Protocols for Publication: Flow Cytometry-Based Immune Assessment

PUBLICATION POLICY: We kindly ask you to acknowledge the Core in all the publications derived through the use of the facility or in collaboration with the staff.

Immunophenotyping: This assay was performed by the UCLA Immune Assessment Core. 0.5 $X10^6$ cells per cocktail were stained for 20 min at 4°C with the 1:1000 LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies). Cells were then washed once with FACS buffer (PBS supplemented with 2% FBS) and stained with fluorochrome-conjugated antibodies (BioLegend, BD Biosciences, eBioscience) for 20 min at 4°C. Finally, the cells were washed and re-suspended in 200 µl FACS buffer. At least 100,000 lymphocyte events per sample were acquired using DIVA 8.0 software on LSRFortessa Cell Analyzer (BD Biosciences). Data analysis was performed using FlowJo VX.0.7r2 (Tree Star) by gating on live cells based on forward versus side scatter profiles, then gating on singlets using forward scatter area versus height, followed by dead cell exclusion using Live/Dead exclusion stain, and then cell subset-specific gating.

Intracellular cytokine staining of antiviral lymphocytes: This assay was performed by the UCLA Immune Assessment Core. PBMCs were resuspended at $1x10^7$ viable cells/ml. 200 µl of cells were plated per well in 96-well plates. After overnight rest at 37°C, viral antigens were added with 10 µg/ml brefeldin A (Sigma) and the cells were incubated for 6-18 hours at 37°C. Cells were then washed with PBS and stained with 1:1000 LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies) for 20 min at 4°C. After washing with FACS Buffer (PBS supplemented with 2% FBS), cells were stained for surface markers for 20 min at 4°C, washed with FACS Buffer, and centrifuged at 1500 RPM for 5 min. Cells were incubated for 45 min at 4°C in the dark using FoxP3 fix/perm buffer (eBioscience) at 4°C in the dark, washed twice with perm buffer (eBioscience) and centrifuged at 2000 rpm for 6 min. Cells were then stained with cytokine-specific antibodies for 30 min at 4°C. Following two washes with perm buffer, cells were re-suspended in FACS buffer and 100,000 $CD3^+$ T cell events per sample were collected using DIVA 8.0 software on LSRFortessa Cell Analyzer (BD Biosciences). Data analysis was performed using FlowJo VX.0.7r2 (Tree Star) by gating on live cells based on forward versus side scatter profiles, then gating on singlets using forward scatter area versus height, followed by dead cell exclusion using Live/Dead exclusion stain, and then cell subset-specific gating.

Direct and indirect allo-stimulation: This assay was performed by the UCLA Immune Assessment Core. Cells were resuspended in 0.5 ml RPMI 1640, supplemented with 10% human AB serum, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin. 0.5 $X10^6$ PBMCs were stimulated with $1X10^6$ irradiated T cell depleted allo-APCs or allo-spleenocytes (direct allo-stimulation), or 4 µg of donor antigens (cell-free membrane fraction prepared from about 1 X 10^6 donor cells) (indirect allo-stimulation) for 15 hours at 37°C in a 5% CO_2 incubator in 48-well plates. Cells were then washed with PBS and stained with 1:1000 LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies) according to the manufacturer's instructions. After washing with FACS Buffer (PBS supplemented with 2% FBS), cells were stained for surface markers for 30 min, washed with 2 ml of FACS Buffer and centrifuged at 1500 RPM for 5 min. Cells were incubated for 40-50 min in Fix/perm buffer at 4°C in the dark, washed twice with perm/wash buffer and stained with cytokine-specific antibodies for 40-50 mins at 4°C. Following two washes with Perm/Wash buffer, cells were re-suspended in 300 µl FACS buffer and 100,000 $CD3^+$ T cell events per sample were collected using DIVA 6.0 software on LSRFortessa Cell Canlyzer (BD Biosciences). Data analysis was performed using FlowJo VX.0.7r2 (Tree Star) by gating on live cells based on forward versus side scatter profiles, then gating on singlets using forward scatter area versus height, followed by dead cell exclusion using Live/Dead exclusion stain, and then cell subset-specific gating.

Lymphocyte proliferation assay (LPA): This assay was performed by the UCLA Immune Assessment Core. PBMCs were isolated from whole blood using Ficoll-Paque PLUS (GE Life Sciences. Isolated cells were

washed with PBS, re-suspended in RPMI with 1% FBS and labeled with 15 µM CFSE (Life Technologies) for 10 min at 37°C. The reaction was then quenched with 10 volumes of complete media (RPMI 1640, supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin) for 10 min in the dark. Cells were then re-suspended in complete media for plating at 1 x 10^6 cells/ml. Cells were plated separately with mitogen and antigens and incubated for 4 days and 6 days, respectively. After the incubation, cells were stained with anti-CD3 and CD4 (BD Biosciences and Biolegend) for 20 min at 4°C in the dark. Cells were also stained for CD8 and CD19 (all mitogen- and PWM-stimulated samples, respectively). Cells were then washed and re-suspended with FACS buffer. At least 100,000 lymphocyte events per sample were collected using FACS DIVA 8.0 software on FACSCanto II or LSRFortessa Cell Analyzer (BD Biosciences). Data analysis was performed using FACS DIVA 8.0 software or FlowJo VX.0.7r2 (Tree Star).

