Running head: SDMA Defines Anti-Sm Autoantibodies in SLE

Symmetrical Dimethylarginine as the Central Antigenic Determinant of Anti-Smith Autoantibodies in Systemic Lupus Erythematosus

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ABSTRACT

Objective

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease causing multi-organ damage. The most specific autoantibody response in SLE, present in 20-30% of patients, targets the Sm-protein and has been shown to recognize a linear Sm-derived B cell epitope containing a post-translational modification (PTM) of arginine termed symmetrical dimethyl arginine (sDMA). As autoantibodies to other PTM-modified proteins are often promiscuous, we aimed to determine the specificity and cross-reactivity of anti-Sm antibodies.

Methods

Specificity and promiscuity/cross-reactivity of anti-Sm IgG were measured by ELISA in anti-Sm+ SLE patients (n=12), anti-Sm- SLE patients (n=12) and healthy donors (HD) (n=12) using peptides containing either sDMA or unmodified arginine. Inhibition and cross-reactivity were determined using competitive ELISA and affinity purification. Recognition of endogenous EBNA1 by anti-Sm IgG was performed by Western blot using lysates of EBV-bearing lymphoblastoid cell lines.

Results

The sDMA residue is recognized by anti-Sm+ SLE patients regardless of the peptide amino acid sequence, with a modest impact of amino acids flanking sDMA on recognition. Most notably, IgGs targeting sDMA comprise the overall majority (~90%) of the anti-Sm antibody repertoire and are highly cross-reactive between $SmD3_{108-122}$ and several sDMA-containing viral-derived epitopes, including full-length EBNA1.

Conclusion

Our data implicate that the majority of anti-Sm IgGs target the sDMA residue irrespective of its Sm-context, thus representing a prototypic anti-modified protein antibody (AMPA) response. These antibodies are highly promiscuous, recognizing several sDMA-modified

proteins, including naturally occurring viral sDMA-expressing proteins. These data indicate a potential link between viral infection and tolerance breach in anti-Sm+ SLE patients.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease characterized by immune cell dysfunction, leading to widespread inflammation and tissue damage.[1] Autoreactive B cells are central to SLE pathophysiology, a concept further emphasized by the recent successes of anti-CD19 CAR-T cell therapy in achieving clinical remission.[2] The antibodies produced by these self-reactive B cells are known as anti-nuclear antibodies (ANAs), a heterogeneous group of autoantibodies that recognize different chromatin components (e.g. double stranded DNA (dsDNA), nucleosomes) or RNA-associated proteins (e.g. Smith (Sm), Ro60). Although ANAs can be present in various autoimmune diseases, anti-dsDNA and anti-Sm antibodies are most specific to SLE, and have therefore been included among the EULAR-American College of Rheumatology (ACR) criteria for classification of this disease.[1, 3]

The Sm protein is composed of eight subunits with molecular weights varying between 9 and 29.5 kDa. It is part of the spliceosome complex, involved in the processing of nuclear pre-mRNA.[4] Several antigenic epitopes have been characterized on the Sm protein, with key targets spanning amino acids (AA) #95–119 on the SmD1 and #108–122 on the SmD3 subunits.[5, 6] Intriguingly, these regions are rich in RGG/RG repeats, which mediate RNA binding, and contain arginines (Arg) that can undergo post-translational modification (PTM) to form symmetrical dimethylarginine (sDMA) residues *in vivo*.[4] These modifications appear to have immunological relevance, as previous studies suggest that the introduction of sDMA significantly enhances the antigenicity of synthetic SmD1/3-derived peptides.[5, 6]

In the current study, we aimed to further explore how sDMA residues influence the recognition of the Sm protein by autoantibodies in SLE. We show that antibodies targeting sDMA are common in anti-Sm+ SLE patients and represent the overall majority of the anti-Sm antibody repertoire. We furthermore demonstrate that anti-sDMA antibodies are highly cross-reactive and can also bind to sDMA on other proteins, including several virus-derived epitopes.

MATERIALS AND METHODS

Patients and healthy subjects

Serum was obtained from 24 patients (12 anti-SmD+, 12 anti-SmD-, measured by Enzyme-Linked ImmunoAssay) with a clinical diagnosis of SLE, and fulfilling the 2019 ACR classification criteria, from the Rheumatology outpatient clinic at the Leiden University Medical Center (LUMC; Leiden, The Netherlands).[3] This study was approved by the Leiden Den Haag Delft medical ethics committee. Serum from 12 HDs was obtained from the LUMC Voluntary Donor Service, with approval from the LUMC Biobank. All subjects have given written informed consent.

Enzyme-linked immunosorbent assays

Biotinylated peptides were synthesized by the Peptide and Tetramer Facility Immunology at the LUMC (detailed sequences in Supplementary Table 1). Standard capacity 384-well streptavidin microplates (Microcoat) were coated with 1µg/mL of the respective peptide in phosphate-buffered saline (PBS; pH~7.6) with 0.1% bovine serum albumin (BSA) for 1 hour (37°C). Plates were washed and incubated with sera (1:200) in PBTT (PBS/1%BSA/50mM Tris/0.05%Tween-20, pH~8.0), for 1 hour (37°C). All washing steps were performed by

washing 4x with 0.05% Tween-20 (Sigma-Aldrich) in PBS. After incubation, the plates were washed and incubated with 1µg/mL anti-IgG horse-radish peroxidase (Bethyl).

Anti-Sm IgGs were determined using the protocol described above, with some adjustments. Clear flat bottom 384-well microplates (Corning) were coated overnight (4°C) with 0.8µg/mL non-recombinant Sm (Diarect). Prior to serum incubation, plates were blocked with PBT (PBS/1%BSA/50mM Tris, pH~8.0) for 1 hour. All procedures were conducted at room temperature (RT).

