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Systemic sclerosis and primary biliary cholangitis share an antibody population with identical specificity

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Abstract

Anti-centromere (ACA) and antimitochondrial antibodies (AMA) are specific for limited-cutaneous systemic sclerosis (lcSSc) and primary biliary cholangitis (PBC), respectively, and can coexist in up to 25 and 30% of SSc and PBC patients. Here, we evaluated whether anti-centromeric protein A (CENP-A) antibodies cross-react with mitochondrial antigens. To this end, sera from two lcSSc patients (pt1 and pt4), one of them (pt4) also affected by PBC, were used as the source of ACA, previously shown to recognize different groups of amino acids (motifs) in the CENP-A region spanning amino acids 1–17 (Ap1–17). Pt1 and pt4 Ap1–17-specific IgG were purified by affinity-chromatography on insolubilized Ap1–17-peptide column and tested by western blotting with nuclear and cytoplasmic protein extract from HeLa cells. Immunoreactive proteins were identified by mass spectrometry and validated by immunodot. The results showed that affinity-purified SSc/PBC pt4 anti-Ap1–17 and not SSc pt1 anti-Ap1–17 Ab, specifically cross-reacted with the *E2* component of the mitochondrial pyruvate dehydrogenase complex (PDC-E2), the major mitochondrial autoantigen in PBC. Sequence homology analysis indicated that the motif A-x-x-P-x-A-P recognized by pt4 anti-Ap1–17 IgG and shared by CENP-A and PDC-E2, is also expressed by some members of the *Human Herpesvirus* family, suggesting that they may trigger the production of these cross-reacting antibodies.

Keywords: systemic sclerosis, primary biliary cirrhosis, anticentromeric protein A antibodies, anti-mitochondrial M2 antibodies, antigenic specificity, common epitope

Abbreviations: Ab: antibodies; ACA: anti-centromere antibodies; AMA: antimitochondrial antibodies; ANA: anti-nuclear antibodies; Ap1–17: CENP-A region spanning amino acids 1 to 17; AU: arbitrary units; CENP-A: anti-centromeric protein A; CMV: *cytomegalovirus*; EBV: *Epstein–Barr virus*; HHV: *Herpesviruses*; IVIG: human IgG preparations for intravenous use; lcSSc: limited-cutaneous systemic sclerosis; nPDC: native mitochondrial pyruvate dehydrogenase complex; PBC: primary biliary cholangitis; PDC-E2: *E2* component of the mitochondrial pyruvate dehydrogenase complex; PDPL: peptide phage library.

Introduction

Systemic sclerosis (SSc) and primary biliary cholangitis (cirrhosis) (PBC) are chronic autoimmune disorders of unknown etiology. SSc leads to widespread microvascular damage and fibrosis of the skin and internal organs, not including the liver [1], while PBC causes progressive destruction of the intrahepatic bile ducts, eventually culminating in liver cirrhosis and failure [2]. Both SSc and PBC mostly affect women [3] and usually appear during middle age [4, 5]. Several studies have reported a coexistence of SSc and PBC (PBC/SSc overlap), with a prevalence ranging between approximately 1.4 and 17% [6]. PBC is mainly associated with limited cutaneous SSc (lcSSc), and patients with PBC/SSc overlap have a slower rate of liver-disease progression compared to patients with PBC alone [7].

Anti-centromere antibodies (ACA) are SSc-specific autoantibodies (Ab) found in around 90% of lcSSc cases, with centromeric protein A (CENP-A) and centromeric protein B being the main target proteins [8, 9]. ACA are also detected in up to 30% of PBC cases, the prevalence is higher in patients affected by PBC/SSc overlap than in those with PBC alone [7].

Anti-mitochondrial Ab (AMA), especially the AMA-M2 subtype, is detected in 90–95% of patients with PBC [10] and is therefore considered a serological hallmark of the disease, regardless of the presence (25–50% of cases) or absence of anti-nuclear Ab [11]. Moreover, AMA titers may predict PBC onset in a preclinical phase [12]. Despite their association with these clinical features, the origin of ACA and AMA is still unknown. The microbial infection has been suggested to play a role in the induction of AMA, as suggested by the detection of antiviral Ab in several AMA+ patients [13].

