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Detecting Cytokine Release from Single Human T-cells

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Abstract

The cytokine production by leukocytes correlates with body's ability to mount an immune response and therefore has high diagnostic value. In the present study we employed microfabricated surfaces to capture T-cells from minimally processed human blood, arrange these cells into a single cell array and then detect interferon (IFN)- γ released from individual cells. The fabrication of cell capture surfaces started with coating a silane-modified glass slide with a uniform layer of poly(ethylene glycol) (PEG) hydrogel. The hydrogel-coated slide was lyophilized and then incubated with a mixture of monoclonal anti-IFN- γ and anti-CD4 antibodies (Abs). To define sites for single cell attachment, PEG hydrogel microwells (20 μ m diameter) were photolithographically patterned on top of the Ab-containing hydrogel layer. This micropatterning process resulted in fabrication of PEG hydrogel microwells with Ab-decorated bottom and non-fouling walls. To minimize the blood volume requirement and to precisely define shear stress conditions, the engineered surface was enclosed inside a PDMS-based microfluidic device. Introduction of red blood cell (RBC) depleted whole human blood followed by controlled washing led to the isolation of individual CD4 T-cells within PEG microwells. Mitogenic activation and immunofluorescent staining performed inside the microfluidic chamber revealed IFN- γ cytokine signal co-localized with specific T-cells. The device and process presented here will be expanded in the future to enable multi-parametric functional analysis of immune cells organized into high density single cell arrays.

INTRODUCTION

T-cells play a critical role in the immune response against viral and bacterial infections. A particular T-cell subset, T-helper cells (CD4 T-cells), regulate immune cell recruitment and proliferation through the production of a wide variety of cytokines.¹ Importantly, CD4 T-cells of identical morphology and surface markers are further categorized into T-helper 1 (Th1), Th2, Th17 solely based on the types of cytokines they secrete.^{2, 3} Specific T-helper subtypes play an important role in the immune response. In particular, Th1 phenotype of CD4 T-cells – characterized by production of IFN- γ – correlates with vigorous immune response and protection against infections and is also monitored to determine vaccine efficacy.^{4, 5}

The functional heterogeneity of T-cells necessitates development of tools that help determine cytokine production of individual cells. In immunology, single cell level cytokine detection is traditionally performed using either intra-cytoplasmic cytokine staining (ICS) coupled with polychromatic flow cytometry (FC) or enzyme-linked immunospot (ELISpot) assay.^{6, 7} FC-

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based analysis relies on detection of cytokines accumulated inside fixed and permeabilized cells and therefore has limited options for time-course experiments or post-detection processing of cells. ELISpot on the other hand can be used for detecting cytokines released by live cells. This approach is based on seeding and activating immune cells in micro-titer plates coated with anti-cytokine Abs. Because most of immune cells are anchorage independent, they can be aspirated after completion of the experiment, leaving behind cytokine spots that correlate with single cells. Therefore, while detecting cytokine release, ELISpot assay does not connect cytokine production to specific cells. Because multiple leukocyte subsets may be responsible for production of the same cytokine (e.g. IFN- γ may be produced by CD4 or CD8 T-cells as well as by monocytes), ELISpot approach necessitates extensive pre-processing of the blood sample to isolate the desired cell subset prior to analysis.

Microfabrication and surface engineering may provide an alternative solution for isolation and analysis of cells.^{8, 9} A number of microfabrication-based approaches for isolating single cells has been described including docking within microfluidic channels¹⁰, entrapment within PDMS-based microwells^{11, 12} and capturing on ligand-decorated micropatterned surfaces.^{13–16} Of particular note are recent reports by Love and co-workers who described a PDMS microengraving technique that allows to trap single immune cells and to detect cell-secreted proteins.^{12, 17, 18} Despite these recent advances, significant challenges remain to be addressed in the development of devices for functional analysis of single immune cells: 1) rapid purification of the desired leukocyte subsets from a complex heterogeneous sample such as whole blood is required, 2) localized, on-chip, detection of products secreted by the single leukocytes needs to be demonstrated, 3) cell-capture surfaces need to be integrated with microfluidics to minimize sample volume requirement.