All ELISAs were developed using ABTS (Sigma-Aldrich) and H_2O_2 (Merck) and measured on an iMark Microplate Absorbance Reader (BioRad). Samples were assessed with technical duplicates.

ELISA-based inhibition assays

Inhibition assays were performed as described previously, with some modifications.[7] Briefly, sera (1:200) were preincubated with indicated concentrations of blocking peptide for 2 hours (37°C), prior to addition to the ELISA plates as described above. Inhibition percentages were calculated by subtracting the mean background OD (from HDs) and dividing the OD at the highest blocking concentration by the OD of the unblocked sample.

Antibody affinity purification

NeutrAvidin Agarose resin (Thermo Scientific) was coupled with 0.1µg/µL $SmD3_{108-122}$ (sDMA or Arg) for 1 hour at RT with shaking. The resin was transferred to a 96-well filter plate (Orochem) and incubated with serum (1:4) in PBS for 2 hours at RT with shaking. Unbound fraction was removed by centrifugation at 500×g for 1 minute. Bound fraction was eluted twice with 25µL 100mM formic acid for 5 minutes with shaking, followed by centrifugation at 500xg for 2 minutes. The eluate was neutralized to pH~7 using Tris-HCL (pH~9.2)

Cell lines

EBV-infected JY or DR7 B cell lines (ATCC) or EBV-negative BCL-6/Bcl-xL transduced immortalized B cells (IMM) were cultured in IMDM containing 10% FCS, 100U/mL penicillin/streptomycin, and 2mM GlutaMax. Whole cell extracts were made by incubation for 30 min on ice in PBS with 0.5% IGEPAL (Sigma-Aldrich), 150mM NaCl, and protease inhibitor cocktail (Roche). Lysates were centrifuged at 12.000×g for 20 minutes, supernatants were used for further analysis.

Western blot

750µg cell lysate was diluted in 4x Laemmli buffer (Bio-Rad) and heated for 5 minutes (95°C). Samples were resolved by SDS-PAGE using a 10% polyacrylamide gel (Bio-Rad), transferred onto PVDF membrane, blocked overnight (4°C) in 5% milk/PBS/0.05%Tween, and incubated for 1 hour with either an anti-sDMA antibody (1:500; Sigma-Aldrich; clone SYM11), anti-EBNA-1 antibody (1:1.000; Sigma-Aldrich; clone 1H4), or affinity purified anti-sDMA IgGs (~1µg/mL). After three washes with PBS/0.05%Tween, blots were incubated with either anti-rat IgG-AF680 (1:5.000; Invitrogen), HRP-conjugated anti-rabbit Ig (1:1.000; DAKO) or HRP-conjugated anti-human IgG (1:1.000; DAKO). Blots were visualized using Pierce ECL substrate according to manufacturer's instructions. Images were taken using iBright FL1500 Imaging System (Invitrogen).

Statistical analysis

Statistical tests were performed in GraphPad Prism version 10.1.0. Paired comparisons were calculated using Wilcoxon signed-rank test and comparisons across multiple groups using a

Friedman test, corrected for multiple testing using Bonferroni-Dunn method. *P* values <0.05 were considered statistically significant

RESULTS

$SmD3_{108-122}$ epitope recognition depends exclusively on sDMA in anti-Sm+ SLE patients

To validate the requirement of an sDMA residue for recognition of the $SmD3_{108-122}$ epitope by anti-Sm+ SLE patients, we assessed IgG reactivity against the full-length Sm protein as well as $SmD3_{108-122}$ variants containing either sDMA, asymmetrical dimethyl arginine (aDMA), or unmodified arginine.[5] We tested sera from anti-Sm+ SLE, anti-Sm− SLE, and HDs. Consistent with previous studies, the $SmD3_{108-122}$ epitope was recognized exclusively by anti-Sm+ SLE patients, and only when containing sDMA (Figure 1A).[5, 6] Increasing the number of sDMA residues did not significantly enhance $SmD3_{108-122}$ recognition by these patients (Supplementary figure 1). A scrambled peptide of the same epitope was also included and surprisingly, was still specifically recognized by anti-Sm+ SLE sera (Figure 1B), demonstrating that the sDMA residue is the principal determinant of $SmD3_{108-122}$ epitope recognition. Still, a slight decrease in binding to the scrambled epitope was observed. We hypothesized that the flanking amino acids may influence sDMA recognition, potentially via steric hindrance or by contributing directly to antibody binding. Replacing the AAs flanking the sDMA residue with isoleucines significantly reduced recognition (Figure 1C), potentially explaining the reduced reactivity toward scrambled $SmD3_{108-122}$ and suggesting the importance of a flanking glycine in the binding of at least a subset of sDMA-specific IgGs.

Next, to evaluate whether IgGs targeting sDMA residues also recognize the full-length Sm protein, we isolated antibodies by affinity purification with $SmD3_{108-122}$ (sDMA or Arg) (Figure 1D). Significantly higher amounts of sDMA-specific IgGs were isolated from anti-Sm+ SLE patients compared to the unmodified control (Figure 1E), and only the anti-sDMA

antibodies exhibited binding to the full-length Sm protein (Figure 1F). Overall, our findings indicate that anti-Sm+ SLE patient sera recognize the $SmD3_{108-122}$ epitope primarily through its sDMA residue, and that these anti-sDMA IgGs are capable of targeting the full-length Sm protein. These observations are particularly relevant as autoantibodies in rheumatoid arthritis (RA) directly recognize citrulline (another PTM of arginine) in a variety of epitopes.[7, 8] Consequently, we continued to investigate whether anti-Sm antibodies represent a bona fide anti-PTM response.

