

## Original article

## Enrichment of malondialdehyde–acetaldehyde antibody in the rheumatoid arthritis joint

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## Abstract

**Objective.** To characterize the expression of malondialdehyde–acetaldehyde (MAA) adducts and anti-MAA antibody in articular tissues and serum of patients with RA.

**Methods.** Paired sera and SF were examined from 29 RA and 13 OA patients. Anti-MAA antibody, RF, ACPA and total immunoglobulin were quantified. SF-serum measures were compared within and between disease groups. The presence and co-localization of MAA, citrulline and select leukocyte antigens in RA and OA synovial tissues were examined using immunohistochemistry.

**Results.** Circulating and SF anti-MAA antibody concentrations were higher in RA vs OA by 1.5- to 5-fold. IgG ( $P < 0.001$ ), IgM ( $P = 0.006$ ) and IgA ( $P = 0.036$ ) anti-MAA antibodies were higher in paired RA SF than serum, differences not observed for total immunoglobulin, RF or ACPA. In RA synovial tissues, co-localization of MAA with citrulline and CD19<sup>+</sup> or CD27<sup>+</sup> B cells was demonstrated and was much higher in magnitude than MAA or citrulline co-localization with T cells, monocytes, macrophages or dendritic cells ( $P < 0.01$ ).

**Conclusion.** Anti-MAA antibodies are present in higher concentrations in the RA joint compared with sera, a finding not observed for other disease-related autoantibodies. Co-localization of MAA and citrulline with mature B cells, coupled with the local enrichment of anti-MAA immune responses, implicates MAA-adduct formation in local autoantibody production.

**Key words:** rheumatoid arthritis, anti-citrullinated protein antibody (ACPA), malondialdehyde–acetaldehyde adducts, oxidative stress, autoimmunity, synovial fluid.

## Rheumatology key messages

- Antibody to malondialdehyde–acetaldehyde adducts is enriched in RA synovial fluid.
- Co-localization with citrullinated protein and mature B cells suggests malondialdehyde–acetaldehyde adducts contribute to RA pathogenesis.

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## Introduction

Tissue exposure to elevated levels of reactive oxygen species leads to lipid peroxidation and cell damage, resulting in the formation of several well-characterized protein adducts. Chief among these is malondialdehyde (MDA), a ubiquitous product that is pro-inflammatory and implicated in several systemic inflammatory diseases [1–4]. MDA spontaneously degrades into acetaldehyde (AA) [5]. MDA and AA are both highly reactive aldehydes, which subsequently react to produce malondialdehyde-AA (MAA) adducts targeted primarily to lysine residues. While MDA and AA are unstable, MAA adducts are composed of extremely stable ring structures and are highly immunogenic [6–9].

Importantly, MAA protein adducts promote robust inflammatory responses [10], eliciting anti-MAA antibody formation in addition to the expression of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-6 and IFN- $\gamma$  [2, 11]. Moreover, MAA adducts act as potent haptens, even in the absence of exogenous adjuvant, and have been shown in animal models to promote loss of tolerance to native carrier proteins following immunization [7, 12].

Our group recently demonstrated that MAA adducts are expressed in inflamed synovial tissues in RA, and that these protein modifications were not detectable in synovial tissues from patients with OA [13]. Moreover, MAA adducts were co-localized with both citrullinated proteins and CD45<sup>+</sup> cells in inflamed RA synovium. We also found that circulating anti-MAA antibody concentrations were higher in RA patients than in age- and gender-matched healthy controls and these concentrations were positively associated with several measures of disease severity and/or activity. Targeting epitopes distinct from those bound by ACPA, we identified robust associations of circulating anti-MAA antibody with ACPA as well as RF even after accounting for group differences in other factors. Taken together, this preliminary work suggests that MAA adduct formation could play a role in the generation of neo-epitopes in RA and subsequent tolerance loss and disease progression that characterizes the condition.

Although MAA adducts are expressed in RA synovium and co-localize with citrullinated protein, prior work used only a non-specific CD45 leucocyte marker. Thus, it is unknown whether MAA modification occurs in the context of B cells, possibly acting as a driver of local B cell activation and autoantibody production, or whether this co-localization occurs in the context of other cell types altogether. In this study, we examined paired SF and serum samples from RA and OA patients. We hypothesized that anti-MAA antibody responses would be higher in RA patients than OA patients regardless of sample type. More importantly, we hypothesized that among RA patients anti-MAA antibody concentrations would be greater in SF than in simultaneously collected sera. With prior work demonstrating the co-localization of MAA adducts and citrullinated epitopes with leucocytes, we also hypothesized that the leucocytes would be composed primarily of mature CD27<sup>+</sup> B cells that have been speculated to drive synovial immunoglobulin production [14]. Taken

together, these results would further elucidate the potential pathogenic role of MAA modifications in RA.

## Methods

### Study subjects

Paired SF and serum samples from RA patients, drawn on the same day, were obtained from the institutional review board-approved National Data Bank for Rheumatic Diseases (NDB) Biorepository. The NDB Biorepository is housed at the University of Nebraska Medical Center with University of Nebraska Medical Center institutional review board approval covering both storage and use of samples. SF samples were obtained from knee arthrocentesis performed as part of routine rheumatological care. Patients provided written consent prior to sample acquisition. RA patients satisfied the ACR 1987 classification criteria for RA [15]. Paired SF and serum samples from OA patients from the NDB Biorepository, also collected on the same day as part of routine rheumatology care, were used for comparisons.

