J Clin Exp Hematopathol
Vol. 49, No. 1, May 2009

Original Article

Immunohistochemical Discrimination of Plasmacytoid Dendritic Cells from Myeloid Dendritic Cells in Human Pathological Tissues

Yuriko Nishikawa,[1)] Hiaki Sato,[1)] Takashi Oka,[2)] Tadashi Yoshino,[2)] and Kiyoshi Takahashi[1)]

Until now, no method has been available to discriminate mature plasmacytoid DC (pDC) from myeloid DC (mDC) immunohistochemically. In this study, we report that these DC-subsets can be distinguished in routine pathological sections. Immature and mature monocyte-derived DCs (MoDCs) were S100 calcium binding protein B (S100B)$^+$, while pDCs generated from pDC-precursors were S100B$^-$. In contrast, both mature MoDC and pDC were fascin$^+$. Epidermal Langerhans cells (LCs) were S100B$^+$/fascin$^-$. Although the majority of DCs were S100B$^+$/fascin$^+$ in the dermis with nonspecific inflammation, dermal DCs were mostly S100B$^-$/fascin$^+$ in psoriasis vulgaris, in which type I interferon secreted by pDC-precursors is thought to play a major role. S100B$^+$/fascin$^+$ DCs were accumulated in the superficial lymph node (LN), while they were scarce in the deep LN. In the superficial LN with dermatopathic lymphadenitis, a large number of S100B$^+$/fascin$^+$ DCs were accumulated in the T-zones, where numerous LC-derived DCs are accumulated. In contrast, almost all DCs were S100B$^-$/fascin$^+$ in the superficial LN with Kikuchi's lymphadenitis, in which numerous pDC-precursors are known to be present. In contrast to the superficial LN, the deep LN contained numerous S100B$^-$/fascin$^+$ DCs and a few S100B$^+$ DCs. Thus, the distributions of S100B$^+$ DC or S100B$^-$/fascin$^+$ DC correspond to the putative distribution of mDC or mature pDC, respectively. 〔*J Clin Exp Hematopathol 49(1) : 23-31, 2009*〕

Keywords: dendritic cell, myeloid dendritic cell, plasmacytoid dendritic cell, S100 calcium binding protein B, fascin

INTRODUCTION

Dendritic cells (DCs) are specialized cells that bridge the innate and the adaptive immune systems and act as antigen-presenting cells with the capacity to initiate primary T-cell responses and to efficiently stimulate memory responses.[1-3] It has been generally accepted that DCs are heterogeneous in origin and classified into two major subsets, myeloid DC (mDC) and plasmacytoid DC (pDC), and that there are significant differences in functional role between these DC-subsets.[4,5]

mDCs originate from myeloid progenitors that are ontogenically closely related to the monocyte/macrophage-lineage.[4,5] Immature mDCs have a strong activity to capture foreign antigens and a weak or absent ability to stimulate primary T-cells, and are distributed throughout the peripheral tissues except for the brain.[4,6] After catching a foreign antigen, immature mDCs migrate to the T-zone of a lymph node (LN) via lymphatics, where they differentiate into mature DCs, which are generally called interdigitating cells, with a strong ability to stimulate naïve T-cells and to present the processed antigen to T-cells to initiate the T-cell-mediated immune response to the antigen.[4,6] Langerhans cells (LC) in the skin are one of the major representative types of mDC.[7] On the other hand, pDCs originate from pDC-precursors in the peripheral blood, which display plasmacytoid appearance and express Toll-like receptor (TLR)-7 and TLR-9, that recognize viral single-stranded RNA or bacterial and viral non-methylated CpG DNA, respectively.[8-11] It has been suggested that pDC-precursors directly enter the T-zone of inflamed lymphoid tissues through high endothelial venules (HEVs), and mature into interdigitating cells after secreting a large amount of type-I interferon upon stimulation with bacteria or viruses through these TLR.[12,13]

It has been also reported that the occurrence of mDCs and pDCs in LNs is different in different types of lymphadenitis. For example, a large number of LC-derived mature DCs (i.e., interdigitating cells) are accumulated in the paracortical area in dermatopathic lymphadenitis (DPL).[14] Numerous pDC-

Received : November 13, 2008
Revised : December 1, 2008
Accepted : December 18, 2008
[1)]Graduate School of Health Sciences, Okayama University and
[2)]Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
Address correspondence and reprint request to Kiyoshi Takahashi, M.D., Ph.D., Graduate School of Health Sciences, Okayama University, Shikata-cho 2-5-1, Okayama-city, 700-8558, Japan
E-mail : dend@md.okayama-u.ac.jp

precursors have been reported to be present in Kikuchi's lymphadenitis (KL ; histiocytic necrotizing lymphadenitis), suggesting the presence of a large number of pDCs in KL.[15,16] Thus, mDCs and pDCs are largely different from each other in origin, in function, and in distribution, but no method has been available to distinguish these two subsets immunohistochemically.

In routine human formalin-fixed, paraffin-embedded sections, S100 calcium-binding protein B (S100B) and fascin are well-known general histochemical markers for DCs.[17-19] However, we recently found that the distribution of $S100B^+$ DCs is largely different from that of $fascin^+$ DCs (unpublished observations). Our preliminary data suggested that such differences could reflect the distributional difference between mDCs and pDCs.

In the present study, we demonstrate that S100B is expressed in experimentally-induced mDCs but not in experimentally-induced pDCs, while fascin is expressed in mature DCs of both subsets. In this study, we precisely investigate DCs immunohistochemically in human formalin-fixed, paraffin-embedded tissue-sections by two-color immunofluorescence microscopy for S100B and fascin. The results strongly suggest that this method makes it possible to discriminate pDCs from mDCs in human routine pathologic tissue-sections.

