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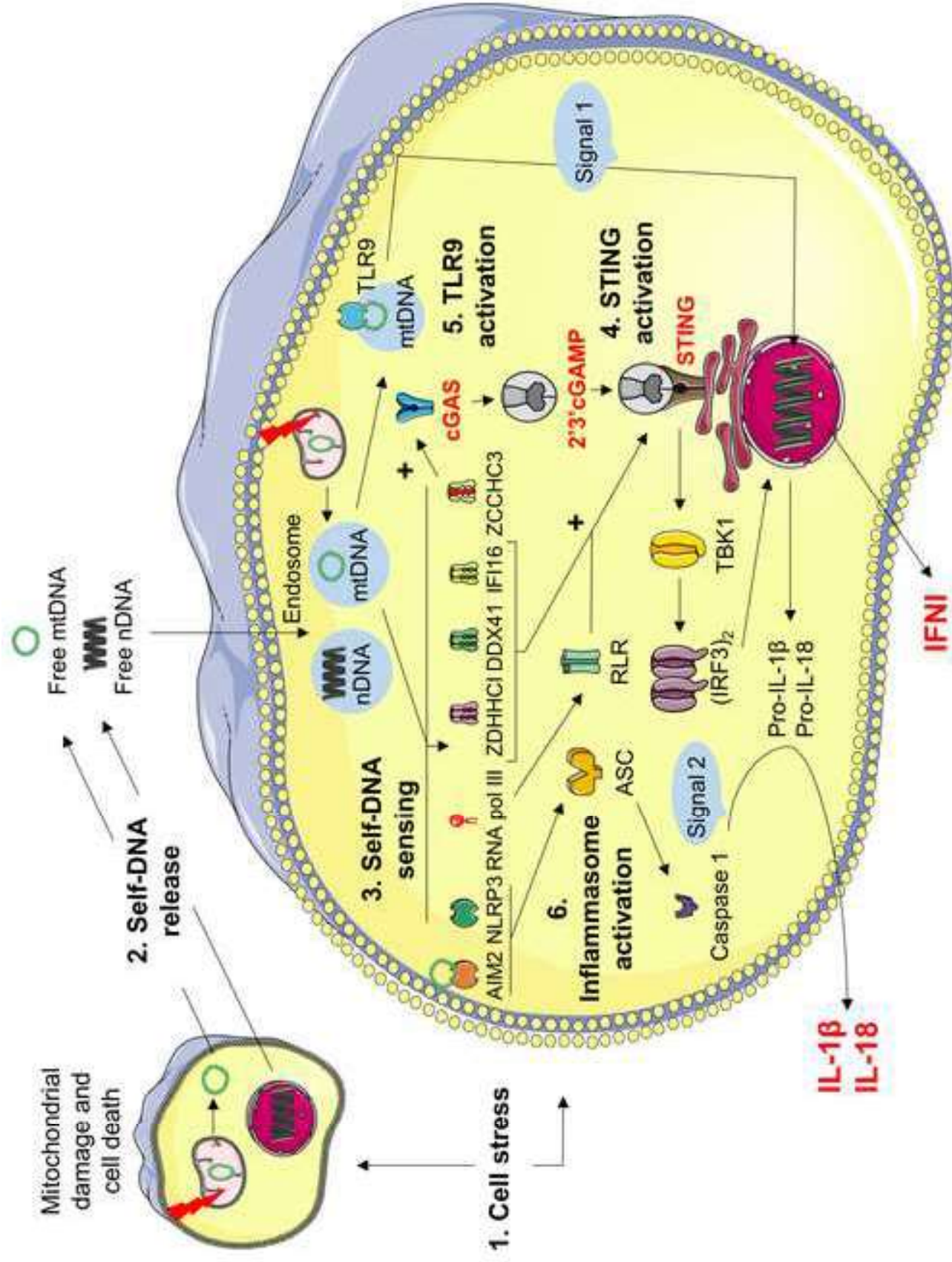
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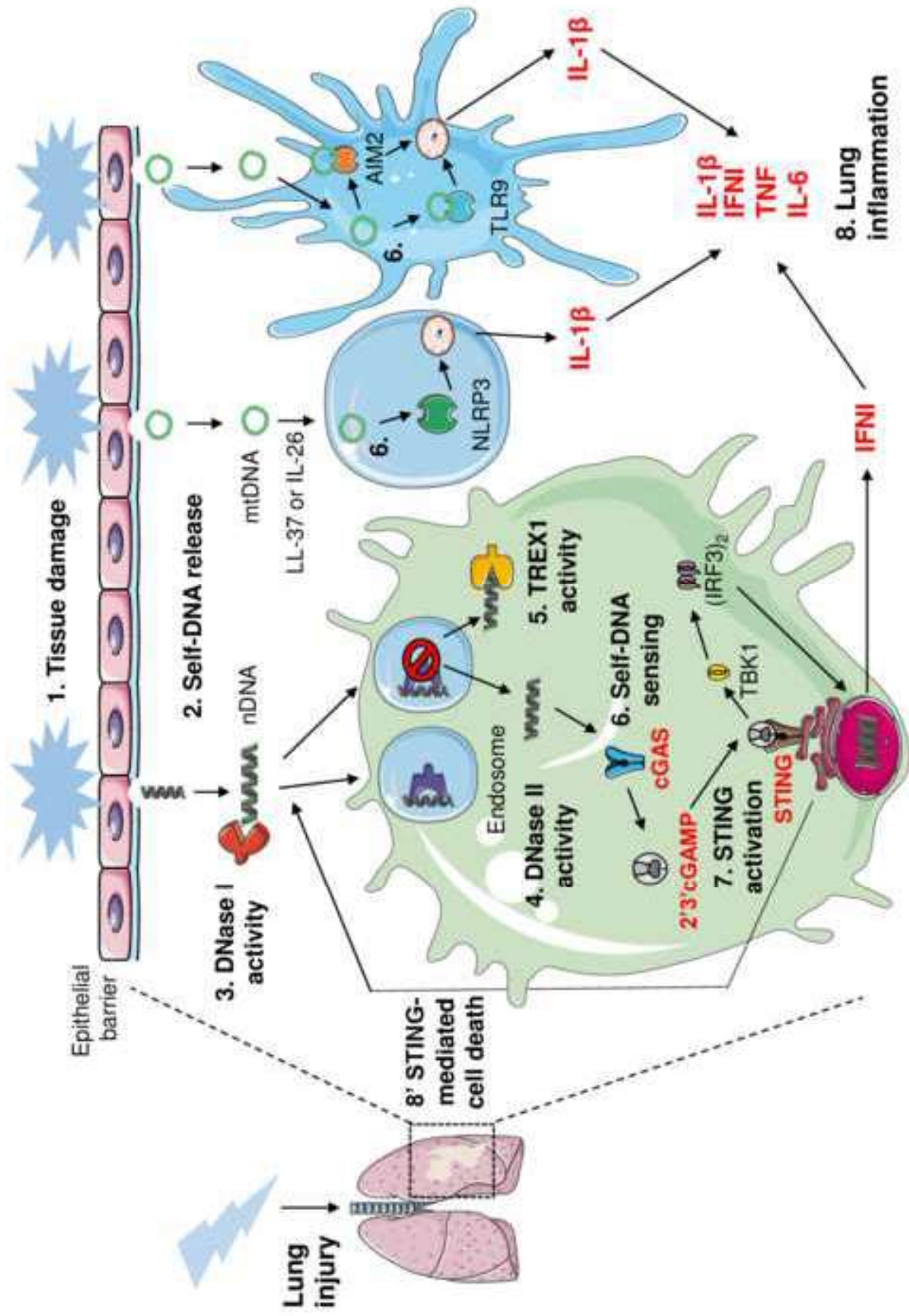
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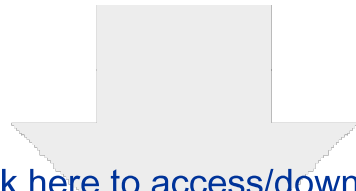
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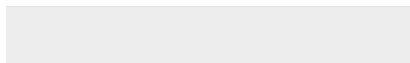
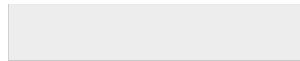