### Anti-MAA antibodies

Paired SF and sera from RA and OA patients were tested for the presence of IgM, IgG and IgA antibodies to the MAA epitope, as previously described [13]. Briefly, aqueous human albumin (Talecris Biotherapeutics, Research Triangle Park, NC, USA) was modified with 1 mM of AA and 2 mM of MDA (Sigma-Aldrich, St Louis, MO, USA) to form the 1,4-dihydropyridine albumin adduct. Native albumin and MAA-modified albumin were coated on 96-well ELISA plates at a concentration of 2  $\mu$ g/well and incubated overnight at 4 °C. We have previously shown that these two antigens coat the wells equally using limiting dilutions and an anti-albumin antibody for detection [16]. This was again confirmed prior to these experiments. To create a standard curve, known concentrations of human IgM, IgG or IgA were also coated and incubated overnight. Plates were washed with PBS-tween using a 405 TS Microplate washer (BioTek Instruments, Winooski, VT, USA), blocked with 2% bovine serum albumin, and incubated with patient SF or sera for 1 h. Reactivity to anti-MAA antibodies was determined using secondary horseradish peroxidase-conjugated goat anti-human antibody specific for IgM Fc5 $\mu$  fragment specific, IgG (Fc $\gamma$ -specific) or IgA ( $\alpha$ -chain-specific) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and developed using tetramethylbenzidine substrate. Absorbance was determined at 450 nm using an Epoch Plate reader (BioTek) and analysed using Gene 5 Software (BioTek). Data are expressed in arbitrary units (AU) of MAA antibody relative to the standard curve. We have previously demonstrated that MAA and citrullinated epitopes represent distinct antibody targets, finding no evidence of cross-reactivity between the anti-MAA and anti-CCP antibody assays used in the present study [13].

### RA-specific autoantibodies

RF and ACPA concentrations were determined on paired SF and sera samples from RA patients. RF (positivity

>15 IU/ml) was measured in house using a BNII Nephelometer Siemens Healthcare Diagnostics Inc (Newark, DE, USA). ACPAs were measured using: a second-generation anti-CCP antibody ELISA (positivity >5 U/ml) (Diastat; Axis-Shield Diagnostics, Dundee, UK); and a bead-based multiplex antigen array [17, 18]. Using the BioPlex platform, serum and SF samples were evaluated for 38 individual analytes including 30 antigen-specific ACPAs and eight unmodified or native peptides. To assess global ACPA responses, we utilized an ACPA score, defined as the sum of normalized fluorescence divided by the total number of analytes examined, in addition to the number of ACPA analytes found in high concentration (defined as >2 s.d. above the mean observed in RA patient sera) [13].

#### Total immunoglobulins and CRP

Total immunoglobulin (IgA, IgM and IgG, mg/dl) concentrations were determined in SF and sera as per manufacturer protocol (Ready-SET-Go! Kit, Affymetrix eBioscience, Santa Clara, CA, USA). Absorbance was determined at 450 nm using an Epoch Plate reader (BioTek) and analysed using Gene 5 Software (BioTek). High sensitivity CRP was measured on banked sera using nephelometry (Siemens).

#### Confocal microscopy

To elucidate the specific immune cells involved in MAA-citrulline-leucocyte co-localization, formalin-fixed paraffin-embedded synovial tissues from three RA and three OA patients were examined using immunohistochemical staining for the presence of both MAA and citrullinated proteins, stains that demonstrate negligible reactivity in OA [13]. Paraffin embedded sections were rehydrated and subjected to antigen retrieval techniques [13]. Sections were blocked and incubated with an IgG rabbit polyclonal antibody to MAA [19] and an IgM mouse mAb to protein-bound citrulline [20]. Leucocyte markers were also examined for (i) B cells using an IgG1 mouse mAb to human CD19 (Dako, Carpinteria, CA, USA) and an IgG rabbit polyclonal antibody to Alexa Fluor 594-conjugated CD27/TNFRSF7 (Bioss, Woburn, MA, USA); (ii) T cells using an IgG rabbit polyclonal antibody to Alexa Fluor 647-conjugated CD3 (Bioss); (iii) monocytes/macrophages/dendritic cells using an IgG rabbit polyclonal antibody to Alexa Fluor 594-conjugated CD14 (Bioss); and (iv) an IgG rabbit polyclonal antibody to Alexa Fluor 647-conjugated CD137 (Bioss), expressed on activated T cells, dendritic cells, natural killer cells and granulocytes but not B cells. Primary antibodies that were not directly labelled were detected using Cy2 Fab fragment goat anti-rabbit IgG (H&L, Jackson ImmunoResearch), Cy3 Fab2 fragment goat anti-mouse IgM  $\mu$ -chain specific, and Cy5 rabbit anti-mouse IgG Fc fragment-specific secondary antibodies (Jackson ImmunoResearch). Rabbit IgG, mouse IgG kappa and mouse IgM (Sigma-Aldrich) were used as isotype controls. Recognizing that Cy2 goat anti-rabbit secondary antibody could bind directly labelled rabbit anti-CD3, -CD14, -CD27 and -CD137 antibodies, resulting in a false-positive

staining, additional studies were carried out to examine this possibility. A checkerboard analysis was set up wherein each of the individual labelled rabbit antibodies was incubated with the Cy2 goat anti-rabbit secondary antibody, and evaluated at each of the three wavelengths for staining. Sections were mounted in fluoromount-G, and subjected to confocal microscopy using a Zeiss 710 Meta confocal laser-scanning microscope. Images were analysed using Zen 2012 software (Zeiss) and quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

