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Automated manufacture of ΔNPM1 TCR-engineered T cells for AML therapy

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Acute myeloid leukemia (AML) is a heterogeneous malignancy that requires further therapeutic improvement, especially for the elderly and for subgroups with poor prognosis. A recently discovered T cell receptor (TCR) targeting mutant nucleophosmin 1 (ΔNPM1) presents an attractive option for the development of a cancer antigen-targeted cellular therapy. Manufacturing of TCR-modified T cells, however, is still limited by a complex, time-consuming, and laborious procedure. Therefore, this study specifically addressed the requirements for a scaled manufacture of ΔNPM1-specific T cells in an automated, closed, and good manufacturing practice-compliant process. Starting from cryopreserved leukapheresis, 2E8 CD8-positive T cells were enriched, activated, lentivirally transduced, expanded, and finally formulated. By adjusting and optimizing culture conditions, we additionally reduced the manufacturing time from 12 to 8 days while still achieving a clinically relevant yield of up to 5.5E9 ΔNPM1 TCR-engineered T cells. The cellular product mainly consisted of highly viable CD8-positive T cells with an early memory phenotype. ΔNPM1 TCR CD8 T cells manufactured with the optimized process showed specific killing of AML *in vitro* and *in vivo*. The process has been implemented in an upcoming phase 1/2 clinical trial for the treatment of NPM1-mutated AML.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by clonal expansion of myeloid precursor cells resulting in a highly heterogeneous malignant disease with poor prognosis. Standard-of-care treatment consists of chemotherapy to induce morphologic remission, followed by a consolidating treatment, often comprising allogeneic stem cell transplantation.[1,2] However, relapses occur with high frequency and are associated with a very poor outcome (survival rates rapidly dropping with increasing age) and require the development of novel strategies.[1,3,4]

The vast majority of AML cases carry defined genetic mutations, the most common one affecting nucleophosmin 1 (NPM1) encoding a multifunctional chaperone protein.[5] 30% of patients with AML exhibit a 4 base pair insertion causing a frameshift mutation in the C terminus to generate mutated NPM1 (ΔNPM1).[6,7] The resulting C-terminal CLAVEEVSLRK sequence eliminates the nucleolar localization signal, which results in the translocation of the nuclear ΔNPM1 protein to the cytoplasm.

Especially for the treatment of hematological malignancies, adoptive cell transfer therapies have made tremendous progress in the past decade. Besides chimeric antigen receptor (CAR) T cells, therapeutic approaches based on T cell receptors (TCRs) are on the rise. TCR-engineered T cells represent a promising and efficacious therapeutic option due to their ability to bind intracellular target molecules in the context of the major histocompatibility complex with a high affinity. van der Lee *et al.* have previously isolated a potent ΔNPM1-specific TCR targeting the HLA-A*02:01-binding CLAVEEVSL neoantigen.[8]

While the development of T cell therapies is progressing rapidly, the manufacture of a functional and robust cell product in required quantities still remains challenging.[9] Conventional techniques include cumbersome and cost-intensive multistep processes depending on several devices and operators, sometimes using open handling steps, and, consequentially, resulting in higher risk of errors.[9,10] Therefore, there is a need to further evolve reliable and automated manufacturing processes producing high-quality cellular products for immunotherapy.

In the past, various processes for the generation of engineered T cells have been established.[10–18] In this work, we adapted our successful manufacturing process for CAR T cells to the specific requirements

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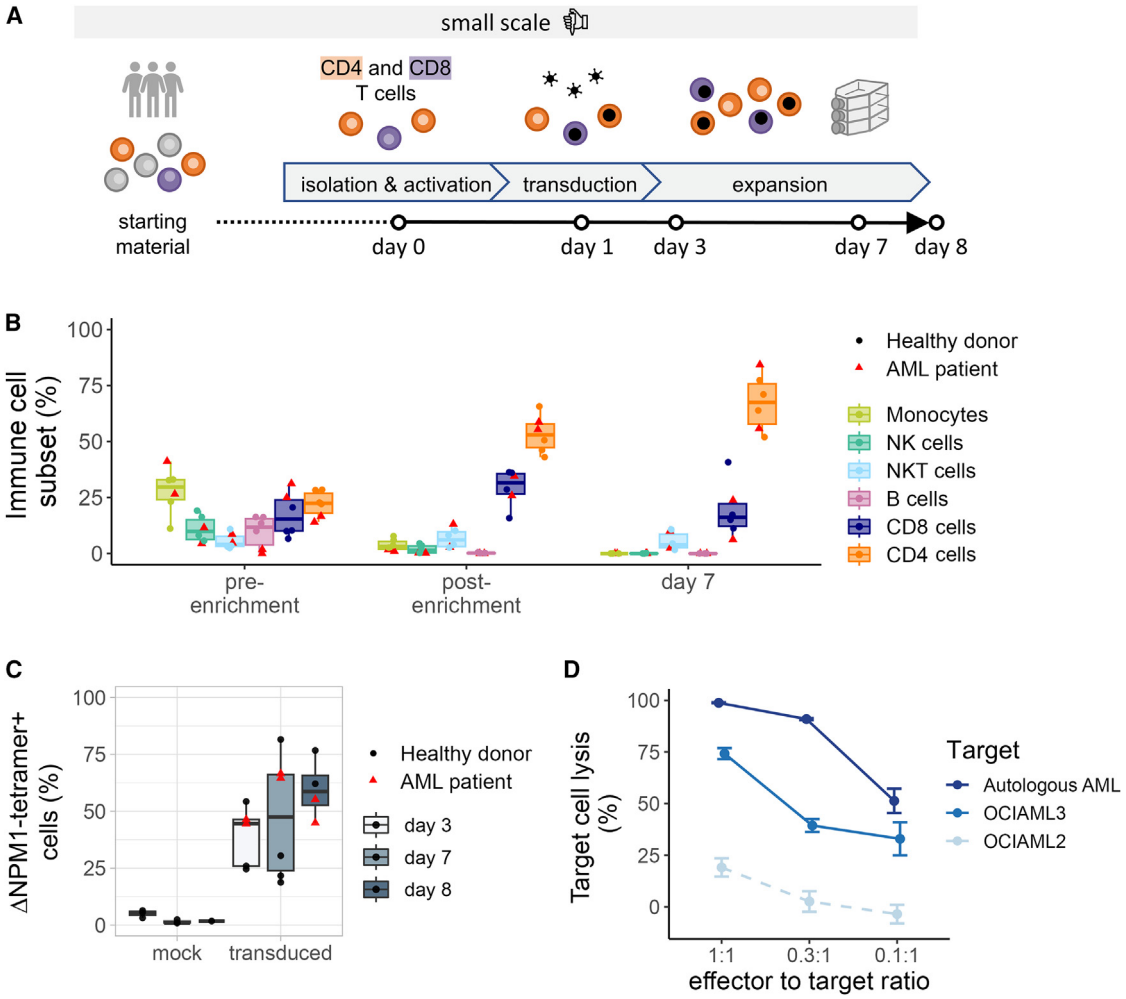


