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Malondialdehyde-Acetaldehyde Adducts and Antibody Responses in Rheumatoid Arthritis-Interstitial Lung Disease

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Abstract

Objective: Compare serum anti-malondialdehyde acetaldehyde (MAA) antibodies and MAA expression in lung tissues of patients with rheumatoid arthritis (RA)-associated interstitial lung disease (ILD) with controls.

Methods: Anti-MAA antibody (IgA, IgM, IgG) concentrations were measured in validated RA-ILD cases and compared to RA patients with chronic obstructive pulmonary disease (COPD) and RA patients without lung disease. Associations of anti-MAA antibody with RA-ILD was assessed using multivariable logistic regression. Lung tissues from patients with RA-ILD, other ILD, emphysema, and controls (n=3/group) were stained for MAA, citrulline, macrophages (CD68), T cells (CD3), B cells (CD19/CD27), and extracellular matrix proteins (type-II collagen, fibronectin, vimentin). Tissue expression and co-localization with MAA was quantified and compared.

Results: Among 1823 RA patients, 90 had prevalent RA-ILD. Serum IgA and IgM anti-MAA antibody concentrations were higher in RA-ILD than RA+COPD or RA alone (p=0.005). After adjusting for covariates, the highest quartiles of IgA (OR 2.09; 95% CI 1.11-3.90) and IgM (OR

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2.23; 95% CI 1.19-4.15) anti-MAA antibody were significantly associated with the presence of RA-ILD. MAA expression was greater in RA-ILD lung tissues than all other groups ($p<0.001$) and co-localized with citrulline ($r=0.79$), CD19+ B cells ($r=0.78$), and extracellular matrix proteins (type-II collagen [$r=0.72$] and vimentin [$r=0.77$]) to the greatest degree in RA-ILD.

Conclusion: Serum IgA and IgM anti-MAA antibody is associated with ILD among RA patients. MAA is highly expressed in lung tissues in RA-ILD where it co-localizes with other RA autoantigens, autoreactive B cells, and extracellular matrix proteins, underscoring its potential role in RA-ILD pathogenesis.

Keywords

rheumatoid arthritis; interstitial lung disease; biomarkers; pathogenesis

INTRODUCTION

Interstitial lung disease (ILD) is a major determinant of poor long-term outcomes in patients with rheumatoid arthritis (RA), who already suffer from premature mortality. Median survival following RA-ILD diagnosis has been reported to be as short as 3 years(1), and trends in mortality related to RA-ILD do not appear to be declining(2). The estimated prevalence of clinically apparent ILD is 5-15% in RA patients with up to 30% having subclinical disease on high-resolution computed tomography(1-4). Contributing to the wide-ranging epidemiologic estimates is the difficulty in establishing the diagnosis of RA-ILD, which relies on a multidisciplinary evaluation that often includes pulmonary function testing, high-resolution computed tomography (HRCT) of the chest, and/or lung biopsy(5, 6). With a poorly understood pathogenesis and the development of clinical symptoms well after radiologic or physiologic abnormalities have established(4, 7), delays in diagnosis of RA-ILD are commonplace. These delays in detection may be particularly harmful if substantial irreversible decline occurs before effective management or other preventative strategies are initiated.

Recognizing the diagnostic uncertainties and associated diagnostic delays, there have been efforts to identify biomarkers capable of accurately identifying patients with, or at risk of developing, RA-ILD. Candidate biomarkers have included Krebs von den Lungen-6, matrix metalloproteinase-7 (MMP-7), interferon-γ-inducible protein-10 (IP-10), pulmonary and activation-regulating chemokine (PARC), surfactant protein D (SpD), antibody to citrullinated-heat shock protein-90 (cit-hsp-90), and a *MUC5B* promoter variant(8-12). While these have shown promise and have provided important insight into putative pathways driving disease, the availability of these measures has yet to be translated into clinical practice. Of the biomarkers reported to date, some appear to lack specificity for RA-ILD, while others have been subject to limited testing in RA patients with other lung disease (such as chronic obstructive pulmonary disease [COPD]) or have not been applied more broadly to large RA patient populations. Thus, there exists a need for ongoing identification and characterization of biomarkers for RA-ILD(13).

The pathophysiology of RA-ILD encompasses multiple complex, interrelated processes - inflammation, autoimmunity, fibrosis, and oxidative stress(6, 14). Malondialdehyde-

acetaldehyde (MAA) adducts are highly immunogenic products of oxidative stress with the potential to facilitate tolerance loss in the absence of adjuvant(15). Antibody responses to MAA have been described by our group in RA patients and are associated with both anti-citrullinated protein antibody (ACPA) responses and disease activity(16). Additionally, MAA co-localizes with citrulline and immune cells in RA synovium. Moreover, both MAA and anti-MAA antibody expression are enriched in RA synovial tissues(16, 17). Beyond its potential contributions to articular disease, MAA has been demonstrated to stimulate inflammation and fibrosis in airway epithelial cells in animal models and *in vitro*(18, 19). Recognizing the pro-inflammatory and pro-fibrotic properties of MAA and our observations of increased anti-MAA antibody responses in RA, we hypothesized that MAA expression and anti-MAA antibody concentrations would be increased in RA-ILD. We tested this hypothesis by comparing circulating anti-MAA antibody concentrations in patients with RA-ILD to other RA patients, including those with other chronic lung conditions. Additionally, we examined MAA expression in lung tissues from RA-ILD, other ILD (non-RA ILD), emphysema, and normal tissues, assessing co-localization with other RA autoantigens as well as immune cells that have been consistently implicated in RA pathogenesis.

