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Distribution of Dendritic Cells and Regulatory T-Cells in Cutaneous Lymphomas

Changyoung Yoo · Young Seon Hong[1] Baik Kee Cho[2] · Sang Ho Kim[3] Sang In Shim[4] · Chang Suk Kang[4]

Department of Hospital Pathology, St. Vincent's Hospital, Suwon; [1]Department of Internal Medicine, Seoul St. Mary's Hospital; [2]Department of Dermatology, Yeouido St. Mary's Hospital; [3]Department of Pathology, Songeui Campus; [4]Department of Hospital Pathology, Yeouido St. Mary's Hospital, The Catholic University of Korea College of Medicine, Seoul, Korea

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Corresponding Author
Chang Suk Kang, M.D.
Department of Hospital Pathology, Yeouido St. Mary's Hospital, The Catholic University of Korea College of Medicine, 62 Yeouido-dong, Yeongdeungpo-gu, Seoul 150-713, Korea
Tel: +82-2-3779-1312
Fax: +82-2-783-6648
E-mail: cskang@catholic.ac.kr

Background : Dendritic cells (DCs) play an important role in immune reactions. This study was designed to identify the distribution patterns of DCs and regulatory T-cells (Tregs) in cutaneous lymphomas. **Methods :** Immunohistochemistry was used to determine langerin expression on Langerhans cells, CD11b on inflammatory DCs, CD209 and CD11c on dermal DCs, CD303 on plasmacytic DCs, and Foxp3 on Tregs in 81 cases of cutaneous lymphomas. **Results :** Various DCs and Tregs were identified in most cutaneous lymphomas. Plasmacytic DCs, inflammatory DCs and Tregs were identified mainly in tumor areas, whereas dermal DCs were distributed both in the tumor and stromal areas. Among DCs, dermal DCs were most prominently identified in the cutaneous lymphomas not only in the tumor area but also in the stroma. The intense stromal infiltration of dermal DCs was consistent finding in T-cell lymphomas. Diffuse large B-cell lymphoma (DLBCL), not otherwise specified also showed intense stromal infiltration of dermal DCs, but stromal infiltration in DLBCL, leg type was relatively scant. **Conclusions :** The results suggest that all types of DCs and Tregs are involved in cutaneous lymphoma tumor immunity. Among them dermal DCs may play a dominant role.

Key Words : Dendritic cells; T-Lymphocytes, regulatory; Lymphomas, cutaneous

The depth of understanding of dendritic cells (DCs) has increased greatly, largely due to studies involving inflammatory conditions. As a result, several DC subtypes have been identified.[1,2] DCs have many important roles, such as antigen capture, processing, and presentation to primitive T cells, so DCs are involved in innate and adaptive immunity or in T cell tolerance, and thereby have an influence on the progression of several diseases.[2,3] In the skin, several kinds of DCs have been identified, which are involved not only in various inflammatory conditions, but also in the occurrence of skin cancers.[4-7]

Every type of DC originates from hematopoietic stem cells induced by several cytokines and transcription factors.[1,2] Langerhans cells (LCs) are distributed in the suprabasal layer of the skin and play an important role in immune tolerance.[4,7-9] Dermal DCs (dDCs) act as a primary sentinel in participation with CD4+ T cells, CD8+ T cells, and regulatory T cells (Tregs).[1,4] Plasmacytic DCs (pDCs) secrete type I interferon in the presence of inflammatory stimuli, especially viral infections.[7,10,11] Inflammatory DCs (iDCs) appear only in the presence of inflammation[1,2] and express mediators that can be used as surface markers for immunohistochemical detection.[1,2,4,5,7,8]

Traditionally, cutaneous lymphomas have been separated from non-cutaneous lymphomas because of different clinical course. Cutaneous lymphomas include diverse disease entities, which may provide fertile ground for studies about DCs. This study was designed to determine the distribution patterns of several types of DCs in various cutaneous lymphomas and to scrutinize their immunological meaning. Additionally, because Tregs are

involved in immune response regulation, we investigated the infiltration grade of Tregs to understand immune regulation in the context of DC physiology.

