Aus der Medizinische Klinik mit Schwerpunkt Pneumologie
und Infektiologie der Charité Universitätsmedizin
und dem Institut für Immunologie des Fachbereichs
Veterinärmedizin der Freien Universität Berlin

The hypomorphic HAQ variant of STING affects cGAS-dependent cytokine expression in response to bacterial infection and is associated with susceptibility to Legionnaires' disease in humans

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Juan Sebastián Ruiz-Moreno
Mikrobiologe aus Bogotá, Kolumbien

Berlin 2019
Journal-Nr.: 4108

Aus der Medizinische Klinik mit Schwerpunkt Pneumologie und Infektiologie der
Charité Universitätsmedizin und dem Institut für Immunologie des Fachbereichs
Veterinärmedizin der Freien Universität Berlin

The hypomorphic HAQ variant of STING affects cGAS-dependent cytokine expression in response to bacterial infection and is associated with susceptibility to Legionnaires' disease in humans

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Juan Sebastián Ruiz-Moreno
Mikrobiologe aus Bogotá, Kolumbien

Berlin 2019
Journal Nr.:4108

Gedruckt mit Genehmigung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan: Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachterin: Univ.-Prof. Dr. Susanne Hartmann
Zweiter Gutachter: Prof. Dr. Lothar H. Wieler
Dritter Gutachter: Prof. Dr. Bastian Opitz

Deskriptoren (nach CAB-Thesaurus): Man, pneumonia, *Legionella pneumophila, Streptococcus pneumoniae,* legionnaire´s disease, cytokines, immune response, immunity, polymorphism

Tag der Promotion: 15.05.2019

Table of contents

List of abbreviations

AIM2	absent in melanoma 2
AM	alveolar macrophage
c-di-AMP	cyclic di-adenosine monophosphate
BIR	baculoviral inhibition of apoptosis protein repeat
BMM	bone marrow-derived macrophages
BYCE	buffered charcoal-yeast extract medium
CAP	community-acquired pneumonia
CARD	caspase activation and recruitment domain
Ccl5	C-C motif chemokine ligand 5
CDN	cyclic dinucleotide
CLR	C-type lectin receptor
CRD	carbohydrate-recognition domain
DAI	DNA-dependent activator of IRFs
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
ECDC	European center for disease prevention and control
ER	endoplasmic reticulum
cGAMP	cyclic GMP-AMP
cGAS	cGAMP-synthase
c-di-GMP	cyclic di-guanosine monophosphate
HiB	*Haemophilus influenzae* type B
ICU	intensive care unit
IFI-16	IFN-γ-inducible protein 16
IFN	Interferon
IFNAR	IFNα/β receptor
IL	interleukin
IL-1R	IL-1 receptor
IP-10	interferon gamma-induced protein 10
IPD	invasive pneumococcal disease
IRAK4	interleukin-1 receptor-associated kinase 4
IRF	interferon regulatory factor
IRG1	immune-responsive gene 1
ISG	interferon stimulated gene
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
JAK1	janus kinase 1
LCV	*Legionella*-containing vacuole
LD	legionnaires' disease
LGP2	laboratory of genetics and physiology 2
LRR	leucine-rich repeat

MARCO	macrophage receptor with collagenous structure
MAVS	mitochondrial antiviral signaling protein
MBL	mannose-binding lectin
MDA-5	melanoma differentiation-associated gene-5
Mincle	macrophage-inducible C-type lectin
MyD88	myeloid differentiation primary response 88
NAIP5	NLR family apoptosis inhibitory protein 5
NEMO	NF-kappa-B essential modulator
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCV	pneumococcal conjugate vaccine
PID	primary immunodeficiencies
Ply	pneumolysin
PRR	pattern recognition receptor
PYD	pyrin domain
qRT-PCR	quantitative real-time PCR
RIG-I	retinoic acid-inducible gene I
RIP2	receptor interacting protein 2
RLR	retinoic acid-inducible gene-I-like receptor
SAVI	STING-associated vasculopathy with onset in infancy
SNP	single gene polymorphism
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
T4SS	type IV secretion system
TBK1	TANK-binding kinase 1
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TMEM173	transmembrane protein 173
TNFα	tumor necrosis factor-α
TNFR1	TNFα receptor 1
TRIF	TIR-domain-containing adapter-inducing interferon-β
TYK2	tyrosine kinase 2
URT	upper respiratory tract
WT	wild type

1. Introduction

1.1 Pneumonia

Pneumonia is an infection of the lower respiratory tract that induces an inflammatory condition in the lungs resulting in accumulation of fluid in the alveoli, consequently leading to a constriction of gas exchange. For those cases in which pneumonia develops in persons with no recent history of hospitalizations and little contact with the health care system, the term community-acquired pneumonia (CAP) has been coined [1]. Despite being extensively studied over the last hundred years, pneumonia remains a significant cause of morbidity and mortality in adults and a leading cause of death in children under 5 years of age. Annually, the worldwide incidence of pneumonia in children ≤ 5 years old is estimated to be over 120 million of which approximately 1.3 million cases are fatal [2, 3]; in adults, the annual incidence varies between 1.6 and 13.4 cases per 1000 habitants with and estimated fatality of 3 to 24% [4, 5]. Great efforts have been made over the lasts decades in order to decrease the incidence, morbidity and mortality of pneumonia. Yet, in spite of the increased hygiene, availability of antibiotic treatments and the widespread use of vaccines (e.g. pneumococcal polysaccharide and conjugate vaccines [PCVs], *Haemophilus influenzae* type B [HiB] vaccine), the burden of the disease remains high, especially in low- and middle-income countries, where approximately 90% of cases arise [1, 3, 6].

1.1.1 Causes

Although vaccination with PCVs and HiB vaccine have considerably decreased their occurrence, *Streptococcus pneumoniae* and *H. influenzae* remain the single most common causes of bacterial CAP in all age groups worldwide [1, 7]. Additionally, different reports have documented an increasing number of severe cases of pneumonia caused by multi-drug resistant strains of *Staphylococcus aureus* and *Klebsiella pneumoniae*, being the latter most commonly associated with hospital settings [8, 9]. Other bacteria that have been recognized as etiological agents of pneumonia are *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila*, a Gram-negative bacillus that causes and atypical form of pneumonia referred to as Legionnaires' disease (LD) [1]. Viruses are likewise frequently identified as causative agents of CAP

especially in children less than 5 years of age. Among the viruses known to cause pneumonia, circulating strains of influenza A and B virus become one of the most common causes of CAP during influenza seasons and outbreaks. Other viruses such as the respiratory syncytial virus, rhinovirus, parainfluenza virus, adenovirus and coronavirus are also commonly reported in patients with CAP [1, 3, 6]. Fungi account for only a small proportion of CAP and nosocomial pneumonia cases; however they are an important cause of mortality in a growing population of immunosuppressed patients. While species from the genus *Aspergillus* are the most frequent cause of fungal lung infections, other fungi such as *Cryptococcus spp.* and *Pneumocystis jirovecii* are also among commonly identified isolates [10].

Some of the most severe cases of CAP are in many instances caused by sequential or concurring infection with multiple pathogens. Coincident or subsequent infection with *S. pneumoniae* following influenza is of common occurrence and known to markedly exacerbate the course of pneumonia. During the 1918 Spanish influenza pandemic and the 2009 H1N1 pandemic, the vast majority of severe cases and deaths were associated with a secondary bacterial infection, most commonly with *S. pneumoniae* [11, 12].

1.1.2 Diagnosis and treatment

Observation of physical signs and chest imaging are frequently the most readily means to diagnose pneumonia. CAP patients normally exhibit cough, production of sputum, fever, breathing difficulty and chest pain. In addition to these, white blood cell count and screening of C-reactive protein, may aid in the diagnosis. However, in some cases patients with pneumonia, in particular those who are elderly, might not cough, produce sputum, present fever or develop and elevated white blood cell count [1, 13]. Likewise, although chest radiography has been considered the gold standard for diagnosing pneumonia, detection of new lung infiltrates or consolidations might be difficult to achieve in patients with chronic lung disease or obesity. Furthermore, this method might not be a completely reliable guide in discriminating bacterial and non-bacterial CAP [1, 14]. It remains virtually impossible to pinpoint the specific cause of CAP based only on clinical observations. A set of laboratory tests that include, *inter alia*, Gram´s staining, sputum and blood culture, antigen tests and polymerase chain reaction (PCR), are therefore necessary in order to ultimately determine the causative agent, especially in critically ill CAP patients [1, 6].

After a presumptive diagnosis of CAP has been made, scoring indexes, such as the CURB-65 index (calculated from a measurement of confusion, blood urea nitrogen, respiratory rate, and blood pressure in patients ≥65 years of age) are regularly employed to assess disease severity and promptly decide whether or not a patient requires hospitalization or admission to the intensive care unit (ICU) [15]. Empirical treatment is often necessarily initiated due to costs and time that specific diagnostic tests might involve, especially in severe cases for which immediate action might be critical for patient survival. Outpatients with signs of bacterial pneumonia are initially treated with amoxicillin–clavulanat, and if infection with *Legionella spp.* is suspected, azithromycin might be additionally administered. In cases were influenza pneumonia is considered, oseltamivir is usually the treatment of choice. For hospitalized or ICU patients, guidelines dictate commencement of therapy with beta-lactam antibiotics (cefotaxime, ceftaroline or ceftriaxone) and a macrolide (azithromycin). If the incidence of influenza is high in the community, therapy with oseltamivir is usually employed, which could be discontinued if PCR tests for influenza virus result negative. Additional treatment with ceftriaxone plus either vancomycin or linezolid is administered in cases where secondary bacterial infections are present. Patients at risk or with confirmed *S. aureus* infection are treated with linezolid or vancomycin; and if *Pseudomonas spp.* is in consideration anti-pseudomonal beta-lactam antibiotics are administered [1, 16, 17].

1.2 *Legionella pneumophila*

Bacteria from the genus *Legionella* are ubiquitous microorganisms found in freshwater environments where they engage in parasitic or commensal relationships with free-living amoeba. Named in 1976 following the investigation of a large pneumonia outbreak in members of the American Legion who were attending an annual meeting in Philadelphia, *Legionella spp.* is a Gram-negative accidental intracellular pathogen and the causative agent of a respiratory disease known as legionellosis [18, 19]. This condition may present itself as a self-limiting, non-pneumonic disease with flu-like symptoms, in which case is regarded as Pontiac fever, or it may give rise to a more common and severe pneumonic form known as Legionnaires' disease (LD). Since the Philadelphia outbreak, approximately 60 species of *Legionella* have been discovered, and although approximately half of them are known to cause disease, *Legionella pneumophila* and more precisely *L. pneumophila* serogroup 1 is presently the most isolated microorganism from infected patients [20-22].

1.2.1 Epidemiology

Appropriate estimation of the worldwide incidence and other epidemiological parameters of LD remains elusive as a result of under-reporting and under-diagnosing caused by the lack of unified and proper definitions, diagnostic methods and surveillance systems in most countries. LD is a notifiable disease in only a handful of countries and in the EU, where surveillance has been in use since 1995 [23]. According to the European centre for disease prevention and control (ECDC), 6560 confirmed cases were reported in Europe in 2016, 71% of which were community-acquired, although a significant proportion remained travel-related. The notification rate reached 1.4 cases per 100000 inhabitants and the case fatality was approximately 8.2%. LD seemed to have a seasonal variation, having its peak between the months of June and October, when humid and warm conditions are more common. Importantly, of all notified cases, 69% were reported in Spain, France, Germany and Italy alone. Similar numbers were described in the US in the year 2016 in which 6100 cases were reported and the death rate was 9% [24, 25]. These figures might however give an underestimation of the current incidence of LD. In a report from the Competence Network for Community Acquired Pneumonia in Germany, exhaustive testing for *Legionella spp.* applied to samples from pneumonia patients showed that this pathogen is in fact one of the most frequent causes of CAP in Germany, with an estimate of 15000 to 30000 cases of sporadic *Legionella*-induced pneumonia occurring every year [26].

Transmission usually occurs by inhalation of infectious aerosols or aspiration of contaminated water. Although rare, exposure to potting soil or compost has been associated with LD caused by *L. longbeachae*. Given its natural niche (i.e. parasite or commensal of amoeba) and its ability to produce biofilms, *L. pneumophila* exhibits resistance against different chemical, acid, osmotic or thermal insults, allowing it to thrive in a wide range of aquatic natural environments and man-made water systems such as evaporative condensers and air-conditioning cooling towers where the risk of infection might be particularly high [23].

Known risk factors commonly associated to LD are age above 50 years, smoking, chronic respiratory and cardiovascular diseases, diabetes, cancer, and immunosuppression [20].

1.2.2 Diagnosis and treatment

Due to it being the fastest and frequently one of the most sensitive tests available, the urinary antigen test is regularly the method of choice for diagnosing LD. However, this method has

proven insufficient in detecting strains other than *L. pneumophila* serogroup 1 and its widespread use has led to a slight yet potentially significant underestimation of cases attributable to other strains of *L. pneumophila* or other *Legionella* species [22]. Quantitative real-time PCR (qRT-PCR) has become the most reliable molecular method for the diagnosis of LD thanks to its superior sensitivity, specificity and celerity. Conserved segments of the 16S subunit or the *mip* gene are the usual targets in most laboratories using this method [26, 27]. Nonetheless, isolation and culture of *Legionella spp.* continues to be the gold standard method for diagnosis. Retrieval of a culture isolate additionally increases the possibility of identifying and solving environmental sources of *L. pneumophila.* Ideal culture samples are obtained from sputum, bronchial aspirates, bronchoalveolar lavage or pleural fluid [20, 22]. In order to minimize the effect of competing respiratory microbiota, samples can be treated with antibiotics to which *L. pneumophila* is naturally resistant (e.g. vancomycin or cycloheximide) or be briefly exposed to heat or acid conditions. Samples are subsequently cultured in buffered charcoal-yeast extract medium (BYCE) supplemented with α-ketoglutarate and L-cysteine, and maintained at 35°C and 2-5% CO_2 [22].

Considering that a definitive diagnosis of LD normally requires periods of time and resources that might not always be attainable, treatment against *Legionella* is usually initiated as soon as pneumonia of bacterial origin is presumptive and scoring systems suggest elevated severity. Thus, empirical antibiotic therapy with a fluoroquinolone or a macrolide, which can be used in combination in severe cases, may be started even before a definitive diagnosis is made [17, 28].

1.2.3 Pathogenicity

Thanks to its innate ability to replicate within free-living amoeba, *L. pneumophila* has acquired a diverse arrange of virulence factors that enable pathogen replication inside alveolar macrophages (AMs) as well. Once *L. pneumophila* reaches the lungs, it adheres to the surface of alveolar macrophages via the flagellum, pili and several surface proteins prior to its internalization through conventional phagocytosis [29]. It is at this point when the key virulence factor of *L. pneumophila*, the Dot/Icm type IV secretion system (T4SS), starts to play its most important role. Following phagocytosis, this macromolecular protein complex, consisting of approximately 27 proteins, translocates ~300 effector molecules that promote the formation of the *Legionella*-containing vacuole (LCV) [30]. Establishment of the LCV is

crucial for bacterial replication inside macrophages and a prominent example of how effector proteins manipulate the host machinery in order to ensure intracellular pathogen survival. While the fate of most phagocytosed particles is to be digested along the endocytic pathway, the phagosome containing *L. pneumophila* circumvents the formation of the phagolysosome and instead evolves into a large vacuole that, following transient association with mitochondria and acquisition of markers and vesicles from both the secretory and endocytic pathway, resembles the rough endoplasmic reticulum (ER) (Fig 1) [29, 31, 32]. The LCV is hence able to sustain the growth and replication of the bacteria until their numbers are sufficiently high as to permit initiation of cell lysis, bacterial egress and launch of a new round of infection [29, 33].

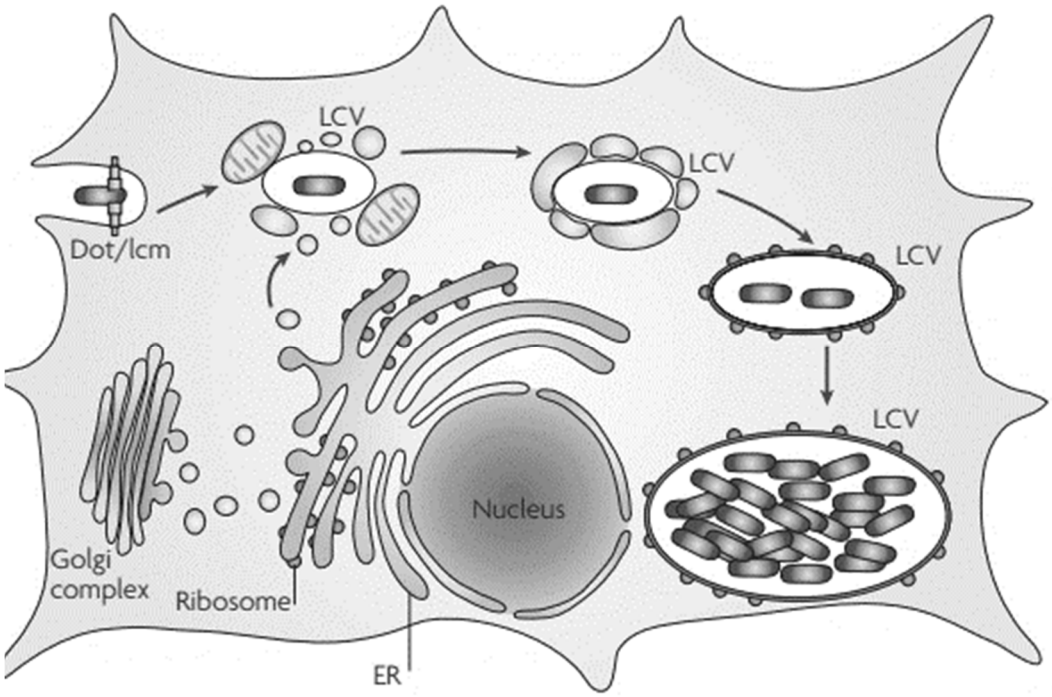


Figure 1. The *Legionella*-containing vacuole. Upon phagocytosis, *L. pneumophila* translocates a myriad of effector proteins through its type IV secretion system that mediate formation of the LCV. This process involves evasion of lysosome fusion, transient association with mitochondria and sequestration of ER vesicles. Bacteria are then able to thrive and multiply inside the LCV before host cell lysis occurs. Figure adapted from [31].

In order to initiate and maintain LCV development and to provide the best conditions for bacterial egress and transmission, the large arsenal of effector proteins translocated into the host must subvert several processes which are pivotal for proper cell functioning. Endocytosis, cell trafficking, autophagy, cell death, protein turnover and remodeling of the host cytoskeleton are just some of the most important cell functions hijacked by effector

molecules from *L. pneumophila* [34]. In addition to the T4SS, *L. pneumophila* harbors a number of secretion systems which complement the function of the Dot/Icm system and are required for full pathogenicity and persistence. These comprise the Lsp (T2SS) system, Lss (T1SS) system, Lvh (a second T4SS) system and a twin-arginine translocation system. Mutations in one of these systems significantly limit the range of hosts that *L. pneumophila* can infect or the conditions at which it can replicate as well as interfere with cell invasion and biofilm formation [29, 35, 36].

1.3 *Streptococcus pneumoniae*

S. pneumoniae was discovered and recognized as a causative agent of pneumonia more than a century ago. Its identification began an intensive research endeavor that has been fundamental to some of the most important clinical and biological discoveries [37]. This bacterium, frequently denoted as the pneumococcus, is an extracellular Gram-positive pathogen with a widespread presence in human population and various degrees of invasiveness that reflect serotype diversity. Considered a frequent colonizer of the upper respiratory tract (URT), *S. pneumoniae* is an important etiological agent of bacterial invasive infections such meningitis and bacteremia, and a leading cause of CAP [38]. Presently, more than 90 serotypes have been described based on the polysaccharide structure of the capsule. Not only do these serotypes differ in their polysaccharide composition, but they also show great variation in their capacity to colonize the nasopharyngeal airway, their potential to invade tissues and the type of illness to which they are associated. The existence of such a diverse and considerable group of serotypes, constitutes one of the biggest challenges in the development of strategies against *S. pneumoniae* [38, 39].

1.3.1 Epidemiology

The nasopharyngeal environment remains one of the primary ecological niches of *S. pneumoniae* and its colonization is a key event in the transmission of the bacterium to other hosts. Carriage of *S. pneumoniae* is highest in children 2-3 years of age and declines thenceforth to 10% in adults; nonetheless, carriage might be higher in child caretakers and parents with small children at home. Although in most cases it remains asymptomatic and resolves within weeks to months, colonization occasionally progresses to pneumococcal disease, with children and the elderly at higher risk. Disease progression can either lead to

mild infections such as otitis media or to more invasive and severe infections such as CAP and/or invasive pneumococcal disease (IPD) generally defined as pneumococcal septicemia or meningitis [38, 40]. The number of cases of IPD reported in the US has dramatically decreased following the introduction of the 7- and 13-valent PCVs in the years 2000 and 2010 respectively. The occurrence of reported cases of IPD in children decayed from 100 cases per 100000 inhabitants in 1998 to 9 cases per 100000 inhabitants in 2015. Likewise, the incidence of IPD in adults declined from 16 cases per 100000 inhabitants in 1998 to 7 cases per 100000 inhabitants in 2015 [41]. The introduction of high-valency PCVs in Europe similarly resulted in a significant reduction of the incidence of IPD; nevertheless, the amount of reported cases ascribable to serotypes not included in these vaccines has increased by 115% compared with the period of time preceding the introduction of the heptavalent PCV, therefore suggesting serotype replacement in the population [42]. Not including data from Germany (as this country has a voluntary laboratory-based surveillance system and does not report to the ECDC), 21986 cases of severe pneumococcal disease were reported in 2016 in Europe. Among cases for which clinical presentation was known, 37% corresponded to confirmed cases of pneumonia. The notification rate was 5.4 cases per 100000 inhabitants and the fatality reached 17% from cases with known outcome. During this year, ten serotypes accounted for 64% of reported cases; these serotypes were, in order of frequency, 8, 3, 12F, 22F, 19A, 9N, 15A, 10A, 33F and 11A [43]. In Germany, the notification rate between 2007 and 2014 reached 2.8 cases per 100000 inhabitants per year. This parameter, nevertheless, has to be carefully considered given the voluntary character of the surveillance system in this country. Reflecting the trends reported in the US and Europe, the occurrence in Germany of serotypes included in PCVs has vastly decreased, yet the proportion of serotypes not included in these vaccination programs has escalated, underscoring the importance of including other serotypes in future immunization schemes [44].

Recognized risk factors associated with IPD are chronic pulmonary and cardiovascular diseases, alcohol abuse, recent influenza infection, asthma, diabetes mellitus and exposure to cigarette smoke [45].

1.3.2 Diagnosis and treatment

Once radiologic evidence and clinical symptoms suggest the existence of pneumonia, a definitive diagnosis (necessary in cases of elevated severity) of infection with *S. pneumoniae* is typically achieved by Gram-staining and isolation of the bacteria from sputum samples.

Inadequate sputum sampling and previous antimicrobial therapy however, frequently hinder the sensitivity of this method. Isolation of pneumococci from blood samples offers a definitive albeit limited diagnosis considering that only in 10% of pneumococcal pneumonia cases *S. pneumoniae* can be recovered from blood cultures [46]. Given the limitations of microbiological methods in the diagnosis of *S. pneumoniae*, several antigen detecting techniques have been developed, which allow for a more rapid diagnosis with greater sensitivity. One such technique is the detection of teichoic acid (C-polysaccharide) from *S. pneumoniae* in urinary samples through an immunochromatographic test. This assay offers a higher sensitivity and the antigen can be detected weeks after commencement of the disease even when antimicrobial therapy has been previously administered. On the other hand, the high sensitivity of this assay could be a drawback, as detection of the antigen can also occur in individuals with asymptomatic nasopharyngeal carriage of pneumococci, for which reason, results of this test should be carefully considered when attempting a diagnosis of pneumococcal pneumonia in children [40, 46]. PCR-based methods have also been introduced offering greater performance in terms of specificity. Detection of the *lytA* gene or the Spn9802 gene fragment through qRT-PCR from blood or lower respiratory tract specimens, respectively, are reliable assays, able to reproducibly discriminate between true events of pneumococcal pneumonia and cases of asymptomatic carriage of *S. pneumoniae* [47, 48].
As soon as a patient is diagnosed with pneumococcal CAP, antimicrobial therapy with broad-spectrum antibiotics is continued until the isolated strain is subjected to antibiotic sensitivity tests. This not only allows the administration of a more targeted therapy, which increases the likelihood of successful outcome, but also the identification of antibiotic-resistant strains. The type of antibiotic and the route of its administration usually depend on the severity of the disease. In cases of low severity, oral administration of amoxicillin is preferred. Patients with moderate severity can similarly be treated orally with amoxicillin together with a macrolide. Cases of high severity pneumococcal CAP are routinely managed with parenteral administration of β-lactamase stable antibiotics like co-amoxiclav in combination with a macrolide such as clarithromycin [17, 40].

1.3.3 Pathogenicity

Colonization of the URT paves the way for the dissemination of *S. pneumoniae* to others organs as well as its transmission to other hosts. Upon reaching the nasopharyngeal cavity, the pneumococcal capsule, by virtue of its negatively charged surface, prevents entrapment and

mucociliary clearance, allowing the bacteria to access the underlying epithelial cells [49]. Different enzymes expressed during early colonization stages, additionally protect bacterial integrity against lysozyme by modifying the peptidoglycan cell wall [50]. Access to nasopharyngeal epithelial cells, favors the production of a thinner capsule allowing for a heightened adherence capacity. Some of the most important proteins that mediated adherence to epithelial cells are PavA, PavB and CbpA which interact with components of the extracellular matrix and cell surface receptors. Moreover, *S. pneumoniae* expresses different glycosidases such as neuraminidase A, which further enhances adherence by cleaving sugar components from glycoconjugates thus exposing additional points of anchorage [51]. Increased pneumococcal burdens in the nasopharynx can take place as a result of damage to the respiratory epithelium and induction of inflammatory cytokines triggered by previous or concurrent viral infections [49].

Depending on the pneumococcal strain and the lifestyle, immune status and age of the host, colonization may either remain asymptomatic or develop into an invasive disease. Translocation of *S. pneumoniae* deeper into the lower respiratory tract commonly occurs by aspiration and requires the bacterium to exert extensive tuning of its gene expression programs in order to endure the changing microenvironment. In this sense, *S. pneumoniae* cannot only adjust the thickness of its capsule to allow for an enhanced adherence or an improved protection against phagocytosis and complement binding, but also upregulate the expression of virulence factors such as adhesins, glycosidases and a vast array of transporters which aid the bacterium in anchoring to surrounding respiratory tissues, acquiring essential nutrients and resisting the action of antibiotics, among other functions [40, 49, 52, 53].

Given its involvement in most of the pathological effects observed during pneumonia, pneumolysin (Ply) is perhaps one of the most important and extensively studied pneumococcal virulence factors. This pore-forming toxin exhibits a wide spectrum of virulence mechanisms that range from induction of cell lysis and apoptosis to stimulation of strong inflammatory responses that might compromise the stability and functionality of the alveolar tissue [54]. Ply has even been suggested to induce damage to the host DNA and interfere with the regulation of cell cycle [55].

Progression to invasive disease is facilitated by the lytic action of Ply, but also by the interaction of phosphorylcholine or the choline-binding protein A with host receptors, through which *S. pneumoniae* promotes its own endocytic engulfment and its subsequent passage through the endothelium into the blood stream [49].

1.4 Innate immunity

The immune system in vertebrates has been traditionally conceived as being composed of two defense strategies whose name reflect the nature and the promptitude with which they are deployed: the innate and the adaptive immune systems. Despite being described as two distinctive strategies with unique attributes, the innate and adaptive immune responses are now commonly conceived as being highly interrelated, with the former strongly influencing the induction of the latter [56].

The skin and the mucosal epithelia provide physical and chemical protection against pathogens and constitute the very first line of defense of the innate immune system. Not only do these barriers physically impede the entry of pathogens into the organism, they also constantly produce a wide array of proteins with antimicrobial properties, maintain low pH levels that can be unfavorable for potential pathogens and provide a niche for resident microbiota that competes with invading microbes for resources [57].

Pathogens that succeed in breaching epithelial defenses, face further and more complex layers of protection that involve recognition of microbial components and initiation of defense mechanisms. A group of serum proteins constantly produced in the liver and altogether known as the complement system constitutes one of such layers. Until triggered, the components of this system circulate as inactive precursors. Activation occurs through either of three initiation pathways which launch a cascade of catalytic reactions that result in the attachment of several complement proteins to the microbial surface. Binding of these proteins promotes the formation of membrane attack complexes which induce pathogen cell lysis and death. Activated complement proteins also trigger the initiation of inflammatory responses and more importantly, serve as opsonins stimulating and facilitating phagocytosis, an essential mechanism of pathogen ingestion and destruction [58].

Cells which actively indulge in phagocyting microbes are called professional phagocytes; these cells include tissue macrophages, normally the first population of immune cells that come into contact with pathogens, neutrophils, which are later recruited to the site of infection, as well as dendritic cells (DCs). Phagocytes utilize a collection of receptors that directly or indirectly recognize and mediate microbial uptake. Receptors such as Dectin-1 and the scavenger receptor-A, directly bind to molecules which are commonly found on the surface of invading microbes. Opsonin receptors, on the other hand, sense host proteins (e.g. complement proteins, antibodies, etc.), previously bound to microbial surfaces and thus indirectly mediate recognition and phagocytosis of pathogens [59].

Apart from the defense mechanisms thus far mentioned, and which are readily available for immediate action, the innate immune system heavily depends on induced cellular responses which consist of more evolutionarily elaborate mechanisms that rely on the recognition of so-called pathogen-associated molecular patterns (PAMPs) and elicitation of signaling-transduction pathways that culminate (in most cases) in the expression of genes with important functions in immune defense mechanisms. Of these mechanisms the inflammatory response is perhaps the most important as it allows the recruitment, to the site of infection, of immune cells that actively fight pathogens and mediate subsequent innate and adaptive responses. Recognition of PAMPs is mediated by a heterogeneous group of receptors collectively known as pattern recognition receptors (PRRs) [56, 60].

1.4.1 Pattern recognition receptors

Unlike the adaptive branch of the immune system which depends on randomly-generated and highly specific receptors, the innate immune response relies on germ-line encoded sensors known as PRRs that recognize a wide spectrum of conserved molecular patterns which are unique to different groups of microbes and play pivotal roles in maintaining their survival. Typical examples of conserved molecular patterns, otherwise known as PAMPs, include components of the bacterial cell wall. While some PRRs are localized on the plasma membrane, some others are expressed on endosomal surfaces as wells as in the cytosol, therefore ensuring a complete surveillance within and around the cell [60]. Engagement of these receptors results in the expression of antimicrobial peptides, type I interferons (IFNs), and various chemokines and cytokines of which interleukin (IL)-1β, IL-6 and the tumor necrosis factor-α (TNFα) are of vital importance for the development of inflammatory responses. PRRs are classified in a variety of receptor families, each with varied biochemical properties, locations and cognate ligands [61].

1.4.1.1 Toll-like receptors

The first and most comprehensively described group of PRRs is the Toll-like receptor (TLR) family. Its name derives from the Toll proteins that were discovered in *Drosophila* fruit flies, where they were shown to play dominant roles in embryo development and in immune responses against fungi [62]. In vertebrates, where this receptor family is only involved in

innate immune defenses, several TLRs have been described. To this date 10 TLRs have been identified in humans (TLR1-10) and 12 in mice (TLR1-9 and TLR11-13; with murine TLR10 being a pseudogene). They can roughly be subdivided in terms of their localization and the ligands they recognize. In this fashion, human TLR1, 2, 4, 5, 6 and 10 are primarily found on the plasma membrane and mediate recognition of different microbial cell wall components; TLR3, 7, 8 and 9 are expressed in the lumen of endosomes where they sense nucleic acids from phagocytosed microbes or aberrantly localized nucleic acids from the host. TLR4, however, has been also found associated with endosomal membranes acting as sensor of viral envelope constituents [63]. Structurally, TLRs are characterized by the presence of leucine-rich repeats (LRRs) in the extracellular region and a Toll/interleukin-1 receptor (TIR) cytoplasmic domain that is also present in the IL-1 receptor (IL-1R). Binding of the ligand to the LRR domains triggers the homodimerization of the receptor (an exceptional case is TLR2 which forms heterodimers with TLR1 or 6) allowing the cytosolic TIR domains of the TLR dimer to serve as docking places for the central adaptor proteins MyD88 (Myeloid differentiation primary response 88) or TRIF (TIR-domain-containing adapter-inducing interferon-β). Once activated, MyD88 and TRIF signal through the transcription factors NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and IRF3 (Interferon Regulatory Factor 3) inducing the expression of pro-inflammatory cytokines and type I IFNs respectively [64].

1.4.1.2 Nod-like receptors

Unlike TLRs which remain associated with cell membranes, the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) carry out their functions in the cytosolic space. As exception to the rule, the recently described NLRX1 has been found localized in the outer mitochondrial membrane where it operates as a negative regulator of antiviral defense mechanisms [65]. Members of this broad protein family share a common domain structure comprised of a C-terminal LRR domain, a central NOD domain and an N-terminal protein-protein interaction domain. It is based on the structure of this last domain that the NLR family is further classified into 4 subfamilies: NLRA (the only member of this subfamily contains an acidic transcriptional activation domain), NLRB (subfamily with only one member comprised of a baculoviral inhibition of apoptosis protein repeat (BIR) domain), NLRCs (consisting of 6 members with a caspase activation and recruitment domain [CARD]) and NLRPs (the largest subfamily characterized by a pyrin domain [PYD]) [66].

The NLRC and NLRP subfamilies are the largest and best described groups of NLRs. Within the NLRC subfamily, NOD1 and NOD2 have been widely studied and characterized. These receptors mediate recognition of peptidoglycan fragments from the cell wall of intracellular and extracellular bacterial pathogens, upon which, the receptor interacting protein 2 (RIP2) is recruited to the CARD domain of the NOD sensor, resulting in the activation of NF-κB and the subsequent induction of pro-inflammatory cytokines. Interestingly, NOD2 has been reported to additionally respond to single-stranded viral RNA and to subsequently induce a type I IFN response [67, 68].

NLRP3 has also been the focus of extensive research due to its distinctive features: i) a myriad of compounds that range from nucleic acids and proteins to uric acid and silica crystals have been observed to induce the activation of NLRP3 and ii) rather than inducing the transcriptional activation of pro-inflammatory mediators, NLRP3 triggers the assembly of a heteromeric multi-protein complex known as the inflammasome. This protein complex activates proteases required for the proteolytic activation of the immature forms of IL-1β and IL-18 and is thus of critical importance for inflammatory responses mediated by these cytokines. NLRP1, NLRC4 and the non-NLR protein absent in melanoma 2 (AIM2) have also been implicated in inflammasome assembly upon recognition of certain ligands [69].