Anti-sDMA IgGs are cross-reactive to viral sDMA-containing epitopes

In addition to the Sm protein, emerging evidence suggests that several viral proteins may carry sDMA residues as well.[9, 10, 11] To determine whether anti-sDMA IgG from anti-Sm+ SLE patients can recognize viral sDMA-containing epitopes, we synthesized peptides of several viral epitopes, each containing sDMA at a specified position or unmodified arginine as control. We assessed epitopes derived from Epstein–Barr virus (EBV) nuclear antigen 1 (EBNA1) (Figure 2A), EBNA2 (Figure 2B), Hepatitis C virus (HCV) nonstructural protein 3 (NS3) (Figure 2C), and infectious bursal disease virus (IBDV) RNA polymerase VP1 (Figure 2D). All viral epitopes were recognized by anti-Sm+ SLE patients, but only when sDMA was present, similar to the $SmD3_{108-122}$ epitope. To assess whether anti-sDMA IgGs were cross-reactive between $SmD3_{108-122}$ and viral sDMA-containing epitopes, we performed an inhibition assay. Anti-Sm+ SLE serum was preincubated with various concentrations of $SmD3_{108-122}$ (sDMA or Arg) before assessing binding to viral epitopes by ELISA (Figure 2E). This resulted in an average inhibition ranging from 95% to 65%, depending on the viral epitope measured, indicating that anti-sDMA IgGs are highly cross-reactive, as long as the epitope contains sDMA (Figure 2F, Supplementary Figure 2).

Next, we sought to determine whether anti-sDMA IgG could also recognize sDMA residues within endogenous viral proteins. To this end, we selected EBNA1 as a model antigen. To confirm the presence of sDMA on endogenous EBNA1, we performed western blotting on lysates from EBV- and EBV+ B cell lines using anti-sDMA (SYM11) and anti-EBNA1 (1H4) monoclonal antibodies (Figure 2G, Supplementary figure 3A/B). The alignment of both signals at the same molecular weight supports the presence of sDMA on EBNA1. Similarly, blots stained with pooled affinity-purified anti-sDMA IgG from multiple SLE patients showed a signal that appeared at the same molecular weight as the anti-EBNA1 band in the EBV+ lysate (Figure 2H, Supplementary figure 3C/D), suggesting that patient-derived anti-sDMA IgGs can bind endogenous EBNA1. Overall, our results indicate that IgGs targeting sDMA are highly cross-reactive to other sDMA-containing epitopes, including several viral-derived proteins.

sDMA forms the predominant target of the anti-Sm response in SLE patients

We noticed a strong correlation between the levels of IgG reactive to $SmD3_{108-122}$ and full-length Sm (Figure 3A). Therefore, we sought to determine to what extent IgGs targeting sDMA-containing epitopes contribute to the overall anti-Sm IgG response in SLE patients. To assess this, we conducted an inhibition assay by incubating anti-Sm+ SLE serum with various concentrations of $SmD3_{108-122}$ (sDMA or Arg) prior to detection by ELISA (Figure 3B). In order to confirm that anti-sDMA IgGs were effectively blocked, we first tested the blocked serum for recognition of $SmD3_{108-122}$–sDMA. At the highest blocking peptide concentration, $SmD3_{108-122}$–sDMA effectively inhibited recognition in most samples (Figure 3C), whereas blocking with $SmD3_{108-122}$–Arg had no effect on recognition (Figure 3D), indicating that nearly all sDMA-specific IgGs were successfully blocked (Figure 3E). In turn, we evaluated the blocked sera for recognition of full-length Sm (Figure 3F). Blocking anti-sDMA resulted in a

significant reduction in Sm recognition (Figure 3G), with an average inhibition of 90% (Figure 3H). No effect in Sm recognition was observed when anti-Sm+ sera were blocked with the arginine control peptide (Figure 3G/H) or in anti-Sm- SLE patients and HDs (Supplementary Figure 4).

To extend these findings, we tested whether IgGs targeting sDMA-containing viral epitopes could similarly inhibit Sm recognition using a comparable inhibition assay. This time, anti-Sm+ SLE sera were preincubated with the respective viral epitopes (sDMA or Arg), which resulted in approximately 80–90% inhibition, indicating significant cross-reactivity between viral sDMA-containing epitopes and the Sm protein (Figure 3J, Supplementary Figure 2). For comparison, anti-Sm+ SLE sera were also blocked with the cross-reactive PPPGMRPP epitope, previously described as an early target in the anti-Sm response.[12] Notably, the PPPGMRPP epitope was not differently recognized by anti-Sm+ SLE sera compared to anti-Sm- SLE or HDs (Figure 3K) and inhibition with this peptide had no effect on Sm protein recognition (Figure 3L). Taken together, our findings strongly suggest that the sDMA residue forms the predominant target of the anti-Sm response in SLE patients.

DISCUSSION

This study sought to deepen the understanding of the specificity and antigenic determinants of the anti-Sm autoantibodies response in SLE. Several antigenic targets have been identified on the Sm protein, with $SmD1_{95\text{-}119}$ and $SmD3_{108–122}$ being recognized as major epitopes. [5, 6] Building on previous studies, our findings now demonstrate that the sDMA residue is the primary antigenic target, independent of the AA sequence in which it is embedded. This conclusion is supported by the sustained recognition of scrambled $SmD3_{108–122}$ and other sDMA-containing viral epitopes by anti-Sm+ SLE patient sera. Thus, the anti-Sm response

represents a prototypic anti-PTM response, resembling the anti-modified protein antibody (AMPA) response described in RA.[8]

