Expression of Cyclic GMP-AMP Synthase in Patients With Systemic Lupus Erythematosus

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cGAS Expression in Patients with Systemic Lupus Erythematosus.

Short title for running head: cGAS in SLE

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ABSTRACT

OBJECTIVES
Type I interferon (IFN-I) is implicated in the pathogenesis of Systemic Lupus Erythematosus (SLE) and ‘interferonopathies’ such as Aicardi-Goutieres Syndrome. A recently discovered DNA-activated IFN-I pathway, cyclic GMP-AMP (cGAMP) synthase (cGAS) is linked to AGS and mouse models of lupus. The aim of this study was to determine whether the cGAS pathway contributes to IFN-I production in SLE patients.

METHODS
SLE disease activity was measured by SELENA-SLEDAI. cGAS and interferon stimulated gene (ISG) mRNA expression was quantified by quantitative PCR. cGAMP levels were monitored by Multiple Reaction Monitoring with Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS).

RESULTS
cGAS expression in peripheral blood mononuclear cells (PBMC) was significantly higher in SLE patients compared to normal controls (n=51 and 20 respectively, p<0.01). cGAS expression and the IFN score were positively correlated (p<0.001). cGAS expression in PBMC showed a dose response to IFN I stimulation in vitro, consistent with it being an ISG. Targeted measurement of cGAMP by MS/MS detected cGAMP in 15% (7/48) of SLE patients but not in any of the normal (n=19) or rheumatoid arthritis (n=22) controls. Disease activity was higher in SLE patients with cGAMP versus those without cGAMP detected.

CONCLUSION
Increased cGAS expression and cGAMP in a proportion of SLE patients indicates that the cGAS pathway needs to be considered as a contributor to IFN-I production. Whereas higher cGAS expression may be a consequence of exposure to IFN-I, detection of cGAMP in patients with increased disease activity indicates potential involvement of this pathway in disease expression.
INTRODUCTION

The type I IFNs (IFN-I) are strongly implicated in the pathogenesis of systemic lupus erythematosus (SLE) based on the following key findings: a) two thirds of SLE patients have a blood interferon (IFN) signature; b) gene variants that enhance IFN-I production are increased in SLE; c) therapeutic administration of IFN-I for virus infection or cancer induces SLE in a small percentage of patients and d) preliminary findings indicate that IFN-I receptor blockade by a biologic leads to improvement in clinical parameters of SLE. How, where and when IFN-I is initially stimulated in lupus patients is uncertain. In vitro studies, including our own (3), have shown that IFN-alpha (IFN-α) is induced by immune complexes (IC) containing (ribo)nucleoprotein antigens (4, 5). However: i) Crow and Niewold have shown that the serum from a significant proportion of SLE family members without autoantibodies induce Interferon stimulated genes (ISGs) in responder cells (6) ii) data from clinical trials using biologics targeting IFN-α led Petri and colleagues to suggest that other IFNs may well be involved (7) iii) microarray analysis of skin from SLE (and dermatomyositis) patients revealed that the interferon stimulated gene (ISG) signature was correlated with IFN-β and IFN-γ but not IFN-α (8). Finally, Chiche et al (9) reported that complex IFN signatures in SLE are not restricted to the IFN-α signature but might also involve IFN-β and IFN-γ. These findings strongly suggest that other interferon pathways are activated, especially prior to the production of high affinity IgG autoantibodies.

Type I IFNs include 13 IFN-α proteins, one IFN-β, and several less explored members including IFN-ε, IFN-κ, IFN-τ, and IFN-ω. IFN-β can be produced by almost any cell following stimulation by (viral) nucleic acids. Release of this cytokine serves to prime or amplify IFN-I by other cells, especially plasmacytoid dendritic cells (pDCs) that are the main producers of IFN-α. Monogenetic disorders associated with aberrant production of IFN-I and SLE-like features (‘interferonopathies’) revealed that responsible mutations activate pathways that induce IFN-β rather than IFN-α (10, 11). This observation is consistent with the idea that elevated levels of IFN-I other than IFN-α can contribute to SLE pathogenesis. Similar observations have been made in mouse models including one mouse model of lupus (12, 13).

