Longitudinal gut microbiome analyses and blooms of pathogenic strains during lupus disease flares

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ABSTRACT
Objective Whereas genetic susceptibility for systemic lupus erythematosus (SLE) has been well explored, the triggers for clinical disease flares remain elusive. To investigate relationships between microbiota community resilience and disease activity, we performed the first longitudinal analyses of lupus gut-microbiota communities.

Methods In an observational study, taxonomic analyses, including multivariate analysis of B-diversity, assessed time-dependent alterations in faecal communities from patients and healthy controls. From gut blooms, strains were isolated, with genomes and associated glycans analysed.

Results Multivariate analyses documented that, unlike healthy controls, significant temporal community-wide ecological microbiota instability was common in SLE patients, and transient intestinal growth spikes of several pathogenic species were documented. Expansions of only the anaerobic commensal, Ruminococcus (blautia) gnavus (RG) occurred at times of high-disease activity, and were detected in almost half of patients during lupus nephritis (LN) disease flares. Whole genome sequence analysis of RG strains isolated during these flares documented 34 genes postulated to aid adaptation and expansion within a host with an inflammatory condition. Yet, the most specific feature of strains found during lupus flares was the common expression of a novel type of cell membrane-associated lipoglycan. These lipoglycans share conserved structural features documented by mass spectroscopy, and highly immunogenic repetitive antigenic-determinants, recognised by high-level serum IgG2 antibodies, that spontaneously arose, concurrent with RG blooms and lupus flares.

Conclusions Our findings rationalise how blooms of the RG pathobiont may be common drivers of clinical flares of often remitting-relapsing lupus disease, and highlight the potential pathogenic properties of specific strains isolated from active LN patients.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Lupus nephritis (LN) is a serious illness, yet despite improvements in therapeutic interventions many patients progress to renal failure the causes of lupus flares are unknown.

WHAT THIS STUDY ADDS
⇒ In these first longitudinal studies, the gut communities in systemic lupus erythematosus patients, unlike those of healthy controls, were inherently unstable in composition over time.
⇒ Within lupus microbiota communities, intestinal expansions (or blooms) of several individual species occurred in different patients but not controls, and most common were blooms of Ruminococcus (blautia) gnavus (RG) that arose in more than 40% of LN patients during disease flares.
⇒ The R. gnavus strains from LN patients have genomes with genes that may provide competitive advantages contributing to bloom events, and these strains were distinguished by a novel highly immunogenic lipoglycan, implicated in interactions with the host, and induction of spontaneously arising high-level specific serum IgG-antibodies concordant with disease flares.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ An antibody biomarker assay for the novel RG strain-associated lipoglycan could be an aid to earlier diagnosis of LN, and better therapeutic decision-making, and therapeutic targeting of pathobiont strains could provide a means to treat LN flares without the immunosuppression inherent to all current treatments.

INTRODUCTION
The interconnected multidimensional commensal microbial communities that we harbour within us provide myriad functions critical for both nutrition and general health. Our commensals also serve as early sparring partners, essential for the development and maintenance of the most fundamental layer of cellular defenders from the innate and adaptive immune systems. Mounting evidence suggest that imbalances, termed dysbiosis, within gut commensal communities are common in patients with diverse inflammatory and autoimmune diseases (reviewed in Rosser and Mauri1).

Lupus is an archetypal systemic autoimmune disease associated often with inadequate clinical responses to treatment and recurrent flares of disease.2 Lupus nephritis (LN) is one of the most prevalent severe disease manifestations occurring in ~40% of patients.3 Patients with class III or IV LN have poor prognoses, and more than 40% of...
patients progress to end-stage kidney disease within 15 years of diagnosis.4–6

To search for a potential disease driver, in earlier cross-sectional studies of the gut microbiome, we characterised the communities within a female cohort with heterogeneous organ system involvement and clinical feature. We found direct correlations between systemic lupus erythematosus (SLE) Disease Activity Index (SLEDAI) and dysbiotic shifts associated with reduced community diversity and richness.7 In contrast, in health our gut communities exhibit remarkable dynamic stability. As the resilience of SLE microbiota communities over time has not previously been examined, in pilot studies we investigated the temporal stability of longitudinal faecal samples from 16 lupus patients and 22 healthy subjects. We here provide the first evidence of previously unsuspected features of the intimate relationships between dynamic microbiota instability in SLE and immune responses to blooms of a common pathobiont.

RESULTS
Gut dysbiosis is pronounced in patients with high disease activity

To study the gut microbial communities in sequential samples from disease-affected individuals, we examined 2–6 faecal samples from 16 individual patients, with 44 samples obtained over 24–291 weeks (online supplemental tables S1 and S2). For comparisons, we also evaluated 72 sequential samples from 22 healthy adult female control (CTL) volunteers. To assess the diversity within these bacterial communities, faecal DNA was used to generate 16S rRNA gene-amplicon libraries.

