Supporting Information for

On-chip activation and subsequent detection of individual antigen-specific T cells

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Materials and Methods

Reagents. HL-1 medium was obtained from Lonza (Walkersville, MD). Human male AB serum was purchased from Omega Scientific (Tarzana, CA, USA) and heat-inactivated. Monoclonal antibodies certified for ELISpot were used to capture IFN-γ, and IL-17 (Pierce Endogen, Rockford, IL, USA or eBioscience, San Diego, CA, USA). Polyclonal goat-anti-human antibodies were used to capture IgG (Zymed, CA, USA). For detection of IFN-γ and IL-17, monoclonal antibodies (Endogen or eBioscience) were labeled by conjugating the antibodies with NHS ester-activated fluorescent dyes, and purified by spin column (Invitrogen, Carlsbad, CA, USA). The average degree of labeling with the commercial kits was 3-4 dyes per antibody. Anti-CD3 (OKT3, ATCC) and anti-CD28 (clone 28.2, BD Biosciences, San Jose, CA) monoclonal antibodies were biotinylated as needed according to manufacturer's instructions (Pierce Endogen). Polydimethylsiloxane (PDMS) elastomeric stamps were prepared using Sylgard 184 obtained from Dow Corning (Midland, MI, USA). Unconjugated streptavidin was obtained from Rockland or Invitrogen.

HLA DR1*0401 monomers/tetramers. The construction of the expression vectors used to generate soluble HLA-DR*0401 molecules has been described previously[1] (Benaroya Research Institute, Seattle, WA). Briefly, chimeric cDNA was generated from the extracellular coding regions of the DRB1*401, then DRA1*0101 chains were attached to leucine zipper motifs, followed by a site-specific biotinylation sequence on the DRβ chain. These cDNAs were subcloned into Cu^{2+} inducible Drosophila expression vectors, then co-transfected into Schneider S-2 cells. Drosophila cells were cloned by limiting dilution, and then screened for MHC class II molecules. The MHC class II monomers were purified, biotinylated, and loaded with peptides (5:1 peptide/MHC molar ratio) at pH 6.0. The Influenza A hemagglutinin p306-318 (PKYVKQNTLKLAT) and myelin oligodendrocyte glycoprotein (MOG) p97-109 (E107S) (TCFFRDHSYQEEA) were used as cognate peptides. Tetramers were prepared as needed by mixing a 4:1 solution of biotinylated monomers with appropriate streptavidin reagents (Invitrogen or Beckman Coulter).

Human peripheral venous blood. Peripheral venous blood was obtained from healthy subjects in compliance with institutional review board protocols. PBMCs were separated using density centrifugation on Ficoll-HyPaque (GE Healthcare). PBMCs were frozen at a concentration of 1-3x10^7/ml in 10% dimethylsulfoxide (Sigma-Aldrich)/90% fetal calf serum (Atlanta Biologicals). After thawing the PBMCs were washed in phosphate buffered saline (PBS).

Antigen-specific T-cell Clones. The MOG-reactive T cell clone used in this study was derived from single-cell sorting of MOG p97-109-HLA DRB1*0401 class II tetramer (TMr) positive CD4+ T cells by FACS as previously accomplished for an influenza peptide[1]. Briefly, peripheral blood mononuclear cells from an HLA DRB1*0401 +Multiple Sclerosis patient were loaded with MOG peptide (10 μg/ml of MOGp 97-109) and cultured 14 days in the presence of IL-2. Cells were stained with PE-labeled MOG-p97-109 peptide loaded TMr and fluorochrome-labeled anti-CD25 and anti-CD4 mAbs. CD4+CD25+TMr cells were single-cell sorted into 96-well plates using a FACSAria cell sorter (BD Immunocytometry Systems, San Jose, CA, USA). Clones thus obtained were expanded for 18 days by stimulation with irradiated allogenenic PBMCs, 5 μg/ml phytohemagglutinin-P (Remel, Lenexa, KS), and 10 U/ml IL-2 (Tecin, NCI, Frederick, MD). The HA-reactive T cell clone was produced in a similar manner using HA peptides (HA p306-318) and appropriate tetramers. Expanded clones were frozen at a concentration of 1-3x10^7/ml in 10% dimethylsulfoxide (Sigma-Aldrich)/90% fetal calf serum (Atlanta Biologicals).

