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The role of macrophages in the pathogenesis of mycosis fungoides

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Summary

Background. Macrophages are classified into classically activated (M1) and alternatively activated (M2) macrophages. Decrease in macrophage number in tumour tissue with treatment has been reported.
Aim. The aim of this study was to determine whether treatment has an effect on the number of dermal M1 and M2 macrophages in patients with mycosis fungoides (MF).
Methods. In total, 21 patients (8 women, 13 men; age range 42–73 years) were included in this study. We determined markers for dermal M1 (inducible nitric oxide synthase and CD68) and M2 (markers: CD163 and CD206) macrophages using double immunohistochemistry to reduce the error rate, and then counted the cells.
Results. The number of dermal M1 cells was significantly lower pretreatment compared with post-treatment ($P < 0.01$). The numbers of dermal M2 cells were also numerically decreased by treatment. These results did not change significantly after exclusion of the patients who had recurrence ($n = 2$). There were no statistically significant differences between groups classified by stage, lesion type or treatment outcome.
Conclusion. Macrophage numbers are decreased in MF after treatment of tumour tissue.

Introduction

Macrophages are widely distributed immune system cells, which have prominent roles in cytokine/chemokine production and receptor expression.[1,2] Classically activated macrophages (M1 cells) are related to inflammation and tumour inhibition, and produce inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1b, IL-6 and IL-23, and high levels of inducible nitric oxide synthase (iNOS). Alternatively activated macrophages (M2 cells) are related to tumour cell growth and produce IL-10. They are also associated with high expression of the class A scavenger receptor CD204, and with mannose receptor, dectin-1, CD209 and CD163.[3,4]

Macrophages in the tumour microenvironment are termed tumour-associated macrophages (TAMs) and generally exhibit an M2 phenotype. TAMs produce a variety of cytokines that act in two contradictory ways, such as suppressing tumour cells and promoting tumour progression. Both M1 and M2 macrophages express CD68 antigens, which are used as markers for detection of TAMs and macrophages.[3–5]

Numerous studies have reported presence of CD68-positive macrophages in glioma,[6] hepatocellular carcinoma[7] and malignant melanoma;[8] of CD163-positive macrophages in leimyosarcoma,[9] Hodgkin lymphoma[10] and rectal cancer;[11] and of CD206-positive macrophages in renal cell carcinoma.[12] In addition, low level iNOS secretion has been correlated with poor prognosis in renal cell[13] and colorectal[14] cancers.

Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma (CTCL) and generally

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progresses slowly through patch, plaque and tumour stages.[15,16] Progression is seen in only a few cases over years, followed by lymph node and visceral involvement.[3] Epidemiological studies have shown that the annual growth rate of this disease is 0.3 cases per 100 000 of the population.[17] The aetiology of MF is not yet known.[18] MF is generally a low-grade malignant lymphoma that has a prolonged survival period.[15] It is is predominantly a disease of elderly people and is twice as common in men as in women.[15]

The aim of present study was to determine whether treatment for MF is associated with a difference in the number of dermal M1 and M2 macrophages, using double immunohistochemistry with macrophage markers in skin lesions from patients with MF.

Methods

This study approved by our institutional review board (5 October 2012, number 12/38), and all participants provided written informed consent.

Patients and samples

The study was performed in the Dermatology Department (Kirikkale University Medical School) between June 2012 and March 2014. Exclusion criteria for this study were age < 18 years, pregnancy or lactation, and receipt of immunosuppressive drug therapy.

In total, 21 patients diagnosed with MF (8 women, 13 men; age range 42–73 years) were enrolled in this study. Of the 21 patients, 15 patients with patch lesions were treated with narrowband ultraviolet (UV)B phototherapy and topical corticosteroids, while 6 patients with plaque lesions were treated with a combination of psoralen/UVA (PUVA) photochemotherapy and acitretin.

After 6 months of treatment, 16 patients had complete clearance and 3 patients had partial remission, while the remaining 2 patients initially had complete remission but later reported recurrence.

Biopsies

Pretreatment and post-treatment biopsies were obtained from the same lesion. Because of ethical concerns, we were not able to obtain biopsies from healthy skin for comparison. Additional biopsies were also taken from any patients with recurrence.

