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Identification and isolation of antigen-specific cytotoxic T lymphocytes with an automated microraft sorting system

Detailed Materials and Methods

Materials

The following materials were obtained from Sigma-Aldrich Corp. (St. Louis, MO): γ-butyrolactone (GBL), propylene glycol monomethyl ether acetate, iron(II) chloride tetrahydrate, iron(III) chloride anhydrous, iron(III) nitrate nonahydrate, ammonium hydroxide solution, oleic acid, toluene, styrene, acrylic acid, benzoyl peroxide, gelatin from bovine skin (type B), dimethyl sulfoxide (DMSO) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). SU-8 photoresist was purchased from MicroChem Corp (Newton, MA). Sylgard 184 silicone elastomer kit (PDMS) was acquired from Dow Corning (Midland, MI). Polycarbonate and Delrin were purchased from McMaster-Carr (Elmhurst, Illinois). NdFeB (Grade N52) permanent magnets were obtained from K&J Magnetics, Inc. (Plumsteadville, PA). Ethanol was purchased from Decon Labs (King of Prussia, PA). The following items were obtained from Thermo Fisher Scientific (Waltham, MA): Dulbecco's phosphate buffered saline (DPBS) (Gibco), CTS AIM V media (Gibco), CellTrace Far Red DDAO-SE (Molecular Probes), Cell Tracker Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes), LIVE/DEAD Fixable Violet Dead Cell Stain (Molecular Probes), SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase (Life Technologies), nuclease-free water (Ambion) and RNase Inhibitor (Ambion). Ficoll-Paque PLUS was from GE Healthcare (Little Chalfont, United Kingdom) and Lymphoprep was from Axis-Shield PoC AS (Oslo, Norway). Interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-15 (IL-15), interleukin-21 (IL-21), Fms-related tyrosine kinase 3 ligand (Flt3-ligand), stem cell factor (SCF) and tumor necrosis factor alpha (TNF-α) were purchased from Peprotech. Granulocyte-macrophage colony stimulating factor (GM-CSF; Leukine®) was from Sanofi (Paris, France) and interferon alpha (IFN-α; Intron® A) was from Schering Corporation (Kenilworth, NJ).

GemCell Human Serum AB was purchased from Gemini Bio-Products (West Sacramento, CA). Purified anti-human CD3 antibody (OKT3 clone) was purchased from eBioscience, Inc. (San Diego, CA). Costar 6 and 24 well ultra-low attachment plates as well as all tissue culture flasks were manufactured by Corning, Inc. (Corning, NY). MACS BSA Stock Solution, CD34 Microbead Kit UltraPure human, $CD8^+$ T cell Isolation Kit, autoMACS rinsing solution, preseparation filters and columns for magnetic separation were purchased from Miltenyi Biotec (Auburn, CA). PE-Cy7-anti-CD8, FITC-anti-CD4, FITC-anti-CD14, FITC-anti-CD16 and FITC-anti-CD19 antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). APC-labeled influenza M1 tetramer and APC labeled negative tetramer were purchased from MBL International (Woburn, MA). The influenza M1 peptide (M1p, GILGFVFTL) was synthesized by Peptide 2.0 (Chantilly, VA), and the PR1 peptide (PR1, VLQELNVTV) was synthesized by Bio-Synthesis, Inc. (Lewisville, TX). Ten percent ultrapure methanol-free formaldehyde was purchased from Polysciences, Inc. (Warrington, PA). The RNeasy Micro Kit, OneStep RT-PCR Kit and PCR Purification Kit were purchased from Qiagen (Hilden, Germany). The Fast Start High Fidelity PCR System was purchased from Roche Diagnostics (Basel, Switzerland). All other reagents were from Thermo Fisher Scientific (Waltham, MA).

