TREX1 Deficiency Triggers Cell-Autonomous Immunity in a cGAS-Dependent Manner

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TREX1 Deficiency Triggers Cell-Autonomous Immunity in a cGAS-Dependent Manner

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Cytosolic detection of DNA is crucial for the initiation of antiviral immunity but can also cause autoimmunity in the context of endogenous nucleic acids being sensed. Mutations in the human 3’ repair exonuclease 1 (TREX1) have been linked to the type I IFN-associated autoimmune disease Aicardi–Goutiéres syndrome. The exact mechanisms driving unbridled type I IFN responses in the absence of TREX1 are only partly understood, but it appears likely that accumulation of endogenous DNA species triggers a cell-autonomous immune response by activating a cytosolic DNA receptor. In this article, we demonstrate that knocking out the DNA sensor cyclic GMP–AMP synthase completely abrogates spontaneous induction of IFN-stimulated genes in TREX1-deficient cells. These findings indicate a key role of cyclic GMP–AMP synthase for the initiation of self-DNA–induced autoimmune disorders, thus providing important implications for novel therapeutic approaches. The Journal of Immunology, 2014, 192: 000–000.

The recognition of nucleic acids is a fundamental mechanism of the host to sense the presence of pathogens. Intracellular dsDNA arising during viral or bacterial infection constitutes a very potent danger signal that stimulates innate immune responses characterized by the production of proinflammatory cytokines and type I IFNs (1). Cyclic GMP–AMP synthase (cGAS) has been identified as a cytosolic DNA receptor responsible for the induction of an antiviral and proinflammatory gene expression profile (2–4). Upon dsDNA binding, cGAS catalyzes the production of the second messenger molecule cGAMP (5–7), a unique cyclic dinucleotide molecule containing one 2’–5’ phosphodiester linkage in addition to a canonical 3’–5’ linkage (5–8). Cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) subsequently stimulates the endoplasmic reticulum–localized protein STING to induce cytokine expression through activation of the transcription factors IRF3 and NF-κB via the protein kinase TBK1 (9). In addition to the secretion of de novo–produced cytokines within the virus-infected cells themselves, we have recently identified an additional layer of cGAS-triggered intercellular signal propagation (10). This pathway is based on the horizontal spread of cGAMP via gap junctions into bystander cells, where again STING is activated and antiviral gene expression is induced.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate; cGAS, cyclic GMP–AMP synthase; CMA, 10-carboxymethyl-9-acridanone; GLuc, Gaussia luciferase; ISO, IFN-stimulated gene; MEF, mouse embryonic fibroblast; qPCR, quantitative PCR; TREX1, 3’ repair exonuclease 1.

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Cell stimulation

Cocultures of MEFs (0.3 × 10^6/ml) with human fibroblasts (0.2 × 10^6/ml) were performed overnight. For quantitative PCR (qPCR) analysis, cocultures of all HEK-derived cells (0.5 × 10^6/ml) with MEFs (0.3 × 10^6/ml) were incubated overnight. DNA-stimulated cocultures of all HEK-derived cells (0.15 × 10^6/ml) with MEFs (0.15 × 10^6/ml) were performed in a 96-well format and transfected with 50 ng reporter plasmid (p-IFN-β-Gaussia luciferase [GLuc]), which served as stimulus and reporter at the same time. Direct stimulation of MEFs (0.3 × 10^6/ml) was performed by transfection of IFN stimulatory DNA (13) or triphosphate RNA (both 1.33 ng/ml), or by addition of 10-carboxymethyl-9-acridanone (CMA; 500 µg/ml) to the culture medium. After 16 h, supernatants were collected and murine CXCL10 (IP10) was measured via ELISA (R&D Systems).

Immunoblotting

Blots were incubated with anti-cGAS (Sigma), anti-TREX1 (C-11; Santa Cruz Biotechnology), anti-pTBK1 or anti-pIRF3 (both Cell Signaling) as primary and anti-rabbit IgG-HRP and anti-mouse IgG-HRP as secondary Ab or β-actin-IgG–HRP (all from Santa Cruz Biotechnology)

qPCR

qPCR analysis was performed as previously described (10). All gene expression data are presented as relative expression to mmβ-Actin (murine cells) or hsGAPDH (human cells). Primer sequences are available upon request.

CRISPR/Cas9-mediated knockout cell line generation

TREX1−/− MEFs were transfected with 200 ng of an mCherry-Cas9-gRNA expression plasmid targeting murine cGAS. After FACS sorting of mCherry+ cells, limiting dilution cloning was performed, and after 10 d, growing clones were selected by bright-field microscopy and genotypes were determined.