The samples were processed for routine examination and embedded in paraffin wax. Haematoxylin and eosin-stained slides of samples from patients with MF were evaluated, and any blocks considered to contain tumour were identified for immunohistochemical analysis.

Immunohistochemistry

Tissue was cut into sections 4 μm thick and placed onto slides coated with poly-L-lysine for immunohistochemical detection of CD68, CD163, CD206 and iNOS expression, using the streptavidin–biotin immunoperoxidase method. The slides were placed in an automatic immunostainer (Bond Max; Leica Microsystems, Wetzlar, Germany). The paraffin wax was melted, then samples were dewaxed at 72 °C, rinsed in 96% alcohol and hydrated in Bond Wash solution. Monoclonal mouse antibodies were used as primary antibodies in all cases: anti-CD68 (Dako, Glostrup Denmark) and anti-iNOS (Abcam, Cambridge, UK) for detection of M1 macrophages, and anti-CD163 (Thermo Fisher Scientific, Waltham, MA, USA) and anti-CD206 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) for M2 macrophages. As this was a double immunohistochemistry staining process, both antibodies in each set were used together.

The chromogens used for antibody visualization of antibody were 3,3′-diaminobenzidine (DAB) for iNOS–CD163 and 3-amino-9-ethylcarbazole (AEC) for CD68–CD206 antibodies. Antigen retrieval from tissue for antibodies to be used with DAB was carried out using citrate buffer at 100 °C for 20 min for anti-iNOS antibody and 10 min for anti-CD163, then anti-iNOS (diluted 1 : 500) and anti-CD163 (diluted 1 : 50) were added together to the slides, which were incubated at room temperature for 15 min. Incubation and washing were performed in an autostainer, in accordance with the instructions of the antibody manufacturers, then uncovering of the antigens for the antibodies to be used with AEC was performed using EDTA solution at 100 °C for 20 min for anti-CD68 and 10 min for anti-CD206. Both anti-CD206 (diluted 1 : 50) and anti-CD68 (undiluted) antibodies were added together to the slides, which were incubated at room temperature for 12 min. A secondary kit (ChromoPlex™ Dual Detection Kit; Leica, Wetzlar, Germany) was used for detection of all primer antibodies; the slides were incubated and washed in the autostainer according to the manufacturer's instructions, then the chromogens were added. After further incubation and washing, the tissue sections were mounted in glycerol on glass slides, which were examined under a light microscope with a × 10 objective lens for the presence of M1 and M2 cells.

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Stained cells were counted in 10 microscopic areas for each section and averaged.

Statistical analysis

All data were analysed with SPSS (Statistical Package for the Social Sciences) software for Windows (v18.0; IBM SPSS, Armonk, NY, USA). Statistical analysis was performed using the Wilcoxon signed rank test for comparison of values. Differences in M1 and M2 cell number between re-formed groups according to stages, lesion types and treatment outcomes were analysed using Mann–Whitney *U*-test and Kruskal–Wallis test. *P*-values of < 0.05 were considered statistically significant.

Results

Patient demographics

Patients with MF comprised 38% men and 61.9% women. Of the 21 patients, 7 (33.3%) were aged 40–49 years, 8 (38.1%) were aged 50–59 years and 6 (28.6%) were aged 60–70 years (Table 1).

Table 1 Demographics of the patients with MF (n = 21).

Characteristic	Result
Sex	
Female	8 (38.1)
Male	13 (61.9)
Age, years	
Mean ± SD	54.48 ± 8.08
Range	42–70
Age range, years	
40–49	7 (33.3)
50–59	8 (38.1)
60–70	6 (28.6)

Data are *n* (%) unless otherwise specified.

Disease stage

Of the 21 patients, 8 (38.1%) had stage 1a disease and 13 (61.9%) had stage 1b disease. There were 15 patients (71.4%) with patch-type lesions and 6 (28.6%) with plaque-type lesions.

Treatment produced partial remission in 3 patients (14.3%) and complete remission in 19; however, 2 of these patients (9.5%) later had recurrence, thus complete remission was actually achieved in 16 patients (76.2%).

