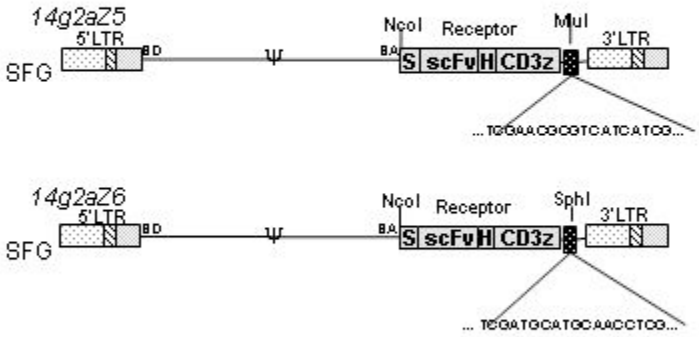
Supplementary File 1: Distinguishable CAR Vectors



Supplementary Figure 1. Design of GD2-specific CAR vectors. Two GD2-specific CAR vectors (Zeta-5, panel a, and Zeta-6, panel b) were prepared from SFG, a Moloney-based retroviral vector lacking env and most of gag-pol, but retaining the packaging sequence (ψ). The chimeric antigen receptor consists of human Ig signal peptide (S), the single-chain variable fragment (scFv) from 14g2a, a GD2-specific monoclonal antibody, the hinge region of IgG1 (H), and the transmembrane and intracellular portions of CD3-Zeta (CD3z). Short unique noncoding 3' oligonucleotides sequences between the receptor open-reading frame and the 3'LTR allowed monitoring of the two different transduced cell populations (CTL vs ATC) after infusion into the same patient. The primers and probes used to amplify and distinguish the14g2aZ5 from the 14g2aZ6 sequences by Q-PCR are listed below:

ID	Sequence
MP1449FZeta5	5'- CAA GGA CAC CTA CGA CGC CCT TC - 3'
MP1036RZeta5	5'- GAC TAA TCC GGA TCG ATG ATG ACG CG - 3'
MP1018Zeta5	5' VIC- CCC CCT CGC TAA CA –MGBNFQ
KM3190FZeta6	5'- ACA GCC AGC TCG ATG CAT G - 3'
KM3256RZeta6	5'- AGT CAA AAC TAG AGC CTG GAC CA - 3'
KM3220Zeta6	5' VIC-TCC GGA TTA GTC CAA TTT GTT AAA GAC AGG ATA TCA-TAMRA

Details of PCR analysis and quantitation

To compare the persistence of CAR-ATC and CAR-CTL in peripheral blood, we used a single set of Q-PCR standards (Zeta-5 and Zeta-6 transduced Jurkat cells), against which all of the patient samples were compared. Furthermore, we rotated Zeta-5 and Zeta-6 vectors between CTL and ATC for each succeeding patient. Finally, we compared our Q-PCR estimates of the percentage of transduction of the infused CAR-ATC and CAR-CTL, with the percentage of ATC and CTL that were CAR positive by immunofluorescence.

We made our Jurkat standards by transducing cells with each vector (either Zeta-5 or Zeta-6) and then cloning single cell by FACS sorting. We used Q-PCR to estimate the

