STAT3 Activation and Infiltration of Eosinophil Granulocytes in Mycosis Fungoides

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Abstract. *Eosinophil granulocytes have been implicated in anticancer immunity but recent data indicate that eosinophils can also promote cancer. Herein, we studied eosinophils in skin lesions from 43 patients with mycosis fungoides (MF). The presence of eosinophils correlated with disease stage: 78% of patients with advanced disease displayed eosinophil infiltration, whereas this was only seen in 11% of patients with patches ($p<0.01$), and in 48% of those with plaque disease. Importantly, 72% of patients with positive staining for phospho-signal-transducer-and-activator-of-transcription (pY-STAT3) in malignant T-cells also stained positively for eosinophils, whereas this was only observed in 28% of pY-STAT3-negative patients ($p<0.01$). Notably, malignant T-cells expressed eosinophilic activation and trafficking factors: High-mobility group BOX-1 protein (HMGB1) and interleukin 5 (IL5). STAT3 siRNA profoundly inhibited IL5 but not HMGB1 expression. In conclusion, these data suggest that malignant T-cells orchestrate accumulation and activation of eosinophils supporting the notion of STAT3 being a putative target for therapy.*

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Eosinophils are innate immune effector cells best known for their role in the pathogenesis of allergic diseases such as asthma and hypereosinophilic syndrome, as well as for their ability to provide host defense against parasites [reviewed in (1)] The role of eosinophils in relation to cancer is less clear but the prevailing view has been that eosinophils are part of the antitumor defense (1, 2). For instance, the presence of tumor-infiltrating eosinophils was associated with a favorable prognosis of patients with colorectal malignancies (3) and a decreased risk of death in patients with gastric cancer (4). Notably, a series of novel cancer therapies directly exploit the anticancer properties of eosinophils (5), underlining the clinical importance of the tumor-suppressive function of eosinophils in these types of cancer. However, recent studies have indicated that eosinophils might also play a role in tumor progression – in particular in hematological malignancies (6-9). Thus, eosinophil infiltration was associated with poor overall survival in Hodgkin's disease and certain types of leukemia (8, 9) and recent data indicate that eosinophils are also linked to a poor prognosis in some non-hematological cancer, including cervical squamous cell carcinoma and small cell esophageal carcinoma (1, 7).

Although these data strongly suggest that eosinophils have a role in tumor progression and clinical outcome, relatively few studies have addressed why and how eosinophils support tumor growth. Yet several mechanisms have been proposed by which eosinophils may directly or indirectly stimulate tumor growth or inhibit antitumor immunity: Firstly, eosinophils promote type-2 T-helper cell (Th2) responses and inhibit Th1 responses (2), favoring a cytokine environment inhibiting antitumor cytotoxicity by

$CD8^+$ T-cells. In support, Willemien *et al*. showed how infiltration of eosinophils leads to an imbalance in the Th2/Th1 ratio and induction of tumor promotion (10). Secondly, eosinophils in concert with other myeloid cell types stimulate tumor-promoting inflammation dominated by regulatory T-cells (Treg) and M2 macrophages (11). Thirdly, eosinophils produce vascular endothelial growth factors (VEGFs) and other pro-angiogenic cytokines known to play a role in tumor progression in cancer (12).

Cutaneous T-cell lymphoma (CTCL) is characterized by the expansion of malignant T-cells in a chronic inflammatory environment. In the predominant clinical variant, *mycosis fungoides* (MF), skin lesions initially present as flat erythematous patches or plaques resembling benign inflammatory skin disorders. As the disease progresses, the lesions may develop into overt tumors and the malignant T-cells may spread to the lymph nodes and internal organs (13). Patients diagnosed in early stages often experience an indolent disease course and have a favorable prognosis with a life expectancy similar to that of a control population. However, in a subgroup of patients diagnosed with early CTCL the disease follows a more aggressive and occasionally fatal course (13, 14). The malignant T-cells found in CTCL are characterized by an ectopic expression and activation of (i) signaling molecules including the src kinases, B lymphocyte tyrosine kinase and hemopoietic cell kinase (15-17), Janus kinase-3 (JAK3), signal transducer and activator of transcription 3 (STAT3), and STAT5 (18-20); (ii) immune modulators such as fork head box P3, programed cell death 1 (PD-1)/PD-1 ligand, microRNAs and suppressor of cytokine signaling 1 (21-27); (iii) angiogenesis factors such as VEGFA and VEGFC (20, 28, 29); and (iv) expression of a Th2 cytokine profile associated with an over-production of interleukin (IL)-5, -6, -10, -15, -17A, and -17F, with IL5 being the predominant cytokine (30-32). In addition to the malignant T-cell clone and stromal cells such as keratinocytes and endothelial cells, skin lesions include non-malignant T-cells, dendritic cells, M2 macrophages and occasionally eosinophils. Importantly, the presence of eosinophils is associated with advanced disease (33-36), suggesting the possibilities that eosinophil infiltration is (i) part of an (unsuccessful) antitumor response, (ii) involved in tumor promotion, or (iii) an epiphenomenon due to the expression of eosinophilic activation factors and chemokines. The role of eosinophils in the pathogenesis of CTCL is thus an open question. Moreover, it is largely unknown what drives homing of eosinophils to a cancer site and whether crosstalk takes place between malignant T-cells and eosinophils. Accordingly, the present study was undertaken to elucidate the relationship between aberrant activation of malignant T-cells and infiltration of eosinophils in MF.

