Differences in the gut permeability marker levels, and abundance and nuclear antigen reactivity of fecal immunoglobulin A (IgA) subclasses in systemic lupus erythematosus patients and healthy controls

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Running title: Gut inflammation and fecal IgA in systemic lupus erythematosus

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Abstract

Objective: Systemic lupus erythematosus (SLE) is characterized by the production of anti-nuclear autoantibodies. Our preclinical studies showed a correlation between pro-inflammatory features of the gut mucosa and autoimmune progression in lupus. The current study was aimed at comparing stool samples of SLE patients and healthy controls (HC) for the gut permeability and inflammatory markers, and the abundance and nAg reactivity of immunoglobulin A (IgA) and its subclasses.

Methods: The abundance of gut inflammation and permeability markers, Calprotectin and Zonulin, and IgA and IgA subclasses (IgA1 and IgA2) in cross-sectional stool samples from 21 SLE patients and 21 HC subjects were determined by quantitative ELISA. The nuclear-antigen (nAg) reactivities of fecal IgA and IgA-subclasses were determined by employing dsDNA and nucleohistone (NH) specific ELISA.

Results: The abundances of Zonulin, total IgA, and IgA1- and IgA2- subclasses were observed to be significantly higher in the fecal samples of SLE patients compared to HCs. SLE patients also showed higher nAg reactivity titers of fecal IgA, IgA1 and IgA2. Importantly, dsDNA- and NH- reactive fecal IgA1:IgA2 ratios were higher in SLE patients. Furthermore, the abundance and nAg reactivity of fecal IgA, IgA1 particularly, showed strong correlation with the fecal Zonulin levels in SLE patients.

Conclusion: Higher amounts of gut permeability protein and nAg-reactive IgA are produced in the intestine, likely in the proximal gut as indicated by the IgA1:IgA2 ratio, in SLE patients. These features of SLE patients, and perhaps at-risk subjects, could serve as biomarkers of systemic autoimmune activity. However, additional studies using longitudinal samples from first degree relatives are needed.

Introduction

Systemic lupus erythematosus (SLE), which predominantly affects women, is an autoimmune disease that arises when abnormally functioning B lymphocytes produce autoantibodies to nuclear antigens (nAg). High levels of circulating autoantibodies and immune-complexes (IC), and IC deposition in the kidney causing glomerulonephritis are the hallmarks of lupus [1, 2]. Autoantibody production and gender bias in SLE is caused by a combination of genetic and environmental factors including diet and gut microbes [1-11]. Our preclinical studies using lupus-prone mice have shown: 1) that minor dietary deviations can alter the composition of gut microbiota and the lupus-like disease progression [9], 2) a potential contribution of pro-inflammatory immune response, including higher frequencies of plasma cells, initiated in the gut mucosa to the disease process and gender bias [12, 13], and 3) higher frequencies of IgA autoantibody producing cells in the gut mucosa, and higher abundance and nAg reactivity of fecal IgA at pre-seropositive and -clinical stages [14]. Importantly, higher gut permeability and microbial translocation have been implicated in the initiation and/or progression of autoimmunity in at risk subjects [15-19]. These and other reports showing the involvement of gut microbiota in systemic autoimmune progression in lupus [6-8, 20, 21] suggest the need to determine if pro-inflammatory/gut permeability factors and IgA are produced and released at higher levels in the gut lumen/ feces of lupus patients.

IgA is the most abundant Ig isotype released into the gut lumen and it plays an important role in the protection against microbial infections as well as in maintaining a healthy gut microbiota [22-24]. In humans, two subclasses of IgA (viz: IgA1 and IgA2) are produced. In healthy human subjects, while the serum IgA antibodies are mostly IgA1, IgA antibodies secreted by intestinal

B cells are composed of about 30-50% IgA1 and 50-70% IgA2 [25]. Fecal IgA from healthy individuals, mostly produced against microbial antigens, is known to be highly poly-reactive and can recognize a variety of antigens including some host antigens [26, 27]. With respect to SLE, it has been shown that in addition to the differences in gut microbiota composition, the levels of pro-inflammatory marker, calprotectin and total IgA in stool samples of SLE patients are relatively higher compared to that of healthy controls (HC) [5]. However, it is unknown if IgA subclasses are differently produced in the gut of lupus patients, and if their fecal IgA have higher nAg reactivity.

