CHAPTER THREE

STING is the predominant receptor for cgamp

Results

Cyclic GMP-AMP (cGAMP), as the first cyclic di-nucleotide found in metazoan, was recently identified as a second messenger molecule that plays an essential role in the cytosolic DNA sensing pathway. In mammalian cells, cGAMP is produced by the DNA sensor, cyclic GMP-AMP synthase (cGAS) from ATP and GTP when it binds to cytosolic double stranded DNA. cGAMP binds to and activates an adaptor protein, STING, which will further activate the downstream signaling cascade and trigger the production of Type-I interferons and inflammatory cytokines. cGAMP has been proven to be a very important molecule in the defense of multiple infectious diseases as well as in the development of self-immune diseases. cGAMP can also be served as an effective adjuvant that can boost antigen-specific antibody production and T cell response. So it is intriguing to investigate if there is any other receptor for cGAMP except STING in cells that may have novel functions. To do so, we make use of the next-generation sequencing techniques and check whether cGAMP will induce any gene expression profile changes when delivered into STING depleted cells. Our conclusion here is that in multiple cell types investigated, STING is the predominant receptor for cGAMP and innate immune response.

cGAS induced Type-I interferons and inflammatory cytokines in STING dependent manner in 293T reconstitution cell line.

In 293T cells, as there is non-detectable expression of cGAS or STING protein, the cytosolic DNA sensing pathway is absent from receptor-adaptor level. However, we can reconstitute this pathway by introducing cGAS and STING protein into 293T cells. We used lentivirus to stably express STING protein in 293T cells (293T\_STING cell line). In this cell line, with transfection of cGAS plasmid, the DNA sensing pathway would be activated and would further induce IRF3 dimerization (Figure 2-1A, lane 3) and IFNb1 production (Figure 2-1B). Transfection of pcDNA3 vector with MAVS coding sequence was used to compare between cytosolic DNA sensing pathway with RNA sensing pathway. To choose a proper amount of vector for activation, we did a titration test and used IFN-b, CXCL10 and TNF-a as indicators for transcriptional activation. Interestingly, increasing amount of cGAS encoding plasmid was toxic to cells and resulted in lower production of IFN-b and CXCL10 mRNA (Figure 2-1C and D). For MAVS overexpression activated RNA sensing pathway, more MAVS would induce much higher IFN-b mRNA level, while the induced CXCL10 and TNF-a mRNA levels were comparable to those induced by cGAS overexpression (Figure 2-1D and E). For optimal cGAS-STING pathway activation, in subsequent sample preparations, we used 0.1ug/ml.

To set up better negative control, we evaluated the effect of transfecting pcDNA3 vector with mouse cGAS catalytic domain mutant coding sequence (G198A, S199A, cGAS\_mt) versus pcDNA3 empty vector (pcDNA3\_EV). As expected, the catalytic domain dead mutant was null functional and the protein did not alter any significant gene expression, comparing with pcDNA3\_EV transfection (Figure 2-1F).

In 293T\_STING cell line, we evaluated the gene expression changes after cGAS activation (by cGAS plasmid transfection). From Figure 2-2A, we can see that a number of genes were induced by cGAS transfection. 263 genes were induced by cGAS activation with induction ratio larger than or equal to 2.0 and induced expression RPKM larger than or equal to 1.0. Among them, 115 genes were induced with ratio larger than 4.0. From ontology analysis of the genes induced, we can see that genes induced by cGAS activation included genes related to cellular responses to virus and bacterium (Type-I interferons and inflammatory cytokines) (Table 2-1). Meanwhile, genes involved in myeloid cell differentiation were also induced (including ZFP36, MAFB, JUN, CSF1 etc.). Furthermore, we also noticed the induction of genes regulating cell apoptosis, which was in consistent with the observation that cells underwent obvious cell cycle arrest and cell death after cGAS expression plasmid transfection. Unexpectedly, a massive induction of histone mRNA also showed up in the list (Table 2-1), of which the mechanism and physiological significance still remains to be further explored.