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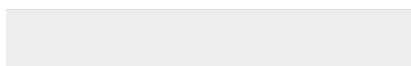
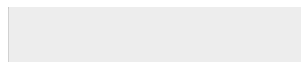
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# Self-DNA Sensing in Lung Inflammatory Diseases

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## Abstract

Self-DNA sensing by the immune system is emerging as a key contributing response for the pathogenesis of cancer and autoimmune diseases. Recent studies are establishing release of nuclear and mitochondrial DNA to also drive lung inflammatory diseases. Here, we review the latest advances of self-DNA sensing and signaling, and their impact on understanding basic mechanisms of innate immunity and how this influences lung inflammation. Among a dozen of DNA sensors, cGAS/STING, inflammasomes and Toll-Like Receptor pathways are central to sensing nucleic acids. We propose a key role for STING pathway in self-DNA sensing in inflammatory lung conditions and identify major remaining questions in understanding and controlling self-DNA sensing and innate immune activation.

## **Self-DNA Sensing in Lung Inflammation?**

The relevance of nucleic acid sensing pathways is well documented for host control of infection and autoimmune diseases. How they contribute to the onset of sterile inflammation is only starting to be appreciated. Uncontrolled lung inflammation may evolve into chronic respiratory diseases such as chronic obstructive pulmonary disease (**COPD**) (see Glossary), progressive lung diseases affecting the alveoli, such as emphysema, chronic bronchitis and asthma, or interstitial lung diseases (**ILD**), all raising major global health concerns. ILD may develop after environmental particle exposure (e.g. pneumoconiosis), infection, or autoimmune response. While progressive idiopathic pulmonary fibrosis (**IPF**) is typically of undefined origin. Among the main causes of lung inflammation are the exposure to pollutants, allergens or pathogens, as well as the process of senescence, inducing cell stress and death with DNA release. The recent advances in understanding self-DNA sensing and signaling pathways sheds a new light on inflammatory lung diseases. Here we discuss how self-DNA derived from cell stress or cell death acts as a danger signal activating nucleic acid sensors such as the recently characterized cGAS/STING pathway and inflammasomes, and how these responses contribute to lung inflammatory diseases or asthma. Thus, DNA sensing pathways are more broadly involved than anticipated in lung inflammation, and DNase I might represent a new therapeutic option to control pathological DNA sensing activation.

### **Self-DNA as a Danger Signal**

#### *Self-DNA Derived from Cellular Stress*

Although DNA provides our genetic information, ‘mislocalized’ self-DNA is a trigger for endogenous inflammation. Cells respond to stress by activating survival pathways, initiating cell death, and eventually eliminating damaged cells by **efferocytosis** [1]. During mitochondrial stress, activation of the **mitochondrial permeability transition** pore allows linear mitochondrial DNA (**mtDNA**) fragments to leak into the cytosol, triggering a pro-inflammatory type I IFN responses [2, 3]. Further, the initiation of apoptosis is accompanied by mtDNA release into the cytosol via increased permeability of the mitochondrial outer membrane [4]. Released mtDNA activates the DNA sensor **cGAS** independently of caspases 3, 7 or 9, while the caspase cascade suppresses mtDNA-induced type I IFN response, as shown in primary mouse embryonic fibroblasts or human peripheral blood cells [5, 6]. The role of ‘mislocalized’ nuclear DNA is less well characterized.



### *Cell Death as a Major Source of Self-DNA*

Although cell death can result in an inflammatory response if poorly controlled. Controlled cell death such as cell apoptosis enables the homeostatic elimination of aged cells without triggering inflammation. For example, removal of apoptotic bodies by efferocytosis prevents deleterious inflammation and hastens tissue repair. Apoptosis however can and does contribute to lung diseases such as acute lung injury or COPD. Here ineffective efferocytosis by macrophages can cause apoptotic cells to undergo **secondary necrosis** resulting in the release of damage- or danger-associated molecular patterns (**DAMP**) such as high-mobility group box-1 protein (HMGB1), heat shock proteins, histones, ATP, uric acid or mitochondrial peptides, that trigger immune responses [7]. It is now clear that self-DNA also serves as a DAMP, as described in major trauma patients in the absence of open wounds or gastrointestinal injuries [8] and our mechanistic understanding of self-DNA contributing to host DAMP driven responses has significantly increased in the last ten years. Beyond apoptosis and necrosis other types of cell death have been characterized; these include pyroptosis, necroptosis and **NETosis** which all contribute to self-DNA release. **Pyroptosis**, or caspase 1-dependent cell death, is inherently inflammatory, inducing the rapid formation of plasma-membrane pores that disrupt the cellular ionic gradients and allow water influx, cell swelling and osmotic lysis [9, 10]. Caspase-activated DNase cleaves DNA between nucleosomes resulting in characteristic oligonucleosomal DNA fragments of approximately 180 bp [9, 10]. Necroptosis, a regulated form of necrosis, involves receptor-interacting serine-threonine kinase 3 (RIPK3) and Mixed Lineage Kinase domain Like pseudokinase (MLKL), and is induced by death receptors such as TNFR1, by interferons, and also by intracellular RNA and DNA sensors [11]. Indeed, DNA sensing by DNA-dependent activator of IFN regulatory factor (DAI) initiates RIPK3-mediated necroptosis through NF- $\kappa$ B and type I IFN pathway in mice [12]. Thus, cell death is a source of self-DNA which can further activate cell death inducing pathways [13].

