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# Autoreactive B cells against malondialdehyde-induced protein cross-links are present in the joint, lung, and bone marrow of rheumatoid arthritis patients

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Autoantibodies to malondialdehyde (MDA) proteins constitute a subset of anti-modified protein autoantibodies in rheumatoid arthritis (RA), which is distinct from citrulline reactivity. Serum anti-MDA IgG levels are commonly elevated in RA and correlate with disease activity, CRP, IL6, and TNF- $\alpha$ . MDA is an oxidation-associated reactive aldehyde that together with acetaldehyde mediates formation of various immunogenic amino acid adducts including linear MDA-lysine, fluorescent malondialdehyde acetaldehyde (MAA)-lysine, and intramolecular cross-linking. We used single-cell cloning, generation of recombinant antibodies ( $n = 356$  from 25 donors), and antigen-screening to investigate the presence of class-switched MDA/MAA+ B cells in RA synovium, bone marrow, and bronchoalveolar lavage. Anti-MDA/MAA+ B cells were found in bone marrow plasma cells of late disease and in the lung of both early disease and risk-individuals and in different B cell subsets (memory, double negative B cells). These were compared with previously identified anti-MDA/MAA from synovial memory and plasma cells. Seven out of eight clones carried somatic hypermutations and all bound MDA/MAA-lysine independently of protein backbone. However, clones with somatic hypermutations targeted MAA cross-linked structures rather than MDA- or MAA-hapten, while the germline-encoded synovial clone instead bound linear MDA-lysine in proteins and peptides. Binding patterns were maintained in germline converted clones. Affinity purification of polyclonal anti-MDA/MAA from patient serum revealed higher proportion of anti-MAA *versus* anti-MDA compared to healthy controls. In conclusion, IgG anti-MDA/MAA show distinct targeting of different molecular structures. Anti-MAA IgG has been shown to promote bone loss and osteoclastogenesis *in vivo* and may contribute to RA pathogenesis.

Rheumatoid arthritis (RA) is a chronic and potentially disabling autoimmune disease that causes inflammation of synovial joints (1). A hallmark of the major seropositive subset is the presence of anti-citrullinated protein autoantibodies (ACPA) targeting proteins with the posttranslational modification (PTM) converting arginine to citrulline. Autoantibodies against other PTMs, that is, carbamylated and acetylated proteins, can also be found in a subset of RA patients but have recently been postulated to primarily reflect the presence of cross-reactive antibodies targeting these protein modifications in addition to citrulline (2–4). However, we and others have shown elevated levels in RA of another nonoverlapping set of PTM-antibodies directed toward proteins containing malondialdehyde (MDA)/malondialdehyde acetaldehyde (MAA) modifications (2, 5–9). Anti-MDA/MAA immunoglobulin G (IgG) are recently gaining more attention in RA due to their association with disease activity and inflammation as well as mechanistic studies suggesting direct pathogenic properties through modulation of osteoclasts (2, 5, 6, 10).

MDA modification is formed by the release of the highly reactive aldehyde MDA during lipid peroxidation under conditions of oxidative stress which is mediating modifications primarily on the  $\epsilon$ -amino group of lysine by a Schiff base reaction (11). Notably, MDA is a major epitope in atherogenic oxidized low-density lipoprotein (LDL) but have also been detected in a range of proteins, including in the RA synovium (5, 6). In combination with acetaldehyde, MDA can form MAA adducts characterized by a fluorescent dihydropyridine (DHP)-lysine ring structure (12–14). Acetaldehyde required in the MAA modification *in vivo* is suggested to have an exogenous source, such as smoking, alcohol, or infection (15–19). Both MDA and MAA adducts have been reported to be commonly targeted by human antibodies (5, 6, 11, 20, 21). However, the MAA type of adducts have been suggested to be especially immunogenic in the context of autoimmunity and a preferential target for disease-associated autoantibodies over MDA-adducts (5, 7–9, 22). Yet, these reactivities can be difficult to

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separate, and free MDA can spontaneously break down to acetaldehyde to mediate formation of MAA (23–25) which proves problematic when investigating antibody reactivity to *in vitro* modified antigens (26–28). In addition, MDA and MAA form intraprotein cross-links that can, besides changing the structure and functionality of proteins, create additional epitopes recognized by autoantibodies (5, 11, 24, 27, 29, 30).

In healthy individuals, anti-MDA/MAA IgM have been suggested to be expressed by innate-like B cells and promote immune homeostasis by mediating clearance of modified proteins and cell debris (31). A high proportion of total IgM at birth constitutes of anti-MDA/MAA IgM and B cells expressing germline-encoded anti-MDA/MAA are present in human cord blood (32–34). However, it has not been clear which B cell subset is responsible for the elevated IgG anti-MDA/MAA during inflammation and autoimmunity. While ACPA have been shown to arise years before disease onset in risk individuals, anti-MDA/MAA autoantibodies seem to increase closer to disease onset which implicates a difference in immune regulation between the two sets of antibodies (2, 7). Moreover, break-of-tolerance toward citrullinated antigens in RA have been hypothesized to occur in the mucosal surface in the lungs, which is supported by a strong epidemiological association with smoking as well as our latest results revealing ACPA+ B cells in the lungs of risk individuals before disease onset (35). Yet, the presence of autoreactive B cells toward other disease-associated antigens such as MDA have not been studied before.

In the current study, we investigated the presence of MDA/MAA reactive B cells from lungs of both individuals at risk of developing RA and newly diagnosed untreated RA patients and from bone marrow (BM)-derived plasma cells of patients with long standing disease. Our findings were compared to previously identified anti-MDA IgG from synovial B cells of established RA patients. By single-cell methodology we generated human mAbs for characterization of their binding specificities. We used biochemical methods to determine the clonal molecular fine specificity of different B cells toward MDA- and MAA-modified linear and cross-linked proteins and peptides.

## Results

### Anti-MDA B cells identified in joint, lung, and BM of RA patients

We have previously identified anti-MDA positive B cells in RA synovial tissue and fluid (5), and now we explored if MDA reactivity is also present in other compartments, that is, the lung and BM in different donors and different B cell subsets (Fig. 1A).

First, we investigated MDA reactivity in B cells in bronchoalveolar lavage (BAL) of ACPA+ individuals at risk of developing RA and ACPA+ newly diagnosed untreated RA patients. By generating recombinant mAbs from single B cells followed by ELISA screening of 98 clones from ten individuals (ACPA+ risk individuals, ACPA+ early RA, and ACPA- early RA), we identified three MDA-reactive clones (Fig. 1B).

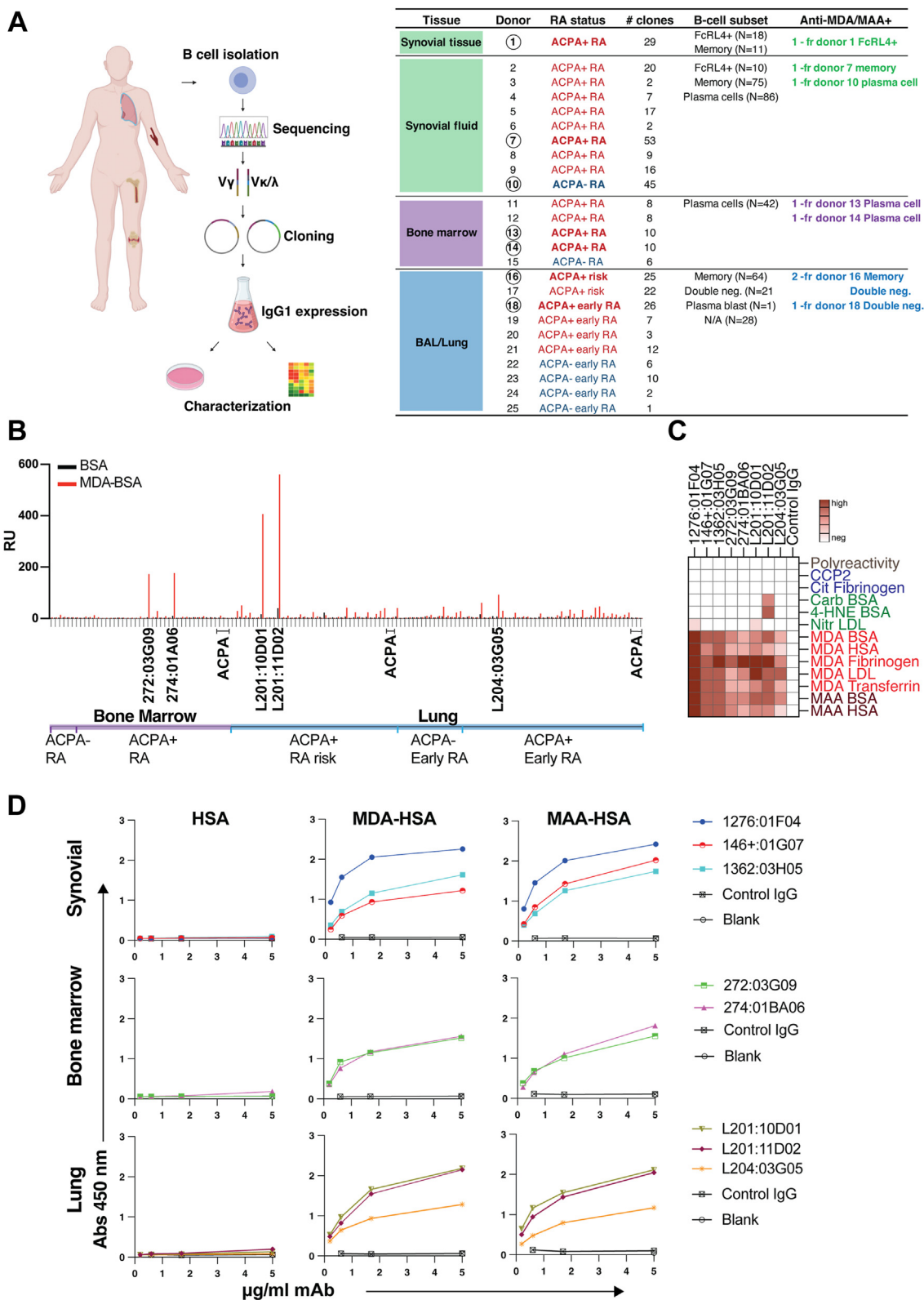
Retrospective analysis of the flow cytometry surface markers of the sorted cells was used to determine the B cells phenotype. Interestingly, two of the MDA+ clones were derived from an ACPA+ individual at risk of developing RA, one MDA-reactive switched memory (CD27<sup>+</sup>IgD<sup>-</sup>) IgG4+ B cell and one MDA-reactive double negative (CD27<sup>-</sup>IgD<sup>-</sup>) IgG1+ B cell. The third lung MDA-reactive clone was derived from an ACPA+ newly diagnosed untreated RA patient and was also coming from a double negative (CD27<sup>-</sup>IgD<sup>-</sup>) IgG1+ B cell.

