Expression of the Autoantigen Topoisomerase-1 is Enriched in the Lung Tissues of Patients With Autoimmune Interstitial Lung Disease: A Case Control Study

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**Background.** Among the autoimmune rheumatic diseases, it is striking that autoantibodies targeting ubiquitously expressed proteins (e.g., topoisomerase-1) associate with specific clinical complications (e.g., interstitial lung disease [ILD]). It has been proposed that enriched antigen expression in inflamed target tissue may play a role in focusing the autoimmune response. We sought to determine whether topoisomerase-1 expression is enriched in lungs from patients with autoimmune/inflammatory diseases relative to normal lung.

**Methods.** We used a 99-core lung tissue microarray (TMA) containing lung tissue from 40 patients with autoimmune inflammatory ILD (cases) and 46 control subjects with normal lungs. We stained the TMA with antibodies to compare topoisomerase-1 and CD8 expression between patients and control subjects and evaluated whether expression is enriched in specific cell types. Staining was analyzed, and statistical comparisons were performed.

**Results.** Cases were more likely to have global topoisomerase-1 expression (53% vs 21%; \(P = 0.003\)), specifically in pneumocytes (47% vs 16%; \(P = 0.003\)) and stromal/immune cells (32% vs 5%; \(P = 0.002\)) compared with control subjects. CD8 cell density (223 cells/mm\(^2\) vs 102 cells/mm\(^2\); \(P = 0.018\)) was significantly higher in topoisomerase-1-positive lung tissues compared with topoisomerase-1-negative lung tissues. Interestingly, topoisomerase-1 expression was significantly more common in scleroderma compared with normal lung (67% vs 21%; \(P = 0.036\)) and was present more frequently in pneumocytes in these patients (67% vs 16%; \(P = 0.018\)).

**Conclusions.** Pulmonary expression of topoisomerase-1 is increased in the setting of autoimmune ILD relative to normal lung, specifically in pneumocytes. This may contribute to the amplification of pulmonary disease in patients with scleroderma with a loss of tolerance to topoisomerase-1.

**INTRODUCTION**

Interstitial lung disease (ILD) is commonly identified in patients with systemic autoimmune connective tissue diseases and is associated with significant morbidity and mortality (1). Though the specific mechanism of autoimmune ILD has not been defined, ILD associated with connective tissue diseases is more likely to be responsive to immunosuppressive therapy than in other forms of parenchymal lung disease (e.g., idiopathic pulmonary fibrosis), suggesting that an active immune-mediated response is driving tissue damage.

Studies performed on myositis muscle biopsies have shown that multiple myositis-specific autoantigens (Mi-2, PARP, DNA-PK, and U1-70) are abundantly expressed in inflamed muscle but are only detected at low levels in normal muscle (2). These findings suggest that high-level antigen expression may contribute to focusing and amplifying the autoimmune response on muscle tissue and, more generally, that high autoantigen expression in inflamed tissue may drive an organ-specific immune response (2). As in autoimmune myositis, in which specific autoantibodies associate with muscle inflammation, in scleroderma, antibodies to topoisomerase-1 associate strongly with ILD (3,4). We therefore

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hypothesized that topoisomerase-1 expression is enriched in
inflamed lung tissue compared with normal lung tissue and that
this may focus the autoimmune response on the lung in patients
who have lost tolerance to this protein.

MATERIALS AND METHODS

Case selection and tissue microarray construction. This study, including tissue collection, was approved by the
Johns Hopkins Institutional Review Board (NA_00093689). The Johns Hopkins Pathology Database was searched for
surgical tissue sections obtained between 2002 and 2014 using the following keywords: rheumatology, scleroderma,
systemic sclerosis, lupus, myositis, sarcoidosis, NSIP, UIP, ANCA, vasculitis, undifferentiated connective tissue
disease, and mixed connective tissue disease. A total of 610 potential cases were identified. The clinical records were
then manually reviewed and cases were selected if the clinical documentation was supportive of one of the following
autoimmune or inflammatory conditions: systemic sclerosis, lupus, Sjogren syndrome, myositis, rheumatoid arthritis, vas-
culitis, undifferentiated or mixed connective tissue disease, overlap rheumatic disease, or nonspecific interstitial pneu-
monia (NSIP) or usual interstitial pneumonia (UIP) with an autoantibody associated with an autoimmune connective tis-
ue disease. All cases with a history of autoimmune/connective tissue diseases and supportive clinical documentation in the
medical records were included in the TMA. The medical records of the confirmed cases were then reviewed in more
detail to extract the available clinical information pertinent to the patient’s diagnosis and to determine autoantibody status
when available. Findings in at least three of the five following categories were considered supportive for diagnosis: clinical
presentation, physical examination, radiographic features, serology, and/or pathology.

