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Nanotopography-Guided Migration of T Cells

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T cells navigate a wide variety of tissues and organs for immune surveillance and effector functions. Although nanoscale topographical structures of extracellular matrices and stromal/endothelial cell surfaces in local tissues may guide the migration of T cells, there has been little opportunity to study how nanoscale topographical features affect T cell migration. In this study, we systematically investigated mechanisms of nanotopography-guided migration of T cells using nanoscale ridge/groove surfaces. The velocity and directionality of T cells on these nanostructured surfaces were quantitatively assessed with and without confinement, which is a key property of three-dimensional interstitial tissue spaces for leukocyte motility. Depending on the confinement, T cells exhibited different mechanisms for nanotopography-guided migration. Without confinement, actin polymerization-driven leading edge protrusion was guided toward the direction of nanogrooves via integrin-mediated adhesion. In contrast, T cells under confinement appeared to migrate along the direction of nanogrooves purely by mechanical effects, and integrin-mediated adhesion was dispensable. Therefore, surface nanotopography may play a prominent role in generating migratory patterns for T cells. Because the majority of cells in periphery migrate along the topography of extracellular matrices with much lower motility than T cells, nanotopography-guided migration of T cells would be an important strategy to efficiently perform cell-mediated immune responses by increasing chances of encountering other cells within a given amount of time. The Journal of Immunology, 2012, 189: 2266–2273.

Chemokines bound on the surfaces of such fibrous structures have been suggested to be primary sources for the guidance (3, 6), but it is also possible that nanoscale topography of fibrous structures itself may guide the migration of T cells. Although how chemokine gradients direct migration of leukocytes has been extensively studied using various in vitro model systems (8–10), little attention has been paid to how nanoscale topographical features affect leukocyte migration.

Surfaces containing nanoscale topographical structures can be an ideal platform for the study of nanotopography-guided migration of T cells because surface topography and biochemical compositions of the surfaces can be independently controlled. Indeed, nanostructured surfaces have been useful in elucidating how strongly adhering cells such as epithelial cells and mesenchymal cells sense and respond to nanoscale topographical features in ECMs (11, 12). When these cells were plated onto substrates containing nanoscale ridges/grooves, they exhibited an elongated morphology and migrated along the axis of the nanogrooves because of the preferential actin polymerization in that direction (11, 13, 14). However, leukocytes including T cells exhibit different modes of migration compared with mesenchymal and epithelial cells: so-called amoeboid migration. They weakly adhere onto the surfaces, do not form focal adhesion, and exhibit much higher motility, typically ~100-fold faster than that of mesenchymal cells (15, 16). Thus, their responses to surface nanotopography are likely to be distinct from that of mesenchymal cells; nevertheless, how leukocytes behave on nanostructured surfaces has not been systematically investigated to date.

In this study, we sought to understand how T cell motility is regulated by nanoscale topography in the form of regular ridges/grooves. The nanopatterned surfaces of polyurethane acrylate (PUA) were fabricated by UV-assisted capillary force lithography, which has proved useful to control cell–biomaterial interfaces with good biocompatibility (17). Then, the mean velocity (Vmean) and directionality of T cells migrating on the nanostructured surfaces were quantitatively analyzed. Three biomechanical factors determining amoeboid cell motility (15)—integrin-mediated adhesion, actin polymerization-driven leading edge protrusion, and myosin
II-mediated contractility—were systematically varied with and without confinement. Confinement is an emerging property of three-dimensional interstitial spaces essential for leukocyte motility, and two-dimensional–confined surfaces (18) and one-dimensional channels (19, 20) have been successfully used to mechanistically study three-dimensional leukocyte migration. Depending on the confinement, T cells exhibited different mechanisms for nanotopography–guided migration. Without confinement, leading edge protrusion was guided toward the direction of nanogrooves via integrin-mediated adhesion. In contrast, the nanoscale ridge/groove structures acted as railroads for T cells under confinement, and integrin-mediated adhesion was dispensable for this process. Therefore, surface nanotopography itself may play a prominent role in generating migratory patterns for lymphocytes under many physiological/pathological circumstances. Because the majority of cells in periphery migrate along the topography of ECMs with much lower motility than T cells, nanotopography–guided migration of T cells would be an important strategy to efficiently perform cell–mediated immune responses by increasing chances of encountering other cells within given amount of time.

