Induction of langerin⁺ Langerhans cell-like cells expressing reduced TLR3 from CD34⁺ cord blood cells stimulated with GM-CSF, TGF- β 1, and TNF- α

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(Received 1 May 2016; and accepted 12 August 2016)

ABSTRACT

Langerhans cells (LCs), a subset of dendritic cells (DCs), reside in body surface presenting antigens from various pathogens and activate immune system after migrating to vicinal lymph nodes. We recently demonstrated that the E-cadherin interaction allowed peripheral blood (PB) CD14⁺ cells to differentiate into LC-like cells that closely resemble primary LCs. Here, with a combination of GM-CSF, TGF- β , and TNF- α , we induced LC-like cells from umbilical cord blood (UCB)-derived CD34⁺ cells and compared them with those induced from PB CD14⁺ cells. In contrast to PB CD14⁺ cell-derived LC-like cells with an undetectable surface level of toll-like receptor (TLR)4 and an unresponsiveness feature to bacterial lipopolysaccharide (LPS), CB CD34⁺ cells-derived LC like cells expressed a low, but apparent, surface level of TLR4 and a reduced level of intracellular TLR3. Consistent with this result, they responded to bacterial LPS, but poorly to poly(I:C) reflecting viral RNA. These findings suggest that LC-precursors from circulating PB CD14⁺ cells seem to be arranged in the outer barrier of skin, while LC-precursors from local undifferentiated UCB-derived CD34⁺ cells may be arranged in the inner barrier of mucosal tissues and work together to combat against external pathogens as well as internal malignancies throughout body surface.

Upon invasion, various pathogens such as bacteria or viruses must encounter the first-line barrier of the mucosal compartments and the skin immune system constitutes a part of the most important and potent surface barrier organized mainly by Langerhans cells (LCs), a type of innate dendritic cells (DCs). LCs are antigen-presenting cells of self-renewing in the steady state and recruited from blood-borne precursors (15). Also, it has been demonstrated that LCs can be replaced by bone marrow-derived cells when they are in the circumstances of transplanta-

tion and inflammation (6, 13). Moreover, it has just been reported that CD1c⁺ DCs more likely become LC-like cells with high langerin, Birbeck granules, and E-cadherin (E-cad) expression when cultured with granulocyte macrophage colony-stimulating factor (GM-CSF), transforming growth factor β 1 (TGF- β 1), and bone morphogenetic protein 7 (4, 16). However, the critical factors regulating their homeostasis and direct precursors have not yet been identified.

The skin LCs, residing within the stratum spinosum of human skin (12), play an important role in the recognition of invaded pathogens and activation of other components of immune system following migration to vicinal lymph nodes (11). However, because of the limited availability of primary LCs from human skin, LCs have been induced *in vitro* from peripheral blood monocytes (PBMos) in the presence of cytokines such as IL-4, GM-CSF, and

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TGF- β 1 (17). Nonetheless, any protocol reported to date have not yet succeeded to induce LCs with all features of primary LCs *in vivo*; most of the efforts have been ended up with the cells that still missed some of the characteristics (10). For example, primary LCs in the skin present a particular C-type of lectin, langerin, on their surface but not, another C-type lectin DC-specific intercellular adhesion molecular-3-grabbing non-integrin (DC-SIGN), whereas *in vitro*-induced LCs tend to express significant levels of DC-SIGN together with langerin (8, 9, 18). Also, the expression of some of the toll-like receptors (TLRs) was different from that observed on the primary LCs (2).

Accordingly, the *in vitro* induced cells are deemed as "Langerhans cell-like (LC-like)" cells (7, 21). To overcome these problems, we have recently reported a novel *in vitro* induction protocol of LCs from CD14⁺ PBMs, which includes co-culture of LC-like cells with keratinocytes (KCs) expressing E-cad or the utilization of a unique culture plate coated with recombinant E-cad molecule for the induction (14). The cells induced under these culture conditions expressed a high level of E-cad as well as undetectable level of DC-SIGN and TLR4 on their surface so that they did not respond to lipopolysaccharide (LPS), a known TLR4 ligand. These phenotypes are very similar to epidermal LCs sampled from human skin tissues (14).

