Stimulator of Interferon Genes in Classical Dendritic Cells Controls Mucosal Th17 Responses to Cyclic Dinucleotides for Host Defenses Against Microbial Infections in Gut

Song Liu1,2†, Qiuyuan Xia2,3†, Xiwen Wu2,4†, Feng Sun1,2, Qiongyuan Hu2,4, Jie Wu2,4, Meng Wang1,2, Qiu Rao2,3 and Wenxian Guan1,2*

1Department of General Surgery, Nanjing Drum Tower Hospital, Nanjing, China, 2School of Medicine, Nanjing University, Nanjing, China, 3Department of Pathology, Jinling Hospital, Nanjing, China, 4Department of General Surgery, Jinling Hospital, Nanjing, China

Cyclic dinucleotides are bacterial signal transducers that bind to host intracellular protein, stimulator of interferon genes (STING) encoded by Tmem173. In this study, we demonstrate that STING triggers adaptive immune responses that control Th17 differentiation. Cyclic dinucleotides recognition enables classical dendritic cells (cDCs) that predominantly express CD103 to induce Th17 lymphocytes in an IL-6/IL-1β-dependent manner in gut. STING expression in human lamina propria is associated with the severity of mucosal inflammation and clinical disease activity in patients with Crohn’s disease. Mice deficient in Tmem173 fail to mount Th17 responses to cyclic dinucleotides or prevent immune evasion of enteroinvasive pathogens. In summary, STING in mucosal cDCs controls Th17 subspecification that is essential for host defenses against microbial infection in gut-associated immune system.

Keywords: stimulator of interferon genes, dendritic cell, Th17, Salmonella, cyclic dinucleotides, Crohn’s disease

INTRODUCTION

The interaction between intestinal microbiome and host immunity plays a critical role in various autoimmune diseases, including Crohn’s disease (1, 2). Microbial nucleic acid belongs to pathogen-associated molecular patterns that can be recognized by specific sensors in dendritic cells to activate adaptive immunity (3, 4). However, the mechanism that links innate immunity triggered by microbial nucleic acid and functional properties of T cell population in gut-associated mucosal system remains to be elucidated.

Cyclic diguanylate monophosphate (c-di-GMP) is a newly identified second messenger in multiple species of bacteria that is released into cytosol of host cells during microbial infection (5). c-di-GMP is recognized by stimulator of interferon genes (STING) that leads to IRF3 or NF-κB activation (6–8). Mucosal dendritic cells are heterogeneous in origin and function (9, 10) and can induce Th17 cells as well as inducible regulatory T cells as parts of adaptive immune responses to intestinal microbiota (11, 12). However, the underlying signaling mechanism by which this is achieved remains elusive (13). The clinical significance of bacterial cyclic dinucleotides recognition by STING in host antimicrobial defenses awaits full elucidations.
In this study, we show that mucosal classical dendritic cells (cDCs) defined by the transcription factor Zbtb46 predominantly express CD103. Mucosal cDCs induce Th17 generation through a STING-dependent recognition of foreign cyclic dinucleotides. STING in lamina propria participates in mucosal inflammation and systemic disease activity in human Crohn’s disease. STING-triggered mucosal Th17 responses can prevent immune evasion of enteroinvasive pathogens and are therefore crucial for host antimicrobial defenses in gut.

**MATERIALS AND METHODS**

**Ethics**

This study was carried out in accordance with the recommendations of Guidelines for Clinical Trials by the Ethics Committee of Nanjing Drum Tower Hospital. The protocol was approved by the Ethics Committee of Nanjing Drum Tower Hospital. All subjects gave written informed consent in accordance with the Declaration of Helsinki. This study was carried out in accordance with the recommendations of Guidelines for Animal Experiment by the Ethics Committee of Nanjing Drum Tower Hospital. The protocol was approved by the Ethics Committee of Nanjing Drum Tower Hospital.

**Human Samples**

Intestine samples from eight adult patients diagnosed as Crohn’s disease were collected from grossly identifiable lesions as well as grossly uninvolved segments during definitive bowel resections. Intestine samples from four adult patients during ileostomy closure served as controls. A written content was obtained from each participant before surgery.

Pathological slides were prepared by fixing specimens in formaldehyde and cutting into 5-µm sections and staining with hematoxylin and eosin. For immunohistochemistry, slides were treated with anti-STING antibody (D2P2F; 13647; Cell Signaling) according to the manufacturer’s recommendations. Pixel intensities associated with anti-STING staining were converted to optical densities using Image J software (US National Institutes of Health). An expert gastrointestinal pathologist that was blinded to patient identity reviewed all slides and calculated the pathological score of each participant. The pathological scoring system ranged from 0 (normal) to 15 (most severe of inflammation) including six parameters: cellular infiltration (0–3), loss of goblet cells (0–3), crypt abscess (0–3), epithelial erosion (0–1), hyperemia (0–2), and thickness of mucosa (0–3) (14).

For immunoblotting, the preparation of tissue and protocol were in accordance to published routine method (15). Antibodies for immunoblotting included IRF-3 (D83B9; 4302; Cell Signaling), p-IRF3 (D6O1M; 29047; Cell Signaling), and β-actin (8H10D10; 3700; Cell Signaling). For patients with Crohn’s disease, their preoperative Crohn’s disease activity index (CDAI) score was calculated according to previous literature (16).

**Mice**

C57BL/6, Il17aGFP, Zbtb46GFP, Zbtb46DTR, and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Tmem173−/− (Sting−/−) mice on C57BL/6 background were generated using CRISPR/Cas9 technique in Nanjing Biomedical Research Institute of Nanjing University. All mice were maintained in H. heparicus- and Pasteurella-free or specific pathogen-free environment at MARC (Model Animal Research Center of Nanjing University). All animal experiments in this study were undertaken when mice were 6–10 weeks old with protocols approved by the Institutional Subcommittee on Research Animal Care at Nanjing University.

