Supplementary Material

Flow cytometry

Flow cytometric analysis was performed on single cell suspensions for determination of transduction efficiencies and surface expression, phenotypic characterization, cytotoxicity and alloreactivity assays. Prior to staining of murine blood samples, unspecific binding was prevented by incubation with mouse Fc block reagent (2.4G2; BD Biosciences PharMingen) for 10 min at room temperature. Red blood cells were lysed using 1x Pharm Lys solution (BD Biosciences PharMingen). The following mouse anti-human antibodies were used (all BD Biosciences PharMingen): fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) conjugated CD3 (SK7), peridinin chlorophyll protein (PerCP) conjugated CD4 (SK3), PerCP or APC conjugated CD8 (SK1), PE or APC conjugated CD20 (L27), PE or PE Cy7 or PerCP Cy5.5 or APC conjugated CD34 (8G12), FITC conjugated CD45RA (L48), PE conjugated CD56 (B159), PE or APC conjugated CD62L (DREG-56). For PBMC donor screening, a PE conjugated anti-HLA-A2 antibody was purchased from AbD Serotec (Duesseldorf, Germany). The following anti-mouse antibodies were used: Pacific Blue conjugated CD3ε (500A2) (hamster anti-mouse), PerCP Cy5.5 conjugated CD4 (RM4-5), FITC conjugated CD8 (53-6.7) (both rat anti-mouse), APC conjugated NK-1.1 (PK136, mouse anti-mouse), (all BD Biosciences PharMingen) and PE Cy7 conjugated CD45.1 (A20) (eBioscience, San Diego, CA). Control stains were performed using isotype-matched fluorochrome conjugated monoclonal antibodies. To exclude dead cells, 7-Aminoactinomycin D (7AAD) was used in some experiments. For quantitative analysis, liquid counting beads (BD) were added prior to measurement. Analyses were performed on a

FACSCalibur using the CellQuestPro Software or a FACSCanto II using the FACSDiva software (both BD Biosciences PharMingen).

Western Blot

For protein expression studies, Western Blot analysis was performed on protein lysates of gene-modified HuT 78 and primary T cells. Primary antibodies used were as follows: a rabbit anti-CD20 for detection of CD20 in a 1:2000 dilution (Cat. Nr. 610067, Neomarkers, Fremont, CA), a rabbit anti-CD34 for detection of tCD34 in a 1:200 dilution (sc-9095, Santa Cruz Biotechnology). Detection of PLC-γ by a mouse anti-PLC-γ antibody (Cat. Nr. 612464, BD Biosciences) in a 1:1000 dilution was used as a loading control. Primary antibodies were detected by horseradish-peroxidase (HRP) conjugated secondary antibodies: goat anti-mouse (sc-2005) or goat anti-rabbit HRP (sc-2004) in a 1:5000 dilution (both Santa Cruz Biotechnology). Molecular weights of detected proteins were determined according to a prestained protein marker (Fermentas, St. Leon-Rot, Germany).

Alloreactivity assay

Alloreactivity of freshly isolated CD3+ T cells and gene-modified, immunoselected T cells from the same donor (HLA-0201+) was tested at day 0 and day 9 after stimulation, respectively. Gene-modified T cells were cultured in X-Vivo 10 media with supplements described above in the presence of 20 U/ml IL-2 for 2 days after immunoselection. Cells were

labeled with 2.5 μM carboxy-fluorescein succinimidyl ester (CFDA SE cell tracer kit, Invitrogen, Karlsruhe, Germany) and incubated at a 1:1 ratio with freshly isolated, 15 Gy irradiated third party PBMCs for 6 to 8 days in the presence of 20 U/ml IL-2. Cells were harvested, stained with corresponding antibodies (anti-CD3, anti-CD8 and anti-CD34) and analyzed by flow cytometry for CFSE signal dilution. For quantification, liquid counting beads were included into the analysis and acquisition was stopped after 2,500 gated bead events.

Isolation of genomic DNA and qPCR analysis

Genomic DNA was extracted from animal blood samples using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Prior to isolation, red blood cells were removed by incubation with an ACK lysis buffer (Lonza, Belgium). Real-time quantitative PCR analysis to determine gene-modified donor T cell frequencies was performed using a LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany). The primer sequences used for donor cells (targeting the deleted Rag1 sequence not present in recipient animals) were as follows: forward 5´ TCCAGCACACTTTGTGAAA 3´ and reverse 5´ GGGATCAGCCAGAATGTGTT 3´. The universal probe library (UPL, Roche Applied Sciences) probe # 51 was used. For the detection of proviral integration (target: tCD34) the following primers were used: forward 5´ GTGAAATTGACTCAGGGCATC 3´ and reverse 5´ GGGATCAGCCAGAATGTGTT 3´. The UPL probe was probe # 1. Mouse β-actin qPCR was used to control input DNA. Primers and probe were described previously.[1]

Histopathologic analysis

Sections were prepared and stained with hematoxylin and eosin using standard methods. CD20 immunostaining was performed using a commercially available primary antibody (Clone L26, DAKO, Glostrup, Denmark). Tissue sections were heat pre-treated and antibody binding was detected using the Dako REAL Detection System (Alkaline Phosphatase/RED, K5005, DAKO).

Supplementary References

1. Brown, BD, Venneri, MA, Zingale, A, Sergi Sergi, L, and Naldini, L (2006). Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat Med* **12**: 585-591.