Filopodial Morphology Correlates to the Capture Efficiency of Primary T-Cells on Nanohole Arrays

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Nanostructured surfaces emerge as a new class of material for capture and separation of cell populations including primary immune cells and disseminating rare tumor cells, but the underlying mechanism remains elusive. Although it has been speculated that nanoscale topological structures on cell surface are involved in the cell capture process, there are no studies that systematically analyze the relation between cell surface structures and the capture efficiency. Here we report on the first mechanistic study by quantifying the morphological parameters of cell surface nanoprotrusions, including filopodia, lamellipodia, and microvilli in the early stage of cell capture (<20 min) in correlation to the efficiency of separating primary T lymphocytes. This was conducted by using a set of nanohole arrays (NHAs) with varying hole and pitch sizes. Our results showed that the formation of filopodia (e.g., width of filopodia and the average number of the filopodial filaments per cell) depends on the feature size of the nanostructures and the cell separation efficiency is strongly correlated to the number of filopodial fibers, suggesting a possible role of early stage mechanosensing and cell spreading in determining the efficiency of cell capture. In contrast, the length of filopodial filaments was less significantly correlated to the cell capture efficiency and the nanostructure dimensions of the NHAs. This is the first mechanistic study on nanostructure-based immune cell capture and provides new insights to not only the biology of cell-nanomaterial interaction but also the design of new rare cell capture technologies with improved efficiency and specificity.

KEYWORDS: CD4 T-lymphocytes, Cell Capture and Separation, Quartz Nanohole Arrays, Streptavidin Functionalization, Filopodia, Contact Guidance.

INTRODUCTION

T-lymphocytes (T-cells) are the central participants in the adaptive immunity to various diseases; e.g., pathogenic infections, neoplastic malignancies and autoimmune diseases, including AIDS.1–5 T-helper cells (CD4 T-cells) and cytotoxic T-lymphocytes (CD8 T-cells) are the major T-cell subsets that regulate immune cell recruitment and proliferation through a program of cytokine secretion.

In human immunodeficiency virus (HIV) infections,1,2 isolation and enumeration of CD4 T-cells from human whole blood samples is the key biomarker to diagnose and monitor the progression of early HIV infection to AIDS.3 Consequently, the ability to separate and quantify specific types of T lymphocytes including CD4 T-lymphocytes from a complex cell mixture and peripheral whole blood is critical for clinical management of AIDS.3,10,11 To date, several technologies including cell size-based separation,12,13 the use of immuno-magnetic beads,14,15 and microfluidic technologies3 have been developed to improve the efficiency of capturing the target cells from a mixed population. Recent reports on the interaction of cells with solid surface nanostructures have shown that...
nanometer-scale topography not only influences diverse cell behavior such as cell adhesion, motility, proliferation and differentiation, but represents a new route to capture and separate rare cell populations. These interesting findings have motivated us to develop a novel nano-platform for separating CD4 T-lymphocytes from primary mouse splenocytes via high affinity streptavidin (STR)-biotin conjugation with immuno-functionalized silicon nanowire arrays. Despite the remarkable improvement in the capture efficiency using nanostructure-based cell capture devices, the underlying mechanism that allows nanostructured-surfaces to achieve significantly enhanced cell separation efficiency as compared to planar substrate remains elusive.

Here we report on the study of how the morphologies of filopodia or microspikes, which are needle-like actin-rich protrusions from the cell surface, are correlated to the cell capture efficiency using a periodic 2D nanohole array model surface. Filopodia are involved in a wide variety of functions including absorption, secretion, cellular adhesion, and mechnotransduction. Because cell capture is a rapid process and the cells captured by the immobilized antibodies can interact with the substrate surface and rest/spread quickly within ~20 min, we suspect the early stage cell-substrate interaction plays a critical role in the cell capture process. We prepared a set of nanohole arrays (NHAs) with the hole size ranging from 140 to 550 nm and observed that the nanostructures indeed directs the formation of filopodia (~100–300 nm in diameter) in the case of CD4+ T-lymphocytes in contact with biofunctionalized NHA substrates that further correlates to the efficiency of CD4+ T-cell separation. While cross-sectional imaging indicates that cell microvilli, which were previously suspected to be playing a crucial role in enhancing cell capture efficiency, were not observed at the interface between cell and nanostructured surface. Although this may not exclude the role of microvilli in the process of initial contact between immune T-cells and the nanostructures, our results suggest that the subsequent cell spreading and actin-rich filopodia formation is a more favorable mechanism that leads to significant enhancement of rare cell capture efficiency.

