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Developing Better Biomarkers for Connective Tissue Disease—Associated Interstitial Lung Disease: Citrullinated Hsp90 Autoantibodies in Rheumatoid Arthritis

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Interstitial lung disease (ILD) describes a heterogeneous group of parenchymal pulmonary conditions that sometimes defy easy categorization (1). This frequently lethal collection of disorders is often characterized by signs and symptoms of systemic inflammation, findings that suggest that immunity causes or, at least, contributes to the development of pulmonary disease. Because lungs interface constantly with the outside world, it is not surprising that the barrier function of the airways requires not only constant mechanical removal of foreign antigens (e.g., cough reflexes, a mucociliary elevator) but also an actively engaged and sophisticated set of immune defenses.

Although the lung is particularly adept, relative to sterile body compartments such as the peritoneum, at dampening overly exuberant inflammatory responses (such as through indoleamine 2,3-dioxygenase-expressing dendritic cells) (2), the lung can eventually suffer from a loss of immune tolerance and become a large target for autoimmune injury. In particular, available evidence suggests that B cell immunity can contribute to autoimmune ILD after the loss of self tolerance following occurrence of one or more of the following events: 1) the inhalation of neoantigens, 2) the generation of cross-reactive autoantibodies in a systemic disorder, or 3) the expression of neopeptides in airway or pulmonary tissue after bronchial or lung injury. With respect to the latter cause of autoantigen generation, the posttranslational process of citrullination, the enzymatic conversion of arginine to citrulline, has now been strongly identified as an important target for autoimmunity in both developing and established rheumatoid arthritis (RA) (3–5).

In this issue of *Arthritis & Rheumatism*, Harlow and colleagues reasoned that, because lung explants from patients with RA-associated ILD are known to possess increased citrullinated proteins, novel autoantibodies (directed against citrullinated proteins) could be developed that might distinguish RA patients with ILD from RA patients without ILD (6). RA-

associated ILD is a type of connective tissue disease (CTD)–associated ILD that is, itself, a major subset of ILD conditions (7). RA-related lung disease has protean manifestations and can present as pleuritis, vasculitis, pneumonitis, pulmonary fibrosis, and nodulosis. CTD-associated ILD, considered generally as a group of inflammation-associated lung disorders, can be difficult to categorize and treat. Experts in the field have advocated for greater clarity in CTD-associated ILD disease classifications to better explain pathogenesis, enhance prognostication, and develop treatments (1).

Identifying new antibodies against citrullinated proteins in RA-associated ILD may be useful in elucidating disease etiology, predicting phenotype, presaging acuity, selecting immunotherapies, and tracking treatment responses. Such progress has already been noted with the study of autoantibodies in ILD associated with inflammatory myopathies. In these conditions, a modification of transfer RNA synthetases in the lung has been postulated to lead to the generation of antisynthetase autoantibodies; these antibodies may be an important factor leading to autoimmune inflammation in the lung and muscle in this condition (8,9). Because ILD is often detected when the diagnosis of myopathy is first made, it has been postulated that the autoimmune injury in the lung is the initiating event in the disease (10).

A number of antisynthetase antibodies have been characterized as being strongly predictive of ILD in inflammatory myopathies, including anti-Jo-1, anti-PL-7, anti-PL-12, anti-EJ, anti-OJ, anti-KS, anti-YRS, and anti-Zo (11). The coexistence of anti-SSA/Ro and anti-Jo-1 antibodies is predictive of more severe and extensive pulmonary fibrosis (12). The anti-CADM140 antibody has been identified in inflammatory myopathy patients and exhibits a strong association with rapidly progressive ILD (13). Patients with these conditions have received benefit from several immunomodulatory approaches, including, most recently, rituximab (14).

Ex vivo analysis of autoantibodies or sera from antibody-positive patients can be used to explore disease pathogenesis. This approach proved useful for examining anti-Jo-1–positive patients with ILD; sera from these patients induced expression of intercellular adhesion molecule 1 in human lung endothelial cells (15). This could be one mechanism explaining the predisposition to ILD in antisynthetase-positive patients. Thus, the progress made in understanding the role of autoantibodies in ILD associated with inflammatory myopathies can be similarly exploited in patients with CTD-associated ILD. The current work by Harlow and colleagues (6) certainly advances this scientific knowledge in patients with RA-associated ILD.

