Autoantibodies targeting LINE-1-encoded ORF1p are associated with systemic lupus erythematosus diagnosis but not with disease activity

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Abstract

Objective

Long Interspersed Element 1 (LINE-1) is an endogenous retroelement that constitutes a significant portion of the human genome and has been implicated in the pathogenesis of systemic lupus erythematosus (SLE). The LINE-1 RNA chaperone protein ORF1p was recently identified as an SLE autoantigen. Here we analyse ORF1p for qualities underlying SLE autoantigen status, compared anti-ORF1p antibodies to markers of SLE disease activity, and performed screening for antibodies against LINE-1 reverse transcriptase ORF2p.

Methods

ORF1p was examined in epithelial cell lines treated with cytotoxic lymphocyte granules and UV irradiation. Anti-ORF1p and anti-ORF2p antibodies were assayed by ELISA and analysed in two SLE cohorts.

Results

We found that ORF1p localises to cytoplasmic RNA-containing blebs in apoptotic cells, and is a substrate of the cytotoxic protease granzyme B (GrB). Anti-ORF1p antibodies were present in 4.2% of healthy controls, compared to 15.8% (p=0.0157) and 15.5% (p=0.036) of subjects in the two SLE cohorts. Anti-ORF1p antibodies were not associated with SLE disease activity nor peripheral blood markers of interferon (IFN) activation. Anti-ORF1p titres demonstrated stability over serial time points. Anti-ORF1p antibodies were not associated with anti-DNA, anti-RNP, or other SLE autoantibodies. There was no difference in anti-ORF2p ELISA results in controls versus SLE patients.

Conclusion

LINE-1 ORF1p is a component of apoptotic blebs and a substrate for GrB. Anti-ORF1p antibodies are enriched in SLE subjects but are not associated with dynamic markers of disease activity. These data support a potential role for LINE-1 dysregulation in SLE pathogenesis.

Key words

autoantibodies, autoantigens, systemic lupus erythematosus, long interspersed nucleotide elements

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Background

Systemic lupus erythematosus (SLE) is a rheumatic disease characterised by several key immunologic features, including impaired clearance of apoptotic debris, activation of B cells, and upregulation of type I interferon (IFN-I) (1). Upregulated IFN-I signalling has contributed to recent attention on retroelements as endogenous triggers of autoimmunity (2, 3). Long Interspersed Element 1 (LINE-1) constitutes 17% of our genome (4). While most LINE-1 insertions are incomplete and inactive, a subset of LINE-1 insertions have replicative capacity (4). LINE-1 is unique among retroelements in its ability to carry out autonomous replication through expression of two proteins: ORF1p is an RNA-binding protein, and ORF2p has endonuclease and reverse transcriptase functions. ORF1p, ORF2p and LINE-1 RNA generate ribonucleoprotein (RNP) complexes with additional proteins, including several known autoantigens (5).

LINE-1 expression activates components of the innate immune system, leading to an IFN-I response (6, 7). Enhanced LINE-1 expression in SLE tissues (2) suggests that LINE-1 could be one driver of IFN expression in SLE. More recently, anti-ORF1p antibodies have also been identified in SLE, and reported to associate with markers of lupus disease activity (3). These findings led us to examine the antigenic features of ORF1p in greater detail, to understand potential origins of its status as an autoantigen, and prompted us to evaluate whether ORF2p is also an autoantigen.

Methods

Patient cohorts

Sera/plasmas from 68 healthy controls and 158 SLE patients in the "Study of biological Pathways, disease Activity and Response markers in patients with systemic lupus Erythematosus" (SPARE) cohort from Johns Hopkins were screened for the presence of autoantibodies to ORF1p and ORF2p. Sera from 90 SLE patients from Emory University (EU) were analysed by ELISA in identical fashion. All studies using human samples were approved by the institutional review boards of each university. SPARE is a prospective observational cohort that has been extensively described (8, 9). Enrolled patients were followed over a 2-year period. To assess disease activity, the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and the Physician Global Assessment (PGA) were completed at each visit, along with serologic laboratory monitoring. Study participants also underwent whole blood gene expression analysis at baseline using the Affymetrix GeneChip HT HG-U133+PM array, and BAFF gene expression by quantitative PCR (8, 9). IFN, neutrophil and plasma cell (PC) transcriptional signatures were identified using modular analysis developed by Chaussabel *et al*. (10).