**Bone Marrow Chimeras and Diphtheria Toxin (DT) Depletion**

Recipient C57BL/6 mice received whole body irradiation (970cGy). A total of 1 × 10^7 bone marrow cells from Zbtb46GFP or Zbtb46GFP mice were injected into recipient mice via tail vein. The mice were housed for 8 weeks before subsequent experiments. For DT depletion, 40 ng DT (Sigma-Aldrich, St. Louis, MO, USA) per gram of body weight was injected (i.p.) twice on days 1 and 3 before experiment to specifically deplete the classical DC subsets.

**In Vivo Stimulation Assay**

To activate STING signaling pathway in vitro, 200 nmol/mouse c-di-GMP (VacciGrade) (Invivogen, San Diego, CA, USA) at 37°C in 200 rpm shaker for 30 min. After addition of 5 mM EDTA (Boston Bioproducts, Boston, MA, USA), all cells were passed through a 70 µm nylon cell strainer (Fisher Scientific). A final OptiPrep density centrifugation at ρ = 1.055 g/ml(Axis Shield, Oslo, Norway) yielded DCs.

To harvest cells from small intestine lamina propria (SILP), the small intestine was cut into four pieces and inverted onto polyethylene tubes (Becton Dickinson, Franklin Lakes, NJ, USA). After washing three times with calcium- and magnesium-free PBS (Lonza, Walkersville, MD, USA), the mucus and epithelium were removed by 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and 33 μg/ml Liberase TL (Roche, Indianapolis, IN, USA) in DMEM (Gibco Life Technology, Gaithersburg, MD, USA) containing 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) at 37°C in 200 rpm shaker for 30 min. After addition of 5 μM EDTA (Boston Bioproducts, Boston, MA, USA), all cells were passed through a 70 µm nylon cell strainer (Fisher Scientific). A final OptiPrep density centrifugation at ρ = 1.055 g/ml(Axis Shield, Oslo, Norway) yielded DCs.

To harvest cells from small intestine lamina propria (SILP), the small intestine was cut into four pieces and inverted onto polyethylene tubes (Becton Dickinson, Franklin Lakes, NJ, USA). After washing three times with calcium- and magnesium-free PBS (Lonza, Walkersville, MD, USA), the mucus and epithelium were removed by 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and 33 μg/ml Liberase TL (Roche, Indianapolis, IN, USA) in DMEM (Gibco Life Technology, Gaithersburg, MD, USA) containing 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) for 100 min at 37°C in a 5% CO2 humidified incubator. All cells digested from tissue were passed through a 70 µm nylon cell strainer (Fisher Scientific) and then centrifuged (density gradient centrifugation) with OptiPrep (ρ = 1.055 g/ml) to yield DCs or Percoll (3 ml of 44% Percoll overlaid upon 3 ml of 67% Percoll) to yield T cells. Purified DCs and T cells were
then stained and analyzed by FACSCalibur flow cytometer (BD Bioscience) followed by FlowJo software (Tree Star).

**DC and T Cell Activation**

Dendritic cells in lamina propria were freshly prepared and sorted by flow cytometry (CD11c\(^+\)MHCI\(^{II}\) as gate for DCs). Murine STING agonists (c-di-GMP) (Invivogen, San Diego, CA, USA) in designated concentration were incubated with DCs (3.5 \(\times\) 10\(^4\) well) in 96-well plates for 3 days. Lipofectamine 2000 (Life Technologies, Invitrogen, Carlsbad, CA, USA) was used for transfection of c-di-GMP. Same volumes of c-di-GMP and Lipofectamine 2000 (i.e., 1:1 volume ratio) were mixed and incubated in vitro for 5 min at room temperature. The complex was subsequently added into cell culture system for transfection according to the manufacturer's instruction. The cell pellet was collected for gene expression measurement.

CD4\(^+\) T cells freshly prepared from SILP and sorted by FACSCalibur (BD Bioscience) (CD3\(^+\) as gate for T cells) were cultured (2.5 \(\times\) 10\(^5\)/well) in 96-well plates for 3 days. The supernatant and cell pellets were collected for protein and gene expression measurement, respectively.

**OT-II Naive CD4\(^+\) T Cells and DCs Coculture Assay**

Naive CD4\(^+\) T cells were freshly prepared from spleen of transgenic OT-II mice and sorted as CD4\(^+\)CD44\(^-\)CD62L\(^+\) cells. Mucosal dendritic cells were freshly prepared from SILP and gated as CD11c\(^+\)MHCI\(^{II}\) cells.

To activate DCs, 200 \(\mu\)g/ml chicken ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) was added into the culture medium in vitro. Agonists of STING (c-di-GMP) (50 \(\mu\)g/ml) (Invivogen, San Diego, CA, USA), anti-IL-6 (25 ng/ml), recombinant IL-6 (20 ng/ml), anti-IL-1\(^{\beta}\) (50 ng/ml), and recombinant IL-1\(^{\beta}\) (20 ng/ml) (R&D Systems, Minneapolis, MN, USA) were incubated with OT-II naive CD4\(^+\) T cells (2.5 \(\times\) 10\(^5\)/well) and DCs (3.5 \(\times\) 10\(^5\)/well) in 96-well plates for 3 days. The supernatant was collected for protein production measurement.