MATERIALS AND METHODS

Materials

Eighty one cases of formalin-fixed paraffin embedded tissues (FFPET) diagnosed as cutaneous lymphomas at the Catholic University Hospital between 1999 and 2008 were used in this study. They included 11 diffuse large B-cell lymphomas, leg type (DLBCLLEG), 14 DLBCL, not otherwise specified (DLBCLNOS), 16 extranodal NK/T-cell lymphomas, 16 anaplastic large cell lymphomas, and 24 peripheral T-cell lymphomas. Clinical data including age, gender, clinical stages, and international prognostic index (IPI) were collected from existing medical records. Approval was obtained from the Institutional Review Board of the Catholic University of Korea, College of Medicine. Paraffin blocks, which were well-preserved and had been diagnosed by a full immunohistochemistry study, were selected. The cases were reviewed and reclassified according to the 2006 World Health Organization/European Organization of Research and Treatment of Cancer classification.[12]

Immunohistochemistry

Among the specific markers for DCs,[1,2,4,5,7,8] langerin for LCs, dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin (CD209), and CD11c for dDCs, CD11b for iDCs, and CD303 for pDCs were selected for immunohistochemistry. Foxp3 were used for the Tregs (Table 1). Proteinase K (Dako, Glostrup, Denmark), pepsin, and boiling in a pressure cooker with pH 6.0 citrate buffer were applied separately for antigen retrieval according to antibody type.

Langerin, CD209, CD303, and Foxp3 showed the best staining results with the boiling method using citrate buffer (pH 6.0) in the pressure cooker, whereas CD11b and CD11c showed satisfactory results with proteinase K treatment. After antigen retrieval, subsequent applications of primary antibodies, secondary Envision antibody (Dako), and diaminobenzidine (Dako) were applied as standard immunohistochemistry procedures.

Staining interpretation

An arbitrary criterion was used in this study because DCs and Tregs are minor components of an immune reaction and the standardized criteria for the gradation of their infiltration status was not found in preexisting reports. The infiltration status of DCs and Tregs was simplified into a two grade system: low grade included cases that seldom showed infiltration (approximately 0 to 3 cells in high power field [HPF]). High grade included cases that showed infiltration of more than three cells in HPF. After ten HPF were examined, the highest number of cells was determined to be the relevant cell number. Because the cellular density of dDCs stained with CD209 was relatively high when compared with other DCs and Tregs, less than ten cells were grouped into the low grade and more than ten cells were considered high grade. Staining was interpreted as positive when the membrane staining pattern with or without cytoplasmic staining was obvious for LCs, iDCs, dDCs, and pDCs. Only the nuclear staining pattern was regarded as positive for Tregs.

Statistical analysis

The infiltration status of DCs and Tregs was compared based on diagnostic entities and clinical or histological parameters such as age, gender, stage, and IPI using a chi-square test. Spearman's

Table 1. Antibodies used in this study

Antibodies	Cells stained	Staining patterns	Clonality	Sources	Dilutions
Langerin/CD207	Langerhans cells	Membrane	Monoclonal	Abcam, Cambridge, UK	1 : 500
CD11b/ITGAM	Inflammatory dendritic cells	Membrane	Monoclonal	Lifespan Biosciences, Seattle, WA, USA	1 : 200
CD11c/integrin α_x	Dermal dendritic cells (dDCs)	Membrane	Monoclonal	American Diagnostica Inc., Greenwich, CT, USA	1 : 200
CD209/DC-SIGN	dDCs	Membrane	Monoclonal	Abcam	1 : 40
CD303/BDCA-2	Plasmacytoid dendritic cells	Membrane	Polyclonal	Abcam	1 : 500
Foxp3	Regulatory T cells	Nucleus	Polyclonal	Abcam	1 : 500

ITGAM, integrin alpha M; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; BDCA, blood dendritic cell antigen.

correlation coefficient was used to determine the correlations among various DCs and Tregs. The Kaplan-Meier method was used for the survival analysis. p-values < 0.05 were considered statistically significant. The statistical analysis was performed using the SPSS ver. 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Case characteristics

The patient age distribution was from 12 to 102 years; 45 patients were males and 36 were females. In the stage DLBCL, NOS showed a tendency to be a higher stage than DLBCL, LEG ($p = 0.040$) and T-cell lymphomas showed a tendency to be a higher stage than B-cell lymphomas ($p = 0.003$), when compared by linear-by-linear association. In the case of IPI, T-cell lymphomas also showed a higher risk than B-cell lymphomas ($p = 0.004$). The follow-up periods ranged from 1 to 107 months.

