

# Monoclonal antibodies RB139 and RB142 recognize citrullinated LL37 by immunofluorescence in histological sections in Systemic lupus erythematosus (SLE) and Rheumatoid arthritis (RA)

Roberto Lande<sup>1</sup>, Stefano Alivernini<sup>2</sup>, Barbara Tolusso<sup>2</sup>, Francesca Spadaro<sup>3</sup>, Mario Falchi<sup>4</sup>, Raffaella Palazzo<sup>1</sup>, Immacolata Pietraforte<sup>5</sup>, Carlo Chizzolini<sup>6</sup>, Kostantino Giannakakis<sup>7</sup>, Francesca Romana Spinelli<sup>8</sup>, Cristiano Alessandri<sup>8</sup>, Fabrizio Conti<sup>8</sup>, Elisa Gremese<sup>2</sup>, Loredana Frasca<sup>1</sup>

*1 Istituto Superiore di Sanità, National center for drug research and evaluation, Viale Regina Elena 299, 00161, Rome, Italy; 2 Division of Rheumatology, Fondazione Policlinico Universitario A. Gemelli, IRCCS, Rome, Italy; 3 Istituto Superiore di Sanità, Confocal Microscopy Unit, Core Facilities, Rome, Italy; 4 Istituto Superiore di Sanità, National AIDS Center, 00166, Rome, Italy; 5 Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy; 6 Dept of Pathology and Immunology, Centre Médical Universitaire (CMU), Geneva University, 1 Rue Michel Servet, Geneva, Switzerland; 7 Department of Pathology, Sapienza University, Rome, Italy; 8 Division of Rheumatology, Internal medicine and medical specialties, University Sapienza, Rome, Italy*

## Abstract

LL37 is a natural antibiotic, but is also a molecule with pleiotropic functions as well as an immune-modulator. LL37 is produced by epithelial cells and is present in neutrophils' granules. LL37 alone, or in complex with DNA, can activate inflammatory pathways in psoriasis, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). In this work, we describe the capacity of two recombinant monoclonal antibodies, RB139 and RB142, previously shown to specifically recognize LL37 in its citrullinated form (cit-LL37) by ELISA, to detect LL37 by immunofluorescence in human inflamed tissues derived from SLE and RA patients. Such antibodies represent previously unavailable tools to detect the presence, the citrullinated state and the exact localization of cit-LL37 in human tissues in health and disease.

## Introduction

Human cathelicidin antimicrobial peptide (Uniprot P49913), also called CAP18 or FALL39, is encoded by the human gene CAMP. The mature form, peptide LL37, corresponds to the COOH-terminal part of the molecule (res. 134-170), and it exerts antimicrobial activity and several immune modulatory functions (Zanetti, 2005; Hancock *et al.*, 2016). LL37 has been shown to undergo post-translational modifications (PTMs) by citrullination and carbamylation; both PTMs are irreversible and change the functions of the original molecule (Kilsgård *et al.*, 2012; Koro *et al.*, 2016).

We have previously shown that the monoclonal antibodies RB139 and RB142 recognize by ELISA citrullinated LL37 (cit-LL37) but do not cross-react with native or carbamylated LL37 (carb-LL37) (Lande *et al.*, 2020a; Lande *et al.*, 2020b). Citrullination and carbamylation are two PTMs that can be found in systemic lupus erythematosus (SLE) (Navarro Quiroz *et al.*, 2019). However, both PTMs seem very diffuse in rheumatoid arthritis (RA), as anti-carbamylated and anti-citrullinated protein antibodies (anti-carP and ACPA) are characteristic of RA (Kissel *et al.*, 2020). Here we used these antibody tools to detect the presence and the localization of cit-LL37 in SLE-affected kidney and RA-affected synovia, by immunofluorescence in histological sections with laser scanner confocal microscopy (LSCM). Both SLE and RA

are characterized by a neutrophilic inflammation (Cecchi *et al.*, 2014; Frangou *et al.*, 2019). Thus, to address whether cit-LL37 is present in the sites of neutrophil infiltrates, we also stained the biopsies for myeloperoxidase (MPO), a reliable marker of neutrophils (Cecchi *et al.*, 2014; Frangou *et al.*, 2019).