#### Statistical analysis

Results are expressed as means (s.d.). RA vs OA comparisons were made using Fisher's exact test for categorical variables and Student's t-test or the Wilcoxon rank sum test as appropriate for continuous variables. Correlations were examined using Spearman's rank coefficients. SF and sera concentrations for individual tests were examined in RA patients using a Wilcoxon signed-rank test with the exception that concentrations of individual ACPA analytes were compared using significance analysis of microarrays [18, 21]. In sensitivity analyses, anti-MAA (IgA, IgM, IgG), RF and anti-CCP antibody were normalized by calculating the proportion of total immunoglobulin that each of these constituted separately in SF and serum. We then compared these values by compartment (SF vs sera for each measure) using the same approach as detailed above. Proportions examined included IgA anti-MAA/total IgA immunoglobulin; IgM anti-MAA/total IgM; IgG anti-MAA/total IgG; anti-CCP/total IgG; and RF/total IgM.

These analyses, with the exception of significance analysis of microarrays, were performed using Stata v14 (Stata Corp., College Station, TX, USA).

In confocal studies, the Fiji plugin, Coloc 2 in ImageJ was used to quantify the magnitude of co-localization of MAA or citrulline with different cell types using five regions of interest from each image. Pearson's correlation coefficient was calculated based on the overlap of two colors to generate an R-value. R-values from the five regions of interest were averaged for images from three patients and compared using a one-way analysis of variance with Tukey's *post hoc* test to account for multiple comparisons. Samples from OA patients and those stained with isotype controls demonstrated very little if any reactivity [13].

## Results

Paired SF and sera were available from 29 RA and 13 OA patients. Group comparisons demonstrated that anti-MAA antibody concentrations were universally higher in SF from RA patients vs OA patients, differences that achieved significance for both IgM and IgG anti-MAA ( $P \leq 0.001$ ) but not for IgA ( $P = 0.124$ ). Likely owing to limited sample sizes available and significant variability in these measures in RA samples, RA-OA differences did not achieve significance in sera ( $P = 0.095$  IgA,  $P = 0.161$  IgM and  $P = 0.695$  IgG) (Table 1). Anti-CCP antibody, RF, ACPA score and the number of ACPA analytes observed in high concentration were significantly higher in RA than OA patients regardless of sample type ( $P < 0.05$  for all)