MATERIALS AND METHODS

Materials

Colloidal polystyrene suspensions (200, 300, 430, and 750 nm in diameter) were purchased from Thermo Scientific (Fremont, CA, USA). Cr etchant (CR-7) was supplied by Cyantek Corporation (Fremont, CA, USA). N-methyl-2-pyrrolidone, (3-aminopropyl)-triethoxysilane (APTES), glutaraldehyde (GA), streptavidin (STR), and osmium tetroxide were purchased for Sigma-Aldrich (St. Louis, MO, USA). C57BL/6 mice were supplied by Nara-Biotech (Seoul, Republic of Korea). Biotinylated anti-CD4 mAb (clone GK 1.5), FITC (518 nm emission)-labeled mAb-CD3, PE (575 nm emission)-labeled mAb-CD4 (clone: RM4-4), and PerCP (690 nm emission)-labeled mAb CD19, and fluorescence dye labeled-CD3, CD4, CD8, and CD19-mAbs were purchased from eBioscience Inc. (San Diego, CA, USA). All other chemicals were of analytical grade.

Nanohole Array (NHA) Fabrication

To produce the NHA, the liquid state of colloidal polystyrene nanoparticles (PS NPs, 200, 300, 430, and 750 nm in diameter) monolayer was first carefully deposited on the quartz (QZ) substrate using a modified self-assembly technique (Fig. 1(A)) we developed previously. To create space between the spin-coated PS NPs, their sizes were first reduced by O₂ plasma (O₂/Ar = 35/10 sccm, RF power of 100 W and bias power of 50 W) for 10 s; 200 nm PS, 23 s; 300 nm PS, 35 s; 430 nm PS, 47 s. To ensure the uniformity of the PS monolayer deposition, the PS monolayer was thoroughly washed (with ethanol and water) four times. After washing, the surfaces were coated with APTES (12.5% GA, 1% APTES in water) at 30 °C. The coated APTES surfaces were then left for 3 h. The obtained NHA substrate was immediately immersed in streptavidin solution (1% STR-biotin) for 1 h and then washed again to remove unbound antibodies. Finally, to create a nanohole array pattern, the NHA substrates were exposed to plasma (O₂/Ar = 35/10 sccm, RF power of 100 W and bias power of 50 W) for 10 s; 200 nm PS, 23 s; 300 nm PS, 35 s; 430 nm PS, 47 s.

Figure 1. (A) Schematic diagram of nanohole array (NHA) fabrication using polystyrene (PS) nanoparticles on a planar quartz substrate. ((B)–(E)) Scanning electron microscope (SEM) images of NHAs (140, 200, 270, and 550 nm in diameter) prepared with four different feature sizes of PS nanoparticles (200, 300, 430, and 750 nm in diameter). Scale bar is 0.5 μm. Insets show the enlarged images of each NHA. These figures also represent the cross-section view images of each NHA shown in the lower part of each SEM image. (F) Schematic diagram showing sequent surface functionalization with APTES, GA, and STR on NHA substrates. (G) showing cell suspension (~30 μl), which is containing CD4, CD8, B, NK, and NKT-cells, loading process onto NHA substrates, and (H) revealing the washing process to remove unbound T-cells from the NHA substrates using a 3D-rocker.
PS, and 60 s; 750 nm PS, respectively, to control the diameter and spacing for preparing the nanohole arrays perforated into continuous metal film. To fabricate the nanohole pattern, the reactive ion etching (RIE, Sorona Inc., Pyeongtaek, Republic of Korea) process was then performed for 40 s (CF$_4$/Ar = 40/5 sccm, RF power of 100 W, and bias power of 50 W) after 25-nm Cr metal onto the coated surface of PS NPs, and the PS NPs were subsequently removed by ultrasonicication in N-methyl-2-pyrrolidone. Finally, the Cr metal layer was removed via a lift-off process using Cr etchant (Fig. 1(A)). The typical diameters/widths of the resultant nanoholes were about 140/60, 200/100, 270/160, and 550/200 nm with depths of 260, 270, 320, an 330 nm, respectively as shown in Figures 1(B)–(E). The period of hole-arrays was determined by the initial period of hcp nanoholes arrays and the diameter of holes could be easily tuned by the O$_2$ etching time. Moreover, our proposed method provides a flexible and versatile route to the fabrication of QNP arrays on a flat QZ substrate, with potential application in optics, electronics, sensing, and as building blocks for more complex nanostructures.