While the two clones from the risk individual showed signs of T cell help and affinity maturation based on the somatic hypermutations (19 and 28 total SHM), the RA patient derived clone had low number of SHM (five mismatches). Notably, low SHM together with the double negative phenotype of this MDA+ B cell, may suggest that it originates from extra-follicular responses.

Next, we investigated MDA reactivity in BM plasma cells from RA patients with long standing disease by screening 44 generated mAbs from five patients (four ACPA+ and one ACPA-). We identified two anti-MDA IgG1 expressing clones among CD138+ CD19+ plasma cells from two different ACPA+ patients (Fig. 1B). As expected, these anti-MDA+ clones carried high SHMs, consistent with what was seen among BM plasma cells. Notably, the five novel anti-MDA clones could be compared with three previously described RA-patient derived MDA-reactive clones originating from various B cells subsets; a germline-encoded IgG1+ synovial fluid CD27+ memory B cell, a synovial fluid IgG+ plasma cell, and a synovial tissue FcRL4+ IgA+ memory B cell, both carrying SHM (5). Sequence analysis disclosed a variety in gene usage and complementarity-determining region 3 sequences between the clones (Table 1). When analyzing the full repertoire of obtained lung and BM paired sequences from the patients, we found that one of the MDA lung clones (L201:10D01) was present in three copies that all originated from IgG1+ double negative cells, while the other clones did not originate from expanded clonotypes in the patients (Fig. 2).

### The MDA-reactive B cells display broad MDA- and MAA-reactivity

Next, when investigating antibody binding to different oxidation-associated antigens, we found that all the identified clones bound both MDA- and MAA-modified proteins. A range of different MDA-proteins were recognized including albumin, transferrin, and LDL (Fig. 1C). While the clones bound MDA-LDL, no preferential binding was detected for MDA-APO B100, the protein component of LDL particles, over MDA-APO A1 (Fig. S3). Interestingly, the germline-encoded 1276:01F04 clone displayed the strongest binding to all MDA- and MAA-modified protein antigens (Fig. 1, C and D). None of the identified MDA-reactive clones recognized citrullinated or native antigens (Figs. 1C and S1) or MDA/MAA-modified calf thymus DNA (Fig. S2). However, the lung clone, L201:11D02, which originates from an IgG4 memory B cell, displayed cross-reactivity also to 4-hydroxynonenal-modified and carbamylated protein. MDA/MAA and carbamylation



**Figure 1. Identification of RA B cell reactivities to MDA- and MAA-modified proteins.** A, illustration of malondialdehyde (MDA)/malondialdehyde acetaldehyde (MAA)-reactive B cell identification from 356 single-cell derived monoclonal antibodies from 25 donors. B, ELISA screening of recombinant human IgG1 mAbs expressed from single B cells from bone marrow plasma cells (indicated by purple line) and lung B cells (blue line). mAbs were screened at 5 µg/ml for binding to bovine serum albumin (BSA) or MDA-BSA. C, summary of reactivity patterns of eight identified MDA/MAA+ clones. Citrulline reactivity was assessed with CCP2 and citrullinated fibrinogen ELISA, polyreactivity by the soluble membrane protein (SMP) assay, MDA/MAA reactivities and oxidative control reactivities were assessed with ELISA. Heatmap shows mAb reactivity scores from 0 to 5 with red being high binding. D, titration of MDA-reactive mAbs to MDA- and MAA-modified human serum albumin (HSA). 4HNE, 4-hydroxynonenal; Abs 450 nm, absorbance at 450 nm; Carb, carbamylated; CCP2, cyclic citrullinated peptide 2; MDA, malondialdehyde; Nitr, nitrated.

**Table 1**  
Features of MDA-reactive B cells

Clone name	Tissue	Patient	B Cell	Subclass	VH	V $\lambda$ / $\kappa$	$\gamma$ -chain CDR3	$\lambda/\kappa$ -chain CDR3	VH SHM	VL SHM	Total SHM
1362:03H05 (5)	Synovium	ACPA- RA	Plasma cell	IgG1	3-53	KV4-1	ARDRRGWSGYSLRYGMDV	QQYVSTPYT	13	12	25
146+:01G07 (5)	Synovium	ACPA+ RA	Fc $\gamma$ RL4+	IgA	3-33	KVD3-15	ARARRGDGYNQARYYFDY	QQYIKWPPPEYT	1	18	19
1276:01F04 (5)	Synovium	ACPA+ RA	Memory	IgG1	4-39	LVI-51	VRVRGYFDY	GTWDSLSVWV	0	1	1
272:03G09	BM	ACPA+ RA	Plasma cell	IgG1	4-34	KVI-5	ARGLYMLTLRTVNRRTSSPRPVWYFDL	QQHQTYEPT	49	31	80
274:01BA06	BM	ACPA+ RA	Plasma cell	IgG1	3-30-3	LW2-11	AKMTIGEHS	CSYTGYSWV	26	19	45
L201:10D01	BAL/Lung	ACPA+ Risk	Double neg <sup>a</sup>	IgG1	1-3	KVI-27	ARSIFFSGWGYYYGMDV	QKYNASPLT	13	6	19
L201:11D02	BAL/Lung	ACPA+ Risk	Memory <sup>b</sup>	IgG4	3-49	KVI-5	TRIPFASYEYFGMDV	QQHHSFWT	14	14	28
L204:03G05	BAL/Lung	Early ACPA+ RA	Double neg <sup>a</sup>	IgG1	4-59	KVI-5	ARGITMVRGVKPELFDY	QQYNSYPLT	3	2	5

Abbreviation: CDR3, complementarity-determining region 3.

<sup>a</sup> CD27<sup>-</sup> IgD<sup>-</sup>.<sup>b</sup> CD27<sup>+</sup> IgD<sup>+</sup>.

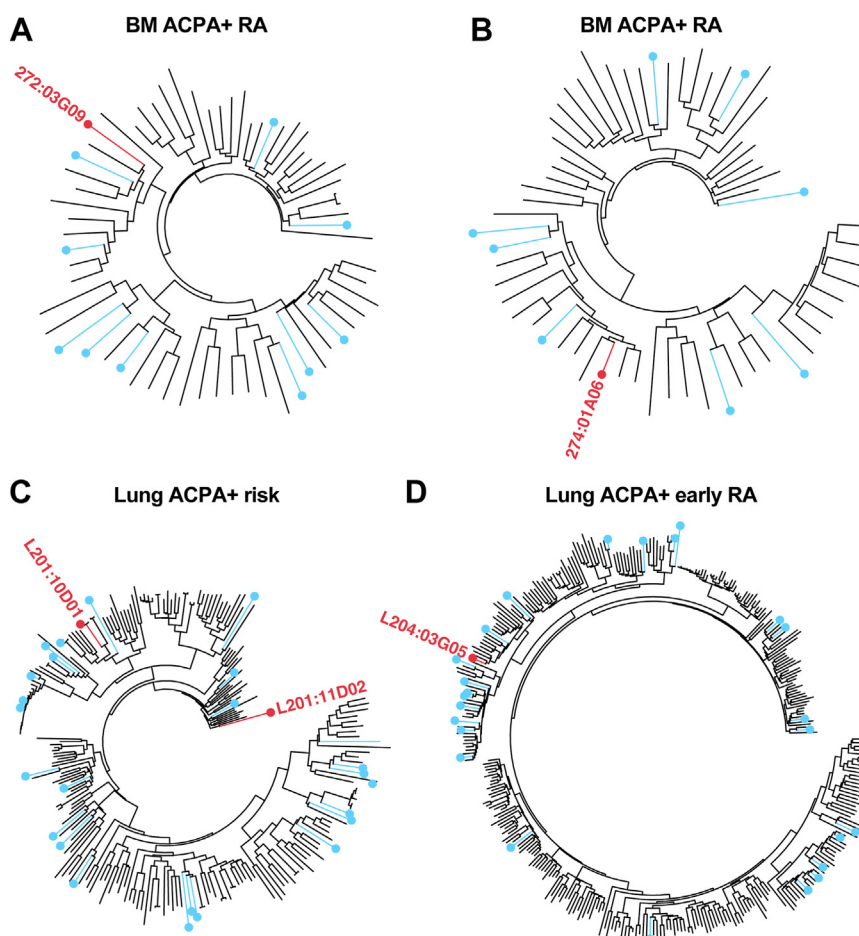
cross-reactivity has previously been reported for an isolated anti-MDA-LDL clone (36), yet, noticeably, only one out of eight clones identified here displayed this wider “polyreactivity profile”.

### The MDA clones display differential binding to different MDA structures

To investigate if binding of the anti-MDA clones differed toward different MDA molecular amino acid structures, we first set up methods to discriminate between different MDA/MAA modifications, including linear monomeric MDA and MAA as well as intramolecular cross-links. For detection, we utilized the fact that MAA-adducts are characterized by the fluorescent DHP-lysine ring structure. We confirmed that MAA-modification also occurred when using a standard MDA protocol by measuring fluorescence of MDA-modified fibrinogen at 399/471 nm as well as by mass spectrometry (MS) analysis. This can be explained by the ability of MDA to spontaneously form MAA-adducts *in vitro* (26). Moreover, using an MAA protocol with addition of acetaldehyde will enhance the formation of MAA/DHP-lysine residues but will also accelerate the generation of MDA-adducts.

We investigated binding to linear MDA/MAA epitopes without cross-linking and MDA-modified RA-relevant peptides from fibrinogen and histone 4 containing modifiable lysine residues. Fluorescence measurements and MS confirmed that both MDA and MAA-modification occurred (Fig. 3 and Table S3). Importantly, out of the analyzed clones, only the germline-encoded 1276:01F04 clone bound the MDA/MAA peptides (Fig. 3, F and H). To further confirm this we also modified the lysine derivative 6-aminocaproic acid (6ACA) with MDA/MAA to form the equivalent to free MDA/MAA-lysine that subsequently was coupled to human serum albumin (HSA). Fluorescence measurement demonstrated the presence of MAA in the MDA-hapten-HSA (Fig. 3A). Yet, in the 6ACA reaction we expect both free MDA-lysine and MAA-lysine to be formed and hence we will have mix of MDA- and MAA-hapten-HSA coupling. SDS-PAGE gel electrophoresis confirmed absence of cross-linking for the MDA/MAA-modified hapten-HSA (Fig. 3B). By normalizing the ELISA coating concentration based on antigen fluorescence, comparable amounts of MAA-adducts for the linear MDA/MAA-hapten-HSA and regular modified MDA-HSA were ensured. Our results confirmed that the germline-encoded clone 1276:01F04 recognize linear MDA/MAA, and only this clone maintained significant binding to the MDA/MAA-6ACA-HAS conjugate (Figs. 3C and S7).