In these analyses, patients who fulfilled ACR criteria for renal involvement at any time8 were designated as LN (online supplemental tables S1A and S2A). Renal disease activity was designated inactive or active, based on urinary protein creatinine ratio (PCR)<0.5 or >0.5, respectively, as per the SLEDAI domain. The other patients were referred to as non-renal (online supplemental tables S1B and S2B). In continuity with our earlier microbiome studies,9 those with an overall SLEDAI score of >8 were designated as high disease activity, with others as low disease activity. All patients with multiple samples were included. Pilot studies demonstrated results for 16S rRNA libraries were highly reproducible based on principal coordinated analyses (online supplemental figure S1).

Reiterating our earlier results,9 high SLEDAI scores and active LN were both associated with decreased richness/diversity of communities compared with CTL subjects, reflected in several measures of α-diversity (online supplemental figures S2–S5). Medications did not correlate with differences in microbiome diversity (online supplemental table S4A and S4B).

Greater lupus disease activity is associated with greater community β-diversity

Based on principal coordinates analysis, communities from patients displayed significant differences overtime in β-diversity compared with CTL using Jensen-Shannon divergence dissimilarity metric (multivariant distance Welch’s W, p=0.001)9 (figure 1A). Furthermore, the communities associated with higher disease activity (eg, active LN in figure 1C, purple) was farther from the communities representing CTL individuals (figure 1C, red) than the distance between CTL and lupus with no LN (green) or inactive (blue). There were significantly greater differences for communities associated with high disease activity (p=0.001) (figure 1B), and patients with active LN (p=0.001) (figure 1C).

Instability over time is a common feature of lupus gut microbiota communities

We next assessed the stability of gut microbiota over time by comparisons of community-wide multivariate analyses in the multiple libraries from each of the 16 lupus-affected individuals and of 9 healthy subjects with multiple samples, without assumptions based on time-intervals. Using a proven approach for estimating averages within group dissimilarity,9 for each of the CTL subjects we found limited differences in overall composition among the sampled communities. In contrast, there was significantly raised level of variance between communities at different time points within an individual patient (Kruskal-Wallis analysis of variance, p=0.03) (figure 1D), which was found in both non-renal (two tailed, p=0.03), and renal (LN) groups (p=0.02) (figure 1D). However, we found no overall correlations between community variance; with disease duration, the periods between sample collection, the maximal disease activity, the range of disease activity in different visits (online supplemental tables S1 and S2), or the medications taken by individual subjects (online supplemental table S4). Notably, the variance within the renal and non-renal groups were not significantly different (p=0.379, NS), indicating that impaired community composition resilience is common in SLE.

There was no relationship between variance, a marker of microbiota instability, and the intervals between sampling (online supplemental figure S6). Taken together, these findings suggest the gut communities in lupus patients are inherently unstable overtime. We, therefore, next sought to investigate whether within these unstable community milieus there were specific bacterial species that underwent major dynamic shifts.

Dynamic blooms of individual bacterial species are common within lupus microbiota communities

In-depth analyses of the sequential microbiota libraries revealed that in several individual patients there were transient often pronounced blooms of amplicon sequence variants (ASV)-defined taxa within both Veillonella and Fusobacterium genera, which were not detected in any healthy subjects (online supplemental figures S7 and S8). Whereas a Veillonella genus bacterium can represent local gut outgrowths due to translocations from the oral cavity into the intestine, or even overt infections,10 the Fusobacterium genus are generally considered oral pathogens.11 Notably, there were neither temporal associations with Veillonella nor the Fusobacterium expansions with specific clinical features or organ involvement, nor with overall flares of lupus disease activity (online supplemental figures S7 and S8, online supplemental tables S1 and S2).

Ruminococcus gnavus blooms are concordant with lupus disease flare episodes

To follow-up our earlier findings,7 we investigated the abundance of Ruminococcus (blautia) gnavus (RG). A study of 16 samples showed RG abundance as determined by 16S rRNA amplimer analysis and by shotgun cloning were highly correlated (online supplemental figure S9). Consistent with an earlier estimate from a large population-based survey,12 we found a mean 0.15% RG abundance in the CTL libraries (online supplemental table S3). There was an overall trend for an increase in abundance of RG in SLE libraries that did not attain significance (Wilcoxon, p=0.076) (online supplemental figure S3A), while in many SLE samples, RG was undetectable or low abundance (online supplemental table S1A and S1A). However, there were marked increases in RG abundance in patients with high SLEDAI scores of 8 or greater (Wilcoxon, p=0.01) (online supplemental figure S5B), and RG abundance was increased in those with active
renal disease (Wilcoxon, p = 0.02) (online supplemental figures S5C and S8). Furthermore, when examined in a continuous distribution, there was a weak direct correlation between disease activity and RG abundance (Spearman, r = 0.320, p = 0.034). Overall, these findings reaffirmed associations between RG expansions within lupus microbiota communities in those experiencing active renal disease, although repeated studies of a limited patient set can evoke concerns about statistical analyses.

