Normal and Cancer Fibroblasts Differentially Regulate Cytokine Genes and TWIST1 and TOX Expression in Cutaneous T-cell Lymphoma

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Research article

Keywords: cutaneous T-cell lymphoma, mycosis fungoides, tumor microenvironment, fibroblasts, biomarkers

DOI: https://doi.org/10.21203/rs.3.rs-98530/v1

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Abstract

Background: Mycosis fungoides (MF) is a primary cutaneous T-cell lymphoma (CTCL) that transforms from mature, skin-homing T cells and progresses in the skin. The role of the skin microenvironment in MF development is unclear, but recent findings in a variety of cancers have highlighted the role of stromal fibroblasts in promoting or inhibiting tumorigenesis. Stromal fibroblasts are an important part of the cutaneous tumor microenvironment (TME) in MF. Here we describe studies into the interaction of TME-fibroblasts and malignant T cells to gain insight into their role in CTCL.

Methods: Myla cell is a CTCL cell line that retains expression of biomarkers TWIST1 and TOX that are frequently detected in CTCL patients. MyLa cells were cultured in the presence or absence of normal or MF skin derived fibroblasts for 5 days, trypsinized to detached Myla cells, and gene expression analyzed by RT-PCR for MF biomarkers (TWIST1 and TOX), Th1 markers (IFN-g, TBX21), Th2 markers (GATA3, IL-16), and proliferation marker (MKI67). Purified fibroblasts were assayed for expressed genes VIM and ACTA2. Cellular senescence assay was performed to assess senescence.

Results: Normal fibroblasts co-cultured with MyLa cells suppressed expression of the CTCL biomarkers TWIST1 (p < 0.0006) and TOX (p<0.03) in MyLa cells. In contrast, MyLa cells cultured with MF fibroblasts retained high expression of TWIST1 and TOX. Normal fibroblasts increased expression of IFN-g (p < 0.03) and TBX21, and decreased expression of GATA3 (p < 0.02) and IL-16 (p < 0.03) in MyLa cells, whereas MF fibroblasts suppressed IFN-g and TBX21 and increased TWIST1 and TOX expression in MyLa cells. Furthermore, expression of MKI67 in MyLa cells was suppressed to a greater degree by normal fibroblasts compared to MF fibroblasts.

Conclusions: Skin fibroblasts represent important components of the microenvironment in MF. In co-culture model, normal and cancer fibroblasts in MF have differential influence on T cell phenotype in modulating expression of Th1 cytokine and CTCL biomarker genes to reveal distinct role with implications in MF progression.

Background

Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of T cell malignancies that develop from the proliferation and transformation of mature skin-homing T cells, the most common types include mycosis fungoides (MF) and Sézary syndrome (SS) (1–3). MF is an indolent variant that progressively advances in the skin. Skin histology of early patch MF lesions show a low tumor burden with T cell infiltration characterized by Th1 cytokine bias, with increased expression of IL-2 and IFN-γ (3–6). In addition, Th1 chemokines such as CXC chemokine ligand (CXCL) 9 and CXCL10 are also expressed in lesional skin of early CTCL, when epidermotropism of tumor cells is remarkable (7). SS is an aggressive variant of CTCL characterize by erythroderma, lymphadenopathy and circulating malignant T cells in the blood. SS can have eosinophilia, a high level of IgE and chemokine ligand (CCL) 17 in patients (8, 9). MF and SS share similarities in gene expression and a subset of MF progresses to SS. Immune analysis of
the skin in SS shows a Th2 cytokine profile (10) and the malignant T cells exhibit a Th2 cytokine pattern with increased IL-4(11). From gene profiling studies, a unique gene expression phenotype of SS has been uncovered(12). Gene expression changes in SS, such as decreased in the ability to expressed IFN-γ, and an increased in unique biomarker genes identified in SS such as TWIST1, and TOX are frequent and represent important features of CTCL (13–15).