ELISA

A plasmid encoding N-terminal His-tagged ORF1p from the LINE-1 insertion in the X-linked retinitis pigmentosa locus (L1RP, GenBank accession number AF148856) was used to express recombinant His-tagged ORF1p. The protein was purified by Nickel-IDA affinity chromatography, concentrated by dialysis and treated with RNase. ORF2p expressed in E coli was purified from inclusion bodies using a nickel column (Supplementary Fig. S1). For each ELISA, 50 ng of protein was applied to replicate wells overnight, then blocked with 5% milk-PBS-T for 2 hours at room temperature (RT). Sera were applied at 1:1000 dilution in 1% milk-PBS-T for 2 hours at RT. For each serum, background correction was performed using an empty (protein-free) well subtracted from the OD of protein-coated wells. Anti-human HRP-labelled antibody was used at 1:10,000 dilution for 1 hour at RT. After washing, samples were developed using KPL SureBlue reagent (SeraCare) then stopped using 1N HCl. Plates were read at 450 nm using a Perkin Elmer Victor 3 plate reader. A positive reference serum was used to generate a standard curve to calculate unknown sample arbitrary units (AU) and calibrate across plates.

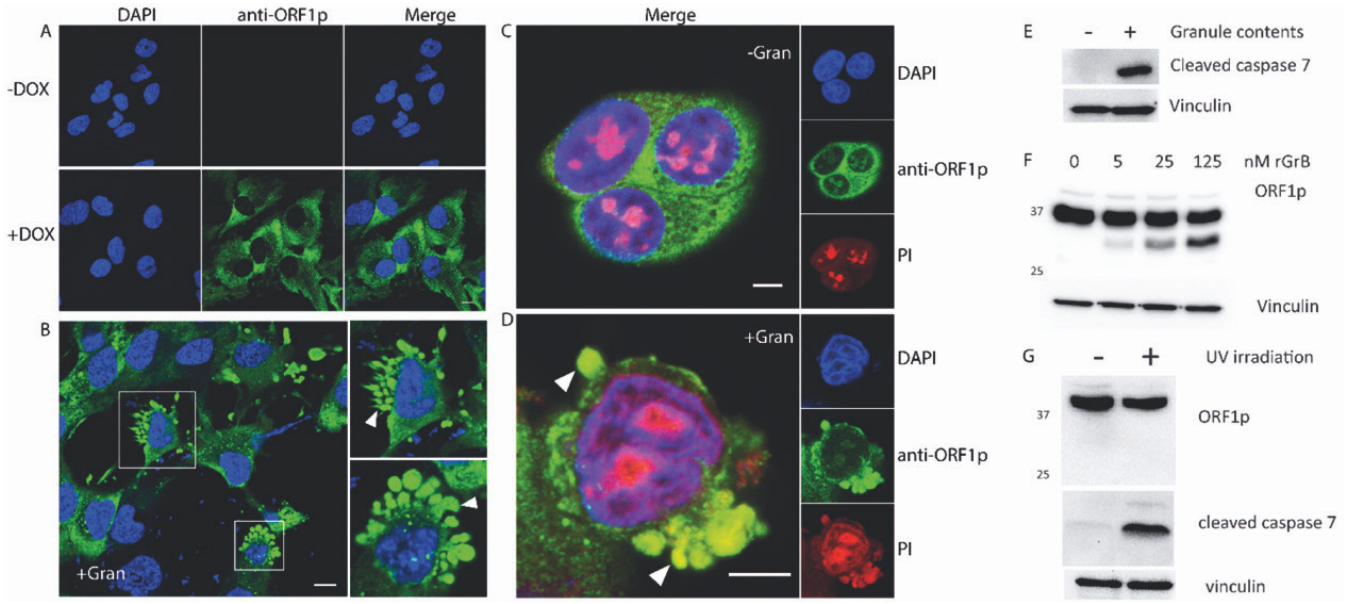

Fig. 1. ORF1p redistributes to surface blebs in apoptotic cells and is cleaved by granzyme B but not caspases.
A: Untreated (-DOX) and doxycycline-induced (+DOX) L1-RPE stained with DAPI (blue, left) and anti-ORF1p antibody (green, middle).
B: Doxycycline-induced LINE-1-RPE treated with NK cell granule contents (+Gran) for 4 hours, then stained as in (A). Apoptotic cells with ORF1p-containing surface blebs are highlighted (right panels).
C: Untreated MCF7 cells stained with DAPI (blue), anti-ORF1p (green) and PI (red).
D: MCF7 cells treated with NK cell granule contents, then stained as in (C). Apoptotic blebs (arrowheads) demonstrating positivity for PI and ORF1p. Scale bars in A, B: 10 μm; scale bars in C, D: 5 μm. Results are representative of 3 experiments.
E: MCF7 cells exposed to control buffer (-) or Granule contents (+) then harvested and probed for cleaved caspase 7 by Western blot.
F: MCF7 cell lysates incubated with recombinant GrB (rGrB) and analysed by Western blotting for ORF1p. G: MCF7 cells exposed to UV light to induce apoptosis, then analysed at 16 hours for ORF1p and cleaved caspase 7 expression by Western blotting. The results are representative of 3 separate experiments.

