Deterministic Migration-Based Separation of White Blood Cells

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Functional and phenotypic analyses of peripheral white blood cells provide useful clinical information. However, separation of white blood cells from peripheral blood requires a time-consuming, inconvenient process and thus analyses of separated white blood cells are limited in clinical settings. To overcome this limitation, a microfluidic separation platform is developed to enable deterministic migration of white blood cells, directing the cells into designated positions according to a ridge pattern. The platform uses slant ridge structures on the channel top to induce the deterministic migration, which allows efficient and high-throughput separation of white blood cells from unprocessed whole blood. The extent of the deterministic migration under various rheological conditions is explored, enabling highly efficient migration of white blood cells in whole blood and achieving high-throughput separation of the cells (processing 1 mL of whole blood less than 7 min). In the separated cell population, the composition of lymphocyte subpopulations is well preserved, and T cells secrete cytokines without any functional impairment. On the basis of the results, this microfluidic platform is a promising tool for the rapid enrichment of white blood cells, and it is useful for functional and phenotypic analyses of peripheral white blood cells.

1. Introduction

White blood cells (WBCs) or leukocytes are some of the major cellular components of blood and play important roles in immune responses. Peripheral WBCs consist of several subpopulations such as lymphocytes, monocytes, and polymorphonuclear cells. In many human diseases, the function and/or phenotype of WBCs deviate from the normal status. Therefore, functional and phenotypic analyses of peripheral WBCs provide useful clinical information. For example, interferon-γ (IFN-γ) release from T cells, one of the major components of lymphocytes, has been used for the diagnosis of tuberculosis.[2,3] However, the separation of WBCs from peripheral blood requires a time-consuming, inconvenient process. In peripheral blood, WBCs make up a small portion of blood cells, comprising one cell among a thousand red blood cells (RBCs), hence their separation faces technical challenges. Current techniques for separating WBCs include density-gradient centrifugation and selective lysis of RBCs, and these methods entail laborious and time-consuming procedures. Moreover, these methods often expose the cells to nonphysiological and stress-inducing environments (e.g., osmotic shock), which can lead to unintended adverse effects on the immunophenotype or function of WBCs.[4,5] Alternatively, WBCs can be sorted from RBCs based on their intrinsic physical differences in magnetic...
susceptibility,[6] dielectric properties,[7] size,[8–14] and rheological properties.[15–17] Microfluidic devices have proven effective for such application, by providing precise cell control over fluidic and separation forces as well as a simple separation setup.[10] The devices can be classified into two major categories: dilute-blood separators which require blood dilution prior to separation, and whole-blood separators which enable the separation of WBCs from undiluted whole blood. In dilute-blood approaches, WBCs can be isolated from RBCs upon the application of a magnetic field,[6] an electric field[7] or inertial effects.[10,11] The limitation of the approaches is that numerous blood cells (≈4 to 6 million cells per µL) in whole blood and their interactions can complicate the separation processes typically designed for single cells, resulting in significant blood dilution and low separation throughput.

Whole-blood approaches, on the other hand, are capable of processing undiluted whole blood and include cross-flow microfiltration,[8,9] deterministic lateral displacement (DLD)[12,13] and WBC margination.[15,16] Both cross-flow microfiltration and DLD techniques incorporate a sheath-flow design to align blood cells in the separation process,[8,9,12,13] thus resulting in an increase in operational complexity. WBC margination provides a simpler separation approach based on a hemodynamic phenomenon that WBCs preferentially localize near the vessel wall, while RBCs migrate to the vessel center. However, a major challenge arises from the dependence of WBC margination on channel geometry, hematocrit, flow rate, and RBC aggregation, which can restrict separation throughput and cause variability in separation efficiency. Important considerations to accelerate the clinical translation of microfluidic WBC separators thus include the improvement of separation throughput and the achievement of operational simplicity.

