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ORIGINAL ARTICLE

Th1- and Th17-polarized immune infiltrates in eosinophilic fasciitis—A potential marker for histopathologic distinction from morphea

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Background: Morphea (localized scleroderma) and eosinophilic fasciitis (EF) are rare fibrosing disorders which may present a diagnostic challenge. While histopathologic features are often distinct, in some cases there may be overlap. T-cells contribute to etiopathogenesis of both autoimmune conditions. We sought to determine whether T-cell immune polarization enables histopathologic distinction.

Materials & Methods: We retrospectively examined clinicopathologically confirmed cases of morphea (n = 12) and EF (n = 8) using immunohistochemistry for CD3, CD8, and dual staining for CD4 with T-bet, GATA-3, STAT-3 or BNC-2 (transcription factors reported to be specific and mutually exclusive for Th1, Th2, Th17 and Th22 cells, respectively) to characterize the T-cell infiltrate.

Results: No significant difference in CD3+ cells was identified (P = .195), however, the CD4/CD8+ T-cell ratio was significantly greater in morphea compared to EF (1.2 and 0.6, respectively; P = .034). Th1/Th2 was significantly lower in morphea compared to EF (1.7 and 2.7, respectively; P = .027). The percent of Th17+ cells was significantly higher in EF (P = 0.041). No significant difference in percent of Th22+ cells was identified.

Conclusion: Morphea and EF may be histopathologically distinguished based on helper T-cell subtype polarization. These findings offer novel insight into our understanding of disease pathogenesis and support a role for Th1/Th2 immune regulation and Th17 inhibition in anti-fibrotic therapeutic strategy.

KEYWORDS

eosinophilic fasciitis, helper T-cell, immune, morphea, Th17

1 | INTRODUCTION

Morphea (localized scleroderma) and eosinophilic fasciitis (EF) are rare fibrosing disorders that can pose a diagnostic challenge. Distinction between these two conditions is necessary for guiding patient management. However, their pathophysiologic bases remain elusive, and histomorphological differentiation may be difficult due to significant overlap in some cases.[1] Specifically, morphea and EF may both show dermal inflammation consisting of lymphocytes, plasma cells and eosinophils, and fibrosis with extension into the subcutis and fascia.[1]

Both of these diseases are postulated to be autoimmune, with T-cell mediated immunity playing a major role in their pathogenesis. Animal and serological studies have shown polarized immune responses in localized and systemic scleroderma and EF. Helper T-cell subtype 1 (Th1) and helper T-cell subtype 2 (Th2) have been implicated in the pathogenesis of both scleroderma and EF.[2,3] CD4/CD8 ratio analyses have demonstrated CD4 excess in morphea and CD8 excess in EF.[4–6] Limited data is available on the role of the more recently identified helper T-cell subtypes, Th17 and Th22, in fibrosis. Th17- and Th22-related cytokines have been shown to be increased in the peripheral blood of morphea patients.[7,8] However, no prior reports delineate the expression of Th17 and Th22 in EF. Thus, to our knowledge, no systematic side-by-side comparison of the helper T-cell subtype in these two conditions in human skin tissue has been performed.

In this study, we sought to determine whether T-cell immune polarization enables histopathologic distinction between morphea and EF. Our findings have potential implications for guiding anti-fibrotic therapy in patients with morphea and the subset of EF patients requiring prolonged corticosteroid therapy for which standard treatment protocols are lacking.[9]

2 | MATERIALS AND METHODS

2.1 | Case selection

After obtaining an Institutional Review Board approval (protocol #P002524), we retrospectively examined all available specimens of clinicopathologically confirmed cases of morphea and EF diagnosed at Massachusetts General Hospital over 3 and 6 year intervals (between September, 2012 and January 2015, and September 2006 and September 2012), respectively. Electronic medical records were searched for pertinent clinical and demographic information (age, gender and skin biopsy site). None of the morphea patients fulfilled criteria for systemic scleroderma: Raynaud's phenomenon, acrosclerosis and visceral organ involvement (eg, dyspnea) were absent.[10] Patient records were searched for evidence of concomitant autoimmune disease and peripheral eosinophilia at the time of skin biopsy.