**TABLE 1** Demographics, disease characteristics and biomarker values for RA and OA patients

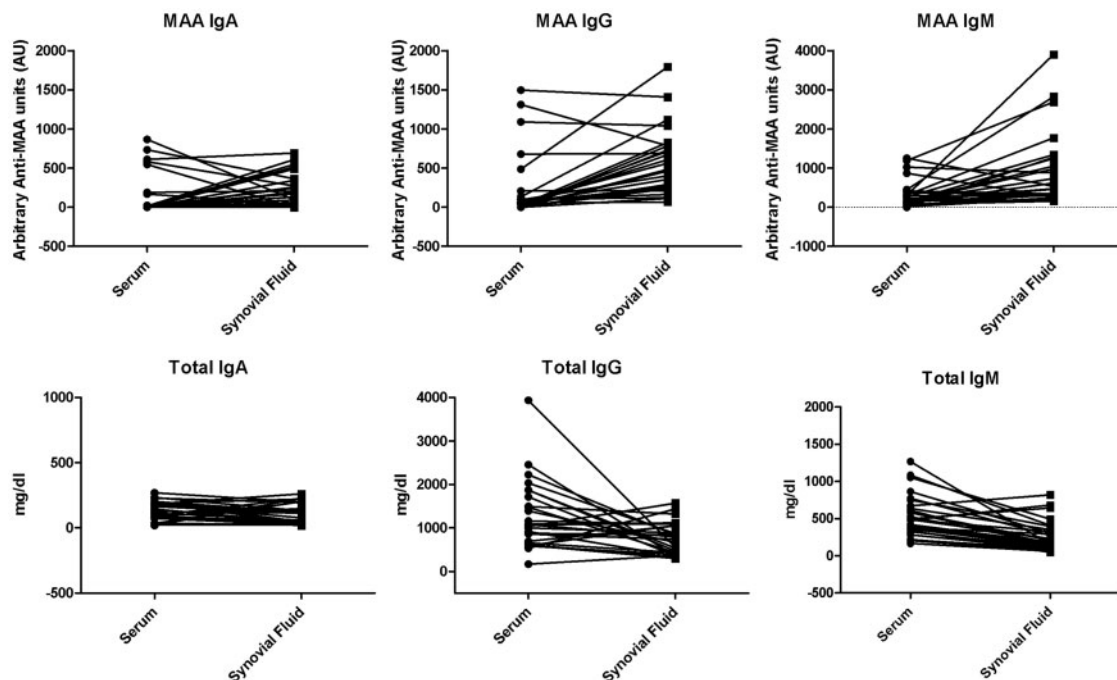
Variables	RA (n = 29)	OA (n = 13)	P-value
Demographics and disease characteristics			
Age, years	61 (13)	69 (8)	0.03 <sup>a</sup>
Male, %	45	38	0.75
Caucasian	93	92	1.00
Disease duration, years	14 (8)	11 (8)	0.39 <sup>a</sup>
Pain (0–10)	5.7 (2.4)	5.9 (2.6)	0.84 <sup>a</sup>
HAQ (0–3)	1.4 (0.6)	0.6 (0.4)	<0.001 <sup>a</sup>
Fatigue (0–10)	4.8 (2.9)	4.0 (2.9)	0.44 <sup>a</sup>
ESR, mm/h	43 (26)	8 (6)	<0.001
Nodules, %	34	—	—
MTX, %	62	—	—
Prednisone, %	52	—	—
Serum biomarkers			
IgG anti-MAA, AU/ml	219 (408)	62 (72)	0.695
IgM anti-MAA, AU/ml	464 (621)	226 (248)	0.161
IgA anti-MAA, AU/ml	129 (259)	82 (80)	0.095
Total IgG immunoglobulin, mg/dl	1235 (781)	958 (612)	0.295
Total IgM immunoglobulin, mg/dl	529 (51)	309 (216)	0.009
Total IgA immunoglobulin, mg/dl	128 (71)	61 (54)	0.003
Anti-CCP antibody, U/ml	225 (259)	1.2 (0.3)	<0.001
Anti-CCP positive, %	86	0	<0.001
RF, IU/ml	920 (1900)	10.5 (0.0)	<0.001
RF positive, %	83	0	<0.001
ACPA score	57 (41)	11 (3)	<0.001
Number of ACPA in high concentration	3.2 (3.6)	0.2 (0.6)	<0.001
SF biomarkers			
IgG anti-MAA, AU/ml	663 (651)	142 (193)	<0.001
IgM anti-MAA, AU/ml	1148 (1768)	380 (717)	0.001
IgA anti-MAA, AU/ml	193 (205)	81 (98)	0.124
Total IgG immunoglobulin, mg/dl	953 (934)	925 (1498)	0.146
Total IgM immunoglobulin, mg/dl	255 (197)	103 (86)	0.001
Total IgA immunoglobulin, mg/dl	104 (77)	20 (9)	<0.001
Anti-CCP antibody, U/ml	123 (102)	1.0 (0.3)	<0.001
RF, IU/ml	52 (72)	10.5 (0.0)	0.012
ACPA score	54 (47)	10 (2)	<0.001
Number of ACPA in high concentration	1.1 (1.7)	0.0 (0.0)	0.003

All values presented as means (s.d.) unless otherwise stated. All continuous comparisons in Table 1 are the Wilcoxon rank-sum test except as noted. All categorical comparisons are Fisher's exact test. <sup>a</sup>Student's t-test.

(Table 1). Pooling data from all 42 patients, SF anti-MAA antibody concentrations were correlated with circulating CRP ( $r=0.351-0.400$ ,  $P<0.05$ ) (data not shown). Correlations of both SF and serum anti-MAA antibody isotypes are shown separately for RA and OA in supplementary Table S1, available at *Rheumatology* Online. Among RA patients, correlations between SF and serum values for each individual anti-MAA antibody isotype were low to modest in magnitude with  $r$  values of 0.257 ( $P=0.179$ ) for IgA, 0.374 ( $P=0.046$ ) for IgG and 0.295 ( $P=0.121$ ) for IgM.

Paired SF and sera from RA and OA patients were then compared for anti-MAA antibody concentrations. Among OA patients, mean anti-MAA antibody concentrations were slightly higher in SF than serum for the IgG isotype [142 (193) vs 62 (72) AU/ml;  $P=0.05$ ]. There were no significant differences between SF and serum values measured

in OA patients for either the IgM [380 (717) vs 226 (248) AU/ml;  $P=0.701$ ] or IgA [81 (98) vs 82 (80) AU/ml] isotype (Table 1). In contrast, significantly higher concentrations of IgG anti-MAA [663 (651) vs 219 (408) AU/ml,  $P<0.001$ ], IgM anti-MAA [1148 (1768) vs 464 (621) AU/ml,  $P=0.006$ ] and IgA anti-MAA [193 (205) vs 129 (259) AU/ml,  $P=0.036$ ] antibodies were present in the SF as compared with sera in those with RA (Fig. 1). To determine whether the relative increases in SF concentration were due to the select presence of anti-MAA antibody, or simply reflected an increase in total antibody, total immunoglobulin levels were compared. In contrast to anti-MAA antibody, concentrations of total immunoglobulin were significantly lower in SF than serum in RA cases for IgG [953 (934) vs 1235 (781) mg/dl,  $P=0.041$ ], IgM [255 (197) vs 529 (51) mg/dl,  $P<0.001$ ] and IgA [104 (77) vs 128 (71) mg/dl,  $P=0.039$ ] (Fig. 1). Likewise, and again in contrast to the observation