### *Mislocalized Nuclear and Mitochondrial DNA: Dr. Jekyll and Mr. Hyde in the Immune System?*

In eukaryotic cells, the ‘good’, hereditary DNA material is confined to the nucleus and mitochondria, while cytosolic or extracellular DNA molecules are usually a result of cell damage and are responded to by the cell as a danger signal. This non-nuclear/mitochondrial DNA can induce strong inflammatory responses. In the case of cell free-mtDNA its high inflammatory capacity relates to its **hypomethylated CpG motifs** having a bacterial origin



which results in a response analogous to non-self [8, 14]. Mammalian mtDNA is detected by several sensors such as Toll-like receptor 9 (TLR9), NLRP3, but also cGAS/STING; in response to systemic mtDNA injection these can cause lung and liver inflammation in mice [8, 14, 15]. Dying cells releasing DAMPs may activate bystander cells, which in turn recruit immune cells to the inflammation site [16]. These powerful responses are inherently regulated, for example the intracellular location of nucleic acid sensors prevents the excessive recognition of extracellular self-DNA released by dying cells and self-DNA is rapidly degraded in the extracellular space. However, several mechanisms enable the internalization of extracellular self-DNA, including IgG-bound DNA interacting with their specific FcγRIIa receptor, or HMGB1-bound DNA interacting with RAGE, the receptor for advanced glycation end products, in human monocytes in vitro [17]. Moreover, the endogenous antimicrobial peptide LL37 can break innate immune tolerance to self-DNA by transporting extracellular DNA into the cytoplasm of human monocytes where it triggers the STING pathway, or into human plasmacytoid dendritic cells (pDC) endosomal compartment where it triggers TLR9, inducing a pro-inflammatory type I IFN response in vitro [18, 19]. Another route for DNA to enter the cells is through binding to the cytokine IL-26. This complex is then shuttled to the cytosol of human myeloid cells, activating STING and NLRP3 **inflammasome** mediated inflammatory responses [20]. Thus, mislocalized self-DNA can induce striking tissue inflammation and injury, although the cellular and sub-cellular sources of self-DNA released in the cytosol or in the extracellular milieu warrant further investigation

## **Nucleic Acid Sensors: Checkpoints in Innate Immunity**

### *TLR Pathways*

DNA is established as a potent activator of inflammatory responses; and, the discovery of TLR9 offered a first molecular mechanism for the recognition of hypomethylated CpG-rich DNA [21]. TLR9 as well as DExD/H box helicases recruit MyD88 to activate type I IFN production in response to DNA in human pDCs (Figure 1) [21-23]. The intracellular localization of TLR9 in the endosomal compartment is crucial to gain access to pathogen-derived DNA, while also regulating the detection of self-DNA [24]. TLR9 may also sense single-strand ssDNA present in endosomes through its PD2' deoxyribose backbone, independently of the presence of CpG-motives, as shown in murine DC [25]. While the endosomal nucleic acid sensors TLR3, TLR7 and TLR8 recognize dsRNA and ssRNA respectively, their role in self-nucleic acid sensing is not clear. Indeed, these TLRs are mobilized against viral infections that may trigger cell stress

and/or cell death, and further induce self-DNA release into the cytosol. Although TLRs were the first host receptors discovered as key players in DNA/RNA sensing in the early 2000's, alternative nucleic acid sensing pathways such as cGAS/STING or inflammasomes have now emerged as crucial not only for host-defense but also as critical checkpoints to initiate innate and inflammatory responses.

### *The cGAS/STING Pathway*

In 2013, cGAMP synthase (cGAS) was identified as a cytosolic DNA sensor inducing type I IFN by producing the endogenous, second messenger cGAMP upon DNA recognition and cGAS oligomerization [26-29]. However, cGAMP requires the endoplasmic reticulum protein STING to trigger the type I IFN pathways in human and murine cells [30, 31] (Figure 1). After binding cGAMP STING traffics to the ER-Golgi intermediate compartment (ERGIC) and the Golgi apparatus [32]. Here, STING recruits and activates the cytosolic kinase **TBK1**, an IKK related kinase, leading to the activation of the interferon regulatory factor 3 (IRF3). IRF3 translocates to the nucleus inducing type I IFNs and other pro-inflammatory cytokines [33, 34]. While STING signaling is independent of TLR9 [35], STING may activate the transcription factor NF- $\kappa$ B in response to DNA damage through a non-canonical STING activation by IFI16, independently of cGAS [36]. In parallel, STING also promotes type III IFN pathway activation in response to exogenous DNA stimulation in human embryonic kidney cells [37]. Indeed, the DNA protein kinase (DNA-PK) component Ku70 binds to DNA and forms a complex with STING to induce the production of IFN- $\lambda$ 1 in a STING-dependent manner, suggesting a role for a STING/type III IFN axis in the late phase of antiviral responses to DNA viruses [37]. Indeed, the involvement of STING in antiviral and antibacterial responses, but also in autoimmune responses has been well documented [35, 38-40].

Although cGAS is the best characterized DNA sensor upstream of STING, other cytosolic DNA sensors, acting independently or modulating cGAS activation, may trigger STING activation (see Box1 and Figure 1), but their role in inducing a robust IFN response is still controversial [41, 42]. STING signaling is central to the production of pro-inflammatory cytokines in response to self-DNA derived from necrotic or inappropriately apoptosed cells [43-45]. However, a dual, noncanonical function of cGAMP in inflammasome activation was recently reported linking cGAS/STING and the inflammasomes; specifically, cGAMP increased the expression and the activation of Absent In Melanoma 2 (AIM2) and NLRP3 inflammasomes *via* STING and type I IFN induction in murine macrophages and induced IL-1 $\beta$  release in the

airways in vivo [46]. DNA-mediated inflammasome activation is initiated in a cGAS/STING-dependent unique lysosomal cell death program upstream of NLRP3, and the cGAS-STING-NLRP3 axis has been proposed as the default DNA-induced inflammasome response in human myeloid cells [47]. Thus, although STING signaling induces type I IFN and pro-inflammatory responses, recent evidence demonstrates a coordinated role for STING and inflammasomes in self-DNA sensing.

