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Plasmacytoid Dendritic Cells (Natural Interferon-α/β-Producing Cells) Accumulate in Cutaneous Lupus Erythematosus Lesions

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Plasmacytoid dendritic cell (P-DC) precursors in peripheral blood produce large amounts of interferon (IFN)-α/β when triggered by viruses. However, when incubated with interleukin-3 and CD40 ligand, the same precursors differentiate into mature DCs that stimulate naïve CD4$^+$ T cells to produce Th2 cytokines. We recently reported that P-DCs accumulate in nasal mucosa of experimentally induced allergic rhinitis, supporting a role for this DC subset in Th2-dominated inflammation. Here we examined whether P-DCs accumulate in cutaneous lesions of lupus erythematosus (LE), a disorder associated with increased IFN-α/β production. Our results showed that P-DCs were present in 14 out of 15 tissue specimens of cutaneous LE lesions, but not in normal skin. Importantly, the density of P-DCs in affected skin correlated well ($r_s = 0.79$, $P < 0.0005$) with the high number of cells expressing the IFN-α/β-inducible protein MxA, suggesting that P-DCs produce IFN-α/β locally. Accumulation of P-DCs coincided also with the expression of L-selectin ligand peripheral lymph node addressin on dermal vascular endothelium, adding further support to the notion that these adhesion molecules are important in P-DC extravasation to peripheral tissue sites. Together, our findings suggested that P-DCs are an important source of IFN-α/β in cutaneous LE lesions and may therefore be of pathogenic importance. *(Am J Pathol 2001, 159:237–243)*

Plasmacytoid dendritic cells (P-DCs) constitute a subset of DC precursors in human peripheral blood and organized lymphoid tissue. Their morphology resembles that of Ig-secreting plasma cells and they can be uniquely identified in blood and tissues by their high levels of interleukin-3 receptor α chain (CD123) combined with other cell markers such as CD45RA, CD68, and HLA-DR.[1–5] For many years, pathologists have observed these plasma cell-like cells (previously known as plasmacytoid T cells or plasmacytoid monocytes) within secondary lymphoid tissues, but without having any knowledge of their function. Only recently, experimental studies have suggested that these cells play an important role in the initiation of immune responses.[1,3,4] In the presence of interleukin-3 and CD40L, P-DCs developed into mature DCs and induced naïve T cells to produce Th2 cytokines, suggesting a role in T-cell-mediated allergic responses.[6] However, it subsequently became clear that circulating P-DC precursors are identical to natural interferon (IFN)-α/β-producing cells, which produce large amounts of these type I IFNs when triggered by certain viruses and bacterial stimuli.[2,7–10] Paradoxically, whereas interleukin-3-stimulated P-DCs induced naïve CD4$^+$ T cells to produce Th2 cytokines, virus-triggered P-DCs activated naïve CD4$^+$ T cells to produce IFN-γ and interleukin-10.[6,11] Thus, P-DCs apparently could have multiple functions in innate as well as adaptive immunity, and might integrate these two arms of the immune system.

Knowledge about P-DCs has primarily been acquired from *in vitro* experiments; therefore, testing of current ideas about the function of these cells will depend on studies in their natural environment—that is, *in situ*. Interestingly, we have recently shown that P-DCs accumulate in nasal mucosa during experimentally induced allergic rhinitis. This finding demonstrates that P-DCs are able to migrate to mucosal effector sites, and suggests that these cells may be directly involved in a Th2-dominated inflammatory reaction.[5] The aim of the present study was to examine whether P-DCs also can be involved in inflammatory disorders associated with elevated IFN-α/β production.

Lupus erythematosus (LE) is an autoimmune disease ranging from a benign chronic cutaneous form [discoid LE (DLE)] to a severe multisystemic disease [systemic LE (SLE)].[12] Although the etiology of DLE and SLE remains elusive, increased production of IFN-α/β has been suggested as a pathogenic factor. In SLE patients, especially those with active disease, elevated blood levels of IFN-α

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Table 1. Tissue Specimen Category, Inflammation Score, and Immunohistochemical Findings

