



The cGAS/STING Pathway Detects *Streptococcus pneumoniae* but Appears Dispensable for Antipneumococcal Defense in Mice and Humans

Juan Sebastian Ruiz-Moreno,^a Lutz Hamann,^b Lei Jin,^c Leif E. Sander,^{a,d} Monika Puzianowska-Kuznicka,^{e,f} John Cambier,^g Martin Witzernath,^{a,d,h} Ralf R. Schumann,^b Norbert Suttrop,^{a,d,h} Bastian Opitz,^{a,d} CAPNETZ Study Group

^aDepartment of Internal Medicine/Infectious Diseases and Pulmonary Medicine, Charité–Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

^bInstitute of Microbiology and Hygiene, Charité–Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

^cDepartment of Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, University of Florida, Gainesville, Florida, USA

^dGerman Center for Lung Research (DZL), Germany

^eDepartment of Human Epigenetics, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

^fDepartment of Geriatrics and Gerontology, Medical Centre of Postgraduate Education, Warsaw, Poland

^gDepartment of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA

^hCAPNETZ Stiftung, Hannover, Germany

ABSTRACT *Streptococcus pneumoniae* is a frequent colonizer of the upper respiratory tract and a leading cause of bacterial pneumonia. The innate immune system senses pneumococcal cell wall components, toxin, and nucleic acids, which leads to production of inflammatory mediators to initiate and control antibacterial defense. Here, we show that the cGAS (cyclic GMP-AMP [cGAMP] synthase)-STING pathway mediates detection of pneumococcal DNA in mouse macrophages to primarily stimulate type I interferon (IFN) responses. Cells of human individuals carrying *HAQ TMEM173*, which encodes a common hypomorphic variant of STING, were largely or partly defective in inducing type I IFNs and proinflammatory cytokines upon infection. Subsequent analyses, however, revealed that STING was dispensable for restricting *S. pneumoniae* during acute pneumonia in mice. Moreover, explorative analyses did not find differences in the allele frequency of *HAQ TMEM173* in nonvaccinated pneumococcal pneumonia patients and healthy controls or an association of *HAQ TMEM173* carriage with disease severity. Together, our results indicate that the cGAS/STING pathway senses *S. pneumoniae* but plays no major role in antipneumococcal immunity in mice and humans.

KEYWORDS innate immunity, cGAS, STING, *Streptococcus pneumoniae*, pneumonia, type I interferons, single-nucleotide polymorphism, human

Pneumonia kills more children under 5 years of age than any other disease worldwide, causing approximately 2 million deaths annually (1), and probably even more in elderly as well as immunocompromised individuals. *Streptococcus pneumoniae* is a leading cause of community-acquired pneumonia (CAP) (2, 3). This bacterium alone caused an estimated 826,000 deaths and 14.5 million cases of serious illness globally in 2000 (1). A better understanding of the bacterial interaction with the host, the antibacterial immune response, and individual risk factors might help in the development of novel preventive and/or therapeutic approaches to lower the high morbidity and mortality associated with *S. pneumoniae*.

The interaction of *S. pneumoniae* with the host usually starts with the asymptomatic

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Address correspondence to Bastian Opitz, bastian.opitz@charite.de.

colonization of the upper respiratory tract. Pneumonia only develops when pneumococci get access to the lower respiratory tract by aspiration and resist prompt elimination by the immune system (2, 3). The innate immune system relies on so-called pattern recognition receptors (PRRs) expressed by macrophages, dendritic cells, and other pulmonary cells to sense infections (4, 5). These PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and cytosolic DNA sensors (4, 6, 7). Among those, TLR2, TLR9, NOD2, NLRP3, AIM2, and Mincle have been identified to mediate inflammatory responses and antibacterial defense during pneumococcal pneumonia (8–18). In addition, we recently demonstrated that a STING-dependent cytosolic DNA-sensing pathway activates type I interferon (IFN) responses to *S. pneumoniae* infection in murine macrophages (19).

STING is both an adapter molecule mediating cytosolic DNA recognition and a direct receptor for the bacterial cyclic dinucleotides (CDNs) cyclic-di-AMP (c-di-AMP) and cyclic-di-GMP (c-di-GMP) (20–23). In the STING-dependent cytosolic DNA sensing pathway, cyclic GMP-AMP (cGAMP) synthase (cGAS) has been identified as the primary receptor of microbial and endogenous DNA (24). cGAS directly binds to double-stranded DNA (dsDNA) and subsequently catalyzes the production of the second messenger 2'3'-cGAMP, which in turn activates STING (25).

Recent studies found that *TMEM173*, the gene encoding STING, is polymorphic (26, 27). The most common allele besides the wild-type (WT) allele *R232* is *HAQ TMEM173*, which contains a haplotype comprised of three nonsynonymous single-nucleotide polymorphisms (SNPs), *R71H-G230A-R293Q*. Ectopically expressed HAQ STING exhibited diminished capability to activate type I IFN production in response to endogenous and bacterial CDNs or *Listeria monocytogenes* infection compared to that of WT STING (26, 27). In contrast, the frequent *R232H* SNP impairs activation by bacterial CDNs but not by endogenous 2'3'-cGAMP (27, 28). In addition, rare gain-of-function mutations in *TMEM173* have been associated with autoinflammatory syndromes (29, 30).