### *The Emerging Roles of Inflammasomes in Self-DNA Sensing*

The first inflammasome reported as a DNA sensor was AIM2, essential for host response to bacterial and viral infections, as shown in vitro and in vivo in mice [48]. AIM2 contains a N-terminal pyrin domain (PYD) and a C-terminal HIN domain. Upon dsDNA binding, AIM2 auto-inhibition is alleviated, leading to its oligomerization, recruitment of the adaptor ASC via PYD-PYD interactions, proteolytic maturation of the caspase-1 substrate pro-IL-1 $\beta$ , and release of biologically active IL-1 $\beta$  [49]. Thus, compared to other DNA sensors, AIM2 is specialized in inflammasome assembly and activation, rather than promoting a type I IFN response. During infection with intracellular pathogens such as *Francisella tularensis* or *Listeria monocytogenes*, bacterial DNA escapes into the cytosol and activates AIM2 leading to inflammation [48, 50]. Both AIM2 and STING have been shown to contribute to elevated self DNA-induced arthritis in DNase II deficient mice, relative to wild-type mice [51]. The relevance of these sensors was revealed using triple deficient mice in DNaseII/IFNAR/STING or DNaseII/IFNAR/AIM2, as compared with DNaseII/IFNAR double deficient mice, unveiling a new role for AIM2 as a co-sensor of endosomal nucleic acids in arthritis in mice [51]. However, the role of AIM2-like receptors (ALR) has been questioned since mice lacking all 13 ALR genes were able to elicit functional type I IFN responses to transfected DNA ligands, DNA virus infection, or lentivirus infection [42]. The limited contribution of ALR to self-DNA sensing was inferred from the fact that ALR deficiency could not rescue mice deficient for DNase III three prime repair exonuclease I (Trex1) from lethal autoimmune disease characterized by increased circulating DNA and elevated production of type I IFNs relative to wild-type mice [42]. However, AIM2 detects self-DNA released upon perturbation of nuclear envelope integrity [52] and AIM2 and ASC form **speckles** co-localizing with dsDNA breaks in the nucleus upon ionizing radiation exposure [53]. AIM2 also detects self-DNA released through exosomes that enter the cytosol of phagocytes, inducing IL-1 $\beta$  maturation [54]. Indeed, exosomes play a key role in sensing

extracellular self-DNA as they act as vehicles for the delivery of inflammatory self-DNA, while protecting DNA from degradation by extracellular nucleases [49].

NLRP3 has also been associated with self-DNA sensing (Figure 1). Indeed, the NLRP3 inflammasome is activated in response to transfected bacterial or host DNA [55], although the exact mechanism by which NLRP3 activation in response to DNA occurs is still unclear. NLRP3 activators such as nigericin or ATP cause mitochondrial dysfunction, mtDNA oxidation and release of oxidized mtDNA into the cytosol where it binds to NLRP3 and stimulates NLRP3 inflammasome assembly [56]. mtDNA is crucial for the assembly and activation of the NLRP3 inflammasome and *de novo* synthesis of mtDNA, induced following engagement of TLRs, is required for NLRP3 signaling in murine macrophages [57]. CMPK2, the rate-limiting enzyme that supplies deoxyribonucleotides for mtDNA synthesis, is necessary to produce oxidized mtDNA fragments in response to NLRP3 activators [57]. In parallel, STING-mediated lysosomal cell death initiates a potassium efflux that activates NLRP3 [47].

The crosstalk between the inflammasomes and type I IFN responses also include negative regulation. Indeed, NLRC3 is a negative regulator of STING, binding both STING and TBK1 and preventing their interaction while preventing STING trafficking to the perinuclear region in human cells *in vitro* [58]. AIM2 activation by intracellular DNA antagonizes STING pathway as STING activation is increased in murine macrophages and DCs deficient for AIM2 [59]. Another negative regulation involves cleavage of cGAS by caspase-1 or other pyroptotic caspases; this impairs cGAMP production during DNA virus infection [60]. Further, the cleavage of gasdermin D by caspase-1 suppresses cGAS-driven type I IFN response to cytosolic DNA via membrane pore formation and intracellular K<sup>+</sup> efflux [61]. Thus, inflammasomes are direct or indirect actors in self-nucleic acid sensing and a complex positive and negative regulatory interplay exists between the inflammasomes and cGAS/STING pathway.

Overall, once self-nucleic acids are released upon cell damage, the availability and activation of the different nucleic acid sensors, TLRs, cGAS/STING pathway or inflammasomes act as checkpoints to determine the upregulation of further inflammatory responses.

## **Self-DNA and Lung Diseases**

### *STING-Associated Vasculopathy with Onset in Infancy (SAVI)*

Gain-of-function mutations in the STING-coding gene *TMEM173* resulting in a constitutive activation of the STING pathway lead to STING-associated vasculopathy with onset in infancy

(SAVI), symptomatic in the first 8 weeks of life [62]. Beside cutaneous vasculopathy, a major feature of SAVI is interstitial lung disease [63]. The expression of STING in bronchial epithelium, alveolar type 2 pneumocytes, and alveolar macrophages might explain the specific lung pathology seen in SAVI. SAVI is considered as an interferonopathy characterized by the constitutive activation of the STING pathway with elevated type I IFNs and ISGs in the peripheral-blood mononuclear cells of SAVI patients, relative to healthy controls [62]. Although STING is closely associated with IRF3, mutant mice with a gain of function STING N153S mutation lacking IRF3 also developed lung disease, myeloid cell expansion and T cell cytopenia, more marked for CD8<sup>+</sup> T cells than for CD4<sup>+</sup> T cells, relative to wild-type littermates, demonstrating that the STING N153S mutation triggered lung disease in a IRF3-independent manner in mice [64]. Further, spontaneous lung disease in STING N153S expressing mice develops independently of cGAS, IRF3/IRF7 and type I IFN IFNAR1 pathway, since STING N153S mice crossed to mice deficient for these genes developed the lung disease [65]. However, intercross of STING N153S expressing mice to mice deficient for adaptive immunity prevented spontaneous lung disease, indicating that T cells are essential for lung disease pathogenesis in these mice [65]. Because SAVI is associated with high mortality risk and significant morbidity [62], this diagnosis should be considered in children with ILD that are resistant to steroids and immunosuppressive therapies, so as to avoid progressing to irreversible lung damage with limited treatment options [62].

### *Self-DNA in Chronic Lung Inflammation and Fibrosis*

Lung exposure to different sources of airborne pollutants present in the environment can induce pulmonary inflammation, which may lead to chronic inflammation or COPD, characterized by remodeling of the airways with **emphysema** and **dyspnea** [66]. Among inhaled toxicants, cigarette smoke is a major cause of COPD, often associated with exposure to dust, infection in early life, genetic predisposition or allergies [67]. Cigarette smoke contains over 5000 compounds, and understanding their individual contribution to lung inflammation is complex [67]. Cigarette smoke exposure induces necroptotic cell death in cultured human bronchial epithelial cells, with increased dsDNA and mtDNA release in the extracellular milieu [68]. *In vivo*, cigarette smoke also induces dsDNA and mtDNA release in the bronchoalveolar space in mice [68]. In cystic fibrosis patients, free DNA concentrations in the airway fluids correlate with airflow obstruction, fungal colonization and CXC chemokine concentrations suggesting that free DNA might be involved in the decline of lung function, although further studies are warranted [69]. Further, high concentrations of extracellular mtDNA have been reported as a

putative negative predictive factor for fatal outcome in IPF patients [70]. Indeed, mtDNA concentrations have been found to be increased in the bronchoalveolar lavage and plasma of IPF patients relative to control subjects without parenchymal lung disease, displaying robust associations with disease progression and reduced event-free survival [70]. Consequently, circulating mtDNA has been proposed as a possible prognostic biomarker for degree of disease severity in IPF patients, although further investigations will be required [71].