Cytosolic nucleic acid sensors play a crucial role in the detection of pathogens that have breached membrane barriers (14). Amongst the most important cytosolic DNA sensors recently identified is Cyclic GMP-AMP synthase (cGAS) (15, 16). Binding of double stranded DNA (dsDNA) to cGAS causes a conformational change in the active site of cGAS, which utilizes ATP and GTP to synthesize the cyclic dinucleotide, cGAMP (16, 17). cGAMP then functions as an endogenous second messenger binding directly to the adapter protein, ‘stimulator of interferon genes ‘(STING), culminating in the activation of the transcription factor, interferon regulatory factor 3 (IRF3) and synthesis of IFN-β (18). Given the central role played by IFN-I in the pathogenesis of SLE, we examined cGAS expression and cGAMP production in SLE patients. Whereas increased cGAS expression could be secondary to exposure to IFN-I, detection of cGAMP in a subset of SLE, especially those with high SLEDAI, suggests that the cGAS pathway is activated and may contribute to disease in some SLE patients.

METHODS

Patients and Statistical Analysis.

Patients who fulfilled the American College of Rheumatology (ACR) criteria for SLE and gave informed consent to participate were included in this study. Samples were...
collected in compliance with the Declaration of Helsinki. Ethical approval was granted by the University of Washington (Approval number 39712). The history, classification criteria, laboratory testing and damage accrual data were measured using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI)(20). At the time of their clinic visit, whole blood samples were collected and disease activity measured using the SELENA revision of the Safety of Estrogens in Lupus Erythematosus National Assessment–SLEDAI score (SELENA-SLEDAI)(21). The SELENA-SLEDAI measures disease activity (range 0–105) within the preceding 10 days and includes 24 clinical and laboratory variables weighted by organ system. Damage accrual was evaluated using the SDI which assigns a numerical value to organ da(20)mage regardless of attribution. ACR criteria positive rheumatoid arthritis (RA) (22) were used as an inflammatory control population.

Three cohorts of SLE patients were studied: an initial cGAS test cohort (Cohort I), where stored cDNA that had been obtained from 51 SLE patients and 20 normal controls were tested for cGAS expression by quantitative PCR (qPCR); an initial test cGAMP cohort (Cohort II, where cells from freshly obtained peripheral blood mononuclear cells (PBMC) from SLE, RA and normal controls (n=17, 10 and 9 respectively) were tested for cGAMP by Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC MS/MS) and a third cohort (Cohort III) where cGAS and cGAMP were examined in the same SLE patients (total number tested = 48 SLE, 22 RA and 19 normal controls). Some cohort II patients were included in Cohort III, depending solely on at least 25 million cells being available for study.

Quantitative real-time PCR (qPCR) for cGAS and ISGs.
Total RNA was isolated from PBMC using the RNeasy mini kit with a DNase treatment step (Qiagen, Valencia, CA). cDNA was generated using 100 ng RNA with the high-capacity cDNA RT-kit using random primers (Applied Biosystems, Foster City, CA). Reactions in duplicate were run on an ABI StepOne Plus using the primers: cGAS: Forward '5'-GAAGAAACATGGCGGCTATC-3'; Reverse :5'-TGAGGGTTCTGGGTACATACG-3'; 18S: Forward, 5'-GAGGGAGCCTGAGAAACGG-3', Reverse, 5'-GTCGGGAGTGGTAATTGC-3'. A two-stage cycle of 95°C for 15 s and 60°C for 1 min was repeated for 40 cycles followed by a dissociation stage. The ‘IFN score’ was calculated from the combined expression of three ISGs (Mx1, CXCL10 and PKR) as described (23). Threshold cycle values were set as a constant threshold at 0.2, and fold changes in gene expression were then calculated using the 2^(-ΔΔCT) method.

