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Intracellular DNA sensing pathway of cGAS-cGAMP is decreased in human newborns and young children

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ABSTRACT

Newborns are highly susceptible to DNA virus infections, which may result from the characteristics of neonatal innate immune systems. Here we analyzed for the first time the development of innate immune sensing and signaling of intracellular DNA virus infection in human newborns and young children. Both mRNA and protein expression of cGAS, an intracellular DNA sensor, were shown to be significantly reduced in neonatal peripheral blood mononuclear cells (PBMCs). In addition, cGAS expression in neonatal PBMCs could be induced upon herpes simplex virus type 1 (HSV-1) or interferon-α (IFNα) stimulation. Furthermore, production of the second messenger cGAMP and activation of the transcriptional factor IRF3 was severely decreased in neonatal cord blood mononuclear cells (CBMCs) or PBMCs compared with adults. In contrast, the downstream signaling STING-TBK1-IRF3 appeared to be functional in neonatal PBMCs, as demonstrated by the fact that IRF3 phosphorylation and IFNβ production in these cells could be activated by cGAMP. Intriguingly, decreased expression of cGAS in neonatal cells can be rescued by DNA demethylation, with concomitant enhancement in IFNβ induction by HSV-1. Thus, cGAS restoration or STING stimulation by small molecules during infancy might improve the age-dependent susceptibility to DNA virus infection.

1. Introduction

Newborns and infants are shown to have a higher susceptibility to infection and a lower response to vaccination than adults (Basha et al., 2014). Mechanistically, it is supposed to be due to the immaturity of both innate and adaptive immune systems (Levy, 2007; Vosters et al., 2010). Notably, adaptive immune responses in neonates are hampered by a lack of pre-existing memory and decreased Th1-type responses. Meanwhile, innate immune sensing and signaling in newborns are not fully developed, allowing rapid dissemination of infections (Belderbos et al., 2009; Kumar and Bhat, 2016). Since the adult resistance to certain virus is also acquired without pathogen infection, age-dependent susceptibility appears not to be mainly mediated by an adaptive cellular or humoral immune response, but lies in the innate immune system (Pott et al., 2012). Further, the observed impairments in neonatal innate cytokine production could result from either reduced expression of pathogen pattern recognition receptors (PRRs) or altered signaling pathways from these receptors (Slavica et al., 2013; Tapping, 2009).

The past two decades has brought great strides in our understanding of innate immune recognition mechanism in humans. It is now well-known that activation of the innate immune system is based on the recognition of conserved microbial structures (such as DNA, RNA and LPS) by a limited number of membrane and cytosol resident receptors including Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) (Orzalli et al., 2015; Takeuchi and Akira, 2009). Among 10 well-characterized TLRs, the endosomal TLR9 are responsible for sensing DNA and mainly contribute to extracellular viral recognition. In the cytosol, cGAS synthesize the second messenger molecule cGAMP upon DNA virus infection and activate the ER-localized molecules STING (Brubaker et al., 2015; Liu et al., 2015). Dimerized STING then activate the transcriptional factors including IRF3/7, NF-κB and AP-1 via the corresponding upstream kinases, and induce antiviral gene expression such as IFN-I. Recently, it was shown that intracellular sensing pathway of cGAS-STING is critical for the immune responses against DNA virus infection (Cai et al., 2014; Ishikawa et al., 2009; Li et al., 2013; Lio

Abbreviations: CBMCs, cord blood mononuclear cells; CBP, CREB-binding protein; cGAS, cyclic GMP-AMP synthase; cGAMP, cyclic GMP-AMP; EBV, Epstein-Barr virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCMV, human cytomegalovirus; HSV-1, herpes simplex virus type 1; IFN, interferon; IFN-I, type I interferon; IRF3, interferon regulatory factor 3; IRF7, interferon regulatory factor 7; NF-κB, nuclear factor kappa B; NK, natural killer cells; PBMCs, peripheral blood mononuclear cells; PRRs, pattern recognition receptors; RLRs, RIG-I-like receptors; RSV, respiratory syncytial virus; STING, stimulator of interferon genes; TB, mycobacterium tuberculosis; TLRs, Toll-like receptors; VZV, varicella-zoster virus

