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Research paper

Panning of multiple subsets of leukocytes on antibody-decorated poly(ethylene) glycol-coated glass slides

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Abstract

The antibody (Ab) array format provides a unique opportunity to pan and characterize multiple leukocyte subsets in parallel. However, the questions of reproducibility and robustness of leukocyte panning on Ab arrays need to be answered for this technology to become an immunophenotyping tool. The present study sought to address several of these questions, including: (1) purity of leukocyte subsets captured on Ab regions, (2) dynamics of leukocyte binding, (3) elimination of non-specific cell adhesion, and (4) standardization of cell washing conditions. Abs for CD4 T-cells, CD8 T-cells, CD36 monocytes, and CD16b neutrophils were dispensed onto standard glass slides containing a thin film of poly(ethylene glycol) (PEG) hydrogel. PEG gel coating was highly effective in eliminated non-specific cell adhesion on the surface. Incubation of the Ab arrays with red blood cell (RBC) depleted whole blood resulting in antigen-specific panning of leukocyte subsets on the respective Ab domains. A flow through chamber was employed to determine optimal shear stress conditions for removal of non-specifically attached cells. The purity of the four subsets remaining on the surface after washing was determined by Wright staining and immunofluorescence, and was found to be as follows: CD4 T-cells (99.2±0.3%), CD8 T-cells (98.7±0.3%), CD36 monocytes (97.2±0.9%), and CD16b neutrophils (99.1±0.6%). In conclusion, the methods described in this study allow to separate whole blood into pure leukocyte subsets with minimal sample preparation and handling. These approaches will be valuable in the future development of Ab arrays as tools for quantitative immunophenotyping of leukocytes.

Keywords: CD4 T-cells; CD8 T-cells; Monocytes; Neutrophils; Antibody; Array

1. Introduction

Leukocytes are blood constituents that serve as key clinical indicators in the diagnosis and monitoring of injuries, infections, malignancies or autoimmune disorders (Turgeon, 1999). Leukocytes are a heterogeneous mixture of several cellular subsets defined by morphology and antigenicity. Monitoring of subset population proportions and enumerating absolute leukocyte counts provides critical clinical information regarding patient care. For example, enumeration of $CD4^+$ T-lymphocytes is a key step in detection and evaluation of infections such as human immunodeficiency virus (HIV) or malaria

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(Hanscheid, 1999; Brando et al., 2000; Norris and Rosenberg, 2002). In patients treated for bacterial infections, levels of neutrophils are reflective of treatment efficacy (Houwen, 2001). Leukemia is often assessed by interpretation of patterns and intensity of cell surface antigen expression of several leukocyte types (Jennings and Foon, 1997; Campana and Behm, 2000).

The gold standard for the analysis of leukocyte subset proportions is flow cytometry. The flow cytometry techniques have been employed in both basic research and clinical settings for the quantitative and multi-parametric leukocyte population analysis. While flow cytometry is the method of choice for leukocyte immunophenotyping, it has several shortcomings: a given cell can only be observed once, and cell morphology observation and kinetic analysis options are limited. In contrast to flow cytometry, cells affixed to glass slides can be visualized using light microscopy, observed over extended periods of time, stained multiple times and archived. The desire to perform multi-parametric analysis of cells immobilized on the glass slides has spurred the development of laser-scanning cytometers or image cytometers: light microscopy-based tools that seek to mimic flow cytometry in terms of informational content.

In addition to the innovative instrumentation, the field of image cytometry will benefit from "smart" glass surfaces that enable precise spatial arrangement of cells for optical imaging. One way to achieve such spatial arrangement is to decorate the surface with cell-specific ligands. In immunology, modifying surfaces for antigen-specific leukocyte capture is a well-established method for both positive and negative cell selection. First proposed by Mage (Mage et al., 1977) and Wysocki (Wysocki and Sato, 1978), this method has traditionally been used to capture a single leukocyte subset on Ab-decorated substrates from a heterogeneous mixture (Chang, 1983; Williams et al., 1986; Small et al., 1994; Bousso et al., 1997). Recently, the concept of leukocyte panning was coupled with protein microarraying to create Ab arrays for capturing multiple leukocyte populations on the same surface (Belov et al., 2001). In their work, Belov and co-workers printed Ab arrays for immunophenotyping of several types of leukemias.

The Ab array format provides a unique opportunity to transition from single to multiplexed leukocyte panning and to capture multiple cellular subsets in parallel. However, several questions pertaining to reproducibility and robustness of leukocyte panning on Ab arrays need to be answered for this technology to become an immunophenotyping tool. The success of an image cytometry platform depends on the isolation of high purity leukocyte subsets on respective Ab domains. To this end, this study addresses: (1) optimization of washing conditions to remove non-specifically bound cells, (2) characterization of dynamics of leukocyte adhesion to Ab regions, and (3) elimination of non-specific leukocyte adhesion through surface modification. Although every leukocyte subset plays an essential role in human immunity, four antigen-specific leukocytes, CD4 T-cells, CD8 T-cells, CD36 monocytes, and CD16b neutrophils, were characterized in our experiments. Abs for specific leukocyte antigens were coated on a thin film of poly (ethylene glycol) (PEG) hydrogel. Wright staining and immunostaining were used to confirm phenotype of the leukocyte subsets captured on the respective Ab domains of the same glass slide. Immunostaining of surface-bound cells pointed to the following purity for the four surface bound leukocyte subsets: 99.2±0.3% (CD4 T-cells), 98.7±0.3% (CD8 T-cells), 97.2±0.9% (CD36 monocytes), and 99.1±0.6% for CD16b neutrophils. The proposed technology provides a rapid and simple method for separating whole blood leukocytes into pure constituent subsets with minimal sample preparation and handling.

2. Materials and methods

2.1. Materials and reagents

Phosphate-buffered saline (PBS) without calcium and magnesium was purchased from Gibco (Grand Island, NY). Antibody panel for leukocyte capture consisted of the following: purified mouse anti-human anti-CD16b (clone: CLB-gran11.5), biotinylated mouse anti-human anti-CD4 (RPA-T4) and anti-CD8 (RPA-T8) obtained from BD Pharmingen (San Diego, CA), biotinylated mouse IgG2a (OX34) from Serotec Antibodies (Raleigh, NC), biotinylated anti-human anti-CD36 (SMO) from Ancell Co. (Bayport, MN). Antibodies used for immunostaining of surface bound cells or flow cytometry analysis were as follows: anti-human anti-CD3-FITC (UCHT1), anti-CD4v4-PE (L120), anti-CD4-PE Cy5 (SK3), anti-CD8-PE (HIT8a), anti-CD8-PE Cy5 (HIT8a), anti-CD36-PE (CB38), anti-CD14-PE (MϕP9), anti-CD14-PerCP (MϕP9), and CDw125-PE (A14) (BD Pharmingen), anti-CD66b-FITC (80H3) (Serotec Antibodies). Corresponding isotype controls were from BD Pharmingen and Serotec Antibodies. Na_4EDTA, $KHCO_3$, NH_4Cl, poly(ethylene glycol)diacrylate (PEG-DA) (MW575), 2,2′-dimethoxy-2-phenyl-acetophenon (DMPA), anhydrous toluene, sodium azide, bovine serum albumin (BSA), 0.1% poly-L-lysine, glovebag and Wright stain (modified) were from Sigma-Aldrich (Saint Louis, MO). Silane adhesion promoter, 3-acryloxypropyl trichlorosilane, was from Gelest, Inc. (Morrisville, PA). Regular (3×1 in.^{2}) glass slides were

purchased from Fisher Scientific (Pittsburgh, PA). FAST® slides were from Whatman Schleicher and Schuell (Keene, NH).