MATERIALS AND METHODS

Antibodies

We used nonconjugated mouse monoclonal antibodies (mmAb) to human antigens, that included anti-CD1a, anti-CD3, anti-CD4, anti-CD8, anti-fascin, anti-CD123a, anti-HLA-DR (Dako Cytomation, Carpinteria, CA), and anti-Langerin (Monosan, Sanbio, Uden, Netherlands), and rabbit polyclonal antibody to S100B[20] and to lysozyme (Dako Cytomation). We also used biotin-conjugated mmAb to human CD123a (BD Pharmingen, San Diego, CA), fluorescein isothiocyanate (FITC)-conjugated mmAb including anti-HLA-DR (Becton Dickinson, San Jose, CA) and anti-CD86 (BD Pharmingen), and phycoerythrin (PE)-conjugated mmAb including anti-BDCA2 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD3 (Becton Dickinson), anti-CD4 (Becton Dickinson), anti-CD86 (Becton Dickinson) and anti-HLA-DR (Becton Dickinson).

Preparation of cells

1) pDC-precursors

In order to generate mature pDCs experimentally, pDC-precursors were isolated from the human tonsil by a method modified from one described previously.[21] Briefly, a human tonsil obtained from a patient with chronic tonsillitis was cut into 2-mm^3 cubes, and a single-cell suspension was obtained by gentle pressure with a glass homogenizer. After the removal of dead cells and erythrocytes by Ficoll-Hypaque density gradient centrifugation, cells were suspended in RPMI1640 (Nissui, Tokyo, Japan) containing 10% fetal calf serum (FCS), penicillin-streptomycin (10 mg/L, Gibco Life Technologies GmbH, Karlsruhe, Germany) and kanamycin sulfate (60 mg/L, Meiji, Tokyo, Japan) (complete RPMI1640).

The fresh tonsillar-cell suspension (10^8/mL) was incubated with biotin-conjugated mmAb to CD123a (20 mg/mL) for 30 min on ice. The cells were then washed with phosphate-buffered saline (PBS) containing 2% FCS, and incubated with FITC-conjugated streptavidin (50 mg/mL) and PE-conjugated anti-BDCA2 (20 mg/mL) for 30 min on ice. After being washed in PBS containing 2% FCS, the cells were resuspended in RPMI1640 containing 2% FCS (1 mL). $CD123a^+$ $BDCA2^+$ pDC-precursors were sorted by FACS Aria (BD Biosciences, San Jose, CA), and suspended in complete RPMI1640.

2) pDC

pDCs were generated from purified $CD123a^+$ $BDCA2^+$ pDC-precursors by the modified method described previously.[22] Briefly, pDC-precursors were incubated in complete RPMI1640 in the presence of recombinant human interleukin (IL)-3 (50 ng/mL ; R&D Systems, Abingdon, UK) and CD40-ligand (CD40L) recombinant protein (50 ng/mL ; GenWay Biotech, San Diego, CA) for 3 days at 37°C in 5% CO_2 and humidified air.

3) Monocyte-derived DCs (MoDCs)

MoDCs were generated from peripheral blood monocytes purified as described previously.[23] Immature MoDCs were generated by treatment of monocytes with human recombinant granulocyte-macrophage colony-stimulating factor (100 ng/mL ; PEPRO TECH EC LTD, London, UK) and IL-4 (20 ng/mL ; R&D systems) for 6 days. Mature MoDCs were generated by stimulation of immature MoDCs with tumor necrosis factor-α (20 ng/mL, PEPRO TECH EC LTD) and CD40L recombinant protein (50 ng/mL) for 4 days. LC-type MoDCs were generated by treatment of monocytes with granulocyte-macrophage colony-stimulating factor (100 ng/mL), IL-4 (20 ng/mL), and transforming growth factor-β (50 ng/mL, PEPRO TECH EC LTD) for 6 days as described previously.[24]

4) Mesenteric lymph nodal cells

A suspension of nonadherent mononuclear leukocytes was obtained from mesenteric LNs from patients with colon cancer without cancer-metastasis as described above. The leukocyte mixture was cultured in complete RPMI1640 for 3 days at 37°C in 5% CO_2 and humidified air.

Flow cytometric analysis

The pDCs and MoDCs were analyzed by flow cytometry. Cells were stained with rabbit polyclonal anti-S100B and mouse monoclonal anti-fascin. Rabbit polyclonal antibody was detected by FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and mmAbs ware detected by PE-conjugated donkey $F(ab')_2$ fragment to mouse IgG (Jackson ImmunoResearch Laboratories). For detection of the intracellular proteins S100B and fascin, the IntraPrep Permeabilization Reagent (Immunotech SAS a Beckman Coulter Company, Marseille, France) was used according to the manufacturer's instructions. The cells were treated with anti- S100B (20 mg/mL) and anti-fascin (20 mg/mL) and then incubated for 30 min on ice. After washing, the cells were incubated with FITC-conjugated goat anti-rabbit IgG (10 mg/mL) and PE-conjugated donkey $F(ab')_2$ fragment to mouse IgG (10 mg/mL) for 30 min on ice. The cells were then washed and fixed in 4% formaldehyde. They were analyzed by flow cytometry.

Tissue preparation

Skin tissues were obtained from 3 patients with solar keratosis and 3 patients with psoriasis vulgaris. Skin-draining LNs without pathological changes were obtained from a patient with skin malignant melanoma. Mesenteric LNs without pathological changes were obtained from a patient with colon cancer. Superficial LNs from 5 cases of DPL and 5 cases of KL were also examined. Tissues were routinely fixed with 10% formalin and embedded in paraffin, and 4-mm sections were examined immunohistochemically.

Immunochemistry

1) Pretreatment

Cultured cells were smeared on glass slides with a Cytospin (Sakura, Tokyo, Japan) and immediately fixed with 4% paraformaldehyde solution for 5 min, washed with PBS, and air-dried. Smeared cells were examined by two-color immunofluorescence microscopy as described below. Some smeared cells were stained with Giemsa. Dewaxed paraffin sections were immersed in citrate buffer (0.01 mol/L citrate, pH6.0), microwaved for 15 min at 600 W, and then cooled at room temperature for 60 min. All preparations were immersed in 10% normal goat serum for 30 min to block non-specific staining.

2) Two-color enzyme immunohistochemistry

To simultaneously detect two different antigens in tissue-preparations with mmAbs, we performed two-color enzyme immunohistochemistry by using immunoperoxidase and immunoalkaline-phosphatase methods. The immunoperoxidase method was performed by using DAKO Envision System Peroxidase (Dako Cytomation) as described previously.[25] After immunoperoxidase staining for the first antigen, sections were washed with PBS, immersed in citrate buffer, and microwaved for 15 min at 600 W. Sections were then rinsed with PBS and incubated with mmAb to a second antigen at 4°C overnight. After washing with PBS, sections were stained by immunoalkaline phosphatase methods using Dako Cytomation LSAB+ System-AP (Dako Cytomation) according to the manufacturer's instructions.