In the study by Harlow et al (6), the authors employed a unique “reverse immunophenotyping” methodologic approach as a mechanism of biomarker discovery. They took this approach because of the strong rationale for the role of citrullinated proteins in RA, coupled with the difficulty in obtaining diseased lung tissue as a primary antigenic source. Instead of using lung tissue to generate citrullinated proteins, the investigators subjected cells from an immortalized erythroleukemia cell line to deimination in vitro. They subsequently isolated citrullinated proteins that were preferentially recognized by RA-associated ILD patient sera, and by so doing, identified citrullinated Hsp90 α and citrullinated Hsp90 β as candidate target proteins.

The investigators then established an enzyme-linked immunosorbent assay-based screening system using citrullinated and uncitrullinated Hsp subunits as substrate antigens from prokaryotic (P) and eukaryotic (E) expression systems. They evaluated 58 RA patients with ILD and compared them with 27 RA patients without ILD. The sensitivity/specificity for use of anti-Hsp90 β -P to identify RA-associated ILD was 0.24/0.19, while the sensitivity/specificity for use of anti-Hsp90 β -E to identify RA-associated ILD was 0.96/1.0. The sensitivity of the anti-citrullinated Hsp90 α antibodies was lower (only 6 of 58 RA-associated ILD patients were seropositive), but no patients without ILD demonstrated this antibody, thus indicating high specificity for this biomarker in the diagnosis of RA-associated ILD. Interestingly, both anti-citrullinated Hsp90 antibody-positive and -negative patient subgroups demonstrated a similar range of computed tomography abnormalities, and therefore the low frequency of RA-associated ILD patients who were positive for these autoantibodies cannot be easily attributed to simply representing a form of ILD atypical for what is seen in RA in general practice.

As with ILD associated with inflammatory myopathies, additional studies, from different cohorts of patients, need to be performed to confirm and expand on how the autoantibodies described in the study by Harlow and colleagues (6) discriminate RA patients with ILD from RA patients without ILD as well as patients with other CTDs; the fact that these investigators were able to distinguish RA-associated ILD from mixed CTD and idiopathic pulmonary fibrosis using these biomarkers suggests that immune responses to citrullinated Hsp90 may have a specific role in the pathogenesis of RA-associated ILD, compared with ILD from other etiologies.

A wide variety of anti-citrullinated protein antibodies (ACPAs) have now been described in RA, including autoantibodies to citrullinated vimentin, fibrinogen, type II collagen, histones, and α -enolase (16–18). There is also growing evidence that ACPAs directly contribute to the pathogenesis of joint disease in RA through immune complexes as well as through direct targeting of synovial antigens (19–21), and antibodies to citrullinated vimentin have been demonstrated to activate osteoclasts (22). However, additional work is needed to determine the specific role of ACPAs, and in particular the autoantibodies to citrullinated Hsp90 identified in the study by Harlow and colleagues (6), in the development of extraarticular manifestations of RA, including RA-associated ILD. Are these autoantibodies directly targeting specific targets in the lung and leading to local tissue inflammation and injury? Are they part of immune complexes that injure the lung as part of a circulatory-based process, or are they an epiphenomenon related to another immune process (T cell or otherwise) that is truly leading to lung disease?

To address these issues, it would be helpful to identify where autoantibodies to citrullinated Hsp90 may be generated. Are they generated outside of the lung and then target the lung due to the development of citrullinated Hsp90 within the lung, perhaps due to factors that may lead to increases in citrullinated Hsp90 within the lung? Or, could immune responses to citrullinated Hsp90 be locally generated within the lung? To support this latter point, Rangel-Moreno and colleagues have demonstrated that plasma cells generating rheumatoid factor (RF) and anti-citrullinated fibrinogen antibodies are present in inducible bronchus-associated lymphoid tissue in the lung, a finding that suggests that the lung can locally

produce RA-related autoimmunity, and that this locally generated autoimmunity may directly contribute to lung injury (23). Of note, while identification of autoantibodies to citrullinated Hsp90 in the serum of these patients implies that this is directly related to systemic autoimmunity, it is still possible that the lungs of these patients initially generated these autoantibodies as a result of local immune responses, and that over time, the autoantibodies became systemic because of antigen–antibody responses in regional lung lymphatic channels (24).