copy number of each transgene in Jurkat-Zeta-5 and Jurkat-Zeta-6 cells by extracting DNA from these cells and serially diluting it with DNA obtained from non transduced Jurkat cells. To estimate the copy number per Jurkat cell, we used specific primers to amplify in parallel serially diluted plasmid DNA encoding Zeta-5 and Zeta-6, respectively. The experiments were repeated in triplicate using three different DNA samples from the Jurkat. We assumed that the human genome contains 3.0 x 10^9 bp (haploid), corresponding to 3.3 pg DNA (Celera Genomic estimation). The copy number per cell was then estimated using the formula: transgene copy number detected by Q-PCR √ cell number containing the quantity of DNA used for each reaction. By this calculation we estimated 6.47±0.35 copies/cell for Jurkat-Zeta-5 and 10.60 ± 0.17 copies/cell for Jurkat-Zeta-6. This difference in copy number between Jurkat-Zeta-5 and Jurkat-Zeta-6 does not distort subsequent calculations about the relative levels or persistence of transduced cells representing each subset, since we alternated the Zeta-5 and Zeta-6 vectors between ATC and CTL with each succeeding patient. This copy number may have been higher than we found in transduced CTL and ATC (3.9 – 7.2), leading us to *underestimate* the percentage of positive cells persisting in blood. In fact, however, as shown in text Figure 1a, the percentages of both CAR-CTL and CAR-ATC are comparable when measured either by Q-PCR or by immunofluorescence staining of expressed receptor.

To further validate our estimates of Zeta-5 and Zeta-6 positivity, we amplified conserved sequences in the ScFv coding region. We chose primers and probes spanning the coding sequence in ScFv that is conserved between Zeta-5 and Zeta-6. We compared the signal (copy number) obtained from PBMCs, using this conserved sequence, with the copy number obtained by adding the individual data from Zeta 5 and Zeta 6 amplifications of the same samples. We studied 14 samples from four of our treated patients. Logistic regression showed a Y = 1.878 + 25.21 and an R2 = 0.7029, representing good a correlation between the levels obtained with the conserved-sequence primers and the summation of the unique Zeta-5 and Zeta-6 sequences. This further supports our overall estimates of CAR-CTL and CAR-ATC levels in peripheral blood.

SUPPLEMENTARY RESULTS
ADDITIONAL CHARACTERISTICS OF CAR-ATC AND CAR-CTL

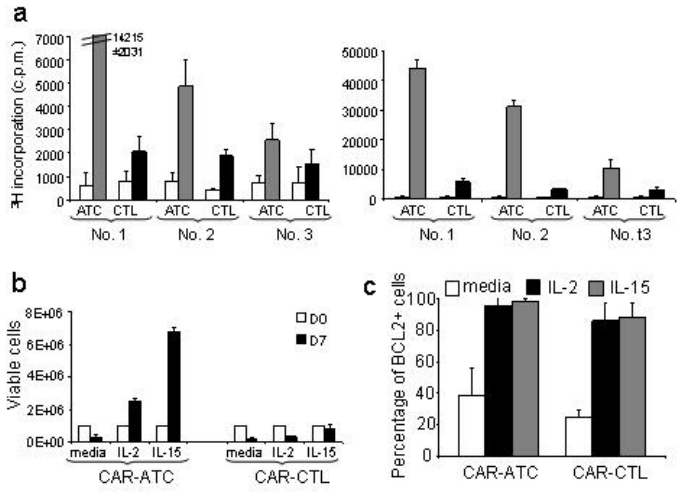
Supplementary Table 1. Cell doublings between culture initiation and freezing

Infused cells	At culture initiation (median [range])	At freezing (median [range])	Median no. of doublings
CTL	8.8×10^6 ($6 \times 10^6 – 1.5 \times 10^7$)	1.5×10^9 ($2.5 \times 10^8 – 1.4 \times 10^{10}$)	8
ATC	7.6×10^6 ($1.8 \times 10^6 – 3.2 \times 10^7$)	3.8×10^8 ($1.7 \times 10^8 – 5.5 \times 10^9$)	6

Antigen independent proliferation and survival by CAR-CTL and CAR-ATC

We added IL-2 and IL-15 cytokines to determine if the CAR-CTL and CAR-ATC retained the same capacity for antigen-independent proliferation and survival, or if differences in this capability could explain the longer persistence of CAR-CTL *in vivo*. We thawed remaining aliquots of CAR-ATC and CAR-CTL that had been given to 3 of our subjects, and cultured the cells with or without IL-2 (50 U/ml) or IL-15 (5ng/ml). We compared their proliferation using thymidine incorporation, and their expansion by counting trypan blue-excluding viable cells. In addition, we evaluated BCL2 expression – as a marker of apoptosis resistance – using FACS analysis.