**NHA Surface Functionalization**

NHA substrates (7 mm × 7 mm) were carefully cleaned with H$_2$O$_2$:H$_2$SO$_4$ (1:1) for 10 min to remove all of the organic materials and impurities on the surface. We then washed the substrates using a three-step cleaning process (acetic acid, isopropyl alcohol, and distilled water) and dried them with air. The NHA surface was treated with O$_2$ plasma for 20 s to confer the hydroxyl groups on the NHA surface after piranha cleaning process (96%H$_2$SO$_4$:30%H$_2$O$_2$ = 1:1) for 10 min. Next, the surface was applied by a three-step surface functionalization process using 1% (v/v) (3-aminopropyl)-triethoxysilane (APTES) in ethanol for 30 min at room temperature, 12.5% (v/v) glutaraldehyde (GA) in distilled water for 4 hrs on a 3D-rocker, and 50 µg/mL streptavidin (STR) in phosphate buffered saline (PBS) overnight in an incubator (37 °C, 5%CO$_2$) as shown in Figure 1(F).

**Cell Preparation**

The CD4$^+$ T-lymphocytes to be separated were mouse CD4$^+$ T-cells from whole mouse splenocytes, which contain CD4$^+$ T, CD8$^+$ T, natural killer (NK), natural killer T (NKT), and B-cells. These splenocytes were prepared from the spleens of C57BL/6 mice as described previously.$^{19}$ A certain quantity of cells (~10$^5$ cells/mL) was counted using a conventional hemocytometer (Hauser Scientific, USA). Prior to loading the cell suspension in the culture medium, the cell population with a final volume of ~30 µl was first reacted with biotinylated anti-CD4 mAb and incubated at 4 °C for 20 min (Fig. 1(G)). Following incubation for 20 min with STR-conjugated NHA substrates at 4 °C, unbound cells were removed by rinsing with phosphate buffered saline (PBS), while separated CD4$^+$ T-cells could bind to STR-NHA surfaces due to adhesion enabled by the STR-biotin interaction. This process was repeated three times for 10 min on a 3-D rocker to completely remove non-specifically unbound cells from the NHA substrates (Fig. 1(H)). Remnant of unbound cells were then collected and transferred to the tube for enumerating the population by standard flow cytometry (Becton Dickinson, NJ, USA).

**Fluorescence Activated Cell Sorter (FACS) Analysis**

To enumerate the remnant of unbound cell population, FACS analysis was performed. First, the non-specifically unbound T-lymphocytes after STR-NHA separation platform were collected by FACS falcon round-bottom tubes (eBioscience, USA). The collected T-lymphocytes were then stained by FITC (518 nm emission)-labeled mAb-CD3, PE (575 nm emission)-labeled mAb-CD4, and PerCP (690 nm emission)-labeled mAb CD19 at 4 °C for 20 min. After reaction, the stained T-lymphocytes were washed for removing the unbounded antibody using centrifuge (1500 rpm, 5 min, twice) and then fixed by 4% paraformaldehyde in PBS for 15 min. Finally the fixed cell suspension were transferred to the flow cytometer (FACS, Calibur, Beckton Dickinson, USA) and analyzed using CellQuest™ Pro software (BD Bioscience, USA) for the separation efficiency of the targeting CD4$^+$ T-lymphocytes.