Materials and Methods
Fabrication of nanoscale ridge/groove surfaces
Glass coverslips were rinsed with ethanol in an ultrasonic bath for 10 min, washed in a flow of distilled water, and dried in a drying oven. To increase the adhesion between the PUA nanostructures and the glass surfaces, we coated the glass surfaces with an adhesion promoter (Minuta Tech). A small amount of the PUA precursor (Minuta Tech) was drop-dispersed onto the surface, and a PUA mold containing engraved nanoscale ridge/groove patterns was directly placed on each coated surface. The PUA precursor spontaneously moved into the cavity of the mold by means of capillary action and was subsequently cured by exposure to UV light (λ = 250–400 nm, 100 mJ/cm²) for ~30 s through the transparent backplane. After the curing process, we peeled the molds from the surfaces using a pair of sharp tweezers.

T cell preparation
DO11.10 TCR transgenic mice were purchased from Jackson Laboratories and bred in the animal care facility in Pohang University of Science and Technology Biotech Center under pathogen-free conditions. All experiments involving mice were approved by the Institutional Animal Care and Use Committee at Pohang University of Science and Technology Biotech Center. DO11.10 CD4+ T cell blasts were prepared by stimulating cells from the spleens and lymph nodes of DO11.10 TCR transgenic mice with 100 mg/ml streptomycin (Invitrogen), and 1–2 U/ml IL-2 (PeproTech), maintaining 37˚C and 5% CO2 for live cell imaging. Time-lapse microscopy was immediately initiated with images recorded at intervals of 15 s for 15 min. At each time interval, two images (differential interference contrast [DIC] and red fluorescence with EX BP 550/25 and EM BP 605/70) were recorded in rapid succession. The trajectory of the T cells was analyzed using the track object function of MetaMorph (Universal Imaging, Molecular Devices). For interference reflection microscopy (IRM), ×100 (NA = 1.3; Plan-Neofluar) objective lens was used, and fluorescence filters were replaced with a linear polarizer, a narrow band-pass filter (EX BP 633/10), a beamsplitter (20/80), and a crossed analyzer (21).

Characterization of agarose solution/gel
Viscosity of 1% ultra-low–melt agarose was measured using SV-10 Vibroviscometer (Malvern Instruments). Stiffness of 0.5% low-melt agarose gel blocks was determined by steel ball indentation as described elsewhere (22). In brief, a steel ball (0.3 mm in diameter and 0.9 mg in mass) was placed onto the agarose gels and indentation depth was measured by microscopy. Young’s modulus was calculated based on the Hertz theory:

\[ Y = \frac{3(1-v^2)f}{4r^2} \]

where \( f \) is the weight of the steel ball, \( d \) is the indentation depth of the substrate, \( r \) is the radius of the steel ball, and \( v \) is the Poisson ratio, assumed to be 0.45.

Scanning electron microscopy
Cells were fixed in PBS with 4% paraformaldehyde and 2% sucrose at room temperature for 20 min. After washing these cells with PBS twice, we fixed them in the second fixative (3% paraformaldehyde and 2.5% glutaraldehyde in a 0.1-M cacodylate buffer supplemented with 1% sucrose and 5 mM CaCl₂, pH 7.4) at room temperature for 20 min. After washing the cells twice with a 0.1-M cacodylate buffer, we postfixed them with 1% osmium tetroxide in a cacodylate buffer for 1 h. Then the cells were dehydrated in a series of ethanol solutions (from 30 to 99.5%) and finally in hexamethyldisilazane. After air-drying, samples were coated with platinum sputtering and imaged using Philips XL30S.