Device preparation

Fabrication of microraft arrays

Microraft arrays, consisting of PDMS microwells containing releasable, magnetic elements were created using previously described methods.[1-3] Briefly, photolithography was used to fabricate an array of SU-8 pillars (200 × 200 × 200 μm, spaced 100 μm apart) on a glass slide and used as a template to generate a PDMS microwell array. Each array contained 4900 microwells (70 × 70

array). Each microwell array was coated with a solution of poly(styrene-*co*-acrylic acid) (PS-AA) in GBL co-mixed with γFe_2O_3 nanoparticles and degassed under vacuum to remove air bubbles.[2] The array was submerged in a jar of the same solution and pulled out by a stepper motor at a rate of 1 mm/s. This procedure of dip-coating resulted in a droplet of solution deposited within each microwell. The array was baked at 95°C for 12 h period producing a hard, concave magnetic element, or microraft, within each microwell. The microraft array was subsequently adhered to a polystyrene cassette using PDMS.

Microscopy setup

An MVX10 MacroView upright microscope (Olympus, Center Valley, PA) equipped with an ORCA-Flash 4.0 CMOS camera (Hamamatsu, Bridgewater, NJ) was used to acquire bright field and fluorescence micrographs. The MVX10 MacroView was fitted with a PS3H122 Motorized Focus Drive and a H138A motorized XY translational stage manipulated by a ProScan H31 Controller and a PJ2J100 joystick (Prior Scientific Inc., Rockland, MA). A Lambda 10-3 optical filter changer was utilized to control an emission filter wheel (LB10-NWE), an excitation filter wheel with SmartShutter (LB10-NWIQ) and a stand-alone SmartShutter shutter (IQ25-SA) (Sutter Instrument, Novato, CA). A sedat filter set (89000 – ET – Sedat Quad; Chroma Technology Corp, Bellows Falls, VT) containing 5 excitation filters (350 ± 50 nm, 402 ± 15 nm, 490 ± 20 nm, 555 ± 25 nm, 645 ± 30 nm), 4 emission filters (455 ± 50 nm, 525 ± 36 nm, 605 ± 52 nm, 705 ± 72 nm) and a multiband dichroic enabled measurement of fluorescence in the blue, green, red and far red wavelengths. A lumen 200 arc lamp (Prior Scientific Inc., Rockland, MA) provided light for fluorescence excitation. All microscopy equipment was controlled by custom software written in MATLAB (MathWorks, Natick, MA) and using a Micro-Manager (Open Imaging, San Francisco, CA) core.[4, 5] A custom-made incubator was made to fit around the

microscopy set-up to regulate temperature, humidity and CO_2 concentration during time-lapse microscopy experiments.

Preparation of microraft arrays for the cytotoxicity assays

Prior to experiments on the microraft array, the array was treated for 5 min in a plasma cleaner (Harrick Plasma, Ithaca, NY). The array was then washed with 100% ethanol and subsequently washed ×5 with PBS. The microraft array was coated in 0.1 wt% bovine gelatin in PBS and incubated at 37°C for ≥2 h. The gelatin solution was aspirated and the array was washed ×3 with PBS before plating cells.

Effector and target cell generation

Generation of dendritic cells (DCs) from $CD34^+$ progenitors

Dendritic cells were differentiated from $CD34^+$ cells using a modification of a previously described method.[6] De-identified cryopreserved leukapheresis products from healthy donors treated with G-CSF to mobilize $CD34^+$ cells were obtained from the Hematopoietic Progenitor Cell Laboratory at UNC Hospitals. On Day 1, a cryopreserved leukapheresis product was thawed, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS density gradient centrifugation, and $CD34^+$ cells were isolated using the CD34 Microbead Kit UltraPure (Miltenyi Biotec). $CD34^+$ cells were plated at 3 mL/well in 6 well ultra-low attachment plates at a concentration of 3×10^5/mL (3mL/well) in CTS AIM V media with 10% human AB serum (complete media, CM), supplemented with 800 U/mL GM-CSF, 100 ng/mL Flt3-ligand and 50 ng/mL SCF. Cells were incubated for 72 hours in a 5% CO_2 incubator at 37^{o}C. Once differentiation was initiated, immature DCs were handled with polypropylene transfer pipets (as polystyrene serological pipets could induce early maturation of the DCs). Cells

were maintained at a concentration of < 0.8 × 10^6/mL. On days 4 and 6, cells were counted, 500 µL of CM was added to each well to maintain a 3 mL volume, and fresh cytokines were added to final concentrations of 800 U/mL GM-CSF, 100 ng/mL Flt3-ligand, 50 ng/mL SCF and 500 U/mL IL-4. On days 8 and 10, cells were counted and split to maintain a concentration < 0.8 × 10^6/mL and 800 U/mL GM-CSF, and 500 U/mL IL-4 were added to each well. Immature DCs were cryopreserved on Day 12 in FBS with 10% DMSO.

