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► **To cite this version:**

Johannes Hartl, Lee Serpas, Yueyang Wang, Ali Rashidfarrokhi, Oriana Perez, et al.. Autoantibody-mediated impairment of DNASE1L3 activity in sporadic systemic lupus erythematosus. *Journal of Experimental Medicine*, 2021, 218 (5), <10.1084/jem.20201138>. <hal-03439379>

HAL Id: hal-03439379

<https://hal.science/hal-03439379v1>

Submitted on 26 Nov 2021

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Autoantibody-mediated impairment of DNASE1L3 activity in sporadic systemic lupus erythematosus

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Abstract

Antibodies to double stranded DNA are prevalent in systemic lupus erythematosus (SLE), particularly in patients with lupus nephritis, yet the nature and regulation of antigenic cell-free DNA (cfDNA) are poorly understood. Null mutations in the secreted DNase DNASE1L3 cause human monogenic SLE with anti-dsDNA autoreactivity. We report that >50% of sporadic SLE patients with nephritis manifested reduced DNASE1L3 activity in circulation, which was associated with neutralizing autoantibodies to DNASE1L3. These patients had normal total plasma cfDNA levels but showed accumulation of cfDNA in circulating microparticles. Microparticle-associated cfDNA contained a higher fraction of longer polynucleosomal cfDNA fragments, which bound autoantibodies with higher affinity than mononucleosomal fragments. Autoantibodies to DNASE1L3-sensitive antigens on microparticles were prevalent in SLE nephritis patients and correlated with the accumulation of cfDNA in microparticles and with disease severity. DNASE1L3-sensitive antigens included DNA-associated proteins such as HMGB1. Our results reveal autoantibody-mediated impairment of DNASE1L3 activity as a common non-genetic mechanism facilitating anti-dsDNA autoreactivity in patients with severe sporadic SLE.

Introduction

The serological hallmark of systemic lupus erythematosus (SLE) is the production of antibodies (Abs) to nuclear antigens, with immune complex formation leading to systemic immune activation and tissue inflammation. Anti-dsDNA IgG Abs can be found in up to 70-80% of SLE patients, of which ~40% have high titers rarely seen in other autoimmune disorders (Rekvig, 2015; Tan et al., 1966). High titers of anti-dsDNA IgG correlate with disease severity (Banchereau et al., 2016) and often accompany disease flares, becoming especially evident when involving the kidney (Pisetsky, 2016; Yung and Chan, 2015). Despite the clinical relevance of anti-dsDNA Abs and numerous studies supporting the pathogenicity of this reactivity, the nature of antigenic DNA and the mechanisms that prevent its recognition by autoreactive B cells still remain largely elusive.

The development of anti-dsDNA Abs involves T cell help, making it unlikely that the "naked" protein-free dsDNA is the initiator of the response. In fact, Abs to chromatin often precede the onset of anti-dsDNA Abs, implicating protein-bound DNA in the initial breakdown of tolerance, followed by the spreading of autoreactivity towards "naked" dsDNA (Munroe et al., 2016). In turn, the antigenic chromatin may exist as small soluble fragments or comprise larger extracellular structures such as neutrophil extracellular traps (NETs) (Garcia-Romo et al., 2011; Hakkim et al., 2010; Lood et al., 2016) and/or microparticles (MP). Indeed, 1-2 μm -sized MP that are derived from the cell membranes of apoptotic cells are found in the plasma of healthy subjects and SLE patients (Dieker et al., 2016; Nielsen et al., 2012). Moreover, MP can carry apoptotic cell DNA and expose it on their surface (Casciola-Rosen et al., 1994; Radic et al., 2004; Ullal et al., 2011). This would effectively create an "ideal" source of antigenic DNA since it is accessible to autoreactive B cells, relatively resistant to degradation, and is associated with proteins that can provide potential T cell epitopes (Beyer and Pisetsky, 2010). In addition to the physical form of DNA, the length of DNA is likely to influence its antigenicity. Anti-dsDNA Abs were proposed to bind longer fragments of naked dsDNA with higher avidity (Ali et al., 1985; Papaliam et al., 1980), although this was shown to depend on assay conditions (Pisetsky and Reich, 1994). Overall, the precise physical form of cfDNA that serves as an antigen in anti-dsDNA responses, and its relationship with DNA size, are not well understood.

The availability of self-DNA to autoreactive B cells can be limited by extracellular deoxyribonucleases (DNases) that digest it (Elkon, 2018; Soni and Reizis, 2018). DNASE1L3 and DNASE1 are homologous extracellular DNases that are responsible for the majority of DNase activity in the circulation (Napirei et al., 2009). DNASE1L3 contains a unique positively charged C-terminal peptide that facilitates digestion of membrane- and/or protein-associated DNA including intact chromatin (Napirei et al., 2005; Sisirak et al., 2016; Wilber et al., 2002). Homozygous null mutations in *DNASE1L3* cause pediatric-onset familial SLE characterized by prominent anti-dsDNA Abs and renal involvement (Al-Mayouf et al., 2011; Batu et al., 2018; Belot and al., 2020; Carbonella et al., 2017; Ozcakar et al., 2013). In some of these cases, the disease initially manifested as hypocomplementemic urticarial vasculitis syndrome (HUVS) (Jara et al., 2009) but almost invariably progressed to severe SLE with glomerulonephritis (Carbonella et al., 2017; Ozcakar et al., 2013). A coding polymorphism of *DNASE1L3* that creates a hypofunctional protein DNASE1L3 (R206C) (Ueki et al., 2009) is associated with several seropositive autoimmune diseases including SLE (Acosta-Herrera et al., 2019). Furthermore, DNASE1L3-deficient knockout (KO) mice rapidly develop anti-chromatin and anti-dsDNA Abs followed by SLE-like disease with renal involvement (Sisirak et al., 2016; Soni et al., 2020; Weisenburger et al., 2018). Thus, DNASE1L3 represents a conserved extracellular DNase that maintains tolerance to self-DNA in a non-redundant manner. DNASE1L3 was shown to digest genomic self-DNA in apoptotic MP (Sisirak et al., 2016), as well as reduce the level of polynucleosomal DNA fragments in the circulation (Chan et al., 2020; Serpas et al., 2019). However, it is unclear how these two activities are related to one another or relevant for the role of DNASE1L3 in preventing SLE. Moreover, the relevance of DNASE1L3 or its DNA substrates to sporadic SLE beyond the rare genetic DNASE1L3 deficiency has never been established.

This study addresses the relevance of DNASE1L3 and its DNA substrates to sporadic SLE, with an emphasis on lupus nephritis (LN). We show that patients with LN often manifest reduced activity of DNASE1L3 in the circulation, which is associated with auto-Abs to DNASE1L3 itself. Furthermore, reduced DNASE1L3 activity in LN patients is associated with cfDNA accumulation in MP, as well as with autoreactivity towards DNA and DNA-associated proteins on the surface of MP. In aggregate, these results

establish DNASE1L3 as a novel self-antigen in patients with spontaneous SLE with renal involvement, and the resulting deficiency of functional DNASE1L3 as a likely cause of cfDNA abnormalities which in turn facilitate anti-DNA responses.

RESULTS

The activity of DNASE1L3 is reduced in SLE patients with renal involvement

Initial experiments were done to establish a reliable assay for DNASE1L3 activity in the circulation. Unlike its homolog DNASE1, DNASE1L3 efficiently digests native chromatin in intact nuclei (Napirei et al., 2005). Using purified recombinant DNASE1L3, we confirmed its superior ability, relative to DNASE1, to digest DNA in intact nuclei isolated from Jurkat T cells (Fig. 1A). High concentrations of DNASE1 produced a smear pattern of nuclear DNA digest, consistent with inefficient random cleavage (Fig. 1B). In contrast, even low concentrations of DNASE1L3 produced a characteristic nucleosomal ladder, suggesting efficient internucleosomal cleavage of chromatin (Fig. 1B). The distinctive ability of DNASE1L3 to digest DNA in nuclei was used to measure its specific enzymatic activity in human plasma. Whereas control human plasma efficiently digested DNA in nuclei, plasma from DNASE1L3-deficient patients failed to do so in the same dilution range (Fig. 1C). A lower dilution of plasma from DNASE1L3-deficient patients was able to digest nuclei in our assay, likely reflecting the activity of DNASE1 and/or non-specific inhibition of detection, e.g. by plasma IgG (Sidstedt et al., 2018). Plasma from an SLE patient who was heterozygous for the hypomorphic R206C variant of DNASE1L3 also showed a noticeable reduction (Fig. 1D), confirming the specificity and sensitivity of this assay. To measure the relative activity of DNASE1L3 in patient plasma, we set the IC₅₀ value for pooled healthy control plasma (N=15) at 100%, the value for DNASE1L3-deficient patient plasma at 0% and considered any activity below two standard deviations of the control (81%) as reduced.

We then evaluated DNASE1L3 activity in several groups of SLE patients (patient demographics and average values for key readouts are provided in Table 1). Note that “renal SLE” indicates past or present renal disease and “LN” indicates renal SLE with persistent proteinuria (urine protein/creatinine ratio or UPCR>0.5) accompanied by positive serologies (hypocomplementemia and/or elevated anti-dsDNA Abs) at the time of sampling. Average DNASE1L3 activity was not significantly decreased in 14 SLE

patients without current or previous renal involvement compared to healthy controls (median activity 92%, Fig. 1E). In contrast, 57% of patients with a history of renal disease (N=37) showed a decrease in DNASE1L3 activity (median activity 69%) with a significant reduction compared to both healthy controls and SLE patients who never had renal disease (Fig. 1E). Within the renal SLE group, DNASE1L3 activity was lower in those with active proteinuria than in those with resolved proteinuria (i.e. inactive renal disease) defined as UPCR<0.5 at the time of sampling (Fig. 1F). Notably, plasma activity of DNASE1L3 did not correlate with the degree of proteinuria (Fig. S1), arguing against protein loss through the kidney as a cause of reduced DNASE1L3 activity. To further evaluate the clinical significance of reduced DNASE1L3 activity, we evaluated 15 mothers of children with heart block and high anti-Ro/SSA Abs, most of whom were clinically asymptomatic or had only minimal symptoms of SLE but did not meet formal classification criteria (Table 1). Only three showed a reduction in DNASE1L3 activity, two of whom were the only patients who progressed to SLE with renal disease (Fig. 1E, arrows). Collectively, these data reveal a significant reduction of circulating DNASE1L3 activity in sporadic SLE patients with renal disease.

Reduced DNASE1L3 activity is associated with antibodies to DNASE1L3

The known hypomorphic allelic variant of *DNASE1L3*, DNASE1L3 (R206C), is predominantly found in subjects of European ancestry, and indeed, only two patients in our SLE cohort carried this variant. However, reduced DNASE1L3 activity was observed in >50% of our renal SLE patients across all ethnic groups, prompting a search for a non-genetic cause. To test whether auto-Abs to DNASE1L3 might contribute to its decreased activity, we examined patients' plasma by ELISA using purified DNASE1L3 as the antigen (Ag). No reactivity to DNASE1L3 was observed in plasma samples from healthy controls (Fig. 2A-C) or from DNASE1L3-deficient patients (data not shown). In contrast, 17 out of 40 (43%) evaluated patients with renal SLE showed a mean anti-DNASE1L3 antibody level greater than the threshold O.D. value of 0.44 (mean OD+2 S.D. of healthy controls) (Fig. 2A). Only 3 out of 17 (18%) plasma samples from non-renal SLE patients and one anti-Ro+ mother with renal disease were positive in this assay (Fig. 2A, B). The same plasma samples that showed strong reactivity to DNASE1L3 (Fig. 2C) had minimal binding to the FLAG epitope present in this protein, or

to immobilized DNASE1 (Fig. S2A). To rule out any differences in immobilization between DNASE1 and DNASE1L3, we incubated plasma from SLE patients with high anti-DNASE1L3 titers with a concentration range of DNASE1 or DNASE1L3 and then tested the reactivity to plate-bound DNASE1L3 by ELISA. Representative examples in Figure 2D show that IgG binding was competed out by DNASE1L3 but not by DNASE1. Taken together, these data suggest a specific autoantibody response predominantly directed towards DNASE1L3.

The binding of plasma IgG to recombinant DNASE1L3 was further confirmed by reducing SDS-PAGE followed by Western blot, in which the reactivity was observed only in plasma samples that showed anti-DNASE1L3 binding by ELISA (Fig. S2B). In addition, DNASE1L3 was incorporated into a bead array that allows simultaneous analysis of reactivity to multiple antigens. Anti-DNASE1L3 Ab levels measured by bead array strongly correlated with those determined by ELISA ($r=0.80$, $p<0.0001$, $N=25$, Fig. S2C). C1q and dsDNA, antibodies to which are closely linked to LN (Yung and Chan, 2015), were also included in the bead array to assess autoreactivity to these antigens. However, neither anti-C1q nor anti-dsDNA Ab levels showed a significant correlation with anti-DNASE1L3 reactivity (Fig. S2D, E). Thus, Ab reactivity to DNASE1L3 in sporadic SLE could be demonstrated by three different methods and appears to be distinct from other autoreactivities.

Importantly, the levels of anti-DNASE1L3 Abs strongly correlated with reduced plasma activity of DNASE1L3 (Fig. 2E). Moreover, positive anti-DNASE1L3 binding in ELISA reliably identified patients with DNASE1L3 activity below the threshold (Fig. 2E horizontal black dashed line) (sensitivity: 71%, specificity: 89%, PPV: 83%, NPV: 81%). To verify that Abs to DNASE1L3 can inhibit its activity, we purified IgG from the plasma of three LN patients with positive anti-DNASE1L3 binding by ELISA (similar to LN1 and LN3 in Fig. 2C). Pre-incubation of DNASE1L3 with IgG from these patients, but not with IgG from 3 healthy controls, decreased enzymatic activity by an average of 30% in our assay (Fig. 2F and Fig. 2G, left panel). Similar inhibitory effects were also observed when nuclear DNA and “naked” genomic DNA were used as DNA substrates (Fig. 2F), suggesting that the Abs primarily target the core catalytic activity of DNASE1L3. In contrast, no inhibition of DNASE1 activity was observed with the same IgG concentration (Fig. S2F and 2G), and only minor inhibition was achieved at a 10-fold

higher concentration (Fig. S2F). Overall, these data suggest that the reduced DNASE1L3 activity in sporadic human SLE is caused primarily by specific Abs that develop against this protein.

DNASE1L3 controls the amount of DNA in microparticles

We next sought to determine the impact of reduced DNASE1L3 activity on cfDNA in sporadic SLE patients. Given that DNA in circulating MP was proposed as a physiological substrate of DNASE1L3 (Sisirak et al., 2016), we sought to characterize MP-bound and soluble cfDNA in normal human plasma. Plasma from healthy donors was subjected to several centrifugation steps to isolate a MP fraction free of cell debris and platelets. Resulting MP were then stained for plasma membrane and for DNA, using a membrane-permeable dye, and examined by confocal microscopy. Figure 3A shows representative examples of 1-2 μm -sized particles composed of plasma membrane and containing dsDNA, suggesting that our centrifugation method indeed isolates human MP with dsDNA. Similarly, a fraction of AnnexinV⁺ MP isolated from human plasma stained positive for DNA by flow cytometry (data not shown).

The fraction of MP-associated cfDNA was estimated by purifying DNA from MP and MP-depleted fractions of 15 healthy controls and quantifying genomic DNA by qPCR for *Alu* repeats in the genome. The concentration of MP-associated cfDNA ranged from 0.4-3.0 ng/ml plasma (Fig. 3B), corresponding to 17-48% (median 23%) of total cfDNA in the circulation (Fig. 3D). The three control samples with DNASE1L3 activity on or slightly below the threshold (Fig. 1E) showed the fraction of MP-associated DNA (28-37%) that was slightly above the median. In contrast, the distribution of cfDNA was strongly skewed towards the MP fraction in three out of four DNASE1L3-deficient patients (Fig. 3C), representing 31-86% (median 69%) of total cfDNA (Fig. 3D). Of note, three of these four DNASE1L3-deficient patients were children diagnosed with HUVS prior to progression to SLE, suggesting that the observed cfDNA redistribution is not a consequence of severe SLE and/or anti-dsDNA reactivity. In our cohort of sporadic SLE patients, only those with renal SLE showed a significant increase in the MP cfDNA fraction (renal median 62%, non-renal median 33%; Fig. 3D), mirroring the changes observed with genetic DNASE1L3 deficiency. In contrast to the observed increase in MP-associated cfDNA, the total concentration of cfDNA was not significantly increased in

any group, highlighting the specific effect of DNASE1L3 on cfDNA accumulation in MP (Fig. 3E). The concentration of soluble cfDNA was slightly decreased in renal SLE compared to controls (2.2 vs 5.0 ng/ml), but the difference was not significant (p=0.08). Importantly, the fraction of MP-associated cfDNA showed a significant inverse correlation with DNASE1L3 activity (Fig 3F); thus, patients with reduced DNASE1L3 activity harbored on average 67% of cfDNA in the MP fraction. The fraction of MP cfDNA (Fig. S3A) showed a significant correlation with anti-dsDNA Ab titers (Fig. S3); nevertheless, multiple patients with a high fraction of MP-associated cfDNA had low or absent anti-dsDNA Ab titers, ruling out the coincidental isolation of DNA-containing immune complexes with MP. In summary, while a substantial fraction of total cfDNA in healthy humans is associated with MP, the representation of this fraction is regulated by DNASE1L3 and is significantly increased in sporadic SLE patients with reduced plasma DNASE1L3 activity.