To overcome the spontaneous formation of MAA in the MDA protocol, we next utilized the chemical reducing agent sodium cyanoborohydride (SCBH) to block the formation of MAA-adducts (27). We MDA-modified fibrinogen with the addition of SCBH and validated MDA modification without any fluorescent MAA epitopes (Fig. 4A). Results were confirmed with immunoassays (Fig. S4) and MS (Tables S1 and S2). Indeed, SCBH blocked the formation of MAA-adducts forming a solely MDA-modified antigen. However,



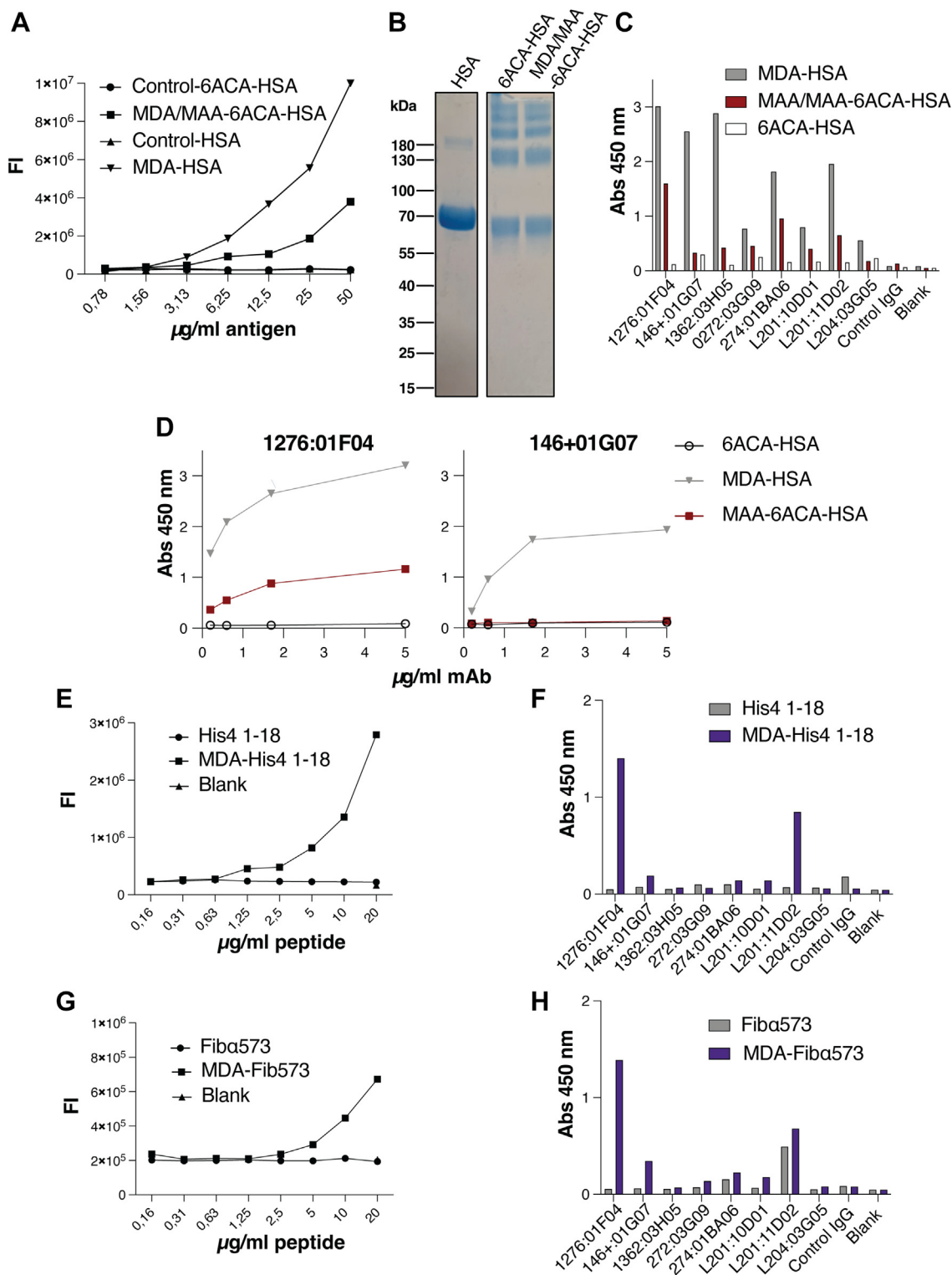
**Figure 2. Phylogenetic trees of clonal relationships between expressed mAbs.** Phylogenetic trees from single-cell derived immunoglobulin VH-VL nucleotide sequences from the four patients with identified anti-MDA+ clones in the lung or bone marrow. *A*, sixty-three single-cell bone marrow plasma cells from an ACPA+ established RA patient. *B*, fifty-eight single-cell bone marrow plasma cells from ACPA+ established RA patient. *C*, two hundred thirty-three single-lung B cells from an ACPA+ individual at risk of developing RA. *D*, three hundred fifty single-lung B cells from an ACPA+ newly diagnosed untreated RA patient. Phylogenetic trees were constructed by performing multiple sequence alignment of the combined heavy-light chain (VDJ-VJ) variable region sequences (58) and visualized using R package and ggtree (59). Sequences that were expressed and screened as mAbs are highlighted in blue and anti-MDA positive clones in red. N.B. the L201:10D01 was found in three copies, while the other anti-MDA clones were singletons. ACPA, anti-citrullinated protein autoantibodies; MDA, malondialdehyde; RA, rheumatoid arthritis.

the MS analysis showed less MDA modification for the SCBH treated fibrinogen in comparison with the MDA only treated fibrinogen (Fig. 4A, Tables S1 and S2). SDS-PAGE analysis visualized that cross-linking of fibrinogen occurred for both the fluorescent MDA/MAA-modified fibrinogen and the nonfluorescent SCBH treated solely MDA-modified fibrinogen (Fig. 4B).

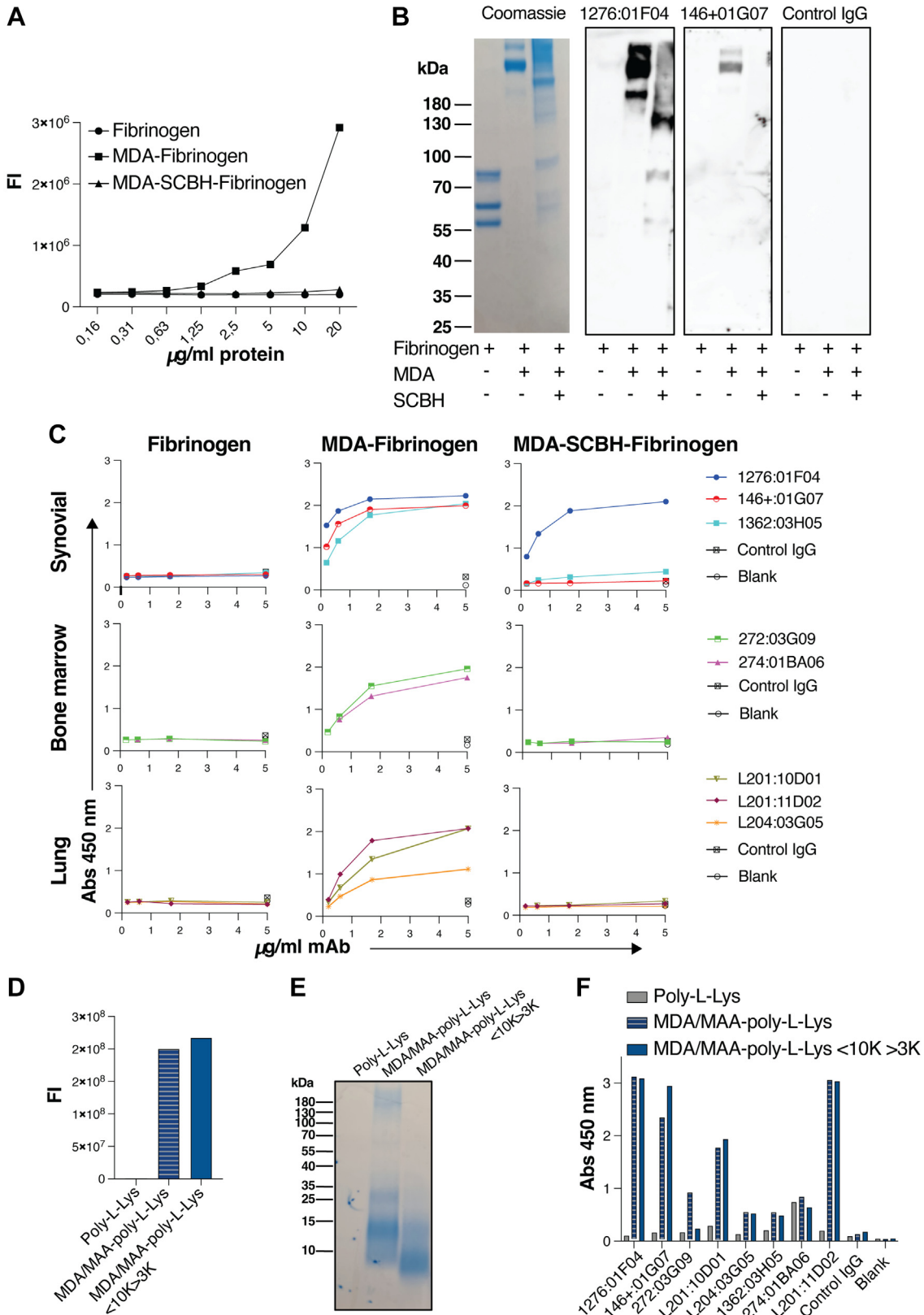
When investigating the binding of the MDA-reactive mAbs to MDA-modified and MDA-SCBH-modified fibrinogen and HSA by Western blot and ELISA the results showed that out of the eight analyzed clones, only the germline encoded synovial clone 1276:01F04 bound to MDA-SCBH-modified fibrinogen and HSA (Fig. 4C). The Western blot analysis also visualized mAb binding to MDA/MAA cross-linked proteins. The analyzed synovial clones bound cross-linked MDA/MAA modified fibrinogen and HSA (Figs. 4B and S5) whereas only the germline encoded mAb 1276:01F04 bound to MDA-SCBH-cross-linked fibrinogen. Hence, we conclude that a majority of the anti-MDA/MAA clones preferentially recognize MAA-mediated intramolecular cross-linked adducts.

To investigate if the mAbs recognized MDA/MAA-modified cross-linked antigens independent of amino acid composition, we produced an artificial cross-linked MDA/MAA-modified antigen utilizing poly-L-lysine. The poly-L-lysine was modified with MDA/MAA and filtered to enrich for cross-linked MDA/MAA-modified lysine smaller than 10 kDa but larger than 3 kDa. All analyzed clones bound the MDA/MAA-modified poly-L-lysine which further demonstrates that the antibodies target the MDA/MAA-adducts independently of the antigen context or a longer amino acid epitope (Fig. 4D). Intriguingly, we observed some variation in signal between the different clones and one clone, 272:03G09, seem to have more reactivity to the larger poly-L-lysine than to the smaller molecular weight cross-linked polymers. Hence, within the MAA-reactive subset of clones there may be further preferential binding to different adducts. Notably, MDA-reactive clones did not bind other types of protein cross-linking by 1-ethyl-3-(3-dimethylaminopropyl) [EDC]/N-hydroxyl succinimide ester [NHS] or paraformaldehyde (Fig. S6).