Here, we demonstrate that the cGAS/STING axis detects *S. pneumoniae* infection, and that this sensing is attenuated in cells of individuals carrying *HAQ TMEM173* but not *R232H TMEM173*. Experiments in a mouse model of pneumococcal pneumonia as well as analyses aimed at elucidating a potential association between *HAQ TMEM173* or *R232H TMEM173* and pneumococcal pneumonia indicate, however, that the cGAS/STING pathway is largely dispensable for antipneumococcal defense in mice and humans.

RESULTS

Type I IFN production during pneumococcal infection is dependent on the cGAS-STING pathway. In order to assess the relevance of STING for the induction of type I IFNs upon *S. pneumoniae* infection, bone marrow-derived macrophages (BMDMs) derived from WT and *Tmem173*^{-/-} mice were infected with *S. pneumoniae* or a capsule-deficient isogenic mutant or were stimulated with pneumococcal DNA. In confirmation of previously published gene-silencing experiments from our group (19), type I IFN induction in response to *S. pneumoniae* or bacterial DNA was impaired in STING-deficient cells (Fig. 1A and B). Moreover, expression of the IFN-stimulated gene *Ccl5* was likewise dependent on STING (Fig. 1C). We next investigated the involvement of cGAS in *S. pneumoniae*-induced type I IFN responses. We found that short interfering RNA (siRNA)-mediated silencing of cGAS reduced type I IFN responses to pneumococcal infection or stimulation with *S. pneumoniae* DNA (see Fig. S1 in the supplemental material). Furthermore, subsequent experiments using BMDMs from *cGas*^{-/-} mice showed that cGAS deficiency leads to a strongly reduced type I IFN induction upon *S. pneumoniae* infection or DNA stimulation (Fig. 1D and E). As expected, however, *cGas*^{-/-} cells were not attenuated in responding to cGAMP. Moreover, expression of *Ccl5* was also reduced in cGAS-deficient cells after pneumococcal infection (Fig. 1F). To elucidate the relevance of the STING-dependent pathway for activation of type I IFN production during pneumococcal pneumonia *in vivo*, we intranasally infected WT and STING-deficient mice with *S. pneumoniae*. Expression of *Ifnb* and *Ccl5* was almost

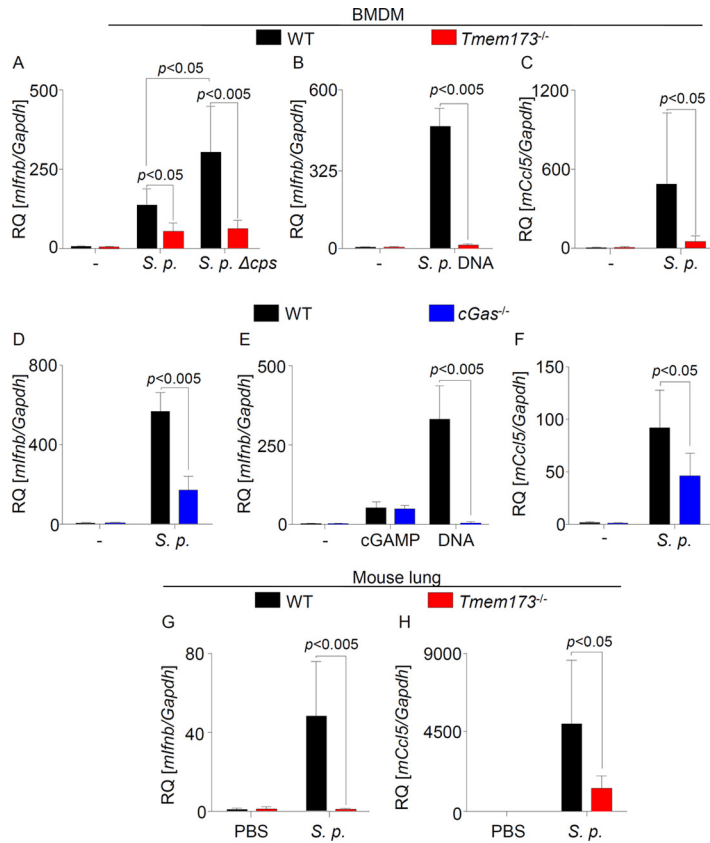


FIG 1 cGAS and STING mediate type I IFN responses to *S. pneumoniae* infection. (A and B) WT and *Tmem173*^{-/-} mouse BMDMs were left untreated, were infected with *S. pneumoniae* (*S. p.*) or its Δ *cps* isogenic mutant strain, or were stimulated with pneumococcal DNA for 6 h. Expression of *Ifnb* was quantified by qRT-PCR. (C) WT and *Tmem173*^{-/-} BMDMs were left untreated or were infected with *S. pneumoniae*, and expression of *Ccl5* was quantified by qRT-PCR. (D to F) BMDMs from *cGas*^{-/-} mice were infected with *S. pneumoniae* or stimulated with synthetic DNA or cGAMP, and expression of *Ifnb* (D and E) and *Ccl5* (F) was quantified by qRT-PCR. (G and H) WT and *Tmem173*^{-/-} mice were intranasally infected with *S. pneumoniae*, and the expression of *Ifnb* and *Ccl5* in the lungs was measured 48 h postinfection by qRT-PCR. Data from *in vitro* analyses are shown as means \pm standard deviations (SD) from three independent experiments, measured in technical duplicates. Mouse data represent analyses of 7 mice. Statistical analyses were performed with the Mann-Whitney U test. Comparisons with a *P* value of <0.05 were considered significant. RQ, relative quantification.

completely or partly inhibited in lungs of mice lacking STING compared to organs from WT animals (Fig. 1G and H). Altogether, these results demonstrate that type I IFN responses induced by *S. pneumoniae* in mice are dependent on the cGAS-STING pathway.

