

RB137 recognizes LL37 in neutrophil-extracellular trap-like (NET) structures in systemic lupus erythematosus and rheumatoid arthritis inflamed tissues by immunofluorescence in histological sections

Roberto Lande¹, Stefano Alivernini², Barbara Tolusso², Francesca Spadaro³, Mario Falchi⁴, Raffaella Palazzo¹, Immacolata Pietraforte⁵, Carlo Chizzolini⁶, Kostantino Giannakakis⁷, Francesca Romana Spinelli⁸, Cristiano Alessandri⁸, Elisa Gremese², Fabrizio Conti⁸, Loredana Frasca¹

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

Besides being a natural antibiotic, the human cathelicidin LL37, produced by epithelial cells and neutrophils, is an important immune-modulator. 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 the recombinant monoclonal antibody RB137, previously shown to specifically recognize LL37 in its native form by ELISA, to detect LL37 by immunofluorescence in human tissues derived from SLE and RA patients. In these tissues, LL37 decorates DNA filaments resembling neutrophil-extracellular-trap (NET) structures. This antibody represents a valuable tool to detect the presence, the native state and the exact localization of 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 peptide LL37, which corresponds to the COOH-terminal part of the molecule (res. 134-170), represents the mature form, which 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 can affect the functions of the original molecule (Kilsgård *et al.*, 2012; Koro *et al.*, 2016).

We have previously shown that the monoclonal antibody RB137 recognize by ELISA native LL37, and it does not cross-react to citrullinated (cit-LL37) nor to carbamylated LL37 (carb-LL37) (Lande *et al.*, 2020a; Lande *et al.*, 2020b). Citrullination and carbamylation are two PTMs that may occur in SLE (Navarro Quiroz *et al.*, 2019). However, both PTMs seem very diffused in RA, as anti-carbamylated and anti-citrullinated protein antibodies (anti-carP and ACPA) are characteristic of RA (Kissel *et al.*, 2020). Here we used RB137 to detect the distribution of LL37 in SLE kidney and RA synovia, by immunofluorescence in histological sections, by using 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 the efficiency of the antibody in recognizing neutrophils (as LL37 is contained in neutrophil granules and represent an additional maker to identify these cells and NETs) (Cecchi *et al.*, 2014; Frangou *et al.*, 2019), we have used LSCM to address whether RB137 could identify neutrophils. Finally, since both SLE and RA neutrophils have been described to be prone to a NET release process called NETosis, we looked for NET-like structures associated with LL37 in SLE and RA tissues.

Materials & Methods

Antibodies: The ABCD_RB137 antibody was produced by the Geneva Antibody Facility as a mini-antibody 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 xilene (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). The antibody RB137 was added at 1:50 dilution 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 μ m.

Results

We first used RB137 in combination with the antibody detecting myeloperoxidase (MPO), as MPO is one of the most used markers of neutrophils. As expected, a consistent neutrophil infiltrate was present in SLE-affected kidney (Fig. 1a). RB137 detected LL37 in the proximity or in the same area of the MPO staining. This indicates that the antibody recognized LL37 expressed in neutrophils (MPO-positive cells) and LL37 was possibly released in the neutrophil-derived granules in the proximal environment.

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 SLE biopsies with RB137 and could detect DNA filaments, reminiscent of NET-like structures, “decorated” by LL37 (Fig. 1b).

In subsequent experiments, we addressed the presence of native LL37 in the joints of RA patients, and we also found that LL37 co-localized with MPO (Fig. 2). This was expected as it is well known that neutrophils infiltrate RA inflamed joints (Cecchi *et al.*, 2014). We observed that some structures resembling NET could be occasionally detected in the same joints (Fig. 2). Thus, it is possible that LL37 is released with NETs also in the RA-affected joints. Native LL37 decorates the DNA filaments that likely represent NET structures, suggesting that LL37 is really attached to the DNA. As citrullination and carbamylation of LL37 decrease the cationic charge of the peptide, it is likely that the diminished electrostatic interactions with the anionic DNA, as shown for citrullinated histones, would instead show a different picture, where cit-LL37 and carb-LL37 co-localize less with the DNA (Navarro Quiroz *et al.*, 2019). Thus, RB137 appears to recognize native LL37 in pathogenic conditions characterized by neutrophilic inflammation, and can detect LL37-DNA complexes formation in tissue by LSCM.

In conclusion, the monoclonal antibody RB137 seems to be a valuable tool to detect native LL37 in the inflamed kidney and joints of SLE and RA, respectively. Since it recognizes specifically native LL37 and not LL37 modified by citrullination and carbamylation (Lande *et al.*, 2020a; Lande *et al.*, 2020b), RB137 can be used to localize native LL37 in SLE and RA and in general in chronic or acute conditions in which neutrophil infiltrates are present and NET-like structures are formed.

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Conflict of interest

The authors declare no conflict of interest.