Case no.	Specimen category	Inflammation score	Cell density related to phenotype*	
			$CD123^{high}$ $CD45RA^{+}$	MxA^{+}
1	DLE	3	40	488
2	DLE	2	226	2679
3	DLE	4	680	4989
4	DLE	4	763	6472
5	DLE	3	239	1975
6	DLE	2	166	2840
7	DLE	3	217	1687
8	DLE	3	15	154
9	DLE	3	46	1649
10	DLE	3	127	2025
11	SLE	1	33	2000
12	SLE	1	14	911
13	SLE	4	103	2294
14	SLE	0	0	965
15	SLE	2	10	1525
16	Normal control	0	0	5
17	Normal control	0	0	0
18	Normal control	0	0	0
19	Normal control	0	0	0
20	Normal control	0	0	0
21	Normal control	0	0	10
22	Normal control	0	0	0

*The tissue density (cell/mm^2) of P-DCs ($CD123^{high}CD45RA^{+}$ cells) and MxA^{+} cells were determined in adjacent sections. See Materials and Methods for detailed description of experimental protocols.

have been found,[13–15] and increased expression of the IFN-α/β-inducible protein MxA has been detected in cutaneous lesions of SLE and DLE.[16] In view of the data derived from recent *in vitro* experiments, we hypothesized that P-DCs might be an important source of IFN-α/β in LE. To investigate this possibility we examined whether P-DCs accumulate in skin lesions of DLE and SLE associated with local expression of MxA.

Materials and Methods

Tissue Material

The study was performed on routine formalin-fixed, paraffin-embedded biopsy specimens (n = 15) obtained from the archives of the Department of Pathology, Rikshospitalet. Based on available clinical information, 10 patients were diagnosed as having DLE whereas 5 had symptoms characteristic of SLE. All included biopsy specimens were histologically examined by an experienced pathologist and found to display features characteristic for LE except in one case of SLE (no.13 in Table 1) that showed a mixed histopathological picture compatible with both LE and lichen planus. A scoring system was established to grade the extent of mononuclear cell infiltration. The following scores were assigned: 0, almost no mononuclear cells present; 1, very few, loosely scattered single cells (no aggregates) in papillary dermis and/or around some adnexal structures/vessels; 2, few and only small aggregates of mononuclear cells in papillary dermis and/or around less than half of adnexal structures/vessels; 3, prominent but discontinuous aggregates of mononuclear cells in papillary dermis and/or around at least half of adnexal structures/vessels; and 4, massive and confluent accumulation of mononuclear cells in papillary dermis and around all adnexal structures/dermal vessels.

Control skin material was obtained at plastic surgery (breast reduction) from three patients and at autopsy from four cadavers, all without dermatological disease. The control samples were handled and fixed as described above. Comparable immunostaining quality for the CD45RA marker in all tissue specimens showed that the antigenicity was well preserved after autopsy (see below). Samples of palatine tonsils were obtained from patients operated for recurrent tonsillitis, handled, and fixed as described above.

Serial paraffin sections were cut at 6 μm, dewaxed, immersed in a 10 mmol/L citrate buffer (pH 6.0) and microwaved for 4 $\times$ 5 minutes at 160 W. The buffer was allowed to cool at room temperature, thereafter the sections were washed in phosphate-buffered saline for 2 $\times$ 3 minutes and immediately immunostained (see below). Microwaving for antigen retrieval was not needed for MECA-79 immunostaining (see below).

Skin Organ Culture

After removing the subcutaneous tissue from a normal control skin specimen obtained at plastic surgery (no.17 in Table 1), it was divided in two, each piece having a diameter of 1 cm. One piece was injected with 1 $\times$ 10^6 IU recombinant human (rh) IFN-α-2b (Introna, Schering-Plough, Madison, NJ) and incubated overnight in a 12-well plate (Corning Costar, Corning, NY) containing RPMI 1640 supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, 1 mmol/L L-glutamine, and 3 $\times$ 10^5 IU/ml rhIFN-α-2b at 37°C in humidified CO_2/95% air. The other piece was incubated in the same buffer without IFN-α. After incubation, both tissue samples were handled exactly as described above.

