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Supplementary appendix

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First Flare of ACPA-positive Rheumatoid Arthritis after SARS-CoV-2 infection

Appendix Methods and Table

Methods

Antibodies

Anti-citrullinated proteins ACPA were detected by two Enzyme-linked immuno sorbent assay (ELISA) tests: anti-CCP2 (Immunoscan RA CCP+ SFAR Euro-Diagnostica, Arnhem, the Netherlands) and anti-CCP3 (QUANTA Lite® CCP3 IgG ELISA, Inova Diagnostics, San Diego, CA, USA)

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence on HEp-2 cells (Kallestad HEp-2 Cell Line Substrate, 12 well slides, Bio-Rad Laboratories, Hercules, CA, USA) at a screening dilution of 1:160.
Anti-ENA and anti-dsDNA antibodies were detected by commercially available kits (EliA dsDNA; Phadia, Uppsala, Sweden; now part of Thermo Fisher Scientific).

Human IgM Rheumatoid Factors were detected with a commercially available ELISA kit (FR-LISA IgM, Théradiag, Croissy-Beaubourg, France) according to the manufacturer's instructions.

Anti-SARS-CoV-2 Spike Protein IgG antibodies were detected with a commercially available ELISA kit (ELISA SARS-CoV-2 IgG, EuroImmun, Lübeck, Germany).

Serum IL6 was quantified with the Human IL-6 Elisa kit II from BD Biosciences (San José, CA, USA) with a limit detection of 2.2 pg/ml.

HLA genotyping:

DNA was extracted from peripheral blood using LabTurbo (Taigen, Taiwan). The sample was typed for HLA-A, -B, -C, -DRB1 and –DQB1 loci by next-generation sequencing in Illumina MiniSeq using NG-Mix® kit (Reagent Production Unit, EFS, France), according to the manufacturer's instructions.

T-cell proliferation assay. Mononuclear cells from patients were isolated from 20 ml of heparinized blood by centrifugation through Ficoll-Histopaque (Sigma Aldrich, St. Quentin-Fallavier, France). Cells were cultured at a density of 10^6 cells/ml in RPMI 1640 with 10% self-serum in the presence of 1 µg/ml of **human PAD4***, **human PAD2*** or **human fibrinogen**** (native or citrullinated) or PHA (phytohemagglutinin) or 5 microg/ml of **PAD4 synthetic peptide (P8)***. After 6 days of culture at 37°C, proliferative response to proteins was evaluated using the colorimetric bromodeoxyuridine kit (Roche Diagnostics, Meylan, France) (19). Positive T cell responses were defined by optical densities (OD) higher than twice the control.

Flow Cytometry. 250 µL aliquots of whole blood were incubated at 37°C for 4 hours with or without 2 µg of PAD4, PAD2 and peptide 8 from PAD4. Incubation was performed in the presence of brefeldin (5 ug/mL). Post incubation, an adapted IntraPrep (Beckman Coulter, Marseille, France (BCMF)) protocol was implemented. Extracellular markers (anti-CD19-ECD, anti-CD4-PC5.5, anti-CD27-A750 and anti-CD8-KrO) were first added to whole blood and incubated for 30 minutes in the dark at room temperature (RT). 400 µL of Optilyse C (BCMF) were then added to the tubes. After 10 minutes, 500 µL of Intraprep reagent 1 (BCMF) were used to enable a strong fixation of the cells prior to the permeabilizing step. After 15 minutes, tubes were centrifuged (300 g, 5 min) and the supernatant discarded. 4 mL of PBS were added to the cell pellet and another centrifugation step was carried out (300g, 5 min). Intracellular markers (anti-IFN□-FITC, anti-CD154-PE, 1 anti-IL4-PC7, anti-IL10-A647, anti-TNF-A700, anti-IL17-PB) were added and 300 µL of Intraprep reagent 2 (BCMF) were used to enable cell permeabilization. Tubes were left in the dark at RT for 60 minutes. 4 mL of PBS were added into the tubes and a last centrifugation step carried out (350g, 5 min). 250 µL of PBS were finally added. Cell suspensions were analyzed on a Cytoflex flow cytometer (Beckman Coulter, Miami, USA). Anti-IL10-A647 was from Biolegend (San Diego, USA), all other conjugated antibodies were from BCMF.

Human PAD4* and PAD2 was produced in a baculovirus expression system and purified (Proteogenix, Schiltigheim, France). **Human fibrinogen**** (Merck Millipore, Darmstadt, Germany) was incubated in 1 M Tris HCl (pH7.4), 100 mM CaCl2, 50 mM

dithiothreitol buffer at a concentration of 1 mg/ml with rabbit PAD2 protein (Sigma Aldrich, St. Quentin-Fallavier, France).

Synthetic peptide 8 from human PAD4*** p8: DPGVEVTLTMKAASGSTGDQ**,** a 20 mer peptide previously associated with RA (11) was synthesized using the solid-phase system and purified (>70%) (Neosystem, Strasbourg, France).

Table :
Progression of inflammation markers and autoantibodies.

Year 2020	April 27	May 1	May 11	June 8	July 15
Sars-Cov-2 symptoms	YES	YES	YES	NO	NO
RT PCR Sars-Cov-2	UK	Positive	Negative	UK	UK
Sars-Cov-2 serology	UK	Negative	Positive	UK	Positive
Rheumatoid arthritis symptoms	UK	NO	NO	YES	YES
C reactive protein (mg/l)	UK	1	1	1.5	18
Sedimentation rate (mm)	UK	UK	UK	UK	97
IL6 (N<2 pg/ml)	UK	2,2	UK	18	31
Rheumatoid Factor N< 20	UK	Negative	UK	23	21
ACPA Anti CCP-2 kit N<25	UK	27	UK	UK	76
ACPA Anti CCP-3 kit N<20	UK	450	UK	450	625
Anti nuclear antiboldies	UK	UK	UK	> 1/1280	> 1/1280
Anti SSA (N <10 U/mL)	UK	UK	UK	UK	240
Anti SSB (N <10 U/mL)	UK	UK	UK	UK	79
Anti PAD4 antibodies	UK	Positive	UK	UK	Positive

UK: unknown, ACPA : anti-citrullinated antibodies, PAD4 : Peptidyl arginine deiminase, N normal