Neither CAR-ATC not CAR-CTL proliferated in the absence of cytokines. In their presence, however, both subsets proliferated, although, the levels of proliferation were was significantly higher for CAR-ATC than for CAR-CTL (IL-2, $P = 0.02$; IL-15, $P = 0.002$) (**Supplementary Fig. 2a**). The enhanced proliferation of CAR-ATC in vitro was paralleled by a significantly increased CAR-ATC number compared to CAR-CTL after 7 d of culture in IL-2 or IL-15 ($P < 0.01$) (**Supplementary Fig. 2b**). Finally, IL-2 and IL-15 cytokines upregulated BCL2 expression equally in both CAR-CTL and CAR-ATC (**Supplementary Fig. 2c**). In summary, since the CAR-ATC prepared for infusion show superior intrinsic proliferative and survival capacities, compared to CAR-CTL, and equivalent expression of the anti-apoptotic marker BCL2, these data strengthen our argument that the superior survival of CAR-CTL versus CAR-ATC *in vivo* is most likely due to the effects of antigen-stimulation.



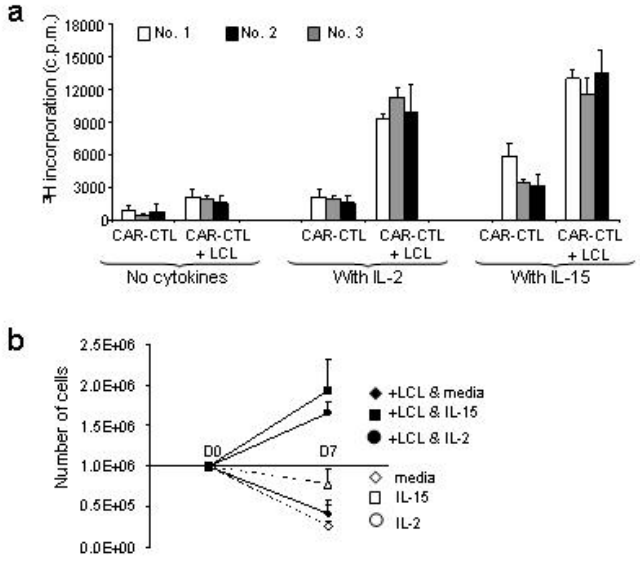
Supplementary Figure 2. Antigen independent proliferation and survival of CAR-ATC and CAR-CTL. Panel 2a shows thymidine incorporation by CAR-ATC (gray bars) or CAR-CTL (black bars) in the presence of IL-2 (left graph) or IL-15 (right graph). White bars show the proliferation of ATC or CTL in the presence of media only. Mean and s.d. measurements of triplicate wells from 3 donors are shown. Panel 2b shows the expansion of CAR-ATC or CAR-CTL in the presence of IL-2 or IL-15. Viable cell numbers are shown on the vertical axis. The white bars show the cell number at day 0 (when cell culture was initiated) and the black bars the cell numbers after 7 days of culture. Data are mean ± s.d. from 3 patients. Panel 2c shows the percentage of CAR-ATC or CAR-CTL expressing BCL2 on day 7 of culture in media only (white bars) or in the presence of IL-2 (black bars) or IL-15 (gray bars). Data are mean ± s.d from 3 subjects.

CAR-CTLs Need Continued *in Vivo* Antigen Stimulation for Persistence.