Repeat biopsies were taken from the two patients who had recurrence, and epidermotropism was detected in the specimens, confirming that a complete cure had not been achieved in these patients, and they were therefore placed on maintenance treatment.

Immunohistochemistry

The immunohistopathological staining of sections stained with iNOS–CD68 and CD163–CD206 for detection of dermal M1 and M2 macrophages, respectively, before and after treatment are shown in Figs 1 and 2.

The number of dermal M1 cells was significantly lower pre-treatment compared with post-treatment ($P < 0.01$). The numbers of dermal M2 cells were also numerically decreased after treatment, but this was not significant. The results did not change significantly after exclusion of the patients who had recurrence (Table 2).

Discussion

TAMs, which originate from blood monocytes, are recruited and differentiated in a tumour mass by various signals produced by neoplastic and stromal cells. This differentiation is a result of the absence of M1-orienting signals. TAMs play a pivotal role in tumour growth, progression and dissemination by producing proangiogenic molecules, vascular endothelial growth factor, IL-8 and fibroblast growth factor. A high rate of TAMs is associated with a poor prognosis, and recent studies indicate that an abundance of TAMs correlates with the metastatic process in tumours.[2–5]

In this study, we used a double immunohistochemistry technique, using markers for M1 and M2 cells.

CD68-positive stained macrophages have been reported to be relatively nonspecific for melanoma, angiosarcoma, lymphoma and schwannoma,[19] and Strojnik *et al.*[6] reported that CD68-positive macrophages correlated with poor prognosis in glioma.

CD163 has an anti-inflammatory function and its expression level is much higher in TAMs than in monocytes.[20] Studies have reported that intratumoral CD163-positive macrophages correlate with poor prognosis in a variety of tumours, including melanoma and leimyosarcoma.[8,9] Sugaya *et al.*[3] showed that the numbers of CD163-positive cells in lesional skin of patients with CTCL, atopic dermatitis or psoriasis were significantly higher than those in normal skin, and CD163 was a more abundant marker of TAMs in the skin compared with CD68.[3] Similar results were obtained from tumour tissue of patients with malignant melanoma.[8] By contrast, Harris *et al.*[21] noted that the numbers of CD163-positive cells were lower than those of CD68-positive cells in tumour tissue of

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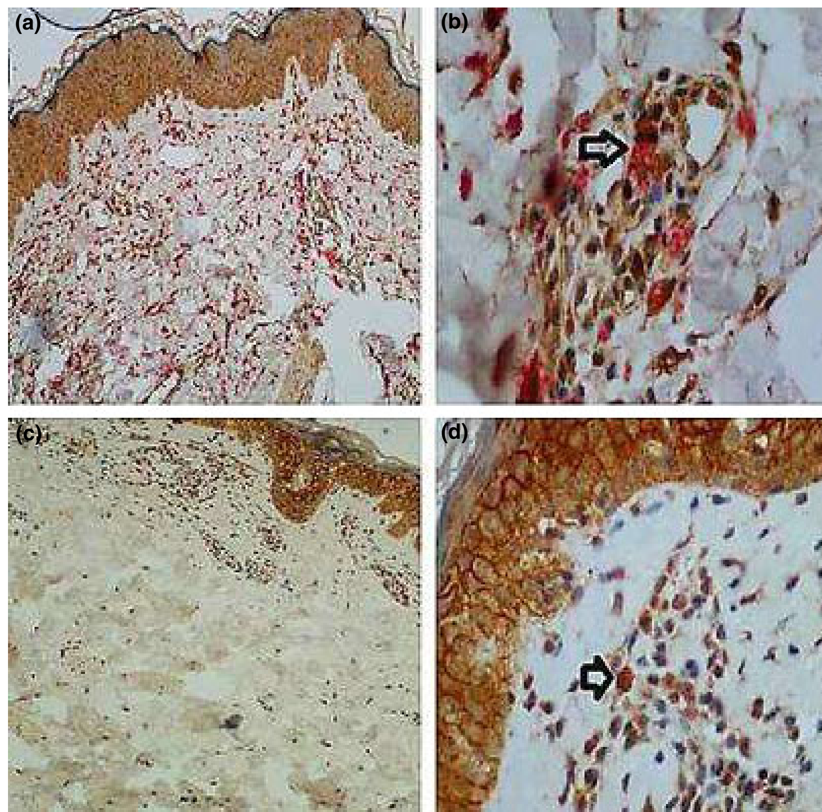