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AMA can also be detected in up to 25% of SSc patients [14, 15], with a higher prevalence in ACA+ patients (33–64% of cases) [14, 16].

Until now, ACA and AMA have been considered discrete Ab populations, because attempts to demonstrate cross-reactivity between ACA and AMA were unsuccessful [17].

We previously showed using the phage library peptide library (PDPL) and bio-panning process, that SSc patients' Ab specific for the CENP-A immunodominant epitope spanning amino acids 1–17 (Ap1–17), displayed heterogeneous specificity in that they recognize different groups of amino acids (motifs) within that amino acids segment [18].

Panning a PDPL with anti-Ap1–17 Ab from a SSc/PBC patient (pt4) resulted in the identification of a novel CENP-A binding motif (<^{9}KPxxPxxR^{16}>) defined by the alignment of specific phage clone (pc) insert sequences, including that of pc4.33 [18].

Here, we evaluated at the molecular level any cross-reactivity between pt4 anti-CENP-A Ap1–17 Ab and mitochondrial antigens.

We show that SSc/PBC pt4-derived anti-Ap1–17 Ab recognize an antigenic motif expressed also on the *E2* component of mitochondrial pyruvate dehydrogenase complex (PDC-E2), the immunodominant autoantigen of PBC, demonstrating for the first time that AMA and ACA are not discrete Ab populations in some SSc patients.

Materials and methods

Cells

HeLa cells were obtained from the American Type Culture Collection (ATCC CCL-2) and cultured and maintained in DMEM (Dulbecco's Modified Eagles Medium), supplemented with 10% (v/v) fetal calf serum and 5 mM L-glutamine without antibiotics.

Reagents and antibodies

Electrophoresis reagents were purchased from Bio-Rad Laboratories (Segrate, Milan, Italy). Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Anti-Ap1–17 Ab from pt1 and pt4 were previously purified and characterized [18], and are available in our laboratory. Pt1 and pt4 anti-Ap1–17 Ab recognize different amino acid motifs within the CENP-A amino-terminal immunodominant epitope, as demonstrated by the lack of reactivity of pt1 anti-Ap1–17 Ab with pc-expressing peptides specific for pt4 anti-Ap1–17 Ab, including pc4.33-derived peptide pep4.33 [18].

Polyclonal human IgG preparations for intravenous use (IVIG, Intratec®) were purchased from Biotest (Dreieich, Germany).

Horseradish-peroxidase (HRP)-conjugated xeno-Ab to human IgG (Fc portion) were purchased from Jackson Immunoresearch Laboratories (Avondale, PA, USA).

Western blotting

HeLa nuclear and cytoplasmic extracts were prepared as previously described in reference [19]. Proteins (40 µg/lane) were resolved by 12.5% SDS–PAGE under reducing conditions and transferred onto polyvinylidene fluoride (PVDF) Immobilin P filters (Millipore, Bedford, MA, USA). Immune detection was performed as previously described [18].

Immunodot

Anti-M2 Ab were detected using a line-dot immunoassay from Alphadia (Liver Profile 10 Ag Dot for BlueDiver, Mons, Belgium), according to the manufacturer's instructions. Every single strip contained 10 dots for the simultaneous detection of Ab against different antigens, including M2, and had built-in positive and negative controls. Dot intensity was determined using Dr DOT® Software, and converted into Dot arbitrary units (AU) ranging from 0 (negative result) to 100 (highly positive result), using the following formula: [(intensity of a specific antigen–intensity of the negative control)/(intensity of the positive control–intensity of the negative control)] × 100. Values less than 5 AU were considered negative.

Sequence analyses

To define the pt4 anti-Ap1–17 Ab-specific epitope shared by CENP-A and PDC-E2, a sequence homology analysis search was performed among CENP-A$^{1–17}$, PDC-E2 and the peptide pep4.33 expressed by the previously isolated pt4-specific pc4.33, obtained by panning a PDPL with pt4 anti-Ap1–17 Ab [18]. The antigenic motif was defined by aligning amino acid sequences using CLUSTAL OMEGA at EMBL's European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalo/). Viral proteins containing the antigenic motif were identified using the Swiss-Prot Protein Sequence Database and the PROSITE tool (https://prosite.expasy.org/scanprosite/).

