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53

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Autophagy generates citrullinated peptides in human synoviocytes: a possible trigger for anti-citrullinated peptide antibodies

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Abstract

Objectives. Autophagy may represent a functional processing event that creates a substrate for autoreactivity. In particular, autophagy may play a role in the pathogenesis of RA, since autophagy is a key cellular event involved in the generation of citrullinated peptides, with consequent breakage of tolerance. Thus, in RA, autophagy may be the common feature in several situations (including smoking, joint injury and infection) that may drive the adaptive responses to citrullinated self-proteins. The aim of this study was the analysis, *in vitro*, of the role of autophagy in the generation of citrullinated peptides and, *in vivo*, of the relationship between autophagy and the production of anti-CCP antibodies (Abs).

Methods. For autophagy induction, fibroblast-like synoviocytes, primary fibroblasts and monocytes were stimulated with tunicamycin or rapamycin. Peptidyl arginine deiminase activity was tested by enzyme-linked immunosorbent assay, and protein citrullination was evaluated by western blotting. The main citrullinated RA candidate antigens, vimentin, α-enolase and filaggrin, were demonstrated by immunoprecipitation. The relationship between autophagy and anti-CCP Abs was analysed in 30 early-active RA patients.

Results. Our results demonstrated *in vitro* a role for autophagy in the citrullination process. Cells treated with tunicamycin or rapamycin showed peptidyl arginine deiminase 4 activation, with consequent protein citrullination. Immunoblotting and immunoprecipitation experiments, using specific Abs, identified the main citrullinated proteins: vimentin, α-enolase and filaggrin. *In vivo*, a significant association between levels of autophagy and anti-CCP Abs was observed in treatment-naïve early-active RA patients.

Conclusion. These findings support the view that the processing of proteins in autophagy generates citrullinated peptides recognized by the immune system in RA.

Key words: autophagy, citrullination, PAD, rheumatoid arthritis, anti-CCP antibodies

Rheumatology key messages

- Autophagy may play a role in the pathogenesis of RA.
- Processing of proteins in autophagy may generate citrullinated peptides in RA.
- A significant association between levels of autophagy and anti-CCP antibodies was observed in RA patients.

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Introduction

RA is an autoimmune disease characterized by chronic joint inflammation and progressive destruction of cartilage and bone, which may lead to loss of function. RA synovial fibroblasts play a key role in the pathogenesis of RA [1, 2]. Synovial fibroblasts are able to release a large variety of inflammatory cytokines, chemokines and matrix-degrading enzymes, thus contributing to the inflammatory and joint-destructive state in RA [3, 4]. During cellular stress, large quantities of proteins are damaged, resulting in their unfolding/misfolding, polyubiquitination and, possibly, aggregation. The efficient removal of these toxic factors can help to relieve the cell of stress and reinstate homeostasis. A fundamental mechanism involved in this action is autophagy [5, 6].

Autophagy is an essential homeostatic process by which cells break down their own components [7]. During autophagy, parts of the cytoplasm and organelles are encapsulated in double-membraned vacuoles called autophagosomes, which finally fuse with lysosomes to degrade the incorporated material using acidic hydrolases [8]. The autophagy machinery is thought to have evolved as a stress response that allows unicellular eukaryotic organisms to survive during harsh conditions, probably by regulating energy homeostasis and/or by protein quality control.

According to an emerging hypothesis, perturbations in autophagy have also been implicated in autoimmune diseases [9, 10]. In particular, activation of the mammalian target of rapamycin, a kinase that represents a key player in autophagy regulation, has been demonstrated in SLE [11]. Moreover, in a recent study, we described a significant disparity in autophagic propensity between T lymphocytes from healthy donors and those from patients with SLE, the latter being resistant to autophagy induction [12].

Interestingly, citrullination in the autophagosomes may favour catabolism of the proteins, as charged residues of the proteins are eliminated. Thus, a central role for autophagy in citrullination of peptides by antigen-presenting cells has been hypothesized [13–15]. In RA patients, anti-CCP antibodies represent a marker of the disease [16] and identify a subset of patients that is characterized by an aggressive disease course, including bone erosion [17]. A number of environmental conditions, such as smoking or infections, are associated with RA [14]. Autophagy in the antigen-presenting cell may be the common feature in several types of stress (including smoking, joint injury and infections) that may drive a response to citrullinate proteins. Recently, the RA synovium was reported to exhibit a highly increased endoplasmic reticulum (ER) stress-associated gene signature [18], and TNFα was shown to further increase the expression of ER stress markers in synovial fibroblasts [19]. Kato *et al*. [20] identified a dual role for autophagy in the regulation of stress-induced cell death in RA synovial fibroblasts. Thus, at present, the exact role of the autophagic process in the progression of RA is still unknown.

In this paper, we focused on the role of autophagy in the generation of citrullinated peptides in human synovial fibroblasts and monocytes, analysing the effect of autophagy on the activity of peptidyl arginine deiminase (PAD), the enzyme involved in the conversion of arginine to citrulline [21]. We also verified the possible relationship between autophagy and the production of anti-CCP antibodies (Abs) in early-active RA patients naïve to any treatment.

Methods

Patients

This study included 30 consecutive early RA patients [10 men and 20 women, mean (S.D.) age 49 (14) years, mean (S.D.) disease duration 26 (15) weeks], attending the Rheumatology Unit of the Sapienza University of Rome, and 20 healthy donors [9 men and 11 women, mean (S.D.) age 45 (11) years]. All the patients, fulfilling the 2010 ACR/EULAR criteria [1], were recruited after written informed consent. All of them were naïve to treatment. Exclusion criteria were: previous use of steroids and/or DMARDs, diabetes mellitus, renal failure, cardiovascular disease, cancer, metabolic syndrome and IBD.

The mean (S.D.) tender joint count of RA patients was 5.3 (4.4); the mean (S.D.) swollen joint count 3.4 (3.6); the DAS 28 3.36 (1); the ESR 37.7 (30) mmHg/h; and the CRP 17.6 (26) mg/l. The local ethical committee of the Sapienza University of Rome approved the study.

Synovial tissue collection

Synovial tissue was collected from RA and OA patients undergoing total knee replacement in London, after obtaining their informed consent. Ethical approval was granted by the East London & The City Research Ethics Committee 3 (LREC07/Q0605/29). The patients' mean age was 73.25 years (range 73–79 years); 50% of patients were female.

