STING Mediates Cytosolic Dna-Induced Muc5ac Mrna Expression in Human Bronchial Epithelial Cells

Masaya Ohta

Nihon Shika Daigaku Niigata Seimei Shigakubu Daigakuin Niigata Seimei Shigakubu Kenkyuka

Yutaka Nishida

Gunma University Graduate School of Medicine

Hisako Yagi

Gunma University Graduate School of Medicine

Aikira Aizawa

Gunma University Graduate School of Medicine

Takahito Oyanagi

Niigata University Graduate School of Medical and Dental Sciences

Akihiko Saitoh

Niigata University Graduate School of Medical and Dental Sciences

Satoshi Yamada

Gunma University Graduate School of Medicine

Takashi Ishige

Gunma University Graduate School of Medicine

Yasuko Kobayashi

Gunma University Graduate School of Medicine

Hirokazu Arakawa

Gunma University Graduate School of Medicine

Takumi Takizawa (✉ takizawt@gunma-u.ac.jp)

Gunma University <https://orcid.org/0000-0003-2169-0787>

Research

Keywords: cytosol, epithelial cells, immunity, lung, mucins

Posted Date: June 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-37814/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

STING mediates cytosolic DNA-induced *MUC5AC* mRNA expression in human bronchial epithelial cells

Masaya Ota^{1,2}, Yutaka Nishida¹, Hisako Yagi¹, Akira Aizawa¹, Takahito Oyanagi^{1,2}, Akihiko Saitoh², Satoshi Yamada¹, Takashi Ishige¹, Yasuko Kobayashi¹, Hirokazu Arakawa¹, Takumi Takizawa^{1*}

¹ Gunma University Graduate School of Medicine, Department of Pediatrics

² Niigata University Graduate School of Medical and Dental Sciences, Department of Pediatrics

*Corresponding author

3-39-22 Showa-machi, Maebashi, Gunma, 371-8511, Japan

Phone: +81-27-220-8203; Fax: +81-27-220-8216; Email: takizawt@gunma-u.ac.jp (TT)

Short title: STING mediates *MUC5AC* expression

Abstract

Background: Non-autologous and autologous cytosolic DNA are recognized as danger signals by cytoplasmic sensor molecules that activate signal-transduction pathways. An important molecule in cytosolic DNA sensing is stimulator of interferon genes (STING), an endoplasmic reticulum protein activated by cyclic GMP–AMP (cGAMP) produced in response to cytosolic DNA. STING is important for innate immune responses to cytosolic DNA in immune cells; however, knowledge about its role in bronchial epithelial cells is limited.

Methods: We stimulated NCI-H292 cells with poly(dA:dT) and silenced STING and other regulatory proteins, and then determined *MUC5AC* mRNA expression levels.

Results: Cytosolic DNA increased the expression of a major respiratory mucin protein, *MUC5AC*, in the human respiratory epithelial cell line NCI-H292 in a STING-dependent manner. Introducing poly(dA:dT) into the cytoplasm induced *MUC5AC* and interferon- β (*IFN* β) expression. Silencing *STING* by RNA interference decreased poly(dA:dT)-induced *MUC5AC* mRNA expression but increased *IFN* β mRNA levels. Furthermore, cGAMP treatment increased *MUC5AC* expression but not *IFN* β expression. In contrast, silencing retinoic acid-inducible gene-I (*RIG-I*), which is a component of a different nucleic acid-sensing system, suppressed poly(dA:dT)-induced *IFN* β expression and increased *MUC5AC* expression.

Conclusions: Unlike its role in other cell types, in human bronchial epithelial cells, STING is central to cytosolic DNA-induced *MUC5AC* expression, whereas *IFN* β expression is dependent on RIG-I. Our data indicate a functional interaction between the STING and RIG-I pathways, suggesting the existence of intricate and cell-specific cytosolic DNA-sensing systems.

Keywords: cytosol, epithelial cells, immunity, lung, mucins

Introduction

Mucus secretion plays a pivotal role in eliminating pathogens from the airway epithelium [1]. *MUC5AC*, a representative mucin protein in the airways, is primarily secreted from goblet cells [2]. Although *MUC5AC* deficiency does not affect the clearance of bacteria in mice, its overexpression increases clearance [3], indicating that its secretion into the airways is protective. *MUC5AC* production is upregulated during lung inflammation caused by multiple disorders, including asthma and airway infections [4]. Proinflammatory cytokines, including IL-

4, IL-13, and transforming growth factor (TGF)- α , can induce *MUC5AC* expression [5], as can external agents such as cigarette smoke and virulent microorganisms [6].

Microorganisms trigger immune responses through pathogen-associated molecular patterns that are sensed by pattern-recognition receptors (PRRs) of host innate immune systems [7, 8]. An example of PRRs are the TLRs; the double-stranded poly(I:C) is a ligand for TLR3 that enhances *MUC5AC* expression at both the mRNA and protein levels [5]. In addition to TLRs, other PRRs for cytosolic RNA or DNA have been identified, including DEAD box polypeptide 41 [9], RNA polymerase (RNAP) III, retinoic acid-inducible gene-I (RIG-I), DNA-dependent activator of IFN-regulatory factors, and absent in melanoma 2 [10, 11]. Cytosolic DNA, derived from genomes (both nuclear and mitochondrial) and pathogenic microorganisms, is sensed as a danger signal, which activates innate immunity through these cytosolic DNA- and RNA-sensing mechanisms [12, 13]. Stimulator of interferon genes (STING) is an endoplasmic reticulum protein involved in cytosolic nucleic acid-sensing systems. It was first identified as a critical mediator of the production of proinflammatory cytokines, including type-I IFNs, in response to viral DNA [14]. STING is activated by cyclic GMP–AMP (cGAMP) produced by cGAMP synthase (cGAS), which senses cytosolic DNA [15]. STING has been implicated in innate immune responses to cytosolic DNA in multiple cell types [16, 17]. However, little is known about its role in bronchial epithelial cells, which are responsible for front-line pathogen defenses in the lungs. We investigated the role of STING in *MUC5AC* expression and unexpectedly found that STING and RIG-I serve different roles in human bronchial epithelial cells. Our results provide insights into the understanding of lung homeostasis and inflammation.

