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INVESTIGATIVE REPORT

Distribution and Maturation of Skin Dendritic Cell Subsets in Two Forms of Cutaneous T-cell Lymphoma: Mycosis Fungoides and Sézary Syndrome

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Dendritic cells (DCs) critically regulate immune responses and the "immune-surveillance" of tumours. This study retrospectively analysed the distribution and maturation status of DC-subsets in T-cell lymphoma of the skin. Mycosis fungoides and Sézary syndrome ($n=25$) were investigated immunohistochemically for DC subsets, based on C-type lectin receptor expression: Langerhans' cells (langerin/CD207$^+$, DEC-205/CD205$^+$), dermal DCs (DC-SIGN/CD209$^+$, CD205$^+$) and plasmacytoid DC (BDCA-2/CD303$^+$). Maturation status was assessed by double-labelling for CD83 and CD208/DC-LAMP. DCs were interspersed between the neoplastic infiltrate, and a marked increase in numbers of all three subsets was noted, DC-SIGN$^+$ dermal DCs constituting the majority. Substantial numbers of plasmacytoid DCs were consistently observed. Most DCs in epidermis and dermis were phenotypically immature. Amongst the relatively few mature DCs in the dermis, langerin$^+$ cells predominated. There was a positive correlation between the histological intensity of the tumour infiltrate and DC numbers. It is possible that mature DCs reflect ongoing anti-tumour immune responses, and immature DCs the induction of tumour tolerance. ***Key words: cutaneous T-cell lymphoma; plasmacytoid dendritic cells; Langerhans' cells; dermal dendritic cells; C-type lectins; mycosis fungoides; Sézary syndrome.***

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Mycosis fungoides (MF) is the most common type of epidermotropic primary cutaneous lymphoma. Its clinical course ranges from an indolent premalignant syndrome, through a patch and plaque stage, to an aggressive tumour stage and systemic involvement (1). The major histological findings in MF consist of a dermal, mostly helper T-cell phenotype (CD4$^+$), patchy to band-like dense infiltrate, epidermotropism of solitary lymphocytes or lymphocytes arranged in so-called Pautrier's microabscesses, and moderate to marked dermal fibrosis (2). Possible contributing stimuli to the pathogenesis of MF involve chronic inflammation and clonal abnormalities in the neoplastic T cells. Infectious agents and occupational exposure have been considered as aetiological factors, but supporting evidence is not readily available. Alterations in cellular immune responsiveness in relation to MF is possibly mediated by Langerhans' cells (LCs) (3–5). Sézary syndrome is also included in the classification of cutaneous T-cell lymphomas. It is defined historically by the triad of erythroderma, generalized lymphadenopathy, and the presence of neoplastic T cells (Sézary cells) in skin, lymph nodes and peripheral blood. The histopathological features of skin lesions are indistinguishable from those of MF.

Dendritic cells (DCs) play a pivotal role in the immunobiology of cutaneous lymphoma (6–9). They function as sentinels beneath the body surfaces. Depending on their maturation stage DCs can either: (*i*) act as professional antigen-presenting cells, which have evolved to monitor the environment, detect pathogens and trigger T-cell activation to initiate immune responses; or (*ii*) induce peripheral tolerance in steady state (10). Different subsets of DCs (11) may subserve different functions, as indicated by the differential expression of C-type lectin receptors. These surface receptors operate as components of the antigen capture and uptake machinery of DCs, including LCs (12). Epidermal LCs and the three subsets of dermal DCs, i.e. (*i*) langerin/CD207$^+$, DEC-205/CD205$^+$ DCs, (*ii*) langerin$^-$, DC-SIGN/CD209$^+$, CD205$^+$ DCs, and (*iii*) langerin$^-$, BDCA-2/CD303$^+$ plasmacytoid DCs (pDCs) have characteristic C-type lectin expression profiles. Langerin/CD207 is specifically produced by LCs in the epidermis and localizes on the cell surface and within Birbeck granules (13). Migration and transport of antigen from the skin to the lymph nodes is an important feature of LCs (14). The DC-SIGN/CD209 receptor is involved in cross-talk between DCs and T cells and binds HIV virions and other types of microbes (15). Blood DC Antigen 2 (BDCA-2) is specifically expressed by pDCs and has been reported to play a role in antigen uptake as well as in inhibition of interferon (IFN)-α/β production (16). The immune system has the potential to eliminate neoplastic cells, but tumours