In our article we suggest that continued stimulation of native-antigen receptor of the virus-specific cells *in vivo* allows them to persist for 6 months and beyond. In this supplementary file we provide data from 3 additional lines of investigation that support this assertion.

i) *In vitro* evidence that chimeric EBV-CTL need continued antigenic stimulation. To show the continuing dependence of CAR-CTL on native receptor stimulation, we thawed residual aliquots of CAR-CTL that had been given to 3 different individuals, and plated them in culture with or without irradiated LCL (E:T ratio, 4:1) in the presence of IL-2 (50 U/ml) or IL-15 (5 ng/ml). We compared their proliferation by thymidine incorporation (**Supplementary Fig. 3a**), and their survival by counting viable cells using trypan blue exclusion (**Supplementary Fig. 3b**).

As expected, CAR-CTL proliferated significantly better in the presence of LCL, irrespective of the cytokine provided ($P < 0.002$). As we also discuss and demonstrate in **Supplementary File 5**, these CAR-CTL do not proliferate or survive in response to stimulation of their chimeric receptors alone.



Supplementary Figure 3. Requirement for continuous antigen stimulation in CAR-CTL proliferation and survival. Panel a shows the thymidine incorporation of CAR-CTL

stimulated with or without irradiated LCL (at an E:T ratio of 4:1) in the absence or in the presence of IL-2 or IL-15. Bars show the mean and s.d. measurements of triplicate wells representing 3 donors. Panel b shows the change in numbers of viable CAR-ATC in the presence (closed symbols) or absence (open symbols) of LCL (at an E:T ratio of 4:1) in media (diamond) or IL-2 (circle) or IL-15 (square). The number of cells counted with trypan blue exclusion on day 0 and day+7 are shown on the vertical axis. Data are means $\pm$ s.d. from 3 donors.

ii) *In vivo* evidence that chimeric EBV-CTL need continued native receptor stimulation to survive.

We have previously shown in a xenogeneic model that EBV-CTL transduced with a CAR provide sustained antitumor activity only if their native receptors are stimulated by EBV-LCL. In these experiments, SCID mice were engrafted with EBV negative $CD30^+$ tumor cells and treated with EBV-CTL expressing a CAR targeting the CD30 molecule. We found that control of tumor growth was sustained long term in mice only in mice that also received costimulation from autologous EBV-infected cells.[1]

iii) Evidence that virus-specific CTL only persist in humans if antigen is present.

In an earlier clinical study in the setting of post-transplant infection,[2] we adoptively transferred an autologous CTL monoculture that was prepared with the same EBV-APC as used in the current study, but which also coexpressed CMV and adenoviral antigens. The CTLs prepared in this way contained EBV, CMV and adenovirus specific CTL. We observed persistence of the autologous CMV and EBV-CTL in the latently infected CMV and EBV seropositive recipients. By contrast, adenovirus specific CTL only persisted when there was concomitant adenoviral infection of the recipient, and hence antigen stimulation. Since CMV-, EBV- and adenovirus-specific CTL all received the same in vitro stimulation and costimulation from the same antigen-presenting cells, and since all subset contained both CD4 and CD8 effector and effector memory cells, these data indicate that *in vitro* activation and co-stimulation events are insufficient alone to produce long-term survival *in vivo*; rather, antigen also needs to be present.

References

[1] Savoldo et al, Blood. 2007 Oct 1;110(7):2620-30.
[2] Leen et al., Nat Med. 2006 Oct;12(10):1160-6.

Lymphodepletion by CD45 Mab

In an effort to increase the in vivo expansion of the CAR-ATC and CAR-CTL, we lymphodepleted 5 individuals prior to cell infusion. For this purpose we used two unconjugated rat antihuman CD45 MAb, YTH54.12 and YTH25.4, which are of the IgG2b subclass, bind to noncompeting but proximate CD45 epitopes present in all CD45 isoforms, and in combination, have a synergistic ability to lyse human hematopoietic and lymphoid cells by complement-mediated and cell-dependent mechanisms.[1,2,3,] Subjects received CD45 MAb (400 μg/kg/dose) as a daily 6 – 8 h infusion for 4 days. Forty-eight to 72 h after the end of the final infusion, CD45 MAb levels were determined, and EBV-CTL were given if the CD45 MAb level was less than 100 μg/L, a CD45 MAb concentration that is below the concentration needed to induce complement-mediated lysis *in vitro*.[3] In fact all serum antibody levels at the time of CAR-CTL/ATC infusion were <10 μg/L. We have found this approach to be a successful means of inducing lymphocyte depletion in adults with nasopharyngeal cancer (Louis et al manuscript in revision), capable of inducing