**Fig. 1** Antibody to malondialdehyde-acetaldehyde in RA SF and serum

Concentrations of anti-MAA antibody (upper panels) and total immunoglobulin (lower panels) isotypes from paired SF and sera from 29 patients with RA. SF concentrations were higher than paired serum values for all anti-MAA antibody isotypes [mean (s.d.); P-value]: IgA anti-MAA [193 (205) vs 129 (259) AU/ml;  $P = 0.036$ ], IgG anti-MAA [663 (651) vs 219 (408) AU/ml;  $P < 0.001$ ] and IgM anti-MAA [1148 (1768) vs 464 (621) AU/ml;  $P = 0.006$ ]. In contrast, total immunoglobulin concentrations were lower in SF than paired serum: IgA [104 (77) vs 128 (71) mg/dl;  $P = 0.039$ ], IgG [953 (934) vs 1235 (781) mg/dl;  $P = 0.041$ ] and IgM [255 (197) vs 529 (51) mg/dl;  $P < 0.001$ ].

for anti-MAA antibodies, concentrations of other RA-specific autoantibodies were also lower in SF than serum, including differences for anti-CCP antibody [123 (102) vs 225 (259) U/ml,  $P = 0.001$ ], RF [52 (72) vs 920 (1900) IU/ml,  $P < 0.001$ ] and the number of elevated ACPA analytes [1.1 (1.7) vs 3.2 (3.6),  $P < 0.001$ ] (Fig. 2). There was no significant difference in ACPA scores ( $P = 0.177$ ). SF-serum differences in IgG anti-MAA ( $P = 0.001$ ), IgM anti-MAA ( $P < 0.001$ ), IgA anti-MAA ( $P = 0.012$ ), RF ( $P = 0.003$ ) and anti-CCP antibody ( $P = 0.048$ ) remained consistent following normalization to total immunoglobulin levels and these results did not change when the analysis was limited to the 25 anti-CCP antibody positive RA cases (data not shown). Among the four seronegative RA cases, median concentrations of anti-MAA antibody were higher in SF than serum in this group: IgG (373 AU/ml vs 0 AU/ml), IgM (1122 AU/ml vs 246 AU/ml) and IgA (40 AU/ml vs 0 AU/ml). Of the 30 multiplex ACPA analytes examined in RA patient samples, SF-sera differences were observed for 13 analytes, all of which were found in higher concentration in serum than in SF (supplementary Fig. S1, available at *Rheumatology* Online).

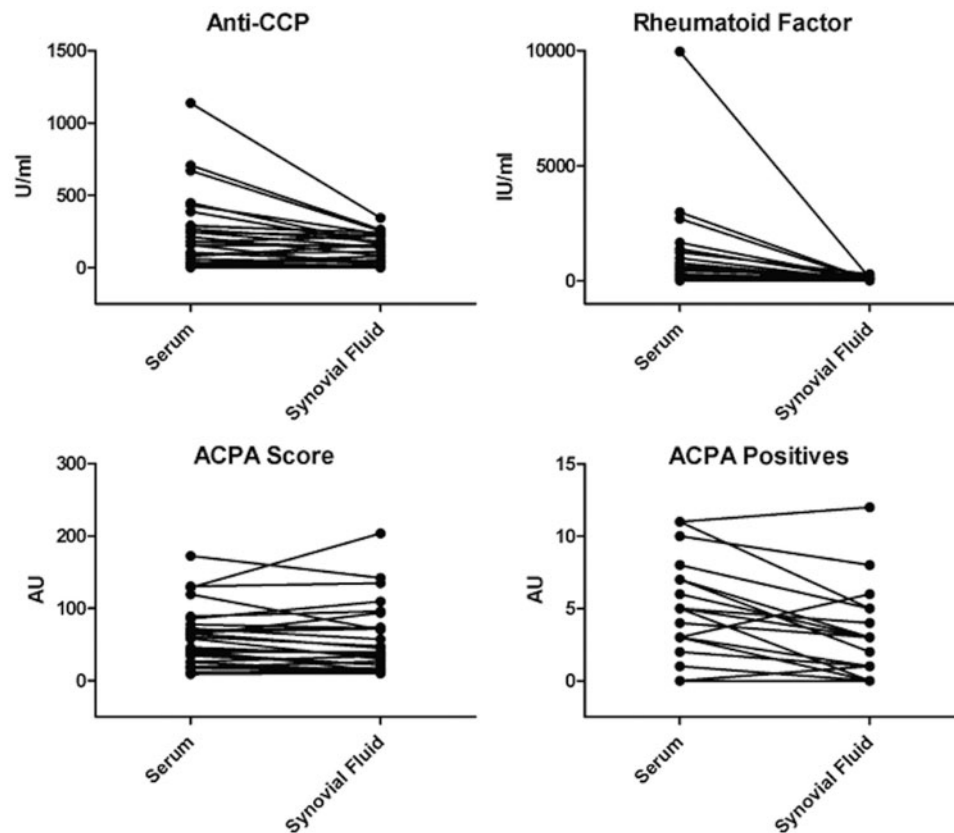
Previous studies in our laboratory have demonstrated co-localization of MAA adducts, citrullinated proteins and CD45 (a non-specific marker expressed on several leucocyte cell types) in RA synovial tissues, findings that were