Transmission electron microscopy
For transmission electron microscopy (TEM), nanostructured surfaces fabricated on polyethylene terephthalate films were used. Nanostructured surfaces formed on polyethylene terephthalate films were coated with 10 μg/ml ICAM-1 and used. T cells on nanostructured surfaces were fixed with PBS supplemented with 3% paraformaldehyde (Electron Microscopy Sciences) and 2% sucrose (Sigma) for 10 min at 4˚C, washed in PBS, and further fixed with 0.1 M cacodylate buffer (sodium cacodylate trihydrate; Sigma) containing 1% paraformaldehyde, 2.5% glutaraldehyde (Sigma), 1% sucrose, and 5 mM CaCl₂ for 10 min at 4˚C. Prefixed samples were washed with 0.1 M cacodylate buffer and postfixed with 0.4% osmium tetroxide (Sigma) for 15 min at 4˚C. Then fixed samples were treated with 0.5% uranyl acetate for 10 min at 4˚C, washed and exchanged in graded ethanol (30, 50, 70, 80, 90, 99.7% for 5 min each), immersed into hexamethyldisilazane (Sigma) for 20 min, and dried in the air. Dried samples were embedded in epoxy resins, followed by thermal curing at 65˚C for 4 h. Thin sections with thicknesses in the 80- to 120-nm range were obtained using an RMC PowerTome XL microtome and imaged by Hitachi 7600.

Results
Morphology of T cells on nanoscale ridge/groove surfaces
To study the effect of nanotopography on directionality of T cell migration, we fabricated nanoscale ridge/groove structures on glass coverslips by UV-assisted capillary force lithography using a UV curable polymer, PUA (17). PUA flat surfaces were fabricated for control experiments. Highly uniform nanoscale ridge/groove structures were neatly formed over the 18- or 25-mm coverslips

Live cell imaging and data analysis
A modified Zeiss Axio Observer Z1 epifluorescence microscope with an ×40 (numerical aperture [NA] = 1.30; Plan-Neofluar) objective lens and a Roper Scientific CoolSnap HQ CCD camera was used for imaging. The T cell–seeded surfaces were mounted on the microscope stage equipped with a Chamlide TC incubator system (Live Cell Instrument, Seoul, Korea) maintaining 37˚C and 5% CO₂ for live cell imaging. Time-lapse microscopy was immediately initiated with images recorded at intervals of 15 s for 15 min. At each time interval, two images (differential interference contrast [DIC] and red fluorescence with EX BP 550/25 and EM BP 605/70) were recorded in rapid succession. The trajectory of the T cells was analyzed using the track object function of MetaMorph (Universal Imaging, Molecular Devices). For interference reflection microscopy (IRM), ×100 (NA = 1.3; Plan-Neofluar) objective lens was used, and fluorescence filters were replaced with a linear polarizer, a narrow band-pass filter (EX BP 633/10), a beamsplitter (20/80), and a crossed analyzer (21).
with few defects (see Supplemental Fig. 1). Nanostructured surfaces with dimensions of 350-nm ridge/700-nm groove/500-nm height, which represent the dimensions of the topographical structure of ECMs (23, 24), were used for the bulk of the experiments, and the effect of groove dimension was assessed separately. Nanostructured and flat PUA surfaces were coated with various concentrations of ICAM-1, which is a ligand for the T cell integrin LFA-1, to assess the role of integrin-mediated adhesion. To minimize the nonspecific binding of adhesion molecules in serum-containing media, surfaces were blocked with casein (25). First, the morphology of T cells seeded onto various types of PUA surfaces was analyzed by scanning electron microscopy (SEM). About 40% of the T cells on the surfaces coated with ICAM-1 extended lamellipodia on the polarized leading edges (Fig. 1A, 1B, upper row), whereas >90% of T cells on the surfaces blocked with casein did not form lamellipodia (Fig. 1B, lower row). Interestingly, the lamellipodia of T cells on nanostructured surfaces were much narrower than that of T cells on flat surfaces (Fig. 1C), and often aligned along the direction of the nanoscale grooves/ridges, indicating that actin polymerization-driven leading edge protrusion is guided by the nanotopographical structures. As shown in a cross-sectional TEM image (Fig. 1D), T cells on nanostructured surfaces rarely touch grooves (<10% of grooves examined) and mostly made contact with ridges.