1.4.1.3 C-type lectin receptors

Lectins comprise a broad group of carbohydrate-binding proteins involved in multiple cellular processes. C-type lectin receptors (CLRs) are a type of calcium-dependent lectins (hence the label “C-type”) which mainly recognize sugar moieties from fungi, but whose recognition range has been also observed to encompass carbohydrates from mycobacterium and different viruses and parasites [70, 71]. CLRs share a common carbohydrate-recognition domain (CRD) and are confined almost exclusively to the plasma membrane, with the mannose-binding lectin (MBL) being the exception as this protein remains in a soluble extracellular form serving as activator of the lectin pathway of the complement system. Depending on the receptor, carbohydrate biding to the CRD may result in stimulation of phagocytosis, initiation of distinct signaling pathways that culminate in the production of pro- and anti-inflammatory mediators or even activation of inflammasome assembly [72, 73].

1.4.1.4 RIG-I-like receptors

The retinoic acid-inducible gene-I-like receptor (RLR) family contains only 3 members all of which remain circumscribed to the cytosol where they perform important functions in antiviral defenses. The three known members of this family are RIG-I (retinoic acid-inducible gene I), MDA-5 (melanoma differentiation-associated gene-5) and LGP2 (laboratory of genetics and physiology 2). RIG-I and MDA-5 rely on a helicase domain for viral RNA recognition and a CARD domain for downstream signaling. Discrimination of self vs. non-self RNA is thought to be mediated by recognition of structural features present in viral RNA (e.g. virus-specific sequence motifs, or 5′ triphosphate modifications). Following RNA recognition, RIG-I and MDA-5 induce the mitochondrial antiviral signaling (MAVS) adaptor protein which promotes activation of the transcription factors IRF3, IRF7 and NF-κB to regulate the subsequent expression of type I IFNs and pro-inflammatory cytokines. RIG-I also serves as an indirect sensor of AT-rich cytosolic DNA through a mechanisms that involves RNA-polymerase III-mediated transcription of DNA into 5'-triphosphate-dsRNA [74-76]. While LGP2 shares extensive sequence conservation with MDA-5 and RIG-I, it lacks the CARD domain required for signaling, and thus, does not act as a sensor but rather as a regulator of RLR signaling [77].

1.4.1.5 Innate recognition of cytosolic DNA by the cGAS-STING pathway

Although the immunostimulatory properties of aberrantly located DNA (i.e. cytosolic or endosomal DNA of microbial or host origin) have been known for over half a century, it wasn't until the last decade that the first DNA-sensing PRRs were first identified [78, 79]. TLR9 was the first receptor to be implicated in DNA recognition. This endosomal sensor induces expression of inflammatory cytokines and type I IFNs upon recognition of unmethylated CpG DNA motifs characteristic of several microbial genomes [80]. A TLR9-independent mechanism of type I IFN induction was later reported to be mediated by the TANK-binding kinase 1 (TBK1) and IRF3, in response to cytosolic DNA, suggesting the existence of novel receptors in the cytoplasm [81]. In succeeding years, the first putative cytosolic DNA receptor was described and designated as DNA-dependent activator of IRFs (DAI). DAI was found to bind DNA and to upregulate IFN expression in response to poly(dA:dT). However, further experiments challenged the relevance of this protein in DNA-dependent immune responses and pointed to the existence of other, more relevant receptors

[82, 83]. Following the discovery of DAI, numerous reports brought into focus the existence of several receptors with varied degrees of influence in the context of different infectious diseases. Among these receptors, IFI-16 (IFN-γ-inducible protein 16) and DDX41 (DEAD-Box Helicase 41) appeared to have a significant role in innate immune responses in certain cell types, although their relevance in a broader infection context still remains controversial [83-85]. A further cytoplasmic DNA sensor, known as AIM2, was interestingly shown, in four independent publications, to assemble an inflammasome complex and activate a cell death response upon cytosolic DNA recognition [86-89].

As a result of an intense research activity aimed at exposing further and more relevant cytosolic DNA receptor candidates, the adaptor protein stimulator of interferon genes (STING) was concurrently discovered. STING (also known as MITA, MPYS or ERIS and encoded by the *TMEM173* gene) is a transmembrane protein located predominantly in the ER. Despite not being a direct DNA receptor, STING is widely accepted as a critical mediator of DNA-dependent immune responses, serving as an adaptor protein for several cytosolic DNA receptors [90-92]. Upon its activation, STING translocates from the ER to the Golgi apparatus where it forms aggregates that facilitate the recruitment and activation of TBK1. This kinase then proceeds to catalyze the phosphorylation of STING residues, which results in the recruitment and further phosphorylation of IRF3. Activated IRF3 subsequently translocates to the nucleus where it drives the expression of type I IFNs (Fig. 2) [93]. Furthermore, STING-dependent TBK1 activation has been found to additionally promote the expression of pro-inflammatory cytokines through the NF-κB transcription factor [94].

Remarkably, STING has been shown to additionally serve as a direct sensor of cyclic dinucleotides (CDNs) such as cyclic di-adenosine monophosphate (c-di-AMP) and cyclic di-guanosine monophosphate (c-di-GMP) whose function as second messengers is crucial for prokaryotic signaling cascades [95, 96]. Succeeding experiments along the lines of this newly found function of STING led to the discovery of 2'3'-cyclic GMP-AMP (cGAMP) as the first metazoan CDN, and cGAMP-synthase (cGAS) as the enzyme responsible for its production upon direct recognition of dsDNA [97, 98]. cGAS discovery was a major breakthrough as it provided almost unequivocal evidence for a major cytosolic DNA receptor and significantly improved the understanding of the recognition pathways that take place upon DNA sensing in the cytoplasm. Binding of DNA, whether from the host or a pathogen, triggers a conformational change that activates the catalytic domain of cGAS and allows the binding of ATP and GTP resulting in the synthesis of cGAMP. This CDN acts as a strong agonist of STING which upon activation elicits the reactions that were previously described leading to

type I IFN induction (Fig. 2) [99]. Recognition of DNA by cGAS is sequence-independent, yet apparently, it is largely influenced by the length of the cognate DNA segment [100].

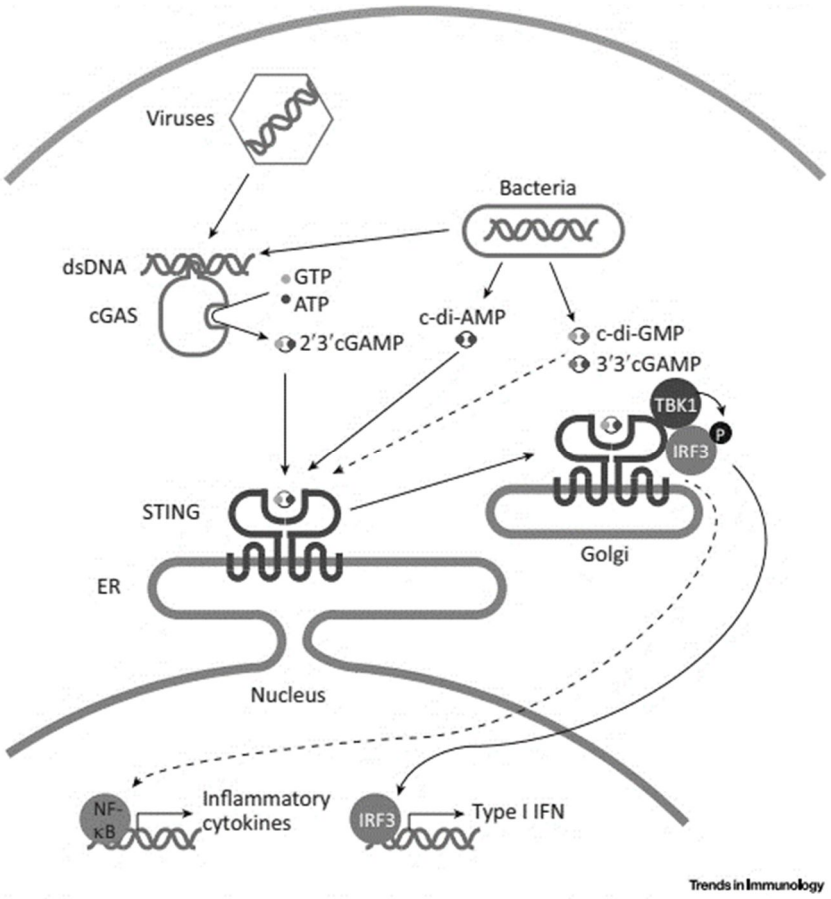


Figure 2. cGAS-STING is a major pathway for the recognition of cytosolic DNA. Upon recognition of DNA, the catalytic domain of cGAS becomes activated and carries out the synthesis of cGAMP which in turn binds to and stimulates the adaptor protein STING. Activation of STING results in its dimerization and translocation to the Golgi apparatus where it mediates the phosphorylation of TBK1 and IRF3. The latter then acts as a transcription factor inducing the expression of type I IFNs. Source [101].

1.4.2 Type I IFNs

IFNs are a large family of glycoproteins produced as a result of PRR recognition of a wide variety of stimuli. This group of cytokines has been typically classified in three groups: type I IFNs which are synthesized by virtually all groups of cells and are comprised by several gene products of which IFNβ and different subtypes of IFNα are by far the best characterized (the terms type I IFN and IFNα/β will therefore be used indistinctively throughout this document); type II IFN comprised of a single member, IFNγ, produced by a restricted group of immune

cells and recognized as a potent modulator of macrophage activity and adaptive immune responses; and type III IFNs, a less well described group of IFNs with properties similar to those of the type I IFN family but with limited activity since their receptor is mostly confined to epithelial cells [102, 103].

Commonly recognized for their ability to orchestrate antiviral defense mechanisms, type I IFNs are potent modulators of immune responses acting as inducers of gene transcription programs that result in the production of hundreds of proteins with diverse functions in innate and adaptive responses against viruses, but also against other types of pathogens [103]. Following the induction of type I IFNs through a broad range of signaling events, some of which have been briefly discussed before, this group of cytokines stimulates in an autocrine or paracrine fashion cells that express the IFNα/β receptor (IFNAR). This receptor, whose expression is as ubiquitous as that of its cognate ligands, is composed of two subunits, IFNAR1 and IFNAR2. Upon its engagement, IFNAR recruits and activates the Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which in turn activate members of the signal transducer and activator of transcription (STAT) protein family. In the canonical IFNAR signaling pathway, activated STAT1 and STAT2 associate with IRF9 forming a heterodimeric protein complex known as IFN-stimulated gene factor 3 (ISGF3) which following its translocation to the nucleus binds to specific sequences known as IFN-stimulated response elements (ISREs) thus inducing the expression of interferon stimulated genes (ISGs) [103, 104]. IFNα/β have been also found to stimulate the activation of STAT3, 4 and 5 which are associated with other cytokine-mediated pathways, therefore broadening the spectrum of genes whose expression can be regulated by type I IFNs. In this way, type I IFNs also promote the expression of genes that encode chemokines, cytokines, anti- and pro-apoptotic mediators and antibacterial effectors [105].

Pioneering studies using the intracellular pathogen *Chlamydia spp.*, revealed that not only are type I IFNs induced during infections with these bacteria, but also they contribute to bacterial restriction and clearance [106, 107]. Subsequent studies showed however, that the role of these cytokines in different bacterial infections is not always protective. Depending on the bacterial pathogen and the nature of the infection, these cytokines may result in beneficial or detrimental outcomes for the host. In the former case for example, IFNα/β have been observed to promote the suppression of exacerbated inflammatory responses or direct the expression of important chemokines during infections caused by *Streptococcus pyogenes* or *Helicobacter pylori* respectively. Additionally, type I IFN signaling is a critical mediator of pro-inflammatory responses and antibacterial defenses during infections with *Streptococcus*

agalactiae [108]. Furthermore, IFNα/β stimulate cell-intrinsic immune responses that promote clearance of *L. pneumophila* through a mechanism that will be described below [109].On the other hand, numerous studies have described how during certain bacterial infections, the immunosuppressive and pro-apoptotic properties of type I IFNs result in detrimental outcomes for the host [103, 110]. Mice deficient in different components of the type I IFN signaling cascade show enhanced resistance during infection with *Listeria monocytogenes* [111]. Moreover, IFNα/β have been observed to undermine resistance mechanisms against *Mycobacterium tuberculosis* in mice and to be linked with disease progression in human patients [112, 113]. In any case, detection of bacterial DNA in the cytoplasm is an important event during host immune responses and appears to significantly contribute to the initiation of type I IFN responses during several bacterial infections [110].

1.4.3 Innate immune responses of the lung

The respiratory tract is permanently in contact with the outside environment and is therefore regularly subject to potential infectious and non-infectious hazards. Respiratory immune responses are required to promptly fight external threats while invoking regulatory mechanisms in order to avoid exacerbated inflammatory responses that might not be compatible with the gas exchange function of the respiratory tract [114]. The pulmonary epithelium constitutes the first line of defense as it provides a physical barrier and actively participates in pathogen clearance through the production of antimicrobial molecules and the secretion of mucus which traps foreign particles to be later eliminated through mucociliary action [115]. In the event that additional layers of protection are needed, several types of resident cells such us AMs, bronchial/alveolar epithelial cells and dendritic cells, equipped with a distinct arrange of PRRs, are ready to mediate recognition of pathogens and coordinate targeted defense responses. Among the PRRs expressed by lung resident cells, TLRs are considered important albeit redundant mediators of respiratory immune responses [116]. TLRs with nucleic acid-sensing properties, TLR3 and to a lesser extent TLR7 and 9, significantly contribute to antiviral responses [117, 118]. On the other hand, TLR2, 4 and 5 seem to have a predominant role in sensing different bacterial components, although TLR9 has also been documented to influence defense responses against certain lung bacterial pathogens [116, 119]. Other PRRs with considerable influence on respiratory immune responses are the NLRs NOD1, NOD2, NLRP3, NLRC4 and the NLR family apoptosis inhibitory protein 5 (NAIP5); the RLRs RIG-I and MDA5 and bitter and sweet taste receptors

which have been recently described to be expressed by respiratory epithelia and to participate in immune defense mechanisms [116, 119, 120].

While the adaptor protein STING has been shown to promote immune responses against different respiratory viruses such as Influenza A virus and coronavirus, full engagement of the cGAS-STING pathway has only been observed during infections with adenovirus [121-123]. Similarly, different respiratory bacterial pathogens are known to activate STING, but observation of *in vivo* cGAS activation has remained restricted to infections with intracellular pathogens such as *M. tuberculosis* [124, 125].

PRR activation in the lung leads to the production of a myriad of mediators that can trigger cell-autonomous defense mechanisms, recruit fast-responding effector cells such as neutrophils and activate lung resident lymphoid cell populations, ultimately leading to either, pathogen clearance and tissue repair, or prolonged inflammatory conditions [114, 126]. Of these mediators, type I IFNs are considered crucial in the initiation of inflammatory lung responses, and its contribution has been deemed protective or detrimental depending on the respiratory pathogen involved [127].

Containment and resolution of inflammatory responses in the lung is as crucial as fighting the infection itself. Due to the detrimental nature that these responses might have on lung tissues, the respiratory immune response has evolved anti-inflammatory mechanisms that counteract exacerbated inflammatory responses and promote tissue repair. Among these, the anti-inflammatory cytokine IL-10, and lipid mediators such as lipoxins, resolvins, and protectins, play central roles in controlling inflammatory defense processes in the lung [128].

1.4.3.1 Innate immune recognition of L. pneumophila

Recognition of cell wall components and flagellin from *L. pneumophila* occurs through TLR2 (in cooperation with TLR1 and 6) and TLR5 respectively, and is mediated by the adaptor protein MyD88 [129, 130]. Endosomal TLR9 has additionally been reported to detect *Legionella* DNA in mice, although its role, together with that of TLR5, has been considered redundant [131, 132]. TLR activation results in the production of important pro-inflammatory cytokines and the subsequent secretion of chemokines required for the recruitment of neutrophils and monocytes that contribute to bacterial clearance [133].

Mice deficient in NOD1 and 2 or their signaling mediator RIP2, display diminished recruitment of neutrophils and decreased bacterial clearance, suggesting that the signaling pathway subordinated to these receptors also contributes to the innate immune responses

against *L. pneumophila* [134]. Apart from being recognized by TLR5, flagellin, delivered through the T4SS, additionally elicits the assembly of the NAIP5/NLRC4 inflammasome. This protein complex has been observed restricting bacterial growth by promoting fusion of the lysosome with the LCV and inducing a type of caspase-1-dependent cell death called pyroptosis [135-137]. The non-canonical caspase 11 inflammasome has likewise been shown to become activated during infections with *L. pneumophila* and its intervention appears to significantly restrict the growth of this bacterium. The NLRP3 inflammasome, on the other hand, seems to play a rather redundant role and its activation appears relevant only when the activation of the NAIP5/NLRC4 inflammasome is circumvented (i.e. during infection with flagellin-deficient *L. pneumophila* mutants) [138, 139].

Cytosolic recognition of *Legionella* DNA also partakes in the immune responses against this pathogen and results in the production by AMs of type I IFNs through a mechanism that is dependent on STING, IRF3 and the bacterial T4SS [140, 141]. Furthermore, previous evidence suggests that DNA from *L. pneumophila* is initially sensed by the cGAS receptor [125]. As illustrated by different studies using single-knockout and double-knockout mice, IFNα/β and IFNγ are critical mediators of immune defenses against *L. pneumophila* [109, 140, 142]. Type I and II IFNs are master regulators of gene expression and strong inducers of cell-intrinsic defense mechanisms in macrophages during *L. pneumophila* infection. Whereas type I IFNs are most likely produced by AMs, and presumably surrounding cells as well, IFNγ appears to be primarily secreted by resident lymphoid cells [143]. The concerted action of these cytokines, results in the induction of ISGs such as the immune-responsive gene 1 (IRG1) which, upon recruitment to the LCV, catalyzes the production of the bactericidal metabolite itaconic acid [109]. Equally crucial for the antibacterial responses elicited against *L. pneumophila*, the pro-inflammatory cytokine TNFα is produced by monocytes and neutrophils which are recruited to the site of infection. This cytokine acts on the TNFα receptor 1 (TNFR1) and, as in the case of type I and II IFNs, stimulates AMs to restrict bacterial growth within the LCV [144, 145].

1.4.3.2 Innate immune recognition of S. pneumoniae

Constituents of the pneumococcal cell wall such as lipoteichoic acid and lipoproteins are primarily sensed through TLR2 [146]. Endosomal TLR9 likewise serves as receptor for pneumococcal DNA and promote anti-pneumococcal responses [147]. Although TLR4 is a well-known receptor of lipopolysaccharides from Gram-negative bacteria, it has also been

suggested to be involved in the recognition of Ply. However, engagement of TLR4 during pneumococcal infections could also be the consequence of binding of host ligands released as a result of Ply-mediated cell damage [148, 149]. The use of single TLR knockout mice in different studies has demonstrated that though important, the involvement of TLR2, 4 and 9 during anti-pneumococcal responses seems to be partly redundant. In contrast, MyD88 appears to be a central player in the development of innate immune defenses against *S. pneumoniae*, as mice lacking this adaptor protein are highly susceptible to pneumococcal infections [150-152].

Phagocytosed pneumococci are recognized by NOD2 following the Ply-mediated delivery of peptidoglycan fragments into the cytosol. Activation of this receptor, results in the production of important chemokines that promote macrophage recruitment and bacterial clearance [153]. The NLRP3 inflammasome is required for the Ply-dependent maturation of IL-1β and IL-18 in macrophages and dendritic cells [154-156]. Interestingly, NLRP3 is differentially activated by distinct Ply variants and is one of the first identified cases in which a virulence factor polymorphism strongly influences the activation of host immune receptors. In addition to its important contribution to bacterial clearance, NLRP3 has been shown to promote alveolar barrier integrity through a mechanism that seems to be independent of inflammasome formation [154, 157]. Other receptors with proven ability to sense and mediate immune responses against *S. pneumoniae* are the CLR DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) and the scavenger receptor MARCO (Macrophage receptor with collagenous structure) [158, 159]. A further CLR involved in the recognition of *S. pneumoniae* is the Macrophage-inducible C-type lectin (Mincle). Although a recent publication found no evidence to suggest that this protein is involved in antibacterial responses during *S. pneumoniae* infection [160], a subsequent study revealed that Mincle deficiency in mice led to dysregulation of cytokine production and increased pneumococcal burden [161].

As in the case of several other bacterial infections, challenge with *S. pneumoniae* is widely accepted to stimulate the generation of type I IFNs. Lung epithelial cells do not substantially contribute to the production of these cytokines during pneumococcal infection, and instead are stimulated in a paracrine fashion by IFNα/β produced by AMs and potentially DCs [162, 163]. Production of these cytokines in these cells requires bacterial uptake and expression of Ply and depends on cytosolic recognition of pneumococcal DNA, the adaptor protein STING and the transcription factor IRF3 [163]. Different studies have examined the role of type I IFNs during pneumococcal infections with contrasting results. Whereas some studies using

mice lacking the IFNAR receptor have shown that type I IFNs are crucial for bacterial resistance and host cell survival [108, 164], other reports examining the invasive serotype 1 strain or post-influenza pneumococcal infections have concluded that this cytokine family can in fact exert detrimental effects for the host [165, 166].

1.4.4 Effect of host gene variations on innate immunity

Human infectious diseases exhibit significant inter-individual phenotypic variability to the extent that a particular infectious disease might remain asymptomatic in some individuals but result life threatening to others. The pathogen has been traditionally viewed as the main cause of disease and the major determinant of its outcome; however, accumulating evidence indicates that host genetic variants are as crucial in defining the course of the disease [167]. A paradigm of human genetics of infectious diseases was proposed in which susceptibility to a specific infectious disease was considered to be the consequence of multiple genetic variants making small contributions to the observed phenotype, whereas rare single-gene deficiencies led to an elevated vulnerability to numerous infections [168]. The latter case corresponds to what has been labeled as primary immunodeficiencies (PIDs) whose cause is rooted in mostly monogenic inborn errors that follow a Mendelian pattern of inheritance. To date, more than 350 such deficiencies have been characterized, with several of them, such as Tyk2 and STAT1 deficiencies, affecting various aspects of innate immunity and conferring enhanced susceptibility to a wide range of infections [169]. Other PIDs, however, have challenged the established paradigm as they were shown to result in an increased susceptibility to a narrow group of infections or even to a specific pathogen. For instance, different mutations affecting genes which encode certain components of the complement system or the IFNγ-mediated immune responses lead to severe susceptibility to a restricted group of diseases such as infections with bacteria from the genus *Neisseria* or *Mycobacterium* respectively [170].
With the advent of genetic epidemiology approaches and genome-wide association studies, it became possible to ponder the influence of genetic variation on predisposition or resistance to infectious diseases. It was progressively evident that major loci and gene polymorphisms, which are maintained at an appreciably frequency in certain populations and do not cause any known immunodeficiencies or pathological disorders, can be associated with an increased/decreased risk of contracting a given infectious disease [171]. Numerous polymorphisms such as single-nucleotide polymorphisms (SNPs) in genes encoding PRRs or their downstream signaling partners have been shown to be linked with protection or

susceptibility towards different pathogens including *L. pneumophila* and *S. pneumoniae* [172]. Case-control studies using samples from an LD outbreak occurred in The Netherlands in 1999 revealed that a common stop codon polymorphism in TLR5 and a SNP in TLR6 are both associated with an increased susceptibility to pneumonia caused by *L. pneumophila* [173, 174]. Surprisingly, two SNPs in the gene encoding TLR4 which have been previously observed to be linked with heightened susceptibility to different bacterial infections, were found in similar analyses to confer protection to LD [175]. A more comprehensive research activity has brought to light both rare genetic immunodeficiencies and common polymorphisms that associate with susceptibility to pneumococcal disease. Patients with PIDs related to MyD88, IRAK4 (interleukin-1 receptor-associated kinase 4) or NEMO (NF-κB essential modulator) deficiencies, usually suffer from recurrent life-threatening infections with *S. pneumoniae* [176, 177]. Moreover, several polymorphisms in TLR-NF-κB signaling components and in the MBL have been suggested to result in increased predisposition or protection to pneumococcal disease [168, 178-180].

1.4.4.1 Genetic heterogeneity in the cGAS-STING pathway

Genetic analyses of children who presented systemic inflammation, cutaneous vasculopathy and an exacerbated type I IFN signature, led to the recent classification of a novel PID involving STING. This immunodeficiency, termed as STING-associated vasculopathy with onset in infancy (SAVI), is caused by rare gain-of-function mutations in *TMEM173* (the gene encoding STING), which result in chronic overproduction of IFNα/β and the manifestation of severe auto-inflammatory disorders in early infancy [181]. Further gain-of-function mutations in *TMEM173*, different than those accounting for the onset of SAVI, were recently shown to be the cause for the development of familial chilblain lupus, a rare autoimmune cutaneous disorder previously shown to also occasionally originate from loss-of-function mutations in important cytosolic nucleases [182]. Interestingly, *TMEM173* also exhibits significant genetic heterogeneity reflected in the existence of different alleles with widespread presence in healthy population. The second most frequent allele after the wild type (WT) R232 variant is HAQ STING, which contains a haplotype composed of three non-synonymous SNPs (rs11554776, rs78233829 and rs7380824) that give rise to the amino acid substitutions R71H-G230A-R293Q, hence the name HAQ (Fig. 3). When ectopically expressed, HAQ STING showed significant defects in activating type I IFN production in response to endogenous and bacterial CDNs as well as *L. monocytogenes* infection [183, 184]. Moreover, individuals

carrying the HAQ allele in homozygosity showed diminished production of STING in B-cells and in organs such as the skin, esophagus and lung. Transgenic mice expressing an equivalent variant to human HAQ likewise exhibited low levels of STING expression and additionally showed a weak IFN induction in response to CDNs.

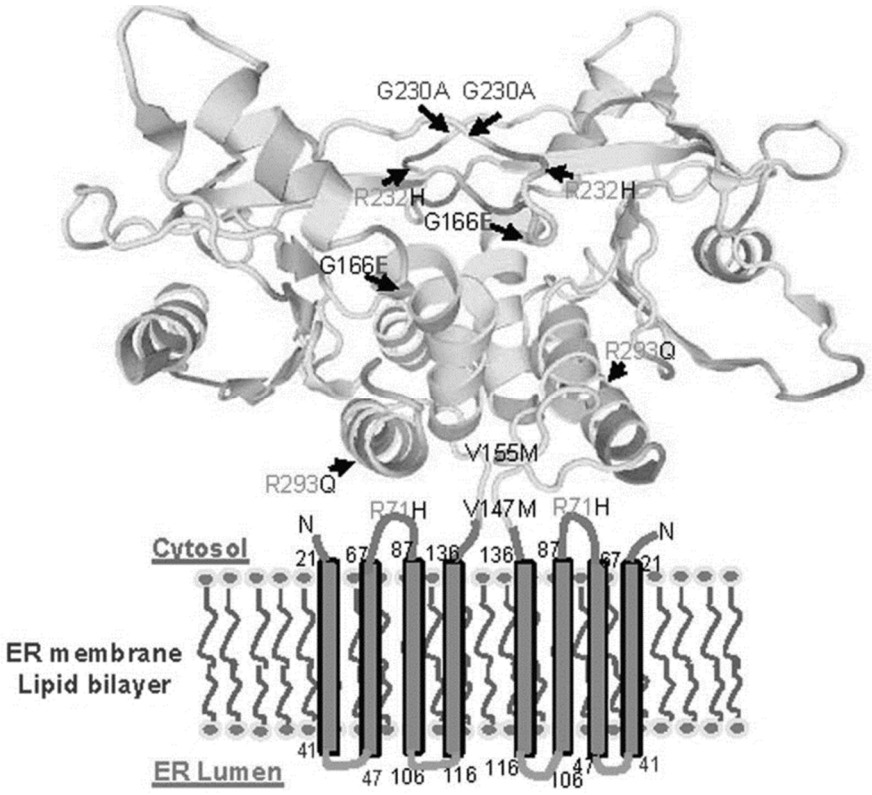


Figure 3. Structure of a STING dimer showing the positions at which amino acid substitutions occur as a result of the most common *TMEM173* polymorphisms. The G230A and R232H substitutions occur in the binding pocket where CDNs tether to STING. The R293Q substitution sits at the bottom of this region. Located in a predicted cytosolic loop, the R71H substitution occurs bellow the binding pocket. Other substitutions shown correspond to mutations associated with auto-inflammatory disorders involving STING. Figure adapted from [185].

Genotype data analyses from the 1000 Genomes Project (phase III) revealed that the frequency of the HAQ allele in homozygosity was highest in East Asian population (~16%), followed by South Americans, South Asians and Europeans with ~8, ~7 and ~3%, respectively. Intriguingly, the analysis did not provide evidence of the presence of homozygous HAQ individuals in African population [186].

The third most common allele, R232H (rs1131769), has been found to be defective in recognizing bacterial CDNs, although interestingly, its activity appears to remain unchanged when challenged with 2'3'-cGAMP in the DNA-sensing pathway. Unlike the HAQ allele, the R232H variant in homozygosity has a more homogeneous distribution in different human populations, with its frequency ranging from ~1 to ~3% in all populations analyzed [183, 184, 186, 187].

In spite of the varied palette of polymorphisms thus far reported for cGAS in databases, only one study has examined the presumptive association of genetic variants of this protein with human disease. The rs311678 SNP, which is localized in the third intron of the cGAS gene, was observed to result in a diminished protein expression and was found associated with protective phenotypes during infection with human papilloma virus in Chinese population. Other cGAS variants examined in the study however, had no effect on protein expression nor were they associated with this diseased [188].

The effect of genetic variants involved in the STING-signaling pathway on bacterial infections and their potential association with increased or decreased risk of contracting infectious diseases in humans has not yet been addressed.

2 Aim of the study

S. pneumoniae and *L. pneumophila* are two of the most common causes of bacterial CAP, with the former remaining as a leading cause of mortality and morbidity in children under 5 years of age. Innate immune responses against these and other pathogens heavily depend on the recognition of microbial components by so-called PRRs. Engagement of these receptors triggers the activation of various signaling events that culminate in the production of inflammatory mediators that activate antimicrobial defense mechanisms. Among those mediators are pro-inflammatory cytokines and type I IFNs, both of which play fundamental roles during bacterial infections. Most, if not all components of the innate branch of the immune response, exhibit extensive genetic heterogeneity that significantly influences predisposition of the host to several infectious diseases.

Among the different sensing mechanisms stimulated during infections with *S. pneumoniae* or *L. pneumophila*, STING-mediated cytosolic recognition of bacterial DNA appears central for the initiation of type I IFN responses. STING is an important signaling adaptor in the cytosolic recognition of microbial DNA and additionally serves as a direct sensor of bacterial CDNs. Upstream of STING the enzyme receptor cGAS has been recognized as the primary sensor of DNA in the cytosol during infections with several pathogens. Following DNA recognition, cGAS produces the CDN cGAMP which binds and activates STING. Previous studies have reported the presence of genetic heterogeneity in the gene encoding STING. The second most common allele, HAQ, has been described as a loss of function allele, defective in activating type I IFNs. Additionally, the third most common variant, R232H, has been found to be defective in sensing bacterial CDNs but active in mediating signals downstream of cGAS-dependent DNA recognition. Nevertheless, the influence of STING genetic variants in primary infections and the effect of these variants on susceptibility to infectious diseases in humans have not yet been addressed.

The aims of the present study were: i) to investigate the role of the cGAS-STING pathway in the mediation of immune defenses against *L. pneumophila* and *S. pneumoniae* in both mice and humans, ii) to evaluate the impact that HAQ and R232H STING have on antibacterial immune responses and iii) to determine whether carriage of either of these alleles is associated with *L. pneumophila* or *S. pneumoniae*-induced pneumonia in humans.

3. Publications

3.1 First publication

Title: The common HAQ STING variant impairs cGAS-dependent antibacterial responses and is associated with susceptibility to Legionnaires' disease in humans

Authors: **Juan Sebastián Ruiz-Moreno**, Lutz Hamann, Javeed A. Shah, Annelies Verbon, Frank P. Mockenhaupt, Monika Puzianowska-Kuznicka, Jan Naujoks, Leif E. Sander, Martin Witzenrath, John C. Cambier, Norbert Suttorp, Ralf R. Schumann, Lei Jin, Thomas R. Hawn, Bastian Opitz &CAPNETZ Study Group

Journal: PLOS Pathogens

Received: March 8, 2017

Revised: December 6, 2017

Accepted: December 18, 2017

DOI: 10.1371/journal.ppat.1006829

PLOS | PATHOGENS

RESEARCH ARTICLE

The common HAQ STING variant impairs cGAS-dependent antibacterial responses and is associated with susceptibility to Legionnaires' disease in humans

Juan S. Ruiz-Moreno[1], Lutz Hamann[2], Javeed A. Shah[3,4], Annelies Verbon[5], Frank P. Mockenhaupt[6], Monika Puzianowska-Kuznicka[7,8], Jan Naujoks[1], Leif E. Sander[1,9], Martin Witzenrath[1,9,10], John C. Cambier[11], Norbert Suttorp[1,9,10], Ralf R. Schumann[2], Lei Jin[12], Thomas R. Hawn[3], Bastian Opitz[1,9]*, CAPNETZ Study Group[¶]

1 Department of Internal Medicine/Infectious Diseases and Pulmonary Medicine, Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany, **2** Institute of Microbiology and Hygiene, Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health Berlin, Berlin, Germany, **3** Department of Medicine, University of Washington, Seattle, Washington, United states of America, **4** VA Puget Sound Health Care System, Seattle, Washington, United states of America, **5** Department of Medical Microbiology and Infectious diseases, Erasmus University Medical Center, Rotterdam, The Netherlands, **6** Institute of Tropical Medicine and International Health, Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany, **7** Department of Human Epigenetics, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, **8** Department of Geriatrics and Gerontology, Medical Centre of Postgraduate Education, Warsaw, Poland, **9** German Center for Lung Research (DZL), Germany, **10** CAPNETZ STIFTUNG, Hannover, Germany, **11** Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, Colorado, United States of America, **12** Department of Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, University of Florida, Gainesville, Florida, United States of America

¶ Membership of the CAPNETZ Study Group is provided in the Acknowledgments.
* bastian.opitz@charite.de

OPEN ACCESS

Citation: Ruiz-Moreno JS, Hamann L, Shah JA, Verbon A, Mockenhaupt FP, Puzianowska-Kuznicka M, et al. (2018) The common HAQ STING variant impairs cGAS-dependent antibacterial responses and is associated with susceptibility to Legionnaires' disease in humans. PLoS Pathog 14(1): e1006829. https://doi.org/10.1371/journal.ppat.1006829

Editor: Dario S. Zamboni, University of São Paulo FMRP/USP, BRAZIL

Received: March 8, 2017

Accepted: December 18, 2017

Published: January 3, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the Deutsche Forschungsgemeinschaft (www.dfg.de) (GRK1673/B5 to JSRM and BO; GRK1673/A5 to RRS, SFB/TR84 project A1/A5 to BO, SFB/TR84 project C3/C6 to MW, and SFB/TR84 project B1 to NS; and SPP1580/OP86/10-1 to BO), the German

Abstract

The cyclic GMP-AMP synthase (cGAS)-STING pathway is central for innate immune sensing of various bacterial, viral and protozoal infections. Recent studies identified the common HAQ and R232H alleles of *TMEM173/STING*, but the functional consequences of these variants for primary infections are unknown. Here we demonstrate that cGAS- and STING-deficient murine macrophages as well as human cells of individuals carrying HAQ *TMEM173/STING* were severely impaired in producing type I IFNs and pro-inflammatory cytokines in response to *Legionella pneumophila*, bacterial DNA or cyclic dinucleotides (CDNs). In contrast, R232H attenuated cytokine production only following stimulation with bacterial CDN, but not in response to *L. pneumophila* or DNA. In a mouse model of Legionnaires' disease, cGAS- and STING-deficient animals exhibited higher bacterial loads as compared to wild-type mice. Moreover, the haplotype frequency of HAQ *TMEM173/STING*, but not of R232H *TMEM173/STING*, was increased in two independent cohorts of human Legionnaires' disease patients as compared to healthy controls. Our study reveals that the cGAS-STING cascade contributes to antibacterial defense against *L. pneumophila* in mice and men, and

Ministry for Education and Research (www.bmbf.de) (CAPSyS; grant TP4 to MW), the Ministry of Science and Higher Education, Poland (MEIN; www.bmbf.de) (PBZ-MEiN-9/2/2006 – K143/P01/2007/1 to MPK), and the National Institutes of Health (www.nih.gov) (grants R21AI099346 and T32AI022295 to JCC). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

provides important insight into how the common HAQ *TMEM173/STING* variant affects antimicrobial immune responses and susceptibility to infection.

