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Original Paper

A profile of pro-inflammatory cytokine expression in human Delta-1-induced monocyte-derived Langerhans cell-like dendritic cells after stimulation with Toll-like receptor ligands

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Abstract

Monocyte-derived Langerhans cell-like dendritic cells (Mo-LCs) are involved in epidermal disorders such as psoriasis in murine models. However, the roles of Mo-LCs in the pathogenesis of psoriasis in humans remain unclear. Also, the contribution of notch ligand delta-like 1 (DLL-1), expressed on keratinocytes, to Mo-LC functions requires clarification. Here, we established a new method of stimulating Mo-LCs derived from $CD14^+$ monocytes with immobilized human DLL-1 to generate induced Mo-LCs (DI(+)Mo-LCs). The DI(+) Mo-LCs were compared to the dendritic cells derived from monocytes (Mo-DCs) cultured with interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and M1 macrophages (Mϕ) derived from monocytes cultured with GM-CSF. The DI(+)Mo-LCs were found to produce significant amounts of *IL15*, *IL23A*, and interferon-β (*IFNB1*) in response to the Toll-like receptor (TLR)3 agonist Polyinosinic-polycytidylic acid (Poly(I:C)) or TLR4 agonist lipopolysaccharide (LPS) despite their low expression of tumor necrosis factor (*TNF*). In conclusion, we have established a new method to generate DI(+)Mo-LCs. We have also discovered that DI(+)Mo-LCs have a unique capacity for producing *IL15* and *IL23A*, which are related to the pathogenesis of psoriasis. Our data contribute to a better understanding of the roles of Mo-LCs in epidermal defense and pathogenesis.

Key words: monocyte-derived LC-like dendritic cells, $CD14^+$ monocytes, immobilized DLL-1, IL-15, IL-23

Introduction

Langerhans cells (LCs) are the dendritic cells (DCs) of the epidermis; they originate from the stem cells that have moved to the epidermis during embryogenesis. Recently, monocyte-derived LC-like dendritic cells (Mo-LCs) appearing during inflammation have been reported[1]. The Mo-LCs are involved in the pathogenesis of psoriasis, a chronic inflammatory skin disease, in some murine models[2,3]. However, because the murine models cannot fully capture the complexity of human psoriasis lesions, the role of Mo-LCs in human psoriasis pathogenesis remains to be elucidated[4].

Although there are many methods of generating Mo-LCs[5,6], they produce Mo-LCs with different properties[7–9]. Thus, the optimal method of Mo-LC generation remains unidentified. Notch ligand delta-like 1 (DLL-1), a keratinocyte surface antigen, is expressed in the epidermis[10] and known to promote LC differentiation. Hoshino *et al*. established a method to generate Mo-LCs from peripheral blood monocytes via the initial stimulation with immobilized DLL-1, transforming growth factor-β1(TGF-β1), and granulocyte-macrophage colony-stimulating factor (GM-CSF)[11]. A model that conforms to the actual Mo-LC differentiation from inflammatory monocytes infiltrating into the epidermis is essential[3],

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and DLL-1-induced Mo-LCs (DI(+)Mo-LCs) suit the model because DLL-1 is expressed in the epidermis. Because DLL-1 affects the differentiation and function of some types of DCs[12, 13], it can also have an essential role in the functions specific to Mo-LCs. Also, DLL-1 is crucial for the monocyte differentiation into Mo-LCs. The blockade of notch signaling was found to prevent the generation of LCs[6, 11], and DLL-1 was found to inhibit the differentiation of monocytes to macrophages[12]. However, the specialized materials used for immobilizing DLL-1 are difficult to obtain; this may explain the small number of subsequent reports on DI(+)Mo-LCs. Thus, a different approach is required to facilitate further studies on DI(+) Mo-LCs. Here, we present a new method of generating Mo-LCs from monocytes by immobilizing DLL-1 using uncoated plates with hydrophobic surfaces that were usually used for suspension cell culture. This new method of DLL-1 immobilization was based on the method of attaching the first antibodies to uncoated plates in the general protocols of enzyme-linked immunosorbent assay (ELISA). The new method was successful in producing DI(+) Mo-LCs sufficient for conducting experiment.