Staining patterns

LCs were distributed mainly in the Malphigian layer. Relatively large numbers of dDCs stained with CD209 were identified in the majority of cases either in intratumoral or stromal areas. dDCs were distributed in the vicinity of the stromal tumor margin. The number of iDCs and pDCs varied and were identified mainly in intratumoral areas. Interestingly, pDCs were found more frequently near blood vessels. The majority of Tregs were found in intratumoral areas (Fig. 1).

The dDC distribution patterns are worth special mention. Stromal dDCs were consistently found in various types of T-cell lymphomas. In DLBCL, dDC stromal distributions were prominent in DLBCLNOS, but dDCs were distributed mainly in the intratumoral areas in DLBCLLEG (Fig. 2).

Frequencies and correlations of DCs and Tregs

Frequencies of DCs and Tregs

As seen in the staining patterns, the dDC distribution patterns were statistically significant when tested by linear association with the chi-square test ($p = 0.011$). The distribution patterns of other DCs and Tregs were not statistically significant (Table 2).

Correlations of various DCs and Tregs

In the statistical correlation test using Spearman's rho correlation for the nonparametric variables, statistical significances were found between LCs and pDCs ($p = 0.020$), iDCs and intratumoral dDCs ($p = 0.023$), and dDC intratumoral and stromal areas ($p = 0.012$) (Table 3).

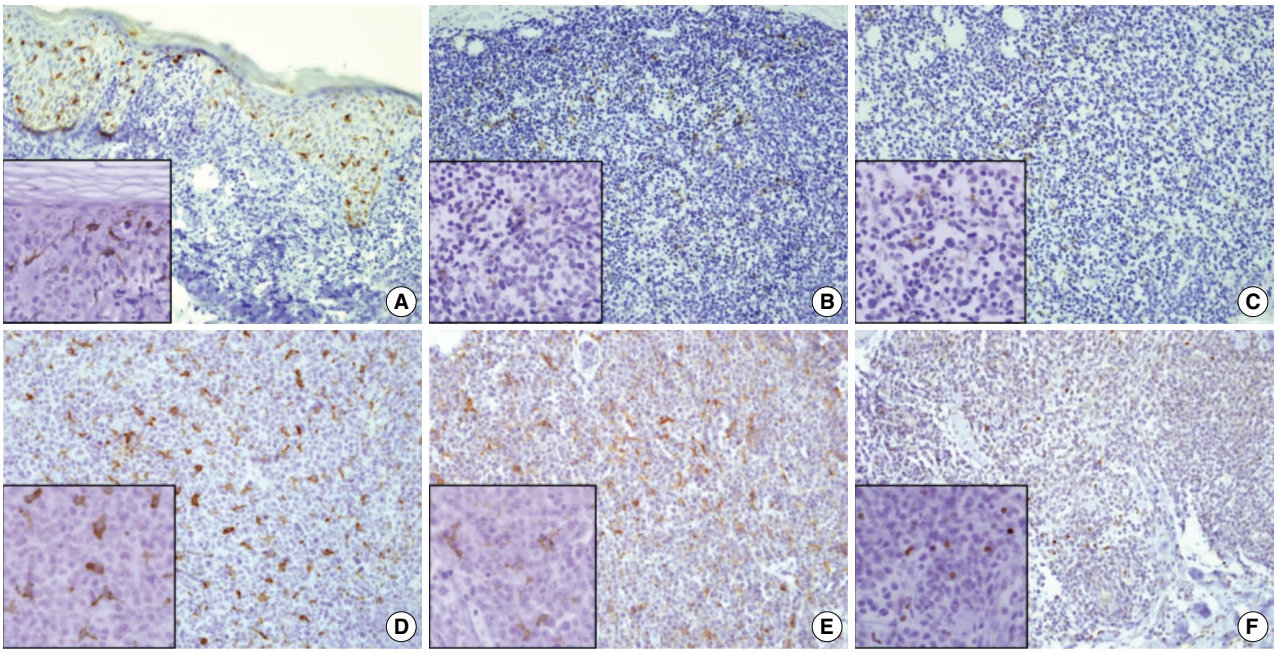

Fig. 1. Immunohistochemical findings of dendritic cells (DCs) and regulatory T-cells (Tregs). (A) Langerhans cells show a limited distribution in the epidermis. (B) Inflammatory DCs show faint membranous staining and (C) dermal DCs (dDC) stained with CD11c also show faint membranous staining. (D) dDCs stained with CD209 and (E) plasmacytic DCs show a dendritic form with membranous staining. (F) Tregs show nuclear staining.

