Immunity against post-translationally modified proteins in autoimmune diseases

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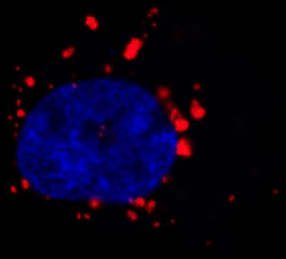

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Post-translationally modified proteins bind and activate complement with implications for cellular uptake and autoantibody formation

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Abstract

Introduction

Autoimmune diseases, such as rheumatoid arthritis (RA), are characterized by the presence of autoantibodies including those targeting self-proteins modified by post-translational modifications (PTMs). The complement system is known for its role in innate immune defense, but also in clearing debris and induction of antibody responses. We therefore hypothesized that complement could directly bind to PTMs and target PTM-modified proteins for clearance, or stimulate (chronic) inflammation and development of anti-PTM autoimmunity.

Methods

Six PTMs were investigated: nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE). We used mass spectrometry and plate-bound assays to analyze binding of serum proteins to PTM-modified proteins. The impact of complement activation on cellular uptake was studied in phagocytosis assays. The relationship between complement SNPs, and presence of anti-PTM autoantibodies was analyzed in 587 RA patients.

Results

Mass spectrometry analysis revealed a strong binding of complement to proteins modified with Ca, Ac, MAA and AGE but not to Nt and Cit. These observations were confirmed by plate-bound assays revealing that Ca-, MAA- and AGE-modified proteins activated the classical pathway, without involving antibodies. Ac activated the lectin pathway through ficolin-3. Complement activation on Ca-, Ac-, MAA- and AGE-coupled beads enhanced phagocytosis. SNPs in complement genes, associated with higher complement activity, were strongly associated with the presence of anti-PTM antibodies in RA patients.

Conclusion

Proteins containing the PTMs Ca, Ac, MAA or AGE activate complement. These complement opsonized PTMs increase phagocytosis and may lead to the development of anti-PTM antibodies.

Introduction

In patients with rheumatoid arthritis (RA) autoantibodies against self-proteins that have undergone a post-translational modification (PTM) are used as diagnostic and prognostic markers (1). These include anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein (anti-CarP) antibodies. Remarkably, these anti-PTM antibodies are present before onset of clinical symptoms and correlate with increased joint destruction over time (2, 3). RA patients benefit from B-cell targeted therapy, suggesting that B-cell mediated (anti-PTM) autoimmunity is playing a pathogenic role (4). However, what triggers the induction of autoantibody responses is currently unknown. Importantly, how the PTMs themselves contribute to inflammation is currently unknown. Unraveling the impact of PTM-driven immune activation on formation of anti-PTM antibody responses and on (chronic) inflammation would allow the design of specific early interventions.

In healthy individuals, proteins are frequently subjected to PTMs. There are more than 650 PTMs described affecting many aspects of protein functions (5). Some PTMs are essential for cellular processes and some take place outside the cell, maintaining homeostasis. A well-known example is phosphorylation, which is typically a reversible process. Other PTMs are largely irreversible such as glycosylation and glycation. Some irreversible PTMs alter the primary structure and lead loss of protein function forming neoantigens. In case long lived proteins are subjected to such irreversible PTMs, they may persist in the body, exposed to the immune system to be detected. While PTMs will occur in all individuals, only a subset of the individuals will produce autoantibodies against certain PTM-modified proteins (6, 7). To date, there is no understanding on how the human body processes these PTM-modified proteins and how these PTM-modified proteins impact (chronic) inflammation and autoimmunity. However, it is likely that PTMs that disrupt protein function are targeted for clearance. In addition to Ca and Cit, implicated in RA, several other PTMs have been reported to be immunogenic (8-10). One immunological system that is particularly known for its clearance function is the complement system.

Binding of complement recognition molecules such as C1q (11) (e.g. to cellular debris, dead cells and immune complexes), results in complement activation, opsonization of the target and cell-mediated clearance. Individuals that lack the early components of the classical pathway (e.g. C1q), frequently develop the autoimmune disease systemic lupus erythematosus (SLE), in part as a consequence of defective clearance (11, 12). In case of aberrant recognition or opsonization, a strong immune response against components of the accumulating debris may occur, including the generation of anti-PTM antibodies. Such a breach in tolerance has been described for several PTMs in the context of RA (13), SLE (14), cardiovascular diseases (15) and autoimmune hepatitis (16). We set out to

identify triggers that induce anti-PTM autoantibodies. Since the complement system is known for its role in clearance of debris and for stimulating adaptive immune responses, we hypothesize that complement can bind directly to PTM-modified proteins and impact on clearance and immune activation. Opsonized PTM-modified proteins can then be recognized by cells expressing complement receptors (CRs). CR3- and CR4-expressing phagocytic cells, such as macrophages and neutrophils, could then clear these opsonized PTM-modified proteins. B-cells specifically express CR2 (CD21) which is known to bind to complement opsonization fragment C3d, enabling co-ligation of the BCR and CR2, thus lowering the threshold for B-cell activation (17, 18).

Whether an individual will clear a PTM-modified protein or mount an autoreactive B-cell response is dependent on both genetic and environmental factors (19, 20). Certain combinations of single nucleotide polymorphisms (SNPs) in complement genes (the so called complotype), may result in high or low complement-activating profiles (19), that may predispose to the breaking of tolerance in only some individuals. In addition, several SNPs in complement activating protein C1q were described to be associated with development of RA (21) and SLE (22-24).

In this study we focus on six PTMs: nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde acetaldehyde adducts (MAA) and advanced glycation end-products (AGE). Each of these PTMs and/or antibodies against these PTMs are associated with a variety of disease such as RA, diabetes and coronary artery disease (1, 25-28). Additionally, some of these PTMs are structurally very different. Nt for example is a modification of tyrosine, Cit of arginine and Ca, Ac, MAA and AGE are all modifications of the amino acid lysine. We studied the capacity of PTM-modified proteins to bind and activate complement and enhance cellular binding and phagocytosis. Additionally, the relationship between SNPs in complement genes and positivity for anti-PTM antibodies was analyzed to obtain insight into the relevance of these findings in patients.

Material and Methods

Protein modification

Modified proteins were produced by either enzymatic or chemical reactions as previously described with minor adaptations for some of the modifications. For all modifications, fetal calf serum (FCS, Bodinco, Alkmaar, the Netherlands), IgG- and IgA-depleted fibrinogen (Fib, F4883, Sigma) and human serum albumin (HSA, A1653, Sigma) were used as backbones. Fibrinogen was depleted of IgG and IgA using protein G Plus/Protein A agarose suspension (IP05, Invitrogen).

For nitration 6 equal amounts of peroxynitrite (14042-01-4, Cayman Chem) were used directly from the stock solution to create an end concentration of 6 mM (29) for 1mg/mL protein. After addition of every aliquot the mix was directly vortexed for 30 seconds and left on ice for one minute before adding the next aliquot. After addition of the last aliquot the samples were incubated for 1 hr at 37°C. Citrullinated proteins were created by incubating 1 mg protein in a volume of 1 mL MilliQ containing 0.1 M TRIS-HCl pH 7.6, 5 mM DL-DTT (D0632, Sigma), 1U peptidyl arginine deiminase 4 enzyme/100µL (PAD enzyme, 1584, Sigma) and 10 mM $CaCl_2$ (21097, Sigma) (30). The mixture was incubated for 6 hr at 53°C. After incubation, citrullinated protein was spun down and stored at -80°C. For carbamylation of proteins, first a 2 M potassium cyanate (KOCN, 215074, Sigma) solution in PBS was prepared (3). 4 mg/mL protein was added to 2 M KOCN in a 1:1 volume-to-volume ratio and incubated for 12 hr at 37°C. Acetylation was performed using protein at a concentration of 1 mg/mL in 0.1 M Na_2CO_3. 50 µL of acetic anhydride (100042, Merck) and 200 µL of pyridine (109728, Merck) were added per 10 mL of protein solution (31). Proteins were incubated for 5hr at 30°C. After incubation, the reaction was stopped using 200 µL (per 10 mL solution) of 1 M TRIS. Samples were concentrated using Amicon Ultra – 15 centrifugal filter unit (10 kDa, UFC901024, Sigma) and buffer was exchanged to PBS using Zeba Spin desalting columns (89890, Thermofisher). For malondialdehyde-acetaldehyde adducts, first an malondialdehyde solution was prepared using 0.5 M 1,1,3,3,-Tetramethoxypropane (108383, Sigma) and 0.3% hydrochloric acid (1003171000, EMSURE) and incubated for 12 min at 37°C in a water bath (32). Meanwhile, 20 mg/mL protein was prepared and diluted 1:5 with 20% MilliQ, 20% freshly prepared MDA solution, and 4% acetaldehyde (402788, Sigma); pH was adjusted to 4.8 and MilliQ was added till 5 times volume of protein was reached. The solution was then incubated for 2 hr at 37°C in a water bath. Advanced glycation end-products were created by incubating 2 mg/mL protein with 1:100 diluted 3.3 M glycolaldehyde (23147-58-2, Sigma) (33). The solution was filtered using a 0.2 µm Whatman filter (10462200, GE Healthcare) and incubated for 10 days at 37°C while shaking. Following incubation, all (modified) proteins (except for Cit, which was spun down) were extensively dialyzed against PBS using 10 kDa cut-off SnakeSkin dialyzing tubes (88243, Thermofisher). All modifications were stored at -20°C until used. Protein concentrations were measured using Bradford protein assay (500-0006, Biorad) or Nanodrop.

The presence of PTM on the proteins was verified in an ELISA-based assay using anti-PTM specific antibodies. To detect Nt, mouse anti-nitrotyrosine (ab61392, abcam) was used. Mouse anti-citrulline (F95, MABN328, MilliPore) was used to detect Cit, rabbit anti-CBL (STA-078, biolabs) to detect Ca, rabbit anti-acetyllysine (ADI-KAP-TF120-E, Enzo Lifesciences) to detect Ac, goat anti-MAA-HRP (ab20703, abcam) to detect MAA and mouse anti-AGE (4B5, kindly provided by Dr. H de Boer, LUMC, Leiden, The Netherlands) (34) to detect AGE. Additionally, to verify that the PTM of interest is solely on the

modified proteins, mass spectrometry analysis was performed as described before and peptide-spectrum matches (PSMs) were determined to assess the concurrent presence of all six PTMs in each protein preparation (35, 36).