After thawing, immature DCs were expanded in CM, GM-CSF and IL-4. Final differentiation of immature DCs into mature DCs was initiated 4 days prior to peptide-pulsing by plating the immature DCs at a concentration of 1.25 – 1.5 × 10^6/mL in fresh CM with GM-CSF, IL-4 and 20 ng/mL TNF-α. The following day, 20 ng/mL TNF-α was replenished. Forty-eight hours prior to peptide pulsing the cells, GM-CSF, IL-4 and TNF-α were replenished, and 1000 U/mL IFN-α and 1000 U/mL IL-6 were added to the cultures. TNF-α was added 24 hours later, and the cells were peptide-pulsed the following day.

For peptide-pulsing, matured DCs were pelleted and resuspended at a concentration of 1 × 10^6 live cells/mL in CM supplemented with GM-CSF, IL-4 and TNF-α. Peptides (M1p or PR1) were co-incubated with DCs at a concentration of 2 µg/mL of peptide and incubated for at least 18 hours. DCs were washed once with CM to remove excess peptide prior to use for T cell stimulation or in bulk cytotoxicity assays.

Generation of cytotoxic T lymphocytes (CTLs)

PBMCs were isolated from another $CD34^+$ leukapheresis product from the same donor using Ficoll-Paque PLUS. PBMCs were suspended at a concentration of 10 × 10^6/mL in CM and plated in two T175 culture flasks (5 × 10^8 cells/flask). Monocytes were allowed to adhere to the

flasks for 2 hours, and non-adherent cells (NADs) were collected. $CD8^+$ cells were isolated from 5×10^8 NADs by magnetic separation using the $CD8^+$ T cell isolation kit (Miltenyi Biotec).

The cytotoxic T lymphocyte (CTL) culture was initiated by incubating 1×10^7 $CD8^+$ T cells with 5×10^5 M1p pulsed DCs (20:1 CTL:DC ratio) in 10 mL CM supplemented with 30 ng/mL IL-21.[7] Cultures were incubated for 3 days without disturbing the co-incubated cells, after which the cells were fed with CM supplemented with 10 ng/mL IL-7 and 10 ng/mL IL-15 every 2 days, keeping the concentration of live cells at 1×10^6/mL. Conditioned media (media already in the culture) was kept as 1/3 of the final culture volume each time the cells were fed. CTLs were restimulated with DCs pulsed with 2 μg/mL M1p at a 50:1 CTL:DC ratio 11 days after initiation of the culture in media supplemented with IL-21, IL-7 and IL-15. After 3 days, cells were plated at a concentration of 1.25×10^6/mL and fed every 2 to 3 days with CM supplemented with IL-7 and IL-15. One hundred U/mL IL-2 was added 19 days after initiation of the culture.[7] CTLs were restimulated 21 and 34 days after culture initiation, and the CTLs were cryopreserved in aliquots 41 days after initiation of the culture.

Bulk T cell culture activity measurements

Tetramer analysis

For tetramer staining, 1×10^6 cells (bulk culture) or 1×10^5 cells (CTL clones) were washed with DPBS + 0.5% BSA and resuspended in 100 μL DPBS + 0.5% BSA. Ten μL of APC-labeled M1p/HLA-A*02:01 tetramer or negative tetramer was added to each sample. PE-Cy7-anti-CD8 antibody and a FITC labeled lineage mix of CD4, CD14, CD16 and CD19 antibodies were added, and the samples were incubated at 4°C for 30 minutes. The cells were washed and resuspended in DPBS + 0.5% BSA + 1 μg/mL DAPI. Sample acquisition was performed on a

FACSCanto flow cytometer (BD Biosciences) and data was analyzed using FlowJo version 7.6.5 (FlowJo LLC, Ashland, OR).