To identify the potential set of genes induced by cGAMP independent of STING, we stimulated 293T wild type cells (293T\_WT) with cGAS plasmid transfection. As the result shown in Figure 2-2B, few genes were induced by cGAS activation in 293T cells in absence of STING. In Table 2-2, we can see that only 11 genes were induced with a ratio larger than or equal to 2.0 and induced RPKM larger than or equal to 1.0, among which, 2 of them with inducing ratio larger than or equal to 4.0. However, none of the listed genes overlapped with the list of genes induced by cGAS in 293T\_STING cells. And when looking into each gene in the list, not only the induction ratios were low, the expression level of these genes were low even in the stimulated cells. So we regard them as experimental variations. This result leads us to the conclusion that in 293T cells, the genes induced by cGAS activation was totally dependent on the presence of STING protein, which makes STING the predominant target for cGAMP produced by cGAS.

In this set of RNA-Sequencing experiment, we also checked the genes induced by the activation of cytosolic RNA sensing pathway. To activate RNA-sensing response, we transiently over-expressed the key adapter protein, MAVS, in 293T\_STING cell line. From Figure 2-3A, we can see that 91 genes were induced, 62 of which were induced with a ratio greater than or equal to 4.0. GO analysis showed that most of the induced genes were related to immune response against virus or bacterium. By comparing the gene lists induced by cGAS and MAVS respectively (Figure 2-3B and C), we can see that cGAS induced a broader spectrum of inflammatory cytokines than MAVS did. Representing gene expression values (RPKM) from RNA-sequencing result are shown in Figure 2-4 and part of them are validated by qPCR (Figure 2-5A). Interestingly, the induction of histone genes and apoptosis related genes (growth and DNA damage related, GADD genes) observed by cGAS induction was absent by MAVS activation (Figure 2-5B to E). These results indicate that cGAS activation (cytosolic DNA sensing pathway activation) may trigger more intense and broader immune response than RNA sensing pathway activation does.

cGAMP activates primary lung fibroblast and bone marrow derived macrophages in STING dependent manner.

In order to test the function of cGAMP in presence or absence of STING under more physiological conditions, we further checked the genome wide gene expression changes in response to cGAMP treatment in primary lung fibroblasts (LF) and bone marrow derived macrophages (BMDM). In LF and BMDM, activation of cGAS-STING pathway by cGAS-encoding plasmid transfection was not efficient (data not shown), so we evaluated three different methods to deliver cGAMP into the cells to directly activate STING. As shown in Figure 2-6A and B, adding relatively high concentration of cGAMP into the culture medium only induced very small amount of IFN-b and CXCL-10 production. Delivery of cGAMP along with Lipofectamine 2000 increased cytokine induction but the activation was still weak. In comparison, an even very low concentration of cGAMP (0.1uM) could induce very high level of IFN-b and CXCL-10 production when it was delivered with Digitonin to partially permeabilize cell membrane. We also did a titration and time course test on cGAMP concentration for optimal activation. As shown in Figure 2-6A to D, 0.1uM of cGAMP was already saturated for maximum activation and increasing amount of cGAMP did not further elicit higher production of IFN-b. So in preparation for RNA-sequencing samples, we used digitonin to permeabilize the cells and delivered 0.1uM of cGAMP. We treated cells from wild type and STING Goldenticket mice and compared the IFN-b and CXCL-10 mRNA induction (Figure 2-6E and F) before sending the samples for next-generation sequencing.

In wild type primary lung fibroblasts (Figure 2-7A), 844 genes were sufficiently induced (induction ratio greater than or equal to 2.0 and induced RPKM greater than or equal to 1.0). Among these induced genes, most of them are related to immune defense response, including inflammatory cytokines, chemokines, antigen processing and presentation related genes and adaptive immune response regulations. However, in lung fibroblast from STING Goldenticket mice, only 74 genes were upregulated at minor levels in response to cGAMP treatment (Figure 2-7B) and most of the genes were only expressed at very low levels. As the majority of the induced genes were unannotated genes and did not overlap with the gene list induced in wild type cells, they were considered to be caused by experimental variations.