Results

Spontaneous IFN-stimulated gene expression in TREX1-deficient cells

As previously reported, antiviral gene expression in the absence of TREX1 is a cell-autonomous phenomenon that is operational in the absence of additional stimulation (16). Consistent with this notion, both BMDMs and MEFs from TREX1-deficient mice cultured in vitro displayed high levels of IFN-stimulated genes (ISGs; IP10, ISG15, and USP18) compared with their TREX1-competent counterparts (Fig. 1A and 1B). At the same time, phosphorylation of TBK1 and IRF3 were readily detected in the lysates of TREX1-deficient MEFs, and production of IP10 at the protein level was markedly elevated in cell culture supernatants of TREX1-deficient MEFs (Fig. 1C and see later).

ISG expression in the context of TREX1 deficiency is cGAS dependent

Given the fact that previous reports have indicated the presence of extranuclear DNA in the context of TREX1 deficiency, we set out to evaluate the role of the DNA sensor cGAS within this process (13, 17). To this effect, we targeted the first exon of cGAS in TREX1-deficient MEFs using the CRISPR/Cas9 system to generate TREX1/cGAS double-deficient cells. After gene targeting, we selected several TREX1-deficient cell clones that were either cGAS-competent or cGAS-deficient (Fig. 2A). We thus recovered one cGAS-competent, TREX1-deficient MEF cell line (#1) and one cell line comprising one in-frame deletion encompassing 51 bp within the N-terminal part of cGAS next to one out-of-frame deletion (#2). Moreover, we obtained two cell lines each harboring two reading frame disrupting deletions (#3 and #4), thus predicted to be cGAS knockout cell lines (Fig. 2A).

The analysis of cGAS and TREX1 confirmed the genotypes of these cell lines at the protein level, with cell lines 3 and 4 displaying no cGAS expression (Fig. 2B). Notably, when we analyzed spontaneous induction of IP10 and other ISGs within cGAS-competent and cGAS-deficient, TREX1-deficient MEFs, we observed that both cell lines lacking functional cGAS (#3 and #4) showed no upregulation of ISGs both at the mRNA level and at the protein level (Fig. 2B–D). In contrast, cGAS-competent, TREX1-deficient MEFs (#1 and #2) showed strong upregulation of IP10 both at the mRNA level and at the protein level. Interestingly, the TREX1-deficient cell line expressing the N-terminally truncated cGAS protein (#2) also displayed a robust, spontaneous ISG response, albeit at a lower extent. Consistent with these results, both cGAS-deficient, TREX1-deficient MEF cell lines were unresponsive to additional dsDNA (ISD) stimulation, yet responded normally to stimulation with the direct STING ligand CMA (18) or the RIG-I ligand 5′-triphosphate RNA (Fig. 2C). Altogether, these results showed that cGAS is critically required for the upregulation of ISGs triggered by TREX1 deficiency.

![Figure 1](http://www.jimmunol.org/Downloadedfrom)
TREX1-deficient cells trigger bystander cell activation in a cGAS-dependent fashion

Previously, we have shown that upon dsDNA stimulation, cGAS-produced cGAMP is transferred through gap junctions to activate STING in neighboring cells (10). As an indirect measure of cGAMP production within TREX1-deficient cells, we next examined whether TREX1-deficient MEFs would also elicit bystander cell activation. Indeed, a series of experiments unequivocally revealed strong gap junction–dependent activation of bystander cells by TREX1-deficient cells. First, the presence of TREX1-deficient MEFs, but not WT MEFs, caused marked and specific upregulation of ISGs (IFIT2 and ISG15) and IFN-β in coculture experiments with human fibroblasts (Fig. 3A). This effect was blocked by transwell separation, as well as gap junction inhibition by carbenoxolone treatment (Fig. 3B and 3C). Given the fact that human-specific primer sets were used, the observed antiviral response could be attributed to human fibroblasts directly, thereby indicating that a stimulatory signal was provided from the murine TREX1-deficient cells to the human fibroblasts in trans. Second, HEK STING cell lines (10) that lack functional gap junction communication (Connexin43 and Connexin45 double-knockout cells) were unresponsive toward the TREX1-deficient MEFs, yet showed normal induction of ISGs when transfected with the RIG-I stimulus 5′-triphosphate RNA (Fig. 4A). This gap junction–dependent in trans activation was also observed in coculture experiments using WT MEFs or TREX1-deficient MEFs and HEK STING cells in conjunction with plasmid DNA transfection and examination of IFN-β reporter activity (10) (Fig. 4B and 4C). Third, the ability of TREX1-deficient MEFs to induce bystander cell activation was dependent on cGAS, given that cGAS-deficient, TREX1-deficient cells failed to elicit such a response (Fig. 5). Taken together, these data clearly define cGAS as the major regulator of ISG induction in response to self-DNA accumulating in the absence of TREX1 and reveal that cGAMP-mediated bystander cell activation could be a novel mechanism of autoimmune propagation.