Natural killer cell cytotoxicity (NKCC) assay: This assay was performed by the UCLA Immune Assessment Core. Effector PBMCs were isolated from whole blood using Ficoll-Paque PLUS (GE Life Sciences), washed with FACS buffer (PBS supplemented with 2% FBS), and re-suspended in complete media (RPMI 1640, supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin) at 5 x 10^6 cells/ml. Cultured 1 x 10^6 K-562 target cells were labeled with 1 µM CFSE (Life Technologies) for 10 min at 37°C, then quenched with 10 volumes of complete media (RPMI 1640, supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin) for 10 min in the dark. Cells were then re-suspended in complete media for plating at 1 x 10^5 cells/ml. Different E:T (effector:target) ratios were tested, ranging from 50:1 to 6:1. After 4 hours, cells are stained with Sytox Red (Lefie Technologies) and acquired using FACSDiva software on FACSCanto II (BD Biosciences). Data analysis is performed FACS DIVA 8.0 software or FlowJo VX.0.7r2 (Tree Star).

Granulocyte/monocyte oxidative burst assay: This assay was performed by the UCLA Immune Assessment Core. Heparinized whole blood was collected and cooled to 0°C for 10 min. The assay was performed using the PHAGOBURST kit.following manufacturer's recommendations (AlleleBiotec). Briefly, 100 µl of whole blood was incubated with 20 µl of wash solution (negative control), 20 µl of pre-cooled E. coli bacteria, 20 µl of chemotactic peptide (fMLP, low control), and 20 µl of phorbol 12-myristate 13-acetate (PMA, high control). Substrate (DHR) solution was then quickly added at 20 µl for and incubated for 10 min at 37°C for oxidization. Pre-warmed 1x lysing solution was then added at 2 ml per sample tube, vortexed, and incubated at room temperature for 20 min. The cells were washed with 3 ml wash solution DNA staining solution was added at 200 µl to each sample and incubated at 4°C for 10 min, protected from light. Samples were acquired within 30 min of staining incubation using FACSDiva software on FACSCanto II (BD Biosciences). Data analysis was performed FACS DIVA 8.0 software or FlowJo VX.0.7r2 (Tree Star).

Protocols for Publication: Real-Time Cell Lysis and Cell Proliferation

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Tumor specific Cytotoxic T lymphocyte or NK cell killing assay: This assay was performed by the UCLA Immune Assessment Core using the xCELLigence RTCA MP System. Tumor cells were seeded in an E-plate at 2 x 10^4 cells/well in triplicate in normal tissue culture medium and allowed to attach overnight. Cytotoxic T lymphocytes, NK cells, or PBMCs were then added to the well at 1:1, 2:1, 5:1 and 10:1 effector:target ratios. Electrical impedance in each well was monitored for 40 hours. Normalized cell index were plotted against incubation time. Lysis of tumor cells is indicated by reduction of cell index.

Cell proliferation and/or proliferation inhibition assay: This assay was performed by the UCLA Immune Assessment Core using the xCELLigence RTCA MP System. Adherent cells were seeded at various cell concentrations in an E-plate and allowed to attach overnight. Cell stimulants and/or proliferation inhibitor were added at different concentrations with proper negative and positive controls. Electrical impedance in each well was monitored for 40 hours. Normalized cell index were plotted against incubation time. Cell proliferation is associated with increasing cell index while proliferation inhibition is associated with decreased cell index compared to wells without proliferation inhibitors.

Protocols for Publication: Immunoassays

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Luminex xMAP® Immunoassay: This assay was performed in the UCLA Immune Assessment Core. Human 38-plex (or mouse 32-plex) magnetic cytokine/chemokine kits were purchased from EMD Millipore and used per manufacturer's instructions. Briefly, 25 µl undiluted (or for mouse, 1:2 diluted) samples were mixed with 25 µl magnetic beads, and allowed to incubate overnight at 4°C while shaking. After washing the plates twice with wash buffer in a Biotek ELx405 washer, 25 µl of biotinylated detection antibody was added and incubated for 1 hour at room temperature. 25 µl streptavidin-phycoerythrin conjugate was then added to the reaction mixture and incubated for another 30 minutes at room temperature. Following two washes, beads were resuspended in sheath fluid, and fluorescence was quantified using a Luminex 200™ instrument.

ELISA: This assay was performed in the UCLA Immune Assessment Core. The kit was purchased by [insert company name here] and run following manufacturer's recommendations.