Bulk stimulation of total PBMCs. A 96-well round-bottom plate (Costar 3799, Corning Inc, Corning, New York, USA) was coated with streptavidin (200 μg/mL) for 2 h, rinsed, coated with biotinylated anti-CD3 (OKT3, 1-10 μg/ml as specified) and anti-CD28 (1 μg/ml) for 2 h at 37˚C, and then briefly washed with PBS. A suspension of total PBMCs was loaded at a concentration of 250,000/well in HL-1 complete media + 5% Human serum.

Preparing Poly-lysine glass slides. Poly-L-lysine slides were prepared according to published protocols available online (http://cat.ucsf.edu/pdfs/PolylysineSlides.pdf). Briefly, 3"×1" glass slides

(Corning) were cleaned in 2.5 M NaOH in 60% ethanol for 2 h. After thoroughly washing with deionized water, slides were submerged in 0.001% poly-L-lysine solution (diluted in 0.1×PBS) for 1 h. Coated slides were further washed with water, dried, and stored in a desiccator until use.

Immobilization of anti-cytokine antibodies on poly-lysine glass slides. Capture antibodies were immobilized on glass slides functionalized with poly-lysine for 2 h at 25°C (25 μg/mL total protein concentration in a buffer comprising 50 mM sodium borate, 8 mM sucrose, and 50 mM NaCl (pH 9.0)).[2] The slides were blocked with bovine serum albumin (1% w/v in PBS) for 1 h at at 25°C, washed three times with PBS, dipped in water, and spun dry.

Preparation of microwell arrays. Dense arrays of microwells were fabricated by casting poly(dimethylsiloxane) (PDMS) against silica wafers bearing arrays of SU-8 posts defined by photolithgraphy.[3] The PDMS stamps were designed to fit on a 3"x1" glass slide, and were approximately 20x60 mm in size. Each array comprised 24 x 72 blocks of microwells (50x50x50 μm^3) (84,672 total wells). Immediately prior to use, the surface of the PDMS was oxidized using a plasma cleaner/sterilizer (PDC-32G, 100W Harrick Plasma, USA) for 30 s, and then immersed in sterile PBS.

Functionalization of PDMS stamps for on-chip activation. The oxidized PDMS arrays were functionalized by coating their surface with streptavidin (200 μg/ml in PBS) for 2 h at 37°C. Then, the PDMS stamps were coated with either biotinylated OKT3 (10 μg/ml), or biotinylated MOG p97-109 and HA p306-318 loaded HLA DRB1*0401 monomers (10 μg/ml) at 4°C for overnight. Finally, the PDMS stamps were coated with biotinylated anti-CD28 Ab (1 μg/ml) at 37˚C for 2 h. The functionalized PDMS stamps were blocked with 0.5% (w/v) bovine serum albumin (in PBS) for 30 min at 25°C before loading cells.

Single-cell stimulation of total PBMCs. A suspension of total PBMCs was diluted to $1x10^5$ cells/ml in serum-containing media. 0.5-1 ml of the suspension was dispensed onto OKT3-functionalized PDMS stamps. The cells were allowed to settle for 5 to 10 min, and then, the excess suspension was aspirated from the surface. Visual inspection of the slabs by microscopy confirmed that the wells contained ~1-2 cells/well with a loading efficiency of 50-70%. The stamps with loaded cells were cultured in HL-1 complete media, supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (Lonza, Walkersville, MD, USA), and 5% heat-inactivated human serum for 6, 18, 48 or 72 h.