**Integrin-dependent nanotopography-guided migration of T cells**

Flat PUA surfaces coated with four different concentrations of ICAM-1 solution (0, 2, 10, and 50 μg/ml) were prepared and characterized. First, relative amounts of ICAM-1 bound to flat PUA surfaces coated with various concentrations of ICAM-1 solution were assessed using anti-ICAM-1 and fluorophore-conjugated secondary Ab. Mean fluorescence intensity of each surface was measured and plotted after subtracting mean fluorescence intensity of uncoated PUA surfaces (Fig. 2A). Then, adhesion of T cells on flat PUA surfaces coated with various ICAM-1 concentrations was assessed by IRM. T cells were plated on PUA surfaces coated with various concentrations of ICAM-1, and DIC and IRM images were sequentially acquired (within 1 s of an interval) 30 min after

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**FIGURE 1.** Ultrastructures of T cells examined by electron microscopy. (A) Representative SEM images of T cells on nanostructured (left column) and flat (right column) surfaces in the presence (upper row) and absence (lower row) of ICAM-1. (B) Effect of surface topography and adhesiveness on lamellipodia formation at leading edges. (C) Effect of surface topography on width of lamellipodia of T cells on ICAM-1–coated surfaces. (D) A representative cross-sectional TEM image of a T cell on a nanostructured surface coated with ICAM-1. (Mann–Whitney U test, ***p < 0.0001).
plating. Only cells in close proximal to the surfaces (within 100 nm) generate dark region in IRM images (21); thus, percentage of adhering cells on each substrate could be precisely evaluated by comparing DIC and IRM images (Fig. 2B). In addition, adhesion areas of T cells were measured from IRM images. Percentage of adhering cells (Fig. 2B) and adhesion areas (Fig. 2C) increased as ICAM-1 concentration increased, meaning that adhesion strength of T cells depended on ICAM-1 concentration.

Migration of T cells on flat/nanostructured PUA surfaces coated with various concentrations of ICAM-1 were recorded by time-lapse microscopy. Final concentration of 1% ultra-low–melt agarose, which has viscosity of 3.60 ± 0.02 mPa s at 37°C (close to the viscosity of 40% glycerol at room temperature), was added to minimize convection as previously described (25). Time-lapse images were quantitatively analyzed as shown in Fig. 3A. Δx is the displacement of T cells along the ridge/groove direction, and Δy is the displacement of T cells perpendicular to the ridge/groove direction. From the displacement information, Vmean and dx, directionality of migration along the direction of ridge/groove of nanostructures, were calculated using the equations shown in Fig. 3A. Directionality index dx is defined as a ratio of Vx and (Vx + Vy) such that the dx values will range from 0 to 1, and will be close to 0.5 if T cells migrate randomly. Conversely, dx values >0.5 or <0.5 indicate that the direction of T cell migration is biased to either parallel or perpendicular to the direction of nanoscale ridge/groove, respectively.

The Vmean of T cells on flat surfaces had a biphasic relationship with the concentration of ICAM-1 (Fig. 3B), which is characteristic of the haptokinetic migration of mesenchymal cells in two dimensions (26, 27). In contrast, the Vmean of T cells on nanostructured surfaces gradually increased as the ICAM-1 concentration increased (Fig. 3D). This difference might reflect the fact that the effective ICAM-1 density of T cells on nanostructured surfaces is ~3-fold lower than that on flat surfaces because T cells on nanostructured surfaces only contact ridges, as illustrated by TEM images (Fig. 1D).

The average dx values of T cells on the flat surfaces were close to 0.5 regardless of the ICAM-1 density (Fig. 3C), meaning that the migration of T cells on the flat surfaces was not biased toward any direction. In contrast, the average dx values of T cells on the nanostructured surfaces gradually increased from 0.49 to 0.64 as the ICAM-1 concentration increased from 0 to 50 μg/ml (Fig. 3E). The increase in the average dx values from 0.49 to 0.64 may appear to be a mild increment, but it is a substantial change in the directionality of migration as shown by the trajectories of individual T cells from the origin (Fig. 3F) and actual locomotion of T cells (see Supplemental Videos 1, 2).