measurable levels of IL-15 in serum during the recovery phase, and expansion of infused cells. In children with neuroblastoma; however, the effects were less striking, with only 44 – 91% (median, 60%) lymphocyte depletion and no significant rise in IL-7 or IL-15 after treatment. These limited activities are consistent with the lack of any effect of CD45 infusions on AUC measures for CAR-CTL or CAR-ATC in the CD45 versus the non-CD45 group.

References

[1]Wulf GG, Luo KL, Goodell MA, Brenner MK. Anti-CD45-mediated cytoreduction to facilitate allogeneic stem cell transplantation. Blood 2003;101:2434-2439.

[2]Brewer Y, Taube D, Bewick K et al. Effect of graft perfusion with CD45 monoclonal antibodies on incidence of kidney allograft rejection. Lancet 1989935-937.

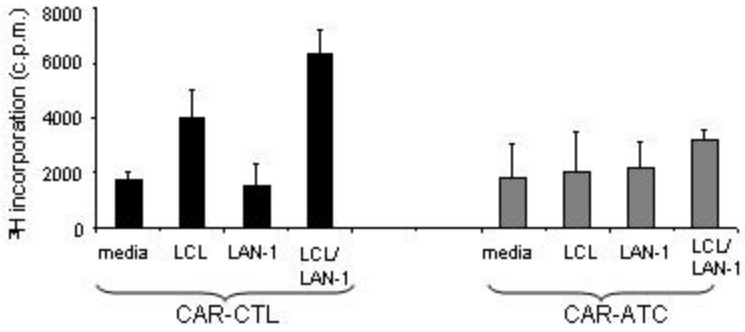
[3]Krance RA, Kuehnle I, Rill DR et al. Hematopoietic and immunomodulatory effects of lytic CD45 monoclonal antibodies in patients with hematologic malignancy. Biol Blood Marrow Transplant 2003;9:273-281.

CAR-CTL But Not CAR-ATC Can Be Enriched Long Term After Infusion by (Selective) Stimulation of the Native Receptor

Chimeric receptor engagement has a limited effect on T cell proliferation in CAR-CTL and CAR-ATC.[1] However, *selective* stimulation of the native receptor by EBV antigen allows enrichment of CAR-CTL for 6 months or more after infusion, enabling us to detect them in cultures of PBMC (Text Fig. 4). In this supplementary file we show how *nonselective* native receptor stimulation by OKT3 cannot enrich for any residual CAR-ATC which therefore remain undetectable once the immediate infusion period is past.

Cross-linking our GD2-specific chimeric T cell receptor alone fails to produce all the stimulatory and costimulatory signals required for sustained T cell survival, activation and proliferation.[2] As a consequence, both CAR-ATC and CAR-CTL can kill target cells expressing the chimeric receptor antigen and release IFN-γ, but engagement of the chimeric receptor alone is insufficient for either CAR-CTL or CAR-ATC to proliferate or survive long term, precluding serial passage of PBMC on GD2-expressing cells. We illustrate this point below, showing how GD2 expressing CAR-CTL and CAR-ATC fail to proliferate or survive on exposure to $GD2^+$ target cells. CAR-CTL respond with increased thymidine uptake only to stimulation of their native receptor by EBV-LCL, but not by stimulation of their chimeric receptor by LAN-1. CAR-ATC do not increase uptake of thymidine after either stimulus (**Supplementary Fig. 4**).