To investigate for temporal changes in RG abundance in these patients, we closely examined the sequential libraries of individual donors. These surveys included nine LN patients and the other seven SLE patients who never had renal manifestations but did have inflammatory arthritis, cutaneous disease and/or other disease features (online supplemental table S1A and S1B). For RG, we found a remarkable stability within the healthy female CTLs over time (online supplemental figure S8A). Among the 16 patients, 11 patients exhibited low or undetectable RG levels with limited variation over time (online supplemental figure S10B). Of these 11 patients, for patient S134 that had quiescent disease during our study (online supplemental table S1A), also had low or undetectable RG in all four time points sampled. Notably, while four LN patients had RG blooms during disease flares (figure 2) another five LN patients also displayed stable low RG abundance pattern despite episodes of active renal disease documented at one or more of the sampling time points.

**Figure 1** Dysbiosis and longitudinal instability in SLE microbiota communities compared with healthy individuals. (A) Principal coordinates analysis (PCoA) was used to estimate β-diversity between groups using the Jensen-Shannon divergence (JSD) dissimilarity metric. Commensal communities from SLE patients were heterogeneous, with most exhibiting significant distance variance from CTL (by multivariate distance Welch W test, p = 0.001). (B) Compared with CTL, variance in diversity was greatest within the SLEDAI high subgroup, and (C) the subset with active renal disease. (D) To compare the overall dynamics of shifts in faecal communities sampled overtime in different subjects, subject variances were computed based on JSD using the average multivariate dissimilarity estimation method reported in. Variance between these three groups was significantly different (Kruskal-Wallis ANOVA, p = 0.03). Lupus patients with a history of nephritis were assigned to the lupus nephritis (LN) group based on ACR criteria, whereas the patients in the non-renal group were without a documented history or laboratory findings of LN. Lupus patients have more unstable gut microbiota than healthy individuals. Variance of gut microbiota was significantly different in the renal lupus group, compared with healthy subjects (two-sided Mann-Whitney U test, p = 0.02). The non-renal lupus group was significantly different than the healthy subjects (p = 0.03). Notably, the overall variance in the renal group was not different from the non-renal group (p = 0.379, NS). ACR, American College of Rheumatology; ANOVA, analysis of variance; CTL, control subjects; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index.
sustaining not all LN flares are associated with RG blooms (online supplemental figure S8B), which could mean other, undetected, disease drivers are involved.

Strikingly, in four LN patients (S107, S47, S120 and S78) (~44% of the LN patients followed over time) dramatic changes in RG abundance were detected, with RG bloom reaching a mean of 9-fold higher abundance compared with other time point sample(s) from the same donor. Such an RG bloom, with temporal concordance with clinical disease flare, was also identified in patient (S61) who was without renal involvement but had inflammatory polyarthritis (figure 2A). When only the samplings from these five patients with an episode of RG bloom were examined, the level of disease activity significantly correlated with RG abundance (r = 0.320, p = 0.03). Of the seven SLE patients without renal involvement, S61 had a baseline RG abundance of 0.6% with a bloom to 4.6% at the subsequent evaluation, while all other non-renal SLE patients had RG abundance of at undetectable levels to at most 0.4% at different time point. These findings may rationalise that RG blooms can contribute to the clinical pattern of relapsing-remitting disease activity that occurs in many lupus patients despite close clinical monitoring and treatment.

**Isolation and characterisation of RG strains from LN patients at disease flare**

To more closely investigate the nature of the RG blooms documented in patients, we sought to characterise individual RG isolates from the LN patients. From samples of two active LN patients, S47 and S107, obtained at time of LN clinical flares with concurrent RG blooms, colonies were initially selected based on RG-specific 16S rRNA PCR-based assay. Analysis of the whole genome sequences from these 27 isolates from the LN patients documented that these represented independent non-identical RG strains. By multidimensional analyses these distributed into four groups (online supplemental figure S11), and the representative strains (S47-18, S107-48, S107-61 and S107-86) were selected for further analysis (figure 3, online supplemental figures S11 and S12).

**RG strain genomes from LN patients include genes implicated in inflammatory bowel disease pathogenesis**

Comparisons were performed between genomes of all currently known RG isolates, which included nine strains (RJX1120-RJX28) isolated from Crohn’s Disease patients, a form of inflammatory bowel disease (IBD), and two from antibiotic-treated infants (RJX1118, RJX1119) previously reported to together represent a separate clade. Yet by our analysis these IBD-associated strain genomes distributed throughout the branches of a dendrogram of all currently available RG genomes (figure 3B and online supplemental figure S10). Whereas the strains from donors, S107-86 and S107-48 were also not related to each other, the genomes of the S47-18 and S107-48 strains, although from different lupus donors, displayed a high level of relatedness (figure 3B, online supplemental figures S11 and S12).