Multicolor Immunofluorescence Staining

The tissue density and phenotype of P-DCs was determined by a multicolor immunostaining technique as described elsewhere.[5] Briefly, a mouse monoclonal antibody (mAb) of the IgG2a subclass specific for human CD123 (IgG2a, clone 7G3, 1 μg/ml; Pharmingen, San Diego, CA) was combined with mouse mAbs to either: CD45RA (IgG1, clone L48, 1.25 μg/ml; Becton Dickinson Immunocytometry Systems, San Jose, CA), HLA-DR (IgG1, clone TAL.1B5, 10 μg/ml), or CD68 (IgG3, clone PG-M1, 7 μg/ml; both from DAKO, Glostrup, Denmark) overnight at room temperature. A mixture of Cy3-labeled (red) goat anti-mouse IgG2a (1.5 μg/ml) and fluorescein isothiocyanate (FITC)-labeled (green) goat anti-mouse IgG1 (5 μg/ml) or FITC-labeled goat anti-mouse IgG3 (20 μg/ml; all from Southern Biotechnology, Birmingham, AL) was next applied for 3 hours at room temperature. In some experiments, rabbit antiserum to human cytokeratin

(1/100; authors' laboratory) and 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-labeled (blue) goat anti-rabbit IgG (15 μg/ml; Vector Laboratories, Burlingame, CA) were added to the primary and secondary antibody mixtures, respectively. Immunostaining of T cells and mast cells was performed on selected sections. Rabbit anti-human CD3 (1 μg/ml, DAKO) combined with mouse mAb to CD45RA (IgG1) were applied overnight at room temperature, followed by Cy3-labeled anti-rabbit IgG (0.3 μg/ml; Sigma, Saint Louis, MO) and FITC-labeled goat anti-mouse IgG1. To identify skin mast cells, both a mouse mAb to mast cell tryptase (IgG1, clone AA1, 2 μg/ml) and a rabbit anti-human c-*kit* (CD117) (1 μg/ml, both from DAKO) were combined with anti-CD123 (IgG2a), followed by a mixture of either FITC-labeled goat anti-mouse IgG1 or Alexa Fluor 488-labeled (green) goat anti-rabbit IgG (1 μg/ml, Molecular Probes, Eugene, OR) and Cy-3-labeled goat anti-mouse IgG2a. To ensure efficiency of the antigen retrieval procedure, routine formalin-fixed tonsil sections were always included as a positive control.

In situ IFN-α/β production was evaluated by immunostaining for MxA, an IFN-α/β-inducible intracellular protein well established as a surrogate marker for local IFN-α/β production.[16–18] Tissue sections were incubated overnight at room temperature with mouse mAb to human MxA (IgG2a, clone M143, 1.5 μg/ml; courtesy of Dr. O. Haller, Freiburg, Germany), followed by Cy3-labeled goat anti-mouse IgG (0.8 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) for 3 hours at room temperature. The sections were finally mounted with a medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories) for nuclear (blue) fluorescent staining. As a positive control for MxA expression, sections from IFN-α-stimulated skin specimens were immunostained in parallel.

Vascular L-selectin ligand peripheral lymph node addressin (PNAd) expression was determined by applying a combination of mAb MECA-79 (rat IgM, 2 μg/ml; courtesy of Dr. E. C. Butcher, Stanford, CA) and FITC-labeled pan-endothelial marker *Ulex europaeus* lectin-1 (10 μg/ml; Vector Laboratories) overnight at room temperature, followed by Cy3-labeled goat anti-rat IgM (1.5 μg/ml; Jackson ImmunoResearch) for 3 hours at room temperature.

In all staining experiments, negative controls were obtained both by omission of primary mAbs and by incubation with irrelevant isotype- and concentrated-matched primary mAbs.

Evaluation of Immunohistochemical Results

P-DCs and MxA$^+$ cells were counted in adjacent tissue sections in a fluorescence microscope (model E800; Nikon Corp., Tokyo, Japan). P-DCs were identified as CD123^{high}CD45RA$^+$ cells, and enumerated at ×400 magnification per tissue unit as defined by an ocular grid representing a section area of 0.058 mm^2. Because of the high density of MxA$^+$ cells in many tissue sections, images were recorded with a Hamamatsu C-5810 3-charge-coupled device cooled video camera (Hamamatsu Photonics KK, Hammatsu-City, Shizuoka-ken, Japan) allowing enumeration on a monitor screen including a section area of 0.040 mm^2 as described elsewhere.[19] A short exposure time (0.16 seconds) was used for red emission, to enable counting of only brightly stained cells with a discernible nucleus visualized with DAPI.

