Folliculotropic Mycosis Fungoides with Skewed T-cell Receptor CDR3 Motif: Suggestive of Lipid-antigen Selection?

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Folliculotropic mycosis fungoides (FMF), a variant of mycosis fungoides (MF) with distinct clinical features, is characterized by infiltration of malignant T cells in hair follicles. This raises the hypothesis that antigens in the hair follicle may contribute to the pathogenesis of FMF. T-cell receptor β gene (*TRB*) sequences as well as dendritic cell subsets in patients with FMF ($n = 21$) and control patients with MF ($n = 20$) were studied to explore this hypothesis. A recurrent usage of the TRB junctional genes *TRBJ2-1* and *TRBJ2-7* was found in patients with FMF compared with those with MF. These genes contribute to an amino acid motif in the complementarity-determining region 3 (CDR3) of the T-cell receptor. This motif was previously found in T cells stimulated by lipids bound to CD1 on antigen-presenting cells. Additional immunohistochemical analysis revealed abundant CD1c- and CD1a- expressing dendritic cells in FMF. The combined findings support a role for lipid-antigen selection in FMF.

Key words: folliculotropic mycosis fungoides; mycosis fungoides; cutaneous T-cell lymphoma; T-cell receptor β gene; complementarity-determining region 3; CD1.

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Folliculotropic mycosis fungoides (FMF) is a variant of mycosis fungoides (MF). Although FMF shares some clinical similarities with conventional MF, FMF presents with other clinical features that are atypical for conventional MF. A pronounced infiltration of malignant T cells in the skin adnexa, most frequently the hair follicles, is typical of FMF (1, 2). FMF seems to have a more aggressive clinical course than conventional MF and to respond poorly to treatment (3, 4) although a subgroup of patients with FMF with different clinicopathological features may have a more favourable prognosis (5, 6). Whether FMF represents a distinct cutaneous T-cell lymphoma entity remains a matter of discussion (3–6).

MF is thought to arise from skin-resident effector memory T cells, unlike other types of cutaneous T-cell lymphoma, such as Sézary syndrome, which probably arises from central memory T cells (7). Berger et al. (8) have shown that cutaneous T-cell lymphoma cells proliferate upon direct membrane contact with dendritic cells, and that inhibition of this binding blocks cell proliferation. The authors proposed that antigen-presentation through major histocompatibility complex (MHC) class II molecules, expressed by dendritic cells, and specific recognition of antigen by the T cell receptor, expressed by cutaneous T-cell lymphoma cells, resulted in cell proliferation (8).

Antigen-stimulation of normal T cells is mediated by the T-cell receptor after an antigen has been presented to T cells by antigen-presenting cells. Antigens bind to the heterodimeric T-cell receptor variable region (9, 10). The immunoglobulin-like fold of the T-cell receptor is composed of 3 loops contributed by the variable region of each of the respective chains. These 3 loops of each chain comprise the complementarity-determining regions, CDR1, CDR2 and CDR3, in close proximity to each other, creating an antigen binding site (9, 10). While the CDR1 and CDR2 regions of the various chains are germline-encoded and show little sequence variation, the CDR3 loops at the centre of the binding site are created by gene rearrangement and show a much more diversified sequence, thus enhancing antigen-specific binding (10). Analysis of the T-cell receptor CDR3 can therefore be instructive for the study of antigen specificity of T cells and T-cell lymphoma. Both peptide and lipid antigens can be presented to T cells. Peptide antigens are presented to T cells through major histocompatibility complex (MHC) class II, expressed by specialized antigen-presenting cells, or MHC class I molecules, expressed by a diversity of other cell types. Lipid antigens stimulate T cells through cluster differentiation 1 molecules (CD1), a set of invariable MHC class I-like surface molecules expressed predominantly by dendritic cells and B cells (11). Two main types of CD1-expressing dendritic cells are recognized in the skin. Langerhans cells express CD1a, whereas dermal dendritic cells express mostly CD1c and, to a lesser extent, CD1b (12).