The pathology slides were reviewed by a board certified pathologist (M.K.H.) to confirm that the histology was consistent
with collagen vascular disease rather than a superimposed infec-
tion or coexisting malignancy. For each case, the most inflamed
region was selected for TMA inclusion (Supplemental Figure 1). For each control, histologically normal lung tissue was included
from uninvolved regions of lung cancer surgical resection speci-
mens from patients without a history of autoimmune disease. The
99-core TMA was ultimately comprised of lung tissue from:
1) 40 cases (Supplemental Table 1), 2) 46 control subjects, 3) seven
case duplicates and two control duplicates, and 4) four cores from
extrapulmonary tissue (to orient the TMA).

Immunohistochemistry. Immunohistochemical staining
with commercial anti-topoisomerase-1 and anti-CD8 antibodies
was optimized (by L.C-R.) before staining the TMA. After staining,
the slides were reviewed by M.K.H., L.C.-R., and Z.M. to assess
the quality of the staining, and then the slides were submitted to
pathology to be read by T.R.C. with F.A.’s oversight. The pathol-
ologists remained blinded to case/control status throughout. Each
tissue core was scored as positive (1) or negative (0) based on the
presence or absence of topoisomerase-1 staining. Topoisomer-
ase-1 expression in distinct cell types (bronchiolar cells, combined
stromal and immune cells, pneumocytes, perivascular cells, and
lymphoid aggregates) was also assessed. The total number of
CD8 cells per core and CD8 cell density (cells/mm²) were quanti-
fied using digital image analysis (Halo Software, Indica Labs).

Statistical analysis. We performed a cross-sectional analysis
to evaluate differences in topoisomerase-1 expression
between cases and controls. χ² or Fisher’s exact tests were
performed to 1) compare topoisomerase-1 expression in cases
and control subjects, 2) determine whether topoisomerase-
1–positive expression is enriched in distinct cell types in cases
versus control subjects, 3) compare topoisomerase-1 in the
lung tissue from patients with scleroderma versus those with
other autoimmune rheumatic diseases (lupus, Sjogren syn-
drome, myositis, rheumatoid arthritis, mixed connective tissue
disease, overlap connective tissue disease, and undifferenti-
ated connective tissue disease), and 4) evaluate whether the
intensity of topoisomerase-1 staining varied between cases and
control subjects and across disease states. For our analysis we
grouped the three patients with mixed connective tissue disease
with one patient who was anti-nuclear antibody (ANA) negative
with overlapping features of scleroderma and dermatomyositis,
as these patients were clinically similar. CD8 cell number and
density (cells/mm²) were compared between cases and control
subjects using the Wilcoxon Mann-Whitney test. Student’s t tests
were used for parametric data to examine differences between
the means of continuous variables between two groups. When
duplicate cores from the same patient were present, one core
was randomly selected for inclusion in all analyses. A P value
of less than 0.05 was considered statistically significant. All statisti-
cal analyses were performed using Stata version 14.2 (Stat-
aCorp, College Station, TX).