Methods

Study Population and Samples

Serum analyses were conducted among participants within the Veterans Affairs Rheumatoid Arthritis (VARA) registry(20). The VARA registry is a multi-center prospective observational study of US Veterans with RA fulfilling the 1987 American College of Rheumatology (ACR) criteria(21) that includes patients from 13 sites. Participants provided informed consent prior to enrollment, all sites obtained local institutional review board approval, and this study obtained approval from the VARA Scientific Ethics and Advisory Committee. At enrollment, participants' demographics, smoking status, education, disease onset, medications, and comorbidities were recorded. At enrollment and follow-up visits, ACR core measures including the multidimensional Health Assessment Questionnaire (MDHAQ)(22), 28-joint tender and swollen joint counts, patient and provider global assessments were collected, acute phase reactants were measured, and composite disease activity measures were scored (e.g. 28-joint Disease Activity Score [DAS28])(23).

Lung tissues were obtained from the National Heart, Lung, and Blood Institute Lung Tissue Research Consortium (https://ltrcpublic.com/). Samples (n=3/group) were obtained following a standard protocol from individuals with RA-ILD, ILD (non-RA; non-specific interstitial pneumonia [n=2] and idiopathic pulmonary fibrosis [n=1]), emphysema (pathologic diagnosis), and controls who underwent transplant procedures, lung volume reduction surgery, or biopsies. The latter control samples were typically collected during evaluation of suspected malignancy and had normal surrounding tissues.

Characterization of lung disease in VARA

International Classification of Diseases (ICD), 9th and 10th revision, codes (ICD-9: 515, 516.3, 516.8, 516.9, 714.8; ICD-10: M05.1, J84.1, J84.9, J99.0) were used for initial ILD

case finding within the VARA registry(2, 24-26). Inpatient and outpatient visit diagnoses in the Corporate Data Warehouse were queried within the VA informatics and computing infrastructure (VINCI)(27). Medical record review was performed within the Compensation and Pension Record Interchange (CAPRI) for all participants with ≥2 outpatient or ≥1 inpatient diagnostic codes for ILD. Diagnoses by provider specialty (pulmonologist, rheumatologist, and other physician), imaging findings (CT and chest x-ray), lung pathology, pulmonary function test (PFT) results, and corresponding dates were abstracted. Participants were classified as RA-ILD if they had a pulmonologist diagnosis and imaging findings of ILD or if they had a non-pulmonologist physician diagnosis plus two of the following: CT or chest x-ray findings interpreted by the reading radiologist as ILD, pathology from a lung biopsy consistent with ILD, or interpretation of PFTs as restrictive by the reading pulmonologist. COPD (clinical diagnoses of chronic bronchitis and emphysema) diagnoses were extracted from medical records and recorded in the VARA registry by treating rheumatologists at the time of VARA enrollment. Patients were categorized into one of three mutually exclusive groups: 1) RA-ILD (with or without comorbid COPD), 2) those with COPD in the absence of ILD, 3) neither RA-ILD nor COPD. Recognizing that pathophysiologic processes, radiologic and physiologic abnormalities, and clinical symptoms precede a formal diagnosis of ILD (resulting in diagnostic delays), a two-year span following VARA enrollment (time of serum collection) was used for classifying prevalent ILD (4, 7). We excluded those with indeterminate ILD (physician diagnosis, CT evidence, or biopsy findings but not fulfilling the aforementioned algorithm) (Supplemental Figure 1).

Measurement of serum and tissue analytes

Anti-MAA antibodies (IgA, IgM, and IgG isotypes) were measured by ELISA in VARA participants using banked serum from enrollment, and reported in relative units (RU) as previously described(16). We categorized anti-MAA antibody values into quartiles to assess trends over the range of values as well as dichotomizing the anti-MAA antibody isotypes into high vs. low concentrations, with the upper three quartiles being considered high (approximating the frequency of other RA-related autoantibodies including both anti-cyclic citrullinated peptide antibody [anti-CCP] and rheumatoid factor [RF]). Anti-CCP antibodies were measured using a second generation ELISA while RF was measured by nephelometry(28).

Lung tissues were stained for MAA using an in-house MAA-specific rabbit polyclonal antibody that was labeled with a Zenon 405 reporter (Molecular Probes, Eugene, OR) and citrullinated proteins using a citrulline-specific mouse IgM monoclonal antibody, clone F95 (Millipore, Temecula, CA). A Cy™3-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgM, μ chain specific (Jackson Immuno Research, West Grove, PA) was used as the detection antibody for the F95. Immune cell types (macrophages, T cells, and B cells) were stained using antibodies to CD68 (polyclonal ALEXA FLUOR 594), CD3 (polyclonal ALEXA FLUOR 647), CD19 (polyclonal ALEXA FLUOR 647), and CD27 (polyclonal ALEXA FLUOR 594) (Bioss, Woburn, MA). Tissues were incubated with isotype controls using a rabbit IgG conjugated to ALEXA FLUOR 594 or 647 (Bioss). Based on prior analyses of paired lung and synovial tissues(29), we also stained for extra-cellular matrix

proteins: type II collagen (polyclonal ALEXA FLUOR 488), vimentin (polyclonal ALEXA FLUOR 647), and fibronectin (polyclonal ALEXA FLUOR 555) (Bioss). Tissues were imaged using a confocal laser scanning microscope and staining was quantified using pixel densities, as in prior studies(16, 17).

Statistical analyses

Baseline characteristics were compared between those with RA-ILD, RA+COPD, and RA alone using chi-square or ANOVA. Anti-MAA antibodies were compared between groups using Kruskal Wallis test with Dunn's post-hoc including a Bonferroni correction. Two multivariable logistic regression models assessed the association between anti-MAA antibody and RA-ILD status (combining RA+COPD with RA alone as the comparator group because there were not significant differences in anti-MAA antibody concentration between these groups in unadjusted comparisons) with covariates being specified *a priori*. The first (model A) adjusted for known patient characteristics associated with RA-ILD: age, sex, race, and smoking status. The second (model B) included covariates from model A in addition to RA-specific factors reported to be associated with ILD: anti-CCP antibody positivity and disease activity (DAS28)(1, 30-32). Anti-MAA antibody isotypes were tested in separate models because of collinearity. Missing data were handled by complete-case analysis with complete data available for >98% of participants.

