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CONCISE COMMUNICATION

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Integrin α5 regulates motility of human monocyte-derived Langerhans cells during immune response

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Abstract

Langerhans cells (LCs) are mainly present in the epidermis and mucosa, and have important roles during skin infection. Migration of LCs to lymph nodes is essential for antigen presentation. However, due to the difficulties in isolating and culturing human LCs, it is not fully understood how LCs move and interact with the extracellular matrix (ECM) through their adhesion molecules such as integrin, during the immune responses. In this study, we aimed to investigate LC motility, cell shape and the role of integrin under inflammatory conditions using monocyte-derived Langerhans cells (moLCs) as a model. As a result, lipopolysaccharide (LPS) stimulation increased adhesion on fibronectin coated substrate and integrin α5 expression in moLCs. Time-lapse imaging of moLCs revealed that stimulation with LPS elongated cell shape, whilst decreasing their motility. Additionally, this decrease in motility was not observed when pre-treated with a neutralising antibody targeting integrin α5. Together, our data suggested that activation of LCs decreases their motility by promoting integrin α5 expression to enhance their affinity to the fibronectin, which may contribute to their migration during inflammation.

KEYWORDS
cell adhesion, cell motility, inflammation, integrins, Langerhans cells, live cell imaging

Kaori Saito (Otsuka) and Fumitaka Fujita contributed equally to this work.

1 | BACKGROUND

Langerhans cells (LCs) are tissue-resident antigen-presenting cells (APCs), which are mainly present in the skin epidermis and mucosa. When infections occur, the local LCs take up and process microbial antigens to become fully functional APCs, migrate through the basement membrane and dermis and finally move towards lymph nodes.[1] Motility plays an important role in LCs to achieve immune functions and maintain skin homeostasis.[2,3]

Cell adhesion is reported to be closely related to cell motility in several types of cells such as cancer cells,[4] smooth muscle cells[5] and leucocytes,[6] mediated by extracellular matrix (ECM). In skin, ECM components such as collagen, laminin and fibronectin form the interstitial dermal matrix and the basement membrane, and contribute to the maturation and differentiation of APCs, such as LCs[7] and dendritic cells (DCs).[8] ECM receptor integrins, are considered to play important roles in LC-ECM interaction and LC migration.[9–11] Price et al. reported that integrin α6 was involved in migration of skin APCs in mice.[9] In another study, up-regulation of integrin α4 was observed in mice LCs after hapten application,[10] but its contribution to LC migration and the precise mechanism remains unclear.

2 | QUESTIONS ADDRESSED

Isolating and culturing primary human LCs remains a challenging task, limiting our understanding of the intricate cellular dynamics during immune responses. In this study, we aimed to uncover the LC movements on fibronectin, one of the ECM proteins, under lipopolysaccharide (LPS) stimulation by investigating LC motility, cell shape and the role of integrin under inflammatory conditions using human monocyte-derived Langerhans cells (moLCs) as a model.

3 | EXPERIMENTAL DESIGN

3.1 | Differentiation of moLCs

Experiments using human peripheral blood mononuclear cells (PBMCs) were approved by the ethics committee of Osaka University (approval number 2019-1). Human whole peripheral blood was purchased from the Japanese Red Cross Society in accordance with the *Guidelines on the Use of Donated Blood in R&D, etc*. PBMCs were isolated[12] and moLCs were generated from human $CD14^+$ monocytes[13] as previously reported. $CD14^+$ monocytes were cultured for 7 days in RPMI 1640 medium (11875-093, Life Technologies) containing 1% penicillin, 1% streptomycin (161-23-181, FUJIFILM Wako Pure Chemical Industries), and 10% feral bovine serum (FBS; 172-012, Sigma-Aldrich), supplemented with 100 ng/mL granulocyte-macrophage colony-stimulating factor (100-08, Shenandoah Biotechnology), 20 ng/mL IL-4 (200-04, PeproTech) and 20 ng/mL transforming growth factor-β1 (PHG9211, Life Technologies). Fresh medium containing cytokines was added on Days 2 and 5. The non-adherent cells were defined as moLCs.

3.2 | Immunocytochemistry

Expression of the LC surface marker CD1a and CD207 was detected by immunocytochemistry. Day 0 monocytes and Day 7 moLCs were washed with PBS, fixed with 4% paraformaldehyde (PFA), blocked with 0.1% Triton X-100, 10% bovine serum albumin (BSA) in 0.1% Tween20 in PBS for 1 h at room temperature followed by incubation with primary antibody overnight at 4°C and secondary antibody for 2 h at room temperature. Primary and secondary antibodies used are shown in Table S1. Images were acquired using a confocal microscope (FV3000, Olympus).

