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A comprehensive analysis of antigen-specific antibody responses against human cytomegalovirus in patients with systemic sclerosis

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ABSTRACT

Anti-human cytomegalovirus (HCMV) antibodies are considered triggers of systemic sclerosis (SSc), but such a hypothesis has been assessed in limited sub-dominant epitopes. Our aim was to systematically assess the potential association of HCMV antibodies targeting most immunodominant and subdominant viral antigens, as this would reveal immunopathogenic associations. Our study included 110 SSc patients, 60 multiple sclerosis (MS) patients, and 51 healthy controls (HC). Anti-HCMV abs were tested by immunoblotting. IgG anti-HCMV was broader in SSc and MS compared to HC. Anti- UL57 and UL55 were more frequent in SSc versus MS forms. Reactivity to multiple viral antigens was more frequent in SSc than MS forms. Anti-viral antibodies levels were higher in specific autoantibody-positive SSc patients compared to seronegative cases. In conclusion, more prevalent and/or stronger antigen-specific HCMV responses are noted in SSc compared to controls, implying a role of these viral responses in SSc development.

1. Introduction

Systemic sclerosis (SSc) is characterized by microvasculopathy, fibrogenesis and activation of the immune system leading to the production of a range of disease-related and other autoantibodies (autoAbs) [1–3]. Clinically, two main forms of the disease are widely recognized, limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc) disease. The pathogenesis of the disease is incompletely understood, and activated fibroblasts may be induced by inflammatory cells, and originated from endothelial-, epithelial- and adipocyte-to-mesenchymal cell transition [4–8]. The etiology of the disease is not known, but several environmental factors, including pathogens, have been considered potential triggering factors [9–12].

Amongst pathogens, epidemiological and immunological studies implicate human cytomegalovirus (HCMV) in the development of SSc. Thus, elevated serum levels of anti-HCMV antibodies (abs) were found in SSc [13–16]. HCMV can infect many cell types and HCMV infected cells after epithelial-to-mesenchymal cell transition can activate TGF-β1, a master fibrogenic cytokine involved in SSc [17]. HCMV can cause vasculopathy similar to that of SSc. For instance, CMV infection causes fibrointimal formation in mice deficient in interferon-γ receptor [18]. Molecular mimicry has also been proposed as an inflicting mechanism. Indeed, anti-UL94 abs, detected in SSc [13], cross-react with NAG-2, an autoantigen expressed on the surface of endothelial cells [19]. In addition, anti-HCMV response specific for the mimicking peptides can induce endothelial cell (EC) damage, EC activation and fibrogenesis [19–21]. However, UL94 and NAG-2 are subdominant antigens in SSc patients with no difference in titres of anti-UL94 abs between SSc patients and healthy controls (HCs) [13]. Furthermore, data have been limited to short peptidyl sequences [19].

We ourselves have previously investigated immune responses against HCMV antigens other than UL94 that had better chances to be involved in SSc and found no difference in ab responses against HCMV UL44 and UL57 antigens between anti-HCMV(+) SSc patients and anti-HCMV(+) HCs [22]. However, we did find a strong ab response to HCMV UL83 antigen [23], although we did not detect cross-reactivity with SSc-associated autoantibodies.

In the present paper, we used a large cohort of SSc patients to study the immune responses to six prominent HCMV antigens, namely UL57, UL83, UL55, UL44, p38 and UL99 to determine the frequency and

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Table 1
Major demographic and clinical characteristics of 110 patients with systemic sclerosis (SSc), including 59 with limited cutaneous SSc (lcSSc) and 51 with diffuse cutaneous SSc (dcSSc), 60 patients with multiple sclerosis (MS) and 51 healthy controls (HC).

	SSc (n = 110)	lcSSc (n = 59)	dcSSc (n = 51)	MS (n = 60)	HC (n = 51)	$p_{SSc\ vs\ MS}$	$p_{SSc\ vs\ HC}$	$p_{lcSSc\ vs\ dcSSc}$	$p_{lcSSc\ vs\ HC}$	$p_{dcSSc\ vs\ HC}$
Age	56.1 ± 13.8	59 ± 13.8	52.7 ± 13	52.8 ± 8.8	52.5 ± 11.5	NS	NS*	**0.016***	**0.009***	NS
Sex						NS	0.054**	NS	0.025**	NS
Males	20 (18.2%)	8 (13.6%)	12 (23.5%)	13 (21.7%)	17 (33.3%)					
Females	90 (81.8%)	51 (86.4%)	39 (76.5%)	47 (78.3%)	34 (66.7%)					
Pulmonary fibrosis	37 (33.6%)	6 (10.2%)	31 (60.8%)	NA	NA	NA	NA	< **0.001****	NA	NA
Ulcers	51 (46.4%)	24 (40.7%)	27 (52.9%)	NA	NA	NA	NA	NS	NA	NA
Pulmonary arterial hypertension	16 (14.5%)	7 (11.9%)	9 (17.6%)	NA	NA	NA	NA	NS	NA	NA
GI involvement				NA	NA	NA	NA	NS	NA	NA
Upper	63 (57.3%)	31 (52.5%)	32 (62.7%)							
Upper & Lower	4 (3.6%)	3 (5.2%)	1 (2%)							
None	43 (39.1%)	25 (42.4%)	18 (35.3%)							
Arthritis	25 (22.7%)	15 (25.4%)	10 (19.6%)	NA	NA	NA	NA	NS	NA	NA
Serositis	9 (8.2%)	6 (10.2%)	3 (5.9%)	NA	NA	NA	NA	NS	NA	NA
Telangiectasia	66 (60%)	33 (55.6%)	33 (64.7%)	NA	NA	NA	NA	NS	NA	NA
Calcinosis	15 (13.6%)	7 (11.9%)	8 (15.7%)	NA	NA	NA	NA	NS	NA	NA
Renal crisis	3 (2.7%)	1 (1.7%)	2 (3.9%)	NA	NA	NA	NA	NS	NA	NA
Disease overlap	11 (10%)	5 (10.2%)	5 (9.8%)	NA	NA	NA	NA	NS	NA	NA
Anti-Topo I antibodies	43 (39.1%)	14 (23.7%)	29 (56.9%)	NA	NA	NA	NA	**0.001****	NA	NA
Anti-CEN antibodies	30 (27.3%)	25 (42.4%)	5 (9.8%)	NA	NA	NA	NA	< **0.001****	NA	NA
Anti-RNApol III antibodies	12 (10.9%)	6 (10.2%)	6 (11.8%)	NA	NA	NA	NA	NS*	NA	NA
HCMV positivity	102 (92.7%)	54 (91.5%)	48 (94.1%)	54 (90%)	36 (70.6%)	NS**	< **0.001****	NS**	**0.010****	**0.004****