2.2 | Histopathological and immunohistochemical evaluation

The original glass slides were reviewed for all available cases. Presence of eosinophils, fascial involvement and epidermal and eccrine gland atrophy were noted for each. Histopathologic inclusion criteria for morphea and eosinophilic fasciitis cases were as follows: Morphea cases were characterized by dermal expansion and sclerosis with involvement of subcutaneous septae, a perivascular and dermal/subcutaneous junction ("advancing edge") inflammatory infiltrate composed predominantly of lymphocytes and plasma cells with variable eosinophils, and atrophy of epidermis and eccrine glands. In contrast, EF cases were characterized by deep dermal to subcutaneous sclerosis with involvement of the fascia and a perivascular inflammatory infiltrate composed predominantly of lymphocytes and plasma cells with variable eosinophils; alternating layers of inflammation and parallel hyaline fibrosis were present.

After verification of appropriate controls, immunohistochemistry was used to characterize the dermal T-cell infiltrate. Skin biopsy specimens were stained for CD3, CD8 and CD4 in combination with T-bet, GATA-3, STAT-3 or BNC-2, which are transcription factors that have been reported to be specific and mutually exclusive for Th1, Th2, Th17 and Th22 cells, respectively. Immunohistochemical stains were performed on 5 μm sections cut from formalin-fixed paraffin-embedded tissue and placed on positively charged slides (Superfrost Plus; Thermo Fisher Scientific, Waltham, Massachusetts) using the commercially available Dako autostainer plus platform (Dako North America; Carpinteria, California). Standard immunoperoxidase technique according to the manufacturer's protocol was utilized with monoclonal antibodies to CD3 (1:400; Dako), CD4 (1:20; Biocare Medical, Concord, California), CD8 (1:100; Dako), T-bet (1:750; Invitrogen, Carlsbad, California), GATA-3 (1:225; Sigma-Aldrich; St. Louis, Missouri), STAT-3 (1:100; LSBio, Seattle, Waltham) and BNC-2 (1:500; Sigma-Aldrich).

Positively stained dual-labeled cells were counted manually in 3 high power fields (HPF) by 2 dermatopathologists who were blinded as to whether the specimen was classified as morphea or EF (O.V.N. and R.M.N.), as previously described.[11,12] The high power fields evaluated were those with the most dense lymphoid infiltrate. The following 3 parameters were examined: density of T-cell infiltrate (average per 3 HPF), proportion of CD4-positive T-cells relative to CD8-positive T-cells, the Th1/Th2 ratio, and percent of helper T-cell subtypes of CD3+ T-cells. Cells simultaneously staining for CD4 (cytoplasmic) and T-bet (nuclear) represented cells differentiating along the Th1 pathway, while those simultaneously staining for CD4 (cytoplasmic) and GATA-3 (nuclear) represented cells developing along the Th2 pathway. Similarly, cells simultaneously staining for CD4 (cytoplasmic) and STAT-3 (nuclear) represented cells differentiating along the Th17 pathway, while those simultaneously staining for CD4 (cytoplasmic) and BNC-2 (nuclear) represented cells developing along the Th22 pathway.

2.3 | Statistical analysis

Statistical analysis was performed using Student's *t*-test to evaluate the relative frequency of T-cell subsets in samples of morphea vs EF patients. The Fisher's exact test was used to compare categorical data. *P*-values < .05 were considered statistically significant.

3 | RESULTS

20 cases were identified for which the archived tissue blocks were available and for which sufficient tissue was present in the block for cutting and staining. Of those, 12 were diagnosed as morphea cases and 8 as EF. Patient clinical and demographic information and histopathological and immunohistochemical stain results are presented in Table 1. There was no significant difference in gender or mean age between morphea and EF patients (58% vs 50% female, $P = .731$; 56.6 vs 52.3 years, $P = .693$, respectively). Skin biopsies were obtained from the trunk in the majority of morphea cases (8 trunk, 2 upper extremity and 2 lower extremity) which was significantly greater than for EF cases (1 trunk, 2 upper extremity and 5 lower extremity) ($P = 0.015$) and no biopsies from the head and neck were obtained for either group. Eight morphea patients had concomitant autoimmune disease including vitiligo, diabetes mellitus, lichen sclerosus, hypothyroidism, inflammatory bowel disease and rheumatoid arthritis, and 3 EF patients had concomitant autoimmune disease including diabetes mellitus, rheumatoid arthritis, and polyarteritis nodosa ($P = .362$). Two morphea patients had peripheral eosinophilia (3.8% and 5.0%) at the time of skin biopsy, 8 morphea patients had values within the laboratory reference range (0%-3%), and no laboratory data was available for 2 patients. Four EF patients had peripheral eosinophilia at the time of skin biopsy ranging between 3.8% and 34.8%, 2 EF patients had values within the laboratory reference

TABLE 1 Clinical, demographic, histopathological and immunohistochemical findings in morphea and eosinophilic fasciitis patients[a]

