

SUPPLEMENTARY METHODS

Flow cytometry

CTL immunophenotypes were determined using fluorescence-conjugated monoclonal antibodies (mAbs) against human Fc γ (see above), CD3, CD8, CD4, CD45RO, CD45RA, CD197 (CCR7), CD279 (PD-1), CD152 (CTLA-4), CD57, CD127 (all from BD Pharmingen, Heidelberg, Germany), TIM-3 and LAG-3 (eBioscience, Hatfield, UK). A PerCP-labeled antibody was used to detect CD19 on target cells, and antibodies against CD16, (FcRIII), CD32 (FcRII) and CD64 (FcRI) were used to stain Fc receptors (all from BD Pharmingen). For each sample, 20,000 cells were analyzed with FACS Canto and FACS Diva Software.

Cell lines

REH and SupB15 are CD19+ human leukemia cell lines, and ML-2 is a CD19-negative leukemia cell line. THP-1 is a monocytic cell line. All cell lines were from ATCC (Manassas, VA, USA).

ELISPOT analysis

96-well filter plates (MultiScreen, MAHAS4510, Billerica, MA, USA) were coated overnight with 10 μ g/ml anti-IFN- γ Ab (catcher mAB91-DIK; Mabtech, Nacka Strand, Sweden). Effector cells were then plated and stimulated with the respective target cells. As a positive control, cells were nonspecifically stimulated with phorbol myristate acetate (PMA) 25 ng/ml (Sigma-Aldrich, Munich, Germany) and ionomycin 1 μ g/ml (Sigma-Aldrich). After 18–24 h, the plates were washed and incubated with secondary biotin-conjugated anti-IFN- γ mAb (detector mAb 7-B6-1 biotin, Mabtech). After incubation with avidin:biotinylated HRP complex (Vectastain Elite ABC Kit (standard), PK6100; Vector Laboratories, Burlingame, CA, USA), plates were developed with AEC substrate (Sigma-Aldrich). Spot-forming units (SFUs) were quantified using a CTL-Immunospot[®] Analyzer and the Immunospot[®] Software.

Intracellular cytokine assay

CTL were seeded at 5×10^5 cells per tube and stimulated with 5×10^5 target cells for 6 hours. To block cytokine secretion, 10 μ g Brefeldin A (Sigma-Aldrich) was added after 2 hours. The cells were then stained with a CD3-specific antibody, followed by permeabilization and intracellular staining for IFN- γ and TNF- α (BD Pharmingen) according to the manufacturer's recommendations. In some experiments, medium containing 25% autologous serum was used to block Fc-receptor binding.

Cytotoxicity assay

CD19CAR CTL and non-transduced EBV-CTL were labeled with Violet Proliferation Dye 450 according to the manufacturer's (BD) recommendations and then cocultured with target cells for 24 hours. After staining with 15 ng 7AAD (BD) for 10 minutes, viable tumor cells were quantified by flow cytometry via gating on VPD450 and 7AAD-negative cells. % cytotoxicity was calculated by $100 - (\% \text{viable tumor cells in experimental well} / \% \text{viable tumor cells in medium}) \times 100$.

CD107a degranulation assay

CTL (1×10^5 /well) were incubated with the indicated target cells in the presence of Monensin (eBioscience) (1 μ l/ml) and CD107a-PE antibody (Biolegend) (100 ng/ml) for 3 hours at 37°C and 5% CO₂. The cells were washed, then incubated with PerCP-labeled CD3-specific antibody for 15 minutes, and fixed in 1% PFA. Autologous EBV-LCL were used as a positive control. Autologous NK cells were isolated with the NK cell isolation kit (Miltenyi, Bergisch Gladbach, Germany), and autologous monocytes were isolated with the RosetteSep (Stemcell Technologies, Cologne, Germany) according to the manufacturer's recommendations. To block Fc-receptor binding 25% autologous serum was added prior to coincubation. An alternative CD19-specific CAR with a modified extracellular domain lacking Fc-receptor binding sites, as suggested by Hudecek et al.²⁹, was used as a control.

Human anti-mouse antibodies (HAMA) analysis

Patient serum was analyzed for the presence of HAMA 2, 6 and/or 12 months after CTL infusion by ELISA, performed by the Lademannbogen laboratory in Hamburg, Germany. Briefly, pre-coated mouse IgG is used to capture human anti-mouse antibodies in the serum, followed by colorimetric detection using peroxidase-labeled mouse IgG.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. In vitro flow cytometry detection of CD19CAR T cells via the Fc γ hinge domain. CD19 ζ -transduced EBV-CTL, positively selected by FACS, were mixed with unstained PBMCs at the indicated ratios. Samples were then stained with FITC-labeled goat antimouse IgG Fc γ -specific antibody staining of Fc γ hinge domain of the CD19 CAR.

Supplementary Figure 2. In vitro restimulation of circulating CD19CAR T cells from patient peripheral blood. PBMC were isolated from peripheral blood samples of P004 taken 2 weeks and 2 months post CTL infusion and restimulated with donor-derived, LCL to selectively expand circulating donor CTL. IFN- γ Elispot analysis (A and B) revealed reactivity

to expanded CTL not only with autologous LCL, but also with CD19-expressing leukemia cells (SupB15, REH). Flow cytometry staining with FLAG-labeled sCD19 detected a population of CD19CAR CTL at 2 weeks (C).

Supplementary Figure 3. Effector memory phenotypes of EBV-CTL given to P004 both prior to adoptive transfer (upper panel) and post adoptive transfer after *in vitro* restimulation (lower panel).

Supplementary Figure 4. Lack of CD19CAR CTL persistence by flow cytometry. Staining of PBMC purified from peripheral blood with CD3, CD8 and goat antimouse IgG Fc γ -specific antibodies (performed in all patients) or FLAG-labeled sCD19 (performed in addition in patients P007, P010, P019, and P022 after LCL vaccination) failed to detect circulating CD8+ T cells coexpressing the CD19 ζ CAR at all time-points. Shown here are representative results from P007 and P010.

Supplementary Figure 5. P004 (A, B) and P010 (C, D) were investigated for the presence of anti-CAR specific T cells by Elispot analysis. CD19CAR CTL and non-transduced CTL from the same donor as well as donor LCL were used to stimulate PBMC obtained from the patients 1-2 weeks and 1-2 months after CTL infusion. As expected, interferon- γ responses were raised against the LCL target. No functional responses against non-transduced and CD19CAR expressing CTL were detected.