**Flow Cytometry**

Isolated cells were incubated in 10% serum and Fc receptor-blocking antibody (clone NOD-15; catalog number 625801; BioLegend) for 15 min at 4°C and then stained with fluorescent-conjugated antibodies. PE/cy7-conjugated CD11c (HL3; 558079; BD PharMingen), BV510-conjugated 1-A/1-E (M5/114.15.2; 107635; BioLegend), APC/cy7-conjugated CD11b (M1/70; 101225; BioLegend), PE-conjugated CD103 (M290; 557495; BD PharMingen), and APC-conjugated B220 (RA3-6B2; 103212; BioLegend) were used for DC staining. APC-conjugated CD3 (145-2C11; 100311; BioLegend), Pacific Blue-conjugated CD4 ( GK1.5; 100428; BioLegend), PE-conjugated CD62L (MEL-14; 12-0621-81; eBioscience), and FITC-conjugated CD44 (IM7; 553133; BD PharMingen) were used for T cell staining. Cells were analyzed on FACSCalibur flow cytometer (BD Bioscience) followed by FlowJo software (Tree Star).

**DCs Sorting Strategy**

Cells from lamina propria, MLNs, or spleen were prepared according to the previous step. FSC/SSC gating was initially employed to exclude debris and identify cell population of interest, followed by DAPI\(^{-}\) to exclude dead cells and/or CD3\(^-\) to exclude T cells. For DCs from lamina propria, CD11c\(^{int\;hi}\)MHCI\(^{II}\) were subsequently used to yield all DCs, which were then separated into CD103\(^+\) or CD103\(^-\) subpopulations according to their CD103 expression. The co-expression of CD11b and CD103 on DCs were analyzed as well (Figure S1 in Supplementary Material). For DCs from MLNs or spleen, CD11c\(^{+}\)CD103\(^+\) was subsequently used to identify CD103\(^+\) DCs in respective organs (Figure S2 in Supplementary Material).

**Confocal Imaging**

To visualize the vascular system, 100 \(\mu\)g Alexa Fluor 647-conjugated wheat germ agglutinin (WGA) ( Molecular Probes, Invitrogen, Carlsbad, CA, USA) was injected (i.v.) 7 min before imaging. Mice were sacrificed and tissues were immediately removed, opened by longitudinal incision, and rinsed with PBS. Living tissues were imaged with an A1R-A1 confocal microscope (Nikon, Melville, NY, USA), and 3D reconstructions were accomplished with Volocity software (PerkinElmer, Waltham, MA, USA).

**Salmonella enterica Serovar Typhimurium Infection**

For S. enterica infection, food and water were withdrawn 4 h before the oral gavage with 200 \(\mu\)l PBS containing 1 \(\times\) 10\(^9\) CFU of naturally streptomycin resistant S. enterica (SL1344). Water was immediately resumed, and food was provided 2 h post infection. Mice were sacrificed 24 h post infection, and the small intestine was removed for cell sorting. Liver, spleen, and feces were collected for bacterial load calculation. To measure bacterial load, the liver and spleen were cut into pieces, and homogenized in 5 ml PBS. Feces were homogenized in 5 ml PBS as well. After serial dilution, the homogenate was spread onto streptomycin-containing LB plate that was then incubated at 37°C overnight. CFU was counted and calculated in the next morning. All Salmonella-related experiment was conducted within the BioSafety Level 2 facility of Nanjing University in China.

**Gene Expression and Protein Production Measurement**

Qiagen RNaseq kit was adopted for the extraction of RNA from all cell types and tissues. Subsequently, iScript cDNA synthesis kit (Bio-Rad) was used for synthesis of cDNA, and iQSYBR Green Supermix kit (Bio-Rad) was used for real-time PCR in Bio-Rad CFX96 Real-Time PCR Detection System according to the manufacturer’s instruction. Gapdh served as the house-keeping gene. The endpoint used in the real-time PCR quantification, \(C_t\), is defined by the PCR cycle number that crosses an arbitrarily placed signal threshold. Gene expression was calculated using 2\(^{-\Delta\Delta Ct}\) method, i.e., each well elicited a value of expression ratio by calculating \(2^{-(C_{target}-C_{Gapdh})}\), and each value was presented in bar graph.
Primers for murine samples were as follows: Il6 forward, 5′-tagtctctctctccacccctcc-3′, and reverse, 5′-ttgctttacctgctctctc-3′; Il10 forward, 5′-gcctactctggtctactac-3′, and reverse, 5′-ccgatccgagggatatctggtc-3′; Il7a forward, 5′-ttaacctcctctgtggc-3′, and reverse, 5′-cttctctctgctctgtgc-3′; Tfng forward, 5′-caccactccgagccagtag-3′; Ifng forward, 5′-acagcagctggccaaagttg-3′, and reverse, 5′-tgagccctccgacaggtgac-3′; Il1b forward, 5′-cactcttcaacgcagacacgacg-3′, and reverse, 5′-ggctctctctgctctgtgc-3′; Il4 forward, 5′-gcctttctctgtgcagagac-3′, and reverse, 5′-gactctctctgctctgtgc-3′; Il7a forward, 5′-cttctctctgctctgtgc-3′; Ifng forward, 5′-acagcagctggccaaagttg-3′, and reverse, 5′-tgagccctccgacaggtgac-3′; Il1b forward, 5′-cactcttcaacgcagacacgacg-3′, and reverse, 5′-ggctctctctgctctgtgc-3′; Il4 forward, 5′-gcctttctctgtgcagagac-3′, and reverse, 5′-gactctctctgctctgtgc-3′; Tgfβ1 forward, 5′-gcctttctctgtgcagagac-3′, and reverse, 5′-gactctctctgctctgtgc-3′; and Gapdh forward, 5′-gcctttctctgtgcagagac-3′, and reverse, 5′-gactctctctgctctgtgc-3′.