Here, we have investigated the function of the DI (+)Mo-LCs and the relationship between DI(+) Mo-LCs and the pathogenesis of psoriasis. We found unique characteristics of the DI(+)Mo-LCs derived from $CD14^+$ peripheral blood monocytes in terms of cytokine expression following Toll-like receptor (TLR) stimulation, especially via TLR3 and TLR4. These results agreed with the reports that the LCs and conventional DCs responded differently to TLR3 and TLR4[7-9]. Furthermore, these results showed that the DI(+)Mo-LCs express interleukin-15 (*IL15*) and *IL23A* related to psoriasis pathogenesis upon TLR stimulation.

Materials and methods

Reagents

Recombinant human (rh)DLL1 (BioLegend, San Diego, CA), rhGM-CSF, rhTGF-β1, and rhIL-4 (PeproTech, Cranbury, NJ) were purchased. Polyinosinic-polycytidylic acid (Poly(I:C)), lipopolysaccharide (LPS) from *Escherichia coli* (L4516), and imiquimod (Sigma-Aldrich, St. Louis, MO) were procured.

Immobilizing DLL-1 and preparation of Mo-LCs, monocyte-derived DCs (Mo-DCs), and macrophages (Mϕs)

2 μg of DLL-1 dissolved in 2 ml of phosphate-buffered saline (PBS) was added to uncoated 6-well plates (MS-8006R; SUMILON, Tokyo, Japan), and the plates were centrifuged at 750 rpm for 2 hours at room temperature. Coated 6-well plates with hydrophilic surfaces usually used for adherent cell cultures (#3516; Corning, Glendale, AZ) underwent the same treatment as a comparison. The supernatant was then removed, and 10 μg of DLL-1 dissolved in 1.25 ml of PBS was added. The plates were then centrifuged at 750 rpm for 2 hours at room temperature and washed with PBS.

Samples from 6 healthy volunteers were obtained after they signed an informed consent approved by the Ethics Committee of Showa University (Tokyo, Japan) (Approval number : 314).

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood via Ficoll-Paque (GE Healthcare, Chicago, IL) density gradient (1.077) centrifugation in Leucosep (Greiner Bio-One, Kremsmünster, Austria). After the depletion of platelets, the $CD14^+$ monocytes were isolated using CD14 antibody (Ab)-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The isolated $CD14^+$ monocytes (1×10^6cells/ml) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (FujiFilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GE Healthcare, Chicago, IL), 100 U/ml penicillin G, 100 μg/ml streptomycin (FujiFilm Wako Pure Chemical Corporation, Osaka, Japan), GM-CSF, and TGF-β1 for 7 days. Human leukocyte antigen (HLA)-DR^+, $langerin^+$ cells, designated Mo-LCs, were isolated by fluorescence-activated cell sorting (FACS). The Mo-DCs were cultured with GM-CSF, IL-4, and the Mϕs cultured with GM-CSF were used as the controls and sorted by FACS as HLA-DR^+, $langerin^-$ cells. All the cytokine concentrations were 10 ng/ml[11].

FACS

On day 7, the cells were adjusted to a concentration of 3×10^6 cells/ml and incubated at 4°C for 40 min with appropriate Abs. After washing with ice-cold PBS containing 0.2% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA), the cells were analyzed on a FACSAria II using the FACSDiva software (Becton-Dickinson and Company, Franklin Lakes, NJ). The fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) to HLA-DR (clone L243); allophycocyanin (APC)-labeled mAb to langerin (CD207, clone10E2);

phycoerythrin (PE)-labeled mAb to TLR3 (clone TLR-104); and Brilliant Violet (BV)421-labeled mAb to TLR4 (clone HTA125) (BioLegend, San Diego, CA) were used for flow cytometry.

The activation of Mo-LC, Mo-DC, and Mϕ by TLR agonists

The cells sorted by FACS were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin and in the presence or absence of 5 μg/ml of TLR3 agonists Poly(I:C) or 1 μg/ml of TLR4 agonists LPS or 1 μg/ml TLR7/8 agonists imiquimod for 3 hours.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The qRT-PCR were performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) with 5 ng of cDNA and TaqMan probes (Applied Biosystems, Foster City, CA) for *TLR2* (primer set Hs00610101_m1), *TLR3* (Hs01551078_m1), *TLR4* (Hs00152939_m1), *TLR7* (Hs00152971_m1), *TLR8* (Hs00152972_m1), *TLR9* (Hs00152973_m1), interferon-α (*IFNA2*) (Hs00265051_s1), *IFNB1* (Hs01077958_s1), *IL1A* (Hs00174092_m1), *IL1B* (Hs01555410_m1), tumor necrosis factor (*TNF*) (Hs00174128_m1), *IL6* (Hs00174131_m1), *IL15* (Hs01003716_m1), *IL23A* (Hs00372324_m1), and β-actin (*ACTB*) (Hs99999903_m1). Results were normalized to *ACTB*, which acted as an endogenous reference, and the relative level of each messenger ribonucleic acid (mRNA) was calculated using the $2^{-\Delta CT}$ method.