Tissue staining of MAA, citrulline, immune cells, and extracellular matrix proteins was compared between RA-ILD, other ILD, emphysema, and normal tissue controls via ANOVA with a post-hoc Tukey's to account for multiple comparisons. Co-localization of MAA with immune cells and extracellular matrix proteins was determined using the Fiji plugin, Coloc 2 in Image J, as previously reported(17). To confirm the validity of this approach, we also measured co-localization between MAA and citrulline using Zen blue software (Zeiss, Thornwood, NY) in normal and RA-ILD tissues. Pearson correlations were compared across groups using ANOVA. Results were consistent between both approaches (r Coloc2: normal=0.12, RA-ILD=0.79, p<0.001; r Zen blue: normal=0.19, RA-ILD=0.72, p<0.001). Thus, the remainder of co-localization analyses were completed using Coloc 2 in Image J. P-values <0.05 were considered statistically significant. Analyses were completed using Stata v15.0 (StataCorp, College Station, TX).

RESULTS

Study cohort derivation and characteristics

Of 2695 patients in the VARA registry, 1885 had anti-MAA antibody measurements from a prior study (measured on the entire cohort at that time(16)). Diagnostic code screening and subsequent chart review confirmed 90 prevalent ILD cases; an additional 63 participants were excluded because of indeterminate ILD status (Supplementary Figure 1). Baseline characteristics of the eligible participants (n=1823) in the VARA registry stratified by lung disease status are shown in Table 1. Those with RA-ILD were older, more often male, have at least a high school education, seropositive, and to have received biologic DMARDs or prednisone. Methotrexate use was less frequent in those with RA-ILD. RA patients with

COPD were less likely to be Caucasian, to have a high-school education, and were more likely to be current smokers.

Characteristics of RA-ILD cases are shown in Supplementary Table 1. The vast majority of cases were confirmed based on a pulmonologist diagnosis (97.8%) and CT evidence (94.4%). Restrictive PFTs were present in 60.0% and biopsy confirmation was present for 13.3%. ILD was present for a mean of 2.3 years prior to enrollment and attributed to RA in 93.3% of cases. ILD pattern was reported for only 38.9% of cases, with usual interstitial pneumonia being the most common pattern.

Serum anti-MAA antibody and RA-ILD

Median serum concentrations of IgA and IgM anti-MAA antibody were higher among those with RA-ILD than RA alone (Table 2; all p-values<0.05). Additionally, median serum concentrations of IgM anti-MAA antibody were also significantly higher in RA-ILD patients (median 3582 RU) than patients with RA+COPD (median 2332 RU; p=0.01). IgG anti-MAA antibody was not significantly different between RA-ILD, RA+COPD, and RA alone (p=0.09).

After multivariable adjustment for patient characteristics and RA-related factors, higher quartiles of IgA and IgM anti-MAA antibody remained significantly associated with RA-ILD (Table 3). Notably, inclusion of anti-CCP antibody positivity and DAS28 in multivariable models had minimal impact on the associations between anti-MAA antibody and RA-ILD. High values of IgA anti-MAA antibody, defined by the upper three quartiles, were associated with a more than 2-fold higher odds of RA-ILD (OR 2.09; 95% CI 1.11-3.90 in fully adjusted model) in the absence of a dose-dependent relationship across quartiles (p for trend=0.07). As with IgA isotypes, higher values of IgM anti-MAA antibody were also significantly associated with RA-ILD (OR 2.23; 95% CI 1.19-4.15 in fully adjusted model) but demonstrated a dose-dependent relationship between anti-MAA antibody quartiles and prevalent ILD (p for trend=0.004). The highest two quartiles of IgG anti-MAA antibody trended towards being associated with RA-ILD, though this did not reach statistical significance (p=0.15 and 0.17). We assessed all three isotypes together by categorizing individuals according to the number of positive anti-MAA antibody isotypes. Individuals with 3 positive isotypes had 2.5-fold higher odds of RA-ILD than those with 0-1 positive isotype (OR 2.56; 95% CI 1.29-5.09).

Lung Tissue Patient Characteristics

Mean (SD) age of participants with tissue samples was 56.4 (11.7) years with 75.0% being female. A smoking history was present overall in 66.7% of patients (100% of other ILD and emphysema, 33.3% of normal and RA-ILD). Mean (SD) pack-years of smoking history was 17.5 (14.3). Anti-CCP antibodies and IgM RF were positive in two of three RA-ILD patients. Anti-CCP antibodies, but not IgM RF, were additionally detected in one of three other ILD patients.

MAA and Citrulline Expression in Lung Tissue

MAA expression was highest in RA-ILD lung tissues (Figure 1A and 1B, p<0.001 vs all other groups). Citrulline was also higher in RA-ILD lung tissues (Figure 1C and 1D) relative to normal and other ILD lung tissues (p<0.001), but not significantly different than emphysematous lung tissue (p=0.91). Expression of both MAA and citrulline was highly co-localized in RA-ILD lung tissue (Figure 1E and 1F; r=0.79), significantly higher than in lung tissues from other patient groups (p<0.001 vs normal [r=0.12] and other ILD [r=0.38], p=0.002 vs emphysema [r=0.47]).

Co-localization of MAA and Citrulline with Immune Cells in Lung Tissue

Staining for CD68+ macrophages and CD3+ T cells was higher in all diseased tissues relative to normal lung tissue (Figure 2A; all p<0.01). Macrophage staining was higher in other ILD than in RA-ILD and emphysema (p<0.05). In contrast, CD19+ and CD27+ (memory) B cells were more abundant in RA-ILD lung tissues than tissues from all other groups (p≤0.02). There was minimal to moderate co-localization between MAA and macrophages or T cells (r values 0.12 to 0.54), with no significant differences between lung tissue types (Figure 2B; all p>0.10). In contrast, we observed strong co-localization of MAA with CD19+ B cells, with the highest correlation identified in RA-ILD (r=0.78; p≤0.02 vs all other lung tissues). Co-localization of MAA with CD27+ B cells was more modest (r vales 0.02 to 0.30), with other ILD yielding the highest correlation (r=0.30; p≤0.004 vs RA-ILD and normal, p=0.06 vs emphysema).