The mouse model of DNASE1L3 deficiency was utilized to directly test the role of DNASE1L3 in the regulation of MP-associated DNA. The dual membrane/DNA staining protocol used for human MP (Fig. 3A) was used to confirm the presence of DNA-containing MP in murine plasma (Fig. 4A). DNA-carrying MP in mice appeared slightly smaller than in humans, possibly reflecting differences between species and/or between isolation protocols. To visualize DNA-containing MP in the absence of chemical dyes, we used dual reporter mice expressing a histone 2B-green fluorescent protein (H2B-GFP) fusion and membrane-targeted red fluorescent protein tdTomato. All leukocytes in these mice were double-positive for GFP and tdTomato by flow cytometry (data not shown), and their plasma MP fraction contained multiple tdTomato⁺ MP carrying GFP (Fig. 4B). Next, cfDNA was purified and measured in MP and MP-depleted soluble fractions from the plasma of wild-type (WT) and *Dnase1l3*-deficient mice. To avoid potential secondary effects of anti-DNA Abs, only young male *Dnase1l3*^{-/-} mice without anti-dsDNA reactivity were used. Unlike our findings in humans, the majority of total cfDNA in wild-type mice was contained within the MP fraction (Fig. 4C). Importantly, *Dnase1l3*^{-/-} mice showed a significantly increased DNA concentration in the MP and soluble fractions compared to age- and sex-matched wild-type mice (Fig. 4D). Conversely, *Dnase1l3*^{-/-} mice harbored a normal amount of cfDNA in MP but significantly more cfDNA in the soluble fraction compared to age- and sex-matched wild-type

controls (Fig. 4E). Thus, the loss of DNASE1L3, but not of its homolog DNASE1, causes the accumulation of cfDNA in the MP fraction, implicating reduced DNASE1L3 activity in SLE patients as a likely cause of increased DNA load in MP.

Microparticles carry longer DNA fragments that are more immunogenic

Given that a substantial fraction of human cfDNA is contained within the MP fraction, we tested whether this MP-associated DNA is qualitatively different from that in the soluble fraction. First, we estimated the length of cfDNA fragments in both fractions using the ratio of long to short amplicons recovered by qPCR (Jiang and Lo, 2016). This approach detected an increase in DNA length in the MP fraction compared to the soluble fraction of plasma from healthy controls (Fig. 5A). Consistent with findings in the total cfDNA of DNASE1L3-deficient human patients (Chan et al., 2020), both MP-associated and soluble cfDNA from *DNASE1L3*-deficient patients was longer than in healthy controls (Fig. 5A). To directly test the size distribution of MP-associated cfDNA in healthy controls, we sequenced it and computed the length of resulting fragments aligned to the genome (Jiang and Lo, 2016). No differences in genome coverage or in the end motif frequency were observed between MP-associated and soluble cfDNA (data not shown). However, the predominant DNA peak of mononucleosomal size (~180 bp) was overrepresented in the soluble fraction, whereas larger DNA fragments in the di- to trinucleosomal range (250-600 bp) were significantly overrepresented in the MP fraction (Fig. 5B, C). Thus, MP-associated cfDNA is enriched in longer polynucleosomal fragments compared to soluble cfDNA, and MP from DNASE1L3-deficient patients show lengthening of their cfDNA cargo compared to healthy subjects.

Given the observed enrichment of longer polynucleosomal fragments in MP and the control of MP-associated DNA by DNASE1L3, we tested whether longer DNA fragments might represent more robust antigens. We incubated plasma from SLE patients with high anti-dsDNA titers with mononucleosomes or polynucleosomes, and then tested the binding of plasma IgG to plate-bound DNA by ELISA. A representative example in Figure 5D shows that IgG binding to DNA was competed out by polynucleosomes but not by mononucleosomes. This was observed using plasma samples from 5 patients (Fig. 5E) and with purified IgG fractions from two of these patients (data not shown). In contrast, protein-free DNA fragments (95-831 bp)

efficiently competed out IgG binding to DNA irrespective of their size (Fig. S4A and 5F). Moreover, IgG binding to the same fragments immobilized on a plate was slightly better for smaller fragments, likely because of differences in binding to the plate (Pisetsky and Reich, 1994). Thus, the impact of DNA length on auto-Ab binding is only apparent in solution and when the DNA is in its physiological chromatin state. In addition to serving as a self-antigen, cfDNA may induce innate immune responses such as the production of type I interferon (IFN-I) by plasmacytoid dendritic cells (pDCs), especially when in complexes with neutrophil-derived cationic peptides such as LL-37 (Garcia-Romo et al., 2011; Lande et al., 2011). We therefore incubated different concentrations of polynucleosomes or mononucleosomes with LL-37 and tested the ability of resulting complexes to activate pDCs from healthy donors. We found that polynucleosomes elicited IFN-I production at lower concentration than mononucleosomes (Fig. 5G). Thus, longer chromatin fragments, such as those enriched in MP, show better auto-Ab binding as well as better ability to elicit innate responses.

DNASE1L3-sensitive MP antigens are targeted in SLE with renal involvement

In view of reduced DNASE1L3 activity and the corresponding DNA accumulation in the MP of patients with renal SLE, we tested the functional consequences of these findings for autoreactivity in these patients. We have previously reported that DNASE1L3-deficient mice and ~30% of human sporadic SLE patients harbored IgG binding to the surface of *in vitro*-generated MP. The binding could be abolished by pre-treatment of MP with DNASE1L3 but not with DNASE1, suggesting Ab reactivity against DNASE1L3-sensitive antigens on the surface of MP (Sisirak et al., 2016). We confirmed these observations (Fig. 6A) and standardized the assay for an unbiased determination of DNASE1L3-sensitive binding of plasma IgG to MP antigens (referred to hereafter as “DNASE1L3-sensitive binding”). MP are known to be heterogeneous (Dalli et al., 2013; Nielsen et al., 2011), thus the gating of MP was deliberately broad to include all fractions below 1 μ m in size; indeed, only a fraction of MP showed positive staining with IgG (Fig. 6A-D). Among four patients with genetic DNASE1L3 deficiency, three manifested DNASE1L3-sensitive binding (Fig. 6B). One DNASE1L3-deficient patient for whom longitudinal samples were available developed DNASE1L3-sensitive binding shortly before a severe flare of LN that required temporary dialysis. This was also

accompanied by a marked increase of cfDNA in the MP fraction but not in soluble cfDNA (Fig. 6C).

To test the prevalence of reactivity to DNASE1L3-sensitive antigens on MP in sporadic SLE, we analyzed a well-characterized cohort of SLE patients (N=120), many of whom were used in the initial description of Ab binding to DNASE1L3 described above. Of these patients, 87 (73%) had renal SLE and 62 (52%) had undergone longitudinal sampling. In total, 221 plasma samples were analyzed, revealing that 61 patients (51%) harbored DNASE1L3-sensitive binding during at least one sample collection. The clinical characteristics of sporadic SLE patients organized by the presence of DNASE1L3-sensitive binding are summarized in Supplemental Table 1. As expected, no DNASE1L3-sensitive binding was observed in any of the healthy controls analyzed (N=15). Binding of plasma IgG to MP antigens could be prevented or reversed by the treatment of MP with DNASE1L3 before or after the incubation with patients' plasma, respectively (Fig. 6D). Importantly, patients with DNASE1L3-sensitive binding showed a significant decrease of DNASE1L3 activity in the plasma (Fig. 6E) and a near-uniform increase in cfDNA in the MP fraction (Fig. 6F). Furthermore, DNASE1L3-sensitive binding was strongly associated with renal involvement: 55 of 87 (63%) renal patients compared to 5 of 33 (15%) non-renal SLE patients ($p < 0.0001$) (Fig. 6G). Moreover, patients with DNASE1L3-sensitive binding showed a significant enrichment in proteinuria, low complement (i.e. C3 and C4) levels, positive anti-dsDNA Abs (Fig. 6H), and showed a higher median SLE disease activity index (SLEDAI) at the time of blood sampling (Fig. 6I). In anti-Ro+ mothers, while 8 of 45 (18%) demonstrated IgG binding to MP antigens, this binding was sensitive to DNASE1L3 in only 3 patients (Fig. 6G). Two of these were the only subjects with renal SLE, with one patient harboring Abs to DNASE1L3 (Fig. 2A and Table1).

To address whether DNASE1L3-sensitive binding tracks with clinical response in patients with active LN, samples were obtained from 30 patients at the time of renal biopsy and longitudinally over a 52-week period. Clinical response was defined at 52 weeks as complete (N=8, UPCR<0.5), partial (N=3, UPCR reduced by 50% from baseline but >0.5) or none (N=19). Detailed information on patient characteristics organized by treatment response is given in Supplemental Table 2 and characteristics for each individual patient included in this analysis are shown in Supplemental Table 3.

At baseline, there was no significant difference in the prevalence of DNASE1L3-sensitive binding between combined partial and complete responders (7/11, 63%) and non-responders (13/19, 68%). However, at 52 weeks, only 2/11 (18%) combined treatment responders still manifested DNASE1L3-sensitive binding, while this reactivity remained persistent in all non-responders (13/19, 68%; Fig. 6J). Additionally, at baseline there was no difference in the fraction of cfDNA located in MP, but combined treatment responders demonstrated a shift of cfDNA towards the MP-depleted plasma fraction, while the distribution of cfDNA remained unchanged in non-responders (Fig. 6K). Collectively, these data suggest that Abs to DNASE1L3-sensitive antigens on MP inversely correlate with clinical response to treatment in patients with active LN.

Reactivity towards DNASE1L3-sensitive antigens includes DNA-associated proteins

It was noted in evaluating the reactivity to DNASE1L3-sensitive MP antigens that this did not fully mirror anti-DNA Abs. Specifically, anti-dsDNA IgG were present in >80% of patients with this reactivity, but also in >50% of patients without it (Fig. 6H). Moreover, only one of the three DNASE1L3-deficient patients with DNASE1L3-sensitive binding (Fig. 6B) had anti-dsDNA Abs as assessed by ELISA (data not shown). To further explore the relationship between Abs to dsDNA and MP, we examined a panel of 9G4⁺ monoclonal Abs (mAbs) derived from SLE patients. Abs marked by the 9G4 idiotope are prominent among SLE-associated auto-Abs and bind multiple antigens including DNA and apoptotic cell surface determinants (Pugh-Bernard et al., 2001; Richardson et al., 2013; Tipton et al., 2015). Out of 20 examined 9G4⁺ mAbs, 3 showed DNASE1L3-sensitive binding (Fig. S5A). Two of those also bound dsDNA and chromatin as expected; however, one (74C2) did not. Conversely, other clones (e.g. 88A1) reacted with dsDNA and chromatin by ELISA but did not bind MP (Fig. S5A). Collectively, these data suggest that reactivity to DNASE1L3-sensitive MP antigens partially overlaps with, but is distinct from, anti-dsDNA Abs and likely includes additional antigenic targets associated with DNA.

To further characterize reactivity to MP antigens, we explored the recognition of MP proteins by IgG from SLE patients using lysates of MP harvested from apoptotic Jurkat T cells. SLE plasma samples that had shown DNASE1L3-sensitive IgG binding

to MP by flow cytometry also demonstrated reactivity to multiple MP proteins by Western blot. Most prominently, reactivity to proteins of ~10-15, 29, 35, 45, and 75 kDa could be observed across multiple plasma samples (Fig. 7A and B). We then took a candidate approach and explored the reactivity to histones and high-mobility group B1 protein (HMGB1). HMG proteins are structural transcription factors that can be incorporated into chromatin or released from activated or dying cells; HMGB1 in particular is an important mediator of inflammation and a common target of auto-Abs in SLE (Pisetsky, 2014a; Urbonaviciute et al., 2008; Wirestam et al., 2015). Both histones H2A/H2B and HMGB1 could be identified by Western blot in lysates of Jurkat cell MP, and bands of similar size (~29 kDa for HMGB1, ~13-15 kDa for histones) were detected by SLE plasma samples with DNASE1L3-sensitive reactivity to MP antigens (Fig. 7B). We next analyzed plasma IgG from *Dnase1/3*-deficient mice and observed IgG binding to proteins of similar size as those seen with human SLE plasma (Fig. 7C). This reactivity was age-dependent (Fig. 7D), consistent with progressive autoreactivity and epitope spreading in older *Dnase1/3*-deficient mice (Sisirak et al., 2016).

Although the Western blot analysis confirmed the reactivity of patients' auto-Abs with multiple protein Ag in MP, it did not establish whether this reactivity is DNASE1L3-sensitive. To this end, we used flow cytometry to show that HMGB1 was exposed on the surface of Jurkat cell-derived MP, and that this exposure was sensitive to DNASE1L3 but not DNASE1 (Fig. 7E). Staining for H2A and H2B did not yield a distinct positive MP population (Fig. S5B), likely due to the blockade of histone epitopes by the associated nucleosomal DNA on the surface of MP. Taken together, these data demonstrate that reactivity to DNASE1L3-sensitive antigens on MP targets not only DNA but also multiple proteins, potentially including known SLE-associated self-antigens such as HMGB1 and Histones.

Discussion

In this study we explored the role of DNase activity in SLE, focusing specifically on DNASE1L3, the extracellular DNase implicated in monogenic SLE. It was reported that total DNase activity may be reduced in SLE patients (Martinez-Valle et al., 2009; Sallai et al., 2005), but no correlation with disease severity was observed and the specific DNase was not identified. More recently, a decrease in total DNASE activity

was observed specifically in nephritis patients (Bruschi et al., 2020), although the protein levels of both DNASE1 and DNASE1L3 appeared normal. Using a novel functional assay that is specific for DNASE1L3, we found that more than half of patients with renal involvement show reduced activity of DNASE1L3 in the circulation. Given that DNASE1L3 non-redundantly protects from SLE and that a partial reduction of its activity in DNASE1L3 (R206C) carriers is associated with autoimmunity, its specific reduction in severe sporadic SLE likely contributes to the pathogenesis.

Reduced DNASE1L3 activity strongly correlated with the appearance of activity-neutralizing Abs to DNASE1L3, suggesting that DNASE1L3 is blocked by this newly described auto-Ab. Indeed, the measurement of anti-DNASE1L3 Abs by ELISA provides a robust high-throughput assay to estimate DNASE1L3 activity in sporadic SLE patients in future studies. This mechanism does not rule out other possible scenarios such as reduced DNASE1L3 protein levels, the possibility that could not be explored here due to the lack of sufficiently specific protein detection reagents. Ab-mediated blockade is consistent with the results of (Bruschi et al., 2020), who implicated inhibitory circulating proteins in the reduction of total DNase activity in renal SLE. Notably, auto-Abs preferentially bound DNASE1L3 compared to DNASE1, and thus appear distinct from previously described DNASE1 inhibitory substances (Hakkim et al., 2010) or polyreactive anti-DNASE1 Abs that also bind DNA (Puccetti et al., 1995; Yeh et al., 2003). The observed Ab-mediated inhibition of DNASE1L3 resembles a similar inhibition of the complement component C1Q. Like DNASE1L3, C1Q is required for self-tolerance, as null C1Q mutations in humans cause SLE with a near-complete penetrance (Leffler et al., 2014). In addition, C1Q can itself be targeted by a specific auto-Ab which reduces C1Q levels in HUVS (Jara et al., 2009) and is also found in sporadic SLE (Orbai et al., 2015). The inhibition of DNASE1L3 by specific auto-Abs appears to recapitulate its genetic deficiency by non-genetic means, emphasizing the critical role of DNASE1L3 in tolerance and its relevance for sporadic SLE.

Self-DNA that may activate innate or adaptive immunity in SLE may comprise inflammation-associated forms such as NETs and oxidized mitochondrial DNA (Caielli et al., 2016; Garcia-Romo et al., 2011; Lood et al., 2016), or cfDNA that exists in the steady state. Given the observed effects of DNASE1L3 on the latter (Serpas et al., 2019), we explored its physical nature in circulation, including the association with MP.