Chip stimulation of MOG/HA clones. MOG/HA clones were thawed, diluted to $1x10^5$ cells/ml in serum-containing media, and 0.5-1 ml cell suspension was dispensed onto the surface of MOG/HA DRB1*0401 monomer-functionalized PDMS stamps. The cells were allowed to settle for 5 to 10 min, and then, the excess suspension was aspirated from the surface. Visual inspection of the slabs by microscopy confirmed that the wells contained ~1-2 cells/well with a loading efficiency of 50-70%. The array of microwells then was incubated in HL-1 complete medium at 37°C for 6, 18, or 42 h.

Microengraving. The experiments were performed as previously described,[4, 5] with some modifications as noted. After aspirating excess media from the surface of the array, a PDMS stamp loaded with cells was placed face down on a glass slide bearing immobilized capture anti-cytokine Abs. Then, the assembly was clamped together under light pressure in a hybridization chamber (Die-Tech DT-1001, San Jose, CA, USA), and incubated for 2 h at 37°C. After incubation, the glass slide was removed and placed into a blocking buffer (0.5% BSA, 0.01% Tween 20 in PBS) for 1 h at 25°C; the array of microwells was stored in PBS at 4°C for subsequent imaging (see below).

Imaging and analysis of printed microarrays. After blocking, the glass slides bearing the captured cytokines were washed with PBS for three times, dipped in DI water, and dried by centrifugation. Appropriate antibodies conjugated to fluorescent dyes (IFNγ-Alexa555 and IL-17-Alexa488) (2 μg/mL each) were applied to the slide for 30-60 min at 25°C in the dark. After incubation, the slides were washed with 0.05% Tween 20 in PBS three times, PBS three times, dipped in water, and then spun dry. Images of the microarrays were collected on a commercial microarray scanner (Genepix 4200AL,

Molecular Devices), and analyzed using the accompanying software (Genepix Pro 6.1, Molecular Devices) to extract the median fluorescent intensities of each spot within the array.

Imaging and analysis of cell-loaded arrays. After printing, cells in microwells were stained on-chip for enumeration by fluorescence imaging. The cells in the microwells were fixed with 70% ethanol for 20-30 min. The arrays were then washed with PBS one time, and stained with Hoechst 33342 (1 μM in PBS) for 15 min at 25 ℃. The arrays were rinsed with PBS three times, and then imaged on an automated inverted epifluorescence microscope (Zeiss). The arrays were mounted face-up on the microscope with a coverslip placed on top of the array. Transmitted light and epifluoresence micrographs were collected block-by-block (7x7 microwells per block). The resulting collection of images was analyzed using a custom software program (MabAnalyze, Dr. Bjorn Nilsson, Eli and Edythe L. Broad Institute) to determine the number of cells present in each well.

Data analysis. The data extracted from the printed arrays of cytokines were aligned with the data on the numbers of cells per well extracted from the microscopy data in Excel (Microsoft). The frequency of events was determined from the number of cytokine-positive spots on the printed array, and the numbers of wells containing a cell. Error bars indicated on plots represent the range of frequencies measured from three independent experiments.

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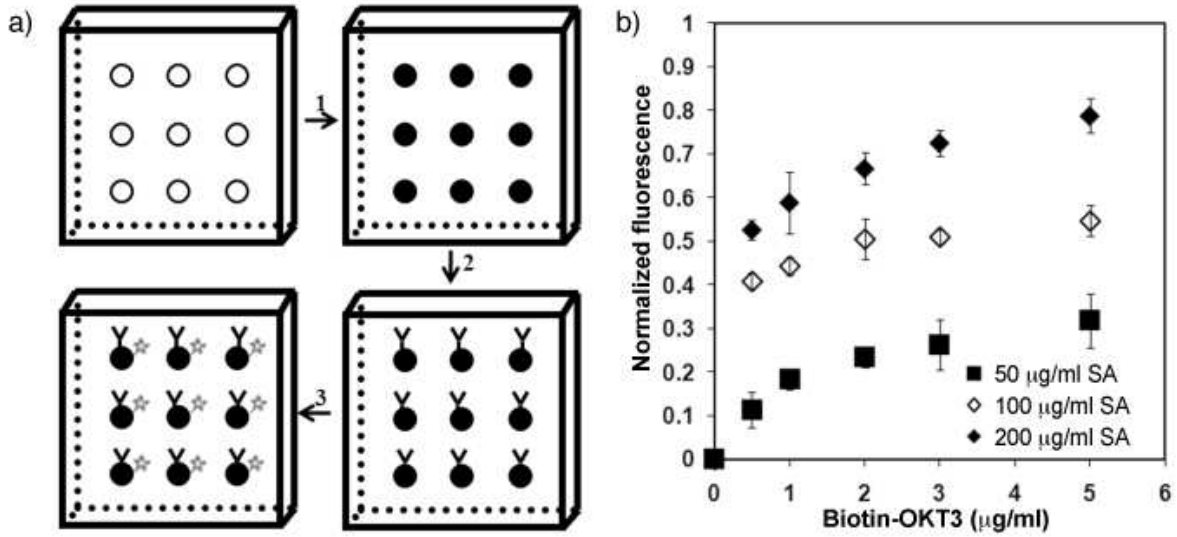