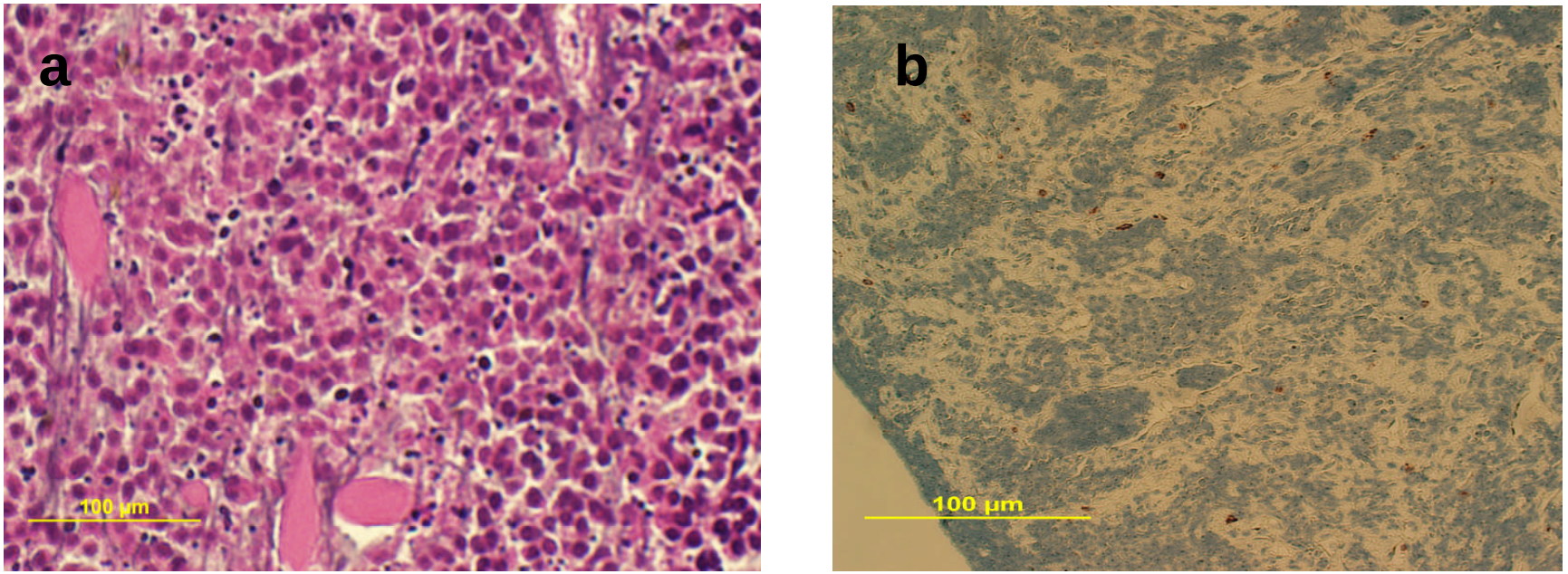
The CAR on ATC may have the same (limited) functionality as the CAR on CTL, but only CAR-CTL can continue to be selectively recruited to proliferate. As a result, only CAR-CTL can be detected long term, and so are the only cell population in which we detect CAR activity for more than a brief period after infusion, CAR-ATC lack a mechanism by which they can be actively recruited.



