**Supplementary Figure 1. Enhanced allogeneic T_{EM} proliferation triggered by alloantibody and complement-activated ECs compared to normal human sera and vehicle control-treated ECs.** Proliferation of CD4+ and CD8+ T_{EM} cells after coculture for 7 days with IFN-γ-primed ECs treated with PRA, normal human sera or vehicle control (gelatin veronal buffer). Data from 2 independent experiments using 2 different PBMC and 2 allogeneic HUVEC donors (mean ± SEM, 1-way ANOVA and Tukey's multiple comparisons test).
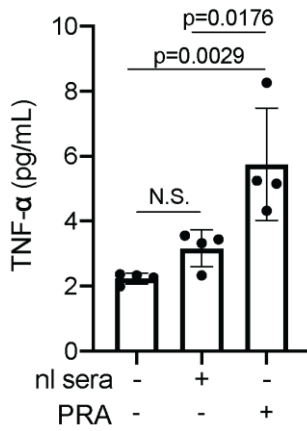
Supplementary Figure 2. Low levels of TNF-α detected in EC:T$_{EM}$ cell cocultures. Media was collected at 24 hours of endothelial cell and T$_{EM}$ coculture and assayed for human TNF-α by ELISA. IFN-γ-primed ECs were treated with PRA, normal human sera or vehicle control (gelatin veronal buffer) prior to coculture with T$_{EM}$. Data shown is representative of 2 independent experiments using 2 different PBMC and 2 allogeneic HUVEC donors (mean ± SEM, 1-way ANOVA and Tukey's multiple comparisons test).

Detailed Materials and Methods

Cell Isolation and Culture

All protocols involving collection of and experimentation with human cells and tissues were approved by the Yale University Institutional Review Board.

Isolation and culture of human ECs: Human umbilical vein ECs (HUVECs) were isolated from de-identified umbilical cords following enzymatic digestion by collagenase. Cells used in all experiments were serially cultured at 37°C on 0.1% gelatin (Sigma)-coated tissue culture plates in Endothelial Cell Growth Medium (EGM2, Lonza) and used for experiments between passage levels 1-6.

Isolation of effector memory CD8+ and CD4+ T lymphocytes: Leukapheresis products were collected from healthy adult volunteers in the Yale blood bank and then de-identified before transfer to the laboratory. Mononuclear cells were enriched by density gradient centrifugations using Lymphocyte Separation Medium (MP Biomedical, 50494X) per the manufacturer's instructions and cryopreserved in 10% DMSO/90% FBS in liquid nitrogen. Thawed cells were washed in RPMI (Gibco) supplemented with 5% FBS, 1.5% L-glutamine, and 1% penicillin/streptomycin. Human CD8+ and CD4+ T cells were isolated from total PBMCs by positive selection with magnetic Dynabeads CD8 (Invitrogen, 11333D) and Dynabeads CD4 (Invitrogen, 11331D), respectively, per the manufacturer's protocol and released with Detachabeads in the Dynabeads Positive Isolation kit (Invitrogen, 11333D and 11331D). Resting effector memory (CCR7$^-$HLA-DR$^-$) T cells were purified from total CD8$^+$ and CD4$^+$ T cells by negative selection using anti-human CCR7 antibody (Biolegend, 353222) and HLA-DR antibody (Biolegend, 307602) by magnetic separation with Dynabeads Pan Mouse IgG (Invitrogen, 11041) to remove antibody-bound cells. The final population was identified as 90-98% resting CD8$^+$ and CD4$^+$ effector memory T cells by flow cytometry analysis.

Antibody and Complement Treatment of ECs

Discarded, de-identified and pooled preparations of high-titer PRA sera from transplant candidates that showed >80% reactivity to class I and II MHC antigens that were collected from the Yale New Haven Hospital tissue typing laboratory. In vitro studies of ECs response to complement were elicited using high titer PRA in gelatin veronal buffer (GVB, Sigma, G6514) to treat serially passaged HUVEC cultures. HUVECs were treated with IFN-γ (50 ng/mL, Gibco, PHC4033) for 48 hours in EGM2 media prior to treatment with PRA to reinduce class I and class II MHC expression. Where indicated, HUVECs were treated with 10μg/mL IL-1 Receptor Antagonist

(Peprotech, 200-01RA), 20μg/mL anti-IL-15 blocking antibody (R&D Systems, MAB247) or DMSO in GVB for 30 minutes, at the indicated concentrations, prior to PRA treatment. PRA was subsequently added to ECs in GVB at a 1:3 ratio for 6 hours. After treatment, ECs were washed with PBS and co-cultured with allogeneic T$_{EM}$ cells as described below.

T Cell Activation Assays

HUVECs were cultured on 96 well round bottom plates and pre-treated with IFN-γ for 48h. Where indicated, HUVECs were treated with 10μg/mL IL-1 Receptor Antagonist (Peprotech, 200-01RA), 20μg/mL anti-IL-15 blocking antibody (R&D Systems, MAB247) or DMSO in GVB for 30 minutes, prior to treatment with PRA sera or GVB control for 6 hours. Where indicated, ECs were treated with normal human serum (MilliporeSigma, H4522) for 6 hours prior to coculture. Freshly isolated human CD8$^+$CCR7$^-$HLA-DR$^-$ and CD4$^+$CCR7$^-$HLA-DR$^-$ T cells were co-cultured with the allogeneic HUVEC at an EC:CD8 and EC:CD4 T cell ratio of 1:30 and 1:20, respectively, in RPMI 1640 media supplemented with 10% FBS, 2% L-glutamine, 1% penicillin. T cell proliferation was assayed by labeling T cells using Cell Trace CFSE Proliferation Kit (Invitrogen, C34554). CD4+CCR7-CFSE^{lo} and CD8+CCR7-CFSE^{lo} T$_{EM}$ were sorted by flow cytometry after 7 days.