Figure S-1. I**mmobilization of streptavidin and biotinylated proteins.** A) Schematic illustration of the process used to generate microarrays of streptavidin (SA) and biotinylated antibodies on a flat slab of PDMS; these arrays were used to assess the relative amounts of proteins immobilized on an oxidized stamp. 1. 200 µL of a solution of SA diluted in PBS was deposited uniformly on the surface of a slab of PDMS, and allowed to incubate for 2 h at 37°C. The surface was then washed with PBS three times. 2. Solutions of biotinylated-OKT3 at different concentrations was arrayed manually onto the slab bearing SA and incubated for 12 h at 4° C in a humidified chamber. The slab was then washed with PBS three times. 3. A solution of fluorescently-labeled SA (Alexa 647, Invitrogen) (2 µg/mL) was applied to the slab uniformly to label the immobilized biotinylated-OKT3. (b) Plot of the relative fluorescent intensities measured from a series of arrays prepared with three different concentrations of SA (50, 100, and 200 µg/mL) and a range of concentrations of biotinylated OKT3. Data points represent an average of three independent determinations, and error bars indicate the range of measured values. The fluorescence measurements were recorded within the linear range of the 16-bit CCD employed, and all measured fluorescence intensities were below the upper limit of detection (a normalized value of 1 here).