Recent studies have established that both the tumor microenvironment (TME) and the activity of stromal cell infiltrating tumors affect cancer phenotypes (16). The contribution of TME to cancer prognosis was highlighted by a recent analysis of 39 malignancies that revealed the TME gene signatures are better predictors of survival than genes expressed in malignant tumor cells (17). The TME is comprised of abundant fibroblasts and immune cells, as well as endothelial cells and extracellular matrix (ECM) components, which closely interact with tumor cells. Crosstalk between the TME and tumor cells can either positively or negatively regulate cancer progression. Fibroblasts have been shown to contribute an important role in maintaining the ECM and regulate epithelial differentiation by stromal–epithelial crosstalk for establishing an invasion-permissive TME (18). In B-cell lymphomas, fibroblasts have a paradoxical correlation with survival outcomes compared to carcinomas (19). In CTCL, fibroblasts are an important component of the TME and have been shown to promote tumorsgenesis by augmenting Th2 and attenuating Th1 immune responses (20). In MF lesional skin, these fibroblast-derived periostin promotes the production of thymic stromal protein (TSLP) (21). TSLP subsequently activates immature myeloid dendritic cells (DCs) to produce the Th2-attracting cytokine C-C Motif Chemokine Ligand 17 (CCL17) (22), suggesting that fibroblasts from CTCL may nurture a Th2-dominant TME in MF lesions through the promotion of TSLP secretion.

A Th1 bias has been described in the skin in early MF, where malignant cells are sparse, but how this immune bias develops is unclear. A Th1 cytokine pattern in the microenvironment may suggest the presence of tumor immunity that inhibit the progression of the malignant cell that is consistent with an indolent clinical course of MF seen in the majority of patients. This observation was supported by the finding that T cell clones isolated from early MF skin lesions lack a Th2-polarized cytokine pattern (23). The interaction of tumor T cells with fibroblasts in MF is not well studied, but normal fibroblasts has variable actions in cancer and can exert suppressive functions against tumor cells (24). With the indolent nature of MF, one hypothesis is an interaction between skin fibroblasts and malignant T cell that influence the malignant cell growth. The underlying propensity immune bias is illustrated when culturing benign host T cells from SS patients in vitro away from the malignant Th2 cells, which leads to an enhanced Th1 cytokine pattern (25). These findings suggest the role of microenvironment in immune bias.

To better understand the interactions between fibroblasts and neoplastic T cells in CTCL, we study immune changes and biomarker regulation using in vitro culture of skin fibroblasts and MF cell. Here we describe one of the first studies using a novel 2-dimensional co-culture method to demonstrate the immune regulation by skin fibroblasts of CTCL cells, and investigates how fibroblasts undergo changes in CTCL.
Methods

Patient Samples:

Fibroblasts were isolated from lesional skin from MF patients, (n=3, stage IIB & IV, Table 1), and de-identified surgical skin remnants from age-matched healthy individuals. Skin specimens were dissociated with 0.25% collagenase I (Worthington Biochemical, Lakewood, NJ) in explant medium (RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Thermo-Fisher Scientific, Waltham, MA)) at room temperature for 1 hour with agitation (Fig. 1). Dissociated cells were filtered through a 40 µm cell strainer (Sigma Aldrich, St. Louis, MO), and then cultured for 2 to 4 days in explant medium, and further passaged to grow sufficient fibroblasts for co-culture experiment.

CTCL Cell Lines:

MyLa cells (26) were a kind gift provided by Dr. Michael Girardi (Yale University). Jurkat, HH and Hut78 cells were purchased from ATCC (Manassas, VA). Jurkat, HH and MyLa cells were grown in RPMI 1640 medium (GIBCO, Gaithersburg, MD) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Thermo-Fisher Scientific, Waltham, MA). Hut 78 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Gaithersburg, MD) supplemented with 10% FBS and 1% penicillin-streptomycin.

2-Dimensional Co-culture Model:

Primary fibroblasts were co-cultured with Myla cells as previously described(27) (Fig. 2). Briefly, normal and tumor fibroblasts (5 × 10^5 cells/ml) were seeded into separate 6-well plates, and cultured in RPMI 1640 medium containing 10% FBS and antibiotics until ~70% confluence was reached. Upon ~70% confluence, Myla cells (3 × 10^5/ml) were added with fresh medium, and cultured with the fibroblasts for 5 days. Myla cells were also cultured in the absence of fibroblasts as a control. After 5 days, co-cultured Myla cells and normal/lesional fibroblasts were trypsinized and re-plated into new 6-well plates for 40 min to allow fibroblasts to adhere to the plastic, leaving Myla cells in suspension. Separated Myla cells along with their no-fibroblast controls were lysed for RNA extraction.

RNA extraction and quantitative RT-PCR:

RNA was purified using the RNeasy Plus mini kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). cDNA was synthesized from 2 µg of total RNA using Maxima H Minus reverse transcriptase (Thermo-Fisher Scientific, Waltham, MA). Real-time PCR quantification was performed with Maxima SYBR Green qPCR master mix (Thermo-Fisher Scientific, Waltham, MA), on a QuantStudio 5 instrument (Applied Biosystems, Foster City, CA).