Parameter	Morphea (*n* = 12)	Eosinophilic fasciitis (*n* = 8)	*P*-value
Female gender	7 (58%)	4 (50%)	.731
Age, mean (range), years	56.6 (26-94)	52.3 (34-76)	.693
Skin biopsy site	8 trunk, 4 extremity	1 trunk, 7 extremity	**.015**
Concomitant autoimmune disease	8 (67%)	3 (38%)	.362
Peripheral eosinophilia	2[b] (20%)	4[c] (57%)	.129
Tissue eosinophilia	1 (8%)	5 (63%)	**.018**
Extension into fascia	3[d] (43%)	8 (100%)	**.004**
Epidermal and/or eccrine gland atrophy	6 (50%)	3 (38%)	.670
CD3 (average per HPF)	81.9	123.6	.195
CD4 (average per HPF)	43.5	44.2	.996
CD8 (average per HPF)	38.0	69.5	.071
CD4/CD8 ratio	1.2	0.6	**.034**
Th1 (average per HPF)	8.6	9.3	.807
Th2 (average per HPF)	5.1	3.5	.224
Th1 (% of CD3+ cells)	10.5	7.5	.706
Th2 (% of CD3+ cells)	6.2	2.8	.084
Th1/Th2 ratio	1.7	2.7	**.028**
Th17 (average per HPF)	3.5	6.3	**.041**
Th22 (average per HPF)	0.5	0.6	.869
Th17 (% of CD3+ cells)	5.0	7.2	.278
Th22 (% of CD3+ cells)	0.7	0.7	.767

Abbreviations: HPF, high power field.

[a]Significant *P*-values are displayed in bold.

[b]Of 10 patients with laboratory data available.

[c]Of 7 patients with laboratory data available.

[d]Of 7 cases with fascia present on the biopsy.

range (0%-3%), and no laboratory data was available for 1 patient. No significant difference in the presence of peripheral eosinophilia was detected (morphea vs EF, $P = .129$).

One morphea case (8%) showed presence of tissue eosinophils compared to 5 EF cases (63%) ($P = .018$). Three morphea cases (25%) showed extension into the fascia (fascia was not present for evaluation in 5 morphea biopsies) while fascia was involved in all EF cases ($P = .004$). One-half of morphea cases (50%) showed epidermal and/or eccrine gland atrophy; 3 EF cases (38%) showed atrophy of epidermis (1 confounded by severe solar elastosis) and none showed atrophy of eccrine glands ($P = .670$).

The counts of dermal cells expressing CD3, CD4, CD8, Th1 differentiation (as evidenced by simultaneous expression of CD4 and T-bet), Th2 differentiation (simultaneous expression of CD4 and GATA-3), Th17 differentiation (simultaneous expression of CD4 and STAT-3) and Th22 differentiation (simultaneous expression of CD4 and BNC-2), Th1/Th2 ratio, and CD4/CD8 ratio are shown in Table 1 and Figures 1 and 2. No significant difference in CD3+ cells between morphea and EF was identified ($P = .195$). However, the ratio of CD4/CD8+ T-cells was significantly greater in morphea compared to EF (1.2 and 0.6, respectively; $P = 0.034$). Although there was no significant difference in the percent of Th1 or Th2 cells, the Th1/Th2 ratio was significantly lower in morphea compared to EF (1.7 and 2.7, respectively; $P = .028$). Similarly, the percent of Th17 cells were significantly lower in morphea compared to EF (3.5 and 6.3, respectively; $P = .041$). No significant difference in the percent of Th22 cells were identified between morphea and EF (0.5 and 0.6, respectively; $P = .869$).

4 | DISCUSSION

Dermal fibrosing disorders are rare and may present a diagnostic challenge. The histopathological differential of fibrosing dermopathy is broad and includes morphea (localized scleroderma) and eosinophilic fasciitis (EF). The clinicopathological distinction of morphea and EF remains important for guiding therapy, given the profound response of EF to steroid treatment or spontaneous resolution.[9,13,14] However, there may be a significant clinical and morphological overlap in these 2 conditions and potential for misdiagnosis.[5,9,15] Indeed some authors have considered these overlapping entities on a clinical and histopathologic spectrum.[16] In our study, no significant difference was identified for patient gender, age, presence of concomitant autoimmune disease and peripheral eosinophilia between morphea and EF groups. We and others show that both may exhibit parallel arrangement of dermal collagen bundles, involvement of the deep dermis and subcutis, and the presence of perivascular lymphoplasmacytic infiltrate with variable eosinophils.[3,5,14,17] Furthermore, morphea cases may show fascial involvement (so called deep morphea, or morphea profunda), and absence of epidermal and/or eccrine gland