Cells

An RPE cell line with tetracycline-inducible LINE-1 (11) was grown in DMEM:F12 medium, and LINE-1 induced using doxycycline (Sigma). MCF7 cells, which express ORF1p constitutively (12), were purchased from ATCC and grown in media per their instructions.

Immunofluorescence

Cells were grown on coverslips, treated as indicated, fixed in 4% PFA and permeabilised in acetone. ORF1p was stained with abcam EPR22227-6 (1:100) (Validated in Supplementary Fig. 2). Propidium iodine was used at 1 μg/mL (Molecular Probes). Coverslips were mounted using ProLong Gold with DAPI (ThermoFisher). Cells were imaged using a Zeiss AxioObserver with 780-Quasar confocal module.

Western blotting

Protein lysates were electrophoresed via SDS-PAGE then transferred via iBlot2 system (ThermoFisher) to nitrocellulose membranes. Blocking was performed with 5%-milk-TBS-T then primary antibody incubation performed at 4C overnight using anti-vinculin (Sigma), anti-ORF1p (abcam), anti-cleaved caspase 7 (Cell Signaling). Blots were incubated with HRP-labelled secondary antibodies (Cell Signaling) for 1 hour at RT, then developed with ECL reagent (ThermoFisher) and imaged using a FluorChem system (ProteinSimple).

Rabbit anti-ORF1p monoclonal validation

Rabbit anti-ORF1p monoclonal (abcam clone EPR22227-6) was validated in immunoprecipitation-Western blotting experiments using mouse anti-ORF1p monoclonal clone 4H1 (13). RPE-LINE-1 cells were treated with Doxycycline at 1 μg/mL for 48 hours and lysates generated from untreated and induced cells. Immunoprecipitation was performed with induced RPE cell lysates using rabbit anti-ORF1p, mouse anti-ORF1p, control rabbit IgG (Cell Signaling), control mouse IgG1 (Cell Signaling), using each antibody at 1 μg/mL for 2 hours, followed by IgG isolation with Protein G Dynabeads (Thermofisher). Immune complexes were recovered from Dynabeads by boiling in GAB, then electrophoresed alongside lysates from untreated and induced cells, and transferred to nitrocellulose membranes. Each membrane was stained with Ponceau S solution, then washed and analysed by Western blotting using complementary anti-ORF1p antibodies at 1:1000 dilution for 1 hour at RT.