2.2. Modification of glass substrates with silane adhesion promoter and PEG hydrogel films

The glass slides were modified with silane coupling agent to ensure covalent attachment of PEG hydrogel films to glass slides. Silane modification followed the procedure described previously (Revzin et al., 2003, 2005). Briefly, glass slides were immersed for 30 min in "piranha" solution consisting of a 3/1 ratio of aqueous solutions of 50% (v/v) sulfuric acid and 30% (w/v) hydrogen peroxide. After removal from the "piranha" bath, glass slides were thoroughly rinsed with deionized (DI) water and dried under nitrogen. Clean glass substrates were then immersed in 2 mM solution of 3-acryloxypropyl trichlorosilane in anhydrous toluene for 10 min. After removal from the silane solution, slides were rinsed in toluene, dried with nitrogen and stored at 4 °C prior to use.

PEG hydrogel coating of the silanized glass slides was performed according to the protocol described elsewhere (Revzin et al., 2003, 2005). In brief, PEG hydrogel films on glass slides were made from the precursor solution of PEG-DA (MW575) with 1% w/v photoinitiator, DMPA. This solution was spun at 700–1000 rpm for 5 s onto the silane-treated glass surface containing terminal acrylate functional groups using a spin-coater (Machine World Inc., Redding, CA). The uniform layer of the PEG-DA precursor solution on glass was then exposed to 365 nm, 15-mW/cm^2 UV light from a light source (Quintel Co., San Jose, CA). The UV exposure, times ranging from 1 to 2 s, caused free-radical polymerization and crosslinking of the diacrylated PEG. The resultant hydrogel films were 2 to 10 μm in thickness as measured by Dektak3 surface profiler (Veeco Instruments, Santa Barbara, CA).

2.3. Printing antibody domains onto PEG hydrogel-coated glass slides

As shown in Fig. 1, spots of multiple antibodies were created by delivering 0.7–1.2 μL of the antibody solution using a pipette. Deposited solution was allowed to air dry for 30 min at ambient temperature, resulting in solvent evaporation and antibody physisorption to hydrogel-coated glass slides. Resulting circular antibody regions were 1.5–2.0 mm in diameter. Composition of the antibody array and number of the printed spots depended on the goals of the experiment. To assess purity of the captured leukocyte subset by flow cytometry, cells were seeded onto redundant 20–30 element arrays of the same antibody, incubated and sheered off the surface according to the protocols described in the succeeding sections of this article. To demonstrate simultaneous isolation of multiple leukocyte subsets on the same surface, anti-CD4, -CD8, -CD36, -CD16b, and a negative control mouse anti-IgG2a Ab were printed in a 5×3 array. The antibody concentrations were as follows: biotinylated anti-CD4 25 μg/mL, biotinylated anti-CD8 6.25 μg/mL, and biotinylated anti-CD36 1.0 mg/mL, biotinylated mouse IgG2a 0.1 mg/mL, and purified anti-CD16b 0.5 mg/mL. While most of the Abs were biotinylated, biotin–avidin conjugation route was not pursued and Ab immobilization occurred by physisorption

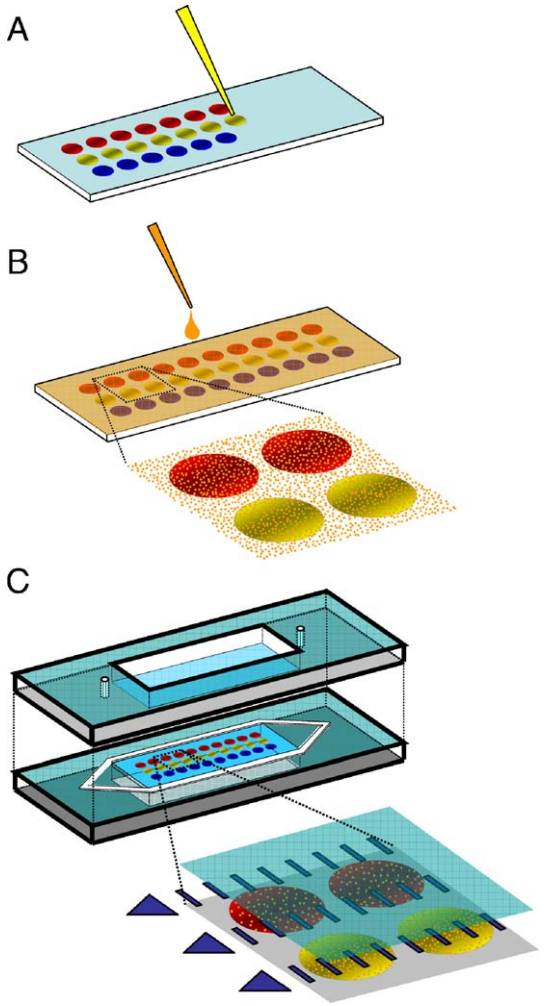

Fig. 1. Experimental process flow. (A) Ab solution is dispensed using a pipette onto PEG gel-coated glass slide, forming an array of Ab spots. (B) Leukocyte suspension is incubated with the surface. (C) After incubating with cells, glass slides are placed into a flow chamber and exposed to precise washing conditions.

as described above. Immediately prior to use, surfaces were gently washed by PBS and used for leukocyte incubation.

2.4. Blood preparation and leukocyte seeding

Samples of peripheral blood were collected into heparinized tubes from healthy volunteers. Red blood cell (RBC) lysing solution was prepared by diluting a stock solution of 10^{-3} M Na_4EDTA, 10^{-2} M $KHCO_3$, 1.7 M NH_4Cl in H_2O, pH=7.3, 1/10 in distilled water. 3 mL of whole blood were then added to 42 mL of the RBC lysis solution were gently mixed for 5 min at ambient temperature and centrifuged at 200×*g* for 7 min at 4 °C to remove lysed RBCs. The pellet was resuspended in 20 mL of PBS and centrifuged again using the same conditions. Thereafter, leukocytes were resuspended in 3 mL of PBS. BSA was not added in the medium, because it led to the non-specific binding to antibody regions in a preliminary study. We did count leukocyte concentration in the cell suspension, but did not check routinely the viability of leukocytes using trypan blue exclusion. However, we confirmed that the cell viability was more than 98% in the first 10 experiments. The leukocyte concentrations in the suspension varied from 2.0×10^6 to 6.0×10^6 cells/mL. In leukocyte seeding experiments, 2 mL of the leukocyte suspension were pipetted onto the antibody array regions of the glass slide. Glass slides were placed into P-100 petri dishes to prevent solution evaporation and incubated at room temperature. In most experiments, leukocytes were incubated for 15 min, but the incubation times varied from 5 to 30 min in time-related studies of cell adhesions as seen in Fig. 3.