To examine whether the ICAM-1–LFA-1 interaction is necessary for the nanotopography-guided migration of T cells or whether it can be replaced with other integrin-mediated binding of T cells on the surfaces, we coated nanostructured PUA surfaces with whole FBS and quantified the dx of T cells. T cells on serum-coated nanostructured surfaces migrated along the direction of

**FIGURE 2.** Characterization of flat PUA surfaces coated with various concentrations of ICAM-1. (A) Surface density of ICAM-1 (a solid line is a guideline to the eye), (B) percentage of adhering T cells, and (C) adhesion area of adhering T cells. Data are representative of two independent experiments (line represents mean; Mann–Whitney U test, *p < 0.05, **p < 0.001, ***p < 0.0001).

**FIGURE 3.** Integrin-dependent nanotopography-guided migration of T cells. The migration of T cells on various types of surfaces was recorded by time-lapse microscopy (objective ×40; Zeiss Axio Observer Z1; NA = 1.30; Plan-Neofluar) and analyzed (MetaMorph). (A) Scheme of quantitative evaluation of the Vmean and dx of T cells. (B) Effect of ICAM-1 concentration on the Vmean of T cells on flat surfaces. (C) Effect of ICAM-1 concentration on the dx of T cells on flat surfaces. (D) Effect of ICAM-1 concentration on the Vmean of T cells on nanostructured surfaces. (E) Effect of ICAM-1 concentration on the dx of T cells on nanostructured surfaces. (F) Representative trajectories of T cells migrating on nanostructured surfaces coated with various concentrations of ICAM-1. (G) Effect of serum coating on the dx of T cells on nanostructured surfaces. (H) Effect of groove dimensions on the dx of T cells on nanostructured surfaces. Data are representative of two independent experiments [line represents mean; Kruskal–Wallis test (B, H), Mann–Whitney U test (C–G), *p < 0.05, **p < 0.001, ***p < 0.0001].
nanogrooves with an average \(d_u\) of 0.6, significantly greater than that of T cells on casein-blocked nanostructured surfaces, suggesting that integrin ligands in serum other than ICAM-1 can also mediate the nanotopography-guided migration of T cells (Fig. 3G).

Lastly, to assess whether the dimension of nanostructures affect the nanotopography-guided migration of T cells, we analyzed the migration of T cells on various nanostructured surfaces with different groove widths: 350-nm ridges/350-nm grooves, 350-nm ridges/700-nm grooves, and 350-nm ridges/1750-nm grooves. The average \(d_u\) values of T cells were almost identical for all three types of nanostructured surfaces as shown in Fig. 3H.

**Integrin-independent nanotopography-guided migration of T cells under confinement**

Recently, confinement has proved to be an essential characteristic of three-dimensional interstitial spaces for leukocyte migration, and one-dimensional channels made of silicone elastomers or two-dimensional surfaces confined with agarose gels have been successfully used to replicate key characteristics of leukocyte migration in three-dimensional interstitial spaces (18–20). Therefore, we also considered the effect of confinement and performed T cell migration assays under confinement by placing agarose gels on top of the nanostructured surfaces.

T cells were seeded on the nanostructured or flat PUA surfaces that were either coated with 10 \(\mu\)g/ml ICAM-1 or blocked with casein. Then, an agarose gel block, which has Young’s modulus of 11.2 ± 2.7 kPa (close to the stiffness of muscle tissues or injured/tumorous soft tissues) (28), was carefully placed on the surface, and time-lapse microscopy was initiated. The \(V_{mean}\) and \(d_u\) values of T cells on different types of surfaces were calculated from the time-lapse images and plotted in Fig. 4A and 4B, respectively. In contrast with the previous experiments performed without confinement, the \(V_{mean}\) values of T cells were not affected by ICAM-1 (Fig. 4A). The average \(d_u\) values of T cells depended only on surface nanotopography, not on the presence of ICAM-1; the average \(d_u\) values of T cells on nanostructured surfaces were ~0.6 for both ICAM-1–coated and casein-blocked surfaces, and these values were significantly greater than the \(d_u\) values of T cells on flat surfaces (Fig. 4B, Supplemental Video 3). The trajectories of representative T cells in each condition shown in Fig. 4C reveal the same trends. Indeed, motility of T cells under confinement were not affected by ICAM-1 coating on nanostructured surfaces; both \(V_{mean}\) values and \(d_u\) values of T cells were not significantly different from each other for ICAM-1 concentration ranging from 0 to 50 \(\mu\)g/ml (Fig. 4D, 4E).