## Materials & Methods

**Antibodies:** ABCD\_RB139 and ABCD\_RB142 antibodies were produced by the Geneva Antibody Facility as mini-antibodies with the antigen-binding scFv portion fused to a mouse IgG2a Fc as described (Lande *et al.*, 2020b).

**Antigen:** Frozen or paraffin-embedded SLE renal biopsies and RA joints biopsies were obtained from Policlinico Umberto I and Fondazione Policlinico Universitario A. Gemelli, (SYNGem cohort), Rome IT.

**Protocol:** Biopsies were saturated with blocking buffer (PBS, 0.05% Tween 20, 4% BSA), for 1 hour at room temperature, and then stained. Biopsies in paraffin were stained after de-paraffination in xylene (5 minutes, two times), followed by passages in: absolute ethanol (3 minutes), 95% ethanol in water (3 minutes), 80% ethanol in water (3 minutes), 70% ethanol in water (3 minutes), and antigen retrieval (5 minutes at 95°C in 10 mM sodium citrate, pH 6.0). Antibodies (RB139 or RB142) were added at 1:50 dilution (all diluted in blocking buffer) (isotype control was added at 1:100 concentration) for 1 hour at room temperature in a humidified chamber. Rabbit anti-human MPO was added at the concentration of 1:25 (Abcam ab208670). Slides were washed three times for 3 minutes under agitation with PBS 0.1% Tween 20 and incubated with donkey anti-mouse or anti-rabbit secondary antibody or mouse anti-goat antibody, conjugated with AlexaFluor 647, Alexa Fluor 568 or 594 or AlexaFluor 488 (Thermo Fisher), depending on the combinations of markers analyzed (in humidified chamber). Slides were washed again, and mounted in Prolong Gold anti-fade media containing a DNA dye (DAPI) (Molecular Probes). Images were taken by a FV1000 confocal microscope (Olympus, Tokyo, Japan), using an Olympus planapo objective 40x or 60x oil A.N. 1.42. Excitation light was obtained by a Laser Dapi 408

nm for DAPI, an Argon Ion Laser (488 nm) for FITC (Alexa 488), a Diode Laser HeNe (561 nm) for Alexa 568, and a Red Diode Laser (638 nm) for Alexa 647. DAPI emission was recorded from 415 to 485 nm, FITC emission was recorded from 495 to 550 nm, Alexa 568 from 583 to 628 nm and Alexa 647, from 634 to 750 nm. Images recorded had an optical thickness of 0.3  $\mu\text{m}$ .

## Results

We first used RB139 in combination with the antibody detecting myeloperoxidase (MPO). As expected, a consistent neutrophil infiltrate was present in the SLE-affected kidney (Fig. 1a). The antibody RB139 detected cit-LL37 in the proximity of MPO staining. Since strict colocalization was not often found with MPO, the staining seems to indicate that the antibody RB139 recognized cit-LL37 expressed in neutrophils that have released their content, as it is possible that LL37 undergoes citrullination outside the cells (Frangou *et al.*, 2019). It has been reported that neutrophils can undergo neutrophil extracellular-trap (NET) or NET-like cell death (NETosis) in SLE, although this issue is currently a matter of debate (Cecchi *et al.*, 2014; Frangou *et al.*, 2019; Boeltz *et al.*, 2019). We colored renal biopsies with RB142 and searched for the possible presence of DNA filaments, which may indicate NET-like structures formation in the inflamed tissues (Cecchi *et al.*, 2014; Frangou *et al.*, 2019). We could detect DNA filaments, reminiscent of NET-like structures (Fig. 1b), with cit-LL37 (detected with RB142) in close proximity of the DNA filaments, although not strictly attached, in SLE-affected kidney (Fig. 1b). This suggests that cit-LL37 is probably released outside neutrophils that undergo a NET-like death process. The lower cationicity, due to citrullination, likely favors the detachment of LL37 from the DNA (Cecchi *et al.*, 2014; Boeltz *et al.*, 2019).