Statistical analysis

The unpaired Student's t-test was performed using the JMP Pro 15 software for Windows (SAS Institute Inc., Cary, NC). A probability (p) value of less than 0.05 was considered statistically significant.

Results

The effect of different DLL-1 treatment on the induction of Mo-LCs from monocytes

We compared the percentage of Mo-LCs derived from the monocytes under three different treatments of DLL-1 (soluble, immobilized on coated plates, and immobilized on uncoated plates). The percentages of cells strongly positive for HLA-DR and langerin were higher when the monocytes were cultured with DLL-1 immobilized on uncoated than coated plates (Figure 1A). Similarly, the percentages of the langerin-positive

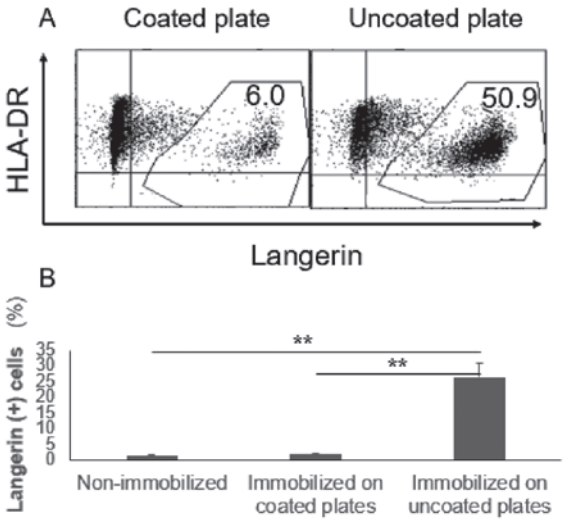

Fig. 1. The comparison of the DI(+)Mo-LCs (HLA-DR$^+$ langerin$^+$ cells) induced from monocytes by various methods

(A) The comparison of the langerin$^+$ cells on the coated and uncoated plates. Two-color flow cytometry of the cultured cells was performed to measure langerin and HLA-DR. The numbers indicate the percentage of the HLA-DR$^+$ langerin$^+$ cells. Similar results were obtained with at least three different donors.

(B) Non-immobilized : the cells were cultured in the presence of 50 ng/ml soluble DLL-1. Immobilized on coated plates : the cells were cultured on the coated plates whose hydrophilic surfaces were pretreated with DLL-1. Immobilized on uncoated plates : the cells were cultured on uncoated plates pretreated with DLL-1. After 7 days of culture, the cells were analyzed by FACS. The samples were from at least three different donors. **, $p < 0.01$.

Mo-LCs derived from the monocytes treated with DLL-1 immobilized on uncoated plates (14%-51%, $p < 0.01$) were significantly higher than those from the monocytes treated with soluble DLL-1 or DLL-1 immobilized on coated plates (Figure 1B).

Comparison of the levels of expression of the TLR genes and TLR proteins in DI(+)Mo-LC, Mo-DC, and Mϕ

We compared the expression of *TLR2*, *3*, *4*, *7*, *8*, and *9* in DI(+)Mo-LCs, Mo-DCs, and Mϕs using qRT-PCR (Figure 2A). Interestingly, *TLR3* expression was significantly higher in DI(+)Mo-LCs than in Mo-DCs or Mϕs ($p < 0.01$), while there was no significant difference in the expression of other TLR genes. The levels of *TLR3* and *TLR4* protein expression on the DI(+)Mo-LCs were much lower than those on the Mo-DCs and Mϕs, according to FACS analysis (Figure 2B).

