Acetylated bacterial proteins as potent antigens inducing an anti-modified protein antibody response

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ORIGINAL RESEARCH

Acetylated bacterial proteins as potent antigens inducing an anti-modified protein antibody response

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ABSTRACT

Objective Gut-residing bacteria, such as *Escherichia coli*, can acetylate their proteome under conditions of amine starvation. It is postulated that the (gut) microbiome is involved in the breach of immune tolerance to modified self-proteins leading to the anti-modified protein antibodies (AMPAs), hallmarking seropositive rheumatoid arthritis (RA). Our aim was to determine whether acetylated bacterial proteins can induce AMPA responses cross-reactive to modified self-proteins and be recognised by human AMPA (hAMPA).

Methods *E. coli* bacteria were grown under amine starvation to generate endogenously acetylated bacterial proteins. Furthermore, *E. coli* proteins were acetylated chemically. Recognition of these proteins by hAMPA was analysed by western blotting and ELISA; recognition by B cells carrying a modified protein-reactive B cell receptor (BCR) was analysed by pSyk (Syk phosphorylation) activation assay. C57BL/6 mice were immunised with (modified) bacterial protein fractions, and sera were analysed by ELISA.

Results Chemically modified bacterial protein fractions contained high levels of acetylated proteins and were readily recognised by hAMPA and able to activate B cells carrying modified protein-reactive BCRs. Likely due to substantially lower levels of acetylation, endogenously acetylated protein fractions were not recognised by hAMPA or hAMPA-expressing B cells. Immunising mice with chemically modified protein fractions induced a strong cross-reactive AMPA response, targeting various modified antigens including citrullinated proteins.

Conclusions Acetylated bacterial proteins are recognisable by hAMPA and are capable of inducing cross-reactive AMPA in mice. These observations provide the first conceptual evidence for a novel mechanism involving the (endogenous) acetylation of the bacterial proteome, allowing a breach of tolerance to modified proteins and the formation of cross-reactive AMPA.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Patients with rheumatoid arthritis (RA) develop anti-modified protein antibodies (AMPAs), including largely multireactive antibodies against citrullinated, carbamylated, acetylated proteins.
⇒ Protein acetylation is present in various bacterial species in the gut.
⇒ It is unknown whether the bacterial proteins can function as AMPA antigens.

WHAT THIS STUDY ADDS

⇒ Acetylated *Escherichia coli*-derived proteins are demonstrated to be recognised by human AMPA and AMPA-expressing B cells; highly acetylated bacterial proteins induce a multireactive AMPA response in mice even without an adjuvant.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Induction of AMPA independently of citrullination can shift the search for aetiological factors in RA from particular pathogens to common mechanisms present in various bacterial species.

INTRODUCTION

Despite considerable efforts, the aetiology of rheumatoid arthritis (RA) remains unknown. Several lines of evidence suggest that exposures at mucosal surfaces and/or dysbiosis may have causal roles in the development of RA.[1] This 'mucosal origins hypothesis' postulates that the autoimmune response underlying RA may start at mucosal sites and then transition to the joints. An important factor in initiating the autoimmune response in RA at mucosal surfaces could be the microbiome which has been reported to be altered in patients with new-onset and established RA.[2]

Anti-citrullinated protein antibodies (ACPAs) have emerged as a characteristic RA feature. These antibodies target epitopes that contain citrulline, a post-translationally modified (PTM) arginine. The range of autoantibody-recognisable PTM residues

in RA has been later expanded by epitopes created by carbamylation or N-ε-lysine acetylation, modifying lysine into homocitrulline and acetyllysine, respectively. Antibodies targeting proteins with any of the three PTM residues (ie, citrulline, homocitrulline, acetyllysine) tend to be present simultaneously in the sera of patients with RA and demonstrate cross-reactivity or multi-reactivity at the monoclonal and polyclonal levels.[3 4] Such cross-reactivity was also observed after immunising mice with model antigens carrying one of the PTMs,[4] indicating that exposure to PTM antigens can induce cross-reactive AMPA responses.