2.5. Analysis of non-specific leukocyte adhesion on PEG hydrogel-coated glass slides

To assess the ability of PEG hydrogel films to prevent non-specific leukocyte adhesion, we compared PEG coating to poly-L-lysine-coated glass slides and commercially available, nitrocellulose-coated FAST® slides. Lysine-modified glass surface served as a positive control while FAST slides represented a commercial product designed to diminish non-specific adsorption. To prepare lysine-modified substrates, glass slides were first exposed to 50% oxygen plasma for 3 min at 150 mW in a plasma chamber (PX-250, March Instruments, Concord, CA). This treatment imparts a partial negative charge onto a glass surface. Glass slides were then immersed in aqueous 0.01% w/v poly-L-lysine solution for 30 min and washed with 1× PBS. FAST® slides were pre-blocked in 10% w/v BSA for 30 min at ambient temperature before cell seeding experiments. Neat PEG hydrogel-coated glass surfaces were used in cell seeding experiments.

Leukocytes were incubated on these surfaces for 15 min at ambient temperature. After incubation period expired surfaces were washed gently by PBS from the backside, followed by observation and enumeration of surface-bound cells using inverted microscope. Cell adhesion on a surface was measured from a cellular image merged with a field finder. For each cell adhesion analysis, three measurements were made and averaged in 1 mm^2 square. All images were obtained at 2× or 20× magnification by a SPOT digital camera (Diagnostic Instruments, Inc., Burlingame, CA) attached to the Nikon Eclipse 2000E inverted microscope.

2.6. Flow chamber for removal of loosely attached leukocytes

Isolation of leukocyte subsets on the glass surface required reproducible and controlled washing conditions which are not provided by manual washing. A chamber designed previously in our laboratory (Tilles et al., 2001) was used to establish precise control of the shear stress applied to surface-bound leukocytes during washing. As shown in Fig. 1B, this flow chamber was designed for a 3×1 $in.^2$ glass slide to be placed between two polycarbonate plates ($50.8\times127\times6.35$ mm^3 (width × length × height)) and secured with screws. Tubing (0.8 mm in diameter) was inserted into the chamber through inlet and outlet holes. A gasket with 100 μm depth was used to create and maintain an enclosed conduit between the upper plate and a glass slide. The effective dimensions of this conduit were $25.4\times76.2\times0.100$ mm^3 (width × length × height). Both plates had a glass window allowing for microscopy observation and leukocyte enumeration experiments to be performed *in situ*. A shear stress at the wall (τ_w) within a parallel plate flow chamber is directly proportional to the flow rate (Q) as described by the equation below:

$$\tau_w = \frac{\mu Q}{\omega h^2} \quad (1)$$

where μ is the viscosity of water (10^{-3} kg/m/s), ω the chamber width (25.4 mm), and h the chamber height (100 μm).

To isolate antigen-specific cells on a surface, such as $CD4^+$ T-cells, $CD8^+$ T-cells, $CD36^+$ monocytes, and $CD16b^+$ neutrophils, the slide after leukocyte incubation was placed and exposed to a constant shear-flow of PBS in this chamber at room temperature. In the present study, we tested three different flow rates of 200, 400, and 2000 μL/min, which is equivalent to 52.5, 105, and 525 dyn/cm^2,

respectively. Using the flow chamber, the slides were subjected to various flow rates in a stepwise manner. To start with, a flow rate of 200 μL/min was applied for the first 15 min and then the flow rate was increased to 400 μL/min for the next 15 min. At the end of 30 min again the flow rate was increased to 2000 μL/min. At the end of each time step interval, 20× images were taken from the same site on each antibody region under microscopy through the window of the chamber, and the numer of cells remained on each antibody domain was counted. More than 90% of all the eliminated cells were removed from the surface within the first 3 min, but a cellular detachment reached a state of equilibrium in 15 min.

2.7. Flow cytometry analysis of purity of captured leukocytes

In order to assess the purity of the captured leukocyte subset, cells were incubated with a redundant Ab array of a single antibody as described in Section 2.4. After flow chamber washing step dislodged and removed loosely bound cells, glass slides with remaining adherent leukocytes were removed from the chamber and exposed to repeated manual pipetting with 500 μL of 1% w/v BSA in 1× PBS solution on a P-100 petri dish. The collected leukocytes on the petri dish were transferred to BD Falcon™ 5 mL polystyrene round-bottom tube for flow cytometry. This washing approach created shear stresses suitable for removal of specifically bound leukocytes while allowing to maintain high cell concentration. Typically, an array of 20 to 30 redundant Ab spots yielded ~0.3 to 0.5×10^6 leukocytes. After detachment, leukocytes were incubated with fluorescent antibodies for 15 min in the dark at ambient temperature, and analyzed by flow cytometry to assess the purity of the leukocyte subsets.

Use of the same antibodies for cell capture and flow cytometry analysis was likely to affect the efficiency of fluoescence Ab labeling due to the target antigen being occupied by the capture antibody. Therefore, in the case of $CD4^+$ T-cell, $CD8^+$ T-cell, and $CD36^+$ monocyte characterizations, Abs were selected to bind to different epitopes of the same antigen. For neutrophil analysis, capturing and labeling Abs were selected for different antigens. The following sets of mouse anti-human antibodies were used for each analysis: anti-CD4 decorated surface, anti-CD4 (clone: RPA-T4) for capture, FITC-CD3, PE-CD4v4 (L120), and PECy5-CD8 Abs for labeling; anti-CD8 decorated surface, anti-CD8 (RPA-T8) for capture, FITC-CD3, PE-CD8 (HIT8a), and PECy5-CD4 Abs for labeling; anti-CD36 surface, anti-CD36 (SMO) for capture, PE-CD36 (CB38), PerCP-CD14 Abs for labeling; assessment from CD16b surface, FITC-CD66b, PE-CDw125 Abs. Stained cells were washed twice with PBS by centrifugation, then resuspended in 1% paraformaldehyde until analysis. Five thousand or more cells were measured on a FACScalibur (BD) flow cytometer running CELLQuest Software (BD Immunocytometry Systems, San Jose, CA) and analyzed with WinMDI 2.5 software. A primary gate (R1) was routinely set to exclude RBCs or debris. To assess purity of the subset of the cells from a surface, cells in the R1 window were counted as a proportion of the total number of events counted. Phenoypic purity of all the cells was determined in a histogram or a two-dimensional dot plot in R1, compared with markers set on the 99th percentile by using isotype-matched irrelevant antibodies as a reference. CD3, CD4, and CD8 were used for evaluating T-cell phenotype while CD66b was for neutrophil identification. For morphological analysis by flow cytometry, further analytical gates were set on lymphocytes or granulocytes within R1 based on a forward and side scatter gram of whole leukocytes.

2.8. Analysis of surface bound leukocytes by Wright stain and immunostaining

Wright staining and immunostaining approaches were used to establish phenotype of leukocytes captured on the surface. Because our fluorescence microscope allowed only two-color analysis, immunofluorescent staining was accompanied by Wright staining. The latter technique defined the generic phenotype (e.g. lymphocytes) while the former method was used for sub-phenotypic identification of the cells (e.g. $CD4^+$ vs. $CD8^+$ lymphocytes).

The manufacturer-suggested Wright staining protocol was modified to increase leukocyte staining time to 20 min. Three differentials, lymphocytes, monocytes, and neutrophils were determined by the same investigator based on cellular and nucleus form, chromatin pattern, and cytoplasmic granules or vacuoles. To assess the three differentials of captured cells on a surface by Wright stain, more than 200 cells were counted on each antibody-coated region or surface.

Immunostaining was used for sub-phenotypic analysis of surface-bound cells with CD4 T-cells defined as $CD3^+$ and $CD4^+$, CD8 T-cells as $CD3^+$ and $CD8^+$, CD36 monocytes as $CD14^+$ and $CD36^+$ cells, while neutrophils captured on anti-CD16b Ab were characterized by immunostaining as $CD66b^+$ and $CDw125^-$.