We confirmed the presence of DNA-carrying MP in normal plasma and established that ~25% and >90% of total cfDNA is MP-associated in humans and mice, respectively. The reasons for this difference between the species are unknown but may involve the shorter time and route of blood circulation in mice, reducing the lifespan of soluble cfDNA. Importantly, MP-associated cfDNA was enriched in longer polynucleosomal DNA fragments, consistent with the detection of such fragments in MP generated *in vitro* (Reich and Pisetsky, 2009). Fragments of naked DNA within the 100-4,000 bp size range were shown to bind anti-DNA Abs equally well in solution (Pisetsky and Reich, 1994). We confirmed these observations but showed that for nucleosome-bound DNA, longer polynucleosomal fragments bind auto-Abs with higher affinity. We also demonstrated the superior capacity of polynucleosomal fragments as inducers of type I interferon production. These data are consistent with the critical role of type I interferon in SLE (Elkon and Wiedeman, 2012) and with its contribution to autoreactivity and disease in DNASE1L3-deficient mice (Soni et al., 2020). Overall, our results highlight the abundance of MP-associated cfDNA and its potential as a self-Ag that requires tight control *in vivo*.

The MP-associated fraction of cfDNA was increased in DNASE1L3-deficient mice and humans, and correlated with reduced DNASE1L3 activity in sporadic SLE patients with nephritis. This was in striking contrast to comparable levels of total plasma cfDNA, which did not show significant differences between patient groups in our study. Although increased total cfDNA has been reported in SLE patients compared to controls (Duvvuri and Lood, 2019), this difference showed no clear association with disease activity or with LN specifically (Atamaniuk et al., 2011; Xu et al., 2018). Sequencing of total cfDNA in SLE revealed certain abnormalities such as aberrant genome representation and shortening due to the presence of anti-dsDNA Abs, but no consistent features serving as biomarkers of the disease (Chan et al., 2014). Our results highlight the relevance of MP-associated cfDNA for SLE pathogenesis, given its striking increase in lupus nephritis relative to healthy and non-LN patients. Moreover, they confirm DNASE1L3 as an essential and specific regulator of MP-associated cfDNA, which therefore represents the physiological substrate of this enzyme. They also help explain the lengthening of total cfDNA in the plasma of *Dnase1l3*-deficient mice (Serpas et al., 2019) and humans (Chan et al., 2020), which apparently reflects the

accumulation of MP-associated polynucleosomal fragments. Thus, auto-Ab-mediated impairment of DNASE1L3 activity in renal SLE patients results in the accumulation of a self-antigen, i.e. MP-associated cfDNA and affiliated proteins.

Auto-Ab from SLE patients were shown to bind endogenous MP (Nielsen et al., 2012) or *in vitro*-generated MP (Ullal et al., 2011), yet the nature of bound Ag and clinical significance of this reactivity remained unclear. We report that Abs to MP are prevalent in patients with SLE and target DNASE1L3-sensitive Ag on the MP surface. We also show that this antibody reactivity is associated with reduced DNASE1L3 activity and cfDNA accumulation into MP, likely representing a consequence of these events. Unlike ELISA-based assays for Abs to DNA and other Ags, the binding to DNASE1L3-sensitive Ag on MP is a physiological interaction that is unbiased with respect to a specific Ag. As such, it represents an integral readout that correlates but does not directly overlap with any single reactivity including the canonical anti-dsDNA ELISA. This likely explains why reduced DNASE1L3 activity and/or DNASE1L3-targeting Abs did not correlate with anti-dsDNA (Fig. S2D and data not shown) but were associated with reactivity to MP (Fig. 6E). Indeed, we demonstrate that the reactivity to MP involves a set of protein Ag carried by MP, with reactivity patterns shared among different patients and even with *Dnase1l3*-deficient mice. One established DNASE1L3-sensitive Ag is the nucleosome (Sisirak et al., 2016), consistent with its exposure on MP surface (Ullal et al., 2011) and the presence of histones in MP (this study); however, pure histones may not be targeted independently of the DNA. Another candidate DNASE1L3-sensitive Ag is HMGB1, a pleiotropic DNA-binding protein that is a common target of auto-Abs in SLE (Harris et al., 2012; Urbonaviciute et al., 2007). The exposure of HMGB1 on apoptotic MP has been described (Pisetsky, 2014b), and here we demonstrate that this phenomenon is controlled by DNASE1L3, presumably by digesting HMGB1-DNA complexes. Thus, by digesting DNA in MP, DNASE1L3 controls the exposure of not only DNA but also multiple DNA-associated proteins, which are targeted by auto-Abs in SLE patients with reduced DNASE1L3 activity.

In conclusion, our data delineate the molecular basis of tolerogenic DNASE1L3 enzymatic activity and describe its impairment in some patients with SLE, most notably those with kidney involvement. Conceptually, our data reveal a novel mechanism of SLE pathogenesis, based on auto-Abs targeting a critical "gatekeeper" of tolerance to

self-DNA. The inhibition of DNASE1L3 activity by such auto-Abs recapitulates the genetic lesion of DNASE1L3-deficient patients by non-genetic means, emphasizing the key role of DNASE1L3 in sporadic SLE. Technically, our work introduces four novel readouts (DNASE1L3 activity, Abs to DNASE1L3, fraction of MP-associated cfDNA, and Abs to DNASE1L3-sensitive Ag) that correlate with each other and with active LN. Of these assays, IgG binding to DNASE1L3-sensitive Ag on MP is a relatively simple, robust, high-throughput assay whose readout showed correlation with LN in both cross-sectional and longitudinal samples. Finally, the reduction of DNASE1L3 activity in SLE patients with nephritis warrants further exploration of DNASE1L3 as a potential therapeutic in this patient group. Our results provide an important caveat to such attempts by describing neutralizing Abs to DNASE1L3; nevertheless, the rationale for the therapeutic use of this secreted enzyme that is fundamentally linked to SLE pathogenesis appears compelling.

Materials and Methods

Human subjects

The study population was composed of four subgroups: *DNASE1L3*-deficient patients (N=4), NYU SLE cohort (N=120), anti-Ro positive mothers of children with neonatal lupus (N=45), and healthy controls (N=15). The NYU SLE cohort was further stratified into SLE patients with (N=87) and without (N=33) renal involvement according to the Systemic Lupus Erythematosus International Cooperating Clinics (SLICC) criteria (Petri et al., 2012).

Sporadic SLE and healthy controls. Blood specimens were obtained from several sources. Patients with SLE and healthy controls greater than 18 years of age were enrolled in the NYU SAMPLE (Specimen and Matched Phenotype Linked Evaluation) Biorepository after signing informed consent approved by the NYU School of Medicine IRB. SLE was assigned using classification criteria as defined by one or more of the following: a) 1997 Revised American College of Rheumatology (ACR) (Hochberg, 1997; Tan et al., 1982), b) Systemic Lupus Erythematosus International Cooperating Clinics (SLICC) (Petri et al., 2012), or c) the most recent ACR/European League Against Rheumatism (EULAR) (Aringer et al., 2019). Plasma for the study of SLE patients was selected randomly to represent the full spectrum of clinical disease. All SLE patients were evaluated for the status of disease activity using a modification of the original SELENA-SLEDAI (Petri et al., 2005) in which proteinuria was scored 4 points only when the urine protein/creatinine ratio (UPCR) increased by more than 0.5 from the previous visit. The modification applied in this study used the SLEDAI 2K definition of proteinuria in which a UPCR of >0.5 is always scored 4 points (Gladman et al., 2002). All other domains of the SELENA-SLEDAI were scored as originally defined. For the longitudinal follow up, a subset of SLE patients undergoing a kidney biopsy to evaluate a UPCR ratio of >0.5 (considered abnormal) was included in the study. A renal response at 52

weeks was considered complete if the UPCR was <0.5 and a partial response if the baseline UPCR was reduced by 50% but did not resolve to <0.5.

Anti-Ro positive mothers of children with neonatal lupus. A second disease cohort evaluated were women enrolled in the Research Registry for Neonatal Lupus (RRNL) (Buyon et al., 1998) after signing informed consent for participation approved by the NYU School of Medicine IRB. All participating mothers in the RRNL included in this study have a child with congenital heart block and/or characteristic rash and have antibodies to at least one component of the SSA/Ro-SSB/La ribonucleoprotein complex including 52kD SSA/Ro, 60kD SSA/Ro or 48B/La as confirmed by ELISA in the laboratory of RMC and JPB (Reed et al., 2012). Many of the women in the RRNL do not have any systemic disease and are only known to have anti-SSA/Ro antibodies because of the work up for their child's illness. Some mothers have an undifferentiated autoimmune disease as they have insufficient criteria for SLE or Sjogren's Syndrome (Shiboski et al., 2017; Vitali et al., 2002) and less than half ever progress to SLE and or SS (Rivera et al., 2009). In this group of RRNL women, three had developed LN.

DNASE1L3-deficient patients. One patient with pediatric HUVS with renal involvement progressing to end stage renal disease was diagnosed and treated at the Istituto Gaslini. Molecular analysis by next generation sequencing was performed in the Laboratory of Neurogenetics and Neuroinflammation at the Imagine-Institute for Genetic Diseases, Paris. The patient was homozygous for the c.289-290delAC *DNASE1L3* mutation, which was reported previously (Batu et al., 2018; Carbonella et al., 2017; Ozcakar et al., 2013) and is predicted to cause premature termination (p.Thr97Ilefs*2). Three patients with pediatric HUVS (ages of onset 9-14 years) were diagnosed and treated at the IRCCS Ospedale Pediatrico Bambino Gesù. All patients had chronic urticarial vasculitis, hypocomplementemia, positive anti-C1Q Ab, negative anti-nuclear Abs and negative anti-dsDNA by laboratory testing; one of the three showed positive anti-dsDNA by in-house ELISA. The patients were subjected to targeted resequencing on the Illumina NextSeq platform for genes associated with interferonopathies. All patients were homozygous for the c.290_291delCA *DNASE1L3* mutation, which was reported recently (Belot and al., 2020) and is predicted to cause the same premature termination as the mutation above. All patients and/or their families signed informed consent for participation approved by the ethics committees at their respective institutions.

Animals

All animal studies were performed according to the investigator's protocol approved by the Institutional Animal Care and Use Committee of New York University School of Medicine. *Dnase1l3*-deficient mice on C57BL/6 (B6) background (*Dnase1l3*^{LacZ/LacZ}) were described previously (Sisirak et al., 2016). Mice carrying a null allele of *Dnase1* (*Dnase1*^{tm1.1(KOMP)Vlcr}) on B6 background were obtained from the Knockout Mouse Project, crossed with wild-type B6 mice and intercrossed to obtain *Dnase1*^{-/-} mice. Mice with doxycycline-inducible ubiquitous expression of H2B-GFP fusion protein (B6;129S4-*Gt(ROSA)26Sor*^{tm1(rtTA^{M2})Jae}*Col1a1*^{tm7(tetO-HIST1H2BJ/GFP)Jae/J}) (Foudi et al., 2009), and mice with constitutive ubiquitous expression of membrane-targeted tdTomato protein (B6.129(Cg)-*Gt(ROSA)26Sor*^{tm4(ACTB-tdTomato,-EGFP)Luo/J}) (Muzumdar et al., 2007), were obtained from Jackson Laboratories (Bar Harbor, ME) and intercrossed. The resulting mice heterozygous for both alleles were given oral doxycycline in water for at least 4-6 weeks to induce the expression of H2B-GFP as described (Foudi et al., 2009). Wild-

type control mice of B6 background were bred in the same animal facility or purchased from Taconic, Inc. and maintained in the same facility.

Mice for cell-free DNA analysis. All mice were age- and sex-matched when comparing groups. Male and female WT mice (57% female and 43% male) between 30 and 450 days of age (median 80 days) were analyzed (Fig. 4C); no difference in the concentration or distribution of cfDNA was observed between mice of different age or sex. *Dnase1l3*-deficient males younger than 60 days were used in order to avoid any confounding effect of SLE-like disease that develops starting at three months (Fig. 4D). One-year old *Dnase1*-deficient females were used in Figure 4E. These mice do not develop any autoreactivity at any age (L.S., unpublished) and thus the age is not expected to impact our findings.

DNASE1L3 protein purification

Preliminary studies showed that the introduction of a 6-a.a. FLAG epitope into a predicted internal loop of the human full-length DNASE1L3 did not affect the activity of DNASE1L3, including its specific ability to digest chromatin. The resulting FLAG-DNASE1L3 ORF was cloned into a lentiviral vector, which was then used to transduce 293T cells followed by batch selection of stable transfectants. FLAG-DNASE1L3-expressing 293T cells were cultured in 150 mm dishes for 7 days, and the supernatants were incubated with magnetic anti-FLAG M2 beads (Sigma-Aldrich) overnight at 4°C. The beads were collected, washed and incubated with 0.25 mg/mL of FLAG peptide, and the eluted protein was quantified by SDS-PAGE with BSA as a standard.

Isolation of microparticles

Human Plasma. To obtain the MP fraction as well as the MP-depleted (soluble) fraction of human plasma, blood was collected in tubes containing EDTA (Becton Dickinson 366643), and blood cells were removed by centrifugation at 1,400 x g for 12 min at 4°C. A second centrifugation step (1,400 x g for 12 min) was used to remove platelets. The resultant plasma (either fresh or stored at -80°C) was centrifuged at 22,000 x g for 60 min to pellet the MP. The supernatant and MP pellet were used to measure cfDNA in the MP depleted (soluble) and MP plasma fraction respectively.

Murine plasma. Mice were anaesthetized by i.p. injection of 100 mg/kg Ketamine/10 mg/kg Xylazine in sterile PBS. Plasma was isolated by cardiac puncture using a 26G syringe and transferred into EDTA-coated tubes (Kent Scientific, 41.1395.105). Blood was centrifuged at 1,000 x g for 10 min and the supernatant was kept on ice. For DNA quantification, the plasma was spun a second time at 1,000 x g for 10 min. For confocal imaging studies, the plasma was spun a second time at 10,000 x g for 10 min to remove platelets. After the second spin the supernatant was centrifuged at 21,130 x g for 60 min at 4°C to pellet microparticles. The supernatant and MP pellet were used to measure cfDNA in the MP depleted (soluble) and MP plasma fraction respectively.

Quantification of cell-free DNA

To extract cfDNA from the MP fraction and MP-depleted fraction, samples were centrifuged as described above. Circulating cfDNA was extracted from 50-400 µl of human plasma or 150-200 µl of murine plasma using the QIAamp DNA Blood Mini Kit (QIAGEN). The amount of DNA in the respective samples was measured by qPCR using *intra Alu human* primers or *inter-B1 mouse* primers. All qPCRs were carried out in 20 µl using FastStart Universal SYBR Green Master mix (Sigma Aldrich 4913914001).

Briefly, the method for *intra Alu human* primers was as follows: initial denaturation of 12 min at 95°C followed by 45 amplification cycles of denaturation at 95°C for 20 s, annealing at 56 °C for 1 min, and elongation at 72°C for 1min (Walker et al., 2003). The mouse *inter-B1* qPCR methods have been reported previously (Zhang et al., 2010). Briefly, each reaction was subjected to an initial denaturation of 8 min at 95°C followed by 40 amplification cycles of denaturation for 30s at 95°C, annealing for 40s at 55°C, and extension for 1 min at 72°C.

Estimation of cell-free DNA lengths by qPCR. Two sets of primers and the respective qPCR programs described by Lou et al. were used to amplify 76 bp (short) or 200 bp (long) of the human Alu repeat sequences (Lou et al., 2015). The concentration of cfDNA as determined by the 76 bp amplicons and 200 bp amplicons were calculated and subsequently the ratio of long to short amplicon products was determined as a measure of cfDNA length in the MP fraction and MP depleted (soluble) plasma fraction.

Cell-free DNA sequencing in human patients

Plasma from four healthy controls (2 males and 2 females) was isolated and centrifuged to separate MP and MP-depleted fractions as described above. DNA was purified from all samples using the QIAamp DSP DNA Blood Mini Kit (QIAGEN 61104) and quantified by qPCR. DNA libraries were prepared using the KAPA HTP Library Preparation Kit (Roche) and purified using AMPure XP purification beads (Beckman Coulter) according to the manufacturer's instructions. Adaptor-ligated libraries were analyzed on an Agilent 4200 TapeStation (Agilent Technologies) using the High Sensitivity D1000 ScreenTape System (Agilent Technologies) for quality control and quantified by Qubit (Invitrogen). The libraries were sequenced for 150 bp for each end in a paired-end format on a Hiseq 4000 or the equivalent Illumina sp300 flow cell.

DNA Sequencing Analysis. The adaptor sequences were trimmed using cutadapt (v1.18) and all of the reads from the sequencing experiment were mapped to the reference genome (hg19/GRCh37.75) using Bowtie2 (v2.2.4). Duplicate reads were removed using Picard tools (v.1.126) and the low-quality mapped reads (MQ<20) were removed from the analysis. The read per million (RPM) normalized BigWig files were generated using BEDTools (v.2.17.0) and the bedGraphToBigWig tool (v.4). The insert sizes and their standard deviations were calculated using Picard tools and used to generate cfDNA length traces shown. Frequencies were calculated by dividing the number of reads from each insert size by the total number of reads acquired per sample. All downstream statistical analysis was performed in the R environment (v3.1.1).