Immunoprecipitation and mass spectrometry

HeLa cells (5×10^{7}) were washed, pelleted, and lysed in 1.5 ml of lysis buffer (50 mmol/L Tris, 4 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40, 1 mmol/L phenylmethylsulfonylfluride), containing a cocktail of protease inhibitors. Following 30 min incubation on ice, the cell lysate was spun at 13 000×g for 30 min at 4°C. The supernatant was then collected, pre-cleared by incubation with protein G Sepharose (PG) (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and transferred to a tube containing 10 µl of packed PG, previously armed with anti-Ap1–17 Ab from pt4 (10 µg). Cell lysates incubated with non-armed PG, and PG armed with anti-Ap1–17 from pt4 not incubated with cell lysate were used as a specificity control. Following a 2 h incubation at 4°C, beads were washed 4 times with PBS, twice with high salt buffer (350 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris, 0.1% bovine serum albumin, 1% NP40), and twice with lysis buffer. Precipitated proteins were eluted in SDS sample buffer, resolved on a non-reducing 4–15% gel gradient SDS–PAGE and stained with Coomassie blue. Bands of interest were directly excised from the Coomassie-stained gel, followed by in-gel digestion with trypsin, and analyzed by nano liquid chromatography–electrospray ionization-tandem mass spectrometry (nLC–ESI-MS/MS) at Cogentech (Milan, Italy). The experiment was performed independently, twice over.

Results

Analysis of the cross-reactivity of SSc anti-Ap1–17Ab with mitochondrial autoantigens

The cross-reactivity of affinity-purified anti-Ap1–17 Ab from the SSc/PBC patient pt4 with mitochondrial antigens was examined by western blot using cytoplasmic and nuclear

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extract from HeLa cells. As shown in Fig. 1, pt4-derived–anti-Ap1–17 Ab specifically reacted with a 17 kDa protein expressed mostly in the nuclear fraction (CENP-A) and with a 70 kDa protein, present only in the cytoplasmic fraction. Instead, anti-Ap1–17 Ab from the SSc patient Pt1, which does not recognize the pt4-specific ACA motif, reacted only with CENP-A. The specificity of the assay was demonstrated by the lack of reactivity of IVIG to both proteins.

To identify the cytoplasmic protein specifically recognized by pt4 anti-Ap1–17 Ab, the 70 kDa band, specifically immunoprecipitated by pt4 anti-Ap1–17 Ab, was excised and in-gel digested with trypsin. Analysis of the resulting tryptic peptides by nLC-ESI-MS/MS identified 20 peptides derived uniquely from PDC-E2 and covering 34% of the PDC-E2 amino acid sequence (Table 1). These results were corroborated by the outcome of a second independent experiment, in which the 19 sequences obtained overlapped the PDC-E2 sequences.

To test the reactivity and specificity of pt4 anti-Ap1–17 Ab against PDC-E2, an immunodot assay was performed against a panel of M2 and autoimmune hepatitis autoantigens, namely M2/native PDC (nPDC), M2/PDC-E2, M2/OGDC-E2, M2/BCOADC-E2, gp210, sp100, LKM1, LC1, SLA, and f-Actin. As shown in Fig. 2, pt4 anti-Ap1–17 Ab reacted strongly and specifically with both nPDC and PDC-E2 (Dot score = 100), while no reactivity was found against the other antigens tested. Neither pt1 Ap1–17 Ab nor IVIG reacted with any of the tested antigens (Fig. 2).

Presence of pt4 anti-Ap1–17 Ab antigenic motif in PDC-E2 and viral proteins

The search for sequence homology among CENP-A$^{1-17}$, PDC-E2, and the pt4-anti-Ap1–17 Ab-specific peptide pc4.33 led to the identification of the motif A-x-x-P-x-A-P (Table 2).