**Surface-Bound T-Cells Preparation Using Scanning Electron Microscopy (SEM) Analysis**

For the FE-SEM analysis, a solution of the cells conjugated with biotin-conjugated CD4 mAbs in RPMI-1640 (500 mL, Invitrogen, NY, USA) was pipetted onto STR-functionalized NHAs (140, 200, 270, and 550 nm in diameter, 7 mm × 7 mm) and STR-planar glass substrates with cell populations ~10$^5$ cells/mL. After incubation at 37 °C and 5% CO$_2$ for 20 min, the separated and immobilized CD4$^+$ T-cells on STR-functionalized NHAs and planar glass substrates were first fixed with 4% GA in the refrigerator for 2 h, followed by a post-fixing in 1% osmium tetroxide for 2 h. The captured T-cells on STR-conjugated substrates were then dehydrated by successive immersion in 25%, 50%, 75%, 95% and 100% ethanol for 5 min at 4 °C and followed by final dehydration with 100% ethanol twice for 10 min at 4 °C. Final dehydration needed to process twice. Dehydrated T-cells were then frozen for 3 h at −80 °C. Subsequently, the cells with substrates were slowly dried for 24 h using a vacuum desiccator. Subsequently, the cells with substrates were slowly dried under vacuum for 24 h. Once dried, the surface-bound T-cells were then sputter-coated with a layer of platinum (~5–6 nm) to prepare conductive samples before performing the FE-SEM measurement. Quantitative characterization of the cellular morphologies including filopodia and...
lamellipodia formation (length, width, and number of the protruded filopodia etc.) for the surface-bound T-cells on STR-functionalized NHAs and planar glass was performed on the FE-SEM images.

RESULTS AND DISCUSSION
T-cell Capture Efficiency on STR-NHAs
Fluorescence activated cell sorter (FACS) revealed that whole mouse splenocytes contained \( \sim 42.7\%\) CD4\(^+\) T lymphocytes (CD3\(^+\)/CD4\(^+\)) and \( \sim 57.3\%\) non-CD4\(^+\) T-cells (Fig. 2(A)). Each type of cells (e.g., CD4, CD8-T, B, NK, and NKT cells) from the suspension of whole mouse splenocytes was quantified using FACS measurement with different surface markers (fluorescence dye labeled-CD3, CD4, CD8, and CD19-mAbs). After completion of the cell separation process via a STR-NHA platform, the percentages of CD4\(^+\) T-cells in the cell suspension with four different NHAs (of 140, 200, 270, and 550 nm in diameter) decreased to \( \sim 2.59\), \( \sim 1.32\), \( \sim 3.10\), and \( \sim 3.99\%\), respectively as shown in Figure 2(A). The cell-capture efficiency, which is defined as the percentage of the target CD4\(^+\) T-cells successfully captured to the total number of cells in the cell suspension (\( \sim 10^5\) cells) initially loaded and analyzed by FACS, is plotted for all four different NHAs in Figure 2(B) (top bar-graph) and also summarized in the table of Figure 2(B) (bottom table). For comparison, the STR-functionalized planar glass substrates as control samples were also tested and the results are shown in Figure 2(B) (top-right of bar graph). The result shows that all STR-functionalized NHA substrates (\( \sim 92.7\%\)) show excellent performance (a factor of \( \sim 1.4\)) in cell capture efficiency as compared to the control samples (\( \sim 65.2\%\)), which is consistent with previous studies. 19, 22 FACS results demonstrated that high yield separation of CD4 T-cells was achieved with...
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all of the STR-functionalized NHAs, and the results were comparable to those from other nonstructure-based cell capture experiments previously reported.19–22 The hypothesis why significantly elevated cell capture efficiency can be achieved on STR-nanostructured surfaces as compared to the planar glass substrates is the following. First, it is likely due to the relatively higher contact area between the cells and the solid surfaces compared to the planar glass substrates as we reported previously.22 Second, it was speculated to be associated with the adhesive and traction force of cells on the substrate, which is unique in the condition of higher degree of dimensionality in STR-NHA surface compared to the planar substrates. Our previous results showed that the surface-bound T-cells on STR-nanostructures have the higher traction force (e.g., adhesion force) due to the three-dimensional (3-D) surface accessibility (e.g., nanohole or nanopillar etc.), while the cells on planar substrates exhibits a lower adhesion force at the same stage, corresponding to 2-D behavior.27 Consequently, it results in elevated cell capture efficiency on the STR-nanostructures with enhanced cell adhesion force occurring at the similar stages (Fig. 2(B)).