As a step towards achieving some of the benchmarks outlined above, we describe a microdevice for capturing single T-cells from minimally processed human blood and for detecting IFN- γ released by single cells. Building on our previous reports of single leukocyte array formation¹⁴ and microdevices for T-cell capture and cytokine detection^{19, 20}, we designed poly(ethylene glycol) (PEG) hydrogel microwells with Ab-decorated attachment sites and non-fouling side-walls (see Figure 1). Importantly, attachment sites contained a mixture of cell-specific anti-CD4 Abs and cytokine-specific anti-IFN- γ Abs. The hydrogel microwells were integrated with a PDMS-based microfluidic device and incubated with human blood, resulting in capture of single CD4 T-cells. On-chip mitogenic stimulation of the T-cell array followed by immunofluorescent staining revealed IFN- γ signal co-localized with individual T-cells. The approach for single cell function analysis described here will be enhanced in the future to enable detection of multiple cytokines secreted from single immune cells arranged into a high-density array.

MATERIALS AND METHODS

Materials and Reagents

Phosphate-buffered saline (PBS, 10x) without calcium and magnesium, paraformaldehyde (PFA), surfactant TWEEN® 20, rabbit anti-mouse IgG antibody (2nd labeling antibody) Na₄EDTA, KHCO₃, NH₄Cl, poly(ethylene glycol)diacrylate (PEG-DA) (MW575), anhydrous toluene (99.9%), sodium azide, bovine serum albumin (BSA), 0.1% poly-L-lysine were purchased from Sigma-Aldrich (Saint Louis, MO). Silane adhesion promoter, 3-acryloxypropyl trichlorosilane, was from Gelest, Inc. (Morrisville, PA). Monoclonal antibodies used for capturing T-lymphocytes and cytokines consisted of the following: purified mouse anti-human CD4 Abs (13B8.2) from Beckman-Coulter (Fullerton, CA), and purified mouse anti-human IFN γ Ab (clone K3.53), biotinylated goat anti-human IFN- γ Ab from R&D Systems (Minneapolis, MN). Mouse IgG2a (OX34) was purchased from Serotec Antibodies (Raleigh, NC). Antibodies used for immunostaining of surface bound cells were anti-CD4-PE

(L120), purchased from BD Pharmingen. Fluorescein conjugated with Avidin FITC was purchased from Pierce (Rockford, IL). Human recombinant IFN- γ was from R&D Systems (Minneapolis, MN). Mitogenic activation reagents: Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich. Cell culture medium RPMI 1640: 1 $\times$, with L-Glutamine was purchased from VWR. Poly-(dimethylsiloxane) (PDMS) and its curing agents were purchased from Dow Corning (Midland, MI).

Immobilization of cell- and cytokine-specific Abs onto hydrogel-coated glass slides

Glass surfaces (25mm $\times$ 75mm) were modified with 3-acryloxypropyl trichlorosilane coupling agent and covered with PEG gel layer as described by us previously.^{19, 21} These PEG gel-coated glass slides were lyophilized for 48 hours to ensure the rapid adsorption and uniform distribution of Abs upon printing. Prior to printing, a mixture of purified anti-CD4 and – IFN γ Abs were dissolved in 1xPBS at a concentration of 0.12 and 0.2 mg/ml respectively and supplemented with Tween20 (0.005% v/v). This Ab cocktail solution was manually pipetted onto the PEG gel-coated surface to create three Ab spots (~1mm, 0.5 μ l print volume) in a row. Alternatively, a manual arrayer (MicroCaster, Schleicher & Schuell, Keene, NH) was used to dispense small print volumes (20–70 nL per spot) of the Ab cocktail solution onto the PEG surface to form a 2 $\times$ 5 array of Ab spots (~500 μ m). After printing, surfaces were air-dried and stored at 4°C prior to further use.