The aim of this study was to analyse the characteristics of the T-cell receptor gene (*TR*) sequence of FMF and to investigate dendritic cells in FMF to find evidence of antigen-selection. The *TR*, including the CDR3 sequence, has been studied previously in conventional MF and Sézary syndrome. These studies have shown broad usage of variable genes without evidence of selection of particular TR β variable region genes (*TRBV*) (13–19). However, no such study has been performed previously in FMF.

METHODS

Patients and clinical data

Patients were registered in the cutaneous T-cell lymphoma database at Oslo University Hospital, comprising all patients diagnosed with cutaneous T-cell lymphoma in the period 1997–2012. Twenty-one patients with FMF were identified from whom skin biopsies were available for analysis. Twenty randomly selected patients with conventional MF from whom skin biopsies were available for analysis were used as control. Diagnoses of all patients were based on clinical examination, skin biopsy, blood cell counts and serum chemistry. All skin biopsies were reviewed by a haematopathologist and diagnoses were made according to the World Health Organization (WHO) classification (1). The clinical presentation of the patients with FMF has been reported elsewhere (4). The study, with reference number 2010/865, was approved by the Regional Ethics Committee of Health Authority Region South-East Norway.

T-cell receptor β *gene rearrangement and sequence analysis*

TRG and *TRB* gene rearrangements were studied in all patients. DNA was isolated from paraffin-embedded or snap-frozen tissue, using the EZ1 tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA quality was verified by PCR amplification of the albumin gene. *TRG* and *TRB* gene rearrangements were detected by multiplex PCR according to the BIOMED-2 protocol provided by van Dongen et al. (20) using a commercially available kit (InVivoScribe Technologies, San Diego, USA). PCR products were size-fractionated on a 3130 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany) and analysed using Gene Mapper v. 4.1 software (Applied Biosystems). All analyses were performed in duplicate. Interpretation of the results was performed as previously recommended by Langerak et al. (21). In short, a clonal *TR* gene rearrangement was diagnosed when the height of one peak on the electrophoregram was at least 3 times higher than that of other peaks resulting from the same competitive PCR product. To confirm the *TRB* sequences, single primers specific for detected *TRBV* family were used for subsequent sequencing (20). Sequences were analysed using the IMGT web-based analysis tool (http://www.imgt.org). *TRBV*, *TRB* diversity (D) and *TRB* junction (J) genes were identified for each sample.

Immunohistochemistry

Immunohistochemical analysis was performed using 5-µm sections from formalin-fixed biopsies taken for diagnostic purposes; 21 biopsies were available from 21 patients with FMF and 20 biopsies from 20 patients with conventional MF. Immunohistochemical staining was performed with primary mouse monoclonal or rabbit polyclonal antibodies against the following markers: CD3 (clone SP7, Thermo Scientific, Waltham, MA, USA), CD4 (clone SP35 Roche, Switzerland), CD5 (clone 4C7, Novocastra, Newcastle upon Tyne UK), CD7 (clone CBC.37, Dako, Denmark), CD8 (clone 4B11 Novocastra), CD1a (clone MTB1, Novocastra), CD1b (NBP1-86559, Novus Biologicals, Littleton, CO, USA), CD1c (clone 2F4, Abcam, UK), CD1d (clone CD1D C3D5, Santa Cruz Biotechnology, Dallas, TX, USA), CD80 (EP 1155Y, Abcam), CD86 (clone B7-2 D-6, Santa Cruz Biotechnology). After pretreatment with PT-Link (Dako), paraffin sections were incubated with primary antibody for 30 min. Bound antibody was subsequently demonstrated applying the En Vision FLEX+ rabbit or mouse linker and the En Vision FLEX/HRP Detection Reagent (Dako) for 30 min. The sections were counterstained with haematoxylin and evaluated in a light microscope. For CD1a/CD1c double immunofluorescence staining, pretreated paraffin sections were incubated with a rabbit anti-CD1a monoclonal antibody (clone L21-A, Abcam) followed by fluorescein isothiocyanate (*FITC*)-conjugated polyclonal swine anti-rabbit antibodies (Dako, code number F0205). After washing, the sections were incubated with a mouse anti-CD1c monoclonal antibody (clone 2F4, Abcam) followed by TRITC-conjugated goat anti-mouse antibodies (ABcam nimber ab 7065). Stained slides were evaluated in a Zeiss fluorescence microscope (Axioplan 2) utilizing the Isis software (MetaSystems, Altlussheim, Germany).

