Journal for ImmunoTherapy of Cancer

Ectopic expression of cGAS in *Salmonella typhimurium* enhances STING-mediated IFN-β response in human macrophages and dendritic cells

Lisette Waanders,[1,2,3] Lieve E H van der Donk,[2,4] Louis S Ates,[4] Janneke Maaskant,[1] John L van Hamme,[2,4] Eric Eldering,[2,3,4,5,6] Jaco A C van Bruggen,[2,3,4,5] Joanne M Rietveld,[2,3,4,5] Wilbert Bitter,[1,2,7] Teunis B H Geijtenbeek,[2,4] Coenraad P Kuijl [1,2,3]

To cite: Waanders L, van der Donk LEH, Ates LS, *et al*. Ectopic expression of cGAS in *Salmonella typhimurium* enhances STING-mediated IFN-β response in human macrophages and dendritic cells. *Journal for ImmunoTherapy of Cancer* 2023;**11**:e005839. doi:10.1136/jitc-2022-005839

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jitc-2022-005839).

LW and LEHvdD contributed equally.

LW and LEHvdD are joint first authors.

TBHG and CPK are joint senior authors.

Accepted 30 March 2023

For numbered affiliations see end of article.

Correspondence to
Dr Coenraad P Kuijl;
c.kuijl@amsterdamumc.nl

Professor Teunis B H Geijtenbeek;
t.b.geijtenbeek@amsterdamumc.nl

ABSTRACT

Background Interferon (IFN)-β induction via activation of the stimulator of interferon genes (STING) pathway has shown promising results in tumor models. STING is activated by cyclic dinucleotides such as cyclic GMP–AMP dinucleotides with phosphodiester linkages 2′–5′ and 3′–5′ (cGAMPs), that are produced by cyclic GMP–AMP synthetase (cGAS). However, delivery of STING pathway agonists to the tumor site is a challenge. Bacterial vaccine strains have the ability to specifically colonize hypoxic tumor tissues and could therefore be modified to overcome this challenge. Combining high STING-mediated IFN-β levels with the immunostimulatory properties of *Salmonella typhimurium* could have potential to overcome the immune suppressive tumor microenvironment.

Methods We have engineered *S. typhimurium* to produce cGAMP by expression of cGAS. The ability of cGAMP to induce IFN-β and its IFN-stimulating genes was addressed in infection assays of THP-I macrophages and human primary dendritic cells (DCs). Expression of catalytically inactive cGAS is used as a control. DC maturation and cytotoxic T-cell cytokine and cytotoxicity assays were conducted to assess the potential antitumor response in vitro. Finally, by making use of different *S. typhimurium* type III secretion (T3S) mutants, the mode of cGAMP transport was elucidated.

Results Expression of cGAS in *S. typhimurium* results in a 87-fold stronger IFN-β response in THP-I macrophages. This effect was mediated by cGAMP production and is STING dependent. Interestingly, the needle-like structure of the T3S system was necessary for IFN-β induction in epithelial cells. DC activation included upregulation of maturation markers and induction of type I IFN response. Coculture of challenged DCs with cytotoxic T cells revealed an improved cGAMP-mediated IFN-γ response. In addition, coculture of cytotoxic T cells with challenged DCs led to improved immune-mediated tumor B-cell killing.

Conclusion *S. typhimurium* can be engineered to produce cGAMPs that activate the STING pathway in vitro. Furthermore, they enhanced the cytotoxic T-cell response by improving IFN-γ release and tumor cell killing. Thus, the immune response triggered by *S. typhimurium* can be enhanced by ectopic cGAS expression. These data show the potential of *S. typhimurium*–cGAS in vitro and provides rationale for further research in vivo.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Stimulator of interferon genes (STING) agonists have shown strong antitumor responses, both in vitro and in vivo tumor models. The stability and the need for intratumor injections of these agonists limit their use in the clinic.

WHAT THIS STUDY ADDS

⇒ The vaccine strain of *Salmonella typhimurium* can be engineered to express cyclic GMP–AMP synthetase, thereby producing STING agonists that activate the type I interferon response in the host.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The use of intrinsic immunostimulatory bacterial vehicles to deliver natural STING agonists creates new opportunities for bacterial-based tumor therapy. It has the potential to overcome the current limitations of STING agonists in cancer therapy.

BACKGROUND

In the last decade, various successful cancer therapies have been developed that are based on reactivation of the immune system. Many of these novel cancer immunotherapies aim to improve the capacity of T cells to kill tumor cells.[1] Nevertheless, 13%–87% of patients show poor tumor sensitivity to such treatments, depending on the type of cancer.[2 3] One of the reasons is that malignant, solid tumors promote an immune-suppressive microenvironment and are often characterized by an abnormal vascularization that restricts entry of therapeutics to the tumor site.[4] To target and disrupt this poorly accessible immune-suppressive microenvironment

is one of the greatest challenges in immunotherapy of solid tumors.

Bacterial immune therapies hold great potential in the treatment of solid tumors because of their natural properties.[5] The immunomodulatory and metabolic characteristics of bacterial species, such as *Salmonella typhimurium*, allow them to penetrate deeply into tumor tissues and even preferentially replicate in this hypoxic environment.[6] In addition, bacteria are recognized by the immune system as foreign and are therefore strong immune activators. Despite preclinical success, a phase I clinical trial with *S. typhimurium* pointed out that the vaccine strain alone is not sufficient to cure the patient and requires optimization.[7] Here, we investigated whether the immune-stimulating properties of *Salmonella* can be improved by ectopic expression of cyclic GMP–AMP synthetase (cGAS).

The enzyme cGAS is a cytosolic surveillance protein that detects double-stranded DNA (dsDNA).[8 9] Normally, there is no dsDNA present in the cytosol of healthy mammalian cells, but this can be introduced by viruses, bacteria, or dead cells. On recognition, mammalian cytosolic cGAS synthesizes cyclic GMP–AMP dinucleotides with phosphodiester linkages 2′–5′ and 3′–5′ (cGAMPs).[10–14] These dinucleotides function as second messengers to activate stimulator of interferon genes (STING), which in turn leads to a signaling cascade that induces interferon (IFN)-β.[8 15] IFN-β is part of the large type I IFN family and binds to the IFN-α/IFN-β receptor in an autocrine manner, thereby inducing hundreds of interferon-stimulated genes (ISGs).

Notably, IFN-β and ISGs have become of great interest in the field of oncology as their expression is strongly correlated to an antitumor immune response.[16] IFN-β has been shown to enhance antigen-presenting capacity of dendritic cells (DCs) and macrophages as well as cross-priming of cytotoxic $CD8^+$ T cells (CTLs).[17–19] In addition, IFN-β and ISGs are also associated with improved natural killer (NK) cytotoxicity and have a synergistic, tumor-suppressive effect in combination with other therapies such as radiotherapy.[16 20–22] STING agonist 5,6-dimethylxanthenone-4-acetic-acid (DMXAA) was successfully used to treat tumor-harboring mice in preclinical models.[23–26] However, it was found that, due to structural differences between murine STING and human STING, DMXAA showed poor results in phase III trials.[11 27–29] Since then, various human-specific STING agonists have been discovered and potentiated for the clinic. Unfortunately, the use of STING agonists in patients is limited by the need to locally administer these medications directly into tumor tissues. There is an urgent need to develop innovative targeting strategies.

Here, we investigated whether *S. typhimurium* can be engineered to activate the STING pathway, thereby combining its hypoxic colonization with its inherent immune stimulatory capacity as well as the induction of type I IFN required for antitumor responses. We engineered *S. typhimurium* to ectopically express murine cGAS, which led to the production of the natural STING agonist cGAMP. A functional needle of the type III secretion (T3S) system was required to inject cGAMPs into host cells. Notably, infection of THP-I macrophages with the cGAS-engineered bacteria induced a strong type I IFN response in a STING-dependent manner. Moreover, infection of human primary DCs with cGAS-engineered *S. typhimurium* induced DC maturation a well as CTL responses. These data strongly suggest that ectopic expression of cGAS in *S. typhimurium* could be an effective way to induce intratumor type I IFN responses leading to effective immune activation and subsequent elimination of the tumor.

METHODS

Cell lines

All THP-I cell lines were cultured in Rosewell Park Memorial Institute-1640 medium, glutaMAX (RPMI-1640, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies) and 1% penicillin/streptomycin (Gibco Life Technologies) . THP-I, harboring an IFN-β promoter–GFP–Firefly–Luciferase reporter, was obtained from Jan Rehwinkel (University of Oxford) and THP-I single clone $SLC19A1^{-/-}$ cells were a kind gift from David Raulet (University of California).[30] HeLa cells, kindly provided by David Holden (Imperial College London), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) glutaMAX (Lonza) supplemented with 10% FBS and 1% penicillin/streptomycin. JeKo-1 B cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS, Trinity Tek) and 1% penicillin/streptomycin. All cell lines were kept at 37°C 5.0% CO_2.

Primary cells

Human $CD14^+$ monocytes were isolated from buffy coats and subsequently differentiated into monocyte-derived DCs as described before.[31] In brief, the isolation from buffy coats was performed by density gradient centrifugation on Lymphoprep (Nycomed) and Percoll (Pharmacia). After separation by Percoll, the isolated monocytes were cultured in RPMI-1640 (Gibco) supplemented with 10% FCS, 2 mM L-glutamin (Invitrogen) and 1% penicillin/streptomycin containing the cytokines IL-4 (500 U/mL) and GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor, 800 U/mL) (both Gibco) for differentiation into DCs. The peripheral blood lymphocyte (PBL) fraction was processed and stored at −80°C. After 4 days of differentiation, DCs were seeded at 1×10^6/mL in antibiotic-free RPMI-1640 in a 96-well plate (Greiner), and after 2 days of recovery, DCs were stimulated or infected as described further.