Figure 1. Generation of ΔNPM1-engineered T cells from AML patient material

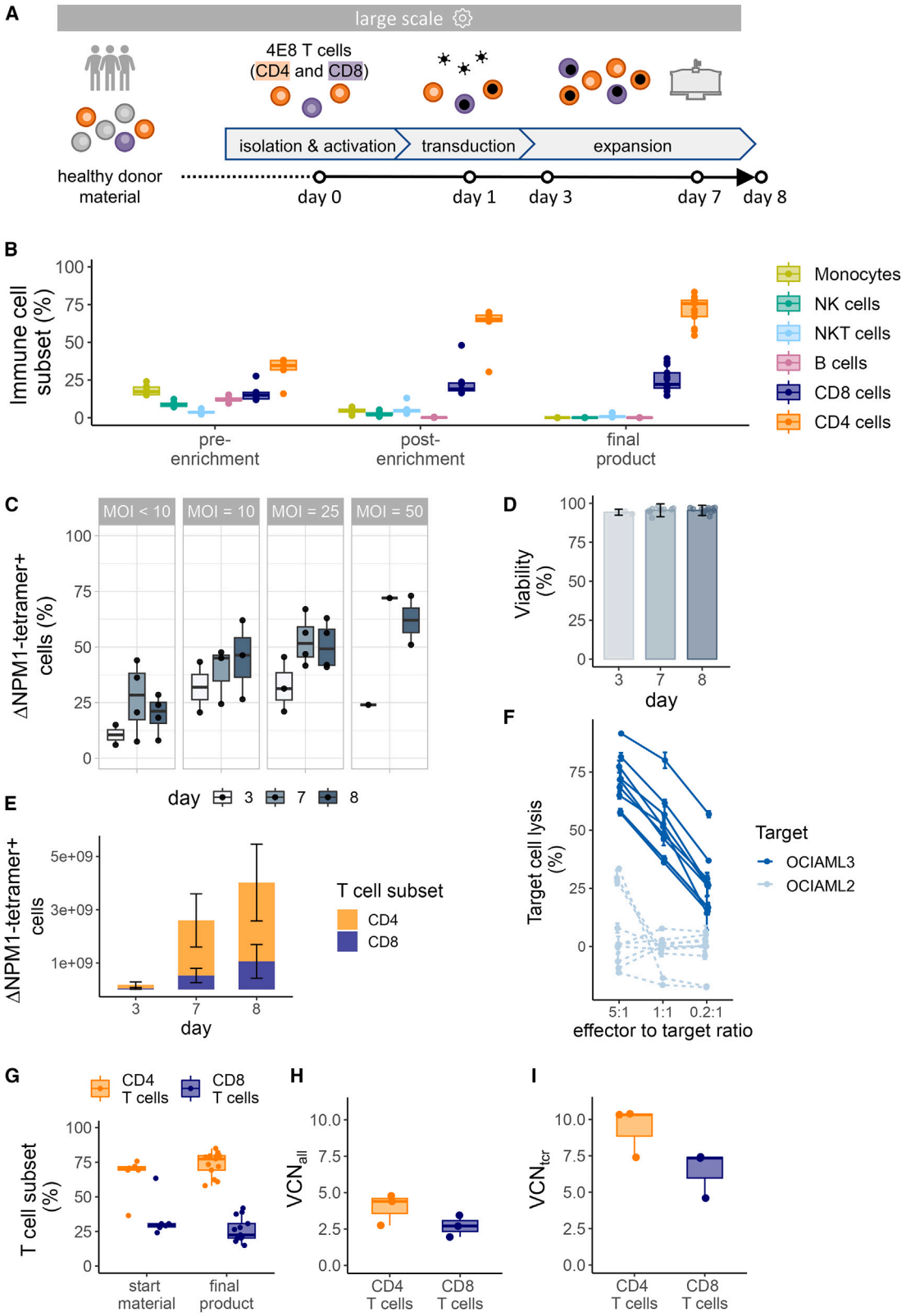
(A) Schematic illustration of small scale production of ΔNPM1-engineered T cells from healthy donors (n = 4) and patient material (n = 2). CD4 and CD8 T cells were isolated and immediately activated at day 0, transduced on day 1, and cultivated until harvest at day 8. (B) Immune cell composition was analyzed using flow cytometry pre- and post-enrichment as well as after expansion. (C) Transduction efficiency was determined by flow cytometry analysis of ΔNPM1-tetramer+ cells on days 3, 7, and 8. (D) Cytotoxicity of the ΔNPM1-engineered T cells was assessed by co-culture with ΔNPM1-positive (OCI-AML3), WTNPM1 (OCI-AML2), or autologous AML cells. Target cell lysis was determined after 24 h (mean ± SEM). (B and C) In all boxplots, median is shown by central line, boxes represent the interquartile range (IQR), and whiskers show distribution up to 1.5 times the IQR from the box.

of TCR-engineered T cells. Starting from frozen leukapheresis (LP), T cells were enriched, activated, transduced, expanded, and formulated in a functionally closed and fully automated system. Here, we produced a good manufacturing practice (GMP)-grade lentiviral vector (LV) encoding a fully humanized version of the ΔNPM1-specific TCR and developed a TCR-specific T cell transduction-large scale (TCT-LS) process allowing the generation of high numbers of TCR T cells for clinical application using the CliniMACS Prodigy platform. Following a specified activity matrix, we were able to shorten the process duration from 12 to 8 days. In-process controls (IPCs) were taken frequently to monitor cellular composition, viability, transduction efficiency, and cellular expansion. Functionality, efficacy, stability, and safety of the final product were assessed *in vitro*, and anti-tumor potential was validated *in vivo*. In 23 independent runs, we continuously optimized conditions and assured robustness and reproducibility of our process, enabling a phase 1/2 clinical trial for the treatment of NPM1-mutated AML.