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may escape immune surveillance by altering tumour immunity (17). The aim of the study was to analyse in more detail the distribution and maturation status of DCs in MF and Sézary syndrome and to characterize DCs subsets, which have as yet been incompletely studied in these disease entities. To determine DC distribution and quantity, immunolabelled cryostat sections of patients' lesions were compared with normal human skin from healthy donors. Also, morphological features were noted. Furthermore, the intra- or peritumoural localization, and the correlation between clinical stage and histological intensity of the infiltrate and DC density were analysed. Finally, staining with dendritic cell maturation markers DC-LAMP/CD208, a lysosome-associated membrane glycoprotein, expression of which increases progressively during *in vitro* DC-differentiation and upon activation with pro-inflammatory stimuli (18), and CD83, one of the most utilized cell surface markers for fully mature DCs (19) was performed to determine their state of maturation and thus to better judge their potential role in the anti-tumour response.

MATERIALS AND METHODS

Tissue specimens

Biopsies of 25 patients with MF ($n=19$) at patch, plaque or tumour stage and Sézary syndrome ($n=6$) seen in the outpatient clinic for cutaneous lymphoma from 1998 to 2003 were studied retrospectively. Clinical information and histopathological data were obtained from the medical charts. The age of the patients ranged from 34 to 91 years, and the female/male ratio was 8/17. Normal skin samples from 14 different healthy donors were included as controls. Skin was obtained, after informed consent, from reductive plastic surgery of breast or abdomen, i.e. from areas not, or rarely, exposed to sunlight. Biopsy specimens were snap-frozen and stored in liquid nitrogen at –196ºC until cryosectioning.

Immunohistochemistry

Frozen tissue sections (8–10 µm thick) were air-dried and fixed in acetone. After blocking slides with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) for unspecific binding, the slides were incubated with the primary antibody (Table SI; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1220). Negative control staining with an IgG isotype (DAKO, Glostrup, Denmark) of irrelevant specificity was performed concurrently. Subsequently, biotinylated polyvalent anti-mouse Ig secondary antibody (Amersham Pharmacia Biotech, UK) was applied, followed by streptavidin-horseradish peroxidase (Amersham). Finally 3´3-diaminobenzidine tetrahydrochloride (Sigma, Saint Louis, MO, USA) was added and the slides were incubated until the desired colour (brown) intensity was reached. Haematoxylin was used for nuclear counterstaining.

Double immunofluorescence

All incubation steps were performed for 1 h at room temperature. Two different protocols were employed with identical results. (A) The primary mouse monoclonal antibody was detected by a biotinylated anti-mouse Ig (Amersham Pharmacia Biotech), followed by a Streptavidin-Texas Red conjugate (Vector Laboratories, Burlingame, CA, USA). Then, an excess of mouse γ globulin (100 µg/ml; Jackson Immunoresearch, Avondale, PA, USA) was used as blocking reagent and, for double-staining, a fluorescein isothiocyanate (FITC)- or Alexa-488-conjugated mouse monoclonal antibody was applied as the last incubation step. (B) The primary mouse monoclonal antibody (Table SI) was visualized by a FITC-Alexa Fluor Signal – Amplification kit (Invitrogen-Molecular Probes, Eugene, OR, USA). Then, residual binding sites were saturated with mouse γ globulin (100 µg/ml; Jackson). In the next step a biotinylated antibody (CD83 biotin) was added and revealed by Streptavidin Texas Red (Vector Laboratories). Isotype control was done with biotinylated mouse IgG (e-Bioscience). Stainings were examined with an Olympus BX60 conventional epifluorescence microscope.