Fabrication of PEG gel microwells on top of Ab-containing hydrogel layer

Abs were immobilized on a PEG gel-coated glass slide as described in the previous section. This functionalized substrate was covered with a prepolymer comprised of poly(ethylene glycol)-diacrylate (PEG-DA) and 2% (v/v) 2-hydroxy-2-methyl-propiophenone and spin-coated on a Spintech S-100 (Redding, CA) at 950 rpm for 4 seconds. The prepolymer layer was then exposed to UV light (60 mWcm⁻²) light through a chrome/sodalime photomask for 0.4–0.5 sec using Omnicure 1000 light source (EXFO, Mississauga, Ontario, Canada). Regions of PEG-DA exposed to UV underwent free-radical polymerization and became cross-linked, while unexposed regions were dissolved in DI water. This process resulted in formation of PEG hydrogel microwells with Ab-decorated bottom and non-fouling side walls. Immunofluorescent staining and cell binding experiments were conducted to verify that Ab molecules remained intact during the microwell fabrication process.

Characterization of cytokine capture on Ab-decorated hydrogel surfaces

Studies were performed to characterize whether co-immobilizing cytokine- and cell-specific Abs affected sensitivity of IFN- γ immunoassay. To test this, we printed arrays of Ab spots (500 μ m diameter) comprised of either anti-IFN- γ or a mixture of anti-IFN- γ and anti-CD4 Ab molecules. The coating of glass slides with PEG hydrogel layer and printing of Ab arrays was performed as described in the preceding section of this paper. The hydrogel-coated glass slides with imprinted Ab arrays were then challenged with different concentrations of recombinant human IFN- γ (60, 125, 250, 500, and 750 ng/ml) for 1 hr. After incubation with recombinant cytokine glass slides were washed using copious amounts of 1 $\times$ PBS, then incubated with biotinylated anti-IFN- γ Abs (5 μ g/ml in 1 $\times$ PBS) for 1h at room temperature and washed with 1x PBS again. As the final step in immunofluorescent staining procedure, Ab arrays were incubated with streptavidin-Alexa546 (10 μ g/ml in 1 $\times$ PBS) for 30 min. The fluorescence emanating from Ab microarrays was imaged with a confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss, Inc.) and quantified with an Agilent microarray scanner. GenePix Pro 6.0 data analysis software (Molecular Devices, Downingtown, PA) was used to construct calibration curves of cytokine concentration vs. fluorescence intensity.

To demonstrate that PEG microwells remained open and permitted access to the underlying Ab-containing hydrogel layer the microfabricated surface was incubated with FITC-labeled

anti-mouse IgG Abs (10 $\mu\text{g/ml}$ in $1\times$ PBS) for 30 min and imaged using confocal microscopy. In other experiments, microfabricated PEG gel surfaces were incubated with recombinant human IFN- γ solution (500 ng/ml) for 1 h and then immunofluorescently stained with biotinylated anti-IFN- γ Ab and streptavidin-Alexa 546 in order to demonstrate detection of cytokine inside the microwells.

Design of a microfluidic device

A microfluidic device was designed as described by us previously²⁰ to enable the isolation of T-cells from a small blood sample and to increase the local concentration of secreted cytokines. Briefly, poly(dimethyl siloxane) (PDMS)-based microfluidic devices with imbedded channel architecture were fabricated using standard soft lithography approaches.²² Inlet/outlet holes were then punched with a blunt 16 gauge needle. The microfluidic device contained two flow chambers with width-length-height dimensions of $3\times 10\times 0.1$ mm and a network of independently addressed auxiliary channels. The auxiliary channels were used to apply negative pressure (vacuum suction) to the PDMS mold and reversibly secure it on top of a glass substrate. This strategy allowed to seal a fluid conduit on top of the Ab-containing PEG hydrogel microwells without compromising immobilized biomolecules.