RESULTS

Clinical characteristics of the patients and control
subjects included in the tissue microarray. Of the patients
with autoimmune disease, 68% (27/40) were women, and 53%
(21/40) were Caucasian. Several different patient subsets were
included, specifically patients with rheumatoid arthritis (RA)
(n = 12), scleroderma (n = 6), myositis (n = 5), systemic lupus
erythematosus (n = 4), undifferentiated connective tissue disease
(n = 4), mixed connective tissue disease/overlap (n = 4), Sjogren
syndrome (n = 2), ILD with RA antibodies (n = 2), and vasculitis
(n = 1). Details of the clinical features and pulmonary pathology
are presented in Supplemental Tables 1 and 2, respectively.
Topoisomerase-1 expression is enriched in pulmonary tissue from patients with ILD and is associated with increased CD8+ T-cell infiltration. Topoisomerase-1 expression was compared between cases and control subjects (Table 1). Pulmonary tissue from patients with ILD was more likely to have topoisomerase-1 expression compared with tissue from patients without ILD (52% vs 21%; \( P = 0.003 \)). The increased topoisomerase-1 expression was seen primarily in pneumocytes (47% vs 16%; \( P = 0.003 \)) and stromal and immune cell (32% vs 5%; \( P = 0.002 \)) compartments, rather than bronchiolar cells (26% vs 12%; \( P = 0.150 \)), (Figure 1). There was a slight trend towards greater expression of topoisomerase-1 in patients with UIP versus those with NSIP (67% vs 21%; \( P = 0.20 \)), although the difference was not statistically significant. Cases had significantly more total CD8 cells per tissue core than control subjects, (222 vs 55; \( P < 0.001 \)), although there was no difference between cases and control subjects in CD8 density. In contrast to ILD, normal lung has significantly less tissue area per core because of preserved alveolar airspaces. Interestingly, however, when comparing median CD8 density in cases with pulmonary topoisomerase-1 expression relative to control subjects, there was no difference in CD8 density in cases positive for topoisomerase-1 expression, (250 cells/mm² vs 98 cells/mm²; \( P=0.035 \)); Figure 1). Among histologically normal lung tissue controls, there was a trend toward a higher CD8 density in topoisomerase-1–positive versus topoisomerase-1–negative cores, although the difference was not statistically significant (median: 174 cells/mm² vs 98 cells/mm²; \( P = 0.197 \)).

Lung tissues from patients with scleroderma ILD show increased CD8+ T-cell infiltration and pneumocyte topoisomerase-1 expression relative to normal lung. Because we hypothesized that high levels of topoisomerase-1 expression in the lung could focus the immune response to this tissue, we sought to determine whether scleroderma, or any of the other autoimmune rheumatic diseases represented in the TMA, were driving the association between cases and high pulmonary topoisomerase-1 expression. Similar to prior findings in myositis, patients with scleroderma were more likely than control subjects to express topoisomerase-1 in the lung (67% vs 21%; \( P = 0.036 \)) (Table 2). Patients with scleroderma had significantly higher numbers of CD8 T-cells per tissue core than control subjects (182 vs 55; \( P = 0.006 \)). Among patients with scleroderma, topoisomerase-1 expression was significantly different from control subjects only in pneumocytes (67% vs 16%; \( P = 0.018 \)). Otherwise, there was no significant difference between scleroderma and control subjects in topoisomerase-1 expression in bronchiolar cells, perivascular cells, lymphoid aggregates, or stromal/immune cells. Among cases positive for topoisomerase-1 expression, there was no difference in topoisomerase-1 staining intensity between patients with scleroderma lung disease and control subjects.

Cell type–specific topoisomerase-1 expression in ILD varies with distinct underlying rheumatic disease diagnoses. Although the number of patients in each group was small, in Sjögren syndrome, there was a trend toward more topoisomerase-1 expression in the lung compared control subjects (100% vs 21%; \( P = 0.056 \)), specifically in stromal/immune cells.

### Table 1. Topoisomerase-1 and CD8 expression in lung tissue in cases and controls

|                     | Case      | Controls | \( P \) Value |
|---------------------|-----------|----------|---------------|
| Topoisomerase-1, % (n) | 53 (20/38) | 21 (9/43) | 0.003         |
| Bronchiolar cells    | 26 (10/38) | 12 (5/43) | 0.150         |
| Stromal and immune cells | 32 (12/38) | 5 (2/43)  | 0.002         |
| Pneumocytes          | 47 (18/38) | 16 (7/43) | 0.003         |
| CD8                  |           |          |               |
| Total CD8 cells per core, median (IQR) | 222 (90-441) | 55 (32-82) | <0.001        |
| CD8 cell density, median (IQR), cells/mm² | 200 (77-288) | 131 (78-244) | 0.344         |