Evaluation of immunolabelled sections

Analyses were performed by a semi-quantitative comparison between the intensity of total infiltrating cells of lymphoma specimens and the numbers of DC in lymphoma compared with normal human skin from healthy donors. The arbitrary score for different classes was defined as: 0 = no infiltrate, +/– mild, + moderate and ++ dense infiltrate. Numerical comparison of DC numbers in lymphoma compared with normal human skin from healthy donors (20) was defined as follows: 0 = normal numbers of DC in healthy human skin, – mild decrease, +/– mild increase, + moderate and ++ strong increase in DC numbers in lymphoma specimens, as described in Table SII (available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1220). DC numbers were studied in the epidermis and dermis.

RESULTS

Plasmacytoid dendritic cells

Distribution. The pDC subset was characterized by the expression of BDCA-2. BDCA-2$^+$ cells in MF and Sézary syndrome were increased compared with the skin of healthy donors. The cells were irregularly distributed throughout the dermal infiltrate. A few positively stained cells were also found in the lower part of the epidermis in 14 out of 25 samples (Fig. 1 a, b).

Localization. pDCs were scattered throughout the lymphoid infiltrate, preferentially close to the basement membrane. Their number was dependent on the intensity of the infiltrate. Formation of loose aggregates was noted, but was not a common feature (Fig. 1 a, b).

Maturation stage. Co-expression of BDCA-2 and CD83 could be detected in only a few cases. Most of the pDCs showed an immature phenotype. CD83$^+$ cells were present in lower numbers compared with the numbers of pDCs (Fig. 1 c–e).

Morphology. Morphology of pDCs showed two opposite aspects. One type appeared plasma cell-like. Cells were round-to-polygonal with dendrites missing. The other type showed a much more dendritic shape. Intra-epidermal BDCA-2$^+$ DCs resembled LCs in terms of morphology (Fig. 1, insets).

Langerhans' cells/dermal langerin$^+$ cells

Distribution. LCs showed a mainly linear and regular (network-like) pattern across the epidermis. Intra-

