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Human B cells fail to secrete type I interferons upon cytoplasmic DNA exposure

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ABSTRACT

Most cells are believed to be capable of producing type I interferons (IFN I) as part of an innate immune response against, for instance, viral infections. In macrophages, IFN I is potently induced upon cytoplasmic exposure to foreign nucleic acids. Infection of these cells with herpesviruses leads to triggering of the DNA sensors interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP (cGAMP) synthase (cGAS). Thereby, the stimulator of interferon genes (STING) and the downstream molecules TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) are sequentially activated culminating in IFN I secretion.

Human gamma-herpesviruses, such as Epstein-Barr virus (EBV), exploit B cells as a reservoir for persistent infection. In this study, we investigated whether human B cells, similar to macrophages, engage the cytoplasmic DNA sensing pathway to induce an innate immune response. We found that the B cells fail to secrete IFN I upon cytoplasmic DNA exposure, although they express the DNA sensors cGAS and IFI16 and the signaling components TBK1 and IRF3. In primary human B lymphocytes and EBV-negative B cell lines, this deficiency is explained by a lack of detectable levels of the central adaptor protein STING. In contrast, EBV-transformed B cell lines did express STING, yet both these lines as well as STING-reconstituted EBV-negative B cells did not produce IFN I upon dsDNA or cGAMP stimulation. Our combined data show that the cytoplasmic DNA sensing pathway is dysfunctional in human B cells. This exemplifies that certain cell types cannot induce IFN I in response to cytoplasmic DNA exposure providing a potential niche for viral persistence.

1. Introduction

Type I interferons (IFN I) play a central role in antiviral immunity as they induce the expression of interferon-stimulated genes (ISG) that create an anti-viral state (Schneider et al., 2014). IFN I is potently induced upon cellular encounter of viral nucleic acids (reviewed in Wu and Chen, 2014; Thompson et al., 2011). RNA and DNA species trigger various pattern-recognition receptors (PRR) resulting in activation of distinct downstream signaling cascades, which eventually converge at interferon regulatory factor 3 (IRF3)-induced transcription of IFN I genes. Nucleic acid-sensing PRRs are located in endosomes, in the cytoplasm, and also in the nucleus (Wu and Chen, 2014). Viral RNA can trigger Toll-like receptor 3 (TLR3) in endosomes and the retinoic acid-inducible protein 1 (RIG-I)-like receptors RIG-I and melanoma differentiation-associated protein 5 (MDA5) in the cytoplasm (Kawai and Akira, 2011; Loo and Gale, 2011). Foreign DNA can be sensed by TLR9 and several cytoplasmic DNA sensors, including cyclic GMP-AMP (cGAMP) synthase (cGAS) and interferon-inducible protein 16 (IFI16) (Unterholzner, 2013). Endosomal TLR9 senses unmethylated CpG DNA leading to transcription of, for instance, pro-inflammatory cytokines and costimulatory molecules upon NF-κB activation (Latz et al., 2004). Cytosolic cGAS directly binds to the backbone of dsDNA in a seemingly

Abbreviations: IFN I, type I interferon(s); IFI16, interferon-inducible protein 16; cGAMP, cyclic GMP-AMP; cGAS, cGAMP synthase; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; IRF3, interferon regulatory factor 3; ISG, interferon-stimulated genes; PRR, pattern-recognition receptors; TLR, Toll-like receptor; RIG-I, retinoic acid-inducible protein 1; MDA5, melanoma differentiation-associated protein 5; HSV-1, herpes simplex virus type 1; HCMV, human cytomegalovirus; KSHV, Kaposi's sarcoma-associated herpesvirus; EBV, Epstein-Barr virus; PBMC, peripheral blood mononuclear cells; B-LCL, B-lymphoblastoid cell line(s); FAM, fluorescein amidite; poly(I:C), polyinosinic-polycytidylic acid

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sequence-independent manner and produces the cyclic dinucleotide 2′3′-cGAMP (Sun et al., 2013; Wu et al., 2013). This second messenger molecule activates the central adaptor protein STING (*st*imulator of *in*terferon *g*enes; also termed MPYS, MITA, and ERIS) (Sun et al., 2009; Ishikawa and Barber, 2008; Zhong et al., 2008). Subsequently, TANK-binding kinase 1 (TBK1) and IRF3 are activated and IFN I production is initiated. Also cytoplasmic and nuclear IFI16-dependent DNA sensing has been linked to IFN I production downstream of STING (Unterholzner et al., 2010; Orzalli et al., 2012). IFI16 is required for full activation of the cGAS-STING pathway in human macrophages, keratinocytes, and fibroblasts (Hansen et al., 2014; Orzalli et al., 2015; Jonsson et al., 2017) and in cell types such as fibroblasts and endothelial cells, DNA sensing by IFI16 causes the formation of an inflammasome, which results in caspase-1-dependent maturation of IL-1β and IL-18 (Johnson et al., 2013; Kerur et al., 2011).

Most cell types are assumed to be capable of producing the IFN I IFN-α and IFN-β (Theofilopoulos et al., 2005). Yet, differential expression and functionality of sensors and downstream signaling molecules could create cell type-dependent differences in responsiveness to nucleic acids. Two examples are: (i) hepatocytes were found not to express STING, which hampered efficient innate control of hepatitis B virus infection; moreover, viral control was improved upon introduction of STING into the hepatocytes (Thomsen et al., 2016) and (ii) while macrophages produce IFN I upon infection with human immunodeficiency virus, human T lymphocytes fail to do so (Jakobsen et al., 2013; Berg et al., 2014). These examples suggest that viruses may exploit cell types with a limited intracellular nucleic acid response as targets for persistence.

Herpesviruses are large, enveloped DNA viruses that are characterized by their lifelong persistence following primary infection. To achieve this, herpesviruses establish a delicate balance between viral replication and immune activation (Schuren et al., 2016; Ressing et al., 2015). Activation of cGAS-STING dependent DNA sensing pathways by human herpesviruses has mostly been studied in macrophages, fibroblasts, and endothelial cells. In these cell types, infection with herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV), and Kaposi's sarcoma-associated herpesvirus (KSHV) led to IFN I production (Jakobsen et al., 2013; Ishikawa et al., 2009; Ma et al., 2015; Gao et al., 2013; Li et al., 2013). The human γ-herpesviruses Epstein-Barr virus (EBV) and KSHV exploit the human B cell as a reservoir for persistence. EBV's DNA genome can be sensed by TLR9 (van Gent et al., 2011) and nuclear EBV and KSHV genomes have been reported to trigger IFI16 resulting in inflammasome-mediated IL-1β maturation in B cells (Ansari et al., 2013; Singh et al., 2013). Thus far, it is unclear if and how the cGAS-STING axis contributes to cytoplasmic sensing of the dsDNA genomes of EBV or KSHV in human B cells.

In this study, we therefore investigated if human B cells produce IFN I upon exposure to cytoplasmic DNA that can stimulate the cGAS-STING pathway.

2. Materials and methods

2.1. Isolation of human primary (B) cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient (LUMC pharmacy) from buffy coats of healthy blood donors obtained from Sanquin blood bank Amsterdam. Isolated PBMCs contained about 40–60% $CD3^+$, 10–20% $CD14^+$, 10–15% $CD19^+$ cells, as determined by flow cytometry. The antibodies used for these FACS analyses were: AlexaFluor700-conjugated anti-CD3 (BD Pharmingen, UCHT1), PE-conjugated anti-CD19 (BD Pharmingen, HIB19), AlexaFluor700-conjugated anti-CD20 (Biolegend, 2H7), and PE-Cy7-conjugated anti-CD14 (BD Pharmingen, M5E2).

Anonymized tonsil material was obtained from tonsillectomies. Tonsils were cut into small pieces and passed through a cell strainer.

To obtain $CD19^+$ B cells from the PBMCs or dispersed tonsils, positive selection was performed using CD19 pan B Dynabeads, according to manufacturer's protocol (Invitrogen). Isolated B cells were released using the DETACH a BEAD CD19 kit (Invitrogen). Purity of isolated B cells was determined by flow cytometry. In each experiment, the purity of the $CD19^+$ B cells was 95–99% and the $CD19^+$ cell fractions contained < 4% $CD3^+$ cells and < 0,5% $CD14^+$ cells.

The $CD19^-$ fraction remaining after B cell isolation was used as a control and typically comprised around 40–60% $CD3^+$ cells, 6–30% $CD14^+$ cells, and < 2% $CD19^+$ cells.

Experiments were performed with freshly isolated primary cells, resuspended in IMDM supplemented with 10% heat-inactivated FCS, 2 mM glutamax, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. Cell lines

Next to the primary cells described above, a panel of human B cell lines was used in this study. Long-term culture was possible due to the B cell lines being transformed, either *in vivo* (i.e. the Burkitt's lymphoma (BL)-derived lines 2A8 and BJAB) or *in vitro* (by EBV for the B-lymphoblastoid cell lines (B-LCLs) JY and MRJ; or by the *in vitro* introduction of the B cell lymphoma 6 (BcL-6) and −xL proteins for the B cell clones with receptors specific for Influenza virus or tetanus toxin (Kwakkenbos et al., 2010)). The 2A8 cell line has been selected for loss of EBV genomes from the Akata BL line (Chodosh et al., 1998); the BJAB cell line originates from a rare EBV-negative BL (Menezes et al., 1975).

