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PAPER

Monitoring the status of T-cell activation in a microfluidic system

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Leukocyte adhesion to the endothelium through surface molecules such as E-selectin and intercellular adhesion molecule-1 (ICAM-1) is a critical cellular event reflecting the physiological status of both cell types. Here we present a microfluidic system that can not only easily monitor the interaction between leukocytes and endothelial cells under physiological conditions, but also screen drug candidates for potential modulation of this interaction. Shear stress, which is an important factor for the binding of activated T cells to tumor necrosis factor-alpha (TNF-α)-treated human umbilical vein endothelial cells (HUVECs), was easily controlled by adjusting the flow rate in the microfluidic system. Whole blood of patients with systemic lupus erythematosus (SLE) who have auto-reactive T cells were infused into the activated HUVECs which subsequently showed a higher level of binding compared to a control blood sample from a person without SLE. When these autoreactive T cells were treated with immunosuppressors tacrolimus and cyclosporin A, the binding of the T cells to HUVECs was dramatically decreased. Therefore, this microfluidic system is capable of differentiating the physiological status of T cells or endothelial cells representing different disease conditions, as well as being useful for the identification of novel reagents that modulate the functions of leukocytes or endothelial cells.

Introduction

The interaction of leukocytes with the endothelium by means of cell adhesion molecules (CAMs) is important to maintain the function and migration of leukocytes in the immune system.[1,2] Induced expression and conformational changes of CAMs are significantly modified in several disease conditions such as inflammation and cancer metastasis.[3,4] Moreover, leukocyte adhesion plays a significant role in autoimmune disease and graft rejection when leukocytes attack their hosts or the transplanted tissues. Thus, the monitoring of leukocyte binding to human umbilical vein endothelial cells (HUVECs) has been widely studied with the aim of finding a treatment for autoimmune diseases.[9,10]

Immunomodulators such as cyclosporin A (CsA), rapamycin, and tacrolimus (FK 506) that inhibit T cell activation and proliferation and lead to both down-regulation of inducible CAM expression and blockage of leukocyte–endothelial interactions have been used in a clinical setting to markedly regulate the progression of autoimmune responses.[5–8] Monitoring the qualitative and quantitative aspects of leukocyte binding to endothelial cells is a critical prerequisite step for the development of a novel immunomodulator.

One of the most commonly used adhesion assays involves counting the number of bound T cells on the endothelial cell monolayer in a static state.[2,11–13] T cell binding, however, depends on both the delivery of cells to the endothelial cell surface as well as on intrinsic cell–surface interactions.[14] Moreover, rolling of leukocytes along the vessel wall is an essential prerequisite for arresting them on the lumenal surface of the endothelium and for their subsequent transmigration into tissues.[15] Thus, traditional static adhesion assays are not suitable to measure real leukocyte migration because it cannot mimic physiological adhesion conditions in which the fluid shear stress of blood flow provides conditions for cell rolling and mechanically influences the process of leukocyte activation.[16,17]

To overcome these problems and quantify the biological adhesion of T cells, a parallel plate flow chamber (PPFC), which has defined flow profiles that simulate the features of hydrodynamic forces found in the vasculature, was introduced.[14,18–21] Although a PPFC provides a controlled environment for shear stress, it is still hard to monitor T cell binding in the microsystem because of the large cross-sectional area of the PPFC which requires large volumes of leukocytes and reagents to maintain a physiological level of shear stress. The vascular mimetic microfluidic chamber (VMMC) was previously introduced to optimize leukocyte recruitment and greatly reduces sample size and inlet volume while maintaining compatibility with live cell substrates and high throughput imaging of leukocyte interactions.[16] The VMMC, however, has not been used to study induction of the expression of different CAMs in endothelial cells within the independent flow chamber because studies have focused on the effect of shear stress.