Bulk culture cytotoxicity assay

CTLs were restimulated with M1p pulsed DCs at a CTL:DC ratio of 50:1 four days prior to the cytotoxicity assay, left undisturbed for 3 days, and plated the day before the assay at a concentration of 1×10^6/mL in CM supplemented with IL-2, IL-7 and IL-15. Differentiated DCs were stained with 0.6 μM DDAO-SE in DPBS for 15 minutes at 37°C, followed by incubation in CM for 30 minutes, pelleted and resuspended in CM supplemented with GM-CSF, IL-4 and TNF-α. DCs were plated at a concentration of 1×10^6/mL in a 24 well low binding plate, and incubated overnight with M1p or PR1 peptide was added at a concentration of 2 μg/mL.

For the cytotoxicity assay, DCs were washed and resuspended in CM at a concentration of 0.5×10^6/mL. One hundred μL (50,000 DC target cells) was added to each assay well in a 96 well round bottom plate. CTLs were stained with 1 μM Cell Tracker Green CMFDA dye, following the manufacturer's instructions and resuspended in CM at a concentration of 1×10^6/mL. To initiate the cytotoxicity assay, CTLs were added to the 96 well plate with DCs at either a 1:1 ratio (50,000 CTLs/well) or a 2:1 ratio (100,000 CTLs/well). The final assay volume was 200 μL. Target cells were incubated without CTLs as a control. After a 6 hour incubation, cells were pelleted in the 96 well plate, washed once with DPBS, and resuspended in 200 μL DPBS with 1 μL LIVE/DEAD Fixable Violet Dead Cell Stain per 1 mL DPBS. Cells were incubated for 30 minutes at room temperature, washed with DPBS and fixed in 2% formaldehyde. Cells were analyzed on a Miltenyi MACSQuant flow cytometer (Miltenyi Biotec) with data analysis using FlowJo version 7.6.5.

Single cell cytotoxicity analysis

Restimulation of CTLs and isolation of $CD8^+$ cells prior to plating on the microraft arrays

Cryopreserved CTLs were thawed and restimulated with M1p pulsed DCs at a CTL:DC ratio of 50:1. After 3 days, $CD8^+$ T cells were isolated using the $CD8^+$ T cell isolation kit and plated at a concentration of 1×10^6 cells/mL in CM supplemented with IL-7, IL-15 and IL-2. $CD8^+$ T cells were plated on microraft arrays 2 to 3 days later.

Single T cell cytotoxicity assay

Autologous DCs were pulsed with either M1p or PR1 16-18 h prior to the cytotoxicity assay. Both sets of DCs were stained with 1 μg/mL Hoechst 33342 and 50 nM Sytox Green following the manufacturers' protocols prior to plating. Cells were deposited on microraft arrays in phenol-red free RPMI 1640 supplemented with 10% human AB serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 20 mM HEPES. For each cytotoxicity assay, two microraft arrays were prepared, and M1p pulsed DCs were added to one array while PR1 pulsed DCs were added to the second array at cell:microraft ratios of 30:1. Both arrays were centrifuged at $400 \times g$ for 4 min and the media was aspirated. $CD8^+$ T cells from the M1p culture were stained with 500 nM CellTracker Deep Red and 50 nM Sytox Green following manufacturer's protocols and were added to both arrays at a cell:microraft ratio of 1:1. The arrays were again centrifuged at $400 \times g$ for 4 min and the media was aspirated. Culture media containing 50 nM Sytox Green was placed over each array and the array covered with a cover slip (50 mm diameter). Arrays were then placed on the microscopy stage and imaged every 30 min for 6 h. During microraft array imaging, an incubator housing the microscope was maintained at 37°C, 60% relative humidity and 5% CO_2.

Image acquisition

A customized MATLAB program controlled the microscope to acquire bright field and fluorescence images of the microraft array at designated time points. A graphical user interface (GUI) was designed to permit input of user-selected parameters such as fluorescence channel selection, camera exposure, microraft array geometry, microraft array numbers and time-lapse conditions.[8, 9] A 5% overlap between imaged fields of view was used in all experiments.