Methods

Cell culture and reagents

The human pulmonary mucoepidermoid carcinoma cell line NCI-H292 (ATCC CRL-1848) was maintained in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere with 5% (v/v) CO₂. The NCI-H292 cells were expanded in 24-well plates and maintained in serum-free RPMI 1640 medium for 6 h before stimulation with mithramycin A (Cayman Chemical, Ann Arbor, MI, USA) for 30 min or with ML-60218 (Cayman Chemical) for 2 h. Subsequently, the cells were transfected with 0.5 µg of poly(dA:dT; Thermo Fisher Scientific) using polyethylenimine MAX (Polysciences, Warrington, FL, USA) and incubated for 6 h. The transfected cells were subsequently exposed to 25 µg/mL poly(I: C; Sigma-Aldrich, St. Louis, MO, USA) and 4 ng/mL TGF- α (R&D Systems, Minneapolis, MN, USA) for 6 h at 37°C. For plasmid stimulation, the cells were transfected with 0.5 µg of pcDNA6.2-EGFP in 24-well plates, using FuGENE HD (Promega, Madison, WI, USA). For cGAMP (Thermo Fisher Scientific)-mediated cellular stimulation, cells were incubated in the presence of cGAMP for 16 h.

Gene knockdowns

For the gene-silencing experiments, we used small interfering RNAs (siRNAs) against *STING*, *RIG-I*, interferon regulatory factor 3 (*IRF3*), *RELA*, tripartite motif protein 32 (*TRIM32*), tripartite motif protein 56 (*TRIM56*) (Thermo Fisher Scientific), and DExH-box helicase 29 (*DHX29*) (Dharmacon, Inc., Lafayette, LA, USA) mRNAs, together with a control scrambled siRNA. Before transfection, NCI-H292 cells were seeded into 24-well plates and grown to 60% confluence. The cells were incubated in 500 µL of antibiotic-free RPMI 1640 medium

supplemented with 10% FBS. Then, a mixture comprised 100 μ L Opti-MEM (Thermo Fisher Scientific), 2.5 μ L 20 μ M siRNA for *DHX29* or 1.2 μ L 5 μ M siRNA for the remaining genes, and 1 μ L Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) was added to each well, followed by incubation for another 6 h. The medium was then changed to 500 μ L RPMI supplemented with 10% FBS and antibiotics. After 18 h, the cells were stimulated.

Reverse transcription (RT)-PCR and real-time quantitative PCR (qPCR) experiments

Total RNA was isolated using the ToTally RNA Kit (Thermo Fisher Scientific). RT was performed with 500 ng total RNA and oligo(dT) primers, using SuperScript III (Thermo Fisher Scientific). qPCR was performed with either KAPA SYBR FAST qPCR Master Mix (Roche) or TaqMan gene-expression assays (Thermo Fisher Scientific), using the ABI Prism 7900HT sequence detection system. TaqMan assays were used to detect *MUC5AC* (HS01365601_m1) and *18SrRNA* (4319413E). The sequences of the other primers used for the SYBR Green assays are shown in Supplementary Table 1. Gene-expression levels were normalized to 18S rRNA expression, and fold changes were calculated.

Statistical analyses

All data are expressed as the mean $\pm$ standard error of the mean. The results were analyzed using analysis of variance, followed by a post-hoc Tukey test or Dunnett test. Analyses were performed with SPSS statistics software version 24 (IBM). A *P* value of less than 0.05 was considered to reflect a statistically significant difference.

Results

Cytosolic DNA induced *MUC5AC* and *IFNB1* mRNA expression

Because cytosolic DNA evokes a primary immune response in immune cells [18] and mucin production participates in the primary immune response in bronchial epithelial cells [1], we analyzed whether cytosolic DNA could induce mucin production in bronchial epithelial cells. We transfected H292 respiratory epithelial cells with plasmid DNA, pcDNA6.2-EGFP, and measured the expression of *MUC5AC* mRNA. The plasmid DNA increased *MUC5AC* mRNA expression only in the presence of a lipofection reagent (Fig. 1a), which indicated that the plasmid DNA needed to enter the cells to induce *MUC5AC* expression. The *MUC5AC*-expression levels were comparable to that induced by synergistic stimulation with poly(I:C) and TGF- α [19-21]¹⁹⁻²¹.

Non-methylated CpG present in bacterial DNA can induce a host immune response after binding with TLR9 [22, 23]. We used a synthetic double-stranded DNA, poly(dA:dT), to determine whether plasmid DNA-induced *MUC5AC* expression requires non-methylated CpG. Transfecting poly(dA:dT) increased *MUC5AC* expression with approximately 5-fold greater efficiency than did transfecting a plasmid (Fig. 1b). In addition, *TLR9* was not detected in H292 cells by RT-PCR (data not shown), indicating that DNA-sensing molecules other than TLR9 mediate this effect. Because poly(dA:dT) induces interferons in macrophages and fibroblasts [24-26], we next examined the expression levels of *IFNA* and *IFNB* after poly(dA:dT) stimulation in respiratory epithelial cells. The expression of *IFNA1* did not increase significantly (Fig. 1c), whereas *IFNB1* expression was significantly increased by the stimulation (Fig. 1d).