Although many protocols, including ours (14), allow PBMs to differentiate into the LC-like cells that harbor many of the properties of primary LCs, the utilization of the peripheral blood as a source of LC-like cells has several drawbacks; individual-to-individual variability of successful induction of LC-like cells, invasive nature of sampling peripheral blood from healthy volunteers, and the requirement of large volume of peripheral blood to obtain the sufficient number of LC-like cells for the subsequent analysis. To circumvent these drawbacks, recent works aiming the *in vitro* induction of LC-like cells have utilized CD34⁺ hematopoietic progenitor cells purified from umbilical cord blood (UCB) (3), which can be sampled noninvasively upon normal delivery, is usually wasted, and contains the sufficient amount of CD34⁺ cells for the LC-like cell induction.

In contrast to LC-like cells from PB (peripheral blood)-derived CD14⁺ cells, we found that those from UCB-derived CD34⁺ cells, expressing langerin but not DC-SIGN, when stimulated with a combination of GM-CSF, TGF- β 1 and TNF- α , did express TLR4 and responded to LPS and reduced level of TLR3

with poor responder to double stranded RNAs, such as poly(I:C). Therefore, we are able to induce two distinct types of LC-like cells, expressing langerin but not DC-SIGN. PBMo-derived LC-like cells will not respond to bacterial LPS but react with viral RNA, while UCB-derived LC-like cells are poor responder to viral RNA but respond to bacterial LPS. On the basis of the findings, we will discuss here the critical factors and possible precursors for LC-induction, and speculate the reason for existence of two distinct types of LCs at the very surface compartment throughout our body.

MATERIALS AND METHODS

Umbilical cord blood sampling. UCB and PB were obtained from mothers at full term (over 37 wks term) and healthy volunteers, respectively, with written informed consent at the university hospital. UCB and PB withdrawal procedures followed the institutional guideline. All pregnant volunteers were free of *Toxoplasma gondii*, HIV, cytomegalovirus, hepatitis B and C viruses, and *Treponema pallidum* infection at the time of labor. This study was approved by the Ethical Committee Review Board of Nippon Medical School.

Isolation and culture of CD34⁺ and CD14⁺ cells from umbilical cord blood and peripheral blood. Mononuclear cells were separated from heparinated whole blood (UCB and PB) by a conventional density gradient method (Lymphosepar I; IBL-Japan), and washed three times with 1 $\times$ PBS. CD34-positive and CD14-positive cells were isolated from mononuclear cells derived from UCB and PB, as described previously (3, 19, 22). In brief, CD34⁺ cell population was purified positively from UCB-derived mononuclear cells with magnetic beads (CD34 MicroBeads; Miltenyi Biotec, San Diego, CA) according to the manufacturer's instruction. CD14⁺ cell fraction was separated from PB- or UCB-derived mononuclear cells with magnetic beads (CD14 MicroBeads; Miltenyi Biotec) according to the manufacturer's instruction. Purity of CD34⁺ and CD14⁺ cell populations was about 99 % and 96 %, respectively.

Purified CD34⁺ and CD14⁺ cells were cultured to induce LC-like cells in complete cell medium (CCM) (20) (RPMI1640 supplemented with 10 mM HEPES, 50 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin [all from GIBCO, Waltham, MA], and 10% heat-inactivated fetal calf serum (FCS)) in the presence of different sets of cytokines indicated below. CD14⁺ cells

were cultured in CCM in the presence of 100 ng/mL recombinant human GM-CSF (PeproTech, Rocky Hill, NJ), 10 ng/mL TGF- β 1 (R&D Systems, Minneapolis, MN), and 10 ng/mL recombinant human IL-4 (PeproTech), while CD34⁺ cells were cultured in CCM in the presence of combinations of the following cytokines; 50 ng/mL recombinant human GM-CSF (PeproTech), 50 ng/mL SCF (PeproTech), 50 ng/mL Flt3-ligand (PeproTech), 50 ng/mL TPO (PeproTech), 1 ng/mL TNF- α (PeproTech), 5 ng/mL TGF- β 1, and 10 ng/mL recombinant human IL-4.