3.3 | Morphological measurements

MoLCs were seeded in a six-well plate coated with fibronectin (F2006, Sigma-Aldrich) and treated with 50 ng/mL LPS for 6 h. Phase contrast images were acquired by a fluorescence microscope (BZ-X710, Keyence) and cell area and circularity were measured by ImageJ software.

3.4 | Cell adhesion assay

MoLCs were stained with Hoechst 33342 (H3570, Invitrogen) and incubated in fibronectin-coated 96-well plates for 1 h with LPS treatment, washed with PBS, then fixed with 4% PFA. Whole-well images were acquired by a fluorescence microscope (BZ-X710, Keyence) and analysed by ImageJ.

$$\text{Adhesion efficiency} = \frac{\text{adherent cell number}}{\text{seeded cell number}} \times 100\,\%$$

3.5 | Cell motility assay

The two-dimensional cell motility was assessed by in vitro time-lapse imaging. After overnight incubation on a fibronectin-coated μ-Slide 18 Well (81-816, ibidi GmbH) or a 96-well plate (353-072, Falcon), 20× bright field images of moLCs were acquired every minute for 6 h in the control medium, and another 6 h in 50 ng/mL medium containing LPS. Images were acquired using an Olympus FluoView FV3000 confocal microscope (Olympus) fitted with a temperature-controlled gas-supplied stage incubator (Tokai Hit), in which the conditions were set to 37°C, 5% CO_2. ImageJ was used to track the cells in the field.

3.6 | Flow cytometry

After washing and stimulation by 50 ng/mL LPS for 6 h, cells were suspended in PBS with 2%FBS. Nonspecific binding was blocked with Fc Blocker (422-302, BioLegend) for 10 min and incubated for 30 min with the following antibodies: CD1a-APC, HLA-DR-Texas Red, CCR7-APC-Cy7, CXCR4-Bv421, CD86-FITC, CD83-PE-Cy7, CD49d-PE-Cy7, CD49e-PE, CD49f-Bv421 together with the reagent 7-aminoactinomycin D (7-AAD) for identifying live cells. After labelling, the cells were fixed with 4% PFA, and protein expression was analysed using a BD FACSAria II (BD Biosciences). All data were analysed using BD FACSDiva and FlowJo software. 10000 cells were analysed for each condition. Five individual experiments were performed.

3.7 | Integrin α5 inhibition experiment

To confirm the effect of integrin α5, moLCs were incubated with 2 or 20 μg/mL monoclonal antibody against integrin α5 (clone 8F1, kindly provided by Prof. Kiyotoshi Sekiguchi) for 20 min. Cell nuclei were stained with Hoechst 33342. Fresh medium was exchanged with or without 50 ng/mL LPS. The adhesion and cell motility assay were performed as described above, except that in the cell motility assay, 10× images were acquired by Cell Voyager High-Content Screening System (CV8000, Yokogawa) and analysed by CellPathfinder software.

4 | RESULTS

4.1 | Effect of LPS stimulation on moLCs

To examine cell surface marker expression in moLCs, we first performed immunocytochemistry. We identified that moLCs express the LC surface markers CD207 and CD1a (Figure 1A, right), whilst expression of both was lower in Day 0 monocytes (Figure 1A, left). We also confirmed the expression of other surface markers that were reported to be upregulated in LCs during immune responses,[14–17] through flow cytometry analysis. Similar to LCs, moLCs also showed an increase in the expression of antigen-presenting markers (HLA-DR, CD86) and a slight increase in migration markers (CCR7, CXCR4) when stimulated by LPS (Figure S1).

Since adhesion to fibronectin was involved in murine epidermal LC migration irritated by ultraviolet-B,[11] we coated the culture plate with fibronectin. To investigate the effect of LPS on cell adhesion, the adherent cell number was calculated (Figure 1B). Statistical analysis revealed a significant increase in moLC adhesion efficiency using 50 ng/mL of LPS. Therefore, we decided to apply this concentration in all subsequent experiments. Cell viability analysis (WST-8 assay) indicated that LPS stimulation did not decrease cell viability up to 5000 ng/mL (Figure S2).

The morphology of moLCs dramatically changed from a round shape to an elongated shape when stimulated by LPS (Figure 1C), with a lower value of circularity (Figure 1D) and an expanded area (Figure 1E). It is worth mentioning that even if most of the cells changed their shape when stimulated by LPS, as the median value of circularity dropped from 0.73 to 0.39, there were still 18% of cells in the LPS (+) group whose circularity value was higher than 0.73. The cell area expanded from $622.6 \pm 9.1\,\mu m^2$ to $1107.1 \pm 5.6\,\mu m^2$.