Purity of Captured T-Cells on STR-NHAs

Furthermore, to investigate the purity of the captured T-cells on NHAs and also to assess cell integrity, the captured CD4 T-cells were stained by phycoerythrin (PE)-conjugated anti-CD4 mAb and 4′,6-diamidino-2-phenylindole (DAPI, DB Bioscience, USA). DAPI (blue-350 nm) is a nuclear dye that stains for cells with intact nuclei. The stained cells were subsequently enumerated for the CD4+ T-cells (PE+/DAPI+) out of total cells (DAPI+) bound to the STR-functionalized NHA substrates by fluorescence microscopy (FM) analysis. Figure 2(C) shows fluorescence images of both PE and DAPI-stained CD4+ T-lymphocytes for the STR-conjugated NHAs and planar glass (control sample). As shown in Figures 2(C), (D), STR-conjugated NHAs (140, 200, and 270 nm in diameter) were found to have a high purity (∼80.6±3.3%), which is comparable to that of the control samples (planar glass, ∼77.7±0.7%).

Cellular Morphology of Captured T-Cells

To quantify the morphological properties of the captured CD4+ T-lymphocytes bound to STR-conjugated NHA substrates, FE-SEM analysis using a cell freezing technique were performed. Quantitative characterization of the cellular morphologies (number of extended filopodia and protruded length and width of filopodia etc.) on both STR-functionalized NHA substrates and planar glass (control sample) was performed via analyzing the FE-SEM images. Figure 3(A) shows representative FE-SEM images of CD4+ T-lymphocytes bound on the surfaces of four different-sized NHAs. It was observed that the captured CD4+ T-cells possessed different forms of filopodia (0.1–0.3 μm)20 on the four NHAs with different nanohole sizes. The formation of the flat lamellipodia extending from the captured T-cells was observed only on the surface of NHAs exceeding ∼270 nm, as shown in Figure 3(A). To further examine the interface and binding properties between the captured CD4 T-cells and NHA substrates, gallium ion (Ga+3) milling of the captured CD4 T-cells was performed using focused ion beam (FIB). Figure 3(B) shows the cross-sectional images of the captured CD4+T-cells on four different NHAs, indicating that the captured CD4 T-cells formed tight binding and adhesion on STR-functionalized NHAs. However the insertion of microvilli into nanoholes, a mechanism speculated to account for enhanced cell capture efficiency, are surprisingly rare. Figure 3(C) shows the cell size, which was calculated without considering the area of filopodia and lamellipodia from the cells (n = 117) captured on a 270 nm NHA substrate, displays a Gaussian distribution, implying that non-specifically bound cells were also counted. According to cell purity measurement (Figs. 2(C), (D)) of cell suspension, ∼80.6% of cells bound on the NHAs substrates could be CD4+ T-cells, indicating that approximately 80% of the bound cells (∼2.8–4 μm in cell-diameter) in the size distribution graph could be the right target cells—CD4+ T-lymphocytes. To ensure that the evaluation of the filopodia morphology including the width, length, feature size and