Immunofluorescent staining. To characterize the phenotype, cell surface markers were stained with specific monoclonal antibodies. After wash, cells were suspended in 100 μ L FACS buffer (PBS with 2% heat-inactivated FCS and 10 mM sodium azide). Nonspecific binding was blocked by adding 10 μ g human Ig polyglobin (Nippon Red Cross, Tokyo, Japan) to each sample and the cells were incubated for 20 min at 4°C. Specific monoclonal antibodies (mAbs) conjugated with fluorophores were then added to cells and further incubated on ice for 30 min in the dark. After incubation, cells were washed three times, and re-suspended in an appropriate amount of FACS buffer for FACS analysis. If the secondary staining was required, after the incubation with primary antibodies, cells were washed twice and incubated with secondary antibodies for 30 min on ice. After washing three times, cells were re-suspended in FACS buffer for analysis. Dead cells were excluded by staining with propidium iodide. The fluorescence signals were detected by FACSCanto II instrument (BD Bioscience, San Jose, CA) with FACSDiva software, and the FACS data were analyzed by FlowJo software (TreeStar, Ashland, OR).

The specific mAbs used in this study included FITC- and APC/Cy7-conjugated anti-CD1a (clone HI149; BioLegend, San Diego, CA), FITC-conjugated anti-CD1b (clone SN13; BioLegend), APC-conjugated anti-CD1c (clone 3.9; BioLegend), APC-conjugated anti-E-cad (clone 67A4; BioLegend), FITC-conjugated anti-DC-SIGN (clone 120507; R&D Systems), PE-conjugated anti-langerin (clone DCGM4; Beckman Coulter, Brea, CA), biotin-conjugated anti-TLR2 (clone TLR2.1; eBioscience), biotin-conjugated anti-TLR3 (clone TLR3.7; eBioscience), biotin-conjugated anti-TLR4 (clone HTA125; eBioscience), FITC-conjugated streptavidin (BioLegend), FITC-conjugated anti-CD34 (BD Pharmingen, San Jose, CA), APC-conjugated anti-CD14 (HCD14; BioLegend), and FITC-conjugated anti-CD83 (HB15e; BioLegend).

Intracellular staining of TLR3. To stain intracellular TLR3 molecules, cell surface markers were stained as described above, followed by fixation and permeabilization of cells with Cytofix/Cytoperm solution (BD Biosciences) for 20 min on ice. After washing twice with Cytoperm/Cytowash solution (BD Biosciences), cells were incubated with human poly Ig to prevent nonspecific binding for 20 min at 4°C and further incubated with biotin-conjugated anti-TLR3 antibodies for 30 min on ice in the dark. In case of staining intracellular TLR3, after being washed three times in Cytoperm/Cytowash solution, cells were incubated with FITC-conjugated streptavidin for 30 min on ice in the dark. Cells were washed three times with Cytoperm/Cytowash solution, and re-suspended in FACS buffer for analysis.

Reverse transcription-polymerase chain reaction (RT-PCR). To examine the expression of C-type lectin and TLRs, cDNAs were amplified from total RNA extract from induced LC-like cells as described previously with minor modifications (23). Total RNA was extracted from 3×10^5 LC-like cells using Quick-RNA MicroPrep (Zymo Research, Irvine, CA) according to the manufacturer's instruction. A pool of first single strand cDNA was synthesized from the total RNA by RT-PCR using MultiScribe Reverse Transcriptase and an oligo d(T) primer. cDNA of the genes of interest was PCR-amplified using total single strand cDNA as a template (one 20th of the total reaction volume) and AmpliTaq DNA Polymerase (50 units per 10 μ L PCR reaction; ABI, Waltham, MA). The oligo nucleotide primers used in this study were listed in Table 1. Amplified GAPDH was used as a PCR control to verify the reaction and allow normalization between samples.