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In this study, we designed a simple microfluidic system allowing induction of expression of different CAMs in each independent chamber to result in multiple binding interactions between HUVECs and T cells. This system required the independent immobilization and stimulation of HUVECs to mimic a variety of inflammatory states in each microfluidic channel with inlets and outlets for fluid infusion and exit. The expression of different CAMs was induced by controlling the stimulating time of HUVECs with tumor necrosis factor-alpha (TNF-α) within each channel. The purpose of this study was to develop this microfluidic system to monitor primary leukocyte binding to endothelial cells under physiological conditions, thus creating a powerful screening system for immunomodulators that control T cell binding.

Materials and methods

Fabrication of a microfluidic system

The microfluidic system was fabricated with PDMS (Sylgard, Dow Corning, Midland, USA) using soft lithography.[22] An SU-8 2050 negative photoresist (MicroChem Corp., Newton, MA, USA) was spin-coated on a silicon (Si) wafer at 1000 rpm for 30 seconds to obtain a 165 μm channel height. The wafer was then pre-baked. The transparent mask was generated by a 20,000 dpi high-resolution printer (Han and All Tech., Ansan, South Korea). This mask was used to create patterns on the SU-8 under 365 nm UV light. After post-baking and developing, a mixture of PDMS pre-polymer and curing reagent (10 : 1 ratio) was poured onto the wafer. A degassing process was used to remove air bubbles, followed by curing the PDMS at 80 °C for 2 hours. PDMS channels were obtained from the master and inlets and outlets were punched with blunt-end needles. To complete this microfluidic system, the PDMS surface and a cover glass were placed in a plasma cleaner (Harrick Plasma, NY, USA) and oxidized at 18 W for one minute. After removal from the plasma cleaner, an irreversible bond was spontaneously formed between the two substrates. The fabricated microfluidic channels were 2 mm wide, 165 μm high and 2 cm long, and had inlets and outlets for fluid infusion and exit. The parallel multiple channel was fabricated on a chip to perform multiple bindings (Fig. 1).

The bottoms of the channels were double-coated with 0.1 mg ml^{-1} poly-L-lysine (Sigma, St Louis, MO, USA) and 50 μg ml^{-1} fibronectin (Sigma, St Louis, MO, USA) to form a matrix for cell attachment[23] (Fig. 1).

Endothelial and Jurkat T cell culture

Single-donor human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (BioWhittaker, Walkersville, MD, USA) and cultured in endothelial basal medium-2, supplemented with 2% fetal bovine serum (FBS), hydrocortisone, human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), ascorbic acid, human epidermal growth factor (hEGF), gentamicin–amphotericin, and heparin (Lonza, Walkersville, MD, USA). The HUVECs were detached from the culture flasks with a trypsin–versene mixture (Lonza, Walkersville, MD, USA), resuspended in Endothelial Growth Medium-2 (EGM-2) culture medium and perfused into the microfluidic device at a density of 1×10^6 cells per ml using a 1 ml disposable syringe. They were cultured overnight in a humidified incubator (37 °C and 5% CO_2). Because the expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) was maximal after 4 and 16 hours of stimulation with tumor necrosis factor-alpha[24] (TNF-α, Bio Vision, CA, USA), HUVECs were stimulated with 10 ng ml^{-1} of TNF-α in each channel to induce expression of E-selectin and ICAM-1, respectively. T cells with different cellular status could also be separately infused into each channel. Thus, this microfluidic system can be used to perform multiple binding experiments between HUVECs and T cells in a variety of inflammatory states. All HUVECs used in the experiments were from up to passage number ten. Jurkat T cells were cultured in RPMI 1640 (Lonza) containing 10% FBS, 1% L-glutamine, and 1% penicillin–streptomycin (Lonza). HUVECs and Jurkat T cells were grown in tissue culture-treated polystyrene T-75 flasks (NUNC, Roskilde, Denmark) in a humidified incubator at 37 °C.