cGAMP purification and detection
PBMCs were lysed with 1ml of 80% methanol spiked with 5nM heavy isotope-labeled cGAMP (cGAMP*), containing ^12C, ^15N-perlabeled AMP, as internal standard. Cell extracts were sonicated on ice for 1 min with 20% duty cycle and 1 output settings. Cell debris was pelleted at 14,000rpm for 10 minutes. Methanol extraction solution was transferred to a new tube and evaporated using a speed vac. cGAMP was further purified by Solid phase extraction column (OASIS WAX column from Waters) and re-suspended in 50ul OPTIMA LC/MS water (Thermal Scientific, Odessa, TX) for Mass Spec analysis. For targeted detection of cGAMP, a Multiple Reaction Monitoring (MRM) assay was developed on Waters Xevo TQS Mass Spectrometer coupled with Ultra-Performance Liquid Chromatogram (UPLC).
the assay, two transitions of each target ion were monitored: cGAMP, +675.1/152.1 (parent ion/daughter ion) and +675.1/136.0; cGAMP*, +690.0/152.1 and +690.0/146.0.

Sequencing of TREX1
Mutation screening of TREX1 was performed by polymerase chain reaction (PCR) amplification of genomic DNA segments (sequences available on request) and direct sequencing of the products using BigDye terminator chemistry and a 3130 DNA sequencer (Applied Biosystems) as described previously (24). Mutation description is based on the reference complementary DNA (cDNA) sequences NM_033629 for TREX1.

Statistical Analysis
Categorical variables were compared using Fischer’s exact test, mean data was compared using a t-test or the Wilcoxon Sum-Rank test for normally or non-normally distributed data respectively. Correlations were performed using linear regression. P values less than 0.05 were considered statistically significant.

RESULTS
cGAS expression is increased in a proportion of SLE patients and expression is responsive to IFN-I.
To determine levels of cGAS expression in SLE patients, we quantified cGAS mRNA expression by qPCR using cDNA collected from sequential blood samples from patients at the UW clinic (Cohort I). In this first cohort of SLE patients (n=51) and normal controls (n=20), cGAS expression was significantly higher in SLE patients (the mean relative expression of cGAS in healthy controls was 0.77, SD 0.31, versus 1.17 SD 0.62 in SLE, p=0.004)(Fig. 1A). Almost half of the SLE population (46.9%) had cGAS expression more than 1 standard deviation above the value in healthy controls, and 26.5% were more than two standard deviations above normal. As an inflammatory disease control, we quantified cGAS expression in RA patients. Interestingly, PBMCs obtained from RA patients had lower expression levels of cGAS compared to SLE (Supplementary Figure 1).

Although cGAS does not feature as a highly induced ISG in SLE (9), there is at least one report that cGAS is an ISG (25). To determine whether the increase in cGAS could be explained as a secondary consequence of IFN-I production, we analyzed the correlation between cGAS and the IFN score, a composite score derived from expression of 3 ISGs (Mx1, CXCL10 and PKR). Expression of cGAS and the IFN score were significantly correlated (Fig. 1B).

To clearly determine whether, in fact, cGAS expression could be influenced by exposure to IFN-I, we exposed PBMC isolated from normal controls to increasing concentrations of IFN-α. Following exposure to IFN-α, cGAS expression increased in a dose responsive manner (Fig. 1C) similar to the ISG, CXCL10 (Fig. 1D). Of interest, whereas a marked increase in cGAS expression was observed in some healthy individuals following incubation with IFN-I, PBMC from other normal controls responded in a more limited fashion indicating considerable individual variation (Fig. 1E and F). Overall, these findings suggests that, in SLE patients, cGAS could be elevated as a consequence of exposure to IFN-I and/or that expression of cGAS may be induced by an unknown DNA stimulus, which then primes for enhanced IFN-I responses in a positive feedback cycle. Cytosolic DNA binding to cGAS induces a conformational change and dimerization of cGAS leading to synthesis of the cyclic dinucleotide, cGAMP (16, 17). To determine whether IFN-I induced expression of cGAS...
(without activation by DNA) was capable of inducing expression of cGAMP, we stimulated 4 healthy donors’ PBMC with IFN-α and examined cGAMP production by Mass Spectrometry. We could not detect cGAMP in IFN-I stimulated PBMC from these 4 healthy donors (Supplementary Figure 2), supporting the concept that cGAS must be activated by DNA in order to synthesize cGAMP.