In the present study, we compared the abundance of pro-inflammatory marker calprotectin, permeability protein Zonulin, and total IgA and IgA-subclasses in cross-sectional stool samples from HC subjects and recent onset SLE patients. We also compared the nAg reactivity of fecal total-IgA and IgA-subclasses from stool samples of these groups. We found that, in addition to relatively higher levels of calprotectin and total IgA [5], fecal levels of Zonulin, and IgA1- and IgA2- subclasses are significantly higher in SLE patients. We also found that fecal IgA and its subclasses from SLE patients have significantly higher nAg reactivity compared to that of HCs. These observations suggest that fecal IgA features, nAg-reactivity in particular, could serve as valuable indicators of the systemic autoimmune process in lupus patients, and potentially, in at-risk subjects.

Materials and methods

Study subjects and samples. Cryopreserved stool sample aliquots, collected under the Institutional review board (IRB) approved protocols, were used in this study. Cross sectional stool samples from 21 adult SLE patients and 21 adult healthy controls were analyzed. Samples

were either from the patients with at least 4 of the 11 ACR revised criteria (for the Classification of SLE) or unaffected controls with no known personal or family history of autoimmune disease by self-report (for the classification of HC). The original IRB protocols excluded subjects who received systemic antibiotics or commercial supplemental probiotics. Those with chronic gastrointestinal disorders such as inflammatory bowel disease, irritable bowel syndrome, celiac disease, persistent, infectious gastroenteritis, colitis or gastritis, or chronic diarrhea by self-report were also excluded. All samples were from self-identified African American women.

Extraction and quantitation of inflammation markers and antibodies: Stool aliquots were weighed, suspended in proportionate volume of PBS (10% suspension or 100 mg feces/ml; w/v) by breaking the fecal material using pipette tip, high speed vortexing for 1 min, and followed by shaking at 800rpm for overnight at 4°C. These suspensions were centrifuged at 14,000 rpm for 10 min, upper 2/3 portion of the supernatants were separated and used in ELISA assays for Calprotectin, Zonulin, IgA, IgA1 and IgA2 levels. Initial optimization studies involved the determination of extraction buffer/stool ratio for preparing soluble fecal extracts, the optimum dilution of extracts required for accurate quantitation of various markers, and various other assay conditions. Calprotectin levels were determined in optimally diluted (1:20) extracts using Human- S100A8/S100A9 Heterodimer DuoSet-ELISA kit from R&D Systems. Zonulin levels were determined in similarly diluted extracts using human Zonulin Stool ELISA kit from ALPCO. For detecting IgA, IgA1 and IgA2, in-house sandwich ELISAs with purified, commercially available antibodies and standards for generating standard curves were employed.

For IgA, IgA1 and IgA2 sandwich ELISA, purified anti-mouse IgA, IgA1 and IgA2 monoclonal antibody (0.1μg/well in 50μl) coated wells were incubated with optimum dilution of samples for 2h. Wells were incubated further with biotin-linked polyclonal anti-mouse IgA, IgA1 and IgA2

antibody for 1h, and finally with streptavidin-HRP for 30 min, before developing the reaction using TMB/H_20_2 substrate and read at 450nm. Optimum dilutions of extracts were 1:3000 for IgA, 1:500 for IgA1, and 1:1000 for IgA2. Purified mouse IgA, IgA1 and IgA2 (Athens Research and Southern Biotech) were used in each plate for generating respective standard curves to calculate the concentration in 100mg stool samples. These values were then converted to respective antibody concentration per gram of stool with a multiplication factor of 10.