An overview of all human B lymphocytes used in this study is given Table 1.

The two Bcl-immortalized EBV-negative B cell clones were cultured on irradiated CD40L-expressing murine feeder cells in the presence of recombinant mouse IL-21, as described elsewhere (Kwakkenbos et al., 2010). Prior to use in experiments, cells were carefully removed from the adherent murine feeder cells and resuspended in IMDM containing 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. As the results obtained with the tetanus- and Influenza virus-

Table 1
B cells used in this study.

Cells	Source	EBV status	Immortalization	References
Primary $CD19^+$ B cells	Tonsil	unknown[a]	no	
Primary $CD19^+$ B cells	PBMC	unknown[a]	no	
B cell clones	PBMC	−	yes, *in vitro* (Bcl-6 and −xL)	Kwakkenbos et al. (2010)
B-LCL (JY, MRJ)	PBMC	+	yes, *in vitro* (EBV)	Kremer et al. (2012); Terhorst et al. (1976)
2A8	BL	−	yes, *in vivo*	Chodosh et al. (1998)
BJAB	BL	−	yes, *in vivo*	Menezes et al. (1975)

[a] In EBV carriers, the mean number of latently infected memory B cells in peripheral blood and tonsils is about 110 and 175 per 10^7 memory B cells, respectively (Laichalk et al., 2002).

specific B cell clones did not differ, we only present the results for the Influenza virus-specific clone.

The EBV-negative BL lines 2A8 and BJAB, the EBV-positive B-LCL JY and MRJ, and the monocyte-like THP1 cells were cultured in RPMI medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Prior to use in experiments, THP1 cells were differentiated into macrophage-like cells by treatment with 150 nM phorbol myristate acetate (PMA, Sigma-Aldrich) for 24 h and were left to rest for another 24 h.

2.3. Replication-deficient lentiviruses and transductions

SIN lentiviruses were generated in 293T cells transfected with the lentiviral plasmid encoding mSTING-HA-IRES-GFP driven by a PGK promoter and the vectors pCMV-VSV-G, pMDLg-RRE, and pRSV-REV to provide the helper functions. 2A8 and BJAB cells were exposed to lentivirus-containing supernatant to transfer the genes encoding murine STING and the GFP marker. To enhance transduction efficiency, cells were spin-inoculated (1000xg, 33°C, 2 h) in the presence of 4 ug/ml polybrene. GFP^+ cells were FACS-sorted and this population of cells was propagated and used for experiments.

2.4. Reagents

For stimulation of the cytoplasmic DNA sensing pathway, we used dsDNA (60mer derived from the HSV-1 genome: nucleotides 144,107–144,166 (Berg et al., 2014)) or 2′,3′-cGAMP (Sigma-Aldrich). Unconjugated or fluorescein amidite (FAM)-labeled DNA oligos were annealed in annealing buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA) by heating at 95°C for 5 min, followed by a slow cool-down. Annealed oligos were kept at 4°C until use. For stimulation of TLR9, cells were incubated with 10 µg/ml CpG DNA (ODN 2006, Invivogen) for 24 h. The cytoplasmic RNA sensing pathway was triggered by polyinosinic-polycytidylic acid (poly(I:C), Invivogen). For priming, cells were treated with 1000 U/ml IFN-β (pbl assay science) 24 h prior to stimulation.

2.5. Transfections and transfection efficiencies

Cells were stimulated with dsDNA or poly(I:C) by transfection using Lipofectamine2000 (Invitrogen) as described previously (Jakobsen et al., 2013; Berg et al., 2014; Chiliveru et al., 2014; Christensen et al., 2016; Reinert et al., 2016). The ratio of DNA or poly(I:C) to lipofectamine2000 was 1 µg per 1 µl. Complexes were allowed to form for 20 min before adding them to the cells. In general, 0.3×10^6 cells seeded per well of a 24-well plate were transfected with 4 µg DNA or 2 µg poly(I:C) to introduce stimuli to the cytoplasm. Supernatants and/or cells were harvested for analysis at the indicated time points, typically 6 h or 24 h post-transfection. Transfection efficiencies were determined with the use of FAM-labeled dsDNA and assessing fluorescence by flow cytometry after overnight incubation.

To induce auto-activation of the cytoplasmic DNA sensing pathway in 293T cells (Ma et al., 2015; Diner et al., 2013), we transfected cells with DNA constructs encoding murine STING-HA and human cGAS. Cells were transfected with a total amount of 180 ng plasmid DNA using lipofectamine2000. Ratios of transfected DNA amounts are indicated in figure legends. Cells were used in experiments 24 h post-transfection.

2.6. Flow cytometry

To determine cell surface levels of CD80 or CD86, cells were stained with the following antibodies: anti-CD80-PE or anti-CD86-PE (both BD Pharmingen). Fluorescence was measured in a LSR II flow cytometer (BD Biosciences) and data obtained were analysed using FlowJo software (Treestar).

2.7. cGAMP stimulation

Digitonin-permeabilized cells were stimulated with cGAMP. For this, cells were taken up in digitonin buffer (5 µg/ml digitonin, 50 mM HEPES (pH 7.0), 100 mM KCl, 85 mM sucrose, 3 mM $MgCl_2$, 1 mM ATP, 0,1 mM GTP, 0,1 mM DTT, 0,2% BSA) containing 1 µM cGAMP. Cells were incubated for 10 min at 37°C, before buffer was replaced by culture medium.

2.8. IFN I bioassay

IFN-α/β levels in cell-free culture supernatants were determined in technical duplicates using the HEK Blue IFN-α/β reporter cell line (Invivogen), which in response to IFN I stimulation produces the secreted embryonic alkaline phosphatase (SEAP). Supernatants were plated with 5×10^4 reporter cells in a 96 well plate and incubated for 24 h. SEAP activity in culture supernatants of the reporter cells was determined by colorimetric analysis, measuring OD at 620–655 nm using a plate reader.

2.9. RNA isolation and PCR

RNA was isolated from stimulated cells at the indicated time points by using the High Pure RNA Isolation Kit (Roche) or NucleoSpin RNA kit (Machery-Nagel) according to manufacturer's protocols. cDNA was generated using oligo-dT primers. Abundance of mRNA was quantified in duplicates or triplicates by real-time PCR using SYBR green together with the primers for IFN-β Fw 5′-TCTGGCACAACAGGTAGTAGGC-3′ and Rv 5′-GAGAAGCACAACAGGAGAGCAA-3′, ISG54 Fw 5″-ATGTGCA ACCTACTGGCCTAT-3′ and Rv 5′-TGAGAGTCGGCCCATGTGATA-3′, and GAPDH Fw 5″GCAAATTTCCATGGCACCGT-3′ and Rv 5′-GCCCCACTTGATTTTGGAGG-3′. Alternatively, RNA was directly used in the TaqMan RNA-to-C_T 1-step kit (Applied Biosystems) using the following TaqMan probes: hIFN-β (Hs01077958_s1), hISG54 (IFIT2) (Hs01922738_s1), hTNF-α (Hs01113624_g1), and hβ-actin (Hs99999903_m1). Replicates with C_T values > 1 apart were excluded from analyses. Expression levels were normalized to GAPDH (SYBR green) or β-actin (TaqMan) using the $2^{-\Delta\Delta CT}$ method, and data are presented as fold induction (± SEM) over control samples.

For semi-quantitative reverse-transcription PCR (RT-PCR) analyses, RNA of B cell lines, primary cells, and non- or PMA-differentiated THP1 cells was isolated using the Absolute RNA Miniprep kit (Agilent) or NucleoSpin RNA kit (Machery-Nagel) according to manufacturer's protocols. 300–1000 ng RNA was used for cDNA synthesis using an oligo-dT primer and the SuperScript III Reverse Transcriptase kit (Invitrogen) according to manufacturer's protocol. To assess RNA samples for DNA contamination, the reactions were also performed without reverse transcriptase. Diluted samples were used for amplification with GoTaq G2 FlexiDNA polymerase (Promega). Primer sequences for RT-PCR analyses, expected length of DNA fragments, and number of cycles performed are listed in Table 2. Multiple exon-spanning primers were used to prevent false-positive signals from contaminating genomic DNA. PCR fragments were visualized by 1,5% agarose gel electrophoresis and ethidium bromide staining.

2.10. Western blot analysis

Total cell lysates were generated using RIPA lysis buffer (ThermoFisher) supplemented with 0,2% SDS, protease inhibitor mix (Roche), and 50 U/ml Benzonase (Sigma-Aldrich). Lysates were denatured using Laemmli sample buffer with 20 mM DTT. Proteins were separated on Bio-Rad premade Criterion Tris-HCl gels (4–20% gradient)

Table 2
RT-PCR primers.