**Role of cytoskeletons in the nanotopography-guided migration of T cells**

To better understand how nanotopographical features can influence the migration of T cells, we inhibited actin polymerization and actomyosin contractility using pharmacological inhibitors and observed their effects on \(V_{mean}\) and \(d_u\). A titrated amount of latrunculin A, which interferes with actin polymerization by sequestering G-actin, was added to T cells on nanostructured surfaces coated with 10 \(\mu\)g/ml ICAM-1, and T cell responses were observed. When the concentration of latrunculin A exceeded 25 nM, T cell motility almost ceased; however, with 12.5 nM latrunculin A treatment, we could abolish leading edge protrusion by actin polymerization while a substantial fraction (>60%) of T cells was still motile. With this concentration of latrunculin A, T cells exhibited a characteristic hand-mirror shape with uropods and frequent membrane blebbing at the leading edge and the midbody (Fig. 5A, Supplemental Video 4), presumably driven by the contractility of myosin II at uropods as previously described (15, 29). The \(V_{mean}\) decreased by ~60% compared with that in DMSO-treated T cells, and ~40% of latrunculin A-treated T cells were nearly immotile (\(V_{mean} < 3 \mu\)m/min). Interestingly, the average \(d_u\) value of the motile fraction of latrunculin A-treated T cells was ~0.5, meaning that the direction of T cell migration was no longer guided by the direction of nanotopography. In contrast, when we inhibited myosin II activity with 50 \(\mu\)M blebbistatin, the morphology of T cells completely changed; uropods at the trailing edge disappeared, the length of T cells was extended, and narrow membrane protrusions appeared at the leading edge along the direction of nanoscale ridges/grooves (Fig. 5B, Supplemental Video 5). The disappearance of uropods upon blebbistatin treatment agrees with previously published results in mouse T cells (19, 25). Conversely, it was reported that human T cells on ICAM-1 surfaces exhibit extreme uropod elongation in the presence of Mg\(^{2+}\)/EGTA, which constitutively activates LFA-1, when myosin II was blocked or knocked down (30, 31). This discrepancy may be potentially caused by different cell sources and the use of Mg\(^{2+}\)/EGTA. Blebbistatin treatment slightly reduced the \(V_{mean}\), but the average \(d_u\) value was comparable with that of untreated T cells, meaning that myosin II-driven contractility is not essential for the nanotopography-guided migration of T cells in the absence of confinement. Similar results were obtained with nanostructured surfaces coated with a higher concentration of ICAM-1 (50 \(\mu\)g/ml ICAM-1); overall, \(V_{mean}\) values and \(d_u\) values were greater than those of 10 \(\mu\)g/ml ICAM-1, but latrunculin A-treated T cells exhibited a significantly lower average \(d_u\) value than DMSO- and blebbistatin-treated T cells (Fig. 5F). Together, actin polymerization-driven leading edge protrusion.
T cells on nanostructured surfaces coated with 50 μM of cytoskeleton inhibition on the substantially reduced motility; the confinement, T cells treated with latrunculin A exhibited sub-concentration of inhibitor in the media was placed on the T cell treated with inhibitors were applied on casein-blocked nano-structures. Myosin II activity inhibition caused se-

sion, as well as integrin-mediated adhesion, was critical for the nanotopography-guided migration of T cells without confinement.

Identical pharmacological inhibition experiments were performed with T cells under confinement. Suspensions of T cells treated with inhibitors were applied on casein-blocked nano-structures, and an agarose block containing the same concentration of inhibitor in the media was placed on the T cell suspensions. Similar to the results for T cells in the absence of confinement, T cells treated with latrunculin A exhibited substantially reduced motility; the $V_{\text{mean}}$ was reduced by $\sim 65\%$ compared with that of untreated T cells, and $>60\%$ of T cells were nearly nonmotile, that is, $V_{\text{mean}} < 3 \, \mu\text{m/min}$ (Fig. 6A). However, the $d_x$ value of the motile fraction of T cells was comparable with that of DMSO-treated T cells (Fig. 6B); that is, T cells moved along the direction of nanogrooves (see Supplemental Video 6). It appeared that the direction of membrane blebs of T cells was mechanically guided in the direction of nanogrooves because of confinement between the agarose gel and nanoscale ridges/grooves. In contrast, the membrane blebbing of T cells in the absence of confinement predominantly occurred toward the free space; thus, the direction was less affected by surface nanotopography. Myosin II activity inhibition caused severe motility defects in T cells under confinement, whereas it had a minor effect on unconfined T cells; the $V_{\text{mean}}$ was reduced by $\sim 60\%$ compared with that of DMSO-treated T cells, and $>50\%$ of T cells were nearly immotile (Fig. 6A, Supplemental Video 7). A substantial reduction of T cell motility in confined environments upon blebbistatin treatment agrees with previous reports (25, 32).