An array for isolation of multiple leukocytes subsets consisted of anti-CD4, anti-CD8, anti-CD36 and anti-CD16b Abs printed in triplicates (i.e. 4×3 array) in four different regions of the glass slide as shown in Fig. 5B. Leukocytes from RBC depleted whole blood were deposited and incubated onto the surface as described

above. Upon washing away non-specifically bound cells in the flow chamber, 3×1 in.^{2} glass slides with captured leukocytes were cut into 4 pieces using diamond scribing pen. Three glass pieces containing bound cells were placed into 36 mm petri dishes and incubated with FITC and PE-labeled Abs for 1 h at ambient temperature in the dark. The fourth glass piece from the same glass slide was used for Wright staining and subsequent leukocyte differential counts.

Fluorescence imaging of the immunostained leukocytes was performed using a Nikon Eclipse TE2000 inverted microscope (Nikon, Japan) with excitation/emission filters suited for fluorescein (480±30 nm/535±40 nm) and rhodamine (540±25 nm/605×50 nm) analysis. A set of three 20× images such as bright field, fluorescein, and rhodamine, was taken from each antibody spot, and merged when analyzed. To assess the phenotypical purity of captured cells on a surface by immunostaining, more than 200 cells were counted on each antibody-coated region using the merged images.

2.9. Statistical analysis

All data were expressed as means±S.D. of the mean values unless otherwise specified. Results were analyzed by analysis of variance (ANOVA). Scheffe's comparison test was used to compare cell density among 3 groups. Paired *t*-test was used for the comparison of differential counts between lysine surface and blood smear. The level of significance was accepted at $p<0.05$.

3. Results

This paper investigated panning multiple pure leukocyte subsets from RBC depleted whole blood on Ab decorated glass surfaces. Capture of the highly purified CD4 T-cells, CD8 T-cells, CD36 monocytes and CD16b neutrophils on the same glass surface was achieved within 30 min of incubation and verified by immunofluorescence staining and flow cytometry. PEG hydrogel coating of the glass slides was introduced to eliminate background leukocyte adhesion. Protocols for cell incubation and surface washing were optimized. Overall, this study addressed several important aspects of leukocyte panning on Ab arrays and will enable future development of Ab array-based immunophenotyping tools.

3.1. Evaluation of non-specific leukocyte adhesion on PEG hydrogel-coated glass slides

In order to achieve precise localization of leukocytes on Ab domains and to ensure purity of the subset within the domain the problem of non-specific cell adhesion had to be addressed. In our previous work, PEG hydrogels were successfully utilized to prevent unwanted attachment of epithelial cells to glass (Revzin et al., 2003). In the present study, suitability of this surface coating for preventing non-specific leukocyte adhesion was investigated. Three types of surfaces were compared in our experiments: commercial nitrocellulose-coated FAST® slides, PEG gel-modified glass surfaces and glass slides coated with 0.01% v/v poly-L-lysine. The latter surfaces are ubiquitously adhesive and were used as positive controls in the experiments. RBC depleted leukocytes (1×10^6 cells/mL) containing both granulocytes and PBMCs were incubated with the surfaces for 30 min, then washed and enumerated under the microscope. Results, reported as concentration of cells on the surface in Fig. 2A, clearly indicate cell-adhesion resistance of both PEG-coated (0.77 ± 1.08/mm^2) and FAST® glass slides (6.48 ± 2.45/mm^2) when contrasted with cell adhesive lysine-modified surfaces (1150 ± 233/mm^2). These data also indicate that PEG gel coating prevented non-specific leukocyte adhesion more effectively than commercial nitrocellulose-covered glass slides. It is important to note that FAST® glass slides were pre-blocked with high concentration BSA solution prior to cell incubation whereas PEG gel-coated slides required no such surface modification. We hypothesized that given its resistance to non-specific leukocyte adhesion, PEG hydrogel should be an excellent surface for immobilizing antigen-specific Abs and capturing pure leukocyte subsets. Results presented in the later sections of the paper validate this hypothesis.

3.2. Distribution of leukocyte populations on ubiquitously adhesive lysine surfaces

The unique feature of the array format is the possibility to set up several panning experiments in parallel on the same slide. Such an array will contain isotype controls and antigen-specific Ab spots with multiple replicates for statistical significance. In the case of small cell samples of unknown concentration, there will also be a need to include ubiquitously adhesive domains into the array. These domains will enable on-chip determination of cell concentration and subset proportions. Lysine coating imparts a positive charge onto a surface and makes it generically adhesive to negatively charged cells by electrostatic forces. Interactions of RBC depleted whole blood leukocytes with lysine-coated glass surfaces were investigated to determine whether the capture of all cells in suspension was possible and whether the surfaces introduced a bias in the subset distribution of captured leukocytes.

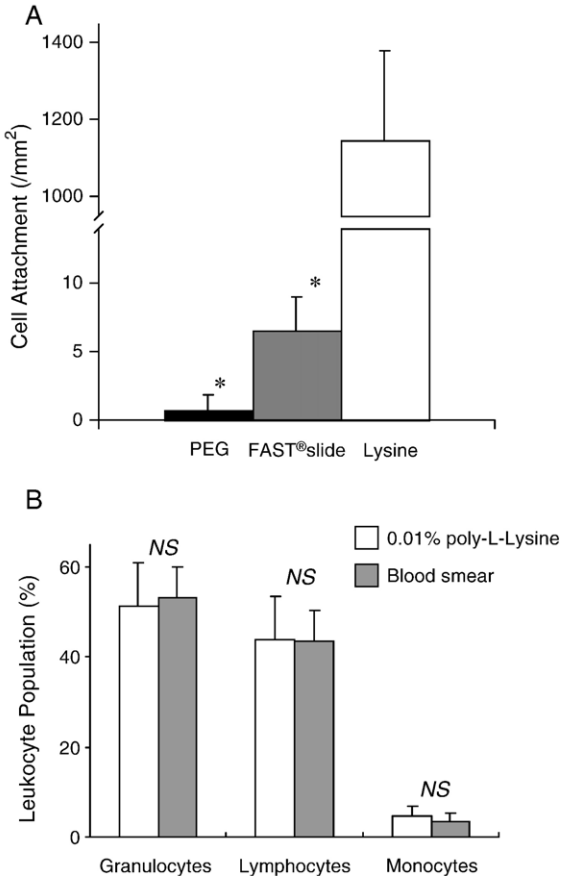

Fig. 2. Elimination of non-specific leukocyte attachment. Leukocytes in suspension (1×10^6 cells/mL) were incubated for 15 min with three types of glass slides: PEG hydrogel-coated, nitrocellulose-coated (FAST slides) and lysine-modified. (A) Comparison of cell surface density on three model surfaces. The results point to excellent leukocyte adhesion resistant properties of PEG hydrogels. The values are expressed as mean ± S.D. *$p < 0.05$ vs. lysine group. (B) Differential count comparison in blood smears and leukocytes captured on a lysine-modified glass surface. Blood drawn from the same donor was analyzed by Wright staining to determine potential bias in subset distribution among leukocytes captured on lysine coating. Blood smear was used as a control. Leukocyte differentials on lysine surfaces and in blood smears compared by paired *t*-test in six different experiments were not statistically significant. The values are expressed as mean ± S.D. The level of significance was accepted at $p < 0.05$. NS: not significant.