Inhaled environmental particulate toxicants such as crystalline silica, asbestos or coal can also lead to chronic pulmonary inflammation [72]. Silica is one of the most commonly inhaled minerals and chronic silica exposure may lead to silicosis with lung inflammation, progressive interstitial fibrosis and cancer [73]. An increased prevalence of occupational exposure to silica has been recently observed to previously unappreciated and new sources of exposure, such as with denim sand blasting, or fracking, hydraulic fracturing in the shale oil and gas industry [73-75]. The inflammatory response following silica exposure involves NLRP3 inflammasome-driven IL-1 $\beta$  production [76, 77]. As silica exposure induces cell death, our group hypothesized that self-DNA release might trigger DNA sensor pathways. Indeed, mouse airway silica exposure induced reactive oxygen species, epithelial and immune cell death, and release of mitochondrial and nuclear self-dsDNA in the airways, triggering STING-dependent type I IFN responses [45]. Moreover, silicosis patients with a history of intense silica exposure exhibited increased circulating dsDNA, as well as CXCL10 in sputum, relative to healthy individuals; in addition, STING was activated in lung tissue from fibrotic ILD patients as seen by STING expression and phosphorylation, relative to the lung of cortisone treated ILD patients [45]. Thus, STING pathway activation might be a hallmark of sterile lung inflammation in humans as evidenced in the mouse.

There also appears to be a crosstalk between STING and NLRP3 pathways following exposure to silica. Indeed, the overexpression of NLRP3 induced after experimental silica exposure is reduced in STING-deficient mice, while silica-induced STING expression and type I IFN response are reduced in mice deficient for NLRP3 [45]. This documented role of self-DNA sensing and cGAS/STING activation after silica exposure anticipates a potential broader implication of STING pathway in lung inflammatory diseases where both DNA release and STING overexpression are present.

Of note, microcrystals of endogenous origin may form after deposition of minerals, cholesterol or uric acids as found in gout. The **Charcot-Leyden crystals**, made of Galectin-10 protein clusters [78], are present in eosinophil and basophil granules, and accumulate in the lung of severe asthmatic patients [79]. Although long regarded as inert crystals, Charcot-Leyden

crystals were recently shown to trigger NLRP3 and IL-1 $\beta$  maturation in vitro in primary human macrophages and in vivo after airway exposure in mice [80]. However, whether Charcot-Leyden crystals induce cell stress and/or cell death resulting in release of nucleic acids and which might trigger STING pathway remains to be explored. Thus, as silica microcrystals were shown to trigger cell death with self-DNA release leading to inflammasome and STING activation, endogenous crystals may also induce cell damage and trigger inflammasome and STING pathways.

### *Self-DNA in Asthma*

Asthma is characterized by airway obstruction and chronic lung inflammation, with intense eosinophil infiltrates, mucus hypersecretion, airway remodeling and hyper-responsiveness [81]. Several mediators including the alarmins IL-1 $\alpha$ , IL-33 and HMGB1, the danger signals ATP and uric acid, and type 2 cytokines IL-4, IL-5 and IL-13 are key players in the pathophysiology of the allergic response [81, 82]. Experimental asthma animal models often rely on allergen exposure in the presence of adjuvant such as aluminum salts (alum), which are rapidly coated by host DNA [83]. Indeed, self-DNA released from dying cells acts as a DAMP that contributes to alum adjuvant activity, as DNase treatment reduced antigen-specific humoral responses in mice immunization with ovalbumin and alum [83, 84]. Further, STING deficient mice showed a reduced antigen-specific IgE response following ovalbumin and alum immunization [83], suggesting that self-DNA and STING activation contribute to alum adjuvant activity independently of type I IFNs pathway [83, 84]. The vaccine adjuvant chitosan also induces mitochondrial stress with mtDNA release into the cytosol in mouse dendritic cells, engaging cGAS/STING pathway to induce type I interferons and protective immunity [85].

However, TLR9 and STING agonists act as vaccine adjuvants inducing a strong type II IFN response, suggesting that combinations of CpG DNA and cGAMP may offer therapeutic applications [86]. Self-DNA might act at several levels, inducing primary B cell responses and IgG1 production, through IRF3-independent mechanisms while stimulating **T helper type 2 responses** through IRF3-dependent mechanisms [84].

In the clinic, increased dsDNA is detected in the bronchoalveolar lavage from asthmatic patients with rhinovirus infection and promotes asthma exacerbation [87]. Recently, prominent ISG and ER stress have been shown in airway epithelial brushings from asthmatic patients independently of viral transcripts, suggesting that type I interferon and possibly DNA sensing pathways might be activated [88]. Indeed, an increased **mtDNA to nDNA ratio**, reflecting mitochondrial dysfunction, was recently reported in blood cells from patients suffering from **asthma-COPD**



**overlap syndrome** relative to healthy non-smokers [89]. Extracellular self-DNA, with increased mtDNA to nDNA ratio, is now considered as a biomarker of chronic lung inflammation in allergic asthma [89]. However, the role of the DNA sensor cGAS/STING *per se* in allergic asthma remains elusive.

House dust mite is one of the clinically relevant and common aeroallergens affecting 80 % of allergic patients [90]. Mouse exposure to house dust mite is a prevalent experimental model of allergic asthma used to induce airway inflammation, accompanied by oxidative stress, cell death and DNA damage in the airways [91]. Self-dsDNA exerts its effect on immune cells mainly through type I IFN dependent pathways [87]. An emerging concept is that the damaged DNA, that eventually leads to the secretion of Th2 cytokines and the induction of allergic inflammation activates specific DNA sensors expressed by epithelial or innate immune cells, including cGAS/STING or other sensors. Although STING is thought to act by inducing type I IFN, the effect of type I IFN in allergic asthma is still matter of debate. Exogenous type I IFN reduces virus induced asthma exacerbation. Several cytosolic DNA sensors may promote STING-dependent signaling pathways [83] (Box1), however whether these DNA sensors contribute to the pathogenesis of asthma is unknown.