IQR, interquartile range. Bold values indicate \( P \leq 0.05 \).
Table 2. Staining comparisons between interstitial lung disease and control lung tissues at global or cell-specific levels

| Autoimmune disease   | Topo+ in Disease vs Controls, % (n) | P Value |
|----------------------|------------------------------------|---------|
| Scleroderma          | 67 (4/6) vs 21 (9/43)              | 0.036   |
| Pneumocytes          | 67 (4/6) vs 16 (7/43)              | 0.0018  |
| MCTD                 | 60 (3/5) vs 21 (9/43)              | 0.056   |
| Stromal/immune cells | 40 (2/5) vs 5 (2/43)               | 0.049   |
| Pneumocytes          | 60 (3/5) vs 16 (7/43)              | 0.055   |
| SLE                  | 50 (2/4) vs 21 (9/43)              | 0.229   |
| Sjogren syndrome     | 100 (2/2) vs 21 (9/43)             | 0.006   |
| Stromal/immune cells | 100 (2/2) vs 5 (2/43)              | 0.036   |
| Pneumocytes          | 100 (2/2) vs 16 (7/43)             | 0.229   |
| Myositis             | 50 (2/4) vs 21 (9/43)              | 0.229   |
| Rheumatoid arthritis | 33 (4/12) vs 5 (2/43)              | 0.017   |
| Stromal/immune cells | 33 (4/12) vs 5 (2/43)              | 0.017   |
| Vasculitis           | 0 (0/1) vs 21 (9/43)               | 1.000   |
| UCTD                 | 50 (2/4) vs 21 (9/43)              | 0.229   |

MCTD, mixed connective tissue disease; SLE, systemic lupus erythematosus; Topo, Topoisomerase-1; UCTD, undifferentiated connective tissue disease.

Bolded P-values are statistically significant.

(100% vs 5%; P = 0.006) and/or in pneumocytes (100% vs 16%; P = 0.036). Patients with mixed connective tissue disease (MCTD) and RA or rheumatoid lung disease were not significantly more likely to express topoisomerase-1 in the lung than control subjects (P > 0.05). They were, however, more likely to have topoisomerase-1 expression enriched in stromal/immune cells but not in other cell types (perivascular cells, bronchiolar cells, lymphoid aggregates, or pneumocytes) (MCTD: 50% vs 5%; P = 0.031; RA or RA lung: 31% vs 5%; P = 0.022).

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

DISCUSSION

Although autoantigens associated with autoimmune connective tissue diseases (eg, topoisomerase-1) are ubiquitously expressed, it remains unclear why they are targeted by the immune response and why these antibodies associate so closely with damage to specific tissues (eg, ILD). In this study, we examined the expression of topoisomerase-1, an autoantigen associated clinically with ILD, in normal and inflamed lung tissue. We determined that topoisomerase-1 is frequently expressed at high levels in patients with inflamed lung disease and is much less frequently expressed at detectable levels in normal lung. We also found that topoisomerase-1 is enriched in patients with scleroderma lung relative to control subjects and that this is specifically observed in pneumocytes.

Inflammation and damage are known to occur in autoimmune connective tissue–associated lung disease. Data from ultrastructural studies have revealed that epithelial and/or endothelial injury precede inflammation and fibrosis, although the inciting event remains unknown. Findings on pathology demonstrate acute, subacute, and chronic lesions within the same specimen, indicating an ongoing cycle of inflammation, damage, and repair. This supports the idea that a tissue autoantigen may be propagating the immune response (5).

Our results demonstrate the novel finding that topoisomerase-1 expression in pulmonary tissue is differential, upregulated in the setting of inflammation/repair, and less frequently expressed in normal lung. This is similar to data in autoimmune myositis, which demonstrate an enrichment of autoantigen expression in inflamed muscle relative to normal muscle. In both cases, the upregulation of autoantigen expression in inflamed target organs (muscle and lung, respectively) may result in organ-specific amplification and propagation of the autoimmune response (eg, ILD) when immune tolerance to the autoantigen is broken. Interestingly, topoisomerase-1 expression is high in inflamed lung acquired from patients who are not positive for anti–topoisomerase-1 antibodies. It is possible that autoantigens are more broadly upregulated in inflamed lung tissue (as is the case with myositis autoantigens in inflamed muscle) (2) and that this high expression sustains/drives the autoimmune response in patients once autoimmunity has been initiated. Additional studies that examine the expression levels of other autoantigens in inflamed lung may provide relevant insights.