Expression of cell adhesion molecules in the microfluidic system

The expression of CAMs such as E-selectin and ICAM-1 in the microfluidic device was confirmed by immunocytochemistry.[2,3] The HUVECs were perfused into the microfluidic device and cultured. Expression of E-selectin and ICAM-1 was induced with 10 ng ml^{-1} of TNF-α. TNF-α-treated HUVECs were washed with phosphate buffered saline (PBS, Gibco, NY, USA) and cells were fixed in 3.7% formaldehyde (Sigma) for 15 minutes at room temperature. To permeabilize the cells, 0.2% Triton X-100 (Fluka, Buchs, Switzerland) was used for 30 minutes on ice. After blocking HUVEC layers with 1% bovine serum albumin (BSA, Amresco, OH, USA), HUVECs were incubated for 1 hour at room temperature with monoclonal anti-E-selectin-fluorescein (Abcam, Cambridge, UK) or monoclonal anti-ICAM-1-fluorescein (R&D Systems, MN, USA) to detect expression of E-selectin or ICAM-1, respectively. After antibody incubation, cells were stained with 1 μg ml^{-1} 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) solution for 2 minutes at room temperature. The entire solution infusion process was performed at 3 dyn cm^{-2} using a syringe pump (New Era Pump System, Inc., NY, USA).

Optimization of the microfluidic system

We investigated the optimal shear stress for Jurkat T cell binding activity in the microfluidic system. First, HUVECs were seeded and cultured within the microfluidic device. TNF-α in complete medium was infused into the channels to induce the expression of cell adhesion molecules. After stimulation, activated and labeled Jurkat T cells were infused within the range of physiological shear stress, followed by prompt washing at 1 dyn cm^{-2} without incubation in the microfluidic system. The shear stress was calculated according to the equation $Q = (\tau_w w h^2)/6\eta$,[9] where Q is the flow rate, τ_w is the wall shear stress, w is the width of the channel, h is the height of the channel, and η is the viscosity of the buffers at 37 °C. Adhesion between Jurkat T cells and HUVECs was observed by confocal microscopy (LSM 510 Meta, Carl Zeiss, Inc., Oberkochen, Germany) using the fluorescence intensity of calcein-acetoxymethylester (calcein-AM) labeled Jurkat T cells. A HeNe2 (633 nm wavelength) laser and a Diode