A 5 ml syringe was connected to silicone tubing (1/32 inch I.D., Fisher), which was attached to the outlet of the flow chamber with a metal insert cut from a 20 gauge needle. A blunt, shortened 20 gauge needle carrying a plastic hub was inserted in the inlet. A pressure-driven flow in the microdevice was created by withdrawing the syringe positioned at the outlet with a precision syringe pump (Harvard Apparatus, Boston, MA).

Capturing T-cells from blood and detecting IFN- γ secreted by single cells

Blood was collected from healthy adult donors through venipuncture under sterile conditions with informed consent and approval of the Institutional Review Board of the University of California at Davis (protocol number 200311635-6). Red blood cells (RBC) were removed with ammonia chloride based erythrocyte lysis solution (89.9 g NH_4Cl , 10.0 g KHCO_3 , and 370.0 mg tetrasodium EDTA in 10 L of deionized water) as described previously²¹. RBC-depleted blood cell suspension was comprised of granulocytes, peripheral blood mononuclear cells and RBC debris. These cells were concentrated by centrifugation and re-suspended in RPMI1640 medium containing 10% FBS and 1% penicillin/streptomycin and lacking L-glutamine and Phenol Red (Mediatech, Herndon, VA). The leukocyte suspension was used immediately for cytokine detection experiments.

In the current study, blood cells were seeded onto two kinds of surfaces: a PEG substrate decorated with Ab spots of 500 and 1000 μm in diameter as well as PEG microwell structure superimposed on Ab spots. In both cases, the procedure for cell capture was similar. Prior to the introduction of cells, a PDMS device containing fluidic and vacuum channels was sterilized by 15 min of UV exposure in a tissue culture hood. The PDMS device was then aligned with the functionalized regions of the surface and sealed with negative pressure applied to the auxiliary channels. Afterwards, sterile $1\times$ PBS was injected into the flow chamber to remove air bubbles. Next, 50 μL of RBC-lysed blood resuspended in phenol red-free RPMI1640 media was added into the inlet reservoir (hub of a 20 gauge needle) and drawn into a microfluidic channel at a flow rate of 10 $\mu\text{L/min}$ (0.3 dyne/cm²). Upon entry of cells into a microfluidic channel, the flow was stopped and cells were allowed to bind for 5–10 min. In order to wash away non-specific cells, the flow rate was increased to between 50 $\mu\text{L/min}$ (1.7 dynes/cm²) and 100 $\mu\text{L/min}$ (3.4 dynes/cm²). The phenotype of captured leukocytes was determined by immunofluorescent staining with FITC-labeled anti-CD3 Abs and PE-labeled anti-CD4 Abs as described in the following paragraph.

To commence cytokine production cell were activated within a microfluidic device as described previously.²⁰ Briefly, captured T-cells were activated *in situ* with a mitogenic solution consisting of PMA (50 ng/ml) and ionomycin (2 μ M) in phenol red-free RPMI1640 media (with 10% FBS). Once mitogenic solution was introduced into the flow chamber, flow was stopped and a surgical clamp was secured around the inlet/outlet tubing to stop the flow and eliminate convective mixing. The microdevice was then kept in a tissue culture incubator (37°C, 5% CO₂ and 90% humidity) for 4 to 15 h.

At the end of the desired activation period, T-cells captured in the microwells were exposed to reagents for detection of IFN- γ production. After flushing away mitogenic solution with 1 $\times$ PBS, microfluidic chambers were filled with biotinylated anti-IFN- γ Abs (5 μ g/ml in 1 $\times$ PBS) for 1h at room temperature. Next, the chambers were again flushed with 1 $\times$ PBS and filled with either streptavidin-Alexa546 (10 μ g/ml in 1% BSA) or neutroavidin-FITC (10 μ g/ml in 1% BSA) for 30 min in order to detect Ab-cytokine complex (Figure 1A). If cell surface staining was desired, a mixture of NeutroAvidin-FITC (10 μ g/ml in 1% BSA) and PE labeled anti-CD4 (1/10 dilution in 1% BSA) was filled in the chambers instead. The fluorescently labeled cytokines and cells were then visualized and imaged with a confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss, Inc.).