## Anti-MAA+ B cells in RA



**Figure 3. mAb reactivity to MDA- and MAA-modified hapten-antigen and peptides reveal protein cross-linking dependence for antigen recognition.** A, MDA/MAA-modified haptens were coupled to HSA, and fluorescence measurements were used to validate MDA/MAA-adducts on MAA-modified 6ACA and MDA-HSA. B, Coomassie stained SDS-PAGE confirms MDA/MAA-hapten coupling to HSA without cross-linking. C, mAb reactivity at 5 µg/ml to modified hapten measured with ELISA. D, mAb serial dilutions against modified antigens coated at normalized concentrations based on fluorescence to ensure similar number of MAA-adducts on each antigen (3 µg/ml MDA-HSA and 12 µg/ml MDA/MAA-6ACA-HSA). E and G, lysine containing biotinylated peptides (Fiba573 or His4 1–18) were modified with MDA and MAA-fluorescence was measured at  $\lambda_{em}$  462 nm and  $\lambda_{ex}$  394 nm. F and H, mAb reactivity to modified peptides were evaluated with ELISA at 5 µg/ml immunoglobulin G. 6ACA, 6-aminocaproic acid; Abs, absorbance; FI, fluorescence intensity; HSA, human serum albumin; MAA, malondialdehyde acetaldehyde; MDA, malondialdehyde.



**Figure 4. mAb binding to MDA/MAA- and MDA-SCBH-modified fibrinogen and MDA/MAA-poly-L-lysine reveal amino acid composition independence for mAb binding.** A, fluorescence intensity (FI) of modified full-length fibrinogen was measured at  $\lambda_{em}$  462 nm and  $\lambda_{ex}$  394 nm in 100  $\mu\text{l}$  protein solution. Lack of fluorescence confirms blocking of MAA-formation with sodium cyanoborohydride (SCBH). B, Coomassie blue stained SDS-PAGE with reduced modified fibrinogen shows protein cross-links both for the MDA-only treated fibrinogen and the MDA-sodium cyanoborohydride treated fibrinogen. Western blot analysis of 3  $\mu\text{g/lane}$  reduced modified fibrinogen with 5  $\mu\text{g/ml}$  mAb. C, ELISA titration of mAbs to MDA-, MDA-SCBH-modified fibrinogen and mock treated fibrinogen. D, poly-L-lysine 4 to 15 kDa was modified with MDA/MAA, and fluorescence was detected at 50  $\mu\text{g/ml}$



## Anti-MAA+ B cells in RA

### MDA-binding is only partially dependent on SHM in affinity matured clones

To investigate the influence of variable region somatic hypermutations and affinity maturation on the MDA reactivity of the different B cell clones, we converted four of the monoclonals to the closest predicted germline sequence. All identified mismatches were replaced, including any identified sites in FRW4; however, the complementarity-determining region 3s were mostly intact as mismatches cannot easily be predicted due to nontemplated nucleotide insertions in the junction. We found that all germline versions maintained a significant level of MAA-binding. Some clones displayed a reduction in binding while others, in particular L204:03G05, showed increased binding in different assays (Figs. 5, S7 and S8). However, none of the clone changed their binding patterns to become more MDA-specific similar to the germline-encoded 1276:01F04. Hence, the MDA reactive B cell clones seem to have specific binding to MDA molecular structures also in their naïve configuration. In summary, a majority, but not all, of the investigated RA-derived anti-MDA clones have distinct recognition of cross-linked MAA residues and originate from different B cell subsets (Fig. 6).

### Polyclonal anti-MAA IgG are present at higher concentrations in RA patients than in healthy controls

To evaluate polyclonal anti-MDA/MAA antibodies, we used MDA/MAA-modified fibrinogen and MDA-SCBH-fibrinogen, containing only MDA, to affinity purify serum IgG from ACPA+ RA patients and healthy controls. By sequential affinity purification, we could fractionate antibodies specific for both MDA/MAA and either MAA or MDA (Fig. 7A). We confirmed that both IgG anti-MDA and IgG anti-MAA with similar binding patterns as seen for the monoclonals were present in both RA patients and healthy controls (Fig. 7, B and C). Interestingly, the yield of anti-MDA/MAA polyclonal Abs from ACPA+ RA patients were higher in comparison with healthy controls (0.55% of total IgG compared to 0.11% of total IgG, respectively) but both MDA and MAA reactive antibodies were present in both RA and controls (Fig. 7D). Yet the proportion of IgG MDA reactive *versus* MAA-reactive antibodies were different between the ACPA+ RA patients IgG pool and serum IgG from healthy controls (29% and 40% of all anti-MDA/MAA IgG were anti-MDA, in RA *versus* controls; Fig. 7E). Hence, potentially pathogenic anti-MAA IgG may be part of common immune responses also in healthy individuals but are significantly elevated in rheumatoid arthritis.

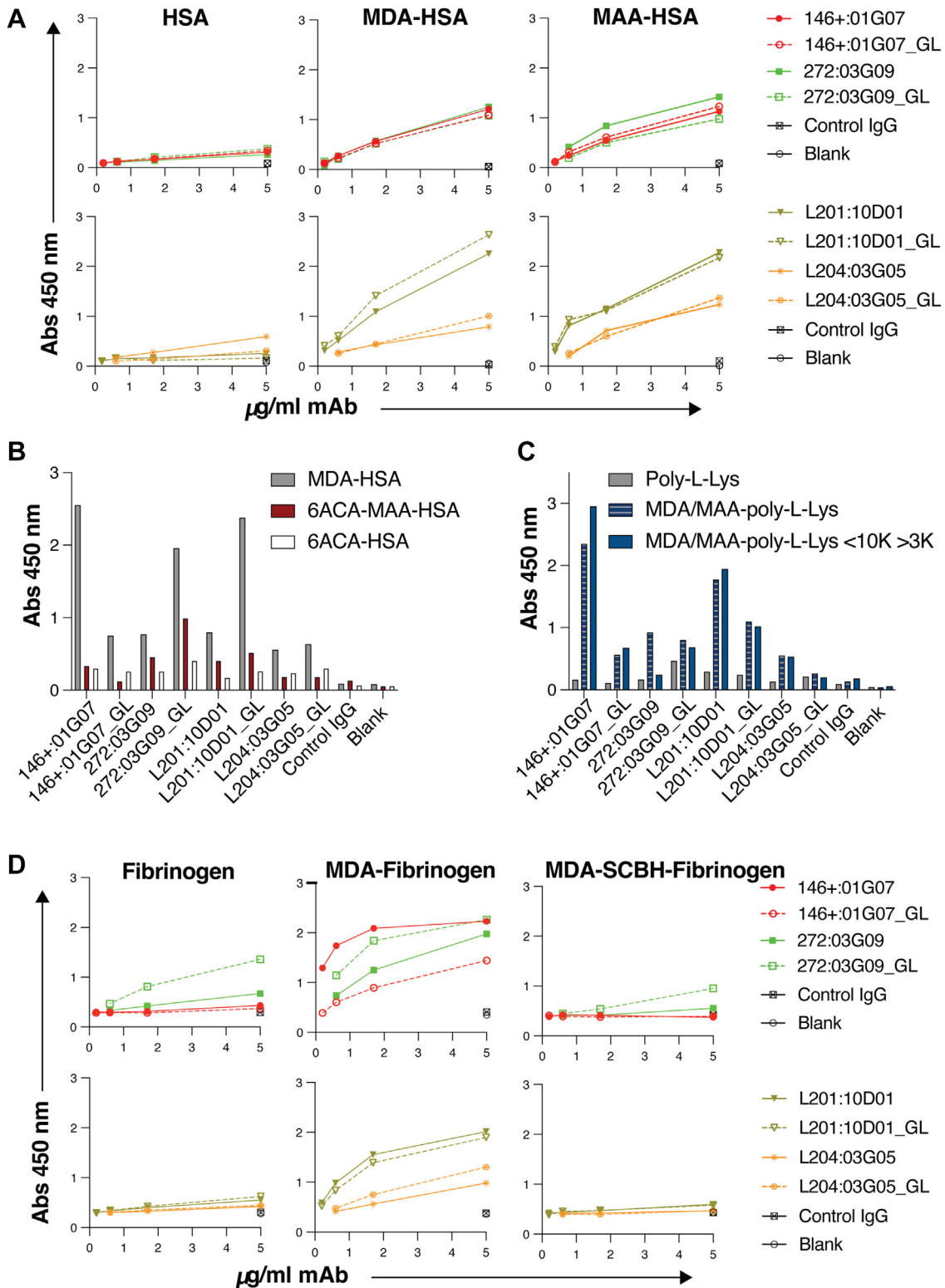
## Discussion

Autoreactivity to MDA adducts is associated with high disease activity in RA and we have recently shown that anti-MDA IgG may contribute to disease by inducing glycolysis pathways in osteoclasts and trigger bone erosion (37). Hence, it can be

postulated that MDA autoreactive antibodies adds an extra layer of inflammation in the pathogenesis of RA. In this report, we demonstrate for the first time that MDA-reactive B cells are present in the lungs of both ACPA+ individuals at risk of developing RA and in newly diagnosed RA patients. We also show the presence of anti-MDA secreting BM plasma cells in established RA patients. Together with previous results from synovial B cells (5), this shows that MDA positivity is common in different B cell compartments and tissues, and that anti-MDA IgG can be secreted both locally in the synovium and systemically. By generating mAbs from paired variable Ig-genes from single B cells, we could perform an in-depth biochemical characterization of the binding specificities of RA anti-MDA. Notably, one clone from synovial memory ACPA+ patient B cell recognized linear MDA-modified antigens, whereas the other clones required MAA-modified cross-linked antigens for recognition.

