A clinical microchip for multiplexed evaluation of single immune cell functions

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Supplementary Figures 1-8

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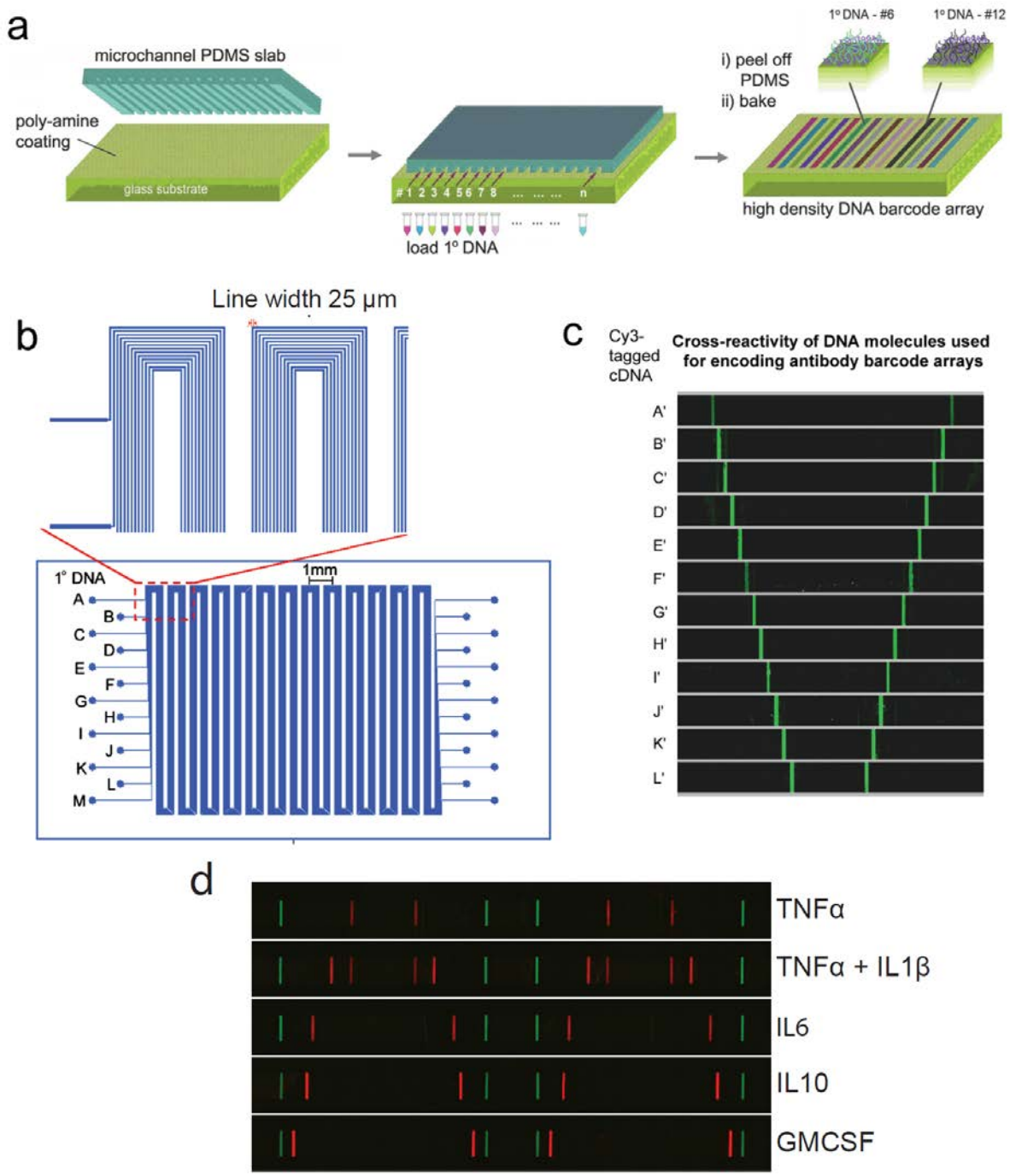
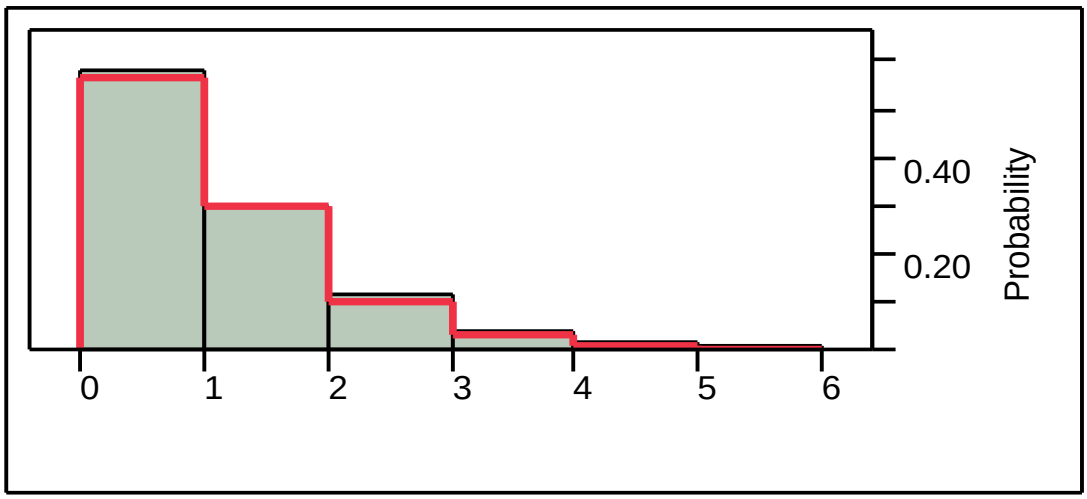
Supplementary Methods

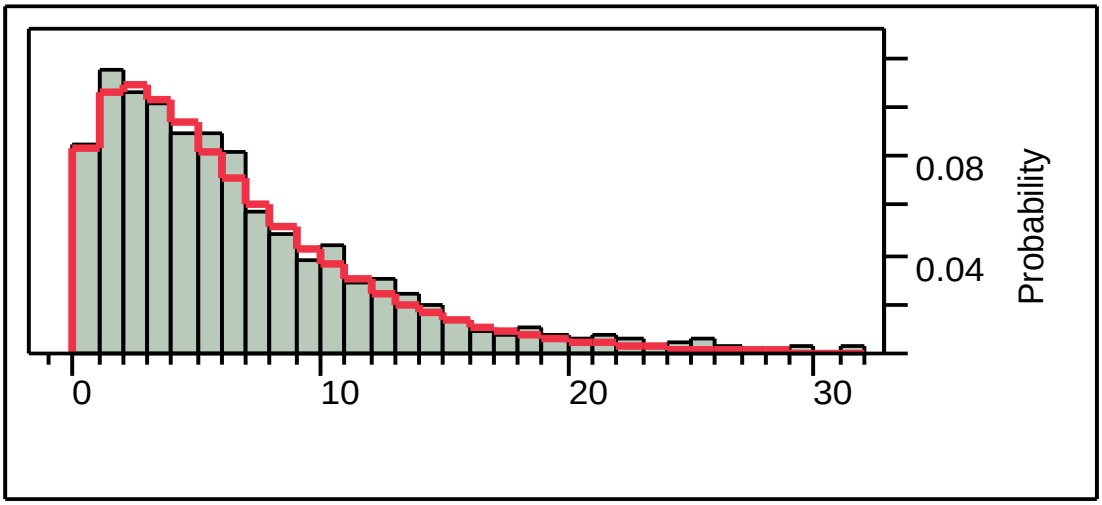
Supplementary Figures



Supplementary Figure 1 Fabrication of the DNA barcode array. (a) Schematic illustration of the microchannel-guided flow patterning techniques. **(b)** AutoCAD design of the photolithographic mask used for patterning a high-density DNA barcode array. A-M are the labels of primary DNA molecules. **(c)** Test result for hybridization to individual fluorescent Cy3-labeled complementary DNA showing negligible cross-reactivity (<1% in photon counts). **(d)** individual channels flowed by different recombinant proteins.

