Chapter 5

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

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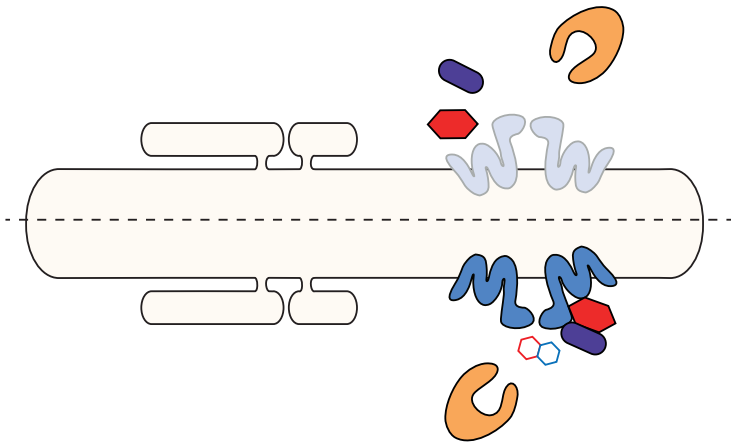


Abstract

Cytoplasmic exposure to foreign DNA or mislocalized self-DNA can trigger secretion of type I interferon (IFN). The DNA sensors interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP (cGAMP) synthetase (cGAS) recognize intracellular DNA and induce type I IFNs via the STING-TBK1-IRF3 axis. DNA sensing is protective as part of an anti-viral immune response, but may also be pathological by promoting auto-inflammatory and auto-immune diseases. B cells exert multiple functions such as antibody secretion, antigen presentation, and pathogen sensing. B cells sense CpG-containing DNA via the endosomal Toll-like receptor 9, thereby can contribute to anti-viral immune responses, but also to auto-immune diseases.

We report here that human B cells fail to secrete type I IFN upon cytoplasmic DNA exposure, although they possess the DNA sensors cGAS and IFI16 and the signalling components TBK1 and IRF3. Primary B lymphocytes and most B cell lines tested appear to lack the central adaptor protein STING that activates TBK1 and IRF3. B cells expressing STING also did not secrete type I IFNs upon dsDNA or cGAMP stimulation. Our data suggest that the cytoplasmic DNA sensing pathway may be dysfunctional in human B cells. Unresponsiveness of these cells to cytoplasmic DNA may render B cells attractive targets for infection by DNA viruses.

Introduction

Type I interferons (IFNs) play a central role in antiviral immunity as they induce the expression of interferon-stimulated genes (ISG) that create an anti-viral state [1]. Most cell types are believed to be able to produce the type I IFNs IFN-α and IFN-β [2]. They are encoded by 12 IFN-α genes and the IFN-β gene in humans. Type I IFNs are potently induced by viral nucleic acids (reviewed in [3,4]). Different RNA and DNA species trigger several pattern-recognition receptors (PRRs) resulting in activation of distinct downstream signalling cascades, which eventually converge and induce interferon regulatory factor 3 (IRF3)-induced transcription of type I IFN genes. The nucleic acid-sensing PRRs are located in endosomes, the cytoplasm, and even in the nucleus [3]. Viral RNA triggers Toll-like receptor 3 (TLR3) and the retinoic acid-inducible protein 1 (RIG-I)-like receptors, RIG-I and melanoma differentiation-associated protein 5 (MDA5), present in endosomes and the cytoplasm, respectively [5,6]. Foreign dsDNA is sensed by the endosomal TLR9 and several cytoplasmic and nuclear DNA sensors including interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP (cGAMP) synthetase (cGAS) [7]. TLR9 senses unmethylated CpG DNA and induces the transcription of pro-inflammatory cytokines through NF-κB [8]. The cytoplasmic DNA sensor cGAS directly binds to the backbone of cytosolic dsDNA in a sequence-independent manner and produces the cyclic dinucleotide 2'3'-cGAMP [9,10]. cGAMP acts as a second messenger molecule and activates the central adaptor protein STING (stimulator of interferon genes; also known as MPYS, MITA, and ERIS) and in turn, TANK-binding kinase 1 (TBK1) and IRF3 are activated [11,12,13]. Nuclear and cytoplasmic IFI16-dependent DNA sensing has also been linked to type I IFN production via STING activation, but the underlying molecular details remain enigmatic at this point [14]. In certain cell types, such as fibroblasts and endothelial cells, IFI16 forms an inflammasome upon DNA sensing, which results in caspase-1-dependent IL-1β maturation [15,16].