Materials and Methods

Cell lines. The malignant (MyLa2059, MF3675) and non-malignant (MF1850) T-cell line were obtained from patients with CTCL (31). The cells were cultured in RPMI-1640 media containing 2 mM l-glutamine and 100 mg/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Malignant T-cell lines were supplemented with 10% fetal bovine serum (Life Technologies, Roskilde, Denmark). The non-malignant part was supplemented with 10% human serum (Bloodbank, Copenhagen University Hospital, Denmark) and 10^3 U/ml IL2.

Antibodies and reagents. The antibody against actin was purchased from Sigma-Aldrich (#A4700, St. Louis, MO, USA), the antibodies against phosphor(TYR705)-STAT3 (pY-STAT3) (#0036-100/STAT3-9E12), phospho-STAT5 (pY-STAT5) (#9351), and total STAT3 (#4904) were from Nano-Tools (Teningen, Germany) and Cell Signaling Technology (Boston, USA).

Patients and Ethics. The study includes formalin-fixed and paraffin-embedded biopsies from patients diagnosed with CTCL during the period from 1979-2004. Samples were drawn from the archives of the Departments of Pathology at Copenhagen University Hospital and Bispebjerg Hospital and have been described in detail elsewhere (37). For immunohistochemistry, 43 cases were selected for analysis. Nine patients had early patch lesions, 25 displayed infiltrated plaques and nine patients demonstrated advanced disease with tumors or transformation to large T-cell lymphoma. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg (journal no. 01 284225) and the Danish Data Protection Agency (Datatilsynet, journal no. 2005-41-5930) (37).

Histological review of biopsies. For all 43 cases, formalin-fixed, paraffin-embedded tissue samples were reviewed by histology and immunohistochemistry using as a minimum cluster of differentiation (CD)-3, CD4, CD8, CD30, CD56, T-cell intracellular antigen-1, granzyme B and anti-pY-STAT3 and pY-STAT5 antibodies (25). The number of eosinophils was evaluated in three hotspot areas at ×40 magnifications in hematoxylin and eosin stained sections (25). Intravascular eosinophils were not included. Classification was performed by two of the authors (LMG and ER) in accordance with the World Health Organization and European Organization for Research and Treatment of Cancer (38).

Enzyme-linked immunosorbant assay (ELISA). The concentration of IL5 and HMGB1 in the cell culture supernatant was measured by ELISA (#DY205 Human DuoSet; R&D System, Minneapolis, MN, USA and #ABIN368346 antibodies-online, Aachen, Germany) according to the instructions provided by the company (39).

Protein extraction and western blot. Protein extraction and western blotting were performed as descried earlier (28, 40, 41). To ensure an equal loading of protein, the concentration in each lysate was measured by Bio-Rad protein Assay (Bio-Rad, Hercules, CA, USA).

Transient transfection with small-interfering RNA (siRNA). Cells were transfected with STAT3 siRNA (#D-001810-01-05) and non-target control#1 (#D-001810-01-05) (Thermo Scientific, Lafayette, CO, USA) using 0.125 nmol on 2.0×10^6 cells with an Amaxa Nucleofector (Lonza, Walkersville, MD, USA), as described previously (42).

Quantitative polymerase chain reaction (qPCR) (Taqman). Total RNA was purified with miRNeasy Mini Kit (#217004, Qiagen, Valencia, CA, USA) cDNA was transcribed from 1,000 ng RNA using High-Capacity cDNA Reverse Transcription Kit (#4368813, Applied Biosystems, Foster City, CA, USA). For analysis of *IL5* (#Hs01548712) and *HMGB1* (#Hs01923466) expression, real-time PCR was performed using Taqman Gene Expression assay (Applied Biosystems, Foster City, CA, USA) according to the manufactures instructions. Glyceraldehyde 3-phosphate dehydrogenase (Taqman Gene expression Assay; Applied Biosystems) was used as reference. Amplification was performed in an MX3005P real-time thermal cycler (Agilent Technologies, Santa Clara, CA, USA) on standard settings. Each experiment included three technical replicates (42).

Statistics. For statistical analysis, a two-tailed Student's *t*-test was used with a significance level of 0.05. The calculations were performed on data obtained from at least three independent experiments including technical replicates. Patient data was calculated by using Fisher's exact test with a significance level of 0.05.