Age data represent mean ± standard deviation. All other data represent number of cases and corresponding percentages in brackets. *p-values were calculated using 2-tailed t-test for Equality of Means, equal variances were not assumed. **p-values were calculated using Pearson Chi-square or Fisher's Exact Test (2-sided) after correcting for continuity. p-values < 0.005 are shown in bold; p-values with a statistical tendency (< 0.100) are also shown. Abbreviations: CEN, centromere; HCMV, human cytomegalovirus; GI, gastrointestinal; NA, non-applicable; NS, not significant; RNApol, RNA polymerase; Topo, topoisomerase.

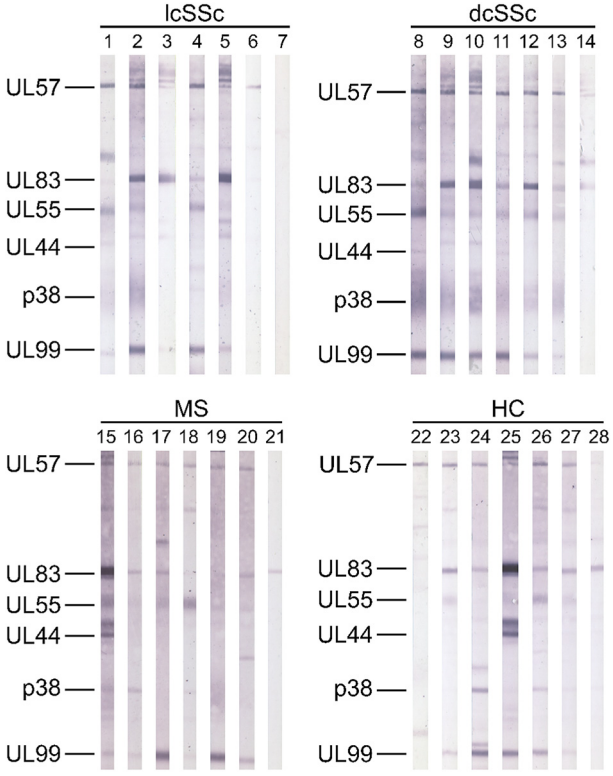

Fig. 1. Antibody reactivity against HCMV antigens by Western immunoblotting in representative limited cutaneous systemic sclerosis (lcSSc, 1-7) patients, diffuse cutaneous systemic sclerosis patients (dcSSc, 8-14), multiple sclerosis (MS, 15-21) patients and healthy controls (HC, 22-28).

magnitude of individual ab response and its relation to clinical and immunological features. In addition, we compared anti-HCMV ab reactivity of SSc patients with that of patients with multiple sclerosis (MS), where the pathogenic link between HCMV and MS is arguably strong.

2. Material and methods

2.1. Patients and controls

One hundred and ten (110) patients with SSc (90 females, 81.8%; median age 55, range 25-87), including 59 with lcSSc (51 females, 86.4%; median age 60, range 34-87) and 51 with dcSSc (39 females, 76.5%; median age 50, range 25-79) were studied. The main demographic, clinical and immunological characteristics of the patients are shown in Table 1. Conventional treatment regimens included low-dose steroids (< 7.5 mg/day), plus azathioprine or methotrexate. All SSc patient had anti-nuclear antibodies by indirect immunofluorescence. Patients fulfilled the American College of Rheumatology criteria for SSc and attended the Out-patient Systemic Sclerosis Clinic of the Department of Rheumatology and Clinical Immunology, at the University General Hospital of Larissa, in central Greece.

Serum samples from 60 demographically matched (age, sex, ethnicity, origin) multiple sclerosis (MS) patients (Table 1, 47 females, 78.3% of total MS; age median 54.5, range 30-69), including 35 relapse-remitting MS (RRMS; 58.3% of total MS; 27 females, 77.1% of RRMS; age median 48, range 39-66) and 25 secondary progressive MS (SPMS; 41.7% of total MS; 20 females, 80% of SPMS; age median 59, range 30-69) were tested as pathological controls. MS patients were followed-up in the Out-patient Clinic of the Department of Neurology, University General Hospital of Larissa. Twenty-two (22) MS patients (36.7%) were not on any treatment. Amongst the remaining 38 patients (63.3%), 13 patients (21.7%) were on interferon-β (including 7 on interferon-beta-1α and 6 on interferon-beta-1β), 7 on natalizumab, 6 on fingolimod, 8 on glatiramer, 3 on teriflunomide and 1 patient on mitoxandrone.

Serum samples from HCs, consisting of 51 age- and sex-matched individuals (Table 1, 34 females, 66.7%; age median 53, range 29-88) were also used. HCs had no family history of any autoimmune disorder and no significant comorbidities including gynecological disorders or other chronic illnesses, including cancer, chronic cardiovascular disease, hypertension, diabetes or depression.

All patients, pathological and healthy controls were white Caucasians. This present study conformed to the principles outlined in the Declaration of Helsinki. A written informed consent was obtained

Table 2
Frequencies of immunoreactive HCMV-specific antigens as detected by Western immunoblotting in sera of 102 positive patients with systemic sclerosis (SSc), including 54 with limited cutaneous SSc (lcSSc) and 48 with diffuse cutaneous SSc (dcSSc), 54 positive patients with multiple sclerosis (MS), including 31 with relapse remitting MS (RRMS) and 23 with secondary-progressive (SPMS), and 36 positive healthy controls (HC).