To investigate the significance of the anti-sDMA response in SLE, we conducted an inhibition experiment by blocking IgG binding to sDMA and evaluating the ability of anti-Sm+ SLE sera to bind to the native Sm protein. We demonstrate for the first time that the majority (>90%) of anti-Sm IgGs specifically target sDMA residues, highlighting a central role of this PTM in the development of the SLE-specific B cell response to Sm. Additional human proteins, such as snRNP70, have been identified to carry sDMA, which may explain the strong association between anti-RNP70 and anti-Sm antibody responses in SLE patients.[11, 13]

Multiple sources have analyzed the initial antigenic epitopes of the anti-Sm response by performing overlapping peptide screens using sera collected before and after SLE diagnosis. The PPPGMRPP motif was identified as a primary target of the anti-Sm response and was suggested to be an initiating factor, as EBV's EBNA-1 (AA #398-404) contains a similar motif.[12] Indeed, numerous viruses have been linked to SLE pathogenesis, particularly EBV.[14] However, the major limitation of these studies is their use of peptides lacking PTMs, thereby failing to capture the full spectrum of the anti-Sm antibody response. Although a potential initiating role cannot be ruled out in this study, we did not observe a significant contribution of antibodies targeting PPPGMRPP to the anti-Sm antibody repertoire in established SLE patients. Nevertheless, molecular mimicry between viral and self-antigens remains a possible and intriguing trigger in the initiation of autoimmunity, and our results suggest that any virus that produces sDMA-containing proteins can trigger such a response. Remarkably, many viral proteins, including those from common viruses (EBNA-1/EBNA-2 from EBV, PB2 from Influenza A), have been reported to contain sDMA.[9, 10] Immunization of mice with an EBNA-1 expression vector induced IgGs that bind to non-recombinant Sm.[15] We now discovered that sDMA-targeting antibodies exhibit significant cross-reactivity, allowing

recognition of multiple sDMA-containing viral epitopes, including endogenous EBNA1. Interestingly, an earlier study on the $SmD3_{108–122}$ epitope did not detect any reactivity in infectious disease controls, including Cytomegalovirus, HCV, or EBV infected individuals.[5] Given that infections with certain sDMA-containing viruses are widespread, these findings suggest that if anti-sDMA IgGs arise in SLE due to viral infection or reactivation, an underlying tolerance defect may predispose these individuals to produce anti-sDMA antibodies, distinguishing them from other infected individuals.[14]

In conclusion, our findings highlight the central role of sDMA residues in the development of anti-Sm autoantibodies in SLE. Furthermore, the high cross-reactivity of Sm antibodies to various sDMA-containing epitopes points to a role of viral infection in tolerance breach towards Sm in SLE.

REFERENCES

1. Suurmond J, Diamond B. Autoantibodies in systemic autoimmune diseases: specificity and pathogenicity. *J Clin Invest* 2015; **125**(6): 2194-2202.
2. Müller F, Taubmann J, Bucci L et al. CD19 CAR T-Cell Therapy in Autoimmune Disease - A Case Series with Follow-up. *N Engl J Med* 2024; **390**(8): 687-700.
3. Aringer M, Costenbader K, Daikh D et al. 2019 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Systemic Lupus Erythematosus. *Arthritis Rheum* 2019; **71**(9): 1400-1412.
4. Thandapani P, O'Connor TR, Bailey L et al. Defining the RGG/RG Motif. *Mol Cell 2013;* **50**(5): 613-623.
5. Mahler M, Fritzler MJ, Blüthner M. Identification of a SmD3 epitope with a single symmetrical dimethylation of an arginine residue as a specific target of a subpopulation of anti-Sm antibodies. *Arthritis Res Ther* 2004; **7**(1): R19-29.

6. Brahms H, Raymackers J, Union A et al. The C-terminal RG Dipeptide Repeats of the Spliceosomal Sm Proteins D1 and D3 Contain Symmetrical Dimethylarginines, Which Form a Major B-cell Epitope for Anti-Sm Autoantibodies. *J Biol Chem* 2000*;* **275**(22): 17122-17129.

7. Ioan-Facsinay A, el-Bannoudi H, Scherer HU et al. Anti-cyclic citrullinated peptide antibodies are a collection of anti-citrullinated protein antibodies and contain overlapping and non-overlapping reactivities. *Ann Rheum Dis* 2011; **70**(1): 188-193.

8. Steiner G, Toes REM. Autoantibodies in rheumatoid arthritis – rheumatoid factor, anticitrullinated protein antibodies and beyond. *Curr Opin Rheumatol* 2024*;* **36**(3): 217-224.

9. Zheng K, Chen S, Ren Z et al. Protein arginine methylation in viral infection and antiviral immunity. *Int J Biol Sci* 2023; **19**(17): 5292-5318.

10. Sun H*,* Tu S, Luo D et al. Protein arginine methyltransferase 5 mediates arginine symmetric dimethylation of influenza A virus PB2 and supports viral replication. *J Med Virol* 2023; **95**(10): e29171.

11. Boisvert F-M, Côté J, Boulanger MC et al. Proteomic Analysis of Arginine-methylated Protein Complexes. *Mol Cell Proteomics* 2003; **2**(12): 1319-1330.

12. Arbuckle MR, Reichlin M, Harley JB et al. Shared Early Autoantibody Recognition Events in the Development of Anti-Sm B/B' in Human Lupus. *Scand J Immunol* 1999; **50**(4): 447-455.

13. Pisetsky DS, Lipsky PE. New insights into the role of antinuclear antibodies in systemic lupus erythematosus. *Nat Rev Rheumatol* 2020; **16**(10): 565-579.

14. Quaglia M, Merlotti G, De Andrea M et al. Viral Infections and Systemic Lupus Erythematosus: New Players in an Old Story. *Viruses* 2013; **13**(2): 277.