STING was central for cytosolic DNA-induced MUC5AC mRNA expression

An increasing body of evidence has demonstrated that STING is essential for immune cell responses to cytosolic DNA [27]. Therefore, to determine whether the same mechanism operates in bronchial epithelial cells, we knocked down STING expression using RNA interference (RNAi; Supplementary Fig. 1a). Knocking down STING suppressed poly(dA:dT)-induced *MUC5AC* expression to approximately one-third of the control value in H292 cells (Fig. 2a). We next investigated the involvement of other pathways known to respond to cytosolic nucleic acids. The RIG-I pathway is activated by single- or double-stranded RNA bearing a 5'-triphosphate. Poly(dA:dT) is transcribed by RNAP III to produce a double-stranded RNA, which can therefore activate the pathway [28, 29]. To determine whether RIG-I is involved in *MUC5AC* expression, we used RNAi to silence *RIG-I* in H292 cells (Supplementary Fig. 1b). Poly(dA:dT)-induced *MUC5AC* expression was not suppressed but was instead enhanced (Fig. 2b). To ascertain whether this pathway might be relevant to *MUC5AC* expression, we inhibited RNAP III using the specific inhibitor ML-60218 [30] at a concentration that blocked the transcription of 5S rRNA (30 μ M) [28] (Supplementary Fig. 2). The inhibition of RNAP III had no statistically significant effect on poly(dA:dT)-induced *MUC5AC* expression (Fig. 2c).

cGAMP is produced by cGAS after cytosolic DNA recognition leads to STING activation [31]. Therefore, we next tested whether cGAMP could induce *MUC5AC* expression. Treatment with cGAMP increased *MUC5AC* expression in a dose-dependent manner (Fig. 2d). The induction depended on STING, as silencing *STING* reduced cGAMP-induced *MUC5AC* expression (Fig. 2e), whereas the induction was increased by knocking down *TRIM 32/56*, a negative regulator of STING [32, 33] (Fig. 2e).

Nuclear factor kappa B (NF-κB) and specificity protein 1 (SP1) regulate MUC5AC mRNA expression

NF-κB and IRF3 have been shown to be activated downstream of STING [34, 35]. We therefore investigated whether poly(dA:dT)-induced *MUC5AC* expression involved these pathways. We knocked down *RELA* [36, 37], which encodes p65 of the NF-κB pathway or *IRF3* [38] (Supplementary Fig. 1e and 1f) and examined *MUC5AC* expression. The expression of *MUC5AC* mRNA was significantly suppressed by *RELA* RNAi, but not by *IRF3* RNAi (Fig. 3a).

Furthermore, we explored whether the transcription factor SP1, which has been shown to play an important role in *MUC5AC* expression [39, 40], is also involved in poly(dA:dT)-induced *MUC5AC* expression. Mithramycin A [21, 41], an inhibitor of SP1, suppressed poly(dA:dT)-induced *MUC5AC* expression in a dose-dependent manner (Fig. 3b).

IFNB1 mRNA expression depended on RIG-I, but not STING and cGAMP

STING was originally identified and annotated based on its central role in the expression of IFNs in response to cytosolic DNA [42]. Therefore, we wanted to know whether STING helps regulate *IFNB1* expression. In contrast to *MUC5AC*, poly(dA:dT)-induced *IFNB1* mRNA expression was not repressed in *STING*-knockdown cells, but rather was significantly increased (Fig. 4a). cGAMP also failed to induce *IFNB1* expression, irrespective of RNAi-based knockdown of *TRIM 32/56* or *STING* (Fig. 4b). In contrast, however, *IFNB1* expression was repressed by knocking down *RIG-I* (Fig. 4c). Therefore, we examined whether RNAP III was involved in the expression of *IFNB1*. Inhibition of RNAP III by ML-60218 failed to suppress *IFNB1* expression (Fig. 4d).

DHX29 was involved in *IFNB1* mRNA expression in response to cytosolic DNA

Because suppressing RNAP III function with the inhibitor did not affect *IFNB1* expression (Fig. 4d), we hypothesized that other molecules are involved in the RIG-I activation. One candidate is DHX29, an RNA helicase expressed in multiple tissues, which can sense intracellular nucleic acids and directly bind to and activate RIG- I [43]. *DHX29* knockdown suppressed poly(dA:dT)-induced *IFNB1* expression (Fig. 5a), whereas it did not suppress *MUC5AC* expression (Fig. 5b).

***IFNB* mRNA expression in response to cytosolic DNA was suppressed by knocking down NF- κ B and IRF3**

We next investigated whether NF- κ B and IRF3 are related to *IFNB1* expression in response to cytosolic DNA signaling. *IFNB1* expression was partially suppressed when the *RELA* or *IRF3* genes were silenced with RNAi (Fig. 6a). In addition, mithramycin A did not affect cytosolic DNA-induced *IFNB1* expression (Fig. 6b).

Discussion

Here, we report that cytosolic DNA induces mucin production in airway epithelial cells, which represent the front-line defense of airway tissues. In addition, we found that STING, whose importance in innate immunity is becoming increasingly recognized, played a critical role in cytosolic DNA-induced *MUC5AC* expression. Although STING is named for its role in inducing interferons in immune system cells, we found that STING was not required for *IFN* gene expression in bronchial epithelial cells. Intriguingly, our data indicate that *IFNB1* expression was instead dependent on RIG-I, which constitutes a different nucleic acid-sensing

pathway. Our findings indicate that tissue specificity may occur among different cytosolic nucleic acid-sensing mechanisms. In other words, common cytosolic DNA stimuli responses use different nucleic acid-sensing systems to induce different target genes. There have been few reports on the selective use of such DNA-sensing mechanisms to date, and elucidation of the molecular mechanisms involved will be important in the future.

Foreign microorganisms are not merely sources of cytosolic DNA, as they also produce cGAMP themselves [44, 45]. We showed that cGAMP loading strongly induced *MUC5AC* expression. These findings suggest an indispensable role for STING in *MUC5AC* expression in the respiratory epithelium in response to infection. Cytosolic DNA can originate from both exogenous sources and self-DNA damage when DNA is released into the cytoplasm, resulting from mitochondrial disorders [46], UV irradiation, and DNA replication/recombination errors [47]. *MUC5AC* can be induced by a wide variety of factors other than respiratory pathogens, including chronic or acute inflammation, cigarette smoke, and air pollutants. Many of these factors are known to evoke mitochondrial disorders through cellular stresses, including endoplasmic reticulum stress and oxidative stress [48, 49]. We therefore propose that mitochondrial DNA induces *MUC5AC* expression through STING activation; this hypothesis is currently being tested. Taken together, our results suggest that STING plays important roles in lung homeostasis and pathogenesis, at least in part by controlling mucin production in the airway epithelium.