To address these restrictions, we developed a microfluidic platform to deterministically induce WBC migration in whole-blood flow and significantly enhance the throughput capability of WBC separation that can be accomplished in a simple microchannel. A major advancement in the microfluidic platform is to incorporate slant array ridges on the top of a planar microchannel to enable the following novel features: (i) controllable and directed migration of WBCs guided by transverse flows that are passively generated by the ridges, (ii) robust migration of WBCs over a wide range of flow rates, hematocrits and channel heights to significantly improve separation performance, and (iii) rapid but gentle separation of WBCs and RBCs are minimal, leading to effective deterministic migration of WBCs with a peak distribution of 75.1 ± 4.3% in outlet 1 at Hct = 40% and q = 100 µL min⁻¹, the effects of both hydrophoresis and blood cell interactions are minimal, resulting in the wide distribution of WBCs ranging from outlets 1 to 5 (Figure 2 c, top). Increasing Hct facilitates enhanced blood cell interactions, leading to effective deterministic migration of WBCs with a peak distribution of 75.1 ± 4.3% in outlet 1 at Hct = 40% and q = 100 µL min⁻¹ (Figure 2a, left and Figure 2c, top). Even at a high Hct of 40%, the CSA ridges created a rotational flow pattern (Figure 1d) where the migration direction of RBCs within the ridges was parallel to the ridge structure and was reversed under the structure, leading to a relatively uniform distribution of RBCs along the channel width (Figure S1, Supporting Information). Deterministic migration is directed by structure-induced transverse flows with directions determined by the ridge pattern.[22] The change in the ridge shape resulted in the change in the migration position (Figure S2 and Section S1, Supporting Information). These results clearly indicate that the deterministic migration is based on the synergistic combination of structure-induced flows and blood cell interactions, and enables precise and predictable control of the migration position of WBCs with a simple change in the ridge pattern.

The discontinuous slant array (DSA) design was developed to improve the deterministic migration process for WBCs (Figure 1a, top and Figure 1b, right). With CSA ridges, we observed undesired deviation from the formation of a single band of concentrated WBCs (Figure 2b, top; Movie S1,
Supporting Information). The deviation process is likely due to the invasion of some of the concentrated WBCs into the ridges and occurs along the deviation path (Figure 1a, bottom and Figure 1b, left). Since the invasion rate can depend on the number of concentrated WBCs, the deviation process becomes significant as Hct increases: 3.6 ± 0.4% and 14.6 ± 2.1% of WBCs were distributed in outlets 4–8 at Hct of 5% and 40%, respectively (Figure 2c, top). The outlet range from 4 to 8 was set for evaluation of WBC loss because the range corresponds to the cut-off for the discharge of a WBC-free RBC stream in the following separation device. To maximize the migration efficiency with minimal deviation, DSA ridges were designed as an array of slant ridges in which each row was shifted horizontally relative to the previous row by \( \delta \), where \( \delta \) is the center-to-center distance between the ridges (Figure 1b, right). The ridge spacing, \( \delta \), was 120 µm and the gap width between the ridges was 20 µm (Figure 1c; Figure S3, Supporting Information). The separate ridges in each row generated localized rotational flows and the center of each rotation migrated laterally according to the shift in the array, which can allow for the focusing of WBCs along the migration path and simultaneously limit the deviation process within the short lengths of the ridges (Figure 1a, top and Figure 1b, right). With DSA ridges, we achieved higher migration efficiency of up to 97.6 ± 0.3% in outlet 1 (Figure 2d, top), while minimizing the deviation process; only 3.3 ± 0.9% of WBCs were distributed in outlets 4–8 even at a high Hct of 40% and a high \( q \) of 100 µL min\(^{-1}\) (Figure 2d, bottom). The output distribution of RBCs was relatively even along the channel width (Figure S1, Supporting Information). The improvement in WBC migration is due to the fact that fluid transport is disconnected between DSA ridges in each row (Figure 1e, middle), thereby terminating the deviation path of WBCs at the short end of the left-most ridges (Figure 2b, bottom; Movie S2, Supporting Information). This novel principle of WBC migration will be characterized by operational and geometric conditions, and further validated for high-throughput separation of WBCs from whole blood and potential use for intracellular cytokine staining of WBCs in the following sections.