Generation of fibroblast-like synoviocytes from RA and OA patients

Fibroblast-like synoviocytes were isolated as previously described [22]. Fibroblast-like synoviocytes were passaged 1:3 using 0.25% trypsin/EDTA (Sigma-Aldrich, Milan, Italy) when ~90% confluent. Synoviocytes were used between passages 4 to 8 in order to avoid contamination from lymphocytes/macrophages [23].

Cultured fibroblasts and monocyte isolation

Primary human fibroblast cultures obtained from skin biopsy were prepared as previously described [24]. $CD14^+$ monocytes were purified from peripheral blood by incubation with anti-CD14-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by sorting with a magnetic device (MiniMacs Separation Unit; Miltenyi Biotec), according to the manufacturer's instructions [25].

Autophagy induction

Fibroblast-like synoviocytes, primary fibroblasts and monocytes were stimulated with 5 μM tunicamycin (from *Streptomyces lysosuperficus*; TNC, Calbiochem, La Jolla, CA, USA) or with 200 nM rapamycin (Sigma-Aldrich), for different times at 37°C. The optimal incubation time with TNC and rapamycin was selected on the basis of preliminary experiments.

After treatments, cells were collected and prepared for experimental procedures as described below.

PAD 4 activity

Briefly, fibroblast-like synoviocytes, primary fibroblasts and monocytes, untreated or treated with 5 μM TNC or 200 nM rapamycin for 5 min, 20 min, 1 h or 4 h at 37°C, in the presence or in the absence of the calcium chelator EDTA, were lysed as described [26]. Equal amounts of whole cellular extracts were analysed by using an Antibody-Based Assay for PAD activity (ModiQuest Research, AC Oss, The Netherlands), a solid ELISA. The optical density measured in a plate reader could be directly correlated to the control enzyme activity present in the test, resulting in a quantitation of the PAD4 enzymatic activity [27].

Detection of PAD4 in LC3-II immunoprecipitates

Fibroblast-like synoviocytes from RA and OA patients, untreated or treated with TNC (5 μM for 4 h at 37°C) or rapamycin (200 nM for 4 h at 37°C), were lysed in lysis buffer. Cell-free lysates were mixed with protein G-acrylic beads (Sigma-Aldrich) and stirred by a rotary shaker for 2 h at 4°C to pre-clear non-specific binding. After centrifugation (500*g* for 1 min), the supernatant was immunoprecipitated with polyclonal anti-microtubule-associated protein 1 light chain 3 beta (anti-LC3-II) (Abgent, San Diego, CA, USA) plus protein G-acrylic beads. A rabbit IgG isotypic control (Sigma-Aldrich) was used. The immunoprecipitates were split into two aliquots. The first one was subjected to western blot analysis, using rabbit polyclonal anti-PADI4/PAD4 Ab (Abcam, Cambridge, UK). The second one was checked, using polyclonal anti-LC3 (MBL Int Corporation, Woburn, MA, USA) or anti-LAMP1 mAb (Santa Cruz Biotechnology, CA, USA). Immunoreactivity was assessed by a chemiluminescence reaction using the ECL Western detection system (Amersham, Buckinghmashire, UK).

Western blot analysis of citrullinated proteins

Fibroblast-like synoviocytes, primary fibroblasts and monocytes, untreated or treated with TNC (5 μM for 4 h at 37°C) or rapamycin (200 nM for 4 h at 37°C) were lysed in lysis buffer. The lysate was centrifuged for 5 min at 1300*g* to remove nuclei and large cellular debris. Equal amounts of total proteins from cell extracts were subjected to 10% SDS–PAGE and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Segrate, MI, Italy). Membranes were blocked with 5% defatted dried milk in Tris-buffered saline, containing 0.05% Tween-20 and probed with rabbit polyclonal anti-citrulline Ab (Millipore, Billerica, MA, USA, cod. #07-377). Immunoreactivity was assessed by a chemiluminescence reaction, as above.

Each PVDF membrane was stripped and reprobed with specific rabbit anti-vimentin mAb (Cell Signaling, Danvers, MA, USA), rabbit anti-α-enolase mAb (ENO1, Abcam), rabbit polyclonal anti-filaggrin (Abcam) and rabbit polyclonal anti-fibrinogen α (Santa Cruz Biotechnology).

Immunoprecipitation experiments

Fibroblast-like synoviocytes, primary fibroblasts and monocytes, untreated or treated with 5 μM TNC or 200 nM rapamycin for 4 h at 37°C, were subjected to immunoprecipitation assays as reported above. The supernatants were immunoprecipitated with goat polyclonal anti-vimentin (R&D Systems, Minneapolis, MN, USA), mouse anti-α-enolase mAb (Abcam) or mouse anti-filaggrin mAb (Abcam), plus protein G-acrylic beads. An appropriate IgG isotypic control (Sigma-Aldrich) was used. The immunoprecipitates were split into two aliquots. The first one was subjected to western blot analysis, using rabbit polyclonal anti-citrulline Ab (Millipore). The second one was checked, using rabbit anti-vimentin mAb (Cell Signaling), rabbit polyclonal anti-α-enolase mAb (Abcam) or rabbit polyclonal anti-filaggrin (Abcam).

Densitometric scanning analysis was performed by Mac OS X (Apple Computer International), using National Institutes of Health Image (NIH Image) 1.62 software. The density of each band in the same gel was analysed.

Analysis of autophagy

Western blot analysis. Fibroblast-like synoviocytes, primary fibroblasts and monocytes, untreated or treated with 5 μM TNC or 200 nM rapamycin for 4 h at 37°C, were lysed in lysis buffer as reported above and allowed to stand for 20 min at 4°C. The cell suspension was mechanically disrupted by Dounce homogenization (10 strokes). The lysate was centrifuged for 5 min at 1300*g* to remove nuclei and large cellular debris. After evaluation of the protein concentration by Bradford Dye Reagent assay (Bio-Rad), the lysate was subjected to 15% SDS–PAGE. The proteins were electrophoretically transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with 5% defatted dried milk in Tris-buffered saline, containing 0.05% Tween-20 and probed with rabbit polyclonal anti-LC3 Ab (MBL International Corporation) or with anti-actin mAb (Sigma-Aldrich). Bound Abs were visualized with HRP-conjugated anti-rabbit IgG (Sigma-Aldrich) or anti-mouse IgG (Sigma-Aldrich) and immunoreactivity assessed by chemiluminescence reaction, as above. Densitometric scanning analysis was performed by Mac OS X (Apple Computer International), using NIH Image 1.62 software. The density of each band in the same gel was analysed, and the densitometric LC3-II/actin ratios are shown. Alternatively, to validate data obtained by using rabbit polyclonal anti-LC3 Ab, we quantified autophagy with rabbit polyclonal anti-p62 Ab (Cell Signaling).