In vitro stimulation of UCB-derived LC-like cells by TLR agonists. UCB-derived LC-like cells were stimulated for 24 h with following TLR agonists; 10 μ g/mL PGN (Fluka Buchs SG, Switzerland) for TLR2, 200 μ g/mL poly(I:C) (Amersham Biosciences, Piscataway, NJ) for TLR3, or 1 μ g/mL LPS (Sigma-Aldrich, St. Louis, MO) for TLR4. Accordingly, the cells were analyzed for their langerin, CD83, and CD86 expression.

Measurement of cytokine production by ELISA. Cytokine production in the supernatant was measured by ELISA kit for human TNF- α , and IL-10 (Biolegend), as well as for human IL-12p40 (R&D Systems).

Statistical analysis. The results were analyzed using

Table 1 Sequences of oligo nucleotide primers used for PCR amplification of C-type lectins and TLR cDNAs

Primer	Nucleotide sequences (5' -> 3')
GAPDH sense	GCC TCA AGA TCA TCA GCA ATG C
GAPDH antisense	ATG CCA GTG AGC TTC CCG TTC
DC-SIGN sense	GAG CTT AGC AGG GTG TCT TG
DC-SIGN antisense	GCA GGC GGT GAT GGA GTC GT
Langerin sense	CGC ACT TCA CTG TGG ACA AA
Langerin antisense	GAA TCC AGG GTG CTG ATG TT
TLR2 sense	CCC TGG GCA GTC TTG AAC ATT
TLR2 antisense	GCC TCC GGA TTG TTA ACG TTT
TLR3 sense	AGG ATT GGG TCT GGG AAC AT
TLR3 antisense	CTG GAA TCT CCT CAA GGA AAA C
TLR4 sense	TGG TGT CCC AGC ACT TCA TC
TLR4 antisense	CTG CAT ATC TAG TGC ACC ATG G

Student's *t*-test and the results are presented as the mean $\pm$ SEM. Differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Difference in the CD14⁺ populations between UCB and PB for the differentiation into LC-like cells

As many studies to date have used the CD14⁺ cell fraction from PB to induce LC-like cells, we first compared the ability of CD14⁺ cells to induce LC-like cells between PB and UCB as a source of this cell fraction. UCB can be obtained non-invasively from healthy pregnant volunteers with CD14⁺ cells being contained at a sufficient rate to induce LC-like cells; in average 18% of the whole UCB from healthy pregnant volunteers (Fig. 1A). As expected, the expression of CD14 and CD34 was almost exclusive; in fact, there were no cells detected by FACS analysis that expressed both CD14 and CD34 in the UCB (Fig. 1A).

In addition, May-Grünwald staining of these cell fractions revealed clear morphological difference between magnetically-sorted CD34⁺ and CD14⁺ cells as shown in Fig. 1B; whereas the former cells showed an irregularly round morphology with dark stained nucleus, CD14⁺ cells were slightly larger in size than CD34⁺ cells and exhibited dendritic processes on their surface. The contamination of other cell types could be excluded by the high purity (> 96%) of the sorted cells. The comparison of the mRNA expression levels of some of the C-type lectin, such as DC-SIGN and langerin, and TLRs revealed significant differences in the expression of TLR4 and TLR2. CD14⁺ cells expressed a high level of TLR4 and less, but significant amount of TLR2 at the transcription-

al level, whereas CD34⁺ cells did not express these two TLRs (Fig. 1C).

Next we attempted to induce LC-like cells using the purified CD14⁺ cells derived from PBMo and UCB in the induction medium supplemented with GM-CSF and IL-4 for 7 days (Fig. 2A). The resultant cells were stained with specific mAbs to examine their phenotype. In the presence of these two cytokines, a significant fraction (> 80%) of the cells induced from PBMo-derived CD14⁺ cells expressed CD1a, a marker for LCs, whereas only a small fraction of the cells induced from UCB-derived CD14⁺ cells expressed cell surface CD1a. The expression of CD1a was significantly increased by the addition of TGF- β 1 in the medium, as more than 75% of the cells induced from UCB-derived CD14⁺ cells expressed the molecule. However, even the addition of TGF- β 1 did not allow UCB-derived CD1a⁺CD14⁺ cells to express langerin on their surface, as less than 1% of the cells expressed langerin, in comparison with more than 20% of PBMo-derived CD1a⁺CD14⁺ cells expressing this molecule (Fig. 2B). These data indicated the inability of the CD14⁺ cells derived from UCB to induce langerin⁺CD1a⁺ LC-like cells.