15. Sundar K, Jacqyes S, Gottlieb P et al. Expression of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) in the mouse can elicit the production of anti-dsDNA and anti-Sm antibodies. *J Autoimmun* 2004; **23**(2): 127-140.

Figure 1: SmD3$_{108–122}$ epitope recognition depends exclusively on sDMA in anti-Sm+ SLE patients

Sera from anti-Sm+ (n=12), anti-Sm- (n=12) SLE patients, as well as HDs (n=12), were analyzed by ELISA for IgG reactivity against: (A) the Sm protein and SmD3$_{108-122}$ peptide variants containing either sDMA, aDMA, or unmodified arginine; (B) SmD3$_{108-122}$ variant with a scrambled sequence; and (C) SmD3$_{108-122}$ variants differing in amino acids flanking the sDMA residue at the indicated positions. (D) Schematic overview of antibody affinity purification, where SmD3$_{108-122}$ binding antibodies were isolated from anti-Sm+ (n=12) and anti-Sm- (n=8) SLE sera using either sDMA or arginine control variants. (E) IgG concentration after affinity purification and (F) antibody binding to the Sm protein were measured by ELISA. Bar graphs show the median with lines connecting data points from individual samples. One representative experiment of three is shown. Data were analyzed using a Friedman test; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Figure 2: Anti-sDMA IgG antibodies from SLE patients are cross-reactive to multiple viral sDMA-containing epitopes.

Sera from anti-Sm+ (n=12), anti-Sm- (n=12) SLE patients, as well as HDs (n=12), were analyzed by ELISA for IgG reactivity against viral sDMA-containing epitopes or corresponding arginine controls: EBV-derived (A) EBNA1$_{34-52}$ or (B) EBNA2$_{335-360}$, (C) HCV-derived NS3$_{1486-1494}$, and (D) IBDV-derived VP1$_{420-439}$. (E) Schematic overview of an inhibition assay, where sera of anti-Sm+ SLE patients (n=6) were incubated with increasing concentrations of blocking SmD3$_{108-122}$ peptide (sDMA or arginine control) and (F) subsequently measured on

the respective sDMA-containing viral peptide ELISA. (G) Lysates from EBV- IMM and EBV+ JY B cell lines were analyzed by western blot for sDMA-modified EBNA1, and (H) subsequently tested for recognition by sDMA-specific IgG antibodies purified from pooled anti-Sm+ SLE sera. EBNA1 is detected at ~88 kDa; additional band at ~15 kDa is consistent with SmD1/3. Bar graphs represent the median, with lines connecting paired samples. One representative experiment of three is shown. Data were analyzed using a Wilcoxon signed-rank test; * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

Figure 3: sDMA forms the predominant target of the anti-Sm response in SLE patients

(A) Correlation of anti-$SmD3_{108\text{-}122}$ OD_{415} with anti-Sm OD_{415} in anti-Sm+ SLE patients (n=12) (data from Figure 1A). (B/F) Schematic overview of the inhibition assay, where sera of anti-Sm+ SLE patients (n=12) were incubated with increasing concentrations of blocking $SmD3_{108\text{-}122}$ peptide (sDMA (red circles) or arginine control (blue squares)) and subsequently measured on (C/D) $SmD3_{108\text{-}122}$ sDMA-peptide ELISA or (G/H) Sm protein ELISA. (J) Inhibition assay where sera of anti-Sm+ SLE patients (n=6) were incubated with respective viral peptides (sDMA or arginine control) and subsequently measured on the respective sDMA-containing viral peptide ELISA. (K) ELISA showing PPPGMRPP peptide binding by sera from anti-Sm+ (n=12), anti-Sm- (n=12) SLE patients, and HDs (n=12). (L) Inhibition assay where sera of anti-Sm+ SLE patients (n=6) were incubated with increasing concentrations of PPPGMRPP or $SmD3_{108\text{-}122}$-sDMA and subsequently measured on Sm ELISA. (E/I/J/L) Bar graphs show percent inhibition at 250 µM relative to the unblocked condition. All bar graphs show the median and line graphs connect individual measurements from the same patient sample. One representative experiment of three independent repetitions is shown. Data were analyzed using simple linear regression or a Wilcoxon signed-rank test; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Supplementary Table 1: An overview of the peptide sequences

Peptide name	Sequence
SmD3$_{108-122}$ – sDMA	AARG-sDMA-GRGMGRGNIF
SmD3$_{108-122}$ – Arg	AARGRGRGMGRGNIF
SmD3$_{108-122}$ – 2x sDMA (1)	AARG-sDMA-G-sDMA-GMGRGNIF
SmD3$_{108-122}$ – 2x sDMA (2)	AARG-sDMA-GRGMG-sDMA-GNIF
SmD3$_{108-122}$ – 3x sDMA	AARG-sDMA-G-sDMA-GMG-sDMA-GNIF
SmD3$_{108-122}$ – sDMA different Arg	AARGRGRGMG-sDMA-GNIF
SmD3$_{108-122}$ – Scrambled	GGM-sDMA-GGIRRAFGNAR
SmD3$_{108-122}$ – G113A	AARG-sDMA-ARGMGRGNIF
SmD3$_{108-122}$ – G111A, G113A	AARA-sDMA-ARGMGRGNIF
SmD3$_{108-122}$ – G113I	AARG-sDMA-IRGMGRGNIF
SmD3$_{108-122}$ – G111I, G113I	AARI-sDMA-IRGMGRGNIF
ENBA-1$_{34-52}$ – sDMA	RGGDNHGRGRG-sDMA-GRGRGGG
ENBA-1$_{34-52}$ – Arg	RGGDNHGRGRGRGRGRGGG
EBNA-2$_{335-360}$ – sDMA	QSSRGQSRGRG-sDMA-GRGRGKGKSRDKQ
EBNA-2$_{335-360}$ – Arg	QSSRGQSRGRGRGRGRGKGKSRDKQ
NS3$_{1486-1494}$ – sDMA	QRRG-sDMA-TGRG
NS3$_{1486-1494}$ – Arg	QRRGRTGRG
VP1$_{420-439}$ – sDMA	GEANCT-sDMA-QHMQAAMYILTR
VP1$_{420-439}$ – Arg	GEANCTRQHMQAAMYILTR