To evaluate whether the motif was expressed by human viruses, the Swiss-Prot database was scanned. Results showed that the motif was mainly expressed by proteins from human herpesviruses (HHV), including HHV-1, HHV-2, HHV-6A, HHV-7, HHV-8, Epstein–Barr virus (EBV), and cytomegalovirus (CMV) (Table 3). The motif was also expressed by proteins from Hepatitis E virus (genotype 70) and viruses belonging to the genus Enterovirus, namely human enterovirus (type 70), rhinovirus (serotype 3 and 14), and poliovirus (type 1, 2 and 3). In addition, it was expressed by proteins from rubella virus, coronavirus NL63, adenovirus F (serotype 40), human immunodeficiency virus (type 2), and papillomavirus (type 18, 48, and 50). Furthermore, in HHV-1, HHV-2, EBV, and HPV viruses, the motif was expressed by viral surface proteins, namely envelope glycoprotein E (HHV-1), envelope protein UL45(HHV-2), envelope glycoprotein GP350 (EBV), and major capsid protein L1 (papillomavirus type 48 and 50).

Discussion

In the present study, the cross-reactivity of anti-CENP-A Ab with mitochondrial autoantigens was investigated. We demonstrated that a subset of anti-Ap1–17 Ab purified from a SSc/PBC patient specifically cross-reacted with PDC-E2, the major PBC-specific AMA autoantigen [36].

Notably, CENP-A-specific anti-Ap1–17 Ab cross-reacting with PDC-E2 did not produce any cytoplasmic reticular pattern at immunofluorescence [18], whereas this had previously been reported to be present in SSc ANA+, expressing anti-M2 Ab [37].

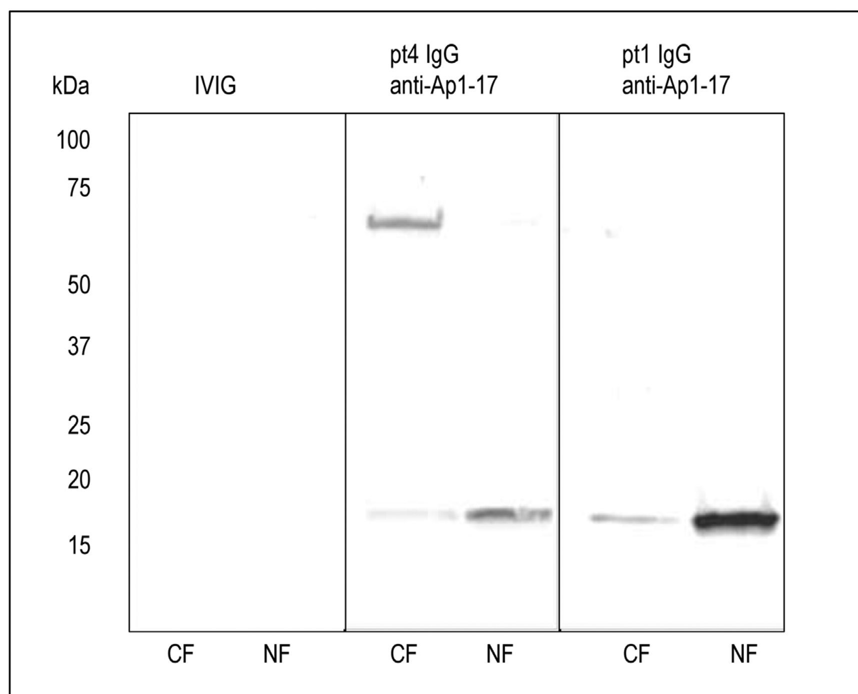

Figure 1. SSc/PBC pt4 anti-CENP-A-derived Ap1–17 antibodies (Ab) specifically cross-react with a cytoplasmic protein measuring approximately 70 kDa. Cytoplasmic (CF) and nuclear fractions (NF) of HeLa cell lysate were loaded on alternative lanes (40 µg/lane) of a 12.5% SDS mini-gel under reducing conditions, transferred to a PVDF filter, and incubated for 3 h with affinity-purified anti-Ap1–17 Ab from pt1, and pt4. IVIG were used as controls. Bound Ab were detected using HRP-conjugated goat anti-human IgG and chemiluminescence detection.