Inhibiting SP1 reduced the expression of *MUC5AC*, which was induced by poly(dA:dT). A body of evidence shows that SP1 plays an important role in *MUC5AC* expression [34]; however, our current results did not delineate whether SP1 was specifically involved in STING-mediated *MUC5AC* expression. In addition, no previous reports have described the activation of

SP1 downstream of STING. As SP1 has been shown to be essential in *MUC5AC* induction by various stimuli, SP1 is likely an essential factor that supports *MUC5AC* basal transcription, as shown for other SP1-regulated genes [50, 51].

STING was not involved in *IFNB* expression in respiratory epithelial cells, consistent with a previous observation made by Sugimoto et al. [43]. Instead, they and we showed that DHX29 and RIG-I played roles in *IFNB* expression [38] (Figs. 4 and 5). Inhibiting RNAP III function did not affect *IFNB1* expression, suggesting that a DHX29–RIG-I pathway rather than an RNAP III–RIG-I pathway regulates *IFNB1* expression in bronchial epithelial cells. Currently, the mechanisms underlying the cell-specificity of STING function are unknown and uncovering them will provide insights into the roles of STING in different tissue types.

When STING expression was knocked down, *IFNB1* expression unexpectedly increased (Fig. 4a). Similarly, RIG-I knockdown increased *MUC5AC* expression (Fig. 2b). These findings suggest an interaction between STING and RIG-I in bronchial epithelial cells; this has been observed in other cell types [52]. Wu et al. [53] recently reported that the STING protein was degraded in an RIG-I-mediated manner and that knocking down *RIG-I* increased STING protein levels by reducing its degradation. Furthermore, RIG-I expression was increased by cytosolic DNA, which was mediated by STING; hence, STING knockdown inhibited the increase in *RIG-I* expression [53]. The increase in *MUC5AC* expression we observed after RIG-I knockdown (Fig. 2b) likely reflects this mechanism, in turn indicating that crosstalk may occur between DNA- and RNA-sensing mechanisms in bronchial epithelial cells.

This study had a few limitations. First, we only used a single type of epithelial cell line. Using normal human bronchial epithelial cells (NHBEs) would have provided results closely reflecting the physiological conditions of the airway but would have shown batch-to-batch

variations in the results. In addition, it would have been more challenging to manipulate gene expression in NHBs than in the NCI-H292 cell line. Nevertheless, our previous findings in NCI-H292 cells helped improve the understanding of airway epithelial cells [54-56]. Second, we focused on changes in *MUC5AC* gene expression, but did not MUC5AC protein expression. This is because understanding the mechanisms of *MUC5AC* gene expression is vital for gaining insights into MUC5AC protein expression. The third limitation is that as a stimulant, we only used cytosolic poly(dA:dT). Using a natural immunostimulant such as a virus would more closely mimic inflammatory airway diseases but using such complex organisms would activate multiple pathways simultaneously. Using poly(dA:dT) enabled us to focus on how *MUC5AC* was expressed by cytosolic DNA.

In summary, our findings suggest that cytosolic DNA induces *MUC5AC* and *IFNB1* gene expression through different pathways, including STING- and RIG-I- pathways, in human bronchial epithelial cells. Our data also suggest functional interactions between these two pathways. Therefore, controlling STING function in the airway epithelium may be useful in chronic lung disease therapy.

List of abbreviations:

DAMP, damage-associated molecular-pattern; FBS, fetal bovine serum; TGF, transforming growth factor; PRRs, pattern-recognition receptors; RNAP, RNA polymerase; RIG-I, retinoic acid-inducible gene-I; STING, stimulator of interferon genes; cGAMP, cyclic GMP–AMP; cGAS, cGAMP synthase; RT, reverse transcription; NF-Kb, nuclear factor kappa B; NHBs, normal human bronchial epithelial cells.

Declarations

274 **Ethics Approval and consent**

275 Not applicable.

276 **Consent for publication**

277 Not applicable.

278 **Availability of data and materials**

279 The datasets supporting the conclusions of this article are included within the article and its
280 additional supplemental files.

281 **Competing interests**

282 The authors have no conflict of interest and no financial support to disclose.

283 **Funding**

284 There was no funding for this study.

285 **Authors' contributions**

286 MO, TT, YN, AA, TO, AS, SY, and HA conceived and designed the experiments; MO, TT, YN,
287 HY, AA, TO, SY, and TI performed the experiments; MO, TT, AA, and HA analyzed the data;
288 MO, TT, SY, TI, YK, and HA drafted the manuscript.

289 **Acknowledgements**

290 We thank Sachiko Hayashi and Kiyoe Ishii for providing technical assistance. We also thank all
291 members of the Department of Pediatrics of Gunma University Graduate School of Medicine for
292 their help and advice. We would like to thank Editage (www.editage.jp) for English language
293 editing.

294

295 **References**

- 296 1. Weitnauer M, Mijosek V, Dalpke AH. Control of local immunity by airway epithelial cells.
297 *Mucosal Immunol* 2016; **9**: 287-98.
- 298 2. Roy MG, Livraghi-Butrico A, Fletcher AA, McElwee MM, Evans SE, Boerner RM,
299 Alexander SN, et al. Muc5b is required for airway defence. *Nature* 2014; **505**: 412.