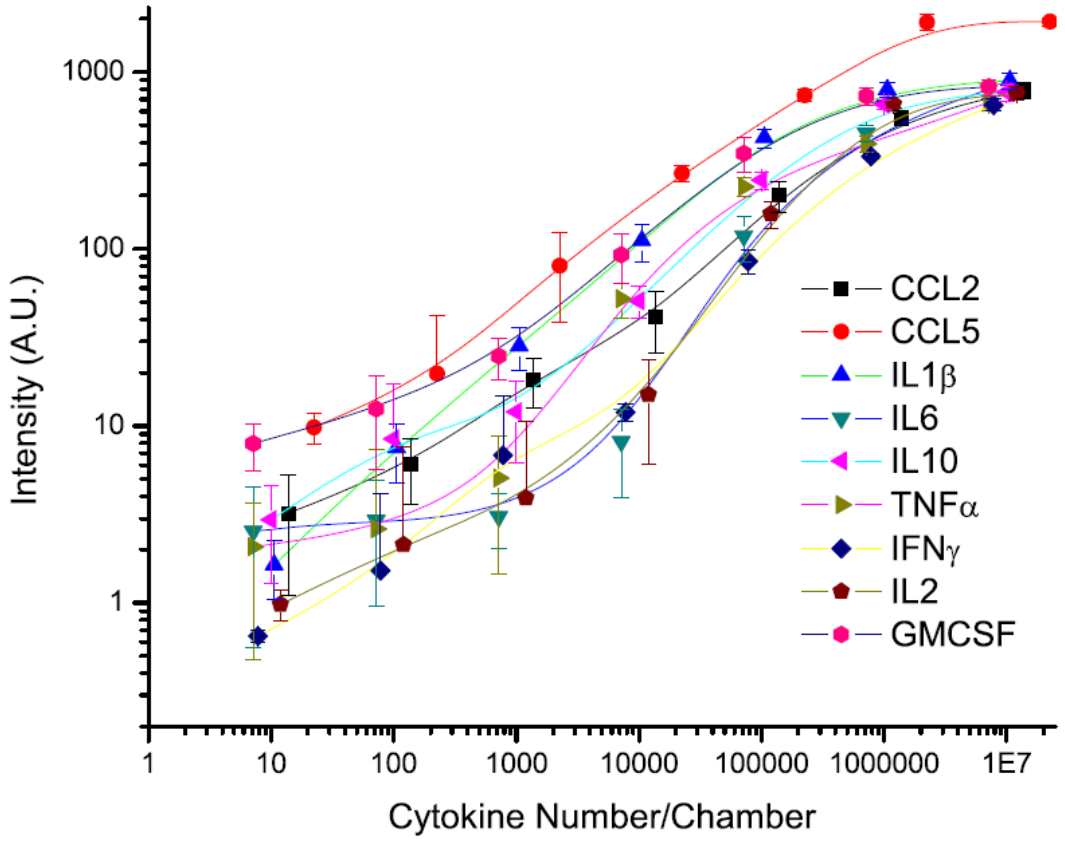


Gamma Poisson (0.61912,1.17662)
Goodness-of-Fit Test p=0.9124

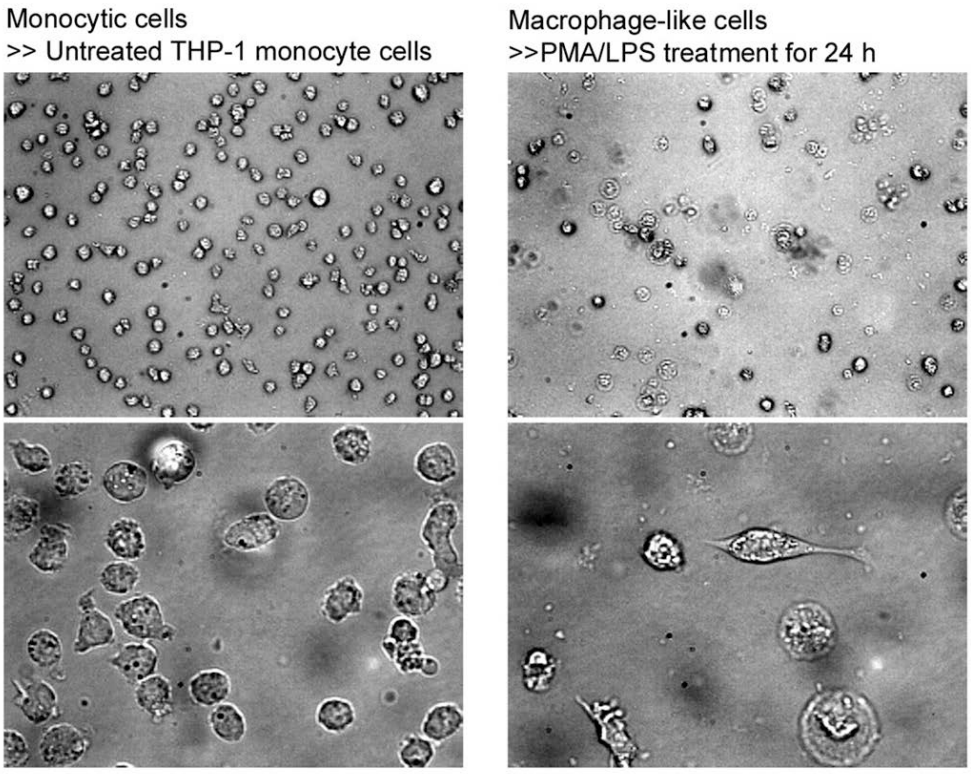


Gamma Poisson (5.89223,4.64461)
Goodness-of-Fit Test p=0.8482

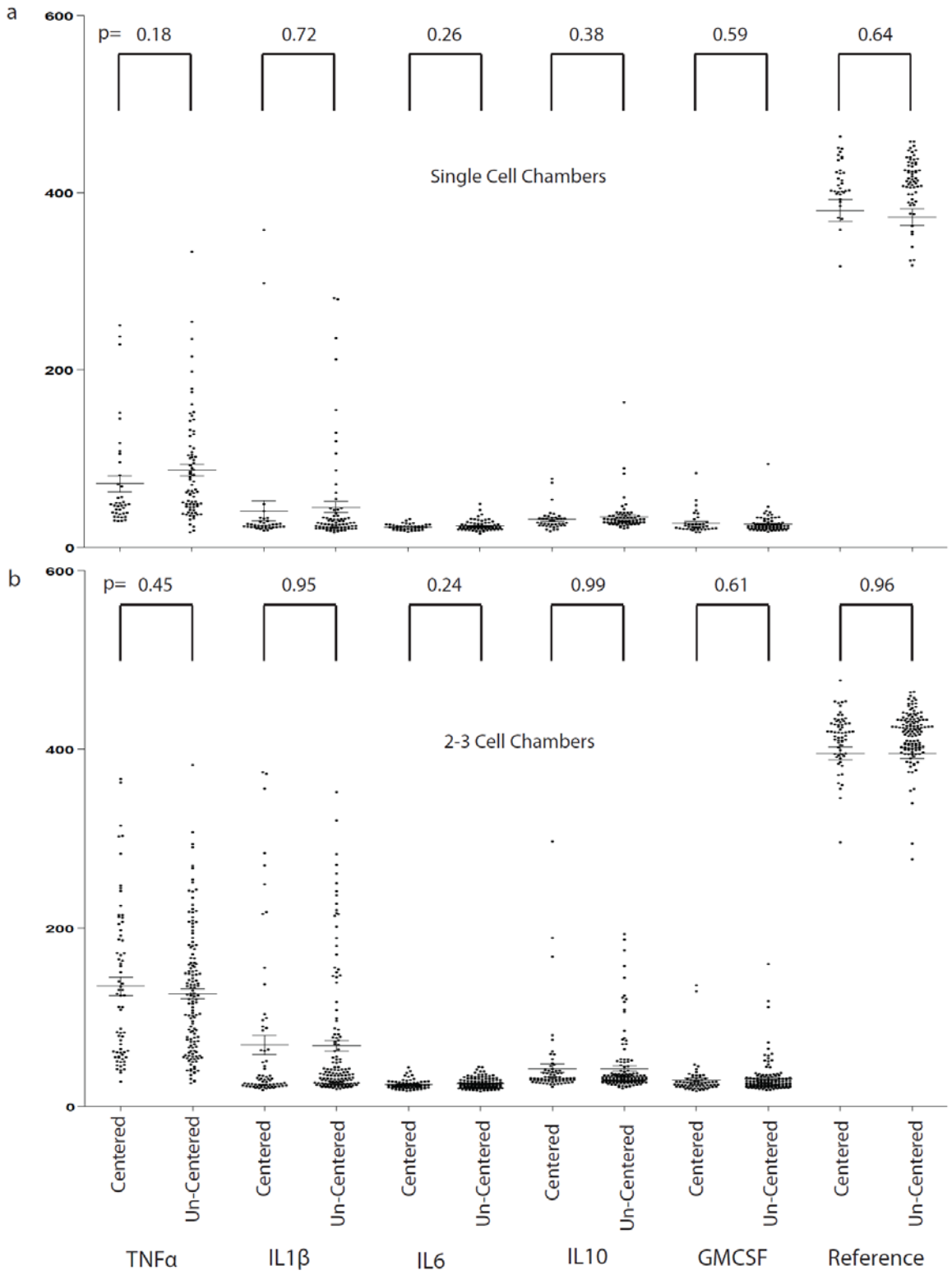
Supplementary Figure 2 Distribution of number of cells across the whole chip, fitted to Gamma Poisson distribution.



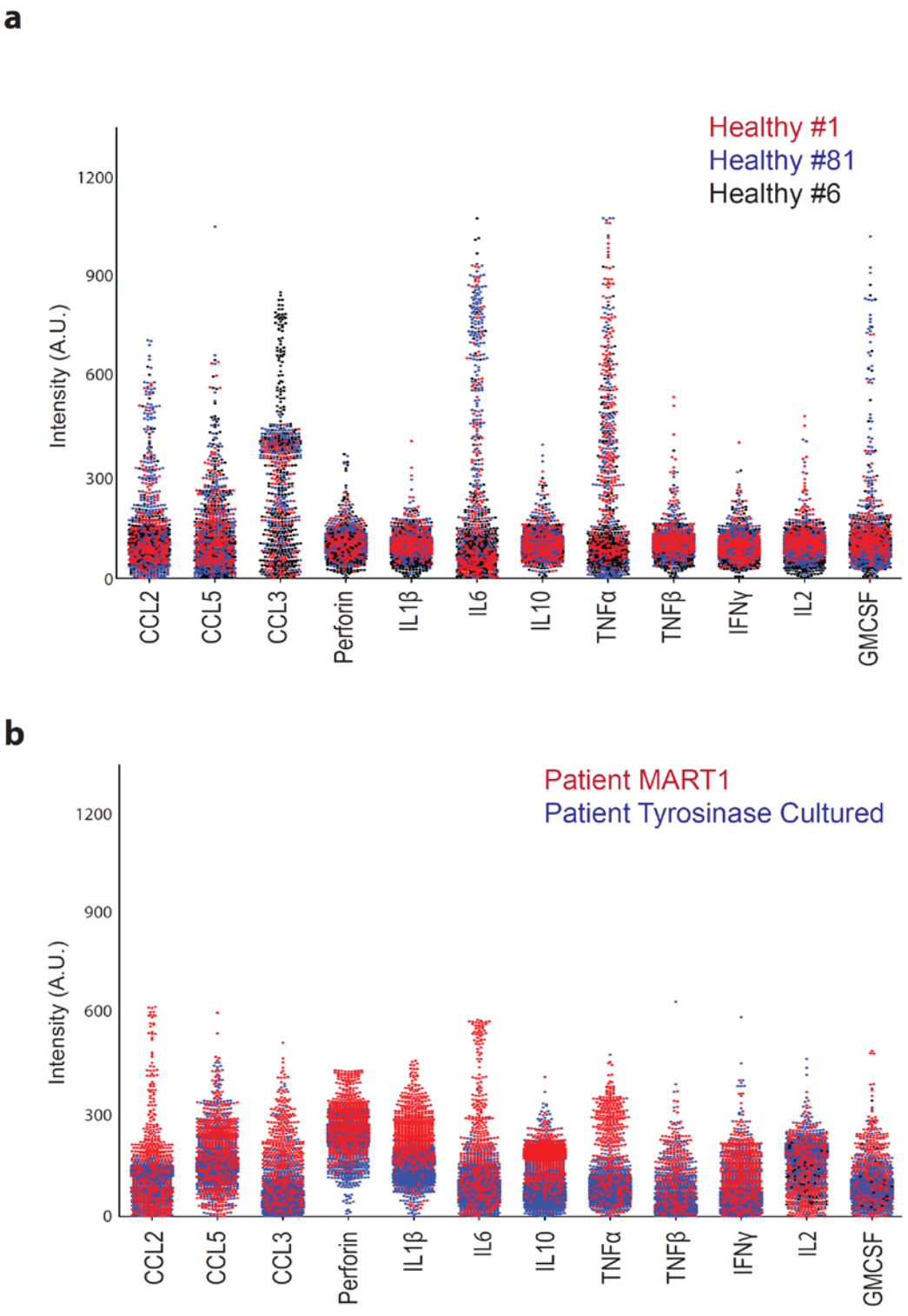
Supplementary Figure 3 Fluorescence quantitation curves for proteins measured from T cells.



Supplementary Figure 4 Morphology change of THP-1 cell upon PMA/LPS activation. The upper panels are lower magnification optical images and the lower panels are viewed at a higher magnification. The morphological change from non-adherent to adherent phenotypes was observed upon PMA/LPS treatment.