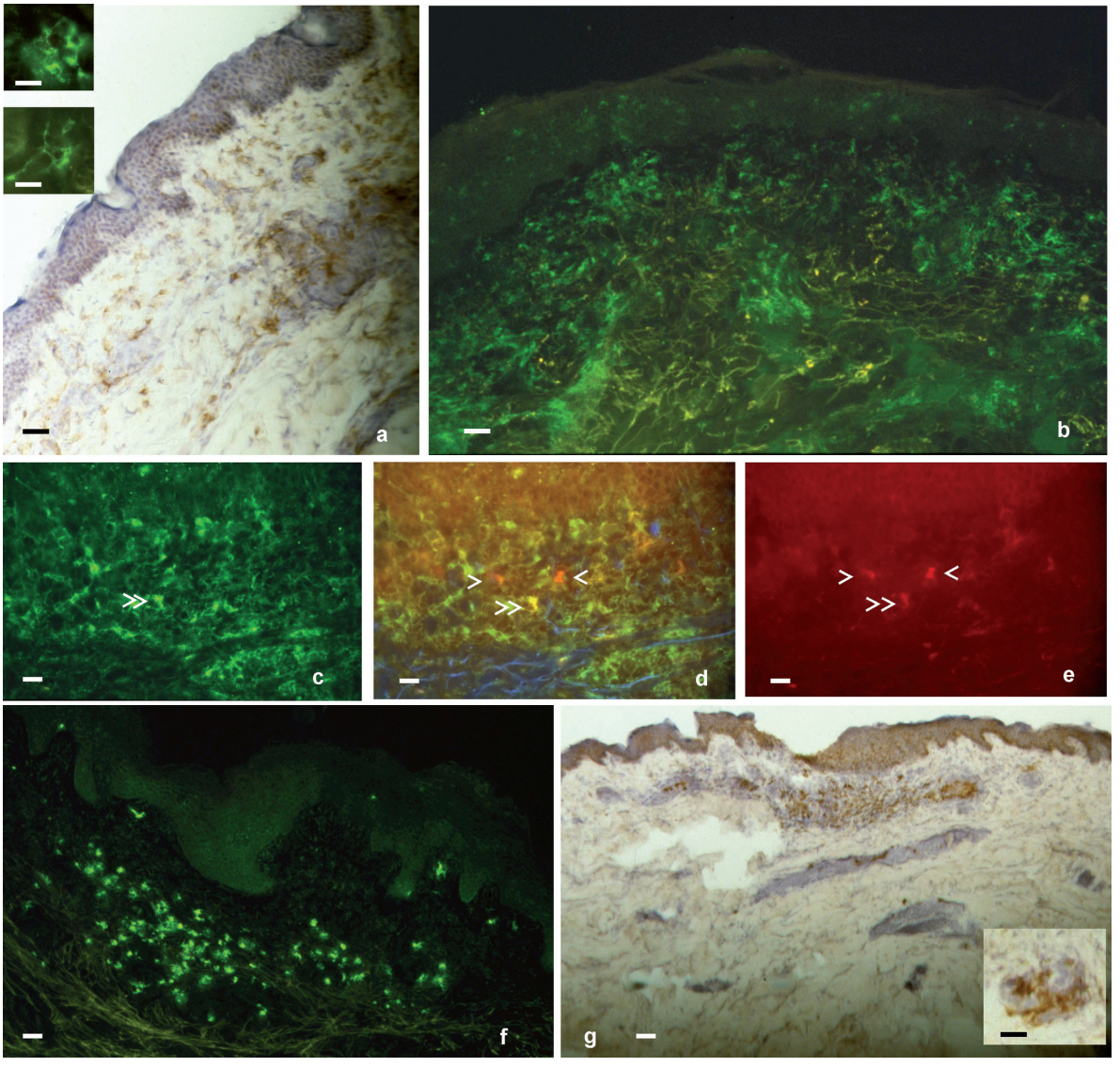

Fig. 1. Plasmacytoid dendritic cells (pDC). Mature dendritic cells. (a, b) BDCA-2$^+$ cells (brown/green) showed an increased number, with irregular distribution throughout the dermal infiltrate. A few positively stained cells were also found in the lower part of the epidermis. The morphology of pDCs showed two opposite aspects: the one looked more plasma cell-like, round and without dendrites (a, *upper inset*); the others had a much more dendritic shape (a, *lower inset*). (c–e) Co-expression of BDCA-2 (*green*) and CD83 (*red*) could just be detected in a few cases. Most of the pDCs showed an immature phenotype. (f, g) An increase in dermal DC-LAMP$^+$ DCs (*green/brown*) cells in the lesional infiltration and in DC clustering is observed. Mature DCs form small-to-large, loose-to-clustered accumulations depending on the intensity of the infiltrate. DC-LAMP$^+$ DCs are predominantly dendritic in shape. Scale bars 50 µm (c, d, e, *insets*), 100 µm (a, b, f), and 200 µm (g).

epidermal clusters of up to four cells were sometimes observed. Epidermal LCs seemed to be slightly increased in number compared with normal human skin (Fig. 2 a, b).

Localization. LCs were mainly lined up along the upper part of the epidermis, close to the stratum corneum, whereas the suprabasal layer, where LCs are typically found, was sparsely populated. Compared with normal skin a marked increase of langerin$^+$ cells was observed in the dermis. There, langerin$^+$ cells were mainly located within the infiltrate of T lymphoma cells and they tended to form clusters. However, some single langerin$^+$ cells were also seen in the infiltrate (Fig. 2 a, b).