TGF- β 1 is required, but not sufficient, for the induction of LC-like cells from UCB-derived CD34⁺ cells, which was improved by the addition of TNF- α

Recent studies have utilized UCB-derived CD34⁺ cells as a possible starting material for the induction of LC-like cells (3). The failure of the induction of LC-like cells from UCB-derived CD14⁺ cells led us to utilize the CD34⁺ cell fraction sorted from UCB for the LC-like cell differentiation and optimization of the culture condition. To this end, the induction medium was supplemented with recombinant cyto-

Fig. 1 Mononuclear cells separated from whole UCB contain distinct populations expressing CD14 and CD34. **(A)** Exclusive expression of CD14 and CD34 on mononuclear cells separated from whole UCB. After separation, mononuclear cells were stained with anti-CD14 and anti-CD34 mAbs, and analyzed by flow cytometry. A representative result from six independent experiments is shown. **(B)** Morphology of magnetically sorted CD14⁺ and CD34⁺ cells from UCB mononuclear cells was examined by May-Grünwald-Giemsa staining under a light microscope. From the top, microscopic images of whole mononuclear cells, purified CD34⁺ cells, and purified CD14⁺ cells are shown in the left column (40X). The area in the black rectangle is enlarged in the right panel (120X). **(C)** Detection of DC-SIGN, langerin, TLR2, TLR3, and TLR4 cDNA by RT-PCR from the total RNA extract of the magnetically sorted CD34⁺ (left lane) and CD14⁺ (right lane) cells. *gapdh* was amplified as a control to verify the reaction. A representative result from five independent experiments is shown.

kines at different combinations, which are depicted in Fig. 3A, and the cell surface expressions of langerin and DC-SIGN were compared. While the combination of GM-CSF and IL-4, usually required for CD14⁺ PBMo to differentiate into CD1a⁺ DCs, made the UCB-derived CD34⁺ cells express DC-SIGN as expected, the addition of GM-CSF, IL-4 and TNF- α , separately or in combination, did not allow the UCB-derived CD34⁺ cells to express langerin (less than 1% of the induced cells expressed this molecule). Similar to LC, cell surface langerin expression was markedly increased and DC-SIGN expression was profoundly diminished in the culture with the combination of GM-CSF and TGF- β 1 (Fig. 2B, left panels). In contrast, the cell surface expression level of langerin was comparable to that observed on PB-derived CD14⁺ cells cultured in the presence of GM-CSF, IL-4 and TGF- β 1 (more than 20% of the PB-derived cells were langerin-positive) (Fig. 2B, right panels). Importantly, contrary to the PBMo, the langerin expression of UCB-derived CD34⁺ cells was markedly suppressed by the presence of an additional cytokine IL-4 (GM-CSF + TGF- β 1 + IL-4; the fourth panel in Fig. 3A). Moreover, the addition of TNF- α significantly augmented the langerin surface expression level (more than 70% were positive, the left bottom panel in Fig. 3A), suggesting that the combinations of the three cytokines (GM-CSF, TGF- β 1 and TNF- α) is optimum to induce langerin expression on the sorted CD34⁺ cells derived from UCB.

To examine if this cytokine combination could induce LC-like cells *in vitro*, cells induced from UCB-derived CD34⁺ cells in the presence of GM-CSF and TGF- β 1 with or without TNF- α were further characterized. Flow cytometry analysis of the cell surface molecules revealed the high expression of CD1a and CD1c, and relatively modest expression levels of CD1b (Fig. 3B). In consistent with our previous study (14), in which TGF- β 1 successfully induced the expression of E-cad on the cells induced from PB-derived CD14⁺ cells, the sorted CD34⁺ cells derived from UCB expressed a significant levels of E-cad molecule on their surface when stimulated with GM-CSF and TGF- β 1 (the middle panel in Fig. 3B). In addition, we examined the expression of TLRs in these cells. The cell surface TLR2 expression level was almost similar to the cells induced with TNF- α , whereas the intracellular expression levels of TLR3 showed marked down-modulation when co-cultured with TNF- α . By contrast, the surface TLR4 expression levels were significantly higher in the langerin⁺ LC-like cells induced from UCB-derived CD34⁺ cells (Fig. 3B) than in the cells de-