3) Two-color immunofluorescence microscopy

Smeared cells or microwaved paraffin sections were treated with both rabbit polyclonal antibody to S100B or lysozyme and mmAb to fascin, CD1a, CD3, CD4, CD8 or Langerin at 4°C overnight. After washing with PBS, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (10 mg/mL) and PE-conjugated donkey $F(ab')_2$ fragment to mouse IgG (10 mg/mL) at 4°C overnight. Some smeared cells were treated simultaneously with FITC-conjugated mmAb and PE-conjugated mmAb at optimal concentrations at 4°C overnight. After washing with PBS, the preparations were immersed in fluorescent mounting medium (Dako Cytomation) and examined with a LSM 510 confocal laser scanning microscope (Carl Zeiss, Tokyo, Japan).

RESULTS

Experimentally induced DCs

1) pDCs

As shown in Fig. 1a, pDC-precursors formed a distinctive small subpopulation of $CD123a^+$ $BDCA2^+$ leukocytes, which comprised approximately 0.8% of the whole leukocytes of the palatine tonsil. The $CD123a^+$ $BDCA2^+$ pDC-precursors were morphologically small, round cells with a clear juxtanuclear Golgi zone exhibiting a plasmacytoid appearance (Fig. 2a). They were negative for S100B and fascin. When they were induced to differentiate into mature DCs by treatment with CD40L and IL-3 for 3 days, they become larger dendriform cells extending fine dendrites (Fig. 2b). Two-dimensional flow-cytometric analysis for S100B and fascin indicated that these DC became positive for fascin, but continued to be negative for S100B (Fig. 1b). Two-color immunofluorescence microscopy also consistently indicated that they were $S100B^-$/$fascin^+$ (Fig. 2c), $S100B^-$/$HLA\text{-}DR^+$ (Fig. 2d), and $HLA\text{-}DR^+$/$CD86^+$ (Fig. 2e). Their expression of CD123a and BDCA2 was significantly down-regulated (Fig. 2f). Thus, we concluded that mature pDCs can be detected as $S100B^-$/$fascin^+$ DCs by two-color immunofluorescence microscopy for S100B and fascin, although they can hardly be detected by CD123a and/or BDCA2.