2.2. Characterization of Deterministic Migration of WBCs

The stability of the deterministic migration can be affected by flow rate, hematocrit levels, cell deformability, and channel geometry. We thus performed parametric studies to determine the optimum migration conditions in the DSA device for application to high-throughput separation. We first considered the effect of flow rate or shear rate on WBC migration in the DSA device. At Hct = 40%, we observed that the peak...
distribution in outlet 1 slightly decreases with increasing $q$ or shear rate (Figure 2d, bottom). We operated the DSA device at $q = 10$ and 100 µL min$^{-1}$, which correspond to average wall shear rates ($\gamma_w$) of $0.13 \times 10^4$ and $1.33 \times 10^4$ s$^{-1}$, respectively. It is important to note that the peak distribution of 10-µm particles in a size similar to WBCs remains almost unchanged over the flow rates (Figure 2g, right), indicating that the rigidity of cells or particles is an important factor in maintaining the stability of the deterministic migration. We then studied the effect of Hct on the migration distribution observed at $q = 100$ µL min$^{-1}$ (Figure 2d, top). There is an optimum Hct (5%) wherein the peak distribution of 97.6 ± 0.3% is higher than the peak distribution at Hct = 0.5% (46.6 ± 5.0%) and at Hct = 40% (83.3 ± 0.7%). This result is likely due to reduced blood cell interactions at a low Hct (0.5%) and the spread of over-crowded WBCs at a high Hct (40%). Though 11.6 ± 1.1% of WBCs were found in outlet 2 at a high Hct of 40% and a high $q$ of 100 µL min$^{-1}$, no significant spread to outlets 4–8 was observed in the DSA device (Figure 2d) and 94.9 ± 1.0% of WBCs formed a tightly focused cell stream, ranging from outlets 1 to 2 (Figure 2b, bottom). These results suggest that WBC migration in the DSA device is much less sensitive to flow rate and Hct as compared with the CSA device (Figure 2c), demonstrating the potential utility of deterministic migration for high-throughput separation of WBCs from whole blood.

To gain further insight into the deterministic migration process, the effect of RBC deformability on the process is explored using whole blood (Hct = 40%) fixed with 0.25% glutaraldehyde. A high concentration of the fixative can lead to complete hardening of blood cells via cross-linking of cellular proteins and thus significantly impair their deformability. As shown in Figure 2e, we observed a markedly scattered distribution of WBCs in the fixated whole blood, clearly indicating that impaired deformability of RBCs results in significant reduction in deterministic migration. WBC margination is known to be a consequence of the difference between lift forces on RBCs and WBCs that results from the high deformability and nonspherical shape of RBCs. In a similar manner, deterministic migration could be attributed to hydrodynamic interactions induced by the rheological properties of RBCs. The interactions seem to be more effective for the deterministic migration of rigid
and large particles (Figure 2g). Comparing the distribution of 10-μm particles in a phosphate buffered saline (PBS) solution and whole blood, we observed effective migration only in the case of whole blood. For hydrophoresis to be effective, the ratio \( r \) of particle size to channel height is a critical factor and typically needs to be \( r \geq 0.5 \).\(^{19–21} \) In a PBS solution, the \( r \) of 10-μm particles is smaller than the critical ratio, resulting in a wide distribution ranging from outlets 2 to 6 (Figure 2g, left). The transition of the peak distribution at a high \( q \) of 100 µL min\(^{-1}\) might be due to the elastic deformation of the poly(dimethylsiloxane) (PDMS) device\(^{28} \) and increased inertial effects.\(^{11,20,29} \) Surprisingly, 10-μm particles in whole blood continued to move toward the left sidewall and the migration process persisted to the range of \( q \) (Figure 2g, right). These results indicate that the deterministic migration process is based on the presence of RBCs and their hydrodynamic interactions. Using polystyrene particles with \( d = 2, 6 \text{ and } 10 \mu m \), we also found that the deterministic migration in whole blood improved with increasing particle size above 10 µm at \( q = 100 \mu L \text{ min}^{-1} \) (see the inset of Figure 2g, right; Figure S4 and Section S1, Supporting Information). This trend might be due to the small size of 2- or 6-µm particles which can facilitate their intrusion into the ridges as shown in Figures S5 and S6 in the Supporting Information, thereby inhibiting deterministic migration. The presence of RBCs and their interactions in the ridges likely allow size-selective entrance of the small particles into the ridges. We next investigated the effect of \( h \) on deterministic migration at \( \gamma_{av} = 1.2 \times 10^{4} \text{ s}^{-1} \) on average, motivated by a recent work\(^{24} \) showing that the channel aspect ratio can affect WBC margination. As \( h \) decreases from 45 to 15 µm, there is a steady increase in the peak distribution in outlet 1 up to 90.4 ± 2.4% (Figure 2f). However, even at \( h = 45 \mu m \), the spread of WBC distribution is narrow and 89.6 ± 2.3% of WBCs remain close to the left sidewall ranging from outlets 1 to 3, indicating that \( h \) is not a significant factor for deterministic migration.