Concentration of IL-10, IL-6, IL-17A, IFN-γ, and IL-4 in cell culture supernatant was detected with commercial enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA, USA).

Statistic Analysis
All statistical tests were performed with GraphPad Prism Software (version 5.01; GraphPad, San Diego, CA, USA). All analyses were two-tailed, and differences were considered statistically significant when p-value < 0.05. Bars indicate mean ± SD. Student’s t-test was used for comparisons between two groups, while Kruskal–Wallis non-parametric test was used for comparisons between multiple groups. Correlation analysis was performed using linear regression model that yielded R² values. Survival rate was compared using log-rank test. All experiments were repeated at least three times.

RESULTS
Small Intestine Classical DCs Express CD103 and Elicit T Cell Responses
Dendritic cells are heterogeneous in origin. Distinct subpopulations of DCs are associated with specific functions in mucosal immunity. Recently, Zbtb46 has been identified as a DC-specific gene and their committed progenitor (17, 18). By using Zbtb46 reporter mice, we revealed a remarkable co-expression pattern of Zbtb46 and CD103 in DCs in SILP (Figures 1A,B), suggesting that the small intestine cDCs predominantly expressed CD103. By contrast, the majority of CD11c+CD103− cells expressed B220 but not CD11b (Figure 1B), suggesting they were predominantly plasmacytoid DCs (pDCs).

To compare the capacity of antigen presentation between cDCs from spleen, MLN and SILP, we established an in vitro coculture system consist of cDCs (defined as CD11c+MHCIICD103+) and OT-II TCR transgenic CD4+CD62L+CD44− naïve T cells. cDCs from SILP elicited significantly stronger immune responses (represented by IL-17A secretion) compared with CD103+ DCs from spleen and MLN upon OVA stimulation (Figure 1C; Figures S1 and S2 in Supplementary Material).

To determine whether mucosal cDCs responded to STING-mediated signaling, we transfected the microbial second messenger c-di-GMP into cDCs and cocultured with OT-II naïve T cells in the presence of OVA. We found that mucosal immunity required cDCs-mediated antigen presentation, since T cells failed to secrete either IL-17A or IL-10 in the absence of cDCs. Furthermore, we found that c-di-GMP-initiated STING signaling induced Th1 (IFN-γ/Th2 (IL-4)/Th17 (IL-17A) immune responses (19–21) while inhibited IL-10 production via OVA-activated cDCs (Figure 1D).

To compare the capacity of inducing Th17 immunity by cDCs or pDCs (defined as CD11c+MHCIICD103-B220+, Figure S1 in Supplementary Material) during STING activation, we transfected c-di-GMP into cDCs or pDCs isolated from SILP and cocultured with OT-II naïve T cells in the presence of OVA. We found that cDCs elicited significantly more IL-17A secretion compared with pDCs. Moreover, c-di-GMP transfection into pDCs failed to mount Th17 immunity in gut (Figure 1E), suggesting cDCs in SILP were the major STING signaling cells involved in Th17 differentiation.

To determine the mechanism of STING-mediated mucosal immunity, we added anti-IL-6 and recombinant IL-6 into the coculture system. Blocking of IL-6 reversed IL-17A induction and rescued IL-10 reduction by c-di-GMP, while recombinant IL-6 was able to synergize with c-di-GMP in inducing Th17 responses (Figure 1F). Similarly, blocking of IL-1β or recombinant IL-1β inhibited or rescued IL-17A production by c-di-GMP (Figure 1F). Together, we identified that STING signaling contributed to Th17 generation through the induction of IL-6/IL-1β secretion by mucosal cDCs.

We next sought to determine the obligatory role of STING in response to cyclic dinucleotides in gut. We generated Tmem173 (Sting)-deficient mice using CRISPR-Cas9 technology (Figure 1G). Tmem173-deficient mucosal cDCs expressed less Il6, Ifnb1, Il1b, and Il23a but more Il10 mRNA in steady state, and more importantly, failed to express higher inflammatory Il6, Ifnb1, Tfng, Il1b, Il23a, and Tgfb1 mRNA levels or lower Il10 mRNA level after being stimulated with c-di-GMP in vivo (Figure 1H). By contrast, Tmem173-deficient mucosal pDCs expressed similar mRNA levels of all above cytokines to C57BL/6 and were unresponsive to c-di-GMP stimulation except for the Tfng and Tgfb1 mRNA expression (Figure S3 in Supplementary Material).

Furthermore, we isolated and stimulated cDCs from SILP of C57BL/6 control and Tmem173-deficient mice with c-di-GMP and cultured them with OT-II naïve T cells in the presence of OVA. We found that Tmem173-deficient cDCs induced significantly less IL-17A in steady state and were unable to induce IL-17A production during antigen-specific T cell activation compared with C57BL/6 control cDCs. By contrast, Tmem173-deficient mucosal cDCs induced significantly more IL-10 secretion during antigen-specific T cell activation. We observed that promotion of Th1 and Th2 responses by c-di-GMP is independent of STING signaling (Figure 1I), although other vaccine adjuvants

Frontiers in Immunology | www.frontiersin.org May 2018 | Volume 9 | Article 1085

Liu et al. STING Controls Th17 in Gut
FIGURE 1 | Continued
Two DT injections significantly decreased IL-17A production during antigen-specific T cell activation by cDCs. CD11c+MHCII+CD103+ cDCs were isolated from either small intestinal lamina propria, spleen, or MLN, and were cocultured with splenic CD4+CD62L+CD44− naïve T cells from OT-II transgenic mice in the presence of OVA for 3 days in vitro. Representative of three independent experiments (n = 4).