Assessment of DNASE1L3 activity

Digestion of DNA substrates with DNases. DNA substrates were incubated with different concentrations of human DNASE1 (Abcam 73430) or human recombinant DNASE1L3 in the presence of 2 mM CaCl₂/MnCl₂ and a total volume of 10 µl for 15 min at 37°C. All dilutions of enzymes were prepared in HBSS (without magnesium and calcium) with 100 ng/ml molecular grade BSA. Following digestion, the reactions were immediately kept on ice. The amount of remaining DNA was measured by qPCR using *Intra Alu human* primers as follows: initial denaturation of 10 min at 95°C followed by 45 amplification cycles of denaturation at 95°C for 15 s, annealing at 60 °C for 15 s, and elongation at 72°C for 15 s. The following DNA substrates were used: 200 ng "naked" Jurkat DNA (ThermoFisher) or nuclei purified from 15,000 Jurkat T cells as described

previously (Nabbi and Riabowol, 2015). The digestion of nuclei was used to assess specific activity of plasma DNASE1L3, while the digestion of naked DNA was used to assess total plasma DNase activity. To assess whether plasma IgG could bind and inhibit enzymatic DNase activity, IgG was purified from healthy controls and three SLE patients with Abs to DNASE1L3 using Protein G beads. Different concentrations of DNases were pre-incubated with 0.1 mg/ml of purified IgG at 37°C for 30 min. Pre-incubated DNase as well as untreated DNase were used to digest DNA substrates as described and the amount of remaining DNA was measured by qPCR. The DNase concentration at which 50% of the input DNA was digested (IC_{50}) was established, and the percent of DNase activity compared to untreated DNase was calculated.

Quantitative DNASE1L3 activity assay. Several dilutions of human plasma were incubated with 15,000 Jurkat T cell-derived nuclei and 2 mM $CaCl_2/MnCl_2$ in a total volume of 10 μ l for 10 min at 37°C. All plasma samples were diluted in HBSS (without magnesium and calcium) with 100 ng/ml molecular grade BSA. The remaining DNA was measured by qPCR using *Intra Alu human* primers as described above. The DNase concentration at which 50% of the input DNA was digested (IC_{50}) was established, and the percent of DNase activity was determined, considering the IC_{50} value for pooled healthy control plasma as 100% and the value for DNASE1L3-deficient patient plasma as 0%.

We have found that total reaction volume, digestion time, digestion solution, batch of nuclei, $CaCl_2/MnCl_2$ concentration, plasma diluent, and polymerase used for qPCR-based DNA quantification can affect the results of this assay. Detailed protocols and troubleshooting tips would be shared with all interested investigators upon request.

ELISA

Anti-DNase ELISA. All plate incubations were done in a hydration chamber unless otherwise specified. Nunc MaxiSorp plates (Invitrogen 44-2404-21) were coated with 50 μ l/well of 2 μ g/ml recombinant human DNASE1 (Abcam 73430), purified recombinant DNASE1L3, or FLAG peptide (Sigma-Aldrich) in PBS overnight at 4°C. Antigen-coated plates were washed with PBS, blocked with 250 μ l/well of PBS with 4% non-fat dry milk (NFDM) for 2-3 hr at RT. Plates were then washed again 3 times with PBS and incubated with plasma diluted in PBS overnight at 4°C. Plates were washed 3 times with PBS containing 1% NFDM, and bound IgG was detected using goat anti-human IgG-alkaline phosphatase conjugate (Sigma Aldrich A1543-1ML) diluted 1:5,000 in PBS containing 1% NFDM. The plate was then washed 3 times with PBS containing 1% NFDM and developed using diethanolamine substrate buffer (Thermo Fisher Scientific) and PNPP phosphatase substrate tablets (Sigma Aldrich).

Anti-dsDNA ELISA. Nunc MaxiSorp plates were coated with 0.01% poly-L lysine (Sigma Aldrich) for 1 hr at RT, washed with PBS and coated with 50 μ l/well of 10 μ g/ml calf thymus DNA (Calbiochem) in PBS, overnight at 4°C. Antigen-coated plates were washed and then blocked with 200 μ l/well of PBS containing 4% NFDM for 3 hr at 4°C. Blocked plates were washed once with PBS + 1% NFDM and coated with diluted serum in PBS and incubated overnight at 4°C. Unbound serum antibodies were washed off with 3 washes of PBS + 1% NFDM. The bound IgG was detected using a goat anti-human IgG-AP-conjugate (Sigma Aldrich A1543-1ML) diluted 1:5,000 in PBS + 1% NFDM. Unbound secondary antibody was washed off with PBS+ 1% NFDM and the plate was developed as described above.

Anti-dsDNA ELISA with DNA fragments of different length. Nunc MaxiSorp plates were coated with 0.01% poly-L lysine (Sigma Aldrich) for 1 hr at RT, washed with PBS and coated with 50 μ l/well of 10 μ g/ml of DNA in PBS, overnight at 4°C. DNA fragments of 95bp, 186bp, 382bp, and 831bp were prepared by PCR and purified using QIAquick Gel Extraction Kit (QIAGEN). Calf thymus DNA (Calbiochem) was used as a control. Antigen-coated plates were washed and then blocked with 200 μ l/well of PBS containing 4% NFDM for 3 hr at 4°C. Blocked plates were washed once with PBS + 1% NFDM and coated with diluted serum in PBS and incubated overnight at 4°C. Unbound serum antibodies were washed off with 3 washes of PBS + 1% NFDM. The bound IgG was detected using a goat anti-human IgG-AP-conjugate (Sigma Aldrich A1543-1ML) diluted 1:5,000 in PBS + 1% NFDM. Unbound secondary antibody was washed off with PBS+ 1% NFDM and the plate was developed as described above.

Competition ELISA with nucleosomes. Anti-dsDNA plates were prepared as described above. During the plate blocking, plasma and inhibitor solutions were prepared separately in PBS. Each plasma sample was prepared at a dilution that yielded an OD_{405nm} of 0.5-0.6 so that we could assess both increases and decreases in the OD. Different concentrations of purified HeLa Mononucleosomes (EpiCypher 16-0002), and purified HeLa Polynucleosomes (EpiCypher 16-0003) were prepared separately and mixed with an equal volume of diluted plasma. This solution was resuspended several times to mix and 50 μ L was immediately added to a freshly blocked dsDNA-coated plate. The plate was then processed as described above.

Competition ELISA with DNases. Anti-DNASE1L3 plates were prepared as described above. During the plate blocking, plasma and inhibitor solutions were prepared separately in PBS. Each plasma sample was prepared at a dilution that yielded a DNASE1L3 ELISA OD_{405nm} of 0.5-0.6 so that we could assess both increases and decreases in the OD. Different concentrations of recombinant human DNASE1 (Abcam 73430) or purified recombinant DNASE1L3 were prepared separately and mixed with an equal volume of diluted plasma. This solution was resuspended several times to mix and 50 μ L was immediately added to a freshly blocked plate coated with 2 μ g/ml DNASE1L3. The plate was then processed as described above.

Competition ELISA with DNA fragments of different length. Anti-dsDNA plates were prepared as described above. During the plate blocking, plasma and inhibitor solutions were prepared separately in PBS. Each plasma sample was prepared at a dilution that yielded an OD_{405nm} of 0.5-0.6 so that we could assess both increases and decreases in the OD. DNA fragments of 95bp, 186bp, 382bp, and 831bp were prepared by PCR and purified using QIAquick Gel Extraction Kit (QIAGEN). Different concentrations of PCR-amplified DNA fragments were prepared separately and mixed with an equal volume of diluted plasma. This solution was resuspended several times to mix and 50 μ L was immediately added to a freshly blocked dsDNA-coated plate. The plate was then processed as described above.

Western blot

IgG binding against antigens on MP. Microparticle proteins from $2-5 \times 10^7$ Jurkat derived MP were separated on a 4-12% Bis-tris western blot gel (Invitrogen NP0326BOX) in reducing conditions and transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Sigma-PVH00005). Afterwards, the membrane was blocked in 5% BSA for 60min at RT and incubated overnight with plasma diluted in blocking buffer. In some instances, incubation with plasma samples was performed using a multiplex

blotting system (LI-COR Biosciences). After washing with TBST, membranes were incubated with HRP-conjugated anti-human IgG Ab (Jackson ImmunoResearch, 1:5,000) or anti-mouse IgG Ab (eBioscience, 1:1,000) diluted in blocking buffer at RT for 60min. The immunoreactive bands were visualized using an Amersham Imager 680 blot and gel imager.

IgG binding to DNases. To assess IgG binding to DNases by Western blot, 2 µg recombinant DNASE1L3 (purified in our lab) or 2-40 µg recombinant DNASE1 (Abcam 73430) were run on a 12.5% separating gel in reducing conditions, and Western blot was performed as above.

IgG binding to HMGB1 on MP. Microparticle proteins from Jurkat derived MP were separated on a 4-12% Bis-tris western blot gel. A polyclonal anti-HMGB1 Ab (Invitrogen PA5-96160) was used as primary Ab (1:1000 dilution) and detected with an HRP-conjugated anti rabbit IgG Ab (Thermofisher G-21234; 1:2,000 dilution).

IgG binding to Histones on MP. Microparticle proteins from Jurkat derived MP were separated on a 4-12% Bis-tris western blot gel. A polyclonal anti-Histone 2A Ab (Cell Signaling Technology, 2578S), or a polyclonal anti-Histone 2B Ab (Cell Signaling Technology, 8135S) was used as primary Ab (1:1000 dilution) and detected with an HRP-conjugated anti rabbit IgG Ab (Thermofisher G-21234; 1:2,000 dilution).

Flow Cytometry

Analysis of DNASE1L3-sensitive IgG binding to MP. MP from Jurkat cells were generated as previously described (Sisirak et al., 2016; Ullal et al., 2011). Briefly, Jurkat cells were cultured in the presence of 1 µM staurosporine (Sigma-Aldrich S440) overnight, harvested, and collected by centrifugation for 5 min at 400 xg. The supernatants were collected and centrifuged at 22,000 x g for 30 min at 4°C to pellet MP. Surface staining of MP was performed as follows: 10⁵ MP were mock-treated or treated with recombinant human DNASE1 (Abcam 73430) or recombinant DNASE1L3. Where specified, MP were treated with DNASE1 and DNASE1L3-containing supernatants, described previously (Sisirak et al., 2016), rather than recombinant proteins. DNase-treated MP were then stained with human plasma at 1:20 dilution in a total volume of 30 µl at 4°C for 90 min. Stained MP were washed, pelleted by centrifugation at 22,000 x g for 30 min at 4°C and incubated with a secondary FITC-conjugated anti-human IgG antibody (Millipore, AP113F; 1:1000). Samples were diluted to stop staining and acquired on an Attune NxT (ThermoFisher) flow cytometer. Representative histograms were generated using FlowJo software (Tree Star).

To determine whether plasma IgG bound to the surface of native MP, the respective histograms were subtracted from a control histogram. The control histogram was generated by merging histograms derived from staining native MP with plasma from healthy controls (N=10). To determine whether IgG binding to native MP occurred in a DNASE1L3-sensitive manner, the histogram derived from staining DNASE1L3-treated MP was subtracted from the histogram derived from staining native MP for each plasma sample using the Overton subtraction method (Overton, 1988) as implemented in FSC Express (De Novo Software).

Analysis of microparticle antigens. 3-4x10⁵ MP were mock-treated or treated with recombinant human DNASE1 (Abcam 73430) or recombinant DNASE1L3. DNase-treated MP were then diluted in HBSS without Magnesium, Calcium, or phenol red (Thermo Fisher Scientific 14-175-095). They were centrifuged at 22,000 x g for 30 min

at 4°C to pellet MP and resuspended in HBSS (for VybrantGreen staining) or PBS for other staining.

Vybrant Green. MP were stained in 400µL HBSS+Vybrant Green (Invitrogen V35004, 1:10,000) for 15min at 37°C. The sample was then kept on ice in the dark and run on an Attune NxT (ThermoFisher) flow cytometer within 60min.

HMGB1 or Histones. MP were stained in 50µL PBS for 105 min at 4°C with HMGB1 (Invitrogen PA5-96160, 1:50), Histone 2A monoclonal (Cell Signaling Technology 12349S, 1:50), Histone 2A polyclonal (Cell Signaling Technology 2578S, 1:50), or Histone 2B (Cell Signaling Technology 8135S, 1:50). After staining, samples were diluted with PBS and centrifuged at 22,000 x g for 30 min at 4°C to pellet MP. They were then stained with AF647 conjugated anti-rabbit IgG (Invitrogen A21245, 1:500) for 45 min at 4°C. Samples were then diluted to 400µL with PBS and run on an Attune NxT (ThermoFisher) flow cytometer. MP gating was calibrated based on 1 µm sizing beads (Invitrogen).

Stimulation of human plasmacytoid dendritic cells

Approximately 80 mL of blood was drawn from healthy controls into EDTA coated tubes (BD 366643). The blood was then split equally between three 50 mL conical tubes and diluted to 40 mL with sterile PBS. 10 mL of Ficoll-Paque (GE Healthcare 17-1440-03) was added to the bottom of the tube using a glass Pasteur pipet. The cells were spun at 400 x g for 22 min with low acceleration and no breaks at RT. Approximately 20 mL of supernatant was aspirated, and the remaining top two layers were transferred into new conical tubes. The cells were spun at 200 x g for 15 minutes at room temperature and washed 3 times with sterile wash buffer (PBS, 2% FCS, 1 mM EDTA). The cells were then counted using an Accuri C6 flow cytometer (BD Biosciences) and diluted to a density of 50,000 cells/µL. Plasmacytoid dendritic cells (pDCs) were enriched from total PBMCs using EasySep™ Human Plasmacytoid DC Enrichment Kit (Stemcell 19062A). After enrichment, the cells were immediately resuspended in full RPMI and transferred into a 96 well plate. 5,000 pDCs were added to each well in 180 µL of full RPMI and antigen (mononucleosomes, polynucleosomes, CpG, etc) was added in a volume of 20 µL for a total reaction volume of 200 µL. The cells were activated for 24 hr at 37°C and 5% CO₂. Following activation, the plate was spun at 1,300 rpm for 10 min. The supernatant was used immediately or stored at -80°C.

Antigen preparation. Purified HeLa Mononucleosomes (EpiCypher 16-0002), and purified HeLa Polynucleosomes (EpiCypher 16-0003) were incubated with LL-37 (InvivoGen ltrl-l37) for 30-40 min at RT in a 20 µL volume and then immediately added to purified pDCs. ODN 2216 (InvivoGen ltrl-2216) was used as a positive control. The pDC/antigen mixture was then resuspended several times without vortexing. ODN 2216 was used at a final concentration of 1 µM. LL-37 was incubated with DNA substrates at a concentration of 500 µg/mL and this mixture was added to pDCs for a final concentration of 50 µg/mL. Mononucleosomes and polynucleosomes were incubated with LL-37 at concentrations between 60 and 20 µg/mL of DNA and the final concentrations after adding this mixture to pDCs were between 6 and 2 µg/mL. Full RPMI: 10% FCS, 100 Units/mL Penicillin & 100 µg/mL Streptomycin (Gibco 15140122), 1X MEM Non-Essential Amino Acids Solution (Gibco 11140050), 2 mM L-glutamine (Gibco 25030081), 1mM Sodium Pyruvate (Gibco 11360070).

Confocal microscopy of microparticles

For human microparticles, blood was drawn into EDTA coated tubes (BD 366643) and centrifuged at 1,000 x g for 10 min. The supernatant was then centrifuged at 10,000 x g for 10 min to isolate platelet-free plasma (PFP). PFP was centrifuged at 21,130 x g for 60 min at 4°C to pellet microparticles. Murine microparticles, were isolated as described in the “*isolation of microparticles*” section of these methods. Microparticle pellets were resuspended in HBSS without Magnesium, Calcium, or phenol red (Thermo Fisher Scientific 14-175-095) and 1-2 million microparticles were used for staining. Samples were stained with DiD (Invitrogen V22887) and Vybrant Green (Invitrogen V35004) in the dark at 37°C for 20 min. They were diluted to 1.5 mL with HBSS to stop the staining. The microparticles were filtered through a 70 µm filter and spun at 21,130 x g for 60 min at 4°C. The pellet was washed again with HBSS, filtered through a 70 µm filter and spun again 21,130 x g for 60 min at 4°C. The pellet was resuspended in 1 µL of HBSS and transferred to a 1.5 coverslip (Corning 2850-22). The sample was kept in a hydration chamber for 40 min and inverted onto a Poly-Prep slide (Sigma P0425-72EA) containing 1 drop of Diamond Antifade Mountant (FischerScientific P36961). Slides were dried for 60 min at RT and stored at 4°C until imaged. The imaging of H2B-GFP/tdTomato double reporter mice was done the same way but without any staining steps. Images were acquired using a Zeiss 880 confocal microscope with the 63x oil objective.

