865 3-4 mice per group and time point). Results from one of two independent experiments are
866 shown.

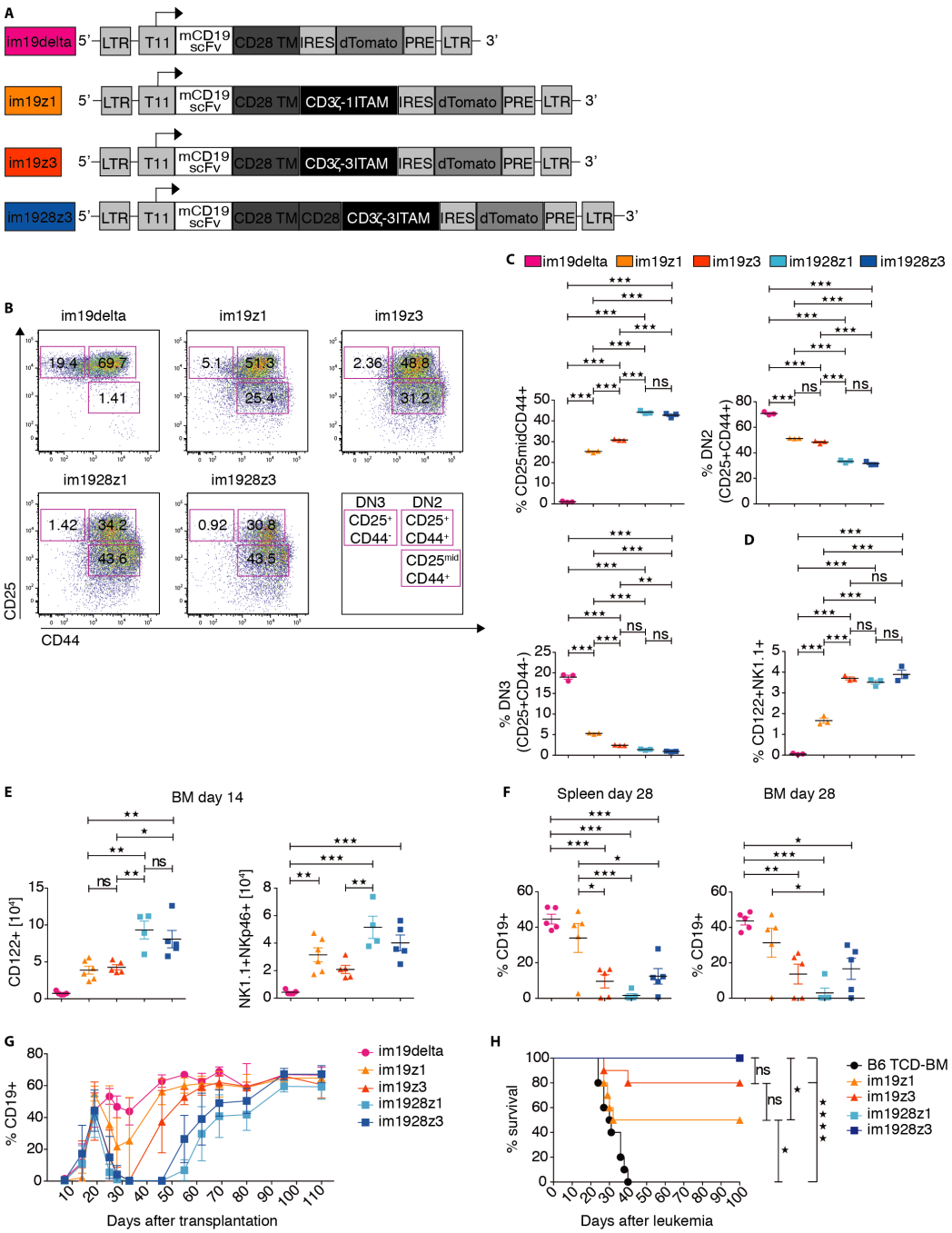

Figure 6. CARs containing a CD28 costimulatory domain induce killer cells with superior functionality (**A**) Design of the im19delta, im19z1, im19z3, and im1928z3 constructs. All CAR constructs were linked to an IRES dTomato cassette. (**B, C**) Representative FACS plots (**B**) and respective $CD25^{mid}CD44^{+}$, DN2 ($CD25^{+}CD44^{+}$) and DN3 ($CD25^{+}CD44^{-}$) populations (**C**) of lymphoid progenitors engineered with the indicated CAR construct (color-coded as indicated) on day 20 of in vitro culture. (**D**) Frequencies of $CD122^{+}NK1.1^{+}$ CARiK cells on day 20 of in vitro culture. Tom^{+} cells were analyzed. (**B-D**) Data from one of two independent

875 experiments measured in triplicates are shown. (**E-G**) Irradiated B6 recipients were
876 reconstituted with 3 x 10^6 B6 TCD-BM and co-transplanted with 8 x 10^6 lymphoid progenitors
877 that had been engineered with the indicated CAR constructs. (**E**). BM cells were analyzed for
878 numbers of $CD122^+$ (left) and $NK1.1^+NKp46^+$ cells (right) within on day 14. (im19delta,
879 im19z3 and im1928z3, n= 5 mice, respectively; im19z1, n= 6; im1928z1, n= 4). (**F)** $CD19^+$ B
880 cells were quantified in BM (left) and spleens (right) on day 28. n=5 mice for each group. (**G**)
881 $CD19^+$ B cells in the peripheral blood were determined in 7-14 day intervals. Analysis at each
882 time point was done on n= 4-5 mice per group. (**C-F)** Analysis was done using one-way
883 ANOVA analysis with Tukey´s post-test. Data represent means ± s.e.m. (**H**) Irradiated B6
884 recipients were transplanted with 3 x 10^6 B6 TCD-BM only (n=10) or additionally with 8 x 10^6
885 CAR-expressing lymphoid progenitors (n= 10). Mice were challenged with 1.2 x 10^6 C1498-
886 mCD19 cells on day 21 after transplantation and monitored for survival. Survival curves were
887 compared using Mantel-Cox (log-rank) test. Significant differences are indicated by *P < 0.05,
888 ****P < 0.0001, ns, not significant.
889