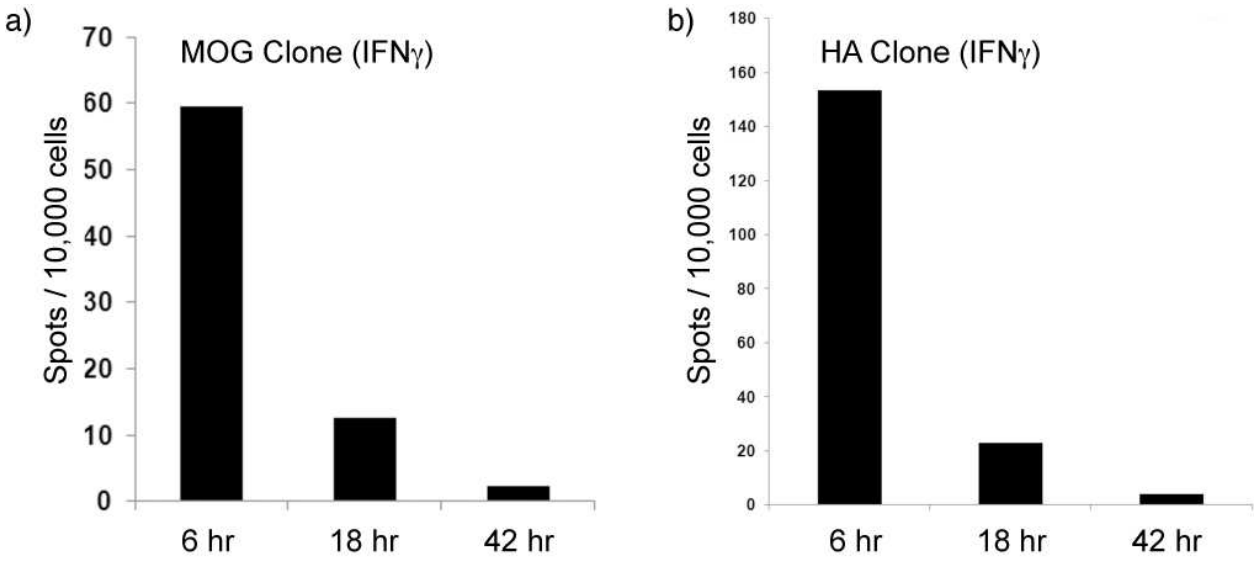

Figure S-2 Specific activation of HA-reactive T cell clone as a function of time. Plots of the frequencies of IFNγ-secreting (a) MOG-specific and (b) HA-specific T cell clones after on-chip stimulation with either MOG-peptide loaded (10 µg/mL) or HA-peptide loaded DRB1*0401 monomer (10 µg/ml) and anti-CD28 (1 µg/ml) for 6, 18, or 42 h.

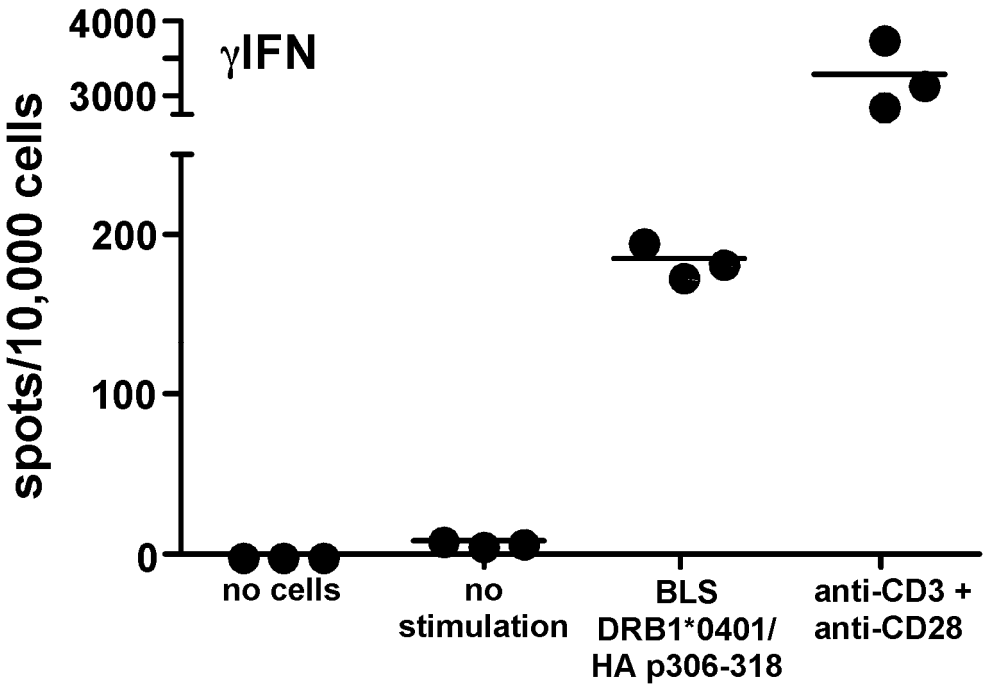

Figure S-3. Analysis of antigen-stimulated HA-reactive T cell clone by ELISpot. The HA-specific clone (300,000 cells/tube) was exposed to stimuli for 42 h in the presence of irradiated 300,000 BLS DRB1*0401 cells (IHWG Cell and Gene Bank, NCBI, NIH), used as antigen-presenting cells and loaded with HA p306-318. The cells were then transferred to ELISpot plates (200,000/well in triplicates) for 16 h to capture secreted IFNγ. For these experiments, anti-CD3 and anti-CD28 (1 μg/ml each) were plate bound during the period of stimulation. These conditions were optimized for measuring the responses from the antigen-specific clones by ELISpot, and therefore allowed a comparison of best case to the microengraving-based approach.