Gene	Primer sequences 5′-3′	Fragment length	# of PCR cycles
cGAS	Fw: GCCCTGCTGTAACACTTCTTAT Rv: GGATAGCCGCCATGTTTCTT	221 bp	35
IFI16	Fw: ACTGAGTACAACAAAGCCATTTGA Rv: TTGTGACATTGTCCTGTCCCCAC	432 bp	25 or 35
STING	Fw: GCTGCTGTCCATCTATTTCTACT Rv: GCCGCAGATATCCGATGTAATA	212 bp	30 or 35
MAVS	Fw: TCCCAAGGAATCCAGATGGT Rv: GGGTCTCCTCATTTCTGCTG	660 bp	30 or 35
MDA5	Fw: GGATATAAAGAATGTAACATTGTTATCCG Rv: ATGAGCATACTCCTCTGGTTTCA	226 bp	35
RIG-I	Fw: CAGTATATTCAGGCTGAG Rv: GGCCAGTTTTCCTTGTC	389 bp	35
TLR3	Fw: TCCCTTTGTCAAGCAGAAGAATTTAATC Rv: AGTTGTATTGCTGGTGGTGG	379 bp	30
GAPDH	Fw: CATCATCCCTGCCTCTACTG Rv: TTGGCAGGTTTTTCTAGACG	404 bp	25

or on hand-casted gels (10%) and transferred onto PVDF membranes. Membranes were blocked and probed with the following specific antibodies: anti-cGAS (HPA031700, Sigma-Aldrich), anti-IFI16 (C-18, sc-6050, Santa Cruz), anti-STING (D2P2F, Cell Signaling), anti-TBK1 (D1B4, Cell Signaling), anti-IRF3 (D6I4C, XP, Cell Signaling or sc9082X, Santa Cruz), anti-actin (C4, MP Biomedicals), anti-vinculin (hVIN-1, Sigma-Aldrich), and anti-HA (3F10, Roche). Membranes were incubated with secondary HRP-conjugated antibodies and ECL substrate subsequently, to visualize bands using films or an ImageQuant LAS 4000 mini Luminescent Image Analyzer (GE Healthcare Life Sciences). To quantify STING protein bands, intensity was determined, corrected for background intensity of an empty lane on the same blot, and normalized to the loading controls actin or vinculin.

3. Results

3.1. Human B cells do not secrete IFN I upon cytoplasmic DNA stimulation

As the γ-herpesviruses EBV and KSHV can persist in human B cells, we were interested if these cells have a functional cytosolic DNA sensing pathway. To investigate whether human B cells secrete IFN I upon cytoplasmic DNA exposure, we first employed primary cells.

Human $CD19^+$ B cells were isolated (> 95% purity) from peripheral blood mononuclear cells (PBMC). For comparison, the $CD19^-$ non-B cell fraction was included in the experiments; the non-B cell fraction typically contained about 5–25% monocytes, cells reported to be capable of producing IFN I upon triggering the cGAS/STING pathway (Liu et al., 2014). Fluorescein amidite (FAM)-labeled foreign dsDNA was introduced into the cytoplasm of cells by lipofectamine2000 transfection. On the basis of FAM-fluorescence, transfections yielded efficiencies of around 45% in primary B lymphocytes and around 17% in the non-B cells (Fig. 1A). Uptake of labeled DNA by human B cells allowed assessment of IFN I secretion in response to cytoplasmic DNA.

Next, we transfected (unlabeled) dsDNA to trigger the cytosolic DNA sensing pathway in the human B and non-B cell fractions and assessed IFN I secretion. To this end, we employed a reporter assay for biologically active IFN I (bioassay) that requires the stimulated cells to have secreted IFN I into their supernatants in quantities sufficient for subsequent detection by reporter cells. To our surprise, the primary human B cells did not secrete IFN I upon triggering with cytoplasmic DNA (Fig. 1B, left). As a control to examine if the TBK1-IRF3 axis required for IFN I induction was intact in these B cells, they were transfected with an alternative upstream activator, the RNA analogue poly(I:C). The primary human B cells did produce IFN I when exposed to the cytosolic RNA mimic (Fig. 1B, left), pointing to functional TBK1-IRF3 signaling. In addition, the non-B cell fraction − likely the monocytes − secreted IFN I in response to both transfected dsDNA and poly(I:C) stimulation (Fig. 1B, right). These observations exclude (i) that the blood donors had a genetic defect in the DNA sensing pathway components and (ii) that the source of cells or the isolation procedure used precluded IFN I production.

To test if low levels of IFN I were induced by DNA transfection into the primary human B cells, we analyzed the levels of interferon-stimulated gene 54 (ISG54) transcripts by quantitative real-time PCR 24 h post-stimulation, to complement the bioassay above. ISG54 is a gene that is transcribed upon stimulation of the IFN I receptor (autocrine IFN I stimulation). Also using this more sensitive read-out, primary B cells were not found to respond to cytosolic DNA stimulation, while a clear induction of ISG54 mRNA was detected following poly(I:C) transfection (Fig. 1C); in non-B cells, ISG54 mRNA was induced by both stimuli. Thus, human primary B cells were capable producing IFN I when poly (I:C) was sensed and the levels were sufficient to induce ISG54 mRNA, but the cells did not secrete IFN I or initiated ISG54 transcription upon exposure to cytoplasmic DNA.

To examine if intracellular dsDNA stimulation of B cells would support NF-κB-mediated responses rather than IFN I production, we assessed surface levels of the costimulatory B7 molecules CD80 and CD86, which are both regulated by NF-κB signaling (Zou and Hu, 2005; Zhao et al., 1996). CpG-mediated TLR9 stimulation of PBMC-derived B cells upregulates CD80 and CD86 (Agrawal and Gupta, 2011), and thus served as a positive control for this pathway. Addition of CpG DNA (ODN2006) indeed induced a pronounced increase in CD80 and CD86 levels at the surface of the primary human B cells, and not on the non-B cells (Fig. 1D, data CD86 not shown). In contrast, dsDNA transfection did not result in upregulation of the activation markers CD80 (Fig. 1D) or CD86 (not shown), suggesting that cytosolic DNA sensing did not initiate NF-κB-dependent signaling in the primary human B cells, in addition to the absence of IFN I secretion and ISG54 induction described above.

EBV persists in human B cells of the large majority of adults and since the EBV status of the blood donors was unknown, a minute fraction of the primary B cells under study could theoretically contain the virus. To avoid any viral interference, we repeated the nucleic acid sensing experiments in truly EBV-negative B cell clones that had been immortalized by Bcl-6 and Bcl-xL expression (Kwakkenbos et al., 2010). Responses were compared to the well-studied THP1 cell line that adopts a macrophage-like phenotype upon PMA-differentiation and produces high levels of IFN I when exposed to cytoplasmic dsDNA (Hansen et al., 2014). Following transfection, we estimated that FAM-dsDNA had entered approximately 50% of the clonal B cells (Fig. 1E, left). This may be an underestimation due to background green fluorescence of a GFP marker already present in the cells. Control THP1 cells were very efficiently transfected with dsDNA (> 98%) (Fig. 1E, right). Exposure to labeled dsDNA in the absence of transfection reagent also resulted in some association of label with THP1 cells. While the levels of FAM fluorescence that reflect DNA entry are difficult to compare, substantial percentages of B cells and THP1 cells appear to have taken up dsDNA following transfection.

Next, we assessed if the B cell clone secreted IFN I after cytoplasmic exposure to dsDNA. We did not detect secretion of IFN I by the B cell clone upon dsDNA or poly(I:C) transfection, whereas in THP1 cells we did (Fig. 1F). CpG DNA stimulation of the B cell clone caused a minute increase in CD86 surface display, likely due to the fact that the B cell clone had already high surface levels of CD86 prior to stimulation