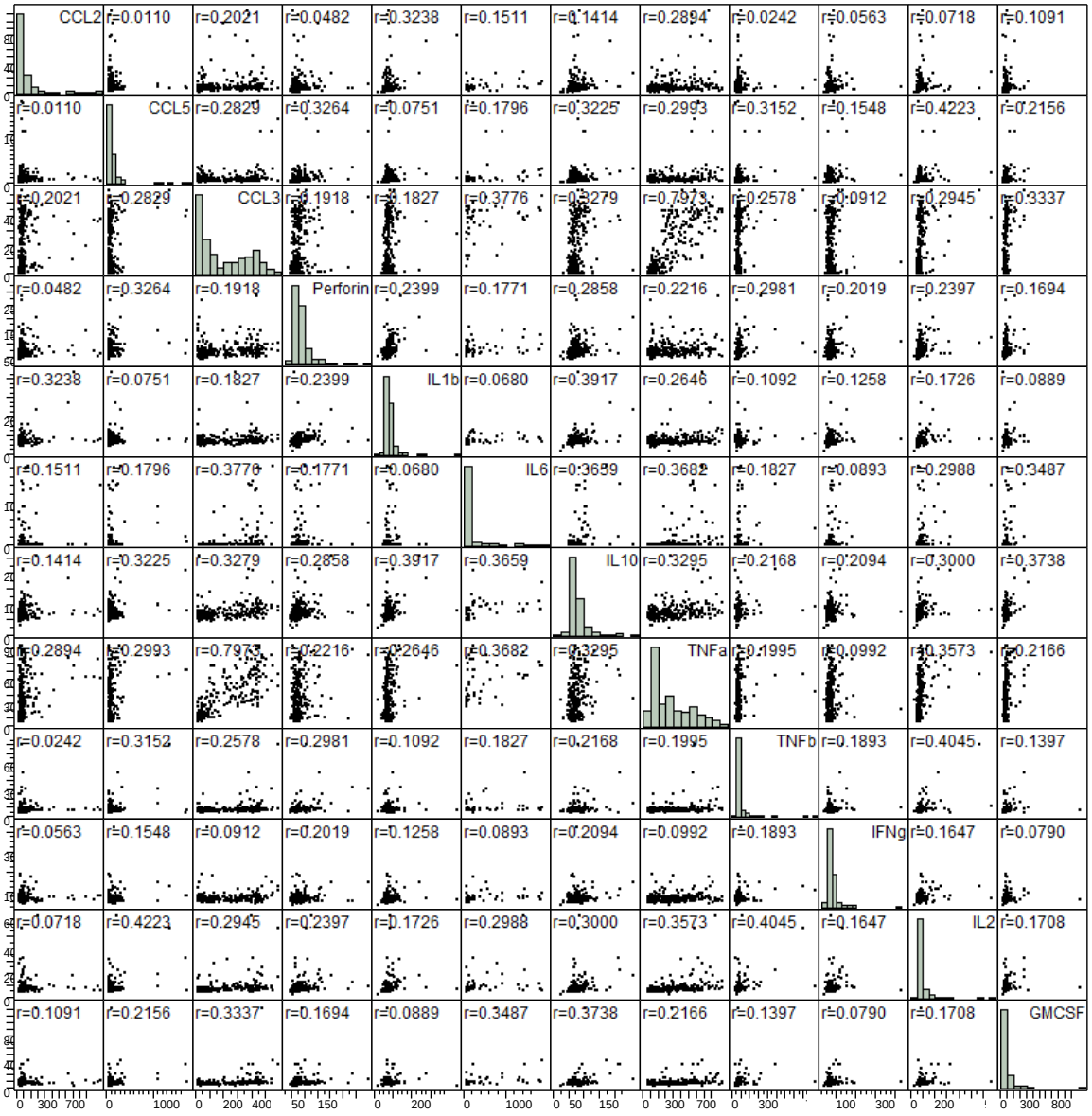


Supplementary Figure 5 Averaged signals detected from cells located in middle of the chamber vs. cells on the edge (p>0.2).



Supplementary Figure 6 Univariate comparison among CTLs from healthy donors (a), CD3+ MART-1-specific CTLs and CD3+ Tyrosinase-specific CTLs from melanoma patients (b).