### *Self-DNA and Respiratory Infection*

Infectious respiratory diseases represent a major global threat. The first line host defense is the airway epithelium. The rupture of this first barrier dysregulates airway tissue homeostasis allowing pathogens to directly activate innate immune cells leading to severe inflammation [92]. However, lung inflammation after pathogen infection is highly regulated to avoid an excessive immune response deleterious for the host. Pathogen-derived nucleic acids such as DNA, RNA or cyclic dinucleotides have recently emerged as important inducers of lung inflammation *via* cGAS/STING or TLR9 pathways [34, 35, 40]. Indeed, nucleic acids derived from bacteria including *Mycobacterium tuberculosis*, or viruses such as HSV-1 activate the cytosolic surveillance system leading to STING activation and type I IFN induction [34, 93]. Further, *M. tuberculosis* actively releases RNA into the cytosol of infected macrophages and a cross-talk between DNA and RNA sensor pathways was recently proposed to trigger host immune response to *M. tuberculosis* [94]. In addition to the presence of nucleic acids derived from the microorganisms, infections might also trigger the release of self-DNA; pathogens may induce host cell stress and/or cell death, leading to self-DNA release in the intracellular or extracellular milieu and activation of the cytosolic surveillance pathway. Indeed, self-DNA is

released after rhinovirus infection and formation of **neutrophil extracellular traps**, influencing type 2 immune responses asthma pathology [87]. Further, host-derived DNA accumulates in the lung of mice infected with influenza A virus, leading to activation of AIM2 inflammasome which however seemed to temper lung inflammation [95]. In addition, there might be an interplay between the responses induced by host-derived and pathogen-derived nucleic acids. As discussed above, our group recently showed that airway exposure to silica microcrystals induced cell death with self-DNA release, as shown in mice and in silicosis patients, which triggers the cGAS/STING pathway, induces a type I IFN response that enhances the expression of ISG, including both cGAS and STING [45]. This conditioned the host response to subsequent *M. tuberculosis* DNA or *M. tuberculosis* infection, leading to exacerbated type I IFN response and type 2 immune response polarization, with increased IL-4, IL-5, Th2 and IL-13 producing alternatively activated macrophages, relative to challenge with *M. tuberculosis* in the absence of silica pre-exposure [96]. Hence, fundamental questions remain as to whether, and how, pathogens induce self-DNA release and cytosolic surveillance response, and conversely, how the response to self-DNA might amplify the pathogenicity and lung inflammation induced by pathogen-derived nucleic acids.

### *Self-DNA, Aging and Senescence*

Aging is the main risk factor for major non-communicable chronic lung diseases including COPD, lung cancer and IPF [97]. While our lifespan increases, the prevalence of lung diseases increases with age, suggesting that aging may contribute to lung inflammatory diseases and reduced resistance to infections [98]. Indeed, the lungs of 18-month old mice have elevated concentrations of pro-inflammatory cytokines together with a resident population of highly activated pulmonary macrophages, as compared to 3-month old, young adult mice [98]. Furthermore, aging is associated with **cell senescence**, a physiological process in which the cGAS-STING pathway has been implicated as an important mediator, since senescing stimuli such as oxidation, radiation, or mitogenic stress, trigger cells to express cytokines including IFN- $\beta$  and IL-1 $\beta$  in a cGAS-dependent manner, while nuclear cGAS suppresses DNA repair in mouse and human cells [99, 100]. Genomic instability is a major driving force of age-related diseases. Although DNA damage is tolerable, cells may elicit cell death with the formation of **micronuclei** to eliminate the damaged genome [99]. cGAS can localize to micronuclei arising from genome instability, as shown in human epithelial cell lines [43, 44]. Sensing of self-chromatin fragments by cGAS and activation of STING in senescent cells promote cell

senescence and inflammation, indeed cGAS interacts with cytosolic chromatin fragments generated in mouse embryonic fibroblasts undergoing oxidative-stress-induced senescence [101]. Further, downregulation of cytoplasmic DNases contributes to cytoplasmic DNA accumulation in senescent cells, inducing a strong and aberrant STING activation [102]. The concentrations of free circulating mtDNA have been reported to increase in elderly people relative to young adults and may contribute to low-grade, chronic **inflamm-aging** [14]. Protection against aging-associated chronic lung diseases was recently reported in seniors carrying a defective STING allele *TMEM173* R293Q, suggesting that decreased STING sensitivity might be associated with healthy aging, likely through reduced inflamm-aging, although this warrants further assessment [103]. With a large and raising global aging population, it is critical to better understand how cells and tissues evolve with age and how this can affect the respiratory system, including the specific role of self-DNA sensing through STING pathway.

### **Nucleases: the Yin and Yang of the Immune System**

While DNA is an essential support of genetic information, DNA accumulation in the cytosol or endosomes of mammalian cells constitutes a danger signal. Nucleases such as DNases I, DNase II or DNase III TREX1 degrade aberrantly localized self-DNA (Figure 2) and disruption of DNases functions lead to severe inflammation [104, 105]. TREX1, the major 3' to 5' DNA-specific exonuclease in mammalian cells [105], is widely expressed as homo-dimeric protein and preferentially degrades ssDNA [106]. Trex1-deficient murine cells and TREX1-mutant human cells display high expression of a subset of ISG transcripts [107]. Trex1-deficient mice develop systemic inflammation characterized by a strong type I IFN response dependent on IRF3, IFNAR1 and RAG2, and succumb after 2-8 months with myocarditis, showing that self-DNA degradation is essential for host integrity [108]. TREX1 maintains immune tolerance to cytosolic self-DNA, and mutations disrupting human TREX1 DNase activity lead to self-DNA accumulation in the cytosol, activating the cGAS/STING pathway, leading to several inflammatory and autoimmune diseases such as Aicardi-Goutiere syndrome or systemic lupus erythematosus [105]. Another key player is the deoxynucleotide triphosphohydrolase (dNTPase) sterile alpha motif and HD domain-containing protein 1 (SAMHD1), by preventing ssDNA release during DNA replication. Indeed, DNA replication is a complex process depending on the coordinated activation of thousands of replication origins. It may be challenged by endogenous or exogenous factors, leading to replication fork slowing or stalling. SAMHD1-deficient cells show deregulated dNTP pools, impairing replication fork progression

and a leakage of ssDNA from the nuclear envelope to the cytosol in human cell lines [109]. SAMHD1 interacts physically with the MRE11 nuclease and stimulates its exonuclease activity. In parallel, SAMHD1-deficient cells show accumulation of ssDNA in the cytosol activating cGAS/STING pathway [110]. Self-DNA can also access the endosomes and activate TLR pathways. The enzyme DNase II is active in lysosome/endosome and DNase II-deficient mice are unable to degrade endosomal DNA leading to autoinflammatory arthritis [51], highlighting the importance of digesting self-DNA (Figure 2) [104]. However, DNases may also favor inflammation, as endosomal TLR9 respond to DNA fragments generated by DNase II; indeed, cleavage of long DNA fragments and bacterial DNA by DNaseII is required for TLR9 response of mouse DCs, demonstrating that DNase II is also required for TLR9 activation [111].