Citrulline co-localized with CD68+ macrophages to a greater degree in RA-ILD (Figure 2C; p=0.04) and emphysema (p<0.001) than in normal lung tissue. There was minimal co-localization of citrulline with T cells (r vales 0.07 to 0.18). There was moderate co-localization of citrulline with CD19+ B cells in both RA-ILD (r=0.53) and other ILD (r=0.44) that exceeded the degree of co-localization observed for emphysema and normal tissues (p<0.01). Co-localization of citrulline with CD27+ (memory) B cells was highly prevalent in diseased lung tissue (all p<0.001 vs normal) but not different between specific types of diseased lung tissues (all p>0.29).

Co-localization of MAA with Extracellular Matrix Proteins

Staining for type II collagen was higher in RA-ILD and other ILD than normal lung tissues (Figure 3A; p≤0.002). However, co-localization of MAA with type II collagen was greater in RA-ILD (r=0.72) compared with other lung tissues (Figure 3B; r=0.12-0.49; all p≤0.02). Fibronectin staining was higher in both RA-ILD and emphysema relative to normal lung tissues (p≤0.03) with only weak co-localization of MAA and fibronectin in RA-ILD (r=0.21). Vimentin staining was higher in all diseased lung tissues compared to normal lung tissue (all p≤0.03), although co-localization of MAA and vimentin was higher in RA-ILD than other ILD (p<0.001) without significant differences compared to other lung tissues (all p≥0.09).

DISCUSSION

ILD complicates the disease course for 5-15% of RA patients(1-4), resulting in potentially devastating complications of functional decline and premature mortality. Enhancing the identification of RA-ILD is an important area of translational research in RA, with serum biomarkers emerging as candidates to fulfill this need. For the first time, we investigated serum anti-MAA antibody as a potential biomarker of RA-ILD and characterized the expression of MAA in lung tissues from RA-ILD patients. We found that IgA and IgM anti-MAA antibody concentrations were higher in RA-ILD patients than in other RA patients, including those with other forms of chronic lung disease (IgM only). In parallel studies, we found MAA adduct expression to be higher in RA-ILD lung tissues than in other chronic lung diseases including other ILD. Importantly, MAA adducts demonstrated marked co-localization with citrulline, CD19+ B cells, and type II collagen that was preferential to RA-ILD lung tissues. This study is among the first to characterize a biomarker for RA-ILD that has leveraged a comparator population incorporating RA patients with other chronic lung diseases that may be overrepresented in RA(33). Together, our findings suggest that MAA modified proteins and resulting immune responses may serve as useful biomarkers for RA-ILD and that MAA modified proteins may contribute to the pathogenesis of RA-ILD.

Serum biomarkers have been increasingly investigated for their potential role in identifying RA-ILD. Protein candidates have included widely used biomarkers in RA (anti-CCP antibody and RF)(30, 32, 34), novel autoantibodies (cit-hsp-90)(11), cytokines/chemokines (MMP-7, IP-10, PARC)(9, 10), and surfactant protein D(10). Oxidative stress represents a potentially relevant biologic pathway that has not been harnessed in prior biomarker studies of RA-ILD. Oxidative stress, a disruption of the balance of free radicals and antioxidants, is believed to be intimately involved with the development of diffuse lung diseases because of the continuous exposure to oxygen, high surface area, and robust blood supply in the lungs. MAA, which is generated from lipid peroxidation during oxidative stress, has the potential to link multiple pathways implicated in RA-ILD pathogenesis - oxidative stress, autoimmunity, inflammation, and fibrosis. MAA induces tolerance loss(15), elicits robust adaptive immune responses (anti-MAA antibody), and upregulates pro-inflammatory and pro-fibrotic pathways(18, 19). Our study importantly begins to characterize lung tissue expression of MAA in different lung disease states as well as serum anti-MAA antibody responses in RA patients with and without lung diseases. Confirming our hypothesis, MAA expression in lung tissue and serum anti-MAA antibody concentrations were highest in RA-ILD patients.

Although we found over 2-fold higher odds of ILD among RA patients with serum IgA or IgM anti-MAA antibody concentrations in the upper three quartiles, it is important to note that these antibodies are not specific for RA-ILD. Anti-MAA antibodies are present in RA patients in the absence of chronic lung disease, as well as other disease states(35). However, specificity of a of a candidate biomarker of RA-ILD may be less important than initial case finding, given that HRCT and PFT are ultimately needed to confirm the presence and subtype of ILD (which influences prognosis). Translating these novel findings of anti-MAA antibody in RA-ILD into clinical practice will require additional work. As several other serum biomarkers have shown promise for identifying RA-ILD, biomarker panels that

include anti-MAA antibody and other analytes are likely to outperform models based on a single analyte. To date, the measurement of anti-MAA antibody has leveraged the use of adducted albumin as the plating antigen, a protein that has no known pathogenic role in RA. Identification of the precise antigenic targets of anti-MAA antibody is likely to allow for improved assay performance in identifying RA patients with ILD. Finally, our current results assessed the ability of anti-MAA antibody to identify established RA-ILD. Future study will need to assess the value of anti-MAA antibody for predicting future RA-ILD risk. This could be of even greater value than identifying prevalent RA-ILD, as it may identify patients with earlier disease that might be more amenable to therapeutic and/or preventative interventions (36), though data specifically in RA-ILD is lacking.