Scatterplot for CTLs from healthy donor controls (n=3)
#1



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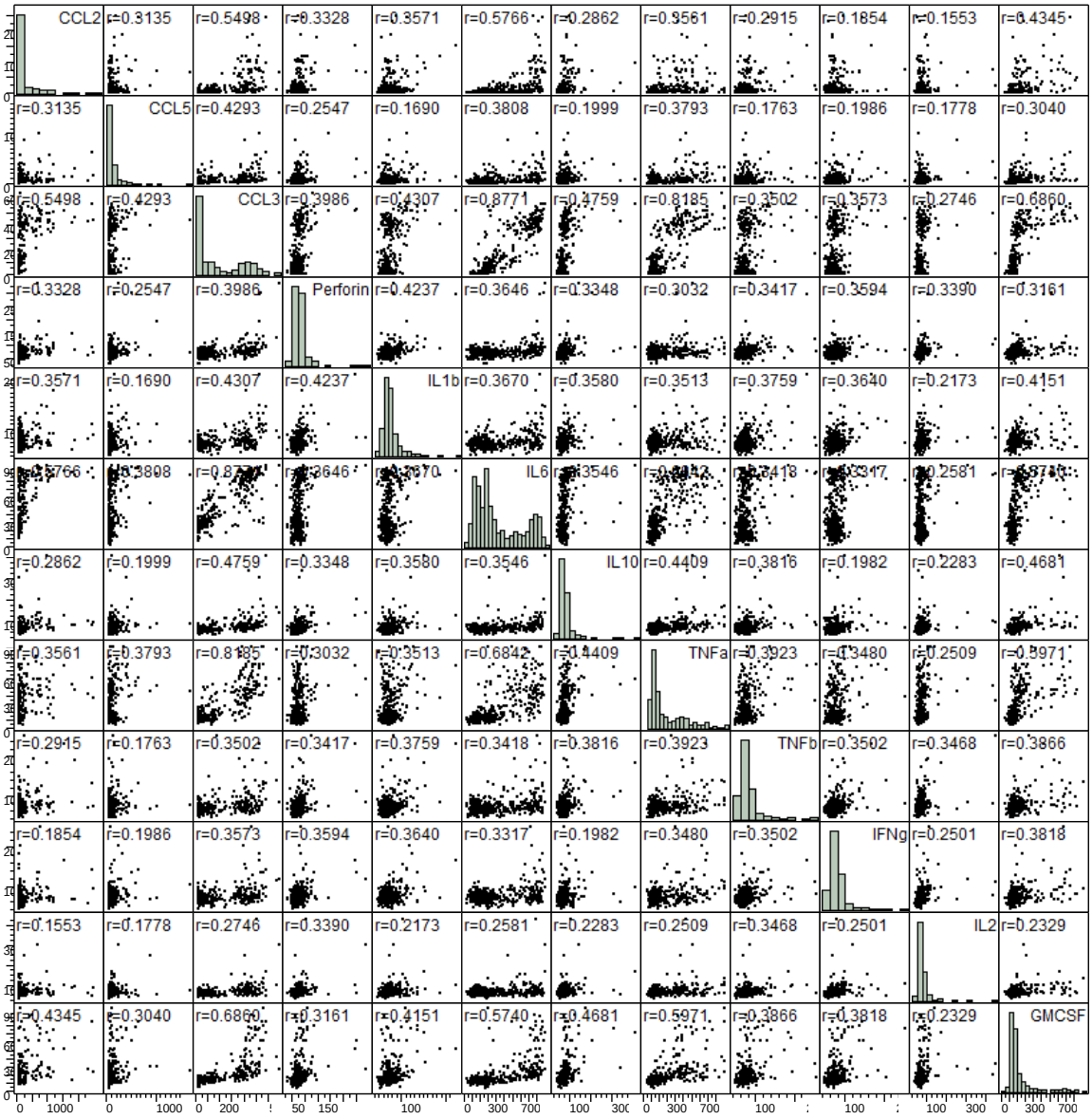
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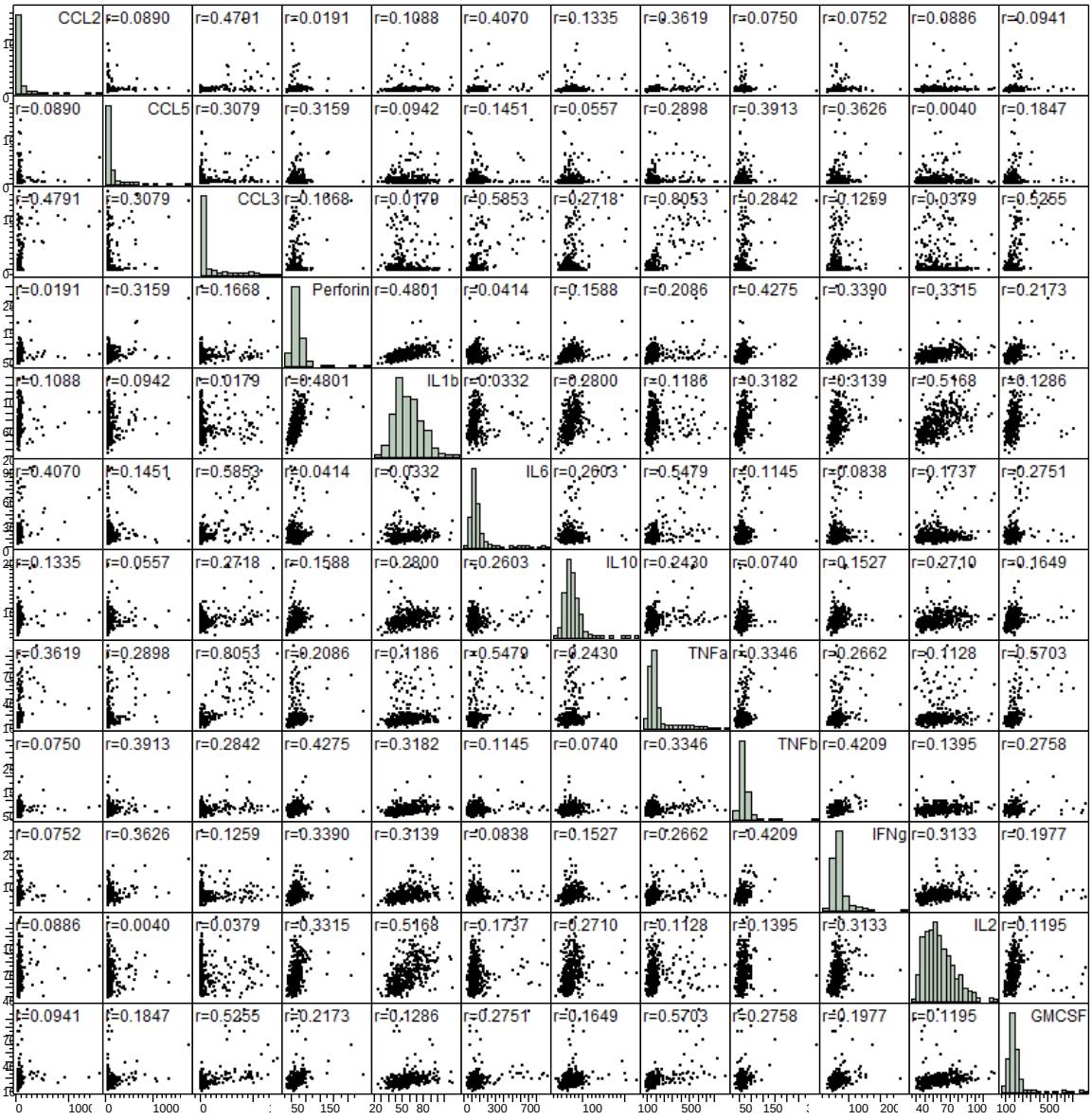