Anti-PTM IgG detection

Anti-PTM IgG antibodies were detected in sera from patients out of three cohorts consisting of patients with RA, SLE or AILD. RA samples were obtained from the Leiden Early Arthritis Cohort (EAC) (37). Details on SLE and AILD cohorts used in this manuscript are previously published in detail. Briefly, SLE samples were obtained from patients visiting the NPSLE clinic of LUMC between 2007 and 2019 with the clinical diagnosis of SLE (14). AILD samples were obtained from patients visiting the Department of Gastroenterology and Hepatology of the LUMC or Department of Gastroenterology and Hepatology between 1996 and 2020 (16). Modified FCS and nonmodified FCS were coated at 10 µg/mL in 0.1 M carbonate-bicarbonate buffer pH 9.6 (CB) on Nunc MaxiSorp plates (430341, Thermofisher) overnight at 4°C. In between each step plates were washed using washing buffer containing PBS and 0.05% Tween (P1379, Sigma). After washing, plates were blocked using PBS/1%BSA (blocking buffer) for 6 hr at 4°C. Following washing, wells were incubated with serum at a 1/50 dilution in PBS/0.05% Tween/1% BSA (PTB) for Nt, Cit, CarP and Ac and at a 1/100 or 1/1000 in PTB for AGE and MAA respectively. For each PTM, a standard of a pool of anti-PTM antibody-positive sera was taken along in serial dilutions on each plate. Human IgG was detected using rabbit anti-human IgG-HRP (P0214, Dako) diluted in PTB and incubated at 4°C for 3.5 hr. After the final wash, HRP enzyme activity was visualized using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck), and absorbance at 415 nm was read using a microplate reader (Bio-Rad). Absorbance was transformed to arbitrary units per milliliter (aU/mL) using the corresponding standard line. Background aU/mL of FCS was subtracted from the aU/mL signal on FCS-PTM to analyze specific anti-PTM antibody reactivity. The cut-off was calculated based on the mean plus two times the SD of the specific anti-PTM antibody reactivity of the healthy controls (HC). Specific signals of HC ten times higher than the mean were excluded in calculating the cut-off.

Binding of serum components to PTM-modified proteins

Binding of serum components to PTM-modified proteins was assessed in an unbiased way using mass spectrometry analysis. For this purpose we biotinylated FCS using EZ-link Sulfo-NHS-SS-biotin kit (21331, Pierce) according to manufacturer's instructions and modified biotinylated FCS as previously described. Biotinylated PTM-modified proteins and biotinylated non-modified proteins were incubated with streptavidin beads (CP01000-CP01N, Bangs Laboratories) at a concentration of 500 µg/mL and 3 µg proteins/µL beads, for 1 hr at 20°C in a shaking heat block at 400 rpm. After incubation, beads were washed twice using PBS/0.05%Tween and once using PBS and centrifuged

for 5 minutes at 10,000 rpm. Next, coupled beads were incubated in 10% normal human serum (NHS) in RPMI in a total volume of 150 µL for 1 hr at room temperature (RT). After incubation, beads were washed and resuspended in 15 µL PBS. Samples were incubated with 1:1 sample buffer with the addition of β-mercaptoethanol (1610737, Bio-Rad and M650, Sigma) for 10 minutes at 95°C. After incubation, samples were centrifuged for 2 min at 13,000 rpm. Samples were run a 4-15% gradient gel (456-1083, Biorad) according to manufacturer's instructions and proteins were visualized using instant stain (ISB1L, Expedeon). Bands of interest were subsequently excised for mass spectrometry analysis.

Analysis of gel bands

Gel slices were washed, subjected to reduction with dithiothreitol, alkylation with iodoacetamide and in-gel trypsin digestion using a Proteineer DP digestion robot (Bruker). Tryptic peptides were extracted from the gel slices and lyophilized.

Peptides were dissolved in 95/3/0.1 v/v/v water/acetonitril/formic acid (FA) and analyzed by online C18 nanoHPLC MS/MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a LUMOS mass spectrometer (Thermo). Samples were injected onto an in-house made precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via an in-house made analytical nano-HPLC column (30 cm × 50 µm; Reprosil-Pur C18-AQ 3 µm). The gradient was run from 10% to 40% solvent B (20/80/0.1 water/acetonitrile/ FA v/v) in 30 min. The nano-HPLC column was drawn to a tip of ~10 µm and acted as the electrospray needle of the MS source. The LUMOS mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 seconds, with an HCD collision energy at 32% and recording of the MS2 spectrum in the orbitrap. In the master scan (MS1) the resolution was 120,000, the scan range 400-1500, at an AGC target of 'standard'. A lock mass correction on the background ion m/z=445.12003 was used. Dynamic exclusion was performed after n=1 with exclusion duration of 10 s. Charge states 2-5 were included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2 Da. The MS scan range was set to 'auto'. The MS2 scan resolution was 30,000 with an AGC target of 'standard' with a maximum fill time of 60 ms. In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.5 (Thermo Scientific), and then submitted to the Uniprot Human minimal database (20596 entries), using Mascot v. 2.2.07 (www.matrixscience.com) for protein identification. Mascot searches were done with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin was specified as the enzyme. Methionine oxidation was set as a variable modification. Carbamidomethyl on cysteine was set as a fixed modification. The false discovery rate was set < 1%.

Subsequently, the abundance as well as abundance ratio (Abundance (Grouped) PTM / Abundance (Grouped) Control) were determined using Proteome Discoverer. Ratios

above 2 were considered enriched and proteins were sorted accordingly. Top 20s of proteins being enriched on the PTM-modified proteins were combined and duplicates removed, resulting in a list of 60 proteins. Proteins were sorted for being enriched on the number of PTMs for which they show a ratio above 2. Next, for each PTM-modified protein with a ratio above 2 data was entered in STRING pathway analysis program (https://string-db.org/) using the ''Proteins with Values/Ranks – Functional Enrichment Analysis'' option. Using the STRING database, a list of biological processes were generated.

Complement activation assay

Complement activation on PTM-modified proteins was assessed by coating PTM-modified proteins or unmodified control on Nunc MaxiSorp plates (430341, Thermofisher) at a concentration of 10 µg/mL in bicarbonate coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation plates were blocked using blocking buffer (PBS/1% BSA) for 1 hr at 37°C. Next, plates were incubated with serial dilutions of NHS in RPMI for 1 hr at 37°C. Then, bound complement components were detected using 1:1000 rabbit anti-human C1q (A0136, Dako), 1:1000 rabbit anti-human C3d (A0063, Dako), 1:333 mouse anti-HuC5b9 (M0777, Dako) or 1:1000 goat anti-human Factor H (A312, Quidel) diluted in PTB buffer. Plates were incubated for 1hr at 37°C whereafter primary antibodies were detected using matched HRP-labeled antibodies; goat anti-rabbit IgG-HRP (P0448, Dako), goat anti-mouse IgG-HRP (P0447, Dako) or rabbit anti-goat IgG-HRP (P0449, Dako) diluted in PTB buffer. Secondary antibodies were incubated for 1hr at 37°C after which plates were washed and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Binding assay of purified components

To assess binding of purified serum components, Nunc MaxiSorp plates (430341, Thermofisher) were coated with PTM-modified proteins or non-modified counterpart at a concentration of 10 µg/mL in bicarbonate coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. As a standard curve, purified serum components (C1q, A099, Complement Technology; or Factor H, A137, Complement Technology) were directly coated on the plate in bicarbonate coating buffer. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation plates were blocked using blocking buffer (PBS/1% BSA) for 1 hr at 37°C. Next, plates were incubated with serial dilutions of purified C1q or purified Factor H and their binding was detected using rabbit anti-human C1q or goat anti-human Factor H (A0136, Dako and A312, Quidel). Primary antibodies were detected using goat anti-rabbit IgG-HRP (P0448, Dako) or rabbit anti-goat IgG-HRP (P0449, Dako) diluted in PTB buffer. Secondary antibodies were incubated for 1 hr at 37°C after which plates were washed

and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Complement activation routes

To study which of the three complement pathways (classical (CP), lectin (LP) or alternative (AP)) were activated by the PTM-modified proteins we performed plate-bound complement activation assays. First we established that we could detect the specific activation of each of the pathways. For this purpose we analyzed the three routes using IgM, LPS and mannan as ligands for the CP, LP and AP, respectively. Nunc MaxiSorp plates (430341, Thermofisher) were coated with human IgM at 2 µg/mL (1838-8, Protos Immunoresearch), LPS at 10 µg/mL (L6386, Sigma) or mannan at 100 µg/mL (M7504, Sigma) in coating buffer (0.1M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. After we verified that we could allocate each pathway correctly, we bound PTM-modified proteins and non-modified proteins at a concentration of 10 µg/mL. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation with the ligands, plates were blocked using blocking buffer (PBS/1%BSA) for 1 hr at 37°C. To analyze activity of only one complement pathway, different complement-deficient/depleted sera and buffers were used. After washing, plates were incubated with serial dilutions (10%, 1%, 0.1%) of NHS or C1q-depleted human serum (A300, Complement Technology) in RPMI to analyze CP, MBL-deficient human serum (collected from a MBL deficient individual) in RPMI to analyze LP, or Factor B-depleted human serum (A335, Complement Technology) in RPMI-5 mM/10 mM MgEGTA ($MgCl_2$*$6H_2O$: 7791-18-6, Sigma and EGTA: E4378, Sigma) to analyze the AP and incubated for 1 hr at 37°C. Sera diluted in RPMI-10mM EDTA were taken along as negative control. After washing, bound C3 (AP) or C4 (CP and LP) was detected using 1:1000 rabbit anti-human C3d (A0063, Dako) or 1:1000 goat anti-human C4 (A305, Quidel) respectively, diluted in PTB Buffer. Additionally, Ficolin-1, -2 and -3 were detected in NHS diluted in RPMI using mouse anti-human Ficolin-1 (clone 7G1,HM2196, Hycult), DIG-labeled mouse anti-human Ficolin-2 (L-ficolin clone GN5, HM2091, Hycult) and mouse anti-human Ficolin-3 (H-ficolin clone 4H5, HM2089, Hycult) to confirm LP activation on acetylated protein as described before (38). Plates were incubated for 1 hr at 37°C whereafter primary antibodies were detected using matched HRP-labeled antibodies: goat anti-rabbit IgG-HRP (P0448, Dako), rabbit anti-goat IgG-HRP (P0449, Dako), goat anti-mouse IgG-HRP (P0447, Dako) or sheep anti-DIG-HRP (1120773910, Roche) diluted in PTB buffer. Secondary antibodies were incubated for 1 hr at 37°C after which plates were washed and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Preparing beads for binding and phagocytosis assays

To assess binding and phagocytosis of complement-opsonized PTM-modified proteins we employed phagocytosis assays using whole blood and differentiated THP-1

macrophages. For this purpose we biotinylated FCS using EZ-link Sulfo-NHS-SS-biotin kit (21331, Pierce) according to manufacturer's instructions and modified biotinylated FCS as previously described. Biotinylated PTM-modified proteins and biotinylated non-modified proteins were incubated with 0.4 µm or 1 µm fluorescent streptavidin beads (CFFR002 or CFFR004, Bangs Laboratories) at a concentration of 300 µg/mL and 5 µg protein per µL beads, for 1 hr at 20°C in a shaking heat block at 400 rpm. Beads were washed 3 times using 10x volume PBS at 10,000 rpm for 5 min. Supernatant was carefully removed to prevent pellets from being disturbed. For phagocytosis assays using differentiated THP-1 macrophages, PTM-modified protein-coupled beads (PTM-coupled beads) were incubated in the presence of RPMI, 10% NHS in RPMI, or 10% NHS in RPMI/10 mM EDTA for 1 hr at 37°C in a shaking heat block (400 rpm). Beads were washed 3 times using 10x volume PBS at 10,000 rpm for 5 min, resuspended at 10 times starting volume. Next, beads were analyzed for presence of C1q, C3c and added to whole blood (1 µm beads, 150 beads/cell) or added to THP-1 macrophages (0.4 µm beads 3000 beads/cell in FCS-free THP-1 medium) to investigate phagocytosis.