Homology comparisons were performed with 199 genes previously reported to be uniquely associated with RG strains isolated from IBD patients but not healthy individuals. Based on a 70% overall sequence homology cut-off, we found 34 of these genes were represented in the genomes of one or more of these four LN RG strains. Indeed, all of the four LN RG strains included 21 of these genes (figure 4 and online supplemental table S5), and many were also assignable to a KEGG orthologue and a putative protein function (online supplemental table S5). All of these LN RG strains contain an anti-oxidant peroxiredoxin gene (PanPhlAn identifier xg001425), postulated to provide a competitive advantage in a host affected by oxidative stress from increased generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which affects both host cells and
 Highly relevant, a recent metabolomics report found that increased serum ROS and RNS levels distinguish LN patients from non-renal SLE and healthy CTL. Further, both the S107-48 and S47-18 RG strains from LN donors contain orthologues of a putative RG PTS sugar transporter and α-L-fucosidase (xg000037 and xg000038), which have been implicated in catabolism of host mucin that is a component of the intestinal barrier, although alone these factors are not currently considered sufficient to cause intestinal injury. Yet we found that the type strains, RG1 and RG2 also share 24 of the 34 above-mentioned IBD-associated genes (figure 4 and online supplemental table S5). Taken together, the LN strains have genes postulated to facilitate better adaptation to an inflamed host, including LN patients, which could contribute to a competitive advantage with other intestinal bacteria. However, these IBD-associated genes were also found in RG strains from IBD, and in healthy donors. While these analyses may rationalise a driver for RG expansions, our results suggesting these particular genes are more commonly distributed in different strains (figure 4 and online supplemental table S5) than previously reported.

Conserved structural features of lipoglycans from three independent RG strains

We, therefore, turned our attention to determine which RG strains produced anon-protein antigen, which had been identified based

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**Figure 3** Whole genome sequencing of *Ruminococcus (blautia) gnavus* (RG) isolates from two lupus patients in clinical flare. Type strain RG2 as well as isolates from patients, S47 and S107, were sequenced and de-novo assembled, while an assembly for RG1, designated *) was obtained from NCBI RefSeq. (A) Long-read assemblies of six RG isolates. From inside to outside, circular tracks show average GC content across 1 kbp windows (black line); assembled contigs (grey ring sections); locations of 1 kbp windows with BLAST hits to type strain assemblies, RG1 (ATCC 29491) and RG2 (CC55_001C); and gene start sites as predicted by Prokka, coloured by strand (+: red, −: blue). (B) Complete-linkage hierarchical clustering of the gene content (orthogroup presence/absence) of newly sequenced RG isolates and publicly available assemblies. Newly sequenced isolates and the type strains RG1 and RG2 are shown in blue. LG producing strains are not in a single cluster. NCBI, National Center for Biotechnology Information.
on recognition by serum IgG-antibodies in a large proportion of patients with active renal disease. In immunoblotting studies, we documented serum antibody reactivity with diagnostic oligobands in extracts of three of the four RG isolated from LN patients, while these antigenic oligobands were not detected in any of the strains from IBD, infants or a healthy adult (figure 5).

In surveys of the structural features of these LN-associated glycans, mass spectrometry (MS) detected very similar overall spectra of the glycan species purified from three independent RG strains (RG2, S107-86 and S47-18) (figure 5A–C). From each of these glycans, an acyl-glycerol lipid anchors was identified, and hence these were then referred to as lipoglycans (LGs). The 100 most abundant mass signals, in the range of 2500–5000 Da, were identified, and depicted as a heatmap (figure 5D) of the ion clusters. A calculated similarity score (figure 5E), indicated very high structural similarity between the LG of these three RG strains ($p<2 \times 10^{-12}$).

The most abundant molecular species had an average mono-isotopic mass of 3632.645 Da (figure 5A–C), which was interpreted as an LG comprised of three fatty acids with acyl chain composition of 47:0, one glycerol, eight hexoses, five N-acetyl-hexosamines and three hexuronic acids. The difference between the major di-acylated LG with a mass of 3394.415 Da (in RG2; calc. mono-isotopic mass: 3394.407 Da; di-acyl LG 31:0) (figure 5A) can be explained by acyl compositions of 31:0, which differed by one less palmitic acid (16:0). The major mono-acylate species in an LG had a 16:0 acyl composition (with 3170.204 Da; mono-isotopic mass: 3170.193 Da; mono-acyl LG 16:0). The strains RG2 and S107-86 also contain a mono-acyl LG carrying a margaric acid (17:0) that was observed at 3184.217 Da in RG2; mono-isotopic mass: 3184.208 Da; mono-acyl LG 17:0) (figure 5A,B). In addition, independent of the acylation status, we also detected LG variants with up to six additional hexoses detected, which were most prominent within the tri-acyl LGs of the RG2 strain (figure 5A, online supplemental table S6).