Fig. 2 Comparison of cellular characteristics in CD14⁺ cells from either umbilical cord blood (UCB) or peripheral blood (PB). Magnetically sorted CD14⁺ population from UCB mononuclear cells failed to express cell surface langerin. CD14⁺ cell fraction was magnetically purified from UCB- and PB-derived mononuclear cells. The purified cells were cultured for 6 days in CCM supplemented with 50 ng/mL GM-CSF and 10 ng/mL IL-4 with (A) or without (B) 10 ng/mL TGF-β1. After the induction, cells were harvested and stained for detecting surface expression of C-type lectins (DC-SIGN and langerin), CD14 and CD1a. A representative result from three independent experiments is shown.

rived from PBMo-derived LC-like cells and primary LCs (4.0–4.5% in this study compared to almost no expression in our previous study (14)), and the higher expression level of TLR4 was not affected by the presence of TNF-α (Fig. 3B). Taken together, characterization of the cells induced from the UCB-derived CD34⁺ cells revealed that GM-CSF and TGF-β1 are the requisite for the induction of cell surface langerin expression, and that TNF-α raised the expression level of langerin, suggesting the successful induction of LC-like cells from UCB-CD34⁺ cells with the combination of the three cytokines.

UCB-derived LC-like cells did not respond to TLR3 agonist

Then, the expression of the three TLRs was confirmed by the RT-PCR performed for amplifying cDNA in these UCB-derived LC-like cells. TLR2 and TLR4 expressions were observed in the cells induced with and without TNF-α, respectively, and

apparent level of mRNA expression of TLR4 was detected in both cells (Fig. 4A). However, the presence of TNF-α profoundly reduced the intracellular expression of TLR3 (Fig. 4A). To confirm these observations, LC-like cells derived from UCB-CD34⁺ cells were treated with agonists of TLR2 (PGN), TLR3 (poly(I:C)), or TLR4 (LPS), and the TLR agonist-induced activation was estimated either by the enhancement of CD83 expression (Fig. 4B; left eight panels) by flow cytometry or by the reduction of the surface expression level of langerin (Fig. 4B; right eight panels), since langerin expression is decreased after activation. When the LC-like cells induced from UCB-CD34⁺ cells in the presence of the three cytokines (GM-CSF, TGF-β1 and TNF-α) were treated with either PGN or LPS, they showed increased expression of CD83 (72.6% for PGN and 69.0% for LPS) and reduced surface langerin expression in mean fluorescence intensity (MFI) (30.5 for PGN and 35.7 for LPS). However, the percent-

Fig. 3 Different combinations of the cytokines affected differently the characteristics of the cells induced from purified CD34⁺ cells. **(A)** CD34⁺ cells were positively selected using magnetic beads from UCB-derived mononuclear cells, and cultured for 15 days in CCM supplemented with a various combinations of the cytokines indicated in the figure. Effects of GM-CSF, IL-4, TNF- α , and TGF- β 1, separately or in combination, on the induction of the C-type lectin expression was examined by flow cytometry using specific antibodies to langerin and DC-SIGN. A representative result from six independent experiments is shown. **(B)** Purified CD34⁺ cells from UCB mononuclear cells were cultured in CCM for 15 days in the presence of GM-CSF and TGF- β 1 without (the left column) or with (the right column) TNF- α . After the induction, cell surface markers and intracellular molecule were stained with specific monoclonal antibodies and their expression was analyzed by FACS. Langerin-positive fraction was gated and mean fluorescent intensity (MFI) of each cell surface molecule is depicted in histograms in the langerin⁺ cell fraction. A representative result from eight independent experiments is shown.