Image processing and analysis

In parallel with the image acquisition, the MATLAB program processed and analyzed the acquired images. The bright field images were used to identify individual microraft locations. Flat-field correction was performed on each bright field image to correct for uneven illumination intensity.[10] Otsu's method was then used to calculate a threshold for each image, and the image was converted to binary.[11] Due to the optical properties of microrafts, the borders of each microraft appear dark in bright field micrographs enabling facile identification of individual microraft borders. The binary images were further processed to fill the interior of each microraft border and remove any resulting objects that were larger than 1.5× or smaller than 0.5× the microraft size eliminating debris. The positions of all microraft were identified at each time point.

Fluorescence images were processed in a similar fashion. A top hat filter was applied with a disk structuring element having a radius twice the nominal diameter of a cell to remove background fluorescence.[12, 13] Otsu's method was utilized to determine a threshold for each image and the threshold was applied to convert each image to binary.[11] The pixel locations and intensity values of each fluorescence image were determined for each individual microraft. Additionally, a watershed algorithm was applied to each image in the far red channel

(corresponding to cells stained with CellTracker Deep Red) to count the number of individual cells on each microraft.[14-16]

Selection of cells for release

The locations of individual microrafts were determined using the image processing and analysis software described above. Microrafts containing a single $CD8^+$ cell were identified from the watershed algorithm using CellTracker Deep Red fluorescence as a marker of $CD8^+$ cells. These microrafts were sorted based on the increase in Sytox Green fluorescence intensity. Automatically identified microrafts were re-screened after gelatin encapsulation (see below) to ensure that a single CellTracker Deep Red-positive cell ($CD8^+$ cell) remained on the microraft.

Single cell transfer and analysis

Microraft/cell release and transfer to a 96-well plate

Upon completion of the cytotoxicity assay, the arrays were overlaid with a thin layer of gelatin as described previously.[1] The gelatin diffused into the cup-shaped microrafts to encapsulate the nonadherent cells ensuring that the cells remained on their microrafts during microraft release and retrieval. Briefly, the culture media above the array was replaced with a thin layer of 5 wt% bovine gelatin in PBS and the array was centrifuged at 400 × *g* for 4 min. The array was then incubated for 10 min at 37°C. The array was washed and then incubated at 4°C for 5 min to solidify the gelatin within the microrafts and cold (4°C) culture media was overlaid onto the arrays. For these experiments, the incubator surrounding the microscope was cooled to 24°C just prior to gelatin overlay of the array.

Microraft isolation was performed by actuating a small needle (10 μm tip, 100 μm base, 5 mm long) to puncture the PDMS of the microraft array and eject individual microrafts as

described previously.[1-3] Released rafts were then captured by a magnetic wand mounted on a computer controlled 3-axis motor and deposited into a 96-microwell plate as described previously.[1]

Preparation of feeder cells for T cell expansion

PBMCs were isolated from 3 different buffy coats using Ficoll-Paque PLUS or Lymphoprep, diluted to 2×10^6/mL in cold DPBS, and irradiated at 35 Gy in an RS 2000 Biological Irradiator (Rad Source Technologies, Inc., Suwanee, GA). After irradiation, equal numbers of PBMCs from each buffy coat were combined in CM supplemented with 20 ng/mL IL-7, 20 ng/mL IL-15 and 200 U/mL IL-2. Irradiated PBMCs were plated at a concentration of 1×10^5/well in 96 well round bottom plates. Cells were plated in 200 μL/well for collecting cells released from the microraft array and 100 μL/well for the parallel limiting dilution assay.

Limiting dilution

$CD8^+$ CTLs, prepared as described for the microraft array cytotoxicity assays, were serially diluted 10-fold in CM from a concentration of 500,000 cells/mL to 5 cells/mL. One hundred μL of the 5 cells/mL dilution was added to each well of a 96 well plate containing irradiated feeder cells, for an expected concentration of 0.5 CTLs/well.