Topoisomerase-1 expression was also enriched in pneumocytes of patients with Sjogren syndrome–associated ILD. It is known that patients with primary Sjogren syndrome may express atypical autoantibodies, including antibodies to topoisomerase-1 (6). In addition, an estimated 9% to 24% of patients with Sjogren syndrome develop ILD (7). Although a larger sample is needed to determine the relevance of this finding, it is possible that ILD in a subset of patients with Sjogren syndrome is associated with high levels of topoisomerase-1 expression in the lung.

It is striking that lung tissue from patients with scleroderma showed significantly higher expression of topoisomerase-1 relative to tissue from control subjects, specifically in pneumocytes. Pneumocytes are surface epithelial cells of the alveoli and form part of the barrier across which gas exchange occurs in the lung. Injury of pneumocytes is known to cause various types of interstitial pneumonias (8). In scleroderma, the disruption of gas exchange is represented clinically by a low diffusing capacity of carbon monoxide on pulmonary function tests and is often a sign of early pulmonary disease (9). Determining whether immune-mediated mechanisms targeting pneumocytes play a biologically important role in such patients would be informative.

We propose that the striking enrichment of topoisomerase-1 expression in pulmonary tissue might focus the anti–topoisomerase-1 immune response onto lung tissue. A proimmune event, such as a malignancy, infection, or trauma from chronic microaspiration of gastric acid, could disrupt tolerance toward an autoantigen in patients who are genetically predisposed to autoimmunity (10–12). Topoisomerase-1 expression would likely amplify and propagate...
the immune response in the lung in patients who have lost tolerance to topoisomerase-1. Our study did not address whether other end organs (eg, skin) are also enriched in topoisomerase-1. As the presence of diffuse skin disease is present in approximately two-thirds of patients with an immune response to topoisomerase-1, comparing topoisomerase-1 expression in patients with limited versus diffuse cutaneous disease would also be of interest.

Our study has several strengths. It is the first study to use a tissue microarray to assess autoantigen expression in patients with a variety of autoimmune connective tissue diseases. The same lung pathologist read all lung biopsy samples in the TMA and was blinded to cases and controls to minimize bias. The number of cases was proportional to the number of controls, minimizing the risk of bias toward finding more histological abnormalities in one group compared with the other. Our study is limited by a small number of cases within each disease subset, which constrained our ability to specifically compare topoisomerase-1 expression between subsets. In addition, tissue microarray has decreased sensitivity relative to the assessment of whole slide sections. Validation of these findings will benefit from a larger cohort of patients with autoimmune ILD. Inclusion of patients with scleroderma with anti-topoisomerase-1 antibodies and control tissues from patients with nonrheumatic lung disease(s) (eg, idiopathic pulmonary fibrosis) would be particularly informative. Future studies using multiparametric approaches, such as RNA sequencing or multiplex immunofluorescence would add significant capacity to examine expression of multiple autoantigens simultaneously and characterize the nature of the immune response associated with autoantigen expression in ILD.

In conclusion, our study demonstrates enriched topoisomerase-1 expression is differential in the setting of autoimmune ILD relative to normal lung. This may contribute to the amplification of autoimmune disease in the lung of patients, such as those with scleroderma, who have a loss of tolerance to topoisomerase-1. Patients with scleroderma are also more likely than control subjects to have enrichment of topoisomerase-1 expression, specifically in the pneumocytes. Further studies aimed at understanding the regulation of antigen expression in tissue and the cognate immune response, as well as defining the role of pneumocytes in the development of scleroderma ILD, may provide insight into disease mechanism.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Casciola-Rosen in the analysis and interpretation of the data. Cottrell in the acquisition of the data.

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