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Table 1. *E2* component of mitochondrial pyruvate dehydrogenase complex (PDC-E2)-derived peptides identified by nanoliquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS) of the 70 kDa protein immunoprecipitated by pt4 anti-CENP-A-derived Ap1–17 antibodies

Peptide		PDC-E2 segment match position	
Numbers	Sequence		
1	KVPLPSLSPTMQAGTIARW	94	112
2	KINEGDLIAEVETDKA	119	134
3	KILVAEGTRD	148	157
4	KILVPEGTRD	275	284
5	RDVPLGTPLCIIVEKE	283	298
6	KGRVFVSPLAKK	353	364
7	RVFVSPLAKK	355	364
8	RVFVSPLAKKL	355	365
9	KGIDLTQVKG	369	378
10	KDIDSFVPSKV	388	398
11	KVAPAPAAVVPPTGPGMAP VPTGVFTDIPISNIRR	397	431
12	RSKISVNDFIIKA	472	484
13	KISVNDFIIKA	474	484
14	KASALACLKV	483	492
15	KVPEANSSWMDTVIRQ	491	506
16	KGVETIANDVVSLATKA	533	549
17	KNFSAIINPPQACILAIGAS EDKLVPADNEKG	575	606
18	KLVPADNEKG	597	606
19	RVVDGAVGAQWLAEFRK	622	638
20	RKYLEKPITMLL	637	648

Interestingly, these data provide a possible explanation of the results previously obtained by Shoji *et al.*, demonstrating that sera from ACA-positive PBC patients all reacted strongly with both PDC-E2 and CENP-A, regardless of the presence of SSc symptoms [38]. The cross-reactivity of ACA with PDC-E2 was previously investigated by Whyte *et al.* [17] but no cross-reactivity was demonstrated. It should be noted that the investigation was performed using affinity-purified Ab with CENP-C; the latter was selected because it was considered a possible primary target of ACA at that time, by the dominant CENP-C-specific IgM response [17].

Great heterogeneity in the fine specificity of anti-Ap1–17 Ab in patients with SSc has already been demonstrated [18]. The fact that SSc/PBC pt4 and not SSc pt1 anti-Ap1–17 Ab recognized PDC-E2 suggests the presence of a subset of ACA that recognize the specific motif, namely AxxPxAP, shared by CENP-A and PDC-E2, and expressed on pc4.33-peptide. Future investigations will define whether a pc4.33 peptide-based ELISA [9] can define a subset of anti-CENP-A positive SSc patients with a high likelihood of developing a PBC overlap syndrome.

The identification of a common antigenic motif between centromeric and mitochondrial autoantigens in SSc/PBC patients shed light on an additional similarity between SSc and PBC features [6]. Indeed, both diseases predominantly occur in genetically predisposed individuals following environmental trigger factors [39, 40]. Susceptibility genes, including HLA-DRB1, -DQB1, IRF5, and STAT4 [41], common to SSc and PBC, have also been identified. Finally, the same viral agents, namely EBV [42, 43] and CMV [42, 44], both belonging to the *Herpesviruses* family, have been suggested to play a role in triggering the development of both diseases, by molecular mimicry [45].

Molecular mimicry refers to the mechanism by which pathogen-derived antigens, sharing sequence similarities with