The cGAS-STING pathway has little effect on production of proinflammatory cytokines during pneumococcal infection of mice. We next investigated whether proinflammatory cytokine production was influenced by the cGAS/STING pathway as well. To this aim, we measured proinflammatory cytokine production in *S. pneumoniae*-infected BMDMs as well as in lungs of mice infected with *S. pneumoniae*. Although the levels of interleukin-1 β (IL-1 β) and IL-6 in the supernatant of STING-deficient cells infected with *S. pneumoniae* were slightly reduced compared with those of their WT counterparts (Fig. 2A to C and Fig. S2), cells from *cGas*^{-/-} mice produced levels of proinflammatory cytokines similar to those of WT cells (Fig. 2D to F). Moreover, production of these proinflammatory cytokines in the lungs of *S. pneumoniae*-infected mice was not affected by STING (Fig. 2G to I). Overall, these results indicate that the cGAS/STING signaling pathway has little effect on proinflammatory cytokine production in infections with *S. pneumoniae*.

Endogenous HAQ STING is impaired in mediating type I IFN responses to *S. pneumoniae* infection or stimulation with pneumococcal DNA. Previous studies

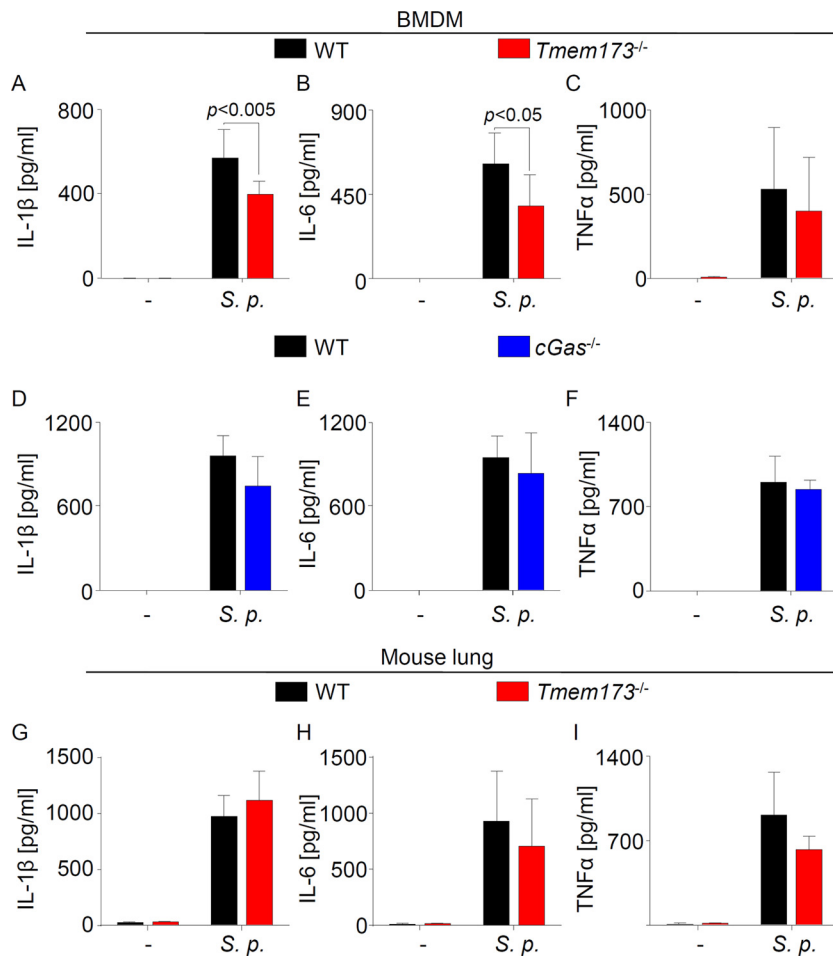


FIG 2 Proinflammatory cytokine production is partly dependent on STING during *S. pneumoniae* infection *in vitro* but not *in vivo*. (A to C) WT and *Tmem173*^{-/-} BMDMs were infected with *S. pneumoniae* for 16 to 18 h, and proinflammatory cytokine production was determined by sandwich ELISA. (D to F) Cytokine protein concentration in the supernatant of *cGas*^{-/-} and WT cells infected for 16 to 18 h with *S. pneumoniae* was measured by sandwich ELISA. (G to I) Cytokine production in whole lung homogenates of *S. pneumoniae*-infected mice was quantified by ELISA. Data are shown as means \pm SD. Data are representative of 4 (A to C) or 3 (D to F) independent experiments carried out in duplicates. (G to I) Data represent analyses of 7 mice. Data were analyzed through the Mann-Whitney U test. Comparisons with a *P* value of <0.05 were considered significant.

showed that HAQ STING was strongly impaired in mediating responses to bacterial CDNs and DNA (26, 27, 31). To assess whether endogenous HAQ STING affects type I IFN and proinflammatory cytokine responses to *S. pneumoniae* and pneumococcal DNA, we screened 564 individuals for the presence of HAQ *TMEM173*, isolated peripheral blood mononuclear cells (PBMCs) from 4 individuals identified as homozygous HAQ *TMEM173* carriers, cultured the cells for 7 days before infection/stimulation to allow differentiation into a macrophage-like state, and compared those cells to cells expressing WT STING. All donors were WT for amino acid position 232 (R232). Intriguingly, cells from HAQ *TMEM173* carriers showed impaired type I IFN expression and production of the IFN-stimulated cytokine IP-10 following *S. pneumoniae* infection or pneumococcal DNA stimulation (Fig. 3A and Fig. S3A). Furthermore, *S. pneumoniae*-stimulated IL-1 β production was also partly reduced in cells expressing HAQ STING (Fig. 3B and Fig. S3B), whereas release of tumor necrosis factor alpha (TNF- α) and IL-6 was not or was only insignificantly affected (Fig. 3C and D and Fig. S3C and D).