	SSc (n = 102)	lcSSc (n = 54)	dcSSc (n = 48)	MS (n = 54)	RRMS (n = 31)	SPMS (n = 23)	HC (n = 36)	$p_{SSc\ vs\ MS}$	$p_{SSc\ vs\ HC}$	$p_{lcSSc\ vs\ dcSSc}$	$p_{lcSSc\ vs\ HC}$	$p_{dcSSc\ vs\ HC}$
UL57 positive	99 (97.1%)	54 (100%)	45 (93.8%)	44 (81.5%)	26 (83.9%)	18 (78.3%)	34 (94.4%)	**0.002**	NS	NS	NS	NS
UL83 positive	70 (68.6%)	39 (72.2%)	31 (64.6%)	30 (55.6%)	18 (58.1%)	12 (52.2%)	32 (88.9%)	NS	**0.031**	NS	NS	**0.022**
UL55 positive	52 (51%)	28 (51.9%)	24 (50%)	18 (33.3%)	7 (22.6%)	11 (47.8%)	18 (50%)	0.052	NS	NS	NS	NS
UL44 positive	17 (16.7%)	8 (14.8%)	9 (18.8%)	9 (16.7%)	6 (19.4%)	3 (13%)	10 (27.8%)	NS	NS	NS	NS	NS
p38 positive	16 (15.7%)	5 (9.3%)	11 (22.9%)	15 (27.8%)	10 (32.3%)	5 (21.7%)	10 (27.8%)	NS	NS	NS	**0.043**	NS
UL99 positive	77 (75.5%)	39 (72.2%)	38 (79.2%)	44 (81.5%)	26 (83.9%)	18 (78.3%)	32 (88.9%)	NS	NS	NS	NS	NS

	$p_{SSc\ vs\ RRMS}$	$p_{SSc\ vs\ SPMS}$	$p_{lcSSc\ vs\ MS}$	$p_{lcSSc\ vs\ RRMS}$	$p_{lcSSc\ vs\ SPMS}$	$p_{dcSSc\ vs\ MS}$	$p_{dcSSc\ vs\ RRMS}$	$p_{dcSSc\ vs\ SPMS}$	$p_{MS\ vs\ HC}$	$p_{RRMS\ vs\ HC}$	$p_{SPMS\ vs\ HC}$
UL57 positive	**0.023**	**0.004**	**0.003**	**0.010**	**0.002**	NS	NS	NS	NS	NS	NS
UL83 positive	NS	NS	NS	NS	NS	NS	NS	NS	**0.002**	**0.009**	**0.004**
UL55 positive	**0.010**	NS	0.080	**0.016**	NS	NS	**0.028**	NS	NS	**0.039**	NS
UL44 positive	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
p38 positive	0.075	NS	**0.026**	**0.017**	NS	NS	NS	NS	NS	NS	NS
UL99 positive	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Data represent number of cases and corresponding percentages in brackets. *p*-values were calculated using Pearson Chi-square or Fisher's Exact Test (2-sided) after correcting for continuity. Underlined *p*-values correspond to higher frequency in the control group. *p*-values < 0.005 are shown in bold; *p*-values with a statistical tendency (< 0.100) are also shown. Abbreviations: NS, not significant.

from all patients and controls. This study was carried out after approval from the Ethical Committee of the University General Hospital of Larissa, Larissa, Greece (Protocol Number: 2406/18-06-2015).

2.2. Anti-HCMV ab testing

Ab responses against HCMV-specific antigens were tested by western immunoblotting [24]. Blot strips with electrophoretically separated CMV extract (CMV strain A169) (EUROIMMUN, Germany) were used as a source of HCMV antigens. Subsequently, the strip membranes were incubated with individual serum samples at a dilution of 1/51 for 30 min. After three 5-min washes, membranes were incubated with predetermined optimal dilutions of alkaline phosphatase (ALP)-conjugated anti-human IgG ab (EUROIMMUN). Ready-made NBT/BCIP (EUROIMMUN) was used as substrate for ALP conjugated antibodies. Membrane strips were evaluated using the EUROLineScan software (EUROIMMUN) to obtain densitometric, quantitative data. Only the amplitude of the curve describing optical density was used for further analysis. Pretests authenticated that the amplitude compared with the integral of the curve was a valid variable; arbitrary units (AUs) of the amplitude were applied. The cutoff value set by the manufacturer at 11–22 for borderline area and > 23 AU for positivity. All ab tests were performed at the Laboratory of Rheumatology and Clinical Immunology, a reference laboratory for autoantibody (autoAb) testing in central Greece.

2.3. AutoAb testing

All SSc sera tested at diagnosis for ANA by indirect immunofluorescence were evaluated for their immunofluorescence pattern. The titer of positive samples was evaluated to extinction. SSc-related autoAbs, in particular anti-Topoisomerase I (anti-Topo I), anti-centromere (anti-CEN) and anti-RNA polymerase III (anti-RNA pol III) antibodies, were also tested by an SSc profile line immunoassay (EUROIMMUN, Lübeck, Germany). This profile testing would enable correlation analysis between anti-HCMV specific abs and SSc-specific autoAbs.

2.4. Statistical analysis

All data are reported as percentages (%). Serum levels variation in each patients group was defined by standard deviation (SD). Differences in categorical data between groups were tested by two-tailed Pearson's chi-square and Fisher's Exact Test. Correction for continuity was applied. Differences in numerical data between groups were tested by the two-tailed Student's t-test. Bivariate analyses of correlations of numerical data between groups were tested by Pearson's Correlation. Multiple regression analysis was performed after corrections for age and sex. *p*-values smaller than 0.05 were considered significant. All statistical calculations were performed with IBM SPSS Statistics 20 software.

3. Results

3.1. IgG anti-HCMV antibodies in patients with SSc

IgG anti-HCMV abs were assessed by Western immunoblotting. Table 1 summarizes frequencies of ab responses to HCMV. Overall, anti-HCMV ab positivity was detected in 102 (92.7%) SSc patients (54 [91.5%] lcSSc patients; 48 [94.1%] dcSSc patients), compared to 54 (90%) MS patients (31 [88.6%] RRMS patients; 23 [92%] SPMS patients), and 36 (70.6%) HC (SSc *vs* HC, $p < 0.001$; lcSSc *vs* HC, $p = 0.010$; dcSSc *vs* HC, $p = 0.004$; MS *vs* HC, $p = 0.018$; SSc *vs* MS, p = ns).