Flow cytometry analysis. Detection of autophagy was performed by using a rabbit polyclonal anti-LC3-II Ab

(Abgent). Briefly, monocyte cells from patients and from healthy donors were fixed with 1 ml of 3% paraformaldehyde solution in PBS for 15 min at room temperature. Cells were washed with PBS and blocked twice by incubating with 0.1 M glycine in PBS for 10 min at RT. Cells were permeabilized with 0.01% saponin in PBS containing 0.2% BSA, and were then incubated with a rabbit polyclonal anti-LC3-II Ab (Abgent) for 1 h at 4 °C, followed by AlexaFluor 488-conjugated anti-rabbit Abs (Life Technologies) for an additional 30 min. After washing, cells were analysed with a Coulter Epics flow cytometer (EPICS Profile, Coulter Electronics, Hialeah, FL, USA). Cells were gated on the basis of forward-angle light scatter and 908 light scatter parameters [28].

Anti-CCP antibodies assay

Anti-CCP Abs were detected with commercial ELISA kits (Phadia, Milan, Italy) in accordance with the manufacturer's instructions. Anti-CCP Abs were considered to be positive when the absorbance was higher than the cut-off of the kit (10 U/ml).

Statistical analysis

Qualitative differences in clinical and baseline autoantibodies between patients were analysed by the Chi-squared and Fisher's exact tests. Differences between groups were analysed using the Mann–Whitney U test, and analysis for matched pairs was done with the Wilcoxon signed-rank test. Correlation analysis was carried out by the Pearson or Spearman test when appropriate. $P < 0.05$ was considered to be statistically significant.

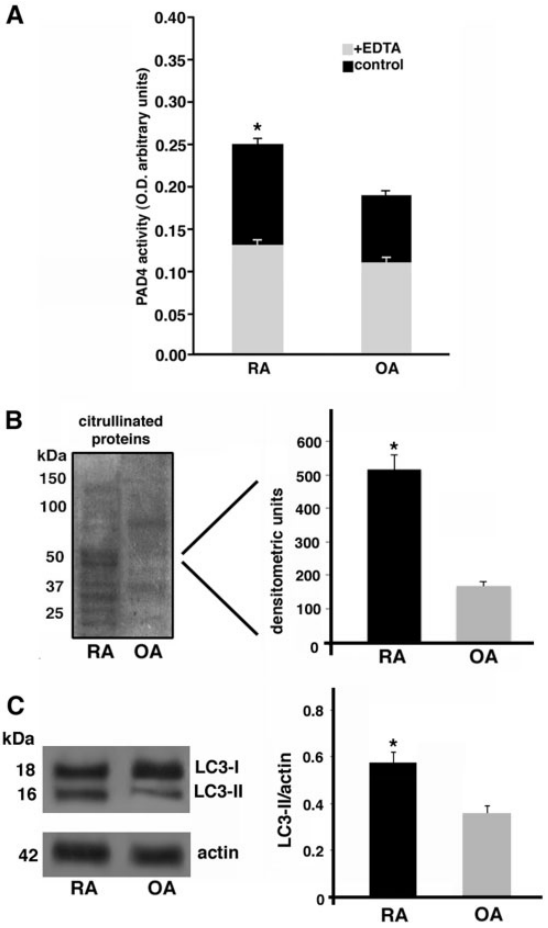
Results

Role of autophagy in activation of the PAD

We preliminary evaluated the activity of PAD4 (an enzyme involved in the conversion of arginine to citrulline [21] and the consequent protein citrullination) in fibroblast-like synoviocytes from RA patients and, as a control, in fibroblast-like synoviocytes from patients with OA, a non-inflammatory arthritis without anti-citrullinated protein Abs. Our results showed a significantly increased basal level of PAD activity (Fig. 1A), with a significant increase in protein citrullination (Fig. 1B) in synoviocytes from RA patients without autophagic stimulation, as compared with those from patients with OA. In parallel with this, a significant increase in levels of autophagy, determined by the autophagic marker LC3-II, was found (Fig. 1C).

To verify the effect of autophagic stimulus on PAD activity, fibroblast-like synoviocytes from RA patients were treated with either TNC or rapamycin. Our results showed that the enzyme was active in a time-dependent manner, even after just 5 min (Fig. 2A). To better define the role of autophagy triggering in PAD activation, we also analysed the effect of TNC or rapamycin on fibroblast-like synoviocytes from OA patients (Fig. 2B), primary fibroblasts (supplementary Fig. S1, available at *Rheumatology* Online) and control monocytes (data not shown). In all of these cells, a relevant induction of PAD activity was observed. Since

Fig. 1 Increase of PAD activity, citrullination proteins and autophagy in RA patients