In our experiments, 250 μL aliquot of 2.0×10^6 cells/mL suspension was incubated with glass pieces 484 mm^2 in area, giving theoretical cell surface concentration of 1033 cells/mm^2. Enumeration of cells captured on lysine-coated glass slides yielded experimental cell concentration of 1150 ± 233/mm^2 (see Fig. 2A), which is similar to the expected theoretical value. The significant standard deviation comes from potential variability in the area of individual glass pieces and concentration of cells.

To determine subset distribution among the leukocytes captured on lysine, three leukocyte differentials (granulocytes, monocytes, lymphocytes) were identified with Wright staining and analyzed by light microscopy. These differential counts were compared to standard blood smears which were assumed to present leukocyte subsets in an unbiased fashion. As seen from Fig. 2B, there was no significant difference between proportions of granulocytes, monocytes and lymphocytes present in standard blood smears and on lysine-coated glass surfaces. Therefore, our results suggest that lysine is a suitable ligand for capturing all leukocytes in an unbiased fashion.

3.3. Dynamics of cell attachment to Ab-modified surfaces

Dynamics of cell adhesion is important in cell panning experiments. The incubation time needs to be optimized to obtain high yield and purity. The goal of this set of experiments was to investigate the effects of incubation time on yield and purity of $CD4^+$ T-cell isolation. Leukocytes (granulocytes and PBMCs) were incubated with lysine and anti-CD4-coated glass surfaces for time periods varying from 5 to 30 min. These glass surfaces were uniformly coated with ligands. The cells captured on both ligands were Wright stained to identify lymphocyte fraction. The Wright stain offered a simpler alternative to immunofluoresce in identifying lymphocyte subsets, however, it did not permit definitive determination of lymphocytes as $CD4^+$. Therefore, flow cytometry analysis was performed on the same sample to determine the proportion of $CD4^+$ T-cells within the lymphocyte population. Based on the overall cell concentration in solution and flow cytometry data, we determined the theoretical number of $CD4^+$ T-lymphocytes available for surface attachment and compared this number to experimental data collected at different time points. The cells captured on lysine and anti-CD4-coated surfaces were counted after different incubation times to determine the overall leukocyte surface concentration, the $CD4^+$ T-cell surface concentration, and the percentage of $CD4^+$ cells in the sample.

The theoretical leukocyte surface density for a given cell solution concentration and surface area of the glass slide was 1550 cells/mm^2. Differential counts of leukocytes captured on lysine indicated that lymphocytes comprised 39.5 ± 3.90% of all cells, while flow cytometry performed in parallel on the same stock solution identified 44.6% of all lymphocytes as $CD3^+CD4^+$ cells. Based on these data, the theoretical surface density of $CD4^+$ T-cells was 273 cells/mm^2. Fig. 3 depicts that the cell surface concentrations achieved on lysine and anti-CD4-coated surfaces after 30 min of incubation were similar to the theoretical values. In fact, the concentration of cells captured on lysine

after 30 min incubation was equivalent to 98% of the theoretical estimate, once again underscoring the possibility of capturing all cells indiscriminately on this ligand. More importantly, Wright stain analysis of the cells captured on the anti-CD4 Ab identified them as pure lymphocytes. As seen from Table 1 high purity was achieved as early as 5 min after incubation, however, at this time point the yield was low at 18%. If the goal of the experiment is to capture a pure population of $CD4^+$ cells as quickly as possible, the 5 min incubation will suffice. However, when the goal is to achieve both purity and high yield of $CD4^+$ T-cell isolation, the incubation time needs to be extended to 30 min where purity and yield were 97.6± 0.67% and 99.3%, respectively. It is important to note that, $CD4^+$ cells were not identified directly in the experiments discussed in this section. However, other studies discussed in this paper involving detachment of the captured cells from the Ab surface for flow cytometry analysis or performing immunofluorescence staining directly on the captured cells clearly identified cells as pure $CD4^+$ T-lymphocytes. Taken together, purity of captured cells on anti-CD4 surfaces was extremely high at all of three different time points, whereas yield of the desired cells depended on the incubation time. Thus, to achieve rapid capture of the desired cells with high purity and yield, we chose 15 min as the optimal incubation time (Fig. 3).

Table 1
Purity of cells incubated with anti-CD4-coated surfaces for different time intervals

Incubation of time (min)	Percentage of leukocytes subsets		
	Lymphocytes	Granulocytes	Monocytes
5	99.7±0.42	0.3±0.42	0
10	98.7±1.38	1.1±1.5	0.3±0.3
30	97.6±0.67	2.42±0.67	0

Purity was estimated by counting three leukocyte differentials determined by Wright staining.
Experiments were repeated four times. Results are reported as the mean±S.D.

3.4. Optimization of cell washing conditions

Washing ligand-modified surfaces after cell incubation is critical for achieving desired yield and purity of captured cells. Sub-optimal washing procedure will either lead to excessive shear stresses (detachment of positively selected cells and lowering of the yield), or will result in low shear stresses (contamination of the desired cells with non-specifically bound cells, thus lowering the purity). Traditionally, washing of surfaces after cell incubation is performed manually, making control of shear stress very difficult and introducing variability in the experimental protocol. In our experiments, a flow chamber was employed to create reproducible and quantifiable shear conditions. The shear stress applied to the cells on the ligand-decorated surface was defined by the dimensions of the flow conduit and the flow rate according to Eq. (1).

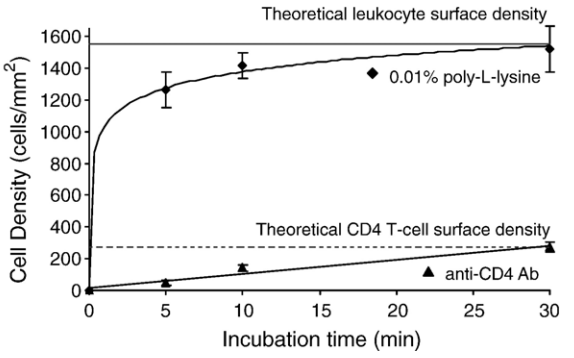

Fig. 3. Dynamics of cell adhesion on glass surfaces uniformly coated with lysine and anti-CD4 Ab. Cells were incubated with the two types of surfaces for varying time periods, washed and counted. Theoretical surface density for all leukocytes and $CD4^+$ T-cells was determined based on the cell concentration in suspension, area of the glass surface and FCM analysis of the lymphocyte fraction of the cell sample. Wright staining was used to identify cells captured on anti-CD4 Ab as lymphocytes. The values are expressed as mean±S.D.

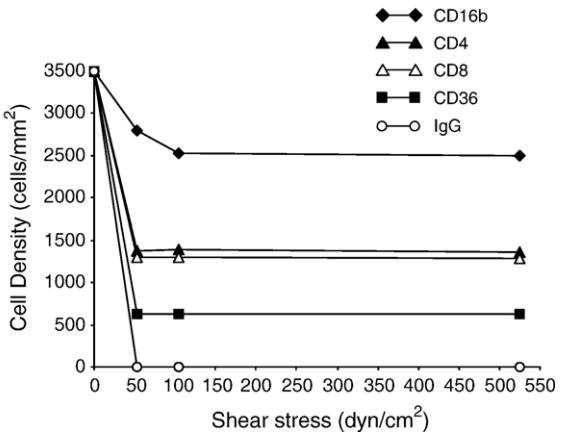

Fig. 4. Analysis of flow conditions required to remove loosely bound cells from the array of five Abs: anti-CD16b, anti-CD4, anti-CD8, anti-CD36, and anti-mouse IgG. Leukocytes were incubated with Ab-presenting surfaces for 15 min minutes and placed into a flow chamber. Flow rate (equivalent to shear stress) was established and changed using a syringe pump. Density of cells remaining on the surface after exposure to a certain flow rate was determined *in situ*, inside the chamber. A flow rate of 400 μL/min, corresponding to 105 dyn/cm^2 shear stress, was selected as optimal in that it removed non-specifically bound cells of all four subsets. Similar results were obtained from three different experiments.