To gain further insights into the composition of the glycolipid anchor structure, we used the earlier described de-O-acyl glycan (there named de-O-acyl LG3)9 for MS2-experiments. Analysis of the doubly charged ion of the core de-O-acyl LG (calc. mono-isotopic mass: 2931.963 Da) was interpreted as indicating the presence of a glycerol–hexuronic acid unit that can also include two or more additional hexoses (online supplemental figure S13).

These MS analyses enabled the formulation of a structural model of the LG species (figure 5F), which covers all abundant MS signals of these RG strains. The assembled LG has a central core structure consisting of 18 carbohydrate moieties, and 1 glycerol with 1–3 fatty acids, which are affixed to the cell wall via these lipids that directly attach to the cell membrane. Additional hexose sugars are attached to the core glycan, which result in further microheterogeneity. Taken together, these studies document the great conservation of structural features of the LGs from these different RG strains, including from unrelated LN donors, with only minor structural variation (figure 5).
LN serum and murine monoclonal antibodies recognise conserved non-protein antigens in RG strains

We, therefore, sought to determine whether the LG of RG strains expressed cross-reactive antigenic determinants. Immunoblots were performed with the serum of a representative active LN patient, S47, obtained during clinical flare (figure 6A–E) and...
we documented whether IgG-reactivity was either present or absent in protease-treated extracts of a large panel of RG strains (figure 6A). In the RG strains, S107-48, S107-86 and S47-18, from the two LN patients, there were IgG-reactive oligobands of the same 20–30 kDa distribution. In contrast, there was neither reactivity with the RG1 type strain from a healthy donor, nor with the nine RG strains from IBD patients, and nor the two RG strains from antibiotic-treated infants (figure 6A–C).

To further evaluate the relatedness of LG antigens, immunoblotting was also performed with the LG purified by hydrophobic interaction column fractionation from the lupus S47-18 strain, which had oligobands with the same apparent MW as those detected in the extracts of the lupus strains, S107-48, S107-86 and S47-18 (figure 6A,B, online supplemental figure S14). Strikingly, preincubation with LG purified from RG2 strain of this same lupus sera resulted in complete inhibition of reactivity with oligobands in the RG extracts, and in the LG purified from the lupus RG strains, while the reactivity of a protease-sensitive (ie, unrelated protein) band in the RG1 strain was unaffected (figure 6D,E). Cumulatively, antigenically related non-protein antigens, attributed to a novel LG were common in LN RG strains but not in the RG strains from IBD patients.21 22

To independently investigate the antigenic diversity of different RG strains, murine monoclonal antibodies (mAbs) were generated by bacterial immunisation with purified RG LG (see the Methods section). The clonally distinct mAbs, termed 33.2.2 and 34.2.2 (online supplemental figure S14), were reactive with purified RG LGs, recognised the same MW non-protein oligoband antigen in extracts of S107-48, S107-86, S47-18 and RG2 strain as LN sera, but were non-reactive with a large panel of unrelated purified bacterial glycans (online supplemental table S7).

High-titre serological responses to RG LGs in SLE with high RG intestinal abundance and disease activity

To investigate the host immune response to the expansion of RG strains overtime, we studied available longitudinal sera from three lupus patients (S47, figure 7A–D; S61, figure 7E–H; and S78, figure 7I–L), including at the time of clinical LN flare (see figure 2C). By bead assay, serum IgG-reactivities were assessed for binding interactions with RG2 strain extract (figure 7A, E, I), with comparisons with purified RG2 strain LG7 (figure 7B, F and J). The near identical reactivity patterns of whole bacterial extracts and the purified LG, confirmed the high immunogenicity of the LG component within the RG2 bacterial extract. Comparable reactivity patterns were documented with the structurally related LG from the S47-18 strain (S47-18 LG) obtained from the S47 LN donor (figures 5A,B and figure 6D,E), although for this LG there were uniformly stronger binding interactions (as IgG-binding curves for S47-18 LG were shifted due to greater reactivity (figure 7C, G and K). By contrast, there was little or no detectable reactivity with the LPS glycan from a Pseudomonas species (figure 7D, H and L). Cumulatively, these data reveal high-titre lupus host LG-specific serum antibody reactivities, with highest detected levels in lupus patients, S47 and S61, at the time of disease flare, which was also concordant with an RG bloom in abundance. For patient S78, limited variations in antibody titres were detected overtime, and there was substantial clinical disease activity at all but one visit (figure 7J, J and K).