Paralleling serum findings, staining for MAA adducted antigens was highest in lung tissues from RA-ILD patients. Importantly, this occurred preferentially in RA-ILD lung tissue, with significantly higher staining than in other ILD and emphysema. In contrast to MAA, citrulline was expressed in both RA-ILD and emphysema. Although the specificity of serum anti-CCP antibodies for RA approaches 96%(37), others have similarly found citrulline and ACPA responses to accompany chronic obstructive lung diseases in the absence of RA(38-40). Given the strong co-localization of MAA with citrullinated antigens in RA-ILD, we postulate that MAA could act as a “second hit” in RA pathogenesis by facilitating tolerance loss to co-localized citrullinated antigens. Although further testing will be needed to address this hypothesis, the co-localization of CD19+ B cells with MAA and citrulline would support the concept that these post-translational changes (both of which likely result from injurious stimuli) conspire in autoantibody generation. This is further supported by preliminary work in animal models suggesting immunization with co-modified (MAA +citrulline) albumin leads to greater ACPA responses than citrullinated-albumin alone(41). Finally, vimentin is an extracellular matrix protein that has previously been shown to be a shared target of citrullination/ACPAs in the synovium and lung (29). While we did not find vimentin expression to be increased in RA-ILD compared to other lung conditions, we observed marked co-localization of MAA with vimentin in RA-ILD lung tissues, co-localization that was significantly more robust than that seen with other ILD.

Our group has previously characterized anti-MAA antibodies in sera from RA and other rheumatic and musculoskeletal disease patients(16, 35). Circulating anti-MAA antibody concentrations are higher in RA patients than those with osteoarthritis, are associated with serum ACPAs, and are enriched within RA synovium(16, 17). As we found in RA-ILD lung tissues assessed in this study, MAA and citrulline co-localized in RA synovium(17). Also paralleling the RA-ILD lung findings from the present study, prior work by our group has shown that MAA and citrulline both co-localize with B cells in the synovium. However, there are differences in B cells implicated by site. In the synovium, MAA and citrulline co-localized most strongly with CD27+ memory B cells(17). In the lung tissues from RA-ILD patients, MAA co-localized most strongly with CD19+ B cells, but not with CD27+ memory B cells. While future work will be needed to elucidate the temporal evolution of immune responses to MAA, it is intriguing that immature B cells are associated most strongly with MAA adduct expression in the lung given the emerging evidence that the lungs may be a site of immune tolerance breakdown contributing to the early development of RA(42).

There are limitations to this study. The male predominance, Veteran status, and lower prevalence of biologic use may affect generalizability. Collection of ILD data was obtained retrospectively and not all data were available within the medical records. This may underestimate the cross-sectional prevalence of ILD in the cohort (4.7%). However, misclassification of ILD cases as non-ILD would bias our results towards the null. Distinguishing between clinical and sub-clinical ILD cannot be definitive based on retrospective classification. By confirming physician diagnoses in the medical records, rather than relying on diagnostic codes or diagnostic testing alone, we believe the majority of ILD cases were clinically evident. Given the low frequency with which ILD pattern (UIP vs. NSIP vs. other) was specified, we were not able to compare anti-MAA concentrations by RA-ILD pattern. Likewise, anti-MAA antibody measurements were not available for all registry participants, which may also have reduced study power. Again, this should not have introduced bias, as antibody measurements were performed on the entire cohort at the time of the prior study without any relation to ILD status. Reflecting the prevalence of seropositivity for RF and anti-CCP antibody, we dichotomized anti-MAA antibody as being in the upper three quartiles. Only increasing IgM anti-MAA antibody quartiles were more strongly associated with the presence of ILD. Further work will be needed to determine clinically important cut-offs for these antibodies. Sample sizes were limited for lung tissue studies, with lung tissues obtained from three individuals with each lung condition, prohibiting multivariable analyses. One of the non-RA ILD patients had detectable ACPAs but was not classified as RA. Given the cross-sectional nature of the study, it is unknown if that patient later developed RA. This potential misclassification of RA-ILD as non-RA ILD would only bias our results towards the null. Lung tissue samples were not matched, so there may be unmeasured confounding.

There are important strengths to this study. We performed detailed review of the medical records to validate ILD diagnoses in RA patients from a well characterized registry that includes robust data including many relevant covariates(20). We evaluated not only serologic anti-MAA antibody concentrations, but also investigated tissue expression of MAA and its co-localization with citrulline, immune cells, and extracellular matrix proteins that have been consistently implicated in disease pathogenesis. Finally, we characterized MAA and anti-MAA immune responses in RA-ILD by using comparators that were free of lung disease in addition to comparators with other chronic lung diseases.