Cellular Senescence:
Cellular Senescence was assessed with the cellular senescence assay kit (Cell BioLabs, San Diego, CA) according to the manufacturer’s protocol that detects senescence-associated β-galactosidase (SA-βGal) in cells.

**Statistical Analysis:**

Two-tailed Student’s *t* test was used to analyze the quantitative PCR data for mRNA expression, with *P* < 0.05 considered statistically significant. All data is the mean of three separate experiments, and results are presented as mean ± standard deviation.

**Results**

*Short-term co-culture does not affect expression of fibroblast markers*

To determine if short-term co-culture of fibroblasts with MyLa cells alters the phenotype of normal fibroblasts, we assessed for changes in fibroblast markers by quantitative gene expression analysis. Mesenchyme-specific genes such as vimentin (*VIM*), alpha-smooth muscle actin (*ACTA2*) and heat-shock protein 47 (*HSP47*) were tested, and were unchanged in normal fibroblasts after co-culture with MyLa cells (Fig. 3A). *ACTA2* is used as a marker for cancer-associated fibroblasts (CAFs) in solid tumors (28), and is associated with worse clinical outcome for several cancers including breast and lung cancers(29, 30). In co-culture studies of MF fibroblasts with CTCL cells, *ACTA2* expression in MF fibroblasts after short-term co-culture was unchanged compared to normal fibroblasts (Fig. 3B). The fibroblasts in MF differ in *ACTA2* expression compared to CAFs from carcinomas, including breast, ovarian, pancreatic, and colorectal cancer where expression of *ACTA2* is elevated.

In addition, we also measured cellular senescence by quantifying SA-βGal activity, and found that co-culture with MyLa cells did not induce a detectable senescence phenotype in normal fibroblasts (Fig. 3C). Therefore, short-term co-culture of MyLa cells with normal fibroblasts does not induce any change in fibroblasts in terms of phenotypic markers expression and proliferation capacity.

*Normal fibroblasts alter expression of CTCL biomarkers in CTCL cells*

Expression of *TWIST1* and *TOX* is frequently increased in tumor T cells from CTCL patients (31, 32). Therefore we assess known CTCL cell lines MyLa, Hut78 and HH for expression of *TWIST1* and *TOX*. Of the three cell lines analyzed, only Myla cells expressed these CTCL biomarker genes (Fig. 3D), indicating that abnormal gene expression similar to that seen in patient-derived T cells is preserved MyLa cells.

To study the influence of the ME in CTCL, MyLa cells were co-cultured with normal fibroblasts (n=3) or MF lesional fibroblasts (n=3), and changes in the expression of CTCL biomarker genes in MyLa cells were assessed. As shown in Fig. 3D, MyLa cells have endogenously high *TWIST1* expression, but after co-culture with normal fibroblasts, expression of *TWIST1* was significantly reduced (*p*<0.0006) (Fig. 4A). *TOX* expression was also suppressed in MyLa cells after co-culture (*p*<0.03) (Fig. 4B).
In contrast, co-culturing MyLa cells with MF lesional fibroblasts increased expression of both TWIST1 and TOX (Fig. 4A-B). As TOX plays an important role in CTCL proliferation (33) and T cell exhaustion (34), the ability of normal fibroblasts to suppress TOX expression in MyLa cells suggests that fibroblasts in the MF TME may have a role in regulating T cell exhaustion and disease progression.

**Normal fibroblasts promote a Th1 phenotype in CTCL cells**

The effect of the co-culture model on the expression of IFNg and TBX21 in MyLa cells was examined because TWIST1 has been shown to limit the expression of IFNg and TBX21 in Th1 cells (35). Co-culture of MyLa cells with normal fibroblasts increased the expression of both IFNg ($p<0.03$) and TBX21 (Fig. 4C-D). TBX21 encodes T box transcription factor (T-bet), a master-regulator of Th1 differentiation (36). Given the modulatory role of TWIST1 in Th1 differentiation (35), the increased expression of IFNg and TBX21 may be secondary to TWIST1 suppression in MyLa cells co-cultured with normal fibroblasts. In contrast, culturing MyLa cells with MF tumor-derived fibroblasts further suppressed expression of IFNg and TBX21 in MyLa cells (Fig. 4C-D). These findings suggest that normal fibroblasts promote Th1 cell transcriptional network in MyLa cells.