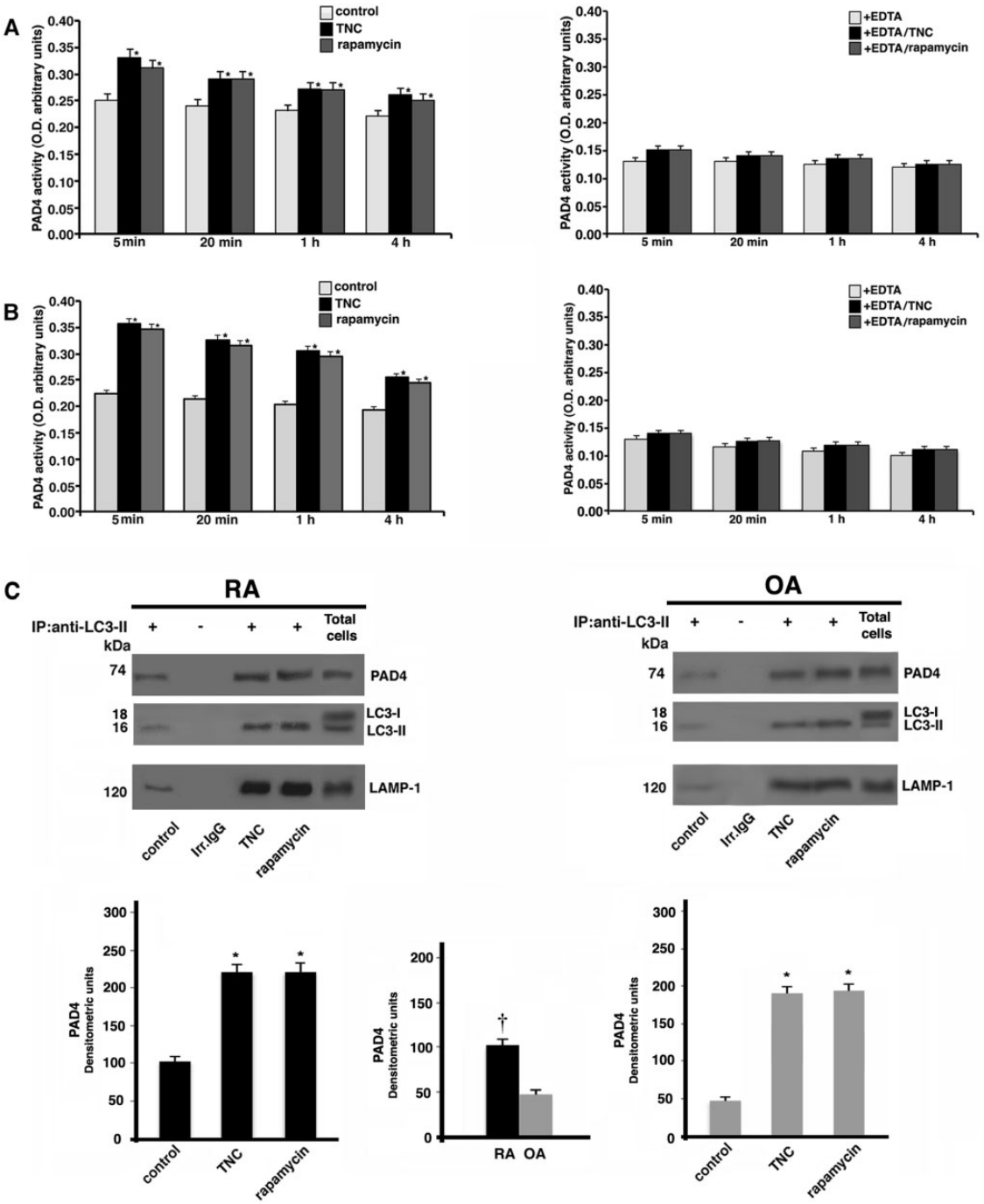


(**A**) Unstimulated fibroblast-like synoviocytes (cells) from RA or OA patients analysed for PAD4 activity. The background was set up by analysing each sample with EDTA [mean (S.D.)]. *P < 0.01 *vs* OA. (**B**) Cells from RA or OA patients were analysed by anti-citrulline Ab; a representative blot is shown. Densitometric analysis of the vimentin band is shown [mean (S.D.)]. *P < 0.01 *vs* OA. (**C**) Cells from RA or OA patients were checked for autophagy by anti-LC3 polyclonal Ab. The loading control was evaluated using anti-actin mAb; a representative blot is shown. Densitometric LC3-II/actin ratios are shown [mean (S.D.)]. *P < 0.01 *vs* OA. LC3: Microtubule-associated protein 1A/1B-light chain 3.

calcium is required for PAD activity, the background of the assay was set up by analysing each sample in the presence of the chelator agent EDTA; as expected, no increase in PAD4 activity was observed following treatment with TNC or rapamycin (Fig. 2A and B, right panels, supplementary S1B, available at *Rheumatology* Online).

In order to clarify whether PAD4 may be present in autophagy vesicles of fibroblast-like synoviocytes from RA and OA patients, we analysed its possible association

FIG. 2 Role of autophagy in activation of the PAD



(**A**) Fibroblast-like synoviocytes from RA patients analysed for PAD4 activity. (**B**) Fibroblast-like synoviocytes from OA patients analysed for PAD4 activity. The background was set up by analysing each sample with EDTA (right panels). Statistical analysis: *P $<$ 0.01 *vs* control. Results are expressed as mean (S.D.); n = 3. (**C**) Fibroblast-like synoviocytes from RA and OA patients, treated with 5 μM TNC or 200 nM rapamycin (4 h at 37 °C) were immunoprecipitated with anti-LC3-II. Immunoprecipitates were analysed for PAD4. Immunoprecipitation was checked by anti-LC3 and anti-LAMP-1. PAD-4 densitometric analysis is shown. Results represent the mean (S.D.) from three experiments. *P $<$ 0.01 *vs* control; †P $<$ 0.01 *vs* OA. PAD: peptidyl arginine deiminase; PAD4: peptidyl arginine deiminase 4; LAMP: lysosomal-associated membrane protein; TNC: tunicamycin.

with LC3-II, which is known to be recruited into autophagolysosomes during the autophagic process [29]. Our findings revealed that PAD4 was present in the immunoprecipitates of LC3-II, following treatment with TNC or rapamycin. Moreover, PAD4 expression was significantly increased in synoviocytes from RA patients as compared with those from patients with OA without autophagic stimulation (Fig. 2C). LC3-II immunoprecipitation was verified by western blot. In the same immunoprecipitates (from RA and OA synoviocytes) we also evaluated the association of lysosomal-associated membrane protein family 1 with LC3-II and observed a positive band of coimmunoprecipitation, which was more evident in cells stimulated with TNC or rapamycin, as a typical marker of autophagolysosomes. No bands were detected in control immunoprecipitation experiments with an IgG having irrelevant specificity (Irr.IgG). These findings suggest that citrullination may occur in autophagy vesicles.

Role of autophagy in generation of citrullinated proteins

The analysis of fibroblast-like synoviocytes from anti-CCP$^+$ RA patients by western blot showed the appearance of numerous bands (corresponding to citrullinated proteins) following autophagic stimulus (Fig. 3A). To better characterize these bands, each PVDF membrane was stripped and reprobed with specific anti-vimentin or anti-α-enolase Abs. As expected, the main citrullinated bands were also stained by these Abs, indicating that autophagic stimuli are able to induce citrullination of vimentin and α-enolase. Interestingly, vimentin showed a double band, due to the presence of a cleaved form of the protein (48 kDa), corresponding to one of the processed citrullinated isoforms of vimentin that are found to be predominantly associated with RA patients [30].