Bacteria

All *S. typhimurium* wild type and mutant strains were cultivated in Lucia broth (LB) growth medium at 37°C and at 200 rpm.[32] *S. typhimurium* SL1344 Salmonella wild-type

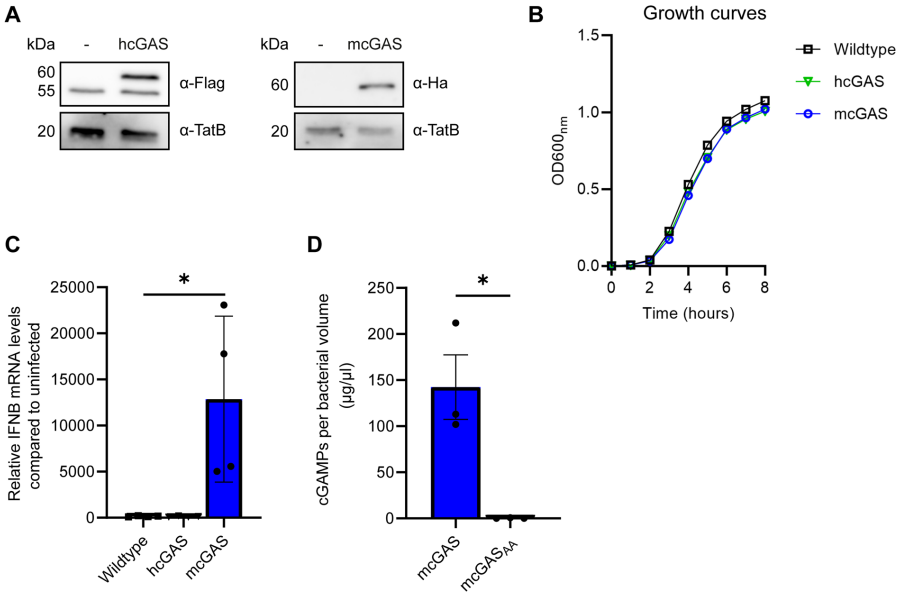

Figure 1 Expression of mcGAS in SL1344 induces IFN-β expression in a human macrophage cell line. (A) Protein expression of SL1344 transformed with pABCON Flag-hcGAS (60.2 kDa) or pMW215 Ha-mcGAS (59.6 kDa) verified with western blot. Equal loading was detected with α-TatB. (B) SL1344 wild type, SL1344 hcGAS and SL1344 mcGAS growth curves in pound. (C) THP-I cells were infected with SL1344 wild type, SL1344 hcGAS and SL1344 mcGAS. IFN-β mRNA levels are determined by qRT-PCR 2.5 hours after infection. IFN-β levels are relative to uninfected THP-I. IFN-β levels: SL1344-wild type mean 147.7, SEM 28.3; SL1344-mcGAS mean 12859, SEM 4499. statistics: one-way analysis of variance, corrected for multiple comparisons, error bars: SEM. (D) Production of cGAMPs by SL1344 mcGAS and SL1344 $mcGAS_{AA}$ quantified. Concentration cGAMPs in bacterial pellets are corrected for OD unit input and bacterial volume. cGAMP levels: SL1344-mcGAS mean 142.3, SEM 35.0; SL1344-$mcGAS_{AA}$ mean 0.29, SEM 0.23. statistics: unpaired t-test, error bars: SEM. P=0.12. *P≤0.05. cGAMP, cyclic GMP–AMP dinucleotides with phosphodiester linkages 2′–5′ and 3′–5′; hcGAS, human cyclic GMP–AMP synthetase; IFN, interferon; mcGAS, murine cyclic GMP–AMP synthetase; OD, optical density; qRT-PCR, Quantitative Reverse Transcription Polymerase Chain Reaction.

SB300, SL1344 Δ*invG* SB161 (referred in the article as SL1344 $SPI\text{-}1_{KO}$), SL1344 Δ*sseD::aphT* M556 (referred as SL1344 $SPI\text{-}2_{KO}$), and SL1344 Δ*sseD::aphT sopBEE2* M716 (referred as SL1344 $SPI\text{-}2_{NEEDLE}$) were kindly provided by Wolf-Dietrich Hardt (ETH Zürich).[33] All *S. typhimurium* strains were transformed via electroporation using gene pulser cuvettes (BioRad) and Eporator (Eppendorf) at 1700 V. *S. typhimurium* 1344 and SL3261 were transformed with pAbcon Flag-human cyclic GMP–AMP synthetase (hcGAS)-mCherry, pMW215 HA-cGAS-mScarlet, or pMW215 HA-$cGAS_{AA}$ and were grown in LB under continuous antibiotic selection with 0.03 µg/mL chloramphenicol (Sigma) for pABCON contructs or 0.1 µg/mL ampicillin sodium salt (Sigma) for pMW215 constructs.

Immunoblot analysis

Expression of proteins was verified by loading bacterial lysates resuspended in sample buffer (50 mM Tris–HCl, 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 5 mM EDTA, and 10% glycerol) on 10% SDS page gel (figure 1A) or 12.5% SDS page gel (online supplemental 3 and 4). Western blot was used to visualize proteins by using α-Ha.11 Monoclonal Mouse IgG1 clone 16B12 (BioLegend) or monoclonal mouse α-flag clone M2 (Sigma) and the loading control polyclonal rabbit α-TatB (a kind gift from Matthias Müller, Albert-Ludwigs-Universität Freiburg). Goat-anti-mouse or goat-anti-rabbit IgG peroxidase-labeled antibodies (American Qualex Antibodies, San Clemente, USA) were used as secondary antibodies and detected with electrochemiluminescence western blotting detection reagent (Amersham Bioscience, Amersham, UK).

Gentamycin protection assay

Gentamycin protection assay was conducted and analyzed according to the protocol of Hapfelmeier *et al*.[33] In short, HeLa cells were exposed to bacteria for 10 min at 37°C 5% CO_2. Infection stocks were plated to determine input colony forming units (CFUs). After infection, cells were washed three times with HBSS (Hanks' Balanced Salt Solution: $CaCl_2$ 0.14 g/L, KCl 0.4 g/L, KH_2PO_4 0.06 g/L, $MgSO_4$ 0.098 g/L, NaCl 8 g/L, Na_2HPO_4 0.048 g/L, $NaHCO_3$ 0.35 g/L, and glucose 1 g/L). DMEM supplemented with 10% FBS was added to cells for 20 min. Afterwards, the medium was replaced with DMEM supplemented with 10% FBS and 100 µg/mL gentamycin (Gibco) and incubated for 30 min. After one wash (PBS, $CaCl_2$ 0.1 g/L, MgCl2x6H_2O pH7.4 0.1 g/L), cells were lysed with 0.2 g sodium deoxycholate in water. Output CFU plates were made and invasiveness was calculated, normalized to wild type of two independent experiments conducted in duplicate.

Macrophage and DC infections

THP-I cells were seeded and differentiated for 24 hours with 25 ng/mL phorbol 12-myristate 13-acetate (Sigma). After 24 hours, the medium was replaced with RPMI-1640 with 10% FBS and kept for 48 hours in an incubator prior to infection. *S. typhimurium* infection stocks were prepared from overnight cultures in LB, of which 1:33 was added to RPMI supplemented with 10% FB on the following day. Strains were kept at 37°C 200 rpm until they reached the exponential phase. Macrophages and DCs were infected with a multiplicity of infection of 30 and incubated for 30 min at 37°C 5% CO_2. Extracellular bacteria were removed from THP-I macrophages by a single wash with PBS, followed by 1-hour incubation in RPMI-1640 supplemented with 10% FBS and 100 µg/mL gentamycin (Gibco). Afterwards, the medium was replaced with RPMI-1640 supplemented with 10% FBS and 10 µg/mL gentamycin and was kept in an incubator at 37°C 5% CO_2 until the time of measurement or processing. Extracellular bacteria were removed from DC cultures by a single wash with PBS, followed by addition of RPMI-1640 10% FCS and 10 µg/mL gentamycin, and were kept in an incubator at 37°C 5% CO_2. A lipopolysaccharide (LPS)-only control was included to help distinguish between TLR4-induced IFN-β production and cGAS/STING-induced IFN-β production. For this, THP-I macrophages and DCs were stimulated with 10 ng/mL LPS from *S. typhimurium* (Sigma). Bacterial input was determined by spotting bacterial suspension and counting of CFU on LB agar plates.

Cytotoxic T-lymphocyte responses: cytokine production

DCs were infected with SL3261 murine cyclic GMP–AMP synthetase (mcGAS) or SL3261 $mcGAS_{AA}$ for 30 min, spun down, and the medium was replaced with RPMI-1640 supplemented with 10% FCS and 100 µg/mL gentamycin. Next, the stimuli LPS or cGAMP (10 µg/mL, Invitrogen) were added to the corresponding wells. The cGAMPs were incubated with transfection reagent Lyovec (Invivogen) according to manufacturer's instructions to ensure intracellular delivery of the cGAMPs prior to adding to DCs. After 16-hour incubation, DCs were cocultured with allogeneic PBLs in Iscove's Modified Dulbecco's Media (Gibco) supplemented with 10% FCS and 1% penicillin/streptomycin, using a 1:8 ratio. As a positive control, T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (BioLegend). After 3 days, IL-2 (20 U/mL, Chiron) was added to the cocultures. After 6 days, PBLs were harvested, plated in 96-well plates, and incubated for 4 hours with brefeldin A (10 µg/mL, Sigma), after which they were analyzed for cytokine production by flow cytometry as described further.

