

The number of regular T cells and immature dendritic cells involved in mycosis fungoides is linked to the tumor stage

Q.-A. ZHANG¹, Z.-Q. CHEN², M.-H. CHEN¹, Z.-D. XU²

¹Department of Dermatology, Huashan Hospital, Fudan University, Shanghai, China

²Department of Pathology, Huashan Hospital, Fudan University, Shanghai, China

Qiao-an Zhang and Zhong-qing Chen contributed equally to this work

Abstract. – BACKGROUND: The balance between immune surveillance and immune escape determines the outcome of patients with primary mycosis fungoides (MF). FOXP3+ regulatory T cells (Tregs) and DC-SIGN+ immature dendritic cells (imDCs) play a central role in regulating the immune state in the progression of MF. However, whether the mechanisms used by these factors depend on MF stage is still undetermined and even controversial.

PATIENTS AND METHODS: FOXP3+ Tregs and DC-SIGN+ imDCs were detected by immunohistochemical staining of formalin-fixed, paraffin-embedded specimens obtained from the lesion biopsies of 89 patients with MF, comprising 69 patients at the patch stage, 12 at the plaque stage, and 8 at the tumor stage. The number of FOXP3+ Tregs and DC-SIGN+ imDCs in each stage was counted and compared.

RESULTS: The expression of FOXP3 and DC-SIGN varied with the MF stage. The number of cells expressing FOXP3 was higher at the patch and plaque stages than at the tumor stage ($p < 0.05$), but no significant difference was noted between the patch and plaque stages ($p = 0.715$). DC-SIGN expression increased continuously, concomitant with tumor progression, through the three stages ($p < 0.05$).

CONCLUSIONS: The predominant factor influencing the immune state is different for each MF stage. Therefore, therapeutic strategies that modulate the antitumor immune responses should be developed depending on MF progression.

Key Words:

Mycosis fungoides, Regulatory T cells, Immature dendritic cells, FOXP3, DC-SIGN.

Introduction

Mycosis fungoides (MF) is the most common form of primary cutaneous lymphoma. Clinically,

patients with MF experience patches that initially develop into plaques and then into tumors. Patients with patch- and plaque-stage MF have excellent outcome. However, the prognosis dramatically worsens when the disease progresses into the tumor stage. The mechanisms underlying the transition from the patch/plaque to the tumor stage are still unknown, but microenvironmental effects have been speculated to play an important role in the process. Recent studies have emphasized the important role of regulatory T cells (Tregs) and dendritic cells (DCs). Some studies suggested that the number of Tregs correlates with the stage and prognosis of MF¹, whereas other studies showed neither a significant difference nor a predictable value of Tregs in prognosis². On the other hand, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) has been considered a differentiation hallmark of immature DCs (imDCs). The increase in the number of DC-SIGN+ imDCs under inflammatory and tumor conditions can induce immunotolerance³. The current study describes the number of FOXP3+ Tregs and DC-SIGN+ imDCs at the patch, plaque, and tumor stages of MF, and aims to identify the predominant immune state in each stage of MF and the stage-dependent mechanism underlying this phenomenon, if any.

Patients and Methods

Patients and Tissues

The study was approved by the local Ethics Committee. Written informed consent was obtained from all subjects enrolled. Diagnosis and staging of MF were performed according to the guidelines of the International Society for Cuta-

neous Lymphomas and the Cutaneous Lymphoma Task Force of the European Organization of Research and Treatment of Cancer. Formalin-fixed and paraffin-embedded specimens of skin biopsies from 89 patients at different stages of MF (69, patch; 12, plaque; 8, tumor) were collected from the archive of the Department of Dermatology, Huashan Hospital, Fudan University, China. For diagnostic purposes, all biopsies were analyzed by conventional microscopy and immunohistology.