Fig. 4 Response to the TLR3 agonist was significantly weakened in LC-like cells induced in the presence of TNF- α from UCB-derived CD34 $^{+}$ cells. **(A)** A significant reduction of mRNA expression of TLR3, but not TLR2 or TLR4, is apparent by RT-PCR from the total RNA extract of the magnetically sorted CD34 $^{+}$ cells cultured with GM-CSF, TGF- β 1 and TNF- α (right lane), when compared with the CD34 $^{+}$ cells cultured with GM-CSF and TGF- β 1 (left lane). *gapdh* was amplified as a control to verify the reaction. A representative result from five independent experiments is shown. **(B)** LC-like cells induced from UCB CD34 $^{+}$ cells either in the presence of two (GM-CSF plus TGF- β 1, left column) or three (GM-CSF, TGF- β 1 plus TNF- α , right column) cytokines were treated with TLR agonists (Top; naïve without agonist, upper middle; PGN for TLR2, lower middle; poly(I:C) for TLR3, and bottom; LPS for TLR4), and tested for the post-activation reduction of surface langerin expression. In the left-most and left-middle columns, surface expression of langerin is shown against surface CD83 expression, and in the right-middle and right-most columns, MFI (as percentage of maximum) of langerin staining is shown in histogram. Staining with isotype control is shown in shade. **(C)** Production of IL-10, IL-12p40 and TNF- α by stimulation of three distinct TLRs, such as TLR2, TLR3, and TLR4 on UCB-derived LC-like cells, with each respective ligand, PGN, poly(I:C) and LPS, was measured by ELISA. Cytokine production from UCB-derived LC-like cells was consistent with each ligand expression, in particular in the case of TLR3. Inclusion of an additional cytokine TNF- α to the basal cytokine combination (GM-CSF and TGF- β 1) markedly reduced the production of three cytokines in UCB-CD34 $^{+}$ derived LC-like cells.

ages of activated CD83⁺ cells were lower and that of langerin⁺ cells was not down-modulated when the cells were treated with poly(I:C), TLR3 agonist, confirming the effect of reduction on the intracellular expression level of TLR3 by the presence of TNF- α (Fig. 4B). As expected, cytokine production via ligand stimulation of UCB-derived LC-like cells was also consistent with each ligand expression, in particular in the case of TLR3 (Fig. 4C).

These results suggest that the inclusion of an additional cytokine TNF- α to the basal cytokine combination (GM-CSF and TGF- β 1) not only enhances the surface expression of langerin, but also markedly reduces the intracellular TLR3 expression in UCB-CD34⁺ derived LC-like cells. Taken together, the induction of LC-like cells from UCB-derived CD34⁺ cells in the presence of a set of the three cytokines (GM-CSF, TGF- β 1 and TNF- α) allow the cells to differentiate into the cells with more primary LC-like characteristics in terms of high langerin and low TLR expressions. Nonetheless, it is of note that the langerin⁺ LC-like cells derived from UCB-CD34⁺ cells showed clear expression of TLR4 and had an ability to respond to LPS but failed to respond poly(I:C) because of their down-modulated TLR3.

Effect of stimulation of UCB-CD34⁺ derived LC-like cells through E-cad-E-cad interaction

We have previously reported that primary LCs from the skin, as well as LC-like cells induced from CD14⁺ PBMs stimulated with GM-CSF, IL-4 and TGF- β 1 and E-cad-coated plate through E-cad-E-cad interaction, showed undetectable level of TLR4 expression, so that they did not respond to LPS, although they expressed apparent level of TLR3 and responded to poly(I:C) (14). On the basis of this observation, we first examined the effect of TNF- α on E-cad expression of the UCB-CD34⁺ derived LC-like cells incubated with GM-CSF and TGF- β 1. Thirteen to 15 days after the incubation, significant increase of E-cad expression was observed on these LC-like cells (Fig. 5A). Then the enhanced level of E-cad⁺ LC-like cells were further incubated on the culture plate pre-coated with purified E-cad molecules for additional 3 days. TLR4 expression on the treated cells was significantly down-modulated with stable langerin appearance (Fig. 5B). Nonetheless, their intracellular mRNA level of TLR4 was still visible and the plate-treated cells with lower TLR4 expression retained the ability to respond to LPS (Fig. 5C). Those LC-like cells induced from UCB-derived CD34⁺ cells, still expressed TLR4 and responded to LPS, although they showed reduced level of TLR3

with poor response to double stranded RNA, such as poly(I:C). Therefore, there seem to be two distinct LC-like cells, expressing langerin but not DC-SIGN. CD14⁺ PBMs-derived LC-like cells will not respond to bacterial LPS but react with viral RNA, while UCB-derived LC-like cells will be poor responders to viral RNA but respond to bacterial LPS.