### 2.3. WBC Separation from Whole Blood

Based on the optimized conditions obtained from the above parametric studies (\( q \geq 100 \mu L \text{ min}^{-1} \), Hct of whole blood and \( h = 23 \mu m \)), we demonstrated high-throughput separation of WBCs from whole blood. To achieve high enrichment of WBCs, we integrated four DSA microchannels in a multistage device, in which they were serially connected to each other with bifurcating regions (Figure 3a). Each DSA microchannel generated a focused WBC stream on
the sidewall through deterministic migration (Figure 3b). At each bifurcation, a WBC-free, RBC stream was passed to the RBC reservoir, while the focused WBC stream was passed through another DSA microchannel, finally creating an enriched WBC population in the WBC reservoir. The multiple separation stages have a beneficial effect on WBC separation. As shown in Figure 2d, WBCs form a more concentrated stream at a low $q$ of 10 µL min$^{-1}$. The discharge of a RBC stream at each bifurcation lowers $q$ in the subsequent separation stage, thereby creating a more concentrated WBC stream. The separation process produced a sorted WBC population in the WBC reservoir, enriching WBCs up to ~50-fold with 80% WBC recovery on average at $q = 150$ µL min$^{-1}$ (Figure 3c,d). The throughput capability is significantly higher than other microfluidic approaches as summarized in Table S1 in the Supporting Information: ~500 times that of a margination-based separator,[15] ~18 times that of a DLD separator,[13] ~15 times that of an inertial microfluidic separator,[11] and ~9 times that of a cross-flow microfiltration.[8] At this condition, the input ratio of WBCs to RBCs (1.7:1000) was significantly improved up to a ratio of 110:1000 in the WBC reservoir. Across the flow rates, we observed the dependence of WBC enrichment on $q$ (Figure 3c), which is likely due to changes in flow patterns with the elastic deformation of the PDMS device under high pressure (3.7 × 10$^5$ Pa)[28] and increased inertial effects[11,20,29] at a high $q$ of 400 µL min$^{-1}$ that corresponds to a Reynolds number ($Re = \rho UL/\mu$) of 5.7, where $\rho$ is the density of the fluid, $\mu$ is its viscosity, $U$ is its average velocity, and $L$ is a characteristic dimension of a channel cross-section. As determined by flow cytometry after staining with a vital dye, the viability of sorted WBCs reached up to 99.8 ± 0.2% ($n = 6$), indicating that the separation process is gentle and noninvasive enough for downstream analysis of WBCs. For the subsequent experiments, we determined an optimal flow-rate condition of 150 µL min$^{-1}$ for the multistage device.

### 2.4. Preserved Lymphocyte Composition in the Separated WBCs

Next, we examined whether the separation process changes the cellular composition of the WBC population. We compared the microfluidic method with conventional separation techniques such as the Ficoll density gradient method[30,31] and the RBC lysis method.[32] The Ficoll density gradient method is the most common way to separate lymphocytes and monocytes from whole blood,[30] and the RBC lysis method is used for brief isolation of WBCs from a small volume of whole blood.[32] After separation, we focused on lymphocytes, major cell types in the immune system, and analyzed the relative frequency of lymphocyte subpopulations such as CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$ T cells, CD3$^+$CD56$^+$ NK cells, and CD3$^+$CD19$^+$ B cells. Total lymphocytes were determined by forward scatter and side scatter in flow cytometry analysis after dead cell exclusion and pan-leukocyte marker identification, CD45-positive gating. The subpopulations of lymphocytes were then defined by the expression of CD3, CD4, CD8, CD56, and CD19 (Figure S7, Supporting Information). The percentages of the lymphocyte subpopulations in the WBCs separated using the microfluidic method were comparable to those in the WBCs separated using the Ficoll density gradient or RBC lysis methods (Figure 3e).