Results

Although eosinophil infiltration is often associated with a good prognosis in patients with solid cancers, recent studies have indicated that the presence of eosinophils is a poor prognostic factor and associated with disease progression in lymphoid cancer including CTCL (33). Herein we studied eosinophil infiltration in a well-characterized cohort of 43 patients with MF (25, 43) and as shown in Figures 1 and 2, the presence of eosinophils in MF lesions was associated with disease stage. Thus, seven out of nine (78%) patients with advanced disease displayed eosinophil infiltration, whereas this was only seen in one out of nine (11%) patients with patches ($p<0.01$), and 12 out of 25 (48%) patients displaying plaque lesions (Figure 1 and Table I). These findings confirm and extended previous studies in CTCL indicating that the presence of eosinophils is a rare event in early disease and, conversely, a common feature of advanced disease (33-36).

Aberrant activation of STAT3 *in situ* is associated with disease progression and advanced stages of MF (19, 44). Figure 3 shows two typical examples of MF lesions: a plaque lesion where T-cells with neoplastic morphology stain negative (Figure 3A) and a case in tumor stage where T-cells of neoplastic morphology stain positively (Figure 3B and C) with a pY-STAT3 antibody.

We next addressed whether STAT3 activation in malignant T-cells was associated with eosinophil infiltration. As shown in Table II, 13 out of 18 (72%) patients with positive staining for pY-STAT3 antibody were positive for eosinophils, whereas this was only seen in 7 out of 25 (28%) pY-STAT3-negative patients ($p<0.01$). Aberrant activation of STAT5 was also seen in a large fraction of these patients (25), but the presence of eosinophils was not significantly associated with aberrant STAT5 activation in T-cells with neoplastic morphology (data not shown). However, the combination of both STAT3 and STAT5 activation was significantly associated with the presence of eosinophils (Table III). Together, these data indicate that aberrant STAT3 activation by T-cells with neoplastic morphology was associated with eosinophil infiltration, whereas STAT5 activation did not appear to have a direct role.

To address whether STAT3 was involved in the downstream induction of cytokines and chemokines attracting eosinophils, we initially carried-out a preliminary screening for spontaneous expression of eosinophil activation

Table I. *Presence of eosinophils in mycosis fungoides lesions in different disease stages from 43 patients. One out of nine patient tissues in the early patch stages were positive for tissue eosinophilia ($p<0.01$). In the later plaque stages, half of the 25 patient tissues stained positively for eosinophil infiltration. In advanced disease, 7 out of a total of 9 patient tissues were positive for eosinophils.*

	Eosinophils	
	Negative	Positive
Patches	8	1
Plaques	13	12
Advanced	2	7

Table II. *Infiltration of eosinophilss in patient samples, as stained with phospho-(TYR705)-Signal-Transducer and Activator of Transcription (pY-STAT3) antibody. Thirteen out of 18 patients positive for pY-STAT3 were also positive for eosinophils, whereas only 7 out of 25 negative for pY-STAT3 were eosinophil-positive ($p<0.01$).*

	Eosinophils	
	Negative	Positive
STAT3 Negative	18	7
Positive	5	13

Table III. *Eosinophil infiltration in patient samples stained with a combination of phospho-(TYR705)-Signal-Transducer and Activator of Transcription (pySTAT3) and pY-STAT5 antibody. A combination of STAT3 and STAT5 activation was significantly associated with the presence of eosinophils. Twelve out of 15 with active STAT3 and STAT5 were also positive for eosinophils, whereas 1 one out of 8 patients negative for both STAT3 and STAT5 were positive for eosinophils ($p<0.005$).*