Both series of cell counts were performed in a systematic manner throughout each parallel section. For CD123^{high}CD45RA$^+$ cells and MxA$^+$ cells every second and every fourth tissue unit was counted, respectively. At least 17 tissue units were included for the CD123^{high}CD45RA$^+$ cells and 11 tissue units for MxA$^+$ cells; this approach provided a cell number sufficiently large to obtain a stabilized mean for both phenotypes.

For endothelial MECA-79 reactivity, both the extent and staining intensity of immunoreactive vessels were evaluated, as previously described.[5] The vessels were divided into two groups according to their smallest outer diameter (<10 μm or ≥10 μm) outlined by *U. europaeus* lectin-1 staining. All detectable vessels throughout the biopsy were counted and graded with regard to the staining intensity for MECA-79 on an arbitrary scale from nil (−) to strong (++). More than 45 vessels with a diameter of <10 μm and 33 with a diameter of ≥10 μm were counted in every specimen.

Statistics

Spearman's rank correlation coefficient was used to evaluate the relationship between the number of P-DCs, MxA$^+$ cells, and the score of the mononuclear cell infiltration.

Results

P-DCs Accumulate in Cutaneous DLE and SLE Lesions

We identified P-DCs in tonsillar tissue (mostly located in T-cell areas) and LE skin as CD123^{high} cells co-expressing CD45RA, CD68, and HLA-DR (Figure 1; a, c, d, e, and f, and data not shown), a phenotype unique for this DC subset.[1–5] P-DCs were present in 14 out of 15 skin samples from DLE and SLE patients, whereas normal control tissue was virtually negative (Figure 2 and Table 1). Large individual variations were observed, some DLE samples displaying particularly high numbers of P-DCs. Interestingly, the number of P-DCs correlated well with the score of the mononuclear cell infiltration ($r_s = 0.65$, $P < 0.009$, $n = 15$). This relationship explained the fact that most SLE samples contained relatively few P-DCs (Table 1). The localization of P-DCs was similar in both types of lesions with predominant accumulation along the dermal-epidermal junction (Figure 1a), around hair follicles (Figure 1c), and perivascularly (Figure 1d). In addition, positive cells occurred scattered in the dermis. Interestingly, when we immunostained for CD123^{high}CD45RA$^+$ P-DCs, many LE specimens (and especially those with high number of P-DCs) contained many cells positive only for