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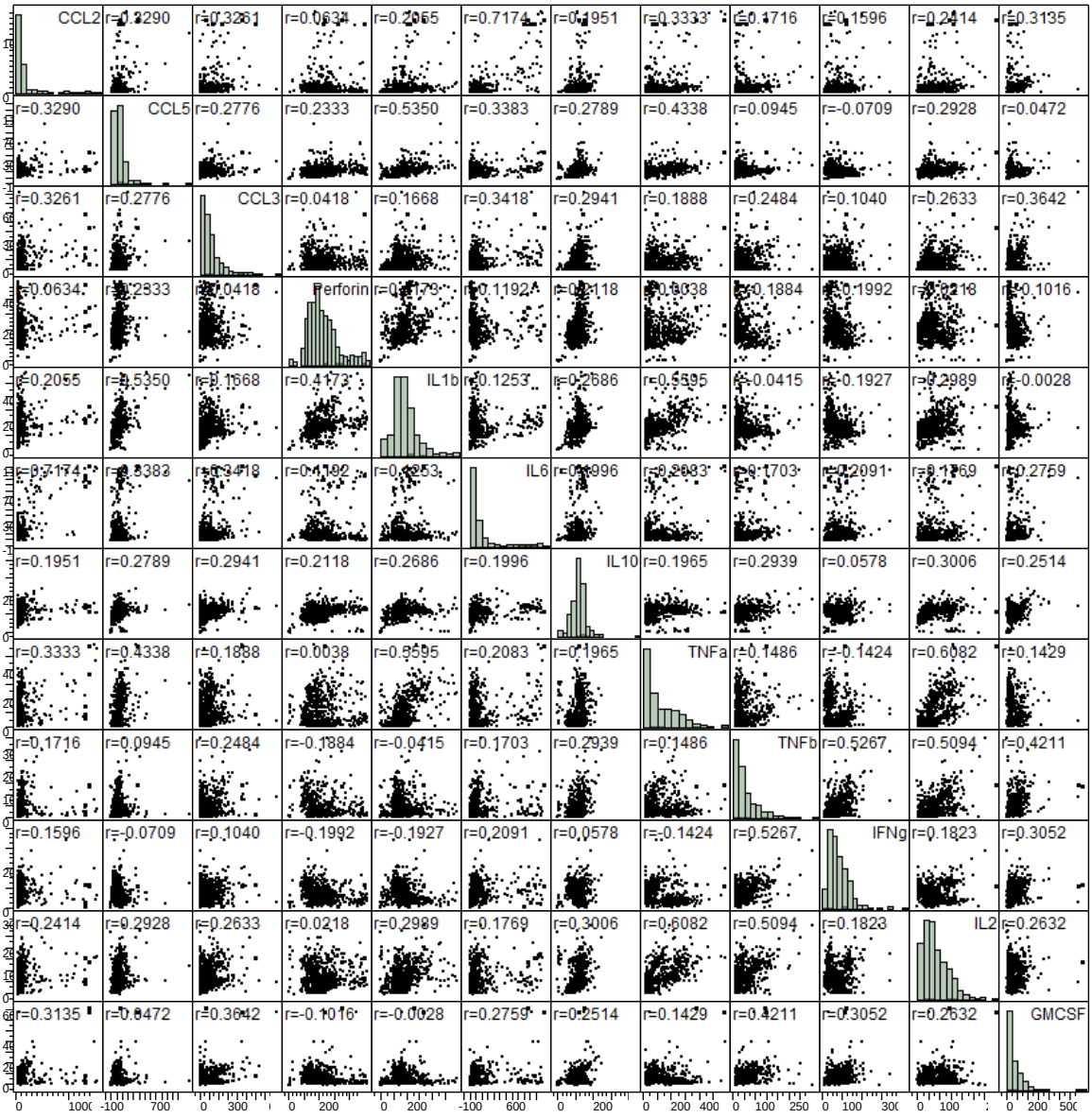
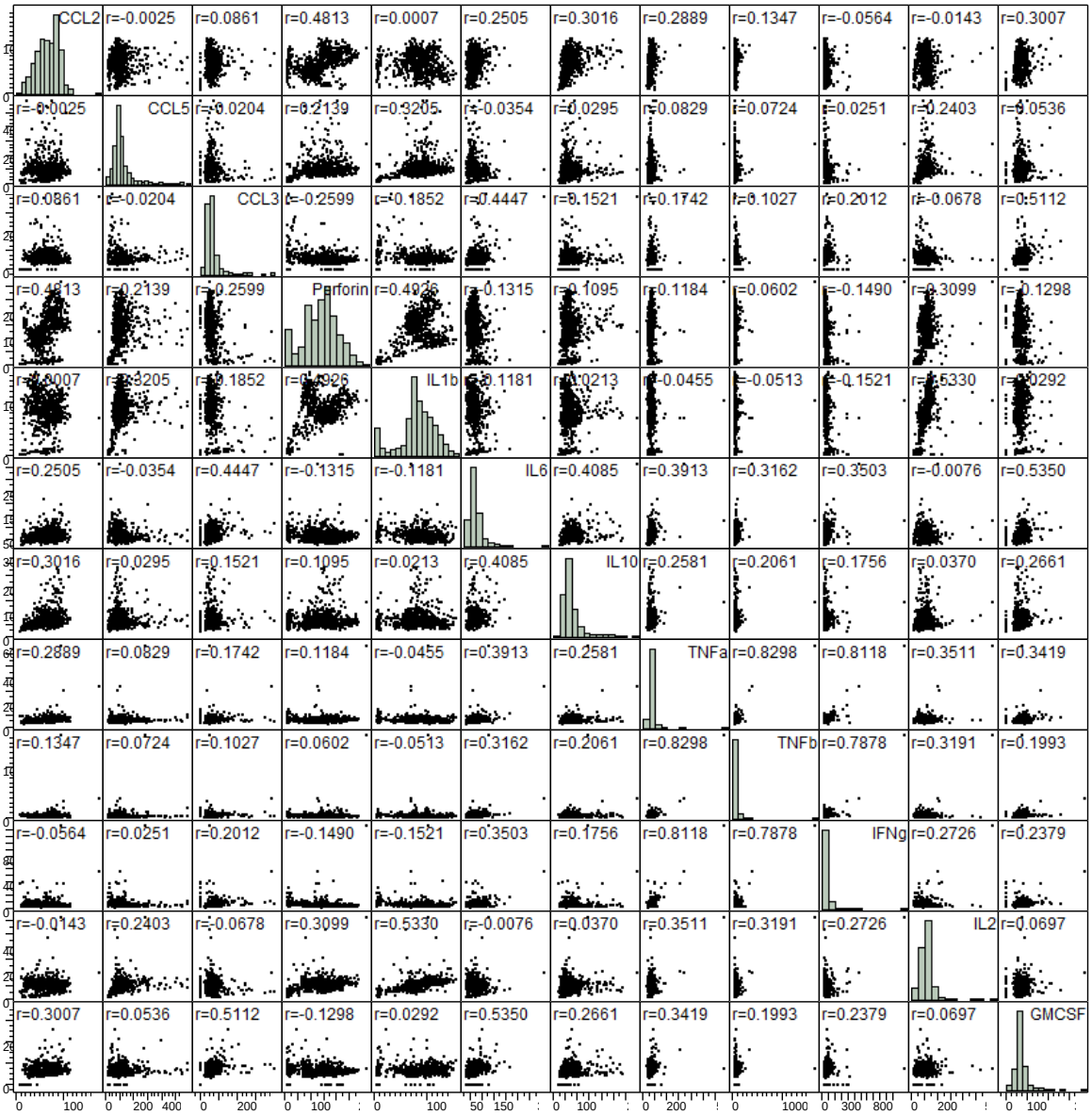
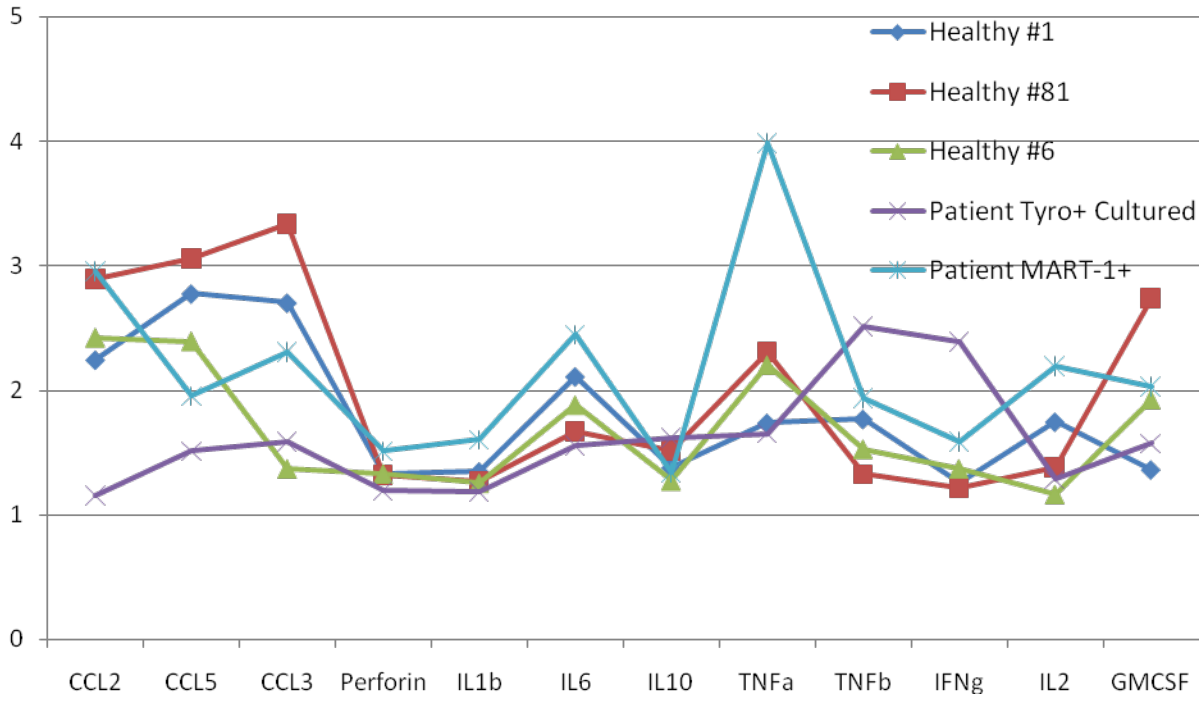
Scatterplot for MART-1 T Cells