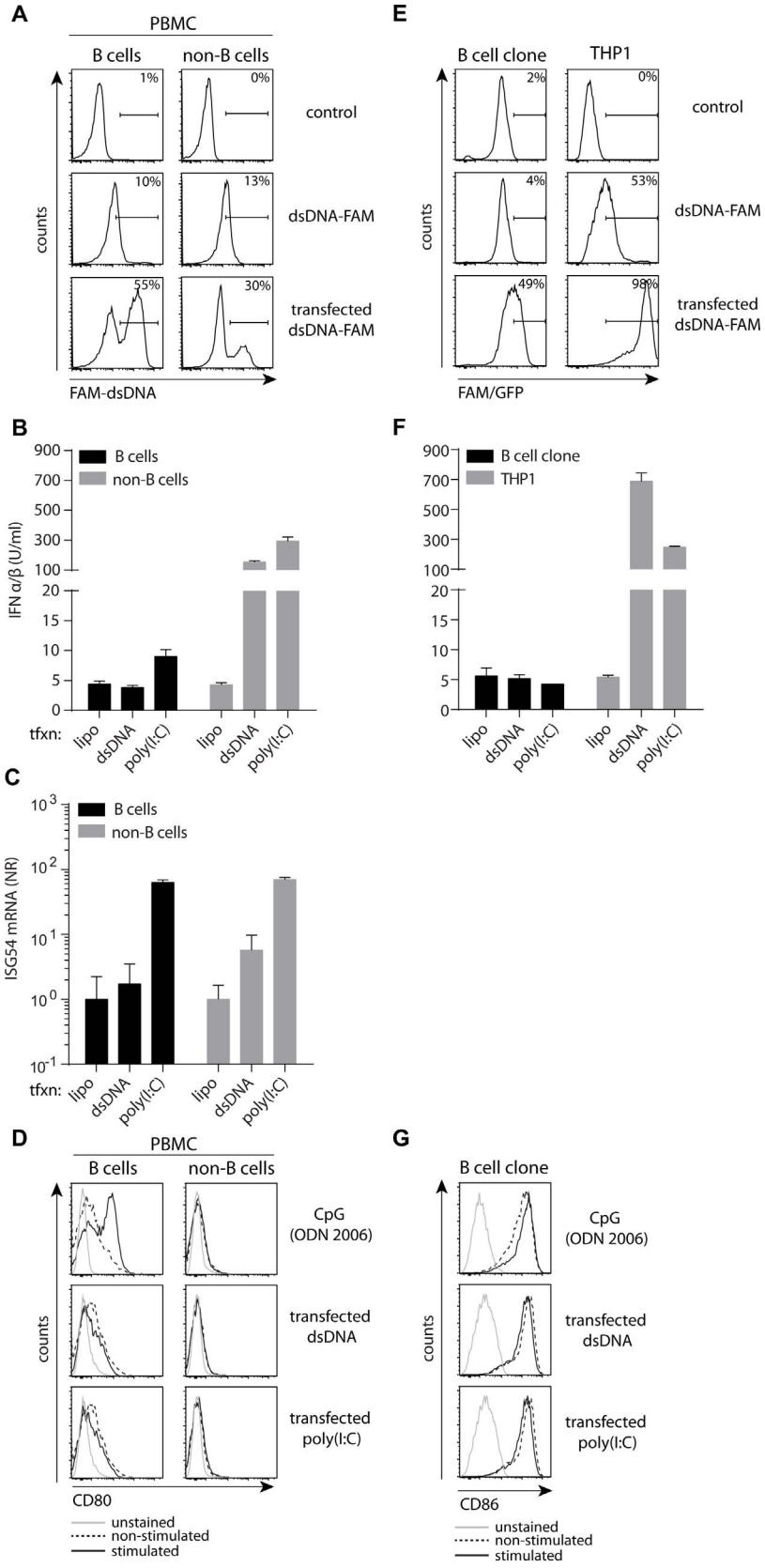

Fig. 1. Human B cells do not secrete IFN I upon exposure to cytoplasmic DNA.

Innate responses were studied in (A–D) primary human PBMC-derived $CD19^+$ B cells and $CD19^-$ non-B cells and (E–G) an EBV-negative human B cell clone specific for Influenza virus and (E–F) macrophage-like PMA-differentiated THP1 cells. (A,E) Efficiencies of introducing stimulatory nucleic acids into the cytoplasm were estimated by flow cytometry using FAM-labeled dsDNA. As a control, cells were treated with FAM-labeled dsDNA alone, left untreated (control, A) or treated with lipofectamine2000 alone (control, E). (B,F) To measure IFN I secretion, a bioassay was performed on supernatants 24 h after transfection with either dsDNA or poly(I:C). One representative of at least two independent experiments is shown. Data are presented as mean ± SEM. (C) Levels of ISG54 mRNA in the cells stimulated as above were measured by qPCR using SYBR green. Data are means (± SEM) of normalized ratios relative to control sample (lipo), using GAPDH as internal reference. One representative experiment performed on cells from two different donors is shown. (D, G) Surface levels of the activation markers (D) CD80 and (G) CD86 were determined by flow cytometry 24 h after stimulation with the indicated nucleic acids (black lines), non-stimulated controls (dashed lines), and background signal without specific antibodies (grey lines).

(Fig. 1G). Transfection of dsDNA or poly(I:C) had no detectable effect on the CD86 levels at the surface of the B cell clone (Fig. 1G). The observed unresponsiveness to cytoplasmic nucleic acid stimuli was not limited to the (Influenza virus-specific) B clone analysed, as we observed a highly similar phenotype using another (tetanus-specific) B cell clone (data not shown).

In conclusion, human primary B cells and EBV-negative B cell clones can sense and signal upon exposure to certain nucleic acids, as

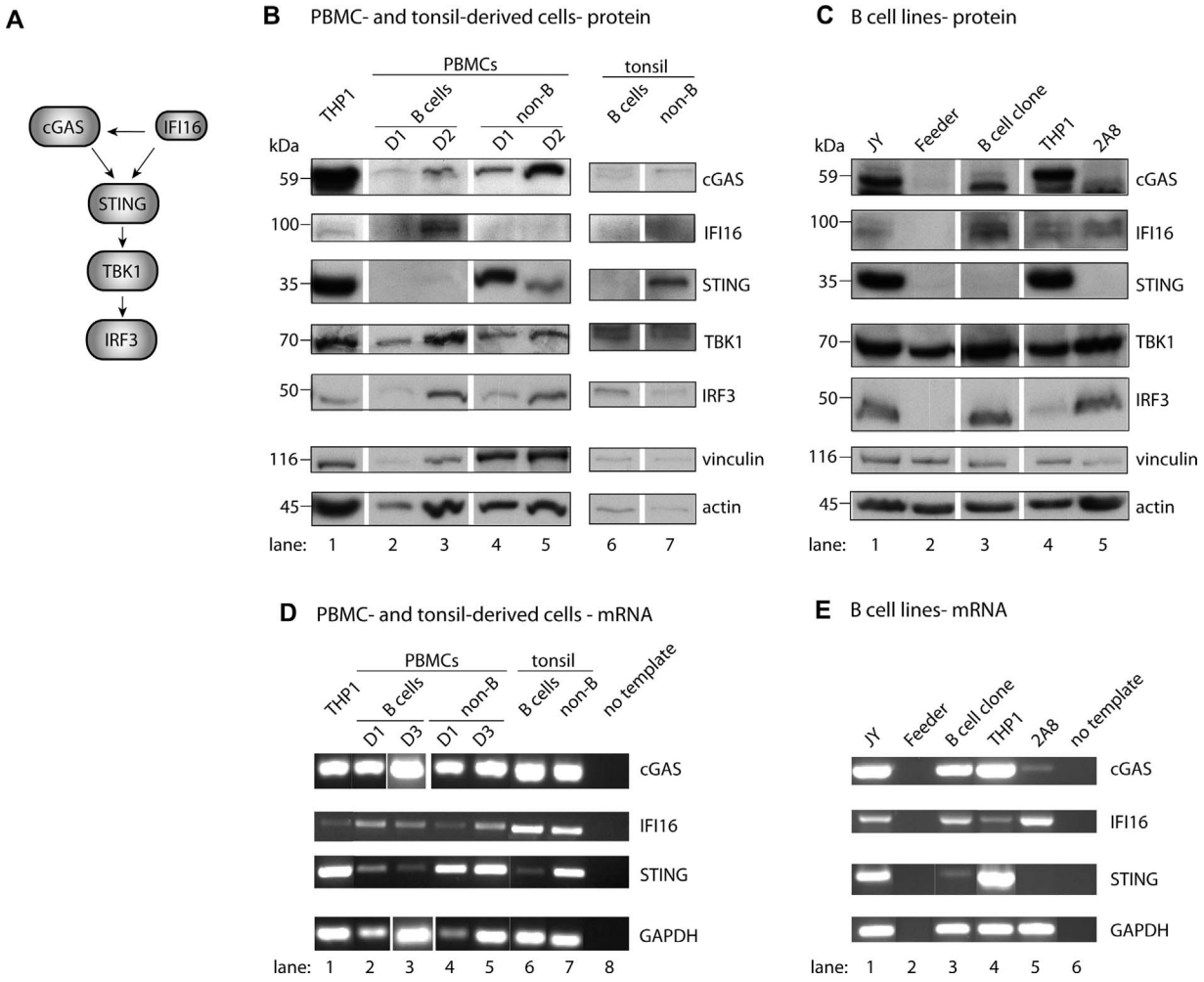

Fig. 2. Primary human B cells and EBV-negative B cell lines are deficient for STING protein.
(A) Schematic overview of key proteins in the cytoplasmic DNA sensing pathway. (B-E) Primary PBMC-derived or tonsillar $CD19^+$ B cells and $CD19^-$ non-B cells (B, D), a B cell clone, the B cell lines JY and 2A8, and THP1 cells (C, E) were analysed on Western blots (B, C) and by RT-PCR (D, E). Representative experiments are shown. Total cell lysates were used to determine levels of cGAS, IFI16, STING, TBK1, and IRF3 and cDNA was analysed for levels of cGAS, IFI16, and STING. As no products were amplified with the primer-pairs used in the murine feeder cell mRNA (Fig. 2D, lane 2), the signals detected for the B cell clones are unlikely to originate from traces of contaminating feeder cells. For Western blot analysis, PMA-differentiated THP1 cells were used, whereas non-differentiated THP1 cells were analysed by RT-PCR. RT-PCR analysis of non- and PMA-differentiated THP1 cells can be found in Supplementary material in Fig. A.1D. Vinculin and actin (B, C) or GAPDH (D, E) served as controls. White spacing indicates where empty lanes separating the samples on the blots have been removed for the figure. (B, D) PBMC-derived cells of two independent donors and tonsil cells of one donor were analysed. (D) The number of cycles completed for the IFI16 and STING PCRs were 25 and 30, respectively. The bands shown for cGAS and GAPDH of the B cells of donor D3 (lane 3) are derived from an independent experiment. (E) 35 cycles were completed for the IFI16 and STING PCRs.

Table 3
Summary of RNA and protein analyses of B cells and non-B cells and IFN I production after cytoplasmic DNA stimulation.