To confirm these findings, the identity of these bands was demonstrated by immunoprecipitation experiments (Fig. 3B). Samples were immunoprecipitated with anti-vimentin or anti-α-enolase Abs and then subjected to western blot analysis, using rabbit polyclonal anti-citrulline Ab, which recognizes citrullinated proteins. The analysis revealed that the bands corresponding to vimentin (double band) and α−enolase were stained by anti-citrullinated protein Ab in cells treated with TNC or rapamycin. Increase in autophagy following treatment with TNC or rapamycin was verified by analysis of LC3-II expression (Fig. 3C).

An evident citrullination following autophagic stimulus was found in fibroblast-like synoviocytes from patients with OA, a non-inflammatory arthritis without anti-CCP antibodies (Fig. 4) or in those from anti-CCP$^-$ RA patients (data not shown), as well as in primary fibroblasts (supplementary Fig. S2, available at *Rheumatology* Online) and monocytes (Fig. 5). Western blot analysis showed the appearance of numerous bands, corresponding to citrullinated proteins. After stripping of the PVDF membranes, the main citrullinated bands were also stained by anti-vimentin and anti-α-enolase in OA fibroblast-like synoviocytes (Fig. 4A); by anti-vimentin, anti-α-enolase and anti-fibrinogen α Abs in human monocytes (Fig. 5A); and by anti-vimentin, anti-α-enolase and anti-filaggrin Abs in primary fibroblasts (supplementary Fig. S2A, available at *Rheumatology* Online). To confirm these findings, the identity of these bands was demonstrated by immunoprecipitation experiments (Figs 4B and 5B; supplementary Fig. S2B, available at *Rheumatology* Online), indicating that these bands were stained by anti-citrulline Abs in cells treated with TNC or rapamycin.

Increase in autophagy following treatment with TNC or rapamycin was verified by analysis of LC3-II expression (Figs 4C and 5C; supplementary Fig. S2C, available at *Rheumatology* Online).

Evaluation of the correlation between autophagy and anti-CCP antibodies in patients with RA

Correlation between the presence of anti-CCP Abs and levels of autophagy was evaluated, as determined by the autophagic marker LC3-II (Fig. 6A) in 30 samples from treatment-naïve patients with early-active RA [1].

The results allowed us to detect an increase in the levels of autophagy in monocytes of anti-CCP$^+$ RA patients compared with anti-CCP$^-$ RA patients, albeit with considerable variability between the various patients (Fig. 6A), highlighting a significant association between levels of autophagy and anti-CCP Abs ($P < 0.0004$), as revealed by Pearson's test.

Autophagy was confirmed by analysis of both LC3-II expression and the degradation of p62 (Fig. 6B). It is well known that the level of the autophagosome adapter protein p62 is inversely correlated with autophagic flux [31].

In the same vein, monocytes from anti-CCP$^+$ RA patients showed the presence of numerous bands, corresponding to citrullinated proteins (Fig. 6C). Again, each PVDF membrane was stripped and reprobed with specific Abs. As expected, the main citrullinated band was stained by anti-vimentin (data not shown). Densitometric analysis revealed an increase in the levels of citrullination in monocytes of anti-CCP$^+$ RA patients compared with in monocytes of anti-CCP$^-$ RA patients (Fig. 6C). A comparison with monocytes from healthy donors is shown in supplementary Fig. S3, available at *Rheumatology* Online.

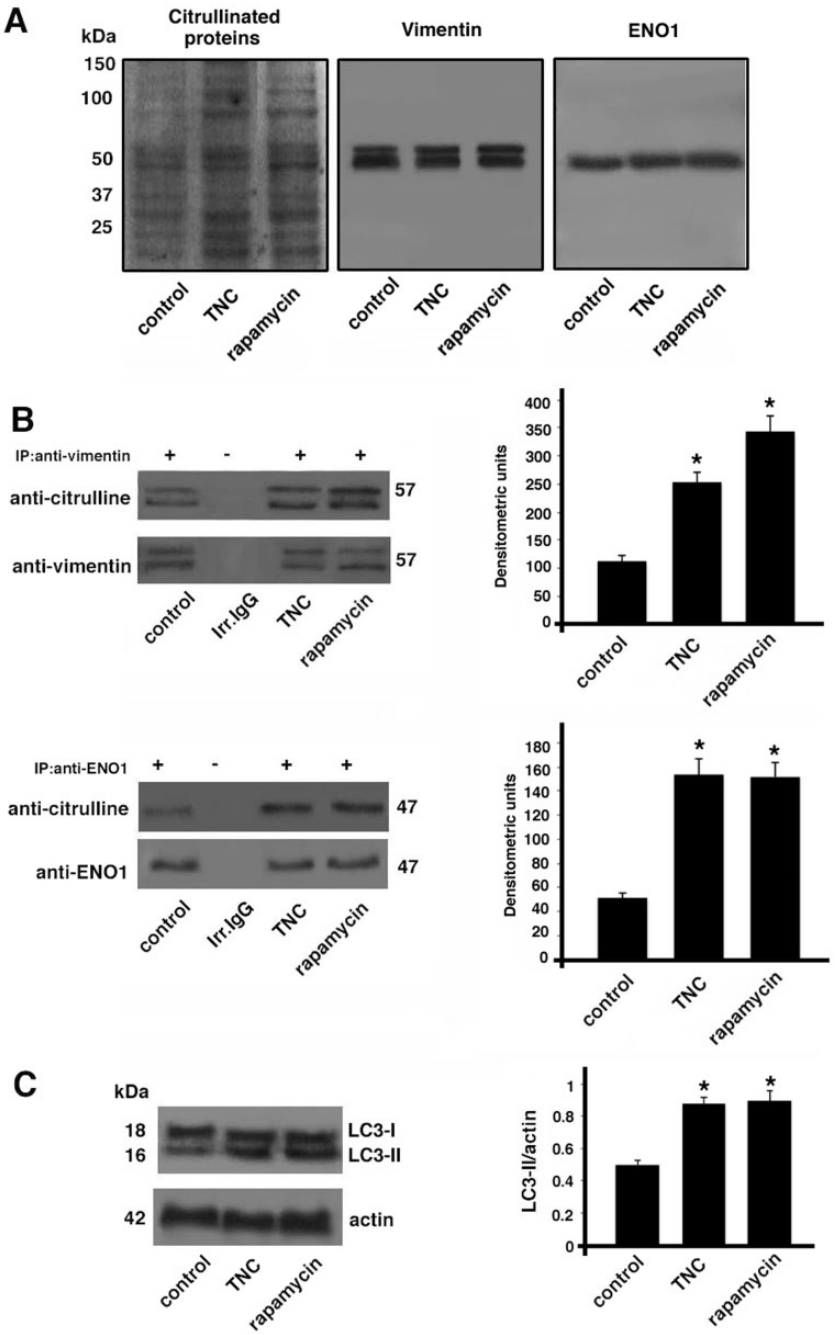
Analysing the relationship between clinical and laboratory parameters, and monocyte levels of autophagy and/or citrullination did not reveal any statistically significant correlation.