Different shear stress values were applied by changing the flow rate settings of a syringe pump.

In the experiments summarized in Fig. 4, a PEG gel-coated glass slide was imprinted with multiple Abs including anti-CD16b, anti-CD4, anti-CD36 and anti-mouse IgG and incubated with RBC depleted whole blood leukocytes. A slide was then exposed to different shear flow conditions and the surface density of cells

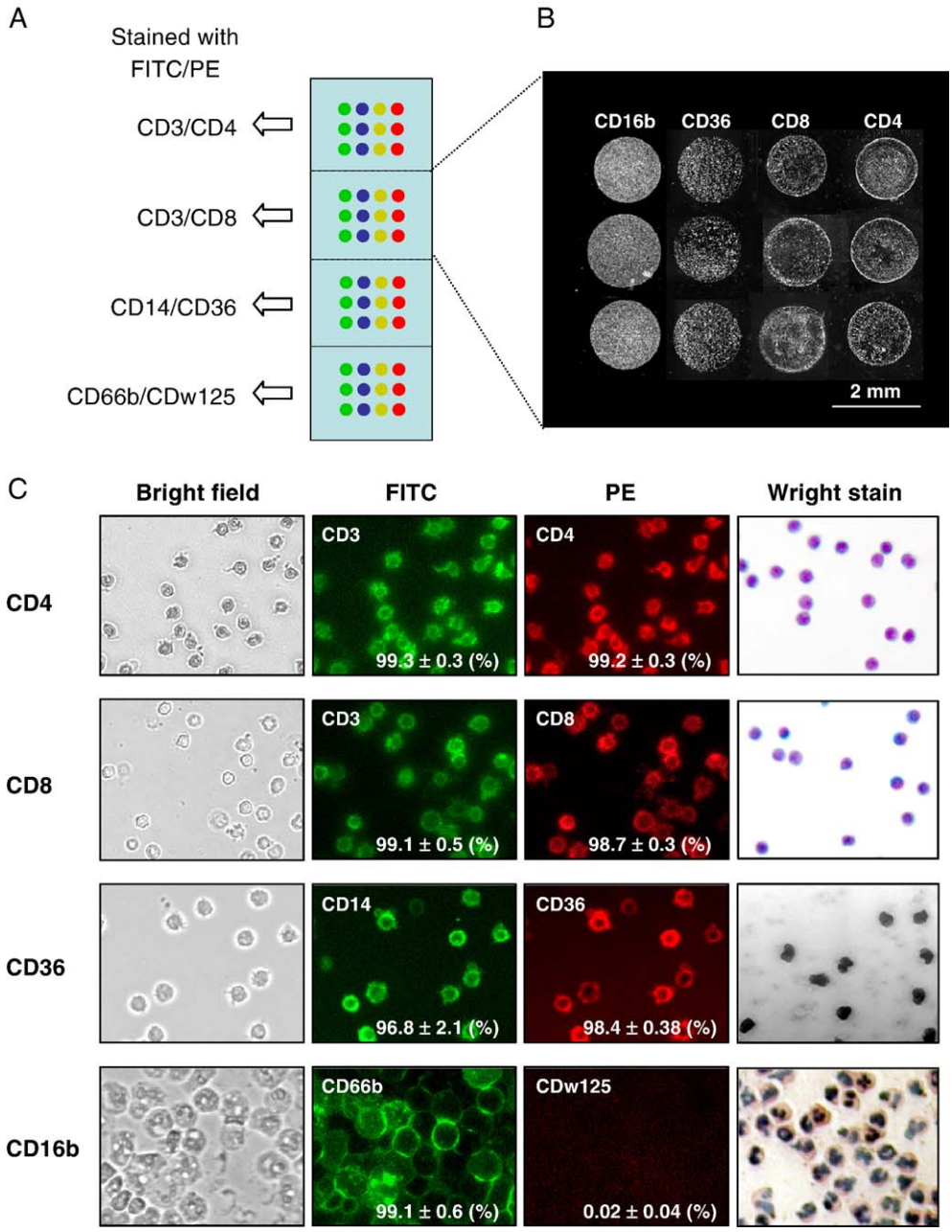

Fig. 5. Capture of leukocytes on an Ab array immobilized on PEG hydrogel coating. (A) Cells immobilized on an array of four Abs printed in three replicates. Individual spots were imaged at 2× magnification and reassembled into a composite picture. (B) Diagrammatic presentation of the immunofluorescent staining experiments. Four regions of the glass slide contained identical 4 × 3 Ab arrays. Each of the four regions was incubated with a staining Abs for phenotype determination. This approached was needed to identify positively and negatively stained cells using a two-color microscopy set-up. (C) Immunofluorescent identification of $CD4^+$ T-cells, $CD8^+$ T-cells, $CD16b^+$ neutrophils and $CD36^+$ monocytes captured on respective Abs within a 4 × 3 array.

Table 2
Summary of immunofluorescent staining of leukocytes captured on antigen-specific Ab domains

Antibody-coated surface	Percentage of positive markers in bound cells		
	CD3	CD14	CD66b
CD4	99.3±0.3	0.4±0.3	0.3±0.5
CD8	99.1±0.5	0.3±0.3	0
CD36	0.3±0.5	96.8±2.1	1.2±0.8
CD16b	0.9±1.2	0.7±1.4	99.1±0.6

Experiments were repeated three to six times. Results are reported as the mean±S.D.

remaining on the Ab spots was enumerated *in situ*, in the flow chamber. As seen in Fig. 4, most loosely bound cells were detached at the shear stress of 52.5 dyn/cm^2 and higher. Since the flow rate of 105 dyn/cm^2 resulted in removal of all loosely bound cells for all subsets, this shear stress was adapted as the optimal washing condition for the removal of loosely (non-specifically) bound cells from the Ab domains. The surface density of cells captured on the isotype (negative) control, anti-mouse IgG Ab, approached zero even at the lowest washing flow rate (shear stress). This behavior was expected and pointed to the specificity of the cell–surface interactions. Similar results were obtained from three different experiments. While the data presented in Fig. 4 stop short of defining yield of the leukocyte subsets, the same washing optimization approach combined with immunofluorescence staining will be used in the future to determine the capture yield.