Scatterplot for Tyrosinase T Cells



Supplementary Figure 7. Protein-protein correlation plot matrices from single cell experiments of CTLs from healthy donor controls, MART1 T cells and Tyrosinase T cells.



Supplementary Figure 8 Ratio of averaged functional intensity of polyfunctional T cells ($n > 4$) to non-polyfunctional T cells ($n \leq 4$).

Supplementary Tables

Supplemental Table 1. Sequences and terminal functionalization of oligonucleotides*.

Name	Sequence	Melting Point
A	5'- AAA AAA AAA AAA AAT CCT GGA GCT AAG TCC GTA-3'	57.9
A'	5' NH3- AAA AAA AAA ATA CGG ACT TAG CTC CAG GAT-3'	57.2
B	5'-AAA AAA AAA AAA AGC CTC ATT GAA TCA TGC CTA -3'	57.4
B'	5' NH3AAA AAA AAA ATA GGC ATG ATT CAA TGA GGC -3'	55.9
C	5'- AAA AAA AAA AAA AGC ACT CGT CTA CTA TCG CTA -3'	57.6
C'	5' NH3-AAA AAA AAA ATA GCG ATA GTA GAC GAG TGC -3'	56.2
D	5'-AAA AAA AAA AAA AAT GGT CGA GAT GTC AGA GTA -3'	56.5
D'	5' NH3-AAA AAA AAA ATA CTC TGA CAT CTC GAC CAT -3'	55.7
E	5'-AAA AAA AAA AAA AAT GTG AAG TGG CAG TAT CTA -3'	55.7
E'	5' NH3-AAA AAA AAA ATA GAT ACT GCC ACT TCA CAT -3'	54.7
F	5'-AAA AAA AAA AAA AAT CAG GTA AGG TTC ACG GTA -3'	56.9
F'	5' NH3-AAA AAA AAA ATA CCG TGA ACC TTA CCT GAT -3'	56.1
G	5'-AAA AAA AAA AGA GTA GCC TTC CCG AGC ATT-3'	59.3
G'	5' NH3-AAA AAA AAA AAA TGC TCG GGA AGG CTA CTC-3'	58.6
H	5'-AAA AAA AAA AAT TGA CCA AAC TGC GGT GCG-3'	59.9
H'	5' NH3-AAA AAA AAA ACG CAC CGC AGT TTG GTC AAT-3'	60.8
I	5'-AAA AAA AAA ATG CCC TAT TGT TGC GTC GGA-3'	60.1
I'	5' NH3-AAA AAA AAA ATC CGA CGC AAC AAT AGG GCA-3'	60.1
J	5'-AAA AAA AAA ATC TTC TAG TTG TCG AGC AGG-3'	56.5
J'	5' NH3-AAA AAA AAA ACC TGC TCG ACA ACT AGA AGA-3'	57.5
K	5'-AAA AAA AAA ATA ATC TAA TTC TGG TCG CGG-3'	55.4
K'	5' NH3-AAA AAA AAA ACC GCG ACC AGA ATT AGA TTA-3'	56.3
L	5'-AAA AAA AAA AGT GAT TAA GTC TGC TTC GGC-3'	57.2
L'	5' NH3-AAA AAA AAA AGC CGA AGC AGA CTT AAT CAC-3'	57.2
M	5'-Cy3-AAA AAA AAA AGT CGA GGA TTC TGA ACC TGT-3'	57.6
M'	5' NH3-AAA AAA AAA AAC AGG TTC AGA ATC CTC GAC-3'	56.9