RESULTS

AML patient-derived ΔNPM1 TCR-engineered T cells are effective *in vitro*

Using peripheral blood mononuclear cells derived from healthy donors and patients with AML, we assessed the purity of the population after magnetic enrichment of CD4 and CD8 T cells and on day 7 after culture (Figure 1A). Analysis for different immune cell subsets showed that T cells, as expected, were the main fraction after



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enrichment, (53.2% ± 8.4% CD4 and 29.5% ± 8.0% CD8) as well as in the final product (67.4% ± 12.5% CD4 and 19.1% ± 12.1% CD8) (Figure 1B). T cells were activated immediately after isolation and lentivirally transduced with the ΔNPM1-TCR on day 1. Transduced T cells revealed transgenic TCR expression ranging from 18.8% to 81.6% (Figure 1C). To assess the cytolytic potential *in vitro*, we co-cultured TCR-engineered T cells on day 8 with ΔNPM1-positive (OCI-AML3) or wild-type (WT)NPM1-expressing (OCI-AML2) cells at different effector-to-target ratios (Figure 1D). As expected, ΔNPM1 TCR-redirected T cells specifically lysed their target cells and spared WTNPM1 cells. Finally, patient-derived TCR T cells were tested against autologous bone marrow cells, which were collected at the time of AML diagnosis and contained HLA-A*02:01-positive NPM1-mutated AML blasts. The patient AML cells were effectively lysed by the autologous ΔNPM1 TCR T cells (Figure 1D). Taken together, these data demonstrate that functional ΔNPM1-specific T cells can be generated from AML patient material and underline the high clinical potential of ΔNPM1 TCR-engineered T cells for the treatment of AML.

Automated manufacturing reproducibly yields clinically relevant doses of ΔNPM1 TCR-engineered T cells

Aiming to generate therapeutic doses, we proceeded to scale up the manufacture of the engineered T cells using the CliniMACS Prodigy platform. All manufacturing runs reported in this study were performed in a single-use disposable tubing set (CliniMACS Prodigy Tubing Set 620; TS 620) installed on the CliniMACS Prodigy. Briefly, maintaining a closed system, cryopreserved material was thawed and sterile connected to a TS 620 containing a large cultivation chamber. In this closed system, T cells are automatically isolated, activated, transduced, expanded, and formulated (Figure S1). Similar to the small scale experiments, T cells were lentivirally transduced on day 1 at defined MOIs and cultivated for another 7 days after transduction. IPCs were taken at days 3 and 7. At day 8, the cellular product was formulated and harvested (Figure 2A).

The robustness and reproducibility of the process were assessed in 13 individual runs using cryopreserved LP from healthy donors. Runs were performed on different devices controlled by varying operators. Consistent with our small scale data, combined selection of CD4 and CD8 T cells resulted in a T cell population dominated by CD4 T cells post-enrichment (61.4% ± 13.9% CD4 vs. 23.6% ± 11.1% CD8) as well as on day 8 at the end of the production process (71.5% ± 9.3% CD4 and 25.3% ± 7.9% CD8, Figure 2B). As expected, transduction efficiency was dependent on the applied LV dose (Figure 2C). As previously observed in small scale experiments, expression of the ΔNPM1 TCR fluctuated during production (6.0%–73.0%). We also observed that the expression peaked on day 7 with, on average, 41.5% ± 19.5% for the different MOIs that were used (MOI = 2.1–50.0). Importantly, intraday comparison showed that percentages of ΔNPM1 tetramer-positive T cells substantially increased with an MOI up to 10 but did not rise noticeably at greater values.