Determination of nAg reactivity titer of fecal antibodies: Antibody titers against nAgs (nucleohistone and dsDNA) in aforementioned fecal extracts were determined by employing in-house indirect ELISA[9, 13] with necessary modifications. Briefly, 0.5μg/well of nucleohistone (Sigma-Aldrich) or dsDNA from calf thymus (Sigma-Aldrich) was coated as antigen (carbonate buffer or DNA-coating reagent from Invitrogen respectively), overnight, onto ELISA plate wells. These wells were then incubated with serial dilutions of the fecal extracts (starting at 1:20 dilution) for 2 hours and followed by 1 hour incubation with biotin-linked anti-human IgA, IgA1, and IgA2 antibody. Streptavidin-HRP was added to the well for 30 min, before developing the reaction using TMB/H_20_2 substrate and the plates were read at wavelength of 450 nm. Known positive and negative control samples identified from initial screening were used in all plates to validate the results for determining a reliable titer. Highest dilution of the sample that produced an OD value of ≥0.05 above background value was considered as the nAg reactive titer of 100 mg feces. These titer values were then converted to per gram nAg reactivity titer with a multiplication factor of 10.

IFA staining of Hep2 cells to detect ANA. Kallestad Hep-2 Complete Kit (Bio-rad) was used to detect ANA reactive fecal IgA following the kit instructions. 1:40 dilution of fecal extracts was employed. Anti-human IgG-FITC from the kit was replaced with anti-human IgA-Alexa flour

488 reagent as the secondary antibody to detect fecal IgA antibody. Relative fluorescence intensities were graded arbitrarily on a 0-4 scale using a fluorescent microscope.

Statistical analysis: Mann-Whitney test using GraphPad Prism was employed to calculate *p*-values. A *P* value of ≤0.05 was considered statistically significant.

Results

Levels of fecal proinflammatory/permeability marker and IgA and its subclasses (IgA1 and IgA2) are higher in SLE patients. Extracts were prepared from frozen stool aliquots of 21 new onset SLE patients and 21 HCs and subjected to quantitative ELISA to determine the levels of calprotectin, zonulin, IgA, IgA1 and IgA2. As observed in **Fig. 1A**, protein levels of the pro-inflammatory marker calprotectin were relatively higher, albeit not significant statistically, in stool samples from SLE patients compared to HC. **Fig. 1B** shows that fecal levels of the gut permeability marker zonulin were significantly higher in SLE patient samples compared to HC samples. As reported before [5], total IgA levels were also significantly higher in fecal samples of SLE patients (**Fig. 1C)**. Importantly, the levels of both IgA subclasses, IgA1 and IgA2, were significantly higher in SLE patient samples compared to that of HCs (**Fig. 1D & 1E**). To assess if SLE patients, compared to HCs, show different degrees of IgA1 and IgA2 secretion in the gut, IgA1:IgA2 ratios were calculated for each sample. As observed in **Figure 1F**, although not significant statistically, IgA1:IgA2 ratios were relatively higher in SLE patients compared to controls. These results show that while the levels of overall IgA and both subclasses are higher in the stool samples of SLE patients, these patients appear to release relatively higher amounts of IgA1 than IgA2 into the gut lumen as compared to HCs.

Fecal IgA and its subclasses show greater nAg reactivity. To determine if fecal IgA of SLE patients have higher nAg reactivity, dsDNA and NH reactive IgA titers of extracts prepared from stool samples were determined by ELISA. As shown in **Figure 2A**, both dsDNA and NH reactive IgA titers were significantly higher in the samples of SLE patients compared to that of HCs. Further, Hep2 cell substrate-based immunofluorescence assay showed similarly higher nAg reactivity by fecal IgA of SLE patients **(Fig. 2B)**. Examination of the nAg reactivities of IgA subclasses revealed that the dsDNA and NH reactive IgA1 and IgA2 antibody titers are higher in SLE patients compared to HCs **(Figure 2C)**. Importantly, differences in the dsDNA and NH reactive titers of SLE patients and HCs were more pronounced with IgA1 than IgA2. Therefore, dsDNA and NH reactive fecal IgA1: IgA2 ratios were determined. As observed in **Fig. 2D**, nAg reactive IgA1:IgA2 ratios were significantly higher in samples from SLE patients than that from HCs. Overall, these results show that the nAg reactivity of fecal IgA is higher with SLE patient samples, and this is more pronounced with IgA1.