Unlike citrullination and carbamylation, N-ε-lysine acetylation of bacterial proteins is widespread across various bacterial species. Acetylation can largely alter the conformation and functional properties of a protein, a mechanism widely used by bacteria to regulate their metabolism.[5] Furthermore, bacteria are reported to affect the acetylome of the host[6] and particular bacterial species express acetyltransferases that function outside of the bacterial cell wall.[7] Moreover, various acetylated proteins can be identified in human faeces, suggesting their presence at mucosal surfaces.[8] Interestingly, acetylation in bacteria can be triggered by the surrounding conditions, such as a lack of amines in the presence of glucose.[9]

In view of this, it is important to know whether acetylated bacterial proteins can function as antigens breaching the tolerance towards PTM-(self)proteins and inducing the characteristic autoimmune responses present in RA. To investigate this possibility, we generated acetylated bacterial proteins and studied whether autoreactive human AMPA (hAMPA) and hAMPA-expressing B cells, derived from patients with RA, recognise these bacterial proteins. In addition, we analysed whether these acetylated bacterial antigens can induce AMPA responses in mice.

METHODS

Generation of acetylated bacterial antigens

E. coli of BL21 strain were cultured overnight in an in-house-made M9 medium. Stimulation of endogenous acetylation was induced as described previously, which led to the generation of endogenously acetylated bacterial proteins (EABPs) and native bacterial proteins (NBPs),[9] and are further detailed in the online supplemental materials.

Chemical acetylation, used to generate chemically acetylated bacterial proteins (CABPs), was achieved by diluting proteins (starting concentration of 1 mg/mL) in 0.1 M Na_2CO_3, then treating with acetic anhydride and subsequently with pyridine. Proteins were incubated at 30°C for 5 hours or overnight while shaking. After incubation, the acetylation reaction was stopped by adding 1 M Tris. Acetylated proteins were purified by exchanging the buffer for phosphate buffer saline (PBS) through Zebra Spin Desalting columns (Thermo Scientific).

Detection of PTM residues by mass spectrometry

Mass spectrometry analysis was used to test EABP, NBP and CABP for the presence of PTM proteins, including acetylation, citrullination and carbamylation: it was performed as described previously and further detailed in the online supplemental materials.[4]

Production of monoclonal hAMPA

hAMPA IgG and IgM sequences were isolated from citrulline-reactive human B cells (using citrullinated fibrinogen in the case of 7E4 IgG and acetylated vimentin peptide HC 55 in the case of 1E3 IgM) and produced in a transitionary Freestyle 293 F-cell (Gibco) transfection system. Recombinant monoclonal antibodies (mAbs) were purified, all as described previously.[3]

Purification of polyclonal hAMPA

Purification of AMPA, recognising acetyllysine, is described in detail in the online supplemental methods. In short, it was purified using a HiTrap Streptavidin HP column (Cytiva) coupled with biotinylated CAcetylP2 on AKTApure purification system and eluted with glycine-HCL buffer of pH 3.5.

hAMPA recognition of acetylated bacterial proteins by ELISA

Detection of acetylated bacterial proteins by ELISA is described in detail in the online supplemental methods. In short, plates were coated with EABP, NBP, CABP and control proteins and peptides: monoclonal and polyclonal hAMPA and monoclonal human non-AMPA were preincubated with horseradish peroxidase (HRP)-conjugated rabbit anti human Ig secondary antibody to increase sensitivity.

hAMPA and rabbit AAPA recognition of bacterial acetylated proteins by western blot

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), 25 µg of each type of bacterial proteins (CABP, EABP, NBP) in PBS was diluted in 4x Laemmli buffer (Bio-Rad) with addition of 1:20 beta-mercaptoethanol. Further, western blot analysis was performed as previously described.[3] In short, the blot was incubated overnight with rabbit anti-acetylated protein antibodies (AAPA) in a form of anti-acetyllysine polyclonal Ig (Enzo Life Sciences), 7E4 AMPA IgG or 1E3 AMPA IgM and then detected with a respective secondary antibody (HRP-conjugated goat anti-rabbit Ig, rabbit anti-human Ig—all from DAKO/Agilent).