RESULTS

T-cell receptor β *gene rearrangement sequence analysis*

Clonally rearranged *TRB* genes were detected in 18 out of 21 patients with FMF and 10 out of 20 patients with MF, clonally rearranged *TRB* gene sequences were detected in 13 out of 18 patients with FMF and 8 out of 10 patients with MF. These results are consistent with what has previously been reported for cutaneous T-cell lymphoma using BIOMED-2 primers (22). TRB PCR products were purified and sequenced successfully in 10 out of 13 patients with FMF and 7 out of 8 patients with MF. The lack of clonal *TRB* gene rearrangements in 2 cases of FMF was probably due to DNA degradation in the archival specimens, as demonstrated by the near absence of PCR products. Negative results with respect to *TRB* gene in the other cases is probably due to lack of sensitivity of the technique, which requires at least 10% neoplastic T cells among all T cells in the sample, which

Table I. *T-cell receptor β (TRB)* gene sequence analysis in folliculotropic mycosis fungoides (FMF) and MF

	TRB				
Case	V-gene	J-gene	D-gene	CDR3 amino acid sequence	Frame
FMF 2	*TRBV6-1*	*TRBJ1-4*	*TRBD1*	CASSEATHRGTNEKLFF	In-frame
FMF 3	*TRBV4-1*	*TRBJ2-2*	*TRBD2*	CASSQLDGAGELFF	In-frame
FMF 5	*TRBV3-1*	*TRBJ2-1*	*TRBD1*	CASSQGSGTGGYN**EQFF**	In-frame
FMF 6	*TRBV24-1*	*TRBJ1-4*	*TRBD2*	CATSCSGGGNEKLFF	In-frame
FMF 9	*TRBV4-1*	*TRBJ2-4*	*TRBD2*	CASSQVFSSGKAKNIQYF	In-frame
FMF 12	*TRBV7-9*	*TRBJ1-5*	*TRBD1*	CASSSDSQVRGAVYSNQPQHF	In-frame
FMF 14	*TRBV7-9*	*TRBJ2-7*	*TRBD1*	CASSLGGQAYY**EQYF**	In-frame
FMF 16	*TRBV12-3 or TRBV12-4*	*TRBJ2-1*	*TRBD2*	CASSLLAGYN**EQFF**	In-frame
FMF 19	*TRBV5-8*01*	*TRBJ2-1*	*TRBD2*	CASRIDN**EQFF**	In-frame
FMF 19	*TRBV5-1*	*TRBJ2-1*	*TRBD2*	CASSGTSSGREN**EQFF**	In-frame
FMF 19	*TRBV12-3 or TRBV12-4*	*TRBJ2-7*	–	CASTPP#YEQYF	Out-of-frame
FMF 21	*TRBV2*	*TRBJ2-7*	*TRBD1*	CASSEALTWLGMSGSY**EQYF**	In-frame
FMF 21	*TRBV10-1*	*TRBJ2-3*	*TRBD2*	CASSP*RGG#TDTQYF	Out-of-frame
MF 1	*TRBV6-5*	*TRBJ2-3*	*TRBD2*	CASSYSFRSGSTDTQYF	In-frame
MF 1	*TRBV10-3*	*TRBJ2-7*	*TRBD2*	CAISVNTSG*V#EQYF	Out-of-frame
MF 2	*TRBV15*	*TRBJ2-1*	*TRBD1*	CATSREAPGDYN**EQFF**	In-frame
MF 2	*TRBV6-5*	*TRBJ2-7*	*TRBD1*	CASSFRVTGEKAS#SYEQYF	Out-of-frame
MF 4	*TRBV5-1*	*TRBJ1-6*	*TRBD1*	CASSLDRRSPLHF	In-frame
MF 7	*TRBV20-1*	*TRBJ1-2*	*TRBD1*	CSARTGGYGYTF	In-frame
MF 8a	*TRBV7-8*	*TRBJ1-2*	*TRBD1*	CASSVSRAGTGGGYTF	In-frame
MF 8b	*TRBV28*	*TRBJ2-6*	*TRBD1*	CASSLLSRGPSGANVLTF	In-frame
MF 16	*TRBV7-2*	*TRBJ2-2*	*TRBD2*	CASSSTSGSLNTGELFF	In-frame
MF 18	*TRBV7-3*	*TRBJ2-1*	*TRBD2*	CASSLWPAGGPSV**EQFF**	In-frame