absent in OA [13]. In this study, we used paraffin-embedded sections from three RA and three OA patients to stain for MAA, citrullinated proteins and markers of other immune cells. In addition to replicating prior results demonstrating co-localization of MAA with citrullinated proteins in RA synovial tissues [13], these analyses showed significantly higher levels of co-localization with CD27<sup>+</sup> B cells than with other cell types as demonstrated in studies using CD137 as a comparator ( $P < 0.01$ ; Fig. 3). Likewise, we observed significantly higher levels of co-localization of MAA and citrullinated proteins with CD19<sup>+</sup> B cells than with T cells (CD3<sup>+</sup>) or monocytes/macrophages/dendritic cells (CD14<sup>+</sup>) ( $P < 0.01$ ; Fig. 4). Reactivities in samples from OA patients and with isotype controls and secondary antibody alone were negligible (supplementary Fig. S2, available at *Rheumatology* Online). In checkerboard analysis, only negligible staining was observed on any of the labelled rabbit antibodies with Cy2 goat anti-rabbit secondary antibody, verifying true co-localization (supplementary Fig. S3, available at *Rheumatology* Online).

## Discussion

These results complement prior studies demonstrating that MAA adducts are expressed in RA synovial tissues

**Fig. 2** Autoantibody concentrations in RA SF and serum



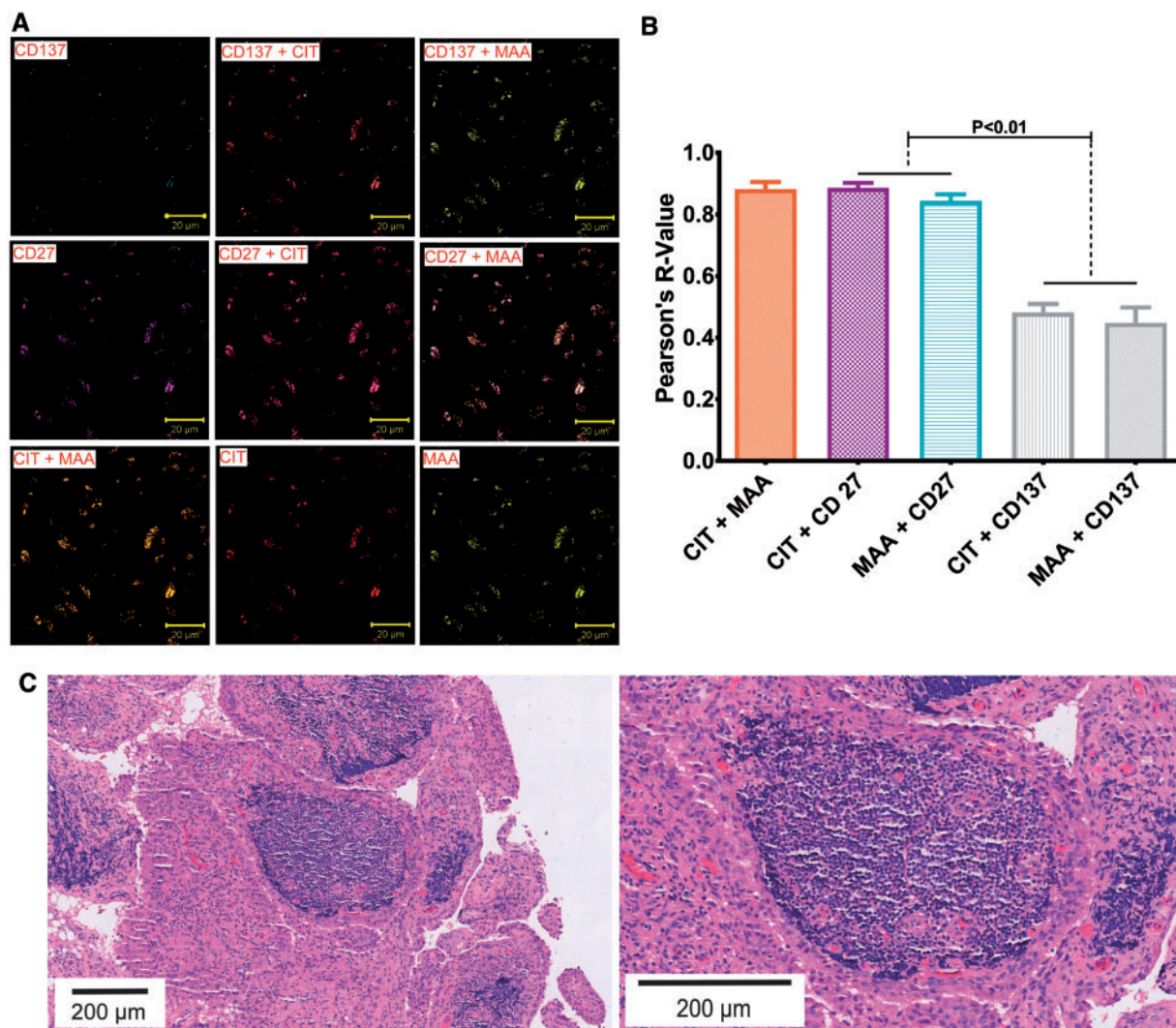
Concentrations of anti-CCP antibody and RF as well as ACPA score and the number of ACPA ( $n=30$  antigen-specific ACPA tested) found in high concentration in paired SF and sera from 29 patients with RA. SF concentrations were significantly lower than paired serum for [mean (s.d.); P-value]: anti-CCP [123 (102) vs 225 (259) U/ml;  $P=0.001$ ], RF [52 (72) vs 920 (1900) IU/ml;  $P < 0.001$ ] and the number of ACPA in high concentration [1.1 (1.7) vs 3.2 (3.6);  $P < 0.001$ ]. There were no differences in ACPA score ( $P=0.177$ ).