Importantly, the strong association of ACPA elevations in RA with smoking and the shared epitope (25), the demonstration of increased citrullination in the lungs of smokers (26), the association between smoking and RA-associated ILD (27), and the potential role of environmental factors influencing increases in Hsp90 all suggest that a combination of genetic and environmental factors plays a crucial role in the development of autoimmunity to citrullinated Hsp90 and RA-associated ILD. Notably, Hsp90 α has been found to be elevated in the serum of patients with smoking-related chronic obstructive lung disease (28). Moreover, patients with the chronic inflammatory lung disease cystic fibrosis can develop an inflammatory arthritis (29); in such patients, high levels of antibody to Hsp90 (not known if citrullinated) have been demonstrated in association with more severe lung disease and higher rates of colonization with *Pseudomonas* species, and it has been hypothesized that infection was related to the development of these anti-Hsp90 antibodies (30).

As such, it is possible that in the appropriate genetic setting, an environmental factor such as smoking, or an alteration of the pulmonary microbiome, leads to increases in citrullinated Hsp90 in the lungs of certain RA patients, and this leads to immune responses to Hsp90 and, potentially, subsequent lung inflammation and injury and the development of RA-associated ILD. For this reason, it will be important to learn whether there is a differential increase in citrullinated Hsp90 in the lungs of RA patients with ILD in comparison with RA patients without ILD, and to evaluate the genetic and environmental factors that may be associated with increased levels of Hsp90.

While much recent focus has been on citrullinated proteins and RA, RF has also been demonstrated to be a risk factor for RA-associated ILD, and importantly, perhaps a more powerful risk factor for RA-associated ILD than ACPAs (31). RF is also present in a majority of patients with RA and elevated ACPA levels, although the specific relationship of RF to auto-antibodies to citrullinated Hsp90 is unknown. Because of these associations, what role might RF play in the relationship between autoantibodies to citrullinated Hsp90 and RA-associated ILD? It is possible that RF potentiates immune injury related to autoantibodies to citrullinated Hsp90, much like it is thought that additional immune factors are necessary for the autoantibody anti-Jo-1 to trigger lung inflammation in myositis-related ILD (15). This will also need to be examined in future studies.

Finally, there are emerging data suggesting that lung disease may be present in patients prior to the development of the clinically apparent joint disease that characterizes RA. In particular, several studies have demonstrated elevations in RA-related autoantibodies and development of, predominantly, airway disease (but occasionally severe ILD) in patients

who were initially without clinically apparent inflammatory arthritis, some of whom later developed RA (32–34).

As such, evaluation of autoantibodies to citrullinated Hsp90 (and perhaps increases in the levels of Hsp90 in the lungs) in patients at various time points in the natural history of RA development, including the phase of disease development during which there are elevations of RA-related autoantibodies in the absence of clinically apparent articular disease, and in early RA in the absence of symptomatic lung disease, would potentially shed light on the pathogenic role of autoimmunity to citrullinated Hsp90 in RA-associated ILD. In particular, if emergence of autoantibodies to citrullinated Hsp90 preceded the development RA-associated ILD, that finding would support the notion that these autoantibodies have a role in the initiation and/or propagation of lung disease. Furthermore, given that a large proportion of patients with RA have some degree of lung disease, even though most do not have severe disease (35), understanding the relationship between autoantibodies to citrullinated Hsp90 and various forms and severity of lung disease in RA is imperative to understanding both the clinical and pathogenic relevance of autoimmunity to citrullinated Hsp90 in RA-associated ILD.

In summary, the RA field benefits from the accumulating knowledge that autoimmunity to citrullinated proteins plays a crucial role in the development of this autoimmune disease. Importantly, it is clear that the lung is an immunologically active organ that is both an important site of autoantigen presentation and a target for autoimmune disease. The current study by Harlow and colleagues (6) creatively and prudently used these issues as a foundation for launching a biomarker investigation and for characterizing a new biomarker that can be used to improve our understanding of RA-associated ILD. Future studies can similarly focus on the process of citrullination as a “hub” for autoantibody discovery in RA-related diseases and for expanding the above-described efforts to use new information to increase our understanding of disease etiology, phenotypes, progression, and treatments. The call for better clarification of CTD-associated ILD conditions is well met by this effort and beckons further work in this arena.

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