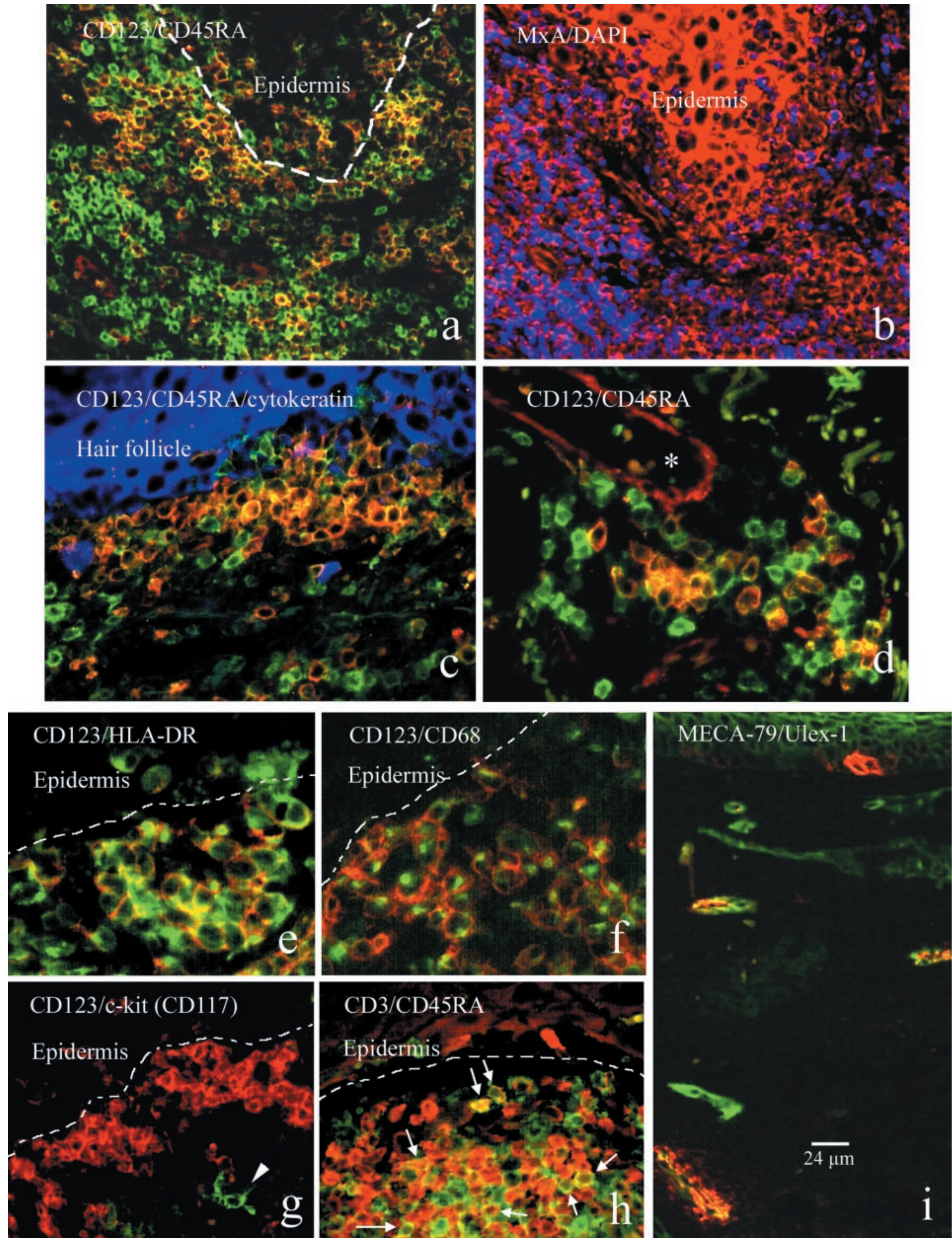

Figure 1. *In situ* phenotypic characterization of $CD123^{high}$ P-DCs and related MxA and PNAd expression. Multicolor immunofluorescence staining for: CD123 (Cy3, red), CD45RA (FITC, green), and cytokeratin (AMCA, blue in **c**) (**a**, **c**, and **d**); human MxA (Cy3, red) with nuclear counterstain (DAPI, blue) (**b**); CD123 (Cy3, red) (**e–g**) and HLA-DR (FITC, green) (**e**), CD68 (FITC, green) (**f**), human c-*kit* (Alexa Fluor 488, green) (**g**); CD3 (Cy3, red) and CD45RA (FITC, green) (**h**); and PNAd revealed with mAb MECA-79 (Cy3, red) and endothelium with *Ulex europaeus* lectin-1 (FITC, green) (**i**) in serial sections (comparable fields in **a** and **b**) of cutaneous DLE lesion. Characteristic accumulation of $CD123^{high}CD45RA^{+}$ cells (yellow staining) along the dermal-epidermal junction (**a**), around hair follicles (**c**), and adjacent to dermal vessel (**asterisk**) with endothelial CD123 expression (**d**); note strong expression of MxA in keratinocytes and infiltrating cells in dermis (**b**) in field comparable to high numbers of P-DCs (**a**). $CD123^{high}$ cells also express HLA-DR (**e**) and CD68 (**f**); the intracellular dot-like staining pattern for CD68 was similar in tonsillar tissue (not shown) and found characteristic for DCs by others.[20] Dermal c-kit^{+} mast cells do not express CD123 (**g**, **arrowhead**); note the large number of naïve ($CD3^{+}CD45RA^{+}$) T cells (yellow staining, some **arrowed**) (**h**). Medium–sized vessels (diameter, ≥10 μm) with strong PNAd expression appears yellow (**i**); note that some keratinocytes also react with MECA as previously noted.[25] Basement membrane of epidermis is indicated by **dashed line**. Original magnifications: ×200 (**a** and **b**); ×400 (**c**, **d**, **g**, and **h**); ×630 (**e** and **f**); ×100 (**i**).