IL-17A production during antigen-specific T cell activation by cDCs or pDCs upon the activation of STING agonist. Cells were prepared as described in panel (D). Representative of three independent experiments (n = 5). IL-17A production during antigen-specific T cell activation by CD103+ cDCs. Cells were prepared as described in panel (D) and were incubated with anti-IL-6, recombinant IL-6, anti-IL-1β, and recombinant IL-1β in the presence of OVA for 3 days in vitro. Representative of three independent experiments (n = 5).

SILP, small intestine lamina propria; MLN, mesenteric lymph node; ND, not detectable; pDC, plasmacytoid DC.

**CD103+ cDCs Are Essential for Mucosal Immunity to Cyclic Nucleotides**

To determine the essential role of cDCs in shaping mucosal immunity, we established Zbtb46 bone marrow chimeric mice in which hematopoietic system was derived from Zbtb46GFP mice. Both epithelium and hematopoietic cells expressed GFP signaling in Zbtb46GFP mice (Figure 3A left), whereas Zbtb46-expressing cDCs in small intestine were clearly identified after bone marrow transplantation in recipient C57BL/6 mice by confocal imaging (Figure 3A right). Similarly, Zbtb46GFP+ bone marrow chimeric mice were developed, in which recipient C57BL/6 mice received whole body irradiation followed by bone marrow cells from Zbtb46GFP+ mice. Two DT injections significantly depleted cDCs population in lamina propria while not affecting CD103+CD11b+CD11c+MHCII+ myeloid phagocytes. Interestingly, foreign cyclic dinucleotides induced CD103+CD11c+MHCII+ subset predominantly expressing CD11b+ (Figure 3B upper panel) even in cDCs-depleted mice (Figure 3B lower panel), implying c-di-GMP might stimulate de novo DC differentiation from precursors.

**CD103+ cDCs Are Essential for Mucosal Immunity to Cyclic Nucleotides**

To determine the essential role of cDCs in shaping mucosal immunity, we established Zbtb46 bone marrow chimeric mice in which hematopoietic system was derived from Zbtb46GFP mice. Both epithelium and hematopoietic cells expressed GFP signaling in Zbtb46GFP mice (Figure 3A left), whereas Zbtb46-expressing cDCs in small intestine were clearly identified after bone marrow transplantation in recipient C57BL/6 mice by confocal imaging (Figure 3A right). Similarly, Zbtb46GFP+ bone marrow chimeric mice were developed, in which recipient C57BL/6 mice received whole body irradiation followed by bone marrow cells from Zbtb46GFP+ mice. Two DT injections significantly depleted cDCs population in lamina propria while not affecting CD103+CD11b+CD11c+MHCII+ myeloid phagocytes. Interestingly, foreign cyclic dinucleotides induced CD103+CD11c+MHCII+ subset predominantly expressing CD11b+ (Figure 3B upper panel) even in cDCs-depleted mice (Figure 3B lower panel), implying c-di-GMP might stimulate de novo DC differentiation from precursors.

**CD103+ cDCs Are Essential for Mucosal Immunity to Cyclic Nucleotides**

To determine the essential role of cDCs in shaping mucosal immunity, we established Zbtb46 bone marrow chimeric mice in which hematopoietic system was derived from Zbtb46GFP mice. Both epithelium and hematopoietic cells expressed GFP signaling in Zbtb46GFP mice (Figure 3A left), whereas Zbtb46-expressing cDCs in small intestine were clearly identified after bone marrow transplantation in recipient C57BL/6 mice by confocal imaging (Figure 3A right). Similarly, Zbtb46GFP+ bone marrow chimeric mice were developed, in which recipient C57BL/6 mice received whole body irradiation followed by bone marrow cells from Zbtb46GFP+ mice. Two DT injections significantly depleted cDCs population in lamina propria while not affecting CD103+CD11b+CD11c+MHCII+ myeloid phagocytes. Interestingly, foreign cyclic dinucleotides induced CD103+CD11c+MHCII+ subset predominantly expressing CD11b+ (Figure 3B upper panel) even in cDCs-depleted mice (Figure 3B lower panel), implying c-di-GMP might stimulate de novo DC differentiation from precursors.

**CD103+ cDCs Are Essential for Mucosal Immunity to Cyclic Nucleotides**

To determine the essential role of cDCs in shaping mucosal immunity, we established Zbtb46 bone marrow chimeric mice in which hematopoietic system was derived from Zbtb46GFP mice. Both epithelium and hematopoietic cells expressed GFP signaling in Zbtb46GFP mice (Figure 3A left), whereas Zbtb46-expressing cDCs in small intestine were clearly identified after bone marrow transplantation in recipient C57BL/6 mice by confocal imaging (Figure 3A right). Similarly, Zbtb46GFP+ bone marrow chimeric mice were developed, in which recipient C57BL/6 mice received whole body irradiation followed by bone marrow cells from Zbtb46GFP+ mice. Two DT injections significantly depleted cDCs population in lamina propria while not affecting CD103+CD11b+CD11c+MHCII+ myeloid phagocytes. Interestingly, foreign cyclic dinucleotides induced CD103+CD11c+MHCII+ subset predominantly expressing CD11b+ (Figure 3B upper panel) even in cDCs-depleted mice (Figure 3B lower panel), implying c-di-GMP might stimulate de novo DC differentiation from precursors.