Cytotoxic T-cell killing assay

Cocultures of DCs and PBLs were performed as described further. After 6 days of coculture, CD19-expressing JeKo-1 B cells were labeled with Cell Trace Violet (CTV) (Thermo Fisher Scientific) according to the manufacturer's instructions. Stimulated PBLs were harvested, counted, and subsequently cocultured for 16 hours with CTV+ JeKo-1 B cells in an effector to target ratio of 4:1. The CD19-targeting bispecific T-cell engager blinatumomab[34] (a kind gift from Eric Eldering) was added in concentrations ranging from 0 to 100 pg/mL where indicated. Viability of the JeKo-1 B cells was assessed using TO-PRO-3 and Mito-Tracker Orange (Invitrogen) using flow cytometry. Specific cell death of the JeKo-1 B cells was assessed by gating the live cells (TO-PRO-3 negative and Mito-Tracker Orange positive) within the CTV^+ population, and the percentage of specific target cell killing was subsequently calculated as 100, the percentage of viable JeKo-1 B cells.

Real-time quantitative PCR analysis

THP-I and HeLa cells were harvested at 2.5 or 5.0 hours post infection. Isolation of mRNA was conducted with NucleoSpin kit (Machery-Nagel) or mRNA Catcher PLUS Purification kit (Thermo Fisher Scientific) according to protocol of the manufacturer. Reverse transcriptase of mRNA to cDNA was conducted with RevertAid First Strand cDNA Synthesis kit (ThermoScientific) or with a reverse-transcriptase kit (Promega) according to protocol of the manufacturer. An input of 30 ng cDNA and total primer concentration of 0.06 µM was used per reaction. SYBR Green Master Mix (BioRad) and white 96-well PCR plates, semiskirted (Applied Biosystems), were used in StepOne Real-Time PCR System (Applied Biosystems). Alternatively for data in figures 2 and 3, PCR amplification was performed in the presence of SYBR green (Thermo Fisher Scientific), primers and cDNA in a 7500 Fast Realtime PCR System (ABI). Comparative delta Ct method was used to determine relative expression levels.

Flow cytometry

To measure DC activation markers, cells were fixated for 30 min with 4% paraformaldehyde (PFA, Electron Microscopy Sciences). For cell surface staining, cells were incubated in 0.5% PBS-BSA (Sigma-Aldrich) containing antibodies for 30 min at 4°C. To measure CTL responses, PBLs were harvested and stained for viability using Fixable Viability Dye eFluor 780 (eBioscience) for 5 min at 4°C and subsequently fixated for 10 min with 2% PFA. After fixation, cells were permeabilized using Perm/Wash solution (BD Biosciences) for 5 min 4°C and incubated with antibodies diluted in Perm/Wash solution for 10 min at 4°C. Single-cell measurements were performed with a FACS Canto flow cytometer (BD Biosciences). FlowJo V.10 software was used to analyze the data. Antibody clones used to analyze DC activation are CD86 (2331 (FUN-1)), CD80 (L307.4), and CD83 (HB15e) (all BD Pharmingen). For each experiment, live cells were gated on FSC (forward scatter) and SCC (side scatter) and analyzed further with the markers mentioned. Antibody clones used for cytotoxic T-cell responses are CD3 (UCHT1),

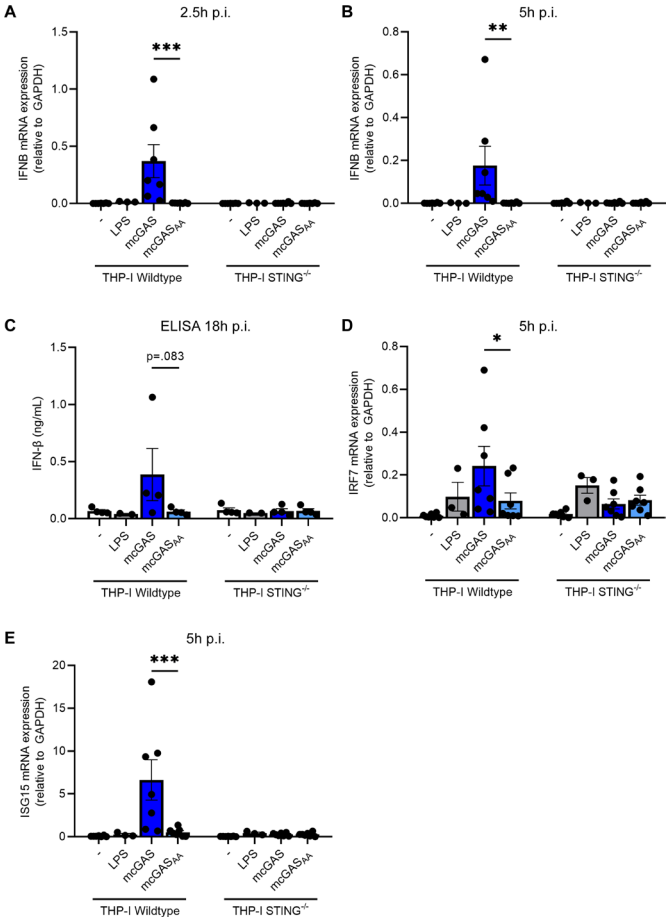

Figure 2 IFN-β induction by mcGAS is mediated via cGAMPs and STING. (A,B) THP-I wild type or THP-I STING$^{-/-}$ were exposed to LPS, SL1344 mcGAS, and SL1344 mcGAS$_{AA}$. Samples were analyzed 2.5 and 5.0 hours p.i. with qRT-PCR. 2.5 hours: SL1344-mcGAS mean 0.37, SEM 0.15; SL1344-mcGAS$_{AA}$ mean 0.0037, SEM 0.00094; 5.0 hours: SL1344-mcGAS mean 0.18, SEM 0.09; SL1344-mcGAS$_{AA}$ mean 0.0017, SEM 0.0009. (C) IFN-β protein expression in the supernatant of different conditions is determined 18 hours p.i.: SL1344-mcGAS mean 0.39, SEM 0.23; SL1344-mcGAS$_{AA}$ mean 0.063, SEM 0.015. (D) IRF7 mRNA expression 5 hours p.i.: SL1344-mcGAS mean 0.24, SEM 0.09; SL1344-mcGAS$_{AA}$ mean 0.08, SEM 0.04. (E) ISG15 mRNA expression in THP-I 5 hours p.i.: SL1344-mcGAS mean 6.63, SEM 2.36; SL1344-mcGAS$_{AA}$ mean 0.52, SEM 0.18. Statistics: two-way analysis of variance, Tukey's multiple comparison test. Error bars: SEM. *P≤0.05, **P≤0.005, ***P≤0.0005. cGAMP, cyclic GMP–AMP dinucleotides with phosphodiester linkages 2′–5′ and 3′–5′; IFN, interferon; IRF7, interferon regulatory factor; LPS, lipopolysaccharide; mcGAS, murine cyclic GMP–AMP synthetase; p.i., post infection; STING, stimulator of interferon genes.

CD8 (RPA-T8), IFN-γ (B27) (all BioLegend), perforin (dG9, eBioscience), and granzyme B (GB11, BD Pharmingen). For each experiment, live cells were selected and gated on CD3 and CD8 expressions. In this CD3^{+}CD8^{+} population, cytokine expression was analyzed.

ELISA

Overnight cultures of SL1344 mcGAS and SL1344mcGAS$_{AA}$ were continued by taking one optical density (OD) and added to 10 mL MgM-MES pH7.4 (170 mM MES (2-(N-morpholino) ethanesulfonic acid), 5 mM KCl, and 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 8 µM $MgCl_2(6xH_2O)$, 38 mM glycerol, and 0.1% casamino acids) supplemented with antibiotics until cultures reached again ~OD 1. Bacteria were pelleted at 10 min, 5000 rpm, and supernatant was taken for cGAMP measurement according to 2′-3′-cGAMP ELISA Kit manufacturer (Cayman chemical). THP-I and DC supernatants were harvested 18–24 hours after stimulation, and secretion of IFN-β was measured by a human IFN-beta DuoSet ELISA (R&D Systems) according to the manufacturer's instructions.

Growth curves

Bacterial $OD600_{nm}$ was measured by using a BioTek Synergy. A bacterial suspension of $OD600_{nm}$ 0.05 in LB with appropriate antibiotics was used as input. Bacteria were incubated at 37°C with linear shaking. LB background was subtracted from $OD600_{nm}$ values.

Constructs and oligos

See online supplemental file 1 for oligos and used constructs.