Immunohistochemical Staining

The formalin-fixed, paraffin-embedded samples were sectioned (4 μm) and picked up on slides coated with lysine. The slides were heated in a drying oven (56°C) for 2 hours, deparaffinized (xylene), washed with alcohol (100% and 95%), and rehydrated in deionized water. Antigen retrieval was performed as follows: the slides were incubated in a microwave oven at 98°C for 20 minutes in Target Retrieval Solution [Tris/ethylene diamine tetra-acetate buffer (pH 9)], allowed to cool to room temperature, and then rinsed with Tris-buffered saline wash buffer. Endogenous peroxidase activity was blocked by incubating the slides for 5 minutes in 0.03% hydrogen peroxide. The sections were rinsed in wash buffer and incubated for 1 hour at 37°C with the monoclonal mouse anti-FOXP3 antibody (dilution 1:50, clone 236A/E7; eBioscience, San Diego, CA, USA) and monoclonal mouse anti-DC-SIGN antibody (dilution 1:100, clone 5D7; Abcam, Hongkong, China) in Tris-HCl buffer antibody diluent. The slides were rinsed in wash buffer and incubated for 30 minutes with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin (EnVision/HRP; Dako-Cytomation, Glostrup, Denmark). The chromogenic reaction was carried out with the chromogen solution (3,3'-diaminobenzidine) for 10 minutes, leading to the generation of brown color. Finally, the slides were rinsed with deionized water, counterstained with hematoxylin, dehydrated, and mounted with toluene-based mounting medium (Thermo Scientific Richard-Allan, Loughborough, Leicestershire, UK).

Evaluation of Immunohistochemical Staining

A blinded evaluation of the clinical data was performed. The tumor-infiltrating FOXP3+ Tregs and DC-SIGN+ imDCs were scored by counting the number of positive cells in 3-8 representative

areas per slide at a magnification of 400 \times , according to the extent of each lesion, and the number of positively stained cells per high-power field [HPF; mean \pm standard error of the mean (SEM)] was recorded.

Statistical Analysis

Statistical analysis was performed using SPSS version 18.0 software packages (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$. Data were presented as mean \pm SEM. One-way ANOVA and the Scheffe model for repeated mean value comparisons were used for analyzing the data obtained from FOXP3 and DC-SIGN immunostaining; the results were used to compare tumor stages. Tumor stage was considered the fixed factor and patients as the random factor.

Results

Results from the staining of FOXP3 and DC-SIGN are summarized in Table I. Representative examples are illustrated in Figure 1. The nuclei of tumor-infiltrating Tregs showed very strong positive staining for FOXP3. The lesions of patch/plaque-stage MF were predominantly infiltrated by FOXP3+ Tregs (an average of 35/HPF and 40/HPF, respectively) in the epidermis and upper dermis, while those of tumor-stage MF displayed significantly lower frequency for FOXP3+ Tregs (an average of 10/HPF) in the deep dermis ($p < 0.05$). However, there was no significant difference in Treg infiltration between the patch and plaque stages ($p = 0.715$). The tumor-infiltrating imDCs stained positive for DC-SIGN; the membrane as well as the cytoplasm showed moderate staining. The lesions of tumor-stage MF were diffusely infiltrated by DC-SIGN+ imDCs (an average of 49/HPF) in the deep dermis and subcutis, while those of plaque-stage MF showed lower frequencies (an average of 21/HPF) in the deep dermis. Patch-stage lesions had the least frequencies, with an average of 8/HPF in the upper dermis. Thus, there was an obvious difference in imDC infiltration between these three stages ($p < 0.05$). Briefly, the data showed stage-dependent increase in the number of Tregs and imDCs in MF (Figure 2). In addition, we found that the epidermotropic lymphocytes at the dermoepidermal junction in the patch stage, referred to as MF cells, were FOXP3 negative (Figure 1B). In the tumor stage, the small,