MDA-reactive germline-encoded natural IgM are hypothesized to be secreted by B-1 cells (31, 32, 34). However, here we demonstrate that MDA+ IgG+ B cells can be identified across different B cells subsets. Previously we have identified both anti-MDA positive cells among synovial fluid memory B cells, plasma cells, and tissue FcRL4+ B cells (5). Here, we report that three out of 98 lung B cell clones and two out of 44 BM plasma cells were reactive to MDA antigens. Numbers correspond to similar frequencies of ACPA-reactive B cells from the same compartments (4, 35, 38). Importantly, seven out of eight MDA clones had SHMs in their variable domains indicating affinity-maturation driven by T cells. In contrast, one of the previously reported synovial CD27+ IgG1+ B cell derived clone, 1276:01F04, was germline encoded and intriguingly this clone has a distinctly different MDA-binding pattern and significantly higher apparent affinity. However, importantly, when the other clones were germline converted, they maintained their reactivity patterns and did not gain an “1276:01F04-profile” with MDA-binding. Instead, they to a large extent maintained their original MAA-binding which may suggest that the SHM and affinity maturation is driven in a different context than the MDA response, perhaps by another type of antigen. Interestingly, two lung clones originate from IgG1+ double negative B cells whereof one had low level of SHM. These cells may represent a preplasmablast phenotype and be a result of extrafollicular responses (39). Interestingly, RA is associated with increased frequencies of circulating class-switched IgG1+ cells with low SHM (<5), which has been suggested to be linked with expansion of the double-negative subset (40, 41). In contrast, another MDA-reactive lung clone was an IgG4+ memory B cell. IgG4 positive B cells are increased in RA and may be an indication of chronic responses (40, 42). Moreover, we have previously identified both IgG4+ ACPA+ BM plasma cells and lung plasmablasts (35, 38). Yet, it is interesting that the IgG4+ anti-MDA was identified in an RA-risk individual without arthritis.

modified antigen before and after size-fractionation with 10 kDa and 3 kDa spin columns. E, Coomassie stained SDS-PAGE visualize cross-linking induced by MDA/MAA-modification. F, ELISA mAb reactivity at 5 µg/ml against MDA/MAA-poly-L-lysine of different sizes. Lys, lysine; MAA, malondialdehyde acetaldehyde; MDA, malondialdehyde; SCBH, sodium cyanoborohydride.

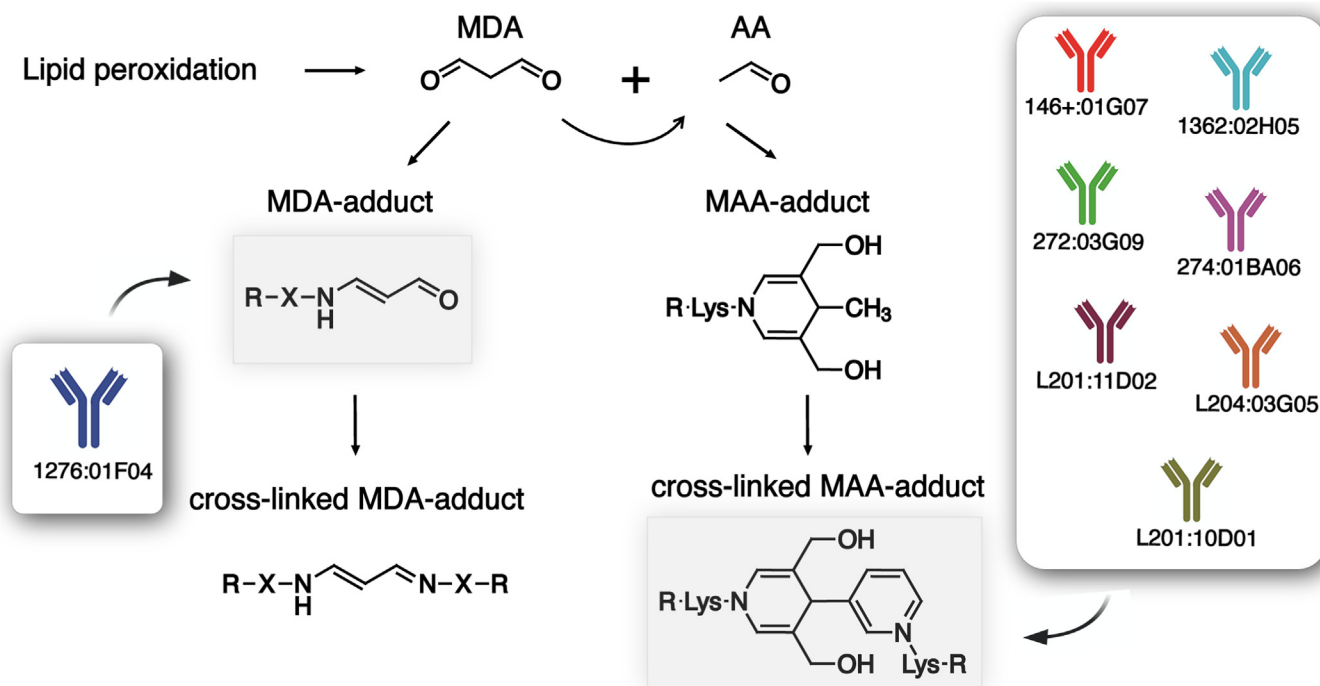


**Figure 5. Germline reverted mAb binding to MDA/MAA modified antigens.** A, titration ELISA of MDA-reactive germline reverted mAbs to MDA- and MAA-modified HSA. B, germline reverted mAb ELISA reactivity at 5  $\mu\text{g/ml}$  to modified MDA/MAA-6ACA-HSA hapten. C, mAb ELISA reactivity at 5  $\mu\text{g/ml}$  against MDA/MAA-poly-L-lysine of different sizes. D, ELISA titration of germline reverted mAbs to MDA-, MDA-SCBH-modified fibrinogen and mock treated fibrinogen. 6ACA, 6-aminocaproic acid; HSA, human serum albumin; MAA, malondialdehyde acetaldehyde; MDA, malondialdehyde; SCBH, sodium cyanoborohydride.

MDA-induced cross-linking of proteins may have especially immunogenic properties and the recognition of distinct types of MDA or MAA adducts may be critical for the pathogenic

functionality of the antibodies (20). However, it is well documented that MDA spontaneously breaks down to acetaldehyde *in vitro*, forming MAA-adducts on proteins and peptides

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**Figure 6. Schematic overview of RA mAb binding to MDA- and MAA-adducts.** Schematic summary of the recognition of distinct MDA-molecular structures by RA-derived monoclonal antibodies. Lipid peroxidation produces MDA that forms MDA-adducts on amino acids, e.g., N-ε-(2-propenyl)lysine when lysine is modified. X = lysine, arginine, glutamine, and histidine in proteins. MDA can spontaneously break down to AA *in vitro* but is likely distributed exogenously *in vivo*. MDA and AA form together MAA-adduct (DHP-lysine) on amino acid lysine in proteins. Both MDA-adducts and MAA-adducts can further react to form intraprotein cross-links (1-amino-3-iminopropene and pyridium DHP). The germline-encoded 1276:01F04 mAb binds linear MDA-adducts. On the contrary, the remaining mAbs bind preferentially to cross-linked MAA-adducts. AA, acetaldehyde; DHP, dihydropyridine; MAA, malonaldehyde acetaldehyde; MDA, malondialdehyde; RA, rheumatoid arthritis.

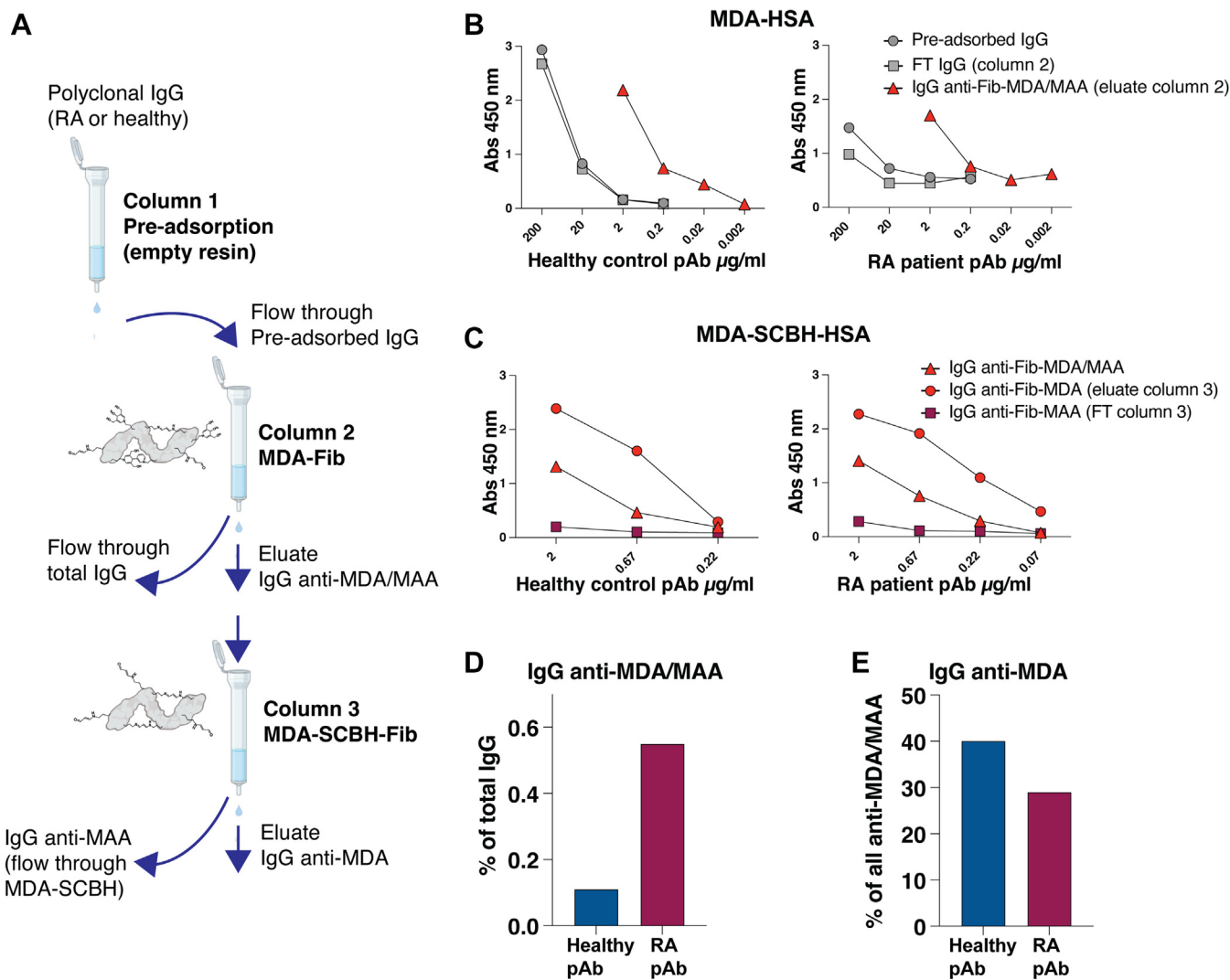
which provided some challenge when studying *in vitro* modified antigens (23). Hence, an important methodological approach in this study was to utilize previously published protocols using the addition of SCBH to block the formation of MAA adducts for production of solely MDA-modified proteins and hapten coupling of MDA/MAA-lysine to a carrier protein (26, 27). Interestingly, both MDA and MAA were found to form interprotein and intraprotein cross-links easily visualized by SDS-PAGE. Our approach made it possible to distinguish MDA/MAA-hapten binding from binding to cross-linked residues. We were surprised to find that binding to the cross-linked epitopes was dominating.