Next, random cell motility assays were performed on a two-dimentional substrate. We observed less movements of moLCs with LPS stimulation (Videos S1 and S2, Figure 1F). Supporting this, the distance travelled by moLCs and their average speed was significantly decreased when stimulated by LPS ($675.5 \pm 48.8\,\mu m$, $0.034 \pm 0.002\,\mu m/s$ for LPS (−), $171.6 \pm 24.0\,\mu m$, $0.017 \pm 0.001\,\mu m/s$ for LPS (+), Figure 1G,H). A similar tendency was observed in transwell assay performed in the absence of chemokine, as the number of cells migrated out of the collagen I gel slightly decreased without the application of chemokine C-C motif ligand 19 (CCL19) (Figure S5E). Conversely, CCL19 dramatically increased the number of migrated cells for LPS (+) (Figure S5F). Together, these results suggested that LPS induced enhanced adhesion, elongated cell shape, expanded cell area, lowered random cell motility but increased migration towards chemokines in moLCs.

4.2 | The role of integrin α5 in regulating moLC motility during the immune response

Considering LPS stimulation also increased cell area and adhesion efficiency, we hypothesised that the decrease in random cell motility was due to increased binding between the fibronectin and its receptor(s), integrins. To investigate the receptor involved in this process, mRNA expression levels of integrins (α1, α2, α3, α4, α5, α6, α7, α8, α9, α10, α11) were assessed by quantitative real-time PCR. Amongst these integrins, integrin α5 had the highest expression in moLCs without stimulation in mRNA level (Table S3). We next confirmed the expression of integrin α4, α5, α6 in protein level by flow cytometry. The mean values of positive population for integrin α4, α5 and α6 were 1.53%, 51.56% and 21.54%, respectively. When stimulated by LPS, the mRNA expression of integrin α5 showed a significant 1.7-fold increase (Figure S3). This increase of expression was also confirmed in protein level by flow cytometry (Figure 2A–C). However, the protein expression of integrin α4 and α6 did not show significant difference after LPS stimulation (Figure S4). To further explore whether integrin α5 was involved in regulating the movement of moLCs during LPS stimulation, neutralizing antibody targeting integrin α5, which was shown to block the function of integrin α5,[18] was used in cell adhesion and motility assays. After pre-treatment with antibody, the cell adhesion efficiency significantly decreased (Figure 2D), suggesting the important role of integrin α5 in moLC adhesion. Furthermore, in 2D random cell migration assay, we found an increase of moLC motility in LPS-treated cells after treatment with anti-integrin α5 antibody (Figure 2E), whilst the migration in 3D