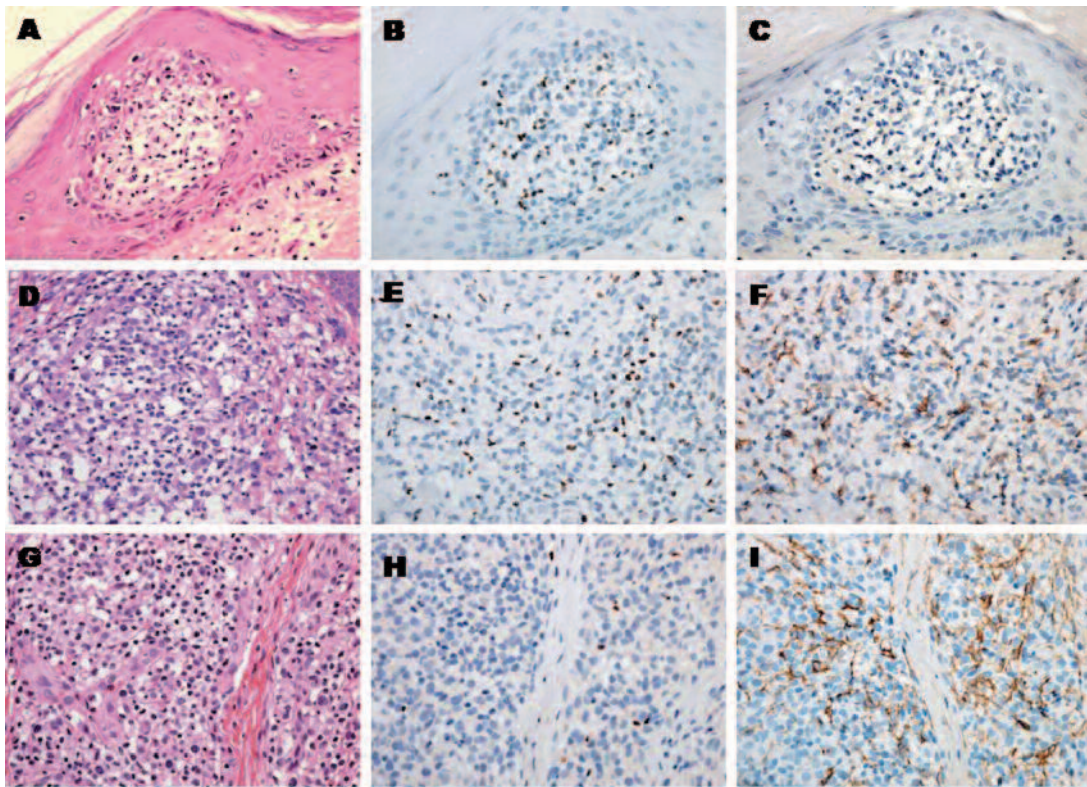


Figure 1. Representative example of hematoxylin and eosin (H&E)-stained and immunohistochemically stained lesions of patch-, plaque-, and tumor-stage MF. In the patch stage, atypical lymphocytes surrounded by a clear “hole” are typically confined to the basal layers of the epidermis (**A**). In the plaque stage, the infiltrating lymphocytes are more dense, and atypical lymphocytes are more common (**D**). In the tumor stage, the infiltrating lymphocytes have a more monomorphic appearance, and atypical cells are dominant (**G**). Immunohistochemical staining with monoclonal mouse anti-FOXP3 and anti-DC-SIGN shows the Tregs and imDCs in the patch (**B**, **C**), plaque (**E**, **F**), and tumor (**H**, **I**) stages of MF. Original magnification: 400 \times .

round, tumor-infiltrating lymphocytes (TILs), but not the medium/large-sized atypical lymphocytes, (Figure 1H) stained positive for FOXP3.

Discussion

MF is a peripheral non-Hodgkins T-cell lymphoma, initially presenting in the skin. Classical MF usually involves three clinical stages: patch, plaque, and tumor. However, not all MF show

this pattern. A subset of patients had persistent patch-stage MF for many years or even permanently stayed at this state without progression. Other MFs progressed into thin and thick plaques. In a minority of patients, the disease eventually developed into tumors, with dissemination to the lymph nodes, blood, and other organs. The underlying mechanisms responsible for such diversities are still unknown. In addition to the individual characteristics of each tumor, the immune microenvironment around tumor

Table 1. Expression of FOXP3 and DC-SIGN in MF patients.