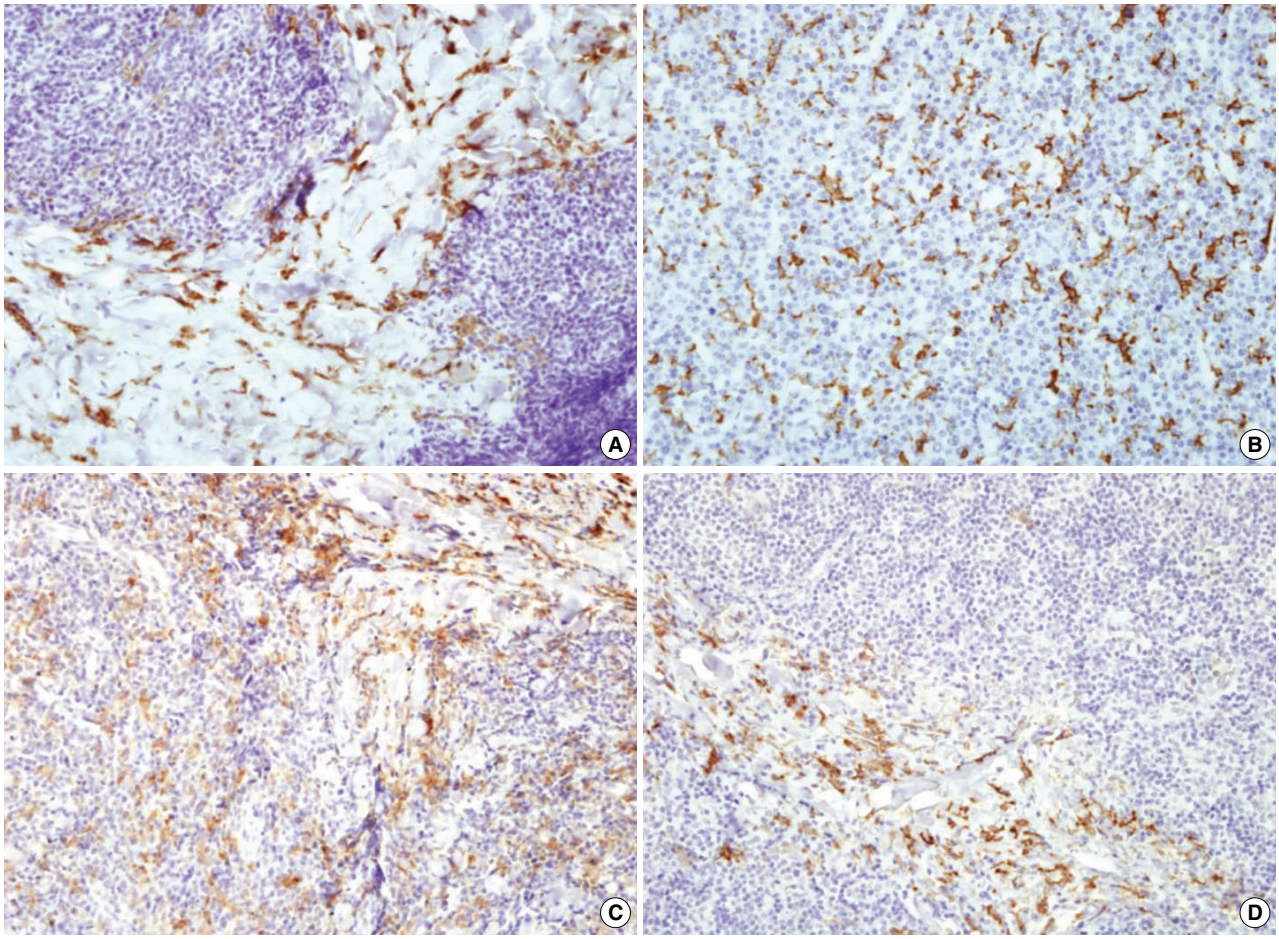

Fig. 2. The distribution patterns of dermal dendritic cells (dDCs) stained with CD209. (A) Intense stromal infiltrations are noted in diffuse large B-cell lymphomas, not otherwise specified, (B) while dDCs are found mainly in the intratumoral area in diffuse large B-cell lymphomas, leg type. (C) In T-cell lymphomas, dDCs are found both in the intratumoral and stromal areas, (D) but intense infiltration in the stromal areas is a more constant finding in various types of T-cell lymphomas.