Furthermore, the MDA clones did not resemble citrulline reactive ACPA in their recognition patterns. ACPA display multireactivity to a range of citrullinated peptides and proteins by recognizing short consensus binding motifs (3, 4, 43, 44), while the multireactive MDA clones in contrast did not display any amino acid epitope specificity. Furthermore, they did not show cross-reactivity to citrullination or to other PTMs. Moreover, our investigations of MDA/MAA-modified poly-L-lysine demonstrate that MDA/MAA-adduct on lysine residues are enough for antibody binding and that no adjacent amino acids are required in the epitope.

Interestingly, some anti-MDA clones have previously been shown to have pathogenic features mediating increased osteoclastogenesis *in vitro* and bone loss *in vivo* in mice (5). This osteoclast promoting effect, particularly studied for the

anti-MAA reactive synovial clone 146+:01G07, was Fc-gamma receptor 1 dependent which suggests immune complex formation for receptor activation (37). Moreover, the clone with linear MDA recognition (1276:01F04) had no effect on osteoclasts, which may suggest that an MAA cross-linked type of adduct is what is targeted by pathogenic anti-MDA antibodies. Hence, it will be intriguing to continue these studies also with the newly identified anti-MAA clones to further elucidate the mechanistic pathways and autoantigen dependence.

We have previously shown by MS that MDA-modified antigens are present in the RA synovium (5), and immunoassays have suggested the presence of MAA-modifications (6). However, the exact mechanism that trigger MDA/MAA modification in the synovium is still unknown as are the precise immunogenic targets. Airway-induced inflammation in the lung has been reported to give rise to MDA-derived modifications (9), and smoking and infection may provide exogenous acetaldehyde. Furthermore, it was recently demonstrated that patients with RA-associated interstitial lung disease have elevated anti-MAA autoantibodies, and MAA-modified protein have been reported to be associated with inflammation and fibrosis in airway epithelial cells (9, 45). Therefore, our identification of MDA/MAA-reactive B cells at mucosal sites in the lung of ACPA+ risk individuals and early RA is particularly interesting. This may suggest that MAA is involved in breaking of tolerance at mucosal sites by smoking and infection and strengthens the concept of mucosal origin



**Figure 7. Purified MDA/MAA polyclonal antibodies from RA patients and healthy controls.** A, schematic overview of the sequential affinity purification procedure using streptavidin sepharose coupled with biotinylated modified fibrinogen (either MDA-fibrinogen or MDA-SCHB-fibrinogen) for purification of IgG anti-MDA/MAA polyclonal autoantibodies (pAb) from IgG from RA patients or healthy controls. IgG fractions were buffer exchanged to PBS between purification steps and IgG concentrations were assessed with spectrophotometer measurement at 280 nm and ELISA. B, ELISA reactivity of purified anti-MDA/MAA-fibrinogen polyclonal IgG antibodies to MDA-modified HSA (containing both MDA and MAA adducts). C, reactivity of purified IgG anti-MDA-fibrinogen or alternatively anti-MAA-fibrinogen polyclonal antibodies to MDA-SCHB-modified HSA (containing only MDA adducts). D, quantitative results from the affinity purification of IgG anti-MDA/MAA. Proportion of anti-MDA/MAA among total IgG in healthy controls and RA patients based on amount of purified IgG in different fractions. E, proportion of anti-MAA among IgG anti-MDA/MAA in healthy controls and RA patients. Fib, Fibrinogen; FT, flow through; HSA, human serum albumin; IgG, immunoglobulin G; MAA, malondialdehyde acetaldehyde; MDA, malondialdehyde; RA, rheumatoid arthritis; SCBH, sodium cyanoborohydride.

for autoimmunity in RA (46). Such a scenario is further supported by the presence of anti-MAA autoantibodies in the sera of RA patients before disease onset. Furthermore, one of the anti-MDA positive B cells with reactivity to cross-linked MAA, the clone G07, originates from an IgA<sup>+</sup> FcRL4<sup>+</sup> synovial tissue B cells (5, 47). The FcRL4<sup>+</sup> B cells are a subset of cytokine producing memory B cells that were originally described to be enriched in mucosa-associated lymphoid tissue (48). However, they are also found in the RA synovium where they express receptor activator of nuclear factor  $\kappa$ B ligand that can stimulate osteoclasts (49). Taking into account the high osteoclast stimulatory capacity of this mAb (5, 37), it represents an interesting interface between the mucosa and the synovium.

In conclusion, we have here shown that autoreactive B cells specific for MDA protein modifications can be found in

the lungs of RA risk individuals and early RA as well as in the BM plasma cell compartment and in the inflamed synovium of RA patients. We have also shown that although there are B cell clones that bind the linear MDA modification, in our study the majority of clones preferentially recognize MAA-cross-linked amino acid adducts independent of the protein backbone. The origin of these autoreactive B cells and their potential role in the RA pathogenesis remains to be elucidated.

## Experimental procedures

### Patient material

All patients gave informed consent to participation. The study was performed in compliance with the declaration of

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Helsinki and approved by the regional ethics review board in Stockholm. The BM derived mAbs originate from one ACPA– and four ACPA+ RA patients fulfilling the 2010 EULAR/ACR RA classification criteria (50) and undergoing hip arthroplasty as previously described (38). The lung-derived mAbs originate from two ACPA+ individuals with musculoskeletal complaints but without arthritis, referred from primary care centers in the Stockholm area as well as four ACPA+ and six ACPA– early untreated RA patients fulfilling the 2010 EULAR/ACR RA classification criteria as previously described (35). Patient sera were tested for ACPA positivity using with CCPlus Immunoscan ELISA (SVAR Life Science).

### B cell isolation

BM from the proximal femur was collected during hip arthroplasty and processed immediately as previously described (38). In brief, BM mononuclear cells were isolated using Ficoll gradient separation (Cytiva) followed by cell surface antibody staining and single-cell flow cytometry sorting of CD19+ CD138+ B cells. Alternatively, BM plasma cells were identified based on the transcriptomics (10X Genomics).

The bronchoscopy procedure and handling of BAL fluid was performed as previously described (35). In brief, collected cells were stained for cell surface markers (CD3, CD14, CD16, CD19, CD27, IgD, and CD38) and CD19+ B cells were single cells flow cytometry sorted.

Single cells Ig sequences were generated as previously described (51) and analyzed using V-QUEST and the international ImMunoGeneTics database (52). To revert the variable heavy and light transcripts back to predicted germline, IgBLAST was used to predict the of closest germline version of the mutated mAbs.

### mAb expression

Ig variable regions of selected clones were synthesized and subcloned into human heavy  $\gamma$ 1 and light  $\kappa$  or  $\lambda$  constant region expression vectors using an off-site service (Integrated DNA Technologies). Recombinant human mAbs were expressed in Expi293 cells (Thermo Fisher Scientific) and purified using protein G affinity chromatography (Cytiva). All expressed mAbs were evaluated using IgG ELISA and SDS-PAGE as previously described (51). Analysis of unspecific polyreactivity was performed at 5  $\mu$ g/ml using the soluble membrane protein assay previously developed (3) to exclude any false positive clones. All expressed mAbs were also screened at 5  $\mu$ g/ml with CCPlus Immunoscan ELISA (SVAR Life Science) following the manufacturer's instructions and with a custom-designed antigen array with citrullinated and native peptides (Thermo Fisher Scientific) (53). MDA-reactive clones and controls were reexpressed in larger scale and further quality controlled by size exclusion chromatography.

### Preparation of MDA-modified antigens

To produce MDA- and MAA-modified protein antigens, MDA was generated as previously described (5) by acid hydrolysis of tetramethoxypropane (Sigma-Aldrich), thereafter

molecular grade bovine serum albumin (BSA) (New England Biolabs), human fibrinogen (Calbiochem), HSA (New England Biolabs) and human transferrin (Sigma-Aldrich) were modified with 50 mM MDA in PBS (pH 7.4) for 6 h at 37 °C, followed by extensive dialysis to PBS. For generation of higher level of MAA-type modifications, 25 mM acetaldehyde (Sigma-Aldrich) was additionally added followed by 2 h incubation at 37 °C with shaking.

The formation of fluorescent adducts were measured as previously described (54) at  $\lambda_{em}$  462 nm and  $\lambda_{ex}$  394 nm by adding 100  $\mu$ l solution to 96-well Nunc high-binding (Thermo Fisher Scientific) ELISA plate followed by fluorescence measurement with Spectramax iD3 (Molecular Devices).

To produce MDA-modified antigens without MAA/DHP residues, a final concentration of 150 mM SCBH (Sigma-Aldrich), was added to the MDA-reaction to block the formation of MAA-adducts as previously described (27). Human fibrinogen (Calbiochem) was citrullinated with PAD4 enzyme (Cayman chemicals) in 100 mM Tris, 10 mM CaCl<sub>2</sub>, 5 mM DTT, 4 h 37 °C, and buffer exchanged to PBS. In addition, commercially available antigens were used: carbamylated BSA, 4-hydroxynonenal-modified BSA, MDA-LDL and Nitrated (Nitr)-LDL, (Academy Bio-Medical). The biotinylated peptides Fiba573 HHPGIAEFPSRKGSSYSKQF and His4 1–18 SGRGKGGKGLGKGGAKRHGSGSK were modified at 70  $\mu$ g/ml in PBS by 50 mM MDA in PBS (pH 7.4) for 16 h at 37 °C followed by buffer exchange to PBS with PD MiniTrap G-10 columns (Cytiva) according to manufacturer's instructions.

For biotinylated full-length antigens, biotin was coupled prior to antigen modification. Antigens were dissolved and dialyzed to Dulbecco's phosphate buffered saline using 10,000 MVCO Slide-A-Lyzer. EZ-link Sulfo NHS-LC-Biotin (Thermo Fisher Scientific) was added to the reaction at 25 $\times$  molar excess and incubated at 2 h in room temperature (RT) followed by dialysis to remove the free biotin.

To make protein antigens without intraprotein MDA-induced cross-links, initial MDA/MAA-haptens were made followed by coupling to HSA as using a modified version of a previously published protocol (26). In brief, 4 mM  $\epsilon$ -aminocaproic acid (6ACA) (Merck), 4 mM acetaldehyde, and 8 mM MDA were dissolved in PBS. The reaction mixture was incubated at 37 °C for 3 days and then purified with Pierce C18 Spin Columns (Thermo Fisher Scientific) according to the manufacturer's instruction. The collected fractions were lyophilized (ScanVac, Labogene) and dissolved in 50  $\mu$ l PBS. The MAA-6ACA was coupled to HSA (Sigma-Aldrich) with EDC as follows. A total of 10 mg/ml HSA was resuspended in 25 mM Mes (Sigma-Aldrich) using Zeba 7K MWCO desalting spin columns (Thermo Fisher Scientific) and incubated for 2 h at RT together with 50 nmol MAA-6ACA plus 10 mM EDC (Sigma-Aldrich) and 40 mM NHS (Sigma-Aldrich). Reaction was extensively dialyzed to PBS using 10 kDa slide-a-lyzer cassettes (Thermo Fisher Scientific) and analyzed with SDS-PAGE.