Stage	Patients (n)	FOXP3+ Tregs (mean \pm SEM)	DC-SIGN+ imDCs (mean \pm SEM)	Representative area
Patch	69	35.77 \pm 2.04	8.65 \pm 0.92	Dermoepidermal junction
Plaque	12	40.83 \pm 10.09	21.17 \pm 4.42	Papillary dermis
Tumor	8	9.88 \pm 3.01	49.38 \pm 7.82	Reticular dermis

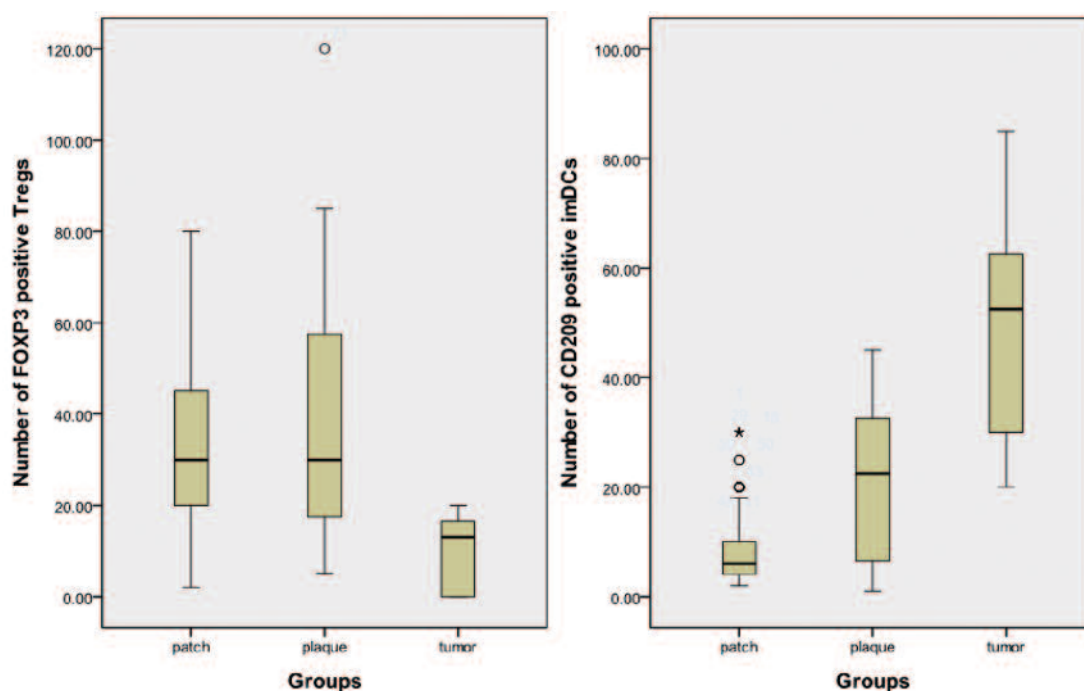


Figure 2. Box plot of the number of FOXP3+ Tregs and DC-SIGN+ imDCs in the different stages of MF. Box plot represents the median (bold line), interquartile range (box), outliers (circle), and extreme observations (star).

cells may play a critical role in disease progression. Tregs and imDCs are two main immune factors that affect the progression of MF^{1,4-6}. However, the correlation of imDCs with tumor stage has not been determined yet, and the correlation between MF stage and Treg number has been controversial^{1,2}.

Our data showed that the number of FOXP3+ Tregs in MF depended on the tumor stage. We observed a markedly higher number of FOXP3+ cells in the early stage than in the later stages of MF. However, the number of DC-SIGN+ imDCs depended on the tumor stage in a manner different from that observed in FOXP3. The number of DC-SIGN+ imDCs steadily increased with tumor progression.

There have been arguments about the malignant characteristic and stage dependency of FOXP3+ Tregs². Data obtained by Gjerdrum et al¹ showed that the atypical neoplastic infiltration was either negative for FOXP3 or only contained occasional weakly positive cells. In addition, they found that patch stage MF or early infiltrated plaques had significantly higher number of FOXP3+ Tregs than cutaneous T-cell lymphoma (CTCL) unspecified or advanced tumor-stage MF. On the contrary, Fried et al² found that a proportion of neoplastic cells in MF expressed FOXP3.