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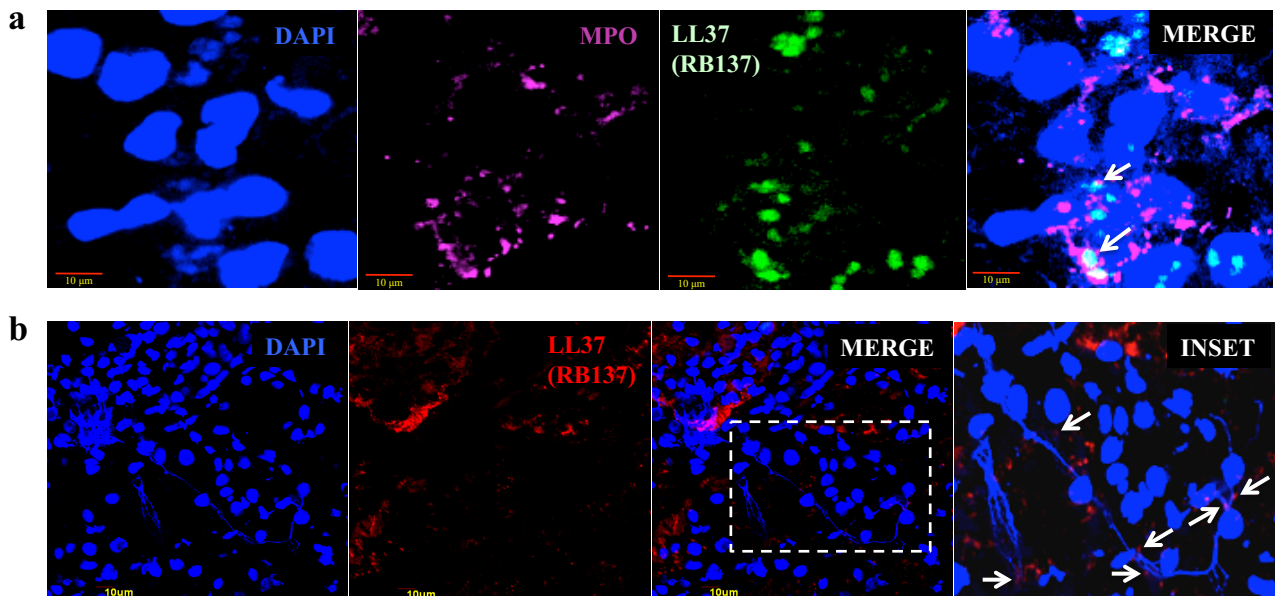


Fig. 1. Native LL37 is present in neutrophil-infiltrated SLE kidney. (a) Neutrophils are identified by MPO staining (magenta) and renal biopsies were also stained with RB137 (native LL37, green) and DAPI (nuclei and DNA filaments, blue). Magnification: 40x; scale bar: 10 μ m. Representative staining of three performed in the same way. Arrows indicate site of co-localization (or very closed proximity) of MPO and LL37. (b) RB137 recognizes native LL37 (red) decorating DNA filaments (DAPI, blue) on NET-like structures in SLE renal biopsies. Magnification: 40x; scale bar: 10 μ m. Arrows indicate co-localization of LL37 and DNA (inset corresponding to the rectangle of the merge panel). One representative staining of six performed in biopsies derived from six different SLE patients.

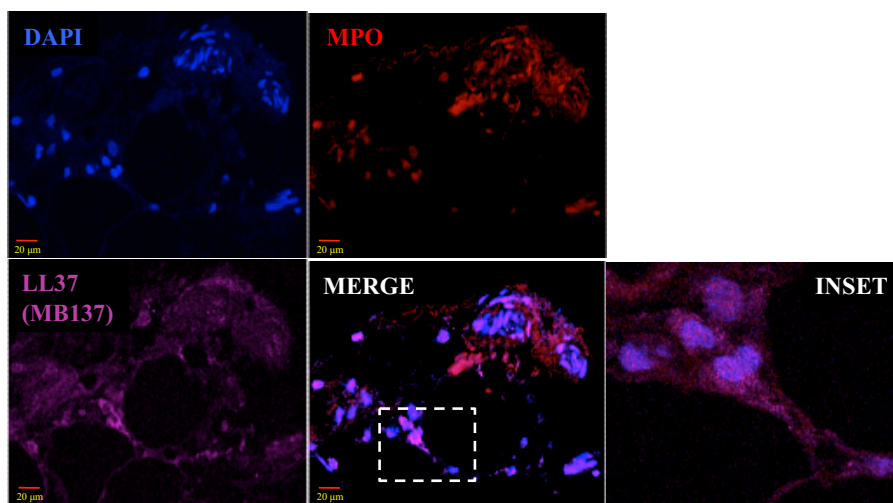


Fig. 2. RB137 identifies native LL37 in RA joints, in the proximity or co-localizing with MPO. RA biopsies were stained as SLE biopsies in Figure 1 and MPO staining (red) identifies the neutrophil infiltrate. LL37 (magenta) is present in the same areas, and DAPI stains the DNA (blue). Magnification: 40x; scale bar: 20 μ m. Inset, corresponding to the rectangle of the merge panel, shows DNA filaments resembling NET-like structures decorated with LL37 and co-stained by MPO. One representative staining of three performed in RA biopsies from different patients.