Maturation stage. CD83 labelling showed a weaker intensity. Therefore, in general, fewer DCs were labelled with CD83 compared with DC-LAMP. Double-labelling with Langerin and CD83 revealed that there were some small clusters of mature LCs in the epidermis, probably forming Pautrier's microabscesses. The majority of LCs in the epidermis was immature, as described for skin of healthy donors, where DC-LAMP$^+$ langerin$^+$ cells can only rarely be detected (20). Most of the CD83$^+$ dermal cells co-expressed langerin. However, these mature dermal langerin$^+$ cells represented only a small portion of the dermal langerin$^+$ cells. In other words, the vast majority of dermal langerin$^+$ cells was immature (Fig. 2 f–h).

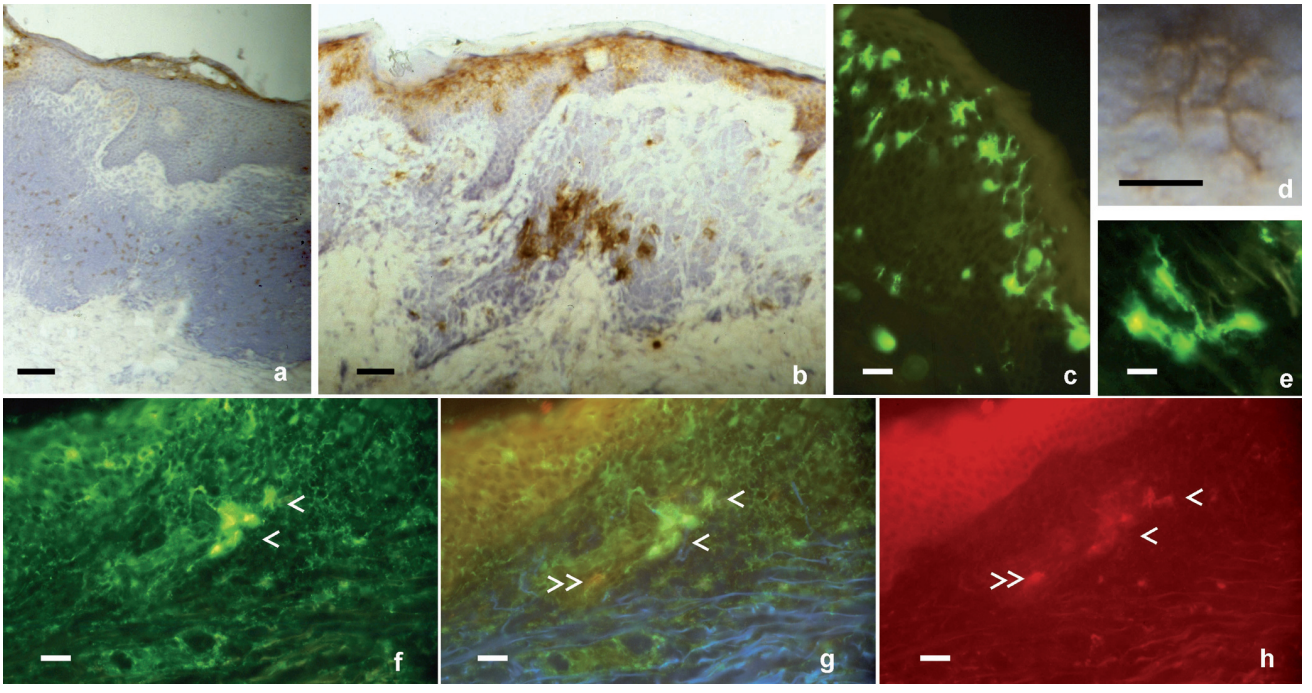

Fig. 2. Langerhans' cells (LC). (a) A marked increase in Langerin$^+$ cells (*brown*) was observed mainly within the dermal infiltrate (*blue*) of T-lymphoma cells. (b) Langerin$^+$ cells formed prominent clusters. (c) Epidermal LC (*brown/green*) cells were predominantly lined up along the upper part of the epidermis and showed a linear and regular pattern. (d, e) Most epidermal LCs and dermal langerin$^+$ cells show typical morphology: dot-like staining of the cytoplasm and dendrites. (f–h) Double-labelling with Langerin (*green*) and CD83 (*red*) revealed that the majority of CD83$^+$ dermal cells co-expressed Langerin (*single arrowheads*). Note, that these mature cells are only a small portion of the dermal langerin$^+$ cells. *Scale bars*: 20 μm (e), 40 μm (d), 50 μm (c, f, g, h), 100 μm (b), and 250 μm (a).