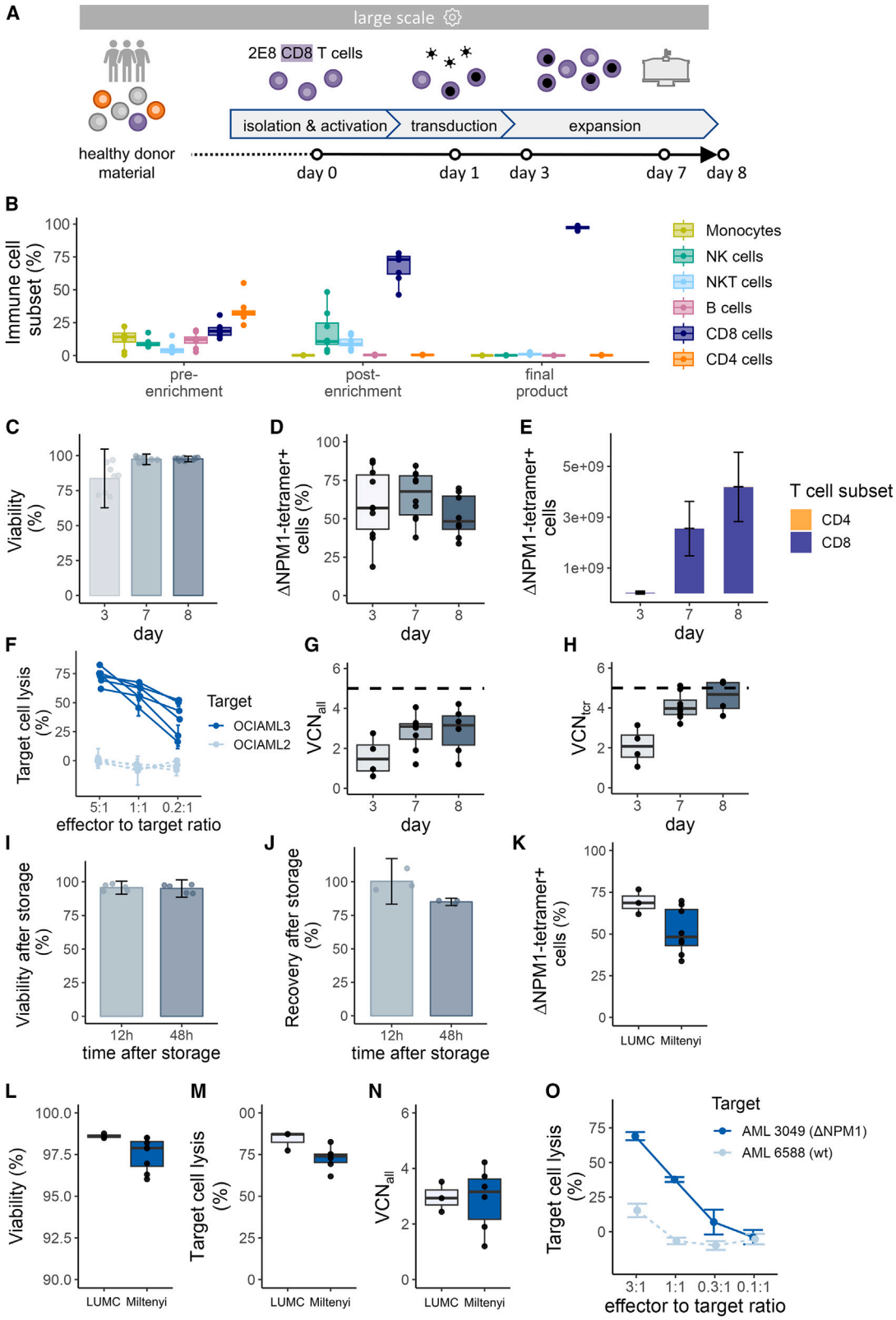
Manufactured T cells were highly viable throughout the manufacturing process (95.3% ± 1.7%; Figure 2D), yielding up to 4.5E9 ± 1.9E9 ΔNPM1 TCR-engineered T cells by day 8 (Figure 2E), and cytotoxicity assays (normalized to CD8-positive, ΔNPM1 TCR-engineered T cells) confirmed that the cellular product specifically lysed ΔNPM1-positive target cells (Figure 2F). However, small scale experiments in which CD4 and CD8 T cells were separately isolated and analyzed indicated that CD8 T cells transduced with the ΔNPM1 TCR LV are significantly more potent than TCR-transduced CD4 T cells, as demonstrated by stronger cytotoxic potential and secretion of more cytokines upon co-incubation with target cells (Figures S2A and S2B). Consequently, the CD4/CD8 manufacturing process (Figure 2A) would result in a cellular product with only a minor fraction of the highly potent ΔNPM1 TCR-engineered CD8 T cells (Figure 2G). Moreover, we also observed that CD4 T cells consistently had a higher number of lentiviral genome integrations (vector copy number [VCN_{all}]: 4.0 ± 1.1 CD4 vs. 2.7 ± 0.7 CD8; VCN per transduced cell [VCN_{tcr}]: 9.4 ± 1.7 CD4 vs. 6.5 ± 1.6 CD8) (Figures 2H and 2I; MOI = 10, n = 3). In conclusion, although total expansion, transduction efficiency, viability, and functionality of the manufactured cells were sufficient for clinical standards, the manufacturing process using combined CD4 and CD8 cells resulted in a cellular product that showed low frequencies of highly cytolytic CD8 T cells with low integration numbers and functionally inferior cytolytic CD4 T cells with high integration numbers. The low fraction of potent ΔNPM1 TCR CD8 T cells and the higher number of viral vector integrations in ΔNPM1 TCR CD4 T cells prompted us to develop a new process to manufacture only ΔNPM1 TCR-engineered CD8 T cells.

Automated manufacture of ΔNPM1 TCR-engineered CD8 T cells robustly yields high doses of potent effector cells

The CD8 TCT-LS process was evaluated in 10 additional runs starting from cryopreserved LPs from healthy donors. In contrast to the previous process, only CD8 T cells were selected, activated, transduced and expanded (Figure 3A). Flow cytometry analysis demonstrated that isolation resulted in predominantly CD8 T cells (67.9% ± 10.9%) with minor fractions of natural killer (NK) and NKT cells that were

Figure 2. Automated manufacturing of ΔNPM1-engineered CD4 and CD8 T cells

Large scale manufacturing of ΔNPM1-engineered T cells from CD4 and CD8 cells of healthy donors (n = 7; 13 runs). (A) Schematic workflow of the manufacturing process. (B) Immune cell composition was analyzed by flow cytometry analysis of the starting material pre- and post-enrichment as well as the final product. (C) Transduction efficiency was measured by flow cytometry of ΔNPM1 TCR+ cells on days 3, 7, and 8. (D) Viability after 7-AAD staining was analyzed by flow cytometry on days 3, 7, and 8. (E) Total yield and distribution of engineered T cells among CD4 and CD8 T cells. (F) Target cell lysis of ΔNPM1- (OCI-AML3) or WTNPM1 (OCI-AML2)-expressing cells after co-culture with effector cells at indicated effector-to-target ratios was examined by flow cytometry after 24 h (mean ± SEM). (G) Cellular composition studies of CD4/CD8 TCT-LS runs by flow cytometry revealed CD4 and CD8 cell content in the starting material and in the final product. (H) Vector copy number (VCN_{all}) and (I) vector copy number per transduced cell (VCN_{tcr}) of ΔNPM1-transduced cells was determined by qPCR analysis in CD4 and CD8 T cells, which were obtained by depleting CD8 and CD4 cells, respectively. (B, C, and G–I) In all boxplots, median is shown by central line, boxes represent the IQR, and whiskers show distribution up to 1.5 times the IQR from the box.



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co-enriched due to CD8dim expression (17.6% ± 15.7% and 9.7% ± 4.7%, respectively). Due to culture conditions supporting T cell growth, however, a final purity of 97.3% ± 1.3% CD8 T cells was reached, with a favorable early memory phenotype (Figures 3B and S3). Although there was some variation in viability at day 3 (83.6% ± 10.5%), viability of T cells was completely restored on day 8 (97.5% ± 1.0%; Figure 3C).

Consistent with previous results, we observed a dynamic TCR expression peaking at day 7 and reaching up to 65.0% ± 15.6% (Figure 3D). Surprisingly, despite the absence of CD4 T cell help, the expansion of CD8 T cells in the CD8 TCT-LS process was not hampered compared to the combined CD4/CD8 process. Importantly, when comparing the numbers of functional ΔNPM1 TCR-engineered CD8 T cells, the CD8 process yielded more than 3 times more cells within the same manufacturing time as the CD4/CD8 process (4.2E9 ± 1.4E9 vs. 1.1E9 ± 0.6E9; Figures 3E vs. 2E). Finally, co-culture experiments confirmed a specific cytolytic potential of the cellular product (Figure 3F).