Extracellular, free circulating DNA also needs to be tightly regulated. Indeed, the most abundant DNase in human serum is DNase I (Figure 2); it plays a key role in degrading DNA derived from apoptotic, necrotic and cells undergoing NETosis [112]. Decreased DNase I activity due to DNase I inhibitors or autoantibodies is associated with impaired DNA clearance and poor prognosis in autoimmune disease such as lupus nephritis [113]. Deficiency in nucleases or DNA mutations (e.g. 8-OHdG) that render DNA resistant to degradation result in the accumulation of cGAS ligands that activate the STING/IRF3 axis [114]. Conversely, treatment with DNase I originally used as a mucolytic is active in treating cystic fibrosis lung disease in the clinic [115]. Furthermore, DNase I intra-peritoneal administration in mice prevented silica induced accumulation of self-DNA in the airways, reducing the silica-induced STING-dependent lung pro-inflammatory response compared to untreated, silica exposed mice [45]. Thus, taken together, these studies suggest that nuclease activities need to be tightly regulated to avoid an over-activation of the cytosolic surveillance system that may trigger inflammation.

### **Concluding Remarks**

While the role of nucleic acid sensing pathways for host control of infection, autoimmune diseases or cancer is well documented, the appreciation of self-DNA sensing in sterile inflammation is just emerging [116] and many questions remain (see outstanding questions). Indeed, self-DNA release is documented in several lung diseases [69-71, 89] and STING pathway activation has recently been associated with experimental and clinical lung inflammation. Specifically, STING is strongly expressed in the lung of ILD patients or after lung exposure to particulate pollutants [45]. Moreover, constitutive STING activation leads to

ILD in SAVI patients [62]. Hence, it is expected to play an important role in the pathogenesis of inflammatory lung diseases. Thus, antagonizing the STING pathway might be promising in treating lung inflammatory pathologies, but further testing is needed. Accordingly, therapeutic approaches targeting STING, both as agonists in oncology [117] and antagonists in autoimmunity [118], are being developed by most major pharmaceutical and biotech companies. However, the safety of such therapies and off-target effects on the immune responses will deserve careful attention.

Conversely, nucleases play a crucial role in maintaining homeostasis, avoiding an excessive inflammatory response. Specifically, free circulating self-DNA has been associated with human ILD or severe asthma and a strict regulation of self-DNA degradation is essential to avoid nucleic acid sensing by the immune system. For instance, cytosolic Trex1 DNase is an important player in self-DNA degradation, but how extracellular self-DNA gets access to the cytosol and ER is not fully understood. DNase I administration, already used in cystic fibrosis [115], can prevent self-DNA accumulation in the airways and STING-dependent lung inflammation in response to experimental silica exposure in mice [45], suggesting a broader potential use for DNase treatment in lung inflammation.

The field is moving fast and the last few months have seen an explosion of high-impact publications regarding cGAS activation mechanisms [116]. For instance, originally considered as a cytosolic DNA sensor, the membrane localization of cGAS was recently reported in human monocytic cells and murine macrophages; indeed cGAS positively charged, phosphoinositide-binding, N-terminal domain interact with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) lipid in the plasma membrane [119], and this may prevent cGAS access to cytosolic self-DNA. How the cGAS N-terminus is released from PI(4,5)P<sub>2</sub> following DNA detection is unknown. Additionally, this location at the plasma membrane may prevent recognition of self-DNA, as mislocalized cGAS deletion mutant unable to bind to the plasma membrane showed a reduced response to viral DNA when compared to self-DNA [119]. The non-enzymatic N-terminal domain also determines cGAS nuclear localization in human DCs [120], and nuclear cGAS, enriched on centromeric satellite and LINE DNA repeats, has a lower affinity for self-DNA than for exogenous DNA [120]. cGAS is deacetylated in response to DNA challenge and cGAS acetylation by aspirin blocks its activity, reducing the concentrations of ISG in cells from patients with **Aicardi-Goutières syndrome** and in *Trex1*<sup>-/-</sup> mice [121]. The GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) interacts with cGAS and primes it for efficient activation [122]. cGAS produces cGAMP upon DNA sensing [27], that may transfer to

bystander cells [16]; the protein SLC19A1, also known as reduced folate carrier 1, was recently shown to import cGAMP and other CDNs into human monocytic cells [123]. As some of these studies were conducted at steady state or after DNA transfection, their impact on cGAS/STING response to ‘physiological’ triggers remains to be investigated.

Nucleic acid sensors such as cGAS/STING detect both self- and microorganisms-derived DNA. Sensing self-DNA might affect host response to infection, and conversely, the activation of nucleic sensors by infection might affect self-DNA sensing. Indeed, self-DNA released after cell damage due to pollutants primes the cGAS/STING pathway, and might exacerbate the host inflammatory response to infection, and conversely. Sterile inflammation after exposure to silica microcrystals inducing cell death with self-DNA release and trigger of cGAS/STING/type I IFN response, enhances cGAS and STING expression and primes the host response to *M. tuberculosis*, leading to exacerbated type I IFN response, type 2 immune polarization and reduced host response to *M. tuberculosis* infection [96]. Whether microcrystals from endogenous origin such as Charcot-Leyden crystals might also induce cell stress with release of nucleic acids and trigger of STING pathway remains to be explored [78-80]. Apoptotic caspases may also regulate virus-triggered host defenses, as activated caspase-3 cleaves cGAS, MAVS and IRF3 in human cells while caspase-3-deficient mice show elevated type I IFNs and increased resistance to vaccinia virus infection [124]. Further, the cGAS-STING pathway is involved in autophagy; STING-containing ERGIC serving as a membrane source for cGAMP induced LC3 lipidation, a key step in autophagosome biogenesis, while cGAMP-induced autophagy contribute to the clearance of DNA and viruses in the cytosol [125].