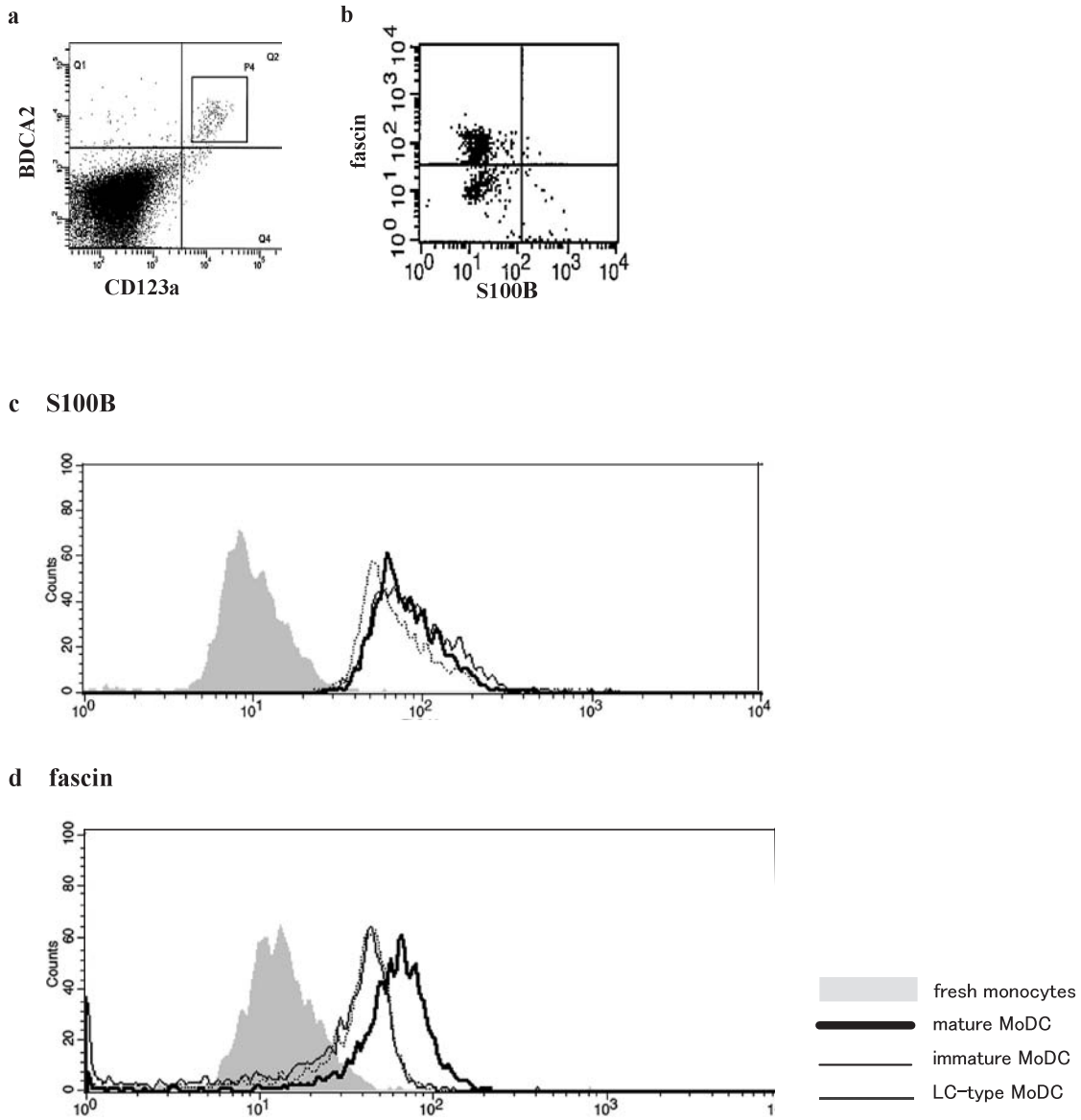

Fig. 1. Flow cytometric analysis of experimentally induced dendritic cells (DC). (***1a***) Two-dimensional flow cytometric analysis of plasmacytoid DC (pDC)-precursor for CD123a and BDCA2. $CD123a^{+}$ $BDCA2^{+}$ pDC-precursors comprised approximately 0.8% of whole leukocytes of the palatine tonsil. (***1b***) Two-dimensional flow cytometric analysis of experimentally induced mature pDC for S100 calcium binding protein B (S100B) and fascin. Mature pDC are $S100B^{-}$/$fascin^{+}$. (***1c***) Single-dimensional flow cytometric analysis of monocyte-derived DC (MoDC) for S100B. All three types of MoDC, immature, Langerhans cell (LC)-type, and mature MoDC are positive for S100B, and there is no significant difference in S100B-immunoreactivity among these three types of MoDC. (***1d***) Single-dimensional flow cytometric analysis of MoDC for fascin. Immature and LC-type MoDC are weakly positive for fascin, but the fascin-expression of mature MoDC is significantly upregulated.

2) mDCs

mDCs were experimentally induced from peripheral blood monocytes. Three types of MoDC were induced : immature MoDCs, LC-type MoDCs, and mature MoDCs. Single-dimensional flow-cytometric analysis indicated that all three types of MoDC were became positive for S100B and that there was no significant difference in S100B-immunoreactivity among them (Fig. 1c). Although immature and LC-type MoDCs were weakly positive for fascin, the

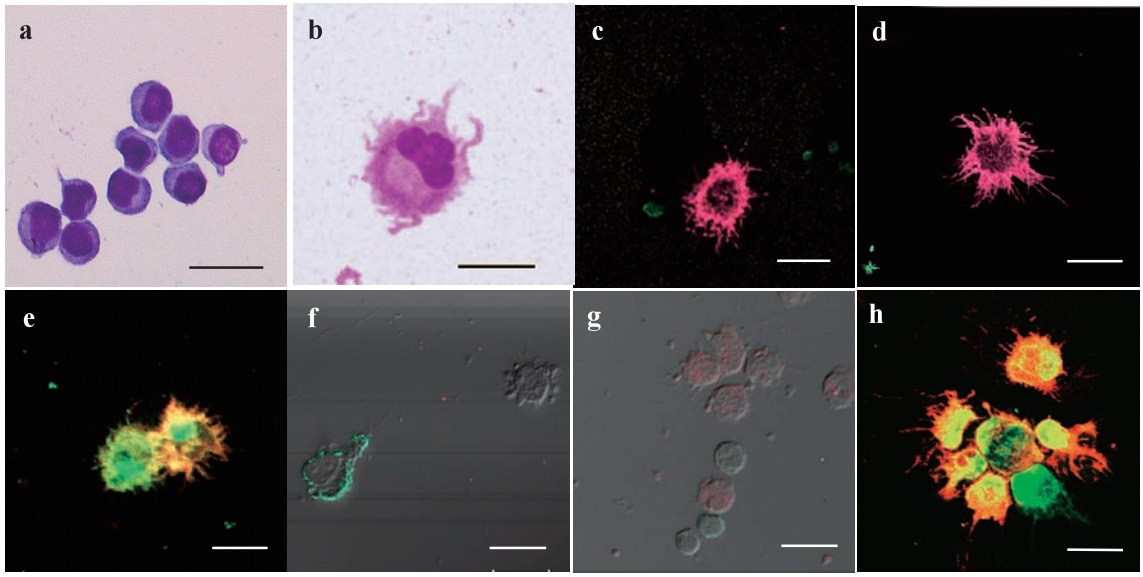

Fig. 2. Morphological analysis of experimentally induced dendritic cells (DC). (***2a***) Plasmacytoid dendritic cell (pDC)-precursors from the palatine tonsil exhibiting plasmacytoid appearance ; Giemsa staining. (***2b***) Mature pDCs induced by treatment with interleukin-3 and CD40L exhibiting extensively dendritic appearance ; Giemsa staining. (***2c-2e***) Two-color immunofluorescence analysis of experimentally induced pDCs for S100 calcium binding protein B [S100B ; fluorescein isothiocyanate (FITC)] and fascin [phycoerythrin (PE)] (***2c***), S100B (FITC) and HLA-DR (PE) (***2d***), and HLA-DR (FITC) and CD86 (PE) (***2e***). pDCs are $S100B^-$ $fascin^+$ $HLA\text{-}DR^+$ and $CD86^+$. (***2f***) Two color immunofluorescence analysis of pDC for CD123a (FITC) and BDCA2 (PE) counter-stained with differential interference contrast microscopy. Mature pDCs are almost negative for either antigen. (***2g***) Two-color immunofluorescence analysis of control monocytes for S100B (FITC) and fascin (PE) counter-stained with differential interference contrast microscopy. Note that control monocytes are completely negative for S100B and fascin. (***2h***) Two-color immunofluorescence analysis of control monocytes for S100B (FITC) and fascin (PE). Mature monocyte-derived DC are $S100B^+$/$fascin^+$ (*yellow*). Every scale bar indicates 20 μm.

expression of fascin was significantly upregulated in mature MoDCs (Fig. 1d). Two-color immunofluorescence microscopy for S100B and fascin consistently indicated that mature MoDCs were positive for S100B and fascin, while fresh monocytes were negative for both antigens (Fig. 2g, 2h).

Tissue-DC **in situ**

1) Epidermis

Epidermal LCs were identified as dendriform cells located in the suprabasal layer of the epidermis. Two-color immunofluorescence microscopy indicated that they were uniformly positive for S100B, HLA-DR, CD1a and Langerin, but negative for fascin (Fig. 3a).