As very high integration numbers may increase the risk of inducing oncogenic events, we analyzed the VCN_{all} and calculated the VCN_{tcr} as recommended in recent US Food and Drug Administration (FDA) guidelines for CAR T products (Table 1).[19] Since the percentage of transduced T cells was determined by tetramer staining of surface-bound engineered TCRs, and since the surface expression of the engineered ΔNPM1 TCR fluctuates over time, even though the number of integrated viral genomes in the total cell population (VCN_{all}) remained constant from day 7 to day 8, the calculated VCN_{tcr} increased. Restimulation of T cells after harvest, however, restored the surface expression of the TCR to similar levels to the peak expression on day 7 of the manufacturing process, demonstrating retention of the transgene (Figure S4). We therefore confirmed that the TCR expression, and hence the VCN_{tcr}, is strongly dependent on the cellular activation status. Consequently, the percentage of transduced cells used to determine the VCN_{tcr} should be analyzed at peak expression of the engineered TCR on day 7, as the number of genomic integrations (VCN_{all}) remains constant at later manufacturing timepoints. (Figures 3G and 3H; Table 1). In order to assess stability of the cellular product, we stored the freshly harvested and formulated cells at a defined effector cell concentration at 4°C. We measured viability and cell recovery from samples collected after 12 and 48 h of storage and observed that the product was stable up to 48 h (Figures 3I and 3J).

Finally, we transferred the process to a GMP manufacturing facility at an academic hospital (Leiden University Medical Center [LUMC]) and confirmed robustness and reproducibility of our 8 day CD8 TCT-LS process in three additional runs, as demonstrated in the comparable results regarding transduction efficiency, viability, potency, and integrated viral genomes (Figures 3K–3N). As expected, large scale-manufactured ΔNPM1 TCR-engineered T cells also lysed primary AML samples (Figure 3O). In summary, the CD8 TCT-LS manufacturing process robustly yielded sufficient viable and functional TCR-engineered T cells to be utilized in a planned phase 1 safety and dose-finding trial in relapsed/refractory AML applying up to 1E7 ΔNPM1 TCR+CD8+ T cells/kg.

Manufactured ΔNPM1 TCR-engineered T cells show efficacy *in vivo*

We used an AML xenograft model to assess and compare the cytolytic potential of ΔNPM1 TCR-engineered T cells manufactured in a CD8 TCT-LS process vs. the conventional CD4/CD8 process including controls depleted of either CD4 or CD8 T cells. Briefly, immunodeficient NOD SCID gamma (NSG) mice were injected intravenously (i.v.) with 1E6 OCI-AML3^{Luc}, randomized after 6 days based on tumor engraftment, and injected i.v. at day 7 with either mock (untransduced) cells or 5E6 ΔNPM1 TCR-engineered T cells derived from the different manufacturing processes (Figure 4A). Tumor burden was monitored twice every week via whole-body luminescence up to 17 days. We detected no anti-tumor effect in animals receiving mock cells, whereas animals receiving ΔNPM1 TCR-engineered T cells exhibited decreased tumor burden, with the CD8-depleted group having the lowest anti-tumor performance among the treatments (Figures 4B, 4C, and S5). Although all treatment groups comprising CD8 T cells showed comparable results, mice injected with ΔNPM1 TCR-engineered T cells obtained from our 8 day CD8-only TCT-LS process had the lowest tumor burden by the end of the study. In summary, our findings emphasize the significance of CD8 T cells in adoptive cellular therapies and demonstrate that the manufacturing of ΔNPM1 TCR-engineered T cells from CD8 cells is not only feasible but also advantageous over a combined CD4/CD8 process.

DISCUSSION

Although treatment options for AML have continually improved during the past decade, the overall mortality of this life-threatening

Figure 3. Automated manufacturing of ΔNPM1-engineered CD8 T cells

Large scale manufacturing of ΔNPM1-engineered T cells from CD8 cells of healthy donors (n = 10; 10 runs). (A) Graphical depiction of the manufacturing process of ΔNPM1-engineered CD8 T cells. (B) Flow cytometric analysis of the cellular composition pre- and post-enrichment and of the final product. (C) Viability after 7-AAD staining on days 3, 7, and 8. (D) Transduction efficiency was analyzed by flow cytometry analysis on days 3, 7, and 8. (E) The yield of ΔNPM1+ CD8 and CD4 cells was measured on days 3, 7, and 8. (F) Cytotoxicity was assessed by co-culturing ΔNPM1-engineered CD8 T cells with ΔNPM1- (OCI-AML3) or WTNPM1 (OCI-AML2)-expressing cells at the indicated effector-to-target cell ratios (mean ± SEM). (G) VCN_{all} and (H) VCN_{tcr} were analyzed by qPCR analysis on days 3, 7, and 8. (I) Viability of the formulated drug product after storage at 4°C for 12 and 48 h (n = 5). (J) Recovery of the formulated cells in percentage after 4°C storage refers to the cell count measured 12 and 48 h post-formulation (n = 3). (K–O) Robustness and reproducibility of manufacturing was confirmed in a side-by-side comparison (n = 10 Miltenyi vs. n = 3 LUMC) of (K) transduction efficiency, (L) viability, and (M) cytotoxicity as well as (N) integrated viral genomes of ΔNPM1 TCR-engineered CD8 T cells manufactured at two different sites by different teams on varying devices. (O) Large scale-manufactured ΔNPM1 TCR-engineered T cells were co-cultured with primary AML samples at indicated effector-to-target ratios, and AML cell lysis was determined by flow cytometry after 24 h (mean ± SEM). (B, D, G, H, and K–O) In all boxplots, median is shown by central line, boxes represent the IQR, and whiskers show distribution up to 1.5 times the IQR from the box.

Table 1. Vector copy number (VCN_{all}) and vector copy number per transduced cell (VCN_{tcr}) manufactured using the CD8 TCT-LS process (n = 10)

Day	VCN_{all} (mean ± SD)	VCN_{tcr} (mean ± SD)
3	1.6 ± 1.0	2.1 ± 0.9
7	2.8 ± 0.9	4.1 ± 0.7
8	2.9 ± 1.1	5.2 ± 1.1

disease is still significant, underlining the need for novel therapies.[20,21] In this context, van der Lee *et al.* identified a novel HLA-A*02:01-restricted TCR targeting a ΔNPM1-specific neoantigen that is present in up to 30% of AML.[8] In accordance with van der Lee *et al.*, we confirmed the cytolytic potential of ΔNPM1 TCR-modified T cells and assessed all process-relevant steps including T cell selection, activation, transduction, and expansion in a small scale setting. Subsequently, we focused on the development of a TCR-specific manufacturing process that is robust, reproducible, scalable, cost efficient, and fulfills all regulatory requirements.[9] To this end, a novel, GMP-compliant, automated, and functionally closed process on the CliniMACS Prodigy was developed and evaluated. In contrast to recent publications,[10–12,14,16] we additionally addressed whether it is possible to specifically isolate and expand CD8 T cells on the Prodigy device and whether therapeutic doses of ΔNPM1 TCR-engineered CD8 T cells can be manufactured within 8 days while meeting clinically relevant specifications.