Figure 1 Immunohistochemical staining of dermal CD68-iNOS positive cells (a,b) before and (c,d) after treatment in lesional skin of patients with mycosis fungoides (MF). (a) Abundant red (3-amino-9-ethylcarbazole; AEC) points represent CD68 + cells. (b) In enlarged size, double-stained [AEC + 3,3′-diaminobenzidine (DAB)] cells represent dermal M1 macrophages. (c,d) Numbers of red and double-stained cells decreased after treatment. (b,d) original magnification × 100.

patients with Hodgkin lymphoma. Consequently, CD163 may not be a specific marker of M2 macrophages in skin; nevertheless, it is still a more sensitive and useful marker than CD68.[3]

The human mannose receptor CD206 is a specific marker of M2 macrophages.[22] A recent study reported that a low number of CD11c-positive macrophages and a high number of CD206-positive macrophages are associated with reduced cancer-specific survival from renal cell carcinoma.[12] Furthermore, studies have shown that CD206 and CD163 expression is increased on macrophages in the microenvironment of hepatic metastatic tumour.[23]

M1 macrophages secrete high levels of iNOS.[4] One study demonstrated that a low level of iNOS secretion and a high level of CD163 expression were associated with more advanced tumour stages in patients with renal cell carcinoma.[13] Another recent study also highlighted a link between high levels of iNOS secretion and increased survival of patients with colorectal cancer.[14]

As mentioned earlier, the existing immunohistochemical data on the identification of M1 and M2 macrophages on various tumours seems to be conflicting. Consequently, we performed double immunohistochemistry for each macrophage type in order to reduce the error rate during microscopic examination, using iNOS–CD68 and CD163–CD206 pairs as marker pairs for detection of dermal M1 and M2 macrophages, respectively.

We found that the number of M1 macrophages was significantly reduced in tumour tissue of patients with MF after treatment. There was also a reduction in M2 cells, but this was not significant. These results were similar after we excluded patients who had recurrence, and these changes in M1 and M2 cell numbers were not affected by stage, lesion type or treatment outcome.

Sugaya *et al.*[3] demonstrated that the numbers of CD163-positive and CD68-positive cells decreased after treatment with topical steroid and ultraviolet light in CTCL. Furthermore, an increased number of

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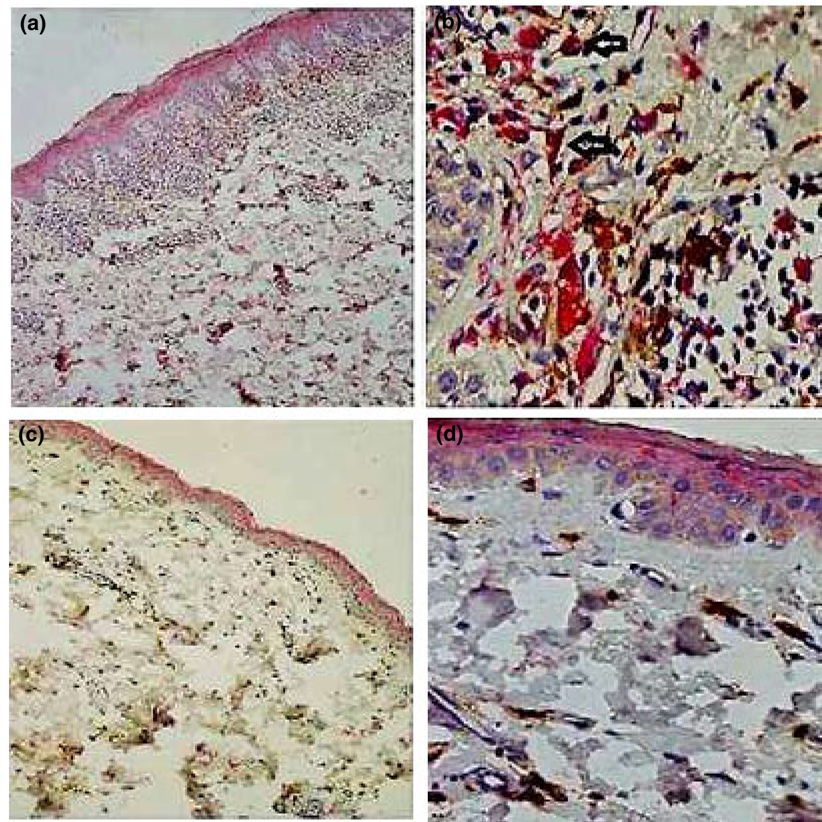