Detection of cGAMP in SLE PBMC
Since increased cGAS expression was insufficient to induce cGAMP, we asked whether cGAS was enzymatically active in SLE patients and, by inference stimulated by ligand. We used mass spectrometry to detect the cyclic dinucleotide, cGAMP, in PBMC in two additional cohorts (cohorts II and III) of SLE patients. cGAMP was extracted from PBMC and the cyclic dinucleotide identified by mass spectrometry using Multiple Reaction Monitoring (MRM) with heavy isotope-labeled cGAMP as internal standard. In the initial optimization of the methodology, we detected cGAMP in DNA transfected (Fig. 2A) (as well as HSV infected, not shown) THP1 cells as has been reported by others (15). When PBMC from 48 SLE, 22 RA and 19 normal controls were tested by the same methods, we detected cGAMP in 7/48 (15%) but in none of the samples from RA or normal controls (Fig. 2B). Of these 7 patients, 5 made samples available for DNA sequence analysis. No TREX1 mutations were detected in these 5 patients.

Patients with detectable cGAMP have higher SLEDAI Scores
To determine what clinical characteristics might distinguish patients with detectable cGAMP, we examined clinical, serologic and treatment variables in SLE patients with (cGAMP+) and without (cGAMP-) cGAMP (Table 1). We first examined duration of disease as we postulated that activation of the cGAS pathway may be an early abnormality that primes cells such as pDC for IFN-α responses. However, we observed no differences in disease duration in cGAMP+ and cGAMP- patients (10.57 versus 12.58 years respectively, p=0.623).

We have shown that antimalarial drugs can attenuate cGAS activation (26). To determine whether drug therapy could explain suppression of cGAMP, we compared the use of hydroxychloroquine, corticosteroid and other therapies. There was a similar proportion of patients prescribed hydroxychloroquine (100 versus 84%, p=0.32), prednisone (57 versus 53%, p=1.00), with comparable dosing regimens and mycophenolate mofetil (28.6 versus 26.8%, p=0.78) in cGAMP+ versus cGAMP- patients respectively. There was a numerically higher proportion of cGAMP+ patients with a history of cyclophosphamide exposure, but this did not reach statistical significance. We also explored severity of disease by examining the SLEDAI and SDI in each patient. Disease activity was significantly higher in cGAMP+ compared to cGAMP- patients (SLEDAI = 7.00±5.09 versus 3.52±2.78 respectively, p=0.0102). Damage, measured by SDI was similar in the 2 groups (0.71 ±1.49 versus 0.95 ±1.14, p=0.624). Regarding specific organ involvement, there were no statistically significant differences between the frequency of active renal, neurologic or articular disease between cGAMP+ and cGAMP- patients. Two (29%) cGAMP+ but no cGAMP- patients had active cutaneous disease at the time tested. There were no distinguishing serological abnormalities demonstrated in cGAMP+ versus cGAMP - patients. The groups were comparable in terms of age and ethnicity. There were no overt infections in the cGAMP positive population. There was a higher proportion of males expressing cGAMP which was not statistically significant with only 4 males in this sample.
DISCUSSION
Aberrant IFN production has long been implicated in SLE pathogenesis and disease activity. As discussed above, recent results suggest that the IFN signature observed in PBMC of SLE patients may not solely be a response to IFN-α suggesting that other Type I and also Type II IFNs may play a role as immune adjuvants in SLE and related systemic autoimmune disorders. Although cGAS appears to be a prominent, if not the dominant cytosolic DNA sensor responsible for inducing IFN-β in response to infections by a variety of pathogens (18), its role in human autoimmune diseases has not been studied in any detail. Here, we describe for the first time, evidence of cGAS/cGAMP activation in human SLE. We show a modest increase in cGAS transcripts in almost half of SLE patients (46.9%) compared to healthy controls. We demonstrate that while such an increase could be explained by a response to IFN-I since cGAS is a weakly induced ISG, we could also detect the product of cGAS enzymatic activity, cGAMP, in ~15% of SLE patients indicating ligand activation of cGAS in a subset of patients.