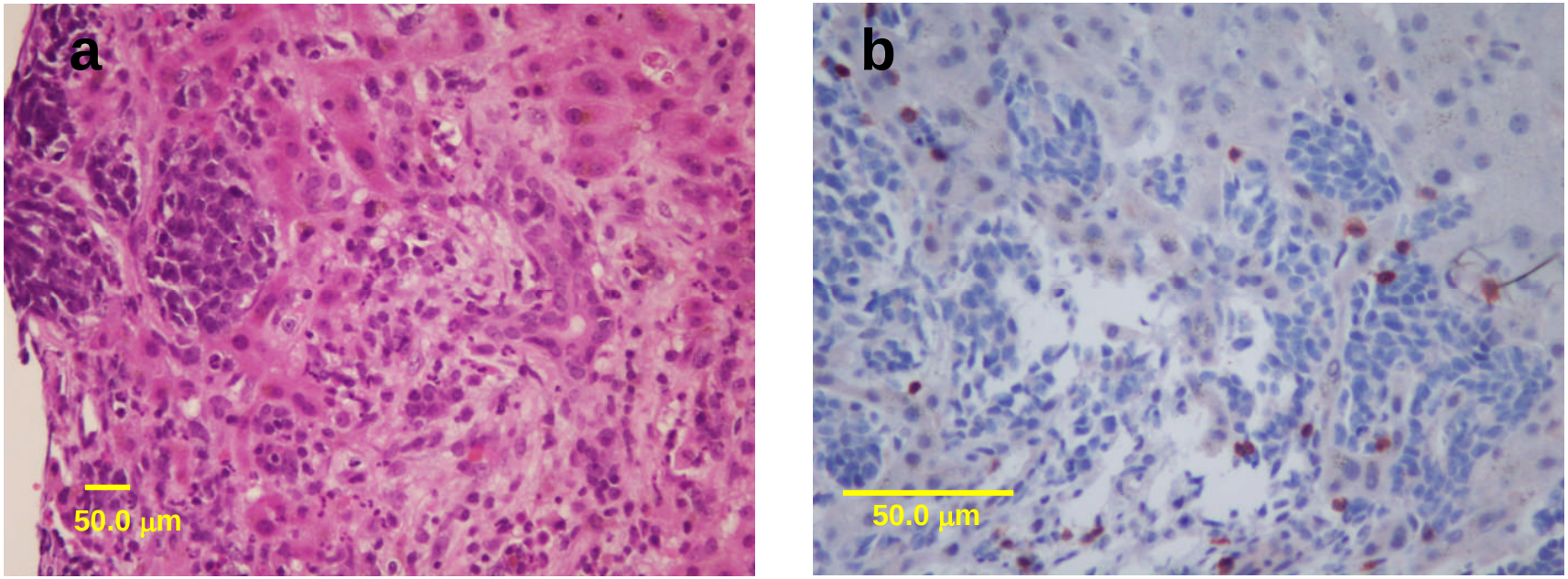
Supplementary Figure 4. CAR-CTL proliferate in response only to stimulation of their native receptor by EBV-LCL. CAR-CTL and CAR-ATC were plated at $1x10^5$/well and irradiated LAN-1 and/or LCL were added at a 4:1 E:T ratio, in the presence of 25 U/ml of IL-2. After 72h of culture, 1 μCi/well of ^3H was added. Cell were harvested after 16 h and proliferation determined by scintillation counter. The figure shows the thymidine incorporation of GD2-specific CAR-CTL (black bars) or CAR-ATC (gray bars) with media alone or after stimulation by EBV-LCL (native receptor) or $GD2^+$ LAN-1 cells (chimeric receptor) Data are mean and s.d. measurements of triplicate wells.

References

[1]Rossig et al, Blood. 2002 Mar 15;99(6):2009-16.
[2]Pule et al Mol Ther. 2005 Nov;12(5):933-41.



Supplementary Figure 5. **Tissue biopsy of the boundary between necrotic and viable tumor and normal muscle.** Panel a is the H&E stain showing clumps of viable neuroblastoma cells infiltrating normal muscle, and areas of tumor necrosis, while panel B shows immunohistochemistry with a CD3 antibody. Infiltrating T cells appear red. (X10/X20)



Supplementary Figure 6. **Tissue biopsy of the boundary between necrotic and viable tumor and normal liver.** Panel a is the H&E stain showing clumps of viable neuroblastoma cells and areas of tumor necrosis, while panel b shows immunohistochemistry with a CD3 antibody. Infiltrating T cells appear red. (X10/X40)