In this study, we analyzed cellular composition, phenotype, transduction efficiency, viability, yield, and cytolytic potency of two different manufacturing protocols: CD4/CD8 and CD8 processes. We confirmed that both processes are robust and reproducibly yielded pure, highly viable, and potent cellular products, but we finally focused on the CD8 T cell-specific process for further development and clinical implementation. This decision was supported by three main findings. First, we observed CD8 co-receptor dependency of the ΔNPM1 TCR, as illustrated by much stronger cytolysis and cytokine release by CD8 TCR T cells in comparison to CD4 TCR T cells. Second, since CD4 T cells are more frequent than CD8 T cells in peripheral blood-derived starting material, the manufacturing process with combined CD4/CD8 T cells limits the total yield of TCR-modified CD8 T cells in the cellular product (CD4/CD8 1.1E9 ± 0.6E9 vs. CD8 4.2E9 ± 1.4E9). Third, we observed that the VCN_{tcr} of the final product is disproportionately affected by the CD4 T cells, as we detected increased numbers of integrated lentiviral genomes in CD4 T cells compared to CD8 T cells coming from the same manufacturing campaign, a phenomenon that is most likely associated with different activation and proliferation kinetics of the defined T cell subtypes during processing.[22] In the following, we elaborate the discussion over these three main points in more detail.

Previous reports on CAR T cells suggest that a manufacturing process containing CD8 and CD4 T cells may be preferred to provide combined help and cytolysis in one cellular product with optimal *in vivo* expansion and persistence.[23–27] Although this could still be the case for production of T cells engineered with a completely CD8-independent TCR, our data demonstrate that in the context of a CD8-dependent TCR, the CD4 T cells compromise the genotoxicity, composition, and yield of functional ΔNPM1 TCR-engineered T cells. Since others showed that a defined CD4/CD8 ratio in CAR T cell products influences not only phenotype and overall CD8 T cell expansion during manufacturing but also the effector function and persistency *in vivo*,[23,25–28] we compared the *in vivo* anti-tumor reactivity of CD8-derived ΔNPM1 TCR-engineered T cells with conventionally produced TCR T cells starting from CD4/CD8 T cells. We observed no difference in anti-tumor reactivity of ΔNPM1 TCR-engineered T cells generated by the CD8 process as compared to the CD4/CD8 counterpart even though the CD4/CD8 mice were injected with higher numbers of T cells as consequence of the normalization based on the frequency of engineered CD8 T cells. Taking into consideration that TCR-engineered T cells are often dependent on the CD8 co-receptor, it is difficult to draw any definite conclusion regarding an optimal CD4/CD8 ratio for specific TCR-engineered T cell products. Although beyond the scope of this study, an extended systematic approach would be required to evaluate whether a defined fraction of CD4 T cells would improve the proliferative capacity of ΔNPM1 TCR-modified CD8 T cells during manufacture and whether such a cellular product would have any advantage regarding persistency and potency *in vivo*.

Unlike previous reported T cell manufacturing processes using the single-use, disposable tubing set TS 520,[10,15,16] this novel process used the TS 620 with an approximately 2-fold larger cultivation chamber. With this large chamber, we not only optimized culture conditions towards high viability, T cell purity, yield of potent TCR-engineered T cells, and optimal transduction efficiencies without exceeding the recommended VCN values but also reduced the manufacturing time from 12 to 8 days, allowing a potential reduction in the vein-to-vein time of a freshly manufactured drug product. Even in this short manufacturing time, suitable numbers of genetically modified T cells could be generated (Figure 3E) to allow similar dosing regimens of TCR-engineered T cells to be applied, as recently described for other TCR T cell therapies (summarized in Table 2), while for higher dosing requirements, an extended 12 day process can be implemented (data not shown).

According to a recommendation of the FDA and the European Medicines Agency, due to an omnipresent risk of oncogenesis, integration studies for lentiviral particles that integrate into the genome of host cells are required and should not exceed 5 integrations per genome for clinical applications.[29,30] In this study, we represented the number of integrations either by VCN_{all}, which denotes the number of viral vector integrations per genome as determined by qPCR in the total cell population independent of transgenic TCR expression, or by VCN_{tcr}, which is calculated using the VCN_{all} corrected by the frequency of ΔNPM1 TCR-expressing T cells. Since we observed that TCR surface expression is highly dependent on the activation state of the T cell (Figure S4), and the expression level is thus strongly modulated during the manufacturing process (Figures 1C, 2C, and 3D), the calculated VCN_{tcr} based on day 8 detection of ΔNPM1 TCR expression exceeded the