**CD103+ cDCs Are Essential for Mucosal Immunity to Cyclic Nucleotides**

To determine the essential role of cDCs in shaping mucosal immunity, we established Zbtb46 bone marrow chimeric mice in which hematopoietic system was derived from Zbtb46GFP mice. Both epithelium and hematopoietic cells expressed GFP signaling in Zbtb46GFP mice (Figure 3A left), whereas Zbtb46-expressing cDCs in small intestine were clearly identified after bone marrow transplantation in recipient C57BL/6 mice by confocal imaging (Figure 3A right). Similarly, Zbtb46GFP+ bone marrow chimeric mice were developed, in which recipient C57BL/6 mice received whole body irradiation followed by bone marrow cells from Zbtb46GFP+ mice. Two DT injections significantly depleted cDCs population in lamina propria while not affecting CD103+CD11b+CD11c+MHCII+ myeloid phagocytes. Interestingly, foreign cyclic dinucleotides induced CD103+CD11c+MHCII+ subset predominantly expressing CD11b+ (Figure 3B upper panel) even in cDCs-depleted mice (Figure 3B lower panel), implying c-di-GMP might stimulate de novo DC differentiation from precursors.

**CD103+ cDCs Are Essential for Mucosal Immunity to Cyclic Nucleotides**

To determine the essential role of cDCs in shaping mucosal immunity, we established Zbtb46 bone marrow chimeric mice in which hematopoietic system was derived from Zbtb46GFP mice. Both epithelium and hematopoietic cells expressed GFP signaling in Zbtb46GFP mice (Figure 3A left), whereas Zbtb46-expressing cDCs in small intestine were clearly identified after bone marrow transplantation in recipient C57BL/6 mice by confocal imaging (Figure 3A right). Similarly, Zbtb46GFP+ bone marrow chimeric mice were developed, in which recipient C57BL/6 mice received whole body irradiation followed by bone marrow cells from Zbtb46GFP+ mice. Two DT injections significantly depleted cDCs population in lamina propria while not affecting CD103+CD11b+CD11c+MHCII+ myeloid phagocytes. Interestingly, foreign cyclic dinucleotides induced CD103+CD11c+MHCII+ subset predominantly expressing CD11b+ (Figure 3B upper panel) even in cDCs-depleted mice (Figure 3B lower panel), implying c-di-GMP might stimulate de novo DC differentiation from precursors.
Figur 2

| Stimulator of interferon genes (STING) triggers Th17 differentiation in gut. (A) Fold change of Ifnb1, Il6, Il1b, Trif, Il10, Il23a, and Tgfb1 gene expression in classical dendritic cells from small intestinal lamina propria in response to STING agonist. PBS or c-di-GMP was injected (i.p.) before sacrifice (n = 5). (B) Confocal imaging (left) and flow cytometry analysis (middle) of IL-17A production together with IL-17A-expressing cells (right) in small intestine lamina propria, Peyer’s patch, colon, mesenteric lymph node and spleen in response to the activation of STING signaling. Il17aGFP/GFP reporter mice were injected (i.p.) with c-di-GMP on day −5, day −3, and day −1, followed by wheat germ agglutinin (WGA) injected (i.v.) 7 min before sacrifice. Fold change was calculated according to flow cytometry analysis. Representative of three independent experiments (n = 3). Scale bars, 50 µm.

(24, 25). We utilized Salmonella infection model to elucidate the role of STING-controlled Th17 responses in antimicrobial infections. Tmem173-deficient mice exhibited significantly decreased survival rate compared with C57BL/6 mice after Salmonella infection (p = 0.01) (Figure 5A). By oral gavage with Salmonella in Il17aGFP/GFP reporter mice, CD4+IL-17A+ T cells increased in
lamina propria (Figure 5B). By analyzing DC subpopulations in lamina propria, we found that cDCs (CD11c^+CD103^−) were recruited by *Salmonella* in C57BL/6 mice. By contrast, cDCs remained similar in lamina propria of *Tmem173*-deficient mice to those found in uninfected littermates (Figure 3B).

To compare the Th17 immunity induced by different DC subpopulations during *Salmonella* infection, cDCs (CD11c^+MHCII^+CD103^−B220^−) or pDCs (CD11c^−MHCII^+CD103^−2B20^+CD11b^−) isolated from SILP of uninfected or *Salmonella*-infected C57BL/6 mice were cocultured with OT-II naïve T cells in vitro in the presence of OVA (Figure S5 in Supplementary Material). *Salmonella*-infected cDCs elicited significantly more IL-17A production compared with *Salmonella*-infected pDCs or uninfected cDCs (Figure 5C). These data indicated that cDCs in SILP were the major STING signaling cells involved in mucosal Th17 differentiation during *Salmonella* infection.

In support of a direct regulation of mucosal DCs function by *Salmonella*, we found that cDCs in C57BL/6 mice expressed significantly more *Il6*, *Il1b*, *Tnf*, *Ifnb1*, *Il23a*, and *Tgfb1* mRNA and less *Il10* mRNA after *Salmonella* infection. However, *Tmem173*-deficient cDCs demonstrated naturally decreased level of *Il6* and *Ifnb1* mRNA and increased level of *Il10* mRNA. More importantly, *Tmem173*-deficient cDCs were unable to respond to *Salmonella* except elevated *Tgfb1* expression (Figure 5D). By contrast, lamina propria pDCs of C57BL/6 and *Tmem173^-/-^* mice demonstrated similar cytokine profiles at steady state and were unresponsive to *Salmonella* infection except elevated *Tgfb1* mRNA expression (Figure S6 in Supplementary Material).

Consistently, *Tmem173*-deficient CD4^+^ T cells produced naturally less IL-17A and failed to respond with the induction of Th17 cells to infection (Figure 5D). By contrast, *Salmonella* induced IFN-γ production by CD4^+^ T cells independent of STING. More importantly, we observed that *Tmem173*-deficient mice were unable to defend against *Salmonella* infection in consequence as indicated by the significantly increased bacterial load in liver, spleen, and feces compared with C57BL/6 mice (Figure 5E). Together, these data indicated that STING signaling was required as an integral mechanism of mucosal immune defenses to foreign pathogens.