Similar results were also observed in primary bone marrow derived macrophages (Figure 2-8A and B). These results indicate that in primary lung fibroblast and bone marrow derived macrophage, STING was the predominant target for cGAMP.

cGAMP activates bone marrow derived conventional dendritic cells in STING dependent manner

Further, we tested the genes induced by cGAMP in bone marrow derived conventional dendritic cells (cDCs). In this set of experiment, we collected and differentiated bone marrow cells into conventionally dendritic cells (cDCs). The treatment method used was similar to the method used for LF and BMDM but with lower optimal dosage of Digitonin.

We found that after 6 or 12 hours’ treatment of cGAMP, innate immune response was sufficiently activated in wild type cDCs (Figure 2-9A and B). While in cDCs from STING Goldenticket mice, the activation or gene induction was minimum (Figure 2-9C and D). And when comparing the induced genes in wild type and STING deficient cells, there was little significant overlap (Table 2-3), indicating that the genes induced in STING deficient cells were probably due to experimental variations.

Meanwhile, in this set of sequencing experiment, we also evaluated the gene induction by cGAMP treatment in interferon alpha receptor knockout (IFNAR KO) cells. As shown in Figure 2-9E and F, in IFNAR KO cDCs, only a subset of interferons and cytokines were induced (including Ifnb1, Ifnas, and certain Isgs). When comparing with genes induction in wild type, the fact that most of the genes were uniquely induced in wild type cells suggest that the majority of the gene induction was secondary response and was dependent on interferon signal.

DNA activates bone marrow derived conventional dendritic cells in cGAS and STING dependent manner

Since in all the types of cells tested above, STING always shows as the predominant target for cGAMP and in absence of STING protein, the function of cGAS (cGAMP) is almost totally abolished. We wanted to further test whether cGAS is the predominant receptor for double stranded DNA.

Firstly, we derived cDC from wild type bone marrow cells and tested different conditions for optimal stimulation condition. We used liposome (Lipofectamine 2000® Transfection Reagent from Invitrogen) to facilitate delivery of stimulatory DNA into cells. As suggested by manufacturer protocol, we used a Lipofactamine 2000 / DNA ratio of 1:3. Interestingly, we found that even at low concentration (3uL/mL), liposome alone could induce considerable amount of CXCL-10 (Figure 2-10C). And at higher concentration (12 to 24uL/mL), liposome induced significant amount of IFN-b production also (Figure 2-10A). Moreover, increasing amount of liposome was very toxic to dendritic cells, as indicated by the amount of total RNA harvested after treatment (Figure 2-10D). Taking all these factors into account, we used 2ug/mL of ISD along with 6uL/mL Lipofectamine 2000 for cDC stimulation for later experiments. To determine the optimal DNA stimulation time in cDCs, we did a time course test and found out that the IFN-b mRNA level reached a peak as soon as 3 hours and dropped dramatically between 6 and 9 hours (Figure 2-10E). Considering we were also looking for the induction of unknown genes that may have different activation dynamics, we collected and sequenced samples that were stimulated for 3 and 6 hours. In Figure 2-10F, we confirmed our samples from wild type, cGAS knockout and STING Goldenticket mice and verified that the induction of IFN-b by ISD (interferon stimulatory DNA) was totally abolished in cGAS or STING null cells.

In cDCs from wild type mice, 3 hours after we transfected interferon stimulating DNA (ISD), Type-I interferons and inflammatory cytokines were highly induced (Figure 2-11A); and even more genes were with higher induction level were observed 6 hours after transfection (Figure 2-11B). In comparison, the gene induction in cells from STING Goldenticket mice or from cGAS knockout mice was much less significant (Figure 2-11C to F). These results suggest that cGAS/STING axis play dominant roles in eliciting innate immune response to cytosolic double stranded DNA.