### 2.5. Functional Analysis of the Separated WBCs

We also examined the function of the separated WBCs. In particular, we analyzed cytokine release from CD4$^+$ and CD8$^+$ T cells, which are helper cells and cytotoxic cells in immune responses, respectively. First, the separated WBCs were stimulated with pan-T cell stimulants, anti-CD3 and anti-CD28 antibodies, and the production of IFN-γ, a representative antiviral cytokine, was assessed by intracellular cytokine staining and flow cytometry analysis (Figure S8, Supporting Information). The relative frequency of IFN-γ$^+$ cells among CD4$^+$ and CD8$^+$ T cells in the WBCs separated using the microfluidic method significantly correlated with those in the WBCs separated using the Ficoll density gradient method (Figure 4a, left). However, the actual percentage of IFN-γ$^+$ cells among CD4$^+$ and CD8$^+$ T cells was significantly higher in the WBCs separated using the microfluidic method than in the WBCs separated using the Ficoll density gradient or RBC lysis method (Figure 4a, right). These data suggest that the microfluidic method does not impair the function of T cells whereas the other methods might.

Next, we examined the function of T cells specific to a viral antigen. In particular, we investigated T cells specific to cytomegalovirus (CMV). CMV exists in a latent status in healthy persons, however, it reactivates in immune-deficient and organ transplanted patients, triggering organ damage.[33–36] Therefore, it is of clinical interest to measure the cytokine-producing capacity of CMV-specific T cells.[37–39] We stimulated the separated WBCs with CMV pp65 overlapping peptide pool and analyzed IFN-γ release. The relative frequency of IFN-γ$^+$ cells among CD4$^+$ or CD8$^+$ T cells in the WBCs separated using the microfluidic method significantly correlated with that in the WBCs separated using the Ficoll density gradient method (Figure 4b, left). Moreover, the actual percentage of IFN-γ$^+$ cells among CD4$^+$ or CD8$^+$ T cells was comparable among all three methods (Figure 4b, right), indicating that the microfluidic method can be used for the evaluation of virus-specific T cell functions in clinical settings.

### 3. Discussion

This work is, to our knowledge, the first demonstration of the migration process of WBCs in microchannels patterned with slant ridges. Deterministic migration is a migration phenomenon of WBCs directed by ridge patterns. The ridge patterns on the channel top generate transverse flows composed mainly of two oppositely flowing currents. Along the ridge patterns, the flow direction is parallel to the patterns, while under the patterns, the flow direction is reversed (Figure 1d,e). WBCs and 10-µm particles are assumed to be
Figure 4. Functional analysis of separated WBCs. a,b) Separated WBCs were stimulated with pan-T cell stimulants, anti-CD3 and anti-CD28 antibodies in panel (a), or CMV pp65 overlapping peptide pool in panel (b) and the IFN-γ release of CD4+ or CD8+ T cells was assessed by intracellular cytokine staining. The relative frequency of IFN-γ+ cells among CD4+ or CD8+ T cells in the WBCs separated using the microfluidic method and the Ficoll density gradient method was analyzed with Pearson’s correlation analysis (n = 11 of independent healthy donors) (the left panel of each figure). The comparisons of the relative frequency of IFN-γ+ cells among CD4+ or CD8+ T cells in separated WBCs using three different separation methods were analyzed with repeated-measures ANOVA and Tukey’s post hoc test (n = 11 of independent healthy donors) (the right panel of each figure).
located under the ridge patterns as a result of blood cell interactions so that their migration position can be determined by the direction of transverse flows under the ridge patterns. Since the transverse flows under the ridge patterns are toward the left sidewall for the CSA and DSA ridges and the channel center for the inverted v-shaped ridges, the migration position can be the same as the flow direction: the left sidewall for the CSA and DSA ridges (Figure 2a) and the channel center for the inverted v-shaped ridges (Figure S2, Supporting Information). We demonstrated the deterministic nature of the migration phenomenon by systematically investigating the parametric effects on the migration performance in terms of flow rate, hematocrit, cell deformability, and channel geometry. In comparing deterministic migration to conventional margination processes,[24–27] there are both similarities and differences between them. The fundamental similarity is that they both are mediated by RBCs and their rheological properties, while the major difference is that the deterministic migration process is much less sensitive to changes in Hct, flow rate and channel aspect ratio than WBC margination processes. The difference is likely due to the reliance of cell migration on the transverse flows and blood cell interactions for deterministic migration and WBC margination processes, respectively. In WBC margination processes, the migration of WBCs across microchannels is believed to be a consequence of hydrodynamic interactions between blood cells which can be significantly affected by little changes in Hct and channel geometry.[24] In contrast, in deterministic migration, the migration of WBCs itself is driven mainly by the transverse flows generated by the ridge patterns so that the cells can reach their migration destination even at high flow rates. The presence of RBCs in the ridges would selectively prevent the entrance of WBCs into the ridges even in microchannels with height on the order of five times the cell diameter, thereby keeping the cells in the migration position. Although these results require further investigation to unveil the exact mechanism underlying deterministic migration, the main focus here is to introduce a novel migration process and to apply it to high-throughput separation.