Notably, these represented high-level titres, detectable in excess of 1:10 000 dilution (figure 7). These antibody responses were overwhelmingly of the IgG2 subclass (figure 7M,N). In addition, to develop a quantitative immunoassay we used the antibody genes from—a monoclonal 33.2.2 anti-LG antibody (online supplemental figure S15) to generate a chimeric anti-LG (online supplemental figure S16), to evaluate IgG anti-LG levels in serum samples in LN patients in a CTL group of previously reported LN trial (figure 7O).23 although microbiome data were
Systemic lupus erythematosus

Figure 7  Serum IgG antibodies to lupus RG strain LGs, parallel gut community abundance and RG blooms with concordant disease flares. To investigate the lupus host immune response overtime to colonisation with RG strains, we studied IgG anti-RG responses longitudinally obtained sera from three lupus patients in detail. (A–D) Depicts IgG responses for patient S47. (E–H) Depicts IgG responses of patient 61. (I–L) Depicts responses of patient S78. (A, E, I) Depicts serum IgG-reactivity with whole bacterial extract of RG2, the index RG strain first identified immunogenic LG.7 (B, F, J) Reactivity with the purified LG from this same RG2 strain (termed RG2 LG) showed very similar reactivity patterns, confirming LG within bacterial extracts is highly immunogenic. (C, G, K) Reactivity with the structurally related purified LG from the S47-18 strain (S47-18 LG). Here, the same relative reactivity patterns are seen, with uniformly stronger levels of IgG-binding curves for purified S47-18 LG. (D, H, L) By contrast, the lipopolysaccharide (LPS) glycan from a Pseudomonas species, displayed little or no IgG reactivity. In each panel, reactivity with serum from a representative healthy female control is shown (CTL). Samples obtained over time are indicated with a sequential designation. Studies were performed with custom bead-based MBI array (Luminex), with previously described methods.7 (M) Strong IgG2 predominance of serum human IgG subclass-specific reactivity with purified RG2 LG, detected by ELISA. RG bloom group are from patients patients; S47 (LN), S78 (LN), S120 (LN) and S61 (polyarthritis without renal involvement)(see figure 2). Representative data are for available sera the 7 non renal patients, at visit associated with highest disease activity. Mann-Whitney two-tailed test. (N) There is a strong correlation between total IgG reactivity and IgG2 subclass restricted serum anti-LG responses, for samples also depicted in panel M (see the Methods section). (O) A quantitative immunoassay that uses a monoclonal chimeric 33.2.2 recombinant antibody standard (see online supplemental figure S15) was used to reevaluate samples from the previously described cross-sectional studies included healthy control (HC) (N=5) and non-renal SLE samples (N=10).7 LN with RG blooms (documented by microbiota analysis), using samples with the highest values, for patients designated SLE61, SLE78 and SLE047. (P) Longitudinal responses of select sets of sera in a longitudinal ACCESS study.23 All assays were performed with serum diluted 1:2000. Where relevant, patients are listed by public identifier from the ACCESS report.23 These samples were obtained at predefined intervals over a period up to 2 years, and we found that 3/8 representative (public identifiers 813123, 641924, 665783) LN patients had anti-LG antibody levels equivalent to those with documented RG blooms (see O). Binding activity of serum IgG antibody to R. gnavus lipoglycan immobilised onto commercial paramagnetic immunoassay beads. Values were deduced by interpolation of serum MFI units into the standard curve depicted in online supplemental figure S16. Results were confirmed in assays repeated at least twice. For these longitudinal samples, microbiota data were unavailable. ACCESS, Abatacept and Cyclophosphamide Combination Therapy for Lupus Nephritis; CTL or HC, healthy female control; LG, lupus nephritis; MFI, mean fluorescence intensity; RG, Ruminococcus (blautia) gnavus; SLE, systemic lupus erythematosus.
Systemic lupus erythematosus

not available. By this approach, IgG antibody levels were detected in human serum samples over a broad range (ie, >10,000 fold difference in activity, −10^3 U/ml to 10^7 U/mL) (figure 7O). The longitudinal samples were obtained at predefined intervals over a period up to 2 years, and we found that 3/8 (public identifiers 813123, 641924, 665783) of these LN patients had anti-LG antibody levels equivalent to those with documented RG blooms, and in several patients detectable serum levels changed substantially over time (figure 7P). Based in part on the magnitude of these responses, we postulate that host immune responses to the novel RG lupus strain-associated LG glycan may contribute to immune-complex mediated pathogenic pathways implicated in lupus disease flares.24

**DISCUSSION**

Here, we report the first longitudinal gut microbiome studies in SLE, which demonstrated temporal instability within these gut microbiota communities, from which RG blooms arose concordant with clinical disease flares (figures 1 and 2). Notably, instability, which is defined by the level of community variance over time, was unrelated to the number of samples from a donor, the timespan of sampling, or the features of the disease activity. This instability was also neither correlated with maximum SLEDAI disease score at sampling, nor span from lowest to highest SLEDAI score in that individual. Impaired gut community stability has not previously been considered. Temporal microbiota instability also did not appear to correlate with the medication ingested, which suggests that community instability could be a feature inherent to this systemic autoimmune disease.