**DISCUSSION**

Our study revealed that STING signaling activates IL-17A while inhibits IL-10 production by adaptive T cells during antigen presentation in gut. Mucosal CD103^+^ cDCs that express the transcription factor *Zbtb46* are required for Th17 development in response to bacterial second messenger in small intestine. In addition, STING-IRF3 signaling correlates with disease activity and mucosal inflammation severity in patients with Crohn’s disease. Since Th17 axis has been implicated as a hallmark of
infectious diseases and inflammatory bowel diseases (IBD), our discovery of STING-controlled Th17 immunity provides new insights into the pathogenesis of IBD and potential interventional target for future therapy.

Dendritic cells are responsible for T cell polarization. The ability of inducing T cell differentiation varies between subgroups of DCs. CD103+ DCs are previously found to mainly induce Tregs and suppress Th17 responses to gut bacteria (26), although inconsistent findings report that CD103+ DCs are capable but dispensable to induce Th17 cells in response to certain commensals (27, 28) We discovered that CD103+ cDCs are indispensable and capable to induce both IL-10 and IL-17A expression upon antigen stimulation, in which STING determines a Th17-dominated outcome of antigen presentation by cDCs in gut although specific mechanism remains unclear. Recent studies have reported that STING activation in T cells triggers apoptosis (29–31), suggesting diverse functions of STING that controls inflammatory events in innate immunity while inhibits lymphocytes proliferation in adaptive immunity. Interestingly, the induction of Th17 populations by cyclic dinucleotides was phenomenal in mucosa-associated immune system, implying that intestinal cDCs utilize STING for mucosal protection.

Th17 differentiation requires a series of cytokines including IL-6, TGF-β, IL-1β, and IL-23. Among them, IL-6 has been well recognized as the critical elements to induce Th17 differentiation (32–34). In addition, IL-1 signaling was reported to be critical for Th17 polarization in mice (35). Nevertheless, the role of IL-1β in Th17 induction seems controversial in human. IL-1β can induce Th17 maturation and simultaneously give rise to Th17/Th1 complex and Th1 cells (36). IL-1β is required for two types of pathogen-induced human Th17 cells with distinct effector functions (37). In our study, we demonstrated that both IL-6 and

**FIGURE 4** Stimulator of interferon genes (STING) recognizes cyclic dinucleotides and correlates with intestinal inflammation. (A) Correlation between severity of intestinal inflammation (H&E staining) and expression level of STING (IHC staining) (left) in human gut was analyzed (right). Human intestine specimens were collected at normal site from non-Crohn’s patients (control) and at para-lesions as well as lesions from Crohn’s patients. The optical density of STING was measured by Image J in IHC staining slides. Each symbol represents an individual patient. (B) Activation of STING signaling in gut of Crohn’s patients. Human intestine specimens were collected as described in panel (A). (C,D) Correlation of cytokine expression in tissue samples (C) and clinical CDAI score (D) to the expression level of STING. The optical density of STING was measured by Image J in IHC staining slides. Each symbol represents an individual patient. Pathological slides, 100× amplification.

Abbreviations: HE, hematoxylin and eosin; IHC, immunohistochemistry; CD, Crohn’s disease; CDAI, Crohn’s disease activity index.
**FIGURE 5** Continued
IL-1β are central regulators of STING-mediated mucosal Th17 immunity.

To elucidate the consequence of STING activation in mucosal cDCs, we identified STING as a target for immune evasion of enteroinvasive pathogens. We demonstrated that *Salmonella* recruits cDCs and induces significant Th17 responses in mucosal immune system that are entirely dependent on STING signaling. Furthermore, the absence of STING is associated with *Salmonella* escape from gut to liver, suggesting that STING is essential for host to prevent bacteria translocation, which is consistent to previous findings in lung (38). Nevertheless, the underlying mechanism by which pathogens regulate STING remains elusive. We hypothesize that other pathogen recognition receptor-mediated signaling could interact with STING to compete for mucosal immunity.

In conclusion, our study discovers that microbial cyclic dinucleotides recognition can drive mucosal Th17 generation. The identification of STING as a decisive element of T cell differentiation may explain why mucosal DCs are able to trigger both immune defenses through Th17 cells and immune regulation by IL-10 to shape individual reaction to gut microbiota. Polymorphisms in signaling pathways that involve Th17 polarization and IL-10 contribute to the risk profiles of IBD and other autoimmune diseases (39). Therefore, the identification of STING-induced Th17 responses and STING-suppressed IL-10 production would help to elucidate the causal relationship between generic variants and nucleic acid recognition by mucosal DCs for host defenses in gut.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

**ETHICS STATEMENT**

This study was carried out in accordance with the recommendations of Guidelines for Clinical Trials by the Ethics Committee of Nanjing Drum Tower Hospital. The protocol was approved by the Ethics Committee of Nanjing Drum Tower Hospital. All subjects gave written informed consent in accordance with the Declaration of Helsinki. This study was carried out in accordance with the recommendations of Guidelines for Animal Experiment by the Ethics Committee of Nanjing Drum Tower Hospital. The protocol was approved by the Ethics Committee of Nanjing Drum Tower Hospital.

**AUTHOR CONTRIBUTIONS**

Conceptualization: WG. Methodology: SL, QX, and QR. Investigation: SL, QX, XX, FS, QH, JW, and MW. Writing—original draft: SL and QX. Writing—review and editing: WG; funding acquisition: SL and WG. Resources and supervision: QR and WG.