The deterministic migration process provides a new separation platform with several advantages over other separation methods. The resulting device used here proved effective not only in rapidly enriching WBCs but also in preserving their functionality for downstream functional assays of WBCs. Using the multistage device, sorting 1 mL of whole blood requires less than 7 min, considerably less time compared with other state of the art microfluidic separators (processing up to ~16.7 µL of whole blood per min)[8,11,13,15] and even with conventional benchtop methods that require a processing time of 90 min for density-gradient centrifugation. Future parallelization is also straightforward and the separation throughput can thus be scaled up for large-scale blood processing. The proposed device exhibits moderate enrichment and recovery performance as compared with existing separators (Table S1, Supporting Information). The moderate recovery is likely due to the cumulative effect of WBC loss at each bifurcation in the separation device. Further improvement in enrichment and recovery performance needs to be addressed to expand the applicability of the separation platform. The separation process is gentle and noninvasive, respecting cell functions and integrity and making it suitable for in vitro analysis of immune responses where preservation of immune cell phenotypes is important. In addition to the gentle separation, the operational simplicity of the separation platform enables the prospect of its widespread use in clinical applications that require WBC separation. As shown in Figure S9 in the Supporting Information, plugging a tubing connected to a blood-filled syringe into the device inlet and turning on a syringe pump are all the operations required for WBC separation. These steps are easy to learn and can be performed quickly even by nonexperts that lack experience in microfluidics.

In the past, information from WBCs was used in a limited fashion for disease diagnosis. Indeed, the counts for total WBCs and for each subpopulation were easily measured and primarily used in clinics. Recently, however, functional information of WBCs has been used for diagnosis in clinics. In particular, functional assays of pathogen-specific T cells have been used for the diagnosis of diseases and the evaluation of immune status. For example, IFN-γ release from mycobacterial antigens-specific T cells is used for the diagnosis of active and latent tuberculosis.[2,3] This method has become popular because it can distinguish tuberculosis infection from BCG vaccination. In addition, the measurement of CMV-specific T cell function is becoming important in the management of immune-deficient and organ transplanted patients because it can assess the risk of CMV reactivation and CMV disease in these patients.[40,41] However, the expansion of T cell functional assays is hampered by the fact that the separation of WBCs from peripheral blood requires a time-consuming, inconvenient process. The clinical application of pathogen-specific T cell function assays will be expanded if there is a rapid and simple method for WBC separation. Therefore, we have focused on T cell function in the current study. Our microfluidic platform provides several advantages over conventional separation approaches, including high-throughput, simple, and biocompatible separation in a portable and low-cost device. Such features, combined with the clinical potential for the functional assay of immune cells, can further extend the clinical application of the separation method presented here.