Interestingly, the average $d_x$ value of the motile fraction of T cells was significantly higher than that of DMSO-treated T cells (Fig. 6B), suggesting that contractile force generated by myosin II was a major driving force of motility against nanotopography under confinement.

### Discussion

T cell migration guided by various fibrous structures has been frequently observed by recent two-photon microscopy of intact tissues (5–7), but the mechanisms for guidance have not been elucidated. biochemical cues such as adhesion molecules and chemokines present on the fibrous structures have been speculated to be important for the guidance (4, 33), but the topographical structure itself may also guide the migration of T cells. Nanostructured surfaces may provide a unique opportunity to address the aforementioned issues because chemical functionality of the surfaces can be controlled independently from the topographical structures.

In this study, we systematically investigated the mechanisms of nanotopography-guided T cell migration by using nanofabricated surfaces containing nanoscale ridge/groove structures with dimensions comparable with the fibrous structures of ECMs. T cell migration on the nanostructured surfaces in the presence and absence of agarose gel confinement was studied to gain insights into the role of nanotopography in T cell migration in three dimensions. Recently, confinement has been identified as a key factor of three-dimensional interstitial spaces for leukocyte migration, and two-dimensional–confined surfaces (18) and one-dimensional channels (19, 20) have been successfully used for the mechanistic investigation of leukocyte migration. For mechanistic investigation, we systematically manipulated integrin-mediated adhesion, actin polymerization-driven leading edge protrusion, and myosin II-mediated contractility, all of which are key factors suggested to determine the diverse modes of amoeboid cell migration (15). Integrin-mediated adhesion was controlled by varying the surface coating. Actin polymerization and myosin II activity were perturbed by pharmacological inhibitors. When a titrated amount of latrunculin A was added, T cells maintained characteristic hand-mirror shapes, but actin polymerization-driven leading edge protrusion was abolished, and motility appeared to be purely driven...
by the membrane blebbing induced by myosin II contractility at uropods. In contrast, when myosin II activity was blocked by blebbastatin, the uropods of T cells disappeared, and T cells formed long leading edges and appeared to move solely by actin polymerization and integrin-mediated adhesion. Interestingly, the mechanism of nanotopography-guided T cell migration in the presence of confinement was completely different from that in the absence of confinement.

In the absence of confinement, leading edge protrusion by actin polymerization and integrin-mediated adhesion were essential for nanotopography-guided T cell migration. Given that integrin-mediated adhesion was essential for the formation of thin two-dimensional sheet-shaped lamellipodia of T cells at the leading edge, as shown by SEM images in Fig. 1, and that lamellipodia of T cells on nanostructured surfaces were aligned to the direction of nanogrooves, preferential lamellipodia formation by actin polymerization toward the direction of nanogrooves and further stabilization of protruded lamellipodia by integrin-mediated adhesion may be key mechanisms of guidance. Interestingly, the proposed mechanism is similar to that of the nanotopography-guided migration of mesenchymal cells in two dimensions (11, 14, 23), and T cells in two dimensions exhibited biphasic responses to the surface density of ICAM-1, which is a characteristic feature of mesenchymal cell migration in two dimensions (26).