Next, we addressed the presence of cit-LL37 in the joints of RA patients (Fig. 2). Also in this case, we found that cit-LL37 staining was in the proximity of the MPO staining. We observed consistent MPO staining in line with the described presence of neutrophils infiltrates in RA inflamed joints (Cecchi *et al.*, 2014).

In conclusion, the monoclonal antibodies RB139 and RB142 represent valuable tools to detect cit-LL37 in the inflamed kidney and joints of SLE and RA patients, respectively. Since they specifically recognize by ELISA cit-LL37 and not native LL37, nor LL37 modified by carbamylation (Lande *et al.*, 2020a; Lande *et al.*, 2020b), both antibodies can be used to localize cit-LL37 in SLE and RA, and possibly in other chronic or acute conditions in which neutrophil infiltrates are present and NET-like structures are formed.

## References

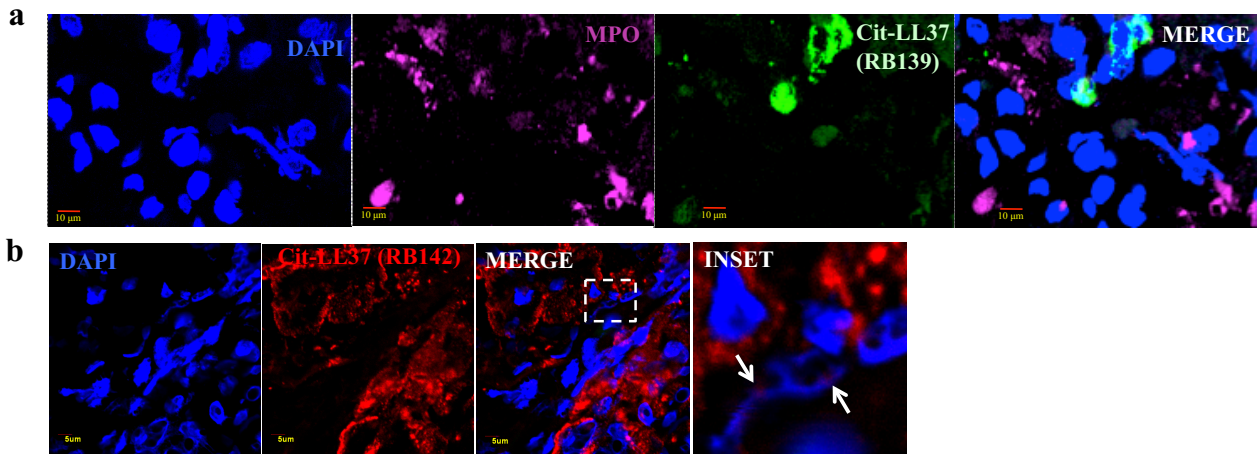
- Boeltz S, Amini P, Anders HJ, *et al.* To NET or not to NET: current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ.* 2019; 26:395-408. PMID: 30622307
- Cecchi I, Arias de la Rosa I, Menegatti E, *et al.* Neutrophils: Novel key players in Rheumatoid Arthritis. *Autoimmun Rev.* 2018; 17:1138-1149. PMID: 30217550.
- Frangou E, Vassilopoulos D, Boletis J, Boumpas DT. An emerging role of neutrophils and NETosis in chronic inflammation and fibrosis in systemic lupus erythematosus (SLE) and ANCA-associated vasculitides (AAV): Implications for the pathogenesis and treatment. *Autoimmun Rev.* 2019; 18:751-760. PMID: 31181324.
- Hancock RE, Haney EF, Gill EE. The immunology of host defence peptides: beyond antimicrobial activity. *Nat Rev Immunol.* 2016; 16:321-34. PMID: 27087664.
- Kilsgård O, Andersson P, Malmsten M, *et al.* Peptidylarginine deiminases present in the airways during tobacco smoking and inflammation can citrullinate the host defense peptide LL-37, resulting in altered activities. *Am J Respir Cell Mol Biol.* 2012; 46:240-8. PMID: 21960546.
- Kissel T, Reijm S, Slot LM, *et al.* Antibodies and B cells recognising citrullinated proteins display a broad cross-reactivity towards other post-translational modifications. *Ann Rheum Dis.* 2020; 79:472-480. PMID: 32041746.
- Koro C, Hellvard A, Delaleu N, *et al.* Carbamylated LL-37 as a modulator of the immune response. *Innate Immun.* 2016; 22:218-29. PMID: 26878866
- Lande R, Palazzo R, Hammel P, *et al.* Generation of monoclonal antibodies specific for native LL37 and citrullinated LL37 that discriminate the two LL37 forms in the skin and circulation of cutaneous/systemic lupus erythematosus and rheumatoid arthritis patients. *Antibodies (Basel)* 2020a; 9:e14. PMID: 32403306.
- Lande R, Palazzo R, Pietraforte I, Chizzolini C, Frasca L. RB139, RB140, RB141 and RB142 antibodies recognize human citrullinated LL37 by ELISA. *Antib. Rep.* 2020b; 3:e189. doi:10.24450/journals/abrep.2020.e189
- Navarro Quiroz E, Chavez-Estrada V, Macias-Ochoa K, *et al.* epigenetic mechanisms and posttranslational modifications in systemic lupus erythematosus. *Int J Mol Sci.* 2019; 20:5679. PMID: 31766160
- Zanetti M. The role of cathelicidins in the innate host defenses of mammals. *Curr Issues Mol Biol.* 2005; 7:179-196. PMID: 16053249.