	Eosinophils	
	Negative	Positive
STAT3+ STAT5+	12	3
STAT3− STAT5−	1	7

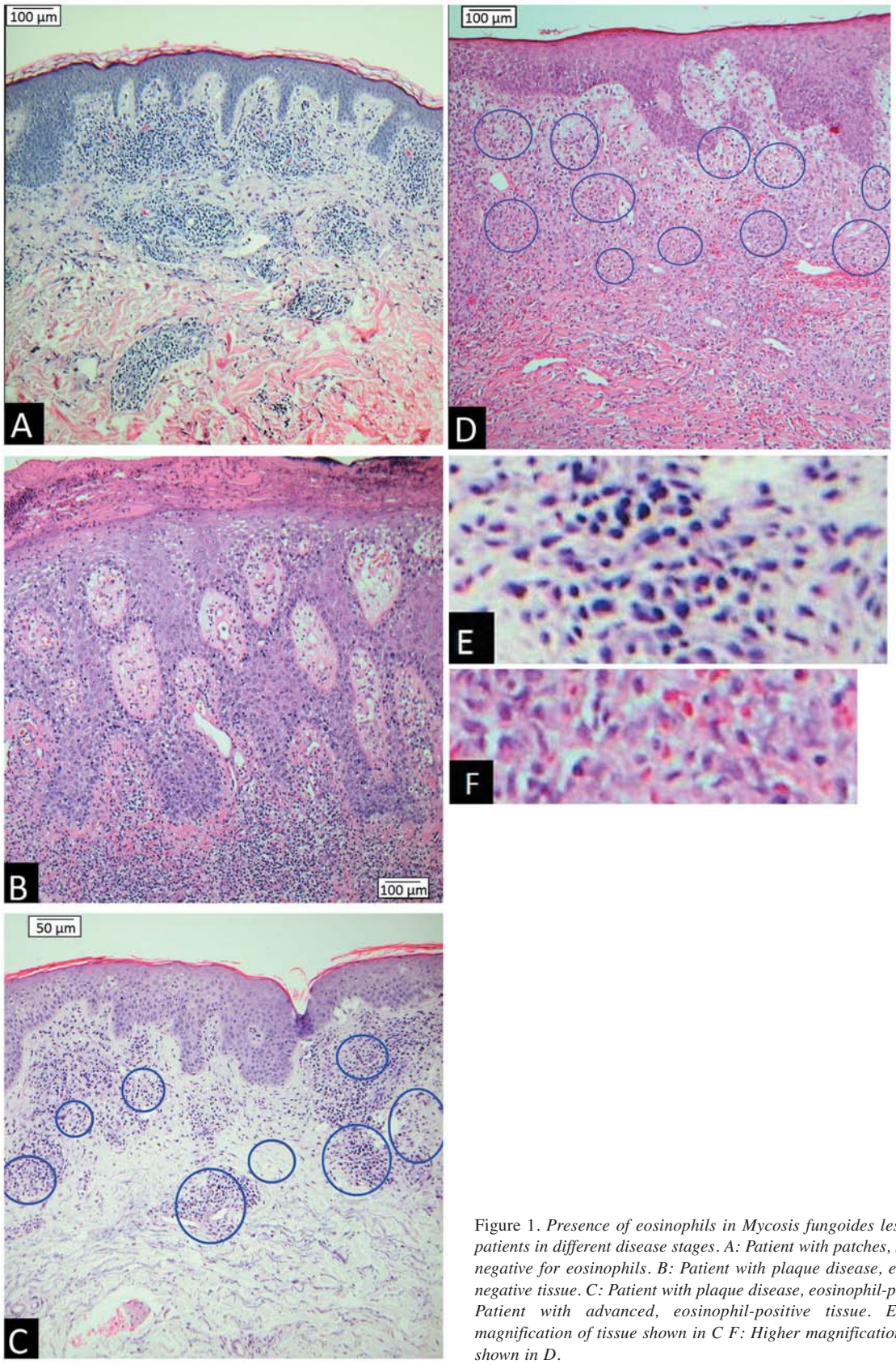


Figure 1. *Presence of eosinophils in Mycosis fungoides lesions from patients in different disease stages. A: Patient with patches, skin tissue negative for eosinophils. B: Patient with plaque disease, eosinophil-negative tissue. C: Patient with plaque disease, eosinophil-positive. D: Patient with advanced, eosinophil-positive tissue. E: Higher magnification of tissue shown in C F: Higher magnification of tissue shown in D.*

and homing factors in pY-STAT3-positive malignant T-cell lines obtained from MF tumors and identified two putative candidates: IL5 which is an important growth, activation and homing factor for eosinophils (45), and HMGB1, an alarmin promoting eosinophil influx in cancer (46). As shown in Figure 4, malignant T-cells (MF2059 and MF3675) spontaneously expressed high amounts of IL5 whereas non-malignant T-cells (MF1850) did not (Figure 4A and B). Both malignant and non-malignant T-cells derived from skin in MF spontaneously expressed and produced high amounts of HMGB1 (Figure 4C and D right column *versus* left column).

Since we previously obtained indirect evidence that STAT3 was involved in IL5 transcription (47), we asked whether aberrant STAT3 activation induced *IL5* and *HMGB1* expression in parallel in malignant T-cells. Accordingly, we took advantage of siRNA-mediated *STAT3* knock-down (48) prior to analysis of *IL5* and *HMGB1* mRNA expression in malignant T-cells. As shown in Figure 5, *STAT3* siRNA treatment induced a profound inhibition of STAT3 protein expression (Figure 5A) and approximately a 50% reduction in expression of *IL5* mRNA (Figure 5B) and protein (Figure 5C) as measured by ELISA analysis of cell culture supernatants. In contrast, *HMGB1* expression was largely unaffected by siRNA-mediated *STAT3* inhibition (Figure 5D), indicating that the two eosinophil activation factors were regulated by distinct STAT3-dependent and-independent pathways.