To induce protein cross-linking on-plate, human fibrinogen (Calbiochem) was coated at 3  $\mu$ g/ml PBS on high-bind half-area 96-well plate (Corning) at 4 °C overnight, followed by

50  $\mu$ l 2 mM EDC (Sigma-Aldrich) and 5 mM NHS (Sigma-Aldrich) in Mes buffer pH 5.5 for 2 h RT or alternatively with 1% paraformaldehyde for 20 min RT.

Modification of poly-L-lysine 4 to 15 kDa (Sigma-Aldrich) was performed in PBS with 100 mM MDA and 50 mM acetaldehyde for 4 h at 37 °C followed by dialysis to PBS. Cleavage was performed with Lys-C (Sigma-Aldrich) 1:100 by mass in PBS over night at 37 °C. Fractionation was performed by first filtering through Amicon 10 kDa spin columns followed by filtering through Amicon 3 kDa spin columns to generate MDA/MAA-modified poly-L-lysine <10 kDa >3 kDa. Finally, the MDA/MAA-modified poly-L-lysine was separated through PD MiniTrap G10 (Sigma-Aldrich) to generate modified poly-L-lysine <3 kDa >700 Da. The reaction was validated by fluorescence and by Coomassie SDS-PAGE protein gel electrophoresis.

### Purification of human polyclonal anti-MDA IgG

To purify MDA/MAA-reactive polyclonal antibodies from human serum, 1 ml Streptavidin Sepharose High Performance resin (Cytiva) was mixed with 1 mg biotinylated and modified full-length fibrinogen for 1.5 h at RT, packed in empty PD-10 columns (Cytiva) and equilibrated with PBS. RA anti-MDA IgG was purified from pooled CCP2-depleted purified IgG from five ACPA+ RA patients. Briefly, IgG was purified from plasma using protein G affinity chromatography, followed by passing through a CCP2 column as previously described (55). The CCP2-depleted IgG was subsequently diluted in PBS to 10 mg/ml and preadsorbed to nonconjugated streptavidin Sepharose to further remove unspecific IgG. The IgG was thereafter purified on the MDA-fibrinogen column and eluted with 0.1 M glycine pH 3 and neutralized Tris pH 9. The eluted anti-MDA/MAA IgG was buffer exchanged to PBS and purified a second time over MDA-SCBH-fibrinogen, resulting in both an anti-MAA IgG enriched fraction in the column flow through and an eluted anti-MDA IgG enriched fraction. Anti-MAA IgG and anti-MDA IgG was similarly purified from healthy individuals using intravenous Ig preparation (IVIg, Privigen).

### MDA-protein and peptide ELISA

Expressed mAbs with adequate amounts were screened for binding to unmodified BSA and MDA-modified BSA (New England Biolab) with ELISA. Antigens were coated at 3  $\mu$ g/ml on high-bind half-area 96-well plate (Corning), blocked with 1% BSA in PBS. Expressed mAbs were screened at 5  $\mu$ g/ml and titrated down to 0.18  $\mu$ g/ml in validation studies. Reactivity was detected with horseradish peroxidase-conjugated Fc-gamma-specific goat (Fab')<sub>2</sub> anti-human IgG (Jackson ImmunoResearch) and developed with 3,3',5,5'-tetramethylbenzidine substrate (Biolegend). All absorbance values were quantified toward an mAb reference (1276:01F04) (5) and presented as relative units (RU)/ml. For peptide ELISA, Streptavidin high-capacity plates (Thermo Fisher Scientific) were coated with biotinylated MDA-modified peptides at 3  $\mu$ g/ml in PBS for 1 h followed by mAb analysis as above.

### Western blot

For SDS-PAGE and Western blot analysis, 3  $\mu$ g of of MDA-BSA or control-treated BSA were reduced and separated on Bolt Bis-Tris 4 to 12% gels with Mes-SDS running buffer and blotted to polyvinylidene fluoride membrane according to the manufacturer's instructions (Thermo Fisher Scientific). The membrane was blocked with 3% BSA and stained with 5  $\mu$ g/ml human IgG1 mAb at 4 °C o/n, followed by detection with horseradish peroxidase-conjugated rabbit anti-human IgG (Jackson ImmunoResearch). Binding was detected by chemiluminescence using Clarity Western ECL Substrate (BioRad).

### Mass spectrometry

Modified proteins and peptides were analyzed using mass spectrometry (see [Supporting Experimental procedures](#)).

### Data availability

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

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*Supporting information*—This article contains supporting information (3, 53, 56, 57).

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*Conflict of interest*—The authors declare that they have no conflicts of interest with the contents of this article.

*Abbreviations*—The abbreviations used are: 6ACA, 6-aminocaproic acid; ACPA, anti-citrullinated protein autoantibodies; BAL, bronchoalveolar lavage; BM, bone marrow; BSA, bovine serum albumin; DHP, dihydropyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl); HSA, human serum albumin; IgG, immunoglobulin G; LDL, low-density lipoprotein; MAA, malondialdehyde acetaldehyde; MDA, malondialdehyde; NHS, N-hydroxyl succinimide ester; PTM, post-translational modification; RA, rheumatoid arthritis; RT, room

temperature; SCBH, sodium cyanoborohydride; SHM, somatic hypermutations.

### References

- Klareskog, L., Catrina, A. I., and Paget, S. (2009) Rheumatoid arthritis. *Lancet* **373**, 659–672
- Grönwall, C., Liljefors, L., Bang, H., Hensvold, A. H., Hansson, M., Mathsson-Alm, L., et al. (2021) A comprehensive evaluation of the relationship between different IgG and IgA anti-modified protein autoantibodies in rheumatoid arthritis. *Front. Immunol.* **12**, 627986
- Sahlström, P., Hansson, M., Steen, J., Amara, K., Titcombe, P. J., Forström, B., et al. (2020) Different hierarchies of anti-modified protein autoantibody reactivities in rheumatoid arthritis. *Arthritis Rheumatol.* **72**, 1643–1657
- Steen, J., Forsstrom, B., Sahlstrom, P., Odowd, V., Israelsson, L., Krishnamurthy, A., et al. (2019) Recognition of amino acid motifs, rather than specific proteins, by human plasma cell-derived monoclonal antibodies to posttranslationally modified proteins in rheumatoid arthritis. *Arthritis Rheumatol.* **71**, 196–209
- Grönwall, C., Amara, K., Hardt, U., Krishnamurthy, A., Steen, J., Engström, M., et al. (2017) Autoreactivity to malondialdehyde-modifications in rheumatoid arthritis is linked to disease activity and synovial pathogenesis. *J. Autoimmun.* **84**, 29–45
- Thiele, G. M., Duryee, M. J., Anderson, D. R., Klassen, L. W., Mohring, S. M., Young, K. A., et al. (2015) Malondialdehyde-acetaldehyde adducts and anti-malondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. *Arthritis Rheumatol.* **67**, 645–655
- Mikul, T. R., Edison, J., Meeshaw, E., Sayles, H., England, B. R., Duryee, M. J., et al. (2020) Autoantibodies to malondialdehyde-acetaldehyde are detected prior to rheumatoid arthritis diagnosis and after other disease specific autoantibodies. *Arthritis Rheumatol.* **72**, 2025–2029
- Mikul, T. R., Duryee, M. J., Rahman, R., Anderson, D. R., Sayles, H. R., Hollins, A., et al. (2017) Enrichment of malondialdehyde-acetaldehyde antibody in the rheumatoid arthritis joint. *Rheumatology (Oxford)* **56**, 1794–1803
- England, B. R., Duryee, M. J., Roul, P., Mahajan, T. D., Singh, N., Poole, J. A., et al. (2019) Malondialdehyde-acetaldehyde adducts and antibody responses in rheumatoid arthritis-associated interstitial lung disease. *Arthritis Rheumatol.* **71**, 1483–1493
- Hardt, U., Larsson, A., Gunnarsson, I., Clancy, R. M., Petri, M., Buyon, J. P., et al. (2018) Autoimmune reactivity to malondialdehyde adducts in systemic lupus erythematosus is associated with disease activity and nephritis. *Arthritis Res. Ther.* **20**, 36
- Papac-Milicevic, N., Busch, C. J. L., and Binder, C. J. (2016) Malondialdehyde epitopes as targets of immunity and the implications for atherosclerosis. *Adv. Immunol.* **131**, 1–59
- Itakura, K., Uchida, K., and Osawa, T. (1996) A novel fluorescent malondialdehyde-lysine adduct. *Chem. Phys. Lipids* **84**, 75–79
- Tuma, D. J., Kearley, M. L., Thiele, G. M., Worrall, S., Haver, A., Klassen, L. W., et al. (2001) Elucidation of reaction scheme describing malondialdehyde-acetaldehyde-protein adduct formation. *Chem. Res. Toxicol.* **14**, 822–832
- Uchida, K., Sakai, K., Itakura, K., Osawa, T., and Toyokuni, S. (1997) Protein modification by lipid peroxidation products: formation of malondialdehyde-derived N(epsilon)-(2-propenyl)lysine in proteins. *Arch. Biochem. Biophys.* **346**, 45–52
- Freeman, T. L., Haver, A., Duryee, M. J., Tuma, D. J., Klassen, L. W., Hamel, F. G., et al. (2005) Aldehydes in cigarette smoke react with the lipid peroxidation product malonaldehyde to form fluorescent protein adducts on lysines. *Chem. Res. Toxicol.* **18**, 817–824
- McCaskill, M. L., Kharbanda, K. K., Tuma, D. J., Reynolds, J. D., DeVasure, J. M., Sisson, J. H., et al. (2011) Hybrid malondialdehyde and acetaldehyde protein adducts form in the lungs of mice exposed to alcohol and cigarette smoke. *Alcohol. Clin. Exp. Res.* **35**, 1106–1113
- Nguyen, T. T., Huynh, N. N., Seubbuk, S., Nilmoje, T., Wanasuntronwong, A., and Surarit, R. (2019) Oxidative stress induced by Porphyromonas gingivalis lysate and nicotine in human periodontal ligament fibroblasts. *Odontology* **107**, 133–141
- Ogawa, K., Tanaka, T., Nagoshi, T., Sekiyama, H., Arase, S., Minai, K., et al. (2015) Increase in the oxidised low-density lipoprotein level by smoking and the possible inhibitory effect of statin therapy in patients with cardiovascular disease: a retrospective study. *BMJ Open* **5**, e005455
- Tuma, D. J., Thiele, G. M., Xu, D., Klassen, L. W., and Sorrell, M. F. (1996) Acetaldehyde and malondialdehyde react together to generate distinct protein adducts in the liver during long-term ethanol administration. *Hepatology* **23**, 872–880
- Antoniak, D. T., Duryee, M. J., Mikul, T. R., Thiele, G. M., and Anderson, D. R. (2015) Aldehyde-modified proteins as mediators of early inflammation in atherosclerotic disease. *Free Radic. Biol. Med.* **89**, 409–418
- Thiele, G. M., Tuma, D. J., Willis, M. S., Miller, J. A., McDonald, T. L., Sorrell, M. F., et al. (1998) Soluble proteins modified with acetaldehyde and malondialdehyde are immunogenic in the absence of adjuvant. *Alcohol. Clin. Exp. Res.* **22**, 1731–1739
- Duryee, M. J. M. J., Klassen, L. W. L. W., Schaffert, C. S. C. S., Tuma, D. J. D. J., Hunter, C. D. C. D., Garvin, R. P. R. P., et al. (2010) Malondialdehyde/acetaldehyde adduct (MAA) is the dominant epitope following MDA modification of proteins in atherosclerosis. *Free Radic. Biol. Med.* **49**, 1480–1486
- Uchida, K. (2006) Lipofuscin-like fluorophores originated from malondialdehyde. *Free Radic. Res.* **40**, 1335–1338
- Slatter, D. A., Murray, M., and Bailey, A. J. (1998) Formation of a dihydropyridine derivative as a potential cross-link derived from malondialdehyde in physiological systems. *FEBS Lett.* **421**, 180–184
- Mooradian, A. D., Reinacher, D., Li, J. P., and Pinnas, J. L. (2001) Malondialdehyde modification of proteins *in vitro* is enhanced in the presence of acetaldehyde. *Nutrition* **17**, 619–622
- Shimomoto, T., Collins, L. B., Yi, X., Holley, D. W., Zhang, Z., Tian, X., et al. (2017) A purified MAA-based ELISA is a useful tool for determining anti-MAA antibody titer with high sensitivity. *PLoS One* **12**, e0172172
- Vay, D., Parodi, M., Rolla, R., Mottaran, E., Vidali, M., Bellomo, G., et al. (2001) Circulating antibodies recognizing malondialdehyde-modified proteins in healthy subjects. *Free Radic. Biol. Med.* **30**, 277–286
- Hill, G. E., Miller, J. A., Baxter, B. T., Klassen, L. W., Duryee, M. J., Tuma, D. J., et al. (1998) Association of malondialdehyde-acetaldehyde (MAA) adducted proteins with atherosclerotic-induced vascular inflammatory injury. *Atherosclerosis* **141**, 107–116
- Zagol-Ikapite, I., Sosa, I. R., Oram, D., Judd, A., Amarnath, K., Amarnath, V., et al. (2015) Modification of platelet proteins by malondialdehyde: prevention by dicarbonyl scavengers. *J. Lipid Res.* **56**, 2196–2205
- Slatter, D. A., Bolton, C. H., and Bailey, A. J. (2000) The importance of lipid-derived malondialdehyde in diabetes mellitus. *Diabetologia* **43**, 550–557
- Grönwall, C., Vas, J., and Silverman, G. J. (2012) Protective roles of natural IgM antibodies. *Front. Immunol.* **3**, 66
- Chou, M.-Y., Fogelstrand, L., Hartvigsen, K., Hansen, L. F., Woelkers, D., Shaw, P. X., et al. (2009) Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J. Clin. Invest.* **119**, 1335–1349
- Grönwall, C., Clancy, R. M., Getu, L., Lloyd, K. A., Siegel, D. L., Reed, J. H., et al. (2016) Modulation of natural IgM autoantibodies to oxidative stress-related neo-epitopes on apoptotic cells in newborns of mothers with anti-Ro autoimmunity. *J. Autoimmun.* **73**, 30–41
- Wang, C., Turunen, S. P., Kumm, O., Veneskoski, M., Lehtimäki, J., Nissinen, A. E., et al. (2013) Natural antibodies of newborns recognize oxidative stress-related malondialdehyde acetaldehyde adducts on apoptotic cells and atherosclerotic plaques. *Int. Immunol.* **25**, 575–587
- Joshua, V., Loberg Haarhaus, M., Hensvold, A., Wahamaa, H., Gerstner, C., Hansson, M., et al. (2023) Rheumatoid arthritis specific autoimmunity in the lung before and at the onset of disease. *Arthritis Rheumatol.* <https://doi.org/10.1002/art.42549>
- Kumm, O., Turunen, S. P., Prus, P., Lehtimäki, J., Veneskoski, M., Wang, C., et al. (2014) Human monoclonal Fab and human plasma