Zonulin levels correlate with fecal IgA abundance and nAg reactivity. Since the fecal levels of Zonulin were significantly higher in SLE patients compared to HCs, we examined if the abundance and nAg reactivity of fecal IgA correlate with the protein levels of this gut permeability protein in each group. Interestingly, while Zonulin levels in the fecal samples of both HC and SLE groups showed significant correlation with total IgA and IgA1 abundances (**Fig. 3A**) nevertheless, samples from SLE group showed an overall better correlation of Zonulin levels with both subclasses of IgA as well as total IgA levels. Importantly, while the Zonulin levels of samples from both HC and SLE groups showed significant correlation with dsDNA reactivity of total IgA, Zonulin levels of samples from SLE group, but not HC group, correlated significantly with nAg reactivity of IgA1. Overall, these observations suggest that Zonulin

dependent higher gut permeability, particularly in the proximal gut, of SLE patients, and the associated pro-inflammatory events could be contributing to the higher abundance and nAg reactivity of fecal IgA.

Discussion

Our recent studies have shown that in a mouse model of spontaneous autoimmune lupus, pro-inflammatory phenotype of gut mucosa at as early as juvenile age correlates with the gender bias observed in lupus-like disease[13, 28]. Pro-inflammatory events of the gut mucosa including higher gut permeability and microbial translocation have been implicated in systemic autoimmunity[15-19]. High levels of a pro-inflammatory factor, calprotectin were detected in the fecal samples of SLE patients[5]. On the other hand, a role of the gut permeability protein Zonulin, which is produced primarily in the small intestine, in autoimmune and inflammatory conditions has also been described[29-31]. However, whether Zonulin is released at higher levels in the gut lumen of SLE patients is not known. Our observations that Zonulin levels are significantly higher in the fecal samples of SLE patients, suggest that SLE patients, and potentially at-risk subjects as well, may have higher gut permeability due to the elevated levels of Zonulin protein and pro-inflammatory response including calprotectin production, which could be associated with this higher gut permeability.

Pro-inflammatory events of the gut mucosa can contribute to the production and release of antibodies, IgA particularly, in the gut lumen. Previous studies have shown that IgA production in the gut under normal and clinical conditions can be different [5, 32, 33]. While a recent report has shown that fecal IgA levels are higher in SLE patients compared to HCs [5], we reported that fecal IgA of lupus-prone mice have higher nAg reactivity long before the clinical disease onset [14]. In agreement with our previous report [5], we found that stool samples from majority of the

21 SLE patients tested in our study had higher amounts of soluble IgA than HCs. More importantly, we report that not only both IgA1 and IgA2 subtypes levels are higher in the fecal samples of SLE patients, but also IgA antibodies in general, and IgA1 in particular, have significantly higher nAg reactivity compared to that of HCs. Although it is not clear if higher fecal IgA or its nAg reactivity have any role in the systemic autoimmunity of SLE patients, IgA nephropathy has been reported in a subset of SLE patients [34]. Furthermore, circulating IgA+ plasma cells with mucosal phenotype have been detected in human SLE [35]. Therefore, it is possible that if higher gut inflammation and IgA production occurs in lupus-prone subjects at pre-clinical stages, as observed in the preclinical model [13, 28], then these IgA producing cells could be contributing to systemic autoimmunity in SLE.

IgA produced in the human intestine is comprised of two subclasses, IgA1 and IgA2 [24, 36]. While human serum contains predominantly IgA1, feces contains both IgA1 and IgA2 with higher amounts of the latter. This is because while plasma cells in the small intestine secrete mostly IgA1, the proportion of IgA2 production increases from the duodenum and this subclass is produced at profoundly higher levels in the colon[24, 25, 36]. Although higher B cell function and antibody production occurs in SLE patients, it is not known whether IgA1 and IgA2 subtypes are produced disproportionately in the gut mucosa of SLE patients compared to HCs. Our observations that while the overall abundances of both IgA1 and IgA2 are higher in the fecal samples of most SLE patients, IgA1:IgA2 ratio is higher in SLE patients compared to HCs suggesting a relatively higher activation of IgA producing B cells in the upper digestive tract of SLE patients, compared to the distal gut.