Trial registration

ClinicalTrials.gov DRKS00005274, German Clinical Trials Register

Author summary

Interferons (IFNs) and pro-inflammatory cytokines are key regulators of gene expression and antibacterial defense during *Legionella pneumophila* infection. Here we demonstrate that production of these mediators was largely or partly dependent on the cyclic GMP-AMP synthase (cGAS)-STING pathway in human and murine cells. Cells of individuals carrying the common HAQ allele of *TMEM173/STING* were strongly impaired in their ability to respond to *L. pneumophila*, bacterial DNA or cyclic dinucleotides (CDNs), whereas the R232H allele was only attenuated in sensing of exogenous CDNs. Importantly, cGAS and STING contributed to antibacterial defense in mice during *L. pneumophila* lung infection, and the allele frequency of HAQ *TMEM173/STING*, but not of R232H *TMEM173/STING*, was increased in two independent cohorts of human Legionnaires' disease patients as compared to healthy controls. Hence, sensing of bacterial DNA by the cGAS/STING pathway contributes to antibacterial defense against *L. pneumophila* infection, and the hypomorphic variant HAQ *TMEM173/STING* is associated with increased susceptibility to Legionnaires' disease in humans.

Introduction

Legionella pneumophila is increasingly recognized as a significant cause of pneumonia in ambulatory and hospitalized patients. This form of pneumonia, commonly referred to as Legionnaires' disease, is associated with high mortality rates, ranging from 8 to 34% depending on the study, despite availability of efficient antibiotic therapies [1]. Known risk factors for Legionnaires' disease include chronic respiratory and cardiovascular diseases, diabetes, cancer, and immunosuppression, although individuals without these predisposing conditions are also affected by Legionnaires' disease [1,2]. Infection occurs following inhalation of *L. pneumophila*-contaminated water droplets. Once in the alveolar compartment, the bacterium is phagocytosed by alveolar macrophages, where it establishes an intracellular replication vacuole. This process requires the Dot/Icm type IV secretion system (T4SS) which injects approx. 300 bacterial effector molecules into the host cytosol [3].

The immune response to *L. pneumophila* in the lung is largely dependent on production of pro-inflammatory cytokines and interferons (IFNs) [4–9]. While IL-1β and TNFα stimulate antibacterial defense by *e.g.* promoting neutrophil recruitment [10,11], type I and II IFNs activate an IRG1- and itaconic acid-dependent macrophage-intrinsic resistance pathway [12]. Infected and bystander macrophages are the main producers of IL-1β, type I IFNs and TNFα, respectively [4,13–15], whereas type II IFN is released by innate and adaptive lymphoid cells [16]. The *L. pneumophila*-induced type I IFN production has previously been shown to depend on the T4SS and cytosolic sensing of bacterial DNA [4,8,13], although detection of the bacterial cyclic dinucleotides (CDNs) cyclic 3′-5′ diguanylate (c-diGMP) has also been implicated in this response [17]. Moreover, we recently showed that inhibition of the endoplasmic

reticulum-associated protein STING (stimulator of IFN genes, also known as MITA, ERIS, MPYS) reduced type I IFN responses to *L. pneumophila* in murine macrophages [4].

STING is encoded by the *TMEM173/STING* gene and functions as both a signaling adaptor in the cytosolic DNA sensing pathway [18,19] and as a receptor for bacterial and endogenous CDNs [20]. Upstream of STING, sensing of DNA in the cytosol additionally requires the cyclic GMP-AMP synthase (cGAS), which binds microbial and host DNA and mediates production of the second messenger cyclic 2'3'-GMP-AMP (2'3'-cGAMP) [21,22]. Recent studies have shown that production of type I IFNs during infections with several bacterial pathogens requires both, cGAS and STING [23–26].

Interestingly, human *TMEM173/STING* exhibits significant heterogeneity [27,28]. The second most common allele besides the WT allele is HAQ, which contains a haplotype comprised of the three non-synonymous single nucleotide polymorphisms (SNPs) R71H-G230A-R293Q. Previous studies indicated that HAQ STING is a loss-of-function variant exhibiting largely reduced capacity to stimulate type I IFN responses [27–29]. Moreover, the third most common allele, R232H, has been found to be defective in sensing bacterial CDNs but not of 2'3'-cGAMP or DNA [28,30]. However, the function of endogenous HAQ and R232H STING in primary infections and the effect of these variants on susceptibility to diseases in humans have not yet been addressed.

Here we tested the hypotheses that i) the cGAS/STING pathway mediates defense against *L. pneumophila* in mice and men, ii) that carriage of HAQ *TMEM173/STING* impairs the antibacterial immune response, and iii) that carriage of HAQ *TMEM173/STING* predisposes to Legionnaires' Disease.

Results

L. pneumophila infection stimulates type I IFN responses *in vitro* and *in vivo* via cGAS and STING

Given the critical role of type I IFNs during *L. pneumophila* infection, we first investigated the role of STING in inducing type I IFN responses in response to *L. pneumophila* as well as *Legionella* DNA in murine bone marrow-derived macrophages (BMDMs). A strong *Ifnb* induction was observed in WT but not in STING-deficient cells in response to two different *L. pneumophila* strains, bacterial DNA as well as our control treatment cGAMP (Fig 1A and 1B). The uptake of *L. pneumophila* into *Tmem173*$^{-/-}$ BMDMs or its replication was, however, not significantly different as compared to WT cells (S1A Fig). As previously shown, *Legionella* lacking the T4SS effector protein sdhA (*ΔsdhA*) [31], which is involved in maintaining the integrity of the *Legionella*-containing vacuole, induced a stronger type I IFN response, whereas a mutant lacking an essential component of the T4SS (*ΔdotA*) was unable to activate STING-dependent *Ifnb* expression (Fig 1B). Moreover, *L. pneumophila*-induced expression of the IFN-stimulated gene *Irg1* and production of the IFN-stimulated chemokine IP-10 were also diminished in *Tmem173*$^{-/-}$ cells (Fig 1C, S1B Fig).

In order to examine the effect of cGAS, BMDMs were first transfected with a siRNA targeting *cGas* or a control siRNA. *cGas*-specific siRNA reduced the expression of its target gene (S2A Fig), and strongly diminished the induction of *Ifnb* and the IFN-induced gene *Irg1* in response to *L. pneumophila* or bacterial DNA (S2B and S2C Fig). As expected, cGAMP-stimulated *Ifnb* expression was not inhibited by *cGas* siRNA, since cGAMP is the second messenger produced downstream of cGAS [21,22]. To further demonstrate the importance of cGAS, we challenged murine cGAS-deficient BMDMs with *L. pneumophila*, DNA and cGAMP. Importantly, *L. pneumophila*- and DNA-induced (but not cGAMP-stimulated) *Ifnb* and *Irg1*

PLOS | PATHOGENS

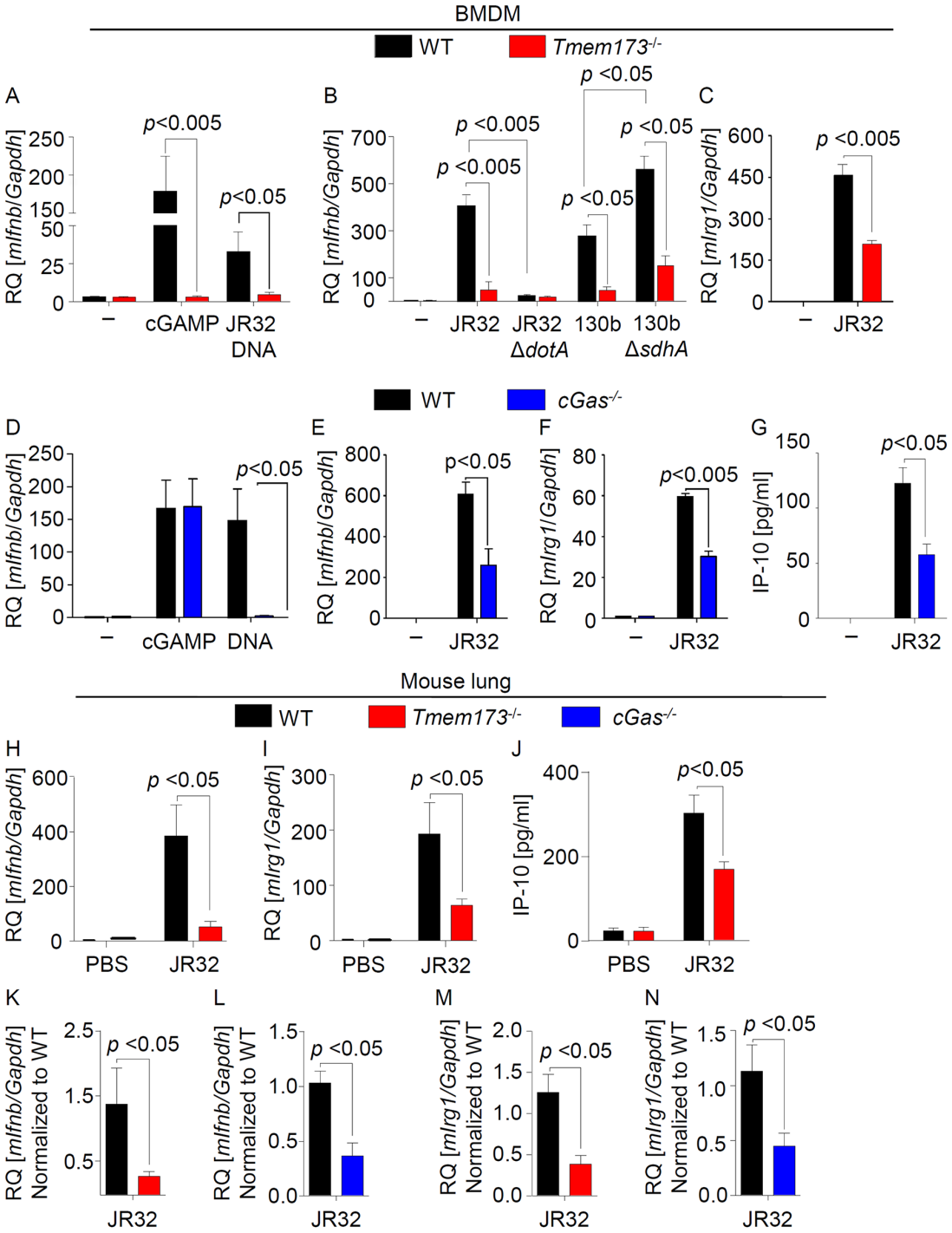


Fig 1. Type I IFN responses during *L. pneumophila* infection are mediated by the cGAS/STING pathway. (A-C) WT and *Tmem173*$^{-/-}$ mouse BMDMs were left untreated or stimulated with 1 ug/ml *L. pneumophila* DNA (JR32 DNA) or 5 ug/ml 2´3-cGAMP (A) or were infected with *L. pneumophila* JR32 WT and 130b WT, or mutant strains deficient for *dotA* or *sdhA* at MOI 10 for 6 h (B, C). Expression of *Ifnb* (A, B) or *Irg1* (C) was measured by qRT-PCR. (D-G) WT and cGAS-deficient BMDMs were stimulated with *L. pneumophila* DNA or 2´3-cGAMP or infected with *L. pneumophila* JR32 WT, and expression of *Ifnb* and *Irg1* was quantified by qRT-PCR (D-F) or production of

IP-10 was measured by ELISA (G). (H-N) WT, STING- and cGAS-deficient mice were intranasally infected with 1×10^6 *L. pneumophila* JR32 WT or instilled with PBS as control (H-J). *Ifnb* and *Irg1* expression in the lungs was assessed 48 (H, I) or 144 h p.i. (K-N) by qRT-PCR, or IP-10 production was measured at 48 h (J). Data are represented as the relative quantification (RQ) of specified mRNAs. Data are shown as the mean + SEM of three to four independent experiments, measured in technical duplicates (Fig. 1A-G) or 6 to 7 mice per group (Fig. H-N). Analyses were performed through the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.

https://doi.org/10.1371/journal.ppat.1006829.g001

expression as well as IP-10 production were reduced in cGAS-deficient BMDMs as compared to WT cells (Fig 1D–1G).

To investigate the relevance of the cGAS/STING pathway for the type I IFN response during lung infection, we intranasally infected WT and *Tmem173*$^{-/-}$ animals with *L. pneumophila*. We observed a strongly decreased induction of *Ifnb* and *Irg1* expression as well as IP-10 production in the lungs of STING-deficient mice 48 h p.i (Fig 1H–1J). Similarly, we found significantly reduced expression levels of *Ifnb* and *Irg1* in lung homogenates from cGAS- and STING-deficient mice 144 h p.i. (Fig 1K–1N). In conclusion, our results show that the cGAS/STING pathway is largely responsible for type I IFN responses to *L. pneumophila* infection in mice.

Production of pro-inflammatory cytokines in response to *L. pneumophila* is partly dependent on cGAS/STING

Next we examined the impact of the cGAS-STING pathway on the production of other pro-inflammatory cytokines in response to *L. pneumophila*. Deficiency of STING or cGAS significantly reduced production of IL-1β and IL-6 and additionally showed some minor effects on TNFα (Fig 2A–2F, S3A–S3C Fig). Moreover, STING-deficient animals produced less IL-1β and IL-6 as well as IFNγ in response to *L. pneumophila* infection of the lung (Fig 2G, 2H and 2J), whereas the effect of STING on TNFα production *in vivo* was not significant (Fig 2I). These data indicate that the cGAS/STING pathway also contributes to the production of pro-inflammatory mediators during *Legionella* infection.

Endogenous HAQ STING is strongly impaired, but not deficient, in mediating type I IFN and pro-inflammatory cytokine responses to *Legionella* infection or stimulation with DNA and cGAMP

Recent studies showed that HAQ STING poorly activates type I IFN responses when ectopically expressed in HEK293 cells [27,28]. In order to examine the activity of endogenous human HAQ STING, we screened about 564 healthy volunteers for the presence of HAQ *TMEM173/STING*. We identified 8 individuals who were homozygous for HAQ (and R232), isolated peripheral blood mononuclear cells (PBMCs) from 4 of them, cultured the cells for 7 days to let the monocytes differentiate into macrophage-like cells, and compared them with cells from persons carrying WT *TMEM173/STING*. In line with our results from STING-deficient murine macrophages (see S1 Fig), we found that replication of *L. pneumophila* was not different in cells expressing WT or HAQ STING (S4 Fig). Interestingly, however, we observed a strong reduction in *Ifnb* expression and in production of the IFN-dependent cytokine IP-10 in cells from homozygous HAQ *TMEM173/STING* carriers as compared to cells from WT allele carriers in response to cGAMP, synthetic DNA, *Legionella* infection, and bacterial DNA, but not following stimulation with the TLR7/8 agonist Resiquimod (R848) (Fig 3A, S5A and S6 Figs). HAQ PBMCs were also partly defective in producing pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα (Fig 3B–3D, S5B–S5D Fig). Moreover, heterozygous carriage of HAQ *TMEM173/STING* also lead to a partial reduction of type I IFN and IL-1β expression,

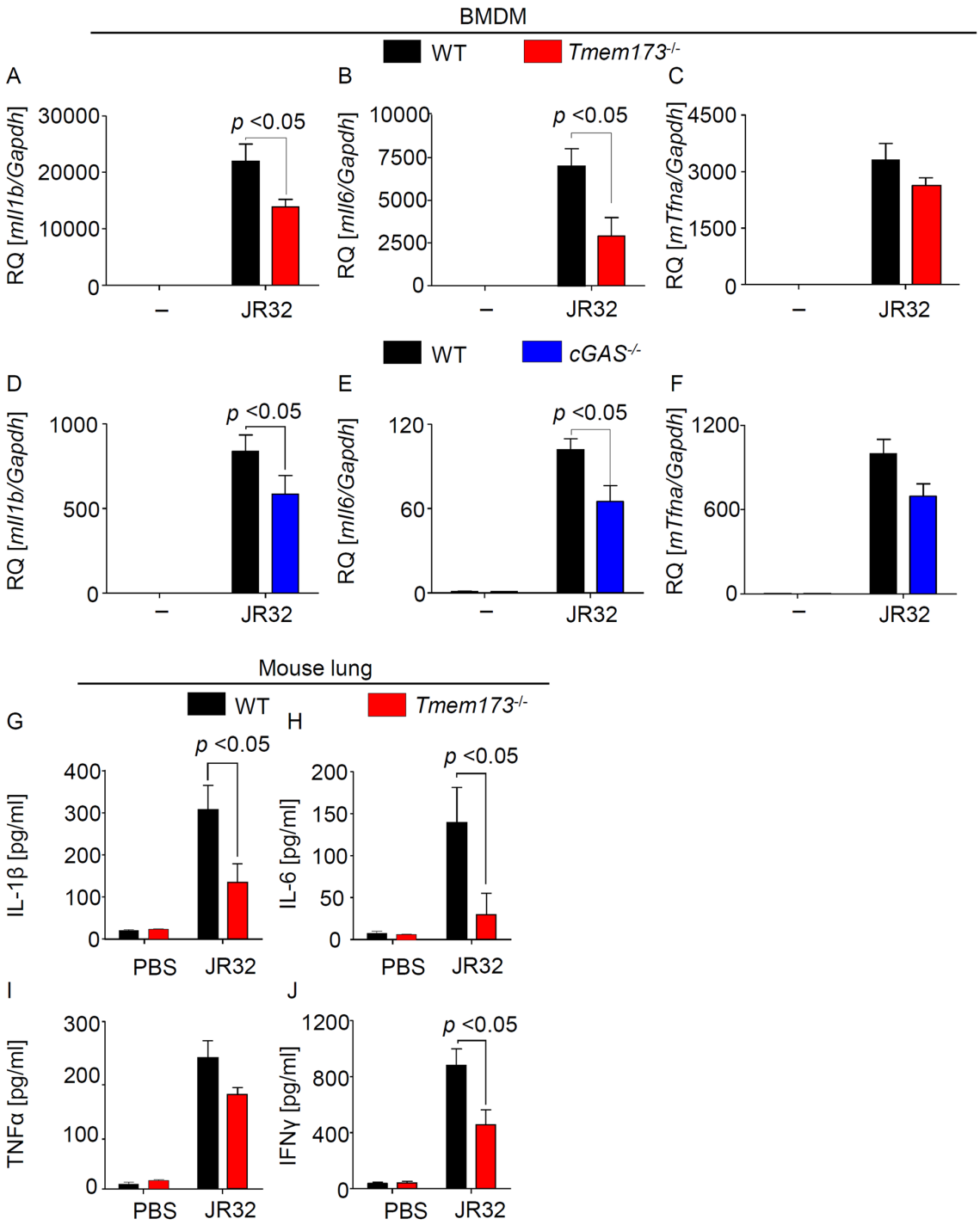


Fig 2. The cGAS/STING axis contributes to the production of pro-inflammatory cytokines during *L. pneumophila* infection. (A-F) WT, *Tmem173*$^{-/-}$ *and cGas*$^{-/-}$ BMDMs were infected for 6 h with *L. pneumophila* WT at MOI 10 and relative cytokine expression was determined by qRT-PCR. (G-J) Cytokine protein concentrations in whole lung homogenates from *L. pneumophila*-infected mice were quantified by sandwich ELISA. Data are shown as mean ± SEM. (A-F) Data representative of 3 to 4 independent experiments carried out in duplicates. (G-J) Data representative of 6 o 7 mice per group. Data were analyzed through the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.

https://doi.org/10.1371/journal.ppat.1006829.g002

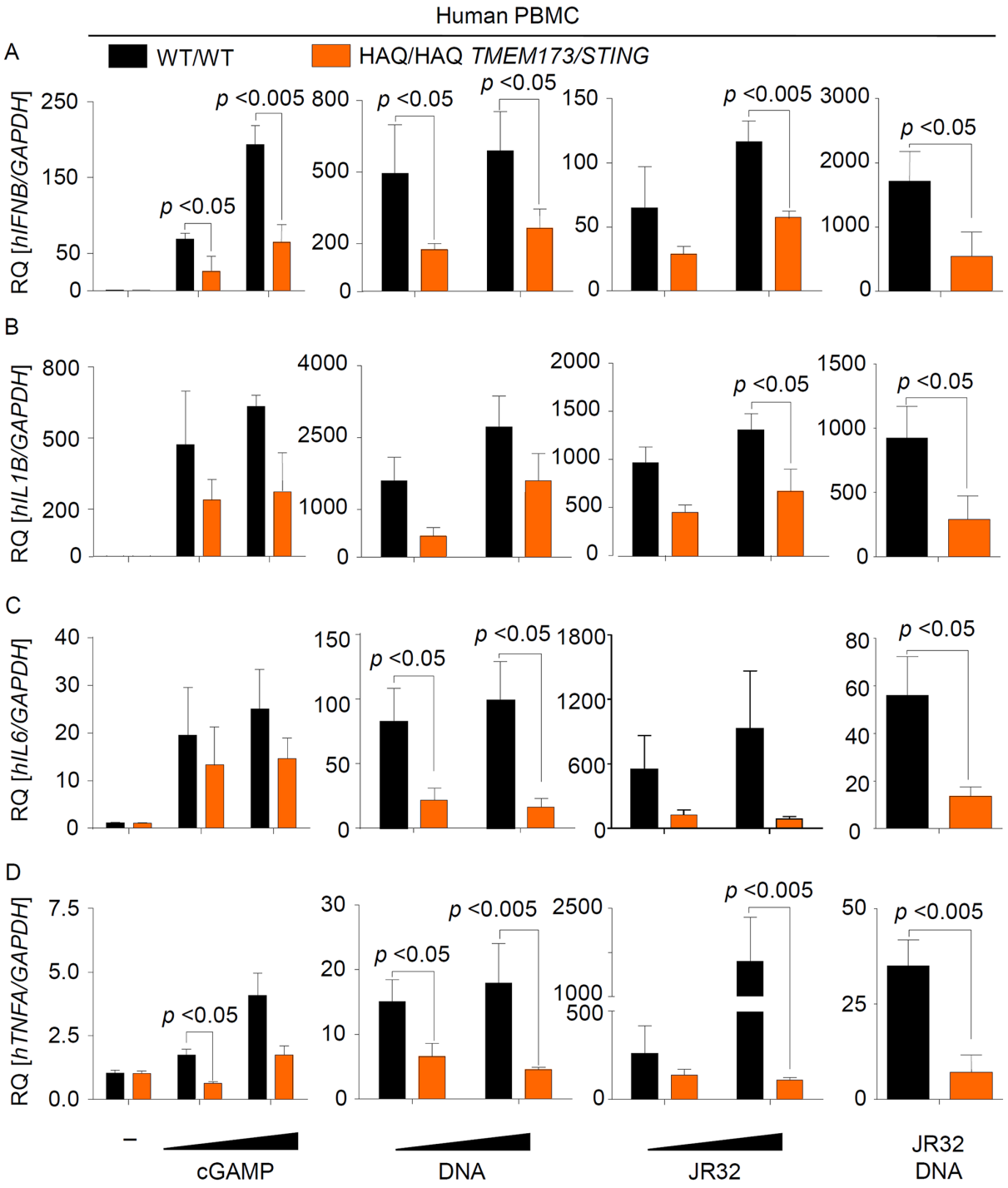


Fig 3. Endogenous HAQ STING is strongly impaired in mounting a type I IFN and proinflammatory cytokine responses against *Legionella* infection or stimulation with DNA or CDNs. (A-D) PBMCs from healthy volunteers (N = 4 for WT and N = 4 for HAQ) were isolated by density gradient centrifugation. 7 d after isolation cells were infected for 6 h with *L. pneumophila* at MOIs 10 and 50 or stimulated for the same period with 1 and 5 ug/ml 2´-3´cGAMP or either bacterial or synthetic DNA at a concentration of 0.2 or 1 ug/ml. RNA was isolated and the expression of *IFNB* (A), *IL1B* (B), *IL6* (C) and *TNFA* (D) was determined by qRT-PCR. Data are shown as the RQ of specified mRNAs. Data represent the mean ± SEM of 4 independent experiments carried out in triplicates. Differences were assessed with the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.

https://doi.org/10.1371/journal.ppat.1006829.g003

which however only reached statistical significance for cGAMP- and *L. pneumophila*-induced *IFNB* induction (S7 Fig).

Homozygous HAQ PBMCs were strongly impaired but not blunted in inducing type I IFN responses to *L. pneumophila* infection or stimulation with DNA or cGAMP (Fig 3A, S5A Fig), suggesting that HAQ STING possesses largely reduced but not blunted activity. In line with this suggestion, THP-1 cells, which have previously been shown to express HAQ STING [30,32] and which we confirmed to carry the HAQ *TMEM173/STING* allele in homozygosity, responded only weakly to DNA, cGAMP and *L. pneumophila* stimulation (2-10-fold increase in *Ifnb* expression, see Fig 4). These type I IFN responses in THP-1 cells were considerably lower as compared to PBMCs expressing WT STING (100-1000-fold *Ifnb* induction, see Fig 3A). Interestingly, however, deletion of cGAS expression by CRISPR/Cas9-mediated genome editing [33] further decreased the type I IFN responses in THP-1 cells (Fig 4). Taken together, our data demonstrate that endogenous HAQ STING is a hypomorphic variant that is strongly impaired (but not deficient) in mediating type I IFN and pro-inflammatory cytokine responses to cGAMP, synthetic DNA, bacterial DNA and *Legionella* infection.

Endogenous R232H STING is partly defective in sensing bacterial CDNs but fully functional in mediating responses to DNA, cGAMP and *L. pneumophila* infection

HEK293 cells expressing a mutated murine STING with an alanine instead of arginine 231 (R231A) respond normally to DNA but not to bacterial CDNs [20]. The corresponding human R232H allele is the third most common *TMEM173/STING* allele [29], and has also been shown to be defective in sensing bacterial CDNs when overexpressed [28,30]. In order to examine the function of endogenous R232H STING and the relevance of c-diGMP sensing for host responses to *L. pneumophila*, we screened healthy volunteers for carriage of this allele, isolated cells from 3 individuals harboring the R232H allele in homozygosity, and compared them with cells expressing WT STING. In agreement with previous studies, we found that human cells expressing R232H STING were partly impaired in sensing cGMP and Rp,Rp-c-diAMPSS (a Rp,Rp-isomer of the di-thiophosphate analogue of the bacterial second messenger c-diAMP) (Fig 5A–5D, S8 Fig). In contrast, expression of the R232H allele did not affect type I IFN or pro-inflammatory cytokine responses to *L. pneumophila* infection or DNA or cGAMP stimulation. Moreover, the R232H SNP did not affect replication of *L. pneumophila* in human cells (S9 Fig). These data indicate that endogenous human R232H STING is partly defective in sensing bacterial CDNs and that recognition of c-diGMP is not critically involved in human cell interactions with *L. pneumophila*.

cGAS and STING contribute to anti-bacterial host defense against *Legionella* infection in mice

Next, we investigated the relevance of the cGAS/STING-dependent pathway for antibacterial defense *in vivo*. cGAS- and STING-deficient mice as well as WT controls were intranasally infected with *L. pneumophila*. 6 days after infection, we observed enhanced (2–3 fold) bacterial loads in the lungs of *cGas*$^{-/-}$ and *Tmem173*$^{-/-}$ mice as compared to WT controls (Fig 6), demonstrating that the cGAS/STING pathway contributes to antibacterial defense against *L. pneumophila in vivo*.

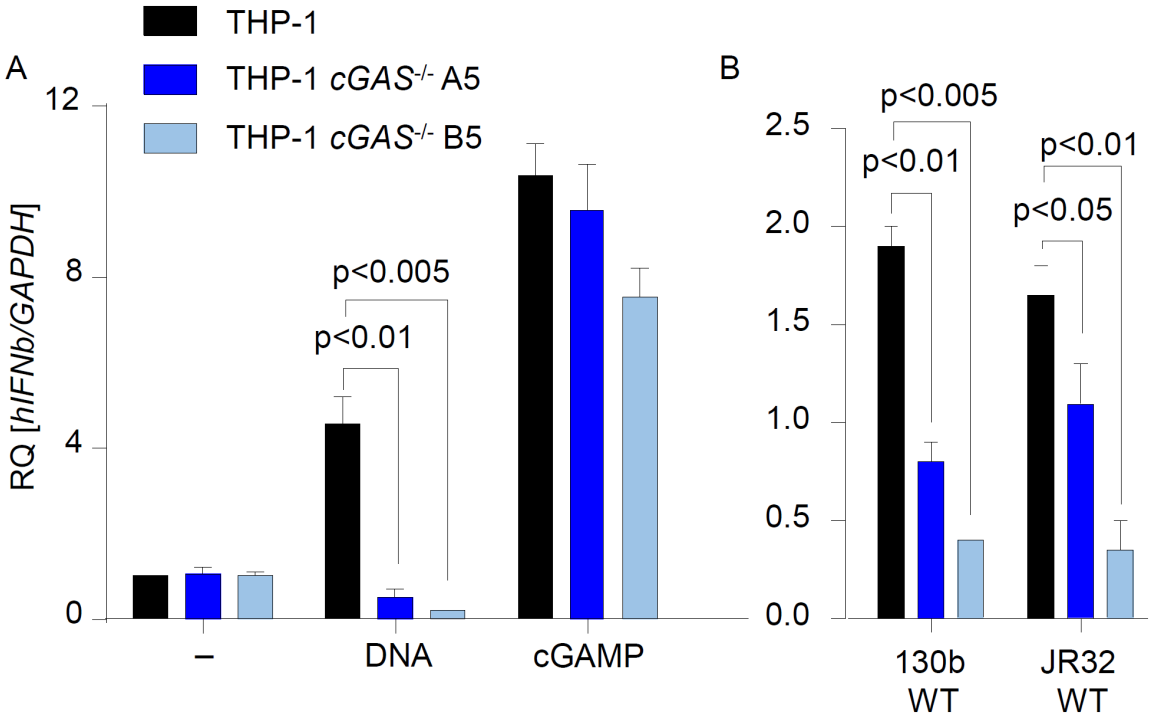


Fig 4. *L. pneumophila* infection and stimulation with DNA or cGAMP induce weak cGAS-dependent type I IFN responses in THP-1 cells. WT THP-1 or cGAS-/- THP-1 clones A5 and B5 were allowed differentiation prior to stimulation with either cGAMP or synthetic DNA (A) or infection with two different strains of *L. pneumophila* (B). *IFNB* expression was determined by qRT-PCR. Data represent mean ± SEM of 2 independent experiments carried out in duplicates. Analyses were performed by employing the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.

https://doi.org/10.1371/journal.ppat.1006829.g004

Carriage of HAQ *TMEM173/STING* might predispose individuals to infection

Finally, we tested for a potential association between HAQ and R232H *TMEM173/STING* carriages and susceptibility towards *L. pneumophila* infection. In an exploratory analysis, allele frequencies and genotypes were compared between 59 Legionnaires´ disease patients and 100 healthy controls of similar age and sex distribution. The frequency of HAQ *TMEM173/STING* (but not R232H *TMEM173/STING*) was significantly increased among cases (0.18) as compared to controls (0.075) (Table 1); an unadjusted analysis showed that carriage of the haplotype almost tripled the odds of being a legionellosis patient in this cohort (p = 0.028; OR 2.69; 95%CI, 1.16–6.27). The HAQ haplotype remained associated with the disease when the analysis was performed with an adjustment for age and gender (p = 0.01; OR 2.70, 95% CI 1.24–5.86; logistic regression with dominant genetic model).