Primer sequences:

Intra-alu human:

FW: 5'-TCACGCCTGTAATCCCAGCA-3', RV: 5'-AGCTGGGACTACAGGCGCCC-3'

Intra-alu human 76bp

FW: 5'-AGACCATCCTGGCTAACACG-3', RV: 5'- GTTCACGCCATTCTCCTGC-3'

Intra-alu human 200bp

FW: 5'- AAAATTAGCCGGGCGTG-3', RV: 5'- AGACGGAGTCTCGCTCTGTC-3'

831bp

FW: 5'-AATGGCCAGTTTCCTGAGGATTGTC-3', RV: 5'-

CCCTGGGCAGCAATAGTTAATG-3'

382bp

FD: 5'-AAGAGTGAAGACCCGTGTGC-3', RV: 5'-CCCCGTCTTCAAAGAACATT-3'

186bp

FW: 5'-GAACCAGCTGATTACCCTGTTATCCCTAC-3', RV: 5'-

CCCTGGGCAGCAATAGTTAATG-3'

95bp

FD: 5'-AAGAGTGAAGACCCGTGTGC-3', RV: 5'-CCAAGTTCACACTAGAGTCAAAGG-3'

Inter-B1 mouse:

FW: 5'-CCAGGACACCAGGGCTACAGAG-3', RV: 5'-CCCGAGTGCTGGGATTAAG-3'

Statistical analysis. Statistical analyses were performed using the Prism software version 8 (GraphPad, La Jolla, CA). Normal distribution of data was not assumed unless otherwise specified. Statistical significance between two unpaired experimental groups was determined by non-parametric Mann-Whitney test unless otherwise specified. For paired groups, normality was assessed using the Shapiro-Wilk test. If the data were normally distributed, a parametric paired t-test was used. If the data were not normally distributed, a non-parametric Wilcoxon matched-pairs signed rank test was used. The

non-parametric Kruskal-Wallis test followed by the Dunn's multiple-comparison test was used for statistical analysis of more than two groups. Contingency tables were analyzed using Fisher's exact test or Chi-square test as appropriate. Correlation between two continuous variables was explored by Spearman correlation. All p-values were two-tailed and differences were considered significant for p values <0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****).

Supplemental material

Supplemental material includes seven figures and three tables. Supplemental Figure 1 shows the correlation of DNASE1L3 activity with renal function in SLE patients. Supplemental Figure 2 presents further characterization of auto-Abs to DNASE1L3 in SLE patients. Supplemental Figure 3 shows the correlation between the MP fraction of plasma cfDNA and anti-dsDNA Ab levels in SLE patients. Supplemental Figure 4 shows the impact of length of protein-free DNA on its binding to anti-dsDNA IgG. Supplemental Figure 5 further characterizes auto-Ab reactivity to microparticle antigens. Supplemental Table 1 describes clinical characteristics of SLE patients with DNASE1L3-sensitive IgG binding to microparticle antigens. Supplemental Table 2 summarizes clinical characteristics of SLE patients responding to treatment, and Supplemental Table 3 describes the characteristics of each such patient.

Author contributions

J.H., L.S., A.R., Y.W., O.A.P., B.S., V.S., C.S., G.J.S. performed experiments, analyzed and interpreted results. A.S.C., I.S. developed and provided reagents. I.C., C.B., S.V., G.M.R., H.M.B., R.M.C., P.M.I., J.P.B. obtained and provided patients' samples. J.P.B. and B.R. conceived and supervised the project. J.H., L.S. and B.R. wrote the original manuscript. All authors reviewed and edited the manuscript.

Acknowledgements

Supported by the NIH grants AR071703 (B.R., J.P.B.), AR070591 (B.R., G.J.S., J.P.B., R.M.C.), CA232666 (O.A.P.), AI100853 (O.A.P., L.S., A.R.), AR069515 (L.S.), and GM136573 (LS). Also supported by the Lupus Research Alliance (B.R.), the Colton

Center for Autoimmunity (B.R., J.P.B.) and the German Research Foundation (DFG) (J.H.). The authors declare no competing financial interests.

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Abbreviations

Ab	antibody
cfDNA	cell-free DNA
dsDNA	double-stranded DNA
HUVS	hypocomplementemic urticarial vasculitis syndrome
LN	lupus nephritis
MP	microparticle
NET	neutrophil extracellular trap
qPCR	quantitative PCR
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
UPCR	urine protein/creatinine ratio

Figure 1. Reduced activity of DNASE1L3 in the plasma of patients with sporadic SLE

(A) Digestion of DNA substrates by human DNases. Recombinant human DNASE1 or recombinant DNASE1L3 were incubated with purified human genomic DNA (left) or intact nuclei from human Jurkat cells (right), and the remaining DNA was quantified by qPCR. Dashed lines indicate the amount of input DNA. Data are representative of three independent experiments.

(B) Digestion of nuclear DNA by human DNases. Indicated dilutions of recombinant human DNASE1 or DNASE1L3 were incubated with intact nuclei from human Jurkat cells, and the resulting DNA was purified and analyzed by agarose gel electrophoresis along with the DNA size markers (bp). Representative of three independent experiments.

(C-D) Validation of DNASE1L3 activity assay in human plasma. Serial dilutions of plasma were incubated with intact nuclei from human Jurkat cells, and the remaining DNA was quantified by qPCR. Shown are representative digestion curves for plasma from a DNASE1L3-deficient patient (panel C) or a patient with a hypomorphic DNASE1L3 (R206C) variant (panel D), along with plasma pooled from healthy controls (Ctrl). Dashed lines indicate 100% (top) and 50% (bottom) of input DNA. Representative of three independent experiments.

(E) Plasma DNASE1L3 activity in SLE patients. DNASE1L3 activity was measured in the assay described in panels C-D and expressed as percent of activity in pooled control plasma. Symbols represent individual subjects, bars represent median \pm interquartile range. Ctrl, healthy controls (N=15); 1L3^{-/-}, patients with genetic DNASE1L3 deficiency (N=4); renal, “ever renal” SLE patients by ACR criteria (N=37); non-renal, “never renal” SLE patients by ACR criteria (N=14); anti-Ro+, anti-Ro Ab-positive mothers of children with neonatal lupus (N=15). Dashed line marks the threshold for reduced DNASE1L3 activity (81%); arrows mark the only two subjects in the anti-Ro+ cohort with renal SLE by ACR criteria.

(F) Plasma DNASE1L3 activity in SLE patients with active renal disease. DNASE1L3 activity was compared between patients with active proteinuria (UPCR>0.5) (N=27) and those with resolved proteinuria (UCPR<0.5) (N=10) from the “renal” SLE group in panel E.

Statistical significance: * $p < 0.05$, ** $p < 0.01$.

Figure 2. Reduced DNASE1L3 activity is associated with neutralizing anti-DNASE1L3 antibodies

(A-C) Anti-DNASE1L3 (1L3) Ab levels in the plasma of sporadic SLE patients as measured by ELISA.

(A) Anti-DNASE1L3 (1L3) Ab OD values in individual patients at a single plasma dilution (1:40); bars represent mean \pm S.D. Patients included healthy controls (N=15); renal (N=40) and non-renal SLE patients (N=17) by ACR criteria; and anti-Ro Ab-positive mothers of children with neonatal lupus (Anti-Ro+, N=18). Dashed line indicates the threshold for a positive anti-DNASE1L3 Ab (mean OD+2 S.D. of control).

(B) Average OD values \pm SD for plasma dilutions (1:40-1:640) in the indicated groups of SLE patients and controls. Significance levels are given for the comparison between “renal” and “non-renal”.

(C) Dilution curves for healthy control plasma (ctrl) and plasma from five SLE patients with LN (LN1-5). Representative of two independent experiments.

(D) Cross reactivity of anti-DNASE1L3 antibodies to DNASE1 as measured in a competition assay. Recombinant DNASE1 (D1, squares) or DNASE1L3 (D1L3 circles) were incubated with plasma at a single dilution, and the binding of plasma IgG to DNASE1L3 was measured by ELISA. Shown are two representative patient samples (LN3 and LN5 from Panel C). Dotted lines represent OD values in the absence of any competitor. Representative of two independent experiments.

(E) Correlation between DNASE1L3 activity and anti-DNASE1L3 (1L3) Ab levels. Symbols represent individual patients with sporadicrenal (red) or non-renal (black) SLE. Dashed lines indicate thresholds for reduced DNASE1L3 activity (black) and positive anti-DNASE1L3 Ab (green), respectively.

(F-G) Inhibition of DNase activity by IgG from patients with anti-DNASE1L3 Abs.

(F) Recombinant DNASE1L3 (1L3) was pre-incubated with purified IgG from plasma of a healthy control (Ctrl) or SLE patient with LN, and used to digest DNA in native nuclei (left panel) or “naked” genomic DNA (right panel). Shown is the amount of remaining DNA after digestion with different concentrations of DNASE1L3. Dashed lines indicate 100% (black) and 50% (gray) of input DNA. Representative of two independent experiments.

(G) Recombinant DNASE1L3 (1L3, left panel) or DNASE1 (right panel) was pre-incubated with purified IgG and used to digest naked DNA (as in Fig. 1A). Shown is the relative enzymatic activity after pre-incubation with IgG from three controls or LN patients relative to the respective enzymes alone. Symbols represent IgG from individual patients; bars represent median \pm interquartile range.

Statistical significance: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 3. DNASE1L3 controls the amount of cell-free DNA in circulating microparticles

(A) The DNA content of microparticles from the plasma of healthy human controls. Microparticles were stained with Vybrant DyeCycle Green for DNA (white) and Vybrant DiD for membrane (blue) and examined by confocal microscopy. Shown are images representative of three independent experiments.

(B-C) Cell-free DNA (cfDNA) content of the microparticle (MP) fraction of human plasma. DNA was purified from the MP fraction, MP-depleted fraction (soluble) and total plasma and quantified by qPCR. Shown are concentrations of cfDNA adjusted to the original plasma volume purified from healthy controls (panel B, N=15) or from DNASE1L3-deficient patients (panel C, N=4).

(D) The cfDNA content of the MP fraction, defined as a percentage of MP-associated cfDNA out of the sum of MP-associated and soluble cfDNA. Shown is the percentage of MP-associated cfDNA in healthy controls (Ctrl, N=15); DNASE1L3-deficient patients (1L3^{-/-}, N=4); renal, “ever renal” SLE patients by ACR criteria (renal, N=44); and non-renal, “never renal” SLE patients by ACR criteria (non-renal, N=15). Symbols represent individual patients, bars represent median \pm interquartile range.

(E) Total concentration of cfDNA in the plasma of patient groups described in panel D.

(F) Correlation of DNASE1L3 activity (Fig. 1E) with the fraction of cfDNA in MP (panel D). Symbols represent individual patients with sporadic SLE. Renal patients are represented by red dots, non-renal SLE patients by black dots, dashed line indicates the threshold for reduced DNASE1L3 activity.

Statistical significance: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. DNASE1L3-mediated control of microparticle DNA is conserved in the mouse

(A) DNA-carrying MP from the plasma of wild-type mice. Microparticles were stained with Vybrant DyeCycle Green for DNA (white) and Vybrant DiD for membrane (blue) and examined by confocal microscopy. Shown are images representative of three independent experiments.

(B) Chromatin in plasma MP visualized using genetically encoded fluorescent probes. MP were isolated from the plasma of mice expressing a histone 2B-green fluorescent protein (H2B-GFP) fusion and membrane-targeted red fluorescent protein tdTomato and examined by confocal microscopy. Shown are images representative of two independent experiments.

(C) Distribution of cfDNA in the plasma of wild-type mice. Shown are concentrations per original plasma volume of cfDNA in the MP fraction and in the MP-depleted (soluble) fraction of wild-type mice (N=42). Symbols represent individual mice, bars represent median \pm interquartile range.

(D) Distribution of cfDNA in the plasma of age and sex-matched wild-type and *Dnase1/3*-deficient mice. Shown are concentrations per original plasma volume of cfDNA in the MP fraction and in the MP-depleted (soluble) fraction of wild-type (WT, N=8) and *Dnase1/3*-deficient (N=7) mice. Symbols represent individual mice, bars represent median \pm interquartile range.

(E) Distribution of cfDNA in the plasma of age and sex-matched wild-type and *Dnase1*-deficient mice. Shown are concentrations per original plasma volume of cfDNA in the MP fraction and in the MP-depleted (soluble) fraction of wild-type (WT, N=8) and *Dnase1*-deficient (N=8) mice. Symbols represent individual mice, bars represent median \pm interquartile range.

Statistical significance: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 5. Microparticles carry longer polynucleosomal fragments with better antigenic properties

(A) Average length of cfDNA in the microparticle (MP) and the MP-depleted (soluble) fraction of plasma from healthy controls (Ctrl, gray, N=7) and DNASE1L3-deficient patients (1L3-/-, blue, N=4) as determined by long to short amplicon ratio. Symbols represent individual subjects; bars represent mean. Unpaired samples were compared using parametric unpaired *t*-test.

(B) Size distribution of cfDNA fragments in the MP and the MP-depleted fractions of plasma from healthy controls as determined by sequencing. Lines represents averages of four healthy controls.

(C) Fraction of cfDNA fragments of the indicated length ranges in the MP (gray) and the MP-depleted (soluble, gold) fraction of plasma from healthy controls as determined by sequencing. Symbols represent individual SLE patients; bars represent mean.

(D-E) The effect of chromatin size on binding to anti-dsDNA Abs in a competition assay using plasma of an SLE patient. Panel D shows representative titration of polynucleosomes and mononucleosomes; dashed line shows binding in the absence of competitor DNA. Panel E shows the inhibition of binding by anti-dsDNA IgG from different SLE patients (N=5) at the maximum inhibitor concentration (10 µg/ml).

(F) Inhibitory capacity of protein-free DNA fragments of the indicated lengths in base pairs (bp) on the binding by anti-dsDNA IgG at the maximum inhibitor concentration (10 µg/ml). Symbols represent individual SLE patients (N=5).

(G) The effect of chromatin size on interferon production by plasmacytoid dendritic cells (pDCs). Mononucleosomes (white) or polynucleosomes (purple) were incubated with antimicrobial peptide LL-37 and used to stimulate pDCs from healthy donors, and interferon-α was measured by ELISA after 24 hours. Each point represents an average of two healthy donors and bars represent standard deviation of the mean. Data are representative of two independent experiments.

Statistical significance: **p*< 0.05, ***p*<0.01, and *****p*<0.0001.

Figure 6. Reactivity to DNASE1L3-sensitive MP antigens is associated with reduced DNASE1L3 activity and renal SLE

(A-D) Auto-Ab binding to DNASE1L3-sensitive antigens on MP. MP from apoptotic Jurkat cells that were untreated or treated with human DNASE1 or DNASE1L3 were incubated with plasma from SLE patients followed by anti-human IgG conjugates.

(A) Histograms of IgG fluorescence using plasma from an SLE patient; representative of 3 independent experiments.

(B) MP staining by plasma from patients with genetic DNASE1L3 deficiency (1L3^{-/-}, N=4) or from a healthy control (Ctrl).

(C) MP binding by IgG from plasma of an DNASE1L3-deficient patient (Patient 4 from panel B) who suffered a severe disease flare with renal failure in May 2010. Top row shows histograms of plasma IgG binding to MP at the indicated dates. Bottom panel shows the concentration of cfDNA in MP, the MP-depleted (soluble) plasma fraction and total plasma at each date.

(D) Autoantibody binding to DNASE1L3-sensitive MP antigens in sporadic SLE patients with lupus nephritis (LN). Jurkat cell-derived MP were treated with DNASE1L3 and stained with plasma (top row) or stained first and subsequently treated with DNASE1L3 (bottom row). Shown are representative histograms of IgG fluorescence in 3 LN patients with reduced DNASE1L3 activity and anti-DNASE1L3 Abs.

(E) Relative DNASE1L3 activity in SLE patients with (N=22) or without (N=29) reactivity to DNASE1L3 (1L3)-sensitive antigens on MP. Symbols represent individual patients, bars represent median \pm interquartile range.

(F) The fraction of MP-associated cfDNA in sporadic SLE patients with (N=25) or without (N=34) reactivity to DNASE1L3-sensitive antigens on MP. Symbols represent individual patients, bars represent median \pm interquartile range.

(G) Reactivity to DNASE1L3-sensitive antigens on MP in the indicated patient groups.

(H) Frequency of the indicated SLEDAI criteria in SLE patients (combined renal and non-renal) with (N=61) or without (N=59) reactivity to DNASE1L3 (1L3)-sensitive antigens on MP. Fisher's exact test was used to prepare contingency tables between the two groups.

(I) SLEDAI index in renal and non-renal SLE patients with (N=61) or without (N=59) reactivity to DNASE1L3 (1L3)-sensitive antigens on MP. Symbols represent individual patients, bars represent median \pm interquartile range.

(J) Reactivity to DNASE1L3-sensitive antigens on MP in LN patients depending on treatment response. Shown is the percent of patients with DNASE1L3 (1L3)-sensitive IgG binding to MP at baseline and after 52 weeks of treatment (orange) for patients with complete (N=8)/partial (N=3) treatment response versus no response (N=19).