While there was a significant correlation between cGAS expression and the IFN-I score in the PBMC of SLE patients suggesting a cause effect relationship, it was of interest that even in a normal control population, there was considerable variation in the cGAS transcript in response to IFN-α. This was not simply explained by the rate of response as the differential response was seen at both 4 and 24 hrs. Whether such variation is due to regulation of cGAS itself or variation in IFN-α receptor and downstream signal transduction pathways remains to be determined. This would be particularly interesting to explore in SLE patients where genetic variants in Tyk2 and IRF5 have been observed (27). Also, we noted that, on average, cGAS expression was lower in RA PBMC compared to SLE. Whether this reflects the often contrasting cytokine profiles in these diseases (28) or drug therapy, remains to be determined.

An important question is why cGAMP was detected in only 15% of SLE patients? The large numbers of cells required and relatively small cGAMP signal observed in a minority of patients could be explained by the fact that cGAMP is rapidly destroyed by phosphodiesterases so that detection may require generation of very high levels of cGAMP. Consistent with this hypothesis, patients with detectable cGAMP had significantly higher SLEDAI scores. In this scenario, cGAS-cGAMP stimulation of IFN-β and other pro-inflammatory cytokines generated by activation of IRF3 and NFkB (18) may be a contributory cause of inflammation and disease activity. Since IFN-β primes cells to enhance IFN-α production (29), we queried whether cGAMP may be more readily detected in recent onset SLE patients prior to the multiple downstream effects caused by IgG containing immune complexes containing nucleoprotein antigens that activate pDC and neutrophils as well as other immune cells (30, 31). However, we observed that this was not the case and that there was no association between cGAMP detection and disease duration. Patients with SLE have changes in the relative proportions of cells in PBMC. We cannot therefore exclude the possibility that some of the findings observed in this study are due to alterations in cell proportions. However, since cGAMP was not detected in any of the normal or diseases controls and was only detected in 7 of a total of 48 SLE patients tested, indicates that it is unlikely that this biologically meaningful signal is explained by alterations in cell subsets. Nevertheless, it will be of considerable interest to determine which cell type(s) synthesize cGAMP as this may provide a clue to its genesis.
Another key question is what activates cGAS to produce cGAMP in the subset of cGAMP+ SLE patients? Amongst the many possibilities that could explain this association are chronic infection with DNA viruses that have long been implicated in SLE (32), endogenous retrovirus DNA that are proposed to activate cGAS in mice (33) or oxidized mitochondrial DNA that stimulates inflammatory cytokines in a STING dependent manner (31). We cannot, however, exclude the possibility that cGAMP generation is a consequence of uptake of nucleoprotein from tissue damage and is therefore a consequence, rather than a cause, of inflammation. It is relevant to note that even when DNA from dying cells is taken into endosomal compartments, DNA can enter the cytosol and activate cGAS-cGAMP as in DNase II deficiency (13).

While almost nothing is known regarding cGAMP expression in human autoimmune disorders, there is clear evidence that the cGAS-STING pathway is implicated in diseases with features of lupus in murine models. Deficiency of the 3/-5’ DNA exonuclease, Trex1 in mice (Trex1 KO) leads to an autoimmune myocarditis and a mild lupus-like systemic autoimmunity (34). Disease is markedly attenuated in Trex1 KO mice that lack the IFN-I receptor as well as when the mice are rendered STING deficient (34). Furthermore, Trex1 KO mice that are deficient in cGAS are fully protected from disease (12, 13). In a second mouse model where DNase II deficiency leads to profound inflammation and autoimmunity, deficiency of cGAS protected the mice from disease (13). It is relevant to point out that 1–2% of SLE patients have mutations in TREX1 which are thought to contribute to the lupus-like syndrome (35, 36). However, we did not detect TREX1 mutations in 5 of the 7 patients from whom DNA was available.