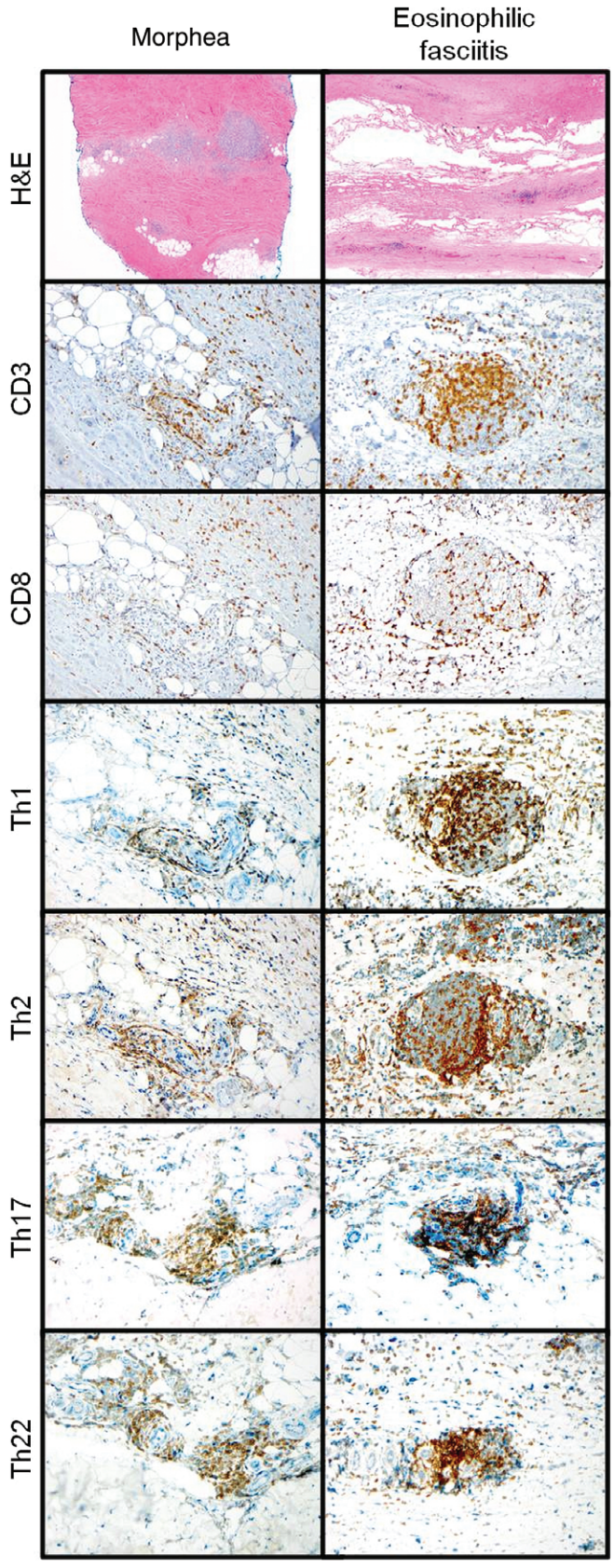

FIGURE 1 Representative hematoxylin & eosin (H&E) and immunohistochemical staining profile of morphea and eosinophilic fasciitis. Dual-labeled helper T-cells (Th) are shown as positive blue/nuclear and brown/cytoplasmic immunoreactivity (eg, T-bet+/CD4+ staining indicates Th1+ cells).

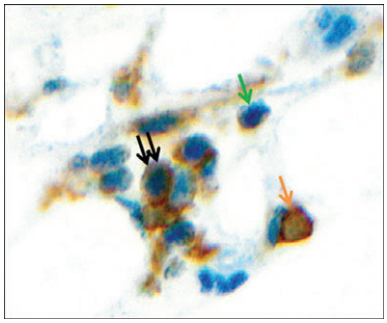

FIGURE 2 High power image representing dual immunohistochemical staining. Cells enumerated as dual-labeled helper T-cell subtype 1 (Th1) were those with both positive nuclear staining (blue chromogen, T-bet nuclear transcription factor) and positive cytoplasmic staining (brown chromogen, CD4), (double arrows). Cells with nuclear staining alone (green arrow) or cytoplasmic staining alone (orange arrow) were not enumerated as Th1+ cells.

atrophy similar to EF as shown in our study and in previous reports.[1,14,16] Therefore, a histopathological marker for differentiating between morphea and EF would be highly valuable.

