SUPPLEMENTAL MATERIAL

for

Alloantigen-specific T regulatory cells generated with a chimeric antigen receptor

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Fig. S1: Expression of a second generation CAR in Tregs does not affect their phenotype or function.

Fig. S2: Treg suppressive capacity induced by CAR stimulation.

Fig, S3: HLA-A2 CAR Tregs transferred into HLA-A2⁺ mice limit tissue-damage caused by PBMCs.
**Supplemental Figure 1.** Expression of a second generation CAR in Tregs does not affect their phenotype or function. A) 14 days after activation, CAR expressing Tregs or Tconvs were analyzed for the indicated markers; representative staining (left) and average mean fluorescence intensity (right). B) Cells were re-stimulated with PMA/ionomycin then stained for IL-2 and IFN-γ. Representative data (left) and averaged data (right) are shown (n=3). C) Suppressive capacity of untransduced or A2-CAR Tregs when stimulated through endogenous TCR was assayed by titrated Treg ratios with HLA-A2 PBMCs stimulated with αCD3/28-coated beads. Division index of CD8+ cells was determined after 96 hours (n=4). D) Cell viability at day 14 post transduction and expansion was determined as the proportion of CD4+ T cells that were viability dye negative (n=4). Significance determined by 2-way ANOVA or by multiple t-tests with Holmes Sidak Comparison. Data represent mean ± SEM.
Supplemental Figure 2. Treg suppressive capacity induced by CAR stimulation.

A) Schematic diagram representing the sources of stimulation and predicted outcomes (indicated by lower case roman numerals) in autologous suppression assays using tetanus-toxoid specific T cell clones from an HLA-A2^+ donor. Responder cells were labeled with CPD, and stimulated by autologous EBV-transformed B cells, which were or were not transduced with HLA-A2, and pulsed with tetanus toxoid. The proportion of divided cells was determined after 5 days (n=1). B) Suppressive capacity of A2-CAR transduced Tregs when stimulated through both endogenous TCR and CAR was assayed by titrating the indicated numbers of A2-CAR Tregs into cultures with HLA-A2^+ PBMCs stimulated with αCD3/28 coated beads (TCR + CAR stimulated). To measure Treg suppression of the same PBMCs stimulated by TCR alone, HER2 CAR Tregs were titrated in (TCR stimulated). Division index of CD8^+ cells is summarized. C) Contact-independent suppressive activity was determined by transwell suppression assays. HLA-A2^+ PBMCs stimulated with αCD3/28 beads were placed in the lower chamber, and A2-CAR Tregs stimulated with HLA-A2^+ K562 cells (1:1 ratio) were in the upper chamber (n=4). Percent suppression of CD8^+ cells is summarized. Data represent mean ± SEM where indicated. Significance determined by multiple t-tests with Holmes Sidak comparison.
Supplemental Figure 3. *HLA-A2 CAR Tregs transferred into HLA-A2+ mice limit tissue-damage caused by PBMCs.* Irradiated A2-NSG mice were injected with PBS (n=2), 1 x 10^7 HLA-A2+ PBMCs alone (n=3) or with 1 x 10^7 A2-CAR Tregs, or A2-CAR Tconv (n=3 for each group). Data shown are the combined results for all mice in one experiment. Mice receiving cells were sacrificed after **A** 2 weeks or **B** 4 weeks. PBS mice were sacrificed after 4 weeks. Liver and lungs were formalin fixed, then hematoxylin and eosin staining was performed of formalin-fixed tissues. Arrows indicate tissue-infiltrating lymphocyte clusters. Original magnification X10; bar represents 200µm; pictures are from one representative experiment and one representative individual per group is shown.