- 300 3. Ehre C, Worthington EN, Liesman RM, Grubb BR, Barbier D, O'Neal WK, et al.
301 Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs.
302 *Proc Natl Acad Sci U S A* 2012; **109**: 16528-33.
- 303 4. Kuyper LM, Pare PD, Hogg JC, Lambert RK, Ionescu D, Woods R, et al. Characterization of
304 airway plugging in fatal asthma. *Am J Med* 2003; **115**: 6-11.
- 305 5. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, et al. Epidermal growth
306 factor system regulates mucin production in airways. *Proc Natl Acad Sci U S A* 1999; **96**:
307 3081-6.
- 308 6. Shao MX, Nakanaga T, Nadel JA. Cigarette smoke induces MUC5AC mucin overproduction
309 via tumor necrosis factor- α -converting enzyme in human airway epithelial (NCI-H292)
310 cells. *Am J Physiol Lung Cel Mol Physiol* 2004; **287**: L420-7.
- 311 7. Rubartelli A, Lotze MT. Inside, outside, upside down: damage-associated molecular-pattern
312 molecules (DAMPs) and redox. *Trends Immunol* 2007; **28**: 429-36.
- 313 8. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur
314 autophagy and immunity. *Immunol Rev* 2012; **249**: 158-75.
- 315 9. Zhang Z, Yuan B, Bao M, Lu N, Kim T, Liu YJ. The helicase DDX41 senses intracellular
316 DNA mediated by the adaptor STING in dendritic cells. *Nat Immunol* 2011; **12**: 959-65.
- 317 10. Corrales L, Woo SR, Williams JB, McWhirter SM, Dubensky TW, Jr., Gajewski TF.
318 Antagonism of the STING pathway via activation of the AIM2 inflammasome by
319 intracellular DNA. *J Immunol* 2016; **196**: 3191-8.
- 320 11. Choi MK, Wang Z, Ban T, Yanai H, Lu Y, Koshiba R, et al. A selective contribution of the
321 RIG-I-like receptor pathway to type I interferon responses activated by cytosolic DNA.
322 *Proc Natl Acad Sci U S A* 2009; **106**: 17870-5.

- 323 12. Wu X, Wu FH, Wang X, Wang L, Siedow JN, Zhang W, Pei ZM. Molecular evolutionary
324 and structural analysis of the cytosolic DNA sensor cGAS and STING. *Nucleic Acids Res*
325 2014; **42**: 8243-57.
- 326 13. O'Neill LA. Immunology. Sensing the dark side of DNA. *Science* 2013; **339**: 763-4.
- 327 14. Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, Hyodo M, et al. STING is a
328 direct innate immune sensor of cyclic di-GMP. *Nature* 2011; **478**: 515.
- 329 15. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor
330 that activates the type I interferon pathway. *Science* 2013; **339**: 786-91.
- 331 16. Dunphy G, Flannery SM, Almine JF, Connolly DJ, Paulus C, Jonsson KL, et al. Non-
332 canonical activation of the DNA sensing adaptor STING by ATM and IFI16 mediates
333 NF-kappaB signaling after nuclear DNA damage. *Mol Cell* 2018; **71**: 745-60.e745.
- 334 17. Kitai Y, Kawasaki T, Sueyoshi T, Kobiyama K, Ishii KJ, Zou J, et al. DNA-containing
335 exosomes derived from cancer cells treated with topotecan activate a STING-dependent
336 pathway and reinforce antitumor immunity. *J Immunol* 2017; **198**: 1649-59.
- 337 18. Stetson DB, Medzhitov R. Recognition of cytosolic DNA activates an IRF3-dependent innate
338 immune response. *Immunity* 2006; **24**: 93-103.
- 339 19. Tadaki H, Arakawa H, Mizuno T, Suzuki T, Takeyama K, Mochizuki H, et al. Double-
340 stranded RNA and TGF-alpha promote MUC5AC induction in respiratory cells. *J*
341 *Immunol* 2009; **182**: 293-300.
- 342 20. Takami S, Mizuno T, Oyanagi T, Tadaki H, Suzuki T, Muramatsu K, et al. Glucocorticoids
343 inhibit MUC5AC production induced by transforming growth factor-alpha in human
344 respiratory cells. *Allergol Int* 2012; **61**: 451-9.

- 345 21. Oyanagi T, Takizawa T, Aizawa A, Solongo O, Yagi H, Nishida Y, et al. Suppression of
346 MUC5AC expression in human bronchial epithelial cells by interferon-gamma. *Allergol*
347 *Int* 2017; **66**: 75-82.
- 348 22. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR9
349 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* 2004;
350 **5**: 190-8.
- 351 23. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor
352 recognizes bacterial DNA. *Nature* 2000; **408**: 740.
- 353 24. Chamilos G, Gregorio J, Meller S, Lande R, Kontoyiannis DP, Modlin RL, et al. Cytosolic
354 sensing of extracellular self-DNA transported into monocytes by the antimicrobial
355 peptide LL37. *Blood* 2012; 120: 3699-707.
- 356 25. Lippmann J, Rothenburg S, Deigendesch N, Eitel J, Meixenberger K, van Laak V, et al.
357 IFNbeta responses induced by intracellular bacteria or cytosolic DNA in different human
358 cells do not require ZBP1 (DLM-1/DAI). *Cell Microbiol* 2008; **10**: 2579-88.
- 359 26. Melchjorsen J, Rintahaka J, Soby S, Horan KA, Poltajainen A, Ostergaard Let al. Early
360 innate recognition of herpes simplex virus in human primary macrophages is mediated
361 via the MDA5/MAVS-dependent and MDA5/MAVS/RNA polymerase III-independent
362 pathways. *J Virol* 2010; **84**: 11350-8.
- 363 27. Burdette DL, Vance RE. STING and the innate immune response to nucleic acids in the
364 cytosol. *Nat Immunol* 2013; **14**: 19-26.
- 365 28. Chiu YH, Macmillan JB, Chen ZJ. RNA polymerase III detects cytosolic DNA and induces
366 type I interferons through the RIG-I pathway. *Cell* 2009; **138**: 576-91.