To validate these findings, we examined another case control cohort (N = 91 Legionnaires´ disease patients and 88 controls) from a flower show outbreak in the Netherlands in 1999 that has been described in detail previously (S1 Table) [34–37]. The HAQ haplotype was present in 23 cases and 12 controls and associated with increased susceptibility to Legionnaires´ disease in an unadjusted analysis (OR 2.24, 95% CI 1.03–5.31; logistic regression with dominant genetic model). In an analysis adjusted for age and gender, the HAQ haplotype remained associated with Legionnaires´ disease (p = 0.013; OR 2.29, 95% CI 1.04–5.24) (Table 2). In contrast,

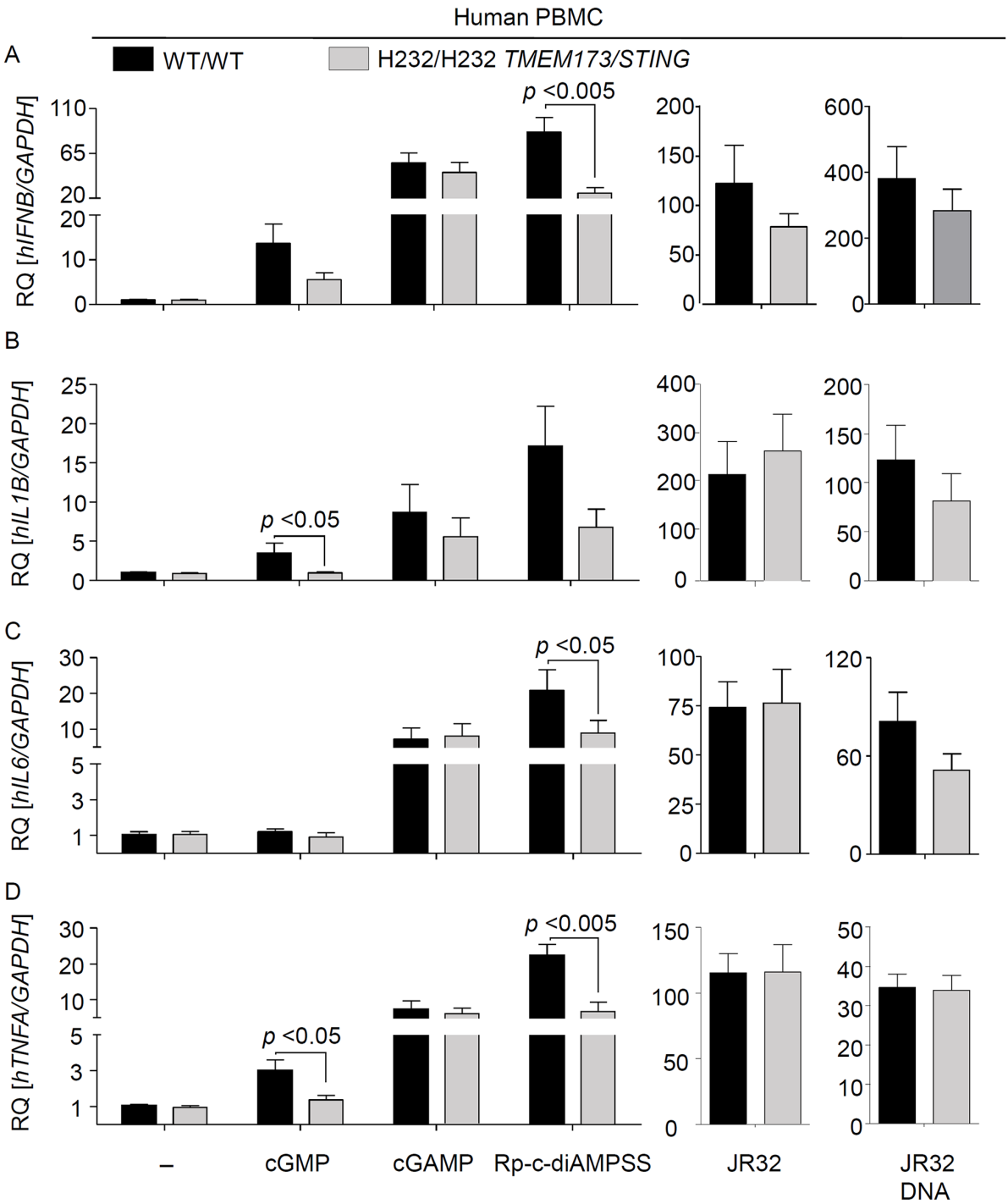


Fig 5. Endogenous R232H STING is partly deficient in sensing bacterial CDN but responds normally to *Legionella* infection or stimulation with DNA. (A-D) PBMCs from healthy volunteers (N = 3 for WT and N = 3 for R232H) were isolated by density gradient centrifugation. 7 d after isolation cells were infected for 6 h with *L. pneumophila* at MOI 10 or stimulated for the same period with 1 ug/ml 2´-3´cGAMP, Rp-c-diAMPSS, cGMP or either bacterial DNA at a concentration of 1 ug/ml. RNA was isolated and the expression of *IFNB* (A), *IL1B* (B), and *IL6* (C) and *TNFA* (D) was determined by qRT-PCR. Data are shown as the RQ of specified mRNAs. Data represent the mean ± SEM of 3 independent experiments carried out in triplicates. Differences were assessed with the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.

https://doi.org/10.1371/journal.ppat.1006829.g005

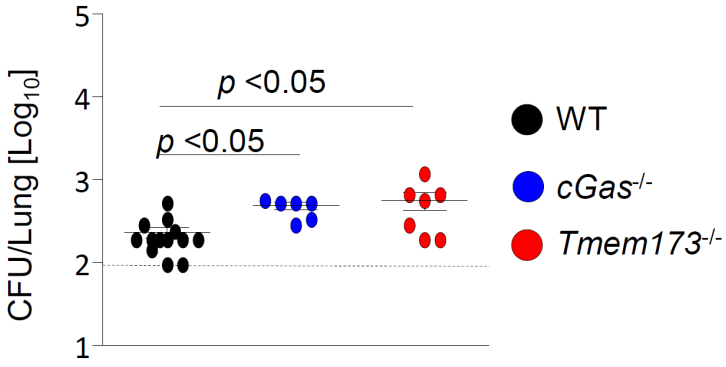


Fig 6. STING contributes to the antibacterial defense in mice infected with *L. pneumophila*. WT, cGAS- and STING-deficient mice were intranasally infected with 1×10^6 *L. pneumophila* WT and the bacterial loads in the lungs were assessed 144 h p.i. Data represent mean ± SEM of 6–13 mice per group. Comparisons were performed with the Mann-Whitney U Test. Comparisons with $p < 0.05$ were considered significant.

https://doi.org/10.1371/journal.ppat.1006829.g006

we did not find a significant association between R232H carriage and susceptibility to infection, although there was a trend towards increased R232H frequency in the Dutch patient cohort that we did not see in the German patients. Together, these data provide evidence in two separate European populations that the HAQ haplotype is associated with increased susceptibility to Legionnaires' disease.

Discussion

Genetic variations in different Toll-like receptors (TLRs) and downstream signaling molecules are known to affect innate immune sensing and susceptibility of human diseases [38–40]. Polymorphisms in the genes encoding TLR4, -5 and -6, for example, have been associated with increased risk of Legionnaires' disease [37,41]. Previous studies also revealed considerable heterogeneity of human *TMEM173/STING* [27,28]. The rare gain-of-function alleles A154S, V155M, V147L are associated with elevated type I IFN production and vasculopathy [42]. In contrast, the common HAQ variant was found to induce less basal type I IFN in the absence of exogenous stimuli as compared to wild-type STING [27,28], and reduced activity in the presence of CDNs [28,29]. Moreover, the R232H isoform of STING was shown to be defective in

Table 1. Distribution of *TMEM173/STING* HAQ and R232H in German patients and healthy controls.

	Controls	Cases	*p*	OR (95% CI)
N	100	59		
Proportion female %(n)	39.0 (39)	35.6 (21)	0.669	
Age median (Q_1 –Q_3)	71.5 (62.75–80)	72.0 (62.5–81.5)	0.551	
TMEM173/STING HAQ Freq.*	0.075	0.178		
Heterozygous %(n)	15.0 (15)	28.8 (17)	0.01	2.70 (1.24–5.86)†
Homozygous %(n)	0	3.4 (2)		
TMEM173/STING R232H Freq.*	0.15	0.11		
Heterozygous % (n)	28.0 (28)	22.0 (13)	0.336	0.69 (0.32–1.46) †
Homozygous % (n)	1.0 (1)	0		

*, HAQ or R232H frequencies = number of individuals with HAQ haplotype/total number of individuals

†, dominant genetic model adjusted for gender and age

https://doi.org/10.1371/journal.ppat.1006829.t001

Table 2. Distribution of *TMEM173/STING* HAQ and R232H in patients and healthy controls from Netherlands cohort.

	Control	Cases	*p*	OR (95% CI)
N	88	91		
Proportion female, %(n)	50 (44)	38,4 (35)	0.128	
Age median (Q_1 –Q_3)	49.6 (35.2–56.1)	64.7 (54.2–71.5)	< 0.001	
TMEM173/STING HAQ Freq.*	0.136 (12/88)	0.252 (23/91)		
Heterozygous % (n)	11.4 (10)	24.2 (22)	0.013	2.29 (1.04–5.24)†
Homozygous % (n)	2.3 (2)	1.1 (1)		
TMEM173/STING R232H Freq.*	9.2 (16/174).	16.1 (30/185)		
Heterozygous % (n)	13.8 (12/87)	16.1 (14/93)	0.09	1.85 (0.9–3.80)
Homozygous % (n)	2.3 (2/87)	9.2 (8/93)		

*, HAQ and R232H frequencies = number of individuals with HAQ haplotype/total number of individuals

†, dominant genetic model adjusted for gender and age

https://doi.org/10.1371/journal.ppat.1006829.t002

sensing bacterial CDNs but not cGAMP or DNA [28,30]. The relevance of endogenously expressed HAQ and R232H STING on the sensing of microbes or DNA, as well as a potential linkage between HAQ *TMEM173/STING* and acute infections, however, had not been addressed before.

Here we demonstrate that endogenous HAQ STING is impaired in mediating type I IFN and pro-inflammatory cytokine production in response to *Legionella* infection, bacterial and synthetic DNA, as well as cGAMP. The fact that PBMCs and THP-1 cells expressing HAQ STING in homozygosity are still able to produce some IFN, and that deletion of cGAS in THP-1 cells further reduces these responses, however, indicates that HAQ STING is a hypomorphic rather than a loss-of-function variant.

Importantly, our analyses of two independent cohorts of patients and healthy controls indicated an association between carriage of HAQ *TMEM173/STING* and Legionnaires' disease. This is the first time that a linkage between HAQ *TMEM173/STING* and susceptibility towards infectious diseases is reported. Our findings indicate that carriage of HAQ *TMEM173/STING* represents a risk factor for Legionnaires' disease. Moreover, considering that STING is involved in the defense against various pathogens like e.g. *Mycobacterium tuberculosis* and HIV, but weakens immunity against *Plasmodium falciparum* infections [43–45], one could speculate that HAQ *TMEM173/STING* carriage might predispose individuals towards several bacterial and viral infections, while at the same time potentially conferring protection against Malaria. Moreover, since STING also plays an important role in the pathogenesis of DNA-/IFN-driven autoimmune diseases [42], carriers of HAQ *TMEM173/STING* might be protected against these conditions.

Similar to most Gram-negative bacteria, *L. pneumophila* produces the second messenger c-diGMP [46,47], and previous studies suggested that sensing of *Legionella* c-diGMP might also play a role in inducing cytokine responses [17]. STING has been identified as a direct sensor of bacterial CDNs [20]. Previous overexpression studies indicated that the third most common *TMEM173/STING* allele R232H encodes for a protein with attenuated ability to recognize bacterial CDNs [28,30]. In agreement to these studies, we found that homozygous carriage of *TMEM173/STING* R232H in primary human cells impaired their ability to sense a bacterial CDN. However we did not observe a reduced cytokine production of R232H cells following infection with *L. pneumophila*, suggesting that sensing of *Legionella* CDNs is not required for the innate immune response to this infection. Moreover, we did not find a significant association between carriage of R232H *TMEM173/STING* and Legionnaires' disease, although the

R232H frequency was enhanced by trend in the Dutch patient cohort as compared to the controls (whereas the opposite trend was observed in the German cohort).

We and others recently showed that type I IFNs activate a macrophage-intrinsic resistance pathway that restricts *L. pneumophila* [4,8,12,48]. Surprisingly, we observed no difference in *Legionella* replication in macrophages from STING-deficient mice or HAQ carriers as compared to control cells. We do not have a definite explanation for this unexpected result, but speculate that small amounts of STING-independently produced type I IFN [49] might be sufficient to control the infection *in vitro*.

While the cGAS-STING pathway has been primarily associated with type I IFN responses to microbial infections, our results illustrate that sensing of *L. pneumophila* by cGAS/STING does not only stimulate type I IFN responses, but also significantly contributes to the production of other pro-inflammatory cytokines such as IL-1β and IL-6 or TNFα. This observation is in line with previous reports demonstrating STING-dependent NF-κB activation [50]. Considering that both IFNs as well as pro-inflammatory cytokines are known to be required for controlling *L. pneumophila* infection in the lung [4–8], a reduced production of these mediators might explain why lack of STING or cGAS in mice or expression of HAQ STING in humans enhances the susceptibility towards *L. pneumophila* infection. The reason for the rather small (but significant) effect of cGAS or STING deficiency on bacterial burden in our mouse model might be that mice are (probably due to an apparently more effective NAIP(5)/NLRC4 inflammasome) generally more resistant to *L. pneumophila* infection that humans.

In summary, we show that cGAS/STING contributes to the antibacterial defense against *L. pneumophila* infection, reveal that the hypomorphic STING variant HAQ negatively affects the antibacterial immune response, and indicate that HAQ *TMEM173/STING* carriage predisposes to Legionnaires 'disease.

Materials and methods

Ethics statement

For healthy volunteers from whom PBMCs were isolated, written informed consent was obtained and the study procedures were approved by the local ethics committee (Charité-Universitätsmedizin Berlin). Samples from the German Legionnaires' disease patients were provided by the CAPNETZ foundation. This prospective multicenter study (German Clinical Trials Register: DRKS00005274) was approved by the ethical review board of each participating clinical center (Reference number of leading Ethics Committee "Medical Faculty of Otto-von-Guericke-University in Magdeburg": 104/01 and "Medical School Hannover": 301/2008) and was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent prior to enrolment in the study. With regard to the Dutch case control study, approval for was obtained from the human subjects' review boards at the University of Amsterdam Medical Center and the University of Washington Medical Center (IRB protocol 1356). All participants gave written informed consent. All animal experiments were carried out in strict adherence to the German law (Tierschutzgesetz, TierSchG), following the approval of the corresponding institutional (Charité-Universitätsmedizin Berlin) and governmental animal welfare authorities (LAGeSo Berlin, approval ID G0440/12).

Bacterial strains

The *L. pneumophila* serogroup 1 strains JR32 and 130b as well as the isogenic mutant strains *ΔdotA* and *ΔsdhA* have been described previously [12,51,52]. Bacterial DNA was purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Murine model of Legionnaires' Disease

Anesthetized 8–16 weeks old, female WT, *cGas*$^{-/-}$ and *Tmem173*$^{-/-}$ mice on C57BL/6 background [53] were intranasally infected with 1×10^6 *L. pneumophila* JR32, and bacterial numbers in the lungs were counted as previously described [4,12].

Cell transfection and infection

Mouse BMDMs were infected with the aforementioned strains of *L. pneumophila* at MOI 10, centrifuged at 200 *g* for 5 min and then incubated for 6 h for qPCR analysis or for 16–18 h for ELISA at 37˚C. Bacterial or synthetic nucleic acids were transfected into the cells using Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) at a concentration of 1 ug/ml. 2'3'-cGAMP was added into the cell media without any transfection reagent at a concentration of 5 ug/ml. Where indicated, BMDMs were transfected 48 h prior to infection with control non-silencing siRNA or with a specific siRNA targeting cGAS using HiPerfect (Qiagen, Hilden, Germany).

Subjects

Human peripheral blood was collected from healthy adult volunteers expressing HAQ/HAQ (N = 4), R232H/R232H (N = 3), WT/HAQ (N = 7) or WT/WT (N = 10) *TMEM173/STING* and belonging to a cohort group (N = 564) collected at the Institute of Microbiology, Charité-Universitätsmedizin Berlin. The association study between the HAQ and R232H haplotypes and Legionnaires' disease was carried out first from DNA samples obtained from 59 German adult patients with confirmed *L. pneumonia*-induced community-acquired pneumonia (CAP). Samples were provided by the CAPNETZ competence network, a German multi-center prospective cohort study for CAP [54]. The control groups consisted of a subgroup of healthy adults of similar age and sex distribution (N = 100) from the PolSenior program, an interdisciplinary project, designed to evaluate health and socio-economic status of the Polish Caucasians aged ≥65 y [55]. Enrollment of the cases and controls from the Legionnaires' disease outbreak in the Netherlands has been described previously [34–37]. Of the 188 cases (all adults) identified in the original investigation of the outbreak, 141 consented for the study. 18 individuals died and no DNA was available for genotyping. 95 cases were available with both DNA and epidemiologic data for STING genotyping. Controls (N = 95, all adults) were drawn from the exhibitioners who worked at the flower show and were at high risk for exposure to *L. pneumophila*. Genomic DNA was purified form peripheral blood leukocytes from 10 ml of blood.

TMEM173/STING genotyping

Genomic DNA from the volunteers belonging to the cohort group collected at the Institute of Microbiology, Charité-Universitätsmedizin Berlin was extracted from buccal mucosa using the Gentra Puregene Buccal Cell Kit from Qiagen, according to the manufacturer's instructions. DNA from cases and controls from the CAPNETZ competence network and the PolSenior program respectively, were isolated from whole blood. Genotyping of *TMEM173/STING* R71H (rs11554776), G230A (rs78233829) and R293Q (rs7380824) in the above mentioned samples was carried out by PCR employing fluorescence-labeled hybridization FRET probes followed by melting curve analysis in a LightCyler 480 (Roche Diagnostics). Primer and probes used were as follows: rs11554776: f-primer: ggagtgacacacgttgg, r-primer: gcctagctgaggagctg, simple probe: LC640-ctggagtggaXItgtggcgcag-PH; rs78233829: f-primer: gggtctcactcctgaatcaggt, r-primer: ccgatccttgatgcaagca, anchor probe: LC640-cagtttatccaggaagcgaatgttggg-PH,

sensor probe: ggtcagcggtctgctgg-FL; rs7380824: f-primer: accctggtaggcaatga, r-primer: gcttagtctggtcttcctcttac, anchor probe: LC640-ggcctgctcaagcctatcctcccgg-PH, sensor probe: cctcaagtgtccggcagaagagtt-FL; rs1131769: f-primer: cccactcccctgcacactt, r-primer: tggataaac tgcccaagcagac, anchor probe: LC640-aggatcgggtttacagcaacagca-PH, sensor probe: ggtgaccat gctggcatc-FL.

Genomic DNA from cases and controls from the Legionnaires' disease outbreak in the Netherlands was isolated from whole blood, and genotyping of selected SNPs was performed using a Fluidigm Biomark 96 x 96 chip (Fluidigm, Inc.). Cluster plots were visually inspected to ensure accurate genotyping calls. SNPs were manually assessed for data quality and only high-quality calls were accepted. 91 cases and 88 controls had high-quality genotyping data available for all three SNPs for analysis. Genotypes were assessed for Hardy-Weinberg equilibrium (HWE) with a Chi-square test comparing observed and expected frequencies in the control population. No SNPs violated HWE ($P < 0.001$).

Infection and stimulation of human peripheral blood mononuclear cells

50 mL of whole blood were drawn from healthy volunteers and peripheral blood mononuclear cells were isolated by gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, Taufkirchen, Germany). Briefly, whole blood was diluted 1:1 with phosphate buffered saline solution (PBS) without calcium or magnesium and layered onto 20 ml Histopaque-1077. The gradient was centrifuged at 800 x g for 25 min at room temperature, and the PBMC were collected from the interface. PBMC were then washed twice with PBS and resuspended in RPMI medium supplemented with 10% FCS and 1% l-glutamine. Cell media was replaced 24 h after plating and half of the media was further replaced every 2 d and the cells were cultured for 7 d before infection or stimulation. Infection with *L. pneumophila* was performed at MOI 10 and 50. Bacterial DNA or synthetic nucleic acids were transfected into the cells using concentrations of 0.2 or 1 ug/ml. 2´3-cGAMP and RpRp-c-diAMPSS were added into the cell media at concentrations of 1 or 5 μg/ml, and R878 was used at a concentration of 1 μg/ml. All procedures used for PBMC infection or stimulation were performed as described before for mouse BMDMs.

Human monocytic THP-1 cells

cGAS-deficient [33] and control THP-1 cells were maintained under normal culture conditions. For induction of cell differentiation into a macrophage-like state, cells were re-suspended in culture medium containing 80 nM phorbol myristate acetate (PMA) for 48 h.

qRT-PCR

Total RNA was isolated from cultured cells or lung homogenates using the PerfectPure RNA purification system (5 Prime) or Trizol (Life Technologies, Darmstadt, Germany), respectively. Total RNA was reverse-transcribed using the high capacity reverse transcription kit (Applied Biosystems, Darmstadt, Germany), and quantitative PCR was performed using TaqMan assays (Life Technologies, Darmstadt, Germany) or self-designed primer sets, on an ABI 7300 instrument (Applied Biosystems, Darmstadt, Germany). The input was normalized to the average expression of GAPDH and relative expression (relative quantity, RQ) of the respective gene in untreated cells or PBS-treated mice was set as 1.

ELISA

Concentrations of IL-1β, IL-6, TNFα and IFNγ were quantified by commercially available sandwich ELISA kits (eBioscience, Frankfurt, Germany) as well as human and mouse IP-10

(Life Sciences, Darmstadt, Germany). Protein concentrations were determined in a FilterMax F5 Multi-Mode Microplate Reader (Molecular devices, Sunnyvale, CA, USA) at 450 nm.

Bacterial replication analysis

Murine BMMs and PBMCs were infected with *L. pneumophila* at MOI 0.1 and intracellular bacterial replication was estimated with a CFU assay. Briefly, 30 min after infection cells were washed with PBS and cell media containing 50 ug/ml gentamycin was supplemented. After 1 h, cells were washed once more and fresh medium was added. Cell lysis was performed by adding 1% saponin 1, 24, 48 and 72 h.p.i and CFUs were estimated by plating different serial dilutions of the resultant cell suspension in buffered charcoal yeast extract (BCYE) agar.

Statistics and genetic analysis

Data analysis was performed using the Prism software (GraphPad Software, La Jolla, CA). Groups were compared using a two-tailed Mann-Whitney U test. The association analysis in the German cohorts was performed through the Chi-Square test for association and calculation of odds ratio using a dominant genetic model (comparing WT individuals (no HAQ haplotype) to those who had 1 or 2 copies of HAQ or R232H). Odds ratios were adjusted for age and gender through a logistic regression analysis using SPSS (IBM Corporation, Armonk, NY). A Fisher´s exact test together with calculation of exact confidence intervals were used if applicable. Differences with $p<0.05$ were regarded as significant. Similarly, the association analysis in the Dutch cohorts was performed with a dominant genetic model using Stata 13 (Stata Corp, College Station, TX) and the user-written package "genass" [56]. The presence of the HAQ or R232H haplotypes was defined as an individual who was heterozygous or homozygous for any of the two STING variants.

Supporting information

S1 Fig. Replication of *L. pneumophila* in murine WT and *Tmem173*-/- macrophages and IP-10 production. (A) WT and *Tmem173*-/- mouse BMDMs were infected with *L. pneumophila* at MOI 0.1, and bacterial loads were analyzed at the indicated time points. Data represent mean ± SEM of 5 independent experiments carried out in triplicates. (B) WT and STING-deficient BMDMs were infected with *L. pneumophila* JR32 for 16–18 h, and production of IP-10 was measured by ELISA. Data represent mean ± SEM of 4 independent experiments carried out in duplicates. Comparisons with a $p < 0.05$ were considered significant.
(PDF)

S2 Fig. siRNA-mediated inhibition of cGAS reduces type I IFN responses against *L. pneumophila* in murine macrophages. BMDMs were transfected with a control siRNA or a siRNA sequence targeting *cGas* 48 h prior to infection; the expression of (A) *cGas*, (B) *Ifnb* and (C) *Irg1* was quantified by qRT-PCR and the input normalized to the average expression of *Gapdh* and the relative expression of the respective gene in untreated cells. Data are shown as mean + SEM of three independent experiments, measured in technical duplicates. Analyses were performed through the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.
(PDF)

S3 Fig. STING deficiency affects *L. pneumophila*-induced production of pro-inflammatory cytokines. (A-C) Cytokine protein production was assessed by sandwich ELISA of supernatants from WT and *Tmeme173*-/- BMDMs infected for 16–18 h with *L. pneumophila* JR32 WT. Analyses were performed through the Mann-Whitney U Test. Data represent mean ± SEM of

4 independent experiments carried out in duplicates. Comparisons with a $p < 0.05$ were considered significant.
(PDF)

S4 Fig. Replication of *L. pneumophila* in human macrophages is not affected by carriage of HAQ *TMEM173/STING*. PBMCs from healthy volunteers (N = 4, per group) were isolated by density gradient centrifugation. 7 d after isolation, cells were infected with *L. pneumophila* at MOI 0.1 and bacterial numbers were counted at the indicated time points. Data represent mean ± SEM of 4 independent experiments carried out in triplicates.
(PDF)

S5 Fig. Cells from homozygous HAQ *TMEM173/STING* carriers are impaired in cytokine production in response to cGAMP, *L. pneumophila*, and bacterial DNA. (A-D) PBMCs from healthy volunteers (N = 4 for WT, N = 4 for HAQ) carrying the WT variant of the *TMEM173/STING* gene or the HAQ allele in homozygosity were isolated as described above and infected for 16 to 18 h with *L. pneumophila* at MOI 50 or stimulated for the same period with 5 ug/ml 2´-3´cGAMP or of 1 ug/ml bacterial DNA. Protein production of (A) IP-10, (B) IL-1β, (C) IL-6 and (D) TNFα, was assessed by sandwich ELISA of cell supernatants. Data are shown as mean + SEM of four independent experiments, measured in technical triplicates. Analyses were performed through the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant. # Not detectable.
(PDF)

S6 Fig. Cells from homozygous HAQ *TMEM173/STING* carriers are competent in responding to TLR7/8 activation. PBMCs from healthy volunteers (N = 3 for WT, N = 3 for HAQ) were isolated by density gradient centrifugation. 7 d after isolation cells were stimulated for 6 h with 1 ug/ml R848. RNA was isolated and the expression of *IFNB* and *IL1B* was determined by qRT-PCR. Data are shown as the RQ of specified mRNAs. Data represent the mean ± SEM of 3 independent experiments carried out in duplicates. Differences were assessed with the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.
(PDF)

S7 Fig. Cells from heterozygous HAQ *TMEM173/STING* carriers are partially impaired in *IFNB* induction in response to cGAMP and *L. pneumophila*. (A-D) PBMCs from healthy volunteers (N = 7 for WT/WT, N = 7 for WT/HAQ) carrying the WT variant of the *TMEM173/STING* gene or the HAQ allele in heterozygosity were isolated as described above and infected for 6 h with *L. pneumophila* at MOI 10 or 50 or stimulated for the same period with 5 ug/ml 2´-3´cGAMP or of 0.2 or 1 ug/ml bacterial DNA. RNA was isolated and the expression of *IFNB*, *IL1B*, *IL6 and TNFA* was determined by qRT-PCR. Data are shown as the RQ of specified mRNAs. Data represent the mean ± SEM of 7 independent experiments carried out in triplicates. Differences were assessed with the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant.
(PDF)

S8 Fig. Carriage of the R232H *TMEM173/STING* affects production of pro-inflammatory cytokines following stimulation with bacterial CDN but not in response to *Legionella* infection or stimulation with DNA. (A-D) PBMCs from healthy volunteers (N = 3 for WT, N = 3 for R232H) carrying the WT or the R232H allele in homozygosity were isolated and infected for 16 to 18 h with *L. pneumophila* at MOI 50 or stimulated for the same period with 5 ug/ml 2´-3´cGAMP, 1 ug/ml Rp,Rp-c-diAMPSS or 1 ug/ml bacterial DNA. Production of (A) IP-10, (B) IL-1β, (C) IL-6 and (D) TNFα was assessed by sandwich ELISA of cell supernatants.

Data are shown as mean + SEM of three independent experiments, carried out in triplicates. Analyses were performed through the Mann-Whitney U Test. Comparisons with a $p < 0.05$ were considered significant. # Not detectable.
(PDF)

S9 Fig. Carriage of the R232H *TMEM173/STING* allele does not affect replication of *L. pneumophila* in human cells. PBMCs from healthy volunteers (N = 3 for WT, N = 3 for R232H) were isolated by density gradient centrifugation. 7 d after isolation, cells were infected with *L. pneumophila* at MOI 0.1 and bacterial numbers were counted at the indicated time points. Data represent mean ± SEM of 3 independent experiments carried out in triplicates.
(PDF)

S1 Table. Demographics of the Netherlands cohort.
(DOCX)

Acknowledgments

We would like to thank the healthy volunteers for donating blood, the CAPNETZ foundation for providing samples from Legionnaires' disease patients, the investigators in the local clinical centers and all practitioners, physicians and respiratory specialists cooperating within the network. Members of the CAPNETZ study group (except the authors) are M. Dreher, C. Cornelissen (Aachen); W. Knüppel (Bad Arolsen); D. Stolz (Basel); P. Creutz (Berlin, Charité); T. Bauer, T. Sabha (Berlin); W. Pankow, A. Lies, D. Thiemig (Berlin-Neukölln); B. Hauptmeier, S. Ewig, D. Wehde, (Bochum); M. Prediger, S. Schmager (Cottbus); G. Höffken, M. Kolditz, B. Schulte-Hubbert, S. Langner (Dresden), T. Welte, G. Barten, M. Abrahamczik, J. Naim, W. Kröner, T. Illig, N. Klopp (Hannover); P. Ravn, A. Vestergaard-Jensen, G. Baunbaek-Knudsen (Hillerød); C. Kroegel, M. Pletz, J. Happe, J. Frosinski, J. Winning, A. Moeser (Jena); K. Dalhoff, J. Rupp, K. Dageförde, K. Franzen, F. Hyzy, H. Schmieg, P. Parschke, P. Thiemann, J. Ahrens, T. Hardel (Lübeck); G. Rohde, J. Drijkoningen (Maastricht); H. Buschmann, R. Kröning, (Paderborn); H. Schütte (Potsdam), T. Schaberg, I. Hering (Rotenburg/Wümme); C. Kropf-Sanchen (Ulm); T. Illmann, M. Wallner (Ulm); and all study nurses. We thank Veit Hornung (Gene Center Munich, Germany) for sharing cGAS-deficient THP-1 cells, and Craig Roy (Yale University, New Haven, USA), Gad Frankel (Imperial College London, UK) as well as Antje Flieger (Robert Koch Institute, Wernickenrode, Germany) for providing bacterial strains. Moreover, we are grateful to Herbert "Skip" Virgin (Washington University School of Medicine, St. Louis, USA) for his kind permission to use *cGAS*$^{-/-}$ mice and Anca Dorhoi (Max Planck Institute for Infection Biology, Berlin, Germany) for providing those animals. We thank Felicia Nguyen and Glenna Peterson for their assistance with genotyping of the Dutch cohort.

Author Contributions

Conceptualization: Bastian Opitz.

Data curation: Annelies Verbon.

Formal analysis: Juan S. Ruiz-Moreno, Javeed A. Shah, Frank P. Mockenhaupt, Thomas R. Hawn.

Funding acquisition: Bastian Opitz.

Investigation: Juan S. Ruiz-Moreno, Javeed A. Shah, Jan Naujoks, Thomas R. Hawn.

Project administration: Bastian Opitz.

Resources: Lutz Hamann, Annelies Verbon, Monika Puzianowska-Kuznicka, Leif E. Sander, Martin Witzenrath, John C. Cambier, Norbert Suttorp, Ralf R. Schumann, Lei Jin, Bastian Opitz.

Supervision: Bastian Opitz.

Writing – original draft: Juan S. Ruiz-Moreno, Leif E. Sander, Thomas R. Hawn, Bastian Opitz.

Writing – review & editing: Bastian Opitz.

References

1. Phin N, Parry-Ford F, Harrison T, Stagg HR, Zhang N, et al. (2014) Epidemiology and clinical management of Legionnaires' disease. Lancet Infect Dis 14: 1011–1021. https://doi.org/10.1016/S1473-3099(14)70713-3 PMID: 24970283
2. von Baum H, Ewig S, Marre R, Suttorp N, Gonschior S, et al. (2008) Community-acquired Legionella pneumonia: new insights from the German competence network for community acquired pneumonia. Clin Infect Dis 46: 1356–1364. https://doi.org/10.1086/586741 PMID: 18419436
3. Isberg RR, O'Connor TJ, Heidtman M (2009) The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. Nat Rev Microbiol 7: 13–24. https://doi.org/10.1038/nrmicro1967 PMID: 19011659
4. Lippmann J, Muller HC, Naujoks J, Tabeling C, Shin S, et al. (2011) Dissection of a type I interferon pathway in controlling bacterial intracellular infection in mice. Cell Microbiol 13: 1668–1682. https://doi.org/10.1111/j.1462-5822.2011.01646.x PMID: 21790939
5. Heath L, Chrisp C, Huffnagle G, LeGendre M, Osawa Y, et al. (1996) Effector mechanisms responsible for gamma interferon-mediated host resistance to Legionella pneumophila lung infection: the role of endogenous nitric oxide differs in susceptible and resistant murine hosts. Infect Immun 64: 5151–5160. PMID: 8945559
6. Sporri R, Joller N, Albers U, Hilbi H, Oxenius A (2006) MyD88-dependent IFN-gamma production by NK cells is key for control of Legionella pneumophila infection. J Immunol 176: 6162–6171. PMID: 16670325
7. Schiavoni G, Mauri C, Carlei D, Belardelli F, Pastoris MC, et al. (2004) Type I IFN protects permissive macrophages from Legionella pneumophila infection through an IFN-gamma-independent pathway. J Immunol 173: 1266–1275. PMID: 15240719
8. Opitz B, Vinzing M, van Laak V, Schmeck B, Heine G, et al. (2006) Legionella pneumophila induces IFNbeta in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. J Biol Chem 281: 36173–36179. https://doi.org/10.1074/jbc.M604638200 PMID: 16984921
9. Naujoks J, Lippmann J, Suttorp N, Opitz B (2017) Innate sensing and cell-autonomous resistance pathways in Legionella pneumophila infection. Int J Med Microbiol. Epub ahead of print
10. LeibundGut-Landmann S, Weidner K, Hilbi H, Oxenius A (2011) Nonhematopoietic cells are key players in innate control of bacterial airway infection. J Immunol 186: 3130–3137. https://doi.org/10.4049/jimmunol.1003565 PMID: 21270399
11. Fujita M, Ikegame S, Harada E, Ouchi H, Inoshima I, et al. (2008) TNF receptor 1 and 2 contribute in different ways to resistance to Legionella pneumophila-induced mortality in mice. Cytokine 44: 298–303. https://doi.org/10.1016/j.cyto.2008.08.015 PMID: 18838275
12. Naujoks J, Tabeling C, Dill BD, Hoffmann C, Brown AS, et al. (2016) IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid. PLoS Pathog 12: e1005408. https://doi.org/10.1371/journal.ppat.1005408 PMID: 26829557
13. Stetson DB, Medzhitov R (2006) Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity 24: 93–103. https://doi.org/10.1016/j.immuni.2005.12.003 PMID: 16413926
14. Lippmann J, Rothenburg S, Deigendesch N, Eitel J, Meixenberger K, et al. (2008) IFNbeta responses induced by intracellular bacteria or cytosolic DNA in different human cells do not require ZBP1 (DLM-1/DAI). Cell Microbiol 10: 2579–2588. https://doi.org/10.1111/j.1462-5822.2008.01232.x PMID: 18771559

15. Copenhaver AM, Casson CN, Nguyen HT, Duda MM, Shin S (2015) IL-1R signaling enables bystander cells to overcome bacterial blockade of host protein synthesis. Proc Natl Acad Sci U S A 112: 7557–7562. https://doi.org/10.1073/pnas.1501289112 PMID: 26034289

16. Brown AS, Yang C, Fung KY, Bachem A, Bourges D, et al. (2016) Cooperation between Monocyte-Derived Cells and Lymphoid Cells in the Acute Response to a Bacterial Lung Pathogen. PLoS Pathog 12: e1005691. https://doi.org/10.1371/journal.ppat.1005691 PMID: 27300652

17. Abdul-Sater AA, Grajkowski A, Erdjument-Bromage H, Plumlee C, Levi A, et al. (2012) The overlapping host responses to bacterial cyclic dinucleotides. Microbes Infect 14: 188–197. https://doi.org/10.1016/j.micinf.2011.09.002 PMID: 21933720

18. Ishikawa H, Barber GN (2008) STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455: 674–678. https://doi.org/10.1038/nature07317 PMID: 18724357

19. Ishikawa H, Ma Z, Barber GN (2009) STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 461: 788–792. https://doi.org/10.1038/nature08476 PMID: 19776740

20. Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, et al. (2011) STING is a direct innate immune sensor of cyclic di-GMP. Nature 478: 515–518. https://doi.org/10.1038/nature10429 PMID: 21947006

21. Wu J, Sun L, Chen X, Du F, Shi H, et al. (2013) Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science 339: 826–830. https://doi.org/10.1126/science.1229963 PMID: 23258412

22. Sun L, Wu J, Du F, Chen X, Chen ZJ (2013) Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 339: 786–791. https://doi.org/10.1126/science.1232458 PMID: 23258413

23. Watson RO, Bell SL, MacDuff DA, Kimmey JM, Diner EJ, et al. (2015) The Cytosolic Sensor cGAS Detects Mycobacterium tuberculosis DNA to Induce Type I Interferons and Activate Autophagy. Cell Host Microbe 17: 811–819. https://doi.org/10.1016/j.chom.2015.05.004 PMID: 26048136

24. Wassermann R, Gulen MF, Sala C, Perin SG, Lou Y, et al. (2015) Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1. Cell Host Microbe 17: 799–810. https://doi.org/10.1016/j.chom.2015.05.003 PMID: 26048138

25. Collins AC, Cai H, Li T, Franco LH, Li XD, et al. (2015) Cyclic GMP-AMP Synthase Is an Innate Immune DNA Sensor for Mycobacterium tuberculosis. Cell Host Microbe 17: 820–828. https://doi.org/10.1016/j.chom.2015.05.005 PMID: 26048137

26. Zhang Y, Yeruva L, Marinov A, Prantner D, Wyrick PB, et al. (2014) The DNA sensor, cyclic GMP-AMP synthase, is essential for induction of IFN-beta during Chlamydia trachomatis infection. J Immunol 193: 2394–2404. https://doi.org/10.4049/jimmunol.1302718 PMID: 25070851

27. Jin L, Xu LG, Yang IV, Davidson EJ, Schwartz DA, et al. (2011) Identification and characterization of a loss-of-function human MPYS variant. Genes Immun 12: 263–269. https://doi.org/10.1038/gene.2010.75 PMID: 21248775

28. Yi G, Brendel VP, Shu C, Li P, Palanathan S, et al. (2013) Single nucleotide polymorphisms of human STING can affect innate immune response to cyclic dinucleotides. PLoS One 8: e77846. https://doi.org/10.1371/journal.pone.0077846 PMID: 24204993

29. Patel S, Blaauboer SM, Tucker HR, Mansouri S, Ruiz-Moreno JS, et al. (2017) The Common R71H-G230A-R293Q Human TMEM173 Is a Null Allele. J Immunol 198: 776–787. https://doi.org/10.4049/jimmunol.1601585 PMID: 27927967

30. Diner EJ, Burdette DL, Wilson SC, Monroe KM, Kellenberger CA, et al. (2013) The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. Cell Rep 3: 1355–1361. https://doi.org/10.1016/j.celrep.2013.05.009 PMID: 23707065

31. Creasey EA, Isberg RR (2012) The protein SdhA maintains the integrity of the Legionella-containing vacuole. Proc Natl Acad Sci U S A 109: 3481–3486. https://doi.org/10.1073/pnas.1121286109 PMID: 22308473

32. Fu J, Kanne DB, Leong M, Glickman LH, McWhirter SM, et al. (2015) STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade. Sci Transl Med 7: 283ra252.