Activation of Ramos B cells expressing acetyllysine-reactive B cell receptors

GFP^+BCR^+ Ramos B-cell lines, expressing a PTM-protein-reactive B cell receptor (BCR) (derived from an hAMPA IgG1 clone 7E4), were generated as described previously.[3] The Ramos cells (0.2×10^6 cells) were stimulated with CABP, NBP and EABP (all 72 µg) for 2 min at 37°C in stimulation medium (RPMI (Gibco)/1% FCS (Bodinco)/GlutaMAX/10 mM HEPES/100 U/mL Penicillin/100 µg/mL Streptomycin (all from Gibco)).

Additionally, stimulation was performed with unmodified and acetylated fibrinogen proteins (12 µg). Further experimental steps and the analysis were performed as described previously.[3] In short, after washing, cells were stained with mouse anti-human pSyk(Y348)-PE mAb (moch1ct, eBioscience) and diluted 1:20 in PBS/0.5% bovine serum albumin (BSA)/0.02% NaN_3. The rate of pSyk expression in Ramos cells was calculated as the percentage and proportion of pSyk+GFP+double positive cells. Gating was based on the human B cell line Ramos, in which the endogenous IGHM, IGHD, and IGLC and activation-induced cytidine deaminase were knocked out (MDL-AID KO), which were stimulated with the citrullinated antigen, and on isotype control staining using mouse IgG1 kappa isotype control-PE mAb (P3.6.2.8.1, eBioscience). Unpaired t-tests were used to analyse the differences.

Immunisation of mice

Mice immunisation with acetylated bacterial proteins is described in detail in the online supplemental methods. In short, 7-week-old female C57BL/6 mice were immunised intraperitoneally, each mouse was immunised four times every 3 weeks with an antigen (100 µg): blood was collected prior to the repeated immunisations, and at the end of the experiment, mice were sacrificed 3 weeks after the last immunisation.

Analysis of AMPA in mouse serum

Detection of mouse AMPA by ELISA and calculation of titres were performed as described previously and further detailed in the online supplemental methods.[10] In short, plates were coated with acetylated, citrullinated, carbamylated or native fibrinogen, and mouse sera were titrated and then detected with HRP-conjugated goat-anti-mouse IgG1 (SouthernBiotech).

Statistics

Statistical tests were performed with Prism V.9 (GraphPad). Differences in titre were tested with Mann-Whitney U tests. Differences between the conditions in the B-cell activation assay were tested with unpaired t-test. A p value <0.05 was considered significant.

RESULTS

Recognition of bacterial proteins by hAMPAs

To investigate the antigenic properties of acetylated bacterial proteins, we used *E. coli* as a model organism and generated three differently acetylated samples: CABP, EABP and NBP (figure 1A). To validate the presence of post-translational modifications, CABP, EABP and NBP were measured by mass spectrometry (accessible via PRIDE archive). As expected, the bacterial protein fraction obtained after chemical acetylation was