**Normal fibroblasts attenuates Th2-dominant microenvironment and reduces proliferation**

Studies have shown that T-bet not only promotes Th1 cell differentiation, but also represses Th2 differentiation by suppressing GATA3 expression (37) and reducing the binding of GATA3 to DNA (35). GATA3 is crucial for the differentiation of naïve CD4+ T cells into Th2 cells. Furthermore, GATA3 deletion permits the development of IFN-g producing cells (38). Therefore, we analyzed whether GATA3 expression in MyLa cells is affected by co-culture with fibroblasts. After co-culture with normal fibroblasts, GATA3 expression was suppressed in MyLa cells ($p<0.02$) (Fig. 5A). In MF, GATA3 is increased, and in MyLa cells after co-culture with MF tumor-derived fibroblasts, GATA3 expression was further upregulated (Fig. 5A).

Several cytokines that are upregulated in advanced CTCL, such as IL-16, can augment the growth of malignant T cells in an autocrine manner (39). IL-16, a potent T-cell chemoattractant, is one of the known markers of MF onset and stage (39). Based on the role of IL-16 as a regulator of T-cell proliferation and migration, we next examined IL-16 expression in MyLa cells in co-culture experiments. After co-culture with normal fibroblasts, a significant suppression in IL-16 expression was observed ($p<0.03$) (Fig. 5B). In contrast, co-culture with MF tumor-derived fibroblasts increased IL-16 expression in MyLa cells (Fig. 5B). We next assessed the role of co-culture on genes important in proliferation by assessing expression of MK167. We observed reduced MK167 expression in MyLa cells when co-cultured with normal fibroblast, whereas little effect on MK167 expression was observed when co-cultured with MF tumor-derived fibroblasts (Fig. 5C). It suggests a differential effect on MK167 by normal fibroblasts versus MF fibroblasts.

**Discussion**
Hallmarks of tumorigenesis describe numerous biologic functions that influence both intrinsic cellular and extrinsic extracellular factors important in the development of cancer (40). Intrinsic events include tumor drivers such as oncogene activation and tumor-suppressor gene inactivation (41), while extrinsic events include interactions of tumor cells with its microenvironment (ME). Studies have shown that tumor growth is preceded by, or is concomitant with, activation of local host stroma (42), which plays a major role in disease evolution and response to therapy (43). Recent studies have described an important role of CAF in a variety of cancers, owing to their abundance in most solid tumors and their diverse tumor-restraining/promoting roles (44-47). The interplay between tumor cells and neighboring CAFs may take place by both paracrine signals (cytokines, exosomes and metabolites) or by the multifaceted functions of the surrounding ECM that may affect growth and play a role in resistance to chemotherapy. These interactions influence proliferation and may play a role in resistance to chemotherapy. The cutaneous TME in MF includes abundant stromal fibroblast; however, their role on the malignant T cell and influence is unknown.

The majority of MF patients have an indolent progression (48), whereas SS is a CTCL with more aggressive rapidly progressive disease. Those patients who present with early stage diagnosis of MF with skin limited disease have an excellent survival (48), whereas SS is a more aggressive CTCL. In early MF, a low burden of malignant T cells characterizes the inflammatory cells in the skin, with a non-malignant reactive Th1 T cells. However in addition to the immune cells, the skin ME background consists of mesenchymal stromal cells, the majority of which are normal fibroblasts (49). As malignant T cell burden increases with MF progression from patch to tumor, changes in the ME can be detected, such as increased angiogenesis and stromal fibroblasts expressing matrix metalloproteinase-2 (MMP2) (50). Therefore as disease stage progresses, the skin architecture is disrupted and the skin ME changes with histologic changes of epidermal fibrosis, and immune cell infiltrate associated with an increased expression of Th2 cytokines and concomitant with declining expression of Th1 factors (3, 51-53). The malignant T cells, which have a Th2 bias, proliferates in the TME in the presence of MF fibroblast. The malignant cell further suppresses an active host immunity from the upregulation of surface CTLA-4 on the malignant T cell (54). Highlighting the importance of this immune shift in disease progression is that restoring cytokines seen in early MF by treating advanced CTCL with IFN-α and IFN-γ is an effective strategy for treatment (55). These observations suggest that normal fibroblasts in the ME in early disease may contribute to altering gene expression of the malignant T cell and play a role in the indolent nature of MF.