Activation of nucleic-acid sensing PRRs must be tightly regulated in order to prevent recognition of cellular "self"-nucleic acids. To this end, the RNA sensor RIG-I recognizes 5'-triphosphates that are present on viral RNA, but absent from cellular mRNA [17]. In contrast, cGAS and IFI16 are triggered by dsDNA irrespective of any apparent sequence or modification. Therefore, they may be triggered by foreign or self-DNA when present in the cytoplasm. The cytoplasm is typically devoid of self-DNA. However, certain genetic defects allow cytoplasmic accumulation of self-DNA, thereby triggering an type I IFN response. Autoimmune diseases like systemic lupus erythematosus (SLE) exhibit an IFN signature, which has been suggested to involve activation of the cytoplasmic and endosomal DNA sensing pathways by accumulating and mislocated self-DNA (reviewed in [18,19]). Mouse models with genetic defects in DNase function are used to study auto-inflammatory diseases. The lethality observed in both DNase II$^{-/-}$ and Trex1$^{-/-}$ (DNase III) mice is abrogated by STING- or cGAS-deficiency [20,21,22,23]. Moreover, DNase II$^{-/-}$cGAS$^{-/-}$ and Trex1$^{-/-}$cGAS$^{-/-}$

did not develop polyarthritis and showed limited induction of auto-antibodies, respectively, suggesting that DNA-driven inflammation may cause auto-immunity [20]. B cells secreting anti-nuclear auto-antibodies contribute to the pathology of SLE and other auto-immune diseases. B cell-receptor-dependent uptake of immune complexes has been implicated in TLR9-dependent DNA sensing and subsequent expansion of autoreactive murine B cells [24,25]. DNA sensing by B cells may not only have pathological effects, but might also be protective in anti-viral immunity.

Herpesviruses are enveloped viruses with a DNA genome that establish latent infection in their hosts. Human B cells are target for infection with the human herpesvirus Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). EBV establishes latent infection in human B cells. Human B cells sense the genome of the herpesvirus EBV by TLR9, which initiates immune activation [26]. Interestingly, EBV infection has been postulated to be a trigger for SLE [27], but it remains mechanistically unclear how EBV infection could contribute to development of this auto-immune disease. IFI16 can be triggered by nuclear EBV or KSHV genomes resulting in inflammasome-mediated IL-1β maturation in B cells [28,29], but whether stimulation of IFI16 could also lead to type I IFN induction was not tested. It is currently unknown whether the dsDNA genome of EBV triggers the cytoplasmic DNA sensing pathway resulting in type I IFNs secretion. Human herpesviruses including herpes simplex virus-1 (HSV-1) and KSHV, but also the reverse-transcribed human immunodeficiency virus (HIV) genome and stem-loop structures of single-stranded HIV DNA are known to trigger the cytoplasmic DNA sensing pathway [30,31,32,33].

Given the central role of DNA recognition in immune responses to DNA viruses and auto-inflammatory diseases, we investigated whether human B cells produce type I IFNs upon exposure to cytoplasmic DNA.

Results

The B-LCL JY does not produce type I IFNs in response to cytoplasmic dsDNA

To investigate whether human B cells secrete type I IFNs upon cytoplasmic DNA exposure, we used both primary B lymphocytes and various B cell lines, as summarized in **Table 1** (s. Material and Methods). In all our experiments, PMA-activated THP1 cells served as a control, since these cells efficiently respond to cytoplasmic DNA exposure. To introduce DNA into the cytoplasm of cells we transfected cells with dsDNA. Fluorescein (FAM)-labeled dsDNA allowed monitoring of DNA-uptake efficiencies by flow cytometry (**Figure 1A**). The control cell line THP1 was efficiently transfected (>90%). Exposure to labeled DNA in the absence of transfection reagent also resulted in labeling of THP1 cells (26%) (**Figure 1A**, left panel). Transfection reagent-independent increase in fluorescence likely reflected cellular uptake or binding of labeled dsDNA to the cells. We examined the EBV-positive human B-lymphoblastoid cell line (B-LCL) JY, a commonly used B cell line first. In JY cells similar

uptake efficiencies were reached as in THP1 cells allowing assessment of type I IFNs secretion by cytoplasmic DNA (**Figure 1A**, right panel). Transfection reagent-independent uptake or binding of labeled dsDNA was low (8%) in JY cells.

Next, we transfected unconjugated dsDNA to trigger the cytosolic DNA sensing pathway in JY and THP1 cells and assessed type I IFNs secretion. In contrast to THP1 cells, JY cells did not secrete type I IFNs upon cytoplasmic DNA exposure (**Figure 1B**). As a control for type I IFN secretion, we transfected the RNA analogue poly(I:C). JY and THP1 cells secreted type I IFNs upon cytoplasmic RNA stimulation (**Figure 1B**). Exposure to dsDNA in the absence of transfection reagent did not trigger a type I IFN response in THP1 cells (data not shown) consistent with the DNA being unable to reach the cytoplasm, in contrast to transfected dsDNA. JY cells appeared able to produce and secrete type I IFNs upon cytoplasmic RNA exposure, but not after DNA stimulation.