(K) Change in the fraction of MP-associated cfDNA in LN patients with a complete (N=8)/partial (N=3) or no treatment response (N=13) at baseline and after 52 weeks of treatment. Symbols represent individual subjects; bars represent median \pm interquartile range.

Statistical significance: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 7. DNASE1L3-sensitive antigens on MP include DNA-associated proteins

(A) Human plasma IgG binding to proteins in MP from Jurkat T cells. Plasma samples from healthy controls (Ctrl), DNASE1L3-deficient patients (1L3 null), sporadic SLE patients (SLE), or purified IgG from a healthy control (Ctrl IgG) and sporadic SLE patient (SLE IgG) were tested by Western blot. The presence (+) or absence (-) of IgG binding to DNASE1L3-sensitive MP antigens as assessed by flow cytometry is indicated.

(B) Histones and HMGB1 as potential antigens on MP as determined by Western blot. Plasma samples from patients with sporadic SLE (SLE) were analyzed for IgG binding to MP proteins as in Panel A. In parallel, MP were probed by anti-Histone 2A (H2A), anti-Histone 2B (H2B), and anti-HMGB1 Abs. Data are representative of two independent experiments.

(C-D) SLE-prone mouse plasma IgG binding to MP proteins as determined by Western blot. In panel C, plasma from a *Dnase1l3*-deficient mouse (1L3 KO mouse, dilutions 1:50, 1:150, 1:450) and plasma from a patient with sporadic SLE (SLE human, dilutions 1:150, 1:500, 1:1500) were tested in parallel. In panel D, plasma from *Dnase1l3*-deficient (1L3 KO) mice prior to (3 months) or after the onset of SLE-like disease (7-10 months) was tested.

(E) DNASE1L3-sensitive exposure of HMGB1 and DNA on the surface of Jurkat MP. MP that were untreated (control) or pre-treated with recombinant human DNASE1 or DNASE1L3 were stained for HMGB1 (top) or DNA (VybrantGreen, bottom) and analyzed by flow cytometry. Data are representative of three independent experiments.

Table 1: Demographics and readouts in different study cohorts.

	Healthy Ctrl (n=15)	DNASE1L3- deficient (n=4)	SLE renal (n=87)	SLE non-renal (n=33)	Anti-Ro+ (n=45)
Demographics					
Female	12 (80%)	2 (50%)	75 (87%)	31 (94%)	45 (100%)
Age	36 (23-59)*	9 (7-14)	37 (20-78)	39 (27-80)	35 (27-64)
Race/ethnicity					
Hispanic/Latino	0	0	25 (29%)	16 (48%)	0
Non-Hispanic/Latino	0	0	53 (61%)	17 (52%)	0
White	5 (33%)	4 (100%)	38 (44%)	23 (70%)	38 (84%)
Black	5 (33%)	0	27 (31%)	7 (21%)	1 (2%)
Asian	1 (7%)	0	21 (24%)	2 (6%)	3 (7%)
Other	1 (7%)	0	2 (2%)	1 (3%)	2 (4%)
Not reported	3 (20%)	-	-	-	1 (2%)
Readouts¹					
DNASE1L3 activity, %	100 (89-100.5, n=15)	0 (n=4)	68 (62-100, n=37)	92 (85-100, n=14)	100 (85-100, n=15)
Anti-DNASE1L3 ELISA, OD	0.15 (0.13-0.26, n=15)	-	0.40 (0.26-0.80, n=40)	0.30 (0.23-0.36, n=18)	0.19 (0.13-0.25, n=18)
cfDNA total, ng/ml	1.5 (1.0-2.1, n=15)	6.9 (3.8-14.6, n=4)	3.8 (1.5-6.0, n=44)	1.3 (0.9-2.7, n=15)	5.5 (2.3-9.2, n=15)
Microparticle cfDNA fraction, %	5.6 (3.2-8.4, n=15)	10.0 (9.0-17.6, n=4)	6.3 (3.0-10.0, n=44)	5.4 (2.6-10.2, n=15)	17.2 (6.5-26.5, n=15)
DNASE1L3-sensitive binding to microparticles ²	23 (18-35, n=15)	69 (40-83, n=4)	62 (39-79, n=44)	33 (26-46, n=15)	49 (43-60, n=15)

Continuous variables are given as median (IQR), categorical variables are given as number of cases (%).

* The age of one control subject was unknown and was not included in the calculation.

¹Not all patients in every group were tested for all readouts. The “n=#” in each of the readouts refers to the number of patients (#) from that group that were tested for this readout.

²Table 1 gives DNASE1L3-sensitive binding at the time cfDNA and plasma activity of DNASE1L3 were measured. Fig. 6F gives the number of patients with DNASE1L3-sensitive binding at any time point.

Supplemental Figure 1. DNASE1L3 activity does not correlate with renal function in patients with renal SLE

DNASE1L3 activity in plasma was measured as in Fig. 1C-E and is expressed as percent of activity in healthy controls. Symbols represent DNASE1L3 activity and urine protein to creatinine ratio (UPCR) in individual patients with renal SLE. Dashed line indicates the threshold for proteinuria (UPCR>0.5).

Supplemental Figure 2. Further characterization of autoantibodies to DNASE1L3 in SLE patients

(A) Antibodies to DNASE1, DNASE1L3, and FLAG peptide in the plasma of renal SLE patients (LN1, LN3, LN5 from Fig. 2C) as measured by ELISA. All antigens were plated at a concentration of 2 µg/ml. Data are representative of two independent experiments.

(B) Antibody binding to recombinant DNASE1L3 assessed by Western blot. Purified recombinant DNASE1L3 was analyzed by denaturing SDS-PAGE and probed by Western blot using plasma samples from SLE patients with (+) or without (-) IgG binding to DNASE1L3 as assessed by ELISA. For sporadic SLE patients with a positive ELISA (SLE, +), both plasma and the purified IgG fraction (SLE IgG, +) were analyzed. Ctrl: Healthy control, 1L3 null: DNASE1L3-deficient patient. Data are representative of three independent experiments.

(C) Correlation between anti-DNASE1L3 (1L3) Ab levels assessed by plate-based ELISA (OD) and by bead-based antigen array (mean fluorescence intensity, MFI). Data points represent individual renal SLE patients.

(D) Correlation between anti-DNASE1L3 (1L3) Ab levels and anti-dsDNA Ab levels as assessed by ELISA. Symbols represent individual patients with SLE (combined renal and non-renal).

(E) Correlation between anti-DNASE1L3 (1L3) Ab levels and anti-C1q Ab levels as assessed by respective bead array assays. Symbols represent individual patients with renal SLE.

(F) Inhibition of DNASE1 activity by IgG from patients with anti-DNASE1L3 Abs. IgG was purified from the plasma of a healthy control (Ctrl, gray) or lupus nephritis patient with anti-DNASE1L3 Abs (LN, gold). Recombinant DNASE1 was pre-incubated with purified IgG at the same concentration as DNASE1L3 in Fig. 2F (left panel) or at a 10-fold higher IgG concentration (right panel) and used to digest “naked” genomic Jurkat DNA. DNASE1 without pre-incubation with IgG (pink) was used as a control. Shown is the amount of remaining DNA after digestion with different concentrations of DNASE1. Dashed lines indicate 100% (black) and 50% (grey) of input DNA.

Supplemental Figure 3. Correlation between plasma cfDNA and anti-dsDNA antibody levels in sporadic SLE patients

Symbols represent individual patients with sporadic SLE; all patients with and without lupus nephritis were included. Anti-dsDNA antibody levels were assessed by ELISA and DNA concentrations were assessed by qPCR.

Supplemental Figure 4. The length of protein-free DNA does not impact binding of anti-dsDNA IgG from SLE patients

(A) The effect of DNA length on binding to anti-dsDNA Abs as measured in a competition assay. Plasma from SLE patients was incubated with increasing concentrations of PCR amplified DNA products of different lengths (95bp, 186bp, 382bp, and 831bp) and the binding of IgG to calf thymus dsDNA was assessed by ELISA. Shown is a representative example of the data quantified for several plasma samples (also used in Figure 5F). These data are representative of the five patients tested.

(B) The effect of DNA length on binding to anti-dsDNA Abs as measured by ELISA. PCR amplified DNA products of different lengths (95bp, 186bp, 382bp, and 831bp) were used as antigen to assess anti-dsDNA antibodies from plasma of SLE patients. Calf thymus DNA, used in standard anti-dsDNA measurements, was used as a control. Shown is a representative example of five patients tested.

In both approaches, data are representative of two independent experiments.

Supplemental Figure 5. Further characterization of microparticle antigens and autoantibody reactivity

(A) Binding of monoclonal antibodies marked by the 9G4+ idiotope (9G4+ mAbs) to MP and nuclear antigens. Recombinant IgG mAb clones were generated based on autoantibody sequences from SLE patients and their reactivity to nuclear antigens, apoptotic cell surfaces, and DNASE1L3-sensitive antigens on MP was determined. Top panel shows the binding of representative clones to MP that were pre-treated with control or DNASE1L3-containing supernatants. Bottom panel lists the original isotype; binding to MP and whether it is DNASE1L3 (1L3)-sensitive; and binding to nuclear/apoptotic cell antigens. ND, not determined.

(B) Exposure of Histone 2A and Histone 2B on the surface of Jurkat MP assessed by flow cytometry. Untreated Jurkat MP were stained in the absence (Background) or presence of monoclonal or polyclonal Abs to Histone 2A (H2) or a polyclonal Ab to Histone 2B (H2B). Data are representative of three independent experiments.

Supplemental tables

Continuous variables are given as median (IQR), categorical variables are given as number of cases (%). Comparisons between continuous variables were performed using nonparametric Mann-Whitney and Fisher's exact was used for comparisons between categorical variables.

Supplemental Table 1: Comparison of clinical characteristics between sporadic SLE patients with DNASE1L3-sensitive IgG binding to microparticle antigens vs. patients with no binding. Shown are demographics, ACR criteria, SLEDAI score, and immunosuppressive treatment in these patients.

Supplemental Table 2: Treatment response in sporadic SLE patients with lupus nephritis. Shown are demographics, renal biopsy characteristics, urine protein to creatinine ratio (UPCR) as well as the change of DNASE1L3 readouts in patients with partial/complete treatment response compared to non-responders. DNASE1L3 readouts were assessed at baseline (time of renal biopsy) and after 52 weeks of immunosuppressive treatment.

Supplemental Table 3: Treatment response in SLE patients with lupus nephritis. Shown are the characteristics of each lupus nephritis patient that had completed 52 weeks of follow-up. Given are demographics, renal biopsy characteristics, treatment dosing, and changes in IgG-binding to microparticle antigens as well as changes to cell free DNA with complete, partial or no treatment response. MP=Microparticle.