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et al., 2016; Paijo et al., 2016). Cells from cGAS- or STING-deficient mice, including fibroblasts, macrophages, and dendritic cells, failed to produce IFN-I and other cytokines in response to DNA virus infection. Furthermore, adult cGAS- or STING-knockout mice were more susceptible to lethal infection with HSV-1 than wild-type mice (Li et al., 2013; Su et al., 2016). The second messenger cGAMP was shown to potently activate the antiviral IFN-I responses in mice and could be used as an adjuvant that boosts the innate immune activation of T cells (Li et al., 2013). Considering the pivotal role of cGAS-cGAMP-STING pathway in antiviral innate immunity, a better understanding of the development of intracellular DNA virus sensing pathway may contribute to preventing and treating neonatal infections (Marodi, 2006).

In recent years, the expression level of TLRs, RLRs and other innate immune receptors was intensively investigated both in the human neonates and newborn mice (Kumar and Bhat, 2016; Nguyen et al., 2010; Pott et al., 2012; Slavica et al., 2013; Tapping, 2009). It was reported that human cord blood natural killer (NK) cells and mouse epithelium have deficient TLR3 expression, which is associated with an inability to respond to poly(I:C) and viral infection (Pott et al., 2012; Slavica et al., 2013). Nevertheless, TLR3 expression was strongly increased during the postnatal period and inversely correlated with rotavirus susceptibility, viral proliferation and histological damage (Pott et al., 2012). In contrast, expression of other virus-sensing TLRs and RLRs in newborns was similar to that of adult blood samples (Pott et al., 2012; Slavica et al., 2013). However, it remains completely unknown whether cGAS expression and the downstream signaling pathway of intracellular DNA virus sensing was decreased in human neonates.

Furthermore, most studies assessing neonatal innate immune responses utilized cord blood as a source of neonatal immune cells, which is more ethically and technically available than neonatal venous blood (Corbett et al., 2010). Very few reports analyzed the evolution of innate immune cell function in the first months of life (Belderbos et al., 2009; Nguyen et al., 2010; Vosters et al., 2010). Here we utilized both CBMCs and PBMCs from the newborns and compared the expression of cGAS and STING with that of adults. Our results revealed for the first time that cGAS expression was severely decreased in neonatal PBMCs throughout the first year of life, leading to a significant reduction in the production of the second messenger cGAMP. Interestingly, expression levels of the endosomal receptor TLR9 and adaptor protein STING were similar to that of adults. In addition, the cGAS-STING axis was functional downstream of STING in PBMCs of 1-month-old newborns, as indicated by IRF3 phosphorylation and IFNβ induction upon synthetic cGAMP treatment. Collectively, these results may provide some evidences to utilize STING agonists in preventing and treating neonatal virus infections.

2. Materials and methods

2.1. Blood samples

Adult fresh blood was obtained from healthy volunteers and umbilical cord blood was collected from healthy full-term neonates at the department of obstetrics of the First People's Hospital of Shangqiu City (Henan, China). Peripheral venous blood were also obtained from a series of 1-, 3-, 12- and 36-month-old children after parental agreement. Children were included in a prospective cohort with regular clinical follow-up. All procedures were approved by the ethics committee of the faculty of medicine at the First People's Hospital of Shangqiu City (Approval Number: 2015-512) and informed consent was obtained. All blood samples were processed as described previously (Jansen et al., 2008).