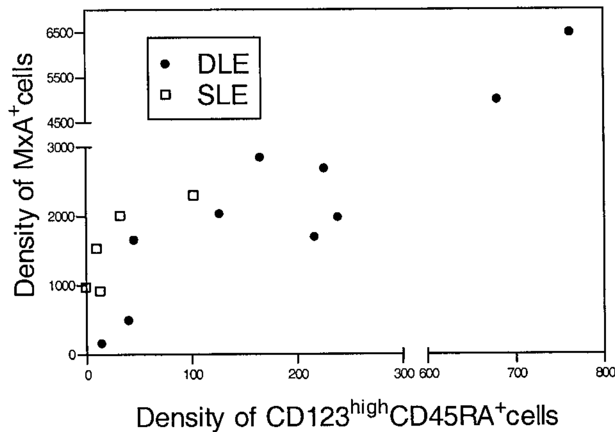

Figure 2. Relationship between the density (cell/mm^2) of P-DCs (CD123^{high}CD45RA$^+$ cells) and MxA$^+$ cells in cutaneous LE lesions (r_s = 0.79, $P < 0.0005$, n = 15). Based on data in Table 1 and Spearman's rank correlation test.

CD45RA (Figure 1; a, c, and d). The majority of these cells were deemed to be naïve (CD3$^+$CD45RA$^+$) T cells (Figure 1h and data not shown).

Because mouse mast cells have been reported to express CD123,[21] we wanted to ensure that the CD123^{high}CD45RA$^+$ cells observed in LE lesions were distinct from mast cells. Antibodies to mast cell tryptase and c-*kit* decorated a population of cells evenly distributed throughout the dermal layer. However, none of these skin mast cells expressed CD123 (Figure 1g), which is in agreement with previous studies in humans.[22–24]

Accumulation of P-DCs Is Related to Expression of MxA

To investigate whether P-DCs produced IFN-α/β locally, we examined the cellular expression of the IFN-α/β-inducible protein marker MxA. All DLE and SLE tissue samples contained MxA$^+$ cells, often in very high numbers, whereas such cells were only infrequently present in the controls (Figure 1b and Table 1). The MxA$^+$ cells were located throughout the epidermis (mainly keratinocytes) and the dermis as well as skin appendages (Figure 1b). Importantly, the number of MxA$^+$ cells was well correlated with the density of P-DCs in adjacent tissue sections of LE lesions (r_s = 0.79, $P < 0.0005$, n = 15) (Figure 2). This finding strongly suggested that P-DCs produced IFN-α/β in these lesions. IFN-α-stimulated skin served as a positive control for MxA expression and contained positive keratinocytes and endothelial cells, whereas unstimulated skin was negative (data not shown).

Endothelial PNAd Is Strongly Expressed in Cutaneous DLE and SLE Lesions

The mechanisms directing emigration of DC precursors to different tissue sites are poorly understood. Accumulation of P-DCs in and around high endothelial venules (HEVs) in secondary lymphoid organs, and especially in inflamed lymph nodes, suggests that these cells extravasate through the specialized HEV endothelium.[1,2] In support of this possibility, circulating P-DC precursors express high levels of L-selectin,[2,3] an adhesion molecule that together with PNAd form a homing receptor-endothelial ligand pair involved in lymphocyte trafficking via HEVs. Interestingly, we have recently reported that accumulation of P-DCs concurred with PNAd expression on the vascular endothelium in experimentally induced allergic rhinitis.[5] Therefore, we investigated vascular PNAd expression by means of MECA-79 immunoreactivity in the LE lesions. All 10 DLE and 4 out of 5 SLE tissue samples contained vessels reactive with MECA-79, whereas normal skin was invariably negative. The majority (>70%) of the MECA-79 reactive vessels had a diameter ≥10 μm, and in some skin lesions >30% of such vessels were strongly positive (Figure 1i).

Discussion

This study demonstrated that P-DCs accumulate in cutaneous DLE and SLE lesions, but not in normal skin. The cells were identified by their high expression of interleukin-3 receptor α chain (CD123) and co-expression of CD45RA, CD68, and HLA-DR. The latter markers discriminate P-DCs from other CD123^{high} cells such as endothelial cells (which are negative for CD45RA) and basophils (which are negative for CD45RA and HLA-DR). Mouse mast cells also express CD123,[21] but human dermal and lung mast cells have previously been shown to lack this receptor.[22–24] In the present study we confirmed the latter observation because dermal mast cells, identified by mast cell tryptase and c-*kit*, were always negative for CD123. The density of P-DCs in LE lesions correlated well with the presence of a high number of cells expressing the IFN-α/β-inducible protein MxA; this strongly suggested that P-DCs produce IFN-α/β locally in both DLE and SLE. Our finding contrasts with the situation in allergic nasal mucosa where MxA-positive cells were virtually absent.[5] Our *in situ* observation thus accords with recent *in vitro* data, suggesting that the function of P-DCs depends on their microenvironment.[11]