Additional experiments using the human monocytic cell line THP-1, which has previously been found to express HAQ STING (28, 32) and which we confirmed to carry HAQ *TMEM173* in homozygosity, showed only a weak type I IFN induction in

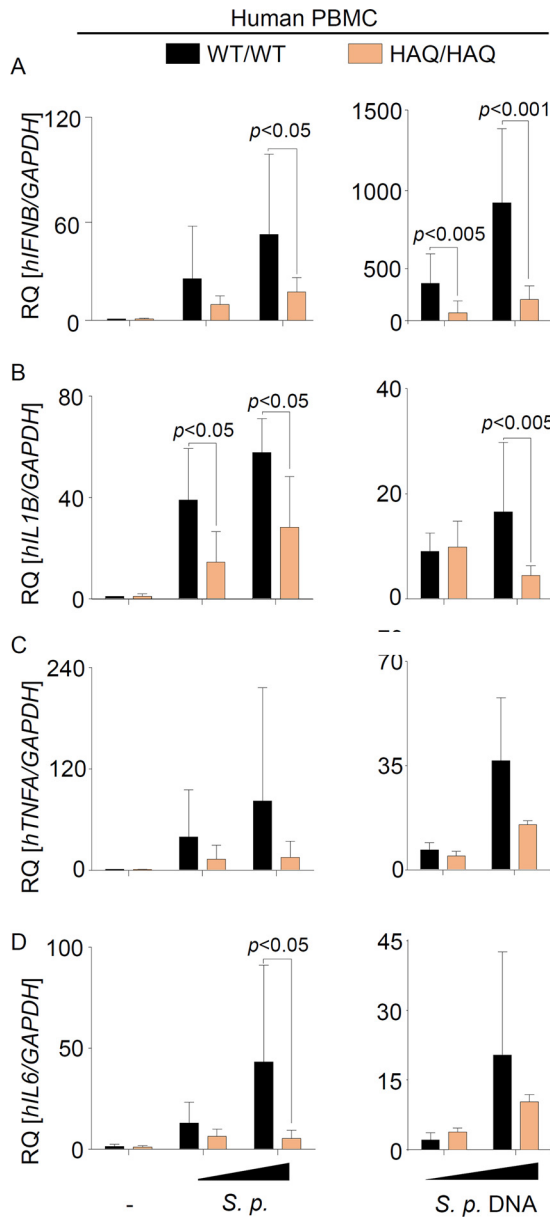


FIG 3 Endogenous HAQ STING is impaired in mediating type I IFN and proinflammatory cytokine responses to *S. pneumoniae* infection. PBMCs from healthy volunteers were isolated, cultivated for 7 days, and subsequently infected with *S. pneumoniae* at a multiplicity of infection of 2.5 or 10 or stimulated with 0.2 or 1 μ g/ml of pneumococcal DNA for 6 h. The expression of *IFNB* (A), *IL1B* (B), *TNFA* (C), and *IL-6* (D) was assessed by qRT-PCR. Data are represented as means \pm SD from 4 independent experiments carried out in triplicates. Comparisons with a *P* value of <0.05 were considered significant.

response to DNA, cGAMP, or *S. pneumoniae* challenge (Fig. S4). Indeed, while WT STING-expressing PBMCs showed a 50- to 1,000-fold *IFNB* induction upon *S. pneumoniae* or DNA stimulation, respectively (Fig. 3A), the bacterium induced only a 9-fold increase in *IFNB* expression in THP-1 cells. Notably, CRISPR/Cas9-mediated deletion of cGAS (33) further weakened type I IFN induction in THP-1 cells (Fig. S4). Altogether, these data reveal that endogenous HAQ STING is a hypomorphic variant that is strongly impaired (but not deficient) in mediating innate immune responses to *S. pneumoniae*.

The STING isoform R232H is functional in mediating immune responses to pneumococcal infection. The R232H variant of STING occurs in approximately 13.7% of the human population. While R232H STING has been shown to respond normally to