3.2. Ab reactivity to individual HCMV antigens

All anti-HCMV positive SSc patients, MS patients and HC were tested for ab reactivity to individual UL57, UL83, UL55, UL44, p38 and UL99 antigens by Western immunoblotting. Fig. 1 illustrates representative anti-HCMV ab testing, whereas Tables 2 and 4 summarize results of ab frequency and magnitude, respectively, in patients and controls.

Reactivity to at least one antigen was found at variable frequencies in anti-HCMV- positive SSc patients, MS patients and HCs (Table 2). The most immunoreactive antigens in SSc patients were UL57 (97.1%), UL99 (75.5%), UL83 (68.6%) and UL55 (51%).

When comparing anti-HCMV antigen-specific reactivities between SSc and HC, anti-UL83 abs were less frequent in SSc (SSc *vs* HC: 68.6% *vs* 88.9%, $p = 0.031$). Reactivities against the remaining 5 HCMV antigens (UL57, UL55, UL44, p38 and UL99) were comparable between SSc patients and HCs.

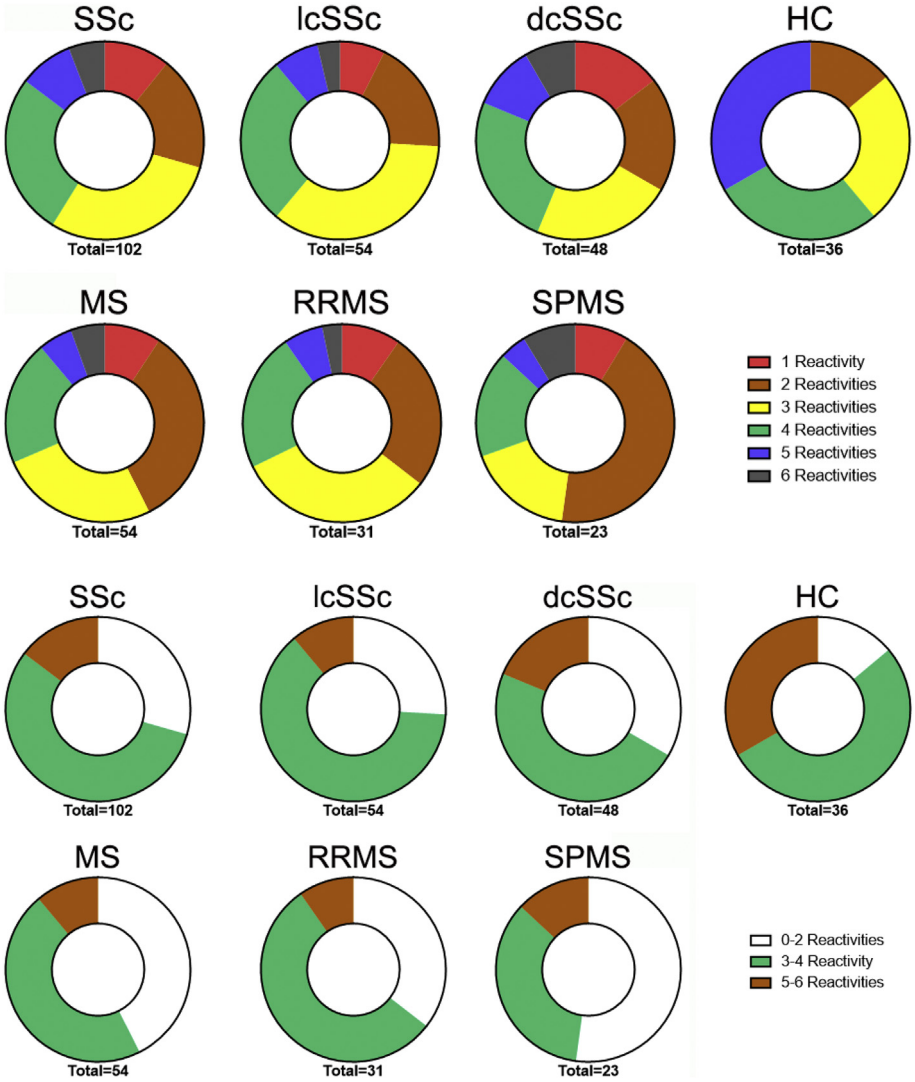

Fig. 2. Pie charts illustrating the proportion of serum samples of anti-HCMV 102 positive patients with systemic sclerosis (SSc), including 54 with limited cutaneous SSc (lcSSc) and 48 with diffuse cutaneous SSc (dcSSc), 54 anti-HCMV positive patients with multiple sclerosis (MS), including 31 with relapse remitting MS (RRMS) and 23 with secondary-progressive (SPMS), and 36 anti-HCMV positive healthy controls (HC) that were reactive with multiple HCMV antigens UL57, UL83, UL 55, UL44, p38 and UL99.

Anti-UL57 reactivity was more frequent in SSc than in MS (SSc *vs* MS: 97.1% *vs* 81.5%, $p = 0.002$). All other reactivities (UL83, UL55, UL44, p38 and UL99) did not vary between SSc and MS.

3.3. Anti-HCMV reactivity in SSc subtypes (lcSSc or dcSSc)

Anti-HCMV ab seropositivity did not differ between lcSSc and dcSSc (91.5% *vs* 94.1%) (Table 1) nor did reactivities against individual HCMV antigens.

When comparing the two SSc subtypes with HCs, reactivities against UL83 were decreased in dcSSc (64.6% vs 88.9%, $p = 0.022$) and reactivities against p38 were decreased in lcSSc (9.3% vs 27.8%, $p = 0.043$). Reactivities against the remaining 4 HCMV antigens (i.e. UL57, UL55, UL44 and UL99) were comparable between SSc subtypes and HCs (Table 2).