In conclusion, we found higher levels of serum IgA and IgM anti-MAA antibody to be associated with RA-ILD in a large cohort of U.S. Veterans with RA. Lung tissue expression of MAA is similarly higher in RA-ILD lung tissue where it co-localizes with citrulline, CD19+ B cells, and extracellular matrix proteins. These findings suggest that MAA immune responses could play an important role in the pathogenesis of RA-ILD and anti-MAA antibodies may be promising serum biomarkers in the identification of this extra-articular disease manifestation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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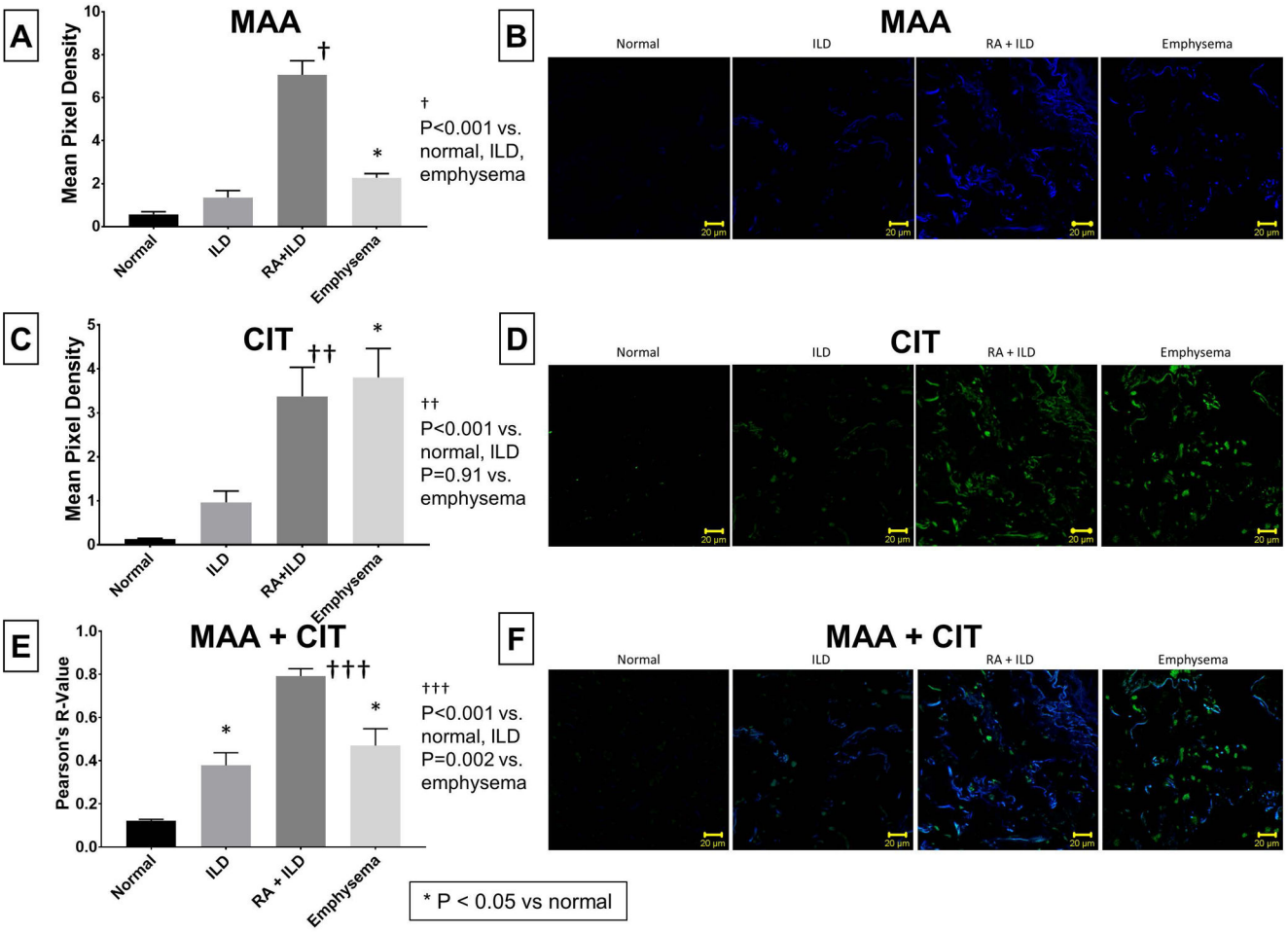

Figure 1. Lung tissue expression of MAA, citrulline, and their co-localization in RA-ILD and other lung diseases.

Expression of malondialdehyde-acetaldehyde adducts (MAA) measured by pixel density (Figure 1A) and representative immunohistochemistry staining of lung tissues for MAA (Figure 1B). Tissue expression (Figure 1C) and immunohistochemistry staining of lung tissues for citrulline (Figure 1D) are also shown. The co-localization of MAA and citrulline was quantified through a correlation coefficient of their staining (Figure 1E) and overlapping immunohistochemistry staining are shown (Figure 1F).

Abbreviations: MAA, malondialdehyde-acetaldehyde adducts; CIT, citrulline; RA-ILD; rheumatoid arthritis interstitial lung disease; ILD, interstitial lung disease.