Morphology. Most of the langerin$^+$ cells in MF and Sézary syndrome showed typical LC morphology: dot-like staining of the cytoplasm and the dendrites with anti-langerin antibodies (Fig. 2d). This goes for epidermal and some dermal langerin$^+$ cells. In contrast, other dermal langerin$^+$ cells revealed to some extent a reduced dendritic shape: they were rounding up and had lost their typical LC morphology (Fig. 2e).

Dermal dendritic cells

Distribution. MF skin lesions, as well as skin of healthy donors, presented equal distribution patterns of dermal DCs as defined by the expression of DCSIGN/CD209. There was no specific staining of dermal DCs in the epidermis. The vast majority of these cells showed a regular distribution across the upper dermis, whereas gradually decreasing numbers of CD209$^+$ cells were observed toward the lower dermal region (stratum reticulare) (Fig. S1 a, b; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1220).

Localization. In all samples DC-SIGN$^+$ cells were densely interspersed throughout the dermal infiltrate. Compared with normal human skin, skin lesions displayed a clearly increased number of dermal DCs. Again, it appeared that they might build a network with direct contacts with each other via dendrites. In three samples a remarkably strong immunolabelling of vessel-like formations was observed. Although not proven, it appeared as if dermal DCs would adhere to the vessel wall and prepare to migrate (Fig. S1c). It must be emphasized, that this staining was specific because isotype controls were totally negative.

Maturation stage. In contrast to the abundant DC-SIGN$^+$ cells in the infiltrate there were few dermal DCs co-expressing a weak CD83$^+$ labelling (Fig. S1 d–f).

Morphology. DC-SIGN+ cells displayed a predominant dendritic shape. Labelling was bright and uniform, with an accentuated surface-membrane staining (Fig. S1, insets).

Mature dendritic cells

As opposed to the double-immunolabelling approach described in the previous paragraphs, these analyses were single-labelling experiments using anti-DC-LAMP antibody (Fig. 1 f, g). The epidermis presented a scattered distribution-pattern of the DC-LAMP$^+$ cells. Mature DCs were very few in number, but consistent in their appearance. Their morphology mainly resembled either LCs, or they were more round-to-oval in shape. In the dermis, mature DC-LAMP$^+$ DCs displayed a predominant dendritic staining and an increased frequency of lesional infiltration and DC clustering compared with normal skin. Mature DCs formed small-to-large, loose-to-clustered accumulations depending on the

intensity of the infiltrate. The stronger the infiltrate the more DC-LAMP$^+$ cells were found.

Clinical correlation

Possible links between the clinical appearance of MF and Sézary syndrome, histological intensity of the neoplastic infiltrate and DC number were investigated. Surprisingly no correlation between clinical stage (patch-plaque-tumour-Sézary syndrome) and the density of the lymphoid infiltrate was found in the particular specimens evaluated in this study. Nor did a correlation of the staining patterns of any of the DC markers with clinical stage become evident. Results summarized in Table SII demonstrate that only a link between the histological intensity of the tumour infiltrate and DC frequencies could be established. In other words, the denser the subepidermal lymphoid infiltrate the higher were the numbers of dermal langerin$^+$ cells, dermal DCs and pDCs in the dermis (Table SII, compare shaded columns). Epidermal LC numbers only marginally exceeded LC numbers in epidermis of healthy donors. It should be noted that no correlation of sex with any of the DC markers was seen.

DISCUSSION

In this study the frequency, distribution and maturation state of DC subsets was semi-quantitatively analysed in MF and Sézary syndrome. Numbers of plasmacytoid DCs, as well as LCs and dermal DCs, were increased in lesional skin compared with healthy human skin. Most DCs were phenotypically immature.