T cell expansion

Microrafts containing selected CTLs were isolated as described above in to 96-well plates containing feeder cells. Cells released from the microrafts and cells plated by limiting dilution were expanded in parallel using minor modifications of the protocol described by Perna el al.[17] OKT3 antibody was added to a final concentration of 50 ng/mL 2 to 3 days after plating the cells. Cells were fed weekly by adding 20 μL of CM supplemented with a 10× concentration of cytokines and OKT3 antibody. Expanding single cell clones were transferred from the 96 well

plates between days 11 and 19 after plating. Clones were transferred to 1 mL of CM supplemented with IL-2, IL-7 and IL-15 in a 24 well plate, and tetramer staining was performed 2 days later. Tetramer positive clones were expanded with a mix of irradiated PBMCs and an EBV-immortalized lymphoblastoid cell line (EBV-LCL) as feeder cells.[17] Cryopreserved PBMCs from the 3 buffy coats used for the feeder plates were thawed and resuspended in cold DPBS at a concentration of 2×10^6/mL. EBV-LCLs (generated by immortalizing human B cells with Epstein Barr Virus (EBV) at the UNC Lineberger Tissue Culture Facility) were resuspended in cold DPBS at a concentration of 1×10^6/mL. PBMCs and EBV-LCLs were irradiated at 35 Gy and resuspended in CM supplemented with IL-2, IL-7, IL-15 and OKT3 antibody. 2×10^6 PBMCs and 0.15×10^6 EBV-LCLs in 2 mL of media were added per well to 3 wells of a 12 well plate, and the expanding CTL clones were pelleted and added to the wells. CTLs were fed 4 days later by pelleting and resuspending in 2 mL CM supplemented with IL-2, IL-7, IL-15. Three days later, $CD8^+$ cells were isolated using Miltenyi's $CD8^+$ T cell isolation kit, and cell pellets were frozen at -80°C for TCR α and β chain CDR3 sequencing.

Analysis of TCR α- and β-chain CDR3 sequences

RNA was isolated from approximately 22,500, 150,000 and 60,000 cells from the B8, D10 and F9 $CD8^+$ T cell clones, respectively, using Qiagen's RNeasy Micro Kit and stored in aliquots at -80°C. Analysis of TCR α- and β-chain rearrangements was performed using multiplex RT-PCR as described by Kim et al.[18] which is a modification of the method described by Seitz et al.[19] Briefly, an aliquot of RNA was thawed, and the initial RT-PCR reaction was performed using the OneStep RT-PCR kit.[18] TCR β-chains were amplified from 1 μL of the RT-PCR product in a run-off reaction, followed by semi-nested PCR using the Fast Start High Fidelity PCR System. Reactions that produced PCR products visible by agarose gel electrophoresis were purified using

the Qiagen PCR Purification Kit and sequenced using the UP primer described by Kim et al.[18] TCR α-chains were amplified by nested touchdown PCR using 1 μL of the RT-PCR reaction as previously described using the Fast Start High Fidelity PCR System.[18] PCR products were analyzed by agarose gel electrophoresis, purified using the Qiagen PCR Purification Kit, and sequenced using the Cα-in primer.[18] Sequencing was performed at the UNC Genome Analysis Facility (University of North Carolina, Chapel Hill, NC), and sequences were analyzed to determine the V and J alleles as well as the CDR3 sequence using IMGT/V-Quest (http://www.imgt.org/IMGT_vquest/vquest).[20, 21] One clone (F9) did not produce readable sequences using the protocol described above, possibly due to poor quality RNA. The TCRβ-chain rearrangement of this clone was analyzed using a modification of another multiplexed RT-PCR protocol for single cell TCRβ analysis.[22] Briefly, an aliquot of RNA was thawed, and multiplex RT-PCR was performed in a 50 μL reaction using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase, 10 U RNase Inhibitor, and 2.8 μL of a mix of 26 primers (external primer set; final concentration of 0.21 μM per primer).[22] PCR products were purified using the Qiagen PCR purification kit, analyzed by agarose gel electrophoresis and sequenced using the internal hCbi primer.[22] Sequencing and sequence analysis were performed as described above.

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