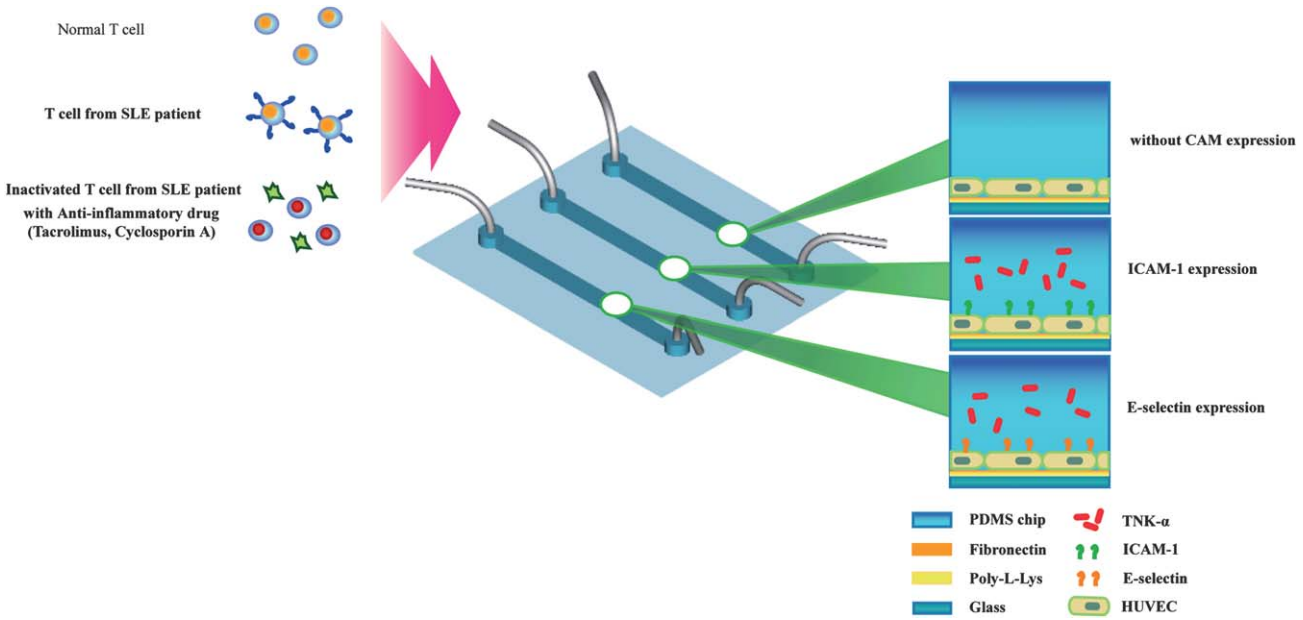

Fig. 1 Scheme of the microfluidic system. The device consists of multiple parallel channels on a chip. Each channel was coated with 0.1 mg ml^{-1} poly-L-lysine and 50 μg ml^{-1} fibronectin. HUVECs were loaded into the device to imitate different inflammatory vascular conditions and stimulated with TNF-α for 4 and 16 hours to induce surface molecule expression. Whole blood, which contains T cells, was infused into the chambers with activated HUVECs with or without drug treatment. T cell binding was observed under fluorescence microscopy after incubation with FITC-conjugated CD3 antibodies to identify primary T cells.

laser (405 nm wavelength) were used for excitation of calcein-AM fluorescence.

The viability of HUVECs in the microfluidic system is also important for the detection of T cell binding because only active and viable HUVECs can bind to T cells in the microfluidic channel. Therefore, HUVEC viability was assessed by their binding activity to Jurkat T cells. HUVECs were infused (3 dyn cm^{-2}), stimulated, and cultured within the microfluidic channels for 12, 24, 36, 48, 60, and 72 hours without changing the medium. After HUVEC cultivation, binding of activated and labeled T cells was observed by confocal microscopy.

Jurkat T cell and primary T cell adhesion

Jurkat T cells (2×10^6 cells) were activated with 10 ng ml^{-1} phorbol myristate acetate (PMA, Sigma) and 1 μM ionomycin (Sigma) at 37 °C for 1 hour in complete media. The activation of Jurkat T cells was suppressed by incubating 2×10^6 cells with 10 nM of the clinically used immunosuppressor, tacrolimus (Sigma), or 1 μg ml^{-1} of cyclosporin A (Sigma) at 37 °C for 1 hour in complete medium. All cells were labeled with 1 μM calcein-AM (Invitrogen, Eugene, OR, USA) at 37 °C for 15 minutes in complete medium. For human primary T cells, whole blood of either SLE patients or normal controls containing primary T cells was treated with 10 nM tacrolimus and 1 μg ml^{-1} cyclosporin A for 1 hour at 37 °C.

Cultured HUVEC monolayers within the microfluidic system were treated with 10 ng ml^{-1} TNF-α as described above. While HUVECs were being stimulated, the same number of activated or drug-treated T cells (2×10^5 cells per channel) and naive or drug-treated white blood cells (2×10^5 cells per channel) were collected and infused at 3 dyn cm^{-2}, followed by washing with PBS at 1 dyn cm^{-2} to remove unbound cells. Adhesion between Jurkat T cells and HUVECs was observed with confocal microscopy (LSM 510 Meta, Carl Zeiss, Inc., Oberkochen, Germany). For the detection of human primary T cells, all cells in each channel were fixed with 3.7% formaldehyde for 15 minutes at room temperature and incubated with FITC-conjugated CD3 T cell-specific antibody (Sigma) for 1 hour at room temperature. After washing with PBS at 1 dyn cm^{-2}, each channel was observed by confocal microscopy.