To examine transcription of the IFN-β gene in JY cells, we determined levels of IFN-β1 transcripts at 6h and 24h after dsDNA or poly(I:C) transfection by quantitative real-time PCR (**Figure 1C**). There was no increase in IFN-β1 transcripts detected after dsDNA stimulation in JY cells. In response to poly(I:C), a low induction of IFN-β1 mRNA levels was detected in JY after 6h, but a robust increase at 24h post-transfection (**Figure 1C**). Upon dsDNA and poly(I:C) stimulation, THP1 cells exhibited very high levels of IFN-β1 mRNA at 6h and at lower levels at 24h. Following the pattern of IFN-β1 induction, transcription of IFN-stimulated gene (ISG)54 was observed to both stimuli in THP1 cells, but only in response to poly(I:C) in JY cells (**Figure 1D**). To test whether dsDNA stimulation resulted in gene expression of pro-inflammatory cytokines in JY and THP1 cells, we examined TNFα transcripts at 6h (**Figure 1E**) and 24h (data not shown). There was no increase detected in JY cells, and also THP1 showed only a very moderate and transient induction of TNFα mRNA to both nucleic acid stimuli at 6h (**Figure 1E**), which was absent at 24h (data not shown).

These data show that the human B-LCL JY did not produce and secrete type I IFN or the pro-inflammatory cytokine TNFα upon cytoplasmic DNA exposure. Poly(I:C) induced production of IFN-β1mRNA and secretion of type I IFNs in JY cells, which in turn stimulated induction of ISG54. This demonstrates that whereas the type I IFN pathway is functional in this B cell line, it is not activated upon cytoplasmic dsDNA stimulation.

EBV-negative B cells do not secrete type I IFN upon cytoplasmic DNA stimulation

To assess if EBV-negative B cells, in contrast to EBV-positive JY cells, produce type I IFNs in response to cytosolic dsDNA exposure, two non-EBV immortalized B cell clones were transfected with dsDNA. The determined transfection efficiency of about 50% may be an underestimation (**Figure 2A**, one clone depicted) due to the GFP marker present in these cells. The B cell clones did not respond to dsDNA or poly(I:C) by type I IFN secretion (**Figure 2B**, one clone depicted) suggesting that the B cell clones were unresponsive to intracellular nucleic acid exposure.

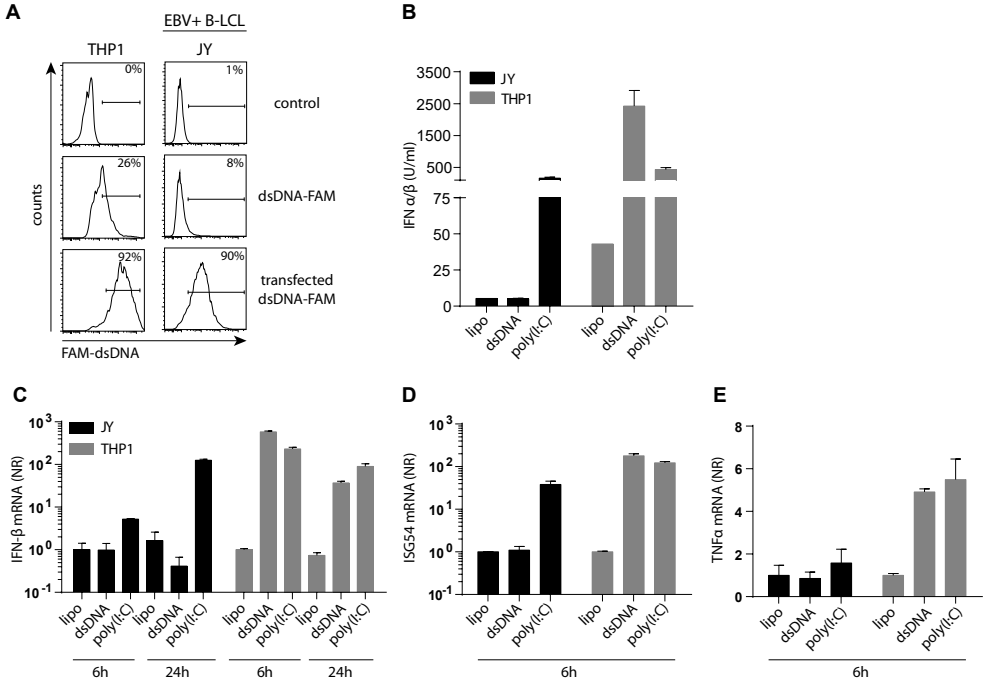
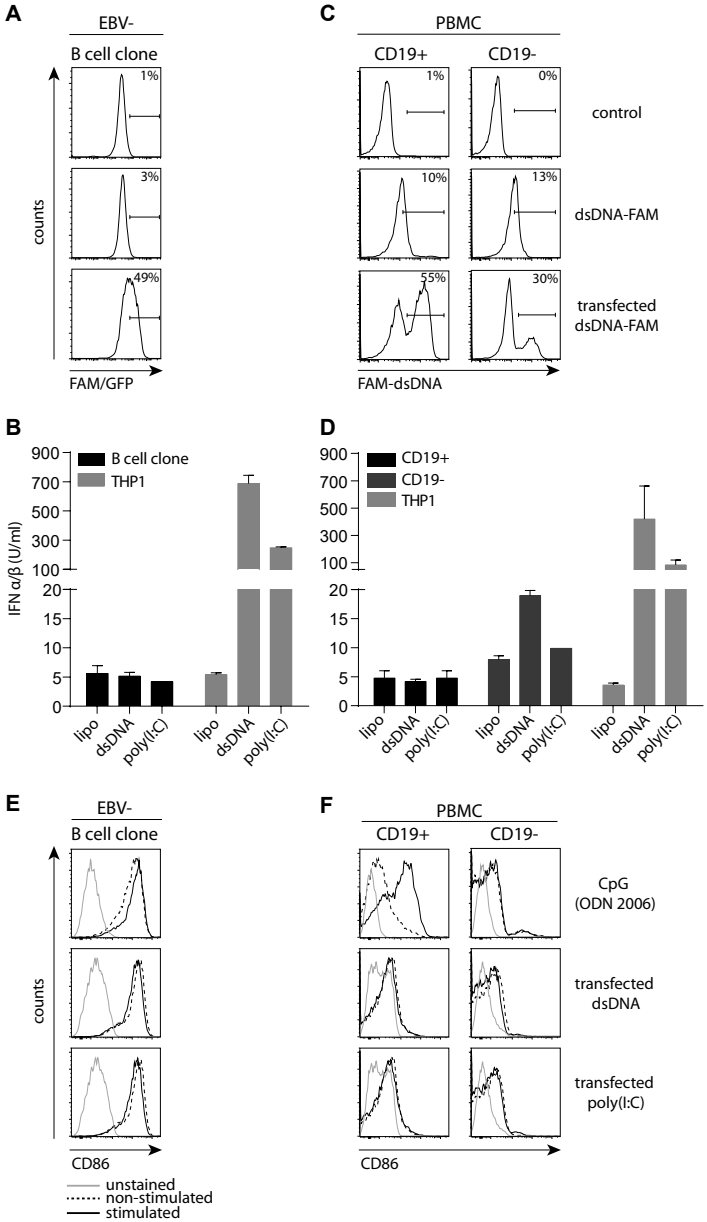