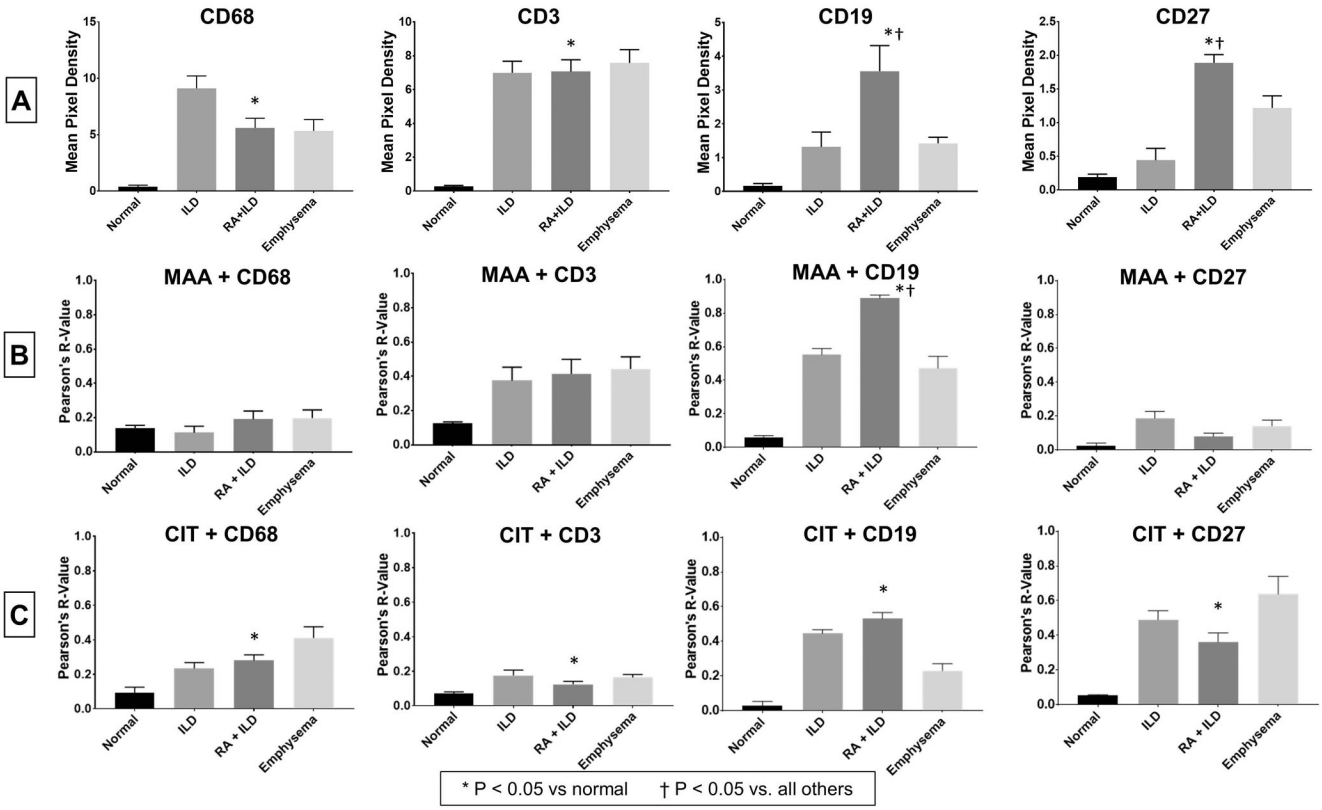

Figure 2. Co-localization MAA with citrulline with immune cells in lung tissue from RA-ILD and other lung diseases.

Figure 2A. Tissue staining for macrophage (CD68), T cells (CD3), and B cells (CD19 and CD27) for RA-ILD, other ILD, emphysema, and healthy control lung tissues. Figure 2B. Co-localization of MAA with macrophage, T cells, and B cells in different lung tissues. Figure 2C. Co-localization of citrulline with macrophage, T cells, and B cells in different lung tissues.

Abbreviations: MAA, malondialdehyde-acetaldehyde adducts; CIT, citrulline; RA-ILD; rheumatoid arthritis interstitial lung disease; ILD, interstitial lung disease.

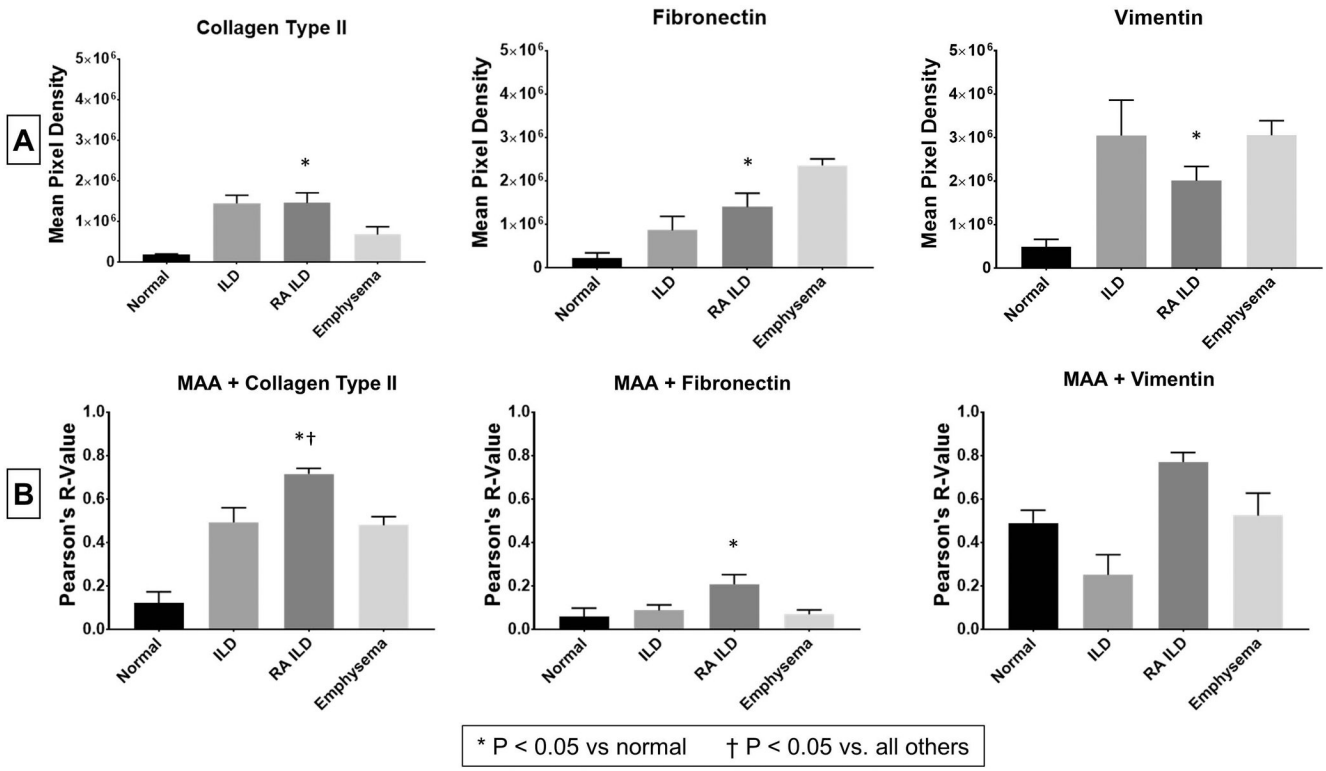

Figure 3. Co-localization of MAA with extracellular matrix proteins in lung tissue from RA-ILD and other lung diseases.