Discussion

In this article, we provide genetic evidence that cGAS is critical for the induction of type I IFNs and ISGs in TREX1-deficient cells, thus formally establishing a role for cGAS as an essential mediator.
of self-DNA–triggered inflammation. Furthermore, we show that TREX1-deficient cells act on bystander cells in a cGAS-dependent fashion to initiate horizontal innate immune signaling, which strongly implies the production and propagation of cGAMP by TREX1-deficient cells. This latter finding raises the possibility that intercellular connectivity might be a decisive pathogenic factor that aggravates and expedites the process of autoimmune-mediated tissue damage. Indeed, it is conceivable that cGAS-expressing cells generate cGAMP and provide it in trans to STING-expressing, but cGAS-incompetent cells, further amplifying antiviral gene expression in the context of TREX1 deficiency. In line with this concept, it is interesting to note that human cGAS and STING do not follow a tight coexpression pattern across different cell types, with cGAS showing a far more restricted expression pattern than STING (19–21). On a similar note, it is also possible that differential expression of connexins contributes to the unique confinement of disease activity to distinct tissues in the context of TREX1 deficiency. Given these findings, we believe that it will be interesting to study the role of in trans signaling in the context of cGAS/STING-driven autoimmunity in vivo. Of note, we cannot formally exclude that additional, putative DNA sensors or modulators participate in the pathogenesis of this autoimmune disease in vivo. Nevertheless, the strong phenotype of cGAS- and TREX1-deficient MEFs demon-

HUMAN FIBROBLASTS

A

Co-culture of WT or TREX1-deficient MEFs with CX334/45WT or CX334/45KO HEK STING cells

B

Co-culture of WT or TREX1-deficient MEFs with HEK STING cells

C

Co-culture of WT or TREX1-deficient MEFs with CX334/45WT or CX334/45KO HEK STING cells

FIGURE 3. TREX1-deficient MEFs trigger antiviral immunity in human fibroblasts. (A) Human fibroblasts were cocultured with WT MEFs or TREX1-deficient MEFs, and after 14 h the induction of human IFIT2, ISG15, and IFN-β was assessed by qPCR. (B and C) Human fibroblasts were cocultured with WT MEFs or TREX1-deficient MEFs in the presence or absence of a transwell system (B), or pretreated with carbenoxolone (200 μM) as indicated (C), and relative expression of human IFIT2 and ISG15 was quantified by qPCR 14 h later. Mean + SEM of four independent experiments is shown (A), whereas (B) and (C) display mean values + SEM of one representative experiment of three independent experiments.

FIGURE 4. Bystander cell activation is gap junction and STING dependent. (A) HEK STING CX334/45WT and HEK STING CX334/45KO cell lines (two distinct cell lines for each genotype) were left untreated, coincubated with WT MEFs or TREX1-deficient MEFs or stimulated with triphosphate RNA, and relative expression of human IFIT2 was analyzed by qPCR after 14 h. (B) HEK cells and HEK STING cells were coincubated with WT MEFs or TREX1-deficient MEFs. Fourteen hours after transfection with p-IFN-β–GLuc, transactivation of the reporter was assessed. (C) HEK STING CX334/45WT and HEK STING CX334/45KO cell lines were coincubated with WT MEFs or TREX1-deficient MEFs and transfected with p-IFN-β–GLuc. After 14 h, transactivation of the reporter was assessed. Mean + SEM of three independent experiments is shown (A) or mean values + SEM of triplicate measurements of one representative experiment of three independent experiments are depicted (B and C).
strate that cGAS operates as a nonredundant DNA sensor in a cell-autonomous manner, at least within these cell types.

With cGAS, a bona-fide DNA sensor at hand, it should now be possible to identify the actual DNA ligands that initiate autoimmune cell in the absence of TREX1. Although retroelement-derived DNA has been identified as a putative source, it might not represent the only substrate that is metabolized by TREX1, and as such serves as a ligand for cGAS (13). Indeed, it is also possible that DNA replication- or DNA damage-dependant by-products contribute to cell-intrinsic type IFN production in TREX1-deficient cells.

Next to TREX1-associated disorders, STING deficiency has also been reported to rescue from lethal inflammation in the context of another disease model, in which endogenous DNA is erroneously sensed by the innate immune system (22). Mice lacking DNase II show a dramatic STING-dependent type I IFN response because of the fact that DNA within the lysosomal compartment of phagocytic cells fails to be degraded and subsequently translocates into the cytoplasm (23). It appears likely that cGAS also plays a pivotal role in this disease setting, yet additional proof is required to substantiate this assumption.

Altogether, our findings establish cGAS as a nonredundant sensor for endogenous DNA species, and thus might have important clinical value for the development of novel therapeutic approaches targeting this receptor in sterile inflammatory conditions.

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Disclosures
The authors have no financial conflicts of interest.

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