Binding assay using whole blood

Fresh heparin blood was collected by staff of the LUMC voluntary donor service. One mL blood was washed using PBS, to wash away the heparin, at 500 g for 5 min. Plasma was removed and blood resuspended in 1 mL RPMI. Five µL washed blood was added to a 96 wells plate and subsequently 1 µL serum/PBS, 1 µL PTM-coupled beads (+/-150 beads/cell; prepared as described before, without serum incubation) and 3 µL RPMI were added resulting in a total volume of 10 µL. The joined volumes were incubated for 1 hr at 37°C to allow binding and uptake of the PTM-coupled beads. 3 µL sample was stained using CD45-FITC (clone 2D1, 345808, BD), CD61-PE (clone VIPL2, 12-0619-42, eBioscience) and CD235a-PE-Cy7 (clone HI264, 349112, Biolegend) to identify leukocytes, platelets and erythrocytes respectively. Samples were acquired using a BD LSR Fortessa equipped with FACSDiva software. Geometric mean fluorescence intensity (GMFI) was determined and normalized to the control condition in the presence of 10% NHS, which was included in each experiment. As a control intravenous immunoglobulin (IVIg) was biotinylated and coupled to the streptavidin beads similar to the biotinylated PTM-modified proteins. Subsequently, data was corrected for the signal on control FCS.

Phagocytosis assay THP-1 macrophages

THP-1 acute monocytic leukemia cells (DSMZ ACC 16) were plated in 6, 24 or 96 wells culture plate at 0.2*10^6 cells/mL. Cells were incubated for 72 hr at 37°C/5% CO_2 in THP-1 medium (Dutch modified RPMI-1640 + 10% FCS + 2% penicillin/streptomycin + 2% Glutamax) in the presence of 400 nM PMA (Sigma, P8139). After 72 hr the medium was removed and fresh THP-1 medium without PMA was added. THP-1 macrophages were subsequently rested for 5 days at 37°C/5% CO_2 before use.

Medium was removed from differentiated THP-1 cells. Serum-free THP-1 medium (RPMI, Pen/Strep/Glutamax) was added to differentiated THP-1 cells. As a negative control, cells were incubated with 10 µM cytochalasin D (Cyto D, Sigma, C8273). Beads were resuspended in serum-free THP-1 medium (with or without 10 µM Cytochalasin D) at 3000 beads/cell and added to the plate. Plates were spun down at 200 g for 1 min in order for the beads to be close to the cells. Phagocytosis took place for 1 hr in the incubator at 37°C/5% CO_2. After incubation, residual beads were washed away and cells were detached using trypsin (25300-054, Gibco). Simultaneously, THP-1 cells were stained using 1/13 CD15-BV10 and 1/40 CD11b-APC antibodies (563141 and 333143, respectively, both BD Biosciences) for 30 min at 4°C in the dark. Samples were acquired using a BD Canto I. Percentage of differentiated THP-1 cells was determined based on expression of CD15 and CD11b. Phagocytosis was determined using GMFI of the APC channel. Data was corrected for Cytochalasin D which was taken along for each condition.

Genetics

587 RA samples from the Leiden Early Arthritis Cohort (EAC) study were sequenced for complement SNPs containing the major alleles rs800292 (*FH*), rs1061179 (*FH*), rs2230199 (*C3*) and rs641153 (*CFB*) and rs665691 (*C1qA*), rs292001 (*C1qA*), rs294179 (*C1qC*) (21, 37). The SNPs in *CFH*, *C3* and *CFB* are part of the complotype (19) and the SNPs in *C1q* have been described as a risk factor for developing RA and SLE (21, 22, 39). We first ran a global test for each anti-PTM antibody with a logistic regression model using the glm function with family binomial (40). These regression models contained all seven SNPs as independent variable, and one of the anti-PTM antibodies (anti-Nt, -Cit, -Ca, -Ac, -MAA, -AGE) as dependent variable. We compared the log-likelihood of these models with a null-model. We used the McFadden pseudo-R-squared (R^2) to estimate which proportion of the variance in anti-PTM antibody presence was explained by the total SNP set.
For each anti-PTM antibody where the genetics significantly improved the model's fit, we ran a logistic regression for each individual SNP against the anti-PTM antibody.

Statistical analysis

Where applicable, statistical analysis was performed using paired t-tests or repeated-measures (RM) one-way ANOVA with Dunnett's multiple comparisons test. Significance was defined as p-value ≤ 0.05. For statistical analysis and graphical representations, GraphPad Prism v10.2.3 was used (San Diego, USA). For analysis on genetics first a logistic regression model was run using the glm function with family binomial and Bonferroni correction using R v4.3.1. For the global genetic test we considered p< 0.008 as significant (correcting the p-value of 0.05 for six tests (one for each anti-PTM antibody) following Bonferroni's correction). When the global trend was positive, we ran the individual SNP tests where a p-value of 0.05 was considered significant (40). Next, the McFadden pseudo-R-squared (R^2) was used to estimate which proportion of the

variance in anti-PTM antibody presence was explained by the total SNP set.

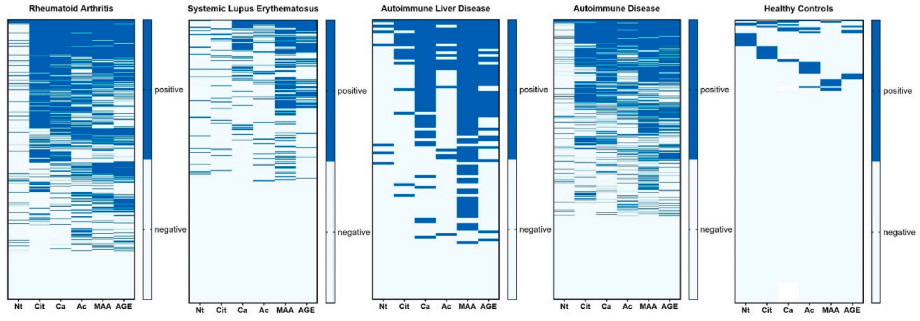
Results

Anti-PTM antibodies occur in many patients suffering from autoimmune diseases. In this study we focused on six PTMs: Nt, Cit, Ca, Ac, MAA and AGE. Fetal calf serum (FCS) was used as a model protein mix for post-translational modification. We confirmed that the desired PTM was present on the modified but not on the non-modified FCS using monoclonal antibodies against each PTM (Supplementary Figure 1A). Next, we confirmed, using mass spectrometry, that the procedures to achieve PTMs indeed resulted only in the desired PTM and did not trigger formation of other PTMs (Supplementary Figure 1B). Since ACPA and anti-CarP are associated with RA we analyzed the presence of anti-PTM autoantibodies against six PTMs in large cohorts of patients suffering from an autoimmune disease.

We screened the sera of 614 RA patients, 360 SLE patients and 106 patients suffering from auto-immune liver disease (AILD) relative to 197 healthy controls for the presence of anti-PTM antibodies (Supplementary Table 1 and Figure 1A). We observed that in all three autoimmune diseases there are substantial numbers of patients positive for anti-PTM antibodies. The clinical associations of positivity for anti-PTM antibodies have been published separately for each cohort (13, 14, 16). Here we focus on the presence and co-occurrence of these antibodies in autoimmune diseases

In RA patients 81,9% were positive for at least one anti-PTM antibody (Supplementary Table 1). In SLE and AILD 47,5% and 80,2% of all patients, respectively, were positive for at least one anti-PTM antibody. Interestingly, the highest percentage positivity was observed for anti-MAA in both SLE and AILD. In RA, anti-MAA was found in 46,7% of all patients and was therefore the second most common anti-PTM antibody in RA next to anti-Cit (49,3%). When all data of all autoimmune diseases were pooled, we observed that antibodies targeting Cit, Ca, Ac, MAA and AGE mostly co-occurred in autoimmune disease (Figure 1B). From these data we conclude that anti-PTM antibodies co-occur in many patients suffering from autoimmune diseases and that different combinations of anti-PTM antibodies exist. These data emphasize on the fact that anti-PTM antibodies are present and therefore relevant in autoimmunity.

A.



B.

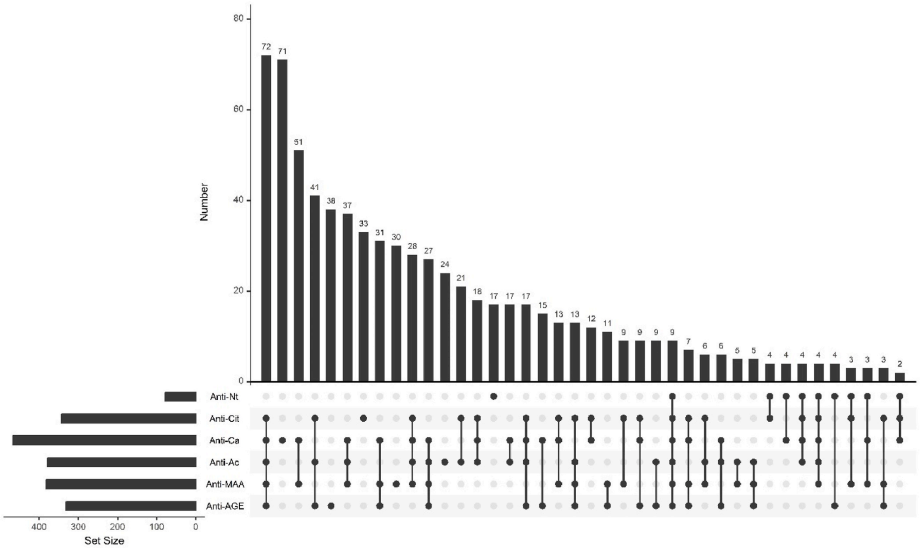


Figure 1: Anti-PTM antibodies against all six PTM-modified proteins are present in autoimmune disease. (A) Anti-PTM IgG positivity for each modification is presented in heatmaps for (from left to right) RA, SLE, AILD and all three auto immune diseases (RA, SLE and AILD) combined and HCs. Positivity was determined by calculating the mean reactivity of the HCs plus two times the standard deviation. (B) Upset plots of prevalence of anti-PTM antibody combinations in autoimmune disease. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; HC, healthy control; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein.*

Complement proteins bind more strongly to PTM-modified proteins compared to their non-modified controls.

To study how PTM-modified proteins could be involved in these chronic autoimmune diseases we set out to screen for plasma proteins that bind to PTM-modified proteins. For this purpose, we incubated PTM-modified-FCS-coupled beads (PTM-coupled beads)

with complement-active normal human serum (NHS). Of note, this NHS was prepared from sera of individuals with no detectable anti-PTM antibodies towards either of the six PTMs. The beads were washed and bound proteins were analyzed by mass spectrometry. We compared the relative abundance of specific proteins, based on the same unique peptides, on the PTM-coupled beads versus the non-modified-FCS-coupled beads and obtained insight into the enrichment of binding to the PTM-coupled beads by evaluating the ratios (Figure 2 and Supplementary Table 2).