Table 2. Frequency of high grade infiltrations of dendritic cells and regulatory T-cells (Tregs)

	No. of cases	LCs (langerin)	iDCs (CD11b)	dDCs (CD11c)	dDCs (CD209, tumor)	dDCs (CD209, stroma)	pDCs (CD303)	Tregs (Foxp3)
DLBCLLEG	11	10 (90.9)[a]	7 (63.6)	6 (54.5)	5 (45.4)	1 (9.1)	7 (63.6)	3 (27.3)
DLBCLNOS	14	10 (71.4)	10 (71.4)	7 (50.0)	6 (42.8)	9 (64.3)	8 (57.1)	7 (50.0)
NKTL	16	9 (56.3)	11 (68.7)	7 (43.7)	11 (68.7)	13 (81.3)	8 (50.0)	2 (12.5)
ALCL	16	15 (93.8)	8 (50.0)	10 (62.3)	14 (87.5)	10 (62.3)	10 (62.3)	7 (43.7)
PTCL	24	19 (79.2)	15 (62.5)	10 (41.7)	7 (29.2)	17 (70.8)	9 (37.5)	10 (41.7)
Total	81	63 (77.8)	51 (62.9)	40 (49.4)	43 (53.1)	50 (61.7)	42 (51.8)	29 (35.8)
p-value[b]		0.818	0.555	0.657	0.652	**0.011**	0.181	0.515

[a]Parentheses represent percentage (%); [b]Statistical significance test was done by linear-by-linear association of a chi-square test.
LCs, Langerhans cells; iDCs, inflammatory dendritic cells; dDCs, dermal dendritic cells; pDCs, plasmacytic dendritic cells; DLBCLLEG, diffuse large B-cell lymphoma, leg type; DLBCLNOS, diffuse large B-cell lymphoma, not otherwise specified; NKTL, extranodal NK/T cell lymphoma; ALCL, anaplastic large cell lymphoma; PTCL, peripheral T-cell lymphoma.

Relationships between DC and Treg infiltration status and clinical and histologic parameters

The infiltration status of DCs and Tregs did not show statistically significant differences when compared by gender, stage, or IPI. For the statistical analysis, the stages were divided into a low stage group, including stages Ia, Ib, IIa, and IIb, and a high stage group including stages III and IV. IPI was also divided into a low risk group including low and low intermediate risks, and

Table 3. Spearman's rho correlation coefficients among dendritic cells and regulatory T-cells (Tregs)

	LCs (langerin)	iDCs (CD11b)	dDCs (CD11c)	dDCs (CD209, tumor)	dDCs (CD209, stroma)	pDCs (CD303)	Tregs (Foxp-3)
LCs (langerin)							
iDCs	- 0.102[a]						
(CD11b)	0.363[b]						
dDCs	- 0.185	- 0.009					
(CD11c)	0.099	0.933					
dDCs	- 0.026	0.252	0.038				
(CD209, tumor)	0.815	**0.023**	0.737				
dDCs	- 0.054	0.027	- 0.035	0.287			
(CD209, stroma)	0.630	0.809	0.756	**0.012**			
pDCs	0.258	- 0.125	-0.037	0.035	0.055		
(CD303)	**0.020**	0.266	0.746	0.757	0.628		
Tregs	- 0.034	0.040	0.035	0.083	0.111	0.050	
(Foxp-3)	0.760	0.726	0.757	0.462	0.323	0.660	

[a]Correlation coefficient; [b]p-value.
LCs, Langerhans cells; iDCs, inflammatory dendritic cells; dDCs, dermal dendritic cells; pDCs, plasmacytic dendritic cells.