2.2. Isolation and stimulation of CBMCs and PBMCs

Cord blood mononuclear cells (CBMCs) and peripheral blood mononuclear cells (PBMCs) from healthy children or adults were isolated by density gradient centrifugation using Ficoll-Paque kits (GE Healthcare). After washing three times with Hanks' balanced salt solution (HBSS) (Gibco), the cells were resuspended in complete medium of RPMI-1640 (Gibco) supplemented with 1% of penicillin/streptomycin (Gibco), 1 × Glutamax (Gibco), 1 × nonessential amino acids (Gibco), 50 μM of β-mercaptoethanol (Gibco) and 10% fetal bovine serum (FBS) (Gibco). Cell viability was estimated by blue trypan to be over 95%. To study the IFN-I and IL-6/8 responses, freshly isolated CBMCs or PBMCs were cultivated individually at 2×10^5 cells/ml and treated by HSV-1 for 24 h, followed by ELISA determination. For all other experiments, equal amount of CBMCs or PBMCs (n = 20 for each group) was pooled together from each individual and subjected to the following studies.

For cGAMP treatment, digitonin permeabilization was used to deliver synthetic cGAMP (Invivogen) into cultured cells as previously described (Sun et al., 2013; Woodward et al., 2010). Briefly, synthetic cGAMP was dissolved in digitonin solution to a final concentration of 200 nM. Culture media was aspirated from cells and replaced with cGAMP/digitonin mix. After incubation at 37 °C for 30 min, plate wells were aspirated again and fresh complete media was added. 6 h later, cells were harvested for western blot and qPCR respectively. For PRDIII-I luciferase determination, mononuclear cells were co-transfected with PRDIII-I luciferase reporters and PTK-Renilla plasmids overnight, followed by cGAMP stimulation for 12 h.

2.3. Cells, compounds and viruses

THP-1 cells (ATCC) were cultured in complete medium of RPMI-1640 (Gibco) supplemented with 1% of penicillin/streptomycin (Gibco), 1 × Glutamax (Gibco), 1 × nonessential amino acids (Gibco), 50 μM of β-mercaptoethanol (Gibco) and 10% fetal bovine serum (FBS) (Gibco). One day prior to the treatment, medium was exchanged and cells were cultivated in fresh medium.

Epigenetic modulators 5-aza-2'-deoxycytidine (5AZADC, A3656) and BIX01294 (B9311) were ordered from Sigma-Aldrich, while cGAMP and Vorinostat (SAHA) were purchased from Invivogen (tlrl-nacga23-5) and Selleck Chemicals (S1047), respectively.

Wild type HSV–1F strain was kindly provided by Chunfu Zheng (Wuhan Institute of Virology) and propagated in Vero cells (ATCC). Viral titers were determined as described previously (Zhang et al., 2016). Unless indicated otherwise, HSV-1 virus was used at 5 multiplicity of infection (MOI).

2.4. In vitro cGAMP activity assay

To isolate cGAMP from CBMCs and PBMCs after HSV-1 stimulation, cells were homogenized in the hypotonic buffer (10 mM Tris-HCl, pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$). After centrifugation at 100,000 rpm for 20 min, the supernatant was heated at 95 °C for 5 min and centrifuged at 12,000 rpm for 5 min to remove denatured proteins. After that, the supernatants were concentrated by drying at 95 °C and aliquoted before quantitation and storing at −80 °C. Then an aliquot of the heat-resistant supernatants was added to 10^6 THP-1 cells and incubated at 37 °C for 6 h to detect the phosphorylation of IRF3 as reported (Gao et al., 2015). For PRDIII-I luciferase determination, THP-1 cells were co-transfected with PRDIII-I luciferase reporters and PTK-Renilla plasmids overnight, followed by cGAMP stimulation for 12 h.

2.5. RNA preparation, PCR array and real-time quantitative PCR (qPCR)

For RNA purification, cell pellets was homogenized in the Buffer RTK provided in the Qiagen RNeasy Mini Kit. RNA was extracted then according to the manufacturer's instructions. All RNA samples were suspended in the RNase-free water provided with the RNA isolation kit. RNA samples were treated with RNase-free DNase I (Ambion) to digest