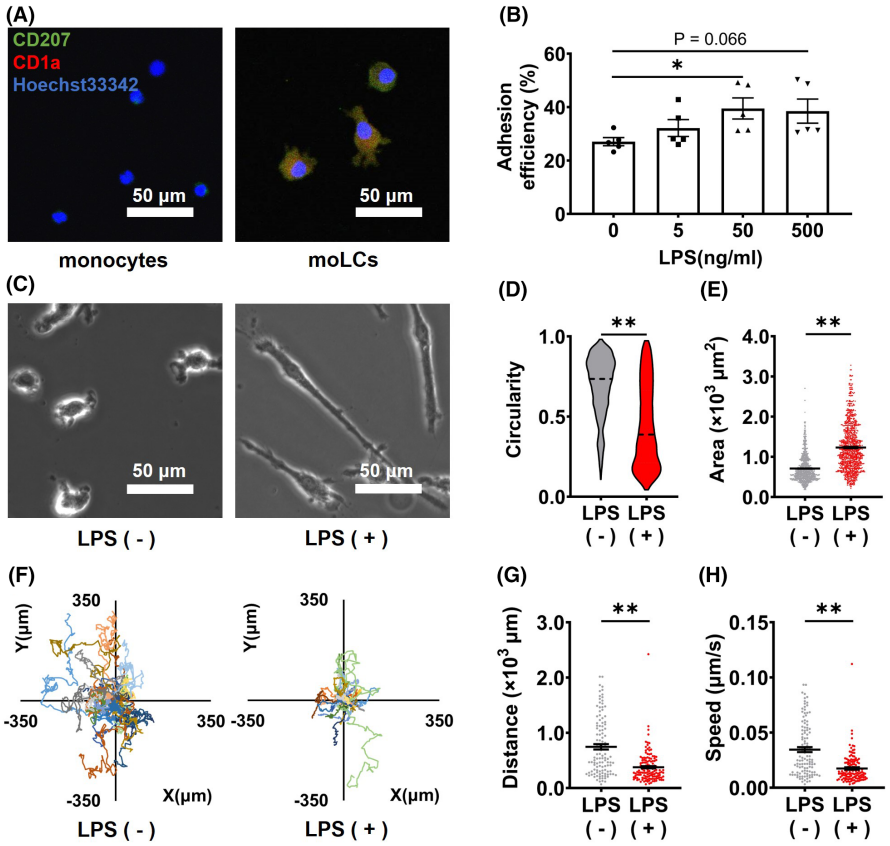

FIGURE 1 Effect of LPS stimulation on moLCs. (A) CD207 and CD1a expression on monocytes and moLCs were visualised by immunocytochemistry. (B) Adhesion efficiency of moLCs. MoLCs were incubated for 1 h on substrate coated with 0.75 μg/cm^3 fibronectin, stimulated by increasing concentrations of LPS. Horizontal lines indicate mean ± SEM, $^*p < 0.05$ (one-way ANOVA, Dunnett's multiple comparisons test), five experiments. (C–E) Effect of 50 ng/mL LPS on the morphology of moLCs. Images were obtained after 6 h stimulation (C). Circularity (D) and cell area (E) of moLCs were evaluated using ImageJ software, $n = 1144$ (395, 366, 383) for LPS (–), $n = 1080$ (374, 374, 332) for LPS (+). Dotted lines (D) indicate medium. Horizontal lines (E) indicate mean ± SEM, $^{**}p < 0.01$ (student *t*-test), three experiments. (F–H) Effect of 50 ng/mL LPS on cell motility for 6 h was evaluated by time-lapse imaging. (F) Representative trajectories of moLCs, $n = 47$ for LPS (–), $n = 51$ for LPS (+). Paths are arranged to show origins at $x = y = 0$. Each line indicates the trajectory of one cell. Accumulated distance (G) and average speed (H) of moLCs were evaluated using ImageJ software, $n = 114$ (47, 31, 36) for LPS (–), $n = 133$ (51, 36, 46) for LPS (+), from three experiments. Horizontal lines indicate mean ± SEM, $^{**}p < 0.01$ (Student's *t*-test).

collagen I gel induced by CCL19 was cancelled by anti-integrin α5 antibody (Figure S5F). These results suggest that integrin α5 is required for the regulation of random motility and chemotactic migration of moLCs when stimulated by LPS.

5 | DISCUSSION

LCs are frontline fighters of the skin immune system against external stimuli, whilst their movements during immune response are still unclear, especially in human beings. It is known that LCs migrate under inflammatory conditions.[19] Several researchers have shown increased displacement and movement of LCs, both in mouse skin[20–22] and human skin equivalents.[23] In our experiment, we focused on the random movements of human moLCs on a fibronectin-coated substrate. As a result, motility of moLCs decreased unexpectedly, accompanied with higher integrin α5 expression and enhanced cell adhesion when stimulated by LPS. The decrease in moLC motility under this observation condition may be due to the strong binding with fibronectin, whilst further research is to be carried out on how this LC–ECM interaction contribute to LC movement in human skin.

In addition, our data suggested the regulation of motility under LPS stimulation was integrin dependent. Leucocytes including DCs and LCs migrate in two ways, adhesion-dependent way or adhesion-independent way.[24] In adhesion-dependent migration where we focused, integrins contributed to the extravasation of leucocytes from blood vessels.[25] Similarly, integrins might regulate the translocation of parasitized DCs across brain endothelial monolayers.[26] In mouse skin, integrin α4 and α6 are possibly involved in LCs' migration in vivo,[9,10] although our result of human moLCs did not reveal the involvement of integrin α4 and α6 (Figure S4). This may be due to the difference in species and experimental methods. Recent study showed that Met signalling regulated LC migration crossing basement membrane, and integrin α5β1 was possibly involved.[27] These results implied the involvement of integrins in breaking through the barriers constructed by cells or the ECM during specific stages of