Results and discussion

Expression of cell adhesion molecules in a microfluidic system

First, the expression of CAMs such as E-selectin and ICAM-1 by HUVECs was confirmed by flow cytometry and immunocytochemistry in the static mode as studied previously (data not shown).[9,19,21] The expression of CAMs was also confirmed in the microfluidic channel by immunocytochemistry, and the upregulation of E-selectin and ICAM-1 was observed in the TNF-α-stimulated channels at 4 and 16 hours by FITC-conjugated antibody (Fig. 2b and c). There was no difference in expression of CAMs between the static state and microfluidic channels (data not shown). This observation implies that the newly designed microfluidic system can be used to culture and stimulate HUVECs, and to monitor T cell binding and adhesion to HUVECs.

Optimization of the microfluidic system

Under physiological conditions, T cells flow over the HUVECs,[25] thus inducing shear stress that provides the driving force for initiating cell rolling and mechanically influences T cell binding.

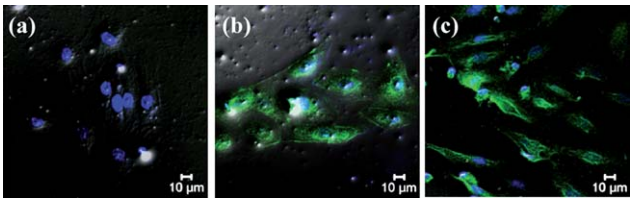

Fig. 2 The expression of cell adhesion molecules such as E-selectin and ICAM-1 was confirmed by immunocytochemistry in the microfluidic device. HUVECs were stimulated with TNF-α for 4 (b) and 16 hours (c) to induce surface molecule expression, followed by FITC-conjugated antibody incubation (green). Control (a) represents HUVECs without TNF-α stimulation. All cells were stained with DAPI (blue) for identification in the field.

Thus, optimization of this shear stress in the microfluidic channel is a critical prerequisite for the detection of T cell binding activity. After stimulation, activated T cells were infused within the range of physiological shear stress (0.1–18 dyn cm^{-2}), followed by prompt washing at 1 dyn cm^{-2} without incubation in the microfluidic system. Jurkat T cells were labeled with 1 μM calcein-AM before perfusion. Maximal binding of Jurkat T cells to HUVECs was observed at an optimal shear stress level of 3 dyn cm^{-2} (Fig. 3). This finding indicated that shear stress was an important factor for T cell binding to endothelial cells. Shear stress depends on the flow rate, geometry of the microfluidic channel and buffer viscosity. Since the size of the microfluidic channel and the buffer did not vary in this experiment, flow rate was the only variable to control shear stress. An increase in the flow rate increases the shear stress. When the T cells were infused at 3 dyn cm^{-2}, their flow rate was 30 times faster compared to T cells infused at 0.1 dyn cm^{-2}. This means that T cells with a flow rate of 3 dyn cm^{-2} have a 30 times lower retention time within the microfluidic channel and a 30 times lower chance of binding if binding only depended on the retention time within the microfluidic channel. However, the T cell binding was 2.5 times higher at an infusion rate of 3 dyn cm^{-2} compared to binding at 0.1 dyn cm^{-2} (Fig. 3a). This observation clearly indicated that shear stress drives the traditional T cell binding method, and that physiological, static state conditions that do not include shear stress are not suitable for the detection of real events of T cell binding to HUVECs.