The EQ(Y/F) F amino acid motif of potentially functional rearrangements is shown in bold. FMF case numbers19 and 21 demonstrated multiple potentially functional clonal rearrangements.

Table II. Immunophenotype of lymphoma cells in patients with folliculotropic mycosis fungoides (FMF) and in patients with mycosis fungoides (MF)

Marker	FMF ($n=21$)	MF ($n=20$)
CD3	21/21	20/20
CD4	21/21	16/20
CD8	0/21	3/20
CD5	15/20	11/18
CD7	5/20	3/17

may not always be the case in MF (22), and due to the fact that all rearranged *TRB* genes cannot be detected with the BIOMED-2 technique (20).

There was no particular selection of *TRBV* or *TRBD* genes in FMF and MF. By contrast, a skewed usage of *TRBJ* genes was seen in FMF and, to a lesser extent, in MF. The highly homologous *TRBJ2-1* or *TRBJ2-7* genes were identified in 5 out of the 10 cases of FMF with potentially functional *TRB* gene rearrangements. The *TRBJ* gene usage bias resulted in the presence of the CDR3 EQ(Y/F)F amino acid motif in FMF (FMF numbers 5, 14, 16, 19 and 21), whereas the motif was only seen in 2 out of 7 patients with MF (MF numbers 2 and 18). Acidic amino acids or amide-containing amino acids resulting in more hydrophilic properties were more frequently present in the CDR3 region in FMF than in MF (**Table I**). Complete nucleic acid sequences of potentially functional rearrangements are available from GenBank (reference numbers KT878518 to KT878536).

Immunohistochemistry

Table II summarizes the immunophenotype of the neoplastic cells in the FMF and MF patients. All cases of FMF and most of MF showed expression of CD4. CD8 was expressed by 3 of the MF cases. CD5 and CD7 were variably expressed. Variable numbers of dendritic cells in epidermis and dermis from FMF and MF patients stained for CD1a, as expected. However, a larger number of dendritic cells stained for CD1c in most cases. CD1c expressing dendritic cells were particularly numerous in the epithelium of adnexa of patients with FMF (**Fig. 1**). The ratio of CD1c- over CD1a-expressing cells is shown in **Table III**. There was no significant difference in the CD1c/CD1a ratio distribution, as analysed by the Wilcoxon-Mann-Whitney test, nor the mean CD1c/CD1a ratio analysed by the Student's *t*-test between patients with FMF and those with MF ($p=0.38$ and $p=0.33$, respectively). No CD1b-expressing or CD1d-expressing dendritic cells were seen in FMF or MF. CD1b was expressed by the basal cell layer of epidermis and adnexa, but not by other epithelial or other cells in FMF as well as in MF. Epithelial cells did not express CD1a, CD1c or CD1d in any of the cases studied. Double immunofluorescence stainings for CD1a and CD1c were performed in 5 randomly selected FMF and MF cases. Single as well as double CD1a- and CD1c-expressing dendritic cells

Table III. CD1c/CD1a ratio in patients with folliculotropic mycosis fungoides (FMF) and mycosis fungoides (MF)