When comparing lcSSc and dcSSc patients with MS and its subgroups, anti-UL57 abs were more frequent in lcSSc compared to MS, RRMS and SPMS ($p \leq 0.01$ for all), while anti-UL55 abs were more frequent in both lcSSc and dcSSc compared to RRMS. In contrast, anti-p38 abs were less frequent in lcSSc compared to RRMS (Table 2).

3.4. Reactivity to multiple HCMV antigens

The concurrent presence of multiple anti-HCMV reactivities is illustrated in Fig. 2 and Table 3. When comparing the presence of antibodies against multiple HCMV antigens in anti-HCMV-positive SSc patients and HCs, we observed that SSc patients were reactive to fewer bands compared to HC. That is, 29.4% of SSc patients were reactive to one or two HCMV antigens, 55.9% were reactive to three or four HCMV antigens and 14.7% were reactive to five or six HCMV antigens compared to 13.9% of HC reactive to one or two HCMV antigens, 52.8% reactive to three of four antigens and 33.3% reactive to five or six antigens ($p = 0.026$). Also, MS patients were reactive to fewer bands compared to HCs. The concurrent presence of anti-HCMV abs was comparable between anti-HCMV-positive SSc and MS patients. In anti-HCMV- positive cases that were analyzed, no zero reactivity was observed.

We then analyzed the overlapping reactivity against the four most immunoreactive HCMV antigens, UL57, UL83, UL55 and UL99, as illustrated in Fig. 3. We observed a significantly higher ratio of quadruple positivity to triple UL57, UL83 and UL99 positivity in dcSSc than RRMS ($p = 0.029$) (Supplementary Table 1).

Table 3
Concurrent presence of anti-HCMV antigen-specific reactivities as detected by Western immunoblotting in sera of 102 positive patients with systemic sclerosis (SSc), including 54 with limited cutaneous SSc (lcSSc) and 48 with diffuse cutaneous SSc (dcSSc), 54 positive patients with multiple sclerosis (MS), including 31 with relapse remitting MS (RRMS) and 23 with secondary-progressive (SPMS), and 36 positive healthy controls (HC).

	SSc (n = 102)	lcSSc (n = 54)	dcSSc (n = 48)	MS (n = 54)	RRMS (n = 31)	SPMS (n = 23)	HC (n = 36)	$p_{SSc\ vs\ MS}$	$p_{SSc\ vs\ HC}$	$p_{lcSSc\ vs\ dcSSc}$	$p_{lcSSc\ vs\ HC}$	$p_{dcSSc\ vs\ HC}$	$p_{MS\ vs\ HC}$	$p_{RRMS\ vs\ HC}$	$p_{SPMS\ vs\ HC}$
0–2 bands	30 (29.4%)	14 (25.9%)	16 (33.3%)	23 (42.6%)	11 (35.5%)	12 (52.2%)	5 (13.9%)	NS	**0.026**	NS	**0.027**	0.084	**0.04**	**0.024**	**0.006**
3–4 bands	57 (55.9%)	34 (63%)	23 (47.9%)	25 (46.3%)	17 (54.8%)	8 (34.8%)	19 (52.8%)								
5–6 bands	15 (14.7%)	6 (11.1%)	9 (18.8%)	6 (11.1%)	3 (9.7%)	3 (13%)	12 (33.3%)								

Data represent number of cases and corresponding percentages in brackets. *p*-values were calculated using Pearson Chi-Square (2-sided). *p*-values < 0.005 are shown in bold. Abbreviations: NS, not significant.

3.5. Magnitude of anti-HCMV responses in SSc patients and HCs

Levels of reactivity to individual HCMV antigens are presented in Table 4 and illustrated in Fig. 4. The responses against UL57 were stronger in SSc, lcSSc and dcSSc compared to HC ($p < 0.001$ for all). In addition, the magnitudes of anti-UL83 and anti-UL99 were higher in SSc and dcSSc than in HC (UL83: SSc *vs* HC: $p = 0.040$ and dcSSc *vs* HC: $p = 0.032$; UL99: SSc *vs* HC: $p = 0.032$ and dcSSc *vs* HC: $p = 0.019$).

Differences in magnitude between SSc and MS patients were observed for UL57, with higher anti-UL57 ab titres in SSc, lcSSc and dcSSc compared to MS and RRMS ($p < 0.001$ for all). In contrast, anti-p38 titres were lower in lcSSc compared to MS and SPMS ($p = 0.020$ and $p = 0.006$, respectively).

When comparing the magnitude of ab responses against HCMV antigens between the two SSc subtypes, anti-p38 magnitude was significantly higher in dcSSc compared to lcSSc. No other differences in magnitudes between the two subtypes were found.

As expected, anti-HCMV antigen reactivities significantly correlated with other anti-HCMV antigen reactivities (Table 5).

3.6. Immunological and clinical associations of antigen specific HCMV ab responses

Few associations were found between anti-HCMV antigen antibodies reactivity and SSc-associated autoantibodies (Supplementary Tables 2a–f). Anti-centromere abs were more frequent in anti-UL83-positive compared to anti-UL83-negative patients ($p = 0.040$, Supplementary Table 2b), while anti-CENPA and anti-CENPB magnitudes were both higher in anti-UL44-positive compared to anti-UL44-negative patients ($p = 0.028$ and $p = 0.002$, respectively, Supplementary Table 2d). Anti-RNApol 55 abs frequency and anti-RNApol 11 magnitude were higher in anti-UL83-positive compared to anti-UL83-negative patients $p = 0.044$ and $p = 0.034$, respectively, Supplementary Table 2b).

When anti-HCMV abs were analyzed for possible association with clinical features, there was only a tendency towards higher frequency of arthritis in anti-UL44-positive compared to anti-UL44-negative SSc patients ($p = 0.067$, Supplementary Table 2d).

4. Discussion

This is the first study to address ab responses against many HMCV antigens in patients with SSc using a whole HCMV extract as antigen source.