Whereas in healthy subjects the pathobiont, RG was consistently documented at stable low abundance over time, in 5 of the 16 lupus patients under investigation, also representing 4/9 of those with documented active LN, dynamic RG blooms were strikingly concordant with periods of raised disease activity. In all but one case, RG blooms were manifest as flares of renal disease. In the sole patient with an RG bloom without a history of renal involvement (S61), the bloom occurred during a disease flare with active inflammatory arthritis involving multiple joints (online supplemental table S2B). We speculate that susceptibility for specific clinical features during RG blooms reflects in part differences in genetic susceptibility of the patient.25

In other diseases, at most only infrequent concordance between specific host-associated microorganisms and pathology has been reported.26 By contrast, evidence of RG expansions in patients with active SLE, and especially LN, has now been found in three US cohorts along with cross-sectional surveys of serum IgG anti-LG studies,2 a Scandinavian cohort,27 a large untreated cohort in China28 and a French cohort.29 The linkage between RG and SLE is also remarkable as RG bloom-associated disease flares occurred in individuals of diverse race and ethnicity (online supplemental table S1).27 Although there are reports of independent studies of the microbiome of lupus patients in which RG expansions were not found, these other reports were underpowered with much smaller cohorts and were also hobbled by including few (or no) patients with substantial disease activity, and of LN in particular.10,30,31

Variations in gut commensal strain genomes are known to at times result in important physiological and functional differences in how microbes interact with the host. In fact, akin to our findings, different strains of the same species have been reported to evoke different host immune responses.28 Indeed, we found that the LN RG strains shared genes previously reported to provide competitive advantages within anaerobic bacterial communities in an inflamed host.35

Marine intestinal colonisation studies have shown that the pathologic potential of RG strains in SLE patients is tied to gut-barrier function,33 and subclinical disturbances in intestinal permeability, found in other conditions,34 may be common in lupus patients.35 Relevant, free transplants from mice with lupus-like glomerulonephritis into germ-free mice are reported to induce an inflammatory autoimmune condition,36 reiterating earlier findings reported for a murine gut pathobiont isolate.37 Notably, in vivo murine colonisation with the LG-expressing RG strains, S107-48 and S47-18, from LN patients, which express the LG previously shown to include potent TLR2 stimulatory activity,38 was also shown to induce severe impairment of the intestinal barrier.39 Neonatal colonisation with the S107-48 strain led to RG translocation into draining mesenteric lymph nodes, and induction of serum IgG anti-DNA antibodies at levels proportional to the induced functional impairment of the gut barrier.40 This may be a fundamental principle, as more recently serum IgG-antibodies in IBD and rheumatoid arthritis patients have been shown to recognise gut commensals, presumably based on similar mechanisms.38,39

RG is a member of the Lachnospiraceae family that plays pleotropic roles in host metabolism and immunity (reviewed in Vacca et al40). Significantly, while our knowledge of the diversity among RG strains is still limited (figure 3B), our examination of nine RG strains from IBD patients found no detectable LG (figure 6A–C), although these studies are far from exhaustive. Hence, the expression of cell wall associated LG distinguished the strains in the blooms in LN from IBD-associated RG strains. We hypothesise that the high-titre circulating anti-LG antibody response in SLE patients, which correlates with anti-DNA antibody levels, and with complement degradation,7 contributes to the systemic and renal pathogenesis of SLE. However, as it remains a possibility that there are patients with Crohn’s disease, or other immune-mediated diseases, that are colonised with RG strains that produce LG, further investigations are warranted.

Our studies document key structural features of the RG LG that display remarkable conservation between different LN-derived strains (figure 5). Cultivation conditions may affect LG production, as only the LGs earlier described as B-series were observed in the current study.7 In any case, immunoblotting of both the earlier LG preparation of strain RG2, and the new LG preparations from RG2, S107-86 and S47-18 strains displayed same reactivity patterns with both LN serum and murine monoclonal IgG-antibody binding interactions.

Whereas bacterial LGs have been little explored, LGs from Corynebacterium glutamicum lipomannan (CgLM)41,42 and the LM from Mycobacterium tuberculosis (reviewed in Mishra et al43) share structural features with the LG observed in RG strains, but there are also distinct features. In CgLM, the glycolipid anchor consists of a diacylglycerol (GroAc) with a glucuronic acid (GlcA) and a mannose (Man) residue attached, and Man GlcAGroAc2 has been isolated as a major glycolipid from C. glutamicum cells.44 In the current study, the LG of RG strains had a very similar anchor structure, but also had a third fatty acid. While the lipoteichoic acid (LTA) of Lactobacillus plantarum, L. casei and Streptococcus lactis also have triacylated glycosylglycerolipids as glycolipid anchors,45 these contain neither hexuronic acids nor the N-acetyl hexosamines found in the RG LG. Hence, as the LG we purified and characterised, so far in three RG strains, has an unique structure with many distinctive features. The genes encoding for the molecular enzymatic pathways responsible for its assembly merit further...
clarification, as this might facilitate experiments to test the influences of these glycans on the pathogenicity of RG strains using gene interference.

