doi:10.24450/journals/abrep.2020.e189 **Antibody Reports**, 2020, vol. 3, e189

RB139, RB140, RB141 and RB142 antibodies recognize human citrullinated LL37 by ELISA

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Abstract
LL37 is a natural antibiotic, active against bacteria and fungi and some viruses. Here we show that three monoclonal antibodies (RB139, RB141, and RB142) are exclusively specific for citrullinated LL37, whereas RB140 recognizes both native LL37 and cit-LL37. None recognizes LL37 modified by carbamylation. These antibodies represent new tools to detect the presence of citrullinated LL37 in body fluids by ELISA in the course of autoimmune and infectious diseases.

Introduction
Human cathelicidin antimicrobial peptide (CAP18 or FALL39, UniProt #P49913) is encoded by the human gene CAMP. The peptide LL37, which corresponds to the COOH-terminal part of the molecule (residues 134-170), represents the mature form, which exerts antimicrobial and immune-modulatory activity (Zanetti, 2005; Hancock *et al.*, 2016). LL37 has been shown to undergo post-translational modifications (PTM), such as citrullination and carbamylation, and both PTM can change the functions of the native molecule (Kilsgård *et al.*, 2012; Koro *et al.*, 2016). Here we describe the production and characterization of four recombinant antibodies (RB139, RB140, RB141 and RB142) able to detect by ELISA a synthetic fully citrullinated LL37 (cit-LL37). Importantly, none of these antibodies react to carbamylated LL37 (carb-LL37).

Materials & Methods
Antibodies: ABCD_RB139, ABCD_RB140, ABCD_RB141 and ABCD_RB142 antibodies (ABCD nomenclature, https://web.expasy.org/abcd/) were produced by the Geneva Antibody Facility (https://www.unige.ch/medecine/antibodies/) as mini-antibodies with the antigen-binding scFv portion fused to a mouse IgG2a Fc (MRB139, MRB140, MRB141 and MRB142). HEK293 adherent cells (growing in DMEM, Gibco #11960044 supplied with 8% FBS) were transiently transfected with the vectors coding for each scFv-Fc. Supernatants (~1-5 mg/l) were collected after 5 days.

Antigen: The antibodies were raised against an N-biotinylated synthetic citrullinated LL37 (cit-LL37), where the 5 arginines were replaced by 5 citrullines (LLGDFF-**Cit**-KSKEKIGKEFK-**Cit**-IVQ-**Cit**-IKDFL-**Cit**-NLVP-**Cit**-TES). This peptide was also used as antigen for ELISA detection. As a negative control, an N-biotinylated scrambled LL37 sequence was used, as well as an N-biotinylated native LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES). Carbamylated LL37 (carb-LL37) (**L***LGDFFR**K***S**K***E**K***IG-K-EF**K***RIVQRI**K***DFLR NLVPRTES, in which the asterisks describe substitutions with homocitrullines) was used to exclude recognition of carb-LL37 (Koro *et al.*, 2016).

Protocol: The whole procedure was carried out at room temperature. Biotinylated peptides at saturating concentration (10 pmol/well) were immobilized on streptavidin-coated ELISA plates (Pierce #15124) for 30 min. Each well was rinsed three times with 100 µl of washing buffer (PBS + 0.5% (w/v) BSA + 0.05% (w/v) Tween20), then incubated for 1 hour with 50 µl of each MRB antibody-containing supernatant diluted in washing buffer (Fig. 1). After rinsing 3 times (100 µl washing buffer), wells were incubated with horseradish peroxidase-coupled goat anti-mouse IgG (Bio-Rad #170-6516, dilution 1:1000, 50 µl per well) for 30 min. After 3 rinses, Tetramethylbenzidine (TMB) substrate (Sigma #T5569) was added (50 µl per well). The reaction was stopped by the addition of 25 µl of 2 M H_2SO_4. The absorbance (OD) was measured at 450 nm, and the absorbance at 570 nm was subtracted. For comparing reactivity of the antibodies to cit-LL37 and carb-LL37, non-biotinylated native LL37, cit-LL37 and carb-LL37 were directly coated on ELISA plates (Costar) and the procedure carried out as above.