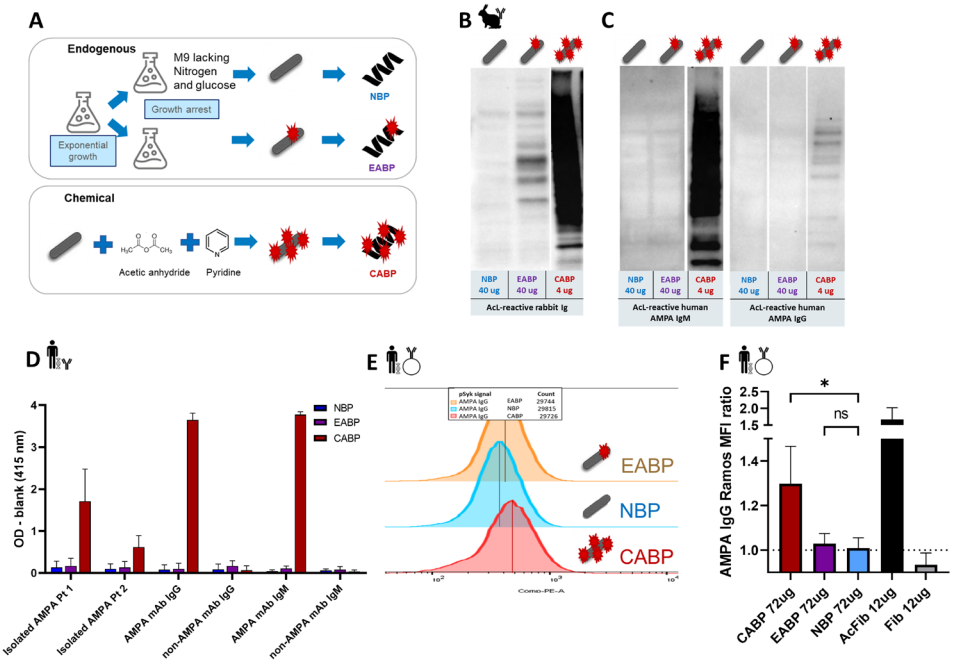

Figure 1 Recognition of acetylated bacterial antigens by hAMPA. (A) Schematic overview of the generation of NBP, EABP and CABP. (B) Western blotting with NBP, EABP and CABP stained with anti-acetyllysine polyclonal rabbit antibody. (C) Western blotting with NBP, EABP and CABP stained with hAMPA mAbs, recognising acetylated proteins: 7E4 IgG and 1E3 IgM. (D) ELISA with polyclonal AMPA isolated with CAcP4 peptide from two patients' sera, AMPA mAb IgG (7E4) and IgM (1E3) controlled by non-AMPA mAb IgG and IgM. Mean values of five representative experiments are shown. (E) pSyk MFI, visualising BCR activation of Ramos B cells expressing 7E4 IgG after incubation with acetylated *Escherichia coli* antigens. (F) Summary of three BCR activation experiments with 7E4 Ramos B cells. Statistical differences are indicated with asterisks, indicating p value: *<0.05, **<0.01, ****<0.0001. AMPAs, anti-modified protein antibodies; BCR, B-cell receptor; CABPs, chemically acetylated bacterial proteins; EABPs, endogenously acetylated bacterial proteins; hAMPA, human AMPA; mAbs, monoclonal antibodies;NBPs, native bacterial proteins; pSyk, Syk phosphorylation; MFI, mean fluorescence intensity.

extensively acetylated. No other modifications could be detected. In contrast, no acetylated or otherwise modified proteins were detected by mass spectrometry in the EABP and NBP fractions. Nonetheless, all protein samples were also tested by western blot to determine whether acetylation could be visualised by a polyclonal anti-acetylated lysine antibody. As shown in figure 1B, CABP contained numerous acetylated proteins. In line with the data obtained by mass spectrometry, acetylated of EABP fraction was far less abundant, as EABP showed a smaller range of acetylated proteins. Almost no reactivity of the antibody towards the proteins in NBP was detected, indicating the induction of bacterial acetylation with both chemical and endogenous methods, with the latter having more limited efficiency.

As both the CABP and EABP samples contained acetylated proteins, we next wished to determine whether hAMPA could also recognise these acetylated antigens. To this end, we first tested monoclonal hAMPA, produced from BCR sequences of patient-derived citrulline-reactive B cells. Two different monoclonal hAMPAs from different patients were analysed by western blotting: these included the extensively studied 7E4 IgG (originally isolated using citrullinated fibrinogen)[3] and 1E3 IgM (originally isolated using acetylated vimentin peptide HC55).[11] Both IgG and IgM mAbs could recognise the acetylated proteins within the CABP sample (figure 1C). In contrast, no recognition of EABP by either of these mAbs was detected by western blotting. In ELISA, CABP was recognised by IgM and IgG mAbs and polyclonal hAMPA, while no clear reactivity was observed to the EABP and NBP fractions (figure 1D). Overall, these data indicate that hAMPA can recognise acetylated bacterial protein fractions and suggest that the level of acetylation is related to the ability of hAMPA to recognise acetylated bacterial proteins.