The limitations of our single-centre observational pilot study included, only 16 SLE patients and 22 healthy subjects were studied over time, and that samples were collected during routine clinical care. Hence, the true occurrence of disease flares with temporally linked RG blooms could have been underestimated, and we could not assess the duration of bloom persistence. In these patients with established disease, we were unable to identify when such anti-pathobiont antibody responses first arose, and how long they may persist. Sequential serum samples time-matched to faecal samples with RG blooms were only available for three subjects. We did study additional LN patients from the ACCESS trial for up to 2 years and here also found a subset with highly elevated anti-LG antibody levels (Figure 7P), although matched microbiome data were unavailable. Hence, longer duration fixed interval sampling studies should be conducted in the future. Hence, longer duration fixed interval sampling studies should be conducted in the future. Whereas medications could have effects on the metabolism and proliferation of commensal species, we excluded patients on cytotoxic agents or antibiotics, and whenever possible samples were obtained before modification of treatment regimen. While all patients received hydroxychloroquine, and many also received prednisone (online supplemental tables S1 and S4), these most commonly used medications in SLE are reported to have little or no effect on the human gut microbiome. As we sought to assess general composition of microbiota communities in each faecal sample, in the initial overview of this new total data set, we treated each data point in the longitudinal study as independent. In some individual patients, we found great variations between some points, and therefore, statistical concerns regarding non-independence were not considered. The design of the current studies, which was inadequate to disentangle the potential influence of diet or OTC medications or prior antibiotic exposure, and we could not consider whether gut microbiota instability predates clinical disease.

In conclusion, an individual RG strain can express different specific types of bacterial glycans, which include the novel LG expressed by lupus strains. Whereas certain RG strains from IBD patients are reported to produce a pro-inflammatory capsular poly-glucorhamnan polysaccharide that is a TLR4 agonist, and these include RJX1120, RJX1121, RJX1123 and ATCC 29149 (RG1). Yet, we found these same IBD strains, as well as RJX1119, RJX22 and RJX24-1128 that are reported to have a capsular polysaccharide were devoid of the LG produced by the LN strains (Figures 4 and 6). Indeed, the LG and the poly-rhamnose capsular polysaccharide are structurally very different (Figure 5) and. Due to the high structural differences of these two glycopolymers very different immunogenicity is very likely, and cross-reactivity unlikely. The genes encoding the enzymatic pathways responsible for the LG assembly merit further clarification. So far it is unclear if they are unique to the LN strains or if (a) central genetic component(s) is/are absent or have been rendered inactive in the non-LG producing strains.

In contrast, other RG strains have been reported to produce a protective or ‘tolerogenic’ capsular polysaccharide postulated to be associated with a separate gene cluster, that conveys homeostatic benefits to the host, although the structure of this putative polysaccharide is currently undefined. Antibodies specific for either of these polysaccharides have not been described. We were unable to isolate a LG from RG1, that was also devoid of the immunoreactive LG oligobands (Figure 4). Disease flares could, therefore, in part be influenced by ecological shifts within a microbiota community that includes a range of RG strains, which differ based on their genomic gene sets and properties of expressed glycans that can contribute to pathogenic potential. With regard to LG producing RG strains, preliminary studies have found a distinct blood transcriptomic profile in SLE patients with RG blooms concurrent with disease flares, that may be relevant to LN pathogenesis.

From a broader perspective, we propose that such RG expansions in a lupus patient may represent a previously unrecognised form of post intestinal-bloom autoimmunity clinical syndrome. In many ways, these RG bloom-associated SLE flares resemble the postinfectious autoimmune syndromes; acute rheumatic fever and post streptococcal glomerulonephritis, which are caused by certain strains of Streptococcus pyogenes, a commensal residing upon mucosal and cutaneous barriers. Yet, we found no evidence that an affected SLE patient is directly symptomatic of an RG gut bloom, as these bacteria, sequestered in the intestine, are not known to cause local tissue injury, and colitis is uncommon in SLE. As for possible roles in SLE pathogenesis, the deposition of IgG2 antibodies in glomeruli has been previously proposed as a specific signature of LN, and we now demonstrate that high-titre IgG2 antibodies commonly arise against a strain-associated novel glycan on an LN-associated gut pathobiont. Further investigations are needed to assess for cross-reactivity with other self-antigens, including those in glomeruli.

We speculate that in a major subset of patients, LN flares are intertwined with intestinal blooms of certain RG strains that may be the cause and the consequence of host systemic inflammation from gut leak of bacterial agonists for innate immune receptors. The origins of the lupus gut dysbiosis, and the specific contribution(s) to autoimmune pathogenesis merit further investigation.

### MATERIALS AND METHODS

Under the supervision of the NYU Institutional Human Subjects Committee, adult females meeting ACR criteria for SLE, were recruited at NYU with exclusion/inclusion criteria as previously described between October 2013 and February 2019. Visit frequency was dictated by clinical need. All patients providing more than one faecal sample were included (see online supplemental materials for further details).

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Systemic lupus erythematosus

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