the number of the filopodial filaments per cell in the early stage of cell adhesion (in our case, only ∼20 min incubation) is statistically sound, we quantified more than ∼55 cells, which were ∼80% of the total bound cells we counted (n = 70) (Figs. 3(D), (E) and 4(A)–(D)). To justify the significance of our correlation results, p values were calculated with neighboring column data. As shown in Figs. 3(D), (E), the protruded filopodia width for 140-nm NHAs (∼80.2 nm in width) exhibits similar trend in size to that of the 200-nm NHAs (∼83.1 nm in width) resulting in statically insignificant difference (Fig. 3(D) and Table I). With further increasing the diameter of NHAs from 270 to 550 nm, filopodia protruding from the T-cells were observed to increase in width (**∗∗∗< P < 0.0001, 126–140 nm in width, Figure 3(E) and Table I). This correlation develops even at the very early stages of adhesion (∼20 min incubation). Hence, this linear correlation (except below <200 nm in NHA diameter) between the extended filopodia width of the captured CD4+ T-cells and the cellular reaction to the NHA topographical structures, may be explained by a phenomenon known as contact guidance, which is used to explain the behavior of endothelial cell and fibroblast filopodia on nanostructured substrates following a long incubation period.28–30 According to this mechanism, the T-cells use filopodia to recognize and sense the surface features of nanotopography, and bind themselves to the substrates. Dalby et al.31,32 reported that filopodia increased in thickness with an increase in the size of the island topography from 13 to 95 nm, which is in good agreement with our results for filopodia width.
Figure 3. (A) Scanning electron microscope (SEM) images of CD4$^+$ T-cells bound on four different sizes of NHA substrates (low- and high-magnification top, tilt, and enlarged tilt view images). (B) Cross-sectional SEM images of surface-bound CD4$^+$ T-cells on four different NHAs (140, 200, 270, and 550 nm in diameter) with enlarged images in selective area marked 1, 2, 3, and 4 in first column of (B). The samples were prepared by Ga$^+$ ion milling with a focused ion beam (FIB). All of the CD4$^+$ T-cells on the NHAs are highlighted in yellow for easy differentiation. (C) Cell size distribution (only cell-body excluding filopodia and lamellipodia) of captured cells ($n$ = 117) randomly bound on streptavidin (STR)-functionalized NHA substrate (270 nm in diameter). Scale bar is 1 $\mu$m. (D) Filopodia width distribution of CD4$^+$ cells bound on the four different STR-functionalized NHAs substrates after only 20 min incubation at 4 °C. $P$ values of $< 0.0001$ (****) considered statically significant. An insignificant statistical difference is represented as NS. (E) Selected filopodia width distribution in which only ~80% of filopodia width taken from (F) of the CD4$^+$ T cells on NHAs substrates, indicating that the filopodia width was not changed between the 140 and 200 nm in diameter and linearly increased with increasing of NHA diameters up to 550 nm.