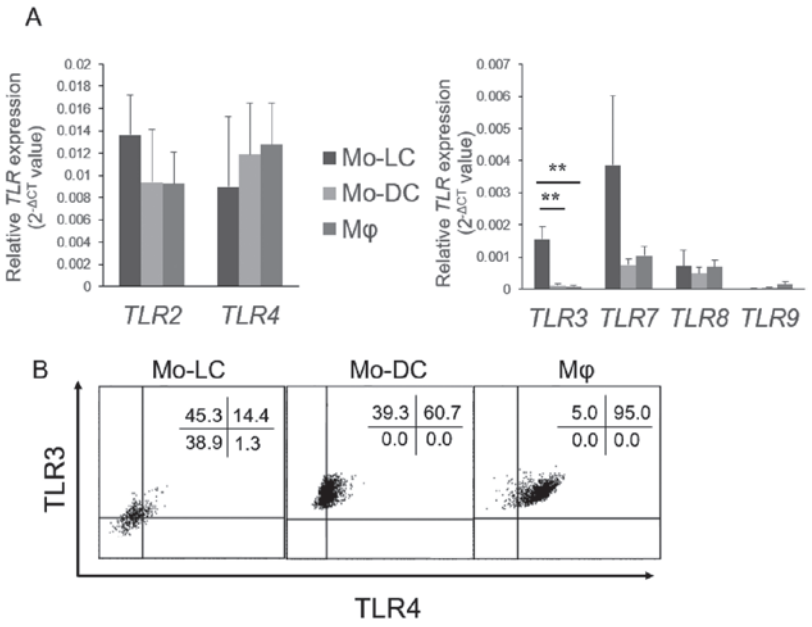

Fig. 2. The expression of the TLRs on Mo-LC, Mo-DC, and Mφ
(A) The mRNA levels of *TLR2*, *3*, *4*, *7*, *8*, and *9* in DI(+)Mo-LC, Mo-DC, and Mφ on day 7 were determined by qRT-PCR. The samples were from at least three different donors. **, $p < 0.01$.
(B) The levels of TLR3 and TLR4 were determined by FACS. Two-color flow cytometry was performed for TLR3 and TLR4. The numbers indicate the percentage of each fraction. Similar results were obtained with at least three different donors.

The comparison of cytokine expression stimulated by TLR3, TLR4, and TLR7/8 agonists

Since the cells differed significantly in the levels of TLR3 and TLR4 (Figure 2), we determined their cytokine expression in response to TLR3 (Poly(I:C)) and TLR4 (LPS) agonists (Figure 3). In addition, the TLR7/8 agonist (imiquimod), used to induce psoriasis in the murine model, was examined. The LPS-stimulated DI(+)Mo-LCs expressed *TNF*, *IL1B*, and *IL6* at significantly lower levels than the Mo-DCs and Mφs. In contrast, the *IL23A* expression by the DI(+)Mo-LCs was significantly higher than by the Mφs. On the other hand, the *IL15* expression by Poly(I:C) induction in the DI(+)Mo-LCs was more robust than in the Mo-DCs ($p = 0.0633$) and significantly more than in the Mφs ($p < 0.05$). Furthermore, the DI(+)Mo-LCs expressed *IFNB1* at a significantly higher level than the Mo-DCs and Mφs ($p < 0.05$). Only a slight weak expression of *IFNA2* and all these cytokines in responses to imiquimod (a TLR7/8 agonist) were observed in any of these cells (data not shown). Altogether, the three types of antigen-presenting cells studied in this report were found to respond to TLR3 and TLR4; however, they displayed different cytokine expression profiles when stimulated by TLR3 and TLR4.

Discussion

The work presented here demonstrates for the first time that DI(+)Mo-LCs can be induced by another method besides the original method[11] and that DI(+)Mo-LCs express psoriasis-related cytokines, *IL15* and *IL23A*, upon stimulation by Poly(I:C) and LPS.

In this study, we described the cytokine expression profile of the DI(+)Mo-LCs since it has not been studied in detail. We mainly compared the stimulation of the DI(+)Mo-LCs, Mo-DCs, and Mφs by TLR3 and TLR4 ligands. Unexpectedly, the DI(+)Mo-LCs had a significantly reduced level of TLR3 and, in particular, TLR4 on their surface membrane, while their expression *TLR3* and *TLR4* were significantly higher than and equal to those in the Mo-DCs and Mφs, respectively. The different results between qRT-PCR and FACS analysis (Figure 2) may be because mRNA expression is not necessarily correlated to protein production. Also, the FACS analysis does not examine the intracellular TLRs but only the TLRs on the surface membrane. Thus, further studies are required to confirm these preliminary findings of the TLR levels.