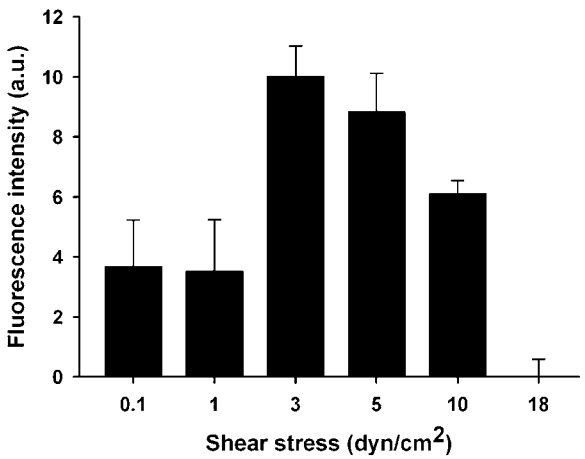

Fig. 3 Shear stress effect on T cell binding to ICAM-1-producing HUVECs. Activated Jurkat T cells were infused over a range of physiological shear stress levels (0.1–18 dyn cm^{-2}), followed by washing promptly without incubation in the microfluidic system. Jurkat T cells were labeled with 1 μM calcein-AM before perfusion.

The viability of HUVECs in the microfluidic system is also important for the detection of T cell binding because only active and viable HUVECs can bind to T cells in the microfluidic channel. This viability was tested by assessing the binding activity of HUVECs to T cells. Because the expression of ICAM-1 was maximal after 16 hours of stimulation, Jurkat T cell binding was expected to reach a maximum at this time. The viability of HUVECs, however, was designed to allow us to assay how long Jurkat T cells were active within the microfluidic channels, and thus HUVECs were infused (at 3 dyn cm^{-2}), stimulated, and cultured within the microfluidic channels for 12, 24, 36, 48, 60, and 72 hours without changing the medium. Because only active and viable HUVECs can undergo binding with T cells in the microfluidic channel, the viability of HUVECs were analyzed by T cell binding using activated and labeled T cells. HUVECs were active for T cell binding for up to 24 hours (data not shown). Thus, freshly activated HUVECs were used in subsequent binding experiments.

Modulation of Jurkat T cell binding in a microfluidic system

First, Jurkat T cell binding was evaluated using the microfluidic system. Because the expression of E-selectin and ICAM-1 was induced by TNF-α for 4 and 16 hours, respectively (Fig. 2), activated Jurkat T cells were infused onto the E-selectin- or ICAM-1-expressing HUVECs at 3 dyn cm^{-2} of shear stress, followed by prompt washing. Activated Jurkat T cells were labeled with 1 μM calcein-AM before perfusion. The binding of T cells to HUVECs was performed without any incubation in the microfluidic system. As expected, we did not observe any binding between activated T cells and unstimulated HUVECs (Fig. 4a) because CAMs which can capture the activated T cells were not expressed (data not shown). However, activated T cells bound to the E-selectin-expressing (Fig. 4b) and ICAM-1-expressing (Fig. 4c) HUVECs under the flow conditions (119.6 ± 8.4 cells per mm^2 and 126.8 ± 8.9 cells per mm^2, respectively).

In order to test that our microfluidic system was suitable for drug screening, we examined the binding affinity changes under conditions that included immunosuppressive agents (Fig. 5).

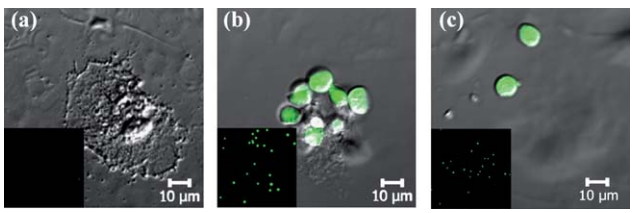

Fig. 4 Adhesion of activated Jurkat T cells to surface molecules expressed by HUVECs within the microfluidic device. T cells activated with 10 ng ml^{-1} of PMA and 1 μM of ionomycin were infused into resting (a), E-selectin expressing (b) and ICAM-1 expressing (c) HUVECs containing microfluidic channels at 3 dyn cm^{-2} of shear stress. Green fluorescent dots represent 1 μM calcein-AM labeled T cells.