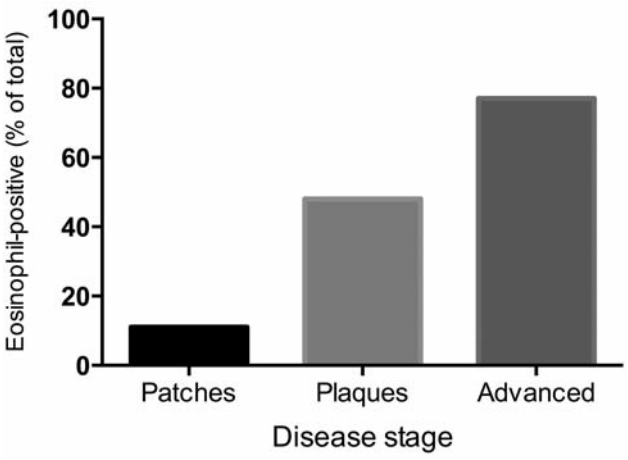
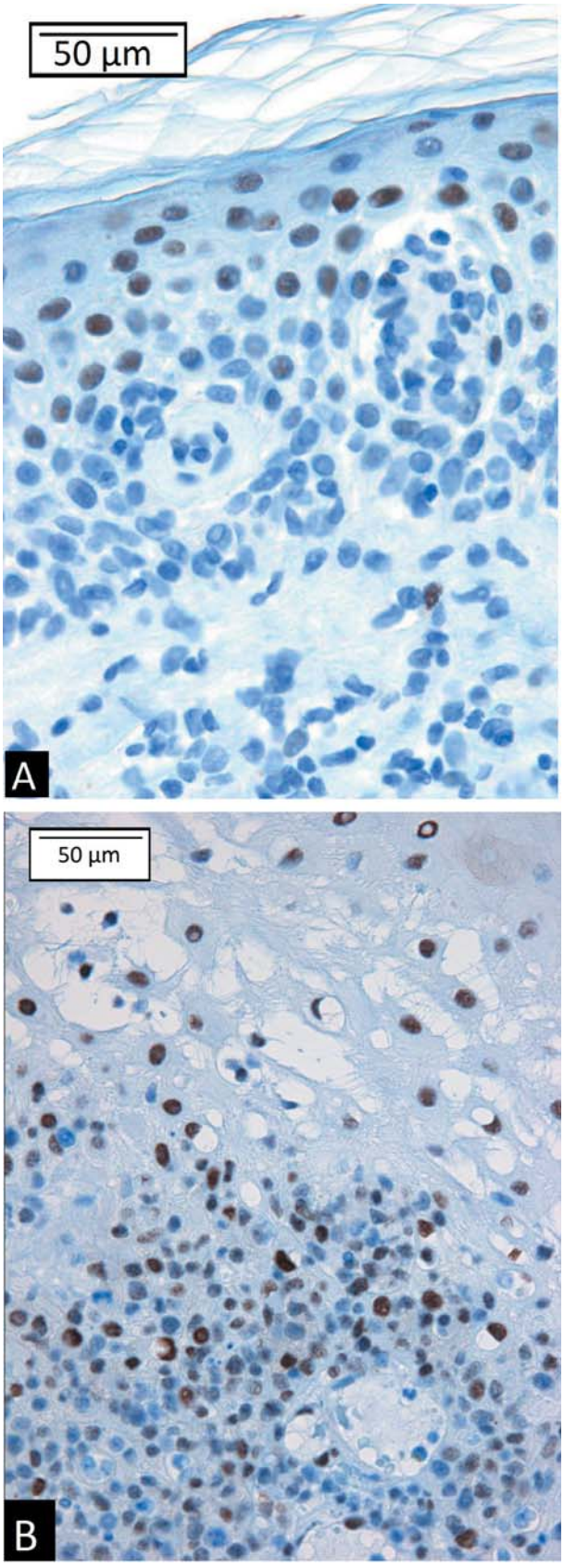


Figure 2. *Presence of eosinophils in Mycosis fungoides lesions from 43 patients in different disease stages. The percentage of patients in the different stages of MF (patch, plaques or advanced disease) positive for eosinophilia are calculated from the total number of patients in each group. A positive correlation between presence of tissue eosinophils and disease stage is demonstrated.*