33. Mankan AK, Schmidt T, Chauhan D, Goldeck M, Honing K, et al. (2014) Cytosolic RNA:DNA hybrids activate the cGAS-STING axis. EMBO J 33: 2937–2946. https://doi.org/10.15252/embj.201488726 PMID: 25425575

34. Boshuizen HC, Neppelenbroek SE, van Vliet H, Schellekens JF, den Boer JW, et al. (2001) Subclinical Legionella infection in workers near the source of a large outbreak of Legionnaires disease. J Infect Dis 184: 515–518. https://doi.org/10.1086/322049 PMID: 11471112

35. Den Boer JW, Yzerman EP, Schellekens J, Lettinga KD, Boshuizen HC, et al. (2002) A large outbreak of Legionnaires' disease at a flower show, the Netherlands, 1999. Emerg Infect Dis 8: 37–43. https://doi.org/10.3201/eid0801.010176 PMID: 11749746

36. Lettinga KD, Verbon A, Weverling GJ, Schellekens JF, Den Boer JW, et al. (2002) Legionnaires' disease at a Dutch flower show: prognostic factors and impact of therapy. Emerg Infect Dis 8: 1448–1454. https://doi.org/10.3201/eid0812.020035 PMID: 12498662

37. Hawn TR, Verbon A, Lettinga KD, Zhao LP, Li SS, et al. (2003) A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. J Exp Med 198: 1563–1572. https://doi.org/10.1084/jem.20031220 PMID: 14623910

38. Schroder NW, Schumann RR (2005) Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis 5: 156–164. https://doi.org/10.1016/S1473-3099(05)01308-3 PMID: 15766650

39. Netea MG, Wijmenga C, O'Neill LA (2012) Genetic variation in Toll-like receptors and disease susceptibility. Nat Immunol 13: 535–542. https://doi.org/10.1038/ni.2284 PMID: 22610250

40. Garantziotis S, Hollingsworth JW, Zaas AK, Schwartz DA (2008) The effect of toll-like receptors and toll-like receptor genetics in human disease. Annu Rev Med 59: 343–359. https://doi.org/10.1146/annurev.med.59.061206.112455 PMID: 17845139

41. Misch EA, Verbon A, Prins JM, Skerrett SJ, Hawn TR (2013) A TLR6 polymorphism is associated with increased risk of Legionnaires' disease. Genes Immun 14: 420–426. https://doi.org/10.1038/gene.2013.34 PMID: 23823019

42. Liu Y, Jesus AA, Marrero B, Yang D, Ramsey SE, et al. (2014) Activated STING in a vascular and pulmonary syndrome. N Engl J Med 371: 507–518. https://doi.org/10.1056/NEJMoa1312625 PMID: 25029335

43. Manzanillo PS, Shiloh MU, Portnoy DA, Cox JS (2012) Mycobacterium tuberculosis activates the DNA-dependent cytosolic surveillance pathway within macrophages. Cell Host Microbe 11: 469–480. https://doi.org/10.1016/j.chom.2012.03.007 PMID: 22607800

44. Yan N, Regalado-Magdos AD, Stiggelbout B, Lee-Kirsch MA, Lieberman J (2010) The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. Nat Immunol 11: 1005–1013. https://doi.org/10.1038/ni.1941 PMID: 20871604

45. Sharma S, DeOliveira RB, Kalantari P, Parroche P, Goutagny N, et al. (2011) Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. Immunity 35: 194–207. https://doi.org/10.1016/j.immuni.2011.05.016 PMID: 21820332

46. Levi A, Folcher M, Jenal U, Shuman HA (2011) Cyclic diguanylate signaling proteins control intracellular growth of Legionella pneumophila. MBio 2: e00316–00310. https://doi.org/10.1128/mBio.00316-10 PMID: 21249170

47. Levet-Paulo M, Lazzaroni JC, Gilbert C, Atlan D, Doublet P, et al. (2011) The atypical two-component sensor kinase Lpl0330 from Legionella pneumophila controls the bifunctional diguanylate cyclase-phosphodiesterase Lpl0329 to modulate bis-(3'-5')-cyclic dimeric GMP synthesis. J Biol Chem 286: 31136–31144. https://doi.org/10.1074/jbc.M111.231340 PMID: 21757706

48. Plumlee CR, Lee C, Beg AA, Decker T, Shuman HA, et al. (2009) Interferons direct an effective innate response to Legionella pneumophila infection. J Biol Chem 284: 30058–30066. https://doi.org/10.1074/jbc.M109.018283 PMID: 19720834

49. Monroe KM, McWhirter SM, Vance RE (2009) Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to Legionella pneumophila. PLoS Pathog 5: e1000665. https://doi.org/10.1371/journal.ppat.1000665 PMID: 19936053

50. Abe T, Barber GN (2014) Cytosolic-DNA-mediated, STING-dependent proinflammatory gene induction necessitates canonical NF-kappaB activation through TBK1. J Virol 88: 5328–5341. https://doi.org/10.1128/JVI.00037-14 PMID: 24600004

51. Sadosky AB, Wiater LA, Shuman HA (1993) Identification of Legionella pneumophila genes required for growth within and killing of human macrophages. Infect Immun 61: 5361–5373. PMID: 8225610

52. Harding CR, Stoneham CA, Schuelein R, Newton H, Oates CV, et al. (2013) The Dot/Icm effector SdhA is necessary for virulence of Legionella pneumophila in Galleria mellonella and A/J mice. Infect Immun 81: 2598–2605. https://doi.org/10.1128/IAI.00296-13 PMID: 23649096

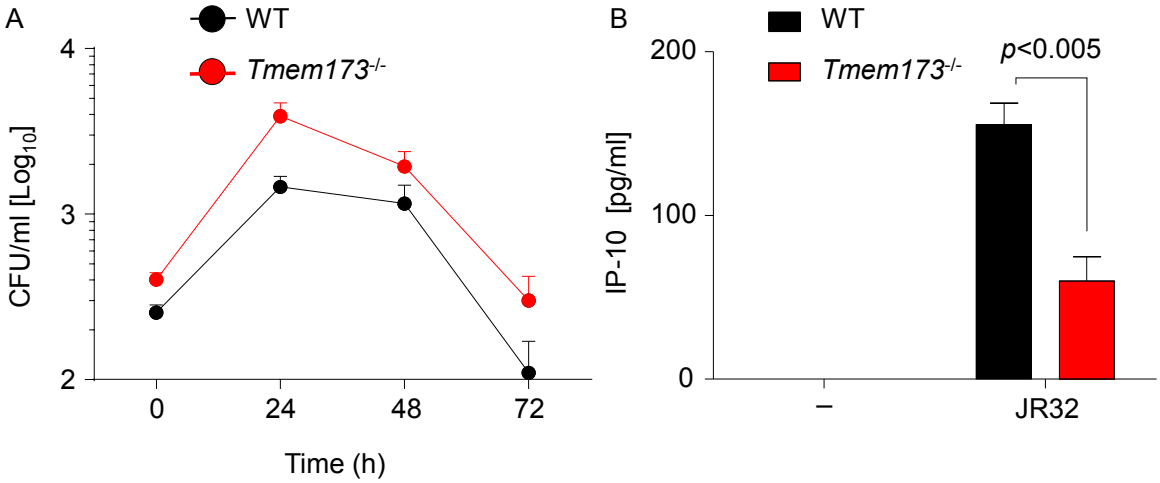
53. Jin L, Hill KK, Filak H, Mogan J, Knowles H, et al. (2011) MPYS is required for IFN response factor 3 activation and type I IFN production in the response of cultured phagocytes to bacterial second messengers cyclic-di-AMP and cyclic-di-GMP. J Immunol 187: 2595–2601. https://doi.org/10.4049/jimmunol.1100088 PMID: 21813776

54. Welte T, Suttorp N, Marre R (2004) CAPNETZ-community-acquired pneumonia competence network. Infection 32: 234–238. https://doi.org/10.1007/s15010-004-3107-z PMID: 15293080

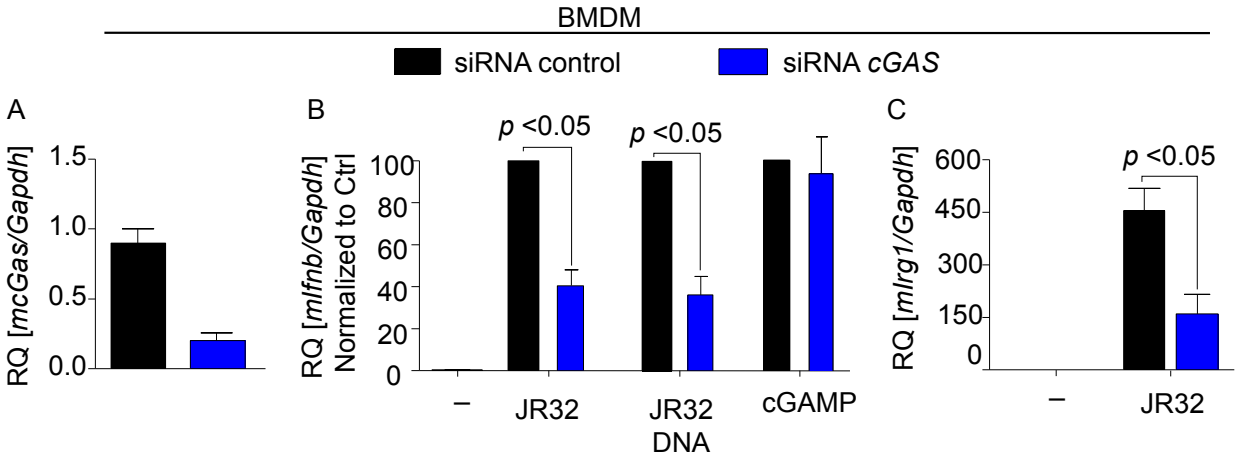
55. Bledowski P, Mossakowska M, Chudek J, Grodzicki T, Milewicz A, et al. (2011) Medical, psychological and socioeconomic aspects of aging in Poland: assumptions and objectives of the PolSenior project. Exp Gerontol 46: 1003–1009. https://doi.org/10.1016/j.exger.2011.09.006 PMID: 21979452

56. Nahid P, Horne DJ, Jarlsberg LG, Reiner AP, Osmond D, et al. (2011) Racial differences in tuberculosis infection in United States communities: the coronary artery risk development in young adults study. Clin Infect Dis 53: 291–294. https://doi.org/10.1093/cid/cir378 PMID: 21765079

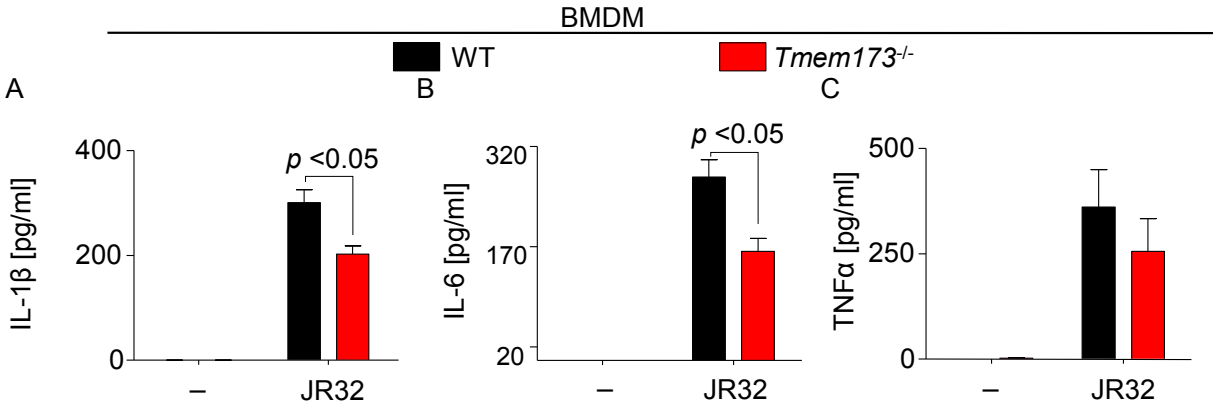
S1 Fig.


A
WT
Tmem173-/-
CFU/ml [Log10]
4
3
2
0
24
48
72
Time (h)
B
WT
Tmem173-/-
p<0.005
IP-10 [pg/ml]
200
100
0
–
JR32

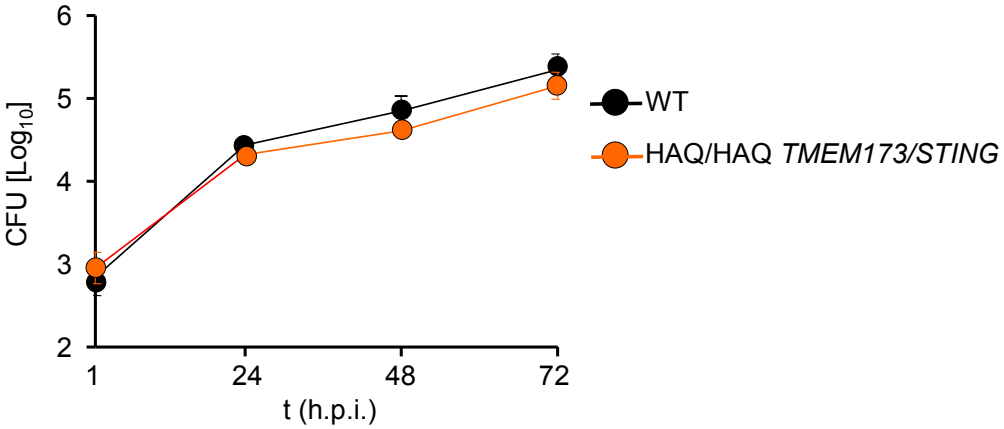
S2 Fig.


BMDM
siRNA control
siRNA cGAS
A
RQ [mcGas/Gapdh]
1.5
1.0
0.5
0.0
B
RQ [mIfnb/Gapdh] Normalized to Ctrl
100
80
60
40
20
0
p <0.05
p <0.05
–
JR32
JR32 DNA
cGAMP
C
RQ [mIrg1/Gapdh]
600
450
300
150
0
p <0.05
–
JR32

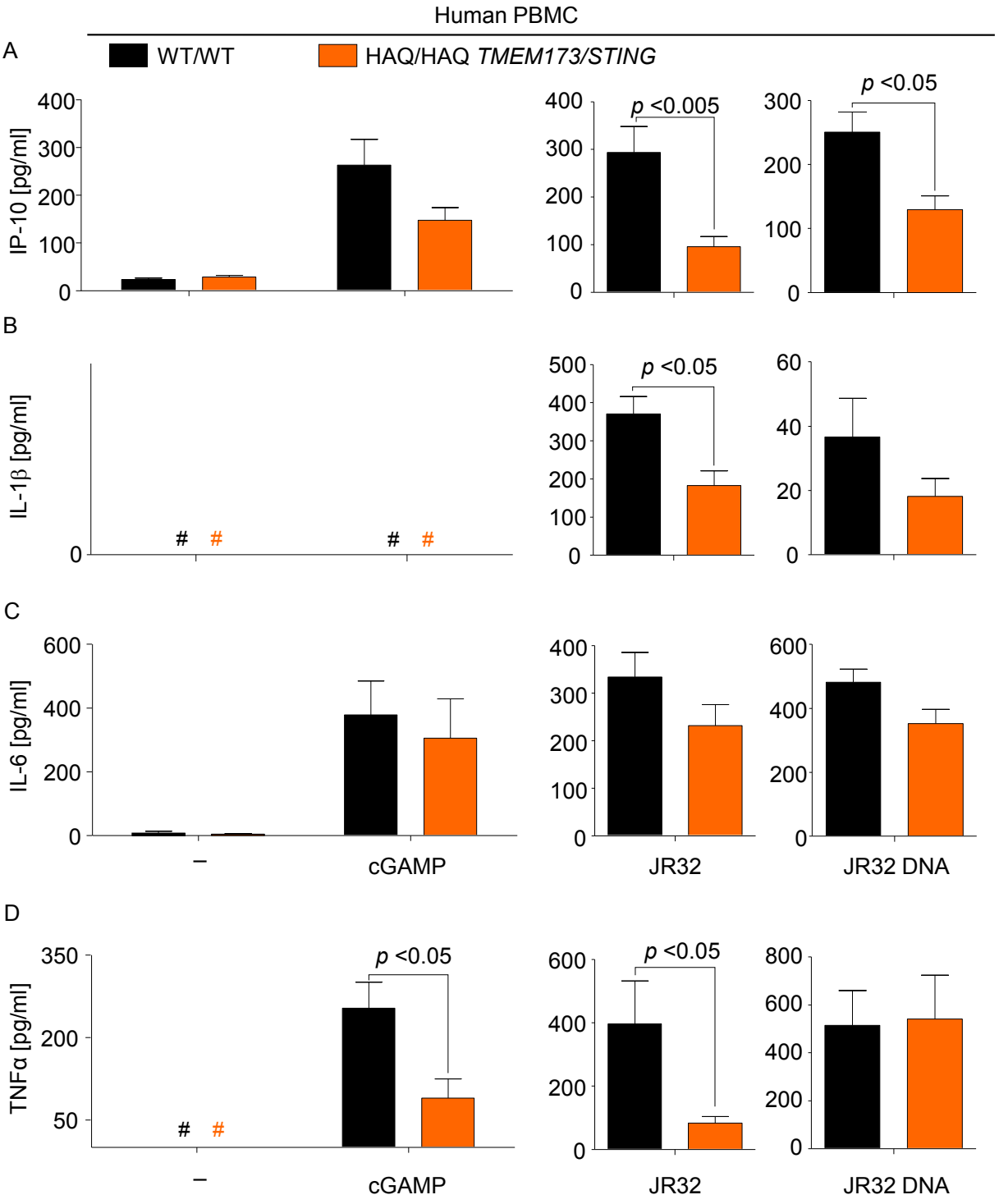
S3 Fig.


BMDM
WT
Tmem173-/-
A
B
C
IL-1β [pg/ml]
400
200
0
p <0.05
–
JR32
IL-6 [pg/ml]
320
170
20
p <0.05
–
JR32
TNFα [pg/ml]
500
250
0
–
JR32

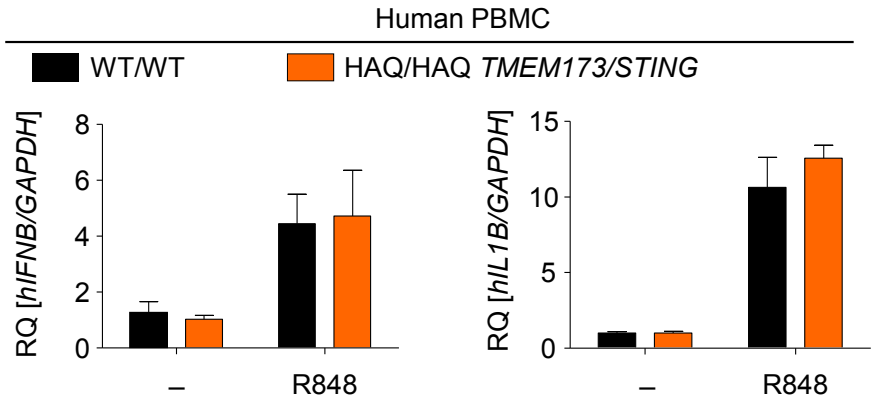
S4 Fig.



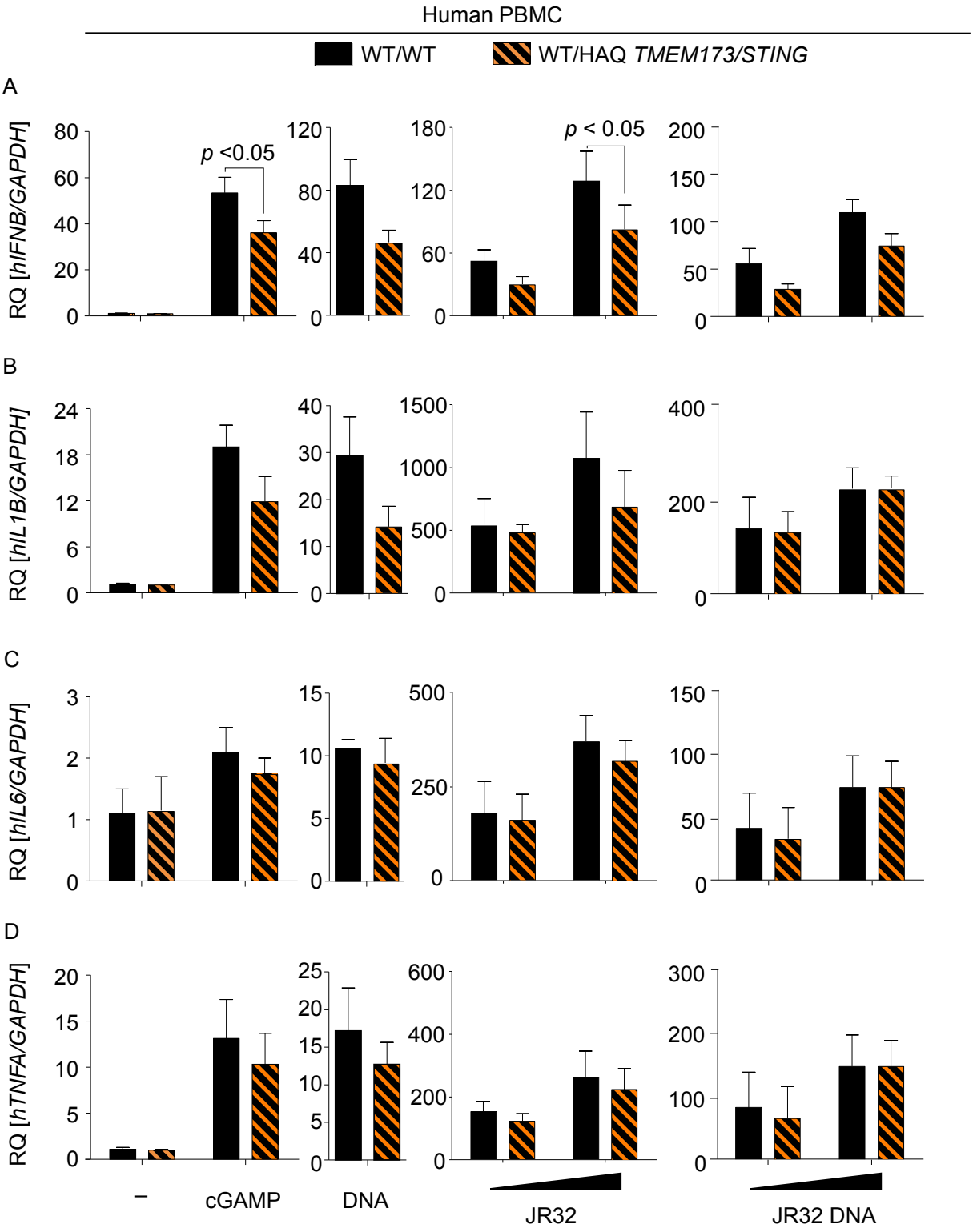
S5 Fig.


Human PBMC
A
WT/WT
HAQ/HAQ TMEM173/STING
IP-10 [pg/ml]
p <0.005
p <0.05
B
IL-1β [pg/ml]
p <0.05
C
IL-6 [pg/ml]
–
cGAMP
JR32
JR32 DNA
D
TNFα [pg/ml]
p <0.05
p <0.05
–
cGAMP
JR32
JR32 DNA

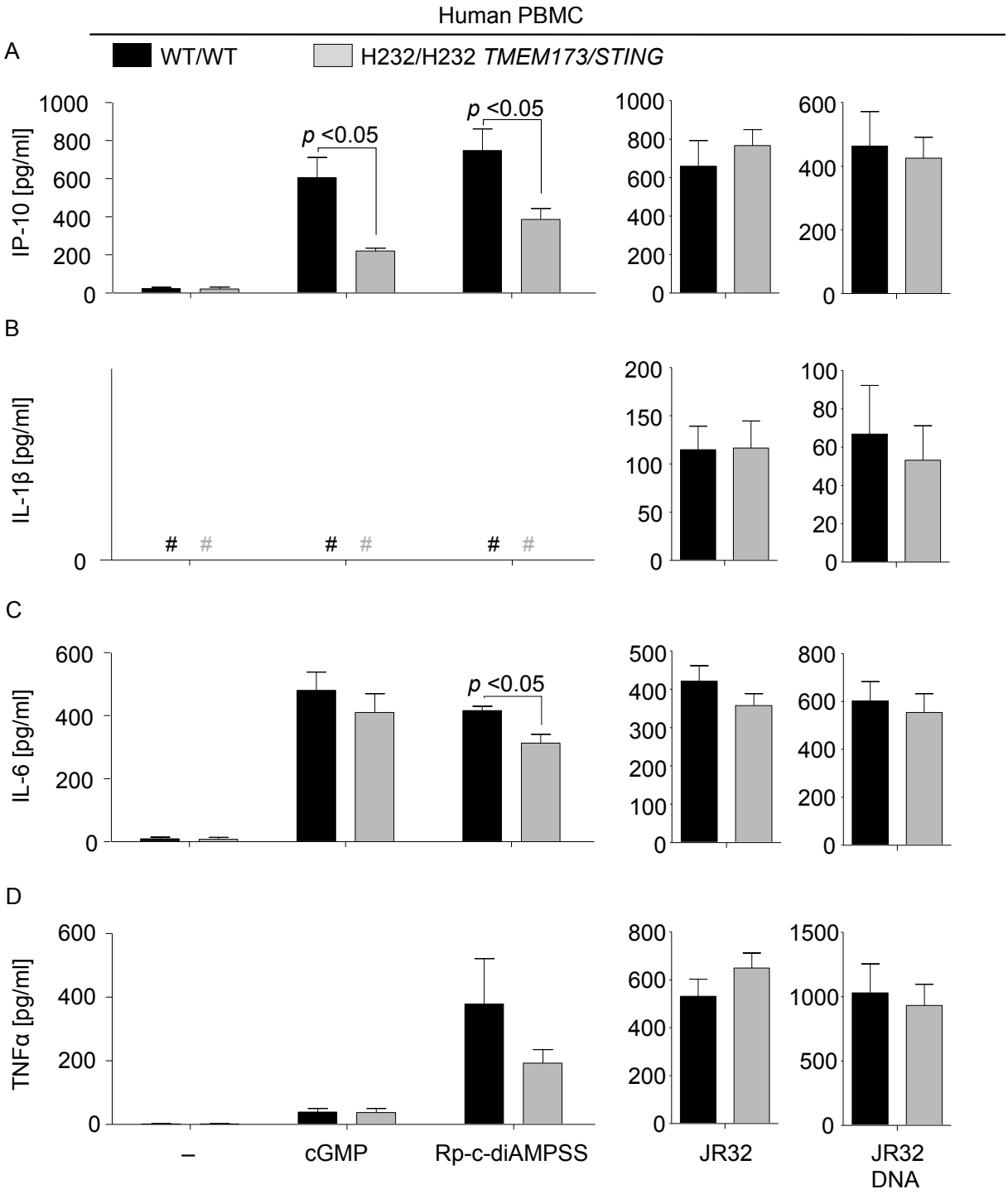
S6 Fig.


Human PBMC
WT/WT
HAQ/HAQ TMEM173/STING
RQ [hIFNB/GAPDH]
8
6
4
2
0
–
R848
RQ [hIL1B/GAPDH]
15
10
5
0
–
R848

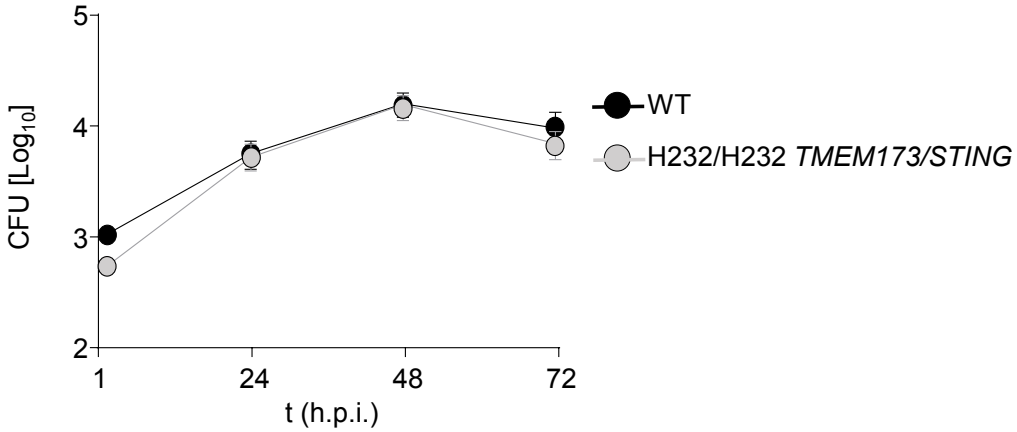
S7 Fig.


Human PBMC
WT/WT
WT/HAQ TMEM173/STING
A
RQ [hIFNB/GAPDH]
p <0.05
p < 0.05
B
RQ [hIL1B/GAPDH]
C
RQ [hIL6/GAPDH]
D
RQ [hTNFA/GAPDH]
–
cGAMP
DNA
JR32
JR32 DNA

S8 Fig.


Human PBMC
A
WT/WT
H232/H232 TMEM173/STING
IP-10 [pg/ml]
p <0.05
p <0.05
B
IL-1β [pg/ml]
#
C
IL-6 [pg/ml]
p <0.05
D
TNFα [pg/ml]
–
cGMP
Rp-c-diAMPSS
JR32
JR32 DNA

S9 Fig.



Supplemental Table 1. Demographics of Netherlands cohort

	Control (N=88)	Cases (N=91)
Male, f*(n)	0.5 (44)	0.61 (56)
Age (median, IQR)	49.6 (35.2 – 56.1)	64.7 (54.2 – 71.5)
Smoker, f (n)	0.31 (27)	0.49 (45)
COPD, f (n)	0.10 (8)	0.07 (6)
Diabetes Mellitus, f (n)	0.04 (3)	0.09 (8)
Cancer, f(n)	0.02 (2)	0.04 (4)
Hx. Transplant, f(n)	0.02 (2)	0 (0)
Autoimmune Dz, f(n)	0 (0)	0.02 (2)
Alcohol Use, f(n)	0.73 (61)	0.35 (32)

*f=frequency

3.2 Second publication

Title: The cGAS-STING pathway detects *Streptococcus pneumoniae* but appears dispensable for anti-pneumococcal defense in mice and humans

Authors: Juan Sebastián Ruiz-Moreno, Lutz Hamann, Lei Jin, Leif E. Sander, Monika Puzianowska-Kuznicka, John Cambier, Martin Witzenrath, Ralf R. Schumann, Norbert Suttorp, Bastian Opitz & CAPNETZ Study Group

Journal: Infection and Immunity

Received: March 29, 2017

Revised: December 11, 2017

Accepted: December 17, 2017

DOI: 10.1128/IAI.00849-17

You have to read this part online

4. Discussion

S. pneumoniae and *L. pneumophila* are common etiological agents of bacterial CAP. The virulence mechanisms by which these pathogens cause disease are substantially different from each other and reflect the biological disparities between these organisms. Indeed, while the former is a Gram-positive extracellular pathogen, well adapted for both asymptomatic colonization and/or pathogenic invasion of human tissues [38], the latter is a Gram-negative bacterium adapted as a parasite of protozoans in freshwater environments and an incidental intracellular pathogen of humans [29]. The mechanisms by which the innate immune system senses and responds to these and other pathogens involves the recognition of microbial molecular patterns by PRRs and the activation of signaling pathways that result in the development of diverse defense mechanisms [60]. Innate immune recognition of *S. pneumoniae* and *L. pneumophila* heavily depends on TLR and NLR sensing, and on the production of pro-inflammatory cytokines. Type I IFN production is also a common feature of the innate immune responses to these pathogens. Production of this group of cytokines has been shown to depend on the cytosolic recognition of bacterial DNA, the presence of key virulence factors from both bacteria and engagement of the adaptor protein STING [140, 151].