* all oligonucleotides were synthesized by Integrated DNA Technology (IDT) and purified via high performance liquid chromatography (HPLC).

Supplementary Table 2. Summary of antibodies used for macrophage experiments.

DNA label	primary antibody (vendor: clone)	secondary antibody (vendor: clone)
A'	mouse anti-hu IFN-γ (eBio: MD-1)	biotin-labeled mouse anti-hu IFN-γ (eBio: 4S.B3)
B'	mouse anti-hu TNF-α (eBio: Mab1)	biotin-labeled mouse anti-hu TNF-α (eBio: Mab11)
C'	mouse anti-hu IL-2 (eBio: MQ1-17H12)	biotin-labeled mouse anti-hu IL-2 (eBio: polyclonal)
D'	mouse anti-hu IL-1α (eBio: CRM8)	biotin-labeled mouse anti-hu IL-1α (eBio: CRM6)
E'	mouse anti-hu IL-1β (eBio: CRM56)	biotin-labeled mouse anti-hu IL-1β (eBio: CRM57)
F'	mouse anti-hu TGF-β (eBio: TB2F)	biotin-labeled mouse anti-hu TGF-β (eBio: 16TFB)
G'	mouse anti-hu PSA (BioDesign: S6)	biotin-labeled mouse anti-hu PSA (BioDesign: S2)
H'	mouse anti-hu IL-6 (eBio: MQ2-13A5)	biotin-labeled mouse anti-hu IL-6 (eBio: MQ2-39C3)
I'	mouse anti-hu IL-10 (eBio: JES3-9D7)	biotin-labeled mouse anti-hu IL-10 (eBio: JES3-12G8)
J'	mouse anti-hu IL-12 (p70) (eBio: BT21)	biotin-labeled mouse anti-hu IL-12 (eBio: C8.6)
K'	mouse anti-hu GM-CSF (BD)	biotin-labeled mouse anti-hu GM-CSF-γ (BD) **
L'	mouse anti-hu MCP-1(eBio: 5D3-F7)	biotin-labeled mouse anti-hu MCP-1(eBio: 2H5)

**** BD OptEIA ELISA Set, Catalog No. 555126.**

Supplementary Table 3. Summary of antibodies used for patient T cell experiments.

DNA label	primary antibody (vendor: clone)	secondary antibody (vendor: clone)
A'	mouse anti-hu IFN-γ (R&D)	biotin-labeled mouse anti-hu IFN-γ (R&D)
B'	mouse anti-hu TNF-α (R&D)	biotin-labeled mouse anti-hu TNF-α (R&D)
C'	mouse anti-hu IL-2 (R&D)	biotin-labeled mouse anti-hu IL-2 (R&D)
D'	mouse anti-hu TNF-β (eBio: 359-238-8)	biotin-labeled mouse anti-hu TNF-β (eBio: 359-81-11)
E'	mouse anti-hu CCL5 (R&D)	biotin-labeled mouse anti-hu CCL5 (R&D)
F'	mouse anti-hu Perforin (Abcam)	biotin-labeled mouse anti-hu Perforin (Abcam)
G'	mouse anti-hu IL-1β (R&D)	biotin-labeled mouse anti-hu IL-1β (R&D)
H'	mouse anti-hu IL-6 (R&D)	biotin-labeled mouse anti-hu IL-6 (R&D)
I'	mouse anti-hu IL-10 (R&D)	biotin-labeled mouse anti-hu IL-10 (R&D)
J'	mouse anti-hu MCP-1(eBio: 5D3-F7)	biotin-labeled mouse anti-hu MCP-1(eBio: 2H5)
K'	mouse anti-hu GM-CSF (eBio: BVD2-23B6)	biotin-labeled mouse anti-hu GM-CSF (BVD2-21C11)
L'	mouse anti-hu CCL3 (R&D)	biotin-labeled mouse anti-hu CCL3 (R&D)

Supplementary Methods

Microchip Fabrication. The control layer was molded from a SU8 2010 negative photoresist (~20um in thickness) silicon master using a mixture of GE RTV 615 PDMS prepolymer part A and part B (5:1). The flow layer was fabricated by spin-casting the pre-polymer of GE RTV 615 PDMS part A and part B (20:1) onto a SPR 220 positive photoresist master at ~2000rpm for 1minute. The SPR 220 mold was ~17 μm in height after rounding via thermal treatment at 80 degree for 60 mins. The control layer PDMS chip was then carefully aligned and placed onto the flow layer, which was still situated on its silicon master mold, and an additional 60 mins thermal treatment at 80^{o}C was performed to enable bonding. Afterward, this two-layer PDMS chip was cut off with access holes drilled. Finally, the microfluidic-containing PDMS slab was thermally bonded for 2 hours at 80 degree onto the barcode-patterned glass slide to give a fully assembled microchip.

Isolation, sorting and expansion of T cells. Briefly, enriched PBMC were applied onto a MART-1 tetramer microarray. After 20 minutes incubation at 37 degree, unbounded cells were washed off and then attached MART-1 specific T cells were released from the array. In order to characterize low frequency antigen-specific T cells, 5 x 10^6 PBMC were expanded with irradiated and tumor antigen peptide-pulsed HLA-A*0201-transfected K562 (K562A2.1) used as antigen presenting cells (APC) at a ratio of 5:1 [27,28]. The PBMC were cultured in AIM-V media plus IL-2 (300IU/ml) and CD3 antobidy (OKT3, 50ng/ml) on day 1, and then kept in culture for 24 days with IL-2 but without OKT-3. Irradiated (75 Gy) K562A2.1 cells were pulsed with 10μg of peptide tyrosinase$_{368-376}$ (YMDGTMSQV). The peptide-pulsed APCs were added weekly. After 30 days, expanded cells were sorted based on the same technique described above with Tyrosinase$_{368-376}$/HLA-A0201 tetramer.

On-chip secretion profiling. First, 200 ul 3% bovine serum albumin (BSA, Sigma) in phosphate buffer saline (PBS, Irvine Scientific) was flowed and dead-end filled into the chip to block non-specific binding. Second, a 200-μl cocktail containing all 12 DNA-antibody conjugates at 5 ug/ml in 3% BSA/PBS buffer was flowed through all microfluidic channels in 1 h at 37 degree. Then, 100μl of 3% BSA/PBS buffer was flowed into the device to wash unbounded DNA conjugated primary antibody solutions. THP-1 Cells were washed, stimulated with PMA/LPS or purified T cells acitivated with tetramer/anti-CD28 antibody or anti-CD3/anti-CD28 antibody in 10% FBS/RPMI-1640 media (ATCC) and loaded on chip within 5 min in order to minimize pre-chip secretion. Then, the pneumatic valves were pressed down by applying 20 psi constant pressure to divide 80 microfluidic channels into 1000 isolated microchammbers. Next, the chip was placed in a cell incubator (~37^{o}C and 5% CO_2) for 24 h (THP-1) or 12 h (T cells) to perform on chip secretion. Afterwards, the chip was removed from incubator and cells were quickly washed of the chip. A 200-μl cocktail containing all biotinylated detection antibodies was then flowed through the microchannels. The antibody concentrations are 5 ug/mL. Then, 200ul of the fluorescent probe solution (100 ng/ml Cy5-labeled straptavidin and 20 ng/ml C3-labeld M' ssDNA) were flowed through to complete the immuno-sandwich assay. Finally, the PDMS slab was peeled off and the microarray slide was rinsed with 1x PBS, 0.5xPBS and DI water twice, sequentially, and spin-dried.