When the cDC cells were treated with only Lipofectamine 2000 alone, significant gene induction was also observed in wild type cells (Figure 2-12A and B). And when comparing the gene induction lists by ISD and liposome alone (Figure 2-12G), the significant overlap suggests that liposome may trigger the activation of DNA sensing pathway in wild type cDC cells. Furthermore, the fact that the gene induction by liposome in cGAS or STING deficient cells was much weaker further proved this conclusion (Figure 2-12C to F). As in vitro cGAS activity assay showed that liposome could not directly activate cGAS protein in absence of DNA, the activation of DNA sensors by liposome could be triggered by the DNA leakage caused by liposome treatment.

cGAS-STING independent gene induction by DNA stimulation in cDCs and pDCs

Though with less intensity and variety of gene induction, we still observed a considerable amount of genes whose expression increased in STING or cGAS deficient cells, especially at 6 hours after ISD transfection. When we compared gene expression changes in STING deficient and cGAS deficient cells (Figure 2-11D and F), there was a significant overlap of genes induced (including interleukins and ifit genes, representing genes are shown in Figure 2-13A and B). This result suggests that there could be other potential but weaker sensors for double stranded DNA that contributed the gene induction in absence of cGAS or STING proteins. An interesting phenomenon was that when looking into the induced genes by ISD independent of cGAS and STING, we found that for majority of these genes, their basal expression levels were much lower in cGAS or STING knockout cDCs than in wild type cDCs. Figure 2-13C lists the expression values of representing genes that were expressed at lower level in cGAS or STING knockout cells. Statistically, there were 127 genes that were induced by 6 hours of ISD stimulation, with induction ratio larger than or equal to 2.0 and induced expression RPKM larger than or equal to 1.0. Among these genes, 104 of them (82%) were in the list of genes whose basal expression levels were 1.5 fold higher in wild type cDCs than in both cGAS and STING knockout cells (Figure 2-13D).

One of the genes that was induced in cGAS or STING knockout cells, Il12b, drew our attention. From Figure 2-14A, where we plotted the RPKM value of Il12b gene from the sequencing results, we can see that in cGAS or STING knockout cDCs, ISD induced significant amount of Il12b expression, though in STING knockout cell, the induction was weaker. To further confirm this, we repeated the stimulation, along with stimulation with cGAMP, HSV and SeV and checked the mRNA level of IFN-b, IL-6 and IL-12b (Figure 2-14B to D). In consistent with previous RNA-sequencing data, cGAMP induced high level of IFN-b and IL-6, but little IL-12b. With ISD stimulation, on the other hand, induced high level of IFN-b and lower level of IL-6, which totally dependent on cGAS or STING, but it also induced IL-12b expression, which was independent of cGAS but seemed partially dependent on STING. Interestingly, though HSV infection only induced low level of IFN-b expression, comparing with ISD stimulation, it elicited very high expression of IL-12b.

To further investigate if the induction of IL-12b by ISD and also to check if there is any synergistic effect between cGAS-STING pathway and TLR9 pathway, we differentiated bone marrow cells from wild type, cGAS knockout and TLR9 knockout mice into cDCs and plasmacytoid dendritic cells (pDCs), and then stimulated the cells with either ISD, CpG DNAs, or in combination, with or without liposome (Figure 2-15A to F). In both cDCs and pDCs, ISD could induce high level of IFN-b and IL-6, which was totally cGAS and liposome dependent. It also promoted the expression level of IL-12b in cDCs, which was independent of both cGAS and TLR9. CpG-A, when delivered with liposome, could activate cDCs or pDCs in cGAS dependent but TLR9 independent manner. This is probably due to that CpG-A can partially activate cGAS with its phosphodiester linkage double stranded DNA region. With CpG-DNA along treatment, cDCs didn’t express much IFN-b or IL-6 but significant amount of IL-12b, while pDCs were strongly activated and expressed high level of IFN-b, IL-6 and IL-12b, which totally dependent on TLR9.

Interestingly, when stimulated with combination of CpG-DNAs and ISD, instead of synergistic effect, we observed that in cDCs, the expression induction of IFN-b and IL-6 by ISD was almost totally abolished after adding CpG-DNAs, especially with CpG-B and CpG-C. This results were in consistent with the discovery that phosphorothioate backbone-modified single stranded DNA can potently inhibit cGAS activity (which will also be discussed in the following chapter).