3.5. Phenotyping leukocytes captured on antigen-specific Ab domains

For panning experiments, 4×3 Ab arrays comprised of anti-CD4, anti-CD8, anti-CD36 and anti-CD16 b were printed (see diagram in Fig. 5A). After incubating with

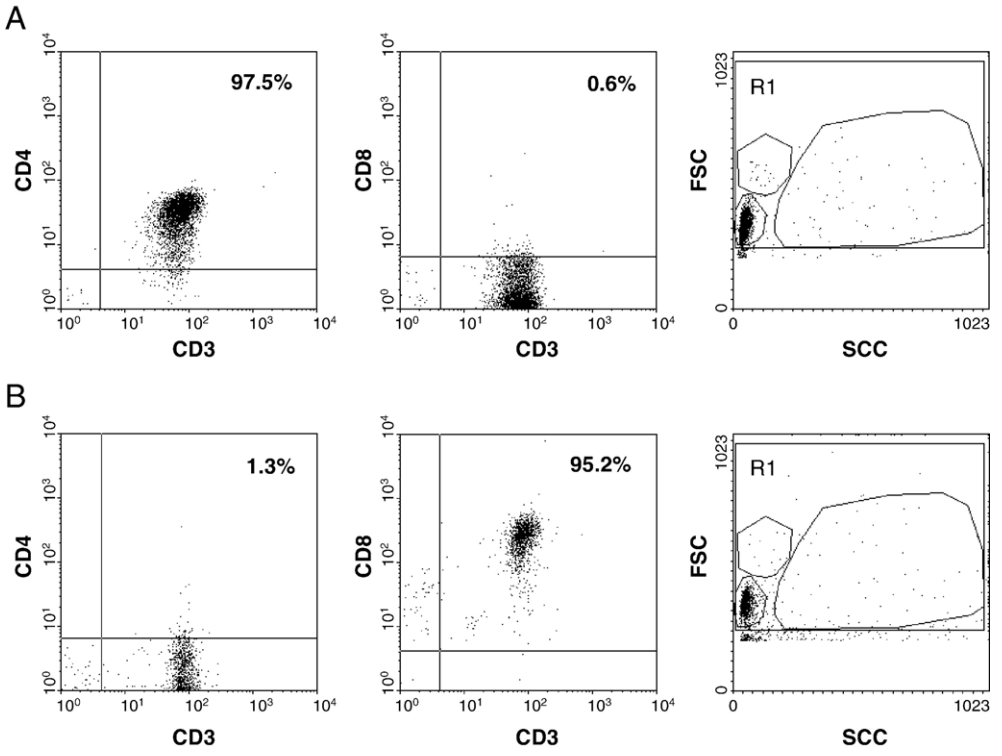

Fig. 6. Flow cytometry validation of purity of captured leukocyte subsets. Cell captured on anti-CD4 or-CD8 Abs were detached from the surface, labeled with fluorescent anti-CD3, anti-CD4, and anti-CD8 Abs and analyzed by flow cytometry. Analytical gates were set on lymphocytes, monocytes, or granulocytes within R1 according to forward and side scatter properties. (A) Three-color analysis of cells detached from anti-CD4-coated surface. CD4$^+$ T-cells comprised 97.5% of the cells, whereas CD8$^+$ T-cells were identified in 0.64% of the cells. The forward and side scatter cytogram showed that most of the cells were localized in the gate of lymphocyte (95.6%). (B) Three-color analysis of cells detached from anti-CD 8 surfaces identified 95.2% of the cells as CD8$^+$ T-cells and only 1.26% of cells as CD4$^+$ T-cells. The forward and side scatter pattern showed that most of the cells were localized in the gate of lymphocyte (92.9%). Of all the cells gated in R1, and the average rate of CD8 positive cells, CD8 T-cells, and presence in the gate of lymphocyte, were 97.0±2.4%, 95.3±1.0%, and 94.8±2.1%, respectively.

cells for 15 min and washing in the flow chamber, the glass slides with leukocytes bound to different capture Abs were incubated with a mixture of fluorescently labeled staining Abs. Staining and capture Abs used to define subset phenotype were specific to different epitopes of the same antigen or different antigens altogether. The cells bound to Ab spots (see Fig. 5B) were incubated with a mixture of FITC and PE conjugated staining antibodies. Captured cells on each antibody domain were also identified by Wright stain to be morphologically pure populations of lymphocytes on the CD4 or CD8 surfaces, monocytes on the CD36 surface, or granulocytes on the CD16b surface (Fig. 5C). As seen from Fig. 5C, 99.3± 0.3% and 99.1±0.5% of cells captured on anti-CD4 and anti-CD8 Abs were stained as $CD3^+$ (FITC). Depending on the binding Ab, the cells were also identified $CD4^+$ (99.2±0.3%) or $CD8^+$ (98.7± 0.3%). In the anti-CD36 regions of the PEG modified glass surface most cells stained with both CD14-FITC (96.8±2.1%) and CD36-PE (98.4±0.4%). Cells on the CD16b surface were all stained all with CD66b-FITC (99.1±0.6%), and very few with CDw125 (0.02± 0.04%), suggesting that contamination by either eosionophils or basophils was rare on the CD16b surface. In addition to purity analysis by immunostaining, contamination by other cells of interest on each surface was summarized in Table 2. Anti-CD4 or CD8 surface had little contamination of $CD14^+$ monocytes (0.3–0.4%) or $CD66b^+$ granulocytes (0.0–0.3%) on themselves. Contamination on anti-CD36-coated surface was derived mostly from $CD66b^+$ granulocytes (3.1%). On anti-CD16b-coated surfaces, most of the cells were CD66b positive (99.1%), but some cells were labeled with CD3 (0.9%) or CD14 (0.7%).

3.6. Validation of subset purity by flow cytometry

For further analysis of isolated cells on surfaces coated with anti-CD4 or -CD8 Abs, multiple redundant spots of the same Ab were deposited onto PEG gel-coated glass slides. After incubation with blood, leukocytes immobilized on Abs were washed in the flow chamber, detached from the surface by pipetting and analyzed using FCM. These analyses served to validate the leukocyte purity data described in the preceding section of the paper. Cytograms shown in Fig. 6A,B were representative of cell analysis from anti-CD4 or -CD8 surface. Anti-CD3, CD4 and CD8 antibodies were used for phenotyping of T-cell subpopulations. As shown in Fig. 6A, three-color analysis indicated that CD4 T-cells were 97.5% of all the cells gated in R1, whereas these cells had a few CD8 T-cells (0.64%). The forward and side scatter cytogram showed that most of the cells were morphologically intact and localized in the lymphocyte gate (95.6%). Similar results were obtained from the other 3 separate experiments. For all the cells gated in R1, the average percentages of $CD4^+$ cells, $CD4^+$ T-cells, and lymphocytes in the gate were 96.4±2.8%, 94.8±2.5%, and 96.0±2.9%, respectively. As for the cells removed from anti-CD8-coated surface, three-color analysis in Fig. 6B indicated that CD8 T-cells were 95.2% of all the cells, whereas these cells had a few CD4 T-cells (1.26%). The forward and side scatter cytogram showed that most of the cells were intact and localized in the lymphocyte gate (92.9%). Similar results were obtained from the other 2 separate experiments. For all the cells in R1, the average percentages of