Dendritic cell subsets and origin of malignant T cells. It has been suggested that MF is a malignancy of skin resident effector memory T cells, whereas Sezary syndrome consists of central memory T cells (21). A possible speculation that this may be a consequence of stimulation by different DC subpopulations could not be substantiated in our study: no differences in numbers and distribution of the DC subsets became apparent between MF and Sézary syndrome.

Plasmacytoid dendritic cells. Amongst other tumours, the presence of pDCs has been reported in melanoma (22). Regarding lymphomas, pDCs were found in the peripheral blood of patients with Sézary syndrome (23). Our observations confirm a very recent report showing a similar distribution pattern of pDCs in skin lesions of MF and Sézary syndrome (24). Importantly, we extend these data, in that we double-labelled pDCs with the maturation marker CD83, and demonstrate directly for the first time that the majority of pDCs is immature.

Similar to LCs and dermal DCs, pDCs are capable of stimulating antigen-specific T-cell proliferation. Upon binding of, for example, viruses and CD40L (25), pDCs mature into efficient antigen-presenting cells and, in the case of virus uptake, or ingestion of CpG DNA produce large amounts of IFN-α. Whereas this cytokine is pathogenic in autoimmune diseases, such as systemic lupus erythematosus (26), it may be beneficial for the host in neoplastic diseases by contributing to the stimulation of innate immune cells and mechanisms such as macrophages, natural killer (NK) T cells and NK cells that kill the tumour. Tumour cell bodies may subsequently be captured by immature DCs. In addition, IFN-α can inhibit tumour growth, differentiation, and vascularization through other pathways, such as induction of G0/G1 arrest, suppression of Rb phosphorylation, and down-regulation of G1 cyclins and cyclin A (27). Indeed, many genes involving cell cycle arrest and apoptosis were shown to be upregulated by IFN-α specifically in cutaneous T-cell lymphoma (CTCL) lines. When these genes become de-regulated, sensitivity of tumour cells to IFN-α is lost (28).

IFN-α production of pDCs is induced during their maturation process; our morphological observations of an increased amount of plasma cell-like (immature) plasmacytoid cells and some dendritic cell-like (mature) pDCs (29) would permit to suspect an ongoing maturation procedure with the release of IFN-α. This was not assessed, however. In a small number of samples it has been shown previously that imiquimod-induced recruitment of pDCs to lesions of CTCL correlated with an increase in the expression of IFN-inducible genes, strongly indicating secretion of this cytokine by the infiltrating pDCs (30). Therefore, it would be of great interest to investigate in more detail the cytokine environment in CTCL in order to determine the role of pDCs in the immune control of tumour proliferation.

Langerhans' cells/dermal langerin$^+$ cells. The conspicuous clustering of clonal CD4$^+$ T cells around LCs in the epidermis in "Pautrier's microabscesses" has previously suggested a dependence of the T cells on interactions with this type of DCs (31). Based on *in vitro* experiments, it has been hypothesized that T helper cells proliferate and become malignantly transformed in response to tumour antigens presented by epidermal LCs (32). The data of this study are in line with previous observations (7) suggesting that immature DCs, mainly of the epidermis, mature and migrate to the papillary dermis to become mature antigen-presenting cells. This is supported by the increased numbers of LCs in the dermis in MF compared with normal skin. In double-labelling experiments, the majority of activated CD83$^+$ dermal cells were contained within the langerin-positive subset of DCs (also shown by Lüftl et al. (7)). Inversely, and in extension of a recent report on LCs in skin lymphoma lesions (5), we show here by double-labelling that most dermal langerin$^+$ cells are immature. It remains therefore unclear whether LCs subserve immunogenic or tolerogenic functions in these lesions. An indication of an ongoing anti-tumour response may be the finding of CD8$^+$ T cells

in the infiltrate of MF (6), which, speculatively, might be activated by LCs presenting unknown lipid antigens on their Langerin receptor (33, 34).