where they co-localize with both citrullinated proteins and leucocytes, and that circulating anti-MAA antibody concentrations are higher in RA patients compared with controls and are independently associated with circulating ACPAs [13]. Although not reaching statistical significance, circulating anti-MAA antibody concentrations were ~1.5- to 5-fold higher in RA patients than OA patients. These serum differences, particularly for the IgG isotype, were similar in magnitude to the differences previously observed to exist between RA patients and healthy controls using much larger cohorts [13], suggesting that the lack of significance relates primarily to the smaller sample sizes available for this study and the variability observed in anti-MAA antibody values. In turn, the moderate and statistically significant correlations observed between SF anti-MAA antibody concentrations and circulating CRP values suggests that at least some of this variability relates to underlying disease activity and varying levels of systemic inflammation.

Concentrations of anti-MAA antibody were ~3- to 5-fold higher in SF samples from RA patients vs OA patients,

differences that were highly significant ( $P \leq 0.001$ ) for the IgG and IgM isotypes. Results from the present investigation further demonstrate that anti-MAA antibodies, particularly IgM and IgG isotypes, are present in concentrations that are ~3-fold higher in RA joint fluids compared with simultaneously collected sera. Disproportionate articular expression appears to be unique to anti-MAA antibody, as similar differences were not observed for total immunoglobulin, RF, or multiple ACPAs, all of which were increased in the circulation relative to SF in patients with RA. It is noteworthy that our results relative to ACPA expression, confirmed in our study using two different approaches of ACPA detection, contrast with prior reports showing preferential expression of select ACPAs in RA joints. In particular, Snir and colleagues [22] observed significantly higher concentrations of ACPA in SF compared with paired sera including anti-CCP antibody, in addition to ACPA targeting citrullinated forms of type II collagen, vimentin,  $\alpha$ -enolase and fibrinogen. Differences in these results may reflect methodological variation, including the disparate assays used and different populations studied.

**Fig. 3** Co-localization of malondialdehyde-acetaldehyde, citrulline and CD27<sup>+</sup> B cells in RA synovial tissue