	Non-B cells			B cells						
mRNA/proteins	THP1 cells	PBMC non-B cells	Tonsil non-B cells	PBMC B cells	Tonsil B cells	B cell clones	2A8	BJAB	JY	MRJ
cGAS	+ +	+ +	+	+	+	+	±	±	+	+
IFI16	+	±	+	+	+	+	+	+	+	+
STING	+ +	+ +	+ +	−	−	−	−	±	+ +	+
TBK1	+	+	+	+	+	+	+	+	+	+
IRF3	+	+	+	+	+	+	+	+	+	+
Response	+	+	NT	−	NT	−	−	−	−	−
Reason				No STING		No STING	No STING	Very low STING	Unknown	Unknown

illustrated by IFN I production and/or upregulation of surface activation markers when transfected with poly(I:C) and/or stimulated with CpG DNA. Despite this, these B cells failed to respond to cytoplasmic dsDNA with IFN I secretion or NF-κB-activation.

3.2. STING protein is absent from primary B cells and EBV-negative B cell lines, yet present in EBV-transformed B-LCL

To elucidate the mechanism underlying the unresponsiveness of human B cells to cytoplasmic dsDNA, we first evaluated if key

Table 4
Summary of RNA and protein analyses of B cells and non-B cells and IFN I production to cytoplasmic poly(I:C) stimulation.

	Non-B cells			B cells					
mRNA	THP1 cells	PBMC non-B cells	Tonsil non-B cells	PBMC B cells	Tonsil B cells	B cell clones	2A8	BJAB	JY
RIG-I	±	+	+	+	+	+	+	+	+
MDA5	+	+	+	+	+	+	+	+	+
MAVS	+	+	+	+	+	−	+	+	+
TLR3	−	+	+	−	−	−	−	++	−
Response	+	+	NT	+	NT	−	−	+	+
Reason						No MAVS	Unknown		

components of the pathway (Fig. 2A) were produced. A summary of all expression data is provided in Table 3.

In total lysates of primary B cells isolated from donor PBMC, the DNA sensors cGAS and IFI16 were present, as were the signaling proteins TBK1 and IRF3 (Fig. 2B, lanes 2 and 3) albeit that their levels displayed some variation. Surprisingly, STING protein − the central signaling hub − was not detected in these cells. A similar expression pattern was observed in primary human B cells from a different source, the tonsil (lane 6). STING deficiency in the B cells was in sharp contrast to the pronounced levels of STING detected in lysates of the non-B cell fractions of the corresponding donors and differentiated THP1 cells (lanes 1, 4, 5, and 7, and Supplementary material in Fig. A.1A), representing positive control cells that supported IFN I production following cytosolic DNA exposure (Fig. 1B and F). These results point towards deficient STING expression in primary human B cells and possibly explain the lack of IFN I production in response to dsDNA in their cytosol (Fig. 1B and C).

Besides the primary B cells, a panel of (transformed) human B cell lines was subjected to Western blot analysis with a focus on the cGAS-STING axis (Fig. 2C, Supplementary material in Fig. A.1B and C). The panel included EBV-negative B cell clones (Influenza virus-specific and tetanus-specific), EBV-negative BL lines (2A8, BJAB), and EBV-positive B-LCL (JY, MRJ). JY and MRJ are EBV-transformed B cell lines harboring viral DNA genomes and producing the nine EBV latency proteins as well as several RNA species. These B-LCL represent EBV-infected B cells in latency stage III, while 2A8 and BJAB are EBV-negative B cell lines, originally derived from (EBV-induced) BL. The murine $CD40L^+$ feeder cells, on which the B cell clones were cultured, were taken along as a control, as small amounts could potentially contaminate the B cell clone lysates. The antibodies used to visualize human cGAS, IFI16, STING, and IRF3 did not cross-react with murine homologs from the feeder cells (lane 2).

All B cell lines tested expressed the proteins cGAS, IFI16, TBK1, and IRF3 (Fig. 2C, Supplementary material in Fig. A.1B and C), as was the case with the primary cells above (Fig. 2B); only in the 2A8 cells, levels of cGAS appeared very low. The adaptor protein STING was absent from the EBV-negative B cell clones (lane 3 and not shown) and 2A8 BL line (lane 5), and strongly reduced in the BJAB cells (Supplementary material in Fig. A.1C, lane 2), thus reproducing the STING deficiency of primary B cells. In contrast, EBV-infected B cells in latency III, represented by JY and MRJ, contained marked amounts of STING proteins (Fig. 2C, lane 1; Supplementary material in Fig. A.1B, lanes 3 and 4). Thus, all components of the STING-dependent DNA sensing pathway appeared to be produced in the EBV-positive B-LCLs, comparable to the THP1 control cells.

To investigate if a deficiency of STING protein in human B cells occurred at the transcriptional or translational level, we additionally examined the mRNA levels of STING for all cells (Fig. 2D and E, Supplementary material in Fig. A.1D and E). Since cGAS and IFI16 protein levels appeared to be low in some B cell lines, we also determined mRNA levels of cGAS and IFI16.

Overall, semi-quantitative RT-PCR analysis confirmed the expression profile of the cytosolic DNA sensors and STING adaptor seen at the protein level (compare panels B and D, and C and E, summarized in Table 3). Please note that for the RT-PCR reactions, undifferentiated THP1 cells were used, explaining the reduced amounts of transcripts compared to proteins for IFI16 (for a side-by-side comparison of PMA-treatment, see Supplementary material in Fig. A.1D). Absence of marked levels of STING mRNA in all B cells except the EBV-transformed B-LCL demonstrates that the lack of STING occurred at the transcriptional level.

In conclusion, both the primary human B cells and the EBV-negative B cell lines tested expressed key components of the cytoplasmic DNA sensing pathway, with the exception of STING. EBV-positive B-LCL appeared to be different, as they had substantial amounts of STING. Absence of the STING protein provides an explanation as to why human primary and EBV-negative B cells did not secrete IFN I upon stimulation with cytoplasmic DNA.

3.3. Absence of MAVS mRNA may underlie unresponsiveness of human B cell clones to cytosolic RNA

Transcriptional regulation of key molecules in the DNA sensing pathway can thus underpin a loss of function. In the experiments above, the responses to poly(I:C) transfection via the cytosolic RNA sensing pathway served as a positive control (Fig. 1B, C), demonstrating responsiveness and ability of the human primary B cells to secrete IFN I. An unexplained observation was that the B cell clones did not produce IFN I upon poly(I:C) stimulation (Fig. 1F). A defect in expression of a key component for RNA sensing (Supplementary material in Fig. A.2A) could, likewise, be responsible. To explore this option, we additionally analyzed the mRNA levels of RIG-I, MDA5, MAVS, and TLR3 (Supplementary material in Fig. A.2B, C, D). Expression data are summarized in Table 4.

Only the PBMC- and tonsil-derived non-B cell fractions contained (some) TLR3 mRNA, but we did not detect it in primary B cells and THP1 cells (Supplementary material in Fig. A.2B and C). As these latter cell types did produce IFN I when stimulated with poly(I:C), the TLR3 expression profile supports that transfected poly(I:C) was sensed via the cytoplasmic RNA sensors RIG-I and MDA5, rather than by the endosomal TLR3 molecules. This is in agreement with the transfection route being more likely to deliver the RNA mimic to the cytosol. Furthermore, TLR3 transcripts were abundant in BJAB cells, yet were also absent from the B cell clones, the JY and 2A8 B cell lines, and THP1 cells (Supplementary material in Fig. A.2C (lane 1) and D). Thus, TLR3 is not likely to participate in RNA sensing in most human B cells lines. As poly(I:C) induced vigorous IFN I production in THP1 cells (Fig. 1F), we deduced that TLR3 did not contribute to a major extent to poly(I:C) sensing upon transfection of the ligand.

The cytoplasmic RNA sensors RIG-I and MDA5 were transcribed in the human B cell lines JY, 2A8, and the B cell clones (Supplementary material in Fig. A.2D). Transcript levels for the signaling adaptor MAVS in PBMC-derived cells − although low − appeared to be sufficient to support the production of IFN I following cytoplasmic RNA sensing