Thus, self-DNA sensing pathways likely contribute to ‘trained immunity’, the concept that innate immune cells build a memory of past stimulations and alter their response to new challenges, emerged in the last ten years [126]. Upon *M. bovis* BCG (Bacillus Calmette-Guérin) vaccination, or after priming with *Candida albicans* or  $\beta$ -glucan, monocytes respond to restimulation with increased cytokine production [127]. Further, viral infection might affect host nucleic acid sensing, as recently shown, viral DNA binds to the inhibitory innate receptor NLRC3, increases its ATPase activity, needed to facilitate its release of STING and TBK1, thus unleashing STING and IFN-I response in vitro [128]. Trained immunity may be triggered not only after vaccination or infection, but also after sterile inflammation.–Indeed, endogenous alarm signals associated with tissue damage and sterile inflammation can induce trained immunity through epigenetic regulation of transcriptional programs [127]. The DAMPs reported to induce priming or trained innate immunity include those in hypercholesterolemia,

modified low and high density lipoproteins, uric acid and monosodium urate crystals, HMGB1, hyperglycemia-induced ROS or advanced glycation end products [127, 129]. Thus, we propose that self-DNA released after sterile damage may be another important trigger for trained innate immunity, and the sGAS/STING DNA sensing pathway that is strongly upregulated upon an initial type I IFN response could convey this effect. In this respect, the ‘Threshold concept’ of cGAS activation is interesting; indeed the formation of ‘ladder-like’ networks between cGAS dimers and DNA molecules, cooperatively stabilized and concentrated in phase-separated liquid droplets, act as microreactors to synthesize high concentrations of cGAMP [116]. Since cGAS is an ISG, any inflammatory priming leading to an early type I IFN response will strongly upregulate cGAS expression, favoring the formation of ‘ladder-like’ networks between cGAS dimers and DNA molecules, and efficient cGAMP formation. The initial type I IFN response might be induced by microbial as well as sterile cell stress triggers, which greatly enhance both cGAS and STING expression [45].

In conclusion, self-DNA derived from cell stress or death acts as a danger signal activating nucleic acid sensors such as cGAS/STING or the inflammasomes, and self-DNA sensing pathways are more broadly involved than anticipated in lung inflammation. Nucleic acid sensors, at the crossroad of immune responses to sterile and infectious insults, might contribute to the ‘trained immunity’ of innate immune cells, conditioning an enhanced response to re-challenge.

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**Box 1. Alternative Cytosolic DNA Sensors**

Although cGAS is the best characterized DNA sensor upstream of STING, other cytosolic DNA sensors may trigger STING activation and modulate inflammatory responses (Figure 1). These include the IFN $\gamma$ -inducible human protein IFI16, member of the DEXDc family of helicases DDX41, DNA-dependent activator of IFN-regulatory factor (DAI) or CCHC-type zinc-finger protein (ZCCHC3) [130-133]. Human IFI16, a PYHIN protein, mediates the induction of IFN $\beta$  *via* the recruitment of STING after DNA sensing [134]. DDX41 binds both DNA and STING in the cytosol for activating TBK1-IRF3-type I IFN pathway in myeloid dendritic cells [131] (Figure 1). DAI binds dsDNA in the cytosol activating TBK1-IRF3 axis [132]. ZCCHC3 is a positive regulator of cGAS/STING pathway through a direct binding of ZCCHC3 to dsDNA, important for cGAS activation following viral infection [133]. Further, the member of the aspartate-histidine-cysteine palmitoyl acyltransferase family ZDHHC1 activates STING in the ERGIC in response to viral DNA, engaging TBK1-IRF3-type I IFN axis [135]. Sox2 is also a direct DNA sensor in neutrophils through TAK1 and TAB2, inducing NF- $\kappa$ B and AP-1 activation [136].

RNA polymerase III (RNA pol III) detects cytosolic DNA, inducing type I IFN through the RIG-I pathway. Indeed, cytosolic B form of dsDNA may be converted into 5'-pppRNA *via* RNA pol III in the cytosol to induce IFN $\beta$  [137]. LSm14a, a member of the LSm family involved in RNA processing, binds to synthetic or viral RNA, but also to DNA, mediating IRF3 activation and IFN $\beta$  induction [138]. Other cytosolic DNA sensors such as leucine-rich repeat in Flightless I interacting protein-1 (Lrrfip1) and DExD/H box helicases (DHX9 and DHX36) also trigger inflammation in response to nucleic acids [22, 23]. DNA sensors such as IFI16 or ZCCHC3 can cooperate or act with cGAS as cosensors [134]. Thus, the cytosolic surveillance system, and in particular cytosolic DNA sensors, is redundant, with several cytosolic DNA sensors being activated and cross-regulating each other.

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### **Figure 1. Nucleic Acid Sensing in Distinct Cellular Compartments**

**1.** Cell stress is characterized by mitochondrial damage, self-mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) release into the cytosol. **2.** Cell stress induces cell death and the release of self-DNA (mtDNA and nDNA) in the extracellular milieu. **3.** Both forms of DNA are found in the cytosol or in endosomal compartments. Ectopic self-DNA is sensed by cytosolic sensors such as cGAS to form 2'3'cGAMP, or by ZCCHC3, IFI16, DDX41, ZDHHC1 that may cooperate with cGAS. RNA pol III recognizes self-DNA to form 5'-pppRNA, sensed by the RIG-I-Like Receptor (RLR) machinery. **4.** After self-DNA recognition, STING is activated and phosphorylated by TBK1, leading to phosphorylation and dimerization of IRF3, and type I IFN response. **5.** In parallel, self-DNA may also be sensed by endosomal TLR9 to provide a first signal activating NF- $\kappa$ B, leading to the release of pro-IL-1 $\beta$  and pro-IL-18. **6.** Self-DNA sensing by cytosolic NLRP3 and AIM2 induces inflammasome activation, cleavage of immature pro-caspase 1 into mature caspase 1, and cleavage of pro-IL-1 $\beta$  and pro-IL-18 into mature IL-1 $\beta$  and IL-18.

### **Figure 2. Proposed Mechanism of Inflammatory Response Induced by Self-DNA in Lung Inflammation**

**1.** Lung damage induced by exogenous (particles, pollutants) or endogenous (senescence) trigger. Epithelial cell barrier, the first line defense of the host organism, becomes leaky. **2.** After cellular stress, self-DNA from mitochondria or nuclei is released into the bronchoalveolar space. Self-DNA is internalized by immune cells such as macrophages, lymphocytes and dendritic cells present at the inflammation site. The entry of extra-cellular self-DNA into immune cells is poorly understood. Cargo molecules such as IL-26 or the antimicrobial peptide LL-37 may shuttle DNA. **3.** Extracellular self-DNA is degraded by extracellular exonuclease DNase I **4.** Inside the cell, DNase II degrades self-DNA present in the endosomal compartment. **5.** Self-DNA escaping from the endosome to the cytosol is degraded by the nuclease TREX1. **6.** Self-DNA accumulation in the cytosol is sensed by cytosolic or endosomal receptors such as cGAS, NLRP3, AIM2 or TLR9. **7.** Intracellular self-DNA activates the cGAS/STING/TBK1/IRF3 pathway leading to type I IFN response. STING activation also leads to NF- $\kappa$ B activation and the production of pro-inflammatory cytokines. **8.** Lung inflammation characterized by type I IFN, IL-1 $\beta$ , IL-6 and TNF in response to self-DNA **8'**.

Self-DNA sensing amplifies cell death in a STING-dependent manner, and further self-DNA release into the bronchoalveolar space.