Table 4. Correlation of dendritic cells and regulatory T-cells (Tregs) with clinical and histological parameters among high grade infiltrations

	No. of cases	LCs (langerin)	iDCs (CD11b)	dDCs (CD11c)	dDCs (CD209, tumor)	dDCs (CD209, stroma)	pDCs (CD303)	Tregs (Foxp-3)
Sex								
M	36	30 (83.3)[a]	24 (66.7)	15 (41.7)	22 (61.1)	20 (55.6)	17 (47.2)	11 (30.6)
F	45	33 (73.3)	27 (60.0)	25 (55.6)	21 (46.7)	30 (66.7)	25 (55.6)	18 (40.0)
p-values[b]		0.282	0.537	0.214	0.196	0.307	0.456	0.378
Stages[c]								
Low	45	36 (80.0)	29 (64.4)	23 (51.1)	23 (51.1)	29 (64.4)	25 (55.6)	17 (37.8)
High	36	27 (75.0)	22 (61.1)	17 (47.2)	20 (55.6)	21 (58.3)	17 (47.2)	12 (33.3)
p-values		0.591	0.758	0.728	0.690	0.574	0.456	0.678
IPI[d]								
Low	53	41 (77.4)	25 (47.2)	31 (58.5)	27 (50.9)	33 (62.3)	30 (56.6)	20 (37.7)
High	28	22 (78.6)	15 (53.6)	20 (71.4)	16 (57.1)	17 (60.7)	12 (42.9)	9 (32.1)
p-values		0.901	0.584	0.251	0.595	0.891	0.239	0.618
Histologic types								
B-cells	25	19 (76.0)	13 (52.0)	16 (64.0)	10 (40.0)	9 (36.0)	15 (60.0)	9 (36.0)
T-cells	56	44 (78.6)	27 (48.2)	35 (62.5)	33 (58.9)	41 (73.2)	27 (48.2)	20 (35.7)
p-values		0.843	0.576	0.654	0.181	**0.004**	0.213	0.836

[a]Parentheses represent percentage (%); [b]Statistical significance test was done by chi-square test; [c]Low stages include stage Ia, Ib, IIa, IIb, and high stages include stage III and IV; [d]Low IPI includes low and low intermediate risks, and high IPI includes high intermediate and high risks.
LCs, Langerhans cells; iDCs, inflammatory dendritic cells; dDCs, dermal dendritic cells; pDCs, plasmacytic dendritic cells; M, male; F, female; IPI, international prognostic index.

a high risk group including high intermediate and high risks. dDC distribution was interrelated with histological types, as seen in the frequency analysis (Table 4).

Survival analysis

No direct relationships between overall survival and infiltration status of LCs ($p = 0.154$), iDCs ($p = 0.876$), dDCs in the tumor area ($p = 0.189$), dDCs in the stromal area ($p = 0.654$), pDCs ($p = 0.737$) and Tregs were observed ($p = 0.457$).

DISCUSSION

DCs are thought to be key components not only in the production and regulation of immune reactions, but also in the occurrence of several diseases.[7] DCs are generated from hematopoietic stem cells and differentiate into several specific types. DCs have a short lifespan, but are continually renewed from bone marrow.[2,7,9]

DCs can be grouped into several categories.[2] Pre-dendritic cells are cells without an immediate dendritic form and DC

function, but with a capacity to develop into DCs with little or no division. This category includes interferon-producing pDCs and monocytes. Conventional DCs (cDCs) already have the DC form and function. Migratory cells, including LCs and dDCs, are representative cDCs that act as sentinels in peripheral tissues. Another type of cDCs is lymphoid-tissue-resident DCs, such as thymic cDCs and splenic cDCs, in which function and life-history are restricted to one lymphoid organ. Another category of DCs is iDCs, which appear as a consequence of inflammation or microbial stimuli. The focus of the current study was on LCs, dDCs, iDCs, and pDCs.

Tregs play an important role in immune homeostasis. Investigations about Tregs have been performed in the field of inflammation, especially as it related to autoimmune diseases. As Th17 is a key effector cell in the propagation of autoimmune diseases, Tregs are known as key components that restrain effector T-cell differentiation. Interaction between Tregs and effector T-cells including Th17 are mediated through cytokines such as interleukin (IL)-6.[13,14] Presently, knowledge about the roles of Tregs in tumors is limited.

The ultimate purpose of this study was to obtain fresh insight about tumor immunity through the distribution patterns of various types of DCs and Tregs. Cutaneous lymphomas are good materials for this purpose because of their diverse disease entities. Proper identification of specific DCs using an immunohistochemical method was the basic tool for this purpose. Unfortunately, identifying the iDCs was somewhat difficult due to faint staining status. Staining of dDCs also fell short of expectations when CD11c was used as a marker. While these antibodies were manufactured for fresh tissues only, they were included in this study based on one report that described the application of these antibodies to FFPET.[6] However, more experience or a method modification may be required to apply these antibodies. A retrial was conducted using CD209 to identify the dDCs. There is no substitution for CD11b to date.