- antibodies to carbamyl-epitopes cross-react with malondialdehyde-adducts. *Immunology* **141**, 416–430
37. Sakuraba, K., Krishnamurthy, A., Sun, J., Zheng, X., Xu, C., Peng, B., *et al.* (2022) Autoantibodies targeting malondialdehyde-modifications in rheumatoid arthritis regulate osteoclasts via inducing glycolysis and lipid biosynthesis. *J. Autoimmun.* **133**, 102903
  38. Hensvold, A., Horuluoglu, B., Sahlström, P., Thyagarajan, R., Diaz Boada, J. S., Hansson, M., *et al.* (2023) The human bone marrow plasma cell compartment in rheumatoid arthritis - clonal relationships and anti-citrulline autoantibody producing cells. *J. Autoimmun.* **136**, 103022
  39. Wang, S., Wang, J., Kumar, V., Karnell, J. L., Naiman, B., Gross, P. S., *et al.* (2018) IL-21 drives expansion and plasma cell differentiation of autoreactive CD11c<sup>hi</sup>T-bet<sup>+</sup> B cells in SLE. *Nat. Commun.* **9**, 1–14
  40. Wang, Y., Lloyd, K. A., Melas, I., Zhou, D., Thyagarajan, R., Lindqvist, J., *et al.* (2019) Rheumatoid arthritis patients display B-cell dysregulation already in the naïve repertoire consistent with defects in B-cell tolerance. *Sci. Rep.* **9**, 19995
  41. Cowan, G. J. M., Miles, K., Capitani, L., Giguere, S. S. B., Johnsson, H., Goodyear, C., *et al.* (2020) Human autoimmunity, a substantial component of the B cell repertoire consists of polyclonal, barely mutated IgG(+ve) B cells. *Front. Immunol.* **11**, 395
  42. Nirula, A., Glaser, S. M., Kalled, S. L., and Taylora, F. R. (2011) What is IgG4? A review of the biology of a unique immunoglobulin subtype. *Curr. Opin. Rheumatol.* **23**, 119–124
  43. Elliott, S. E., Kongpachith, S., Lingampalli, N., Adamska, J. Z., Cannon, B. J., Mao, R., *et al.* (2018) Affinity maturation drives epitope spreading and generation of pro-inflammatory anti-citrullinated protein antibodies in rheumatoid arthritis. *Arthritis Rheumatol.* **70**, 1946–1958
  44. Titcombe, P. J., Wigerblade, G., Sippl, N., Zhang, N., Shmagel, A. K., Sahlström, P., *et al.* (2018) Pathogenic citrulline-multispecific B cell receptor clades in rheumatoid arthritis. *Arthritis Rheumatol.* **70**, 1933–1945
  45. Wyatt, T. A., Kharbanda, K. K., McCaskill, M. L., Tuma, D. J., Yanov, D., DeVasure, J., *et al.* (2012) Malondialdehyde-acetaldehyde-adducted protein inhalation causes lung injury. *Alcohol* **46**, 51–59
  46. Catrina, A. I., Ytterberg, A. J., Reynisdottir, G., Malmström, V., and Klareskog, L. (2014) Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. *Nat. Rev. Rheumatol.* **10**, 645–653
  47. Amara, K., Clay, E., Yeo, L., Ramsköld, D., Spengler, J., Sippl, N., *et al.* (2017) B cells expressing the IgA receptor FcRL4 participate in the autoimmune response in patients with rheumatoid arthritis. *J. Autoimmun.* **81**, 34–43
  48. Falini, B., Tiacci, E., Pucciarini, A., Bigerna, B., Kurth, J., Hatzivassiliou, G., *et al.* (2003) Expression of the IRTA1 receptor identifies intraepithelial and subepithelial marginal zone B cells of the mucosa-associated lymphoid tissue (MALT). *Blood* **102**, 3684–3692
  49. Yeo, L., Lom, H., Juarez, M., Snow, M., Buckley, C. D., Filer, A., *et al.* (2015) Expression of FcRL4 defines a pro-inflammatory, RANKL-producing B cell subset in rheumatoid arthritis. *Ann. Rheum. Dis.* **74**, 928–935
  50. Aletaha, D., Neogi, T., Silman, A. J., Funovits, J., Felson, D. T., Bingham, C. O., *et al.* (2010) 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League against Rheumatism Collaborative Initiative. *Ann. Rheum. Dis.* **69**, 1580–1588
  51. Amara, K., Israelsson, L., Stålesen, R., Sahlström, P., Steen, J., Malmström, V., *et al.* (2019) A refined protocol for identifying citrulline-specific monoclonal antibodies from single human B cells from rheumatoid arthritis patient material. *Bio Protoc.* **9**, e3347
  52. Lefranc, M. P., Giudicelli, V., Ginestoux, C., Jabado-Michaloud, J., Folch, G., Bellahcene, F., *et al.* (2009) IMGT, the International ImMunoGeneTics Information System. *Nucleic Acids Res.* **37**, D1006–D1012
  53. Hansson, M., Mathsson, L., Schleder, T., Israelsson, L., Matsson, P., Nogueira, L., *et al.* (2012) Validation of a multiplex chip-based assay for the detection of autoantibodies against citrullinated peptides. *Arthritis Res. Ther.* **14**, R201
  54. Gonen, A., Hansen, L. F., Turner, W. W., Montano, E. N., Que, X., Rafia, A., *et al.* (2014) Atheroprotective immunization with malondialdehyde-modified LDL is hapten specific and dependent on advanced MDA adducts: implications for development of an atheroprotective vaccine. *J. Lipid Res.* **55**, 2137–2155
  55. Ossipova, E., Cerqueira, C. F., Reed, E., Kharlamova, N., Israelsson, L., Holmdahl, R., *et al.* (2014) Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatoid joint. *Arthritis Res. Ther.* **16**, R167
  56. Alzolibani, A. A., Rasheed, Z., and Al Robaee, A. A. (2014) Acquired immunogenicity of DNA after modification with malondialdehyde in patients with alopecia areata. *Scand. J. Clin. Lab. Invest.* **74**, 312–321
  57. Mistry, N., Podmore, I., Cooke, M., Butler, P., Griffiths, H., Herbert, K., *et al.* (2003) Novel monoclonal antibody recognition of oxidative DNA damage adduct, deoxycytidine-glyoxal. *Lab. Invest.* **83**, 241–250
  58. Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., *et al.* (2019) The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* **47**, W636–W641
  59. Yu, G. (2020) Using ggtree to visualize data on tree-like structures. *Curr. Protoc. Bioinformatics* **69**, e96