FMF		MF	
Patient #	CD1c/CD1a ratio	Patient #	CD1c/CD1a ratio
1	2	1	1.5
2	1	2	0.75
3	4	3	2
4	1	4	4
5	2	5	4
6	2	6	1
7	4	7	1
9	1	8	1
10	1	9	0.75
11	1	10	1
12	3	11	2
13	3	12	2
14	1	13	1
15	4	14	3
		15	1.5
		16	1
		18	2
		19	2
Mean	2.1	Mean	1.75

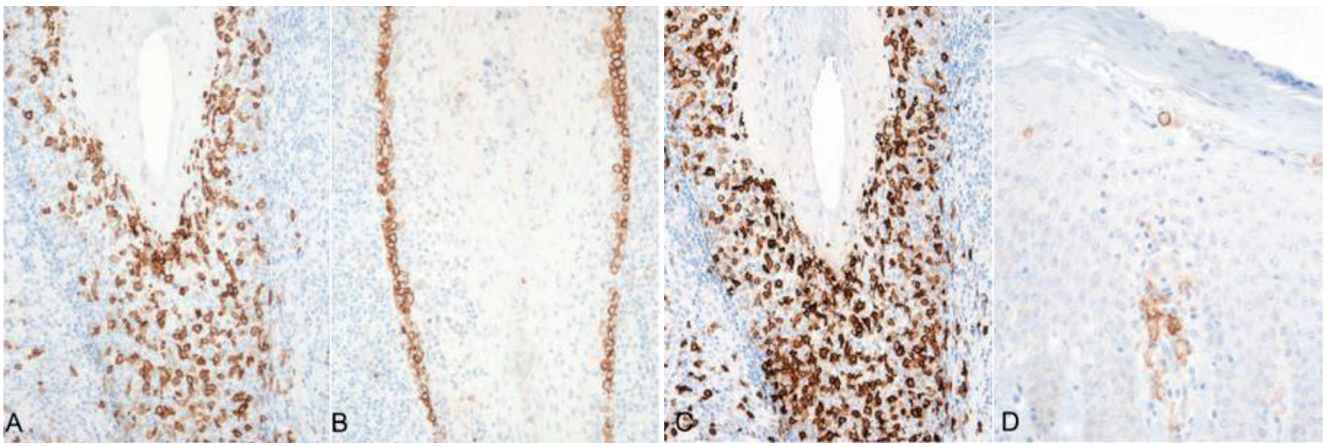

Fig. 1. Expression of CD1a and CD1c in folliculotropic mycosis fungoides (FMF). Skin biopsy of 1 of the patients with FMF (FMF; #5) analysed with immunoperoxidase staining for CD1a (A, ×100), CD1b (B, ×100), CD1c (C, ×100) and CD86 (D, ×200). (A), (B) and (C) are semi-serial sections. CD1a- and CD1c-staining reveal numerous positive dendritic cells within a hair follicle, while CD1b-staining reveals expression only by cells of the basal epithelial layer of the follicle. CD86 is expressed in a small number of dermal dendritic cells.

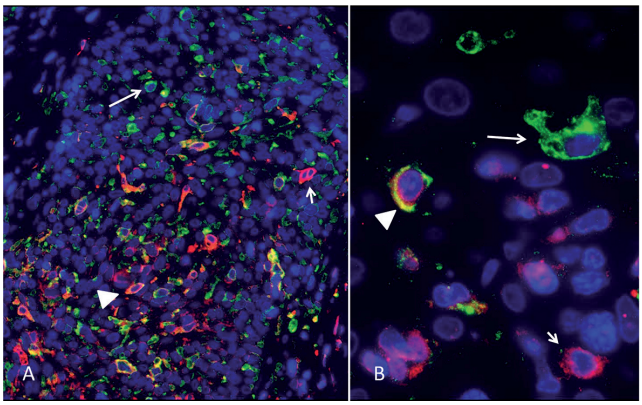

Fig. 2. Double immunofluorescence staining for CD1c and CD1a in folliculotropic mycosis fungoides (FMF). Staining of a hair follicle infiltrated by FMF cells with fluorochrome-labelled anti-CD1a (*green*) and anti-CD1c (*red*). The image shows single CD1a- (*short arrow*), single CD1c- (*long arrow*) positive cells and CD1a/CD1c-double-positive cells (*arrow head*) in an overview of the follicle (A, objective ×20) and at higher magnification (B, oil immersion, objective ×100).