GrB cleavage assays

Cell lysates were incubated at 37C with wild type recombinant GrB and analysed by Western blotting.

Statistical analysis

Fisher's exact test was used for univariate analyses. The two sample t-test was used for normally distributed variables and Mann-Whitney test for non-normally distributed continuous variables. Linear regression evaluated relationships between gene signatures and anti-LINE1 antibodies in the SPARE cohort. Correlation between continuous variables was calculated using Pearson's r coefficient. Statistical analysis was performed using GraphPad Prism and R software.

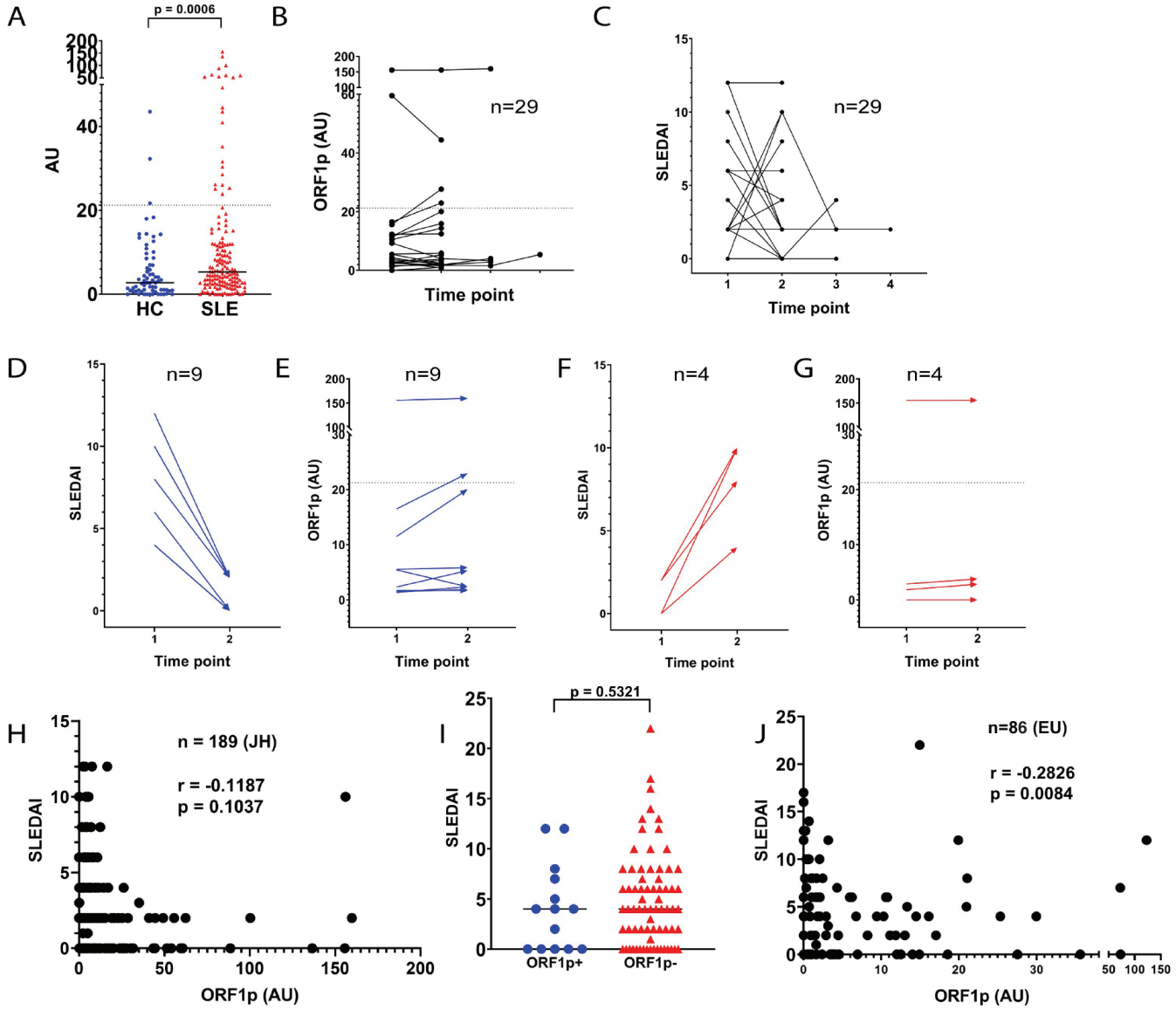

Fig. 2. ORF1p autoantibodies are not associated with SLE disease activity.
A: Anti-ORF1p ELISA performed in healthy control (HC, n=68) and SLE (n=158) JH SPARE cohorts. Median values (solid line) and positivity threshold (dashed line) indicated.
B: Anti-ORF1p results in longitudinal serum samples in 23 JH SLE patients at >1 time point (total n=29 samples).
C: Corresponding SLEDAI values for the 29 longitudinal samples over the same time intervals depicted in (B).
D: Individual time intervals corresponding to drop in SLEDAI of ≥4 points in the JH longitudinal cohort (n=9 intervals).
E: Corresponding changes in anti-ORF1p levels observed during the same 9 intervals in (D).
F: Individual time intervals corresponding to increase in SLEDAI by ≥4 points in the longitudinal JHU cohort (n=4 intervals).
G: Corresponding changes in anti-ORF1p levels observed during the same 4 intervals in (F).
H: Correlation between anti-ORF1p and SLEDAI in 189 total samples in the SPARE cohort. Spearman r and *p*-value indicated.
I: EU SLE patients (n=86) with and without anti-ORF1p antibodies compared for disease activity by SLEDAI.
J: Correlation between SLEDAI and anti-ORF1p level in the EU SLE cohort.
AU: arbitrary units.

Results

ORF1p localises to apoptotic blebs