The etiology of LE remains elusive but increased production of IFN-α/β has been proposed as a pathogenic factor. In support of this hypothesis, IFN-α therapy of patients with nonautoimmune diseases occasionally induces SLE-like syndromes.[26–28] Our finding is therefore of considerable interest because the cellular source of IFN-α/β in DLE and SLE has been unclear. Although P-DCs has been proposed to be one source of IFN-α in SLE, a recent report showed that the number of such precursors was markedly decreased in peripheral blood of SLE patients.[29] Our results may explain this paradox, because we show that these cells have the capacity to migrate into inflamed skin in LE where they most likely produce and secrete IFN-α/β.

The high number of MxA$^+$ cells in cutaneous LE lesions, contrasting our principally negative result for this IFN-α/β marker in allergic nasal mucosa,[5] suggests that inflamed skin afflicted with LE contains specific factors

triggering P-DCs to produce IFN-α/β. Viruses and bacteria are known to induce P-DCs for such production *in vitro*,[2,7–10] and both types of pathogens have been discussed as etiological factors in LE.[30–34] Another possibility of IFN-α/β induction is the increased apoptosis of cells seen in cutaneous LE lesions.[35–37] Båve and colleagues[38] recently showed that apoptotic cells from several human cell lines combined with serum IgG from SLE patients triggered P-DCs to produce IFN-α *in vitro*. The underlying mechanisms remains elusive but several lines of evidence suggest that DNA molecules containing unmethylated CpG can activate human peripheral blood DCs.[39] In particular, CpG oligonucleotides, but not lipopolysaccharide or poly IC, potently triggered survival, growth, activation, and maturation of P-DCs and certain CpG sequence motifs induced IFN-α/β production in these cells (G. Hartmann, personal communication).[40] Furthermore, it has been shown that serum from SLE patients contains complexes of anti-DNA antibodies and double-stranded DNA especially effective in stimulating IFN-α production in P-DCs.[41] Whether this DNA is of human or microbial origin is unknown.

Immature DCs, such as Langerhans' cells, reside in nonlymphoid tissues where they take up antigen and migrate to draining lymph nodes in response to inflammatory signals. P-DCs, however, seem to migrate to lymphoid tissues in the absence of such signals and most likely enter directly from blood via HEVs.[1–3] Therefore, it has been unclear how P-DCs encounter and take up antigen. It has previously been reported that cells with morphological and certain immunophenotypic characteristics of P-DCs are present in some skin disorders,[42–44] suggesting that they may migrate to nonlymphoid tissues in response to certain inflammatory stimuli. This possibility was recently supported by our study on experimental allergic rhinitis where we demonstrated that accumulation of P-DCs in allergic nasal mucosa concurred with strong expression of the L-selectin ligand PNAd on the mucosal vascular endothelium.[5] PNAd is constitutively expressed on HEVs and mediates homing of naïve lymphocytes to organized lymphoid tissue. Similar to naïve lymphocytes, circulating P-DCs also express high levels of L-selectin, which most likely explains their extravasation preference in HEV-containing lymphoid tissue. In this study we found that PNAd also is expressed by dermal endothelial cells in cutaneous LE lesions. Together, our findings suggest that P-DCs are recruited to inflamed peripheral tissues by the same adhesion molecules that normally mediate leukocyte extravasation in secondary lymphoid tissues. The finding that many naïve CD45RA$^+$ T cells were present in LE lesions further supports this notion. We are currently testing this hypothesis by comparing adhesion molecule profiles in allergic rhinitis and cutaneous LE lesions with chronic inflammatory conditions where P-DCs are virtually absent such as nasal polyps, celiac disease, and inflammatory bowel disease (F. L. Jahnsen and colleagues, unpublished observations).

In summary, we found that P-DCs accumulate in cutaneous DLE and SLE lesions. The density of these cells correlated well with the high number MxA$^+$ cells (a surrogate marker for IFN-α/β), suggesting that P-DCs produce these type I IFNs locally in these lesions. P-DCs could therefore be an important source of the increased levels of IFN-α/β found in LE patients and therefore be of pathogenic importance.

Acknowledgments

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