Measurement of Cytokine Production

Media was collected from EC/CD4/CD8 T$_{EM}$ cocultures after 24 hours and analyzed for TNF-α using TNF alpha Human ELISA Kit (ThermoFisher Scientific, 88-7346-22).

Flow cytometry sorting analysis

After 7 days of EC:T cell co-culture, T cell proliferation was assessed by flow cytometry. T cells were harvested and stained with Fixable Viability Dye (eBioscience, 65-0865-14), anti-human CD4 (Biolegend, 317424), anti-human CD8 (eBioscience, 48-0086-42). CFSE dilution was assessed as an indicator of proliferation. At least 100,000 live CD4$^+$CCR7$^-$CFSE^{lo} and CD8$^+$CCR7$^-$CFSE^{lo} T$_{EM}$ for each treatment group were sorted, separated and cryopreserved in 10% DMSO/FBS prior to single cell 32-plex proteomics analysis. All samples were sorted using the BD FACSAria Flow Cytometer and BD FACSDiva Software and analyzed with FlowJo software.

Polyfunctional T cell evaluation by the single-cell 32-plex proteomics

Cryopreserved T cells were thawed and cultured in complete RPMI medium with IL-2 (10 ng/mL, Biolegend) at a density of 1 x 10^6/mL at 37°C. Pilot experiments suggested that cryopreservation did not alter the established functional phenotyping of the T cell response. After overnight recovery, viable T cells were enriched using Ficoll-

Paque Plus (GE Healthcare) and resuspended in complete RPMI media at a density of 1×10^6/mL. Approximately 100 µl of T cell suspension were seeded into a well of 96-well flat-bottom plate precoated with anti-human CD3 (clone OKT3, 10 ug/ml in PBS at 4°C, O/N) with a supplement of soluble anti-human CD28 (clone CD28.2) at a final concentration of 5 µg/mL. After 3h stimulation at 37°C, 5% CO_2, the cells were stained with PE-conjugated anti-CD4 or Alexa Fluor 647-conjugated anti-CD8 antibody at room temperature for 10 minutes, rinsed once with PBS and resuspended in complete RPMI medium at a density of 1×10^6/mL. Approximately 30 µl of cell suspension was loaded into an IsoCode chip containing ~12,000 microchambers pre-patterned with a complete copy of a 32-plex antibody array. After 16-hour-on-chip incubation at 37°C, 5% CO_2, proteins signals from ~1000 live single cell were captured and analyzed by fluorescence ELISA-based assay; the polyfunctional T cells that co-secreted 2+ cytokines per cell were analyzed by the IsoSpeak software across the five functional groups:

Effector: Granzyme B, TNFα, IFN-γ, MIP1α, Perforin, TNFβ;

Stimulatory: GM-CSF, IL-2, IL-5, IL-7, IL-8, IL-9, IL-12, IL-15, IL-21;

Chemoattractive: CCL11, IP-10, MIP-1β, RANTES;

Regulatory: IL-4, IL-10, IL-13, IL-22, sCD137, sCD40L, TGFβ1;

Inflammatory: IL-6, IL-17A, IL-17F, MCP-1, MCP-4, IL-1β.

As previously published [1-5], the Polyfunctional Strength Index (PSI™) of a sample is a single-cell metric defined as the percentage of cells co-secreting 2+ proteins (polyfunctional) in a sample, multiplied by the average mean fluorescence intensity (MFI) of the proteins secreted by these polyfunctional single cells. The PSI of the secretors was calculated as the percentage of polyfunctional cells out of the total secretors of a sample multiplied by the MFI of the proteins secreted by these polyfunctional cells. Advanced visualization algorithms included polyfunctional heatmap, polyfunctional activation topology-principal component analysis (PAT-PCA) and t-SNE were performed using the IsoSpeak software suite.

References:

1.   Xue Q, Bettini E, Paczkowski P, et al. Single-cell multiplexed cytokine profiling of CD19 CAR-T cells reveals a diverse landscape of polyfunctional antigen-specific response. *Journal for ImmunoTherapy of Cancer.* 2017;5(1):85.

2. Bowman N, Liu D, Paczkowski P, et al. Advanced Cell Mapping Visualizations for Single Cell Functional Proteomics Enabling Patient Stratification. *Proteomics.* 2020:e1900270.

3. Fousek K, Watanabe J, Joseph SK, et al. CAR T-cells that target acute B-lineage leukemia irrespective of CD19 expression. *Leukemia.* 2020.

4. Li D, Li N, Zhang YF, et al. Persistent Polyfunctional Chimeric Antigen Receptor T Cells That Target Glypican 3 Eliminate Orthotopic Hepatocellular Carcinomas in Mice. *Gastroenterology.* 2020;158(8):2250-2265.e2220.

5. Parisi G, Saco JD, Salazar FB, et al. Persistence of adoptively transferred T cells with a kinetically engineered IL-2 receptor agonist. *Nat Commun.* 2020;11(1):660.