Analysis of the data revealed strong enrichment of complement proteins binding to four of the six PTM-modified proteins compared to non-modified FCS. Seven of the top 10 hits were complement components including C1q, C4, C3 and Factor H (Figure 2). There were pronounced differences in the specific complement proteins binding to each of the PTM-modified proteins. We observed that proteins modified with the PTMs Ca, MAA and AGE bound proteins of the classical pathway in particular. The Ac-modified protein bound a similar set of complement proteins but did not show enhanced binding for C1q subunit A and C, suggesting a different activation pathway. Noteworthy is that Ac has been described to bind ficolin-3 (38). Indeed, we confirmed in our mass spectrometry analysis that ficolin-3 is enriched on Ac (Supplementary Table 2). The protein containing the PTMs Nt and Cit bound fewer proteins and did not reveal a similar enrichment of complement proteins (Figure 2).

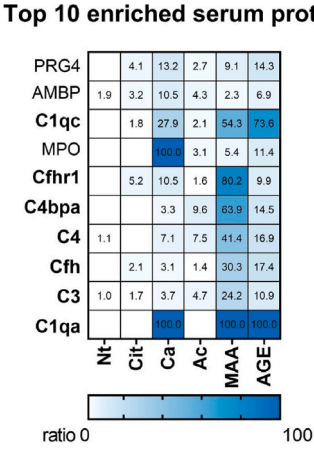


Figure 2: Seven out of top 10 enriched serum components binding to PTM-modified proteins are complement components. Heatmap of top ten enriched proteins binding to PTM-modified proteins compared to its control non-modified protein indicated by ratio abundance PTM / abundance Control. Ratio above two (filled blue boxed) were considered enriched. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylation; AMBP, alpha-1-microglobulin; C1qA, complement C1q subcomponent subunit A; C1qc, complement C1q subcomponent subunit C; C3, complement C3; C4b, complement C4b; C4bpa, C4b-binding protein alpha chain; Ca, carbamylation; Cit, citrullination; Cfh, complement factor H; Cfhr1, complement factor H-related protein 1; MAA, malondialdehyde-acetaldehyde adduct; MPO, myeloperoxidase; Nt, nitration; PRG4, proteoglycan 4.*

STRING pathway analyses were performed on proteins with a ratio above two, revealing that most proteins were involved in protein activation cascades and complement activation (data not shown).

PTM-modified proteins activate the complement system

To investigate whether PTM-modified proteins were able to activate the complement system we performed an ELISA-based assay. Complement binding and activation on PTM-modified proteins was assessed by detecting plate-bound C1q and Factor H as well as deposition of C3 and C5b9, respectively. We determined optimal assay conditions using carbamylated proteins first. Complement was increasingly activated on carbamylated proteins at the level of C1q upon increasing complement active serum concentrations until 1% NHS (Figure 3A). C1q binding was not observed on non-modified protein, indicating that C1q binding is PTM-specific. For all other modifications one concentration (1% or 3% NHS, depending on the read out) was chosen and depicted in heatmaps. Next to Ca, also MAA- and AGE-modified proteins were able to bind C1q.

When analyzing downstream complement activation markers C3d and C5b9, we observed that Ca, MAA, AGE and also Ac were able to activate complement at the level of C3 and C5b9. Likewise, Factor H binding was observed for Ca, Ac, MAA and AGE. Since the FCS used in our experiments is a rich mix of different proteins we next confirmed that the same observations could be made using defined purified proteins. Indeed, using PTM-modified versions of purified human fibrinogen and human serum albumin in the complement activation assays, we observed a very similar complement activation profile indicating that complement activation was PTM-specific and not dependent on the protein backbone where the modifications are present (Figure 3A).

PTM-modified proteins bind complement independently of antibodies

After establishing that complement was activated by PTM-modified proteins, we assessed binding of purified complement components to investigate whether complement components bind directly to the PTM-modified proteins or indirectly through the presence of antibodies in the serum (Figure 3B). Ca, MAA and AGE modified proteins were able to bind purified C1q in a dose-dependent fashion. In addition, we verified whether Factor H was binding directly to the PTM-modified proteins or bound the deposited C3b. Binding of purified Factor H was readily observed for MAA-modified proteins, while Ca-, Ac- and AGE-modified proteins did not bind factor H. These data indicate that for Ca, Ac and AGE the Factor H binding observed in the complement activation assays is mainly indirect and a consequence of C3b binding. Additionally, similar assays were performed using the non-complement plasma proteins human serum albumin (HSA), alpha-2-macroglobulin (A2M) and alpha-1-antitrypsin (A1AT) in order to exclude that not just any plasma protein was binding to the PTM-modified protein (Supplementary Figure 2).

Indeed, we observed that these abundantly present plasma proteins were not binding to the PTM-modified proteins.

Ca, Ac, MAA and AGE activate complement through different complement pathways

Next, we set out to investigate which of the three complement pathways, classical (CP), lectin (LP) or alternative (AP), was activated by the PTM-modified proteins. For this purpose, we employed complement activation assays using serum depleted or deficient for specific complement components and made use of pathway-specific buffers. On top of that, we detected complement activation at the level of C4 and C3 to further discriminate between the pathways. We verified the experimental set up by making use of IgM (CP), mannan (LP) or LPS (AP) as a coating and C1q-depleted serum in RPMI, MBL-deficient serum in RPMI and Factor B-depleted serum in RPMI Mg-EGTA, respectively, as a source of complement. We compared these sera and buffer combination to NHS in RPMI to verify that we could correctly allocate the activation pathway of these positive controls. (Supplementary Figure 3). Next, the PTM-modified proteins were used as the coating and the same set of reagents was used (Figure 3C). With increasing concentrations of NHS, the complement system was activated on FCS-Ca. No complement activation was observed when C1q-depleted serum was used, indicating that the CP was activated on FCS-Ca. For the other PTM-modified proteins a dilution of 1% serum was chosen to assess the CP and LP and 10% to assess the AP. The complement system was not activated on Nt and Cit, as was observed before, and no pathway could be allocated. Similarly to FCS-Ca, also FCS-MAA and FCS-AGE activated complement in the presence of NHS and did not activate complement when using C1q-depleted serum, indicating that the CP was activated and essential on these PTM-modified proteins. For FCS-Ac, complement activation was still observed even when using C1q-depleted serum and MBL-deficient serum. However, earlier reports indicate that acetylated proteins may trigger the LP through binding of ficolins and not MBL (38). We therefore tested ficolin binding to FCS-Ac. Indeed, we observed that ficolin-3 was specifically binding to FCS-Ac, leading to complement activation (Figure 3D). Altogether, these data indicate that the CP was activated on FCS-Ca, -MAA and -AGE and the LP on FCS-Ac.

C1q binding to Ca-, MAA- and AGE-modified proteins was significantly decreased when C1q was preincubated with F(ab')2 fragments of an anti-C1q-head but not anti-C1q-tail antibody (data not shown). Additionally, C1q binding was abrogated only in the presence of 2M NaCl and not in the presence of heparin or low pH (data not shown). These data indicate that C1q binds to Ca, MAA and AGE through its globular heads and that the interaction is an electrostatic interaction.

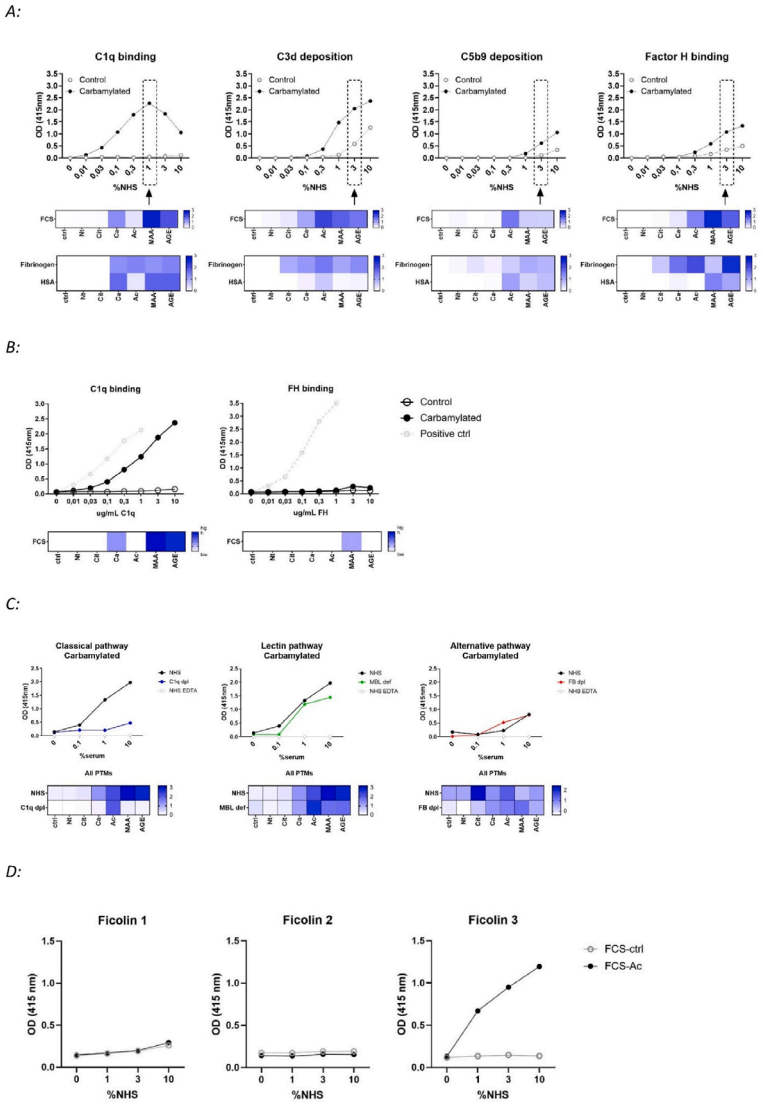


Figure 3: Complement binds directly and is activated on specific PTM-modified proteins. (A) Both complement binding (C1q and Factor H) and complement activation (C3d and C5b9) were measured using ELISA using a pool of complement active serum. (B) Direct binding of C1q and Factor H was assessed using purified C1q and Factor H, respectively. Directly coated C1q and Factor H were taken along as positive controls. (A,B) In the upper figures, plates were coated with carbamylated or control protein and serially diluted serum or purified complement was added and assessed. Below, one concentration was chosen and heatmaps were created to visualize reactivity for all six PTM-modified proteins and non-modified protein. Complement activation pathways (CP, LP and AP) were activated on (C) the different PTM-modified proteins and (D) ficolin detected on acetylated FCS. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ by one-way ANOVA and Dunnet's multiple comparison. Data are an representative of three independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; FB, factor B; FCS, fetal calf serum; HSA, human serum albumin; MAA, malondialdehyde-acetaldehyde adduct; MBL, mannan binding lectin; NHS, normal human serum; Nt, nitrated protein; OD, optical density; PTM, post-translational modification.*

As the mass spectrometry data indicated presence of some of the FH related proteins, we further investigated if next to Factor H also members of the FHR family were binding to PTM-modified proteins (Supplementary Figure 4). We observed that Cit, Ca, MAA and AGE modified proteins bind FHR-1, -3 and -5.