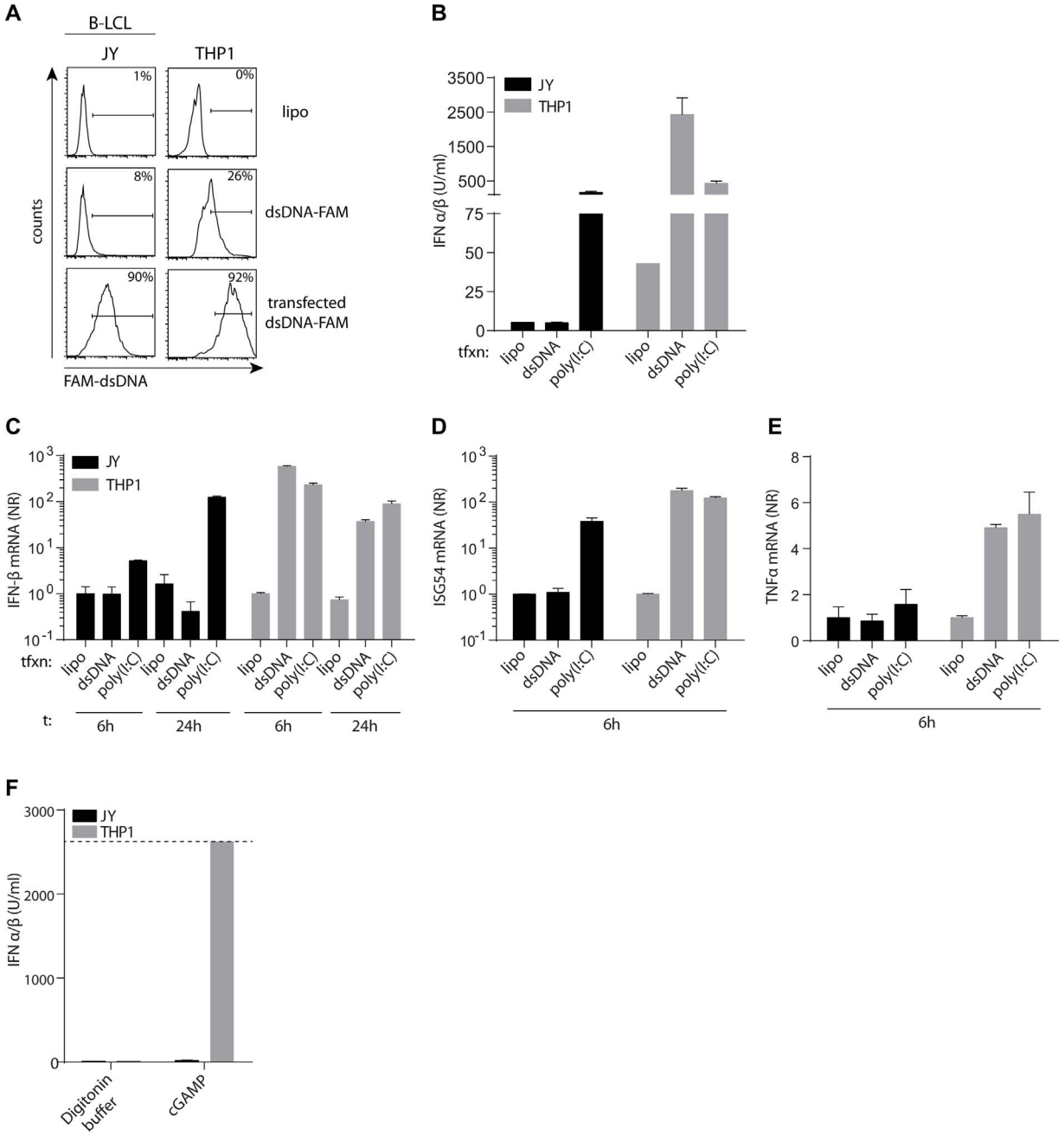

Fig. 3. The human B-LCL JY has STING protein, but fails to produce IFN I upon dsDNA or cGAMP stimulation.
Innate responses were studied in JY and PMA-differentiated THP1 cells. (A) Efficiencies of introducing stimulatory nucleic acids into the cytoplasm were determined as in Fig. 1A. (B–E) Cells were stimulated as in Fig. 1B. (B) One representative of four independent experiments is shown. Data are presented as mean ± SEM. (C-E) RNA was isolated from cells 6 h or 24 h post-transfection and analysed for mRNA levels of (C) IFN-β, (D) ISG54, or (E) TNFα by quantitative real-time PCR using TaqMan Probes. Data are means (± SEM) of normalized ratios relative to control sample, using β-actin as internal reference. One representative of two independent experiments is shown. (F) To stimulate STING directly, cells were stimulated with cGAMP in a buffer containing digitonin. Supernatants were harvested and analysed at 6 h post-stimulation. Data are presented as mean ± SEM. One representative of three independent experiments is shown.

(Fig. 1B and C). Of note, the B cell clones did not produce MAVS mRNA (Supplementary material in Fig. A.2D, lane 3 and not shown). Thus, for the B cell clones, a transcriptional defect in MAVS expression could explain why these cells did not secrete IFN I upon cytoplasmic poly(I:C) stimulation.

3.4. *Despite STING expression, EBV-transformed B cell lines do not produce IFN I in response to cytoplasmic dsDNA or cGAMP*

As described above, the EBV-positive B cell lines JY and MRJ differed from other human B cells in their STING expression. In view of the presence of all major components of the cGAS-STING pathway in the EBV-transformed B-LCL cells (Fig. 2C and Supplementary material in Fig. A.1B), we examined its functionality, i.e. whether the cells produced IFN I upon cytoplasmic DNA exposure. Upon transfection, labeled dsDNA had entered the large majority of JY cells (90% FAM-positive cells), comparable to THP1 cells (92% FAM-positive cells) (Fig. 3A). Despite this, JY cells did not secrete IFN I in response to the introduced dsDNA, in contrast to THP1 cells (Fig. 3B). Yet, in line with expression of the RIG-I, MDA5, and MAVS mRNAs (Supplementary material in Fig A.2D), both JY and THP1 cells secreted IFN I upon poly (I:C) transfection (Fig. 3B). This positive control indicated that both the

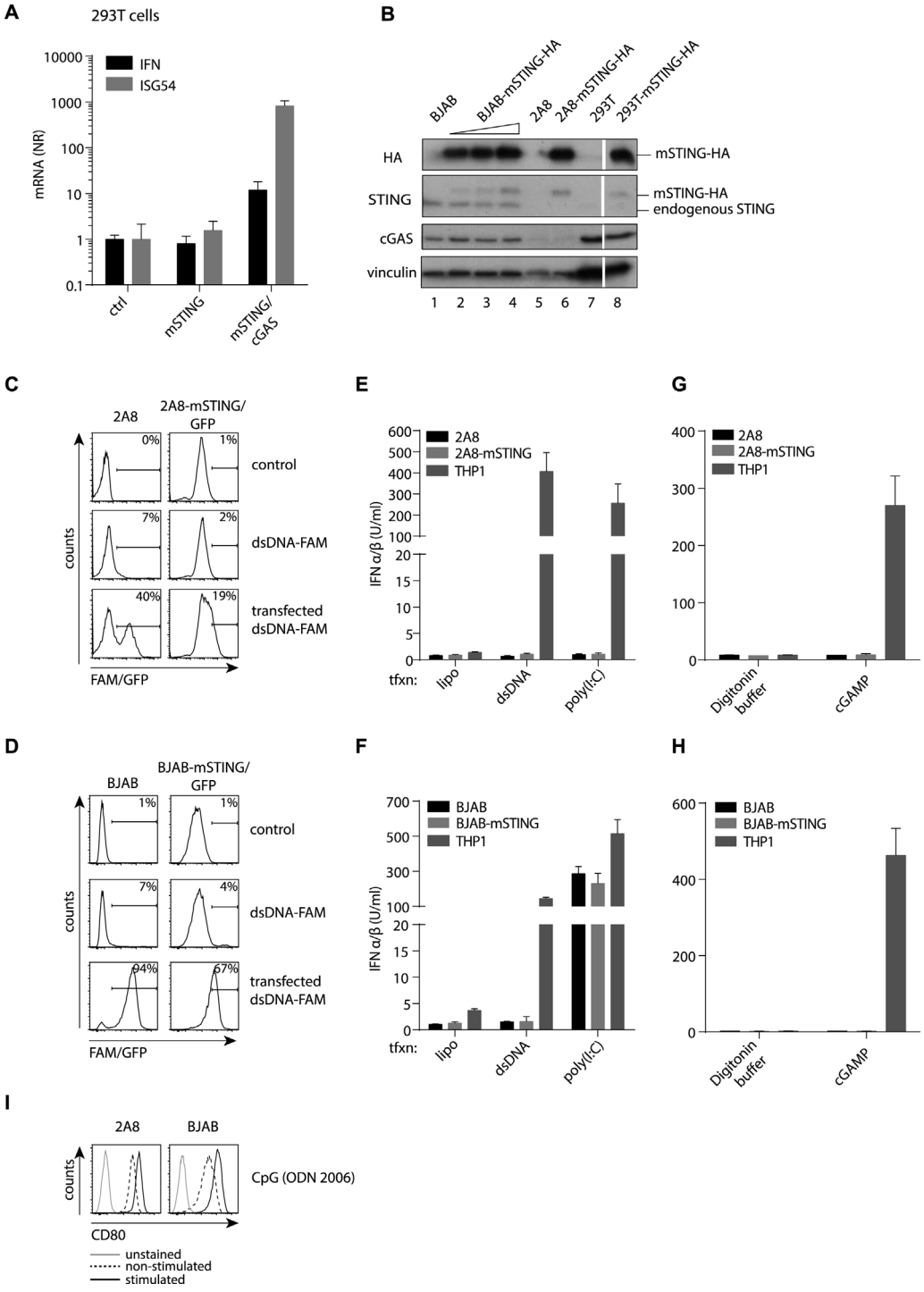

Fig. 4. Reconstitution of STING expression in EBV-negative B cells does not rescue responsiveness to cytoplasmic DNA or cGAMP. HA-tagged murine STING was introduced into 293T cells by transfection (A, B) and into 2A8 (B, C, E, G) and BJAB (B, D, F, H) cells by lentiviral transduction. (A) 293T cells were transfected with 180 ng empty vector (ctrl), 30 ng mSTING plasmid and 150 ng empty vector (mSTING) or 150 ng cGAS plasmid (mSTING/cGAS). 24 h post-transfection, levels of IFN-β and ISG54 mRNA were determined as in Fig. 1C. One representative of two independent experiments is shown. (B) Total cell lysates of mSTING-transduced BJAB and 2A8 cells, and 293T cells transfected with 180 ng mSTING plasmid were analysed for cGAS and STING protein and HA-tag. Vinculin served as loading control. White spacing indicates where empty lanes separating the samples on the blots have been removed for the figure. (C–I) 2A8, 2A8-mSTING, BJAB, BJAB-mSTING, and PMA-differentiated THP1 cells were analysed. (C,D) Transfection efficiency of cells was determined as described in Fig. 1A. (E,F) Cells were stimulated and supernatants analysed as in Fig. 1B. One representative of at least three independent experiments is shown. Data presented are means (± SEM). (G, H) Cells were stimulated as in Fig. 3F and supernatants analysed at (G) 3,5 h or (H) 6 h post-stimulation. One representative of at least two independent experiments is shown. Data presented are means (± SEM). (I) Cells were stimulated and analysed as in Fig. 1D 24 h post-stimulation (black lines) and compared to non-stimulated control cells (dashed lines), which were left untreated. Unstained cells (grey lines) were used to determine background levels.

cytosolic delivery of nucleic acid ligands and the activity of the TBK1-IRF3 axis were effective in the B-LCL.