The current study is a first step to elucidate the regulatory role of fibroblasts in CTCL. Our novel co-culture findings firstly reveal that normal fibroblasts interaction with T cells have an impact on T cell phenotype using the CTCL derived MyLa cell line. We demonstrate for the first time that normal fibroblasts in co-culture can induce gene expression changes in cells from CTCL. The results indicate that fibroblasts in the ME from normal skin may regulate T cells gene expression, which we detected on MyLa CTCL cells. In our studies, normal fibroblasts affect genes important in cytokine regulation and proliferation, which has the ability to inhibit the progression of CTCL. Specifically we show that fibroblasts modulate MyLa cells to alter cytokine expression from Th2 to Th1, and suppressed the expression of MF biomarkers such as
TWIST1 (Fig. 4A) and TOX (Fig. 4B), and inhibit proliferation of MyLa cells (Fig. 5C). The results suggest that signal from normal fibroblasts may recapitulate the ME of early MF skin lesions, creating an environment inhospitable for proliferation. Similar observations was also seen in diffuse large B-cell lymphoma (DLBCL), where stromal gene signature representing fibroblasts and extracellular matrix components has been associated with good survival and creating a ME not conducive for lymphoma progression (19).

Second, we demonstrate that MF fibroblasts from cutaneous tumors differ from that of normal fibroblasts in inducing gene expression changes in MyLa cells. We show that MF fibroblasts promote expression of Th2 cytokine genes and the SS biomarkers TWIST1 and TOX. Whether this is similar to CAF from solid tumors is unclear. ACTA2, which is highly activated in other carcinomas (28-30), is not upregulated in MF fibroblasts compared to their normal counterparts (Fig. 3B). These findings are similar to the previous study conducted in DLBCL patients (56). Lenz et al., identified two sets of stromal gene signatures, stromal-1 and stroma-2, of which stromal-1 gene signature was found to be associated with good survival in DLBCL patients, which includes the genes that are associated with poor survival in other carcinomas (55). The mechanism behind the suppressive effect of normal fibroblasts in CTCL is unclear, and will need further analysis.

Our findings have implication in the understanding tumor progression in MF. In the early stages where malignant cells are low, the skin architecture is preserved with normal fibroblasts, and the immune infiltrate consists primarily of nonmalignant Th1 cells and cytotoxic CD8+ T cells (3, 57). The results from normal fibroblasts with MyLa cells suggest that during the early stage of MF disease, fibroblasts may contribute to the Th1 immune phenotype as supported by normal fibroblasts promoting IFN-γ and TBX21 expression in MyLa cells (Fig. 4C-D). Our findings suggest that as MF progresses to a more advance stage, there are changes associated with ME in the fibroblasts that can affect the malignant T cell as shown by stimulation of CTCL biomarker genes in co-culture (Fig. 4A-B). When fibroblasts from tumor stage lesions were co-culture with MyLa cells, the cytokine genes maintained a Th2 bias that is consistent with malignant CTCL phenotype (3, 58). There was a higher level for TWIST1 and TOX, which was markedly in contrast to that observed when MyLa cells were co-culture with normal fibroblast (Fig. 4A-B). These experiments for the first time demonstrate that fibroblast from normal and fibroblast from MF differ functionally and can affect the gene expression of malignant T cells (Fig. 6).

**Conclusions**

In summary, our results describe novel activity of fibroblasts in MF in the ability to modulate T cell gene expression compared to normal fibroblasts. The finding suggests TME change between normal and advanced MF, and it is the first report of such type of study, which supports that fibroblasts in the ME play a role in disease progression. These novel findings suggest that fibroblast promotes a Th1-dominant ME in early MF patients by augmenting Th1 and attenuating Th2 immune responses. We demonstrate that fibroblasts from advanced stage tumor lesions differ from normal and have unique activity on MyLa cells, and promote Th2 cytokine gene expression in MyLa cells an enhance CTCL biomarker genes.
Whether lesional tumor fibroblasts may protect tumor cells from cytotoxic and genotoxic therapies in CTCL is unclear and will need to be explored further. Future studies identifying pathways important in altering gene expression by skin fibroblasts may lead to the development of novel strategies to identify compounds for the treatment of CTCL.

**Abbreviations**


**Declarations**

**Ethical approval and consent to participate**

This study was conducted under a human research protocol approved by the Institutional Review Board (IRB) of the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR). All participants voluntarily provided written informed consent.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

**Competing interests**

The authors have no competing interests.

**Funding**

This work was supported by Translational Research Institute Special Populations Pilot Award to HKW (NCATS UL1TR003107).

**Author's contributions**

Conception, design and analysis: SJM and HKW; manuscript preparation: SJM, AMH and HKW; sample collection: SJM and AMH. HKW supervised all aspects of the research. All authors approved the final manuscript.
Acknowledgements

The authors would like to acknowledge the support from the UAMS Department of Dermatology and volunteers.

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References


Tables

Table 1: Patient Demographics

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