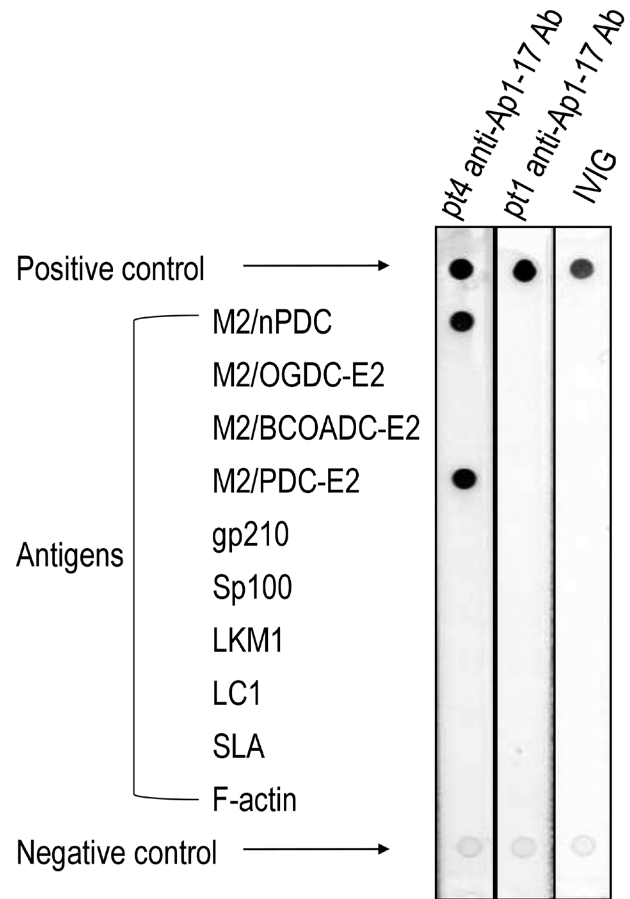

Figure 2. SSc/PBC pt4 anti-CENP-A-derived Ap1–17 antibodies (Ab) specifically cross-react with the *E2* component of the mitochondrial pyruvate dehydrogenase complex (PDC-E2). Purified pt4 anti-Ap1–17 Ab (5 µg/ml) were tested for anti-M2 using the line-dot immunoassay from Alphadia "Liver Profile 10 Ag Dot". Purified pt1 anti-Ap1–17 Ab and IVIG were used as control probes.

Table 2. Definition of the epitope shared between CENP-A and PDC-E2 by the alignment of CENP-A-, PDC-E2-derived peptides with the pt4 anti-Ap1–17 antibodies-specific peptide pep4.33

Denomination	Amino acid sequence
CENP-A$^{1–17}$	MGPRRRSRKPEAPRRRS
PDC-E2$^{331–347}$	PTPQPLAPTPSAPCPAT
pep4.33*	----GIAKKPSAPLQR-
	------:--*-**---
Motif	A--P-AP

*Peptide pep4.33 is expressed by the pt4-specific phage clone pc4.33 isolated by panning a phage display peptide library with purified pt4 anti-CENP-A-derived Ap1–17 antibodies [18].

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Table 3. Viral proteins containing the antigenic motif AxxPxAP, shared by CENP-A and PDC-E2