2) Dermis

DCs were scarcely detected in the uninflamed dermis. However, a considerable number of DCs were detected in the inflamed dermis. We examined 2 different types of dermatitis, nonspecific dermatitis associated with solar keratosis and dermatitis associated with psoriasis vulgaris. Two-color immunofluorescence microscopy for S100B and fascin indicated that there were three types of DC (i.e., $S100B^+$/$fascin^-$ DC, $S100B^+$/$fascin^+$ DC, and $S100B^-$/$fascin^+$ DC) in the inflamed dermis. In nonspecific inflammation associated with solar keratosis, the majority of dermal DCs were $S100B^+$ DC ($S100B^+$/$fascin^-$ and $S100B^+$/$fascin^+$), while $S100B^-$/$fascin^+$ DCs were scarce (Fig. 3b). In contrast, almost all dermal DCs were $S100B^-$/$fascin^+$ in psoriasis vulgaris (Fig. 3c).

3) Superficial LNs

a. Skin-draining LNs

Two-color immunofluorescence microscopy for S100B and fascin indicated that a considerable number of DCs were present in the T-cell areas of the skin-draining LNs and that the majority of them were $S100B^+$/$fascin^+$ (Fig. 3d). There were also relatively small numbers of $S100B^+$/$fascin^-$ and $S100B^-$/$fascin^+$ DCs. CD1a and Langerin were detected on $S100B^+$ DCs, but not on $fascin^+$ DCs, indicating that these LC-associated molecules were expressed exclusively on $S100B^+$/$fascin^-$ DCs (Fig. 3e). Interestingly, $S100B^-$/$fascin^+$ DCs tended to be distributed around the HEVs (Fig. 3f).