Our results showed that the DI(+)Mo-LCs responded mainly to the TLR3 agonist and, to a

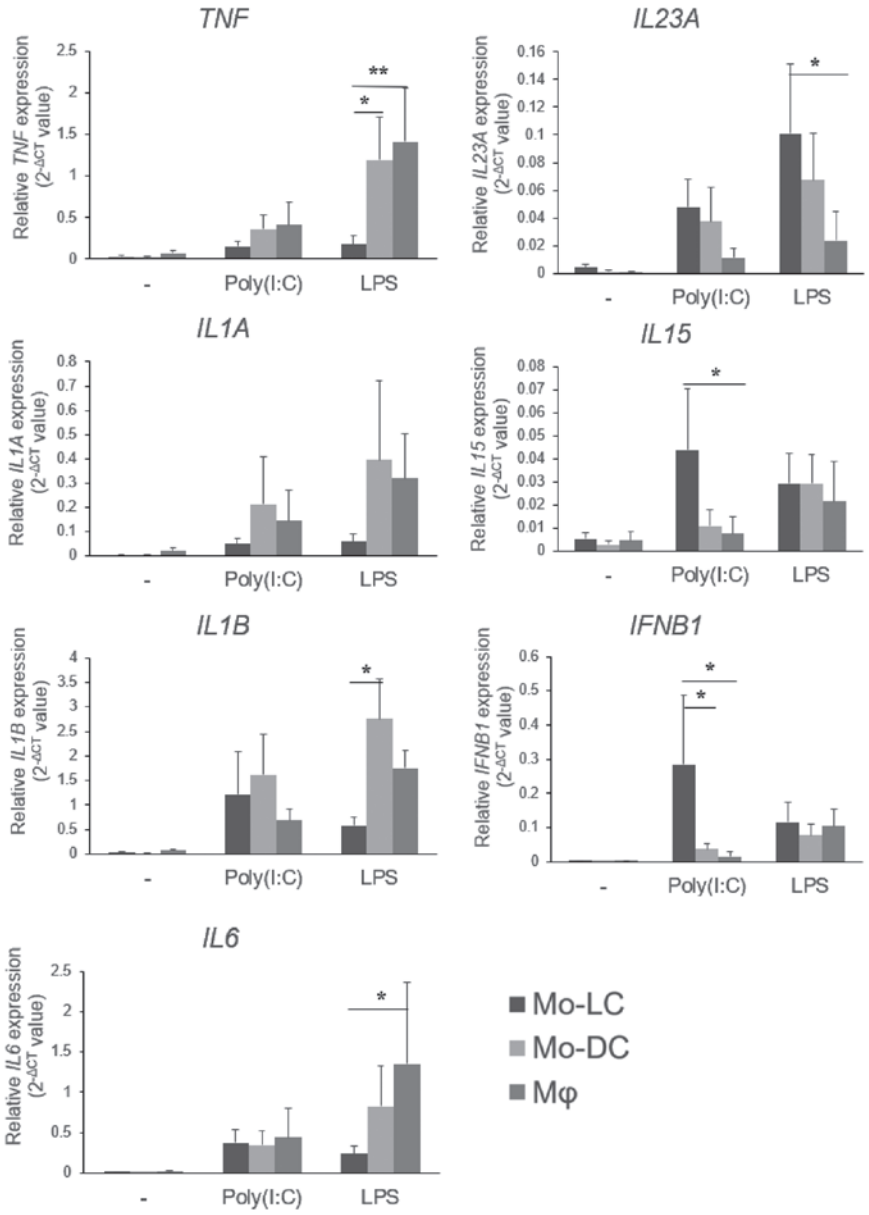

Fig. 3. The cytokine profiles of DI(+)Mo-LC, Mo-DC, and Mφ. The expression of *TNF*, *IL1A*, *IL1B*, *IL6*, *IL23A*, *IL15*, and *IFNB1* mRNA in Mo-LC, Mo-DC, and Mφ after activation by the TLR3 ligand Poly(I:C) or the TLR4 ligand LPS for 3 hours on day 7 was determined by qRT-PCR. The samples were from at least three different donors. *, $p < 0.05$, **, $p < 0.01$.