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

370 30. Wu L, Pan J, Thoroddsen V, Wysong DR, Blackman RK, Bulawa CE, et al. Novel small-
371 molecule inhibitors of RNA polymerase III. *Eukaryotic Cell* 2003; **2**: 256-64.

372 31. Tao J, Zhou X, Jiang Z. cGAS-cGAMP-STING: the three musketeers of cytosolic DNA
373 sensing and signaling. *IUBMB Life* 2016; **68**: 858-70.

374 32. Zhang J, Hu MM, Wang YY, Shu HB. TRIM32 protein modulates type I interferon induction
375 and cellular antiviral response by targeting MITA/STING protein for K63-linked
376 ubiquitination. *J Biol Chem* 2012; **287**: 28646-55.

377 33. Tsuchida T, Zou J, Saitoh T, Kumar H, Abe T, Matsuura Y, Kawai T, Akira S. The ubiquitin
378 ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA.
379 *Immunity* 2010; **33**: 765-76.

380 34. Abe T, Barber GN. Cytosolic-DNA-mediated, STING-dependent proinflammatory gene
381 induction necessitates canonical NF-kappaB activation through TBK1. *J Virol* 2014; **88**:
382 5328-41.

383 35. Liu S, Cai X, Wu J, Cong Q, Chen X, Li T, et al. Phosphorylation of innate immune adaptor
384 proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* 2015; **347**: aaa2630.

385 36. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved
386 mediators of immune responses. *Annu Rev Immunol* 1998; **16**: 225-60.

387 37. Ghosh S, Karin M. Missing pieces in the NF-kB puzzle. *Cell* 2002; **109**: S81-96.

388 38. Ysebrant de Lendonck L, Martinet V, Goriely S. Interferon regulatory factor 3 in adaptive
389 immune responses. *Cell Mol Life Sci* 2014; **71**: 3873-83.

390 39. Jonckheere N, Van Der Sluis M, Velghe A, Buisine MP, Suttmuller M, et al. Transcriptional
391 activation of the murine Muc5ac mucin gene in epithelial cancer cells by TGF-
392 beta/Smad4 signalling pathway is potentiated by Sp1. *Biochem J* 2004; **377**: 797-808.

393 40. Di YP, Zhao J, Harper R. Cigarette smoke induces MUC5AC protein expression through the
394 activation of Sp1. *J Biol Chem* 2012; **287**: 27948-58.

395 41. Jia Z, Zhang J, Wei D, Wang L, Yuan P, Le X, et al. Molecular basis of the synergistic
396 antiangiogenic activity of bevacizumab and mithramycin A. *Cancer Res* 2007; **67**: 4878-
397 85.

398 42. Chen Q, Sun L, Chen ZJ. Regulation and function of the cGAS-STING pathway of cytosolic
399 DNA sensing. *Nat Immunol* 2016; **17**: 1142-9.

400 43. Sugimoto N, Mitoma H, Kim T, Hanabuchi S, Liu YJ. Helicase proteins DHX29 and RIG-I
401 cosense cytosolic nucleic acids in the human airway system. *Proc Natl Acad Sci U S A*
402 2014; **111**: 7747-52.

403 44. Liu Y, Goulet ML, Sze A, Hadj SB, Belgnaoui SM, Lababidi RR, et al. RIG-I-mediated
404 STING upregulation restricts Herpes simplex virus 1 infection. *J Virol* 2016; **90**: 9406-
405 19.

406 45. Ablasser A, Schmid-Burgk JL, Hemmerling I, Horvath GL, Schmidt T, Latz E, et al. Cell
407 intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP.
408 *Nature* 2013; **503**: 530.

409 46. Liu S, Feng M, Guan W. Mitochondrial DNA sensing by STING signaling participates in
410 inflammation, cancer and beyond. *Int J Cancer* 2016; **139**: 736-41.

- 411 47. Hartlova A, Erttmann SF, Raffi FA, Schmalz AM, Resch U, Anugula S, et al. DNA damage
412 primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-
413 microbial innate immunity. *Immunity* 2015; **42**: 332-43.
- 414 48. Rongvaux A, Jackson R, Harman CC, Li T, West AP, de Zoete MR, et al. Apoptotic caspases
415 prevent the induction of type I interferons by mitochondrial DNA. *Cell* 2014; **159**: 1563-
416 77.
- 417 49. White MJ, McArthur K, Metcalf D, Lane RM, Cambier JC, Herold MJ, et al. Apoptotic
418 caspases suppress mtDNA-induced STING-mediated type I IFN production. *Cell* 2014;
419 **159**: 1549-62.
- 420 50. Liu J, Yang H, Liu W, Cao X, Feng X. Sp1 and Sp3 regulate the basal transcription of
421 receptor activator of nuclear factor kappa B ligand gene in osteoblasts and bone marrow
422 stromal cells. *J Cell Biochem* 2005; **96**: 716-27.
- 423 51. Xu HG, Jin R, Ren W, Zou L, Wang Y, Zhou GP. Transcription factors Sp1 and Sp3 regulate
424 basal transcription of the human IRF-3 gene. *Biochimie* 2012; **94**: 1390-7.
- 425 52. Zevini A, Olganier D, Hiscott J. Crosstalk between cytoplasmic RIG-I and STING sensing
426 pathways. *Trends Immunol* 2017; **38**: 194-205.
- 427 53. Wu X, Yang J, Na T, Zhang K, Davidoff AM, Yuan BZ, et al. RIG-I and IL-6 are negative-
428 feedback regulators of STING induced by double-stranded DNA. *PLoS One* 2017; **12**:
429 e0182961.
- 430 54. Kim S, Beyer BA, Lewis C, Nadel JA. Normal CFTR inhibits epidermal growth factor
431 receptor-dependent pro-inflammatory chemokine production in human airway epithelial
432 cells. *PLoS One* 2013; **8**: e72981.