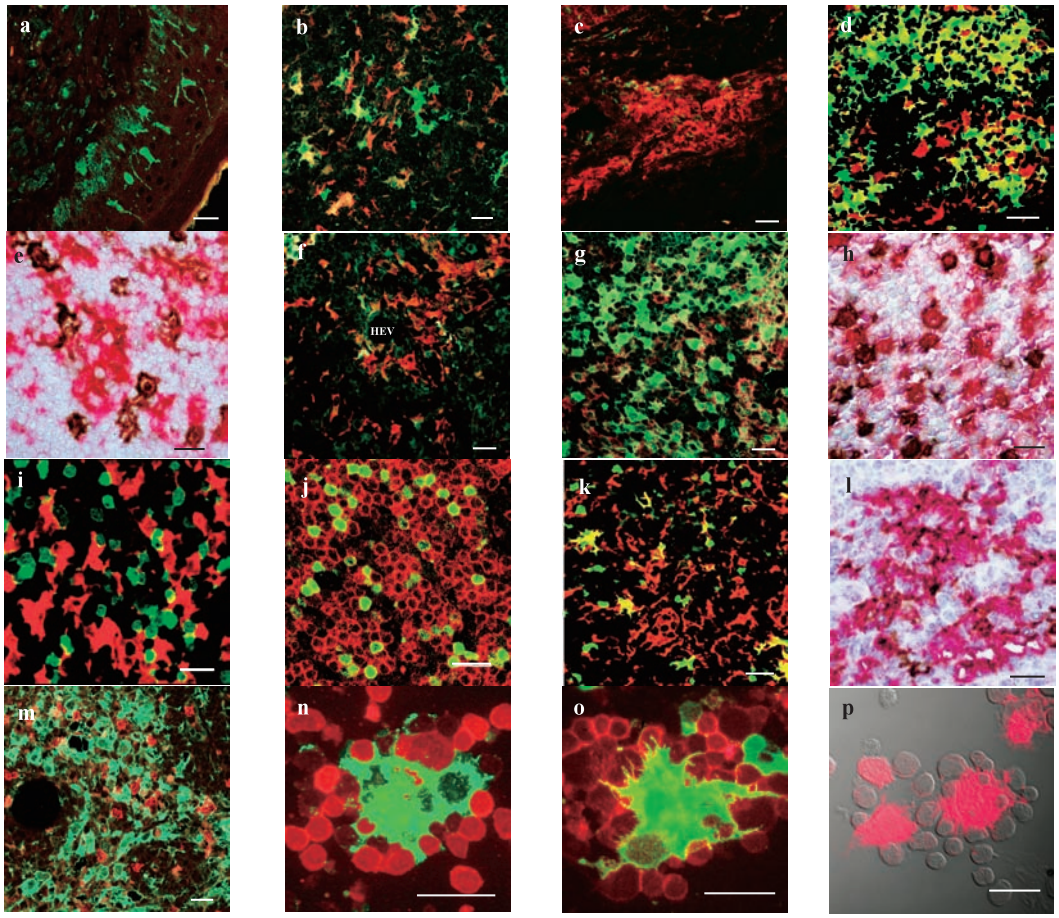

Fig. 3. Two-color immunohistochemical analysis for tissue-dendritic cells (DC). (***3a-3c***) Two-color immunofluorescence microscopy for S100 calcium binding protein B [S100B ; fluorescein isothiocyanate (FITC)] and fascin [phycoerythrin (PE)] of the skin. Epidermal Langerhans cells are $S100B^+$/$fascin^-$ (*green*) (***3a***). The majority of dermal DC in nonspecific dermatitis are $S100B^+$ (*green* or *yellow*) (***3b***), while almost all dermal DC in psoriasis vulgaris are $S100B^-$/$fascin^+$ (***3c***). (***3d***) Two-color immunohistochemical analysis of the skin-draining lymph nodes (LN) for S100B (FITC) and fascin (PE). Note that the majority of DC are $S100B^+$/$fascin^+$ (*yellow*). (***3e***) Two-color enzyme immunohistochemical analysis of the skin-draining LN for CD1a (*brown*) and fascin (*red*). Note that CD1a is expressed $fascin^-$ DC. (***3f***) Two-color immunohistochemical analysis of the skin-draining LN for S100B (FITC) and fascin (PE). Note that $S100B^-$/$fascin^+$ DC (*red*) tend to be present around high endothelial venule. (***3g***) Two-color immunohistochemical analysis of dermatopathic lymphadenitis (DPL) for S100B (FITC) and fascin (PE). Almost all DC accumulated in the paracortical area are $S100B^+$/$fascin^+$ in DPL (*yellow*). (***3h***) Two-color immunohistochemical analysis of DPL for CD1a (*brown*) and fascin (*red*). Note that CD1a is expressed on $fascin^+$ DC. (***3i***) Two-color immunofluorescence analysis of Kikuchis lymphadenitis (KL) for S100B (FITC) and fascin (PE). Note that almost all DC in KL are $S100B^-$/$fascin^+$ (*red*). Note numerous $S100B^-$ cells with lymphoid appearance (*green*) forming complexes with $S100B^-$/$fascin^+$ DC. (***3j***) Two-color immunofluorescence analysis of KL for S100B (FITC) and CD8 (PE). Note that $S100B^+$ lymphoid cells are $CD8^+$. (***3k***) Two-color immunofluorescence analysis of the mesenteric LN for S100B (FITC) and fascin (PE). Note numerous $S100B^-$/$fascin^+$ DC (*red*) and a small number of $S100B^+$/$fascin^-$ DC (*green*) and $S100B^+$/$fascin^+$ DC (*yellow*). (***3l***) Two-color enzyme immunohistochemistry of the mesenteric LN for fascin (*brown*) and HLA-DR (*red*). Note that $fascin^+$ dendriform cells are positive for HLA-DR. (***3m***) Two-color immunofluorescence analysis of the mesenteric LN for fascin (FITC) and lysozyme (PE). Note $fascin^+$ dendriform cells (*green*) are negative for lysozyme. (***3n-3p***) Two-color immunofluorescence analysis of cultured mesenteric LN-cells for HLA-DR (FITC) and CD3 (PE) (***3n***), CD86 (FITC) and CD4 (PE) (***3o***), and S100B (FITC) and fascin (PE) (***3p***). Note that cultured DC from the mesenteric LN form large complex with $CD3^+CD4^+$ T-cells and are mostly $S100B^-$/$fascin^+$. Every bar indicates 20 μm.

b. DPL

Two-color immunofluorescence microscopy for S100B and fascin indicated that the vast majority of DCs were $S100B^+/fascin^+$ (Fig. 3g). In contrast to normal skin-draining LNs, CD1a or Langerin was detected on not only $S100B^+$ DCs but also $fascin^+$ DCs in DPL, indicating that these LC-associated antigens were expressed on $S100B^+/fascin^+$ DCs in DPL (Fig. 3h).

c. KL

Two-color immunofluorescence microscopy for S100B and fascin indicated that almost all DCs in KL were $S100B^-/fascin^+$ (Fig. 3*l*). Interestingly, instead of $S100B^+$ DCs, numerous $S100B^+$ small lymphoid cells were detected around $S100B^-/fascin^+$ DCs. $S100B^+/fascin^-$ small lymphoid cells often formed complexes with $S100B^-/fascin^+$ DCs. Two-color fluorescence microscopy for S100B and CD3 or CD8 indicated that $S100B^+$ small lymphoid cells were $CD3^+CD8^+$ T cells (Fig. 3j). Thus, $S100B^+$ DCs were scarcely detected in KL.

d. Deep LNs

We also examined mesenteric LNs as deep LNs. Two-color immunofluorescence microscopy for S100B and fascin indicated that there were numerous DCs in the mesenteric LNs, and that the vast majority of them were $S100B^-/fascin^+$ (Fig. 3k). These $S100B^-/fascin^+$ DCs were large, extensively dendriform cells extending fine dendrites. Two-color enzyme immunostaining for fascin and HLA-DR or lysozyme indicated that these $fascin^+$ dendriform cells were positive for HLA-DR but negative for lysozyme (Fig. 3*l*, 3m).

A small number of S100B/$fascin^+$ DCs with a dendritic appearance were detected in the T-cell areas. In contrast, $S100B^+/fascin^-$ DCs, which were also detected in the T-cell areas in a small number, tended to be smaller and less dendritic than $fascin^+$ DCs.

Cultured DCs from the mesenteric LNs

Cultured cells obtained from the mesenteric LNs were examined by two-color immunofluorescence microscopy. DCs were clearly detected as $HLA\text{-}DR^{hi}CD86^+$ extensively dendriform cells forming complexes with numerous $CD3^+$ $CD4^+$ T cells (Fig. 3n, 3o). Two-color immunofluorescence microscopy for S100B and fascin indicated that almost all DCs in the mesenteric LN-cultures were $S100B^-/fascin^+$ (Fig. 3p).

DISCUSSION

The mDCs and pDCs serve their own roles in immunity and cooperate to remove exogenous pathogens. When certain exogenous pathogens, such as bacteria and viruses, invade peripheral tissues, the residing immature mDCs are recruited to the inflamed sites, where they capture the exogenous pathogen and then migrate to the draining LN.[1-3,6] The immature mDCs then undergo full maturation and present peptide-MHC-class II or peptide-MHC-class I complexes to naïve T cells, thereby inducing the immune response to the exogenous pathogen.[1-3,6] On the other hand, pDC-precursors in the peripheral blood, which lack phagocytic activity to exogenous pathogens, directly enter the T-zones of inflamed lymphoid tissues via HEV.[8-12] The pDC-precursors produce a large amount of type I interferon, upon stimulation with single-stranded viral RNA or bacterial/viral non-methylated CpG DNA through TLR7 or TLR9, to stimulate mDC-mediated immune responses to the exogenous pathogens. Then, the pDC-precursors differentiate into mature DCs (i.e., pDCs) to regulate the immunological reaction to the pathogen.[8-11] Little is known about the functional role of pDCs in the immune system.

The behaviors of pDCs should be examined to gain a better understanding of their role in the immune system. However, no immunohistochemical method has previously been available to distinguish pDCs from mDCs. Although several antigens, such as CD123a and BDCA2, are specific for pDC-precursors, these antigens are usually absent from the surface of mature pDCs.[21] Therefore, although the distribution of pDC-precursors in peripheral tissues has been examined, the distribution of mature pDCs remains unknown. Thus, we have attempted to establish an immunohistochemical method to distinguish human pDCs from mDCs in routine, formalin-fixed, paraffin-embedded tissue sections.