The present research work shows that type I IFN responses to *L. pneumophila* and *S. pneumoniae*, not only are mediated by STING, but also by the cytosolic DNA receptor cGAS. Activation of the cGAS-STING axis significantly contributes to pro-inflammatory cytokine production during *L. pneumophila* infection, but not following pneumococcal challenge. Moreover, the STING cascade, contributes to antibacterial defenses against *L. pneumophila* in mouse lungs but does not appear to be critically involved in control of pneumococcal lung infection. Interestingly, the common HAQ variant of STING, previously reported to be a loss-of function variant with regard to type I IFN production [186], was observed in this study to behave as a hypomorphic allele, largely defective in its capacity to induce IFNβ and with a reduced ability to mediate pro-inflammatory cytokine production during infection with either of these two pathogens. In consistence with previous publications [183, 184, 187], the R232H STING variant was observed to be partly defective in sensing bacterial CDNs but fully functional in response to 2'3'-cGAMP or DNA as an upstream ligand. Most importantly, case-control analyses revealed that carriage of the HAQ allele is linked with a higher risk of contracting pneumonia caused by *L. pneumophila*, although it seems not to be associated with

enhanced susceptibility to pneumococcal pneumonia. These analyses are thus in line with the aforementioned studies in mice, and together indicate that the STING-dependent signaling cascade is critical for antibacterial defense against *L. pneumophila* in mouse and humans but redundant in *S. pneumoniae* infections. Moreover the R232H variant, although impaired in sensing CDNs, was fully functional in mediating responses to both bacteria. Consequently, the R232H allele frequency appeared not to be associated with pneumonia caused by *L. pneumophila* or *S. pneumoniae*

Together this work provides the first report of an association between STING polymorphisms and susceptibility to infectious diseases, and of mouse infection studies performed in parallel.

4.1 The cGAS-STING pathway mediates type I IFN responses to *L. pneumophila* and *S. pneumoniae*

Type I IFNs have been long acknowledged for their strong influence on the development and regulation of immune responses during viral infections [189]. The role of this group of cytokines, however, goes beyond the sphere of antiviral immune responses, and it is now widely acknowledged to be likewise relevant during bacterial, fungal and parasitic infections [103]. Several publications have previously addressed the activation of type I IFNs during infections with *L. pneumophila* and *S. pneumoniae* and the mechanisms of their induction. In the former case, type I IFN responses were found to be dependent on the T4SS, the cytosolic presence of bacterial DNA and the host adaptor protein STING [140, 190]. Similarly, during pneumococcal infections, the observed type I IFN response appeared to be dependent on bacterial DNA, Ply and the adaptor STING [163, 191].

The results presented here confirm previous reports demonstrating the importance of the cGAS-STING axis in inducing type I IFN responses to *L. pneumophila* [125] and provide novel evidence of the role of the cGAS receptor during pneumococcal infections.

STING- or cGAS-deficient murine bone marrow-derived macrophages (BMM), were largely deficient in their capacity to activate IFNβ or the downstream ISGs Irg1, IP-10 (Interferon gamma-induced protein 10) or Ccl5 (C-C Motif Chemokine Ligand 5), in response to infection with *L. pneumophila* or *S. pneumoniae*, or stimulation with genomic DNA from these pathogens. Moreover, mice lacking STING had a severely impaired induction of *Ifnb* and ISGs, following intranasal infection. *cGas*$^{-/-}$ BMMs showed a normal type I IFN response upon the control treatment with cGAMP, a result that was expected given the fact that cGAS operates upstream of this endogenously produced second messenger CDN. Both WT and

STING-deficient cells induced a slight *Ifnb* expression upon infection with a *L. pneumophila* mutant lacking an important component of the T4SS (*ΔdotA*). This result is consistent with previous observations [140] and highlights the importance of an intact T4SS for the activation of IFN responses during *L. pneumophila* infection. WT BMMs infected with the mutant strain *ΔsdhA* showed an enhanced activation in comparison to WT cells infected with the corresponding background strain. The SdhA protein of *L. pneumophila*, previously thought to be a modulator of innate immune responses against this bacterium [192], is now considered an essential effector for the maintenance of the LCV integrity [193]. It is therefore reasonable to speculate that in the absence of the SdhA protein, a destabilized LCV results in an augmented leakage of bacterial DNA and other PAMPs, leading to enhanced immune responses as compared to infections with WT bacteria. Whether this heightened immune response leads to a stronger antibacterial defense, or to exacerbated inflammatory responses detrimental for the host, remains to be elucidated, if future therapeutic approaches targeting SdhA are envisioned.

Similarly, infection with a capsule-deficient pneumococcal mutant resulted in increased *Ifnb* expression. Considering that *Δcps* pneumococcal strains are more efficiently phagocytosed by BMMs [163], the augmented IFN response induced by these mutants can be explained by an increased exposure of pneumococcal DNA to its receptor in the cytoplasm.

The bacterial CDN c-di-AMP, which is also produced by *S. pneumoniae* [194], has been described to play an important role in sensing *L. monocytogenes* infection by the innate immune system [195]. Moreover, c-di-GMP, which is essential for the intracellular growth of *L. pneumophila* [196], has been recently shown to be positively correlated with *Ifnb* expression [197]. A closer look at the type I IFN responses of *Tmem173*$^{-/-}$ vs. *cGas*$^{-/-}$ cells following infection with *L. pneumophila* or *S. pneumoniae* reveals that cGAS-deficient cells have, in this study, a slightly higher capability of inducing IFN responses compared to their STING-deficient counterparts. This might suggest that in the model presented here, bacterial CDNs play a perceptible albeit minor role in stimulating type I IFN expression. Furthermore, human cells expressing R232H STING were impaired in sensing CDNs but responded normally to *L. pneumophila*, which suggests that sensing of *Legionella* c-di-GMP is not critically involved in innate immune response to this bacterium.

Cells lacking STING are not completely deficient in their ability to induce *Ifnb* expression, implying the existence of an additional STING-independent sensory mechanism which contributes to some extend to the type I IFN response to *L. pneumophila* infection. The RNA receptors RIG-I and MDA5 were described in a previous publication to participate in the type

I IFN response to *L. pneumophila* [192]. The activation of these RNA receptors might therefore account for the residual expression of *Ifnb* in STING-deficient cells. However, while the activation of such receptors was not assessed in this study, it seems safe to speculate that their role during infections with *L. pneumophila* (as well as *S. pneumoniae*) remains negligible. Consequently, DNA appears to be the main PAMP that induces type I IFNs during infection with *L. pneumophila* and *S. pneumoniae*. However, the mechanism by which bacterial DNA is transferred to the host cytosol remains incompletely understood. Prior studies examining the conjugative properties of the T4SS demonstrated that this molecular complex is capable of translocating bacterial nucleic acids [198, 199], indicating that this is the most probable mechanism by which *Legionella* DNA reaches the host cell cytoplasm. A more complicated scenario arises when considering extracellular pathogens such as *S. pneumoniae*. In addition to all the pathogenic processes associated with it, pneumococcal Ply was found to induce host DNA damage and cell cycle arrest [55]. This observation raises the possibility that DNA from the host might in fact contribute to the activation of the cGAS-STING pathway during infections with *S. pneumoniae*. Nevertheless, the fact that Ply alone is not sufficient to induce type I IFN responses and that phagocytosis of pneumococci and acidification of the phagosome are required for IFN-β production [163], suggests that Ply-mediated release of pneumococcal DNA is the main event activating the cGAS-STING cascade during infections with this pathogen.

4.2 Pro-inflammatory responses to *L. pneumophila* but not *S. pneumoniae* are partly dependent on cGAS-STING

As with several other pathogens, a tightly controlled pro-inflammatory response is crucial for mounting antibacterial defenses against *L. pneumophila* and *S. pneumoniae* [200-203]. The evidence in this study suggests that cGAS and STING contribute to the induction of pro-inflammatory cytokines during *L. pneumophila*, but not during pneumococcal infection.
Deficiency of cGAS or STING significantly reduced the expression of *Il1b* and *Il6* and additionally showed some minor effects on *Tnfa* induction during *L. pneumophila* infection. Similar results were obtained after measuring the pro-inflammatory cytokine production in the lungs of WT and *Tmem173*$^{-/-}$ mice infected with this pathogen. The contribution of the cGAS-STING cascade to pro-inflammatory cytokine production observed in this report is in line with previous studies in which STING was found to mediate the activation of NF-κB and pro-inflammatory gene transcription through a TBK1-dependent mechanism [94].

Importantly, STING-deficient mice showed significantly lower levels of IFNγ in their lungs. This cytokine has a significant role in the immune responses against intracellular pathogens [204] and has been recently shown, together with type I IFNs, to markedly influence cell-intrinsic immune responses to *L. pneumophila* [109]. The altered type I and II IFN responses might therefore account for the enhanced bacterial loads observed in the lungs of STING-deficient mice (discussed below).

While *Tmem173*$^{-/-}$ BMMs showed significantly reduced production of IL-1B and IL-6 following pneumococcal challenge, cGAS-deficient cells displayed a pro-inflammatory response that was comparable to that of WT cells. This might suggest the existence of a STING-dependent but cGAS-independent sensing mechanism involved in the activation of pro-inflammatory responses *in vitro*. A potential contribution of bacterial c-di-AMP or alternative cytosolic DNA sensors cannot therefore be excluded.

The fact that cGAS and STING significantly contribute to the activation of pro-inflammatory cytokines to *L. pneumophila*, but appear dispensable for these responses during pneumococcal pneumonia, suggests that other receptors such as TLRs and NLRs have a more preponderant role in recognizing *S. pneumoniae* and largely mediate pro-inflammatory cytokine responses to this bacterium. The cGAS-STING cascade therefore plays a rather redundant and secondary role in inducing inflammatory responses against this extracellular pathogen.

4.3 STING assists the clearance of *L. pneumophila*, but is dispensable for anti-pneumococcal defenses

The induction of type I IFNs results in protective host responses during *L. pneumophila* infection [109, 205]. Conversely, the role of this group of cytokines remains controversial in the case of pneumococcal infections [108, 164-166]. Our experiments with cGAS- and STING-deficient animals add to the existing evidence supporting a protective function of the type I IFN cascade in *L. pneumophila* infections, and again suggest that the role of this pathway is more ambiguous in infections with *S. pneumoniae*.

As discussed below, the reduced production of type I and II IFNs in *Tmem173*$^{-/-}$ mice impairs intracellular antibacterial defense mechanisms in alveolar macrophages [109] thus likely explaining why these animals have an enhanced burden of *L. pneumophila* in their lungs. Although bacterial loads in the lungs of both *cGas*$^{-/-}$ and *Tmem173*$^{-/-}$ were significantly higher as compared to WT animals, these effects were in fact rather small. A reason for this could be that mice (particularly those on a C57BL/6 background as used in this study) are in general

relatively more resistant to *L. pneumophila*, probably as a result of an efficient NAIP5/NLRC4 inflammasome and most likely other immune pathways [206]. Alternatively, the reduced but still existing levels of IFNγ produced by STING-deficient animals could be sufficient to control excessive bacterial growth in *Tmem173*$^{-/-}$ mice.

Assessment of pneumococcal burdens in the lungs, spleen and blood of *Tmem173*$^{-/-}$ and WT mice following intranasal infection revealed no differences. Considering the possibility that the initial infection dose might have been too high to allow detection of differences in bacterial burden between the two mouse strains, a tenfold lower infection dose was administered. Nonetheless, bacterial burdens remained equal between WT and STING-deficient animals, suggesting that STING is not involved in the acute anti-pneumococcal defense. A prior publication in which similar experimental conditions were employed revealed that *Ifnar*$^{-/-}$ mice exhibited higher bacteremia 48 h after pneumococcal intranasal challenge [207]. Discrepancies between the present study and the cited report, might be reconciled if the capacity of cGAS-STING to also regulate the production of other mediators in addition to type I IFNs is considered. Taking into account that STING-mediated signaling results in the activation of different transcription factors [93, 94, 208], it could also be possible that STING might negatively influence anti-pneumococcal responses in addition to promoting production of e.g. type I IFNs. Additional work is required to further decipher the exact functions of the STING pathway and type I IFNs in pneumococcal infections.

4.4 The HAQ and R232H variants of STING lead to reduced cytokine responses in human PBMCs

The second and third most common STING alleles, HAQ and R232H, have been described as deficient in their capacity to induce type I IFN responses. The former variant has been found to be largely defective in inducing immune responses when ectopically expressed in 293MT cells. Individuals carrying the HAQ haplotype in homozygosity have a reduced production of STING in B-cells and different tissues. Similarly, mice expressing an equivalent form of the human HAQ variant show reduced levels of STING production and display a weakened type I IFN response. The R232H allele, on the other hand, appears to be defective in recognizing bacterial CDNs, but is fully capable of responding to 2′3′-cGAMP synthesized upon cGAS-mediated recognition of DNA [183, 184, 186, 187]. The endogenous function of these STING variants during primary infections and their potential association with susceptibility to disease

in humans, have so far not been assessed. In the present study, human peripheral blood mononuclear cells (PBMCs) carrying the HAQ allele in homozygosity were significantly impaired in mediating type I IFN induction and pro-inflammatory cytokines following *L. pneumophila* infection or stimulation with DNA and cGAMP. Interestingly, cells from heterozygous HAQ carriers showed a reduced induction of type I IFNs in response to cGAMP stimulation or *L. pneumophila* infection. Whether this reduction is relevant for antibacterial responses or leads to increased susceptibility to LD remains to be assessed.

It is striking that *Legionella* replication within macrophages from HAQ human carriers, as well as from STING-deficient mice, remained unchanged in comparison to WT cells, given the significant contribution of STING to IFN production. Indeed, type I and II IFNs act on macrophages in an auto-/paracrine fashion to stimulate cell-intrinsic defense mechanisms that restrict *L. pneumophila* growth [109]. It is therefore conceivable that the small amounts of IFNs produced independently of STING are sufficient to control bacterial replication *in vitro*.

PBMCs from HAQ homozygous carriers likewise showed decreased expression of type I IFNs in response to *S. pneumoniae* infection and pneumococcal DNA. Interestingly, an altered STING-dependent cascade had stronger effects on macrophages derived from human PBMCs than on murine macrophages in terms of pro-inflammatory cytokine expression. A differential involvement of alternative PRRs known to sense *S. pneumoniae* between mice and human, might account for this disparity.

Altogether, the data here presented indicate that the HAQ STING variant induces weakened, but not absent IFN responses. The monocytic THP-1 human cell line, which, according to analyses from the present and previous studies [187, 209] carries the HAQ allele in homozygosity, induces *IFNB* expression in response to bacterial infection and DNA. Nonetheless, when compared to WT STING-expressing PBMCs, it is apparent that the extent of if this gene expression is several times lower. Moreover, deletion of cGAS further weakens type I IFN induction in THP-1 cells. These observations support the hypothesis that the HAQ STING allele is a hypomorphic rather than a loss-of-function variant [186].

As in prior studies, the R232H variant seemed to have a reduced response when challenged with CDNs other than 2′3′-cGAMP. However, this allele was functional in response to bacterial infection or stimulation with DNA. This observation further supports the assumption that bacterial CDNs are not required for the elicitation of immune responses during infections with the bacteria examined in this study.

4.5 Carriage of HAQ STING might predispose individuals to *L. pneumophila* infection but not to pneumococcal pneumonia

Host genetic polymorphisms in several components of the innate immune response are widely accepted to have profound effects on infectious disease susceptibility, and their influence on disease outcome might be as relevant as that of the genetic makeup of the pathogen involved [171]. The case-control analyses of two different European cohorts presented in this work, unveiled an association of the HAQ variant of STING with an increased risk of contracting *L. pneumophila*-induced pneumonia. Importantly, this is the first report in which a STING polymorphism is found to be associated with susceptibility to infectious diseases. The hypomorphic nature of this variant with respect to the activation of cytokines which are important for defense responses against *L. pneumophila,* might explain its linkage with increased predisposition to LD. Taking into consideration that STING contributes to the immune responses mounted against several pathogens including *M. tuberculosis*, *L. monocytogenes* and several types of viruses [121-125], it is tempting to speculate that the HAQ allele might be associated with these infections as well. Conversely, this variant could potentially be protective in infections with *Plasmodium falciparum*, as control of this parasite has been shown to be negatively affected by STING in mouse models [210]. This presumptive protective effect of the HAQ variant, might account for the considerable prevalence of this allele in the population [186]. Similarly, carriers of the HAQ allele might be partially protected against auto-immune conditions such as SAVI and familial chilblain lupus in which STING has been recently implicated [181, 182].

Different polymorphisms in genes coding for TLRs, have been previously found to be associated with increased or decreased susceptibility to LD [173-175]. It would therefore be important to test how simultaneous carriage of STING and TLR polymorphisms might affect disease susceptibility and outcome.

Although there was no association between HAQ STING and enhanced predisposition to pneumococcal pneumonia or increased disease severity in non-vaccinated patients, a different picture might arise if vaccinated individuals are taken into consideration. The recently descripted involvement of the cGAS-STING pathway in B-cell responses and the fact that the equivalent HAQ STING murine variant was observed to reduce the efficacy of *S. pneumoniae* vaccination [186, 211], might endorse this hypothesis. Future studies should therefore take

into consideration vaccinated patients and test whether carriage of HAQ STING interferes with the efficacy of pneumococcal vaccination in humans.
Finally, the R232H allele was not found associated with increased predisposition to infection with any of the pathogens examined in this study, further suggesting that bacterial CDNs play no role in immune defenses during bacterial pneumonia.

4.6 Conclusion

CAP is a serious infection of the lower respiratory tract that can be caused, among others, by both extracellular (e.g. *S. pneumoniae*) and intracellular bacteria (e.g. *L. pneumophila*). Previous research has shown that type I IFNs have a significant impact on the antibacterial immune response to both pathogens in the lung [109, 140, 163]. The evidence provided in the present study indicates that induction of type I IFN production during *L. pneumophila* and *S. pneumoniae* infection is mediated by the cGAS-STING cascade. This pathway additionally enhances the production of pro-inflammatory cytokines in human PBMCs following both types of infection, and in *L. pneumophila*-infected mouse macrophages. Moreover, the cGAS-STING pathway contributes to control of *L. pneumophila* but not to pneumococcal defenses in mouse models of bacterial pneumonia. The common HAQ allele of the human *TMEM173* gene encodes for a hypomorphic STING variant, which is strongly impaired in mediating type I IFN and pro-inflammatory cytokine responses upon bacterial infection or stimulation with DNA or CDNs. Conversely, cells expressing the R232H STING variant responded normally to *L. pneumophila* and *S. pneumoniae* infection as well as DNA stimulation, but were impaired in sensing bacterial CDNs. Most importantly, the HAQ variant was associated with an increased risk of developing LD but was not linked to enhanced predisposition to pneumococcal pneumonia in non-vaccinated individuals.
This is the first study to examine the influence of STING polymorphisms on infectious disease susceptibility.
Further studies should fill the gaps that remain to be elucidated and build upon the evidence presented in this research work. For instance, a more detailed understanding of the mechanism by which bacterial DNA reaches the cytoplasm is required. Considering that activation of the cGAS-STING axis has been recently linked with the elicitation of important cellular processes such as apoptosis and autophagy [212, 213], it would be worth examining if these mechanisms play a role downstream of cGAS-STING during infections with *L. pneumophila* or *S. pneumoniae*.

Future studies should also analyze the potential association between STING variants and other infectious diseases such as those caused by *M. tuberculosis*, *Plasmodium spp.* and HIV. Similarly, it would additionally be important to evaluate other polymorphism in genes involved in IFN responses. A functional polymorphism in the *IFNAR* gene has been previously associated with susceptibility to viral disease [214]. The control-case analyses in this report could be improved by, for instance, including ambulatory and hospitalized patients in order to elucidate whether STING alleles are associated with a higher chance of being hospitalized. Given that the influence of genetic variability on disease susceptibility is strongly affected by the age of the host [167], subsequent studies should aim at performing age-stratified analyses with the purpose of evaluating genetic variability and disease risk in specific age groups.

5. Summary

The hypomorphic HAQ variant of STING affects cGAS-dependent cytokine expression in response to bacterial infection and is associated with susceptibility to Legionnaires' disease in humans

Community-acquired pneumonia (CAP) remains one of the most notorious burdens for health worldwide. Among the wide spectrum of etiological agents, *S. pneumoniae* and *L. pneumophila* are considered two of the most common causes of bacterial CAP. Innate immune defenses against these and several other pathogens, largely relies on activation of pattern recognition receptors (PRRs) and initiation of inflammatory responses. Infection with *L. pneumophila* and *S. pneumoniae* has been previously shown to induce type I IFN responses in a manner dependent of STING. This protein is an essential adaptor of the cytosolic DNA sensor cGAS. Interestingly, two allelic variants of the STING-encoding gene *TMEM173*, namely HAQ and R232H, have been described before to have a diminished capacity of inducing type I IFN responses when ectopically expressed. The function of endogenous HAQ and R232H STING variants and their potential association with predisposition to infectious diseases have, however, not yet been assessed.

Here, I show that the cGAS-STING pathway mediates detection of *S. pneumoniae* and *L. pneumophila* infection to primarily stimulate type I IFN responses. Cells of human individuals carrying HAQ TMEM173 were largely or partly defective in inducing type I IFNs and proinflammatory cytokines upon bacterial infection or stimulation with bacterial DNA and cyclic di-nucleotides (CDNs). In contrast, cells expressing R232H STING were partly impaired in their response to CDNs but responded normally to *L. pneumophila* and *S. pneumoniae*. Subsequent analyses revealed that the STING pathway contributed to control of *L. pneumophila* infection but was dispensable for restricting *S. pneumoniae* during acute pneumonia in mice. Moreover, explorative analyses revealed an association of HAQ *TMEM173* with susceptibility to *L. pneumophila* but not with *S. pneumoniae* infection in humans. Our study reveals that the cGAS-STING cascade contributes to antibacterial defense against *L. pneumophila* but not *S. pneumoniae* in mice and humans, and provides important insight into how the common HAQ TMEM173/STING variant affects antimicrobial immune responses and susceptibility to infection.

6. Zusammenfassung

Die hypomorphische HAQ-Variante von STING beeinflusst die cGAS-abhängige Zytokinexpression nach bakterieller Infektion und ist mit einer Anfälligkeit für Legionärskrankheit beim Menschen assoziiert

Ambulant erworbene Pneumonien (CAP) sind weltweit eine wesentliche Ursache für Morbidität und Mortalität. *S. pneumoniae* und *L. pneumophila* sind zwei der häufigsten Verursacher von CAP. Die angeborene Immunabwehr gegen diese und andere Pathogene ist abhängig von der Aktivierung von Mustererkennungsrezeptoren und der nachfolgenden Initiierung von Entzündungsreaktionen. Frühere Arbeiten zeigten, dass Infektionen mit *L. pneumophila* und *S. pneumoniae* die Produktion von Typ I IFNs abhängig von STING stimulierten. STING ist ein essentielles Adaptermolekül des zytosolischen, DNA-erkennenden Mustererkennungsrezeptors cGAS. Vorherige Studien beschrieben zudem, dass häufige genetische Varianten vom STING-kodierenden Gen *TMEM173* vorkommen und dass die Varianten HAQ und R232H nach Überexpression vermindert in der Lage waren, Typ I IFNs zu induzieren. Die Funktion der endogenen HAQ und R232H STING-Moleküle und ihren potentiellen Einfluss auf Empfänglichkeit gegenüber Infektionen waren bisher jedoch noch nicht untersucht worden.

In dieser Arbeit zeige ich, dass *S. pneumoniae* und *L. pneumophila* über cGAS erkannt werden und nachfolgend über STING insbesondere die Produktion von Typ I IFNs induziert wird. Zellen von gesunden, freiwilligen Spendern, die HAQ STING exprimieren, waren stark oder teilweise in ihrer Fähigkeit eingeschränkt, Typ I IFNs bzw. proinflammatorische Zytokine nach bakteriellen Infektionen oder Stimulation mit bakterieller DNA und zyklischen di-Nukleotiden (CDNs) zu produzieren. Im Gegensatz dazu zeigten Zellen von R232H *TMEM173*-Trägern nur auf CDNs eine abgeschwächte Antwort, nicht jedoch auf Infektionen mit *L. pneumophila* und *S. pneumoniae* oder auf Stimulation mit bakterieller DNA. In Pneumoniemausmodellen zeigte sich zudem, dass der STING-Signalweg zur antibakteriellen Abwehr gegen *L. pneumophila* aber nicht gegen *S. pneumoniae* beiträgt. Außerdem konnte in explorativen Analysen von humanen Patientenproben eine Assoziation von HAQ *TMEM173* mit erhöhter Empfänglichkeit für *L. pneumophila*-, nicht jedoch *S. pneumoniae*-Infektionen nachgewiesen werden. Zusammengenommen zeigt die Arbeit, dass die cGAS-STING-

Kaskade zur antibakteriellen Abwehr gegen *L. pneumophila* nicht jedoch gegen *S. pneumoniae* in Mensch und Maus beiträgt. Die Arbeit liefert zudem wichtige Einsichten darüber, wie die häufige HAQ *TMEM173*/STING–Variante antimikrobielle Immunantworten und Empfänglichkeiten für Infektionen beeinflussen kann.

7. References

1. Musher, D.M. and A.R. Thorner, *Community-acquired pneumonia.* N Engl J Med, 2014. **371**(17): p. 1619-28.

2. le Roux, D.M. and H.J. Zar, *Community-acquired pneumonia in children - a changing spectrum of disease.* Pediatr Radiol, 2017. **47**(11): p. 1392-1398.

3. Walker, C.L.F., I. Rudan, L. Liu, H. Nair, E. Theodoratou, Z.A. Bhutta, K.L. O'Brien, H. Campbell, and R.E. Black, *Global burden of childhood pneumonia and diarrhoea.* Lancet, 2013. **381**(9875): p. 1405-1416.

4. Almirall, J., M. Serra-Prat, I. Bolibar, and V. Balasso, *Risk Factors for Community-Acquired Pneumonia in Adults: A Systematic Review of Observational Studies.* Respiration, 2017. **94**(3): p. 299-311.

5. Torres, A., W.E. Peetermans, G. Viegi, and F. Blasi, *Risk factors for community-acquired pneumonia in adults in Europe: a literature review.* Thorax, 2013. **68**(11): p. 1057-65.

6. Tramper-Stranders, G.A., *Childhood community-acquired pneumonia: A review of etiology- and antimicrobial treatment studies.* Paediatr Respir Rev, 2018. **26**: p. 41-48.

7. Don, M., M. Canciani, and M. Korppi, *Community-acquired pneumonia in children: what's old? What's new?* Acta Paediatr, 2010. **99**(11): p. 1602-8.

8. Navon-Venezia, S., K. Kondratyeva, and A. Carattoli, *Klebsiella pneumoniae: a major worldwide source and shuttle for antibiotic resistance.* FEMS Microbiol Rev, 2017. **41**(3): p. 252-275.

9. Self, W.H., R.G. Wunderink, D.J. Williams, Y. Zhu, E.J. Anderson, R.A. Balk, S.S. Fakhran, J.D. Chappell, G. Casimir, D.M. Courtney, C. Trabue, G.W. Waterer, A. Bramley, S. Magill, S. Jain, K.M. Edwards, and C.G. Grijalva, *Staphylococcus aureus Community-acquired Pneumonia: Prevalence, Clinical Characteristics, and Outcomes.* Clin Infect Dis, 2016. **63**(3): p. 300-9.

10. Lamoth, F. and B.D. Alexander, *Nonmolecular methods for the diagnosis of respiratory fungal infections.* Clin Lab Med, 2014. **34**(2): p. 315-36.

11. McCullers, J.A., *The co-pathogenesis of influenza viruses with bacteria in the lung.* Nat Rev Microbiol, 2014. **12**(4): p. 252-62.

12. Palacios, G., M. Hornig, D. Cisterna, N. Savji, A.V. Bussetti, V. Kapoor, J. Hui, R. Tokarz, T. Briese, E. Baumeister, and W.I. Lipkin, *Streptococcus pneumoniae coinfection is correlated with the severity of H1N1 pandemic influenza.* PLoS One, 2009. **4**(12): p. e8540.

13. Nambu, A., K. Ozawa, N. Kobayashi, and M. Tago, *Imaging of community-acquired pneumonia: Roles of imaging examinations, imaging diagnosis of specific pathogens*

and discrimination from noninfectious diseases. World J Radiol, 2014. **6**(10): p. 779-93.

14. Lynch, T., L. Bialy, J.D. Kellner, M.H. Osmond, T.P. Klassen, T. Durec, R. Leicht, and D.W. Johnson, *A systematic review on the diagnosis of pediatric bacterial pneumonia: when gold is bronze.* PLoS One, 2010. **5**(8): p. e11989.

15. Chalmers, J.D., P. Mandal, A. Singanayagam, A.R. Akram, G. Choudhury, P.M. Short, and A.T. Hill, *Severity assessment tools to guide ICU admission in community-acquired pneumonia: systematic review and meta-analysis.* Intensive Care Med, 2011. **37**(9): p. 1409-20.

16. Fiore, A.E., A. Fry, D. Shay, L. Gubareva, J.S. Bresee, and T.M. Uyeki, *Antiviral agents for the treatment and chemoprophylaxis of influenza --- recommendations of the Advisory Committee on Immunization Practices (ACIP).* MMWR Recomm Rep, 2011. **60**(1): p. 1-24.

17. Lim, W.S., S.V. Baudouin, R.C. George, A.T. Hill, C. Jamieson, I. Le Jeune, J.T. Macfarlane, R.C. Read, H.J. Roberts, M.L. Levy, M. Wani, and M.A. Woodhead, *BTS guidelines for the management of community acquired pneumonia in adults: update 2009.* Thorax, 2009. **64 Suppl 3**: p. iii1-55.

18. Fraser, D.W., T.R. Tsai, W. Orenstein, W.E. Parkin, H.J. Beecham, R.G. Sharrar, J. Harris, G.F. Mallison, S.M. Martin, J.E. McDade, C.C. Shepard, and P.S. Brachman, *Legionnaires' disease: description of an epidemic of pneumonia.* N Engl J Med, 1977. **297**(22): p. 1189-97.

19. Rowbotham, T.J., *Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae.* J Clin Pathol, 1980. **33**(12): p. 1179-83.

20. Cunha, B.A., A. Burillo, and E. Bouza, *Legionnaires' disease.* Lancet, 2016. **387**(10016): p. 376-385.

21. Kaufmann, A.F., J.E. McDade, C.M. Patton, J.V. Bennett, P. Skaliy, J.C. Feeley, D.C. Anderson, M.E. Potter, V.F. Newhouse, M.B. Gregg, and P.S. Brachman, *Pontiac fever: isolation of the etiologic agent (Legionella pneumophilia) and demonstration of its mode of transmission.* Am J Epidemiol, 1981. **114**(3): p. 337-47.

22. Mercante, J.W. and J.M. Winchell, *Current and emerging Legionella diagnostics for laboratory and outbreak investigations.* Clin Microbiol Rev, 2015. **28**(1): p. 95-133.

23. Phin, N., F. Parry-Ford, T. Harrison, H.R. Stagg, N. Zhang, K. Kumar, O. Lortholary, A. Zumla, and I. Abubakar, *Epidemiology and clinical management of Legionnaires' disease.* Lancet Infect Dis, 2014. **14**(10): p. 1011-21.

24. European Centre for Disease Prevention and Control. Legionnaires' disease. (2018). In: ECDC. Annual epidemiological report for 2016. Stockholm: ECDC.

25. Dooling, K.L., K.A. Toews, L.A. Hicks, L.E. Garrison, B. Bachaus, S. Zansky, L.R. Carpenter, B. Schaffner, E. Parker, S. Petit, A. Thomas, S. Thomas, R. Mansmann, C. Morin, B. White, and G.E. Langley, *Active Bacterial Core Surveillance for*

Legionellosis - United States, 2011-2013. MMWR Morb Mortal Wkly Rep, 2015. **64**(42): p. 1190-3.

26. von Baum, H., S. Ewig, R. Marre, N. Suttorp, S. Gonschior, T. Welte, and C. Luck, *Community-acquired Legionella pneumonia: new insights from the German competence network for community acquired pneumonia.* Clin Infect Dis, 2008. **46**(9): p. 1356-64.

27. Cristovam, E., D. Almeida, D. Caldeira, J.J. Ferreira, and T. Marques, *Accuracy of diagnostic tests for Legionnaires' disease: a systematic review.* J Med Microbiol, 2017. **66**(4): p. 485-489.

28. Mandell, L.A., R.G. Wunderink, A. Anzueto, J.G. Bartlett, G.D. Campbell, N.C. Dean, S.F. Dowell, T.M. File, Jr., D.M. Musher, M.S. Niederman, A. Torres, and C.G. Whitney, *Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults.* Clin Infect Dis, 2007. **44 Suppl 2**: p. S27-72.

29. Newton, H.J., D.K. Ang, I.R. van Driel, and E.L. Hartland, *Molecular pathogenesis of infections caused by Legionella pneumophila.* Clin Microbiol Rev, 2010. **23**(2): p. 274-98.

30. Zhu, W., S. Banga, Y. Tan, C. Zheng, R. Stephenson, J. Gately, and Z.Q. Luo, *Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of Legionella pneumophila.* PLoS One, 2011. **6**(3): p. e17638.

31. Isberg, R.R., T.J. O'Connor, and M. Heidtman, *The Legionella pneumophila replication vacuole: making a cosy niche inside host cells.* Nat Rev Microbiol, 2009. **7**(1): p. 13-24.

32. Tilney, L.G., O.S. Harb, P.S. Connelly, C.G. Robinson, and C.R. Roy, *How the parasitic bacterium Legionella pneumophila modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane.* J Cell Sci, 2001. **114**(Pt 24): p. 4637-50.

33. Alli, O.A., L.Y. Gao, L.L. Pedersen, S. Zink, M. Radulic, M. Doric, and Y. Abu Kwaik, *Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by Legionella pneumophila.* Infect Immun, 2000. **68**(11): p. 6431-40.

34. Finsel, I. and H. Hilbi, *Formation of a pathogen vacuole according to Legionella pneumophila: how to kill one bird with many stones.* Cell Microbiol, 2015. **17**(7): p. 935-50.