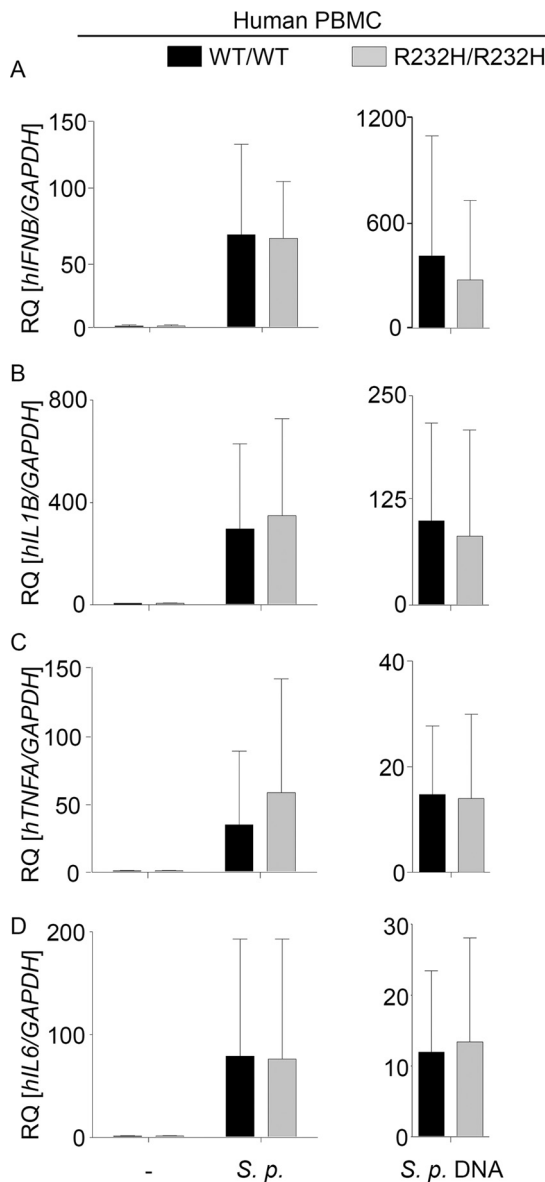


FIG 4 R232H variant of STING responds normally to *S. pneumoniae* infection or stimulation with pneumococcal DNA. PBMCs from healthy volunteers were isolated by density gradient centrifugation. Seven days after isolation, cells were challenged for 6 h with *S. pneumoniae* at a multiplicity of infection of 2.5 or stimulated for the same period with 1 μ g/ml pneumococcal DNA. RNA was isolated, and the expression of *IFNB* (A), *IL1B* (B), *TNFA* (C), and *IL-6* (D) was determined by qRT-PCR. Data are shown as the RQ of specified mRNAs. Data represent the means \pm SD from 3 independent experiments carried out in triplicates. Differences were assessed with the Mann-Whitney U test.

DNA, it was found to be defective in inducing immune responses to bacterial CDN stimulation (27, 28). To evaluate the capacity of endogenous R232H STING in inducing immune responses against *S. pneumoniae* infection or pneumococcal DNA stimulation, we screened healthy volunteers for the presence of this allele, isolated cells from 3 individuals carrying the R232H allele in homozygosity, and compared their response to that of cells from volunteers carrying WT *TMEM173*. In agreement with previous studies (27, 28), cells expressing R232H STING exhibited reduced responses to exogenous CDNs (unpublished data). The presence of the R232H STING, however, did not influence the type I IFN or proinflammatory cytokine response against pneumococcal infection or DNA stimulation (Fig. 4A to D and Fig. S5). These data indicate that recognition of pneumococcal CDNs does not play a major role in mediating immune responses during pneumococcal infection.

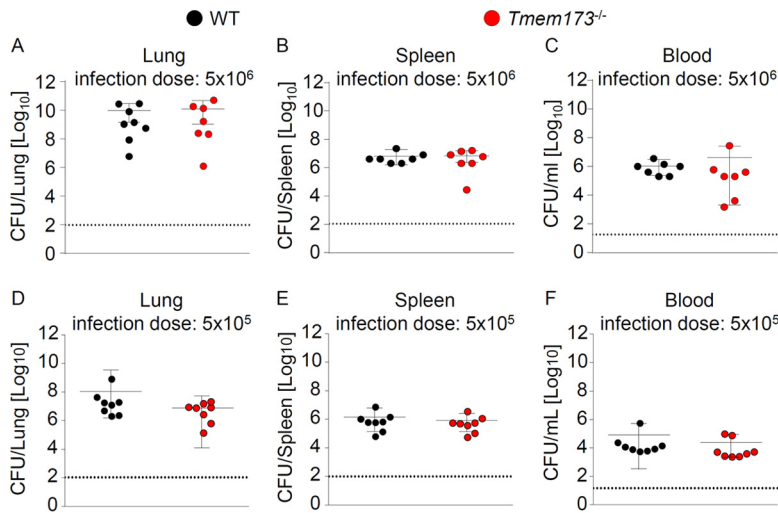


FIG 5 STING does not contribute to antipneumococcal defense in mice. WT and STING-deficient mice were intranasally infected with 5×10^6 CFU (A to C) or 5×10^5 CFU (D to F) *S. pneumoniae*, and the bacterial burdens in the lungs (A and D), spleen (B and E), and blood (C and F) were measured 48 h postinfection. Data represent means \pm SD from 7 or 8 mice per group. Comparisons were performed with the Mann-Whitney U test.

STING is dispensable for the antibacterial defense against *S. pneumoniae* in mice.

In order to evaluate the relevance of STING in the antibacterial defense against *S. pneumoniae* in mice, we intranasally infected WT and *Tmem173*^{-/-} animals with a high infection dose (5×10^6 CFU/mice) and a low infection dose (5×10^5 CFU/mice) and measured bacterial burdens in the lungs, spleens, and blood. We found no differences between bacterial burdens in WT and *Tmem173*^{-/-} animals (Fig. 5A to F). These results indicate that STING is not required for antibacterial immunity against *S. pneumoniae* in mice.

Expression of common STING variants seems to have no major impact on susceptibility of humans to pneumococcal pneumonia.