The interrelationship between lymphomas and DCs have been described in several reports. In non-cutaneous lymphomas, cytokines released from tumor cells inhibit the differentiation of DCs from monocytes,[3] and DCs distributed in intratumoral areas are associated with a favorable prognosis.[15] In cutaneous lymphomas, one report stated that DCs are involved in generating low-grade cutaneous B-cell lymphomas.[16] The distribution patterns of dDCs and different cutaneous lymphomas were interesting findings in this study. Therefore, the importance of dDCs in tumor immunity was the main focus. Based on the results of this study, we suggest that the distribution patterns of dDCs may be closely related with biological behaviors of cutaneous lymphomas.

The staining status of DCs in this study was identical with previous reports,[5-7] whereas CD11b and CD11c for iDCs and dDCs, respectively, were not expressed. Previous reports using DC markers, such as CD1a and factor XIII, did not determine the DC distribution patterns.[6,16] In this study identifying the distribution patterns in cutaneous lymphomas was a valuable result. From a clinical viewpoint, differences in the dDC distribution patterns may be applied in the differential diagnosis between DLBCLNOS and DLBCLLEG, or between T- and B-cell lymphomas. Although one report suggested that intratumoral dDCs are associated with a favorable prognosis for nodal lymphomas,[15] their meaning in DLBCLLEG is uncertain.

Tregs are involved in the immune regulation and play reciprocal effects with conventional T cells.[17-19] Recently, several studies have suggested the interrelationships between DCs and Tregs. Tregs are involved in the regulation of immune reactions in the presence of DC stimuli. In the presence of several cytokines, such as IL-2 and interferon-gamma, DCs expand Tregs, which, in turn, inhibit not only inflammatory conditions, such as autoimmune diseases, but also tumor progression.[17,20-22] Therefore, there is no doubt that a feedback regulatory loop exists between DCs and Tregs.[23] In this study, Tregs showed constant expression as various DCs in most cases but a meaningful relationship with DCs was not found. Other studies besides a morphological analysis such as cytokines which are functionally related, might be necessary.[24,25]

pDCs were objects of interest at the beginning of this study, but unfortunately, no insights about their roles in cutaneous lymphomas were found. On reflection, pDCs could be a key component in tumor immunity.[11,26,27]

The interrelationship between various types of DCs and Tregs is an important thesis. In addition to statistical correlation evidences, a more sophisticated microscopic investigation is needed. In this study, statistically significant correlations were found between LCs and pDCs, iDCs and dDCs in the tumor area, and dDCs in the tumor and stromal areas. When LCs and pDCs were identified in various cutaneous lymphomas and LCs are involved in the immune tolerance,[8] their imaginary roles in this function could be a good additional theme for study. Because the identification of iDCs was limited due to poor staining quality, the correlation was not reliable, suggesting that dDCs in the tumor and stromal areas may have a synergistic effect.

More sophisticated research may be necessary to identify the

clinical significance of DCs. A presentation of the possible links between DCs and clinical parameters was one of the major interests in this study. For this purpose, clinical data, such as age, gender, stages, IPI, and follow-up period were collected, and a statistical analysis was performed to identify correlations between DCs or Tregs and clinical parameters. However, we may have to establish more precise criteria to interpret the infiltration status of various DCs and Tregs, and this requires developing more reliable DC and Treg markers. As seen in this study, CD11b and CD11c were specific markers for iDCs and dDCs respectively, but their practical usefulness was lessened because of limited application. Furthermore, because the cell markers used in the immunohistochemical studies were not completely specific, another tool may be necessary for the morphological verification. Immunoelecton microscopy can be a useful tool for this purpose. The ultrastructures of various types of DCs are not known sufficiently except for dDCs.[28,29] Therefore, ultrastructural studies of various types of DCs would be a good topic for a future study.

In conclusion, although we investigated the immunological and clinical roles of DCs in cutaneous lymphomas using FFPET and showed only vague and fragmentary information about the presence of DCs and Tregs in the various cutaneous lymphomas, interesting points such as the distribution patterns of dDCs, and the statistical correlation between LCs, pDCs, and dDCs were found. The precise mechanisms of these interactions and their clinical meaning have not been established. But, certain influential roles of DCs especially dDCs in the context of immune regulation carried out by Tregs may hold a premier position in tumor immunity.

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