**ACKNOWLEDGMENTS**

The authors acknowledge the scientific contributions from Gefei WANG and Jianan REN for this paper.

**FUNDING**

This study is supported by National Natural Science Foundation of China (81602103), Natural Science Foundation of Jiangsu Province (BK20160114), Distinguished Young Scholar Project of Medical Science and Technology Development Foundation of Nanjing Department of Health (JQX17005), Key Project of Medical Science and Technology Development Foundation of Nanjing Department of Health (YKK16114), Medical Research Program of Jiangsu Provincial Commission of Health and Family Planning (Q2017007), and Wu Jieping Medical Foundation (320.2710.1817).

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01085/full#supplementary-material.

**FIGURE S1** Lamina propria DCs separation strategy by flow cytometry. Cells from small intestine lamina propria were prepared according to the description in Section “Materials and Methods” and were sorted using FACSCalibur flow cytometer (BD Bioscience) and analyzed by FlowJo software (Tree Star). Initially, FSC/SSC gating was employed to exclude debris and identify cell population of interest, followed by DAPI− to exclude dead cells and CD3+ to exclude T cells. Subsequently, CD11b+CD103+ was used to yield all DC populations, which were then separated into CD103+ or CD103− population according to their CD103 expression. The expression of B220 in CD103+ and CD103− populations were analyzed. The co-expression of CD11b and CD103 on DCs was analyzed as well.

**FIGURE S2** Separation strategy of DCs in mesenteric lymph nodes and spleen by flow cytometry. Cells from mesenteric lymph nodes or spleen were prepared according to the description in Section “Materials and Methods” and were sorted using FACSCalibur flow cytometer (BD Bioscience) and analyzed by FlowJo software (Tree Star). Initially, FSC/SSC gating was employed to exclude debris and identify cell population of interest, followed by DAPI− to exclude dead cells. CD11c+CD103− was subsequently used to identify CD103− DCs in respective organs.
C57BL/6 and Tmem173−/− mice were mice inoculated orally with Salmonella 24 h before sacrifice, and pDCs were then isolated from small intestine lamina propria (n = 4).

**REFERENCES**

1. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet (2012) 13:260–70. doi:10.1038/nrg3182
2. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. Gastroenterology (2014) 146: 1489–99. doi:10.1053/j.gastro.2014.02.009
3. Liu S, Feng M, Guan W. Mitochondrial DNA sensing by STING signaling participates in inflammation, cancer and beyond. Int J Cancer (2016) 139: 736–41. doi:10.1002/ijc.29007
4. Liu S, Zhang Y, Ren J, Li J. Microbial DNA recognition by cGAS-STING and other sensors in dendritic cells in inflammatory bowel diseases. Inflamm Bowel Dis (2015) 21:901–11. doi:10.1097/MIB.000000000000229
5. McWhirter SM, Barbalat R, Monroe KM, Fontana MF, Hyodo M, Joncker NT, et al. A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. J Exp Med (2009) 206:1899–911. doi:10.1084/jem.20088274
6. Tanaka Y, Chen ZJ. STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. Sci Signal (2012) 5:ra26. doi:10.1126/scisignal.2005251
7. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science (2013) 339:786–91. doi:10.1126/science.1232458
8. Abe T, Barber GN. Cytosolic-DNA-mediated, STING-dependent proinflammatory gene induction necessitates canonical NF-kappaB activation through TBK1. J Virol (2014) 88:3538–41. doi:10.1128/JVI.00337-14
9. Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, et al. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. J Immunol (2014) 193:3509–13. doi:10.4049/jimmunol.1301812
10. Blaauboer SM, Gabrielle VD, Jin L. MPYS/STING-mediated TNF-alpha, not Toll IFN1, is essential for the mucosal adjuvant activity of (3′-5′)- cyclic-di-guanosine-monophosphate in vivo. J Immunol (2014) 192:492–502. doi:10.4049/jimmunol.1301812
11. Liu et al. STING Controls Th17 in Gut
12. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Immunity (2006) 736–41. doi:10.1016/j.immuni.2005.02.060
13. Best WR, Becktel JM, Singleton JW. Development of a Crohn’s disease activity index. National Cooperative Crohn’s Disease Study. Gastroenterology (1976) 70:439–44.
31. Gulen MF, Koch U, Haag SM, Schuler F, Apetoh L, Villunger A, et al. Signalling strength determines proapoptotic functions of STING. Nat Commun (2017) 8:427. doi:10.1038/s41467-017-00573-w
32. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature (2006) 441:235–8. doi:10.1038/nature04753
33. Mangan PR, Harrington LE, O’Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature (2006) 441:231–4. doi:10.1038/nature04754
34. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity (2006) 24:179–89. doi:10.1016/j.immuni.2006.01.001
35. Chung Y, Chang SH, Martinez GI, Yang XO, Nurieva R, Kang HS, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity (2009) 30:576–87. doi:10.1016/j.immuni.2009.02.007
36. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J Exp Med (2006) 203:1685–91. doi:10.1084/jem.20060285
37. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, et al. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. Nature (2012) 484:514–8. doi:10.1038/nature10957
38. Zelante T, Wong AY, Ping TJ, Chen J, Samato HR, Viganò E, et al. CD103(+) dendritic cells control Th17 cell function in the lung. Cell Rep (2015) 12: 1789–801. doi:10.1016/j.celrep.2015.08.030
39. Hall AB, Tolonen AC, Xavier RJ. Human genetic variation and the gut microbiome in disease. Nat Rev Genet (2017) 18:690–9. doi:10.1038/nrg.2017.63

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.