Virus (worldwide prevalence)	Protein	Amino acid stretch containing motif	
		Starting position	Sequence
HHV-1 (67%) [20]	Envelope glycoprotein E	98	AyaPpAP
	mRNA export factor ICP27	288	AnsPwAP
	Major viral transcription factor ICP4	1153	AmaPgAP
	Large tegument protein deneddylase	2851	AvqPvAP
	DNA-binding protein UL42	441	AarPaAP
	Ribonucleoside-diphosphatereductase large subunit (R1)	271	AvdPhAP
	Protein US8.5.	52	AphPeAP
	Tegument protein VP22	151	AalPdAP
HHV-2 (11.3%) [21]	Envelope protein UL45	9	AyqPlAP
	Transcriptional regulator ICP22	152	AaqPqAP
	mRNA export factor ICP27	288	AnsPwAP
	Major viral transcription factor ICP4 homolog	1181	AmaPgAP
	Thymidine kinase	148	AvgPqAP
	Nuclear egress protein 2	223	AsgPpAP
	Replication origin-binding protein	291	AppPdAP
	DNA primase	682	AcrPrAP
	Tegument protein UL21	257	AppPsAP
	Tegument protein UL47	639	AafPpAP
	Tegument protein VP16	310	AeePgAP
HHV-6A (unknown)	Uncharacterized protein DR2	579	AafPpAP
HHV-7 (90%) [22]	mRNA export factor ICP27	99	AnlPtAP
HHV-8 (6–50%) [23]	Packaging protein UL32	303	AahPeAP
EBV (90%) [24]	Envelope glycoprotein GP350	735	AtsPqAP
	EBV nuclear antigen 3A	463	AqnPrAP
	DNA replication helicase	2	AeePrAP
	mRNA export factor ICP27	151	AraPrAP
	Large tegument protein deneddylase	1143	AsaPgAP
	Major tegument protein (MTP)	851	AttPrAP
CMV (83%) [25]	Uncharacterized protein UL13	330	AviPpAP
*HEV genotype 1 (12.47%) [26]	Protein ORF3	93	AtrPsAP
*HEV genotype 3 (12.47%) [26]	Non-structural polyprotein pORF1	755	AsvPpAP
*Human enterovirus 70 (6.3%) [27]	Genome polyprotein	816	AwaPrAP
*Human rhinovirus 3, 14 (1–21%) [28]	Genome polyprotein	819	AwiPrAP
Poliovirus type 1, 2, 3 (0.01%) [29]	Genome polyprotein	299	AilPlAP
		627	AtnPlAP
Rubella virus (8.8%) [30]	Non-structural polyprotein p200	1016	AspPaAP
	Structural polyprotein (p110)	646	AwvPaAP
Human coronavirus NL63, (1–9.3%) [31]	Replicasepolyprotein 1ab	2207	AvqPtAP
*Human adenovirus F serotype 40 (1–0%) [32]	Protein 33K	107	AgsPtAP
HIV type 2 (1–5%) [33]	Protein Vpr	93	ApcPaAP
*HPV type 48, 50 (9.9%) [34]	Major capsid protein L1	419	AfvPpAP
		422	AyvPpAP
HPV type 18 (1.4%) [35]	Protein E4	30	ApcPwAP

*Non-genotype-specific overall prevalence.

self-antigens of the mammalian host, trigger an immune response against the host antigens [45]. In SSc, CMV has been implicated in triggering the production of SSc-related Ab. Indeed, Ab directed against the viral protein UL94 has been demonstrated to cross-react with the autoantigen NAG2, a cell-surface protein expressed by endothelial cells and fibroblasts [46, 47].

Along this line, a Swiss-Prot database search for human virus proteins containing the motif AxxPxAP led to the location of this motif in proteins from *Herpesviruses*, including EBV and CMV, both characterized by high worldwide prevalences. Notably, the motif AxxPxAP was present in envelope proteins from HHV-1 (glycoprotein E), HHV-2 (protein UL45), and EBV (glycoprotein gp350), suggesting that it could serve as

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a target of cross-reactive Ab specific for CENP-A and PDC-E2. Supporting this hypothesis, there is evidence that EBV-associated envelope glycoprotein gp350, responsible for the initial attachment of the virus to B lymphocytes [48], is the major target for EBV-neutralizing Ab [49]. Finally, the motif was also expressed by HHV-6A, the latter being suggested to contribute to the SSc pathogenesis [50].

Overall, these data reinforce the molecular mimicry model concept and suggest that viral proteins can trigger autoimmunity in genetically predisposed individuals by the induction of an immune response mounted against the non-self antigen and able to cross-react with self-antigens using an epitope spreading mechanism [18, 51, 52].

Future investigations will be focused on the definition of protein subunits from exogenous pathogens that are effectively involved in priming anti-CENP-A Ab cross-reacting with PDC-E2.

Finally, the origin and significance of AMA in ACA-negative SSc patients remain to be defined.

In summary, this study demonstrates for the first time that ACA and AMA are not a different, co-existing Ab population in SSc/PBC patients, and that the concurrent presence of Ab specific for centromeric and mitochondrial antigens in these patients is due, at least partially, to the presence of a single Ab population recognizing an epitope common to CENP-A and PDC-E2.

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Conflict of interests

None declared.

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Data availability

The data that support the findings of this study are available from the corresponding author, F.P., upon request.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Conception and design E.F., F.P. Data acquisition: E.F., E.G., G.B., V.L., P.R., C.F., and G.R. Data analysis and interpretation: E.F., F.P. Permission to reproduce (for relevant content): Not applicable. Clinical trial registration: Not applicable.

The animal research adheres to the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines).

Not applicable.

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