To examine whether cytoplasmic DNA exposure in JY cells resulted in an IFN I response, which was too low to be detected in the bioassay, IFN-β1 and ISG54 transcripts were quantified by real-time qPCR at 6 h and 24 h after dsDNA transfection (Fig. 3C, D). In accordance with the bioassay, introduction of poly(I:C) into both JY and THP1 cells increased IFN-β1 and ensuing ISG54 mRNA levels (Fig. 3C, D; in JY cells: small for IFN-β1 and no increase for ISG54 mRNA at 6 h, both robust at 24 h post-transfection). No relevant increase in IFN-β1 or ISG54 transcripts was detected after dsDNA stimulation in JY cells, in contrast to THP1 cells. Although we noted minor variations in IFN-β1 transcript levels upon dsDNA stimulation in some experiments, the variation observed was in the same range as the one detected between untreated and lipofectamine-treated samples (Supplementary material in Fig. A.3A). In addition, we tested if introduction of cytoplasmic dsDNA into JY cells resulted in gene expression of the pro-inflammatory cytokine TNFα, as an alternative to the IFN I pathway. Upon cytoplasmic DNA stimulation, we observed no increase in TNFα mRNA in JY cells, whereas THP1 cells showed a moderate, but transient, response to transfection of dsDNA as well as poly(I:C) (detectable at 6 h (Fig. 3E), but not at 24 h (not shown)).

These combined data show that − although synthesizing STING − the EBV-positive B cell line JY does not respond to cytoplasmic dsDNA

exposure either through production of IFN I or transcription of the pro-inflammatory cytokine TNFα.

IFN I induces expression of many ISGs via the JAK-STAT pathway, which acts downstream of the IFN I receptor (Schneider et al., 2014). Upregulation of ISGs, including PRRs such as cGAS, IFI16, and other downstream signaling proteins, limits viral replication (Schoggins et al., 2011; Liu et al., 2012). Hence, IFN I primes cells to detect and/or respond to PAMPs in a stronger way. For example, human IFN I-primed macrophages showed enhanced responses to TLR stimuli in comparison to unprimed cells (Siren et al., 2005). Following this rationale, we examined whether the JY cells would respond to dsDNA when in a primed state.

JY and THP1 cells were stimulated with IFN-β for 24 h prior to transfection with dsDNA or poly(I:C). IFN-β treatment of JY cells did not give rise to a dsDNA-induced IFN I response (Supplementary material in Fig. A.3B, left panel). In contrast, enhanced IFN I levels were detected in primed THP1 cells upon dsDNA stimulation in comparison to unprimed cells (Supplementary material in Fig. A.3B, right panel). Correcting for the increased levels observed in transfection reagent-treated cells showed that the levels induced by dsDNA transfection were not higher in a primed state (Supplementary material in Fig. A.3C). Secretion of IFN I following poly(I:C) stimulation was not altered by pre-stimulation in either of the cell lines (Supplementary material in Fig. A.3B and C). As pretreatment with recombinant IFN-β may be detected in the bioassay, we additionally examined IFN-β transcription in response to nucleic acid stimulation. In JY cells, transcription of IFN-β was not elevated upon priming (Supplementary material in Fig. A.3D and E). Similar to our findings for IFN I secretion, IFN-β transcription was slightly increased in THP1 cells in response to nucleic acids upon IFN pre-stimulation, but IFN I treatment alone also elevated transcription (Supplementary material in Fig. A.3D). Correcting for this, in primed THP1 cells the fold induction was not higher (Supplementary material in Fig. A.3E). To conclude, these data indicate that IFN-β-priming of the B-LCL JY did not rescue responsiveness to cytoplasmic dsDNA.

Despite the presence of STING in addition to cGAS, IFI16, TBK1, and IRF3 in the EBV-positive B-LCL, also these human B cells were unresponsive to dsDNA transfection. Sequence analysis revealed that JY cells had the same variant of STING transcribed as the THP1 cells (data not shown), suggesting that JY cells did not carry a mutated form or non-responsive allele of STING. As it is difficult to control levels of cytoplasmic dsDNA introduced by lipofectamine transfection, we tested whether the STING-TBK1-IRF3 pathway could be activated downstream of DNA detection. To this end, we bypassed the DNA sensing step by introducing the STING-specific ligand 2′3′-cGAMP into digitonin-permeabilized JY cells. No IFN I was secreted by cGAMP-stimulated JY cells at 6 h post-stimulation, while THP1 cells produced IFN I in the presence of cGAMP (Fig. 3F). Thus, the high levels of STING present in JY cells did not support an IFN I response even when triggered downstream of the DNA sensors.

In summary, the IFN I pathway is functional in EBV-positive B-LCL when appropriately stimulated (i.e. via the RNA sensing pathway), but also these human B cells did not produce IFN I upon cytoplasmic dsDNA exposure.

3.5. Introduction of STING does not render human B cells responsive to cytosolic dsDNA or cGAMP

As herpesviruses like EBV are known to interfere with innate immunity, the unresponsiveness of JY cells to cytosolic DNA could reflect viral immune evasion. To circumvent potential inhibitory effects of EBV proteins present in the JY cells, we turned to the two EBV-negative human B cell lines 2A8 and BJAB. These cells produced the DNA sensors IFI16 and cGAS, and the signaling components TBK1 and IRF3, but lacked sufficient levels of STING protein (Fig. 2D and E). We assessed if reconstitution of STING could rescue IFN I production upon dsDNA stimulation.

Murine STING (mSTING) is able to reconstitute human STING-deficient cells to respond to cyclic dinucleotides (Diner et al., 2013). To confirm that the mSTING construct we used was functional in human cells, we expressed low levels of mSTING in 293T cells. When mSTING was co-expressed with human cGAS in these cells, IFN-β and ISG54 were induced as determined by qPCR analysis (Fig. 4A). Transfection of cGAS alone did not result in activation of the pathway, while high levels of mSTING did (data not shown). Having shown that the mSTING construct was functional in human cells, we introduced it into human B cells to reconstitute the cGAS-STING pathway. For this, we used lentiviral transduction, since transfection approaches to introduce genes for protein synthesis are inefficient in B cells. We introduced different amounts of C-terminally HA-tagged mSTING into 2A8 and BJAB cells and pure populations of transduced, GFP^+ cells were sorted and analysed by Western blot (Fig. 4B). An HA-reactive antibody revealed high levels of HA-tagged STING in mSTING-expressing lines (lane 2–4, 6, and 8); the BJAB lines transduced with increasing doses of lentivirus showed increasing levels of STING (lane 2–4). Western blots of the mSTING-transduced B cells or -transfected 293T cells showed an additional weak band using a human STING-specific antibody, reflecting some degree of conservation in STING sequence between mouse and man. The bands detected with the HA-reactive antibody migrated at the same height as the weak STING antibody-reactive bands suggesting that these bands represented HA-tagged STING protein.

Upon transfection, the parental 2A8 and BJAB cells were labeled with dsDNA-FAM with efficiencies of around 40% and 90%, respectively (Fig. 4C and D). GFP^+ 2A8- and BJAB-mSTING cells showed an efficiency of around 20% and 70%, which may be an underestimation due to presence of GFP. Upon transfection of poly(I:C) or dsDNA, neither the parental 2A8 cells, nor the 2A8-mSTING cells secreted IFN I, in contrast to control THP1 cells (Fig. 4E). It is unclear why poly(I:C) stimulation of 2A8 cells failed to induce production of IFN I, despite the presence of the cytoplasmic RNA sensors RIG-I and MDA5, as well as the adaptor protein MAVS (Supplementary material in Fig. A.2B, lane 5). Also BJAB and BJAB-mSTING cells did not secrete IFN I in response to cytoplasmic DNA, although these cells responded to poly(I:C) stimulation with IFN I production (Fig. 4F).

To circumvent potential effects of low levels of cGAS in 2A8 and BJAB cells (Fig. 4B), we examined whether the STING-TBK1-IRF3 pathway could be activated by the STING ligand cGAMP in 2A8-mSTING and BJAB-mSTING cell lines, thereby bypassing the need for activation of the DNA sensors. 2A8-mSTING and BJAB-mSTING cell lines did not secrete IFN I upon cGAMP stimulation, while the positive control THP1 cells produced IFN I (Fig. 4G and H). These data indicate that STING reconstitution was not sufficient for EBV-negative B cell lines to respond to the second messenger molecule cGAMP.