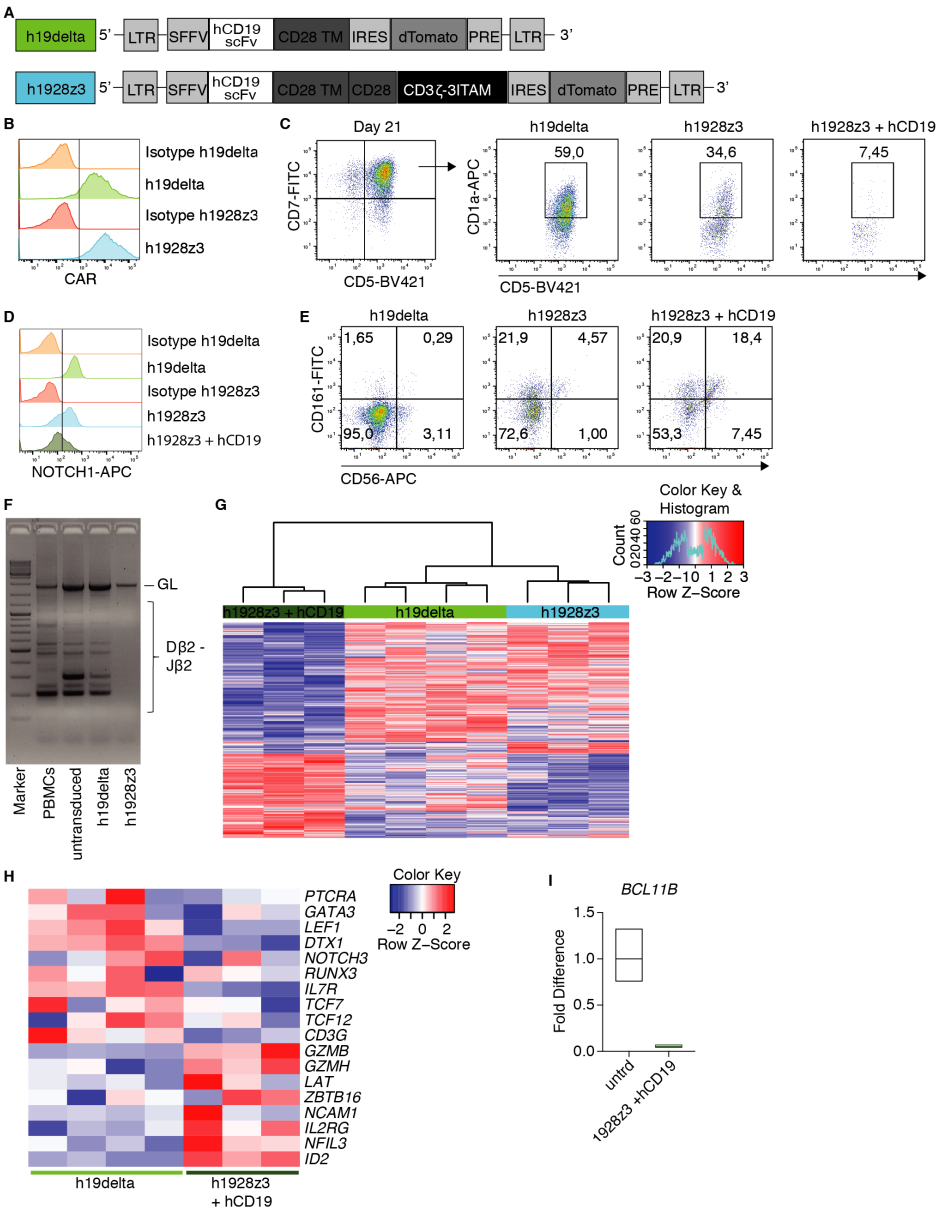

Figure 7. CAR-induced developmental shifting from T cell to NK cell-like differentiation translates to humans

(A) Representation of the lentiviral human CD19 CAR constructs with either CD28 costimulatory and CD3ζ signaling domain (h1928z3) or without signaling domains (h19delta). An IRES dTomato reporter cassette was used. **(B-F)** Human $CD34^+$ CB-derived HSPCs were engineered with respective CAR constructs and consecutively differentiated on OP9-DL1 stromal cells. FACS analyses were performed within the Tom^+ gate on day 21 of coculture. For stimulation, h1928z3 lymphoid progenitors were cocultured with irradiated $hCD19^+$ Daudi cells in a 1:10 ratio from day 4 onwards. Results from one of two experiments are shown. **(B)**

Expression of the CAR constructs on differentiating human HSPCs analyzed by Protein L staining. **(C)** $CD7^{+}CD5^{+}$ engineered human lymphoid progenitor cells were evaluated for CD5 and CD1a expression. Numbers represent percentages in the respective gates. **(D)** Histograms represent NOTCH1 expression on engineered early hematopoietic human progenitors. **(E)** CAR-modified HSPCs were analyzed for CD161 and CD56 expression. **(F)** Human CAR-engineered lymphoid progenitors were evaluated for *TCRB* rearrangement by PCR analysis of genomic DNA on day 18 of culture. Human PBMCs, non-transduced and h19delta-modified progenitors were used as controls. **(G)** Hierarchical clustering of the 500 most differentially expressed (p-value < 0.05) transcripts across lymphoid progenitors expressing the h1928z3 CAR which had been either stimulated with hCD19 or not; h19delta CAR served as signaling-deficient control. **(H)** Heat map showing the relative expression of exemplary transcripts that are either related to T cell or NK cell development. Data are normalized according to expression in each row. **(G, H)** Experiments was performed once. (h1928z3, h1928z3 + hCD19, n= 3; h19delta, n= 4). **(I)** qPCR analysis of *BCL11B* expression in non-transduced or h1928z3-expressing progenitors stimulated with hCD19. Data shows mean of triplicates and upper and lower limit from one experiment performed.