Activation of monoclonal hAMPA-expressing Ramos-B cells by bacterial protein fractions

Next, we set out to investigate whether the modified bacterial proteins could be recognised by human B cells expressing a PTM-directed BCR. To do so, we used a Ramos B-cell line expressing a PTM-protein-directed BCR at the cell surface that is derived for the monoclonal AMPA (7E4).[3] The Ramos cells were stimulated with CABP, EABP or NBP fractions. BCR-mediated activation of the cells was visualised by detection of Syk phosphorylation (pSyk; figure 1E, online supplemental figure 1) downstream of BCR signalling. As shown in figure 1F, the Ramos-B cells were readily activated by acetylated fibrinogen (positive control) as well as by CABP, as demonstrated by increased pSyk expression after exposure to these antigens. In contrast, EABP and NBP did not induce Syk phosphorylation. Together, these data indicate that human B cells, expressing an acetyllysine-reactive BCR, can be activated by acetylated bacterial proteins with sufficient acetylation abundance.

Immunisation of mice and induction of AMPA responses

After these in vitro experiments, we next turned to the in vivo setting to address the question as to whether modified bacterial proteins can induce AMPA responses. To this end, we immunised C57BL/6 mice with CABP, EABP or NBP (figure 2A). As positive control, mice were immunised with acetylated ovalbumin (AcOVA); PBS served as negative control. After four immunisations, sera were tested for antibody responses using ELISA with modified fibrinogen as coating. Repeated immunisation with CABP induced an antibody response, recognising acetylated, but also carbamylated and citrullinated proteins (figure 2B), which was not seen after fewer immunisations (data not shown). The strength of these responses appeared to be equivalent or even exceeded those observed in mice immunised with the positive control AcOVA (figure 2B,C). Immunisation with EABP or NBP did not generate antibody responses to the PTM proteins.

As bacterial protein fractions might act as their 'own adjuvant' due to the likely presence of lipopolysaccharide, we next wished to address the question whether these fractions can induce autoreactive AMPA without the need for additional adjuvancy. Therefore, mice were immunised with CABP and AcOVA without adjuvant. The data obtained indicate that, in contrast to AcOVA, exposure to CABP without adjuvant was sufficient to induce a cross-reactive AMPA response, indicating that modified bacterial proteins can independently induce AMPA responses, when sufficiently modified (figure 2D). Other ELISA backbones (CCP2, other proteins) were not tested due to the limited mouse material. Mice did not develop any visible symptoms or changes in behaviour throughout the experiment.

DISCUSSION

This is the first study demonstrating that the bacterial proteome, when sufficiently acetylated, can induce an autoreactive AMPA response. Although using *E. coli* as model microbe, we did not detect AMPA induction in mice exposed to EABP fractions, strong AMPA responses were observed after exposure to acetyllysine-rich EABP. Clearly, we can only speculate how our observations relate to a potential role of the gut microbiome in the induction of AMPA, it is tempting to hypothesise that under certain conditions, the microbiome-derived antigens might reach a sufficient acetylation level to induce AMPA. Indeed, under certain pathological conditions, increased acetylation levels of the bacterial proteome have been reported in human.[8] Our data, therefore, show a novel concept for the induction of AMPA that might represent one of the routes by which B cell tolerance to PTM-proteins in RA is breached.

AMPAs are known to be present years before the onset of RA.[12] The search for a potential culprit pathogen responsible for autoimmunity in RA has been ongoing for decades and various pathogens have been suspected.[13]