Autoantibody response against nucleic acids and nuclear proteins including histones is the key feature of lupus in humans and rodent models. Our observation that fecal IgA, both IgA1 and

IgA2 subclasses, of SLE patients have higher nAg reactivity suggests that IgA response to gut microbes could be contributing to the autoimmune process in SLE. Of note, IgA produced in the gut mucosa are mostly poly-reactive and previous studies have recognized some degree of self-antigen reactivity of fecal IgA and/or B cell clones from the gut mucosa of healthy individuals [37, 38]. Hence it was not surprising that fecal IgA of many HCs also showed nAg reactivity. Nevertheless, SLE patients have significantly higher nAg reactive fecal IgA than the HCs. Higher IgA production and nAg reactivity of fecal IgA, particularly IgA1, in SLE patients could also be possibly associated with higher gut permeability associated inflammation in SLE patients. Since IgA1 and Zonulin are produced mainly in the small intestine [25, 29, 30, 36], higher correlation of Zonulin levels with IgA and IgA1 abundance and nAg reactivity in SLE patients does support this notion. Of note, leaky gut has been linked to higher vascular deposition of IgA antibodies, leading to cardiovascular inflammation which could be inhibited using the Zonulin inhibitor peptide AT1001[39].

In conclusion, we found, for the first time, that both IgA1 and IgA2 levels are significantly higher in SLE patients compared to HCs. Most importantly, we also found that fecal IgA, both IgA1 and IgA2 subclasses, of SLE patients are more nAg reactive compared to that of HC subjects. Furthermore, fecal IgA1:IgA2 abundance and nAg reactivity ratios are higher in SLE patient samples compared to that of HCs. With respect to potential mechanisms, higher Zonulin-dependent gut permeability and the associated inflammation of proximal intestinal regions could be contributing to these higher fecal IgA and nAg reactivity in SLE patients. Our observations suggest that, in addition to the higher abundance and nAg reactivity of fecal IgA and its subclasses, particularly IgA1, in association with Zonulin levels could serve as a biomarker and be indicative of the severity of systemic autoimmunity in SLE patients. In this regard, it would

be informative to determine if fecal IgA and subclass features and Zonulin alter during active disease flares in SLE patients. Furthermore, although additional studies using longitudinal samples from subjects who are susceptible to SLE are needed, our current observations in the context of our preclinical study [14] suggest the potential biomarker value of fecal IgA features alone or along with Zonulin levels, in predicting the ongoing autoimmune process, if any, in at-risk subjects.

Figure legends

Figure 1: Levels of fecal proinflammatory/permeability marker, and IgA and its subclasses (IgA1 and IgA2) in HC subjects and SLE patients. Optimally diluted PBS extracts of stool samples from HC subjects (n=21) and SLE patients (n=21) were tested for protein levels of calprotectin **(A)**, Zonulin **(B)**, IgA **(C)**, IgA1 **(D)** and IgA2 **(E)** as described under materials and methods section. IgA1: 1gA2 ratios are also shown **(F)**. *P*=values by Mann-Whitney test.

Figure 2: nAg reactivity of fecal IgA and its subclasses. PBS extracts of stool samples from HC subjects (n=21) and SLE patients (n=21) were examined for nAg reactivity as described under the Materials and methods section. **A)** nAg reactivity of total IgA was determined with dsDNA and NH(nucleohistone) coated plates, 2-fold serial dilutions of the extracts, and anti-human-IgA-biotin as the secondary antibody. **B)** Hep-2 slides were stained using 1:40 dilution of fecal extracts and anti-human IgA-Alexa flour 488 reagent and imaged under a fluorescent microscope. Relative fluorescence intensities were graded arbitrarily on a 0-4 scale. Representative images (upper panel) and overall intensities (lower panel) are shown. **C)** nAg reactivity of total IgA was determined as described for panel A using anti-human-IgA1- or IgA2-

biotin as the secondary antibody. **D)** dsDNA reactive IgA1: 1gA2 ratios are shown. *P*=values by Mann-Whitney test.