We used purified YT cell granule contents (GC) (14) to induce apoptosis in both RPE and MCF7 cell lines (Fig. 1A-E). ORF1p expression was cytoplasmic at baseline in both MCF7 and induced RPE lines. Following GC exposure, ORF1p redistributed from the cytoplasm to apoptotic blebs in both cell types (Fig. 1B, D). PI staining identified these blebs as RNA-rich small surface blebs, known to contain Ro antigen in addition to endoplasmic reticulum (15). ORF1p-containing blebs did not contain DNA as visualised by DAPI, differentiating them from larger apoptotic bodies. Our experiments identify ORF1p as a component of apoptotic blebs in LINE-1 expressing cells.

ORF1p is cleaved by Granzyme B but not caspases

The serine protease GrB is a major component of cytotoxic T lymphocyte (CTL) and NK cell granules. Susceptibility to GrB cleavage is a characteris-

tic of many autoantigens, theorised to contribute to antigenicity by creating novel immunostimulatory epitopes via protein cleavage in the setting of cell death (16). To examine this characteristic, MCF7 lysates were exposed to purified recombinant human GrB *in vitro*. This revealed that ORF1p is efficiently cleaved by GrB, resulting in the generation of an ~30 kDa C-terminal fragment (Fig. 1F). To demonstrate specificity of this cleavage, we induced caspase-mediated cell death by UV irradiation, finding that ORF1p was not cleaved following UV-induced apoptosis (Fig. 1G). Together, these experiments confirm that ORF1p is a target of GrB, but not caspase-mediated cleavage.

ORF1p is a lupus autoantigen

We developed an ELISA to screen 189 SLE samples from the SPARE cohort (8). A cohort of 68 healthy controls was analysed for comparison. We found anti-ORF1p antibodies in both lupus and control samples, with a significantly higher prevalence in lupus patients: using a positivity threshold of 2 standard deviations above the mean titre of healthy controls, anti-ORF1p antibodies were found in 15.8% of SLE and 4.2% of healthy subjects (p=0.0157 by Fisher's exact test) (Fig. 2A). The median antibody titre was higher in SLE than control sera (5.273±22.19 *vs.* 2.75±7.792, p=0.0006).