RESULTS

Murine cGAS in *S. typhimurium* induces IFN-β production in macrophages

The cytoplasmic dsDNA sensor cGAS synthesizes cGAMPs.[8 10 12–15] Although both human and murine cGAS produce cGAMPs, they differ in dsDNA recognition. hcGAS is more selective as it is only activated by long dsDNA, while mcGAS is more sensitive.[35] To investigate which mammalian cGAS is most efficient in inducing IFN-β in the host, both were introduced in *S. typhimurium* strain SL1344. Human Flag-cGAS and murine HA-cGAS were cloned in pAbcon and pMW215, respectively. Both cGAS proteins were efficiently expressed in *S. typhimurium* SL1344 (figure 1A). Expression of neither hcGAS nor mcGAS affected bacterial growth as growth curves of transformed SL1344 were similar to SL1344-wild type (figure 1B). Interestingly, whereas expression of hcGAS did not show a significant effect, infection of the human macrophage cell line THP-I with SL1344-mcGAS induced on average 87-fold more IFN-β than SL1344-wild type (figure 1C). Next, we determined the amount of cGAMPs produced by SL1344-mcGAS (figure 1D and online supplemental 2). We used a double-point mutation in the catalytic site of mcGAS (mcGAS$_{AA}$) as negative cGAMP control (online supplemental 3).[8] Notably, SL1344-mcGAS shows a 39-fold higher increase in cGAMP production than SL1344-mcGAS$_{AA}$. Collectively, these data strongly suggest that mcGAS expressed in *S. typhimurium* is well tolerated, produces high amounts

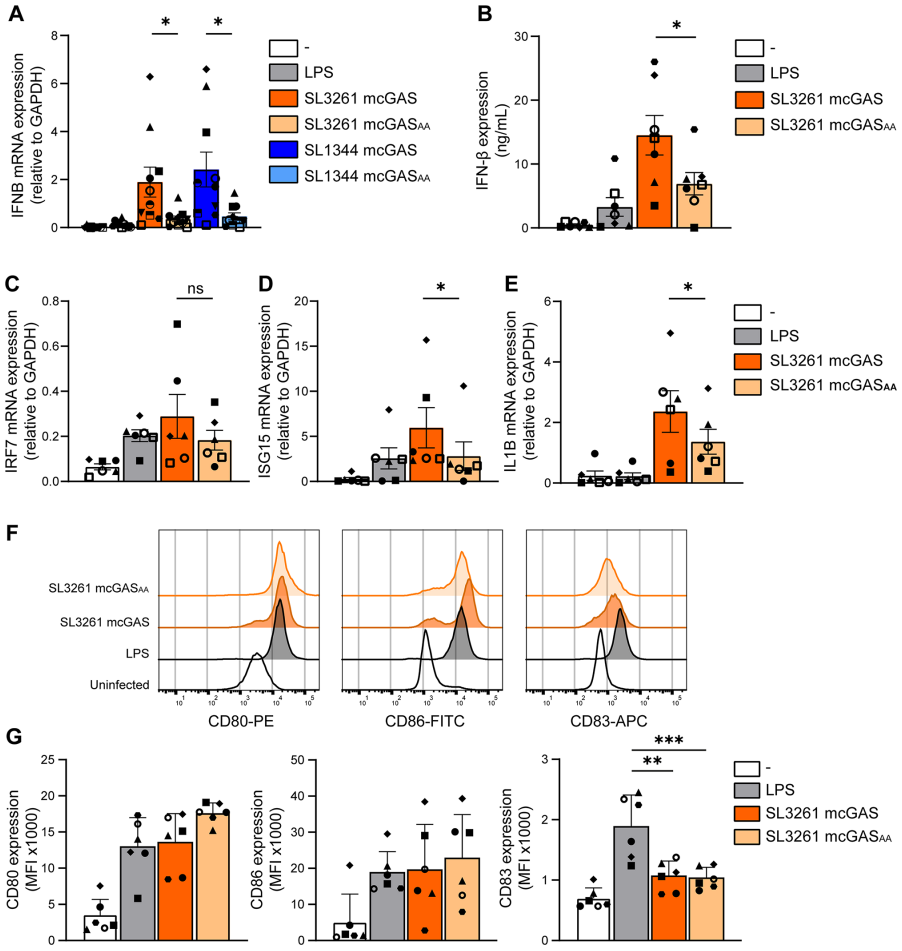

Figure 3 Expression of mcGAS in SL3261 induces IFN-β expression in primary human DCs. DCs were exposed to LPS, SL1344 mcGAS, SL1344 mcGAS$_{AA}$, SL3261 mcGAS, and SL3261 mcGAS$_{AA}$. Samples were analyzed 5 and 18 hours p.i. with qRT-PCR or 24 hours p.i. for protein production and maturation. (A–E) Expression levels of IFNB mRNA 5 hours p.i. (A) and IFN-β protein 24 hours p.i. (B), IRF7 mRNA 18 hours p.i. (C), ISG15 mRNA 18 hours p.i. (SL3261-mcGAS mean 5.96, SEM 2.23; SL3261-mcGAS$_{AA}$ mean 2.81, SEM 1.58) (D), and pro-IL-1B mRNA 18 hours p.i. (E) were determined. (F) Representative histograms of CD80, CD86, and CD83 expression. (G) Cumulative flow cytometry data of CD80, CD86, and CD83 expressions. Data represent 10 donors analyzed in five separate experiments (A) and 6 donors analyzed in three separate experiments (B–E,G), with each symbol representing a different donor. Statistics: two-way analysis of variance, Tukey's multiple comparison test (A,G), and paired Student's t-test (B–E). Error bars: SEM. *P≤0.05, **P≤0.005, ***P≤0.0005. DC, dendritic IFN, interferon; cell; IRF7, interferon regulatory factor; LPS, lipopolysaccharide; mcGAS, murine cyclic GMP–AMP synthetase; MFI, mean fluorescence intensity; ns, not significant; p.i., post infection.

of cGAMPs, and induces an IFN-β response in infected macrophages.

mcGAS-mediated IFN-β expression is STING dependent

As SL1344-mcGAS produces cGAMPs and induces strong IFN-β expression, we investigated the role of STING by infecting THP-I wild type and THP-I STING$^{-/-}$ macrophages. Macrophages were infected with either SL1344-mcGAS or SL1344-mcGAS$_{AA}$, and type I IFN responses were measured by RT-qPCR (figure 2A,B) and ELISA (figure 2C). Stimulation of THP-I with LPS showed no significant effect on IFN-β mRNA levels as compared with uninfected control, indicating that LPS alone does not induce a strong IFN-β response in THP-I macrophages. In contrast, SL1344-mcGAS showed a 100-fold increase of IFN-β mRNA expression compared with mcGAS$_{AA}$ at both time points. Therefore, we conclude that the enzymatic activity of mcGAS is responsible for IFN-β induction in SL1344-mcGAS. IFN-β production was fully abrogated in the THP-I STING$^{-/-}$ macrophages. These results were confirmed on protein level in an IFN-β ELISA (figure 2C). Although IFN-β protein levels were not statistically different, THP-I infected macrophages with SL1344-mcGAS induced an average 6.1-fold increase compared with SL1344-mcGAS$_{AA}$. Again, THP-I STING$^{-/-}$ infected macrophages did not show a difference between SL1344-mcGAS and SL1344-mcGAS$_{AA}$. Next, we investigated the induction of ISG interferon regulatory factor (IRF7) and ISG15 to verify that a functional IFN-β response was induced (figure 2D,E). SL1344-mcGAS infection of wild type, but not THP-I STING$^{-/-}$ macrophages, led to a significant induction of both IRF7 (average 3.0-fold) and ISG15 (average 12.7-fold) in contrast to infection

with SL1344-mcGAS$_{AA}$. These data strongly suggest that cGAMP produced by SL1344-mcGAS induces STING-dependent type I IFN responses in THP-I macrophages.

cGAMPs are transported through SPI-1 needle

In order to induce the STING pathway, cGAMPs should be in the cytosol of the host cell. We therefore set out to determine the molecular mechanism of cytosolic cGAMP delivery by SL1344-mcGAS. Several cyclic dinucleotide transporters have been identified of which mammalian folate carrier SLC19A1 has been shown to be important in extracellular cGAMP uptake by THP-I cells.[30 36] To determine if SLC19A1 is involved in cGAMP uptake, we infected THP-I wild type and THP-I SLC19A1$^{-/-}$ single clones with SL1344-mcGAS and SL1344-mcGAS$_{AA}$. No statistical differences in IFN-β mRNA levels were observed between the THP-I SLC19A1$^{-/-}$ and wild-type macrophages after 5 hours of infection, indicating that SLC19A1 is not the principal transporter responsible for cGAMP transport into the cytoplasm (figure 4A).

To test whether bacterial transporters are important for STING activation, we used mcGAS expressing T3S *Salmonella* mutants to infect HeLa cells.[33] Unlike macrophages, HeLa cells can only be invaded by *S. typhimurium* using the T3S systems encoded by the *Salmonella* pathogenicity island 1 (SPI-1). The T3S systems form a needle-like structure that inject bacterial effectors in host cells.[37] A second T3S system encoded by SPI-2 is important for intracellular survival by maintaining the Salmonella containing vacuole (SCV).[37] To study the role of the T3S systems on IFN-β induction, we made use of four strains: wild-type Salmonella (SL1344), an SPI-1 mutant (SL1344 *SPI-1*$_{KO}$), a SPI-2 mutant (SL1344 *SPI-2*$_{KO}$), and a SPI-1 mutant lacking secreted effectors required for invasion, yet having a functional needle (SL1344 *SPI-1*$_{NEEDLE}$)[33] (figure 4B). We verified bacterial invasion of these T3S system mutants. As expected, SL1344 *SPI-1*$_{KO}$ and SL1344 *SPI-1*$_{NEEDLE}$ CFU counts were strongly reduced by the gentamycin treatment, whereas both wild type and the *SPI-2*$_{KO}$ where protected due to their intracellular localization (figure 4C). In parallel, the IFN-β expression was determined in infected HeLa cells (figure 4D). SL1344 *SPI-2*$_{KO}$ mcGAS induced similar IFN-β levels to wild-type mcGAS, indicating that SPI-2 T3S machinery is not necessary for IFN-β induction. However, SL1344 *SPI-1*$_{KO}$ showed reduced IFN-β expression, demonstrating that SPI-1 is required for STING-pathway activation. Notably, SL1344 *SPI-1*$_{NEEDLE}$ showed similar IFN-β induction as wild-type mcGAS, strongly suggesting that the SPI-1 T3S needle facilitates cGAMP transport from the bacterium to the host.