- 433 55. Kim S, Lewis C, Nadel JA. Epidermal growth factor receptor reactivation induced by E-
434 prostanoid-3 receptor-and tumor necrosis factor-alpha-converting enzyme-dependent
435 feedback exaggerates interleukin-8 production in airway cancer (NCI-H292) cells. *Exp*
436 *Cell Res* 2011; **317**: 2650-60.
- 437 56. Kim S, Lewis C, Nadel JA. CCL20/CCR6 feedback exaggerates epidermal growth factor
438 receptor-dependent MUC5AC mucin production in human airway epithelial (NCI-H292)
439 cells. *J Immunol* 2011; **186**: 3392-400.

Figure legends

Fig. 1. Cytosolic DNA induced *MUC5AC* and *IFNB1* mRNA expression. (a and b) RT-PCR for *MUC5AC* mRNA. (a) NCI-H292 cells were treated with a plasmid (with or without polyethylenimine) or stimulated with TGF- α (4 ng/mL) and poly(I:C) for 6 h (n = 8). (b) Plasmid and poly(dA:dT) (0.5 μ g/well) lipofected into H292 cells (n = 8, $P < 0.001$). (c) *IFNA1* mRNA expression (with or without poly[dA:dT] stimulation) was determined in H292 cells (n = 4, $P = 0.6678$). (d) Expression of *IFNB1* mRNA, with or without poly(dA:dT) (n = 4, $P < 0.001$). The data are presented as fold changes relative to untreated controls after being normalized to 18S rRNA levels. The bars in panels a–d represent means $\pm$ SEMs. *** $P < 0.001$, as determined using Tukey' test.

Fig. 2. *MUC5AC* mRNA expression induced by cytosolic DNA required STING and cGAMP. (a and b) H292 cells were transfected with siRNAs against *STING* (a) or *RIG-I* (b) mRNA, or a control siRNA, for 6 h and then treated with poly(dA:dT) for an additional 6 h. *MUC5AC*-expression levels were determined by RT-PCR and are presented as fold changes relative to that without poly(dA:dT) (A, n = 14, *** $P < 0.001$; B, n = 8, *** $P < 0.001$). (c) H292 cells were treated with 30 μ M ML-60218 for 2 h and then with poly(dA:dT) for 6 h. *MUC5AC*-expression levels were determined by RT-PCR and are expressed as fold changes relative to that in untreated cells (n = 4, $P = 0.111$). (d and e) Effects of cGAMP on *MUC5AC* expression. H292 cells were incubated with cGAMP (3.3 or 10 μ g/mL) for 16 h (n = 4, *** $P < 0.001$). (e) H292 cells were treated with control siRNA, *siSTING*, or *siTRIM32/56* for 6 h before cGAMP stimulation. (n = 4, * $P = 0.012$ and *** $P < 0.001$). The error bars in panels A–E represent means $\pm$ SEMs. *** $P < 0.001$, * $P < 0.05$, as determined using the Tukey test for the experiments shown in panel B and the Dunnett test for the experiments shown in the other panels.

464

465 **Fig. 3. NF- κ B and SP1 were involved in cytosolic DNA-induced *MUC5AC* expression.** (a)

466 H292 cells were transfected with *RELA*, *IRF3*, or control siRNA for 6 h and then stimulated with
467 poly(dA:dT) for 6 h (n = 8). Knocking down the *RELA* gene suppressed *MUC5AC* expression (P
468 < 0.001). (b) Cells were treated with poly(dA:dT) for 4.5 h and stimulated with different
469 concentrations of the SP1 inhibitor mithramycin for 17 h. *MUC5AC* expression was suppressed
470 by mithramycin in a dose-dependent manner (n = 4, $**P = 0.004$ and $***P < 0.001$). The error
471 bars in (a) and (b) represent means $\pm$ SEMs. $***P < 0.001$, $**P < 0.01$ using the Dunnett test.

472

473 **Fig. 4. RIG-I but not cGAMP or STING played a role in *IFNB1* upregulation.** (a) *IFNB1*

474 expression was determined after cells were treated with a control or STING siRNA for 6 h and
475 stimulated with poly(dA:dT) for an additional 6 h. *IFNB1* expression increased after STING
476 knockdown (n = 8, $P < 0.001$). (b) Cells were treated with the indicated siRNAs for 6 h and then
477 with cGAMP for 16 h. *IFNB1* mRNA expression did not increased after incubation with either
478 3.3 or 10 μ g/mL cGAMP, using any of the three siRNAs (n = 4). (c) Cells were transfected with
479 siRNA against *RIG-I* and stimulated with poly(dA:dT). *IFNB1* expression decreased after RIG-I
480 knockdown (n = 4, $P < 0.001$). (d) Cells were treated with the RNA polymerase III inhibitor ML-
481 60218 for 2 h and then stimulated with poly(dA:dT) for 6 h. No change in *IFNB1* expression was
482 observed (n = 4, $P = 0.873$). The bars in panels a–d represent means $\pm$ SEMs. $***P < 0.001$, as
483 determined using the Tukey test for the experiments shown in panel b and the Dunnett test for
484 the experiments shown in the other panels.

485

Fig. 5. DHX29 participated in cytosolic DNA-induced *IFNB1* expression. (a and b) Cells were treated with siRNA for 6 h and stimulated with poly(dA:dT) for 6 h. The mRNA expression levels of (a) *IFNB1* and (b) *MUC5AC* were determined by RT-PCR. *IFNB1* was inhibited by DHX29 knockdown ($n = 12$, $P = 0.003$) (a), whereas a significant difference was not observed for *MUC5AC* expression (b) ($n = 12$, $P = 0.270$). The error bars represent means $\pm$ SEMs. $**P < 0.01$, as determined using the Tukey test.

Fig. 6. NF- κ B and IRF3, but not SP1, were involved in *IFNB1* mRNA expression. (a) Cells were treated with siRNA for 6 h and then stimulated with poly(dA:dT) for 6 h. *IFNB1* expression was suppressed by *RELA* knockdown ($P = 0.003$) and *IRF3* knockdown ($P = 0.002$) ($n = 12$). (b) Cells were treated with poly(dA:dT) for 4.5 h and stimulated with the SP1 inhibitor mithramycin A for 17 h ($n = 4$). The error bars represent means $\pm$ SEMs. $**P < 0.01$, as determined using the Dunnett test.