T-cells have been shown to play a major role in the pathogenesis of dermal fibrosis. They form the predominant inflammatory infiltrate in both morphea and EF, with cytokine driven synthesis of extracellular matrix by fibroblasts resulting in excessive fibrosis.[18,19] EF patients have been shown to have increased levels of Th1 and Th2 related cytokines.[3] In addition, CD4 and CD8 analysis demonstrated an increased CD4/CD8 ratio in morphea and a CD4/CD8 ratio <1 in EF.[4,6,20] Investigation of dermal fibrosis in animal models showed that the absence of T-bet transcription factor was associated with an increase in tissue eosinophils in knock-out mice with bleomycin-induced fibrosis,[2] while overexpression of T-bet led to an exaggerated Th1 inflammatory response.[21] However, Th2-related interleukins released by the CD4 positive cells (IL-4, IL-13), and increased expression of the Th2-associated transcription factor GATA-3 have been implicated in increased fibrosis.[2,22] No prior study has compared the T-cell immune phenotype present in skin biopsies of morphea and EF.

In our study, there was no difference in CD3 positive cells, endorsing that T-cell mediated autoimmunity plays an important role in both of these conditions. However, we demonstrate that the ratio of CD4/CD8 positive T-cells is significantly greater in morphea compared to EF. The Th1/Th2 ratio was significantly lower in morphea compared to EF, suggesting that antibody-mediated immunity may play a significant role in morphea, but less so in EF. These results corroborate the findings in prior studies and support the distinct pathogenesis of morphea and EF, suggest a means for histopathological differentiation, and may guide therapeutic strategies to balance polarized Th1/Th2 immune responses.

Limited data is available in the literature on the role of the more recently identified helper T-cell subsets, Th17 and Th22, in fibrosis. Th17 cells produce IL-17 and TGF-β, known pro-fibrotic cytokines, and Th17 cytokines have been shown to play a pathogenic role in inflammatory and autoimmune diseases.[8] In systemic sclerosis, peripheral blood Th17 and Th22 cells were shown to be increased in

comparison to healthy controls, and higher IL-17 and IL-22 levels were shown to correlate with the presence of interstitial lung disease.[23] Serological studies have shown significantly elevated IL-17 levels in early stage systemic sclerosis in comparison to healthy controls.[7] In morphea, Kurzinski and Torok reported elevated serum levels of Th17 inducers and effectors in 71 pediatric cases.[8] Th22 cells produce IL-22 in conjunction with Th1 and Th17 cells.[24] Significantly, elevated IL-22 expression has been reported in the dermis of patients with morphea as compared to systemic sclerosis and healthy controls.[24] However that study was limited to investigation of 4 morphea cases and IL-22 cytokine expression was assessed as a component of all cells, thus the contribution to the immune phenotype by Th22 cells remains unknown. To our knowledge, no prior studies have investigated tissue levels of Th17 and Th22 in morphea relative to EF.

We report significantly increased Th17 cells in EF as compared to morphea, suggesting that this helper T-cell subtype plays an integral role in EF pathogenesis and supports the rationale for anti-Th17 therapy in combination with systemic corticosteroids. In addition, anti-Th17 therapy may potentially be used as a corticosteroid-sparing alternative in the subset of EF patients requiring prolonged corticosteroid treatment.[9] Of interest, we identified no significant difference in Th22 cells between morphea and EF cases. Furthermore, Th22 cells comprised < 1% of the helper T-cell infiltrate in this study. These findings suggest that this helper T-cell subtype is unlikely to participate in the pathogenesis of morphea despite prior reports of Th22 dominance in systemic sclerosis.[23] Further investigation is warranted to establish the role of Th22 in scleroderma.

Our study was limited by the fact that both of these conditions are rare, therefore the number of cases was relatively small. Additionally, the study was a retrospective analysis. We evaluated the quantity of T-cell subsets present in skin biopsies but did not assess cytokine production levels. Thus, further prospective clinicopathological studies are needed to investigate the immune phenotype of dermal fibrosing conditions, including morphea and EF.

In conclusion, our observations support the role of T-cells in morphea and EF. We describe a distinct polarization in the helper T-cell subtypes in these 2 conditions, which may be a reliable means for histopathologic distinction in cases with morphologic overlap. These findings offer novel insight into our understanding of disease pathogenesis and support a potential role for Th1/Th2 immune regulation and Th17 inhibition in anti-fibrotic therapeutic strategy.

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