In whole blood PTM-modified proteins bind to leukocytes and not to erythrocytes or platelets

To study functional consequences of the PTM-complement interaction we set out to investigate PTM-modified protein binding to cells expressing complement receptors, namely leukocytes, platelets and erythrocytes from whole blood. Therefore, a whole blood assay was performed with PTM-modified FCS coupled to fluorescent beads, with and without serum (Figure 4A). We observed that those PTMs that activated complement (Ca, Ac, MAA, AGE) and Cit were able to bind to leukocytes. For Nt we did not observe binding to leukocytes, erythrocytes or platelets. We observed an increased binding of PTM-modified protein loaded beads in the presence of complement active serum compared to complement inactivated serum. Interestingly, we did not observe a noticeable binding to erythrocytes or platelets. These data indicate that PTM-modified proteins mainly bind to leukocytes, a process that is increased in the presence of active complement.

PTM-modified proteins that activate complement show increased uptake by differentiated THP-1 cells.

Since macrophages are the main phagocytic cells in tissues that express complement receptors, we set out to investigate uptake of PTM-modified proteins by THP-1 cells that were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA). For these experiments, beads containing PTM-modified FCS or non-modified FCS were incubated without serum, with complement-active serum or with complement-inactive serum (EDTA). We first confirmed that also for PTM-modified proteins coupled to beads, the same complement activation profiles were present as observed in the plate-bound assays (data not shown). These sets of beads were added to THP-1 macrophages and uptake was assessed (Figure 4B). Non-modified FCS, Nt and Cit coupled beads were not taken up by differentiated THP-1 macrophages. Ca-, Ac-, MAA- and AGE-coupled beads were taken up by THP-1 macrophages, and uptake was significantly increased in the presence of complement-active serum. When using complement-inactive serum the signal does not decrease to the level of no serum, indicating that the uptake is partially depending on complement activation and partially dependent on serum factors that bind but do not require complement activation. In addition, we have used complement receptor blocking antibodies to study the relative importance of complement activation on the uptake of PTM-coupled beads. We observed that by blocking CR1, -3 and -4 the uptake of complement opsonized PTM-coupled beads is partially inhibited (Supplementary Figure 5). These results indicate that the PTM-modified proteins capable

of activating complement demonstrate enhanced uptake by THP-1 macrophages, which is partially dependent on complement activation.

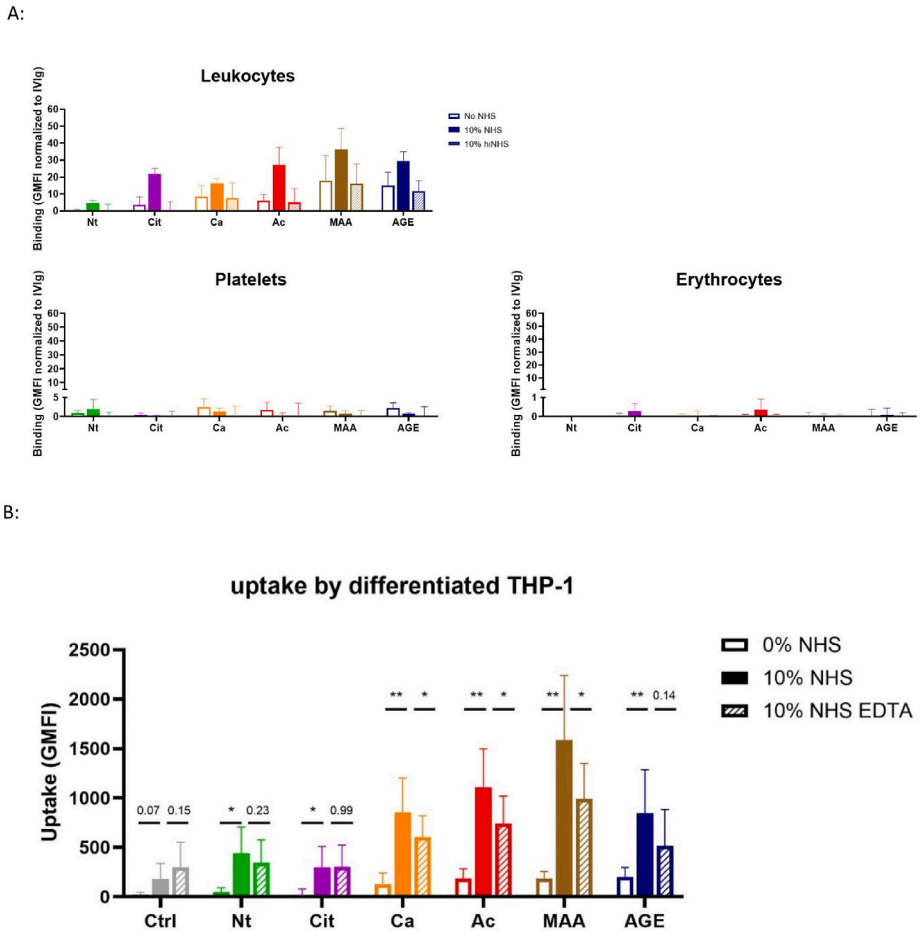


Figure 4: Leukocytes and THP-1 macrophages bind PTM-coupled beads in a complement dependent manner. (A) Binding of PTM-coupled beads to different cell populations from whole blood that express complement receptors (i.e. leukocytes, platelets and erythrocytes) in the presence of RPMI (open bar), RPMI/10% NHS (solid bar) or RPMI/10% hiNHS (striped bar). (B) PTM-coupled beads that were incubated with 0% NHS (open bar), 10%NHS (solid bar) and 10%NHS in the presence of 10mM EDTA (striped bar) and incubated with THP-1 macrophages and phagocytosis was assessed. $^{*}P < 0.05$, $^{**}P < 0.01$ by multiple paired t-tests. Data shown as mean +/- standard deviation and are representative of three independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; GMFI, geometric mean fluorescence intensity; hiNHS: heat inactivated normal human serum; IVIg,* intravenous immunoglobulin; *MAA, malondialdehyde-acetaldehyde adduct; NHS, normal human serum; Nt, nitrated protein.*

Combinations of complement SNPs associate with anti-PTM antibody positivity.
Environmental and genetic risk factors play an important role in the induction of autoantibody responses in RA patients. We therefore hypothesized that individuals that harbor genetic variants of complement genes, associated with a more active complement system, opsonization and activation of immune cells leading to phagocytosis and/or induction of antibody responses may be different from individuals with genetic variants that result in a less active complement system. Therefore, we assessed the association of complement SNPs with positivity for anti-PTM autoantibodies in RA patients. For that purpose, we used samples from RA patients enrolled in the Leiden Early Arthritis Clinic cohort (n = 587). We used genotype data for the complotype SNPs including *CFH*, *CFB* and *C3* (19) as well as SNPs in *C1q* known to associate with risk for developing RA and SLE (21, 22, 39).

The global test, where we ran a model including all SNPs to predict anti-PTM antibody positivity, showed a significant association of the complement SNPs with each of the anti-PTM autoantibodies (Supplementary Table 3). The SNPs together explained 17 to 20 percent of the variance of the individual anti-PTM antibodies.

Since the global tests were statistically significantly different, we further explored which of the individual SNPs were significantly associated with the anti-PTM antibodies (Table 1). Here, we found an association of rs292001 (*C1qA*) with anti-Cit (OR 1.27, 95%CI 1.0-1.6, *P-value 0.05*), rs2230199 (*C3*) with anti-Ca (OR 1.4, 95%CI 1.0-2.0, *P-value 0.04*), rs1061170 (*CFH*) with anti-Ac (OR 1.35, 95%CI 1.0-1.8, *P-value 0.04*), rs1061170 (*CFH*) with anti-MAA (OR 1.38, 95%CI 1.1-1.8, *P-value 0.01*), rs1061170 (*CFH*) with anti-AGE (OR 1.36, 95%CI 1.1-1.7, *P-value 0.02)*. These significantly associated SNPs captured often only part of the proportion of the variance of anti-PTM antibody presence explained by the total SNP set. Possibly the combination of SNPs, also known as the complotype, is relevant for developing antibody responses against PTM-modified proteins. Overall, these data indicate that having genetic variants that are known to result in a more active complement system, is associated with positivity for anti-PTM antibodies.

Table 1: Association between individual complement SNPs with each anti-PTM antibody

	SNP	rs800292	rs1061170	rs665691	rs292001	rs294179	rs2230199	rs641153
Anti-PTM	**Gene**	*CFH*	*CFH*	*C1qA*	*C1qA*	*C1qC*	*C3*	*CFB*
Anti-Nt	OR	3.54	0.77	1.66	1.79	2.36	1.51	0.30
	P	*0.16*	*0.79*	*0.56*	*0.52*	*0.33*	*0.73*	*0.60*
	R2	0.01	0.01	0.01	0.02	0.02	0.15	0.01
Anti-Cit	*OR*	*1.05*	*1.19*	*1.22*	*1.27*	*1.22*	*1.03*	*1.15*
	P	*0.73*	*0.18*	*0.09*	*0.05**	*0.09*	*0.83*	*0.56*
	R2	0.05	0.05	0.05	0.06	0.05	0.19	0.05
Anti-Ca	OR	1.13	0.98	1.13	1.13	1.12	1.4	1.19
	P	*0.35*	*0.85*	*0.32*	*0.34*	*0.35*	*0.04**	*0.48*
	R2	0.05	0.05	0.05	0.06	0.05	0.19	0.05
Anti-Ac	OR	1.02	1.35	0.98	1.09	0.94	1.06	0.96
	P	*0.89*	*0.04**	*0.9*	*0.52*	*0.66*	*0.76*	*0.88*
	R2	0.05	0.05	0.05	0.05	0.05	0.16	0.05
Anti-MAA	OR	0.73	1.38	1.11	1.04	1.03	0.88	0.76
	P	*0.16*	*0.01**	*0.42*	*0.75*	*0.83*	*0.43*	*0.27*
	R2	0.06	0.06	0.05	0.05	0.05	0.18	0.05
Anti-AGE	OR	0.96	1.36	1.12	1.13	1.13	0.88	1.15
	P	*0.78*	*0.02**	*0.36*	*0.31*	*0.3*	*0.42*	*0.55*
	R2	0.05	0.06	0.05	0.06	0.05	0.19	0.05

* significant association

Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; CFB, co-factor B; Cit, citrullinated protein; FH, Factor H; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; OR, odds ratio; PTM, post-translational modification; R2, McFadden Pseudo R squared; SNP, single nucleotide polymorphism.

Discussion

Autoimmune diseases, such as RA and SLE, are characterized by chronic inflammation and the presence of autoantibodies, including autoantibodies that target PTM-modified proteins. What triggers B-cells to generate these autoantibodies is largely unknown. Unravelling B-cell triggers by PTM-modified proteins will provide more specific treatment options as compared to the broad B-cell depletion used nowadays (4). Therefore, we investigated six irreversible PTMs (Nt, Cit, Ca, Ac, MAA, and AGE). We generated these six PTMs on a mix of proteins (FCS) and also generated mock-modified FCS by performing exactly the same procedure but leaving out the key-reagent for the modification. We verified the presence of the PTMs using both mass spectrometry and ELISA (36, 41). Importantly, we not only confirmed that the modified FCS preparations contained the desired PTM, but also that they did not contain any of the other PTMs. AGEs consist of a number of different structures and for the mass spectrometry analysis we therefore focused on carboxymethyl lysine, the key and most common AGE structure (42). Therefore, the number of peptide-spectrum matches (PSMs) are lower as compared to other modifications. However, compared to its non-modified counterpart there is clearly increased modification (Supplementary Figure 1B). Additionally, we verified that equal levels of PTM-modified protein and control protein was coated on the plate (data not shown).