Figure 1 The human B-LCL JY fails to produce type I IFNs in response to dsDNA stimulation. A) Efficiencies of dsDNA transfection in the B-LCL JY and PMA-differentiated THP1 cells were determined by lipofectamine2000 transfection of FAM-labeled dsDNA. As controls, cells were either treated with the transfection reagent or FAM-labeled dsDNA alone. After overnight incubation, cells were analysed by flow cytometry. B) The B-LCL JY and PMA-differentiated THP1 cells were transfected with dsDNA or poly(I:C) using Lipofectamine2000. Cell-free supernatants were harvested 24h post-transfection and analysed for IFN-α/β in duplicates using a bioassay. One representative of four independent experiments is shown. Data are presented as mean ± SD. C-E) The B-LCL JY and PMA-differentiated THP1 cells were transfected as in B). RNA was isolated 6h or 24h post-transfection and analysed for mRNA levels of C) IFN-β, D) ISG54, E) TNFα by quantitative real-time PCR. Data are means (± SD) of normalized ratios relative to control sample, using β-actin as internal reference. One representative of two independent experiments is shown.

Figure 2 Human B cells do not secrete type I IFN upon exposure to cytoplasmic DNA. A) To determine transfection efficiencies, an immortalized B cell clone was transfected with FAM-labeled DNA or as a control treated with FAM-labeled dsDNA alone or left untreated. B) IFN-α/β secretion from tetanus- or influenza-specific immortalized B cell clones and PMA-differentiated THP1 cells transfected with dsDNA or poly(I:C) were determined by bioassay. Cell-free supernatants were harvested 24h post-transfection and analysed for IFN-α/β in duplicates. One representative of two independent experiments is shown for the influenza-specific clone. C) PBMC-derived $CD19^+$ or $CD19^-$ cells were treated as in A). Control cells were treated with lipofectamine2000 alone. D) PBMC-derived $CD19^+$, $CD19^-$ cells,



and PMA-differentiated THP1 cells were treated as in B). Supernatants were harvested 15 h post-transfection. One representative of two independent experiments is shown. E and F) An EBV-negative B cell clone (E) or CD19^{+} and CD19- PBMCs (F) were stimulated with CpG DNA or transfected with dsDNA or poly(I:C) (as in B) and cell surface levels of CD86 were determined by flow cytometry 24 h post-stimulation and compared to non-stimulated control cells, which were left untreated (CpG) or treated with lipofectamine alone (dsDNA and poly(I:C)). Data are depicted as histograms.