## Conflict of interest

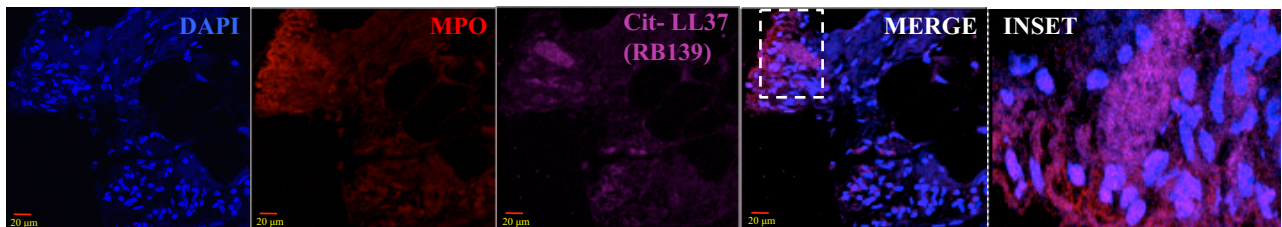
The authors declare no conflict of interest.

## Funding

This work was supported by the following grants: Carlos and Elsie de Reuter Foundation, Centre Médical Universitaire, Geneva, Switzerland to LF; “Ernst et Lucie Schmidheiny” Foundation, Geneva, Switzerland to LF; “Ricerca Finalizzata” (CO-2013-02356463), Ministry of Health, Italy; Swiss National Found to CC (grant 310030–159999) and National Psoriasis Foundation, USA (2019–2021) to LF.



**Fig. 1. Citrullinated LL37 (cit-LL37) is detected in SLE kidney infiltrated by neutrophils.** (a) Neutrophils are identified by MPO staining (magenta) and renal biopsies were also stained with RB139 (cit-LL37, green) and DAPI (nuclei and DNA filaments, blue). Representative staining of three different SLE patients performed in the same way. Magnification: 40x; scale bar: 10  $\mu$ m. (b) RB142 also recognizes cit-LL37 (red) in SLE renal biopsies. DAPI (blue) stains nuclei and DNA filaments. Arrows indicate co-localization of cit-LL37 and DNA (inset corresponding to the rectangle of the merge panel). Magnification 20x; scale bar: 5  $\mu$ m. One representative staining of six performed in biopsies derived from six different SLE patients.



**Fig. 2. RB139 identifies cit-LL37 in RA joints, in the proximity of MPO.** RA biopsies were stained as SLE biopsies in Figure 1, and MPO staining (red) identifies the neutrophil infiltrate. Cit-LL37 (magenta) is present in the same tissues. Magnification: 40x; scale bar: 20  $\mu$ m. Inset is a magnification of the rectangle in the merge panel. Results representative of three stainings performed in three different RA patients.