Discussion

In this study we clarified the role of the autophagic process in the generation of citrullinated peptides and demonstrated a relationship between the autophagic response and the presence of anti-CCP antibodies in patients with treatment-naïve early-active RA.

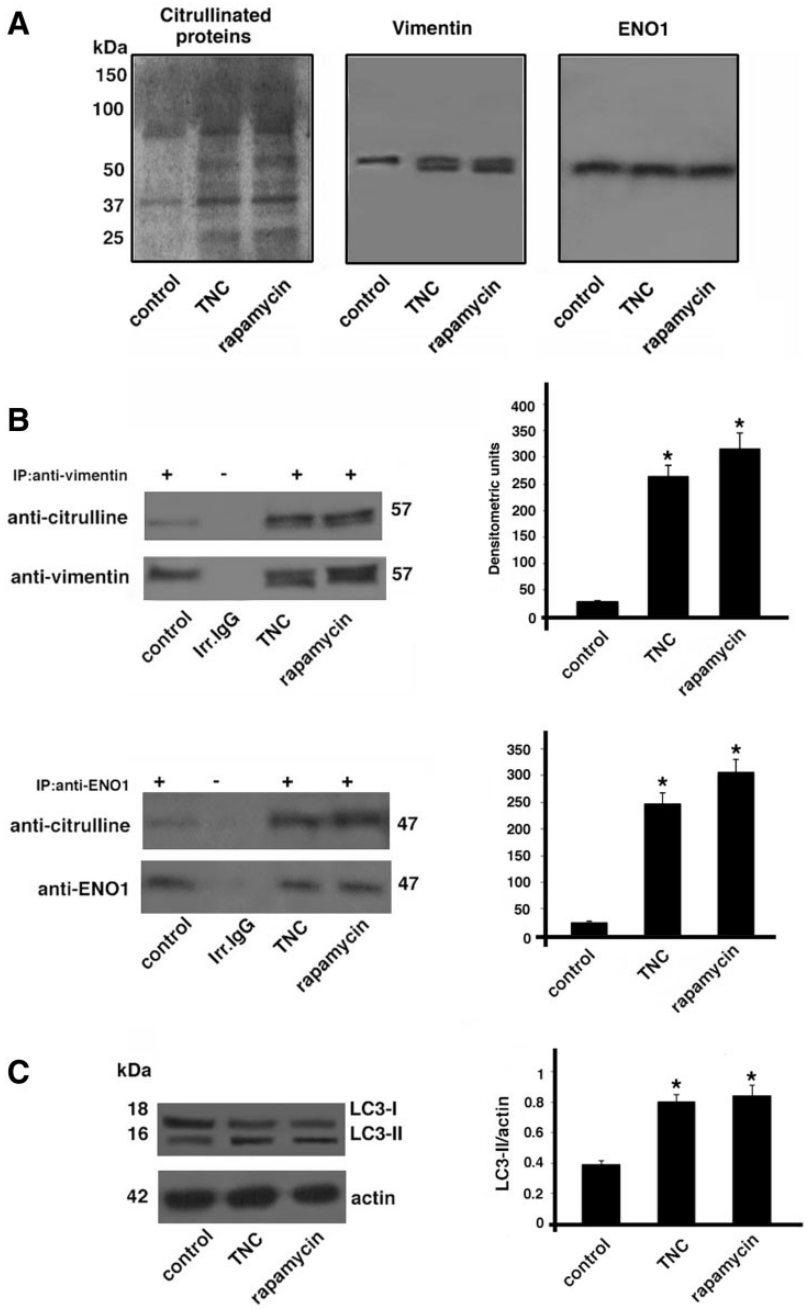
Autophagy may play a role in the pathogenesis of RA [14, 32]. Continued removal of unfolded and misfolded proteins by the proteasome pathway and by autophagy is essential for cell survival. Both pathways were reported to be more active in RA synovial fibroblasts as compared with normal cells [19]. Recently, the RA synovium was

FIG. 3 Role of autophagy in generation of citrullinated proteins in human fibroblast-like synoviocytes from RA patients



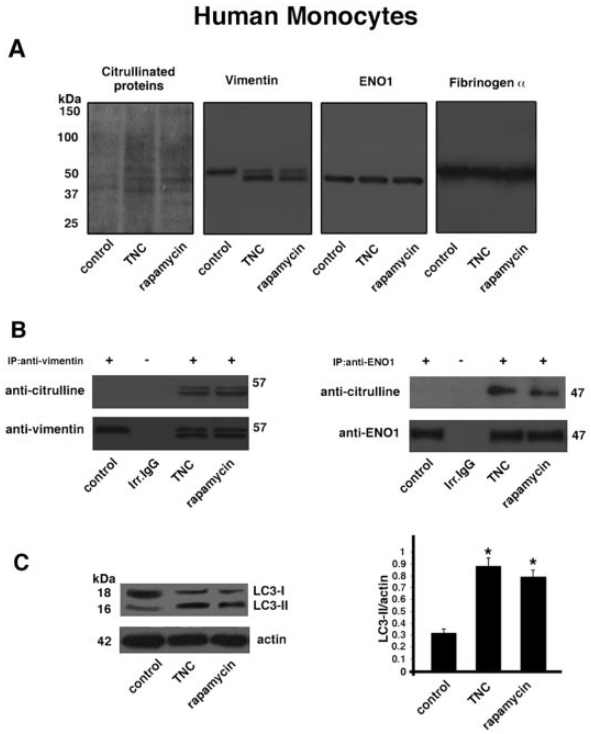
(**A**) Fibroblast-like synoviocytes, untreated or treated with 5 μM TNC or 200 nM rapamycin (4 h at 37 °C) were analysed by anti-citrulline Ab; membranes were reprobed with anti-vimentin and anti-ENO1. (**B**) Fibroblast-like synoviocytes, treated as above, were immunoprecipitated with anti-vimentin or anti-ENO1. Immunoprecipitates were analysed using anti-citrulline Ab. Immunoprecipitation was checked by anti-vimentin or anti-ENO1. Densitometric analyses are shown. Results represent the mean (S.D.) from three independent experiments. *$P < 0.01$ *vs* control. (**C**) Autophagy was checked by anti-LC3 Ab. The loading control was evaluated using anti-actin mAb. Densitometric LC3-II/actin ratios are shown. Results represent the mean (S.D.) from three independent experiments. *$P < 0.01$ *vs* control. ENO1: anti-α-enolase; TNC: tunicamycin; LC3: microtubule-associated protein 1A/1B-light chain 3; Irr.IgG: IgG having irrelevant specificity.