Discussion

The present study provides first evidence that eosinophil infiltration *in situ* is associated with STAT3 activation in T-cells with neoplastic morphology. Thus, eosinophils were seen in more than two-thirds of pY-STAT3-positive tissue specimens of patients with MF, whereas this was only the case in less than one-third of the pY-STAT3-negative specimens ($p<0.05$). In contrast, expression of the active form of STAT5 was not significantly associated with the presence of eosinophils, suggesting that STAT5 did not play an independent role in eosinophil infiltration. However, we cannot exclude the possibility that STAT5 may also contribute to activation and homing of eosinophils *in situ,* as we observed that eosinophil infiltration was more common (80%) in STAT3/STAT5 double-positive patients and, conversely, a quite rare event (13%) in STAT3/STAT5 double-negative patients. Although STAT3 activation in T-cells with neoplastic morphology was significantly associated with the presence of eosinophils *in situ*, it remained to be elucidated whether or not eosinophil infiltration is directly linked to STAT3 activation in malignant T-cells. In an attempt to address this issue, we performed a preliminary screening of malignant T-cells for mRNA expression of known eosinophilic activation and homing factors and chemokines. We found spontaneous high expression of HMGB1, which has not previously been linked to CTCL, and of IL5, which is a well-known cytokine in relation to CTCL (30). Importantly, both factors play a key role in eosinophil infiltration and activation in other types of cancer [reviewed in (49, 50)] and we previously obtained circumstantial evidence that spontaneous production of IL5 is mediated by STAT3 in malignant T-cell lines (47). To address whether the expression of HMGB1 and IL5 were regulated in tandem in malignant T-cells, we used siRNA to directly target *STAT3* that showed a 50% inhibition of *IL5* mRNA expression and IL5 secretion, whereas HMGB1 expression was unaffected, indicating that malignant T-cells express eosinophilic activation and homing factors *via* both STAT3-dependent and independent pathways. Although these data cannot be translated directly to the *in vivo* situation, they provide a possible explanation for a link between malignant activation of STAT3 and eosinophil infiltration. Indeed, IL5 is considered the most important factor regulating production and release of eosinophils from the bone-marrow, and homing and activation of eosinophils in tissues [reviewed in (1)]. Our observation that *STAT3* knock-down did not fully inhibit IL5 production is in keeping with our observation that we did not obtain a complete gene knock-down. Moreover, eosinophil infiltration was only partly associated with STAT3 activation *in situ*. Nevertheless, several studies have linked IL5 expression with accumulation of eosinophils in MF skin lesion including erythroderma, and 'red man skin' syndrome sometimes seen in patients with severe disease (33, 49). Thus, although STAT3 did not appear to be the sole regulator of IL5 expression, a 50% reduction of IL5 production is substantial, making STAT3 a putative target for therapeutic intervention.



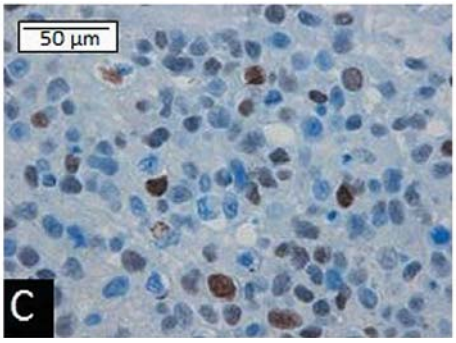


Figure 3. *Mycosis fungoides lesions in different disease stages stained with an anti-phospho-(TYR705)-Signal-Transducer and Activator of Transcription (pySTAT3) antibody. A: Plaque lesion. T-Cells with neoplastic morphology do not stain for pY-STAT3. B: Advanced disease. T-Cells with neoplastic morphology stain positively for pY-STAT3. C: Higher magnification of tissue shown in B.*

Indeed, STAT3 activation is driven by JAK3 in malignant T-cells (50) and a small molecular inhibitor of JAKs abrogated IL5-dependent recruitment, homing, and migration of eosinophils into lung tissue *in vivo* in a mouse asthma model (51), suggesting that the JAK/STAT pathway is engaged at multiple levels during the mobilization, activation and trafficking of eosinophils. This is of particular relevance for patients with severe erythroderma as this condition is associated with severe subjective symptoms, a defect skin barrier, and an increased risk of severe bacterial infections, which is a major cause of death in advanced disease (52).

It is an unresolved paradox why eosinophils have anticancer properties and even a potential for anticancer therapy in some malignancies and a tumor-promoting function in other malignancies (1, 2, 5-10). It has been shown that through the release of toxic substances and other factors eosinophils can elicit apoptosis and stimulate generation of an environment which is hostile to malignant cells (2, 12). At the same time, eosinophils may stimulate a tumor-promoting inflammatory environment, dominated by M2 macrophages, Tregs, and angiogenic factors (11). It is unknown what determines the balance between pro- and antitumor functions of eosinophils. Interestingly, advanced MF is characterized by ectopic expression of cyclooxygenase 2 and release of prostaglandin E_2 (PGE_2) by malignant T-cells (53, 54). As PGE_2 inhibits eosinophil trafficking and degranulation (55), it may be speculated that through simultaneous release of eosinophilic activation factors (IL5 and HMGB1) and inhibitors of degranulation (PGE_2), malignant T-cells stimulate cytokine and VEGF production and at the same time inhibit degranulation and release of toxic substances. As PGE_2 also inhibits eosinophil migration (56), eosinophils may become 'trapped' by PGE_2 at the tumor site, leading to a gradual accumulation of eosinophils within the tumor.