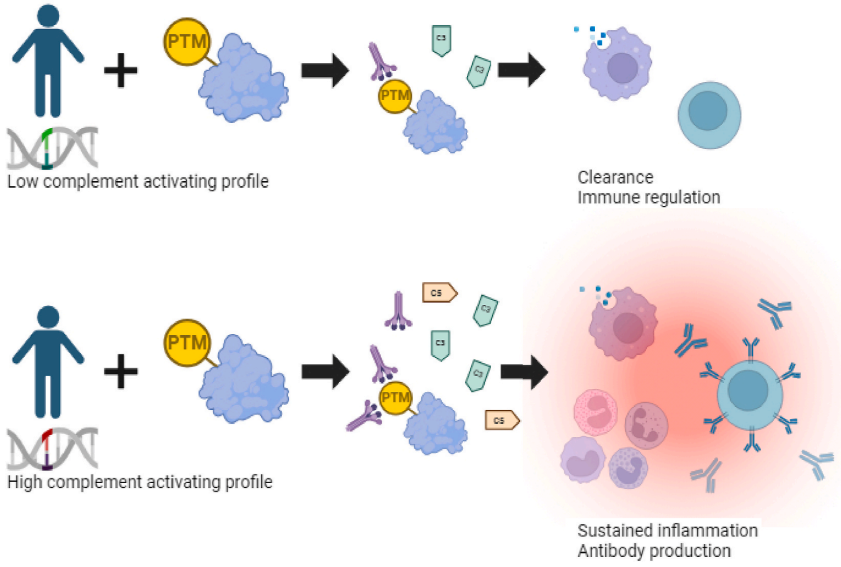


Figure 5: Model.
The so-called complotype of individuals results in a low (upper part) or high (lower part) complement activating profile. Depending on the complotype, complement opsonization of PTM-modified proteins could lead to clearance and immune regulation or (sustained) inflammation and autoantibody production, respectively.

We investigated autoantibody responses against the six PTM-modified proteins in three cohorts of patients suffering from the autoimmune diseases RA, SLE or AILD. In previous publications we established that in these three clinical conditions the presence of anti-PTM antibodies is associated with disease severity and/or response to therapy (13, 14, 16). These studies, and others, indicate that in many autoimmune diseases both PTM-modified proteins and/or anti-PTM antibodies, are present and could contribute to the disease process (36, 43-46). In this work, we hypothesized that complement proteins could directly bind to PTM-modified proteins to enhance clearance but that depending on the complotype this process could lead to (sustained) inflammation and autoantibody production (Figure 5).

Using mass-spectrometry we identified all human serum proteins that were bound to our PTM-modified proteins, therefore providing an unbiased analysis. The data indicated that complement proteins were specifically binding to 4 of the 6 PTM-modified proteins tested, namely Ca-, Ac-, MAA- and AGE-modified proteins. As we, and others, previously established the presence of antibodies against PTM-modified proteins, we needed to make sure that the observed complement activation was not simply the result of anti-PTM antibodies in the human serum (47). Our further experiments, using purified C1q, revealed that indeed there is a direct binding of C1q to the PTM-modified proteins as was previously also indicated for AGE (48). For Ac, we confirmed that, ficolins were

directly binding to the PTM-modified protein (38). Binding of complement recognition molecules, C1q and ficolin, resulted in complement activation for Ca, Ac, MAA and AGE at the level of C3d and C5b9. Complement activation is regulated by Factor H and FHR proteins acting on the AP and amplification loop of all pathways (49). Similar to other studies, we observed direct binding of Factor H (50) and FHR-1, -3 and -5 (51-54) to MAA when using purified proteins. No competition was observed between C1q and factor H for binding to MAA modified proteins (data not shown). Next to MAA, we observed minor direct binding of Factor H to Ca only at high concentrations. Additionally, when employing complement activation assays using C1q depleted serum, no residual complement activation is observed. These data indicate that the binding of FHR-3 and -5 to Ca, MAA and AGE did not induce AP activation.

Interestingly, Ca, Ac, MAA and AGE are all modifications of the amino acid lysine. However, we observed that not all lysine modifications induce similar complement activation pathways and activate complement to the same extent, thereby indicating that not all modifications of lysine behave similarly. In patients, direct complement activation on certain PTMs may contribute to sustained inflammation, in the patients anti-PTM antibodies could trigger additional complement activation (47). Complement activation assays including anti-CarP IgG (WT and LALAPG) showed that with the addition of increasing concentrations LALAPG mutated anti-CarP antibodies (that lack the capacity to activate complement), complement activation by Ca modified FCS itself was inhibited (Supplementary Fig. 6). Addition of the WT anti-CarP antibody further enhanced complement activation, as evidenced by increased levels of C5b-9. These data indicate that complement activation occurs directly on PTM-modified proteins and is further amplified in the presence of anti-PTM antibodies.

We coupled PTM-modified proteins to beads and then examined their binding and phagocytosis by leukocytes, platelets and erythrocytes from whole blood. Additionally, THP-1 macrophages were included to mimic a tissue-resident phagocytic cell population. We observed an increased uptake and binding for complement opsonized Ca-, Ac-, MAA- and AGE-coupled beads for THP-1 macrophages and leukocytes.

While we expected to see predominant binding to erythrocytes because of their high CR1 expression, we observed a clear binding particularly to leukocytes. This interaction is not antigen-specific as all leukocyte types were involved. This process of complement-dependent antigen transport has been described before (55) and would allow accumulation of the PTM-modified proteins e.g. in liver and spleen where they could trigger immune responses. The B-cells and follicular dendritic cells could serve a specific function as they express CR2 (17). Several studies based on mouse experiments indicate a clear enhancement of B-cell activation if next to the BCR also CR2 is engaged (17, 56)

resulting in enhanced antibody responses to complement-opsonized targets. However, in a human setting contradictory results are observed (18, 57).

Genetic and environmental factors are important factors that contribute to disease onset (19, 20). In case of polymorphisms in complement genes, certain combinations, so called complotype, may result in high or low complement activating profiles (19). We observed that some genetic variants that are part of the complotype are associated with the presence of anti-PTM antibodies. Namely, the minor allele of *CFH* rs1061170, being associated with higher complement activity (58), was associated with the presence of anti-Ac, -MAA and -AGE. Moreover, this minor allele was previously shown to alter FH binding abilities to malondialdehyde (50). Others showed that the minor allele of *C3* rs2230199, associated with anti-Ca, bound FH less strongly and was less efficiently inactivated, thereby increasing AP activity (59). Noteworthy is that, studies showed that minor allele of *C1q* SNPs associate with change in C1q serum levels but do not show increase in complement activity (21, 60, 61). Interestingly, the minor allele of *C1qA* rs292001 associated with anti-Cit, contributing to the findings that *C1q* SNPs are described in the context of RA (21). These data suggest a link between complotype and development of anti-PTM antibody reactivity. Inhibiting the complement system systemically, may not be a realistic option to limit complement activation mediated by PTM, because of costs and because of infectious risk associated with systemic complement inhibition. For future purposes we therefore propose to explore bispecific antibodies targeting both the PTM-modified protein and bringing complement inhibitors into close proximity (62).

Future *in vivo* studies should unravel the importance of complement in the induction of anti-PTM antibodies. In such studies complement deficient mice could be used and compared to mice with a fully functioning complement system. We previously established that in mice several anti-PTM antibody reactivities can be observed and induced (63, 64). However, the role of complement has not been studied. Additionally, specified cell populations as well as cells from (at risk) patients should be further investigated to elucidate how complement leads to (chronic) inflammation and autoimmunity.

In conclusion, PTM-modified proteins can bind and activate complement, which increases inflammation and phagocytosis, and may lead to development of anti-PTM antibodies.

Data availability

All data are available upon request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD054220".

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Supplementary files

Additional materials and methods on supplementary figures

Complement activation through anti-PTM antibodies versus PTM-modified proteins

To assess relative complement activation through anti-PTM antibodies compared to PTM-modified proteins complement activation assays were employed using monoclonal IgG antibodies with and without LALAPG mutations. First, carbamylated FCS was coated on Nunc MaxiSorp plates (430341, Thermofisher) at a concentration of 10 µg/mL in bicarbonate coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation plates were blocked using blocking buffer (PBS/1% BSA) for 1 hr at 37°C. Next, plated were incubated with or without IgG antibodies (with and without LALAPG mutations) against Ca. The LALAPG mutation prevents Fc mediated complement activation. Then, plates were incubated with 3% NHS in RPMI for 1 hr at 37°C. Bound IgG and C5b-9 were detected using 1:2000 goat anti-rabbit IgG-HRP (P0448, Dako) and 1:333 mouse anti-HuC5b9 (M0777, Dako), respectively, diluted in PTB buffer. Plates were incubated for 1hr at 37°C whereafter mouse-anti-HuC5b9 was detected using goat anti-mouse IgG-HRP (P0447, Dako). Secondary antibodies were incubated for 1hr at 37°C after which plates were washed and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Binding of Factor H related proteins

To assess binding of Factor H-related (FHR) proteins, modified FCS and non-modified FCS were coated on Nunc MaxiSorp plates (430341, Thermofisher) at 10 µg/mL in coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH 9.6) overnight at RT. The next day, and after each incubation step, plates were washed 5 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After washing, plates were blocked using blocking buffer (PBS/1% BSA) for 30 min at RT while shaking. After blocking, His-tagged FHR proteins 1, 2, 3, 4A and 5 (all produced at Sanquin Research) were diluted to equal molar concentrations, starting the concentration curve at 80 nM for all FHR proteins. As positive and negative controls His-tagged recombinant Factor H (produced in house at Sanquin Research) and His-tagged irrelevant protein FcRn (neonatal Fc Receptor; produced in house at Sanquin Research) were taken along. Since all FHR proteins and recombinant Factor H contained a His tag, FHR proteins could be detected using the same mouse anti-His tag at 1 µg/mL followed by a rat anti-mouse kappa HRP conjugated secondary antibody (Clone RM-19, produced in house at Sanquin Research) at 0.25 µg/mL. Both were incubated for 1 hr at RT while shaking. Plates were developed using TMB and absorbance was read at 415 nm using a microplate reader (Bio-Rad).

Complement receptor blocking experiments

To assess the importance of complement receptors in phagocytosis of complement-opsonized PTM-coupled beads by THP-1 macrophages we analyzed uptake in the presence or absence of complement receptor blocking antibodies. Blocking antibodies against CR1, CR3 and CR4 (CR1, clone J3D3 sc-59022 Santa Cruz; CR3, anti-CD11b, clone ICRF44, 301302, Biolegend; CR4, anti-CD11c, clone 3.9, 301602, Biolegend; CR3/4, anti-CD18, clone 1B4, 373402, Biolegend) were used. Additionally, blocking antibodies against CR2 (not expressed by THP-1, anti-CD21, 552727, BD Pharmingen) and isotype controls (IgG1 21335011 and IgG2 21335021, both Immunotools) were included as controls. THP-1 macrophages were first incubated with Fc block (564220, BD) for 10 min. Without washing, cells were incubated with a combination of all three blocking antibodies against CR1, 3 and 4 or their controls at a concentration of 10 µg/mL for 30 min at 4°C. Additionally, THP-1 macrophages were incubated without blocking antibodies or with Cyto D (C8273, Sigma) as reference conditions. Without washing, beads were added at 3000 beads/cell. Phagocytosis took place for 1 hr in the incubator at 37°C/5% CO_2. After incubation, beads were washed away and cells were detached using trypsin (25300-054, Gibco). Samples were acquired using BD Canto I. Phagocytosis was determined using GMFI of the APC channel.