We demonstrate increased SLE disease activity in a small subset of patients who have activation of the cGAS/cGAMP pathway. These results will need to be examined in larger numbers of patients in whom cGAS, cGAMP, IFN-β, ISGs and other cytokines are prospectively evaluated. We have recently reported that in addition to the inhibition of TLR stimulation, antimalarial drugs such as hydroxychloroquine and quinacrine attenuated cGAS production of cGAMP and IFN-β (26). Although this effect remains to be shown in vivo, our results lend support to the theory that antimalarial drugs provide therapeutic benefit not only by attenuating TLR activation (37) but also by interfering with the cGAS-STING pathway in some patients with SLE.
Figure Legends

Fig 1. Increased expression of cGAS in SLE patients and cGAS response to IFN-I.
Peripheral blood mononuclear cells (PBMC) were obtained from 20 healthy controls (CNT) and 51 SLE patients. A. cGAS mRNA expression was analyzed by qPCR and the results expressed relative to the 18S mRNA. Horizontal bars represent the mean values. B. Correlation between cGAS mRNA expression as calculated in A and the IFN score in the SLE patients. The IFN score was calculated from the average expression of three ISGs (Mx1, CXCL10 and PKR). C and D. PBMC were obtained from normal controls and incubated with Type I interferon (IFN-I) at the doses shown for 4 hours. cGAS expression (C) and CX-CL10 expression (D) was quantified by qPCR and the relative expression normalized to 18S mRNA. cGAS relative expression in different normal controls in response to IFN-I stimulation at 4 (E) and 24 (F) hours. In A, groups were compared by a t test and in B, by linear regression analysis.

Fig 2. cGAMP is detected in a proportion of SLE patients. A. To optimize conditions for detection of the cyclic dinucleotide, cGAMP, THP1 cells were transfected with herring testis (HT)-DNA. After 16 hr, cGAMP was isolated from THP1 cells by a methanol extraction procedure as described in Methods. The abundance of cGAMP was quantitated by mass spectrometry using multiple reaction monitoring (MRM). B. PBMC (20 x 10⁶) obtained from patients with SLE (n=48), RA (n=22) or healthy controls (n=19) were subject to the same isolation procedures as in A. The presence of cGAMP was measured by mass spectrometry using MRM. Heavy isotope-labeled cGAMP was spiked into each sample as an internal standard and the mass spectrum of an internal standard (dotted vertical arrow) was used to determine the peak of the endogenous cGAMP. The X axis shows the retention time of cGAMP daughter ion (+675.1/136.0; And +690.0/146.0 for internal standard cGA*) and the Y axis shows the relative intensity of the cGAMP mass spectrum signal.
TABLE 1. Clinical characteristics of SLE patients with (positive) and without (negative) cGAMP detected in PBMC.

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<th>Positive * (N=7)</th>
<th>Negative* (N=41)</th>
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<td>Gender, male (%)</td>
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<td>Age, years (SD)</td>
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<td>39.58 (14.84)</td>
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<td>Disease duration (SD)</td>
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<td>3.52 (2.78)</td>
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<td>Anti dsDNA positivity (%)</td>
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<td>29 (70.73)</td>
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<td>196.27 (280.99)</td>
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<td>0.71 (1.49)</td>
<td>0.95 (1.14)</td>
<td>0.62</td>
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SLEDAI=SLE Disease Activity Index.
* Number (and percentage)
**= Statistically significant.
References


Fig 1. Increased expression of cGAS in SLE patients and cGAS response to IFN-I.

194x198mm (300 x 300 DPI)
Fig 2. cGAMP is detected in a proportion of SLE patients.