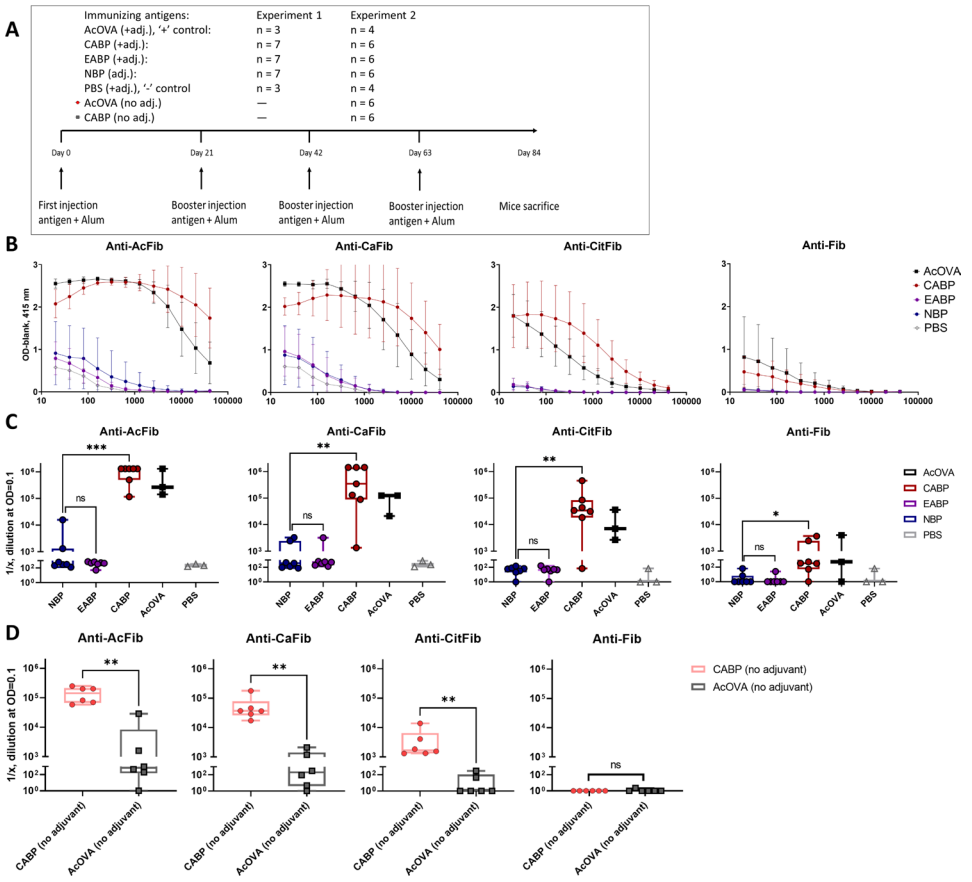

Figure 2 Immunisation of mice with bacterial acetylation proteins. (A) Immunisation scheme of the performed mouse experiments. (B) Reactivity of titrated mouse serum to the modified and unmodified versions of fibrinogen, per immunising antigen. (C) Mice immunised with the antigens with the adjuvant: titres at OD=0.1, as determined by ELISA to modified and native versions of fibrinogen. (D) Mice immunised with CABP or AcOVA without the adjuvant: reactivity of individual mouse serum samples to the modified and unmodified versions of fibrinogen. Statistical differences are indicated with asterisks, indicating p value: *<0.05, **<0.01, ****<0.0001. (A–C) Data are representative of two experiments. AcOVA, acetylated ovalbumin; CABPs, chemically acetylated bacterial proteins; EABPs, endogenously acetylated bacterial proteins; NBPs, non-acetylated bacterial proteins; PBS, phosphate buffer saline.

Nevertheless, it remains challenging to identify a particular species, causally linked with autoimmunity in RA. Based on this and previous work,[10] we suggest that a generic pathogen with abundant acetylation could play a causal role in the breach of tolerance towards acetylated, cross-reactive to carbamylated or/and citrullinated antigens. Later in time, this response can potentially be skewed by other PTM antigens,[10] including citrullinated proteins of the host, which can result in the predominance of ACPA within the AMPA response, as observed in patients with RA.[14] The relevance of this should be further investigated, ideally, in large longitudinal studies in AMPA-positive subjects at risk of RA, which could elucidate how prevalence the skewing from AAPA to ACPA is observed in human individuals.

Acetylation was reported to be present on a variety of proteins in the human gut[8] and this is likely to be observed in patients with RA and healthy donors alike. This raises the question about the conditions that could spur the induction of AMPA responses by acetylated antigens. One of these conditions could include the increased abundance of acetylated antigens, potentially resulting from low protein/high carbohydrate environment in the gut, mimicking the tested conditions for acetylation accumulation.

