Protocols

Tissue reservoirs of antiviral T cell immunity in persistent human CMV infection, Gordon et al., 2017, J Exp Med. 2017 Mar 6;214(3):651-667. doi: 10.1084/jem.20160758. Epub 2017 Jan 27.

Acquisition of human tissues

Human tissues were obtained from deceased (brain dead) organ donors at the time of organ acquisition for lifesaving clinical transplantation through an approved protocol and Material Transfer Agreement (MTA) with LiveOnNY. Organ donors were free of chronic disease and cancer and negative for HIV, hepatitis B, and hepatitis C. The study does not qualify as human subjects research, as confirmed by the Columbia University Institutional Review Board, as tissue samples were obtained from deceased individuals. Donor HLA type, and CMV and EBV serology were available from all donors.

Lymphocyte isolation from human lymphoid and non-lymphoid tissues

Tissue samples were maintained in cold saline and brought to the laboratory within 2–4 h of procurement from the donor. Samples were rapidly processed using enzymatic and mechanical digestion to obtain lymphocyte populations with high viability as described in detail (Sathaliyawala et al., 2013;Thome et al., 2014, 2016). Lymphocytes were isolated from blood using lymphocyte separation media (CellGro; Cell-Genix) and ACK lysis buffer as previously described (Sathaliyawalaet al., 2013). Lymphocytes were either analyzed immediately or cryopreserved for future analysis.

Detection and analysis of CMV-specific T cells

HLA multimers reagents containing epitopes of CMV (CMV-multimers) were obtained from Proimmune and Immudex; the CMV A6801 tetramer was obtained from the National Institutes of Health tetramer core. Staining with multimers was done according to the manufacturer's protocols using HLA-A2 Negative Control (Proimmune) or Immudex Negative Controls. Only samples with >500 CD8+ T cells were included in the analysis. The level of detection of CMVmultimer+CD8+ T cells was 0.1–0.3% of CD8+ T cells. For CMV peptide stimulation, 1–3 × 10^6 mononuclear cells from blood or tissues were plated in 96-well tissue culture plates incomplete RPMI medium (RPMI-1640 containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin,and 2 mM l-glutamine (Sigma-Aldrich) containing 1 µg/ml HCMV pp65 peptide mix and 1 µg/ml HCMV pp65 peptidemix (PepMix HCMV; JPT Peptide Technologies) and incubated at 37°C for 6 h in the presence of BD GolgiStop (monensin).For intracellular staining, surface stained cells were fixed, washed, and resuspended in permeabilization buffer (BD) before staining with anti–IFN-γ.

Flow cytometry analysis

Fluorochrome-conjugated antibodies used for staining. For intracellular staining, surface stained cells were fixed, washed, resuspended in permeabilization buffer (eBioscience), and stained with antiperforin

antibodies.For T cell stimulation, 1–3 × 10^6 mononuclear cells from blood or tissues were plated in 96-well tissue culture plates incomplete RPMI medium containing phorbol-12-myristate-13-acetate (PMA; 50 ng/ml), ionomycin (1 mg/ml) andanti-CD107a and incubated at 37°C for 3 h in the presence of BD GolgiStop (monensin). The cells were washed with PBS and surface stained, fixed, and permeabilized as above, before staining with anti–IFN-γ and anti–IL-2 antibodies. For expansion of CMV-specific T cells, 1–3 × 10^6 mononuclear cells from blood or tissues were plated in 96-well tissue culture plates at 5 × 105/ml in RPMI-1640, 10% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, and 100 µM β-mercaptoethanol in the presence of 0.3 µg/ml HCMV pp65 peptide mix (JPTPeptide Technologies). IL-2 100 U/ml was added on day 2and cells were analyzed at day 9 after stimulation. Stained cells were acquired on a 6-laser LSR II analytical flow cytometer(BD) in the CCTI flow cytometry core and analyzed using FlowJo software (Tree Star).

CMV serology

Serum CMV IgG avidity and CMV IgM testing were performed by Quest Diagnostics.

Detection of CMV genomes

Cells were thawed in IMDM with 2% FBS, counted with Trypan blue, and pelleted. Cells were lysed with 400 µl ZRDuet lysis buffer per 5 × 10^6 cells, and processed with the ZR-Duet DNA/RNA purification kit (Zymo Research). Each 20 µl qPCR reaction contained: 1× Roche Lightcycler480 Probes Hot-start Mastermix, 200 nM primers (Quick-LC purified primers; Eurofins MWG Operon), 100nM Roche UPL hydrolysis probe, and 700 ng template DNA. Cycling conditions on the Lightcycler 480-II qPCR cycler(Roche) were: Taq activation at 95°C for 10 min, followed by 55 cycles of (95°C for 15 s; 60°C for 45 s), using the following primers:

CMV long noncoding RNA β2.7:

- forward, 5’-TGT TCT TCT GGT TCA TTT CCT ATG-3’
- reverse, 5’-CGT GTC CGG TCC TGA TTC-3’
- probe, GGC TGC TG

CMV UL69:

- forward, 5’-CCT ACG ACT TTC GGT TCTTCTC-3’
- reverse, 5’-CGT CCA GTT CGT CGT CAA TAA-3’
- probe, CCT CAG CC)

cellular genomic RNase P primers:

- forward, 5’-GAC GGA CTG CGC AGG TTA-3’
- reverse, 5’-CCA TGC TGA AGT CCC ATGA-3’
- probe, CAG CTC CC

These RNA β 2.7 and UL69 primers were chosen and optimized to ensure that they function with maximum specificity and sensitivity. The genomes of 100 clinical CMV strains were aligned to generate

multiple primers to amplify the most highly conserved regions across the CMV genome. Primers were tested for on sequences from uninfected cells with low levels of “spiked” viral genomes, and primers for were chosen based on specificity and sensitivity that exceeded that used in clinical protocols (Binnicker and Espy, 2013). Total cellular genomes were quantitated using a standard curve of known cellular genome concentration amplified using primers for human RNase P: RPP30. CMV genomes were quantitated using a standard concentration curve of SwaI-linearized HCMV BAC genomes (strain TB40/E, available from Gen-Bank under accession no. EF999921.1), diluted in 5 ng/µl sonicated salmon sperm carrier DNA (Ambion). Each sample was run in 10–13 replicates and scored as “detected” (CMV genome target[s] detected in any replicate) or “not detected”(no CMV genome targets detected in any replicate). The limit of detection for the RNase P and β2.7 genes was 1–2 copies and 100 genomes, respectively. Negative controls included water, 5 ng/µl sheared salmon sperm carrier DNA, or genomes extracted from cultured uninfected primary cells (MRC-5; CCL-171; ATCC).