RESULTS AND DISCUSSION

The present paper describes the development of a microfabricated cytometry platform for capture and functional analysis of T-cells. The cytometry platform consisted of PEG hydrogel microwells with Ab-decorated attachment sites and non-fouling side-walls that were combined with a PDMS-based microfluidic conduit. This microdevice was employed to capture CD4 T-cells from blood, form these cells into single cell arrays and detect IFN- γ released from individual T-cells.

Characterization of IFN- γ immunoassay

The premise of the proposed immunosensing strategy is that co-localization of leukocyte- and cytokine-specific Abs may be employed to both capture pure T-cells from a heterogeneous sample and to detect an important cytokine (IFN- γ) secreted by these cells. Unlike a report by Chen et al.,²³ who first described a concept of Ab spots for cell capture and cytokine detection, we wanted to incorporate Ab-modified surfaces into microfluidic devices and to arrange T-cells into single cell arrays in order to better connect cytokine release to specific cells. A first step towards the development of such a platform was to investigate whether co-printing of cell- and cytokine specific Abs has a detrimental effect on the sensitivity of the IFN- γ immunoassay.

In this set of experiments, arrays of Ab spots (500 μ m diameter) comprised of either anti-IFN- γ Abs or a mixture of anti-CD4 and anti-IFN- γ Abs were printed on hydrogel-covered glass slides and then incubated with varying concentration of recombinant IFN- γ . The microarrays were immunofluorescently stained and analyzed using a laser scanner. The results presented in Figure 2 demonstrate that mixing of IFN- γ Abs with CD4 antibodies had no appreciable effect on the sensitivity of IFN- γ immunoassay. Given the structural similarity of IgG molecules specific to CD4 antigen and IFN- γ , it is expected that the ratio of Ab molecules immobilized on the surface is similar to that of solution. The mechanism of Ab immobilization is by physical incorporation into the hydrogel matrix and the surface concentration of IgG molecules in the gel was determined to be ~ 1.7 ng/mm² in our previous study.¹⁹

Capture of T-cells and detection of secreted IFN- γ on Ab-decorated surfaces

In addition to detecting IFN- γ on surfaces containing a mixture of two Ab types, we wanted to demonstrate capture of T-cells and detection of T-cell secreted IFN- γ on these surface. A

number of studies carried out by us and others have pointed to the benefit of utilizing fluidic chambers in order to precise control shear stress and ensure retention of Ab-bound leukocytes as well as removal of non-specific cells.^{19, 21, 24, 25} The microfluidic devices are particularly useful because blood sample and reagent requirements are minimized while local concentration of secreted molecules is enhanced. Schaff et al. have recently described a strategy for reversibly sealing PDMS-based fluidic chambers on glass substrates by applying negative pressure.²⁶ This novel strategy was employed by us previously to integrate microarrays of cell and cytokine capture Abs into a microfluidic device.²⁰ In the present study PDMS chambers were vacuum suctioned on top of Ab-modified surfaces to create miniature (3 μ L) immunoreaction chambers. All cell seeding, patterning, stimulation and immunofluorescent staining steps were performed inside these microfluidic chambers.

In order to verify T-cell capture on anti-CD4 and anti-IFN- γ Ab regions, hydrogel-coated glass slides with imprinted Ab spots (either 500 or 1000 μ m diameter) were incorporated into a microfluidic device and were incubated with RBC-depleted whole blood. After controlled washing, leukocytes captured on Ab regions were stained with FITC-labeled (green) anti-CD3 Abs to determine a presence of CD3 antigen – a marker common to all T-cells.²⁷ The results of immunofluorescent staining, shown in Figure 3A, revealed that the vast majority of leukocytes captured on the Ab regions were stained with green fluorescence pointing to their T-cell phenotype. These data corroborate our previous results pointing to very high purity of T-cell isolation (<95%) on Ab-modified surfaces enclosed inside microfluidic device.^{19, 21}