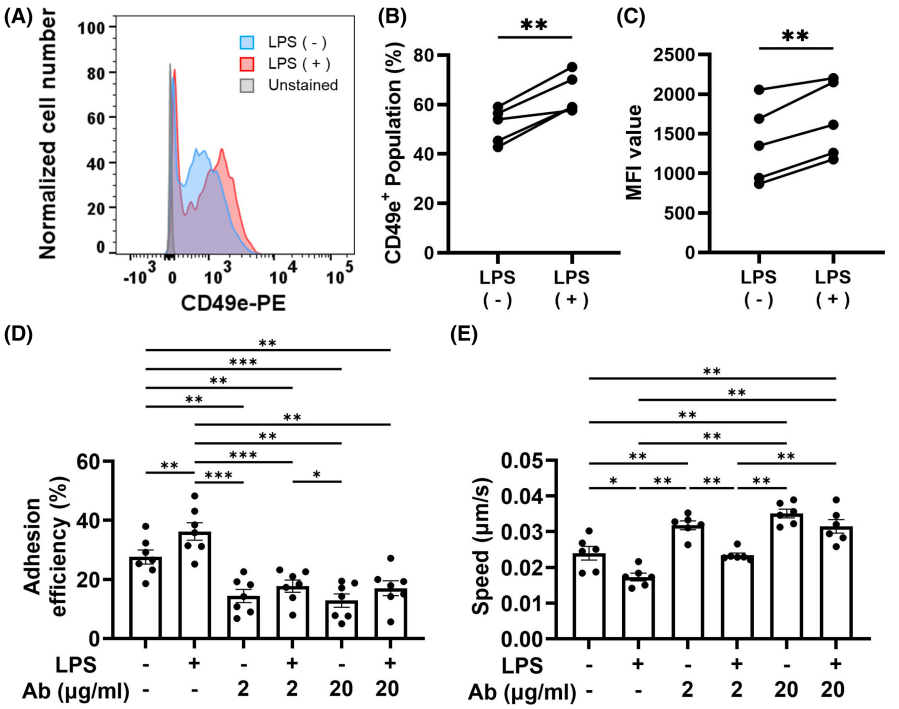

FIGURE 2 Regulation of integrin α5 in moLCs during LPS stimulation. (A–C) Expression of CD49e (integrin α5) in moLCs treated with 50 ng/mL LPS for 6 h. Representative normalized histograms (A), $CD49e^+$ population (B) and mean fluorescence intensity (MFI) (C) were analysed by flow cytometry. $^*p < 0.05$ (Student's *t*-test), five experiments. (D, E) Effect of anti-integrin α5 antibody (Ab) on adhesion efficiency (D) and average speed (E) of moLCs. MoLCs were pre-treated with 2 or 20 μg/mL of Ab for 20 min. Horizontal lines indicate mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (one-way ANOVA, Tukey's multiple comparisons test), seven and six experiments for (D) and (E), respectively.

infection. Taken together, when LCs are activated, increased expression of integrin α5 at the adhesive interface may facilitate crossing through the basement membrane and dermis in human skin.

Next, we confirmed the directional movements of LPS-activated moLCs in collagen I gel towards chemokine, CCL19, by live-imaging (Figure S5B,C), although it is unclear why moLCs move longer distance in the absence of CCL19 than in the presence of CCL19. We also performed transwell assay and observed an increase in their migration towards chemokine CCL19 after a 6-h LPS stimulation. Interestingly, the transwell assay suggested that integrin α5 was also involved in chemotactic migration of moLCs although the mechanism remains unclear. Further study should be carried out to investigate the role of integrin α5 on migration during specific phases of inflammation in vivo.

Additionally, ECM–integrin engagement was reported to disrupt the cadherin-mediated cell–cell adhesion.[28,29] This suggests that the enhanced binding between the ECM and LCs may result in lower attachment between LCs and surrounding keratinocytes, which contributes to LCs migrating out of the epidermis. Furthermore, in macrophages, fibronectin cooperated with TLR2/TLR4 receptor to promote innate immune responses,[30] and the binding of integrin and the ECM is possibly involved in the activation of MAPK signalling.[31] This mechanism may also exist in LCs, which may contribute to their functions in immune response, though further evidence is needed.

6 | CONCLUSIONS AND PERSPECTIVES

Collectively, our study suggested that integrin α5 regulates the motility of LPS-stimulated moLCs, providing valuable insights into the functional mechanisms of moLCs in immune response. This study may provide new possibilities for development of drugs for treatment of skin immune diseases.

AUTHOR CONTRIBUTIONS

F.F., K.O.S., M.M. and Z.G. conceived and designed the experiments. Z.G. performed the experiments and analysed the data. F.F., K.O.S. and Z.G. contributed to the writing and editing of the manuscript. M. Toriyama, H.K., M. Tominaga, K.J.I. and F.F. designed the experiments and discussed the results. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

M.M., K.O.S. and F.F. are employed by the Mandom Corporation. The other authors have no financial conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1:
Video S1:
Video S2:

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