were demonstrated in both lymphoma subtypes (**Fig. 2**). This analysis also confirmed a CD1c/CD1a expression ratio > 1 in both lymphoma types. However, while FMF displayed similar numbers of CD1a single-positive and CD1a/CD1c double-positive cells, MF lesions showed a trend towards a lower number of CD1a single-positive and a higher number of CD1a/CD1c double-positive dendritic cells compared with FMF. CD80 was expressed on T cells, but not on dendritic cells, whereas CD86 was expressed on a small subpopulation of dendritic cells in all cases, as described previously (23).

DISCUSSION

Evidence of antigen-selection has been documented for indolent B-cell lymphoproliferative diseases, such as chronic lymphocytic leukaemia, marginal zone lymphoma, but also for clinically more aggressive B-cell lymphomas, such as mantle cell lymphoma and diffuse large B-cell lymphoma (24). Analysis of the B-cell receptor in these lymphomas often shows restricted usage of immunoglobulin genes or even stereotypic B-cell receptors (25). Restricted immunoglobulin gene usage probably results from selective pressure exerted by antigen-stimulation resulting in growth advantage of lymphoma cells (25). Such antigens can either be exogenous or auto-antigens or a combination of both. A role for antigen-stimulation has also been postulated for T-cell lymphomas, especially MF (26). Langerhans cells have been shown to stimulate cutaneous T-cell lymphoma cells, although the nature of the antigen is unknown (8). *TRB* gene sequence analysis had not hitherto been performed in FMF, although it has been studied in MF (13–18). Restricted use of *TRB* genes was not demonstrated in MF (13–18). Similarly, the limited number of cases of MF in our study does not show selective usage of *TRB* genes. However, our results indicate recurrent usage of the highly homologous *TRBJ2-1* and *TRBJ2-7* genes in FMF, but not of *TRBV* and *TRBD* genes. Usage of these genes results in a EQ(Y/F)F amino acid motif at the COOH-terminal end of the T-cell receptor CDR3 region. A similar restricted usage of *TRBJ2-1* and *TRBJ2-7* genes has been demonstrated in Sézary syndrome and in lesional T cells of psoriasis (19, 27, 28). In contrast to the studies in psoriasis, but similar to the study in Sézary syndrome, recurrent *TRB* CDR3 motifs encoded by D and N nucleotides were not detected in FMF or MF in our study. Of note, *TRBJ2-1* and *TRBJ2-7* genes are also selected for in the peripheral blood of the healthy adult population (29). In contrast to the healthy adult population, other TRBJ genes, such as *TRBJ1-1* and *TRBJ2-3*, are not commonly used in the FMF patient cohort of this study and in Sézary syndrome (19). However, the number of patients is limited and studies of more patients are needed to confirm the data. The T-cell receptor EQ(Y/F)F amino acid motif encoded by *TRBJ2-1* and *TRBJ2-7* genes is associated with T cells that recognize lipid antigens through presentation by CD1 molecules on dendritic cells (30, 31). The motif might facilitate docking of the TCR to the CD1 molecule (30, 31). If proved, the presence of this motif does not necessarily indicate that similar antigens are presented to T cells bearing the EQ(Y/F)F TCR motif, but only that antigens are presented through the CD1 molecules. Most T lymphocytes are stimulated through specific recognition of peptide antigens bound to major histocompatibility complex MHC I or II molecules on the surface of antigen-presenting cells. T lymphocytes can also be stimulated by lipid antigens presented by CD1 molecules (32–34). CD1 molecules are homologous to MHC class I-molecules. In contrast to MHC molecules, CD1 are non-polymorphous (11, 35). In humans, CD1 molecules comprise 5 isoforms, CD1a, CD1b, CD1c (group 1) and CD1d (group 2) and CD1e (intermediate). The group 1 isoforms presents lipids to T cells, while group 2 isoforms present lipids to natural killer T cells (35). CD1 molecules are expressed by antigen-presenting cells including dendritic cells, Langerhans cells and B cells and also on epithelial cells (36). A wide repertoire of self-lipids and foreign lipids, such as microbial lipids, have been found to be presented by the different CD1 isoforms (35).