Furthermore, they found that the number of FOXP3+ Tregs in MF was independent of the tumor stage and unrelated to the disease course. In this our study, immunohistochemical staining revealed that the FOXP3+ cells had small and round nuclei, and infiltrate MF cells with middle-sized nuclei. The analysis of the H&E-stained sections showed that the basilar, linearly arranged MF cells in the patch stage were negative for FOXP3. In plaque-stage MF, only the small lymphocytes with round nuclei within the Pautrier's microabscesses were positive for FOXP3. The atypical, cerebriform lymphocytes were clearly negative, which was in contrast with the findings of Fried et al² that all cells within Pautrier's microabscesses were positive for FOXP3. Gjerdrum et al¹ observed that only 1 of the 86 CTCL types, that is, a CD8+ cytotoxic T-cell lymphoma, displayed positive staining for FOXP3. The other types had cells that were either FOXP3 negative or occasionally weakly positive. Three independent studies⁷⁻⁹ exclusively showed FOXP3 expression in TILs, and not in the malignant T cells, by immunohistochemical staining of 44 CTCL types. On the basis of these findings, we postulate that strong FOXP3 expression was restricted to tumor-infiltrated Tregs rather than to tumor cells. The different clones of anti-FOXP3 antibodies that

each group chose may cause the differences in the results and relevant conclusions on the nature of FOXP3+ lymphocytes. In addition, our data showed a higher number of FOXP3+ tumor-infiltrating Tregs in early-stage than in advanced-stage MF.

However, we found that the distribution of tumor-infiltrating DC-SIGN+ imDCs differed from that of FOXP3+ Tregs. There was a steady increase in imDCs with MF progression through patch, plaque, and tumor stages. Schlapbach et al⁶ revealed significant infiltration of CTCL lesions by DC-SIGN+ imDCs, which closely contacted to tumor cells. Mature and activated DCs were rarely detected in the lesions of CTCL. However, Schlapbach et al⁶ could not determine whether DC-SIGN infiltration was stage dependent because of limited sample size. Our data showed that DC-SIGN+ imDC infiltration in MF is stage dependent, with maximum infiltration in tumor-stage lesions.

As discussed, patch- and plaque-stage MF had higher number of tumor-infiltrating FOXP3+ Tregs than tumor-stage MF. Other studies^{1,8,10} consented that a high frequency of FOXP3+ TILs was associated with improved survival. FOXP3+ Tregs might suppress the proliferation of neoplastic T cells, similar to the effect of cytotoxic T cells. Under these circumstances, new treatment modalities that can influence the number or function of Tregs must be introduced carefully in patients with early-stage MF. However, when MF progresses into the tumor stage, with tumor cells infiltrating into deep dermis and subcutis, the number of tumor-infiltrating Tregs decreased, whereas that of DC-SIGN+ imDCs markedly increased. Using immunohistochemical staining, we found that DC-SIGN+ imDCs were surrounded by malignant MF tumor T cells, which can secrete vascular endothelial growth factor (VEGF). The secreted VEGF enters the bloodstream and stimulates the myeloid-derived imDCs migrating from the bone marrow to the primary tumor site¹¹. MF tumor cells could also suppress the maturation and the antigen-presenting function of imDCs by secreting interleukin (IL)-10 and transforming-growth factor- β (TGF- β), thereby, inducing immune tolerance in tumor antigens¹². DC-SIGN expression is associated with poor prognosis in tumors such as acute lymphoblastic leukemia¹³, while the infiltration of mature DCs in tumors such as melanoma is associated with long survival¹⁴. Therefore, the intratumoral presence of imDCs may facilitate tumor progression

and result in poorer prognosis. Accordingly, therapeutic strategies targeted at tumor-stage MF should focus on DC vaccination to induce imDC maturation.

Conclusions

Our findings suggest that tumor-infiltrated FOXP3+ Tregs play a dominant role in antitumor immunity in the early stage of MF, whereas DC-SIGN+ imDCs play a key role in inducing immune suppression in the late stage of MF. This should be considered during the development of a therapeutic strategy for modulating antitumor immune response.

Conflict of Interest

The Authors declare that they have no conflict of interests..

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