Our data suggest that the amount of acetylated antigens appears to be important: we show that in mice, repetitive exposure and high acetylation abundance appeared to be crucial for the development of a robust and cross-reactive AMPA response. Likewise, impaired barrier function of the gut mucosa may also play a role. Impaired barrier function, also known as 'leaky' gut syndrome, has been hypothesised to contribute to autoimmunity and inflammation in RA.[15] In this scenario, bacterial antigens may be transferred to the tissues and circulation beyond the intestine and thus activate the immune system. Importantly, intestinal bacteria have also been shown to induce immune responses in healthy individuals, suggesting that increased intestinal permeability might not even be required for these immune responses against microbial antigens.[16]

Another interesting aspect is the role of adjuvants for induction of immune responses. Even when using non-self-proteins for immunisation, adjuvants are

generally required to induce a robust response in laboratory animals. Interestingly, even without the additional adjuvant, CABP could induce a robust cross-reactive AMPA response, recognising antigens bearing each of the three PTMs (acetyllysine, homocitrulline and citrulline). This was not observed for AcOVA. The fact that CABP, being a mix of acetylated bacterial proteins, appeared to be a more potent AMPA-inducing antigen could potentially be explained by the adjuvant function of bacterial compounds (such as lipopolysaccharide), the larger variety of acetylated epitopes present in CABP as compared with AcOVA may also be of significance. Nevertheless, this highlights the potent nature of bacterial antigens in inducing potentially autoreactive AMPA responses.

Worth noting that, while developing strong AMPA responses, mice did not develop visible symptoms or behavioural changes that may be associated with arthritis. This is in line with previously published data on mice developing AMPA in the absence of symptoms.[4] Breach of tolerance and development of AMPA can be seen as the so-called 'first hit', while further triggers are necessary for the autoimmunity to transition into disease. It remains to be investigated whether immunisation with acetylated bacterial proteins leads to the onset of symptoms in mice prone to develop arthritis, although it is also conceivable that no arthritis will develop given the recent anti-inflammatory effects of ACPA in murine arthritis models.[17–19]

To the best of our knowledge, this is the first study showing acetylated bacterial antigens to be recognised by human AMPA from patients with RA. We now also show that these proteins can induce AMPA responses in an animal model. However, this study also has several limitations: we did not show the acetylated proteins (earlier shown to be present in the gut[8]) are recognised by hAMPA. Furthermore, despite the difference in acetylation between EABP and NBP (visible on western blotting with rabbit pcAAPA but not on mass spectrometry), only CABP and not EABP showed specific signal in the western blotting with hAMPA, B cell activation assay and immunisation experiments. Nevertheless, EABP was specifically recognised by human AMPA in ELISA and by rabbit AMPA. This may be explained by the importance of the acetylation abundance together with varying method sensitivity. The major limitation of the mouse experiments is the artificial nature of CABP high acetylation and peritoneal route of immunisation; however, repetitive and prolonged exposure to endogenously acetylated bacteria might still be able to trigger an anti-acetyl response in 'real life'. Furthermore, AcOVA is a merely a single protein as opposed to the CABP being a whole protein fraction of a lysate. However, AcOVA is a large protein with a variety of epitopes and we do not expect major immunological implications of using a eukaryotic protein mix instead of a single protein.

CONCLUSION

Overall, our data suggest a conceptual possibility that bacterial acetylated antigens originating from the gut can be involved in the induction of AMPA responses and thus take part in the breach of tolerance in RA. Acetylation, being a generic metabolic process taking place in various bacteria, does not have to be linked with a particular bacterial species but can rather be accumulated as a response to changed conditions. It remains to be investigated whether certain conditions in the gut can contribute to the transfer of acetylated antigens beyond the intestinal lumen and whether this can result in the induction of an AMPA response in susceptible individuals.

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Contributors All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Study conception and design: MV, ASBK, KAJvS, REMT, DvdW. The mouse experiments were supervised and mainly performed by ASBK. Acquisition of data: MV, ASBK, JCK, AdR. Analysis and interpretation of data: MV, ASBK, JCK, AdR, PAvV, TWJH, REMT, DvdW. Guarantor: DvdW.

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Competing interests MV became employed by Pfizer within 36 months after completing work related to this study.

Patient consent for publication Not applicable.

Ethics approval All animal experiments were approved by the Ethical Committee for Animal Experimentation of the LUMC, Leiden. The study with human material was conducted with the approval of the regional ethics committee at Leiden University Medical Center.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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