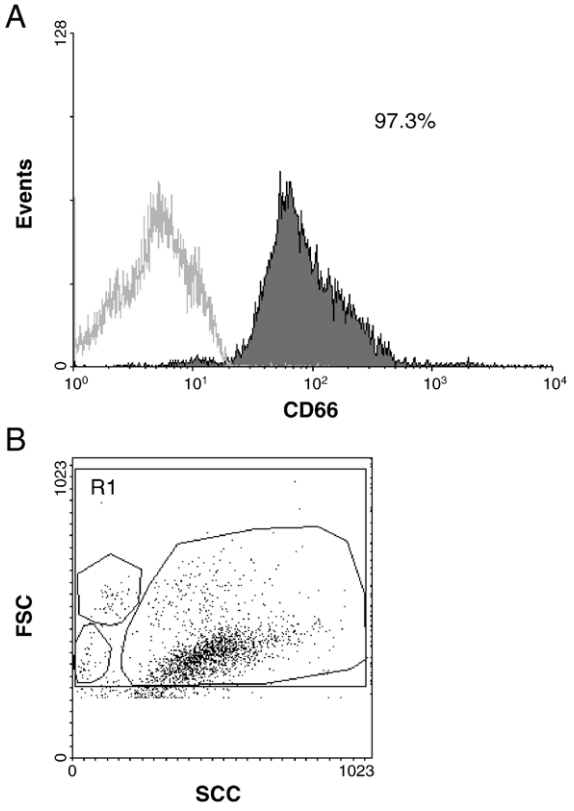

Fig. 7. Phenotypic flow cytometry analysis of cells detached from PEG gel surfaces containing anti-CD16b Ab. Captured cell population was dislodged from the surface stained with fluorescently labeled anti-CD66b Ab and analyzed by flow cytometry. Analytical gates were set on lymphocytes, monocytes, or granulocytes within R1 according to forward and side scatter properties. (A) CD66b histogram indicated that CD66b positive cells were 97.3% of all the cells. (B) The forward and side scatter pattern showed that most of the cells were localized in granulocyte fraction (97.7%). Similar results were obtained from the other 3 separate experiments. Of all the cells gated in R1, the average rate of CD66b positive cells and presence in the gate of the granulocyte were 95.8±2.9% and 97.5±2.8%, respectively.

CD8 positive cells, CD8 T-cells, and presence of lymphocytes in the gate were 97.0± 2.4%, 95.3± 1.0%, and 94.8±2.1%, respectively.

Similar experiments were also performed to analyze purity of neutrophils captured on the redundant arrays of anti-CD16b Ab. Cells isolated on CD16b-coated regions was detached, collected by pipetting, and analyzed by flow cytometer. Since Fig. 5C showed that there were few CDw125$^+$ cells (eosinophil or basophil) on anti-CD16b surface, only FITC-conjugated CD66b was used for evaluation of the phenotype of detached cells. CD66b histogram indicated that CD66b$^+$ neutrophils comprised 97.3% of the cells detached from anti-CD16b Ab (Fig. 7A). The forward and side scatter pattern (Fig. 7B) showed that most of the cells (97.7%) were intact and localized in granulocyte fraction. Similar results were obtained from the other 3 separate experiments. Of all the cells gated in R1, the average rate of CD66b$^+$ cells and presence in the gate of granulocyte were 95.8±2.9% and 97.5±2.8%, respectively. Unfortunately, flow cytometry characterization of monocytes captured on anti-CD36 Ab was not possible due to the small number of cells available for analysis.

4. Discussion

This paper demonstrated possibility of capturing four pure leukocyte subsets from RBC depleted whole blood onto Ab decorated, PEG gel-coated glass slides. Major aspects of the leukocyte panning protocol including non-specific cell adhesion, incubation time and sample washing were optimized, resulting in rapid, simple and reproducible isolation of four pure leukocytes subsets on the same surface. The following purity was obtained: CD4 T-cells (99.2±0.3%), CD8 T-cells (98.7±0.3%), CD36 monocytes (97.2±0.9%), and CD16b neutrophils (99.1±0.6%).

When compared with conventional isolation techniques, such as fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS), the proposed method has several appealing features including simplicity, minimal blood processing, rapid capture (15–30 min), high purity of captured leukocytes, and, most importantly, isolation of multiple pure cell populations on the same surface. This type of cytometry platform will be particularly useful in instances where maximum amount of information needs to be extracted from a small leukocyte sample. When coupled with emerging laser-scanning cytometry tools designed for rapid, multiparametric analysis of surface bound cells, the proposed platform can be used for high throughput immunophenotyping of leukocytes.

Several avenues are also available for removal of captured leukocytes for further analysis. Exposing cells to high shear stress proved effective in dislodging captured leukocytes from the surface. Subsequent flow cytometry analysis proved that detached cells were phenotypically intact. Other technologies, for example, laser capture microdissection may be used to extract specific leukocytes for downstream molecular analysis (Fend et al., 1999; Revzin et al., 2005; Knezevic et al., 2001; Miura et al., 2002; Michener et al., 2002).

In the previous studies with human peripheral blood leukocytes employing MACS the following purity data were reported: 85.7–95.5% in CD4 T-cells (Semple et al., 1993; Stanciu et al., 1996; Busch et al., 2004), 88.3% in CD8 T-cells (Stanciu et al., 1996), 93.8–98.6% in CD14 monocytes (Pickl et al., 1996; Babatz et al., 2003; Motta et al., 2003), and 99–99.1% in CD16 neutrophils (Sjolinder et al., 2000; Zahler et al., 1997). Purity results presented in the present paper are comparable to or better than those observed with MACS. It is important to note that MACS protocols call for density gradient centrifugation to enrich leukocyte fraction prior to isolation whereas no such enrichment steps were necessary in our experiments. Overall, the antibody-coated PEG surfaces enabled rapid leukocytes isolation (30 min) while providing high purity of captured cells.

One has to note that the present study did not take advantage of the microarray robots that dispense small volumes (~1 nL) and print high-density arrays of small (~200 μm diameter) protein spots. We have employed robotic printing for cell capture and patterning in the past (Revzin et al., 2004) but chose to manually dispense relatively large volumes (~1 μL) of Ab solutions resulting in relatively large 1.5–2 mm spots. Ligand domains of this dimensions were particularly suitable for the quantitative studies because a large number of cells could be captured on each spot. In the future, we do not see any obstacles to robotic printing of high density miniaturized arrays of Abs to replicate the leukocyte panning results presented here and to extend to other Abs for high throughput immunophenotyping.

Other important parameters to be addressed in the future are the Ab concentration in the printed domains and binding of Abs to PEG gel coating. In the present study, Abs were used as received from commercial vendors and Ab concentration dependence on the capture yield of specific subsets was not investigated. In addition, Abs were deposited onto PEG hydrogel films by physisorption as opposed to covalent or avidin–biotin linkage. More specific Ab conjugation method to PEG gel will result in a more ordered presentation of Fab domains and will improve binding characteristics of the device.

The present results directly demonstrated rapid isolations of $CD4^+$ and $CD8^+$ T-cells. Additionally, our ongoing research (data not shown) and the published literature reports (Belov et al., 2001) suggest that the numbers of cells captured on antibody arrays may be correlated with leukocyte subset proportions in blood. Especially, HIV infection leads to selective decrease of $CD4^+$ T-cells and resultant decrease of CD4/CD8 ratio (McCune, 2001). In our current device, quantitative analysis of the selective adhesions of $CD4^+$ and $CD8^+$ T-cell can be useful for estimation of the absolute $CD4^+$ counts or CD4/CD8 ratio, which is a key to the initial diagnosis and the clinical follow-up.

The Ab array platform may be broadly applicable in the clinical setting, where the physician often needs to rapidly identify specific cells or microorganisms in biological fluids including blood, bronchoalveolar lavage fluid, cerebrospinal fluid, pleural effusion, ascites, urine, or exudates. Cell panning on Ab arrays will provide a simple and effective means of testing for these clinical scenarios.

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