Although no significant differences in the prevalence of anti-HCMV abs between SSc or SSc cutaneous subtypes and normal controls were found, the magnitudes of anti-UL83, anti-UL57 and anti-UL99 ab responses were increased in SSc and SSc subtypes. Such data point towards the need to check not only differences in the prevalence of anti-HCMV abs in general or individual anti-HCMV abs but also to assess the magnitude of ab response to viral antigens, as this maybe the only discriminating point between cohorts. We and others have previously reported data suggesting that the prevalence and/or magnitude of ab responses to UL57 and/or UL83 (pp65) are more frequent or stronger, respectively in SSc than in controls [22,23,25].

It should be mentioned that HCMV UL83 antigen is important in HCMV infection and responsible for evading host immune cells by blocking antigen presentation, modulating NK cells and suppressing the induction of type I interferons and IL-1β [26]. Apart from being a target of B cells, UL83 is a target of T cells. Peptides derived from UL83 are immunodominant T cell target antigens [27,28], regulating active infection in patients with stem cell transplantation [28]. UL57 is a single-stranded DNA-binding protein, whereas UL99 protein (pp28) polymerizes and is essential for the envelopment of HCMV [29], while it is an immunodominant antigen in HCMV infection [30].

The increased frequency of anti-UL57 and anti-UL55 ab response in

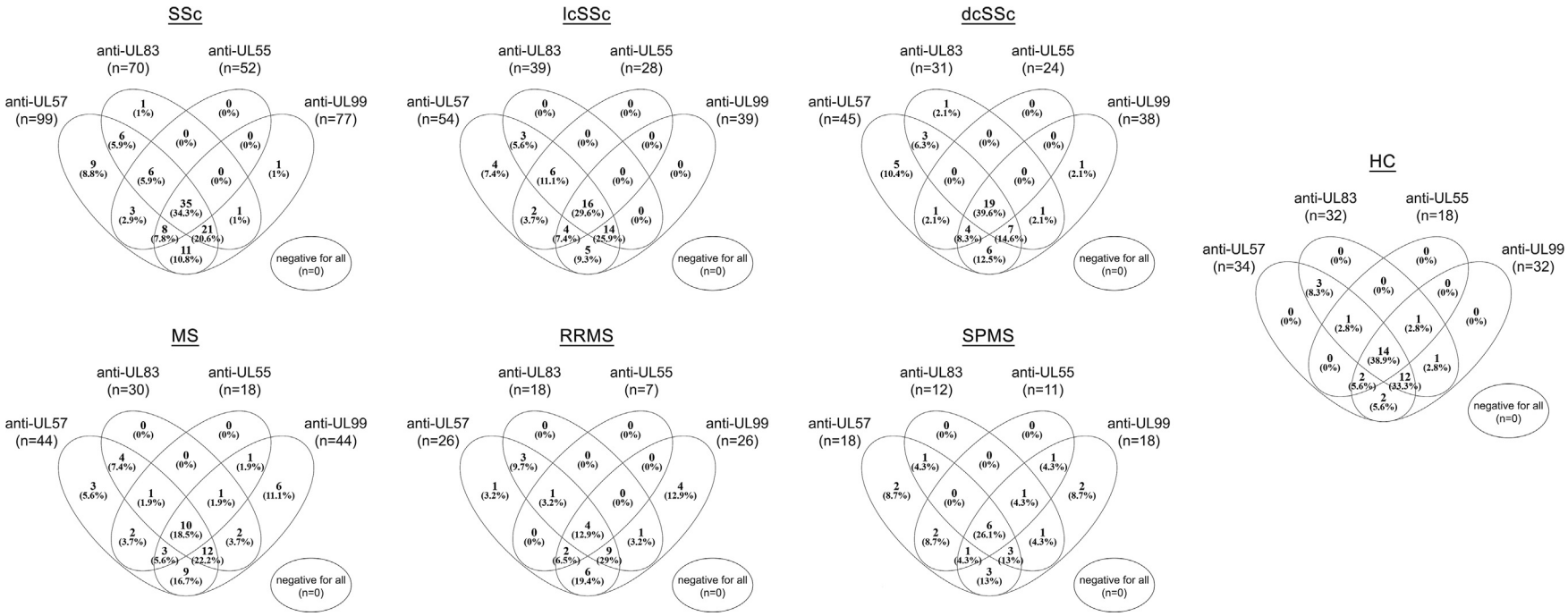

Fig. 3. Venn diagrams illustrating the patterns of anti-UL57, anti-UL83, anti-UL55 and anti-UL99 overlapping reactivities in anti-HCMV positive patients with systemic sclerosis (SSc), including limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) patients, in anti- HCMV positive patients with multiple sclerosis, including relapse remitting MS (RRMS) and secondary-progressive MS (SPMS) patients, and anti-HCMV positive healthy controls (HC).

Table 4
Magnitude of antibody responses against immunoreactive HCMV-specific antigens as measured by Western immunoblotting in sera of band-positive (absolute number of positive cases for individual bands is shown in Table 2) patients with systemic sclerosis (SSc), including patients with limited cutaneous SSc (lcSSc) and patients with diffuse cutaneous SSc (dcSSc), patients with multiple sclerosis (MS), including relapse remitting MS (RRMS) and secondary-progressive (SPMS), and healthy controls (HC).

	SSc (n = 102)	lcSSc (n = 54)	dcSSc (n = 48)	MS (n = 54)	RRMS (n = 31)	SPMS (n = 23)	HC (n = 36)	$p_{SSc\ vs\ MS}$	$p_{SSc\ vs\ HC}$	$p_{lcSSc\ vs\ dcSSc}$	$p_{lcSSc\ vs\ HC}$	$p_{dcSSc\ vs\ HC}$
UL57	64.7 ± 17.4	64.3 ± 17.8	65 ± 17.2	51.7 ± 15.9	49.2 ± 14.2	55.3 ± 14.2	43.4 ± 14.2	**< 0.001**	**< 0.001**	NS	**< 0.001**	**< 0.001**
UL83	77.2 ± 31.2	74.4 ± 30.4	80.8 ± 32.2	65.5 ± 33.3	63.4 ± 31.4	68.7 ± 37.2	63.2 ± 31.4	NS	**0.040**	NS	NS	**0.032**
UL55	41.2 ± 14.8	38.6 ± 10.5	44.3 ± 18.3	39.6 ± 9.7	37.9 ± 5.5	40.7 ± 11.8	41.2 ± 13.4	NS	NS	NS	NS	NS
UL44	66.9 ± 27.6	70.8 ± 23.7	63.6 ± 31.7	65.1 ± 23.3	66.2 ± 23.5	63 ± 28	53.3 ± 27.8	NS	NS	NS	NS	NS
p38	48.4 ± 18.7	37.2 ± 10.3	53.5 ± 19.7	54.3 ± 16.8	49.9 ± 17.7	63 ± 11.7	50.1 ± 16.8	NS	NS	**0.049**	0.092	NS
UL99	62.3 ± 20.9	59.8 ± 20.2	64.9 ± 21.6	56.7 ± 19.9	54.8 ± 21.2	59.4 ± 18.1	53.1 ± 19.7	NS	**0.032**	NS	NS	**0.019**