Results
Antibodies RB139, RB140, RB141 and RB142 bound in a concentration-dependent manner to cit-LL37, but not to the negative control scrambled peptide (Fig. 1). Only RB140 showed significant binding to the native unmodified LL37 (Fig. 1). However, none of the antibodies reacted to carb-LL37 (Fig. 2). This is important in that the two PTM types are similar: for cit-LL37, the arginines are substituted by citrullines; for carb-LL37, leucine/lysines are substituted by homocitrullines, and the two substitution amino acids have very similar structures (Kilsgård *et al.*, 2012; Koro *et al.*, 2016). Thus, these antibodies can represent important tools for studying citrullination of LL37, avoiding confusion with carbamylation of the same protein. Of note, naturally occurring antibodies to citrullinated proteins are in general very likely to be cross-reactive between the citrullinated and carbamylated forms (Kissel *et al.*, 2020). RB142 has since been shown to recognize citrullinated LL37 in human tissue by immunofluorescence and immunohistochemistry (Lande *et al.*, 2020).

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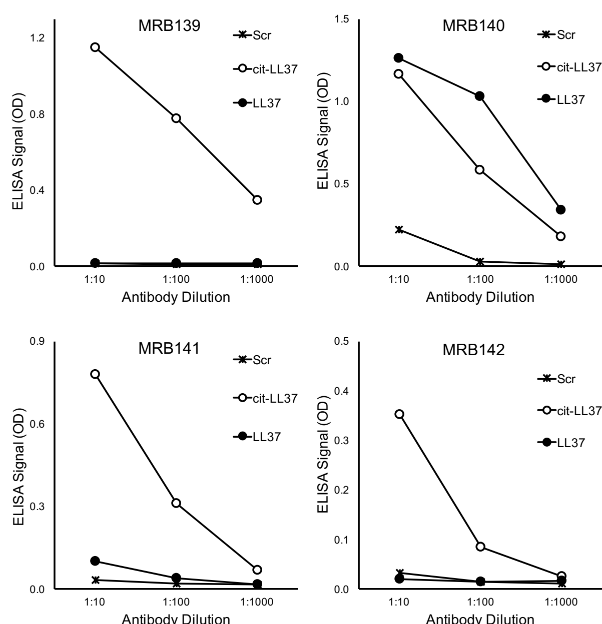

Fig. 1. Specific binding of RB antibodies to the target citrullinated LL37 peptide (cit-LL37), as detected by ELISA. 'Scr' indicates binding to scrambled LL37 and 'LL37' to native LL37.

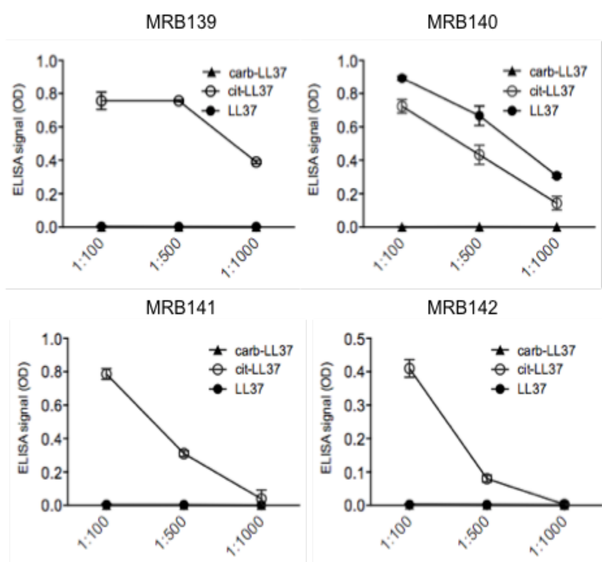

Fig. 2. Specific binding of RB antibodies to the target citrullinated LL37 peptide (cit-LL37), as detected by ELISA. 'LL37' indicates binding to native LL37 and 'carb-LL37' to carbamylated LL37.

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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 L.F.; Ernst et Lucie Schmidheiny Foundation, Geneva, Switzerland to L.F; Swiss National Found to C.C. (grant 310030–159999); and National Psoriasis Foundation, USA (2019–2021) to L.F.

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