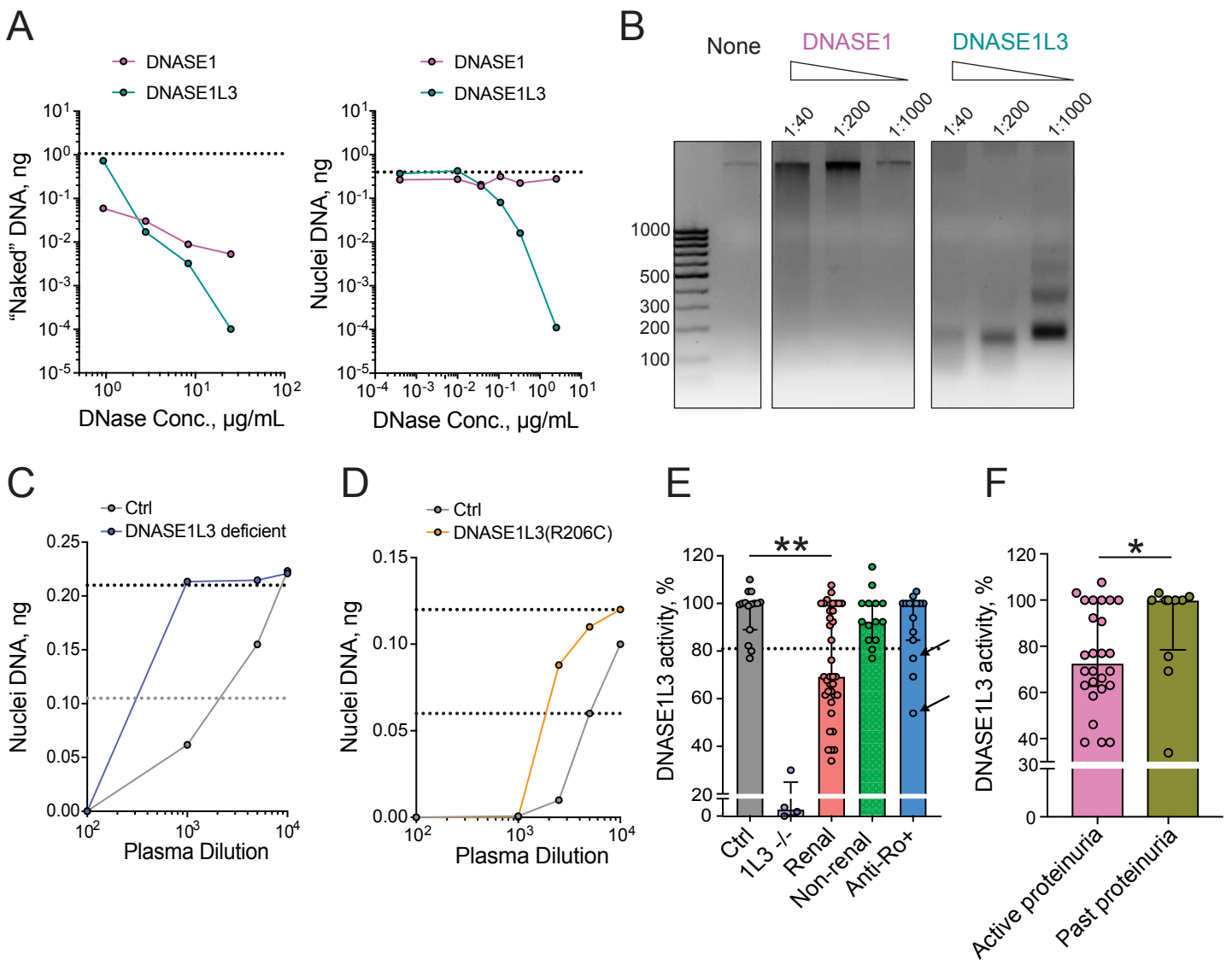


Figure 1

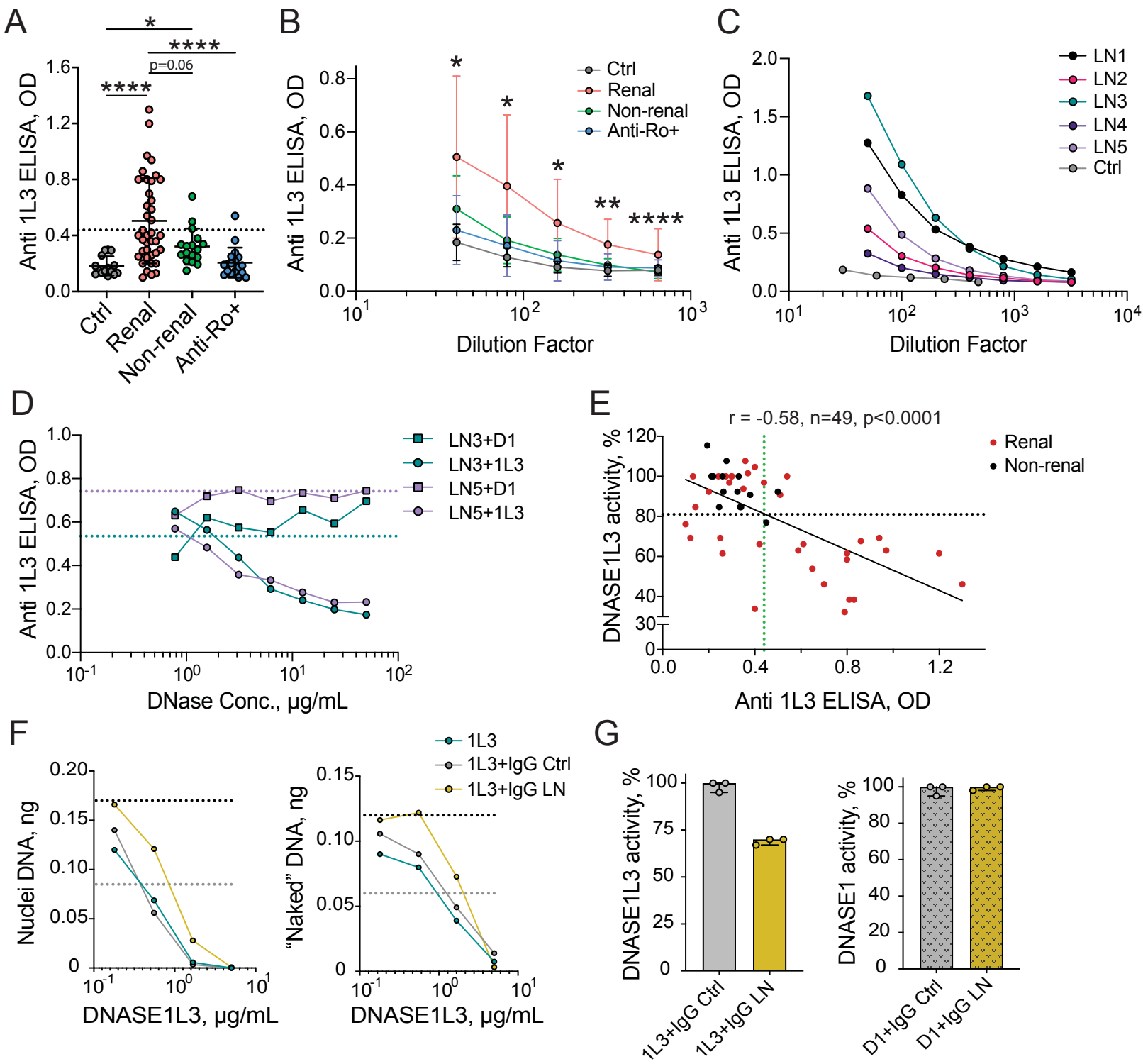


Figure 2

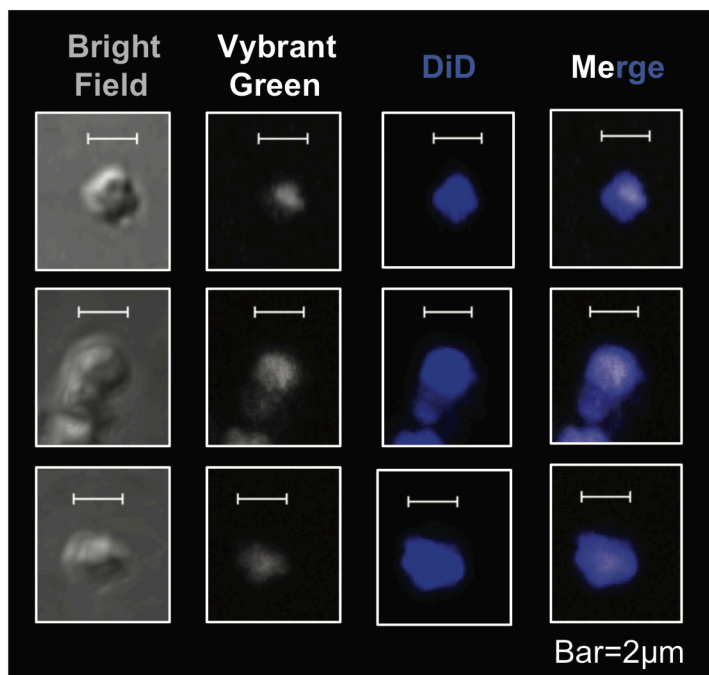
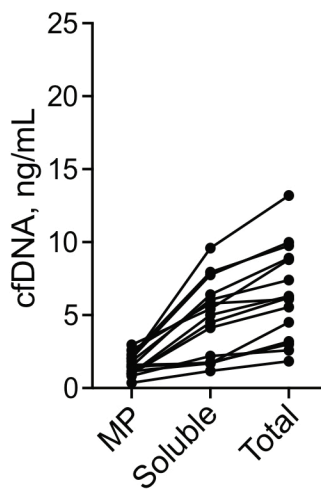
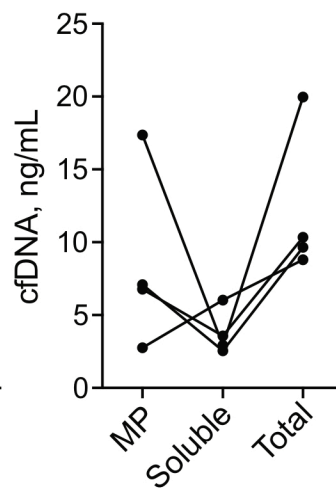
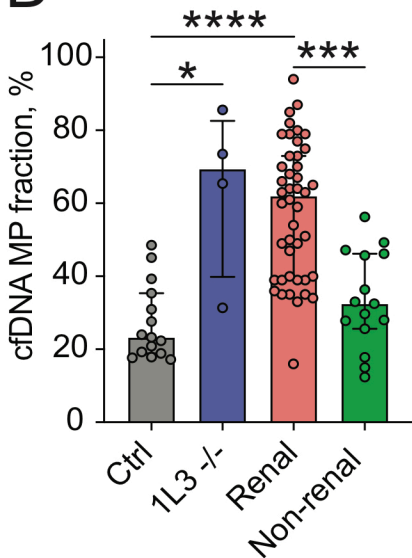
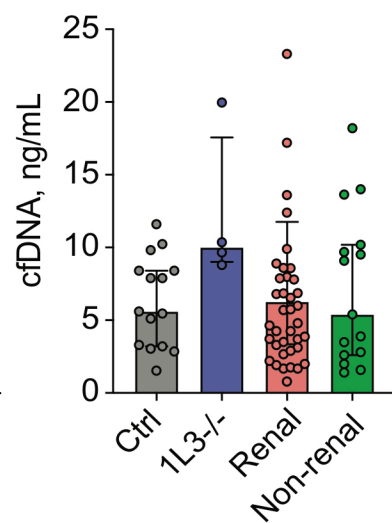
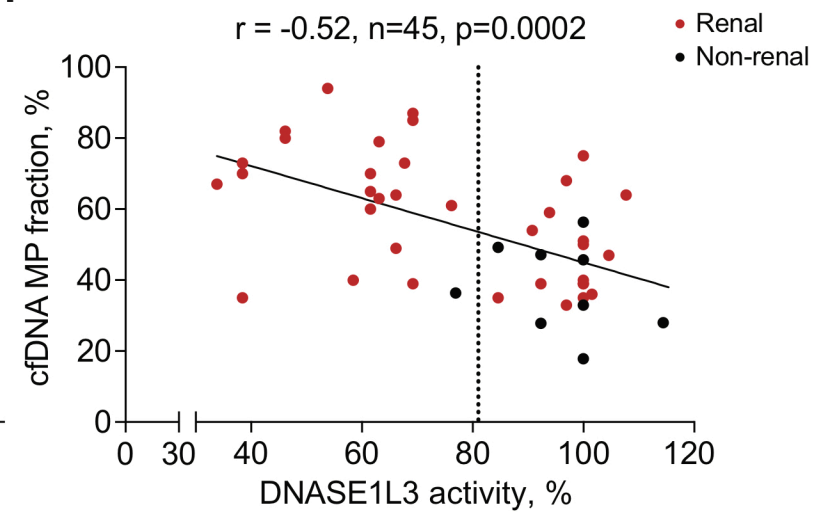
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Figure 3

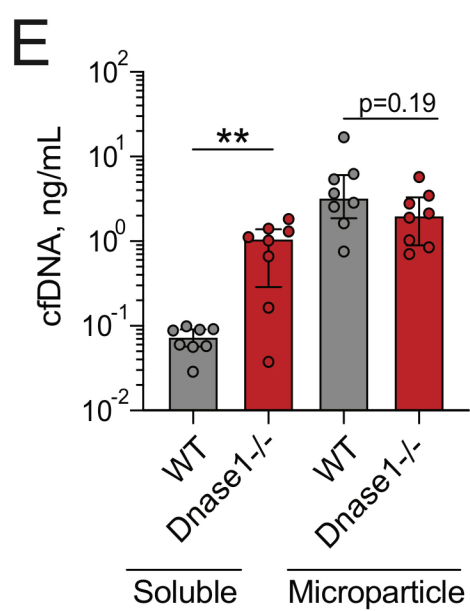
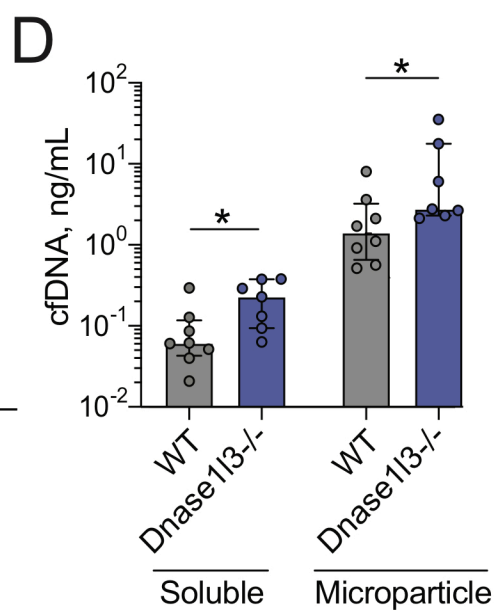
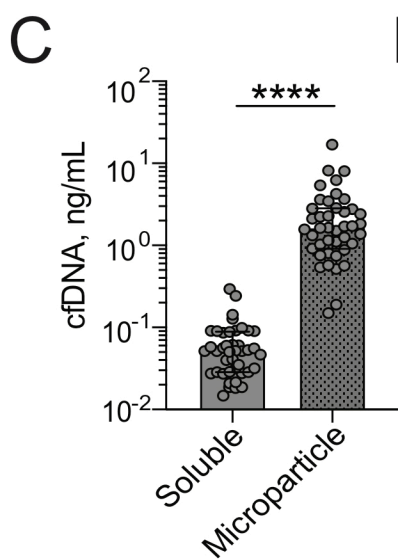
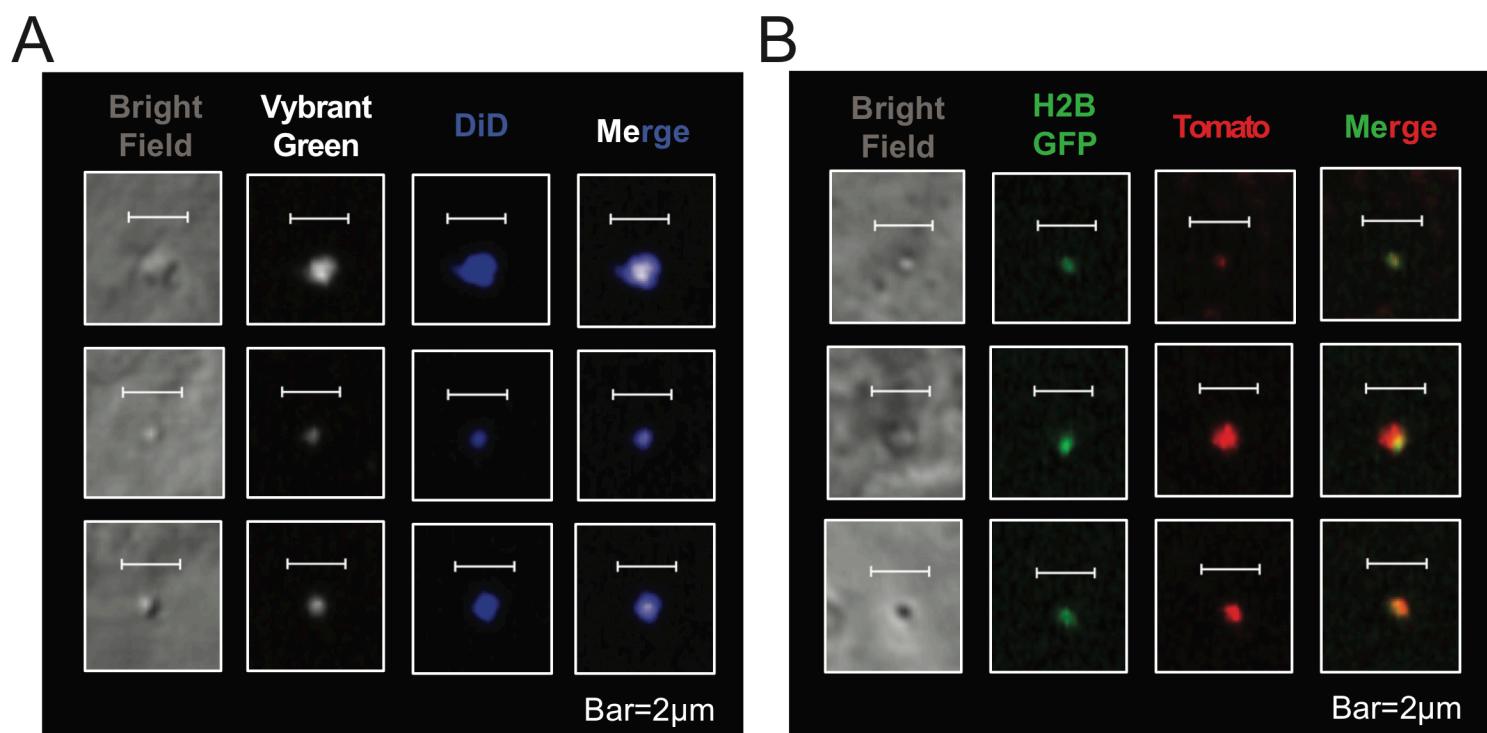