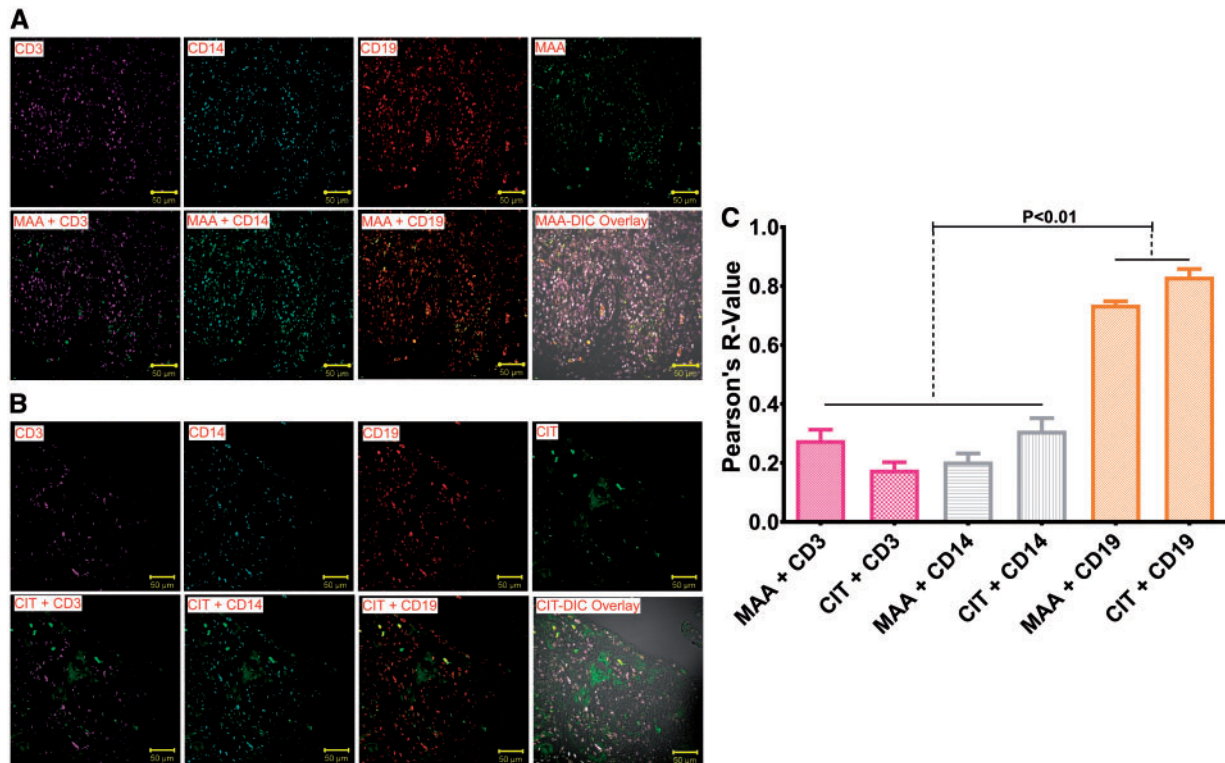


Synovial tissues from three RA patients examined for malondialdehyde-acetaldehyde (MAA), citrulline (CIT), B-cell marker CD27 and/or CD137. Sections subjected to confocal microscopy ( $\times 24$ ). Five regions of interest (ROI) identified per sample. Pearson correlations calculated for marker pairs. R-values from five ROIs averaged ( $n = 3$ ), compared using analysis of variance. **(A)** Row 1: CD137, CD137+CIT, CD137+MAA; Row 2: CD27, CD27+CIT, CD27+MAA; Row 3: CIT+MAA, CIT alone, MAA alone. Scale bars: 20  $\mu\text{m}$ . **(B)** R-values as measure of co-localization; CIT+MAA, CIT+CD27, MAA+CD27 significantly ( $P < 0.01$ ) increased vs CIT or MAA with CD137. **(C)** Haematoxylin and eosin staining of adjacent tissues with follicles, including tissues in **(A)**.

Mechanisms explaining the higher concentrations of anti-MAA antibodies observed in SF relative to serum in RA are unknown. It is possible that anti-MAA antibodies are produced at extra-articular sites and subsequently sequestered in joint tissues. Extravasation through leaky vasculature and subsequent inflammatory cell-mediated sequestration, a mechanism that has been proposed to explain articular targeting of macromolecular prodrugs in RA [23, 24], could be the pathway through which anti-MAA antibodies accumulate in joint tissues. Alternatively, anti-MAA antibodies produced elsewhere

could simply accumulate in the joint as a result of a highly oxidative environment that favours lipid peroxidation and local MAA adduct formation, resulting in antigen-antibody complex formation.

The hypothesis that disease specific autoantibodies are produced locally in the RA joint has been studied for decades. In fact, by tissue biopsy, approximately one-half of RA patients show evidence of synovial ectopic lymphoid structures that contain B cell follicles supporting local germinal cell responses [25, 26]. Results from this study demonstrate the co-localization of MAA adducts with CD19<sup>+</sup>

**Fig. 4** Co-localization of malondialdehyde–acetaldehyde, citrulline and immune cells in RA synovial tissue

Synovial tissues from three RA patients stained for malondialdehyde–acetaldehyde (MAA) with citrulline (CIT), immune cells. Sections subjected to confocal microscopy ( $\times 24$ ). Five regions of interest (ROI) identified per sample; Pearson correlations calculated for paired markers. R-values from the five ROI averaged for patients ( $n = 3$ ), compared using analysis of variance. **(A)** Co-localization of MAA+T cells (CD3), monocytes/macrophages/dendritic cells (CD14)+B cells (CD19). Scale bars: 50  $\mu\text{m}$ . **(B)** Co-localization of CIT+T cells (CD3), monocytes/macrophages/dendritic cells (CD14)+B cells (CD19). Scale bars: 50  $\mu\text{m}$ . **(C)** R-values as measure of co-localization. MAA+CD19 and CIT+CD19 co-localized to a significantly greater magnitude ( $P < 0.01$ ) compared with MAA or CIT co-localization with CD3 or CD14.

and CD27<sup>+</sup> B cells in addition to citrullinated antigens and far less co-localization with other inflammatory cells such as T cells, monocytes/macrophages or dendritic cells. To our knowledge, this is among the first studies to demonstrate co-localization of post-translationally modified proteins implicated in RA with B cells, a finding that adds to a growing body of evidence emphasizing the importance of B cells in RA. These results are consistent with another recent report demonstrating the accumulation of B cells in RA synovial tissues with evidence supporting the active recruitment of CD27<sup>+</sup> B cells to the synovial compartment [27]. Likewise, results from others have suggested that the formation of ectopic lymphoid tissues and the presence of large concentrations of immunoglobulins are attributable to local CD27<sup>+</sup> B cells [14]. It is important to note that tissues used for these studies were taken from patients with well-established and long-standing disease. Whether similar co-localization with CD27<sup>+</sup> cells would be observed earlier in the course of disease, or during the critical pre-clinical period, is a question of significant importance that cannot be addressed with these data.

Other important questions remain to be addressed in light of these findings. Perhaps most importantly, additional investigations will be needed to understand the potential biological relevance of MAA and anti-MAA antibody enrichment in the joint. Based on observations showing that select ACPAs promote osteoclastogenesis [28] and the formation of neutrophil extracellular traps [29], these could represent pathways of interest in future explorations elucidating the downstream effects of anti-MAA antibody production in RA. Likewise, it will be important to clarify whether the co-localization observed occurs on the same peptide/protein, the identity of the antigens that are modified, and how this co-localization impacts the presentation or processing of citrullinated autoantigens or the generation of disease-specific autoimmune responses.

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## Supplementary data

Supplementary data are available at *Rheumatology* Online.

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