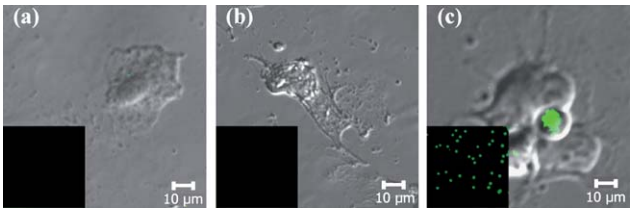

Fig. 5 Application of the microfluidic system to anti-inflammatory drug screening. Jurkat T cells treated with immunosuppressors, 10 nM of tacrolimus and 1 μg ml^{-1} of cyclosporin A, were infused into E-selectin (a) or ICAM-1 (b) induced microfluidic channels at 3 dyn cm^{-2} of shear stress. Activated Jurkat T cells (green) were perfused into the ICAM-1-containing channel as a control experiment (c) after 1 μM calcein-AM labeling.

While HUVECs were stimulated within the microfluidic channels, either activated or inhibited Jurkat T cells were perfused at 3 dyn cm^{-2} of shear stress into the device, which contained E-selectin- or ICAM-1-expressing HUVECs. Jurkat T cells were activated with PMA and ionomycin or inhibited with tacrolimus and cyclosporin A for 1 hour, labeled with 1 μM calcein-AM for 20 minutes, and then washed with PBS. Jurkat T cells that were treated with 10 nM tacrolimus and 1 μM cyclosporin A lost their binding affinity to both E-selectin- (Fig. 5a) and ICAM-1-expressing (Fig. 5b) HUVECs while activated T cells remained bound to the ICAM-1-expressing HUVECs (126.8 ± 8.9 cells per mm^2, Fig. 5c). These results suggested that our microfluidic system could determine the change in binding affinity of Jurkat T cells induced by an anti-inflammatory drug. The observed difference in binding to the T cells was caused by the immunosuppressors which inhibit T cell activation, but any drug candidate affecting T cell activity could be screened by this microfluidic system, which imitates the human circulatory system.

High content drug screening using human primary T cell adhesion

Human primary T cell binding was also evaluated using the microfluidic system (Fig. 6). Whole blood of patients with systemic lupus erythematosus (SLE) (n = 13) who have auto-reactive T cells[25,26] was used. The same number of white blood cells (2 × 10^5 cells) from each untreated sample was infused into the microfluidic system. To identify the T cells, immunocyto-chemistry was performed with FITC-conjugated CD3 T cell-specific antibody. No binding was observed between normal T cells and HUVECs expressing ICAM-1 (Fig. 6a); however, the highly active T cells of SLE patients showed significant binding to ICAM-1-expressing HUVECs (Fig. 6b). Because both normal primary T cells and highly active primary T cells were used, this binding difference clearly confirmed that the microfluidic system could differentiate T cell-binding activity representing different disease conditions. Furthermore, the detection of differences in T cell-binding activities suggested that this system could be applied to drug screening in cases where T cell-binding activity is modified. As shown in Fig. 6c, the binding of T cells from SLE patients in the microfluidic system was dramatically decreased after treatment with immunosuppressors tacrolimus and cyclosporin A.