	$p_{lcSSc\ vs\ MS}$	$p_{dcSSc\ vs\ MS}$	$p_{lcSSc\ vs\ RRMS}$	$p_{dcSSc\ vs\ RRMS}$	$p_{lcSSc\ vs\ SPMS}$	$p_{dcSSc\ vs\ SPMS}$	$p_{MS\ vs\ HC}$	$p_{RRMS\ vs\ HC}$	$p_{SPMS\ vs\ HC}$
UL57	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.065	0.057	**0.017**	NS	**0.021**
UL83	NS	0.074	NS	0.073	NS	NS	NS	NS	NS
UL55	NS	NS	NS	NS	NS	NS	NS	NS	NS
UL44	NS	NS	NS	NS	NS	NS	NS	NS	NS
p38	0.020	NS	NS	NS	0.006	NS	NS	NS	NS
UL99	NS	0.080	NS	0.070	NS	NS	NS	NS	NS

Data are expressed as mean ± standard deviation of arbitrary units (AU) (see Section 2). *p*-values were calculated using 2-tailed t-test for Equality of Means, equal variances were not assumed, or using 2-tailed Mann–Whitney test. *p*-values < 0.005 are shown in bold; *p*-values with a statistical tendency (< 0.100) are also shown. NS, not significant.

SSc compared to MS patients was rather unexpected. There is a strong link between HCMV and MS and someone would argue that MS patients (rather than) SSc patients would have been more frequently reactive. We think that the most important finding of our study, though, is the likely association of anti-HCMV response with autoabs in SSc. Anti-UL83 ab(+) SSc patients had higher frequency of SSc-associated autoAbs, namely, anticentromere and anti-RNA pol III (RNA pol 155) autoAbs, and higher magnitude of anti-RNA pol III (RNA pol 11) ab response, whereas anti-UL44(+) SSc patients had higher magnitude of anti-CENPA and CENPB ab response. It should be mentioned that HCMV UL44 is a processivity factor that binds to DNA and is an immunodominant antigen in HCMV infection [30].

HCMV could initiate an immune response that breaks tolerance in a host with appropriate genetic background. HCMV DNA is large (about 230 kbp) and encodes more than 750 proteins [31] and any of these could lead to break of immunological tolerance in SSc. Of relevance, injection of BALBc mice with a UL83 peptide induces abs against dsDNA and other nuclear antigens, as well as lupus glomerulonephritis, further strengthening the notion that this HCMV antigen is able to induce autoreactive responses and clinical autoimmune rheumatic disease [32]. Also, immunization of female NZB/F F1 mice with HCMV pp65 DNA developed autoAbs against dsDNA and other nuclear components and glomerulonephritis [33]. In addition, immunization of CMV UL55 protein to normal mice induced autoAb response against U1-70 KDa splicesome protein, which is part of anti-riboprotein response seen in autoimmune connective tissue diseases [34]. Yet, a recombinant HCMV UL55 vaccine failed to induce autoAbs in CMV-seronegative individuals [35].

How HCMV induces SSc-specific autoabs remains uncertain. A proposed model of molecular mimicry and antibody cross-reaction as potential mechanisms of HCMV-induced SSc-autoantibodies is illustrated in Supplementary Fig. 1. There are amino acid (aa) sequence homologies between HCMV and human proteins that could lead to the initiation of cross-reactive responses. In an early study, a comparison of the NH2-terminal portion of DNA polymerase I revealed a 5-amino acid sequence homology (aa 121-126) with HCMV UL70 protein [36]. Also, the HCMV UL55 protein has a high aa homology with a large number of human proteins at the penta-, hexa- and hepta-peptide level [37]. HCMV UL44 protein exhibits aa similarity with eukaryotic processivity factor proliferating cell nuclear antigen (PCNA) [38].

Our Blast2p search revealed a significant homology between aa27 –RQVSLRSYDNIPPTS- aa41 part of the immunodominant –TTPGEPLKDALGRQVSLRSYDNIPPTSSSDEGEDDDC- epitope of UL99 [30] with DNA topoisomerase (Scl-70) aa 311 RAVALYFIDKLALRA- aa324 (Supplementary Fig. 1). The second most immunodominant UL99 epitope aa130- CETDDLDEEDTSIYLSPPPVPPVQVVAKRLPRPDTPRT –aa160 [30], also shares a significant degree of local homology between its core epitopic region aa132 –DDLDEEDTSIYLS- 144 and aa398 - DDLFDRLTTTSLN-aa410 Scl-70 (Supplementary Fig. 1). Of interest, no such similarities were found between the UL99 HCMV epitopes and the lcSSc related centromere A autoantigen. Hence, it is worthy to investigate the extent of ab cross-reactivity involving the relevant HCMV and Scl-70 homologues.

Also, amongst various clinical features of SSc only arthritis tends to be more prevalent in anti-UL44(+) compared to anti-UL44(-) patients. No other associations between anti-HCMV antigen antibodies reactivity and SSc clinical parameters were found. This finding further supports the concept that the response to viral antigens may play role (if any) in the break of tolerance and the induction of the disease rather than the perpetuation of the disease.