Figure 3: Zonulin levels correlate with the fecal IgA abundance and nAg reactivity. A) Fecal IgA, IgA1 and IgA2 concentrations of samples from HC subjects and SLE patients were correlated with respective fecal Zonulin concentration values by Pearson's correlation approach. **B)** dsDNA reactivity titers of fecal IgA, IgA1 and IgA2 of HC subjects and SLE patients were correlated with respective fecal Zonulin concentration values by Pearson's correlation approach.

Conflict of Interest statement: Authors do not have any conflict(s) of interest to disclose.

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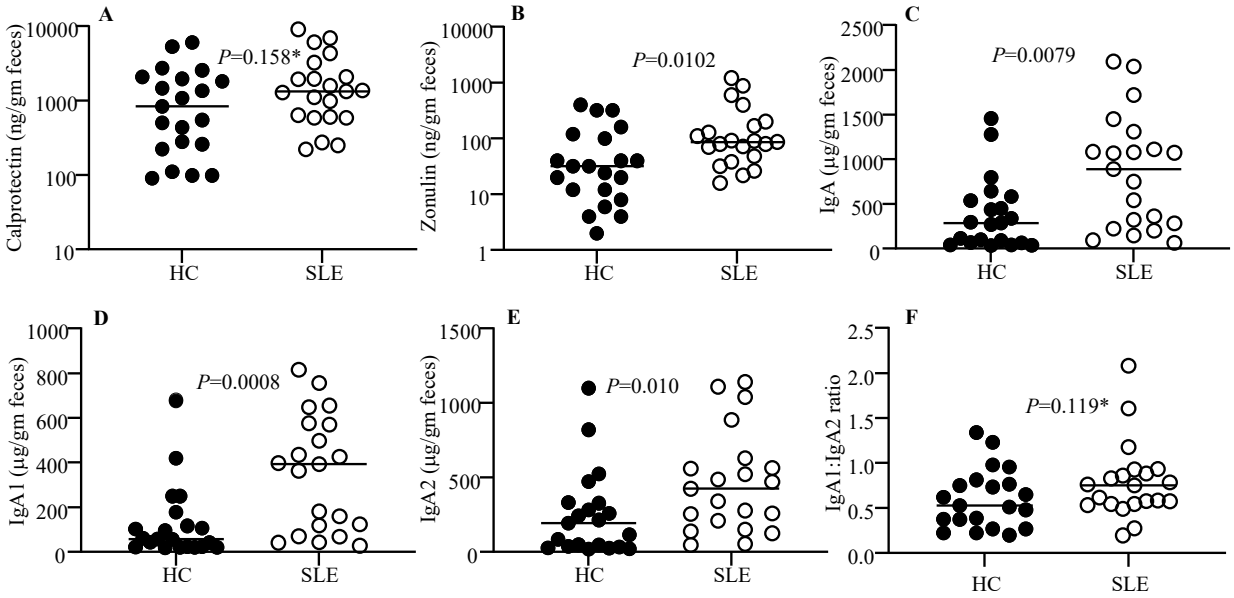
Figure 1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P=0.010
HC
SLE
F
IgA1:IgA2 ratio
2.5
2.0
1.5
1.0
0.5
0.0
P=0.119*
HC
SLE