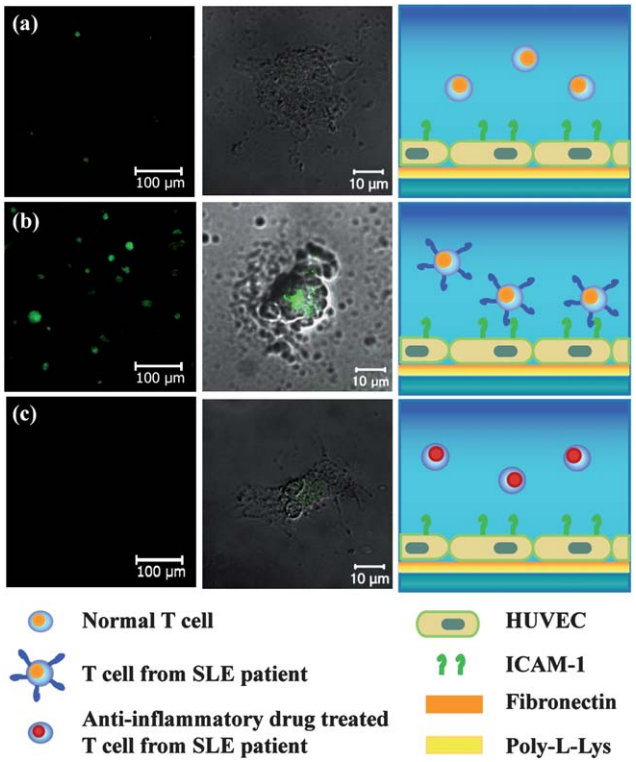

Fig. 6 Adhesion of human primary T cells to ICAM-1-expressing HUVECs within the microfluidic system. Whole blood from a normal person (a), SLE patients (b), and a SLE patient after anti-inflammatory drug treatment (c) was infused into ICAM-1-expressing HUVECs. For anti-inflammatory drug treatment, 10 nM tacrolimus and 1 μg ml^{-1} cyclosporin A were used. All cells were infused with 3 dyn cm^{-2} shear stress and all channels were incubated with FITC-conjugated CD3 antibodies to identify primary T cells.

Although the same number of white blood cells was infused into the microfluidic device for each experiment, the total number of T cells was not constant. The T cell and B cell profiles were analyzed by flow cytometry using CD3 and CD19 to determine the fraction of T and B cells in blood. Although flow cytometry showed that patient 1 had fewer T cells than patient 2 (2683 *vs.* 4110 cells), more binding was detected with blood from patient 1 (203 *vs.* 58 cells per mm^2, Fig. 7). The lack of binding observed for normal T cells (~17% of the total number of white

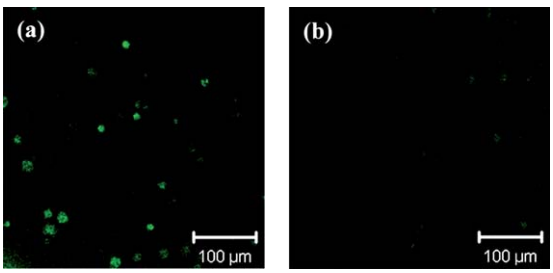

Fig. 7 Adhesion of patient T cells to E-selectin-expressing HUVECs. Patient 1 (a) has less T cells (2683 cells) compared to patient 2 (b) (4110 cells). T cell binding was performed with blood samples from patient 1 (a) and patient 2 (b) as described above using the microfluidic system.

blood cells) (Fig. 6a) also clearly indicated that the binding differences were caused by differences in T cell-binding activity, not by differences in T cell number. These results indicated that the binding activities of the T cells in this system depended on binding activity rather than cell number.

Conclusions

Our microfluidic system successfully differentiated the binding activities of Jurkat T cells and human primary T cells and monitored changes in the binding affinity of T cells from autoimmune disease patients after anti-inflammatory drug treatment. Furthermore, shear stress was controlled within physiological conditions, thus providing an environment for cell rolling and mechanical leukocyte activation. Therefore, this microfluidic system is a powerful screening tool for immunomodulators; it uses human primary cells,[27,28] and requires less time, cost and labor than conventional methods. *In vitro* and animal experiments are still important in drug discovery and development, but our microsystem offers many complementary advantages. Moreover, our microsystem, which imitates the human circulatory system, can be applied to study a variety of cell–cell interactions, such as those in cancer metastasis in which shear stress and E-selectin play important roles. In addition to the diseases investigated in this study, our microfluidic system can be applied to investigate any sort of disease modeling which is caused by abnormal cell–cell interactions.

Acknowledgements

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