Conclusions and discussion

In this project, we analyzed the global gene expression changes triggered by cGAS or cGAMP in different cell types, including 293T cell lines, primary mouse lung fibroblasts, bone marrow derived macrophages, conventional dendritic cells, and plasmacytoid dendritic cells, with or without STING expression and found that in cells lacking STING protein expression, the gene expression changes induced by cGAMP were almost totally abolished. Our results confirmed that STING is the predominant receptor for cGAMP.

In response to DNA or RNA derived from infectious pathogens, different receptors and adaptor proteins are activated and lead to the activation of kinases and transcription factors, including NF-kb, IRF3, IRF7, etc. By comparing the genes induced by cGAS-STING and MAVS, we found that except from type-I interferon genes, interferon-stimulated genes and chemokines that are commonly activated by both cytosolic DNA sensing and RNA sensing pathways, cGAS-STING triggers the increased expression of much more genes, in consistent with reported and our own observations that cGAS-STING pathway also involves in processed including autophagy, cell death, cell senescence, antigen cross-presentations and etc. This suggest that cGAS-STING pathway may serve as a very promising method or target for therapeutic interventions.

The invading DNAs from infectious pathogens provide the stimuli for the host to identify the infection and initiate immune response. Till the discovery of cGAS, many cytosolic DNA sensors, including DDX41(Zhang et al. 2011), IFI16(Unterholzner et al. 2010), and DAI(Takaoka et al. 2007), were reported to recognize intracellular double stranded DNAs. All these putative DNA sensors rely on STING, which does not bind to DNA, to initiate type I interferon production. The binding of STING with cyclic dinucleotides such as c-di-GMP or c-di-AMP to activate TBK1-IRF3, leading to the production of interferon added mysteries to this field (O'Connell et al. 2004). Since cGAS identified as the most important cytosolic DNA sensor, the puzzles were clearly solved. Upon binding of pathogen derived dsDNA or self-generated DNAs, cGAS utilize ATP and GTP to produce the second messenger, cGAMP, which binds with STING to activate the downstream signaling cascade. Although cGAS plays pivotal roles in cytosolic DNA sensing, there are still other putative known or unknown DNA sensors that may also respond to the ligand. In cGAS or STING knockout dendritic cells, when stimulated with ISD, we still observed considerable genes expression changes. One example is IL-12b gene, whose mRNA expression was not induced by cGAMP treatment but was induced by ISD stimulation in cGAS independent manner. So it is possible that there are other DNA sensors contribute to add the immune response on the basis of cGAS or they may take the responsibilities in cells that lack cGAS or STING expression.

Previous research reported that either liposomes or herpesvirus derived nucleic acid free particles could lead to the translocation of STING and thus activation of the downstream signaling (Holm et al. 2012). Here we observed similar result with Lipofectamine 2000 treatment alone without ISD and we showed that this immune response was dependent on both cGAS and STING. So it’s more likely that the activation is still DNA dependent but the presence of liposome or virus-like particles makes the otherwise inaccessible DNA available to cGAS recognition. Interestingly, this activation of cGAS-STING pathway by liposome was absent in cell types like lung fibroblasts or cell lines including 293T, BJ, Hela, etc., which suggests that dendritic cells or macrophages may utilize additional mechanisms to access these DNA ligands.

Material and methods

Mice, Cells and Nucleotides

cGas-/- mice were generated in our lab as previously described (Li et al. 2013). STING Goldenticket mice were from the Jackson Laboratory (Stock No: 017537). All mice used in this study were on C57/B6 background. The mice were bred and maintained under specific pathogen-free conditions in the animal care facility of the University of Texas Southwestern Medical Center at Dallas according to experimental protocols approved by the Institutional Animal Care and Use Committee.