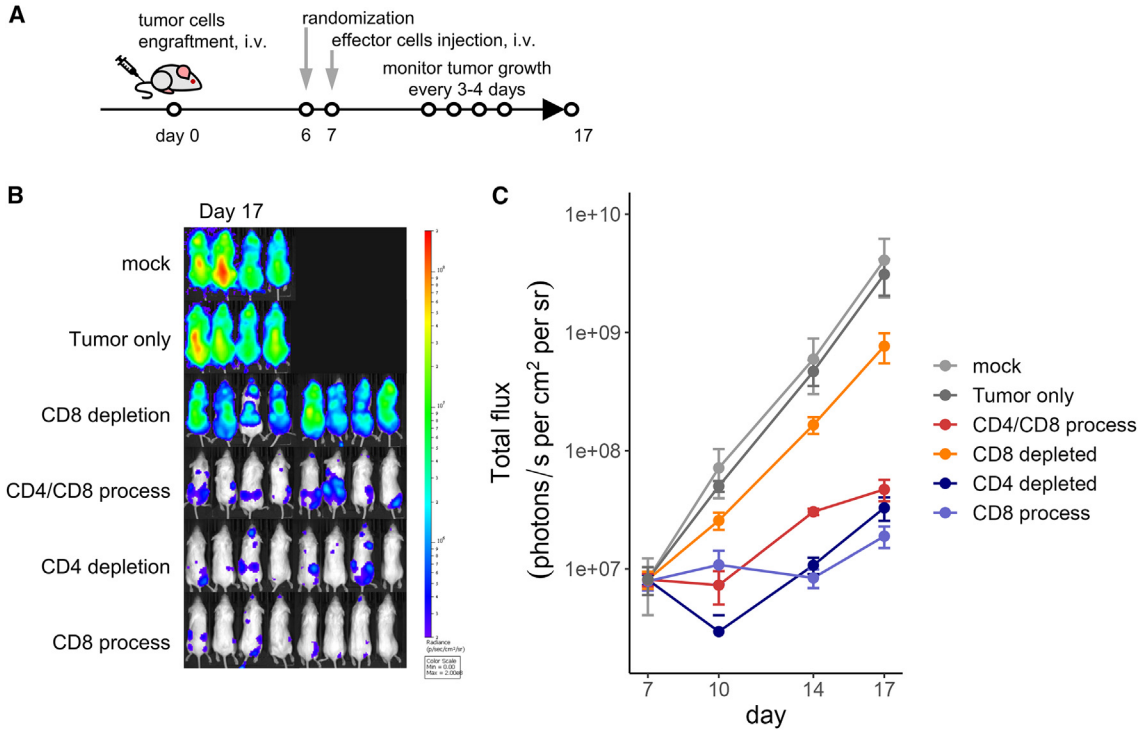

Figure 4. *In vivo* efficacy of ΔNPM1-engineered T cells

(A) Graphical scheme of the *in vivo* experiment workflow. (B and C) NSG mice were injected i.v. with 1E6 OCI-AML3^{Luc}, randomized after 6 days based on tumor engraftment, and injected i.v. at day 7 with either mock (untransduced) T cells or 5E6 ΔNPM1 TCR-engineered cells derived from the different manufacturing processes. Tumor burden (mean ± SEM) was measured over time using an *in vivo* tumor imaging system (IVIS).

value of 5 on certain occasions (Figure 3H). This, however, would be an overestimation of the VCN_{tcr} since the VCN_{all} remains largely constant after day 4 (Figure 3G) and after T cell reactivation (data not shown). More precisely, VCN_{tcr} calculations based on the peak expression of the ΔNPM1 TCR measured mainly between days 5 and 7 resulted in values below 5 and represent a more accurate estimation of the number of integrated viral genomes per modified cell. We therefore propose to calculate the VCN_{tcr} at the time point when peak TCR expression is detected.

In conclusion, here we report on the first TCT-LS process for the manufacture of ΔNPM1 TCR-engineered T cells. We show that across independent donors, devices, manufacturing sites, and operators, this process consistently yielded therapeutic doses of potent TCR-engineered CD8 T cells while maintaining the product's high viability, purity, and favorable early memory phenotype, which is known to support *in vivo* persistence and efficacy.[31–33] This novel state-of-the-art manufacturing process will now be used to manufacture TCR-modified T cells for assessment in a phase 1/2 clinical trial to treat patients with relapsed or refractory NPM1-mutated AML.

MATERIAL AND METHODS

Unless otherwise specified, devices and materials were obtained from Miltenyi Biotec.

Starting material

LP of healthy donors were obtained from UK Köln, MH Hannover, UKW Ulm, or Biomex GmbH (Heidelberg) or the Hemapheresis Center of LUMC (Leiden) and cryopreserved within 24 h post-blood donation. Patient samples were used from the LUMC Biobank from Hematological Diseases. Peripheral blood or bone marrow mononuclear cells were isolated from patients with AML by Ficoll-Isopaque separation and cryopreserved. Use of patient material for this study was approved by the Institutional Review Board of the LUMC (IRB LUMC approval no. B16.039). Materials from patients and healthy individuals were collected after written informed consent according to the Declaration of Helsinki.

Cryopreservation

Cellular composition, cell count, and viability of LP were determined by flow cytometry. LP was diluted with CliniMACS Formulation Solution, and plasma was removed using an extractor after centrifugation of 200 × *g*, 15 min, room temperature (RT; deceleration: 0). Cells were formulated in CliniMACS Formulation Solution and CliniMACS Cryo Supplement to a final DMSO concentration of 7.5% and a white blood cell density of 2E7/mL. 100 mL cell suspension in CryoMACS Freezing bag 500 was cryopreserved in CryoBioSpares cassettes using a Controlled Rate Freezer (Biofreeze BV45, Consarctic) and stored in liquid nitrogen. Prior to

Table 2. Dose levels in different TCR assets from literature

Asset	Dose/kg BW	Total dose (cells)	Citation
HA-1 TCR	3E6–1E7	3E8–1E9	Krakow et al.[34]
NY-ESO-SPEAR T	5E7	5E9	D'Angelo et al.[35]
WT-1 TCR	~2E8 (1E10/m^2)	2E10	Chapuis et al.[36]
NY-ESO-1/LAGE-1	0.5E7–5.1E7	0.5E9–5.1E9	Stadtmauer et al.[37]

BW, body weight.

cryopreservation, a sample was taken, and cell count and viability were confirmed again by flow analysis. For thawing frozen LP, the material was transferred from liquid nitrogen to a 37°C water bath and connected to the CliniMACS Prodigy directly post-thawing. Cellular composition, cell number, and viability were determined by flow cytometry.

Automated manufacture of TCR-engineered T cells

The CliniMACS Prodigy TCT-LS process was developed on the CliniMACS Prodigy using a single-use CliniMACS Prodigy Tubing Set (TS 620) with a large cultivation chamber. CliniMACS PBS/EDTA buffer supplemented with 0.5% HSA (OctaPharma) was used as process buffer, and TexMACS GMP medium supplemented with MACS GMP recombinant human interleukin-7 (IL-7; 12.5 ng/mL) and MACS GMP recombinant human IL-15 (12.5 ng/mL) and with or without 3% human AB serum (Capricorn or Access Biologicals) was used for cultivation. Process parameters were defined by an activity matrix prior to process start. Starting from frozen LP, cells were separated using CliniMACS CD8 and/or CD4 GMP MicroBeads by the system with a labeling incubation for 30 min at 4°C–8°C. All runs were started typically with 1E8–4E8 enriched T cells, which were cultivated at 37°C and 5% CO_2. After enrichment, cells were activated using MACS GMP TCT-LS according to the manufacturer's instructions. 24 h post-stimulation, cells were transduced with GMP-grade LV encoding a fully humanized version of the ΔNPM1 TCR (2.5E9 TU/mL). Culture washing steps, media exchange, and feeding were performed automatically by the system according to the pre-defined activity matrix. IPCs were taken frequently in order to monitor cellular composition, expansion, viability, and transduction efficiency by flow cytometry. Finally, cells were automatically rebuffered and harvested in CliniMACS Formulation Solution.