Our data are validated in a larger cohort, strengthening further the potential role of these viral antigens in the pathogenesis of SSc. A larger prospective study including very early SSc patients is required to enlighten the pathogenic potential of these proteins. Of relevance, injection of BALBc mice with the major tegument protein UL83 induces systemic lupus erythematosus-related dsDNA autoabs, and lupus

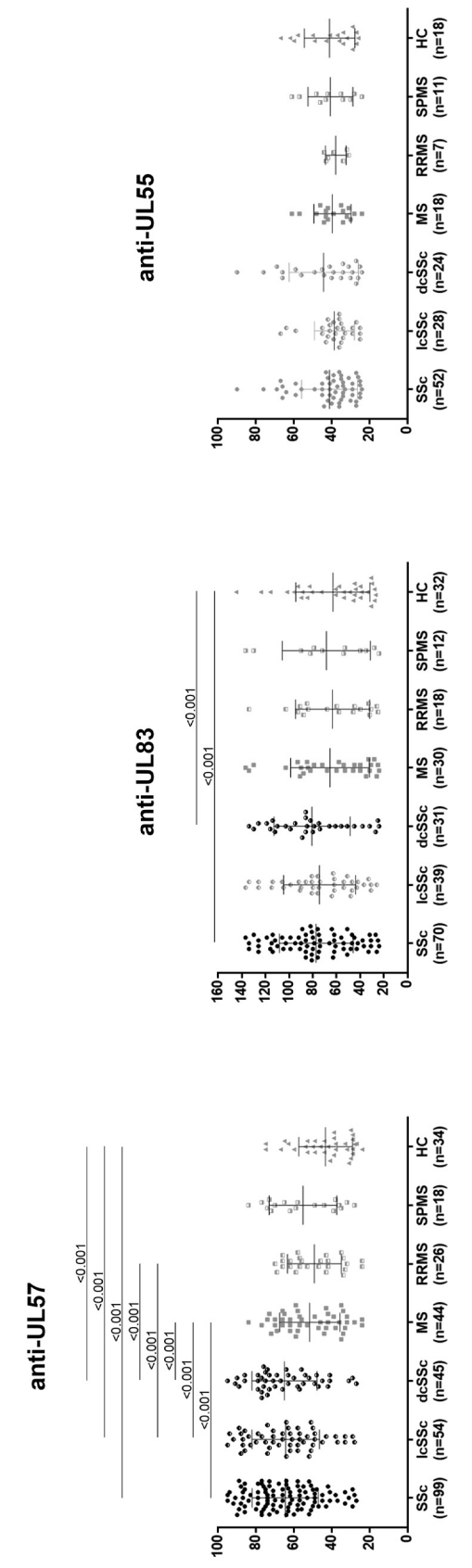

Fig. 4. Scatter plots illustrating antibody titres by Western immunoblotting against individual HCMV antigens in sera of systemic sclerosis (SSc) patients, including limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc), multiple sclerosis (MS) patients, including relapse remitting MS (RRMS) and secondary-progressive (SPMS), and healthy controls (HC).

Table 5
Correlations of anti-HCMV antigen reactivities with other anti-HCMV antigen reactivities in anti-HCMV positive SSc patients. R represents Pearson's correlation coefficient. *p* represent *p* value (2 tailed) associated with the correlation. *p*-values < 0.005 are shown in bold; *p*-values with a statistical tendency (< 0.100) are also shown. Abbreviations: NS, not significant.

		UL57	UL83	UL55	UL44	p38	UL99
UL57	R	1	0.094	0.319	−0.122	−0.266	0.164
	p		0.079	**< 0.001**	**0.023**	**< 0.001**	**0.002**
UL83	R		1	0.234	0.614	0.209	0.293
	p			**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
UL55	R			1	0.155	0.133	0.294
	p				**0.004**	**0.013**	**< 0.001**
UL44	R				1	0.471	0.218
	p					**< 0.001**	**< 0.001**
p38	R					1	0.311
	p						**< 0.001**
UL99	R						1
	p						

glomerulonephritis, further strengthening the notion that this HCMV antigen is able to induce autoreactive responses and clinical autoimmune rheumatic disease [32,33,39,40].

Fresh exposure to or re-activation of HCMV infection may lead to self-targeting immune response, as well as partaking of virus-encoded factors contributing via direct infection of endothelial cells, induction of IL-1b, chemokines and other pro-inflammatory adhesion molecule and selective recruitment of leucocytes and other inflammatory mediators which lead to cellular damage and tissue destruction.

Another HCMV antigen, previously unnoticed in terms of its pathogenic potential is UL99. UL99, a late and abundant outer tegument protein also known as pp28, is encoded by HCMV UL99 ORF [41,42]. pp28 is crucial for the cytoplasmic assembly and efficient infectivity of the virus as essential deletion of the UL99 ORF severely impairs the construction of enveloped virus particles [43,44]. pp28 is also one of the most immunodominant antigens in blood donors and at least two linearized epitopes of the antigen have been identified [30].

A few more points need to be discussed. Analysis of ab reactivity to viral antigens and clinical or laboratory parameters showed that anti-CENPA/CENPB ab reactivity was more frequent in anti-UL83 positive compared to anti-UL83 negative patients, while anti-CENPA and anti-CENPB magnitudes were both higher in anti-UL44 positive compared to anti-UL44 negative patients. Also, anti-RNApol 155 abs frequency and anti-RNApol 11 magnitude were higher in anti-UL83 positive compared to anti-UL83 negative patients, respectively. These may be accidental findings or may require further investigation as they may imply an association of anti-UL83 abs with CENP or RNA pol III autoabs. A combination of increased prevalence and magnitude against specific viral antigens may be instrumental for the loss of immunological tolerance in autoimmune diseases, such as SSc, but this remains to be seen. Finally, amongst various clinical features only arthritis tends to be more prevalent in anti-UL44 positive compared to anti-UL44 negative SSc patients. No other associations between anti-HCMV antigen antibodies reactivity and SSc clinical parameters were found suggesting that response to viral antigens may play role (if any) in the induction of the disease rather than specific clinical phenotypes of the disease.

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Conflict of interests

Thomas Scheper & Wolfgang Meyer are employees of Euroimmun AG. All other authors do not have any conflict of interest

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