4. Conclusion
The deterministic migration principle presents an effective means not only for rapidly enriching WBCs from whole blood but also for preserving their integrity and immunological function, thus enabling reliable and accurate analysis of peripheral WBC functions and phenotypes to further obtain useful clinical information for cell-mediated immune functions. The operational simplicity of the platform also allows nonexpert users to easily learn and perform the necessary separation procedure, simply plugging a tubing connected to a blood-filled syringe into the device inlet. We believe that these new capabilities would facilitate the widespread use of the platform in clinical applications that require WBC separation.
5. Experimental Section

**Microfluidic Device Fabrication and Design:** Master molds for the microfluidic devices were fabricated in SU-8 photoresist (Microchem, USA) via two-step photolithography techniques. The first layer of photoresist was patterned to generate base channel structures using photolithography processes including spin-coating of photoresist, UV exposure through a photomask (Microtech, Korea), development to remove the unexposed areas of photoresist, and baking to solidify the resulting photoresist structures. In the second step, the same processes were repeated to generate ridge patterns on the channel structures. The dimensional details of the ridge patterns are shown in Figure S3 in the Supporting Information. The SU-8 molds were then silanized with trichloro(1H,1H,2H,2H-per-fluoroctyl)silane (Sigma-Aldrich, USA) in a vacuum chamber overnight. Microfluidic devices were made by degassing and pouring PDMS (Dow Coming, USA) onto the SU-8 molds. PDMS cured for 1 h at 75 °C was cut into individual devices and punched to form inlet and outlet holes for fluidic access. Then, each device was irreversibly bonded to a glass slide after oxygen plasma activation for 60 s.

To ensure the passage of blood cells through the microfluidic devices without clogging, $h$ was set to be larger than WBCs and ranged from 15 to 45 µm. $h$ was 21, 25, 26, or 26 µm for $h = 15$, $h = 23$, 31, or 45 µm, respectively. We set the channel length longer than 5.9 cm, the length at which we observed the complete deterministic migration of WBCs at $q = 100$ µL min$^{-1}$. Each outlet channel of the multistage device was designed to have multiple outlet sites (Figure 3a). An optimal outlet site was experimentally determined for each outlet channel to achieve high recovery and enrichment of WBCs. The first three outlet channels from the top were 200 µm wide $\times$ 23 µm high $\times$ 12.9 mm long; the next channel was 200 µm wide $\times$ 23 µm high $\times$ 4.3 mm long; the last outlet channel to the WBC reservoir was 100 µm wide $\times$ 23 µm high $\times$ 8 mm long.

**Microfluidic Setup and Analysis:** Microfluidic devices were vacuumed in a desiccator and filled with 1% bovine serum albumin (BSA) solution (Sigma-Aldrich) to prevent the generation of bubbles and to passivate PDMS surfaces from nonspecific adsorption, respectively. Blood and bead samples were drawn into 2.5 mL glass syringes (Hamilton, USA) or 5 mL plastic syringes (BD Biosciences, USA) and then injected into the devices via a connecting Tygon tubing (Cole Parmer, USA). A syringe pump (KD Scientific, USA) was used to produce a uniform flow rate over a range from 10 to 400 µL min$^{-1}$. To avoid the settling of blood cells or beads at low flow rates of $\sim$10 µL min$^{-1}$, a small magnet was preloaded into the syringes and periodically agitated using an external magnet during experiments. For bead experiments, fluorescent polystyrene beads of 2, 6, and 10 µm in diameter (Invitrogen, USA, and Phosphex, USA) were spiked in whole blood or suspended in 0.1% BSA solution at a concentration over 10$^4$ beads mL$^{-1}$. Fluorescence imaging experiments were performed on an inverted fluorescence microscope (Nikon, Japan). Images were acquired with $\times$10 objective lens magnification using a CCD camera (Nikon) and taken with long exposure times (i.e., $\sim$100 to 400 ms) to record multiple streaklines at a time and to describe the overall spatial distribution of fluorescently labeled WBCs or fluorescent beads in microfluidic flows. For quantitative analysis of the spatial distributions, WBCs or beads were collected at different outlets in the single-stage device and manually counted using a hemocytometer (Invitrogen). The diameters of WBCs were measured using the image analysis software, ImageJ (National Institutes of Health, USA), on bright-field images of the cells acquired under the microscope and were 8.9 ± 0.9 µm ($n = 142$). For fluorescence imaging of WBC trajectories in microfluidic flows, whole blood cells were stained with a cell permeable, nucleic-acid specific fluorescent dye (SYTO-13; Thermofisher Scientific, USA) at room temperature for over 10 min. For blood cell fixation, 15 µL of 50% glutaraldehyde (Sigma-Aldrich) stock was added to 3 mL of whole blood and then incubated with periodic agitation at room temperature. The fixative causes covalent cross-links between cellular molecules, thereby preserving the structure of cells with minimum alteration in morphology and size.[23]