In an attempt to assess a potential association between carriage of the HAQ *TMEM173* allele and an increased susceptibility to pneumococcal pneumonia, we next compared the frequencies of the HAQ *TMEM173* allele in nonvaccinated patients suffering from pneumococcal pneumonia and healthy controls (Table 1). None of the groups analyzed deviated significantly from Hardy-

TABLE 1 Distribution of *TMEM173* HAQ and *TMEM173* R232H in patients and healthy controls

Parameter	Value(s) for:		
	Patients	Control group	Replication control group ^a
No.	87	92	564
Proportion female [% (n)]	48 (42)	53.2 (49)	63.7* (359)
Age [yr; median (range)]	66 (36–94)	72 (35–93)	32.0* (22–74)
<i>TMEM173</i> HAQ [% (n)]			
Heterozygous	24 (21)	20.6 (19)	24.6 (139)
Homozygous	1 (1)	0 (0)	1.4 (8)
Haplotype carrier	25 (22)	20.6 (19)	26.1 (147)
Allele frequency	0.132	0.103	0.137
<i>TMEM173</i> R232H [% (n)]			
Heterozygous	23.0 (20)	29.3 (27)	23.8 (134)
Homozygous	0 (0)	2.2 (2)	1.8 (10)
Haplotype carrier	23.0 (20)	31.5 (29)	25.5 (144)
Allele frequency	0.115	0.168	0.137

^a*, *P* < 0.05 compared to the patient group.

TABLE 2 Distribution of *TMEM173* HAQ and *TMEM173* R232H in patients grouped according to severity of pneumonia

Parameter	Severity ^a			P value ^b
	Low (CURB-65 = 0–1)	Middle (CURB-65 = 2)	High (CURB-65 = 3–5)	
Proportion female [% (n)]	42.6 (23)	45.4 (10)	83.3 (5)	0.37
Age [yr; median (range)]	57.5 (37–94)	74 (36–94)	73 (72–81)	0.004
<i>TMEM173</i> HAQ [% (n)]				
Haplotype carrier	27.8 (15)	13.6 (3)	50 (3)	0.778
<i>TMEM173</i> R232H [% (n)]				
Haplotype carrier	18.5 (10)	36.4 (8)	33.3 (2)	0.256

^aSeverity was defined by following the guidelines of the British Thoracic Society (34). From 87 patients initially included in the study, 5 were not considered for this analysis, as information regarding pneumonia severity was not available.

^bSignificance was evaluated through an ordered logistic regression model.

Weinberg equilibrium. The analysis revealed no difference in HAQ allele frequency between our patient and control cohorts and did not provide evidence for substantially altered odds of pneumococcal disease in HAQ *TMEM173* carriers. Similarly, the frequency of the R232H allele in our pneumococcal pneumonia patient group was not different from that of our healthy control cohorts. We then conducted an ordered logistic regression analysis in order to test whether carriage of HAQ or R232H *TMEM173* is associated with severity of pneumonia. Patients were grouped in three different levels of increasing severity according to the CURB-65 scores (34). As expected, age, but not gender, was significantly associated with increased severity of the disease. On the contrary, carriage of neither HAQ nor R232H *TMEM173* showed a significant effect on pneumonia severity (Table 2). Collectively, our explorative analyses strongly suggest that carriage of HAQ or R232H *TMEM173* is not a major risk factor for acute pneumococcal pneumonia in nonvaccinated individuals. However, due to limited sample size, the possibility of small effects actually being present cannot be excluded.

DISCUSSION

Type I IFNs were discovered more than half a century ago as key antiviral mediators (35) whose activity against viruses largely depends on the transcriptional induction of IFN-stimulated genes with direct antiviral activities (36). In addition, increasing evidence obtained in recent years indicates that type I IFNs also play important roles in bacterial infections. They have been shown to induce the expression of proteins with antibacterial activity (36, 37) and to regulate interleukin (38) as well as chemokine (39, 40) production. According to this pleiotropic function of type I IFNs, they have been demonstrated to be either beneficial or harmful for the host, probably depending on the pathogen and the mode of infection. In models of lung infections, for example, type I IFNs enhance host resistance to *Legionella pneumophila* (37, 41–43), *Chlamydia pneumoniae* (44), and *Pseudomonas aeruginosa* (45) but increase susceptibility to *Mycobacterium tuberculosis* (46) and *Staphylococcus aureus* (47). Several studies also reported that mice lacking receptors for type I IFNs exhibit impaired antibacterial defense against *S. pneumoniae* in models of nasal carriage, pneumonia, and sepsis (48–51), although other studies examining invasive serotype 1 strain (52) or postinfluenza pneumococcal infections indicated that this cytokine family can also have detrimental effects for the host (39, 40).

In this report, we show that production of type I IFNs during *S. pneumoniae* infection depends on recognition of bacterial DNA by the cGAS/STING pathway, and that this pathway is dispensable for antibacterial defense during acute pneumococcal pneumonia in mice. Moreover, we show that expression of the hypomorphic HAQ variant of STING dramatically reduces *S. pneumoniae*-induced type I IFN responses, but that it appears to have no major influence on the susceptibility of nonvaccinated individuals

toward pneumococcal pneumonia or on disease severity. We speculate that the divergent effect of type I IFN versus STING deficiencies on pneumococcal infections can be explained by the capacity of cGAS/STING to also regulate the production of other mediators in addition to type I IFNs (53, 54). Indeed, STING-dependent signaling has been shown to activate various transcription factors, such as IRF3, NF- κ B, and STAT6 (21, 53). STING deficiency might lead to a reduced production of unknown factors that impair antipneumococcal immunity, and this effect might compensate for the defect in type I IFN and IL-1 β production. Moreover, human macrophages expressing HAQ STING were still able to produce small amounts of type I IFNs, which might be sufficient for mediating potentially protective effects on antibacterial immunity.