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Table I. The summary of evaluated filopodia morphology (number, length, and width of filopodia) of CD4+ T-cells bound on the different nanohole arrays (NHA) using SEM analysis. For the comparison, the results of control samples (planar glass substrates) were also included.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number of filopodia</th>
<th>Filopodia length (nm)</th>
<th>Filopodia width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>STDEV</td>
<td>Average</td>
</tr>
<tr>
<td>Control NHA</td>
<td>9.44</td>
<td>3.39</td>
<td>547.5</td>
</tr>
<tr>
<td>140 nm</td>
<td>6.39</td>
<td>1.94</td>
<td>600.3</td>
</tr>
<tr>
<td>200 nm</td>
<td>6.67</td>
<td>2.01</td>
<td>616.8</td>
</tr>
<tr>
<td>270 nm</td>
<td>5.11</td>
<td>1.60</td>
<td>675.9</td>
</tr>
<tr>
<td>550 nm</td>
<td>3.42</td>
<td>1.48</td>
<td>673.4</td>
</tr>
</tbody>
</table>

Notes: *STDEV: Standard deviation; **Control: STR-functionalized planar glass samples without nanostructure; ***NHA: Nanohole arrays.

versus nanohole size shown in Figures 3(D), (E). Another possible explanation on the elevation in filopodia width with increasing the NHA diameters (200–550 nm) is the increasing STR-conjugated surface width (100–270 nm). The amine containing in the STR-conjugated NHA promote the actin extension to guide the development of filopodial filaments in the initial stage of the incubation. Therefore, the filopodia of the captured T-cells start to deliver more actin-rich filament along the shafts of the initial filopodia for covering different widths of STR-NHAs. Therefore, these results suggest that the filopodia of CD4+ T-cells interact with the NHA substrate via an initial high-affinity STR-biotin conjugation followed by guided extension of filopodia on the nanostructures of the NHA (Fig. 3(E)). Furthermore, we also quantified other morphological parameters such as the protrusion length of filopodia and the average number of the filopodial filaments per cell as summarized in Table I and Figures 4(A)–(D). In addition, the cell-capture efficiency (n = 4) is plotted for the four different NHA in Figure 4(E). In Figures 4(A), (B), no statically significant difference (NS, not-significant) was observed in the plot showing the filopodial protrusion length of the captured CD4 T-cells versus nanohole dimensions, indicating that the length of the protruded filopodia is relatively independent of the dimensions of the NHAs in the early stage of cell capture (< 20 min). This result could be due in part to the non-adherent property of T-cells since these cells usually do not adhere, spread or migrate within such a short period of time (~ 20 min). As shown in Figures 2(B) and 4(E), the cell capture efficiencies of STR-functionalized NHAs exhibit statistically insignificant difference (NS, ~ 94.6% and ~ 96.0% for 140 nm and 200 nm, respectively) by increasing the nanohole diameter from 140 to 200 nm. By further increasing the diameters from 270 nm up to 550 nm, the capture efficiency then decreases to ~ 88.1 ± 4.7% (550 nm in diameter) (Figs. 2(B) and 4(E)). Interestingly, by changing the nanohole diameter from 140 to 550 nm, the average number of filopodial filaments per cell (Figs. 4(C), (D)) exhibits the same trend as the cell capture efficiency (Fig. 4(E)), implying that nanostructure-based cell capture process is strongly dependent on the formation of filopodia (e.g., the number of filopodial filaments). Moreover, the surface areas of four different NHAs (7 × 7 mm²), calculated from the depth and bottom diameter of four different NHAs (140, 200, 270, and 550 nm in diameter) obtained by SEM measurement, were determined to be 120.15, 125.64, 125.40, and 90.82 mm², respectively. As shown in Figure 4(E), the deterioration in capture efficiency as increasing the NHA diameters from 200 to 550 nm is strongly correlated a decrease of surface areas from 125.64 to 90.82 mm². This result clearly shows that higher contact area between the cells (including filopodia) and NHA surfaces leads to significant enhancement of the adhesive and traction force of cells. These cellular behaviors are consistent with previous literature reported by Sui-jian and coworkers. They observed that more cells are detached from the flat silicon wafer than from the SiNW array under the same centrifugal force, which suggesting that the adhesion force between the cells and the flat silicon wafer is lower than that between the cells and the silicon nanowire wafer.

Fluorescent Microscopic Analysis
To further verify our finding obtained by SEM analysis, which may result in some damages of the cells during the dehydration process, fluorescent microscopy (FM) analysis was also performed even though the resolution of the FM analysis is relatively lower compared to the SEM analysis. Figure 5(A) shows the FM images of the captured T-lymphocytes, which were stained by rhodamine-conjugated phalloidin (Life technologies, USA) and DAPI for actin filaments and nuclei, respectively, on four different NHAs of 140 ~ 550 nm in diameters. The obtained results are summarized in the Figures 5(B)–(D) (n = 250–300). The average cell area quantified by cytoskeleton actin staining of the capture cells on the NHA ranges from 140 to 550 nm using FM analysis (Fig. 5) exhibits a similar trend as the average number of the protruded filopodia quantified by SEM analysis (Figs. 4(C), (D)). Hence, the FM analysis agrees with our results from the SEM analysis obtained from the dehydrated T-lymphocytes on
the STR-NHA substrates. This observation, which we believe is novel, can be explained by ‘contact guidance’ and ‘cell-adhesion force.’ When the T cells interact with STR-conjugated NHA surfaces, they develop filopodia to sense the surface, and then preferentially adhere to the region with features of similar dimensions. Afterwards the filopodia of the captured T-cells start to extend rapidly on the surface even within a short incubation time (\(~20\) min).