FIG. 4 Role of autophagy in generation of citrullinated proteins in human fibroblast-like synoviocytes from OA patients



(**A**) Fibroblast-like synoviocytes, either untreated or treated with 5 μM TNC or 200 nM rapamycin, (4 h at 37 °C) were analysed by anti-citrulline Ab; membranes were reprobed with anti-vimentin and anti-ENO1. (**B**) Fibroblast-like synoviocytes, treated as above, were immunoprecipitated with anti-vimentin or anti-ENO1. Immunoprecipitates were analysed using anti-citrulline Ab. Immunoprecipitation was checked by anti-vimentin or anti-ENO1. Densitometric analyses are shown. Results represent the mean (S.D.) from three independent experiments. *$P < 0.01$ *vs* control. (**C**) Autophagy was checked by anti-LC3 polyclonal Ab. The loading control was evaluated using anti-actin mAb. Densitometric LC3-II/actin ratios are shown. Results represent the mean (S.D.) from three experiments. *$P < 0.01$ *vs* control. ENO1: anti-α-enolase; TNC: tunicamycin; LC3: microtubule-associated protein 1A/1B-light chain 3; Irr.IgG: IgG having irrelevant specificity.

FIG. 5 Role of autophagy in generation of citrullinated proteins in human monocytes



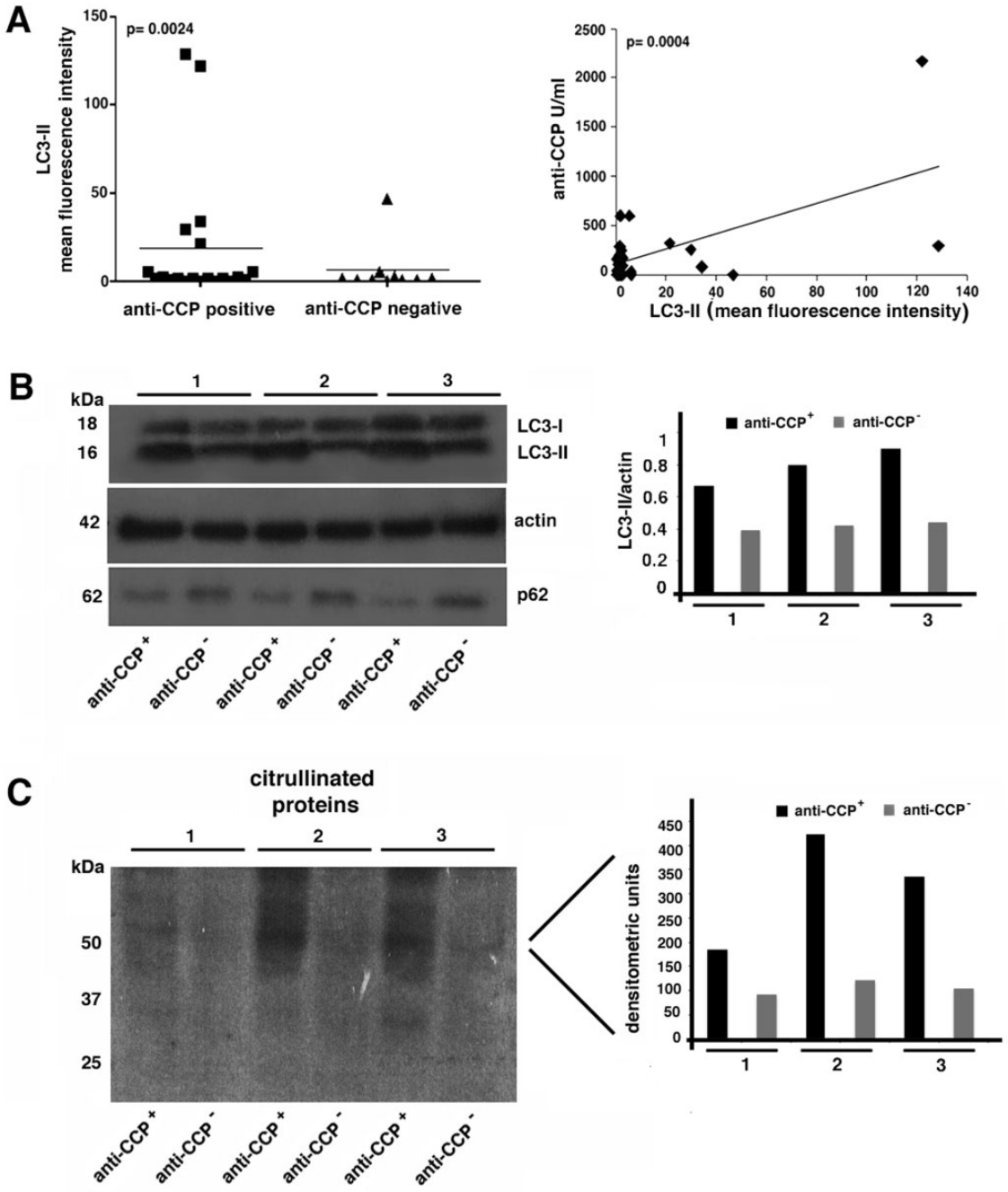
(**A**) Monocytes, obtained from a healthy donor, untreated or treated with 5 μM TNC or 200 nM rapamycin (4 h at 37 °C) were analysed by anti-citrulline Ab; membranes were reprobed with anti-vimentin, anti-ENO1 and anti-fibrinogen α Abs. (**B**) Monocytes, treated as above, were immunoprecipitated with anti-vimentin or anti-ENO1. Immunoprecipitates were analysed using anti-citrulline Ab. Immunoprecipitation was checked by anti-vimentin or anti-ENO1. (**C**) Autophagy was checked by anti-LC3 polyclonal Ab. The loading control was evaluated using anti-actin mAb. Densitometric LC3-II/actin ratios are shown. Results represent the mean (S.D.) from three independent experiments. *$P < 0.01$ *vs* control. ENO1: anti-α-enolase; TNC: tunicamycin; LC3: microtubule-associated protein 1A/1B-light chain 3; Irr.IgG: IgG having irrelevant specificity.