Expression of mcGAS in auxotrophic *S. typhimurium* SL3261 strengthens IFN-β production by primary human DCs

S. typhimurium SL1344 is a virulent strain and is therefore not applicable in a clinical setting. Therefore, we further investigated the potential of mcGAS in the live-attenuated vaccine strain SL3261. SL3261 is derived from SL1344 but is a histidine auxotroph due to a mutation in the *AroA* gene,[32] making it a safe, self-limiting organism. Similar as to SL1344, SL3261 growth is unaffected by ectopic mcGAS expression (online supplemental 4). We compared the IFN-β response on challenge with SL1344-mcGAS and SL3261-mcGAS in primary monocyte-derived DCs from healthy donors (figure 3A). DCs were exposed to LPS, SL1344-mcGAS, SL1344-mcGAS$_{AA}$, SL3261-mcGAS, and SL3261-mcGAS$_{AA}$. After 5 hours of incubation, the cells were lysed and the expression of IFN-β was determined by qRT-PCR. Both SL1344 and SL3261 strains expressing mcGAS showed a significant increase in IFN-β expression in DCs as compared with their respective catalytically inactive mcGAS$_{AA}$ controls. These data indicate that both the virulent and non-virulent *Salmonella* strains with mcGAS expression induce IFN-β expression in human primary DCs.

Due to the clinical relevance of SL3261, we continued with SL3261 and determined the IFN-β production on protein level by ELISA. These data confirmed that SL3261-mcGAS induced significantly higher levels of IFN-β than LPS or mcGAS$_{AA}$ (figure 3B). Next, IRF7 and ISG15 expression levels were measured (figure 3C,D). A trend was observed for IRF7 between SL3261-mcGAS and SL3261-mcGAS$_{AA}$ and a significant twofold higher ISG15 expression is seen in SL3261-mcGAS-infected cells. This modest but significant difference could possibly be attributed to LPS triggering of TLR4 on primary DCs. Indeed, both LPS and SL3261-mcGAS$_{AA}$ induced a strong but short IFN-β pulse, whereas SL3261-mcGAS induced a stronger and sustained IFN-β expression pattern (online supplemental 5 and figure 3A). Furthermore, SL3261-mcGAS induced significantly higher upregulation of the proinflammatory cytokine IL-1β compared with the catalytically inactive control (figure 3E). We also determined the expression of the costimulatory molecules CD80 and CD86 and maturation marker CD83 by flow cytometry (figure 3F,G). In this assay, all strains and mutants induced similar levels of CD80 and CD86, which were comparable to the response to LPS. CD83 expression was significantly lower on DCs when infected with either SL3261-mcGAS or SL3261-mcGAS$_{AA}$. Together, these data show that the vaccine strain activates DCs, but immunomodulatory properties are further strengthened by mcGAS expression.

SL3261-mcGAS-mediated IFN-β production by DCs induces potent cytotoxic T-cell responses

Next, we investigated whether DC activation by SL3261-mcGAS is functional with regard to the induction of CTLs. CTLs are crucial players in antitumor immunity, which can increase their cytotoxic function when exposed to proinflammatory stimuli such as IFN-β.[18] We exposed DCs to LPS, cGAMP, SL3261-mcGAS, and SL3261-mcGAS$_{AA}$ and cocultured these with allogeneic PBLs to analyze their capacity to activate CTLs. After 6 days of coculture, the cytokine production by CD3^{+}CD8^{+} T cells was determined (figure 5A,B). LPS or cGAMP-exposed DCs did

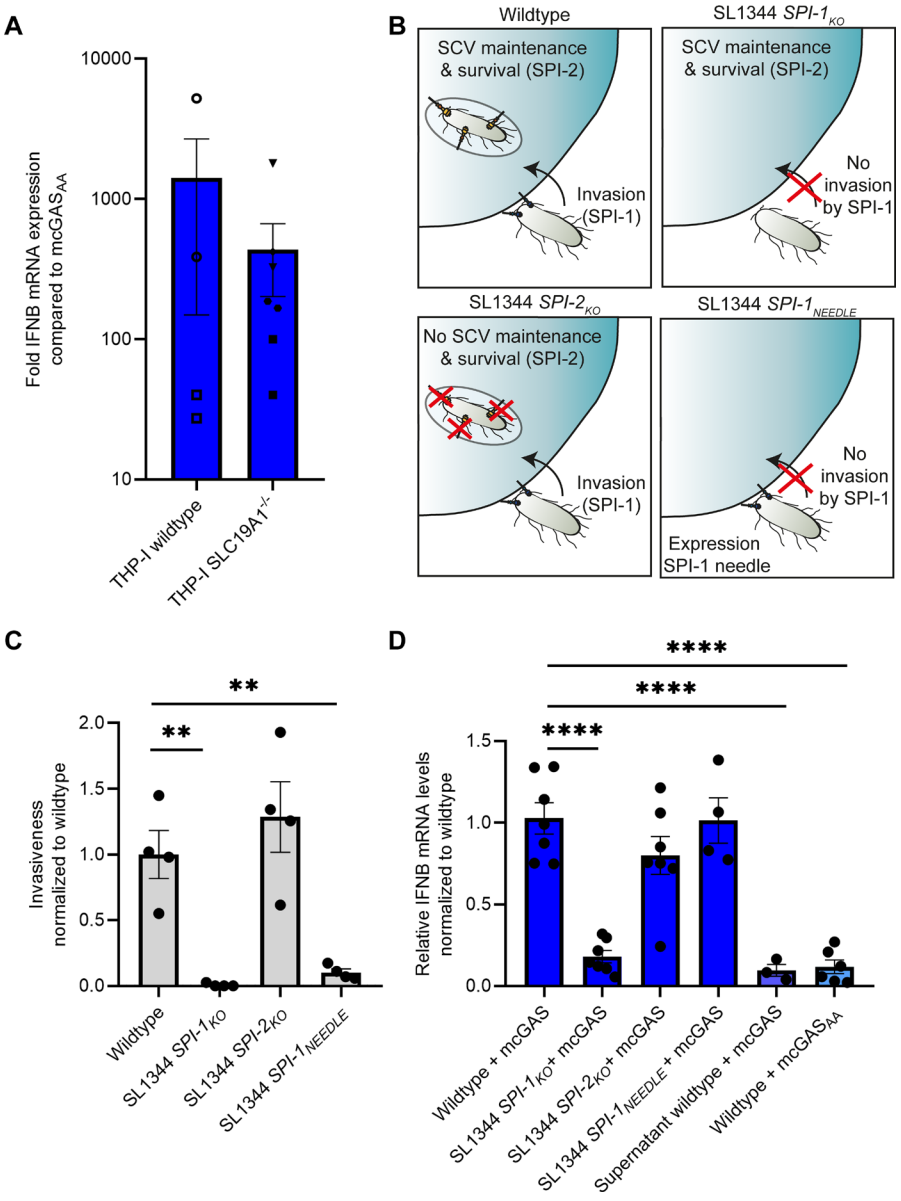

Figure 4 *Salmonella* produces cGAMPs that are injected into host cells by SPI-1. (A) IFNB expression levels of THP-I SLC19A1$^{-/-}$ single clones compared with THP-I wild type 2.5 hours p.i. with SL1344 mcGAS or SL1344 mcGAS$_{AA}$. Results of each THP-I control or SLC19A1$^{-/-}$ single-cell clone is indicated with a similar symbol. (B) Overview of SL1344 SPI-1 or SPI-2 mutants. SL1344 wild type has functional SPI-1 and SPI-2 T3S which enables the bacterium to invade the host and to survive within the SCV. SL1344 *SPI-1*$_{KO}$ is a functional SPI-1 knockout and cannot invade host cells. SL1344 *SPI-2*$_{KO}$ is a functional SPI-2 knockout; it can invade but cannot maintain its presence in SCV. SL1344 *SPI-1*$_{NEEDLE}$ has the SPI-1 needle but does not have the effector proteins that are necessary to invade the host. (C) Verification of invasiveness with gentamycin protection assay of SL1344 T3S mutant strains in HeLa. (D) IFNB levels measured by qRT-PCR 2.5 hours after challenge of HeLa cells. IFN-β levels of different conditions are normalized to wild-type mcGAS. Filtered supernatant of SL1344 mcGAS and SL1344 mcGAS$_{AA}$ are included as control. Statistics: one-way analysis of variance, corrected for multiple comparisons. Error bars: SEM. **P≤0.005, ****P≤0.0001. cGAMP, cyclic GMP–AMP dinucleotides with phosphodiester linkages 2′–5′ and 3′–5′; IFN, interferon; mcGAS, murine cyclic GMP–AMP synthetase; SCV, *Salmonella*-containing vacuole; SPI-1, *Salmonella* pathogenicity island 1; T3S, type III secretion.

not significantly increase IFN-γ production in CTLs in the coculture as compared with unstimulated DCs. However, DCs infected with SL3261-mcGAS induced significantly higher IFN-γ levels in CTLs than DCs exposed to SL3261-mcGAS$_{AA}$ (figure 5C). Interestingly, this difference could be complemented by transfecting cGAMPs into mcGAS$_{AA}$-infected DCs. A similar trend was observed in CTLs for perforin and granzyme B expression levels; incubation of DCs with SL3261-mcGAS led to a slightly higher increase of perforin and granzyme B CTLs as compared with incubation with SL3261-mcGAS$_{AA}$ control (figure 5D,E). Addition of cGAMP rescued the effect in DCs treated with SL3261-mcGAS$_{AA}$. Interestingly, neither LPS nor CD3/CD28 stimulation induced the expression of perforin