In addition to capturing T-cells, we wanted to demonstrate detection of secreted IFN- γ on the same Ab spot. In these experiments, T-cells were bound on Ab spots within microfluidic devices and were then mitotically activated with PMA and ionomycin to commence cytokine production. The flow was stopped during activation to prevent convection and the microfluidic devices were incubated from 4 to 15 hrs under physiological conditions (37°C and 5% CO₂). After activation, T-cell containing regions were stained with biotinylated anti-IFN- γ Abs and streptavidin-Alexa546 (red) complex to reveal cytokine production. Figure 3B depicts a representative IFN- γ signal after 4 h mitogenic activation. The red fluorescence observed in this image clearly points to release of IFN- γ from T-cells and local detection of this cytokine. Increasing time of mitogenic activation to 15 h resulted in much stronger immunofluorescence signal due to IFN- γ release (Figure 3C).

While proving the concept of co-localized capture of both T-cells and secreted cytokines on the same Ab region, the results shown in Figure 3(B,C) also point to diffusion and smearing of the cytokine which makes it difficult to accurately assign IFN- γ signature to specific T-cells. In order to better coordinate cytokine signals with individual cells we sought to organize Ab-bound T-cells into single cell arrays using PEG hydrogel photolithography.

Hydrogel microwells to capture single T-cells and detect secreted IFN- γ

PEG hydrogel photolithography, a micropatterning technique described by us previously,²⁸ was employed in order to define sites of single cell attachment within the Ab domains. One design criterion important for the success of our device was to ensure the purity of captured T-cells. Our previous studies pointed to PEG hydrogel-coated glass substrates with immobilized Abs as optimal surfaces for eliminating non-specific binding of leukocytes.^{19, 21} Therefore, in the present study we constructed surfaces for single cell capture composed entirely of PEG hydrogel.

Figure 4 shows arrays of hydrogel microwells comprised of cell- and cytokine-specific attachment sites and non-fouling side-walls. One concern with this technique was the attachment of PEG hydrogel wells to the underlying hydrogel layer. However, we found adhesion of the microwells to be excellent with no delamination occurring after 4 days of

incubation at 37°C. This attachment is likely due to the presence of unreacted acrylate groups in the first layer of PEG that become covalently linked with the second layer of PEG. There is also a possibility of interpenetration of PEG chains between the two polymer layers. Importantly, physical adsorption of Ab molecules into the first layer of hydrogel did not prevent effective binding of the second gel layer containing microwells. In order to demonstrate the presence of Abs at the bottom of the microwells, surfaces were incubated with FITC-labeled anti-mouse IgG Abs that were immunoreactive to both anti-cell (anti-CD4) and anti-cytokine (anti-IFN- γ) Abs. The green fluorescence observed within the microwells in Figure 4A demonstrates localization of Ab molecules at the bottom of the wells. To further demonstrate capability of cytokine sensing within the microwells, surfaces were incubated with 500 ng/ml IFN- γ solution and then immunofluorescently labeled with biotinylated anti-IFN- γ and streptavidin-Alexa546. As shown in Figure 4B, the red fluorescence signal due to cytokine binding was once again localized to the bottom of the wells. Therefore, the data in Figure 4 (A,B) point to the presence of Ab molecules in the attachment sites and also highlight the non-fouling properties of walls of PEG microwells.