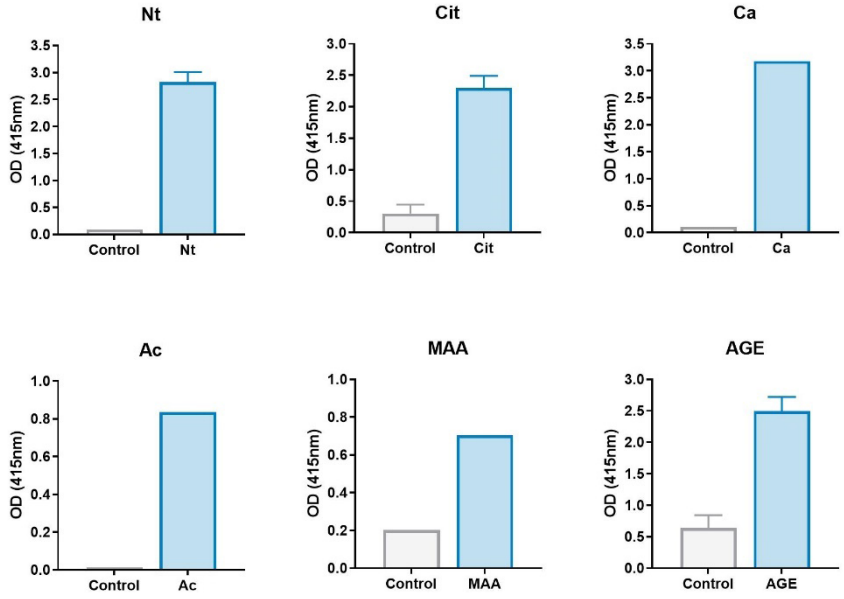
Supplementary Tables and Figures

Supplementary Table 1: Prevalence of anti-PTM antibodies in three autoimmune diseases (RA, SLE and AILD, respectively), and the combination of these autoimmune diseases (AID), compared to anti-PTM antibodies in HCs.

	RA n=614	SLE n=360	AILD n=106	AID n=1080	HC n=197
Anti-Nt n, (%)	54 (8.8)	18 (5.0)	7 (6.6)	79 (7.3)	12 (6.1)
Anti-Cit n, (%)	303 (49.3)	22 (6.1)	18 (17.0)	343 (31.8)	12 (6.1)
Anti-Ca n, (%)	276 (45.0)	53 (14.7)	50 (47.2)	379 (35.1)	8* (4.5)
Anti-Ac n, (%)	278 (45.3)	33 (9.2)	20 (18.9)	331 (30.6)	13 (6.6)
Anti-MAA n, (%)	287 (46.7)	108 (30.0)	72 (67.9)	467 (43.2)	8 (4.1)
Anti-AGE n, (%)	277 (45.1)	67 (18.6)	39 (36.8)	383 (35.5)	7 (3.6)
At least one anti-PTM	503 (81.9)	171 (47.5)	85 (80.2)	759 (70.3)	48 (24.4)
At least two anti-PTMs	407 (66.3)	85 (23.6)	54 (50.9)	546 (50.6)	9 (4.6)

*for 20 HCs data on anti-CarP was missing. *Abbreviations: AGE, advanced glycation end-product; AID, autoimmune disease; AILD, autoimmune liver disease; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; HC, healthy control; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification; RA, rheumatoid arthritis; SLE, systemic erythematosus.*

A:



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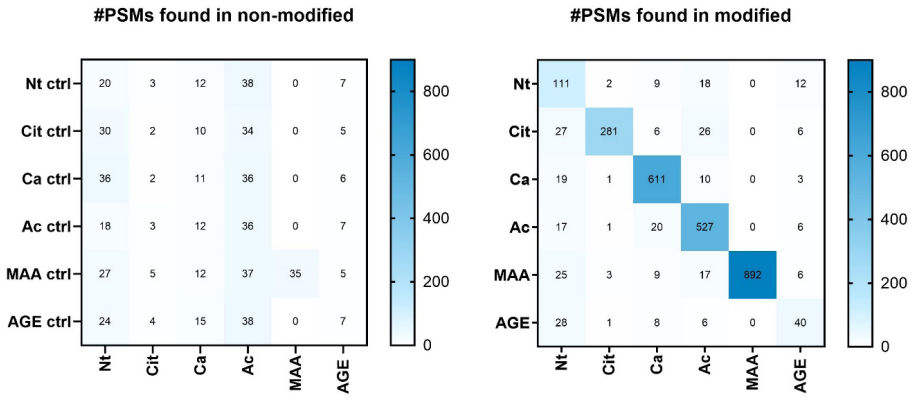


Supplementary Figure 1: Confirmation of PTM on FCS. (A) PTMs were detected using specific antibodies against the PTM or (B) the amount of modification on each modified protein represented by peptide-spectrum matches (PSMs), analyzed using mass spectrometry. Data are representative of three (A) or one (B) independent experiment(s). *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; OD, optical density; PSMs,* peptide-spectrum matches; *PTM, post-translational modification.*

Supplementary Table 2: Combined data of enriched serum components binding to PTM-modified proteins. Top 20 enriched proteins binding to each PTM-modified proteins compared to its control non-modified protein were combined. List is based on ratio abundance PTM / abundance Control determined by Proteome Discoverer. Duplicates were removed and ratio above 2 (bold and underlined) were considered enriched. Complement proteins are depicted in yellow and immunoglobulin fragments in pink. Data are representative of two independent experiments.

	Accession #	Description	Nt	Cit	Ca	Ac	MAA	AGE	# ratio >2
1	Q92954	Proteoglycan 4 OS=Homo sapiens	-	**4,1**	**13,2**	**2,7**	**9,1**	**14,3**	5
2	P02760	Protein AMBP OS=Homo sapiens	1,9	**3,2**	**10,5**	**4,3**	**2,3**	**6,9**	5
3	P02747	Complement C1q subcomponent subunit C	-	1,8	**27,9**	**2,1**	**54,3**	**73,6**	4
4	P05164	Myeloperoxidase	-	-	**100**	**3,1**	**5,4**	**11,4**	4
5	Q03591	Complement factor H-related protein 1	-	**5,2**	**10,5**	1,6	**80,2**	**9,9**	4
6	P04003	C4b-binding protein alpha chain	-	-	**3,3**	**9,6**	**63,9**	**14,5**	4
7	P0C0L5	Complement C4-B	1,1	-	**7,1**	**7,5**	**41,4**	**16,9**	4
8	P08603	Complement factor H	-	**2,1**	**3,1**	1,4	**30,3**	**17,4**	4
9	P01024	Complement C3	1,0	1,7	**3,7**	**4,7**	**24,2**	**10,9**	4
10	P02745	Complement C1q subcomponent subunit A	-	-	**100**	-	**100**	**100**	3
11	P02746	Complement C1q subcomponent subunit B	-	-	**25,4**	**2,1**	-	**100**	3
12	P05155	Plasma protease C1 inhibitor	-	-	-	**2,4**	**52,5**	**11,7**	3
13	P01008	Antithrombin-III	1,0	1,7	**6,8**	1,2	**45,6**	**3,9**	3
14	P00734	Prothrombin	1,6	1,8	**6,8**	0,9	**37,0**	**6,0**	3
15	P05546	Heparin cofactor 2	-	1,9	10,0	**2,4**	**16,8**	-	3
16	P04004	Vitronectin	1,4	1,5	**5,1**	0,8	**17,9**	**4,0**	3
17	P05154	Plasma serine protease inhibitor	-	1,5	**4,9**	1,7	**9,1**	**6,7**	3
18	O43866	CD5 antigen-like L	1,0	**9,8**	1,5	**5,5**	**3,5**	1,1	3
19	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	-	-	**6,3**	1,8	**6,6**	**3,0**	3
20	P02649	Apolipoprotein E	1,3	**4,8**	**2,2**	1,0	**3,8**	1,2	3
21	P01137	Transforming growth factor beta-1	-	2,0	**5,8**	**2,6**	**3,3**	-	3
22	P55056	Apolipoprotein C-IV	**2,2**	1,1	1,8	**2,1**	0,01	**2,0**	3
23	O75636	Ficolin-3	-	-	**100**	**100**	-	-	2
24	Q9BXR6	Complement factor H-related protein 5	-	-	**22,3**	-	**75,5**	-	2
25	B4DPQ0	Complement C1r subcomponent	-	-	-	-	**62,5**	**20,0**	2
26	P09871	Complement C1s subcomponent	-	-	-	-	**23,7**	**5,5**	2
27	P02788	Lactotransferrin	-	1,0	1,4	1,3	**7,2**	**7,7**	2
28	P27918	Properdin	-	-	**6,7**	-	**6,8**	-	2
29	A0A0A0MRJ7	Coagulation factor V	0,8	**2,4**	0,6	0,7	**7,9**	1,0	2
30	P01591	Immunoglobulin J chain	-	-	1,9	**2,5**	**8,2**	-	2
31	P02647	Apolipoprotein A-I	1,8	**2,6**	1,3	0,8	**2,7**	1,6	2
32	Q13201	Multimerin-1	1,9	1,1	**2,7**	**2,3**	0,2	1,6	2
33	P02675	Fibrinogen beta chain	-	1,4	**2,9**	**4,9**	0,1	-	2
34	H0YAC1	Plasma kallikrein (Fragment)	-	**2,3**	0,7	1,0	**3,6**	-	2
35	Q9Y2J8	Protein-arginine deiminase type-2	-	**100**	-	-	-	-	1
36	O00187	Mannan-binding lectin serine protease 2	-	-	-	**100**	-	-	1
37	P20851	C4b-binding protein beta chain	-	-	-	-	**100**	-	1
38	A0A075B6I9	Immunoglobulin lambda variable 7-46	-	-	-	**92,9**	-	-	1
39	Q15485	Ficolin-2	-	-	-	**51,7**	-	-	1
40	P48740	Mannan-binding lectin serine protease 1	-	-	-	**26,1**	-	-	1
41	B1AHL2	Fibulin-1 GN=FBLN1	-	-	-	-	**20,0**	-	1
42	P0C0L4	Complement C4-A	-	-	-	-	**19,6**	-	1
43	P01767	Immunoglobulin heavy variable 3-53	-	-	-	-	**19,1**	-	1
44	P02743	Serum amyloid P-component	-	-	-	-	**16,2**	-	1
45	P01876	Immunoglobulin heavy constant alpha 1	1,5	1,4	1,2	1,1	**4,7**	1,1	1
46	Q14766	Latent-transforming GF beta-binding protein 1	-	1,5	**6,9**	1,4	1,0	-	1
47	P02776	Platelet factor 4	**4,8**	0,9	1,4	1,2	0,1	1,0	1
48	P06312	Immunoglobulin kappa variable 4-1	1,4	1,0	1,3	1,2	1,8	**2,7**	1
49	P04196	Histidine-rich glycoprotein	-	**8,3**	-	1,2	-	-	1
50	P02654	Apolipoprotein C-I	1,5	**2,1**	0,6	1,5	0,8	1,5	1
51	P01042	Kininogen-1	0,8	**2,5**	0,8	1,1	0,6	1,1	1
52	P02751	Fibronectin	0,6	1,3	1,1	**2,7**	0,3	0,7	1
53	P02671	Fibrinogen alpha chain	0,5	1,1	1,1	**2,9**	0,4	-	1
54	P06727	Apolipoprotein A-IV	-	**3,7**	0,8	-	1,1	-	1
55	P62805	Histone H4	**5,4**	-	-	0,2	-	-	1
56	P0DOY3	Immunoglobulin lambda constant 3	-	1,1	1,4	**3,0**	-	-	1
57	P01031	Complement C5	-	-	-	**2,8**	1,9	-	1
58	P02749	Beta-2-glycoprotein 1	-	**4,4**	-	-	-	-	1
59	D6RF35	Vitamin D-binding protein	-	**2,3**	0,3	-	-	-	1
60	P18428	Lipopolysaccharide-binding protein	-	**2,5**	-	-	-	-	1