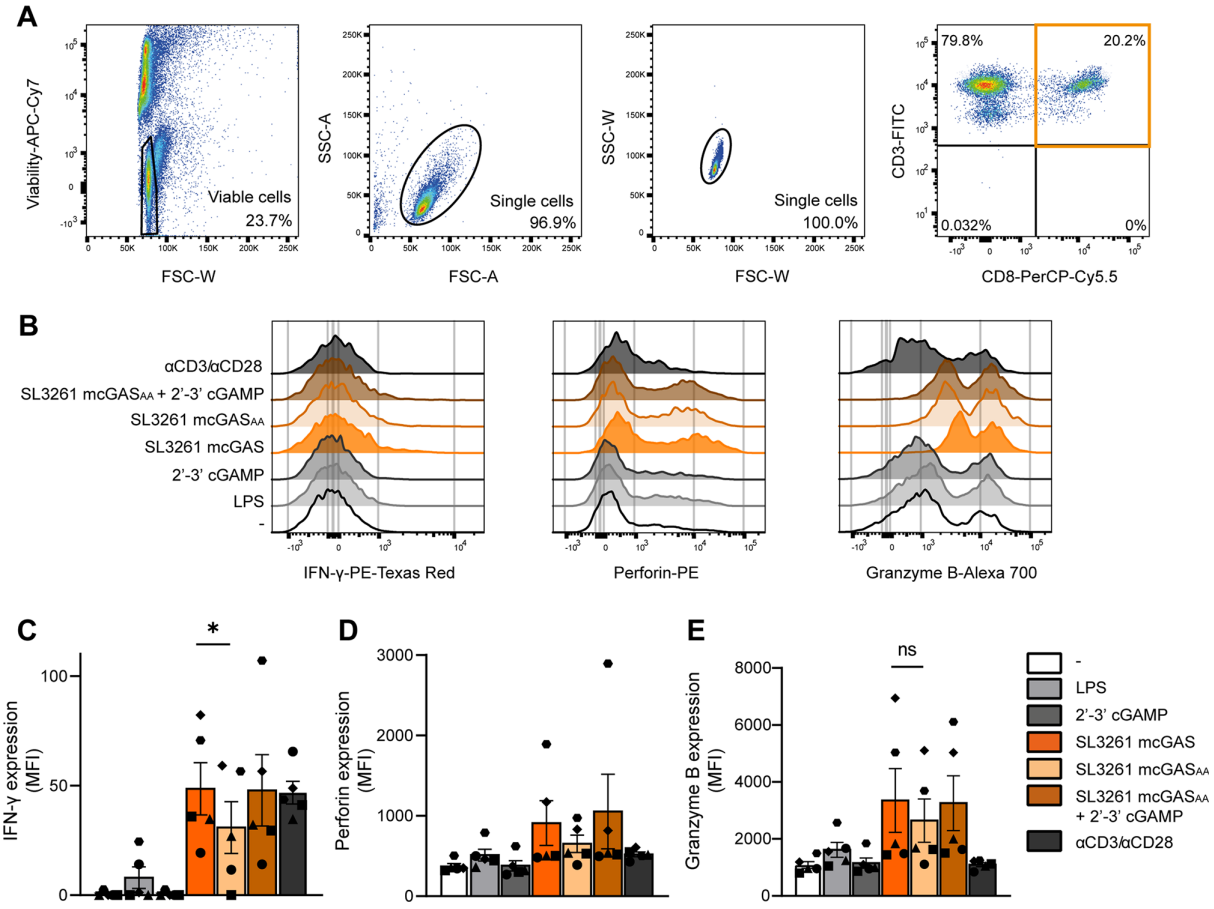

Figure 5 SL3261 mcGAS-mediated IFN-β production by DCs induces cytotoxic T-cell responses. (A–E) DCs were exposed to LPS, cGAMP, SL3261 mcGAS, SL3261 mcGAS$_{AA}$, or SL3261 mcGAS$_{AA}$ supplemented with 2′-3′-cGAMP for 16 hours and subsequently cocultured with allogeneic human PBLs. (A) After 6 days of coculture, the CD3$^+$ CD8$^+$ T cells were gated and analyzed for cytokine production by flow cytometry. (B) Representative histograms of IFN-γ, perforin, and granzyme B expressions. (C–E) Cumulative flow cytometry data of IFN-γ, perforin, and granzyme B expressions. Data represent five donors analyzed in three separate experiments, with each symbol representing a different donor. Statistics: paired Student's t-test. Error bars: SEM. *P≤0.05. FSC-A, forward scatter-area; FSC-W, forward scatter-width; SSC-A, side scatter-area and SSC-W, side scatter-width. cGAMP, cyclic GMP–AMP dinucleotides with phosphodiester linkages 2′–3′; DC, dendritic cell; IFN, interferon; LPS, lipopolysaccharide; mcGAS, murine cyclic GMP–AMP synthetase; MFI, mean fluorescence intensity; ns, not significant; PBL, peripheral blood lymphocyte.

or granzyme B as in SL3261-mcGAS, suggesting that DCs infected with SL3261-mcGAS are more efficient in inducing CTL activation. These data suggest that although *S. typhimurium* already induces CTL activation, mcGAS expression further increases the ability of DCs to stimulate the IFN-γ CTL response.

SL3261-mcGAS-mediated IFN-β production by DCs enhances T-cell capacity to kill malignant B cells

Next, we investigated whether SL3261-mcGAS activates DCs to enhance allogeneic CD8$^+$ T-cell cytotoxicity toward tumor cells by a cytotoxic T-cell killing assay.[38] Infected DCs were cocultured with allogeneic T cells, and activated T cells were subsequently cocultured with CTV-labeled malignant Jeko-1 B cells. To overcome HLA-matching requirements, T cells and malignant B cells were cocultured with the CD3–CD19 bispecific antibody (BsAb) blinatumomab (figure 6A). The BsAb is used in the clinic to reinvigorate T-cell responses against B-cell malignancies.[34] To bring T and malignant B cells into close proximity without overriding the IFN-β effect mediated by DCs on T cells, we cultured them with low concentrations of activating blinatumomab and cytotoxicity toward tumor cells was measured (figure 6B and online supplemental 6). Notably, in the absence of blinatumomab or the presence at a low concentration, SL3261-mcGAS-DC-stimulated T cells were more cytotoxic toward malignant cells than those stimulated by SL3261-mcGAS$_{AA}$-infected or uninfected DCs. SL3261-mcGAS-induced IFN-β production by DCs activated T cells to more efficiently kill malignant tumor cells (figure 6B,C). As expected, in the presence of a high concentration of blinatumomab, all T cells became highly cytotoxic toward B cells irrespective of DC activation condition. These results strongly suggest that IFN-β production by DCs leads to the activation of T cells that are more cytotoxic toward malignant tumor cells.

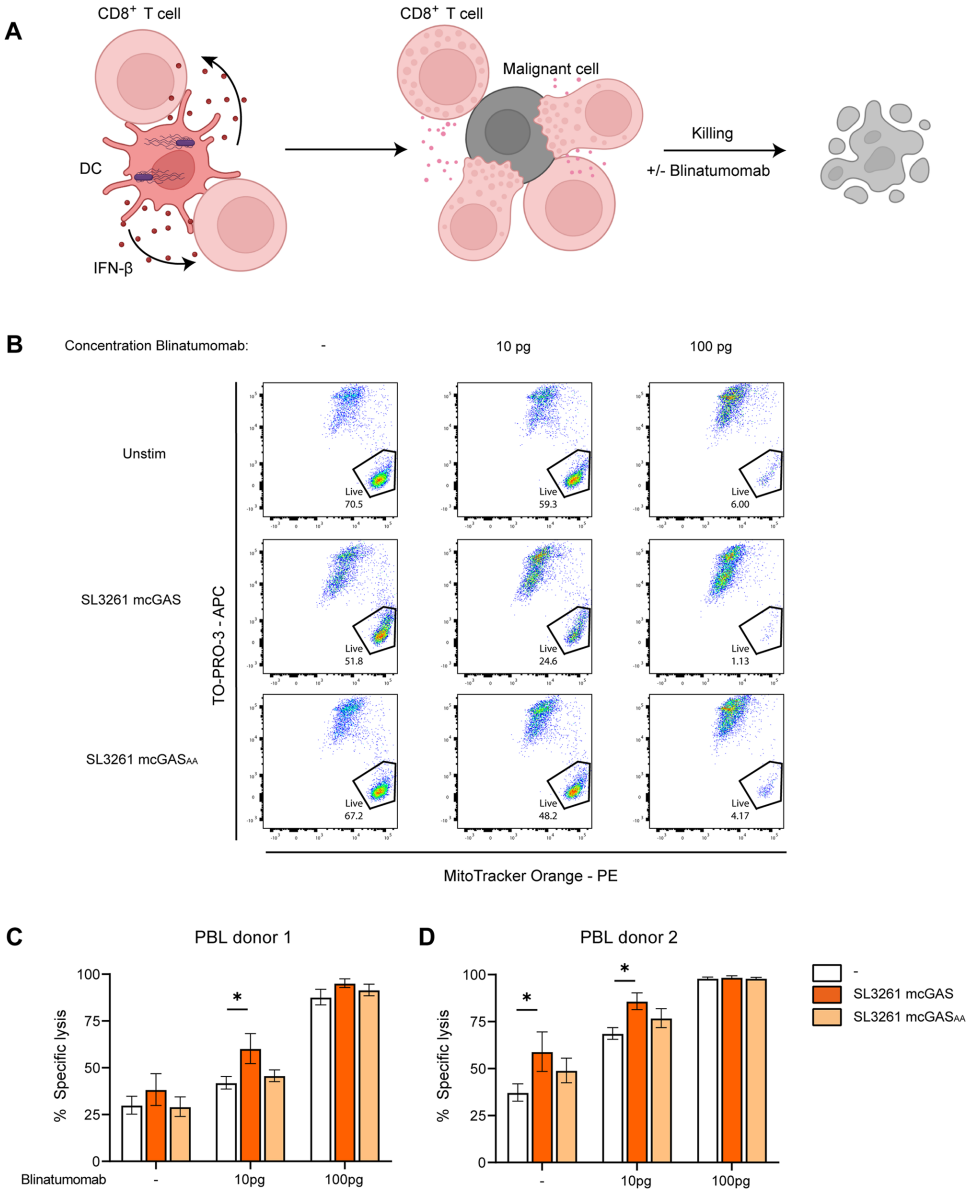

Figure 6 SL3261 mcGAS-mediated IFN-β production by DCs improves antitumor T-cell responses. (A) Graphical representation of the assay. DCs were exposed to medium, SL3261-mcGAS, or SL3261-mcGAS$_{AA}$ and subsequently cocultured with allogeneic human PBLs. After 6 days of coculture, the PBLs were harvested and counted and cocultured with CTV-labeled Jeko-1 B cells in a 4:1 effector to target ratio to assess cytotoxicity by flow cytometry. This figure was created with BioRender.com. (B) Representative dot plots of live/dead staining of CTV$^+$ B cells in coculture with T cells. (C) Cumulative flow cytometry data of cell-specific lysis. For PBL donor 1, data represent five DC donors analyzed in three separate experiments. For PBL donor 2, data represent four DC donors analyzed in two separate experiments. Statistics: two-way analysis of variance, Tukey's multiple comparison test. Error bars: SEM. *P≤0.05. DC, dendritic cell; IFN, interferon; mcGAS, murine cyclic GMP–AMP synthetase; PBL, peripheral blood lymphocyte.