To test whether unresponsiveness to cytoplasmic DNA was a general feature of human B lymphocytes, we expanded our study to primary B cells. Transfection of dsDNA was successful in 55% of the primary $CD19^+$ B lymphocytes and in 30% of the $CD19^-$ PBMCs serving as control (**Figure 2C**). The $CD19^+$ B cells did not secrete type I IFN upon triggering with cytoplasmic DNA or RNA (**Figure 2D**). $CD19^-$ mononuclear cells showed only a slight response to dsDNA, but not to poly(I:C) stimulation (**Figure 2D**). The $CD19^-$ fraction contained about 50% $CD3^+$ cells, 9% $CD16^+$ cells, 4% $CD14^+$ cells and 35% of the cells were negative for these three surface markers as well as for CD19. Type I IFN secretion upon dsDNA stimulation was likely derived from monocytes, although the percentage of monocytes was low in the $CD19^-$ cell fraction. It is unclear why the cells did not secrete type I IFN after exposure to cytoplasmic poly(I:C). By speculation, dsDNA might induce higher levels of type I IFN upon cytoplasmic exposure in comparison to poly(I:C) in monocytes, as observed for THP1 cells. In these experiments the DNA-induced type I IFN levels were low, hence it could be that poly(I:C)-induced levels were not detectable.

We tested whether the primary B lymphocytes and the B cell clones were responsive to another nucleic acid stimulus indicating that the cells were viable at the time point of treatment. To this end, we stimulated the endosomal TLR9 present in B cells with the agonist CpG DNA (ODN 2006). CpG-mediated TLR9 stimulation of PBMC-derived B cells is known to result in upregulation of the activation marker CD86 [40]. The B cell clone tested expressed high cell surface levels CD86 prior to stimulation. A minute increase in the CD86 surface levels was observed upon stimulation with CpG DNA (MFI 15282 (unstimulated) vs 19915 (CpG)), but not with transfected dsDNA or poly(I:C) (**Figure 2E**). Stimulation with CpG DNA, but not transfection of the other two stimuli, resulted in upregulation of the activation marker CD86 in PBMC-derived $CD19^+$ cells (**Figure 2F**, left panel). The $CD19^-$ fraction showed no increase in surface levels of CD86 upon nucleic acid stimulation (**Figure 2F**, right panel). The primary $CD19^+$ and $CD19^-$ cells displayed sensitivity towards the cytotoxic transfection reagent, as a lower percentage of cells (around 25% for both $CD19^+$ and $CD19^-$) was present in the "live gate" in comparison to untreated or CpG-treated cells (about 80% for $CD19^+$ and 50% for $CD19^-$). This demonstrates that the primary B cells were responsive to endosomal DNA exposure suggesting that they were viable at the point of treatment.

In conclusion, primary human B lymphocytes and EBV-negative B cell clones did not secrete type I IFNs upon exposure to cytoplasmic DNA, while primary $CD19^-$ cells responded to transfected DNA.

Absence of STING protein in primary B cells and B cell lines, but not in B-LCL lines

To determine why human B cells did not respond to dsDNA stimulation, we examined the B cells for the following proteins of the cytoplasmic DNA sensing pathway: the DNA sensors cGAS and IFI16, the adaptor protein STING, and the downstream signalling molecules TBK1 and IRF3 (**Figure 3A**). PMA-differentiated THP1 cells having all these proteins served as a control (**Figure 3B-E**).

Although the transformed B-LCL JY did not respond to dsDNA stimulation, these cells produced cGAS, IFI16, STING, TBK1, and IRF3 (**Figure 3B**, lane 1). The B cell clones were grown in the presence of the CD40L-expressing murine feeder cell line. To exclude contamination of the B cell sample with feeder cells, this cell line was tested for cross-reactivity of the antibodies used. In feeder cells, we detected only a clear signal for TBK1, but not for the other proteins tested (lane 2). Therefore, we concluded that proteins detected in the B cell clones were not due to contamination with murine feeder cells. Both B cell clones had cGAS, IFI16, TBK1, and IRF3, but lacked the adaptor protein STING (lane 3 and 4). The EBV-negative cell line 2A8 (lane6) showed the same pattern as the B cell clones, whereas THP1 cells produced all proteins examined (lane 5).

To examine whether transformed B cells were representative for primary B cells, we probed for the proteins involved in the cytoplasmic DNA sensing pathway in primary PBMC-derived $CD19^+$ B cells (**Figure 3C**). In addition, we examined $CD19^-$ cells and total PBMCs. THP1 cells served as positive control. $CD19^+$ B cells from two donors possessed the DNA sensors cGAS and IFI16, and the signalling molecules TBK1 and IRF3, but lacked STING (lane 2 and 3). In the $CD19^-$ cells and total PBMCs cGAS, STING, TBK1, and IRF3 were detected, but IFI16 levels appeared to be low (lane 4, 5, and 6).