Anti-ORF1p antibodies are not dynamic markers of lupus disease activity

We next analysed anti-ORF1p results against clinical and gene expression data. We found no relationship between anti-ORF1p antibodies and disease activity at the time of visit: anti-ORF1p positivity was not associated with higher SLEDAI, higher PGA, low complement proteins, or anti-DNA positivity in a multivariable analysis (Supplementary Table S1). We analysed anti-ORF1p results against 4 peripheral blood gene signatures (8), including the IFN, neutrophil and plasma cell (PC) signatures, and BAFF expression; this analysis demonstrated no statistically significant relationship between anti-ORF1p antibodies and any of the 4 gene profiles (Suppl. Table S2). There was an association between clinical disease activity (SLEDAI) and peripheral blood IFN, neutrophil, PC and BAFF gene signatures in the SPARE cohort (Suppl. Table S3), but anti-ORF1p antibodies demonstrated no relationship to these gene expression markers. Anti-ORF1p was not associated with any specific clinical or serological features of SLE in this cohort (Suppl. Tables S4 and 5).

Longitudinal samples were available for 23 patients in this cohort, separated by a median time of 221 days. These serial samples demonstrated marked stability of anti-ORF1p antibodies over time: only 2 subjects demonstrated a change in anti-ORF1p status from positive to negative (or vice versa), despite the presence of dynamic SLEDAI over these intervals (Fig. 2B-C). We identified longitudinal samples that accompanied a change in SLEDAI of ≥4 points (Fig. 2D-G), finding that these SLEDAI changes were not paralleled by corresponding changes in anti-ORF1p results. Analysing all 189 time points for a correlation between anti-ORF1p level and SLEDAI (Fig. 2H) revealed no significant relationship between these two variables in the SPARE cohort. An additional analysis was performed to examine the subset of SLE patients with high-titre anti-ORF1p autoantibodies (>45 AU, n=13); we again found no relationship between SLEDAI and anti-ORF1p using this higher threshold for autoantibody positivity (SLEDAI 3.46±3.66 anti-ORF1p>45 AU *vs.* 2.22±1.8 anti-ORF1p <45 AU, p=0.1971)

To confirm these findings, we screened a second lupus cohort from EU, which included patients with higher disease activity as measured by SLEDAI (4.826±4.612 EU *vs.* 2.307±2.849 SPARE). The prevalence of anti-ORF1p antibodies was nearly identical in the two cohorts: 15.8% in SPARE *versus* 15.5% in EU. SLEDAI was not higher in anti-ORF1p+ than anti-ORF1p- SLE patients in the EU cohort (median = 4 in both groups, Fig. 2I). Moreover, there was a modest but statistically significant negative relationship between anti-ORF1p levels and SLEDAI in the EU cohort (Fig. 2J). In summary, our analysis of anti-ORF1p antibodies in two separate SLE cohorts demonstrates that these antibodies are associated with SLE disease status, and are stable over time, but do not indicate patients with higher levels of disease activity.