Figure 3A. Tissue staining for extracellular matrix proteins (type II collagen, fibronectin, and vimentin) in RA-ILD, other ILD, emphysema, and healthy control lung tissues. Figure 3B, co-localization of MAA with extracellular matrix proteins in different lung tissues. Abbreviations: MAA, malondialdehyde-acetaldehyde adducts; CIT, citrulline; RA-ILD; rheumatoid arthritis interstitial lung disease; ILD, interstitial lung disease.

Table 1.

Baseline characteristics of Veterans Affairs Rheumatoid Arthritis participants by lung disease status.

	Overall (n=1823)	RA-ILD (n=90)	RA + COPD (n=294)	RA alone (n=1439)	p-value
Age, years	63.5 (11.0)	67.0 (9.9)	65.8 (9.7)	62.8 (11.3)	<0.001
Male sex	90.1	95.6	92.5	89.2	0.05
Caucasian	76.7	76.7	83.7	76.2	0.02
HS education	86.4	91.7	78.9	87.5	<0.001
Smoking status					<0.001
Current	26.1	27.8	31.0	25.1	
Former	53.4	58.9	58.8	52.0	
Never	20.4	13.3	10.2	23.0	
BMI, kg/m^2	28.4 (5.7)	27.8 (5.1)	28.3 (6.1)	28.4 (5.7)	0.67
RDCI score	1.9 (1.5)	3.2 (1.6)	3.9 (1.1)	1.4 (1.2)	<0.001
RA duration	11.1 (11.5)	13.3 (13.1)	11.1 (11.9)	10.9 (11.3)	0.17
SE positive	68.8	65.6	73.0	68.2	0.22
Anti-CCP positive	77.3	86.7	80.3	76.0	0.03
RF positive	79.8	92.2	80.6	78.9	0.009
MDHAQ	0.9 (0.6)	0.9 (0.5)	1.1 (0.6)	0.9 (0.6)	0.004
DAS28	4.0 (1.6)	4.1 (1.4)	4.4 (1.5)	3.9 (1.6)	0.003
Methotrexate	51.9	21.0	47.6	54.7	<0.001
Biologic	22.9	30.0	16.3	23.8	0.005
Prednisone	43.5	63.0	43.1	42.4	0.01

p-values test of group differences by ANOVA or chi-square tests

Abbreviations: RA, rheumatoid arthritis; ILD, interstitial lung disease; COPD, chronic obstructive pulmonary diseases; HS, high-school; BMI, body mass index; RDCI, rheumatic disease comorbidity index; SE, shared epitope; anti-CCP, anti-cyclic-citrullinated peptide antibody; RF, rheumatoid factor; MDHAQ, multidimensional health assessment questionnaire; DAS28, 28-joint disease activity score

Table 2.

Anti-MAA antibody concentrations by lung disease status in rheumatoid arthritis patients.

	RA-ILD (n=90)	RA + COPD (n=294)	RA alone (n=1439)	p-value*
IgA anti-MAA antibody	891 (501, 1624) †	869 (399, 1665) †	689 (323, 1440)	0.005
IgM anti-MAA antibody	3582 (1302, 11141) †±	2332 (888, 5649)	2094 (843,5610)	0.005
IgG anti-MAA antibody	2226 (1353, 3781)	1996 (1039, 3701)	1868 (943, 3415)	0.09

Values represent median (interquartile range) in relative units

* p-value by Kruskal Wallis (unadjusted comparisons)

† p < 0.05 vs RA alone (Dunn's test with Bonferroni correction)

± p < 0.05 vs RA + COPD (Dunn's test with Bonferroni correction)

Abbreviations: RA, rheumatoid arthritis; ILD, interstitial lung disease; COPD, chronic obstructive pulmonary disease; anti-MAA, anti-malondialdehyde acetaldehyde adduct antibodies

Table 3.

Multivariable associations of anti-MAA antibody with RA-ILD

	***Model A*. Age, sex, race, smoking status (n=1820)**		***Model B*. Model A + anti-CCP positivity and DAS28 (n=1792)**	
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**
Quartiles				
IgA anti-MAA				
Quartile 1	Referent	-	Referent	-
Quartile 2	2.27 (1.12, 4.59)	0.02	2.09 (1.03, 4.27)	0.04
Quartile 3	2.20 (1.09, 4.43)	0.03	2.07 (1.02, 4.18)	0.04
Quartile 4	2.26 (1.12, 4.56)	0.02	2.10 (1.04, 4.25)	0.04
p-trend		0.04		0.07
IgM anti-MAA				
Quartile 1	Referent	-	Referent	-
Quartile 2	1.87 (0.91, 3.86)	0.09	1.84 (0.89, 3.81)	0.10
Quartile 3	2.26 (1.11, 4.60)	0.03	2.08 (1.02, 4.27)	0.05
Quartile 4	2.93 (1.49, 5.78)	0.002	2.73 (1.38, 5.41)	0.004
p-trend		0.001		0.004
IgG anti-MAA				
Quartile 1	Referent	-	Referent	-
Quartile 2	1.34 (0.69, 2.61)	0.39	1.33 (0.68, 2.59)	0.41
Quartile 3	1.73 (0.91, 3.27)	0.09	1.61 (0.84, 3.06)	0.15
Quartile 4	1.67 (0.88, 3.18)	0.12	1.58 (0.83, 3.02)	0.17
p-trend		0.09		0.14
Antibody positive				
IgA anti-MAA	2.24 (1.20, 4.18)	0.01	2.09 (1.11, 3.90)	0.02
IgM anti-MAA	2.35 (1.26, 4.38)	0.007	2.23 (1.19, 4.15)	0.01
IgG anti-MAA	1.58 (0.91, 2.75)	0.11	1.50 (0.86, 2.63)	0.15

*Antibodies tested in separate logistic regression models

Abbreviations: MAA, malondialdehyde-acetaldehyde adducts; RA, rheumatoid arthritis; ILD, interstitial lung disease; anti-CCP, anti-cyclic-citrullinated peptide antibody; DAS28, 28-joint disease activity score; OR, odds ratio; CI, confidence interval