reported to exhibit a highly increased ER stress, and TNFα was shown to further increase the expression of ER stress markers in RA sinovial fibroblasts [18]. In addition, it was observed that autophagy induction by proteasome inhibition or ER stress is higher in RA synovial fibroblasts than in OA synovial fibroblasts [20]. In the same vein, a recent study showed increased expression of the autophagy markers LC3-II and beclin 1 in the lining layer of RA tissue, as compared with OA tissue, which is negatively correlated with apoptosis induction in RA [33]. In summary, till now, a dual role of autophagy in the regulation of stress-induced cell death in RA synovial fibroblasts has been reported [20]. Autophagy activation exhibited a protective role against MG132-induced apoptosis and contributed to the apoptosis-resistant phenotype. In contrast, fibroblasts were hypersensitive to autophagy under conditions of severe ER stress induced by thapsigargin, which was associated with imbalance in p62 and autophagy-linked FYVE (zinc finger interacting specifically with phosphatidyl inositol 3 phosphate) protein expression, leading to the formation of polyubiquitinated protein aggregates and non-apoptotic cell death [19, 20].

Here, we demonstrated a third mechanism by which autophagy may play a role in the pathogenesis of RA. Indeed, our results demonstrated *in vitro* a role for autophagy in the citrullination process. Human synoviocytes, treated with a potent ER stress inducer, such as TNC [34, 35], as well as with m-TOR activator rapamycin [36], revealed an activation of PAD4, with consequent generation of citrullinated proteins. To further support the role of autophagy in the citrullination process, we investigated whether PAD4 may be present in autophagy vesicles. We recently observed that, during the autophagic process, LC3-II is recruited into autophagosomes, where it interacts with the glycosphingolipid environment [35]. Here, we revealed that PAD4 was present in the immunoprecipitates of LC3-II, following treatment with TNC or rapamycin. This finding confirms and extends previous data of Ireland [13], who reported that PAD activity can be detected in isolated autophagosomes with LC3-II enrichment, supporting the view that citrullination may occur in these compartments. As a consequence of PAD4 activation, generation of citrullinated proteins was observed. Interestingly, this protein citrullination was significantly increased in fibroblast-like synoviocytes from RA patients, as compared with control cells from OA, a non-inflammatory arthritis without anti-CCP antibodies. In particular, we demonstrated that the main citrullinated RA candidate antigens [37], including vimentin, α-enolase, filaggrin and fibrinogen α, were processed, following autophagic stimulus. Interestingly, in fibroblast-like synoviocytes, one of the processed citrullinated isoforms of vimentin (predominantly associated with RA patients [30]) was identified. Concerning the possible identification of other processed citrullinated isoforms of the candidate proteins, it is important to point out that the reactivity with the synthetic citrullinated peptides may not fully reflect reactivity with the corresponding citrullinated antigens. Conformational aspects and inaccessibility in the complete proteins may affect reactivity of the epitopes identified [38].

In this study, protein citrullination, following TNC or rapamycin incubation, was conclusively demonstrated by immunoprecipitation experiments, which confirmed processing of the main candidate antigens in RA patients [37]. PADs are a family of enzymes that mediate post-translational modifications of protein arginine residues by deimination or demethylimination to produce citrulline. Although the regulation of the activity of PADs *in vivo* remains largely elusive, there is growing evidence that the deregulation of PADs is involved in the aetiology of multiple human diseases, including RA [39, 40]. In fact,

Fig. 6 Correlation between monocyte LC3-II expression and anti-CCP antibodies in treatment-naïve early-active RA patients



(A) LC3-II expression was analysed by flow cytometry in 30 samples from RA patients. A significant correlation between LC3-II (mean fluorescence intensity) and anti-CCP Abs, revealed by the Pearson test, was found. (**B**) Monocytes from anti-CCP$^+$ and anti-CCP$^-$ RA patients were analysed using anti-LC3 or anti-p62 Abs. Samples from three representative anti-CCP$^+$ and three anti-CCP$^-$ RA patients are shown. The loading control was evaluated using anti-actin mAb. Densitometric LC3-II/actin ratios are shown. (**C**) Monocytes from anti-CCP$^+$ and anti-CCP$^-$ RA patients were analysed, using anti-citrulline Ab. Samples from three representative anti-CCP$^+$ and three anti-CCP$^-$ RA patients are shown. Densitometric analysis of the vimentin band is shown. anti-LC3: anti-microtubule-associated protein 1 light chain 3 beta.

autophagy is a key cellular process that may be the common feature in several stressful cellular environments (including during smoking, joint injury and infection), and it may be involved in the breach of tolerance [9] due to an adaptive response to citrullinated self-proteins, triggering ACPA [13, 14]. Interestingly, in our study population (all treatment-naïve patients affected with early-active RA), we observed a significant association *in vivo* between levels of autophagy and anti-CCP Abs. This finding prompts us to hypothesize, also *in vivo*, that in RA patients, who display a significant increase in protein citrullination and LC3-II expression in synovial fibroblasts, autophagy may be able to trigger a biochemical pathway(s) leading to PADs activation, with consequent processing of defined RA-associated citrullinated antigens, and that this in turn may be responsible for the presence of ACPA. We cannot exclude the possibility that other unidentified stimuli may play a role in this process.

In this study, we focused on early-stage RA patients because we assumed that particular period is the best one in which to study the initiating mechanism at the basis of citrullination and RA. Moreover, in order to avoid confounding therapeutic factors, we included only patients not taking medications such as steroids or DMARDs. That was a strict exclusion criterion, since it is well known that some drugs may interfere with the autophagic mechanism [41]. In order to better understand that mechanism and to elucidate the possible therapeutic regulation of autophagy, we are proceeding with the follow-up of patients that underwent a 1-year treatment.

In conclusion, these findings, together with the observed upregulation of autophagy in RA fibroblasts [40], support the view that processing of proteins in autophagy may generate citrullinated peptides recognized by the immune system in early-active RA [42].

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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