In sharp contrast, the nanotopography-guided migration of T cells under confinement did not require integrin-mediated adhesion. Inhibiting actin polymerization had a minor effect on the directionality of migration; although a substantial fraction of T cells was immotile, the motile fraction of T cells exhibited biased membrane blebbing toward the direction of nanogrooves, presumably by mechanical guidance because of confinement. Interestingly, when myosin II activity was inhibited, the motile fraction of T cells exhibited even enhanced directionality toward the direction of nanogrooves, although overall motility was substantially reduced. These results suggest that a mechanism of guidance is mechanical effects of nanotopography. Under agarose gel confinement, it is possible that membranes of T cells contacting nanostructured surfaces were deformed into the nanogrooves, and surface nanotopography may have acted as a railroad for T cells. We attempted to formally prove this possibility by cross-sectional TEM and IRM, but a number of technical issues were difficult to overcome. Fixing cells under agarose confinement was difficult, and during the washing step, we lost most of the cells, so sample preparation for TEM was not possible. Nanostructures used for the experiments themselves generate complex patterns in IRM images because their dimensions were comparable with wavelength of visible light, so interpreting IRM data was not straightforward. However, given that the stiffness of agarose gels used to confine cells is >10 times greater than that of typical cells (34), it is reasonable to speculate that membranes of cells are deformed into the nanogrooves of the surfaces by the confinement of agarose gel. Indeed, a similar mechanism has been suggested as a mechanism underlying the contact guidance of T cells in a three-dimensional collagen matrix (35). However, the nanostructured surfaces used in our experiments mostly present specific proteins that we coated, whereas collagen fibers possess a variety of chemical moieties that can potentially interact with T cells (33); thus, our results provide definitive evidence that nanoscale topography itself can guide the migration of T cells in confined spaces solely via mechanical means.

In tissues, cancer cells and stromal cells including fibroblasts migrate along the direction of fibrillar collagen bundles by taking a mesenchymal cell migration mode (14). In some cases, cancer cells actively modify the orientation of fibers to effectively migrate toward the direction that they desire to move in (36, 37). Overall, following the trajectory of stromal cells and cancer cells with higher speed would be beneficial for T cells to perform functions related to cell-mediated immunity, which requires direct cell–cell contact, and to increase the likelihood of encountering their potential targets. In normal tissues or near-intact tissues where they can be tightly confined by cells and ECMs, they can move along similar pathways as stromal cells and cancer cells via mechanical guidance by ECM nanotopography. Conversely, in inflamed tissues or tumor microenvironments, highly swollen tissues or damaged tissues may not provide sufficient confinement for integrin-independent motility. In such cases, T cells may switch to integrin-mediated modes of migration but still maintain their direction of migration by preferentially protruding their lamellipodia toward the direction of ECM topography.

In summary, we systematically investigated the mechanisms of nanotopography-guided T cell migration by using nanoscale ridge-/groove-structured surfaces and demonstrated that the mechanisms of guidance could be switched depending on the confinement. These results suggest that nanotopography-guided migration of T cells is as robust a mechanism as chemokine-guided migration (18), facilitating cell-mediated immune responses by guiding T cells toward the migratory tracks of other slowly moving cells in tissues, thus increasing the possibility to encounter infected/transformed cells within a given amount of time.

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Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Data**

**Fig. S1.** Fabrication of nanoscale ridge/groove surfaces. (A) Schematic representation of fabrication. (B) Representative image of 25-mm cover slip containing 350-nm ridges/700-nm grooves. (C) Representative SEM image of 350-nm ridges/700-nm grooves.
Supplemental Movie Legends

**Movie 1.** Random migration of T cells on nanostructured surfaces blocked with casein (0 μg/ml of ICAM-1).

**Movie 2.** Nanotopography-guided migration of T cells on nanostructured surfaces coated with 10 μg/ml of ICAM-1.

**Movie 3.** Nanotopography-guided migration of T cells on nanostructured surfaces blocked with casein under confinement.

**Movie 4.** Migration of latrunculin A-treated T cells without confinement. A nanostructured surface coated with 10 μg/ml of ICAM-1 was used.

**Movie 5.** Migration of blebbistatin-treated T cells without confinement. A nanostructured surface coated with 10 μg/ml of ICAM-1 was used.

**Movie 6.** Migration of latrunculin A-treated T cells under confinement. A nanostructured surface blocked with casein was used.

**Movie 7.** Migration of blebbistatin-treated T cells under confinement. A nanostructured surface blocked with casein was used.