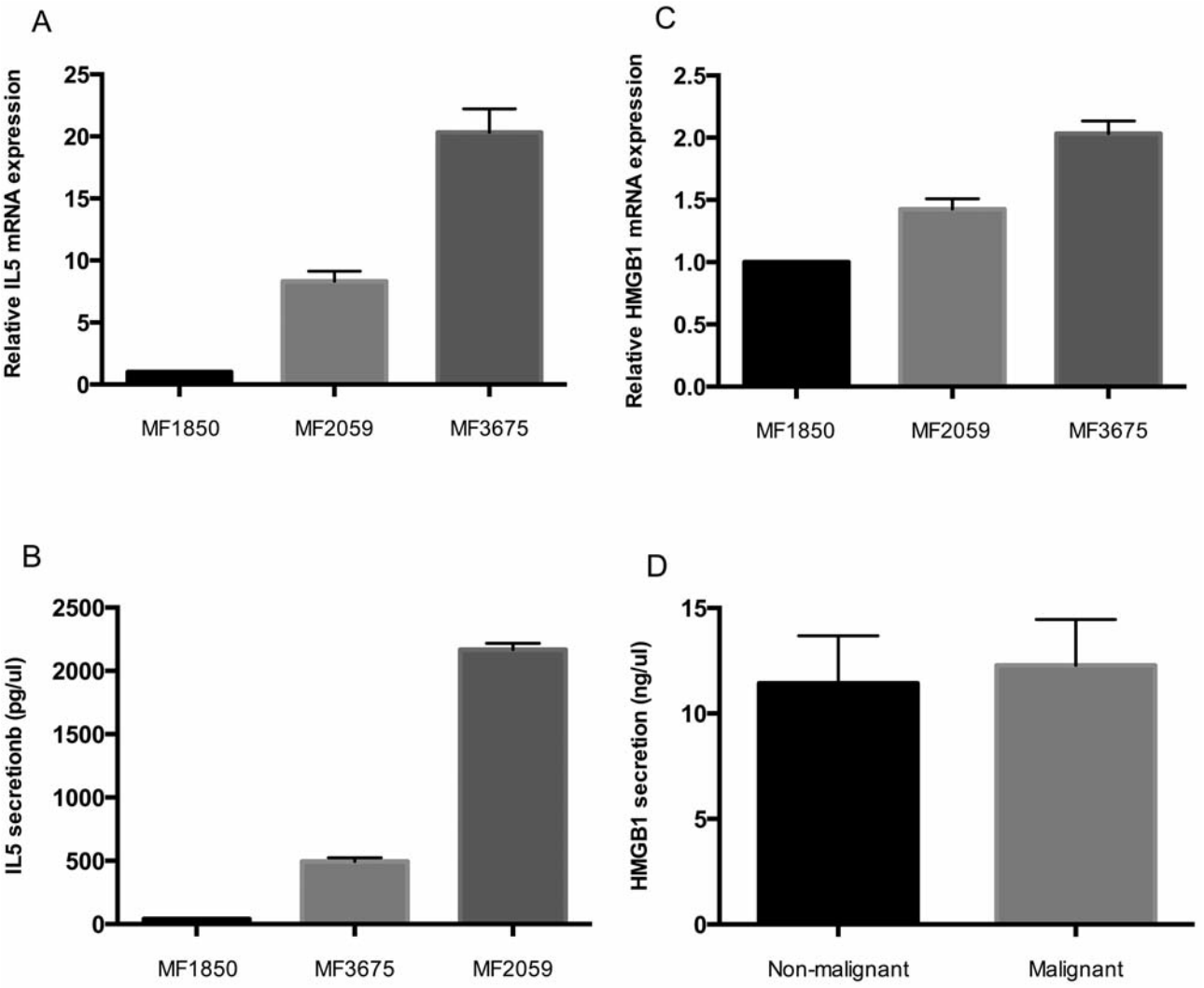


Figure 4. *Expression of interleukin-5 (IL5) and high-mobility group BOX-1 protein (HMGB1) in malignant and non-malignant T-cell lines. RNA from non-malignant (MF1850) and two malignant (MF2059 and MF3675) T-cell lines was purified and gene expression was analyzed by qualitative polymerase chain reaction. The culture supernatant of these cell lines was analyzed for IL5 and HMGB1 protein by enzyme-linked immunosorbent assay (ELISA). A: The two malignant T-cell lines express up to 20-times more IL5 mRNA compared to the non-malignant T-cell line. B: The expression of HMGB1 mRNA was up to two-fold higher in malignant compared to the non-malignant T-cell line. C: The expression of IL5 protein analyzed by ELISA showed a high secretion of IL5 by the two malignant cell lines (up to 2,000 pg/ml) compared to the non-malignant cells, which secreted only 40 pg/ml. D: HMGB1 protein secreted by cells was analyzed by ELISA. Both non-malignant (MF1850) and malignant (MF3675) T-cell lines secreted high amounts of HMGB1 protein (up to 15 ng/μl).*

Accordingly, studies are underway to address whether malignant T-cells stimulate VEGF and cytokine production in eosinophils *via* IL5- and PGE_2-dependent pathways.

In conclusion, we showed that STAT3 activation in T-cells with neoplastic morphology is associated with the presence of eosinophils *in situ* and that malignant T-cell lines produce activators of eosinophils through both STAT3-dependent and -independent pathways. We, therefore, propose that malignant T-cells orchestrate accumulation and activation of eosinophils in CTCL. Taken together, the present findings further strengthen the notion that the JAK3/STAT3 is a putative target for therapy in CTCL.