Both S100B and fascin have been used as general DC-markers in routine, formalin-fixed, paraffin-embedded tissue-sections from humans.[17-19] However, we recently found that the distribution of $S100B^+$ DCs is largely different from that of $fascin^+$ DCs in human tissues.[25] In this study, we found that epidermal LC and mature LC-derived DCs (interdigitating cells) are positive for S100B and that there are few $S100B^+$ DCs, despite the presence of numerous $fascin^+$ DCs in KL, in which numerous pDC-precursors have been reported to be present. These results suggest that S100B is a marker for mDCs but not for pDCs. LCs isolated from the epidermis are initially negative for fascin but acquire this molecule during morphological maturation *in vitro*.[26] Additionally, the expression of fascin in DCs has been correlated with their dendritic morphological maturation.[27] These findings strongly suggest that fascin is a marker for mature DCs in common with mDCs and pDCs.

To confirm this idea, we experimentally induced mDCs from blood monocytes and pDCs from pDC-precursors isolated from the palatine tonsil, and then we immunocytochemically examined these cells. We found that both immature and mature MoDCs were strongly positive for S100B. Although immature MoDCs were weakly positive for fascin, the fascin

expression by mature MoDCs were significantly upregulated. Although LCs *in situ* are completely negative for fascin, LC-type MoDCs were weakly positive for fascin. This discrepancy may indicate that LC-type MoDCs are to some extent more mature than LCs *in situ*.

We found that CD123a$^+$ BDCA2$^+$ pDC-precursors purified from the palatine tonsil had a plasmacytoid appearance and were negative for S100B and fascin and that they were converted to S100B$^-$/fascin$^+$ mature DCs upon stimulation with IL-3 and CD40L. These results clearly indicate that S100B is not a marker for pDCs, whereas fascin is a marker for pDCs. This finding strongly suggests that pDCs can be distinguished from mDCs by the absence of S100B. Because both S100B and fascin can be easily detected immunohistochemically in formalin-fixed, paraffin-embedded tissue sections, we expected that double immunostaining for S S100B and fascin would distinguish pDCs from mDCs in routine pathological sections.

The present finding that LCs in the epidermis were uniformly S100B$^+$/fascin$^-$ is consistent with the fact that LCs are immature mDCs. Although DCs were scarcely detected in uninflamed dermis, a considerable number of them were detected in inflamed dermis. The present findings that dermal DCs were classified into S100B$^+$/fascin$^-$ DCs, S100B$^+$/fascin$^+$ DCs, and S100B$^-$/fascin$^+$ DCs suggests that both immature and mature mDCs and pDCs are present in the inflamed dermis. Consistent with this idea, the presence of pDC-precursors in the inflamed dermis has been reported previously.[28] We supposed that a large number of pDCs are present in the dermis with psoriasis vulgaris, because numerous pDC-precursors are distributed in the dermis with psoriasis vulgaris, and an excess amount of type I interferon secreted by pDC-precursors in the dermis is responsible for the pathogenesis of psoriasis vulgaris.[29] Consistent with this supposition, we detected a large number of S100B$^-$/fascin$^+$ DCs in the dermis with psoriasis vulgaris. In contrast, a relatively small numbers of S100B$^-$/fascin$^+$ DCs were detected in the dermis with nonspecific inflammation associated with solar keratosis.

The present finding that the majority of DCs were S100B$^+$/fascin$^+$ in the skin-draining LNs may indicate that LCs are major sources of DCs in the superficial LN. Our finding that a large number of S100B$^+$/fascin$^+$ DCs were accumulated in the paracortical areas of the LNs, combined with the fact that epidermal LCs are a major source of DCs in superficial LN is consistent with the fact that numerous LC-derived DCs are accumulated in DPL.[14] Interestingly, although CD1a and Langerin were detected exclusively on immature LC-derived DCs (i.e., S100B$^+$/fascin$^-$ DCs) in normal skin-draining LN, these antigens were detected on S100B$^+$/fascin$^+$ DCs in DPL. These findings are consistent with our previous findings that LC-derived DCs in DPL were specifically activated.[30]

A large number of pDC-precursors are present in the superficial LN with KL.[15,16] KL is a rare, benign and self-limiting disorder that characteristically presents with fever and cervical lymphadenopathy.[15] Numerous pDC-precursors are present in the T-cell area around the granuloma,[15,16] suggesting that a large number of pDCs are present in KL. Consistently, almost all DCs detected in KL were S100B$^-$/fascin$^+$ DCs, while S100B$^+$ DCs were scarcely detected. Instead of S100B$^+$ DCs, there were large numbers of S100B$^+$ CD3$^+$CD8$^+$ T-cells. This is consistent with the fact that CD8$^+$ T-cells are significantly increased in KL.[15] These S100B$^+$ T-cells formed complexes with S100B$^-$/fascin$^+$ DCs. In addition, we detected a large number of FoxP3$^+$ regulatory T-cells forming complexes with S100B$^-$/fascin$^+$ DCs (data not shown). These findings suggest that pDCs have an immunoregulatory function in KL. In the present study, we detected numerous S100B$^-$/fascin$^+$ DCs and only a small number of S100B$^+$ DCs in deep LNs. We have usually used S100B by itself as a marker for DCs until recently ; thus, these findings were quite unexpected. The findings that these S100B$^-$/fascin$^+$ DCs in deep LNs were large, extensively dendriform cells negative for lysozyme and positive for HLA-DR strongly suggest that they are not macrophages but true DCs. Moreover, the findings that these S100B$^-$/fascin$^+$ DCs strongly expressed HLA-DR and CD86 and formed large complexes with CD3$^+$CD4$^+$ T-cells confirm that S100B$^-$/fascin$^+$ DCs in deep LNs are mature DCs. Therefore, it is probable that the vast majority of DCs in deep LN are not mDCs but pDCs. It seems likely that pDC-precursors directly enter the T-zone of the deep LNs from the peripheral blood and mature into pDCs. We also detected a small number of S100B$^+$ DCs in deep LNs. We believe that they are mDCs that may originate from immature mDCs distributed in the peripheral tissues such as the mucosa of the gastrointestinal tract. The fact that they were small in number suggests that the presentation of foreign antigens by mDCs is usually inactive in the gastrointestinal system.

In conclusion, we found that double immunostaining for S100B and fascin seems to be very useful to immunohistochemically distinguish pDCs from mDCs in routine pathological sections. We believe that this method will yield new information about DC-mediated immunity.