Figure 4

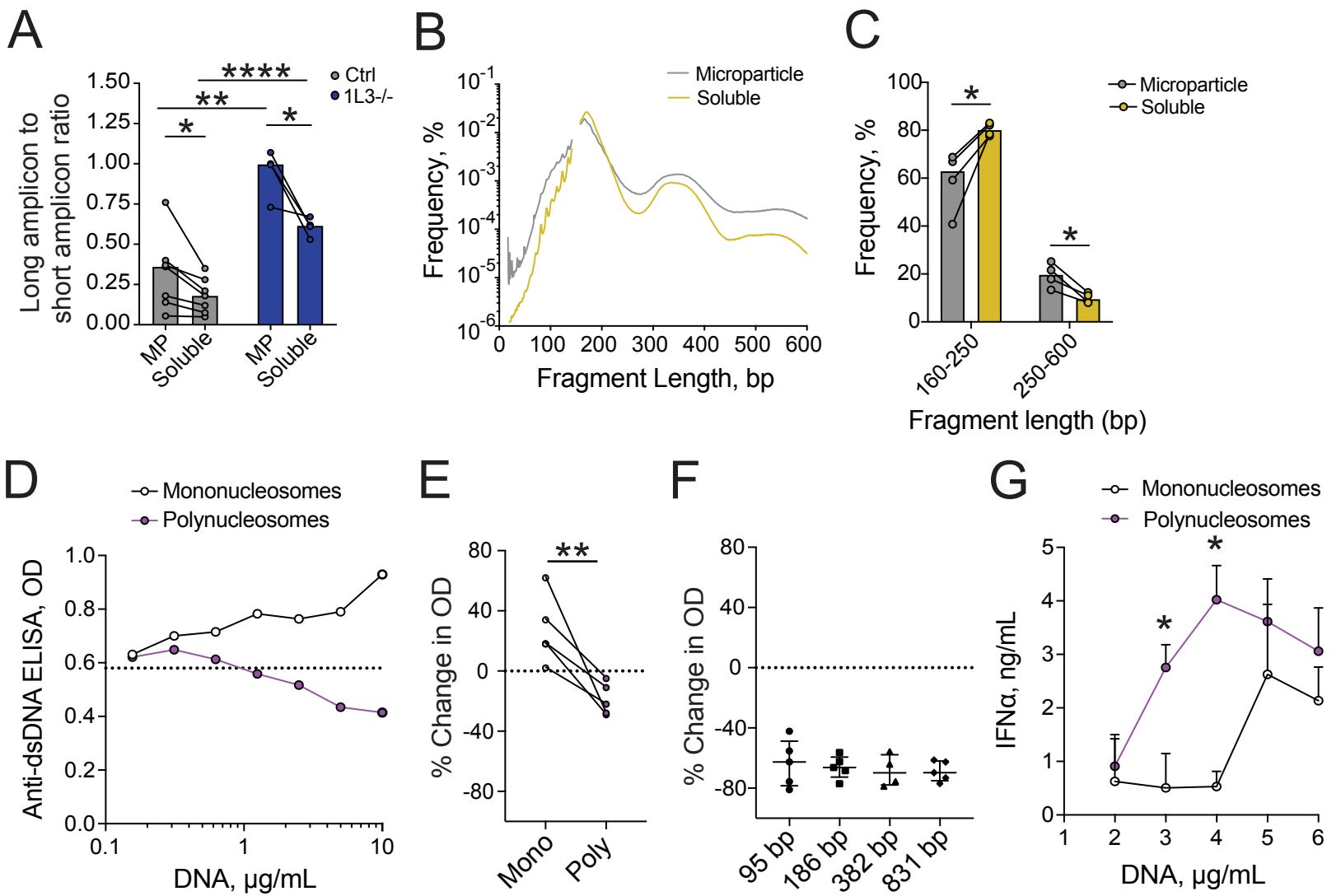


Figure 5

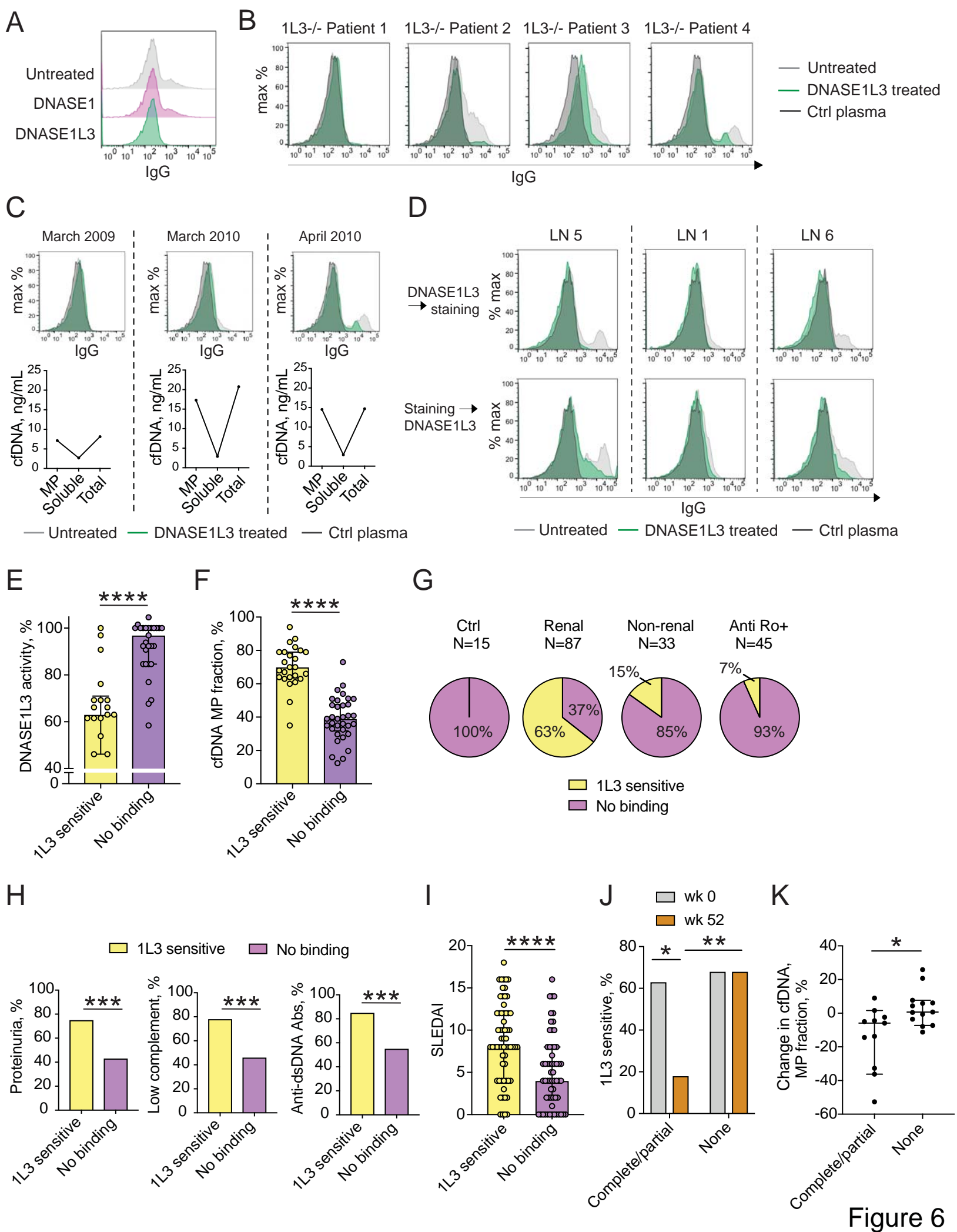


Figure 6

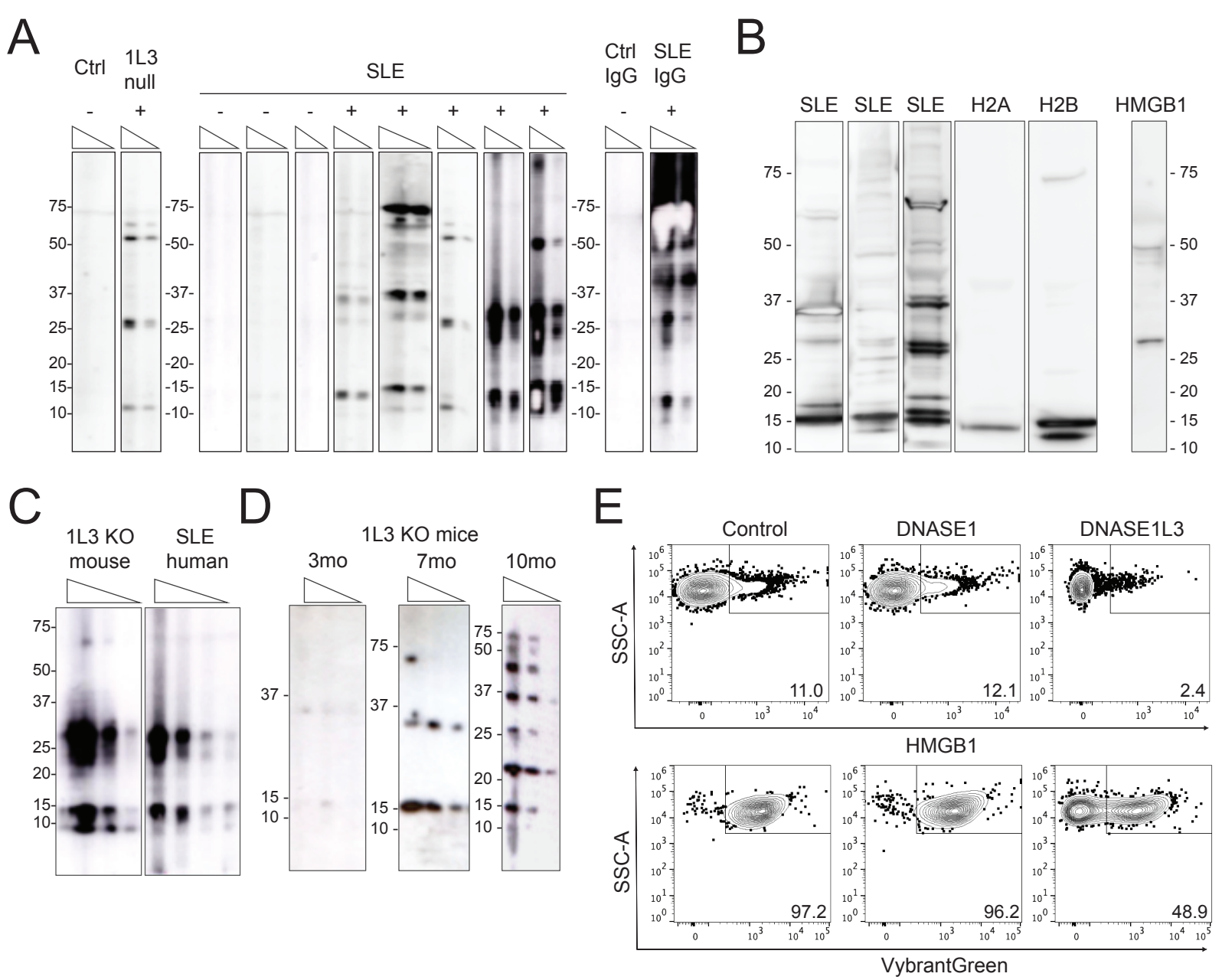
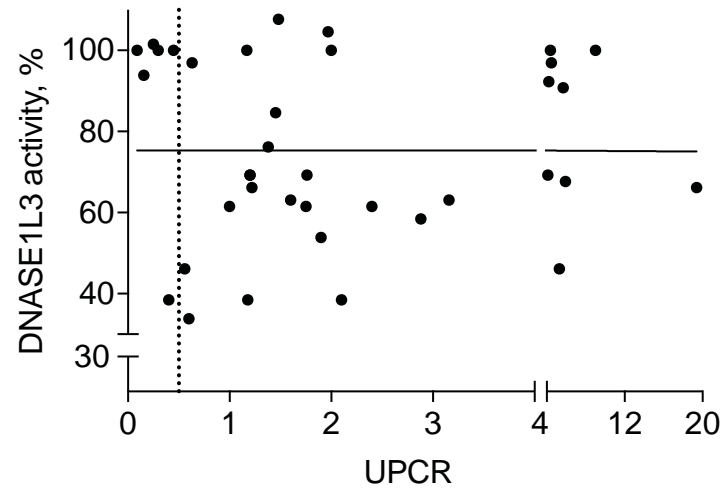


Figure 7

$r = -0.07, n=37, p=0.68$



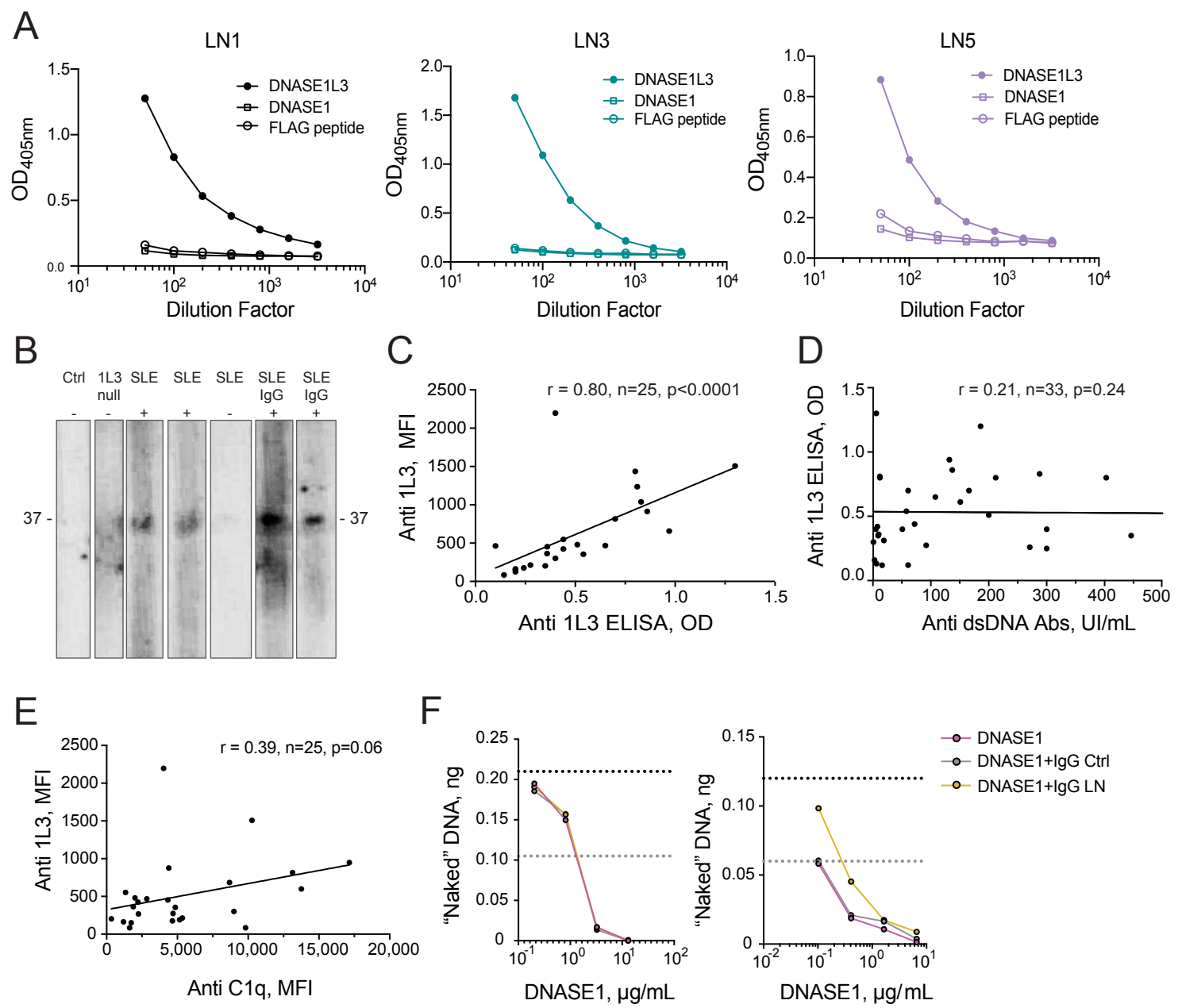


Figure S2

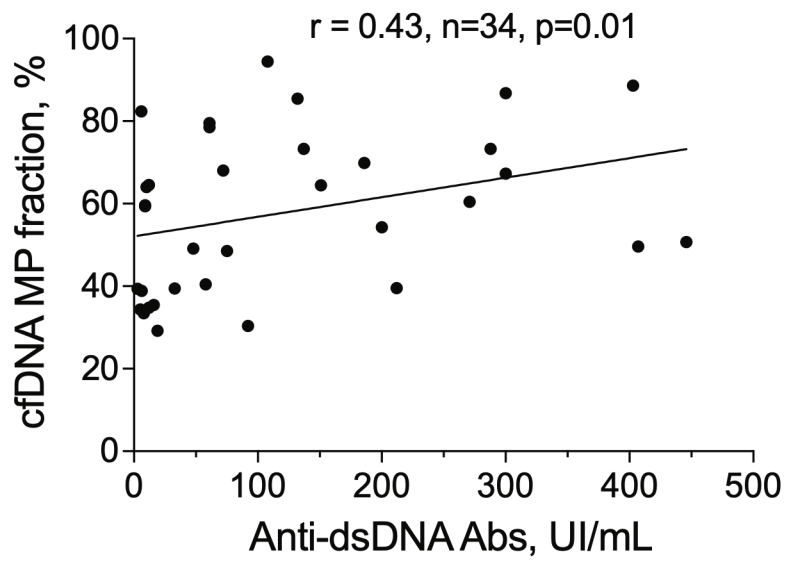


Figure S3

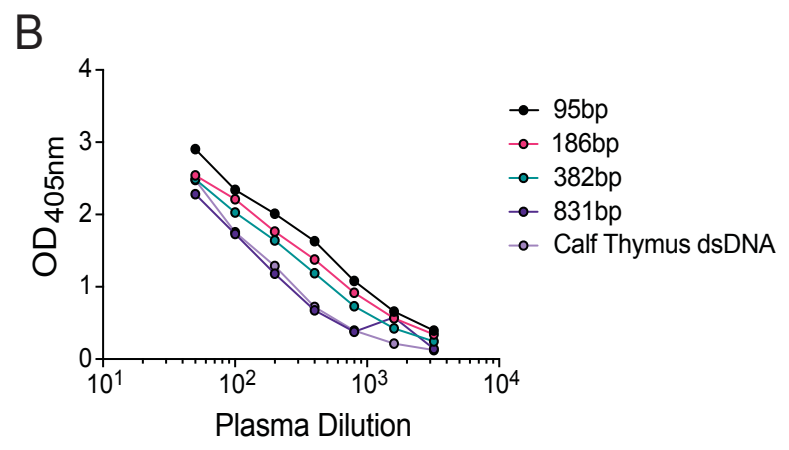
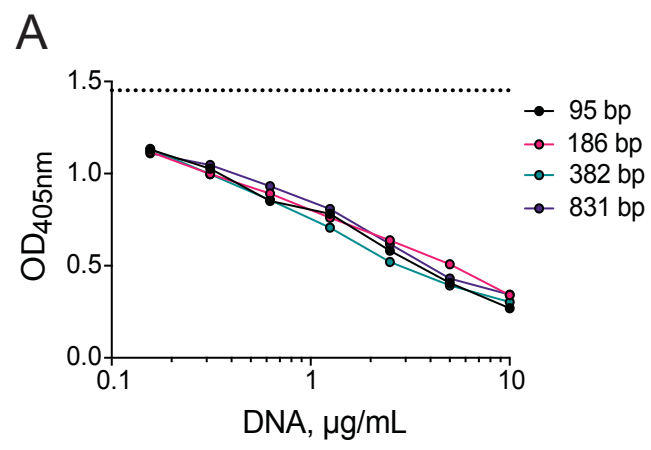
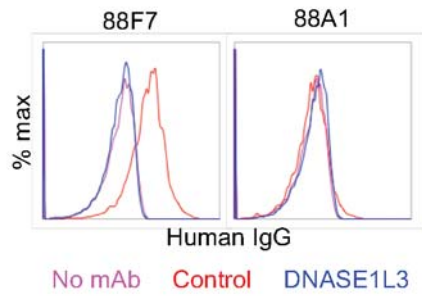


Figure S4

A



Clone	Isotype	MP binding	1L3-sens.	ANA	APC binding	ds-DNA	Ro 52/60	Sm	Chromatin
627 E6	IgG	+	+	+++	++	+++	-	++	+++
88 F7	IgG	+	+	+++	++	++	+	-	++
74 C2	IgM	+	+	+++	++	-	++	+	-
88 A10	IgA	+	-	+++	+	+	+	-	ND
75B8	IgA	-	-	++	-	-	-	-	-
651 E12	IgM	-	-	++	++	+	++	+	+
88 A1	IgG	-	-	++	+	+++	++	ND	++

B