**Filopodia Contact Behavior**

The length of filopodia including lamellipodia (Fig. 4(A)) protruding from primary mouse T-lymphocytes on the...
Figure 5. (A) Fluorescence images and cell size histograms of live primary CD4\(^+\) T-lymphocytes bound on four different NHA substrates (140, 200, 270, and 550 nm in diameters) using confocal microscopy. (B)–(C) Cell size distribution (histogram) of the CD4\(^+\) T-lymphocytes \((n = 250–300)\) on four different NHAs. The results shown in (C) were taken from \(~80\%\) of CD4\(^+\) T-lymphocytes of all bound cells shown in (B). The actin and nuclei were stained by rhodamine-conjugated phalloidin (Life technologies Co., USA) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, BD Bioscience, USA), respectively. \(P\) values were calculated with neighboring column data (\(* P < 0.0282, ** P < 0.0061,\) and NS, not significant). (D) The summary of evaluated filopodia morphology (number, length, and width of filopodia) of CD4\(^+\) T-cells bound on the different nanohole arrays (NHA) using fluorescence measurements. For the comparison, the results of control samples (planar glass substrates) were also included.
NHA surface was \( \sim 0.60 \) to \( 0.67 \) \( \mu \text{m} \) determined by FE-SEM (Figs. 4(A), (B)), which is relatively longer than the intrinsic nanoscale structures (e.g., microvilli) on the surface of human T cells observed on planar coverslip glass (\( \sim 0.25 \) to 0.78 \( \mu \text{m} \)). The cell “filopodia” and “microvilli” are interrelated because the filopodia or microspikes start to protrude when the cells contact the substrate surface via microvilli and started to spread even though their sizes were less than \( \sim 1 \) \( \mu \text{m} \). However, in our study we observed that more specifically filopodia-substrate interaction is correlated to cell capture efficiency and the microvilli at the interface of cell-substrate were not extensively observed. To further confirm the development of the structural proteins of the actin-rich filopodia from surface-bound CD4 T-cells on the NHA substrates, FM analysis of live single CD4\(^+\) T-lymphocyte bound on NHA substrate (\( d = 270 \) nm) was also performed (Figs. 6(A)–(F)). Scanned FM images taken from the top to bottom of the bound CD4 T-lymphocyte clearly exhibit actin-rich filopodial protrusions in the early stage of the cell-substrate interaction (\( \sim 20 \) min, Fig. 6(F)). On the basis of our results, it is clear that the greater the average number of filopodia per cell, the larger the cell adhesion force is on the nanostructured-surface of NHAs. The cell adhesion force is also related to the cell shape, elongation and contact area.\(^{17}\) As a general rule, the cell capture efficiency increases with increasing the adhesion force between the CD4\(^+\) T-cells and the NHA surface as discussed before to explain the difference in the cell capture efficiency between the NHA and control sample. Moreover, as shown in Figure 4(E), the \( \sim 200 \) nm NHAs (140–200 nm in diameter) show the highest separation yield, suggesting that the size of the nanohole (\(< 200 \) nm) is close to the optimum size of filopodial protrusion from the CD4\(^+\) T-cells. This study provides the first quantitative analysis on the effect of cell-nanotopography interaction and the interface structure on the cell capture process. Furthermore, this platform is available to develop the sensor of disease diagnosis (e.g., AIDS, cancer, Alzheimer’s disease etc.) using optimal NHA by detection of target cell with high yield and purity.

**CONCLUSIONS**

We investigated how the formation of filopodia affects the capture and separation efficiency of primary CD4\(^+\) T-lymphocytes from a mixed cell population using a nanohole array (NHA) with a hole diameter ranging from 140 to 550 nm. Our result suggested that the average number of filopodial filaments per cell is strongly correlated to the cell capture efficiency, suggesting a crucial role of early stage cell adhesion and spreading in the process of cell capture. In addition, STR-functionalized NHAs of \(< 200 \) nm in diameter were demonstrated to be the optimal sizes for isolating and separating CD4\(^+\) T-lymphocytes from whole mouse splenocytes, with a high separation yield of \( \sim 95.3\% \). Such a high performance is due in part to the matched dimensions of filopodia fibers, filopodia formation initially stimulated by the STR-formation on surface, and the nanostructures of the NHAs for 3D interaction of the captured cells on the surface followed by guided sensing and adhesion.

**Acknowledgments:** This study was supported by the Priority Research Centers Program and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0019694 and NRF-2013R1A1A2012685, PI: Sang-Kwon Lee), a New Investigator Research Grant from Alzheimer’s Association (PI: Rong Fan), and the U.S. National Cancer Institute Howard Temin Pathway to Independence Award (NIH 4R00 CA136759-02, PI: Rong Fan). This study was also supported by a grant from the Global Excellent Technology Innovation R&D Program funded by the Ministry of Knowledge Economy, Republic of Korea (10038702-2010-01).

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