Flow cytometry analysis and antibodies

Any red blood cells in the starting material were removed with red blood cell lysis solution. Cellular composition was analyzed using fluorescent-labeled antibodies against CD45, CD4, CD3, CD56, CD16, CD19, CD14, and CD8. Phenotype analysis was performed by staining with A*0201/ΔNPM1 TCR-Detection Reagent prior to labeling with fluorescent antibodies against CD45RA, CD4, CD3, CD62L, CD45RO, and CD8. Transduction efficiency was determined by staining with A*0201/ΔNPM1 TCR-Detection Reagent prior to labeling with fluorescent antibodies against CD45, CD4, CD3, and CD8. 7-AAD was added to each panel for dead cell exclusion. All substances were incubated for 10 min at RT in the dark. For analysis of phenotype and transduction efficiency, cells were washed once after staining with PEB Buffer (CliniMACS PBS/EDTA buffer supplemented with 0.5% BSA, centrifugation 5 min, 300 × *g*, RT). Cells were resuspended in PEB Buffer, acquired on a MACS Quant Analyzer 10 using Express Modes for automated gating, and analyzed with the MACS Quantify 2.13 software.

Stability and cell fitness

To assess stability of the drug product during storage between manufacture and potential application, cells were formulated in CliniMACS Formulation Solution after harvest (5E6 TCR-engineered T cells/mL, 70 mL) and stored at 2°C–8°C in a CryoMACS Freezing Bag 250. Samples were taken frequently from this bag after thorough mixing, diluted 1:10 in TexMACS medium, and analyzed after an additional storage for 2 h at 2°C–8°C regarding cell count and viability on a MACS Quant Analyzer 10 and analyzed with MACS Quantify 2.13 software.

ΔNPM1 TCR LV and VCN analysis

Vesicular stomatitis virus G protein-pseudotyped lentiviral particles encoding the ΔNPM1 TCR were produced in house under GMP-compliant conditions, and transducing units were defined by transduction of HEK293T cells and PCR. For automated transduction, the required LV amount was formulated in 10 mL culture medium, transferred to a 150 mL transfer bag, and connected to the tubing set by sterile welding. Genomic DNA was isolated from cells using the DNeasy Blood & Tissue Kit from Qiagen according to the product guidelines. VCN_{all} and VCN_{tcr} analysis was subsequently performed with the MACS COPYcheck Kit and analyzed on a CFX96 Touch Real-Time PCR Detection system (BioRad).

The following formula was used to calculate VCN_{all}:

$$VCN_{all} = \frac{\text{qPCR copy number}/\mu\text{L (gag)}}{\text{qPCR copy number}/\mu\text{L (PTBP2)}} \times 2.$$

The following formula was used to calculate VCN_{tcr}:

$$VCN_{tcr} = \frac{\text{qPCR copy number}/\mu\text{L (gag)}}{\text{qPCR copy number}/\mu\text{L (PTBP2)}} \Big/ \frac{\textit{transduction frequency}\ (\%)}{100\%} \times 2.$$

Reactivation

In order to identify the ability of TCR-engineered T cells to modulate transgene expression upon restimulation, freshly harvested cells were reactivated in a small scale experiment. Therefore, cells were seeded in a 24-well plate with 2E6 cells/well in duplicate. TransAct was added to a final dilution of 1:100 to half of the samples. Cells were incubated for 48 h at 37°C, 5% CO_2, and transduction efficiency was analyzed by flow cytometry.

Cytotoxicity

Cytolytic potential of ΔNPM1 TCR-engineered T cells was assessed by co-culturing with HLA-A*02:01-positive OCI-AML cell (luc+/GFP+) target cells (DSMZ). ΔNPM1-positive OCI-AML3 cells were used to confirm functionality, whereas WTNPM1-expressing OCI-AML2 cells were used to confirm specificity. Mock (untransduced) T cells served as control. 1E4 target cells were co-cultured with ΔNPM1 TCR T cells for 24 h at 37°C and 5% CO_2 at the indicated effector-to-target ratios in 96-well round-bottom plates (Falcon). Specific lysis by ΔNPM1 TCR-engineered T cells was detected by quantitation of viable cells via flow cytometry after adding propidium iodide (1:100) to 70 μL cell suspension and calculated relative to target cells in the absence of T cells.

Cytokine release

70 μL supernatant was taken from co-culture assays and was analyzed with the MACSPlex Cytokine Kit, human, following the manufacturer's instructions. Secretion of cytokines was measured on the MACS Quant Analyzer 10 and analyzed with the MACS Quantify 2.13 software.

In vivo experiments

All animal experiments were performed following institutional guidelines and regulations. *In vivo* functionality of ΔNPM1 TCR-engineered T cells was verified in immune-deficient NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ). Tumor was engrafted by tail vein injection of 1E6 OCI-AML3 cells 7 days prior to treatment in groups of five (mock and tumor only) or eight mice (treated), respectively. 5E6 ΔNPM1 TCR-engineered T cells or mock (untransduced) T cells were administered i.v., and tumor kinetics were monitored frequently using an In Vivo Imaging System (PerkinElmer). Therefore, 100 μL (30 mg/mL) XenoLight D-Luciferin Potassium Salt Bioluminescent Substrate (PerkinElmer) per animal was injected intraperitoneally.

DATA AND CODE AVAILABILITY

Data are available upon reasonable request. All data directly relevant to the study are included in the article. Further data are available upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101224.

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AUTHOR CONTRIBUTIONS

R.d.G., I.C.D.J., and D.L. conceptualized and supervised the study; I.E.Y.O., V.H., S.L., C.B., E.A., C.F., C.H., S.V., L.H., K.T., and D.L. generated and analyzed data; C.Ha. and N.M.-T. specifically supported the process development; G.K., M.G., and T.S. supervised defined aspects of the study; and I.E.Y.O., V.H., R.d.G., I.C.D.J., and D.L. wrote the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

I.E.Y.O., V.H., S.L., C.B., C.F., C.Ha., K.T., N.M.-T., T.S., I.C.D.J., and D.L. are or were employees of Miltenyi Biotec during the time of this study.

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