DISCUSSION

The discovery of STING to be a major contributor to IFN-β levels stimulated research into STING agonists for cancer treatment.[39] The proinflammatory cytokine IFN-β has a large and diverse role in shaping the antitumor response, for example, by promoting DC-antigen cross-presentation, inducing NK cytotoxicity and inhibition of tumor proliferation.[16–18 20] However, important limitations in STING agonists are the rapid enzymatic degradation, membrane impermeability, and the insufficient delivery at the tumor site.[39] Therefore, this study aimed to engineer *S. typhimurium* with ectopic mcGAS expression that has the potential to migrate to tumor tissues and activate the STING pathway locally. These strains might produce the STING agonist for several days, while synthetic STING agonists diffuse away or end up solely in immune cells.

Our study shows that *S. typhimurium*–mcGAS induces a strong, STING-dependent IFN-β response on challenge of THP-I macrophages and primary human DCs. We hypothesized that expression of the cGAS enzyme in *S. typhimurium* would lead to constant cGAMP production

because of the naked bacterial DNA. This has been shown previously in an *E. coli* B21.[40] Interestingly, only murine and not human cGAS showed a striking IFN-β increase. Previously, it has been reported that mcGAS produces more cGAMPs and thereby triggers a stronger IFN-β response than hcGAS.[8 35] Activation of hcGAS is highly dependent on dsDNA length, which could explain suboptimal activity in our bacteria.[9]

We have investigated the mode of transport of cGAMP from the bacteria into the host. The cGAMP molecule is negatively charged and therefore depends on transporters for cell entry. Several of these transporters have been reported.[30 36 41] SLC19A1 has been identified as the dominant cyclic dinucleotide transporter in THP-I cells.[30 36] However, using SL1344-mcGAS on single-cell THP-I SLC19A1$^{-/-}$ clones did not alter IFN-β induction compared with THP-I wild type. Interestingly, we could show that cGAMPs are directly transported into the host cell via the via T3S needle. The non-invasive *S. typhimurium* strain with a functional SPI-1 needle revealed the importance of this T3S system needle in cGAMP transport. Pathogenic Gram-negative bacterial pathogens commonly use the T3S system to modulate the host signaling processes.[42–44] To our knowledge, this is the first time it has been shown that a small effector molecule like cGAMP can be injected into host cells. It seems likely that these results could also be applicable to other small molecules. Indeed, transport of ADP–heptose, the ligand for the innate immune receptor ALPK1, is suggested to be partially mediated by T3S.[45] The reverse transport of small molecules in a T3S-like manner, for example, nutrient uptake from the host, has also been documented.[46] These data add to the literature that T3S is highly versatile in function in that it can modulate the host not only by protein secretion but also by small molecules. This opens up possibilities for tailoring T3S-expressing bacteria to deliver various types of molecules to the host through their secretion needle.

The potential of *S. typhimurium*–mcGAS is further supported by the observation that IFN-β expression is significantly induced in DCs on challenge. IFN-β target gene expression of IRF7 and ISG15 both show a trend toward induction in the mcGAS samples compared with the inactive site mutant mcGAS$_{AA}$. The expression of the proinflammatory cytokine IL-1β was also increased on SL3261-mcGAS challenge; this indicates that IL-1β expression is also stimulated on sustained IFN-β signaling. Overall, a high variability is observed between DC donors, which could be due to different responses to LPS. Strong responders can have high IFN-β or IFN-β target gene expression in mcGAS challenge. However, these donor cells tend also to respond more to mcGAS$_{AA}$ challenge than average. In our experiments, CD83 expression showed lower expression levels than LPS only condition. This suggests active interference of *S. typhimurium* on CD83 activation, a phenomenon which has been described previously.[47 48] Importantly, we find that SL3261-mcGAS is able to prime human DCs to activate CTL responses in primary human PBLs. Addition of cGAMPs could rescue mcGAS$_{AA}$, while cGAMP alone did not induce CTL responses. This suggests synergistic effect of *Salmonella* and cGAMP-mediated STING activation. In a cytotoxic T-cell killing assay, we observed more killing of the B-cell line by CD8$^+$ T cells cocultured with DCs infected by SL3261-mcGAS, whereas killing by CD8$^+$ T cells activated by DCs infected by SL3261-mcGAS$_{AA}$ was significantly lower and to the same level as CD8$^+$ T cells activated by unstimulated DCs. These data support our other data that indeed type I IFN responses induced by SL3261-mcGAS-infected DCs lead to a more efficient CD8$^+$ T-cell activation as well as more cytotoxic capacity toward malignant tumor cells.

A limitation in our study is that we could only show the potential in vitro and not in an in vivo tumor model. To date, two other groups have used engineered bacteria to activate the STING pathway in a tumor mouse model and have shown promising results.[49 50] These studies indicate the potential of engineered bacterial strains to induce IFN-β as an immunotherapy. An important difference between these reported strains and *S. typhimurium*–mcGAS is that our engineered strains produce cGAMPs instead of c-di-AMP. cGAMPs are the strongest endogenous STING activator molecules known to date.[10 13 14] Furthermore, *S. typhimurium*–mcGAS can induce also IFN-β in non-immune cells, such as tumor cells, that could enhance tumor targeting by other immune cells, such as NK cells. Both features might lead to an even stronger antitumor immune response than the reported bacterial strains.

In conclusion, our results show that mcGAS expressing *S. typhimurium* are strong IFN-β inducers in human macrophage cell line THP-I and primary human DCs. This effect is STING dependent and relies on the T3S needle. Importantly, our *S. typhimurium*–mcGAS stimulates the CTL response in vitro, thereby providing grounds for further study in tumor models.

Author affiliations
[1]Department of Medical Microbiology and Infection Control, Amsterdam UMC Locatie VUmc, Amsterdam, The Netherlands
[2]Amsterdam institute for Infection and Immunity, Infectious Diseases, Amsterdam, Netherlands
[3]Cancer Center Amsterdam, Cancer Immunology, Amsterdam, Netherlands
[4]Department of Experimental Immunology, Amsterdam UMC Locatie AMC, Amsterdam, The Netherlands
[5]Amsterdam institute for Infection and Immunity, Cancer Immunology, Amsterdam, Netherlands
[6]The Lymphoma and Myeloma Center Amsterdam, LYMMCARE, Amsterdam, Netherlands
[7]Amsterdam institute for Life and Environment, Vrije Universiteit, Amsterdam, Netherlands

Twitter Coenraad P Kuijl @ckuijl

Acknowledgements We thank Jan Rehwinkel (University of Oxford) for donating THP-I cells and David Holden (Imperial College London) for providing HeLa cells. THP-I SLC19A1 single knockout cells were a kind gift from David Raulet (University of California). We also thank Wolf-Dietrich Hardt (ETH Zürich) for making SL1344 SPI mutants available to us and Matthias Müller (Albert-Ludwigs-Universität Freiburg) for donating and TatB antibody. Maroeska Burggraaf (AmsterdamUMC) kindly provided us the human cyclic GMP–AMP synthetase construct. We thank Melissa

Stunnenberg (AmsterdamUMC) for her expertise of cytotoxic T-cell assays. We acknowledge the Microscopy and Cytometry Core Facility at the Amsterdam UMC, Location VUmc for providing assistance in our microscopy and cytometry work.

Contributors LW, LEHvdD, LSA and CPK designed the experiments; LW, LEHvdD, JM and JLvH performed the experiments; EE, JACvB and JMR contributed materials and scientific discussion; LW, LEHvdD, LSA, TBHG, WB and CPK analyzed and interpreted the data; LW and LEHvdD wrote the manuscript with input from all listed authors. TBGH, WB and CPK supervised all aspects of this study. CPK is acting as guarantor.

Funding This work was supported by funding of the Cancer Center Amsterdam to LW. This research was furthermore funded by the European Research Council (advanced grant 670424 to TBHG). LEHvdD and LSA were supported by the Netherlands Organization for Scientific Research (grant number 91717305).