To assess whether 2A8 and BJAB cells were responsive to another DNA stimulus indicating that the cells were viable at the time of treatment, we stimulated these cells with CpG DNA. Prior to stimulation, the cells expressed high surface levels of CD80, yet the levels further increased upon stimulation with CpG DNA suggesting that the cells were viable and responsive to TLR9-mediated DNA sensing (Fig. 4I).

In conclusion, these data show that EBV-negative human B cells producing STING, similar to the EBV-positive JY cells, do not respond to cytoplasmic DNA or cGAMP stimulation with IFN I secretion.

4. Discussion

In this study, we found that human B cells fail to produce IFN I in response to cytoplasmic dsDNA. In primary B cells and EBV-negative B cell lines, this is correlated with the absence of the adaptor protein STING (Table 3), the central adaptor protein that is required for signal transduction downstream of the cytosolic DNA sensors. Moreover, when STING protein is present, as in EBV-positive B-LCL or STING-

reconstituted EBV-negative B cell lines, human B cells did not secrete IFN I upon dsDNA or cGAMP stimulation. This would implicate that the cGAS-STING pathway in human B cells will not be activated by B cell-tropic DNA viruses, such as EBV and KSHV.

The STING protein was originally identified in murine B cell lines as a molecule involved in apoptosis (Jin et al., 2008) and, shortly thereafter, it was recognized as the central signaling hub for cytosolic DNA sensing (Sun et al., 2009; Ishikawa and Barber, 2008; Zhong et al., 2008). Murine B cells are able to respond to cGAMP stimulation with transcription of IFN I (Tang et al., 2016), which contrasts to our findings on human B cells (Figs. 3F, 4G and H). Thus, STING is present and functional in B cells of mouse, but not of human origin. Murine and human STING proteins exhibit a different activation profile in response to cyclic dinucleotides and synthetic ligands. Murine STING is potently triggered by bacterial 2′2′- and 3′3′- and mammalian 2′3′-linked cyclic dinucleotides as well as the synthetic ligands DMXAA and CMA resulting in IFN I production, while human STING is mainly activated by the mammalian 2′3′-cGAMP (Diner et al., 2013; Yi et al., 2013; Conlon et al., 2013; Cavlar et al., 2013). Hence, human STING responds to a narrower spectrum of ligands, which likely reflects a slightly different role of this protein in innate immune activation. One could speculate that a distinct expression profile in (immune) cells is evolutionarily favored due to differential sensitivity towards cyclic dinucleotide ligands.

Cytosolic DNA sensing plays a crucial role in host immune defense against invading DNA viruses, as exemplified by the lethality of HSV-1 infection in STING-knockout mice (Ishikawa et al., 2009; Ma et al., 2015). The absence or low levels of STING in human B cells may render these cells more permissive for infection, for instance with the herpesviruses KSHV or EBV. Still, human B cells are not devoid of functional DNA sensing. We have reported earlier that EBV genomes can trigger the DNA sensor TLR9 (van Gent et al., 2011). Moreover, IFI16 senses nuclear EBV and KSHV genomes in human B cells resulting in subsequent formation of IFI16 inflammasomes and IL-1β, IL-18, and IL-33 maturation, but not in the release of these cytokines (Ansari et al., 2013; Singh et al., 2013). As yet, it is not known if human B cells support formation of another DNA-induced inflammasome, the AIM2 inflammasome, upon cytoplasmic DNA exposure. In some murine immune cells, AIM2 was found to affect the IFN I production induced by cytosolic DNA sensing (Corrales et al., 2016; Nakaya et al., 2017). This raises questions as to how the different cytoplasmic DNA sensing routes cooperate and what the net effects are of dysfunctionality of the cGAS-STING pathway. All in all, human B cells appear − to some level − equipped to sense invading DNA viruses and initiate innate immune responses, yet they fail to induce an IFN I response upon cytoplasmic DNA exposure, which could be explained by low levels of STING in (primary) EBV-negative B cells.

In contrast to the primary and EBV-negative B cells, EBV-transformed B cell lines have high STING levels, which are comparable to those observed in THP1 cells. It remains to be determined whether EBV latency gene products present in the B-LCL contribute to STING expression in these cells. In a recent report, it has been proposed that enhanced CD40 expression increases levels of STING protein (Yao et al., 2016). One of the latency genes present in EBV-positive B-LCL encodes the latency membrane protein 1, an EBV protein mimicking a constitutively active CD40 molecule, thereby supporting B cell transformation. We propose that this protein might contribute to the elevated STING levels in B-LCL compared to EBV-negative B cells. Nevertheless, the STING-competent EBV-positive B-LCL did not respond to cytoplasmic DNA or cGAMP stimulation (Fig. 3). This may be linked to other EBV gene products present in these B cells, possibly modulating the outcome of activation of the cGAS-STING pathway.

Shortly after elucidation of the cGAS-STING pathway as a major DNA sensing route, the first herpesvirus evasion molecules − acting in latency or during virus production − were identified that perturbed its function. Identification of KSHV proteins, including the KSHV-specific K9/vIRF1 protein, interfering with the cGAS-STING pathway (Ma et al., 2015) argues that this pathway is important in sensing of KSHV. The latency-associated KSHV protein LANA inhibits cGAS and also the tegument protein ORF52, produced during lytic infection, prevents cGAS-mediated cGAMP production *in vitro* and in infected cells (Wu et al., 2015; Zhang et al., 2016). The EBV homologue of ORF52, BLRF2, also prevented cGAMP formation in an *in vitro* assay. Identification of viral proteins inhibiting cGAS function indicates that the DNA sensing pathway plays a role in detection of these viruses. As the cytoplasmic DNA sensing pathway appears dysfunctional in EBV-negative B cells, it seems that viral inhibition of this pathway is not necessary in these B cells. Hence, viral evasion may be required for infection of other cell types. For instance KSHV infection of endothelial cells triggers activation of the cytosolic DNA sensing pathway, which was inhibited by KSHV immune evasion proteins (Ma et al., 2015). Last but not least, in the absence of STING from the same cell, the DNA sensor cGAS might still contribute to induction of innate immune responses against B-lymphotropic viruses such as KSHV or EBV. Recently, two independent groups found that viral particles can transfer the second messenger molecule cGAMP to other cells (Bridgeman et al., 2015; Gentili et al., 2015). Hence, triggering of cGAS in B cells could result in cGAMP production and packaging, and subsequent transfer to other cells by released virions.

EBV-positive B-LCL were unable to mount an IFN I response upon stimulation with dsDNA or the STING ligand cGAMP, despite substantial levels of STING and the other proteins known to participate in the cytosolic DNA sensing pathway (Figs. 2 and 3). In an attempt to unleash the cGAS-STING pathway in JY cells, we pre-stimulated these cells with IFN-β prior to introduction of dsDNA. However, this priming did not result in induction or secretion of IFN I (Supplementary material in Fig. A.3). Similarly, introduction of murine STING into the STING- and EBV-negative cell lines 2A8 and BJAB did not render these cells responsive to dsDNA or cGAMP, although murine STING can reconstitute human cells lacking STING (Fig. 4A and (Diner et al., 2013)). Based on this, our data imply that STING expression in human B cells does not reconstitute production of IFN I upon cytoplasmic DNA or cGAMP exposure. Besides transcriptional regulation of STING levels in human B cells, this additionally suggests that signal transduction was abrogated downstream of the STING protein. A similar phenomenon has been reported for activated human T lymphocytes that produce substantial levels of STING protein (Berg et al., 2014). T cells appeared to sense cytoplasmic DNA leading to formation of the STING signalosome including TBK1 recruitment, but still these cells failed to mount a IFN I response (Berg et al., 2014). We demonstrate that primary B cells and the BJAB and JY cell lines responded to transfected poly(I:C), which is sensed by the cytosolic RNA sensors MDA5 and RIG-I. They signal via the adaptor protein MAVS that, in turn, recruits TBK1 and IRF3 suggesting that signaling involving the proteins TBK1 and IRF3 was supported. This may, therefore, imply that STING failed to activate TBK1 and IRF3 in the B-LCL and BJAB cell lines.

It remains to be determined why human lymphocytes expressing the adaptor STING, the DNA sensors, and signaling intermediates TBK1 and IRF3 fail to induce an IFN I response upon cytoplasmic exposure to DNA. Given the unresponsiveness of T and B cells to cytosolic DNA stimulation, it is tempting to speculate that lymphocytes might possess a safeguard mechanism that prevents them from triggering an IFN I response upon exposure to intracellular DNA. Such a mechanism may present as a lymphocyte-specific inhibitor hampering activation or as an inducible, yet-unknown, factor facilitating signaling. The recent identification of the proteins iRhom2 and TRIF as required for STING activation (Luo et al., 2016; Wang et al., 2016) and NLRP14 as a negative regulator of cytosolic nucleic acid sensing during fertilization (Abe et al., 2017) illustrates that not all players involved in the cytoplasmic DNA sensing pathways might have been identified yet. Therefore, further studies are needed to identify the underlying mechanism of frustrated IFN I induction upon introduction of cytoplasmic DNA observed in human T and B lymphocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2017.08.025.

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