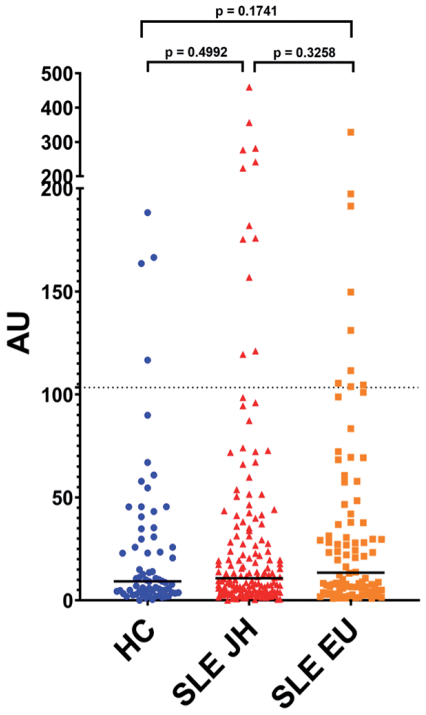

Fig. 3. Anti-ORF2p antibodies are not associated with SLE disease status.
Anti-ORF2p ELISA was performed using healthy control (n=69), Johns Hopkins lupus sera (SLE JH, n=158) and Emory lupus sera (SLE EU, n=90). Assay positivity was established as the mean + 2 standard deviations of healthy control values. Mann-Whitney test was used to compare median values with p-value indicated. AU: arbitrary units.

Anti-ORF2p autoantibodies are not enriched in SLE

Given the presence of anti-ORF1p antibodies, we developed an ELISA assay to determine whether ORF2p might also be a target of the antibody response in SLE. Using conditions identical to the ORF1p ELISA, we found that anti-ORF2p serum reactivity was no different in healthy control or SLE patients, indicating that anti-ORF2p antibodies are not associated with SLE disease status (Fig. 3).

Discussion

Several features unite the array of autoantigens targeted by the immune response in SLE. The LINE-1-expressed

RNA chaperone ORF1p possesses several characteristics of lupus autoantigens, including properties of nucleic acid binding and localisation in RNPs. Here, we have defined qualities that may contribute to ORF1p's status as an autoantigen, through demonstrating that ORF1p is a substrate for GrB, and is a constituent of small surface blebs in apoptotic epithelial cells. These properties are shared by numerous SLE autoantigens, and link the process of cell death to the development of autoimmunity (15-17). Our *in vitro* studies offer a construct for tying the observation of ORF1p expression in lupus tissues (2) to the development of anti-ORF1p antibodies: LINE-1-expressing cells present in the targeted organ may generate immunogenic forms of ORF1p during cell death, and LINE-1 itself can trigger apoptosis through the induction of genetic damage incurred during its active expression and replication (18).

We did not uncover a relationship between anti-ORF1p autoantibodies and lupus disease activity, making our results different from those described in the initial description of anti-ORF1p antibodies by Carter *et al*. (3). Comparing the ELISA assays utilised identifies important differences: Carter *et al*. used more ORF1p, more serum, and an overnight serum incubation. Their assay would therefore identify lower affinity interactions than our ELISA. In addition, increased concentration of IgG, acute phase reactants, and immune complexes are known to generate non-specific background signal in ELISA assays (19), and could lead to an apparent relationship between an autoantibody and lupus disease activity in ELISA assays that do not account for nonspecific background.

Our finding of relatively stable anti-ORF1p antibodies over time suggests that anti-ORF1p antibodies may result from an anti-LINE-1 response that occurred early, in the initiation phase of disease. For example, anti-ORF1p antibodies might develop in patients who share specific, active LINE-1 insertions (4) with the potential to elicit sustained immune activation. Additional study in pre-clinical SLE is required to determine whether these antibodies are indicators of LINE-1 dysregulation occurring early in SLE pathogenesis.

In contrast to the scenario with ORF1p, we found that anti-ORF2p ELISA results were no different in SLE *versus* control subjects. Despite the fact that both proteins are translated from a single transcript, ORF2p protein expression is far lower than ORF1p, and may not reach a necessary threshold in vivo to induce humoral autoimmunity (20).

In summary, we have characterised the LINE-1 RNA chaperone ORF1p as a substrate of GrB and a component of RNA-containing apoptotic blebs. Antibodies against ORF1p, but not ORF2p, are found at a greater prevalence in SLE than control subjects, but did not associate with dynamic markers of SLE activity in two cohorts. Accurately defining LINE-1's contribution to SLE pathogenesis may ultimately inform the development of novel therapeutic approaches to SLE.

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