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the institutional review board of the Amsterdam University Medical Centers, Location University of Amsterdam Medical Ethics Committee and the Ethics Advisory Body of Sanquin Blood Supply Foundation (Amsterdam, Netherlands; code CT23.002.F.NW, study number NVT0257). The participants gave informed consent to participate in the study before taking part. We used buffy coats donated by healthy volunteer donors, obtained in accordance with the ethical principles set out in the Declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iD

Coenraad P Kuijl http://orcid.org/0000-0003-4378-1428

REFERENCES

1 Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol* 2020;20:651–68.
2 Darvin P, Toor SM, Sasidharan Nair V, *et al*. Immune checkpoint inhibitors: recent progress and potential biomarkers. *Exp Mol Med* 2018;50:165:1–11.:.
3 Haslam A, Prasad V. Estimation of the percentage of us patients with cancer who are eligible for and respond to checkpoint inhibitor immunotherapy drugs. *JAMA Netw Open* 2019;2:e192535.
4 Tang T, Huang X, Zhang G, *et al*. Advantages of targeting the tumor immune microenvironment over blocking immune checkpoint in cancer immunotherapy. *Signal Transduct Target Ther* 2021;6:72:72.:.
5 Chorobik P, Czaplicki D, Ossysek K, *et al*. Salmonella and cancer: from pathogens to therapeutics. *Acta Biochim Pol* 2013;60:285–97.
6 Pawelek JM, Low KB, Bermudes D. Tumor-targeted salmonella as a novel anticancer vector. *Cancer Res* 1997;57:4537–44.
7 Toso JF, Gill VJ, Hwu P, *et al*. Phase I study of the intravenous administration of attenuated salmonella typhimurium to patients with metastatic melanoma. *J Clin Oncol* 2002;20:142–52.
8 Sun L, Wu J, Du F, *et al*. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 2013;339:786–91.
9 Luecke S, Holleufer A, Christensen MH, *et al*. CGAS is activated by DNA in a length-dependent manner. *EMBO Rep* 2017;18:1707–15.
10 Ablasser A, Goldeck M, Cavlar T, *et al*. CGAS produces a 2′-5′-linked cyclic dinucleotide second messenger that activates sting. *Nature* 2013;498:380–4.
11 Gao P, Ascano M, Zillinger T, *et al*. Structure-function analysis of sting activation by c[g(2',5')pa(3',5')p] and targeting by antiviral dmxaa. *Cell* 2013;154:748–62.
12 Diner EJ, Burdette DL, Wilson SC, *et al*. The innate immune dna sensor cgas produces a noncanonical cyclic dinucleotide that activates human sting. *Cell Rep* 2013;3:1355–61.
13 Zhang X, Shi H, Wu J, *et al*. Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for sting. *Molecular Cell* 2013;51:226–35.
14 Kranzusch PJ, Lee ASY, Wilson SC, *et al*. Structure-guided reprogramming of human cgas dinucleotide linkage specificity. *Cell* 2014;158:1011–21.
15 Ouyang S, Song X, Wang Y, *et al*. Structural analysis of the sting adaptor protein reveals a hydrophobic dimer interface and mode of cyclic di-GMP binding. *Immunity* 2012;36:1073–86.
16 Borden EC. Interferons α and β in cancer: therapeutic opportunities from new insights. *Nat Rev Drug Discov* 2019;18:219–34.
17 Le Bon A, Durand V, Kamphuis E, *et al*. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. *J Immunol* 2006;176:4682–9.
18 Gutjahr A, Papagno L, Nicoli F, *et al*. The STING ligand cgamp potentiates the efficacy of vaccine-induced CD8+ T cells. *JCI Insight* 2019;4:e125107.
19 Fuertes MB, Kacha AK, Kline J, *et al*. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. *J Exp Med* 2011;208:2005–16.
20 Edwards BS, Hawkins MJ, Borden EC. Correlation between in vitro and systemic effects of native and recombinant interferons-alpha on human natural killer cell cytotoxicity. *J Biol Response Mod* 1983;2:409–17.
21 Deng L, Liang H, Xu M, *et al*. Sting-Dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity* 2014;41:843–52.
22 Wan S, Pestka S, Jubin RG, *et al*. Chemotherapeutics and radiation stimulate MHC class I expression through elevated interferon-beta signaling in breast cancer cells. *PLoS ONE* 2012;7:e32542.
23 Downey CM, Aghaei M, Schwendener RA, *et al*. DMXAA causes tumor site-specific vascular disruption in murine non-small cell lung cancer, and like the endogenous non-canonical cyclic dinucleotide sting agonist, 2'3'-cgamp, induces m2 macrophage repolarization. *PLoS One* 2014;9:e99988.
24 Rauca V-F, Licarete E, Luput L, *et al*. Combination therapy of simvastatin and 5, 6-dimethylxanthenone-4-acetic acid synergistically suppresses the aggressiveness of b16.f10 melanoma cells. *PLoS One* 2018;13:e0202827.
25 Smolarczyk R, Cichoń T, Pilny E, *et al*. Combination of anti-vascular agent - dmxaa and hif-1α inhibitor - digoxin inhibits the growth of melanoma tumors. *Sci Rep* 2018;8:7355.
26 Curran E, Chen X, Corrales L, *et al*. STING pathway activation stimulates potent immunity against acute myeloid leukemia. *Cell Rep* 2016;15:2357–66.
27 Lara PN Jr, Douillard J-Y, Nakagawa K, *et al*. Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent Vadimezan (ASA404) in advanced non–small-cell lung cancer. *JCO* 2011;29:2965–71.
28 Corrales L, Glickman LH, McWhirter SM, *et al*. Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. *Cell Rep* 2015;11:1018–30.
29 Conlon J, Burdette DL, Sharma S, *et al*. Mouse, but not human STING, binds and signals in response to the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid. *J Immunol* 2013;190:5216–25.
30 Luteijn RD, Zaver SA, Gowen BG, *et al*. SLC19A1 transports immunoreactive cyclic dinucleotides. *Nature* 2019;573:434–8.
31 Mesman AW, Zijlstra-Willems EM, Kaptein TM, *et al*. Measles virus suppresses RIG-i-like receptor activation in dendritic cells via DC-SIGN-mediated inhibition of PP1 phosphatases. *Cell Host Microbe* 2014;16:31–42.
32 Hoiseth SK, Stocker BAD. Aromatic-dependent salmonella typhimurium are non-virulent and effective as live vaccines. *Nature* 1981;291:238–9.
33 Hapfelmeier S, Ehrbar K, Stecher B, *et al*. Role of the Salmonella pathogenicity island 1 effector proteins sipa, SopB, SopE, and SopE2 in Salmonella enterica subspecies 1 serovar typhimurium

colitis in streptomycin-pretreated mice . *Infect Immun* 2004;72:795–809.
34 Kantarjian H, Stein A, Gökbuget N, *et al*. Blinatumomab versus chemotherapy for advanced acute lymphoblastic leukemia. *N Engl J Med* 2017;376:836–47.
35 Zhou W, Whiteley AT, de Oliveira Mann CC, *et al*. Structure of the human cGAS–DNA complex reveals enhanced control of immune surveillance. *Cell* 2018;174:300–311.
36 Ritchie C, Cordova AF, Hess GT, *et al*. SLC19A1 is an importer of the immunotransmitter cGAMP. *Mol Cell* 2019;75:372–81.
37 Deng W, Marshall NC, Rowland JL, *et al*. Assembly, structure, function and regulation of type III secretion systems. *Nat Rev Microbiol* 2017;15:323–37.
38 Martens AWJ, Janssen SR, Derks IAM, *et al*. CD3xCD19 dart molecule treatment induces non-apoptotic killing and is efficient against high-risk chemotherapy and venetoclax-resistant chronic lymphocytic leukemia cells. *J Immunother Cancer* 2020;8:e000218.
39 Amouzegar A, Chelvanambi M, Filderman JN, *et al*. STING agonists as cancer therapeutics. *Cancers (Basel)* 2021;13:2695.
40 Lv Y, Sun Q, Wang X, *et al*. Highly efficient preparation of cyclic dinucleotides *via* engineering of dinucleotide cyclases in *escherichia coli*. *Front Microbiol* 2019;10:2111.
41 Cordova AF, Ritchie C, Böhnert V, *et al*. Human SLC46A2 is the dominant cgamp importer in extracellular cgamp-sensing macrophages and monocytes. *ACS Cent Sci* 2021;7:1073–88.
42 Lou L, Zhang P, Piao R, *et al*. *salmonella* pathogenicity island 1 (SPI-1) and its complex regulatory network. *Front Cell Infect Microbiol* 2019;9:270.
43 Figueira R, Holden DW. Functions of the salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology (Reading)* 2012;158:1147–61.
44 Jennings E, Thurston TLM, Holden DW. Salmonella SPI-2 type III secretion system effectors: molecular mechanisms and physiological consequences. *Cell Host Microbe* 2017;22:217–31.
45 Zhou P, She Y, Dong N, *et al*. Alpha-Kinase 1 is a cytosolic innate immune receptor for bacterial ADP-heptose. *Nature* 2018;561:122–6.
46 Pal RR, Baidya AK, Mamou G, *et al*. Pathogenic E. coli extracts nutrients from infected host cells utilizing injectisome components. *Cell* 2019;177:683–96.
47 Aulicino A, Rue-Albrecht KC, Preciado-Llanes L, *et al*. Invasive salmonella exploits divergent immune evasion strategies in infected and bystander dendritic cell subsets. *Nat Commun* 2018;9:4883.
48 Bayer-Santos E, Durkin CH, Rigano LA, *et al*. The salmonella effector sted mediates MARCH8-dependent ubiquitination of MHC II molecules and inhibits T cell activation. *Cell Host Microbe* 2016;20:584–95.
49 Leventhal DS, Sokolovska A, Li N, *et al*. Immunotherapy with engineered bacteria by targeting the STING pathway for anti-tumor immunity. *Nat Commun* 2020;11:2739.
50 Singh AK, Srikrishna G, Bivalacqua TJ, *et al*. Recombinant BCGs for tuberculosis and bladder cancer. *Vaccine* 2021;39:7321–31.