To isolate primary lung fibroblasts, lung tissues from each mouse were minced and digested twice in 5 ml of DMEM with 0.1% collagenase D (Roche) and 0.2% trypsin (Sigma) at 37°C for 45-60 min. After washing with DMEM containing 10% calf serum, cells were seeded in a 10-cm dish in 12 ml DMEM containing 10% FBS in addition to 10% calf serum and antibiotics. Bone marrow cells were collected from femurs and tibiae of mice. To obtain BMDM and cDCs, about 10 million bone marrow cells were cultured in RPMI-1640 (Gibco 21870076) containing 10% FBS (Sigma), antibiotics, 1% NEAA, 1% sodium pyruvate, 1% glutamax, 50uM beta mercaptoethanal and recombinant GM-CSF (20ug/ml, Preprotech, 315-03). After 7 days, attached cells were collected as mature macrophages and cultured in 12-well plates for experiments, and suspension cells were collected as conventional dendritic cells. To obtain pDCs, 100ng/ml hFLT3L was used instead of GM-CSF and the culture time is 10 days. Floating and loosely adherent cells were harvested. Cultures were analyzed by FACS before use.

2’3’cGAMP was synthesized and purified as described (Zhang et al. 2013). Herring testes DNA (HT-DNA) was from Sigma (D6898). ISD was annealed from the forward and reverse DNA oligos, which were synthesized from Sigma using the following sequences: 5’-TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA-3’ and 5’- TGTAGATCATGTACAGATCAGTCATAGATCACTAGTAGATCTGTA-3’; CpG DNAs were synthesized from Sigma with the following sequences: CpG-C: T\*C\*G\*T\*C\*G\*T\*T\*T\*T\*C\*G\*G\*C\*G\*C\*G\*C\*G\*C\*C\*G; CpG-B: T\*C\*C\*A\*T\*G\*A\*C\*G\*T\*T\*C\*C\*T\*G\*A\*C\*G\*T\*T; CpG-A: G\*G\*GGTCAACGTTGAG\*G\*G\*G\*G\*G (\* indicates phosphorothioate backbone-modification).

Stimulation methods

For 293T cells, cells were transfected with indicated amount of pcDNA3 vector with sequence encoding wild type or mutant cGAS, or MAVS for 12 hours for proper activation before proceeding to following experiments. For cGAMP treatment for lung fibroblasts, BMDM and BMDC, the cells was incubated in the following delivering buffer with or without the following buffer: 50 mM HEPES pH 7.0, 100 mM KCl, 3 mM MgCl2, 0.1 mM DTT, 85 mM Sucrose, 0.2% BSA, 1 mM ATP, and 10 µg/mL Digitonin, for 15 minutes and then recovered in normal media for the indicated time. For ISD transfection, indicated amount of DNA was transfected into cells using DNA (µg) to Lipofectamine™ 2000 (µl) ratio of 1:3.

RNA-sequencing process and data analysis

The total RNA from the cells after stimulation was extracted with RNeasy Mini Kit from Qiagen (Cat No.: 74104). Samples are then run on the Agilent 2100 Bioanalyzer to determine level of degradation thus ensuring only high quality RNA is used (RIN Score 8 or higher). The samples were sent to UTSouthwestern Next Generation Sequencing Core for subsequent preparation and sequencing. The Qubit fluorometer was used to determine the concentration prior to cDNA library preparation. 4 µg of total DNAse treated RNA was then prepared with the TruSeq Stranded Total RNA LT Sample Prep Kit from Illumina. Poly-A RNA was purified and fragmented before strand specific cDNA synthesis. cDNA was then a-tailed and indexed adapters were ligated. After adapter ligation, samples were PCR amplified and purified with Ampure XP beads, then validated again on the Agilent 2100 Bioanalyzer. Samples were quantified by Qubit before being normalized and pooled, then run on the Illumina HiSeq 2500 using SBS v3 reagents. The sequencing data was processed using RNA-Seq CLC-Bio analysis for mRNA expression values, represented by Reads Per Kilobase per Million mapped reads values (RPKM).

Quantitative RT-PCR and Primers

Total cellular RNA was isolated using TRIzol. 0.1-1 μg total RNA was used for reverse transcription (RT) using iScript Kit (Bio-Rad). The resulting cDNA served as the template for Quantitative-PCR analysis using iTaq Universal SYBR Green Supermix (Bio-Rad) and ViiTM7 Real-Time PCR System (ABIApplied Biosystems Inc., Foster City, CA). The primers used are listed in Table 2-4.

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