lesser extent, to the TLR4 agonist compared to the Mo-DCs and Mφs. However, DI(+)Mo-LCs' substantial expression of *IL15* and *IL23A* in response to LPS suggested that these cytokine expressions might be significant for the functions of the DI(+) Mo-LCs. On the other hand, the TLR4-stimulated expression of *TNF*, *IL1B*, and *IL6* by the DI(+) Mo-LCs was significantly lower than by the Mo-DCs or Mφs, which has been reported as a common feature of LC[6, 14]. The differential response to TLR4 among the cells in terms of cytokine expression was not explained by the loss of TLR4 on the DI (+)Mo-LCs. Presumably, DLL-1 may affect the Mo-LCs in terms of their cytokine expression profile as well as the loss of TLR4. The above results may suggest that the inflammatory monocytes infiltrated into the epidermis can retain the specific feature of the DI(+)Mo-LCs at the epidermis where DLL-1 is present[10, 11, 15]. To date, numerous reports on the cytokine profiling of the LCs have used cord blood myeloid-progenitor-derived LC (rLC) or monocyte-derived LC without using DLL-1 (DI(−)Mo-LC)[7–9]. According to these reports, there are similarities and differences between the DI(+)Mo-LCs described

here and the LCs from other sources. We observed a substantially lower level of TLR4 on DI(+)Mo-LC here, and Aar *et al*. reported low levels of TLR4 on the rLCs and DI(−)Mo-LCs[14]. While the DI(+) Mo-LCs were found to express *IL15* and *IL23A* after Poly(I:C) stimulation (Figure 3), the expression of these cytokines were not detected by rLCs[16].

Our results raised the question of why DI(+) Mo-LC robustly expresses *IL15* and *IL23A* but weakly expresses *TNF* in response to TLR4. The variation in cytokine expression, which is dependent on the type of DCs, including LC, is complex. Some differences in the downstream signaling of TLR4 may have occurred during the individual differentiation of precursors into mature DCs. Jurkin *et al*. reported a mechanism of DLL-1 in the drastic conversion of inflammatory DC into Mo-LC in the epidermis. Namely, notch signaling by DLL-1 in the keratinocytes represses kruppel-like factor 4 (KLF4) in infiltrating inflammatory DCs, where KLF4 maintains the DC/macrophage phenotypes and depresses runt-related transcription factor 3 (RUNX3), which is crucial for LC differentiation in response to TGF-β1[15]. Furthermore, it is reported that KLF4 or RUNX3 can regulate the expression of *IL23A*, *IL15*, *IFNB1*, and other pro-inflammatory cytokines[17–20]. These reports are partially consistent with our observation of the high expressions of *IL23A*, *IL15*, and *IFNB1* and low expressions of pro-inflammatory cytokine genes by the DI(+)Mo-LCs. Since the effect of notch signaling on cytokine regulation can be complex, further detailed studies are necessary.

Psoriasis, marked with well-demarcated patches of inflammation, is a common skin disease related to the immune system. Because *IL15* and *IL23A* are involved in the pathogenesis of epidermal lesions in psoriasis[4], the cytokine profile of the DI(+)Mo-LCs may have pathogenic roles in epidermal diseases. This result is consistent with Mo-LCs' reported roles in psoriasis in the murine models[2,3]. While these murine models were induced by imiquimod, our results (Figure 3) showed that the DI(+)Mo-LCs were stimulated more by Poly(I:C) and LPS than by imiquimod and that they could be involved in the pathogenesis of psoriasis in humans. Although TLR3 and TLR4 were reported as critical regulators of the inflammation of psoriasis[21], the relationship between the pathogenesis of psoriasis and the stimulation of the Mo-LCs by TLR3 and TLR4 remains to be seen. Further studies using our new method to generate DI (+)Mo-LC might uncover this relationship.

Conclusions

Using a newly established method to generate Mo-LCs, we found that the DI(+)Mo-LCs had a unique capacity to produce cytokines related to the pathogenesis of psoriasis. Our data contribute to a better understanding of the roles of Mo-LCs in epidermal pathogenesis. Further studies using the procedures presented in this report will advance the research for chronic inflammatory skin disease.

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Conflict of interest disclosure

The authors declare no conflicts of interest associated with this manuscript.

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