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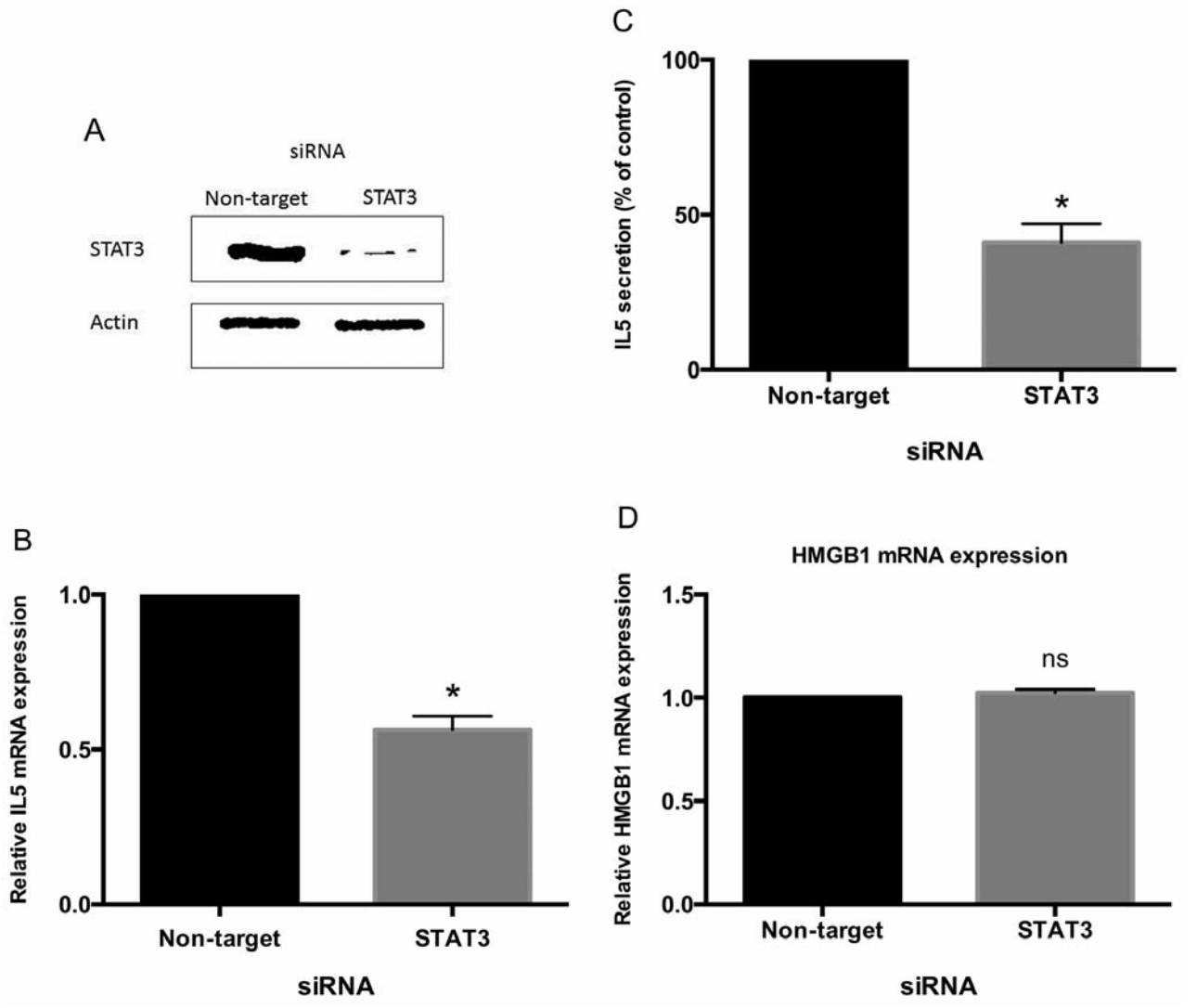

Figure 5. *Expression of Interleukin (IL) 5 and high-mobility group BOX-1 protein (HMGB1) in MF2059 malignant T-cell line after treatment with siRNA against Signal transducer and activator of transcription (STAT3). The malignant T-cell line was treated with siRNA against STAT3 and analyzed for IL5 and HMGB1 expression. A: Representative western blot showing siRNA-mediated knockdown of STAT3 in malignant MF2059 cells. B: RNA was purified and expression of IL5 mRNA was analyzed by quantitative polymerase chain reaction (qPCR). IL5 mRNA expression was significantly reduced (50%; p<0.005). C: Culture supernatant was analyzed post treatment with siRNA against STAT3 to confirm the inhibition seen in IL5 mRNA (p<0.005). D: RNA was purified and expression of HMGB1 mRNA was analyzed by qPCR. There was no significant change in the HMGB1 expression.*

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