Supplementary Figure 1: $SmD3_{108-122}$ epitope recognition containing multiple sDMA residues

Sera from anti-Sm+ (n=12), anti-Sm- (n=12) SLE patients, as well as HDs (n=12), were analyzed by ELISA for IgG reactivity against $SmD3_{108-122}$ peptide variants containing one or multiple sDMA residues. Bar graphs represent the median, with lines connecting paired samples. One representative experiment of three is shown.

Supplementary Figure 2: Line graphs of viral inhibition assay

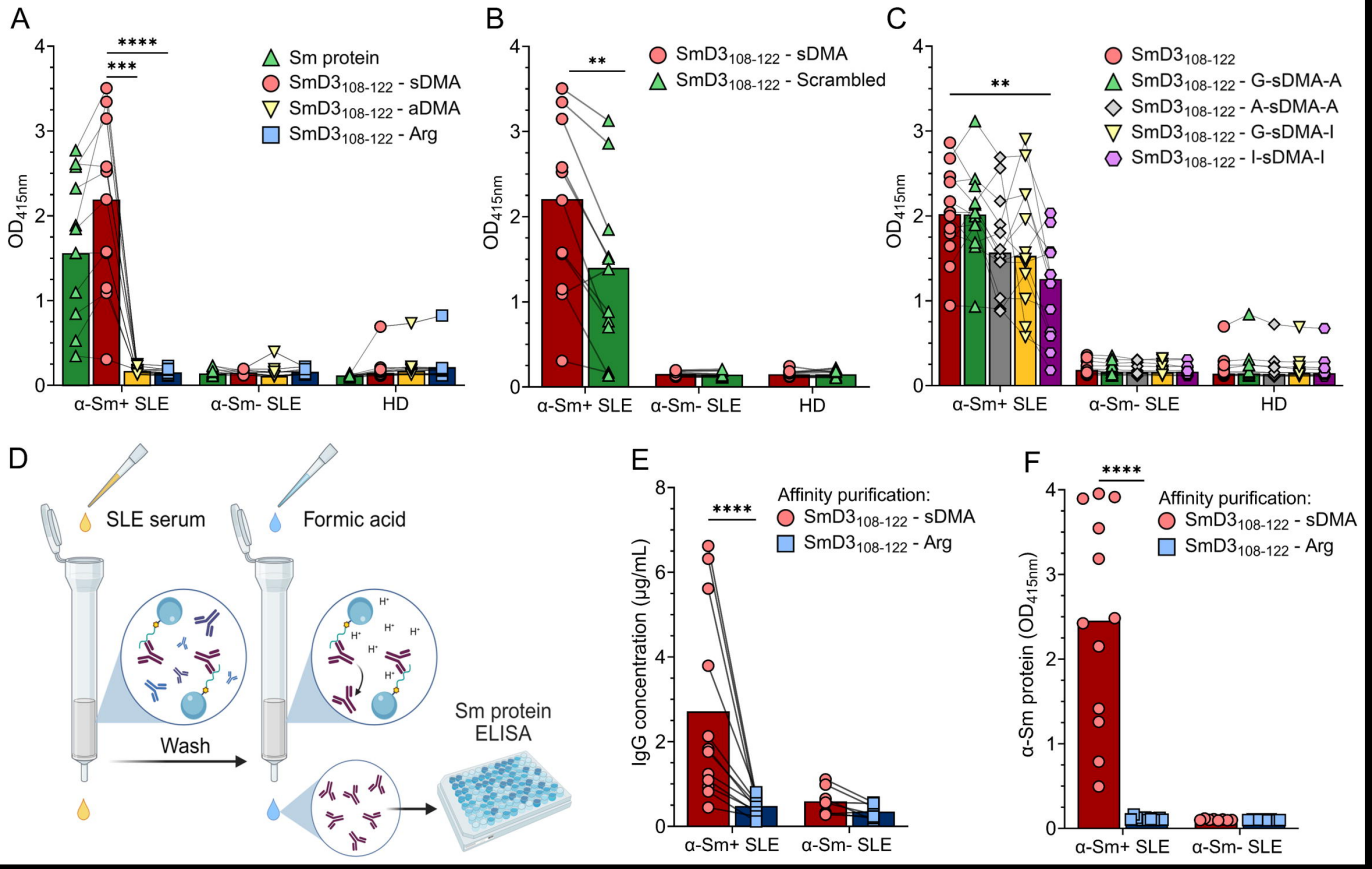
Line graphs corresponding to the bar graphs indicated in Figure 2F and 3J. Sera from anti-Sm+ SLE patients (n=6) were incubated with either increasing concentrations of (A-H) $SmD3_{108-122}$, (I-J, Q-R) $EBNA1_{34-52}$, (K-L, S-T) $EBNA2_{335-360}$, (M-N, U-V) $NS3_{1486-1494}$, or (O-P, W-X) $VP1_{420-439}$ blocking peptide, containing either an sDMA (red) or unmodified arginine (blue) and tested on either the corresponding viral peptide or Sm protein ELISA. Line graphs connect individual measurements from the same patient sample.