35. Bandyopadhyay, P., S. Liu, C.B. Gabbai, Z. Venitelli, and H.M. Steinman, *Environmental mimics and the Lvh type IVA secretion system contribute to virulence-related phenotypes of Legionella pneumophila.* Infect Immun, 2007. **75**(2): p. 723-35.

36. Söderberg, M.A., J. Dao, S.R. Starkenburg, and N.P. Cianciotto, *Importance of type II secretion for survival of Legionella pneumophila in tap water and in amoebae at low temperatures.* Appl Environ Microbiol, 2008. **74**(17): p. 5583-8.

37. Watson, D.A., D.M. Musher, J.W. Jacobson, and J. Verhoef, *A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery.* Clin Infect Dis, 1993. **17**(5): p. 913-24.

38. Henriques-Normark, B. and E.I. Tuomanen, *The pneumococcus: epidemiology, microbiology, and pathogenesis.* Cold Spring Harb Perspect Med, 2013. **3**(7).

39. Jansen, A.G., G.D. Rodenburg, A. van der Ende, L. van Alphen, R.H. Veenhoven, L. Spanjaard, E.A. Sanders, and E. Hak, *Invasive pneumococcal disease among adults: associations among serotypes, disease characteristics, and outcome.* Clin Infect Dis, 2009. **49**(2): p. e23-9.

40. van der Poll, T. and S.M. Opal, *Pathogenesis, treatment, and prevention of pneumococcal pneumonia.* Lancet, 2009. **374**(9700): p. 1543-56.

41. Centers for Disease Control and Prevention. (2017). Pneumococcal Disease, Surveillance and Reporting. https://www.cdc.gov/pneumococcal/surveillance.html. Accessed January 11 2018.

42. Savulescu, C., P. Krizova, A. Lepoutre, J. Mereckiene, D.F. Vestrheim, P. Ciruela, M. Ordobas, M. Guevara, E. McDonald, E. Morfeldt, J. Kozakova, E. Varon, S. Cotter, B.A. Winje, C. Munoz-Almagro, L. Garcia, J. Castilla, A. Smith, B. Henriques-Normark, L.P. Celentano, and G. Hanquet, *Effect of high-valency pneumococcal conjugate vaccines on invasive pneumococcal disease in children in SpIDnet countries: an observational multicentre study.* Lancet Respir Med, 2017. **5**(8): p. 648-656.

43. European Centre for Disease Prevention and Control. (2018) Invasive pneumococcal disease. In: ECDC. Annual epidemiological report for 2016. Stockholm: ECDC.

44. van der Linden, M., G. Falkenhorst, S. Perniciaro, and M. Imohl, *Effects of Infant Pneumococcal Conjugate Vaccination on Serotype Distribution in Invasive Pneumococcal Disease among Children and Adults in Germany.* PLoS One, 2015. **10**(7): p. e0131494.

45. Lynch, J.P., 3rd and G.G. Zhanel, *Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention.* Semin Respir Crit Care Med, 2009. **30**(2): p. 189-209.

46. Song, J.Y., B.W. Eun, and M.H. Nahm, *Diagnosis of pneumococcal pneumonia: current pitfalls and the way forward.* Infect Chemother, 2013. **45**(4): p. 351-66.

47. Azzari, C., M. Cortimiglia, M. Moriondo, C. Canessa, F. Lippi, F. Ghiori, L. Becciolini, M. de Martino, and M. Resti, *Pneumococcal DNA is not detectable in the blood of healthy carrier children by real-time PCR targeting the lytA gene.* J Med Microbiol, 2011. **60**(Pt 6): p. 710-4.

48. Abdeldaim, G.M., K. Stralin, P. Olcen, J. Blomberg, and B. Herrmann, *Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of Streptococcus pneumoniae target genes, with special reference to the Spn9802 fragment.* Diagn Microbiol Infect Dis, 2008. **60**(2): p. 143-50.

49. Weiser, J.N., D.M. Ferreira, and J.C. Paton, *Streptococcus pneumoniae: transmission, colonization and invasion.* Nat Rev Microbiol, 2018. **16**(6): p. 355-367.

50. Davis, K.M., H.T. Akinbi, A.J. Standish, and J.N. Weiser, *Resistance to mucosal lysozyme compensates for the fitness deficit of peptidoglycan modifications by Streptococcus pneumoniae.* PLoS Pathog, 2008. **4**(12): p. e1000241.

51. King, S.J., *Pneumococcal modification of host sugars: a major contributor to colonization of the human airway?* Mol Oral Microbiol, 2010. **25**(1): p. 15-24.

52. Gutierrez-Fernandez, J., M. Saleh, M. Alcorlo, A. Gomez-Mejia, D. Pantoja-Uceda, M.A. Trevino, F. Voss, M.R. Abdullah, S. Galan-Bartual, J. Seinen, P.A. Sanchez-Murcia, F. Gago, M. Bruix, S. Hammerschmidt, and J.A. Hermoso, *Modular Architecture and Unique Teichoic Acid Recognition Features of Choline-Binding Protein L (CbpL) Contributing to Pneumococcal Pathogenesis.* Sci Rep, 2016. **6**: p. 38094.

53. Honsa, E.S., M.D. Johnson, and J.W. Rosch, *The roles of transition metals in the physiology and pathogenesis of Streptococcus pneumoniae.* Front Cell Infect Microbiol, 2013. **3**: p. 92.

54. Mitchell, A.M. and T.J. Mitchell, *Streptococcus pneumoniae: virulence factors and variation.* Clin Microbiol Infect, 2010. **16**(5): p. 411-8.

55. Rai, P., F. He, J. Kwang, B.P. Engelward, and V.T. Chow, *Pneumococcal Pneumolysin Induces DNA Damage and Cell Cycle Arrest.* Sci Rep, 2016. **6**: p. 22972.

56. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nat Immunol, 2015. **16**(4): p. 343-53.

57. Elias, P.M., *The skin barrier as an innate immune element.* Semin Immunopathol, 2007. **29**(1): p. 3-14.

58. Sarma, J.V. and P.A. Ward, *The complement system.* Cell Tissue Res, 2011. **343**(1): p. 227-35.

59. Gordon, S., *Phagocytosis: An Immunobiologic Process.* Immunity, 2016. **44**(3): p. 463-475.

60. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.

61. Palm, N.W. and R. Medzhitov, *Pattern recognition receptors and control of adaptive immunity.* Immunol Rev, 2009. **227**(1): p. 221-33.

62. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.

63. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.

64. Kawasaki, T. and T. Kawai, *Toll-like receptor signaling pathways.* Front Immunol, 2014. **5**: p. 461.

65. Moore, C.B., D.T. Bergstralh, J.A. Duncan, Y. Lei, T.E. Morrison, A.G. Zimmermann, M.A. Accavitti-Loper, V.J. Madden, L. Sun, Z. Ye, J.D. Lich, M.T. Heise, Z. Chen, and J.P. Ting, *NLRX1 is a regulator of mitochondrial antiviral immunity.* Nature, 2008. **451**(7178): p. 573-7.

66. Motta, V., F. Soares, T. Sun, and D.J. Philpott, *NOD-like receptors: versatile cytosolic sentinels.* Physiol Rev, 2015. **95**(1): p. 149-78.

67. Kim, Y.K., J.S. Shin, and M.H. Nahm, *NOD-Like Receptors in Infection, Immunity, and Diseases.* Yonsei Med J, 2016. **57**(1): p. 5-14.

68. Sabbah, A., T.H. Chang, R. Harnack, V. Frohlich, K. Tominaga, P.H. Dube, Y. Xiang, and S. Bose, *Activation of innate immune antiviral responses by Nod2.* Nat Immunol, 2009. **10**(10): p. 1073-80.

69. van de Veerdonk, F.L., M.G. Netea, C.A. Dinarello, and L.A. Joosten, *Inflammasome activation and IL-1beta and IL-18 processing during infection.* Trends Immunol, 2011. **32**(3): p. 110-6.

70. Hardison, S.E. and G.D. Brown, *C-type lectin receptors orchestrate antifungal immunity.* Nat Immunol, 2012. **13**(9): p. 817-22.

71. Hoving, J.C., G.J. Wilson, and G.D. Brown, *Signalling C-type lectin receptors, microbial recognition and immunity.* Cell Microbiol, 2014. **16**(2): p. 185-94.

72. Bermejo-Jambrina, M., J. Eder, L.C. Helgers, N. Hertoghs, B.M. Nijmeijer, M. Stunnenberg, and T.B.H. Geijtenbeek, *C-Type Lectin Receptors in Antiviral Immunity and Viral Escape.* Front Immunol, 2018. **9**: p. 590.

73. Brown, G.D., J.A. Willment, and L. Whitehead, *C-type lectins in immunity and homeostasis.* Nat Rev Immunol, 2018. **18**(6): p. 374-389.

74. Loo, Y.M. and M. Gale, Jr., *Immune signaling by RIG-I-like receptors.* Immunity, 2011. **34**(5): p. 680-92.

75. Chiu, Y.H., J.B. Macmillan, and Z.J. Chen, *RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway.* Cell, 2009. **138**(3): p. 576-91.

76. Ablasser, A., F. Bauernfeind, G. Hartmann, E. Latz, K.A. Fitzgerald, and V. Hornung, *RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate.* Nat Immunol, 2009. **10**(10): p. 1065-72.

77. Rodriguez, K.R., A.M. Bruns, and C.M. Horvath, *MDA5 and LGP2: accomplices and antagonists of antiviral signal transduction.* J Virol, 2014. **88**(15): p. 8194-200.

78. Paludan, S.R. and A.G. Bowie, *Immune sensing of DNA.* Immunity, 2013. **38**(5): p. 870-80.

79. Rotem, Z., R.A. Cox, and A. Isaacs, *Inhibition of virus multiplication by foreign nucleic acid.* Nature, 1963. **197**: p. 564-6.

80. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira, *A Toll-like receptor recognizes bacterial DNA.* Nature, 2000. **408**(6813): p. 740-5.

81. Ishii, K.J., C. Coban, H. Kato, K. Takahashi, Y. Torii, F. Takeshita, H. Ludwig, G. Sutter, K. Suzuki, H. Hemmi, S. Sato, M. Yamamoto, S. Uematsu, T. Kawai, O. Takeuchi, and S. Akira, *A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA.* Nat Immunol, 2006. **7**(1): p. 40-8.

82. Takaoka, A., Z. Wang, M.K. Choi, H. Yanai, H. Negishi, T. Ban, Y. Lu, M. Miyagishi, T. Kodama, K. Honda, Y. Ohba, and T. Taniguchi, *DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response.* Nature, 2007. **448**(7152): p. 501-5.

83. Dempsey, A. and A.G. Bowie, *Innate immune recognition of DNA: A recent history.* Virology, 2015. **479-480**: p. 146-52.

84. Unterholzner, L., S.E. Keating, M. Baran, K.A. Horan, S.B. Jensen, S. Sharma, C.M. Sirois, T. Jin, E. Latz, T.S. Xiao, K.A. Fitzgerald, S.R. Paludan, and A.G. Bowie, *IFI16 is an innate immune sensor for intracellular DNA.* Nat Immunol, 2010. **11**(11): p. 997-1004.

85. Zhang, Z., B. Yuan, M. Bao, N. Lu, T. Kim, and Y.J. Liu, *The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells.* Nat Immunol, 2011. **12**(10): p. 959-65.

86. Fernandes-Alnemri, T., J.W. Yu, P. Datta, J. Wu, and E.S. Alnemri, *AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA.* Nature, 2009. **458**(7237): p. 509-13.

87. Roberts, T.L., A. Idris, J.A. Dunn, G.M. Kelly, C.M. Burnton, S. Hodgson, L.L. Hardy, V. Garceau, M.J. Sweet, I.L. Ross, D.A. Hume, and K.J. Stacey, *HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA.* Science, 2009. **323**(5917): p. 1057-60.

88. Burckstummer, T., C. Baumann, S. Bluml, E. Dixit, G. Durnberger, H. Jahn, M. Planyavsky, M. Bilban, J. Colinge, K.L. Bennett, and G. Superti-Furga, *An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome.* Nat Immunol, 2009. **10**(3): p. 266-72.

89. Hornung, V., A. Ablasser, M. Charrel-Dennis, F. Bauernfeind, G. Horvath, D.R. Caffrey, E. Latz, and K.A. Fitzgerald, *AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC.* Nature, 2009. **458**(7237): p. 514-8.

90. Sokolowska, O. and D. Nowis, *STING Signaling in Cancer Cells: Important or Not?* Arch Immunol Ther Exp (Warsz), 2018. **66**(2): p. 125-132.

91. Ishikawa, H. and G.N. Barber, *STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling.* Nature, 2008. **455**(7213): p. 674-8.

92. Ishikawa, H., Z. Ma, and G.N. Barber, *STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity.* Nature, 2009. **461**(7265): p. 788-92.

93. Tanaka, Y. and Z.J. Chen, *STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway.* Sci Signal, 2012. **5**(214): p. ra20.

94. Abe, T. and G.N. Barber, *Cytosolic-DNA-mediated, STING-dependent proinflammatory gene induction necessitates canonical NF-kappaB activation through TBK1.* J Virol, 2014. **88**(10): p. 5328-41.

95. Burdette, D.L., K.M. Monroe, K. Sotelo-Troha, J.S. Iwig, B. Eckert, M. Hyodo, Y. Hayakawa, and R.E. Vance, *STING is a direct innate immune sensor of cyclic di-GMP.* Nature, 2011. **478**(7370): p. 515-8.

96. Danilchanka, O. and J.J. Mekalanos, *Cyclic dinucleotides and the innate immune response.* Cell, 2013. **154**(5): p. 962-970.

97. Wu, J., L. Sun, X. Chen, F. Du, H. Shi, C. Chen, and Z.J. Chen, *Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA.* Science, 2013. **339**(6121): p. 826-30.

98. Sun, L., J. Wu, F. Du, X. Chen, and Z.J. Chen, *Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway.* Science, 2013. **339**(6121): p. 786-91.

99. Bhat, N. and K.A. Fitzgerald, *Recognition of cytosolic DNA by cGAS and other STING-dependent sensors.* Eur J Immunol, 2014. **44**(3): p. 634-40.

100. Luecke, S., A. Holleufer, M.H. Christensen, K.L. Jonsson, G.A. Boni, L.K. Sorensen, M. Johannsen, M.R. Jakobsen, R. Hartmann, and S.R. Paludan, *cGAS is activated by DNA in a length-dependent manner.* EMBO Rep, 2017. **18**(10): p. 1707-1715.

101. Margolis, S.R., S.C. Wilson, and R.E. Vance, *Evolutionary Origins of cGAS-STING Signaling.* Trends Immunol, 2017. **38**(10): p. 733-743.

102. Pestka, S., C.D. Krause, and M.R. Walter, *Interferons, interferon-like cytokines, and their receptors.* Immunol Rev, 2004. **202**: p. 8-32.

103. McNab, F., K. Mayer-Barber, A. Sher, A. Wack, and A. O'Garra, *Type I interferons in infectious disease.* Nat Rev Immunol, 2015. **15**(2): p. 87-103.

104. Gonzalez-Navajas, J.M., J. Lee, M. David, and E. Raz, *Immunomodulatory functions of type I interferons.* Nat Rev Immunol, 2012. **12**(2): p. 125-35.

105. Rauch, I., M. Muller, and T. Decker, *The regulation of inflammation by interferons and their STATs.* Jakstat, 2013. **2**(1): p. e23820.

106. Sueltenfuss, E.A. and M. Pollard, *Cytochemical Assay of Interferon Produced by Duck Hepatitis Virus.* Science, 1963. **139**(3555): p. 595-6.

107. de la Maza, L.M., E.M. Peterson, J.M. Goebel, C.W. Fennie, and C.W. Czarniecki, *Interferon-induced inhibition of Chlamydia trachomatis: dissociation from antiviral and antiproliferative effects.* Infect Immun, 1985. **47**(3): p. 719-22.

108. Mancuso, G., A. Midiri, C. Biondo, C. Beninati, S. Zummo, R. Galbo, F. Tomasello, M. Gambuzza, G. Macri, A. Ruggeri, T. Leanderson, and G. Teti, *Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria.* J Immunol, 2007. **178**(5): p. 3126-33.

109. Naujoks, J., C. Tabeling, B.D. Dill, C. Hoffmann, A.S. Brown, M. Kunze, S. Kempa, A. Peter, H.J. Mollenkopf, A. Dorhoi, O. Kershaw, A.D. Gruber, L.E. Sander, M. Witzenrath, S. Herold, A. Nerlich, A.C. Hocke, I. van Driel, N. Suttorp, S. Bedoui, H. Hilbi, M. Trost, and B. Opitz, *IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid.* PLoS Pathog, 2016. **12**(2): p. e1005408.

110. Boxx, G.M. and G. Cheng, *The Roles of Type I Interferon in Bacterial Infection.* Cell Host Microbe, 2016. **19**(6): p. 760-9.

111. O'Connell, R.M., S.K. Saha, S.A. Vaidya, K.W. Bruhn, G.A. Miranda, B. Zarnegar, A.K. Perry, B.O. Nguyen, T.F. Lane, T. Taniguchi, J.F. Miller, and G. Cheng, *Type I interferon production enhances susceptibility to Listeria monocytogenes infection.* J Exp Med, 2004. **200**(4): p. 437-45.

112. Stanley, S.A., J.E. Johndrow, P. Manzanillo, and J.S. Cox, *The Type I IFN response to infection with Mycobacterium tuberculosis requires ESX-1-mediated secretion and contributes to pathogenesis.* J Immunol, 2007. **178**(5): p. 3143-52.

113. Berry, M.P., C.M. Graham, F.W. McNab, Z. Xu, S.A. Bloch, T. Oni, K.A. Wilkinson, R. Banchereau, J. Skinner, R.J. Wilkinson, C. Quinn, D. Blankenship, R. Dhawan, J.J. Cush, A. Mejias, O. Ramilo, O.M. Kon, V. Pascual, J. Banchereau, D. Chaussabel, and A. O'Garra, *An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis.* Nature, 2010. **466**(7309): p. 973-7.

114. Iwasaki, A., E.F. Foxman, and R.D. Molony, *Early local immune defences in the respiratory tract.* Nat Rev Immunol, 2017. **17**(1): p. 7-20.

115. Whitsett, J.A. and T. Alenghat, *Respiratory epithelial cells orchestrate pulmonary innate immunity.* Nat Immunol, 2015. **16**(1): p. 27-35.

116. Opitz, B., V. van Laak, J. Eitel, and N. Suttorp, *Innate immune recognition in infectious and noninfectious diseases of the lung.* Am J Respir Crit Care Med, 2010. **181**(12): p. 1294-309.

117. Tengroth, L., C.R. Millrud, A.M. Kvarnhammar, S. Kumlien Georen, L. Latif, and L.O. Cardell, *Functional effects of Toll-like receptor (TLR)3, 7, 9, RIG-I and MDA-5 stimulation in nasal epithelial cells.* PLoS One, 2014. **9**(6): p. e98239.

118. Wang, Q., D.R. Nagarkar, E.R. Bowman, D. Schneider, B. Gosangi, J. Lei, Y. Zhao, C.L. McHenry, R.V. Burgens, D.J. Miller, U. Sajjan, and M.B. Hershenson, *Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses.* J Immunol, 2009. **183**(11): p. 6989-97.

119. Parker, D. and A. Prince, *Innate immunity in the respiratory epithelium.* Am J Respir Cell Mol Biol, 2011. **45**(2): p. 189-201.

120. Lee, R.J. and N.A. Cohen, *Bitter and sweet taste receptors in the respiratory epithelium in health and disease.* J Mol Med (Berl), 2014. **92**(12): p. 1235-44.

121. Ma, Z. and B. Damania, *The cGAS-STING Defense Pathway and Its Counteraction by Viruses.* Cell Host Microbe, 2016. **19**(2): p. 150-8.

122. Holm, C.K., S.H. Rahbek, H.H. Gad, R.O. Bak, M.R. Jakobsen, Z. Jiang, A.L. Hansen, S.K. Jensen, C. Sun, M.K. Thomsen, A. Laustsen, C.G. Nielsen, K. Severinsen, Y. Xiong, D.L. Burdette, V. Hornung, R.J. Lebbink, M. Duch, K.A. Fitzgerald, S. Bahrami, J.G. Mikkelsen, R. Hartmann, and S.R. Paludan, *Influenza A virus targets a cGAS-independent STING pathway that controls enveloped RNA viruses.* Nat Commun, 2016. **7**: p. 10680.

123. Lam, E., S. Stein, and E. Falck-Pedersen, *Adenovirus detection by the cGAS/STING/TBK1 DNA sensing cascade.* J Virol, 2014. **88**(2): p. 974-81.

124. Marinho, F.V., S. Benmerzoug, S.C. Oliveira, B. Ryffel, and V.F.J. Quesniaux, *The Emerging Roles of STING in Bacterial Infections.* Trends Microbiol, 2017. **25**(11): p. 906-918.

125. Watson, R.O., S.L. Bell, D.A. MacDuff, J.M. Kimmey, E.J. Diner, J. Olivas, R.E. Vance, C.L. Stallings, H.W. Virgin, and J.S. Cox, *The Cytosolic Sensor cGAS Detects Mycobacterium tuberculosis DNA to Induce Type I Interferons and Activate Autophagy.* Cell Host Microbe, 2015. **17**(6): p. 811-819.

126. Hartl, D., R. Tirouvanziam, J. Laval, C.M. Greene, D. Habiel, L. Sharma, A.O. Yildirim, C.S. Dela Cruz, and C.M. Hogaboam, *Innate Immunity of the Lung: From Basic Mechanisms to Translational Medicine.* J Innate Immun, 2018.

127. Makris, S., M. Paulsen, and C. Johansson, *Type I Interferons as Regulators of Lung Inflammation.* Front Immunol, 2017. **8**: p. 259.

128. Carlo, T. and B.D. Levy, *Molecular circuits of resolution in airway inflammation.* ScientificWorldJournal, 2010. **10**: p. 1386-99.

129. Archer, K.A. and C.R. Roy, *MyD88-dependent responses involving toll-like receptor 2 are important for protection and clearance of Legionella pneumophila in a mouse model of Legionnaires' disease.* Infect Immun, 2006. **74**(6): p. 3325-33.

130. Hayashi, F., K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, and A. Aderem, *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.* Nature, 2001. **410**(6832): p. 1099-103.

131. Newton, C.A., I. Perkins, R.H. Widen, H. Friedman, and T.W. Klein, *Role of Toll-like receptor 9 in Legionella pneumophila-induced interleukin-12 p40 production in bone marrow-derived dendritic cells and macrophages from permissive and nonpermissive mice.* Infect Immun, 2007. **75**(1): p. 146-51.

132. Archer, K.A., L. Alexopoulou, R.A. Flavell, and C.R. Roy, *Multiple MyD88-dependent responses contribute to pulmonary clearance of Legionella pneumophila.* Cell Microbiol, 2009. **11**(1): p. 21-36.

133. Casson, C.N., J.L. Doerner, A.M. Copenhaver, J. Ramirez, A.M. Holmgren, M.A. Boyer, I.J. Siddarthan, S.H. Rouhanifard, A. Raj, and S. Shin, *Neutrophils and Ly6Chi monocytes collaborate in generating an optimal cytokine response that protects against pulmonary Legionella pneumophila infection.* PLoS Pathog, 2017. **13**(4): p. e1006309.

134. Frutuoso, M.S., J.I. Hori, M.S. Pereira, D.S. Junior, F. Sonego, K.S. Kobayashi, R.A. Flavell, F.Q. Cunha, and D.S. Zamboni, *The pattern recognition receptors Nod1 and Nod2 account for neutrophil recruitment to the lungs of mice infected with Legionella pneumophila.* Microbes Infect, 2010. **12**(11): p. 819-27.

135. Molofsky, A.B., B.G. Byrne, N.N. Whitfield, C.A. Madigan, E.T. Fuse, K. Tateda, and M.S. Swanson, *Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection.* J Exp Med, 2006. **203**(4): p. 1093-104.

136. Lightfield, K.L., J. Persson, N.J. Trinidad, S.W. Brubaker, E.M. Kofoed, J.D. Sauer, E.A. Dunipace, S.E. Warren, E.A. Miao, and R.E. Vance, *Differential requirements for NAIP5 in activation of the NLRC4 inflammasome.* Infect Immun, 2011. **79**(4): p. 1606-14.

137. Amer, A., L. Franchi, T.D. Kanneganti, M. Body-Malapel, N. Ozoren, G. Brady, S. Meshinchi, R. Jagirdar, A. Gewirtz, S. Akira, and G. Nunez, *Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf.* J Biol Chem, 2006. **281**(46): p. 35217-23.

138. Case, C.L., L.J. Kohler, J.B. Lima, T. Strowig, M.R. de Zoete, R.A. Flavell, D.S. Zamboni, and C.R. Roy, *Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. 1851-6.

139. Casson, C.N., A.M. Copenhaver, E.E. Zwack, H.T. Nguyen, T. Strowig, B. Javdan, W.P. Bradley, T.C. Fung, R.A. Flavell, I.E. Brodsky, and S. Shin, *Caspase-11 activation in response to bacterial secretion systems that access the host cytosol.* PLoS Pathog, 2013. **9**(6): p. e1003400.

140. Lippmann, J., H.C. Muller, J. Naujoks, C. Tabeling, S. Shin, M. Witzenrath, K. Hellwig, C.J. Kirschning, G.A. Taylor, W. Barchet, S. Bauer, N. Suttorp, C.R. Roy, and B. Opitz, *Dissection of a type I interferon pathway in controlling bacterial intracellular infection in mice.* Cell Microbiol, 2011. **13**(11): p. 1668-82.

141. Opitz, B., M. Vinzing, V. van Laak, B. Schmeck, G. Heine, S. Gunther, R. Preissner, H. Slevogt, P.D. N'Guessan, J. Eitel, T. Goldmann, A. Flieger, N. Suttorp, and S. Hippenstiel, *Legionella pneumophila induces IFNbeta in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication.* J Biol Chem, 2006. **281**(47): p. 36173-9.

142. Shinozawa, Y., T. Matsumoto, K. Uchida, S. Tsujimoto, Y. Iwakura, and K. Yamaguchi, *Role of interferon-gamma in inflammatory responses in murine*

respiratory infection with Legionella pneumophila. J Med Microbiol, 2002. **51**(3): p. 225-30.

143. Brown, A.S., C. Yang, K.Y. Fung, A. Bachem, D. Bourges, S. Bedoui, E.L. Hartland, and I.R. van Driel, *Cooperation between Monocyte-Derived Cells and Lymphoid Cells in the Acute Response to a Bacterial Lung Pathogen.* PLoS Pathog, 2016. **12**(6): p. e1005691.

144. Fujita, M., S. Ikegame, E. Harada, H. Ouchi, I. Inoshima, K. Watanabe, S. Yoshida, and Y. Nakanishi, *TNF receptor 1 and 2 contribute in different ways to resistance to Legionella pneumophila-induced mortality in mice.* Cytokine, 2008. **44**(2): p. 298-303.

145. Ziltener, P., T. Reinheckel, and A. Oxenius, *Neutrophil and Alveolar Macrophage-Mediated Innate Immune Control of Legionella pneumophila Lung Infection via TNF and ROS.* PLoS Pathog, 2016. **12**(4): p. e1005591.

146. Yoshimura, A., E. Lien, R.R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock, *Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2.* J Immunol, 1999. **163**(1): p. 1-5.

147. Albiger, B., S. Dahlberg, A. Sandgren, F. Wartha, K. Beiter, H. Katsuragi, S. Akira, S. Normark, and B. Henriques-Normark, *Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection.* Cell Microbiol, 2007. **9**(3): p. 633-44.

148. Malley, R., P. Henneke, S.C. Morse, M.J. Cieslewicz, M. Lipsitch, C.M. Thompson, E. Kurt-Jones, J.C. Paton, M.R. Wessels, and D.T. Golenbock, *Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1966-71.

149. Imai, Y., K. Kuba, G.G. Neely, R. Yaghubian-Malhami, T. Perkmann, G. van Loo, M. Ermolaeva, R. Veldhuizen, Y.H. Leung, H. Wang, H. Liu, Y. Sun, M. Pasparakis, M. Kopf, C. Mech, S. Bavari, J.S. Peiris, A.S. Slutsky, S. Akira, M. Hultqvist, R. Holmdahl, J. Nicholls, C. Jiang, C.J. Binder, and J.M. Penninger, *Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury.* Cell, 2008. **133**(2): p. 235-49.

150. Knapp, S., C.W. Wieland, C. van 't Veer, O. Takeuchi, S. Akira, S. Florquin, and T. van der Poll, *Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense.* J Immunol, 2004. **172**(5): p. 3132-8.

151. Koppe, U., N. Suttorp, and B. Opitz, *Recognition of Streptococcus pneumoniae by the innate immune system.* Cell Microbiol, 2012. **14**(4): p. 460-6.

152. Albiger, B., A. Sandgren, H. Katsuragi, U. Meyer-Hoffert, K. Beiter, F. Wartha, M. Hornef, S. Normark, and B.H. Normark, *Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice.* Cell Microbiol, 2005. **7**(11): p. 1603-15.

153. Davis, K.M., S. Nakamura, and J.N. Weiser, *Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of S. pneumoniae colonization in mice.* J Clin Invest, 2011. **121**(9): p. 3666-76.

154. Witzenrath, M., F. Pache, D. Lorenz, U. Koppe, B. Gutbier, C. Tabeling, K. Reppe, K. Meixenberger, A. Dorhoi, J. Ma, A. Holmes, G. Trendelenburg, M.M. Heimesaat, S. Bereswill, M. van der Linden, J. Tschopp, T.J. Mitchell, N. Suttorp, and B. Opitz, *The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia.* J Immunol, 2011. **187**(1): p. 434-40.

155. McNeela, E.A., A. Burke, D.R. Neill, C. Baxter, V.E. Fernandes, D. Ferreira, S. Smeaton, R. El-Rachkidy, R.M. McLoughlin, A. Mori, B. Moran, K.A. Fitzgerald, J. Tschopp, V. Petrilli, P.W. Andrew, A. Kadioglu, and E.C. Lavelle, *Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4.* PLoS Pathog, 2010. **6**(11): p. e1001191.

156. Fang, R., K. Tsuchiya, I. Kawamura, Y. Shen, H. Hara, S. Sakai, T. Yamamoto, T. Fernandes-Alnemri, R. Yang, E. Hernandez-Cuellar, S.R. Dewamitta, Y. Xu, H. Qu, E.S. Alnemri, and M. Mitsuyama, *Critical roles of ASC inflammasomes in caspase-1 activation and host innate resistance to Streptococcus pneumoniae infection.* J Immunol, 2011. **187**(9): p. 4890-9.

157. Kostadinova, E., C. Chaput, B. Gutbier, J. Lippmann, L.E. Sander, T.J. Mitchell, N. Suttorp, M. Witzenrath, and B. Opitz, *NLRP3 protects alveolar barrier integrity by an inflammasome-independent increase of epithelial cell adherence.* Sci Rep, 2016. **6**: p. 30943.

158. Koppel, E.A., C.W. Wieland, V.C. van den Berg, M. Litjens, S. Florquin, Y. van Kooyk, T. van der Poll, and T.B. Geijtenbeek, *Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary Streptococcus pneumoniae infection.* Eur J Immunol, 2005. **35**(10): p. 2962-9.

159. Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason, and L. Kobzik, *The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles.* J Exp Med, 2004. **200**(2): p. 267-72.

160. Rabes, A., S. Zimmermann, K. Reppe, R. Lang, P.H. Seeberger, N. Suttorp, M. Witzenrath, B. Lepenies, and B. Opitz, *The C-type lectin receptor Mincle binds to Streptococcus pneumoniae but plays a limited role in the anti-pneumococcal innate immune response.* PLoS One, 2015. **10**(2): p. e0117022.

161. Behler-Janbeck, F., T. Takano, R. Maus, J. Stolper, D. Jonigk, M. Tort Tarres, T. Fuehner, A. Prasse, T. Welte, M.S. Timmer, B.L. Stocker, Y. Nakanishi, T. Miyamoto, S. Yamasaki, and U.A. Maus, *C-type Lectin Mincle Recognizes Glucosyl-diacylglycerol of Streptococcus pneumoniae and Plays a Protective Role in Pneumococcal Pneumonia.* PLoS Pathog, 2016. **12**(12): p. e1006038.

162. Joyce, E.A., S.J. Popper, and S. Falkow, *Streptococcus pneumoniae nasopharyngeal colonization induces type I interferons and interferon-induced gene expression.* BMC Genomics, 2009. **10**: p. 404.

163. Koppe, U., K. Hogner, J.M. Doehn, H.C. Muller, M. Witzenrath, B. Gutbier, S. Bauer, T. Pribyl, S. Hammerschmidt, J. Lohmeyer, N. Suttorp, S. Herold, and B. Opitz,

Streptococcus pneumoniae stimulates a STING- and IFN regulatory factor 3-dependent type I IFN production in macrophages, which regulates RANTES production in macrophages, cocultured alveolar epithelial cells, and mouse lungs. J Immunol, 2012. **188**(2): p. 811-7.

164. Maier, B.B., A. Hladik, K. Lakovits, A. Korosec, R. Martins, J.B. Kral, I. Mesteri, B. Strobl, M. Muller, U. Kalinke, M. Merad, and S. Knapp, *Type I interferon promotes alveolar epithelial type II cell survival during pulmonary Streptococcus pneumoniae infection and sterile lung injury in mice.* Eur J Immunol, 2016. **46**(9): p. 2175-86.

165. Hughes, C.E., R.M. Harvey, C.D. Plumptre, and J.C. Paton, *Development of primary invasive pneumococcal disease caused by serotype 1 pneumococci is driven by early increased type I interferon response in the lung.* Infect Immun, 2014. **82**(9): p. 3919-26.

166. Nakamura, S., K.M. Davis, and J.N. Weiser, *Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice.* J Clin Invest, 2011. **121**(9): p. 3657-65.

167. Casanova, J.L. and L. Abel, *The genetic theory of infectious diseases: a brief history and selected illustrations.* Annu Rev Genomics Hum Genet, 2013. **14**: p. 215-43.

168. Casanova, J.L. and L. Abel, *Human genetics of infectious diseases: a unified theory.* Embo j, 2007. **26**(4): p. 915-22.

169. Picard, C., W. Al-Herz, A. Bousfiha, J.L. Casanova, T. Chatila, M.E. Conley, C. Cunningham-Rundles, A. Etzioni, S.M. Holland, C. Klein, S. Nonoyama, H.D. Ochs, E. Oksenhendler, J.M. Puck, K.E. Sullivan, M.L. Tang, J.L. Franco, and H.B. Gaspar, *Primary Immunodeficiency Diseases: an Update on the Classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015.* J Clin Immunol, 2015. **35**(8): p. 696-726.

170. Casanova, J.L. and L. Abel, *Human genetics of infectious diseases: Unique insights into immunological redundancy.* Semin Immunol, 2018. **36**: p. 1-12.