Figure 1. Ota et al.

Figure 2. Ota et al.

Figure 3. Ota et al.

Figure 4. Ota et al.

Figure 5. Ota et al.

508 Figure 6. Ota et al.

Figures

Figure 1

Cytosolic DNA induced MUC5AC and IFNB1 mRNA expression. (a and b) RT-PCR for MUC5AC mRNA. (a) NCI-H292 cells were treated with a plasmid (with or without polyethylenimine) or stimulated with TGF- α (4 ng/mL) and poly(I:C) for 6 h (n = 8). (b) Plasmid and poly(dA:dT) (0.5 μ g/well) lipofected into H292 cells (n = 8, P < 0.001). (c) IFNA1 mRNA expression (with or without poly[dA:dT] stimulation) was determined in H292 cells (n = 4, P = 0.6678). (d) Expression of IFNB1 mRNA, with or without poly(dA:dT) (n = 4, P < 0.001). The data are presented as fold changes relative to untreated controls after being normalized to 18S rRNA levels. The bars in panels a–d represent means $\pm$ SEMs. ***P < 0.001, as determined using Tukey' test.

Figure 2

MUC5AC mRNA expression induced by cytosolic DNA required STING and cGAMP. (a and b) H292 cells were transfected with siRNAs against STING (a) or RIG-I (b) mRNA, or a control siRNA, for 6 h and then treated with poly(dA:dT) for an additional 6 h. MUC5AC-expression levels were determined by RT-PCR and are presented as fold changes relative to that without poly(dA:dT) (A, $n = 14$, $***P < 0.001$; B, $n = 8$, $***P < 0.001$). (c) H292 cells were treated with 30 μM ML-60218 for 2 h and then with poly(dA:dT) for 6 h. MUC5AC expression levels were determined by RT-PCR and are expressed as fold changes relative to that in untreated cells ($n = 4$, $P = 0.111$). (d and e) Effects of cGAMP on MUC5AC expression. H292 cells were incubated with cGAMP (3.3 or 10 $\mu\text{g/mL}$) for 16 h ($n = 4$, $***P < 0.001$). (e) H292 cells were treated with control siRNA, siSTING, or siTRIM32/56 for 6 h before cGAMP stimulation. ($n = 4$, $*P = 0.012$ and $***P < 0.001$). The error bars in panels A–E represent means $\pm$ SEMs. $***P < 0.001$, $*P < 0.05$, as determined using the Tukey test for the experiments shown in panel B and the Dunnett test for the experiments shown in the other panels.

Figure 3

NF- κ B and SP1 were involved in cytosolic DNA-induced MUC5AC expression. (a) H292 cells were transfected with RELA, IRF3, or control siRNA for 6 h and then stimulated with poly(dA:dT) for 6 h ($n = 8$). Knocking down the RELA gene suppressed MUC5AC expression ($P < 0.001$). (b) Cells were treated with poly(dA:dT) for 4.5 h and stimulated with different concentrations of the SP1 inhibitor mithramycin for 17 h. MUC5AC expression was suppressed by mithramycin in a dose-dependent manner ($n = 4$, $**P = 0.004$ and $***P < 0.001$). The error bars in (a) and (b) represent means $\pm$ SEMs. $***P < 0.001$, $**P < 0.01$ using the Dunnett test.

Figure 4

RIG-I but not cGAMP or STING played a role in IFNB1 upregulation. (a) IFNB1 expression was determined after cells were treated with a control or STING siRNA for 6 h and stimulated with poly(dA:dT) for an additional 6 h. IFNB1 expression increased after STING knockdown ($n = 8$, $P < 0.001$). (b) Cells were treated with the indicated siRNAs for 6 h and then with cGAMP for 16 h. IFNB1 mRNA expression did not increase after incubation with either 3.3 or 10 $\mu\text{g/mL}$ cGAMP, using any of the three siRNAs ($n = 4$). (c) Cells were transfected with siRNA against RIG-I and stimulated with poly(dA:dT). IFNB1 expression decreased after RIG-I knockdown ($n = 4$, $P < 0.001$). (d) Cells were treated with the RNA polymerase III inhibitor ML-60218 for 2 h and then stimulated with poly(dA:dT) for 6 h. No change in IFNB1 expression was observed ($n = 4$, $P = 0.873$). The bars in panels a–d represent means $\pm$ SEMs. *** $P < 0.001$, as determined using the Tukey test for the experiments shown in panel b and the Dunnett test for the experiments shown in the other panels.

Figure 5

DHX29 participated in cytosolic DNA-induced *IFNB1* expression. (a and b) Cells were treated with siRNA for 6 h and stimulated with poly(dA:dT) for 6 h. The mRNA expression levels of (a) *IFNB1* and (b) *MUC5AC* were determined by RT-PCR. *IFNB1* was inhibited by DHX29 knockdown ($n = 12$, $P = 0.003$) (a), whereas a significant difference was not observed for *MUC5AC* expression (b) ($n = 12$, $P = 0.270$). The error bars represent means $\pm$ SEMs. $**P < 0.01$, as determined using the Tukey test.

Figure 6

NF- κ B and IRF3, but not SP1, were involved in IFNB1 mRNA expression. (a) Cells were treated with siRNA for 6 h and then stimulated with poly(dA:dT) for 6 h. IFNB1 expression was suppressed by RELA knockdown ($P = 0.003$) and IRF3 knockdown ($P = 0.002$) ($n = 12$). (b) Cells were treated with poly(dA:dT) for 4.5 h and stimulated with the SP1 inhibitor mithramycin A for 17 h ($n = 4$). The error bars represent means $\pm$ SEMs. $**P < 0.01$, as determined using the Dunnett test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryDataCombined.pdf](#)