Figure 2 Immunohistochemical staining of dermal CD163−CD206 positive cells (a,b) before and (c,d) after treatment in lesional skin of patients with mycosis fungoides (MF). (a) Red points represent dermal CD206+ cells. (b) Double-stained cells represent dermal M2 macrophages. Numbers of red and double stained cells decreased after treatment. (b,d) Original magnification × 100.

Table 2 Number of M1 and M2 cells before and after treatment in all patients with MF, and after exclusion of the two patients with recurrence.

Group	Before treatment	After treatment	*P**
All patients (*n* = 21)			
M1	2.75 (0.60–7.50)	1.30 (0.25–7.00)	< 0.01
M2	2.00 (0.30–5.10)	1.70 (0.00–6.40)	0.24
No recurrence (*n* = 19)			
M1	2.75 (0.60–7.50)	1.20 (0.25–3.20)	<0.01
M2	2.00 (0.30–5.10)	1.70 (0.00–6.40)	0.11

Data are *n* (range). *Wilcoxon signed rank test.

CD163-positive cells correlated with worse prognosis. They also reported that there was no significant correlation between prognosis and the high number of CD163-positive macrophages in patch or plaque CTCL. In addition, Miyagaki *et al.*[24] reported that high serum levels of CCL18 and high levels of CCL18 expression by dermal macrophages in lesional skin were associated with poor prognosis in CTCL. Supporting this, Gunther *et al.*[25] highlighted that increased expression of CCL18 has an immunomodulatory effect on the pathogenesis of CTCL, and they also observed that serum levels of CCL18 and levels of CCL18 presentation in skin biopsy specimens of patients with MF were higher compared with those of healthy controls. Similarly, we demonstrated that the total number of dermal (M1 + M2) macrophages decreased after treatment, with M1 macrophages showing the greatest decrease. Although we did not evaluate the prognosis of our patients with MF, we found that the differences in M1 and M2 cell numbers were not affected by tumour stage, lesion type or treatment outcome. These results indicate the impact of the immunoregulatory roles of macrophages in the aetiopathogenesis of MF. TAMs play a prominent role in tumour growth, progression and dissemination by producing certain molecules as described above, and we have shown that the number of macrophages decreased with treatment in patients with MF. When therapy was successful, the number of macrophages reduced and their regulatory impact on tumour cells disappeared.

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A potential limitation of our study is that we considered only CD163+ CD206+ cells as being M2 macrophages, even though CD68+ iNOS- cells may also indicate M2 macrophages. However, CD68 (KIP1) is not a sufficiently specific marker for macrophages, as it may also be found on neutrophils, basophils, large lymphocytes, Langerhans cells and myeloid precursors. Our choice of double staining did not affect our results, as we detected M2 cells more specifically with CD163+ CD206+ staining.

Conclusion

The number of macrophages decreased after treatment in patients with MF. This may be attributable to the reduction of tumour activity due to treatment, providing a partial explanation for the disappearance of promoting signals that lead to differentiation of M1 macrophages in the tumour microenvironment. Studies with larger numbers of patients are warranted to confirm our results.

Acknowledgements

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What's already known about this topic?

- A decrease in macrophages in tumour tissue after treatment has been reported.

What does this study add?

- We performed double immunohistochemistry for each macrophage type in order to reduce error rate.
- Macrophage numbers in tumour tissue of MF patients decreased after treatment.

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