As an alternative source of primary human B cells, tonsillar $CD19^+$ B cells were examined for the proteins of the cytoplasmic DNA pathway and compared to tonsil-derived $CD19^-$ cells (**Figure 3D**). In $CD19^+$ cells, the DNA sensors, and the signalling intermediates TBK1 and IRF3 were detected, but STING protein was not (lane 2), while $CD19^-$ tonsillar cells had all proteins examined (lane 3). Thus, $CD19^+$ cells exhibited the same pattern as observed for blood-derived $CD19^+$ cells, respectively.

As JY cells were the only human B cells that appeared to have STING, we tested another B-LCL, MRJ, and compared it to JY and THP1 cells. MRJ displayed the same profile of proteins of the DNA sensing pathway as JY cells (**Figure 3E**, lane 3), and also these cells appeared to be unresponsive to dsDNA stimulation (data not shown).

In conclusion, human B lymphocytes appear to produce the cytoplasmic DNA sensors cGAS and IFI16 and the molecules TBK1 and IRF3. Whereas the EBV-positive B-LCLs JY and MRJ exhibited substantial levels of STING, this protein was undetectable in other human B cells tested. Absence of STING would provide an explanation why the EBV-negative blood-derived $CD19^+$ B cells and the B cell clones did not secrete type I IFN upon exposure to cytoplasmic dsDNA.

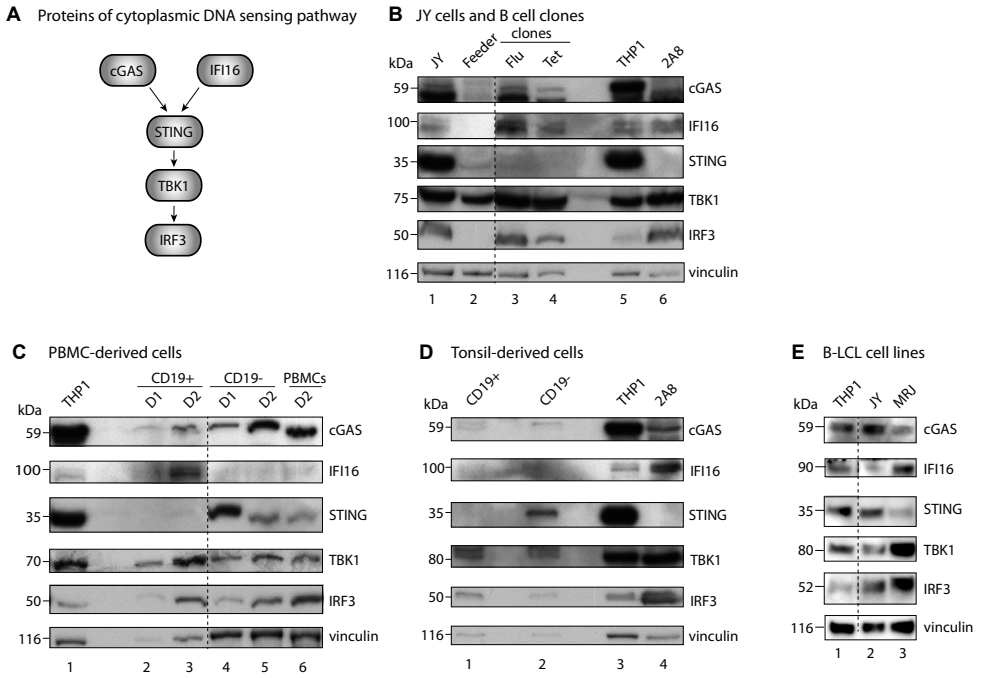


Figure 3 Most human B cells are deficient for STING protein. A) Schematic overview of proteins involved in the cytoplasmic DNA sensing pathway. B-E) Whole cell lysates were analysed for the presence of cGAS, IFI16, STING, TBK1, and IRF3 by Western blot analysis. Vinculin served as loading control. Dashed line indicate position of removed marker lanes. B) Tetanus (Tet)- and influenza (Flu)-specific immortalized B cell clones as well as the cell lines JY, 2A8, and PMA-differentiated THP1 cells were analysed. B cell clones were grown in the presence of murine feeder cells, but the feeder cells did not appear to account for proteins detected in B cell clone lysates. C) $CD19^+$ and $CD19^-$ PBMCs of two independent donors, total PBMCs of one donor and PMA-differentiated THP1 cells were analysed. D) Tonsillar $CD19^+$ and $CD19^-$ cells of one donor as well as the cell line 2A8, and PMA-differentiated THP1 cells were analysed for indicated proteins. E) The B-LCL cell lines JY and MRJ, and PMA-differentiated THP1 cells were analysed by Western blot analysis.