Culture and stimulation of THP-1 cells. THP-1 monocyte cells were (clone TIB 202) cultured in RPMI-1640 (ATCC) medium supplemented with 10% fetal bovine serum (FBS) and 10 μM 2-mercaptoethanol. Cells grown close to the maximum density ($0.8x10^6$ cells/mL) were chosen for the experiment in order to minimize variation of cell cycles. Cells were washed and resuspended in fresh media at $2\text{-}4x10^6$ cells/ml. Aliquots each containing 100 μL of cell suspension were prepared. 1μL of 1mg/mL PMA and 1μL of 20μg/ml LPS were sequentially added into each aliquot to induced monocytic differentiation and stimulate TLR-4 activation. THP-1 cells activated with PMA and LPS exhibited a characteristic morphological change (**Supplementary Fig. 2**).

Melanoma adoptive transgenic T cell transfer clinical trial. Peripheral blood samples were obtained from patients with metastatic melanoma enrolled in a T cell receptor (TCR) transgenic ACT protocol (UCLA IRB# 08-02-020, IND# 13859, clinical trial registration number NCT00910650). A single baseline unmobilized leukapheresis processing two plasma volumes was performed to obtain PBMC, which were isolated by Ficoll-Hypaque (Amersham-Pharmacia) gradient centrifugation, for the generation of TCR transgenic cells and dendritic cell (DC) vaccines. Freshly collected PBMC were activated for 2 days by clinical grade anti-CD3 antibody OKT3 (50 ng/ml, anti-human CD3 FG purified OKT3, eBioscience) and human interleukin-2 (IL-2, 300 IU/ml, aldesleukin, Novartis). Following activation, cells are transduced in two consecutive days with a retroviral vector expressing the MART-1 F5 TCR (MSGV1-F5AfT2AB vector), in plates coated with retronectin (Takara Bio Inc.) as previously described[23]. The clinical grade retroviral vector MSGV1-F5AfT2AB was generated at the Indiana University Viral Production Facility starting from the master cell bank provided by Dr. Steven A. Rosenberg (Surgery Branch, NCI) as previously described[23]. Patients were then admitted to the hospital and conditioned with a non-myeloablative, lymphocyte depleting chemotherapy regimen consisting of cyclophosphamide (60 mg/kg/day for two days, Mead Johnson & Company) and fludarabine (25 mg/m^2/day i.v. daily for five days, Berlex). On study day 0 patients received up to 10^9 MART-1 F5 TCR transgenic T cells infused. MART-$1_{26\text{-}35}$ peptide pulsed DC were generated from cryopreserved PBMC following a one-week *ex vivo* differentiation culture in RPMI 1640 medium supplemented with 5% heat inactivated autologous plasma, 256 IU/ml of GM-CSF (Bayer) and 160 IU/ml of IL-4 (CellGenix) as previously described[24]. On the day after TCR transgenic ACT, patients received an i.d. administration of up to 10^7 MART-$1_{26\text{-}35}$ peptide pulsed DC vaccines at a site close to a lymph node basin, and were started on systemic high dose IL-2 at 600,000 IU/kg i.v. every 8 hours for up to 14 doses as tolerated. Thereafter, patients stayed in the hospital until they had recovered from the chemotherapy-induced pancytopenia (median of 11 days).

Synthesis of DNA-1^o antibody conjugates. As-received antibodies were desalted, buffer exchanged to pH 7.4 PBS and concentrated to 1 mg/mL using Zebba protein desalting spin columns (Pierce). Succinimidyl 4-hydrazinonicotinate acetone hydrazone in *N*,*N*-dimethylformamide (DMF) (SANH, Solulink) was added to the antibodies at variable molar excess of (85:1) of SANH to antibody. Separately, succinimidyl 4-formylbenzoate in DMF (SFB, Solulink) was added at a 20-fold molar excess to 5‘aminated 28mer oligomers in PBS. After incubation for 4 h at room temperature, excess SANH and SFB were removed and both samples buffered exchanged to pH 6.0 citrate buffer using protein desalting spin columns. A 20-fold excess of derivatized DNA was then combined with the antibody and allowed to react overnight at room temperature. Noncoupled DNA was removed using a Pharmacia Superdex 200 gel filtration column (GE) at 0.5 mL/min isocratic flow of PBS.

Fabrication of microfluidic-patterning chips. The microfluidic-patterning chips were made by molding a polydimethylsiloxane (PDMS) elastomer from a master template, which was prepared using photolithography to create a photoresist pattern on a Si wafer. An alternative was to make a silicon “hard” master by transferring the photolithographically-defined pattern into the underlying silicon wafer using a deep reactive ion etching (DRIE) process[1]. The first method offers rapid prototyping, while the second method yields a robust and reusable mold, permitting higher throughput chip fabrication. The typical line width and height are 20μm and 20μm, respectively. The PDMS elastomer-based microfluidic patterning chips were fabricated via a molding process. The mixture of Sygard PDMS (Corning) prepolymer and curing agent (10:1) was stirred, and poured onto the silicon mold which was pre-treated with trimethyl-chloro-silane vapor to facilitate mold release. Next the PDMS

poured on the mold was degassed for 30 min via a house vacuum line, and then cured at 80^{o}C for 1 h. The solidified PDMS slab was cut off the mold, assess holes drilled and then bonded onto a glass slide. Prior to bonding, the glass surface was pre-coated with the polyamine polymer, poly-L-lysine (Sigma-Aldrich), to increase DNA loading. The coating process is described elsewhere[18]. The number of microfluidic channels determines the size of the barcode array. The completely microfluidic patterning chip contained of 13 parallel microchannels designed to cover a large area (3cm×2cm) of the glass slide for creating the DNA barcode microarray.

Patterning of DNA barcode arrays. Using the microchannel-guided flow-patterning approach, we fabricated DNA barcode arrays that were at least 10-fold denser than conventional microarrays. Microcontact printing can generate high density arrays of biomolecules with spot sizes of a few micrometers (μms)[2,3]**,** but extending stamping to large numbers of biomolecules is awkward because of the difficulty in aligning multiple stamps precisely to produce a single microarray. Direct microfluidics-based pattering of proteins has been reported, but DNA flow-patterning with sufficient loading remains less successful compared to conventional spotting methods[4,5]**.** During the patterning of a DNA barcode array, solutions, each containing a different strand of primary DNA oligomers prepared in 1x PBS buffer, were flowed into each of the microfluidic channels. Then, the solution-filled chip was placed in a desiccator to allow solvent (water) to evaporate completely through the gas-permeable PDMS, leaving the DNA molecules behind. This evaporation process took from several hours to overnight to complete. Last, the PDMS elastomer was removed from the glass slide, and the barcode-patterned DNA was fixed to the glass surface by thermal treatment at 80^{o}C for 4 hours. Potassium phosphate crystals precipitated during solution evaporation, but were readily removed by rapidly dipping the slide in deionized water prior to bonding the blood-assay chip to the slide. This did not affect the quality of the DNA barcode arrays.

Supplementary References

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