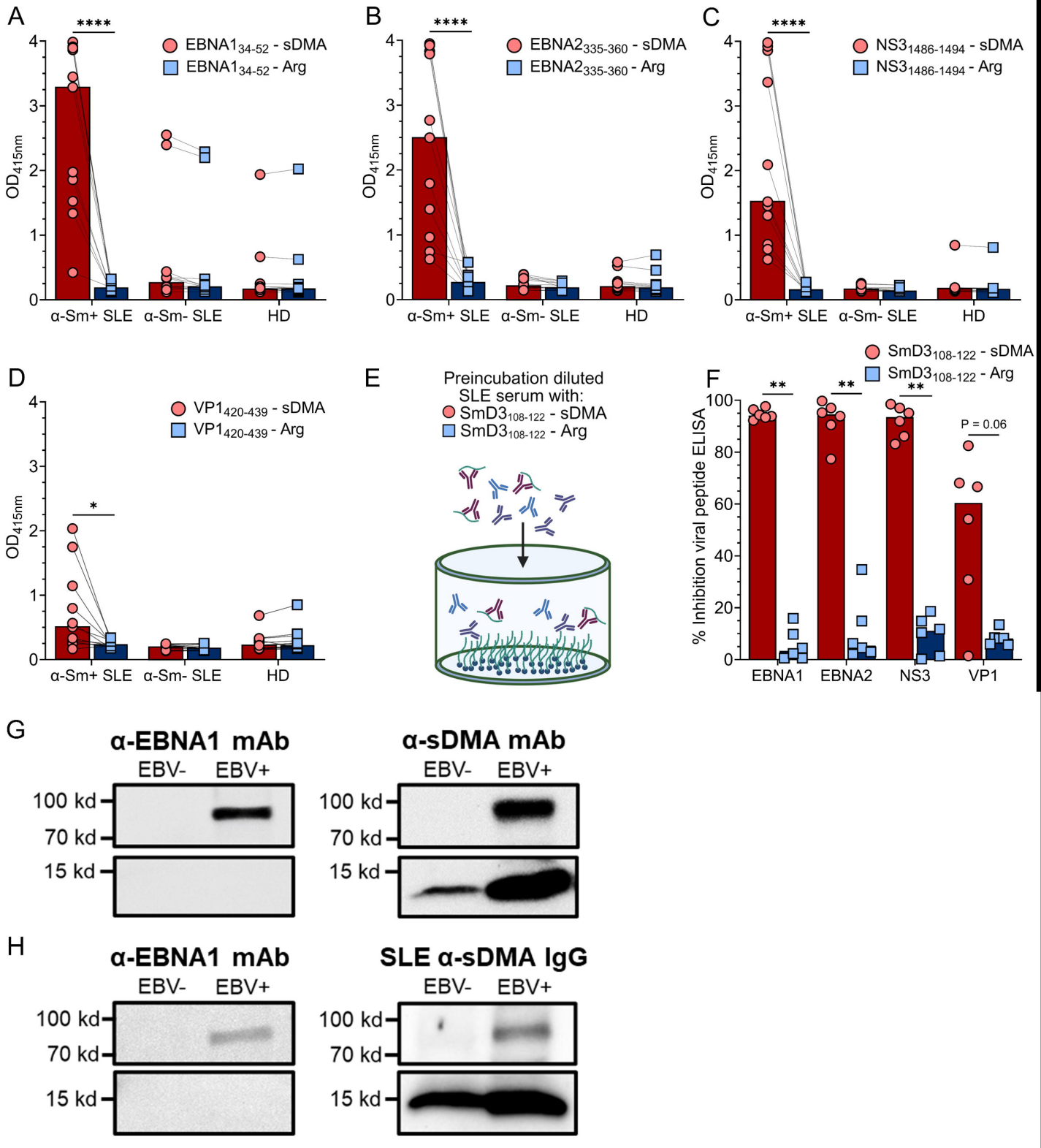
Supplementary Figure 3: Uncropped and unedited blots for figure 2G/H. Panels A and B correspond to Figure 2G; panels C and D correspond to Figure 2H. Arrows indicate the positions where the blots were cropped for the main figure. Lanes: 1, molecular weight ladder; 2, EBV- IMM B cell lysate; 3, EBV+ JY B cell lysate; 4, (A/B) EBV+ DR7 B cell lysate, (C/D) molecular weight ladder.