T cells in psoriasis have recently been proven to be, at least in part, CD1a-restricted and to be activated by neolipid antigens released by increased extracellular phospholipase PLA2 activity (37). In view of this finding and of the skewed presence of the EQ(Y/F)F motif in the T-cell receptor in T cells of psoriasis (28), it is tempting to speculate that FMF is also CD1 restricted. To find further evidence of the role of CD1-restricted antigen presentation in FMF, we studied CD1 expression by immunohistochemistry in archival skin biopsies of FMF and control MF. Although the presence of nume-

rous CD1a-expressing dendritic cells and Langerhans cells is well-documented in primary cutaneous T-cell lymphoma (38), expression of CD1b, CD1c and CD1d is less well documented in these diseases and has not yet been studied in FMF. A high number of CD1a- and CD1c-expressing dendritic cells was found in FMF and MF. However, CD1c-expressing cells were particularly numerous in skin adnexa in FMF. We also demonstrated that a subpopulation of CD1a and CD1c double-positive cells were present in FMF and MF. CD1b expression was limited to the basal cells of the epidermis and skin adnexa in both FMF and MF patients, while CD1d-expressing cells were not detected. Similar to our findings in MF, increased numbers of CD1a- and CD1c-expressing dendritic cells have been reported previously in MF (39, 40). One of these studies also reported an increased number of CD1b-expressing dendritic cells in MF, but did not report expression in epithelial cells (39), a finding that contrasts with our study. Our findings show exclusive expression of CD1b in the basal cell layer of epidermis, but not in dendritic cells. The use of frozen section immunohistochemistry and a different CD1b antibody in the study of Fivenson & Nickoloff (39) probably explains the different results. The exclusive expression of CD1b in the basal cell layer of the skin is similar to what is reported for normal skin in the Human Protein Atlas using yet another polyclonal antibody (www.proteinatlas.org). The presence of CD1d-expressing dendritic cells had hitherto been documented in normal skin only, but not in MF or FMF (12). We did not find increased numbers of CD1d-expressing dendritic cells in MF or FMF. The immunohistochemistry results strengthen the hypothesis that lipid-presentation through CD1-expressing dendritic cells in skin adnexa probably occurs in FMF. It is likely that lipid-presentation by dendritic cells also occurs in MF, as indicated by the presence of CD1-expressing cells in lesional biopsies, albeit at reduced absolute numbers. It is of interest in this respect that 2 out of 7 cases of MF also showed the EQ(Y/F)F amino acid motif in the T-cell receptor β CDR3 region.

In conclusion, our findings show evidence of skewed *TRBJ* usage in FMF, possibly indicating antigen-selective pressure exerted by lipid antigens. Furthermore, the CD1c molecule was most abundantly expressed by dendritic cells in cutaneous lesions of patients with FMF. Therefore, CD1c-expressing dendritic cells might be responsible for antigen-presentation to FMF lymphoma cells. Further testing is required to show that FMF lymphoma cells are stimulated by lipid antigens presented by CD1 proteins, as recently proved for T cells in psoriasis (37). A complete characterization of lipid-specific CD1-restricted T cells has been facilitated by the development of CD1 tetramers loaded with lipid antigens (41). However, these studies need to be performed on cell suspensions using flow cytometry. These studies cannot be carried out on archival formalin-fixed biopsy materials of patients, such as used in our study. Unfixed fresh biopsy samples for making cell suspensions should be collected prospectively for such a study. The study of potential CD1-antigen restriction in FMF and MF is important in view of novel therapeutic possibilities that may result, such as treatment with anti-CD1 antibodies. The use of anti-CD1 antibodies has recently been proposed for the treatment of psoriasis (42).

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