We did not find an association between carriage of *TMEM173* variants with pneumococcal pneumonia or severity of the disease in nonvaccinated patients. However, this might be different in vaccinated individuals, since STING has recently been indicated to affect B cell responses in mice (55), and expression of a mouse equivalent of the HAQ allele reduced the efficacy of pneumococcal vaccination using Pneumovax 23 in mice (31).

In *L. monocytogenes* infection, sensing of bacterial c-di-AMP by STING has recently been shown to trigger type I IFN production (56). Similar to *Listeria* spp. and most other Gram-positive bacteria, *S. pneumoniae* is able to produce c-di-AMP (57). However, our data demonstrating that DNA sensing by cGAS is required for pneumococcus-induced type I IFN production suggest that recognition of c-di-AMP is dispensable for innate immune sensing of *S. pneumoniae*. In line with this conclusion, cGAS-deficient cells exhibited decreased IFN induction in response to *S. pneumoniae* infection. Additionally, cells carrying the R232H allele, which has been reported to affect bacterial CDN recognition (28), were fully capable of responding to *S. pneumoniae* infection. Furthermore, distribution of the R232H SNP was equal in the pneumococcal pneumonia patients and healthy controls analyzed. However, while STING-deficient BMDMs showed a slightly diminished production of IL-1 β and IL-6 upon *S. pneumoniae* infection, cGAS^{-/-} cells exhibited no defect in proinflammatory cytokine production. Thus, we cannot exclude a minor contribution of a STING-dependent but cGAS-independent sensing mechanism in stimulating proinflammatory responses *in vitro*.

Our analyses indicate that deficiency in the STING-dependent pathway had stronger effects on the production of proinflammatory cytokines in human PBMC-derived macrophages than in murine BMDMs. This difference might be explained by the differential expression of other PRRs known to detect pneumococci between murine and human macrophages, which might compensate for the lack of cGAS/STING-dependent sensing.

In summary, we show that cGAS/STING senses pneumococcal DNA during *S. pneumoniae* infection to primarily stimulate production of type I IFNs and to contribute to proinflammatory cytokine production in human cells. Analyses of mice lacking STING as well as individuals expressing common STING variants, however, suggest that this pathway plays no major role in antipneumococcal host defense.

MATERIALS AND METHODS

Ethics statement. Healthy volunteers from whom PBMCs were isolated provided written informed consent, and the study procedures were in agreement with the local ethics committee guidelines (Charité–Universitätsmedizin Berlin). Samples from patients with pneumococcal pneumonia were kindly provided by the CAPNETZ study group. This prospective multicenter study (German Clinical Trials Register DRKS00005274) was approved by the ethical review board of each participating clinical center (reference number of the leading ethics committee of Medical Faculty of Otto-von-Guericke-University in Magdeburg, 104/01; reference number for Medical School Hannover, 301/2008; see www.capnetz.de for participating centers) and was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent prior to enrollment in the study. All animal experiments were approved by institutional (Charité–Universitätsmedizin Berlin) and governmental animal welfare committees (LAGeSo Berlin; approval ID G0440/12).

Bacterial strains. The *S. pneumoniae* D39 strain, belonging to serotype 2, as well as the Δ ply and Δ cps isogenic mutant strains (58), were used for *in vitro* infection assays. *S. pneumoniae* strain serotype 3 (NCTC 7978) was used to induce pneumonia in mice. Bacteria were cultured on Columbia blood agar plus 5% sheep blood for 12 h at 37°C. Culture of the mutant strains also required the addition of 40 μ l of the

following antibiotics on the agar plate: D39 Δ *cps* strain, 50 mg/ml kanamycin; D39 Δ *ply* strain, 1 mg/ml erythromycin.

BMDMs. Bone marrow-derived macrophages (BMDMs) were isolated from femurs and tibiae of WT, *Tmem173*^{-/-}, and *cGas*^{-/-} female mice on a C57BL/6 background. Before infection, cells were grown in RPMI 1640 containing 30% L929 cell supernatant and 20% fetal calf serum (FCS) for 10 days.

Cell transfection and infection. Mouse BMDMs were infected with the above-mentioned strains of *S. pneumoniae* at a multiplicity of infection of 1, centrifuged at 200 \times *g* for 5 min, and then incubated for 6 h at 37°C. Bacterial DNA or synthetic dsDNA (ISD, or interferon stimulatory DNA; Invivogen) was transfected at a concentration of 1 μ g/ml into the cells using Lipofectamine 2000 (Invitrogen). For siRNA-mediated *cGas* downregulation, BMDMs were transfected 48 h prior to infection with control nonsilencing siRNA or with a specific siRNA targeting *cGas* using HiPerfect (Qiagen).