**Collection of Whole Blood and Separation of WBCs:** Peripheral venous blood was obtained from healthy volunteers in ethylenediaminetetraacetic acid (EDTA) tubes and WBCs were isolated by density gradient centrifugation using Ficoll-based, lymphocyte separation medium (Corning, USA), RBC lysis using RBC lysis buffer (Biolegend, USA) or the microfluidic device. The present study was approved by the Institutional Review Board at Korea Advanced Institute of Science and Technology, and all subjects provided informed consent.

**Analysis of Lymphocyte Composition in the Separated WBCs:** Sorted WBCs were stained with the vital dye, Live/Dead Fixable Red Stain dye (Life Technologies, USA) and fluorochrome-conjugated monoclonal antibodies for cell surface proteins. The antibodies used are as follows: anti-CD3-V500 (UCHT1, BD Bioscience), anti-CD4-PerCP-Cy5.5 (SK3, eBioscience, USA), anti-CD8-APC-H7 (SK1, BD Biosciences), anti-CD14-PE-Cy7 (M5E2, BD Bioscience), anti-CD19-V450 (HIB19, BD Bioscience), anti-CD56-BV786 (NCAM16.2, BD Biosciences), anti-CD45-Alexa Fluor700 (HI30, BD Bioscience), anti-CF4a-FITC (HP8, eBioscience), anti-CD235a-APC (HIR2, eBioscience), and anti-CD66b-PE (G10F5, eBioscience). The stained cells were analyzed with an LSR II flow cytometer (BD Biosciences) and FlowJo software (FlowJo, USA).

**Intracellular Cytokine Staining:** Sorted WBCs were stimulated with anti-CD3 (1 µg mL$^{-1}$; BD Bioscience) and anti-CD28 (10 µg mL$^{-1}$; BD Bioscience) or a mix of CMV pp65 overlapping peptides (Peptidevant CMV pp65; Miltenyi Biotech, USA) for six hours. Brefeldin A and monensin were added one hour after stimulation. Then, the cells were stained with the vital dye and fluorochrome-conjugated monoclonal antibodies for cell surface proteins. The stained cells were fixed and permeabilized using BD Cytofix/Cytoperm buffer, (BD Bioscience) and stained for IFN-γ. The antibodies used are as follows: anti-CD3-V500, anti-CD8-APC-H7, anti-CD4-APC-V500 (RPA-T4, BD Biosciences), and anti-IFN-γ-APC (B27, BD Biosciences). The stained cells were analyzed with an LSR II flow cytometer and FlowJo software. The percentage of IFN-γ$^+$ cells in the stimulated tubes was subtracted by the percentage in the unstimulated control tubes.

**Computational Fluid Dynamic Simulation:** To simulate microfluidic flows in the microfluidic devices, 3D finite element models were created over the same dimensions as the devices and were solved using a finite-element method with Comsol Multiphysics (Comsol, USA). Due to high wall shear-rate conditions over 1000 s$^{-1}$, blood was assumed to be an incompressible and Newtonian fluid, having a constant viscosity of 3 cP.[42] No-slip boundary conditions were applied at the channel walls except at the inlet.
and outlet, and the pressure at the outlet was fixed at atmospheric pressure. In the finite element models, the Navier–Stokes equation was solved by assigning a normal velocity condition at the inlet (Figure S10, Supporting Information). The average wall shear rates ($\dot{\gamma}_w$) for the devices were calculated by exporting and averaging the values of the wall shear rate along the perimeter of the selected channel cross-section.

Statistical Analysis: All error bars represent the standard deviation. Associations between two parameters were tested by Pearson’s correlation. Pairwise comparisons of multiple groups were assessed by repeated-measured ANOVA with Tukey’s post hoc test. Two-sided $P$-values were determined in all analyses, and a $P$-value less than 0.05 was considered significant. Statistical analysis was performed using the statistics software SPSS version 20.0 (SPSS, USA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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