Further studies on the migration path of LC precursors into the epidermis (e.g. staining with MIP-3alpha/CCL20 antibody) and of resident LCs from dermal lesions of MF (labelling with CCR7 and Lyve-1 antibodies) to identify lymph vessels may lead to a more precise understanding of the role of LCs in the development of this lymphoma and in an eventual anti-lymphoma immunity, and perhaps also help in the subsequent development of targeted anti-tumour therapies.

A novel aspect must be considered in this context; an unexpected, functionally active population of blood-derived langerin$^+$ dermal DCs has been described recently in mice (reviewed in (14)). The dermal langerin+ cells found in our study may in part belong to this novel population. It must be stressed, however, that at this time a likely human equivalent is still being sought (14).

DC-SIGN$^+$ dendritic cells. DC numbers in the dermis (langerin$^+$ cells, dermal DC, pDC) were found to be related only to the density of the subepidermal tumour infiltrate. Their mainly immature state, which we determined here by double-labelling experiments, in extension of a recent description of DC-SIGN$^+$ DC in skin lesions (24), might be the result of an active tumour immunosuppressive mechanism, e.g. by blocking the maturation process of dermal DCs and thus avoiding the generation of an anti-tumour immune response. Observations from cell culture experiments report the ability of CTCL cells to retard DC maturation. The prolonged survival of the DCs, their ability to proliferate and take up dying CTCL cells may drive the long-term proliferation of CTCL (9). Speculatively, the persistence of malignancy may further be promoted by the induction of tolerance through MHC-II presentation of tumour antigens by immature DC (10). This would require a close contact between those cells and explain the intra-infiltrate-localization of the DC subsets juxtaposed to the CD4$^+$ T-cell clones. A caveat is warranted with regard to CD209$^+$ cells in the dermis. This population may also contain dermal macrophages that express CD163, as shown previously (35, 36). We did not further pursue this issue by double-labelling experiments with both antibodies.

Distribution and state of maturation of dendritic cells. Presence and increased density of DCs in tumours have frequently been found associated with a better prognosis. In melanoma an increased number of mature DCs (DC-LAMP$^+$) was observed in the neoplastic infiltrate (37). They were found in close apposition to T cells (of tumour and/or infiltrate origin). Therefore, it is tempting to assume that their presence stimulates an anti-tumour immune response. This was confirmed in a more recent study that reported a significant correlation between the density of DC-LAMP$^+$ DC infiltrates in sentinel lymph node of melanoma patients with the absence of metastasis in downstream lymph nodes (38). Our observations therefore support the hypothesis of an ongoing anti-tumour immune response in MF lesions, leading to a prolonged disease course. The intensive bystander infiltrate found in MF lesions involves early stages of clonal as well as reactive cells that have the capacity to produce regulating cytokines (3).

Although an anti-tumour immune response is suggested, our findings of an immature state of most DCs are somewhat contrasting. One may speculate that the immature DCs are recruited to the site of inflammation, but their maturation may be largely suppressed by the tumour environment. One may further speculate that this suppression of DC activation may be the cause for an insufficient immune response and, as consequence, for the protracted course of the disease. In a recent study by Schlapbach et al. (24) similar patterns of DC distribution and states of maturation were observed. These authors hypothesized that the preponderance of immature DC may even tolerize against the malignant cells and thereby allow their escape from an immune attack. They substantiated this notion by showing increased numbers of FoxP3$^+$ regulatory T cells in close proximity to the immature dermal DC. On the other hand, the consistently found mature DCs (mainly of the LC type) may reflect some ongoing anti-tumour response. The balance of these pro- and anti-immunogenic observations may shape the appearance of the disease, that is the failure to eradicate the tumour cells and, on the other hand, the relative containment/control of tumour cell growth (at least over long periods). Clearly, further research is needed to better understand the immune response in MF and Sézary syndrome.

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The authors declare no conflicts of interest.

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