To further demonstrate the utility of our micropatterned surfaces for capturing immune cells, Ab-modified PEG hydrogels were incorporated into microfluidic devices as described above and exposed to RBC-depleted blood suspension. Dimensions of the attachment sites were varied to determine the microwell diameter most suitable for capturing single T-cells. Representative results shown in Figure 4C demonstrate capture of immune cells in Ab-decorated PEG hydrogels. Significantly, immunofluorescence analysis revealed that most of the captured cells stained positive (green) after labeling with FITC-anti-CD3 Ab. CD3 antigen is a T-cell-specific marker,²⁷ therefore, our captured cells were indeed T-lymphocytes. Figure 4C also underscored the possibility of controlling the number of captured cells by the area of the attachment site. As shown in this experiment, 20 μ m diameter wells contained single cells while larger microwells captured multiple cells per well. Fabrication of an array of redundant 20 μ m diameter hydrogel wells resulted in reproducible capture of single T-cells from a heterogeneous leukocyte suspension (Figure 4D). This is consistent with our previous reports of high occupancy rate (>90%) and high incidence of single cells (~1.5 cells/per well) in the arrays of PEG hydrogel microwells.¹⁵ However, unlike previous studies employing cell lines and well-defined cell suspensions, the present paper highlights the possibility of capturing primary T-cells from minimally processed whole blood.

Upon organizing primary T-cells into single cell arrays, we proceeded to detect IFN- γ release from these cells. In these experiments, detailed schematically in Figure 1B, T-cells were captured on micropatterned surfaces inside a microfluidic device and then exposed to mitogens (PMA and ionomycin) to commence cytokine production. During this activation period (15 h), flow inside the microfluidic chamber was stopped to avoid possible convective mixing of secreted cytokines from cells at different locations. After completion of mitogenic activation, immune cell arrays were immunofluorescently labeled for CD4 cell-surface antigen and IFN- γ inside the microfluidic devices. Results of a representative experiment are highlighted in Figure 5. As seen from Figure 5A, immune cells did indeed organize into single cell arrays inside the microwells. Importantly, the immune cells stained positive for CD4 marker (red fluorescence in Figure 5B) and also had large circular nuclei consistent with lymphocyte morphology (DAPI nucleus staining not shown here). This evidence points to CD4 T-cells being captured in PEG hydrogel microwells. Significantly, immunofluorescent staining of cellular micropatterns also revealed a “halo”-like IFN- γ signal (green fluorescence) associated with most but not all T-cells. As seen from Figure 5D, mitogenic activation resulted in a strong IFN- γ fluorescence signal that corresponded to 661 ± 75 ng/ml concentration (39 ± 4.4 nM). The cytokine concentration detected from cells is well above the detection limit of the IFN- γ immunoassay reported by us previously to be 20 ng/ml.²⁰ This image also reveals heterogeneity

in the function of T-cells, with some CD4 cells not secreting detectable quantities of cytokine and others producing a strong fluorescence signal.

CONCLUSION

The present study describes a surface micropatterning strategy and a microdevice designed to detect IFN- γ production of individual CD4 T-cells isolated from human blood. The surface engineering strategy consisted of photolithographic patterning of non-fouling PEG hydrogel microwells on top of the hydrogel layer containing cell- and cytokine-specific Abs. This surface micropatterning was designed to allow co-localization of single CD4 T-cells and IFN- γ -sensing Abs. Hydrogel microwells were combined with a PDMS-based microfluidic conduit in order to create a functional microdevice. Incubation of RBC-depleted whole blood in the microdevice followed by mitogenic activation and immunofluorescent staining resulted in capture of single T-cells within the microwells and also revealed IFN- γ signal associated with specific single cells.

While single cell analysis of immune cell function is carried out routinely using flow cytometry or ELISpot instruments, the bioanalytical approach described here offers a number of novel capabilities and advantages compared to the current state-of-the-art: 1) cell purification and cytokine detection can be performed on-chip, in the same microdevice, 2) cytokine production is associated with specific viable T-cells that can hypothetically be retrieved for further downstream analysis or re-cultivation,^{14, 29} 3) the surface micropatterning strategy is very-well suited for creating large-scale and high-density single cell arrays so that thousands of primary cells may be analyzed in parallel, 4) the use of microfluidics allows to minimize blood volume requirement and to control shear stress inside the device.

In the future, the functionality of the microdevice will be expanded to enable detection of multiple cytokines from single cells. This microfabricated cytometry platform will be employed for the analysis of antigen-specific immune and will also have applications in pediatric immunology where multiparametric analysis based on a small blood volume is particularly important.