STING-expressing cells do not produce type I IFNs upon dsDNA or cGAMP stimulation

Despite presence of STING and the other proteins in the B-LCLs JY and MRJ, the cells were unresponsive to dsDNA stimulation. To get an indication whether the STING-TBK1-IRF3 pathway could be activated in B-LCLs, we bypassed the DNA sensing step by stimulating digitonin-permeabilized JY cells with the mammalian STING-activating molecule 2'3'-cGAMP. There was no type I IFN secreted from cGAMP-stimulated JY cells at 6h post-stimulation, while THP1 cells produced type I IFNs in the presence, but not in the absence of cGAMP (**Figure 4A**). This indicates that the STING pathway was not activated by cGAMP in JY cells. Sequence analysis revealed that JY cells had the same variant of STING as THP1 cells (data not shown), suggesting that JY cells did not carry a mutated form of STING.

To exclude potential inhibitory effects of EBV proteins, we turned to an EBV-negative human B cell line. As the EBV-negative B cell line 2A8 had the DNA sensors and the signalling component TBK1 and IRF3, but lacked STING, we assessed if reconstitution of STING induces a type I IFN response upon dsDNA stimulation. We stably introduced C-terminally HA-tagged murine STING in the EBV-negative cell line 2A8 that endogenously produced the sensors cGAS and IFI16, and the signalling molecules TBK1 and IRF3 (**Figure 3B**, lane 6 and **Figure 3D**, lane 4). Murine STING is able to reconstitute human cells lacking STING to respond to cyclic dinucleotides [41]. Two 2A8 cell lines were generated using different amounts of STING-coding lentivirus. The lentiviral vector also encoded GFP, which we used as a marker to FACS-sort transduced cells. Pure populations of GFP^+ 2A8-mSTING cells were used to examine presence of STING-HA by Western blot analysis (**Figure 4B**). The cell lines JY and THP1 displayed high levels of STING detected by a STING-specific antibody, whereas in the untransduced 2A8 cell line no signal was detected (**Figure 4B**). The 2A8-mSTING cells showed a weak signal using the STING-specific antibody, which binds human and murine STING. The 2A8 cell line transduced with a higher dose of lentivirus showed increased levels of STING in comparison to the other transduced cell line. Using an HA-reactive antibody allowed clear detection of HA-tagged STING in 2A8-mSTING lines (**Figure 4B**, lane 2 and 3). 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.

In a next step, we tested the transfection efficiency of 2A8 and 2A8-mSTING cells using the FAM-labeled dsDNA. Upon transfection, 40% of the 2A8 cells were labeled indicating successful uptake of the DNA (**Figure 4C**), while the GFP^+ 2A8-mSTING cells showed a transfection efficiency of around 20%, 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 type I IFNs, in contrast to control THP1 cells (**Figure 4D**). To assess whether 2A8 cells were responsive to another nucleic acid stimulus indicating that the cells were viable at the time point of treatment, we stimulated the 2A8-mSTING cells with CpG DNA. Prior to stimulation, the cells expressed high surface levels of CD86, but the levels slightly increased upon stimulation with CpG DNA suggesting that the cells were viable (**Figure 4E**).

To examine whether the STING-TBK1-IRF3 pathway could be activated in the EBV-negative 2A8-mSTING cell line, we stimulated these cells with cGAMP. Irrespective of STING expression, none of the 2A8 cell lines did secrete type I IFNs upon cGAMP stimulation, while THP1 cells produced type I IFNs (**Figure 4F**). Although direct evidence is missing at this point that mSTING introduced in 2A8 cells was functional and present at adequate levels, these data indicate that exogenous or endogenous STING expression was not sufficient for EBV-positive or -negative B cell lines to sense the second messenger molecule cGAMP. This may suggest that human B cells are unresponsive to dsDNA or cGAMP stimulation, irrespective of the presence or absence of EBV proteins.