171. Chapman, S.J. and A.V. Hill, *Human genetic susceptibility to infectious disease.* Nat Rev Genet, 2012. **13**(3): p. 175-88.

172. Pothlichet, J. and L. Quintana-Murci, *The genetics of innate immunity sensors and human disease.* Int Rev Immunol, 2013. **32**(2): p. 157-208.

173. Hawn, T.R., A. Verbon, K.D. Lettinga, L.P. Zhao, S.S. Li, R.J. Laws, S.J. Skerrett, B. Beutler, L. Schroeder, A. Nachman, A. Ozinsky, K.D. Smith, and A. Aderem, *A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease.* J Exp Med, 2003. **198**(10): p. 1563-72.

174. Misch, E.A., A. Verbon, J.M. Prins, S.J. Skerrett, and T.R. Hawn, *A TLR6 polymorphism is associated with increased risk of Legionnaires' disease.* Genes Immun, 2013. **14**(7): p. 420-6.

175. Hawn, T.R., A. Verbon, M. Janer, L.P. Zhao, B. Beutler, and A. Aderem, *Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease.* Proc Natl Acad Sci U S A, 2005. **102**(7): p. 2487-9.

176. Ku, C.L., C. Picard, M. Erdos, A. Jeurissen, J. Bustamante, A. Puel, H. von Bernuth, O. Filipe-Santos, H.H. Chang, T. Lawrence, M. Raes, L. Marodi, X. Bossuyt, and J.L. Casanova, *IRAK4 and NEMO mutations in otherwise healthy children with recurrent invasive pneumococcal disease.* J Med Genet, 2007. **44**(1): p. 16-23.

177. von Bernuth, H., C. Picard, Z. Jin, R. Pankla, H. Xiao, C.L. Ku, M. Chrabieh, I.B. Mustapha, P. Ghandil, Y. Camcioglu, J. Vasconcelos, N. Sirvent, M. Guedes, A.B. Vitor, M.J. Herrero-Mata, J.I. Arostegui, C. Rodrigo, L. Alsina, E. Ruiz-Ortiz, M. Juan, C. Fortuny, J. Yague, J. Anton, M. Pascal, H.H. Chang, L. Janniere, Y. Rose, B.Z. Garty, H. Chapel, A. Issekutz, L. Marodi, C. Rodriguez-Gallego, J. Banchereau, L. Abel, X. Li, D. Chaussabel, A. Puel, and J.L. Casanova, *Pyogenic bacterial infections in humans with MyD88 deficiency.* Science, 2008. **321**(5889): p. 691-6.

178. Chapman, S.J., C.C. Khor, F.O. Vannberg, A. Rautanen, S. Segal, C.E. Moore, R.J. Davies, N.P. Day, N. Peshu, D.W. Crook, J.A. Berkley, T.N. Williams, J.A. Scott, and A.V. Hill, *NFKBIZ polymorphisms and susceptibility to pneumococcal disease in European and African populations.* Genes Immun, 2010. **11**(4): p. 319-25.

179. Brouwer, M.C., J. de Gans, S.G. Heckenberg, A.H. Zwinderman, T. van der Poll, and D. van de Beek, *Host genetic susceptibility to pneumococcal and meningococcal disease: a systematic review and meta-analysis.* Lancet Infect Dis, 2009. **9**(1): p. 31-44.

180. Khor, C.C., S.J. Chapman, F.O. Vannberg, A. Dunne, C. Murphy, E.Y. Ling, A.J. Frodsham, A.J. Walley, O. Kyrieleis, A. Khan, C. Aucan, S. Segal, C.E. Moore, K. Knox, S.J. Campbell, C. Lienhardt, A. Scott, P. Aaby, O.Y. Sow, R.T. Grignani, J. Sillah, G. Sirugo, N. Peshu, T.N. Williams, K. Maitland, R.J. Davies, D.P. Kwiatkowski, N.P. Day, D. Yala, D.W. Crook, K. Marsh, J.A. Berkley, L.A. O'Neill, and A.V. Hill, *A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis.* Nat Genet, 2007. **39**(4): p. 523-8.

181. Liu, Y.*, et al.*, *Activated STING in a vascular and pulmonary syndrome.* N Engl J Med, 2014. **371**(6): p. 507-518.

182. Konig, N., C. Fiehn, C. Wolf, M. Schuster, E. Cura Costa, V. Tungler, H.A. Alvarez, O. Chara, K. Engel, R. Goldbach-Mansky, C. Gunther, and M.A. Lee-Kirsch, *Familial chilblain lupus due to a gain-of-function mutation in STING.* Ann Rheum Dis, 2017. **76**(2): p. 468-472.

183. Jin, L., L.G. Xu, I.V. Yang, E.J. Davidson, D.A. Schwartz, M.M. Wurfel, and J.C. Cambier, *Identification and characterization of a loss-of-function human MPYS variant.* Genes Immun, 2011. **12**(4): p. 263-9.

184. Yi, G., V.P. Brendel, C. Shu, P. Li, S. Palanathan, and C. Cheng Kao, *Single nucleotide polymorphisms of human STING can affect innate immune response to cyclic dinucleotides.* PLoS One, 2013. **8**(10): p. e77846.

185. Patel, S. and L. Jin, *TMEM173 variants and potential importance to human biology and disease.* Genes Immun, 2018.

186. Patel, S., S.M. Blaauboer, H.R. Tucker, S. Mansouri, J.S. Ruiz-Moreno, L. Hamann, R.R. Schumann, B. Opitz, and L. Jin, *The Common R71H-G230A-R293Q Human TMEM173 Is a Null Allele.* J Immunol, 2017. **198**(2): p. 776-787.

187. Diner, E.J., D.L. Burdette, S.C. Wilson, K.M. Monroe, C.A. Kellenberger, M. Hyodo, Y. Hayakawa, M.C. Hammond, and R.E. Vance, *The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING.* Cell Rep, 2013. **3**(5): p. 1355-61.

188. Xiao, D., W. Huang, M. Ou, C. Guo, X. Ye, Y. Liu, M. Wang, B. Zhang, N. Zhang, S. Huang, J. Zang, Z. Zhou, Z. Wen, C. Zeng, C. Wu, C. Huang, X. Wei, G. Yang, and C. Jing, *Interaction between susceptibility loci in cGAS-STING pathway, MHC gene and HPV infection on the risk of cervical precancerous lesions in Chinese population.* Oncotarget, 2016. **7**(51): p. 84228-84238.

189. Isaacs, A. and J. Lindenmann, *Virus interference. I. The interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 258-67.

190. Stetson, D.B. and R. Medzhitov, *Recognition of cytosolic DNA activates an IRF3-dependent innate immune response.* Immunity, 2006. **24**(1): p. 93-103.

191. Parker, D., F.J. Martin, G. Soong, B.S. Harfenist, J.L. Aguilar, A.J. Ratner, K.A. Fitzgerald, C. Schindler, and A. Prince, *Streptococcus pneumoniae DNA initiates type I interferon signaling in the respiratory tract.* MBio, 2011. **2**(3): p. e00016-11.

192. Monroe, K.M., S.M. McWhirter, and R.E. Vance, *Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to Legionella pneumophila.* PLoS Pathog, 2009. **5**(11): p. e1000665.

193. Creasey, E.A. and R.R. Isberg, *The protein SdhA maintains the integrity of the Legionella-containing vacuole.* Proc Natl Acad Sci U S A, 2012. **109**(9): p. 3481-6.

194. Bai, Y., J. Yang, L.E. Eisele, A.J. Underwood, B.J. Koestler, C.M. Waters, D.W. Metzger, and G. Bai, *Two DHH subfamily 1 proteins in Streptococcus pneumoniae possess cyclic di-AMP phosphodiesterase activity and affect bacterial growth and virulence.* J Bacteriol, 2013. **195**(22): p. 5123-32.

195. Woodward, J.J., A.T. Iavarone, and D.A. Portnoy, *c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response.* Science, 2010. **328**(5986): p. 1703-5.

196. Levi, A., M. Folcher, U. Jenal, and H.A. Shuman, *Cyclic diguanylate signaling proteins control intracellular growth of Legionella pneumophila.* MBio, 2011. **2**(1): p. e00316-10.

197. Abdul-Sater, A.A., A. Grajkowski, H. Erdjument-Bromage, C. Plumlee, A. Levi, M.T. Schreiber, C. Lee, H. Shuman, S.L. Beaucage, and C. Schindler, *The overlapping host responses to bacterial cyclic dinucleotides.* Microbes Infect, 2012. **14**(2): p. 188-97.

198. Segal, G., M. Purcell, and H.A. Shuman, *Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the Legionella pneumophila genome.* Proc Natl Acad Sci U S A, 1998. **95**(4): p. 1669-74.

199. Vogel, J.P., H.L. Andrews, S.K. Wong, and R.R. Isberg, *Conjugative transfer by the virulence system of Legionella pneumophila.* Science, 1998. **279**(5352): p. 873-6.

200. Copenhaver, A.M., C.N. Casson, H.T. Nguyen, M.M. Duda, and S. Shin, *IL-1R signaling enables bystander cells to overcome bacterial blockade of host protein synthesis.* Proc Natl Acad Sci U S A, 2015. **112**(24): p. 7557-62.

201. Asrat, S., A.S. Dugan, and R.R. Isberg, *The frustrated host response to Legionella pneumophila is bypassed by MyD88-dependent translation of pro-inflammatory cytokines.* PLoS Pathog, 2014. **10**(7): p. e1004229.

202. Xu, F., D. Droemann, J. Rupp, H. Shen, X. Wu, T. Goldmann, S. Hippenstiel, P. Zabel, and K. Dalhoff, *Modulation of the inflammatory response to Streptococcus pneumoniae in a model of acute lung tissue infection.* Am J Respir Cell Mol Biol, 2008. **39**(5): p. 522-9.

203. Periselneris, J., R.J. José, and J. Brown, *Targeting Inflammatory Responses to Streptococcus pneumoniae.* New Horizons in Translational Medicine, 2015. **2**(6): p. 167-174.

204. Schoenborn, J.R. and C.B. Wilson, *Regulation of interferon-gamma during innate and adaptive immune responses.* Adv Immunol, 2007. **96**: p. 41-101.

205. Plumlee, C.R., C. Lee, A.A. Beg, T. Decker, H.A. Shuman, and C. Schindler, *Interferons direct an effective innate response to Legionella pneumophila infection.* J Biol Chem, 2009. **284**(44): p. 30058-66.

206. Wright, E.K., S.A. Goodart, J.D. Growney, V. Hadinoto, M.G. Endrizzi, E.M. Long, K. Sadigh, A.L. Abney, I. Bernstein-Hanley, and W.F. Dietrich, *Naip5 affects host susceptibility to the intracellular pathogen Legionella pneumophila.* Curr Biol, 2003. **13**(1): p. 27-36.

207. LeMessurier, K.S., H. Hacker, L. Chi, E. Tuomanen, and V. Redecke, *Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung.* PLoS Pathog, 2013. **9**(11): p. e1003727.

208. Chen, H., H. Sun, F. You, W. Sun, X. Zhou, L. Chen, J. Yang, Y. Wang, H. Tang, Y. Guan, W. Xia, J. Gu, H. Ishikawa, D. Gutman, G. Barber, Z. Qin, and Z. Jiang, *Activation of STAT6 by STING is critical for antiviral innate immunity.* Cell, 2011. **147**(2): p. 436-46.

209. Fu, J., D.B. Kanne, M. Leong, L.H. Glickman, S.M. McWhirter, E. Lemmens, K. Mechette, J.J. Leong, P. Lauer, W. Liu, K.E. Sivick, Q. Zeng, K.C. Soares, L. Zheng, D.A. Portnoy, J.J. Woodward, D.M. Pardoll, T.W. Dubensky, Jr., and Y. Kim, *STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade.* Sci Transl Med, 2015. **7**(283): p. 283ra52.

210. Sharma, S., R.B. DeOliveira, P. Kalantari, P. Parroche, N. Goutagny, Z. Jiang, J. Chan, D.C. Bartholomeu, F. Lauw, J.P. Hall, G.N. Barber, R.T. Gazzinelli, K.A. Fitzgerald, and D.T. Golenbock, *Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome.* Immunity, 2011. **35**(2): p. 194-207.

211. Zeng, M., Z. Hu, X. Shi, X. Li, X. Zhan, X.D. Li, J. Wang, J.H. Choi, K.W. Wang, T. Purrington, M. Tang, M. Fina, R.J. DeBerardinis, E.M. Moresco, G. Pedersen, G.M. McInerney, G.B. Karlsson Hedestam, Z.J. Chen, and B. Beutler, *MAVS, cGAS, and endogenous retroviruses in T-independent B cell responses.* Science, 2014. **346**(6216): p. 1486-92.

212. Liang, Q., G.J. Seo, Y.J. Choi, M.J. Kwak, J. Ge, M.A. Rodgers, M. Shi, B.J. Leslie, K.P. Hopfner, T. Ha, B.H. Oh, and J.U. Jung, *Crosstalk between the cGAS DNA sensor and Beclin-1 autophagy protein shapes innate antimicrobial immune responses.* Cell Host Microbe, 2014. **15**(2): p. 228-38.

213. Tang, C.H., J.A. Zundell, S. Ranatunga, C. Lin, Y. Nefedova, J.R. Del Valle, and C.C. Hu, *Agonist-Mediated Activation of STING Induces Apoptosis in Malignant B Cells.* Cancer Res, 2016. **76**(8): p. 2137-52.

214. Zou, R., G. Zhang, S. Li, W. Wang, J. Yuan, J. Li, Y. Wang, Y. Lin, Y. Deng, B. Zhou, G.F. Gao, and Y. Liu, *A functional polymorphism in IFNAR1 gene is associated with susceptibility and severity of HFMD with EV71 infection.* Sci Rep, 2015. **5**: p. 18541.

8. Funding sources

This work was financially supported by the *Deutsche Forschungsgemeinschaft* (DFG), through the GRK1673 and the SFB/TR84 projects and the grant SPP1580/OP86/10-1.
No conflict of interests arose through the financial support of this research work.

9. Appendix

The next pages contain the declaration of consent that was provided to each and all volunteers who donated DNA, following the guidelines of the ethics committee of the Charité Universitätsmedizin Berlin. In addition to a small interview in which volunteers were informed about the nature of the study and the intended use of the DNA samples, an information leaflet was offered in which the background and aims of the study were adequately covered. The declarations of consent as well as the information leaflets were made available in German or English depending on the native language of the volunteer. Both versions of these documents are reproduced here.

9.1 Einverständniserklärung

Einverständniserklärung, Version 15. Januar 2016

Einverständniserklärung

Untersuchung des Einflusses von Punktmutationen in STING und funktionell verwandten Genen der angeborenen Immunität auf die Erkennung bakterieller Pneumonieerreger

Name, Vorname, Geb.-datum

KENNCODE _____
(auszufüllen durch Studienleitung)

Hiermit erkläre ich,

Name: Inititiale:

Geburtsdatum: Geschlecht:

KENNCODE _____
(auszufüllen durch Studienleitung)

dass ich durch Herrn/Frau Dr._____
Name des/der Studienarzt/Studienärztin, Adresse

Einverständniserklärung, Version 15. Januar 2016

mündlich und schriftlich über das Wesen, die Bedeutung, Tragweite und Risiken der wissenschaftlichen Untersuchung im Rahmen der o.g. Studie informiert wurde und ausreichend Gelegenheit hatte, meine Fragen hierzu in einem Gespräch mit dem/der Studienarzt/Studienärztin zu klären.

Ich habe insbesondere die mir vorgelegte Information verstanden und eine Ausfertigung derselben und dieser Einwilligungserklärung erhalten.

Mir ist bekannt, dass ich meine Einwilligung jederzeit ohne Angabe von Gründen und ohne nachteilige Folgen für mich zurückziehen und einer Weiterverarbeitung meiner Daten und Proben jederzeit widersprechen und ihre Löschung bzw. Vernichtung verlangen kann.

Ich bin bereit, an der wissenschaftlichen Untersuchung im Rahmen der o.g. Studie teilzunehmen.

Ich erkläre mich damit einverstanden, dass im Rahmen dieser Studie erhobene Daten/Angaben verschlüsselt (pseudonymisiert) in Papierform und auf elektronischen Datenträgern aufgezeichnet und verarbeitet, sowie die anonymisierten Studienergebnisse veröffentlicht werden.

Darüber hinaus bin ich mit der Entnahme, Verschlüsselung, Untersuchung sowie Lagerung meines im Rahmen dieser klinischen Studie entnommenen Wangenabstrichs, sowie ggf. des Blutes und der hieraus entnommenen Materialien für den Zweck der Studie durch den/die Studienarzt/-Studienärztin einverstanden. Ich erkläre ausdrücklich, dass ich mit der Entnahme und der Untersuchung der genetischen Materialien einverstanden bin.

_____ _____

Unterschrift des Patienten Datum

_____

Name des Patienten in Druckbuchstaben

Arzt/Ärztin, welche(r) die Einwilligung einholt

Hiermit erkläre ich, den/die o.g. Versuchsteilnehmer/in am ..... über Wesen, Bedeutung, Tragweite und Risiken der o.g. Studie mündlich und schriftlich aufgeklärt und ihm/ihr eine Ausfertigung der Information sowie dieser Einwilligungserklärung übergeben zu haben.

_____ _____

Unterschrift Datum

_____

Name in Druckbuchstaben

_____

Unterschrift Studienleitung

9.2 Declaration of consent

Declaration of Consent

Evaluation of the influence of point mutations in STING and functionally related genes of the innate immune system on recognition of pneumonia-associated pathogens

Surname, first name, date of birth

PASSCODE _____
(Field filled out by principal investigator)

Hereby I declare,

Name: Initials:

Date of birth: Gender:

PASSCODE _____
(Field filled out by principal investigator)

that I by means of Dr._____
Name of the Study doctor, Address

Declaration of Consent

received oral and written information about the nature, the significance, implications and risks of the scientific analyses in the context of the above mentioned study and that I had sufficient opportunity to clarify my questions about this in an interview with the investigator.

I have understood the information presented to me, and I received a copy of this consent form

I am aware that I can withdraw my consent at any time without giving reasons and without adverse consequences for me and that I can disagree with a further processing of my personal data and samples at any time and can demand its deletion or destruction.

I am willing to adhere to the scientific analyses in the framework of the study mentioned above.

I hereby agree that in the framework of this study any collected data about me will be encrypted (pseudonymized), and recorded on an electronic medium. Likewise, I agree that the pseudonymized data will be processed, transmitted, if necessary to other investigators in Charité and published in an anonymous form.

Moreover I agree with the extraction, encryption, analysis and storage of my blood taken in the context of this clinical study as well as any component extracted from it by means of the study doctor, for the purpose of this study.

_____ _____
Signature of Volunteer Date

_____
Name of the volunteer in block capitals

Doctor who obtains the consent

I hereby declare that the study participant was informed in oral and in writing form on ..... about the nature, significance, implications and risks of the above mentioned study; and that a copy of the information and of this declaration of consent was handed over to him/her.

_____ _____
Signature Date

_____
Name in block capitals

_____
Signature of principal investigator

9.3 Probandeninformation

Probandeninformation, Version 1 vom 15. Januar 2016

Probandeninformation

Untersuchung des Einflusses von Punktmutationen in STING und funktionell verwandter Gene der angeborenen Immunität auf die Erkennung bakterieller Pneumonieerreger

Studienleitung (Studienärzte):

Prof. Dr. med. Ralf R. Schumann
Institut für Mikrobiologie und Hygiene
Charité Universitätsmedizin Berlin
Helmut-Ruska-Haus
Rahel-Hirsch-Weg 3
10117 Berlin

Prof. Dr. med. Bastian Opitz
Medizinische Klinik m.S. Infektiologie und Pneumologie
Charité Universitätsmedizin Berlin
Augustenburger Platz 1
13353 Berlin

Probandeninformation, Version 1 vom 15. Januar 2016

Liebe Interessentin, lieber Interessent,

wir führen derzeit eine wissenschaftliche Untersuchung an isolierten Blutzellen von freiwilligen gesunden Probanden durch. Ziel dieser Untersuchungen ist es, den Einfluss von genetischen Variationen in Genen des Immunsystems auf die antibakterielle Abwehr gegen Erreger von Lungenentzündungen zu untersuchen.

Krankheitserreger, die in den Körper eingedrungen sind, werden durch Fühler (Rezeptoren) auf weißen Blutkörperchen erkannt, wodurch eine Bekämpfung dieser Krankheitserreger durch das Immunsystem eingeleitet wird. STING ist ein Molekül, das in verschiedenen Blutzellen vorhanden ist und an der Erkennung von Krankheitserregern mitwirkt. Es ist bekannt, dass STING in verschiedenen Formen vorkommen kann, die auf kleinen Unterschieden im Erbgut beruhen. Wir vermuten, dass diese verschiedenen Formen sich in ihrer Fähigkeit, einzelne Krankheitserreger zu erkennen, voneinander unterscheiden. Nach heutigem Wissensstand haben die Genvarianten in STING bzw. in funktionell verwandten Genen der angeborenen Immunität jedoch keinen Krankheitswert.

Ihre Einwilligung vorausgesetzt, möchten wir etwas Erbgut aus Ihrer Wangenschleimhaut mit Hilfe eines Abstrichs isolieren. Nachfolgender sollen nur die kleinen Teile Ihres Erbguts untersucht werden, die die Information für STING bzw. ggf. weiterer funktionell verwandter Moleküle des Immunsystems enthalten. Diese Untersuchungen werden im Labor des Instituts für Mikrobiologie der Charité-Universitätsmedizin Berlin unter Leitung von Herrn Prof. Schumann durchgeführt. Zugriff auf die Proben haben dabei lediglich Prof. Schumann, Prof. Opitz, sowie die Wissenschaftler Dr. Lutz Hamann und Juan Sebastian Ruiz-Moreno, M. Sc. Alle Proben werden „pseudonymisiert“, das heißt, dass die persönliche Informatiuonen durch eine Zahl ersetzt wird und niemand außer den Studienärzten die Identität des Spenders erkennen kann. Nach spätestens 10 Jahren werden alle Proben vernichtet. Ggf. ist in einem zweiten Schritt geplant, einmalig ca. 50 ml Blut zu entnehmen, um periphere weiße Blutzellen zu isolieren. Hierzu würden wir Sie separat anschreiben, die Blutentnahme würde dann wiederum im Labor des Instituts für Mikrobiologie der Charité-Universitätsmedizin Berlin unter Leitung von Herrn Prof. Schumann durchgeführt Nachfolgend soll im Labor getestet werden, wie gut die Blutzellen in der Lage sind, Bakterien oder bakterielle Bestandteile zu erkennen und eine Immunantwort auszulösen.

Im Rahmen dieser Untersuchungen werden personenbezogene Daten verarbeitet. Diese Daten, wie Name, Vorname, Geburtsjahr, Geschlecht, Kontaktinformationen, werden erhoben und auf der Einverständniserklärung verzeichnet. Wie oben erwähnt, werden diese Informationen dann über einen Kenncode verschlüsselt, das heißt Ihre Probe wird mit einer Zahl versehen, die elektronisch gespeichert wird. Die Pseudonymisierung und Zuordnung zu den persönlichen Daten erfolgt ausschließlich durch die Studienärzte Prof. Schumann und Prof. Opitz. Es ist beabsichtigt, die Ergebnisse der Studie anonym, d.h. ohne Angabe von Daten, die einen Rückschluss auf Ihre Person zulassen, zu veröffentlichen. Die Daten werden nach 10 Jahren gesetzlicher Lagerfrist gelöscht bzw. vernichtet. Sie können jederzeit einer Weiterverarbeitung Ihrer Daten widersprechen. Weder Ihnen, noch den Krankenkassen oder dem Krankenhaus entstehen durch unsere Studie zusätzliche Kosten. Zwar ist, so wie für andere klinische Studien, für diese Studie keine gesonderte Versicherung abgeschlossen worden, jedoch ist Ihr Prüfarzt durch die Betriebshaftpflichtversicherung gegen schuldhaftes Versagen versichert. Wir können Ihnen keinen (für Wangenabstrich) bzw. nur einen kleinen (für die ggf. durchgeführte Blutentnahme) finanziellen Ausgleich für die Teilnahme an der Studie geben. Allerdings können die Ergebnisse der Studie in der Zukunft anderen Patienten helfen. Falls Sie es beantragen, kann Ihnen das Ergebnis der genetischen Untersuchung erläutert werden. Zurzeit ist nicht zu erwarten, dass diese Ergebnisse eine unmittelbare Auswirkung auf die Gesundheit haben. Die Teilnahme an der Studie ist selbstverständlich freiwillig. Sie können jederzeit Ihre Teilnahmeerklärung zurückziehen. Bitte informieren Sie in diesem Fall einen der Studienärzte oder ihre Vertreter. Wie immer Sie sich entscheiden, es wird Ihnen daraus keinerlei Nachteil erwachsen.

Bitte lesen Sie dieses Dokument sorgfältig. Wenn Sie irgendwelche Fragen haben, so wenden Sie sich bitte an den aufklärenden Arzt oder an einen der Studienärzte. Die Adressen und Telefonnummern entnehmen Sie bitte diesem Aufklärungsbogen. Sie erhalten für Ihre Akten eine Kopie des Dokumentes.

Vielen Dank!

Prof. Dr. med. Ralf R. Schumann, Studienleitung

Prof. Dr. med. B. Opitz, Studienleitung

9.4 Information for volunteers

Information for volunteers

Evaluation of the influence of point mutations in STING and functionally related genes on the innate immune response, upon recognition of pneumonia-associated pathogens

Medical doctors involved in the study:

Prof. Dr. med. Ralf R. Schumann
Institut für Mikrobiologie und Hygiene
Charité Universitätsmedizin Berlin
Helmut-Ruska-Haus
Rahel-Hirsch-Weg 3
10117 Berlin

Prof. Dr. med. Bastian Opitz
Medizinische Klinik m.S. Infektiologie und Pneumologie
Charité Universitätsmedizin Berlin
Augustenburger Platz 1
13353 Berlin

Information for volunteers

Dear Sir or Madam,

We are currently conducting a scientific study with isolated blood cells from healthy volunteers. The aim of this study is to assess the influence of genetic variations in genes from the immune system on the antibacterial response against pneumonia-associated pathogens.

Bacteria and viruses that gain access in the body are recognized trough different sensors (receptors) found on white blood cells, through which an immune response against those pathogens is initiated. STING is a molecule, which is present in different blood cells and is involved in the recognition of several microbes.

It is known that STING may occur in different forms, which are originated from small differences in the genome. We hypothesize that those forms differ from each other in their ability to recognize certain microbes.

With your consent, we would like to isolate genetic material from your buccal mucosa with the aid of a swab. Subsequently, only a small portion of your genetic material, containing the information for STING and if necessary, other related immune molecules, will be analyzed.

Some proband will additionally been asked to donate approx. 50 ml blood in a second step. This blood will be used to isolate peripheral white blood cells. The cells will be afterwards tested in the laboratory for their capacity of recognizing bacteria or bacterial components and triggering an immune response.

In the frame of this study, personal data will be processed. Data such as surname, name, date of birth, gender, and contact information will be collected and stored in the declaration of consent. These data will be then encrypted in a passcode. The blood sample, and the results from the experiments will be identified with this code. Results are stored electronically. It is planed that the results of the study will remain anonymous, i.e. without giving any information, which would allow a conclusion of your person to be published. After 10 years of legal storage, the data will be deleted or destroyed. The transmission of data will only take place between the investigators of the study. You can at any time object further processing of your data.

The study will not cause any additional cost for you, your health insurance or for the hospital. Although for this study, like for other clinical studies, no individual insurance was agreed, a liability insurance against culpable failure covers the investigator. We cannot give you a financial compensation for the swab of the buccal mucosa; we will give you however, a small compensation (50 EUR) in case we further need draw blood from you. Nevertheless, the results from this study could in the future help other patients. In case you request it, the results from the genetic analysis could be explain to you. It is currently not expected that these results could have any direct effect on your health. Participation in this study is of course voluntary. You can withdraw at any time your statement of participation. If this is the case, please inform one of the investigators or any of their representatives. Whatever your decision is, it will not cause any disadvantage to you.

Please read this document carefully. If you have any questions, please contact one of the main investigators or any of their representatives. Please refer to the address and telephone number described in this information sheet. You will receive a copy of this document for your records.

Thank you very much!

10. Publications and conferences

10.1 Scientific articles

Patel, S., Blaauboer, S.M., Tucker, H.R., Mansouri, S., **Ruiz-Moreno, J.S.**, Hamann, L., Schumann, R.R., Opitz, B. and Lei, J. *The common R71H-G230A-R293Q Human* TMEM173 *Is a Null Allele.* J Immunol, 2017. 198(2):776-787.

Patel, S., Blaauboer, S.M., Tucker, H.R., Mansouri, S., **Ruiz-Moreno, J.S.**, Hamann, L., Schumann, R.R., Opitz, B. and Lei, J. *Response to Comment on "The Common R71H-G230A-R293Q Human TMEM173 Is a Null Allele"*. J Immunol. 2017. 198(11):4185-4188

Ruiz-Moreno, J.S., Hamann, L., Sander, L.E., Puzianowska-Kuznicka, M., Cambier, J.C., Witzenrath, M., Schumann, R.R., Suttorp, N., Opitz, B. and CAPNETZ Study Group. *The cGAS-STING Pathway Detects* Streptococcus pneumoniae *but Appears Dispensable for Anti-pneumococcal Defense in Mice and Humans*. Infect Immun, 2018. 86(3):e00849-17.

Ruiz-Moreno, J.S., Hamann, L., Shah, J.A., Verbon, A., Mockenhaupt, F.P., Puzianowska-Kuznicka, M., Naujoks, J., Sander, L.E., Witzenrath, M., Cambier, J.C., Suttorp, N., Schumann, R.R., Jin L., Hawn, T.R., Opitz, B. and CAPNETZ Study Group. *The common HAQ STING variant impairs cGAS-dependent antibacterial responses and is associated with susceptibility to Legionnaires' disease in humans*. PLoS Pathog, 2018. 14(1):e1006829.

Caronni, N., Simoncello, F., Stafetta, F., Guarnaccia, C., **Ruiz-Moreno, J.S.**, Opitz, B., Galli, T., Proux-Gillardeaux, V. and Benvenuti, F. *Downregulation of Membrane Trafficking Proteins and Lactate Conditioning Determine Loss of Dendritic Cell Function in Lung Cancer*. Cancer Res, 2018. 78(7):1685–1699.

Hamann, L., **Ruiz-Moreno, J.S.**, Szwed, M., Mossakowska, M., Lundvall, L., Schumann, R.R., Opitz, B. and Puzianowska-Kuznicka, M. *STING SNP R293Q Is Associated with a Decreased Risk of Aging-Related Diseases*. In press.

10.2 Poster presentations

5th TOLL Meeting 2015, “Targeting Innate Immunity” - 30.09-04.10.2015 - Marbella, Spain. “The role of cytosolic DNA sensing pathways in the innate immune response during *Legionella pneumophila*-induced pneumonia”.

SFB-TR 84. 2nd International Conference, “Innate immunity of the lung – Improving pneumonia outcome” - 15-17.09.16 - Berlin, Germany. “The role of cytosolic DNA sensing pathways in the innate immune response during bacterial pneumonia”.

Deutsche Gesellschaft für Immunologie. 46th Annual Meeting of the German Society for Immunolgy - 27-30.09.16, Hamburg, Germany. “Sensing of *Legionella pneumophila* by the cGAS-STING pathway is affected by the HAQ variant of STING”.

10.3 Oral presentations

BRIDGE block seminar - 03.11.14 - Berlin, Germany. “The role of cytosolic DNA sensing pathways in the innate immune response during bacterial pneumonia”.

RKI Symposium for doctoral students “Functional Molecular Infection Epidemiology” - 06.04.16 – Berlin, Germany. “Cytosolic DNA sensing in bacterial lung infection: mechanism and impact of host gene variations”.

Deutsche Gesellschaft für Immunologie and *Deutsche Gesellschaft für Hygiene und Mikrobiologie*. 21st Symposium "Infection and Immunity" – 08.03.17 – Rothenfels, Germany. “Sensing of *Legionella pneumophila* by the cGAS-STING pathway”.

11. Acknowledgments

First and foremost, I want to extend my gratitude to my doctoral advisor Prof. Dr. Bastian Opitz for his supervision and support which guided me throughout the entire development of my project. I am deeply thankful for his patience and his advice at critical points during my studies.

I would also like to acknowledge Prof. Dr. Lothar Wieler for having laid the cornerstone of the IRTG-1673 graduate school, thus granting me the opportunity to initiate my studies in Berlin. Prof. Wieler´s advice was also of immense support for the development of my research project. I would also like to express my gratitude to the coordinators of the IRTG-1673 program, Dr. Esther-Maria Antáo and specially Dr. Elisabeth Otto for her invaluable help with the administrative and logistic matters of my studies.

For her advice and support, I would also like thank my supervisor Prof. Dr. Susanne Hartmann.

Without Prof. Dr. Martin Witzenrath and Dr. Birgitt Gutbier, who provided me access to space, equipment and most importantly with generous support, I wouldn´t have been able to carry out the *in vivo* experiments that were of significant importance for my project. The contribution of Dr. Lei Jin from the University of Florida was of especial importance for these experiments as well.

I also want to extend my gratitude to Dr. Lutz Hamann and Prof. Dr. Ralf Schuman who collaborated with us in the conception, execution and analysis of the gene association studies that proved central for the outcome of my research. These studies were also possible thanks to the contribution of Javeed Shah M.D., Prof. Dr. Annelies Verbon, Prof. Dr. Frank Mockenhaupt, Dr. Thomas Hawn and Dr. Monika Puzianowska-Kuznicka, for whose help I remain deeply grateful.

My gratitude also goes to all other members of Prof. Opitz´s group who made of my stay in the group an enjoyable and unforgettable experience. Especial thanks to Dr. Jan Naujoks, Dr. Elena Kostadinova, Dr. Juliane Lippmann, Dr. Catherine Chaput and Dr. Anne Rabes who helped me in taking the first steps as a PhD student.

Also, I would like to thank Prof. Dr. Leif Sander and his research group with whom I did not only shared a working space, but also pleasant discussions and experiences. Prof. Leif´s advice was invaluable during the preparation of my thesis defense.

I would also like to acknowledge the *Deutsche Forschungsgemeinschaft*, the *Freie Universität* Berlin and the *Charité Universitätsmedizin* for funding and supporting my project.

Finally, and most importantly, I want to thank my girlfriend and best friend Surabhi Goyal for being my constant support and motivation and the source of my serenity during stormy and sunny days. Also, my gratitude goes to my best friends in Berlin and Bogotá, and my parents and siblings for continuously lighting up my path even with an entire ocean between us. To all of you I dedicate this work with my deepest affection.

12. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Juan Sebastián Ruiz-Moreno

Berlin, 15.05.2019

Druck: Mensch und Buch Verlag Berlin