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Figure 1.

(A) Fabrication of hydrogel microwells for capturing single T-cells and secreted cytokines. *Step 1.* Anti-CD4 and anti-IFN- γ Abs are mixed in solution and physisorbed onto a lyophilized, PEG hydrogel-coated glass slide. *Step 2.* Photosensitive PEG prepolymer is spin-coated on top of the Ab-containing hydrogel layer and then exposed to UV through a photomask. *Step 3.* Prepolymer not exposed to UV is removed by development in water, leaving behind microwells with non-fouling walls and Ab-containing attachment sites. (B) Detection of IFN- γ secreted by single T-cells. *Step 1.* PEG hydrogel microwells are enclosed inside a PDMS microfluidic device and are incubated with RBC-depleted human blood. *Step 2.* Controlled washing is used to remove non-specific cells, leaving behind Ab-bound T-cells. *Step 3.* Mitogenic activation induces cytokine production in T-cells. Secreted cytokines become captured in the proximity of cells and are detected using sandwich immunoassay.

Figure 2. Comparing sensitivity of IFN- γ immunoassay. Ab spots comprised of anti-IFN- γ or anti-CD4 and -IFN- γ were printed into PEG hydrogel-covered glass slides and incubated with varying concentrations of human recombinant IFN- γ . Co-immobilizing two Ab types did not affect characteristics of IFN- γ immunoassay.

Figure 3.

Capturing T-cells and detecting IFN- γ release on Ab-containing surfaces. Solution containing a mixture of anti-CD4 and -IFN- γ Abs was pipetted onto a PEG hydrogel-covered glass slide creating ~1 mm diameter Ab spots. An Ab-modified surface was placed inside a microfluidic device, incubated with RBC-depleted whole blood and then mitogenically activated. (A) Immunofluorescent staining with anti-CD3-FITC - reveals that majority of captured cells stain positive (green) for this T-cell marker. T-cells are also stained with DAPI (blue) to reveal morphology of the nucleus. (B–C) Immunofluorescent staining with anti-IFN- γ -biotin and streptavidin-Alexa546 shows detection of secreted IFN- γ in the vicinity of surface bound T-

cells (red fluorescence). Comparison of mitogenic activation lasting 4 h (**B**) and 15 h (**C**) shows a stronger cytokine signal (red) corresponding to longer activation.

Figure 4.

PEG hydrogel microwells containing T-cell capture (anti-CD4) and cytokine detection (anti-IFN- γ) Abs. (A) Micropatterned surfaces were stained with FITC-labeled anti-mouse IgG to show localization of the Abs (green fluorescence) at the bottom of PEG hydrogel microwells. (B) To reveal presence of cytokine-sensing Abs, micro-patterned surfaces were challenged with human recombinant IFN- γ (500 ng/ml) and then incubated with anti-IFN- γ -biotin and streptavidin-Alexa546 (red fluorescence). (C) Hydrogel microwells were enclosed in a microfluidic device and incubated with RBC-depleted human blood, resulting in capture of the cells. This image highlights the possibility to sequester single cells by defining the area of an attachment site. Majority of cells stained positive for CD3 marker (green fluorescence) pointing to T-lymphocyte phenotype. (D) SEM image showing an array of single T-cells residing in 20 μ m diameter hydrogel microwells.

Figure 5.

Detection of IFN- γ production from single T-cells. Cells were captured on micropatterned surfaces inside a microfluidic device and then activated with mitogens for 15h. **(A)** Brightfield image of single T-cells captured from peripheral blood inside the PEG hydrogel microwells. **(B)** Staining with anti-CD4-PE (red) shows that all of the captured cells are CD4 positive T-cells. **(C)** The same image stained with anti-IFN- γ -boitin and neutravidin-FITC shows “halo” of green fluorescence due to secreted cytokine. The IFN- γ concentration in the vicinity of single T-cells was estimated to be 661 ± 75 ng/ml (39 ± 4.4 nM).