REFERENCES

1 Banchereau J, Steinman RM : Dendritic cells and the control of immunity. Nature 392 : 245-252, 1998

2 Steinman RM : Dendritic cells and the control of immunity : enhancing the efficiency of antigen presentation. Mt Sinai J Med 68 : 160-166, 2001

3 Munz C, Steinman RM, Fujii S : Dendritic cell maturation by innate lymphocytes : coordinated stimulation of innate and adaptive immunity. J Exp Med 202 : 203-207, 2005

4 Ardavin C, del Hoyo GM, Martin P, Anjuere F, Arias CF, Marin AR, Ruiz S, Parrillas V, Hernandez H : Origin and differentiation of dendritic cells. Trends Immunol 22 : 691- 700, 2001

5 del Hoyo GM, Martin P, Vargas HH, Ruiz S, Arias CF, Ardavin C : Characterization of a common precursor population for dendritic cells. Nature 415 : 1043- 1047, 2002

6 Becker Y : Immunological and regulatory functions of uninfected and virus infected immature and mature subtypes of dendritic cells -a review. Virus Genes 26 : 119- 130, 2003

7 Silberberg I, Baer RL, Rosenthal SA : The role of Langerhans cells in allergic contact hypersensitivity. A review of findings in man and guinea pigs. J Invest Dermatol 66 : 210- 217, 1976

8 Colonna M, Trinchieri G, Liu Y-J : Plasmacytoid dendritic cells in immunity. Nature Immunol 5 : 1219- 1226, 2004

9 Cox K, North M, Burke M, Singhal H, Renton S, Aqel N, Islam S, Knight SC : Plasmacytoid dendritic cells (PDC) are the major DC subset innately producing cytokines in human lymph nodes. J Leukoc Biol 78 : 1142- 1152, 2005

10 Liu Y-J : IPC : Professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu Rev Immunol 23 : 275- 306, 2005

11 Soumelis V, Liu YJ : From plasmacytoid to dendritic cell : morphological and functional switches during plasmacytoid predendritic cell differentiation. Eur J Immunol 36 : 2286- 2292, 2006

12 Yoneyama H, Matsuno K, Zhang Y : Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. Int Immunol 16 : 915- 928, 2004

13 Yrlid U, Cerovic V, Milling S : Plasmacytoid dendritic cells do not migrate in intestinal or hepatic lymph. J Immunol 177 : 6115- 6121, 2006

14 Geissmann F, Dieu-Nosjean MC, Dezutter C, Valladeau J, Kayal S, Leborgne M, Brousse N, Saeland S, Davoust J : Accumulation of immature Langerhans cells in human lymph nodes draining chronically inflamed skin. J Exp Med 196 : 417- 430, 2002

15 Bosch X, Guilabert A, Miquel R, Campo E : Enigmatic Kikuchi-Fujimoto disease : a comprehensive review. Am J Clin Pathol 122 : 141- 152, 2004

16 Lin CW, Liu TY, Lin CJ, Hsu SM : Oligoclonal T cells in histiocytic necrotizing lymphadenopathy are associated with $TLR9^{+}$ plasmacytoid dendritic cells. Lab Invest 85 : 267- 275, 2005

17 Steinman RM, Pack M, Inaba K : Dendritic cells in the T-cell areas of lymphoid organs. Immunol Rev 156 : 25- 37, 1997

18 Shrestha P, Muramatsu Y, Kudeken W, Mori M, Takai Y, Ilg EC, Schafer BW, Heizmann CW : Localization of Ca^{2+}-binding S100 proteins in epithelial tumours of the skin. Virchows Arch 432 : 53- 59, 1998

19 Ross R, Jonuleit H, Bros M, Ross XL, Yamashiro S, Matsumura F, Enk AH, Knop J, Reske-Kunz AB : Expression of the actin-bundling protein fascin in cultured human dendritic cells correlates with dendritic morphology and cell differentiation. J Invest Dermatol 115 : 658- 663, 2000

20 Takahashi K, Isobe T, Ohtsuki Y, Sonobe H, Takeda I, Akagi T : Immunohistochemical localization and distribution of S-100 proteins in the human lymphoreticular system. Am J Pathol 116 : 497- 503, 1984

21 Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, Buck DW, Schmitz J : BDCA-2, BDCA-3, and BDCA-4 : three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 165 : 6037- 6046, 2000

22 Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ : The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J Exp Med 185 : 1101- 1111, 1997

23 Scandella E, Men Y, Legler DF, Gillessen S, Prikler L, Ludewig B, Groettrup M : CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. Blood 103 : 1595- 1601, 2004

24 Sallusto F, Lanzavecchia A : Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 179 : 1109- 1118, 1994

25 Takahashi K, Nishikawa Y, Sato H, Oka T, Yoshino T, Miyatani K : Dendritic cells interacting mainly with B cells in the lymphoepitehelial symbiosis of the human palatine tonsil. Virchows Arch 448 : 623- 629, 2006

26 Ross R, Ross XL, Schwing J, Längin T, Reske-Kunz AB : The actin-bundling protein fascin is involved in the formation of dendritic processes in maturing epidermal Langerhans cells. J Immunol 160 : 3776- 3782, 1998

27 Al-Alwan MM, Rowden G, Lee TD, West KA : Fascin is involved in the antigen presentation activity of mature dendritic cells. J Immunol 166 : 338- 345, 2001

28 Wollenberg A, Wagner M, Günther S, Towarowski A, Tuma E, Moderer M, Rothenfusser S, Wetzel S, Endres S, Hartmann G : Plasmacytoid dendritic cells : a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. J Invest Dermatol 119 : 1096- 1102, 2002

29 Gilliet M, Conrad C, Geiges M, Cozzio A, Thürlimann W, Burg G, Nestle FO, Dummer R : Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. Arch Dermatol 140 : 1490- 1495, 2004

30 Takahashi K, Asagoe K, Zaishun J, Yanai H, Yoshino T, Hayashi K, Akagi T : Heterogeneity of dendritic cells in human superficial lymph node : *in vitro* maturation of immature dendritic cells into mature or activated interdigitating reticulum cells. Am J Pathol 153 : 745- 755, 1998