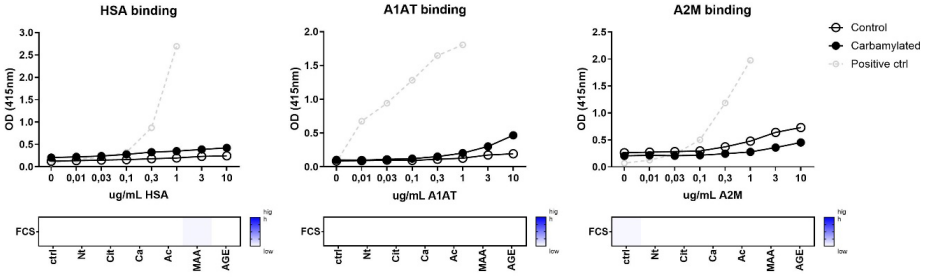
Ratio: Abundance ratio: (Grouped Abundances PTM)/(Grouped Abundances ctrl)

Complement factor

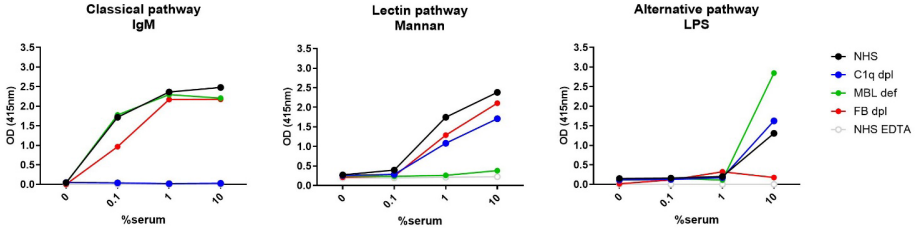
Immunoglobulin fragment

- : no data

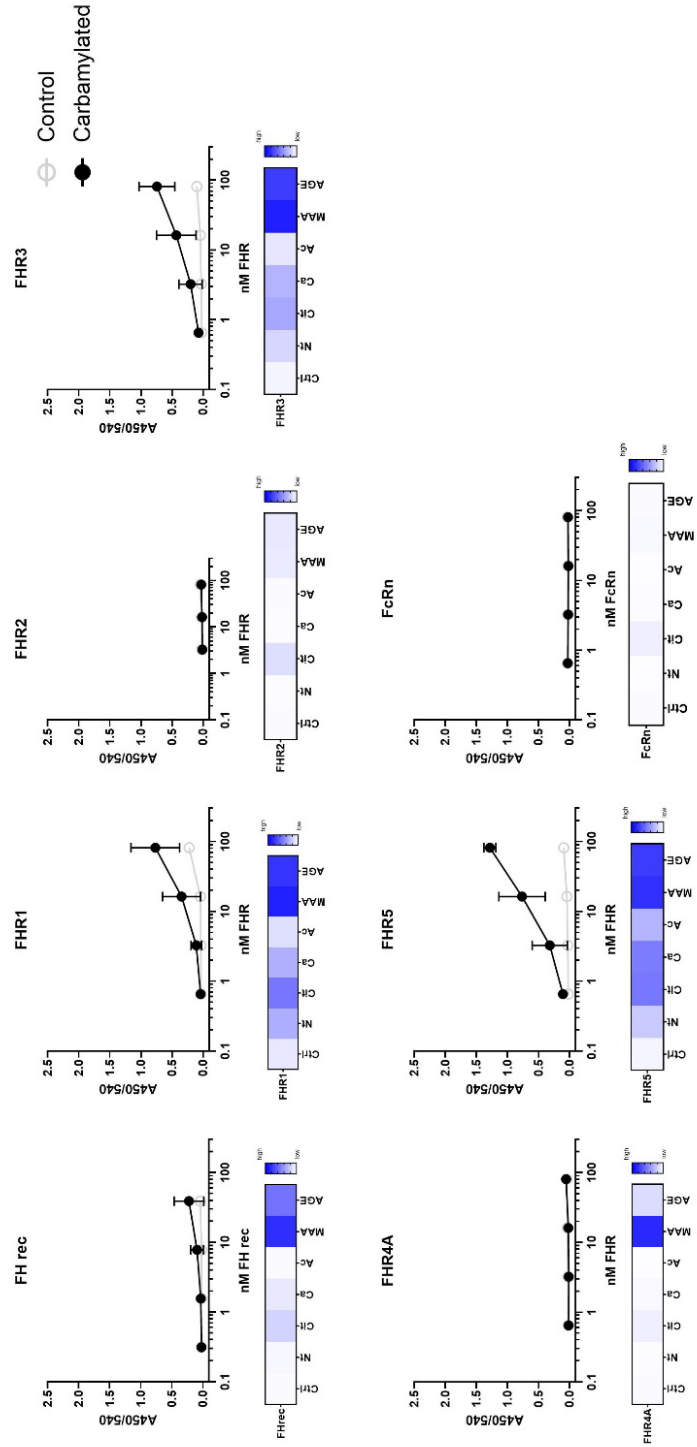
Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification.



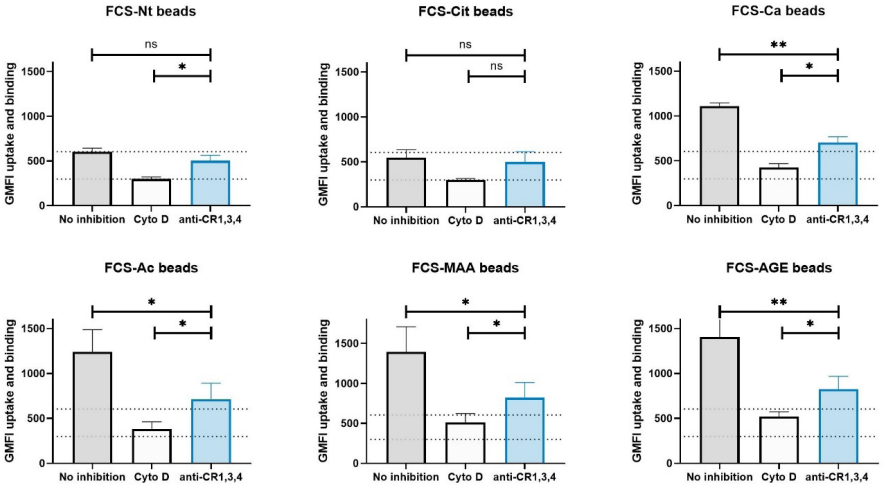
Supplementary Figure 2: Abundantly present serum proteins (HSA, A1AT and A2M) do not bind PTM-modified proteins. Direct binding of HSA, A1AT and A2M to all six PTM-modified proteins was assessed using plate-bound assays. Plates coated with either non-modified FCS (black open symbols) or PTM-FCS (black closed symbols) were incubated with serial dilutions of the purified proteins HSA, A1AT and A2M (left to right). Additionally, plates were coated with each protein (HSA, A1AT or A2M) as a positive control (light grey). Data are representative of three independent experiments. *Abbreviations: A1AT, alpha-1 antitrypsin; A2M, alpha-2-macroglobulin; AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; HSA, human serum albumin; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification.*



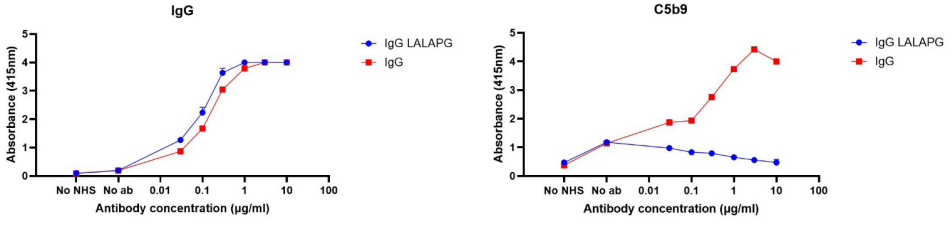
Supplementary Figure 3: Controls for complement activation pathways. Complement activation pathways (from left to right: CP, LP and AP) were activated on IgM, mannan or LPS, respectively, coated ELISA plates. Coated wells were incubated with NHS, C1q-depleted serum, MBL-deficient or Factor B-depleted serum in the presence of RPMI, RPMI/10mM EGTA/5mM MgCl2 or RPMI/10mM EDTA. C4 (CP and LP) and C3 (AP) were analyzed. Data are representative of three independent experiments. *Abbreviations: FB dpl, Factor B-depleted; MBL def, mannan-binding lectin-deficient; NHS, normal human serum.*



Supplementary Figure 4: Factor H and FHR proteins bind to specific PTM-modified proteins. Binding of purified Factor H and FHR-family (FHR-1, -2, -3, -4A, -5) to the PTM-modified proteins was assessed. Graphs are representative of two independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification.*



Supplementary Figure 5: Complement receptor blocking experiments. PTM-coupled beads were subjected to differentiated THP-1 cells in the presence of CR blocking antibodies. After incubation, THP-1 cells were detached and analyzed by flowcytometry. Data are representative of three independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; CR, complement receptor; Cyto D, cytochalasin D; FCS, fetal calf serum. MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein;*



Supplementary Figure 6: Complement activation in the presence or absence of anti-CarP IgG antibodies with or without LALAPG mutation. Binding of IgG (WT or LALAPG) and subsequently complement activation was assessed on carbamylated protein using 3% NHS.

Supplementary Table 3: P-values for the global test of all seven complement SNPs against the individual anti-PTM antibodies.

Anti-PTM	***P-value*** **models improvement**	***McFadden*** **Pseudo-R-squared**
Anti-Nt	*0.002*	0.17
Anti-Cit	*2.5e-34**	0.20
Anti-Ca	*5.9e-32**	0.19
Anti-Ac	*1.4e-23**	0.17
Anti-MAA	*5.7e-35**	0.20
Anti-AGE	*4.0E-34**	0.20

* significant association

Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification; SNP, single nucleotide polymorphism.