Why does our body harbor two distinct subsets of LCs? LCs are thought to be replenished from precursors migrating from the circulation to the site of inflammation in the skin. For example, in case of viral infection, Th1-type immune response is induced to produce cytokines such as IL-12 and IL-18, as well as TNF- α , which facilitates the induction of LCs that mainly respond to viral RNA by expressing TLR3. Indeed, primary LCs isolated from skin tissues matured after Herpes simplex virus type 2 infection via TLR3 stimulation by viral double strand RNA (5). In contrast, when inflammation is caused by bacterial infection, Th2-type immune response is induced in the early phase of infection by the production of pro-inflammatory cytokines including IL-5 and IL-10, as well as IL-4, which facilitates the induction of LCs that express mainly TLR4 to further combat against bacterial infection. Supporting this idea, oral mucosal LCs, exposed unceasingly to the bacterial flora, reportedly expressed TLR4 (1). After stimulation by a lipid A-derivative, oral LCs weakened, rather than augmented, their capability to stimulate T cells by decreasing the expression of co-stimulatory molecule, CD86, and by increasing the expression of co-inhibitory molecules, the B7 family, leading to the tolerogenic state predominating in the oral cavity (1). Taken together, LC-precursor from circulating CD14⁺ PBMs-derived cells appears to be arranged the outer barrier of skin, while LC-precursor from local undifferentiated CD34⁺ UCB-derived cells may be arranged in the inner barrier of mucosal tissues. These skin and mucosa-resident LCs may constitute the outer and inner barrier, respectively, and work together to combat against external pathogens as well as internal malignancies throughout the entire body surface.

Acknowledgments

The authors would like to thank Ms. Masumi Shimizu for helping flowcytometry analysis.

This study was supported in part by grants from the Ministry of Education, Science, Sport, and Culture from the Ministry of Health and Labor and Welfare, Japan (25461715 to H.T.), from the Japanese Health Sciences Foundation, and from the Pro-

Fig. 5 Effect of E-cad-E-cad interaction on TLR4 expression in CD34-derived LC-like cells. Surface expression of TLR4 was significantly reduced, but not lost, on the CD34-derived LC-like cells after E-cad interaction. Positively selected CD34⁺ cells from UCB-derived mononuclear cells were cultured for the duration indicated in the figure in CCM supplemented with GM-CSF and TGF- β 1 with or without TNF- α , and cell surface molecules were analyzed by flow cytometry using specific antibodies. **(A)** Kinetics of the cell surface expression of the E-cad molecule on days 8, 10, 13 and 15 after the induction were shown. **(B)** Magnetically-sorted CD34⁺ cells were plated on the culture vessel pre-coated with purified E-cad molecules and cultured as described above. MFI of TLR4 staining was analyzed in the langerin-positive cell fraction and shown in histograms. Data obtained from the induction culture using the culture plate without E-cad are shown in the upper panels as a negative control of the effect of E-cad interaction. Representative results from three independent experiments are shown. **(C)** Amplification of TLR4 cDNAs from the total RNA extracts of the cells induced from UCB-derived CD34⁺ cells in the presence of GM-CSF and TGF- β 1 without (left lane) or with (right lane) TNF- α . *gapdh* cDNA was amplified as a control for the reaction. A representative result from three independent experiments is shown.

motion and Mutual Aid Corporation for Private Schools of Japan, and from and from MEXT-supported Program for the Strategic Research Foundation at Private Universities, Japan.

DISCLOSURES

The authors have no financial conflicts of interest.

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