Murine model of pneumococcal pneumonia. Female WT and *Tmem173*^{-/-} mice (23, 59), 8 to 14 weeks old on a C57BL/6 background, were housed in individually ventilated cages, with food and water provided *ad libitum*. Mice were anesthetized by intraperitoneal injection of ketamine (1.6 mg) and xylazine (0.5 mg) and intranasally infected with 5×10^5 or 5×10^6 CFU *S. pneumoniae* serotype 3 (NCTC 7978) in 20 μ l of phosphate-buffered saline (PBS). Body weight and temperature were monitored every 12 h. Two days after infection, mice were anesthetized, heparinized, and euthanized through final blood withdrawal. Lungs were then flushed via the pulmonary artery and removed for subsequent analyses. Bacterial counts were assessed by plating serial dilutions of lung or spleen homogenates or blood on Columbia blood agar plates.

Quantitative reverse transcription-PCR (qRT-PCR). Total RNA was isolated from cultured cells or mouse lung homogenates using the PerfectPure RNA purification system (5 Prime) or TRIzol (Life Technologies), respectively. cDNA was synthesized from total RNA using a high-capacity reverse transcription kit (Applied Biosystems), and quantitative PCR was performed using TaqMan assays (Life Technologies) or self-designed primer sets on an ABI 7300 instrument (Applied Biosystems). The input was normalized to the average expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in untreated cells or PBS-treated mice.

ELISA. Protein concentrations of IP-10, IL-6, TNF- α , and IL-1 β were quantified by commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (eBioscience). Protein concentrations were quantified in a FilterMax F5 multimode microplate reader (Molecular Devices) and read at 450 nm.

Subjects. Peripheral blood was collected from 14 healthy volunteers carrying *HAQ*, *R232H*, or WT *TMEM173* and belonging to a cohort group ($n = 564$) assembled at the Institute of Microbiology, Charité University Medical Center Berlin. The association study between the *HAQ* haplotype and pneumococcal pneumonia was performed using DNA samples obtained from 87 patients with *S. pneumoniae*-induced community-acquired pneumonia (CAP) without antipneumococcal vaccination. Samples were provided by the CAPNETZ study group, a German multicenter prospective cohort study for CAP. A detailed description of the CAPNETZ methodology has been provided before (60). The control groups were composed of a subgroup of healthy individuals of similar age and sex distribution ($n = 92$) from the PolSenior program, an interdisciplinary project, designed to evaluate health and socioeconomic status of Polish Caucasians aged ≥ 65 years (61) and the cohort of the Institute of Microbiology, Charité University Medical Center Berlin.

TMEM173 genotyping. Genomic DNA was collected in buccal swabs and isolated by employing the Genra Puregene buccal cell kit from Qiagen by following the manufacturer's instructions. Genotyping of *TMEM173* *R71H* (rs11554776), *G230A* (rs78233829), *R293Q* (rs7380824), and the *TMEM173* *R232H* SNP (rs1131769) was performed by PCR using fluorescence-labeled hybridization fluorescence resonance energy transfer probes (TIB Molbiol) accompanied by melting curve analysis in a LightCycler 480 (Roche Diagnostics). Primers and probes used were the following (where f-primer indicates forward primer, r-primer indicates reverse primer, and uppercase letters within the sequences [e.g., "XI"] indicate a label within the oligonucleotide probe, with "I" indicating a base analogue and "X" the modification): rs11554776 f-primer, ggagtgacacagctgtg; r-primer, gcctagctgaggagctg; simple probe, LC640-ctggagtggaXlItgtggcgcag-PH; rs78233829 f-primer, ggggtctactctgaatcaggt; r-primer, cccgatcctgtgatgcaagca; anchor probe, LC640-cagttttaccaggaagcgaatgtggg-PH; sensor probe, ggtagcggctgctgtg-FL; rs7380824 f-primer, accctggtaggcaatga; r-primer, gcttagctggtctctcttac; anchor probe, LC640-ggctgctcaagcctatcctcccg-PH; sensor probe, cctcaagtgtccggcagaagatt-FL; rs1131769 f-primer, cccactccctgcacact; r-primer, tggataaactgcccaagcagac; anchor probe, LC640-aggatcgggtttacagcaacagca-PH; sensor probe, ggtgaccatgctggcatc-FL.

Infection and stimulation of human PBMCs. Fifty-milliliter aliquots of whole peripheral blood were drawn from healthy volunteers, and PBMCs were isolated by density gradient centrifugation (62, 63). Briefly, whole blood was diluted 1:1 with PBS and layered onto 20 ml Histopaque-1077 (Sigma-Aldrich). The gradient then was centrifuged at 800 \times *g* for 25 min at room temperature, and the PBMCs were collected from the interface. PBMCs were washed twice with PBS and resuspended in RPMI medium supplemented with 10% FCS and 1% L-glutamine. Half of the cell medium was replaced every 2 days, and the cells were kept on culture for 7 days to allow differentiation into a macrophage-like state before infection or stimulation. Infection with *S. pneumoniae* and stimulation with bacterial DNA were performed as described before for mouse BMDMs.

Human monocytic THP-1 cells. *cGAS*^{-/-} (33) and control THP-1 human cells were maintained under standard culture conditions. Cell differentiation into a macrophage-like state was induced by resuspension of cells in culture medium containing 80 nM phorbol myristate acetate (PMA) for 48 h.

Statistics. Data analysis was carried out using Prism software (GraphPad Software). Groups were contrasted using a two-tailed Mann-Whitney U test. The association study was performed employing a chi-square test for association and calculation of odds ratios. A Fisher's exact test, together with calculation of exact confidence intervals, was used if applicable. The analysis of association between allele carriage and disease severity was conducted through an ordered logistic regression using SPSS (IBM Corporation). Differences with a *P* value of <0.05 were regarded as significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00849-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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