Figure 2

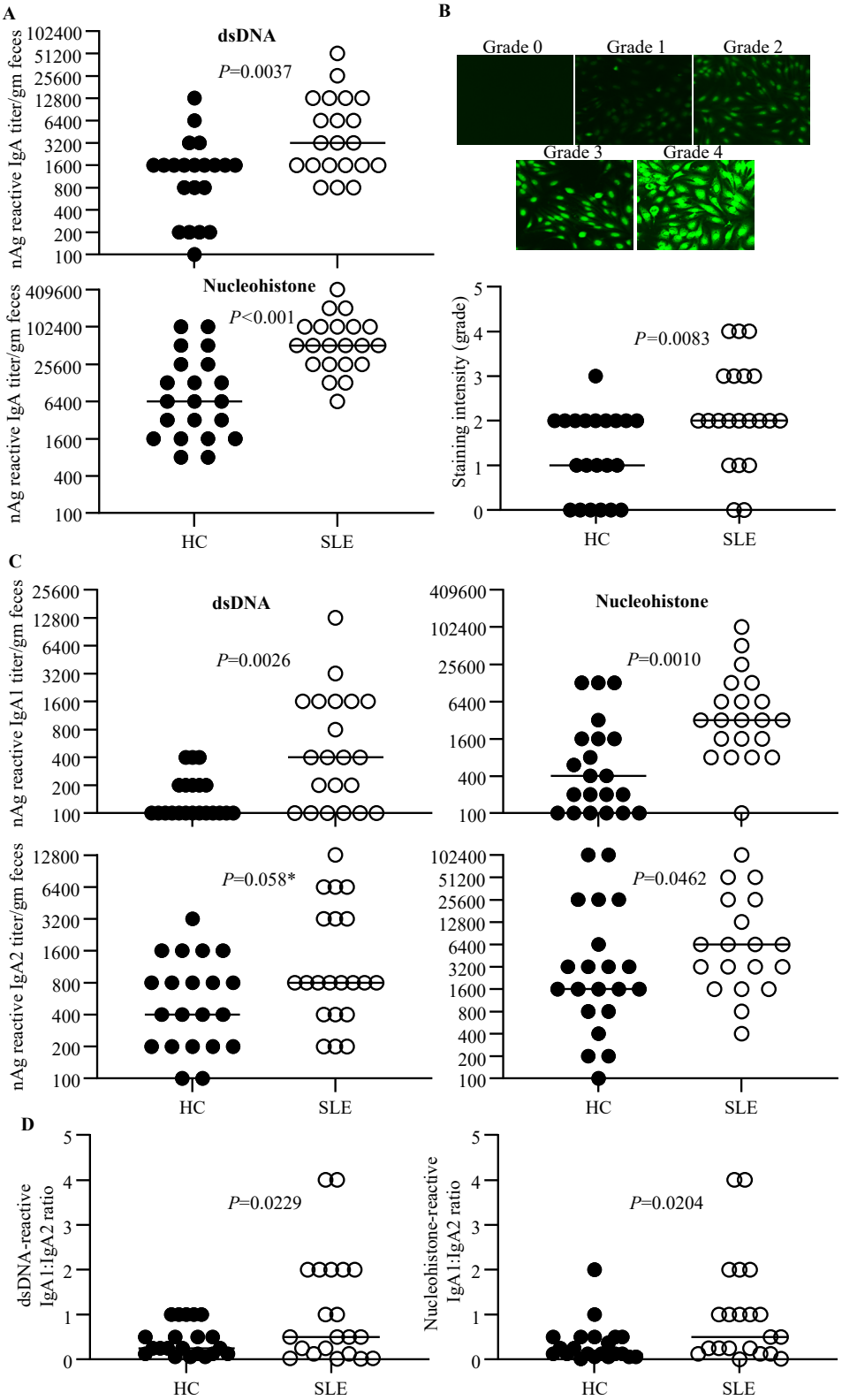
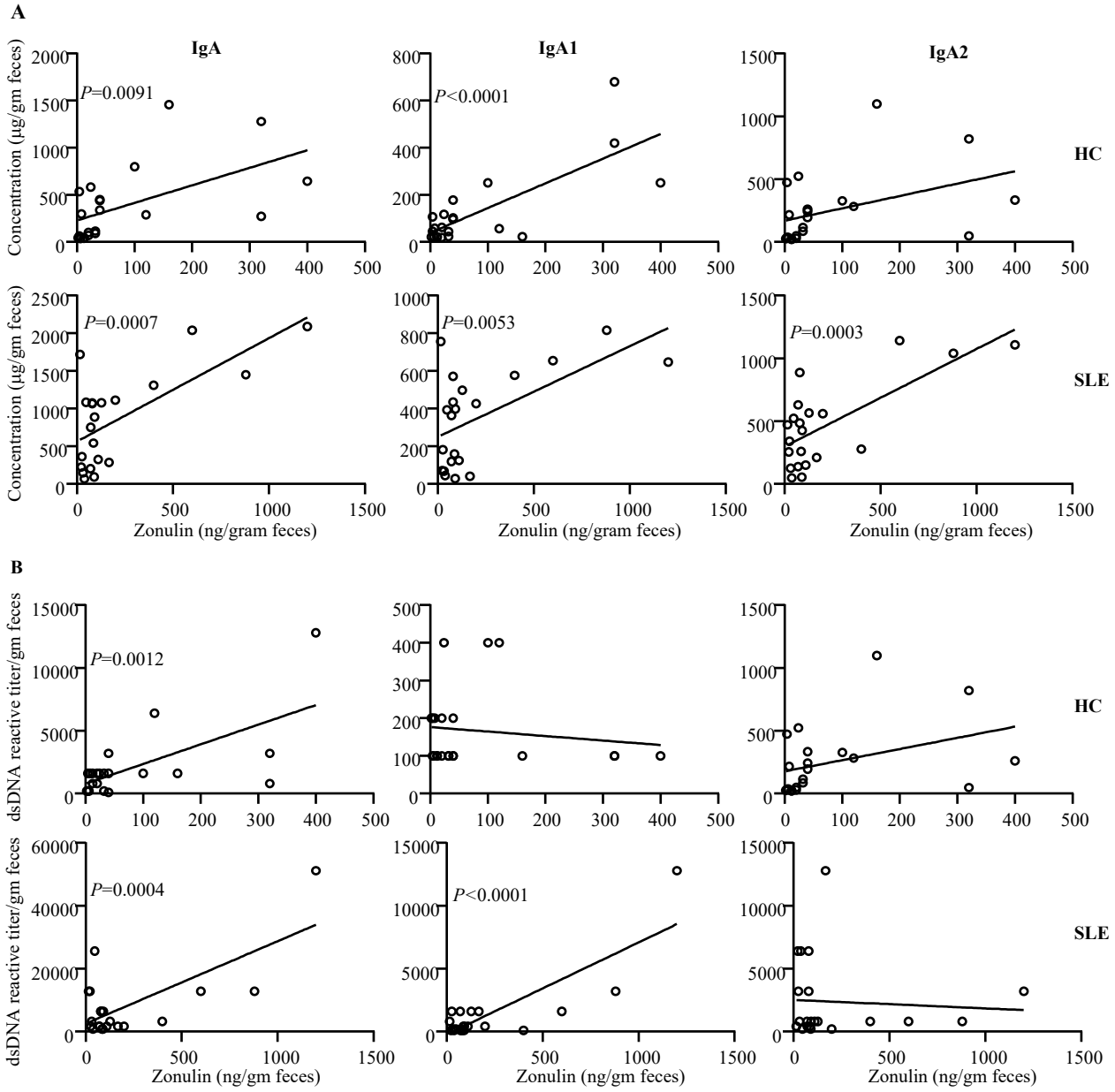

A
dsDNA
P=0.0037
nAg reactive IgA titer/gm feces
102400
51200
25600
12800
6400
3200
1600
800
400
200
100
Nucleohistone
P<0.001
409600
102400
25600
6400
1600
400
100
HC
SLE
B
Grade 0
Grade 1
Grade 2
Grade 3
Grade 4
Staining intensity (grade)
P=0.0083
C
dsDNA
P=0.0026
nAg reactive IgA1 titer/gm feces
Nucleohistone
P=0.0010
nAg reactive IgA2 titer/gm feces
P=0.058*
P=0.0462
D
dsDNA-reactive IgA1:IgA2 ratio
P=0.0229
Nucleohistone-reactive IgA1:IgA2 ratio
P=0.0204

Figure 3


A
IgA
IgA1
IgA2
HC
SLE
Concentration (µg/gm feces)
P=0.0091
P<0.0001
P=0.0007
P=0.0053
P=0.0003
Zonulin (ng/gram feces)
B
dsDNA reactive titer/gm feces
P=0.0012
P=0.0004
P<0.0001
Zonulin (ng/gm feces)