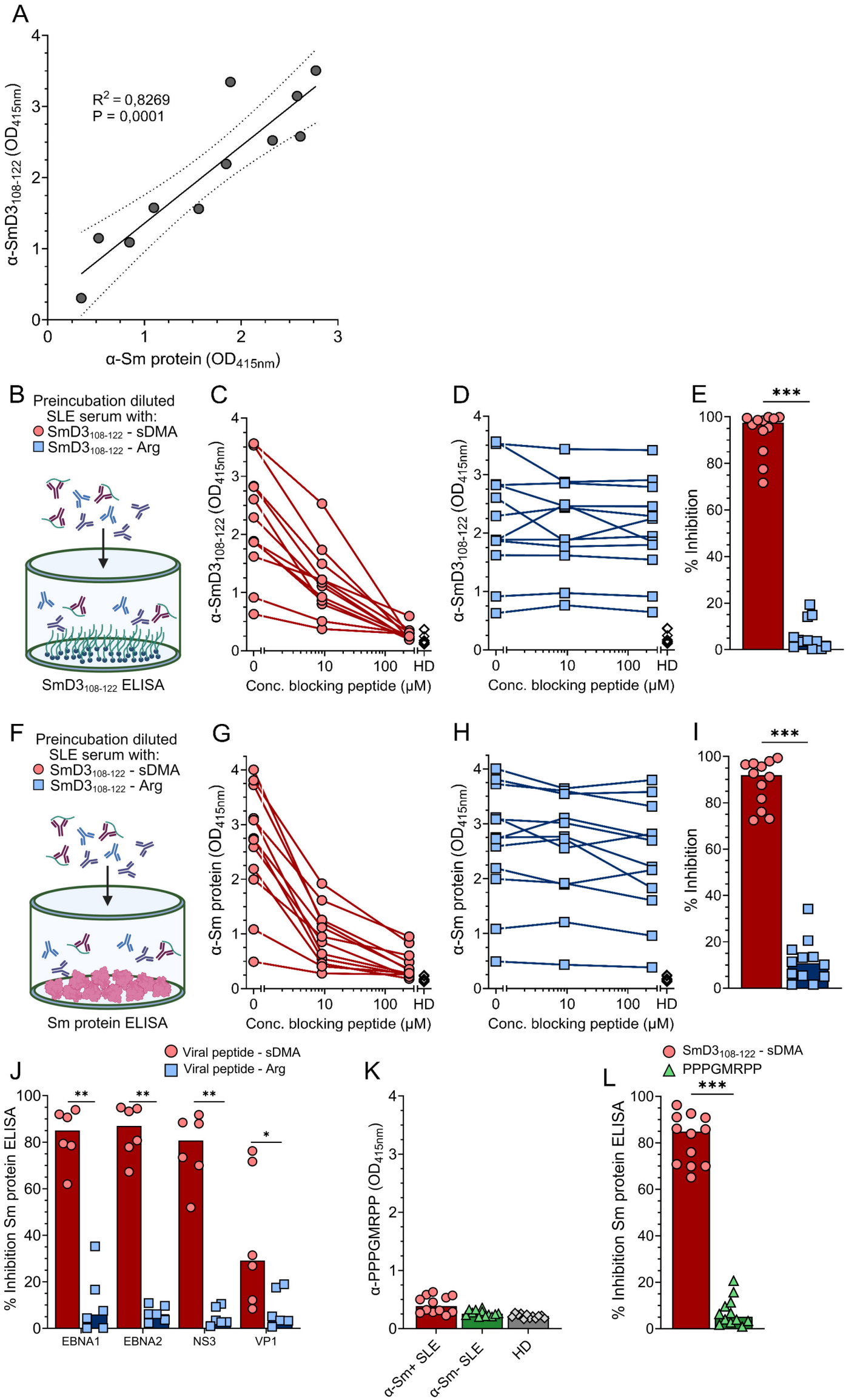
Supplementary Figure 4: Inhibition assay in anti-Sm- SLE patients and HDs

Sera from (A–B) anti-Sm- SLE patients (n=12) and (D–E) HDs (n=12) were incubated with increasing concentrations of blocking $SmD3_{108-122}$ peptide, containing either an sDMA (red

circles) or unmodified arginine (blue squares), and tested on Sm protein ELISA. Line graphs connect individual measurements from the same patient sample.


A
Sm protein
SmD3108-122 - sDMA
SmD3108-122 - aDMA
SmD3108-122 - Arg
OD415nm
α-Sm+ SLE
α-Sm- SLE
HD
B
SmD3108-122 - sDMA
SmD3108-122 - Scrambled
C
SmD3108-122
SmD3108-122 - G-sDMA-A
SmD3108-122 - A-sDMA-A
SmD3108-122 - G-sDMA-I
SmD3108-122 - I-sDMA-I
D
SLE serum
Formic acid
Wash
Sm protein ELISA
E
Affinity purification:
SmD3108-122 - sDMA
SmD3108-122 - Arg
IgG concentration (µg/mL)
F
α-Sm protein (OD415nm)


A
****
EBNA1 34-52 - sDMA
EBNA1 34-52 - Arg
OD 415nm
α-Sm+ SLE
α-Sm- SLE
HD
B
****
EBNA2 335-360 - sDMA
EBNA2 335-360 - Arg
C
****
NS3 1486-1494 - sDMA
NS3 1486-1494 - Arg
D
*
VP1 420-439 - sDMA
VP1 420-439 - Arg
E
Preincubation diluted SLE serum with:
SmD3 108-122 - sDMA
SmD3 108-122 - Arg
F
SmD3 108-122 - sDMA
SmD3 108-122 - Arg
**
P = 0.06
% Inhibition viral peptide ELISA
EBNA1
EBNA2
NS3
VP1
G
α-EBNA1 mAb
α-sDMA mAb
EBV-
EBV+
100 kd
70 kd
15 kd
H
α-EBNA1 mAb
SLE α-sDMA IgG


A
R2 = 0,8269
P = 0,0001
α-SmD3108-122 (OD415nm)
α-Sm protein (OD415nm)
B
Preincubation diluted SLE serum with:
SmD3108-122 - sDMA
SmD3108-122 - Arg
SmD3108-122 ELISA
C
α-SmD3108-122 (OD415nm)
Conc. blocking peptide (μM)
D
α-SmD3108-122 (OD415nm)
Conc. blocking peptide (μM)
E
***
% Inhibition
F
Preincubation diluted SLE serum with:
SmD3108-122 - sDMA
SmD3108-122 - Arg
Sm protein ELISA
G
α-Sm protein (OD415nm)
Conc. blocking peptide (μM)
H
α-Sm protein (OD415nm)
Conc. blocking peptide (μM)
I
***
% Inhibition
J
Viral peptide - sDMA
Viral peptide - Arg
% Inhibition Sm protein ELISA
EBNA1
EBNA2
NS3
VP1
K
α-PPPGMRPP (OD415nm)
α-Sm+ SLE
α-Sm- SLE
HD
L
SmD3108-122 - sDMA
PPPGMRPP
***
% Inhibition Sm protein ELISA