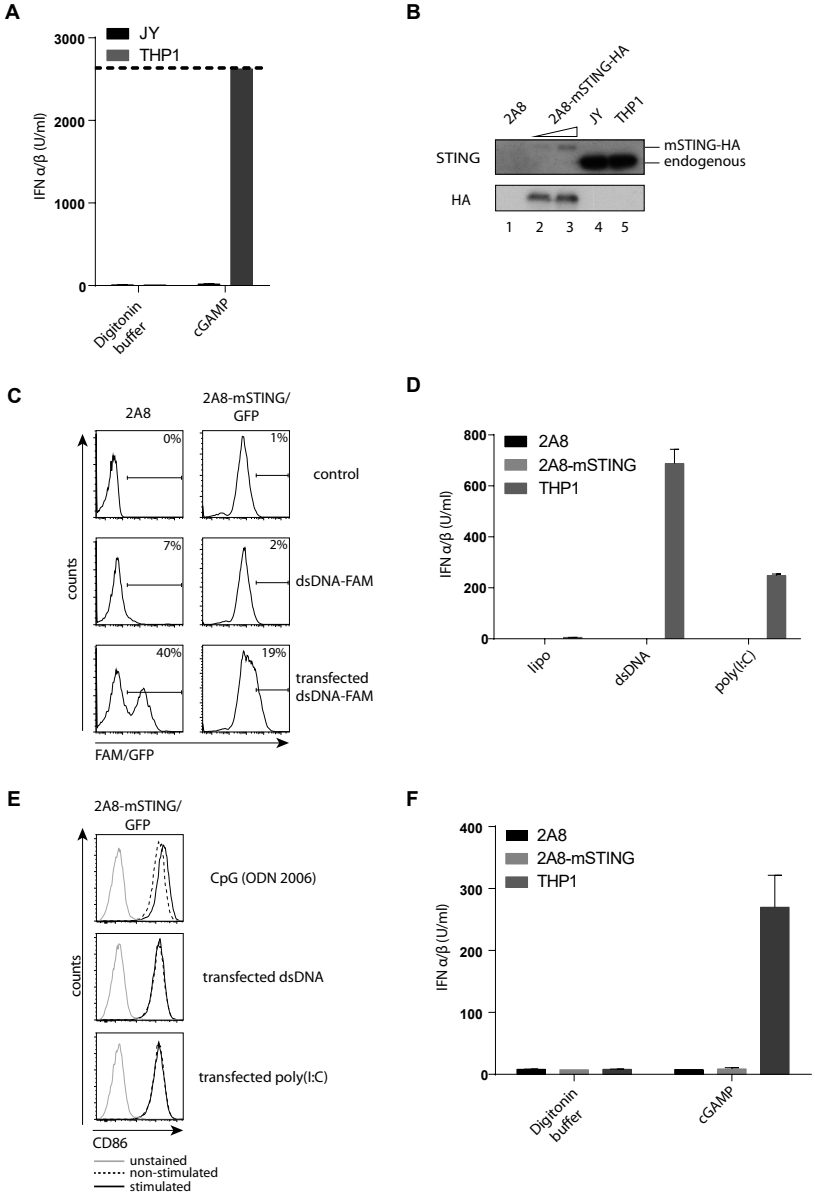


Figure 4 STING-expressing B cells fail to respond to cytoplasmic nucleic acids and cGAMP. A) JY and PMA-differentiated THP1 cells were stimulated with cGAMP using a buffer containing digitonin. Supernatants were harvested at 6 h post-stimulation. Data are mean ± SD of one representative out of three independent experiments. B) The cytoplasmic fraction of 2A8, mSTING-HA/GFP-transduced 2A8 (2A8-mSTING), JY, and PMA-differentiated THP1 cells was analysed for STING and HA-tag expression by Western blot analysis. C) Transfection efficiency of dsDNA into 2A8 and 2A8-mSTING cells was determined by lipofectamine2000 transfection of FAM-labeled dsDNA. As a control, cells were

left untreated or treated with dsDNA-FAM alone. Cells were analysed by flow cytometry after overnight incubation. D) 2A8, 2A8-mSTING, and PMA-differentiated THP1 cells were transfected with dsDNA or poly(I:C) using Lipofectamine2000. Cell-free supernatants were harvested at 24 h post-transfection and analysed for bioactive IFN-α/β in duplicates. E) 2A8-mSTING cells were stimulated with CpG DNA or transfected with dsDNA or poly(I:C) (as in D) and cell surface levels of CD86 were determined by flow cytometry 24 h post-stimulation and compared to non-stimulated control cells, which were left untreated (CpG) or treated with lipofectamine alone (dsDNA and poly(I:C)). Data are depicted as histograms. F) 2A8, 2A8-mSTING, and PMA-differentiated THP1 cells were stimulated with cGAMP using a buffer containing digitonin. Cell-free supernatants were harvested at 3,5 h post-stimulation and IFN-β bioactivity was determined by BioAssay.

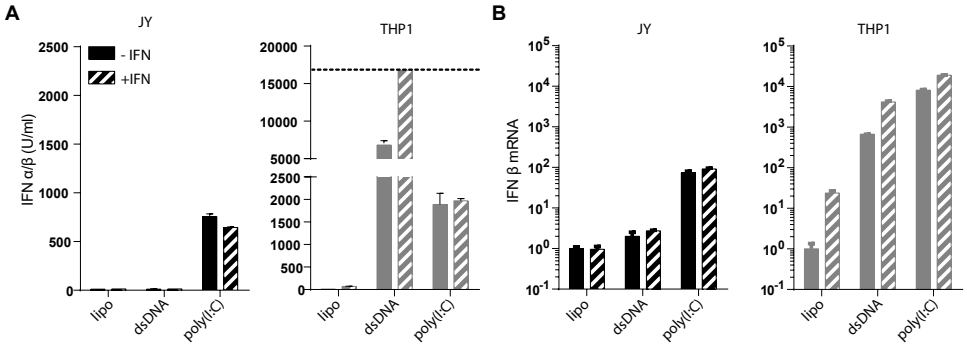


Figure 5 IFN-β priming does not rescue responsiveness to cytoplasmic DNA exposure of the B-LCL JY. A and B) JY (black bars) and PMA-activated THP1 cells (grey bars) were pre-stimulated with 1000 U/ml IFN-β for 24 h. Cells were then transfected with dsDNA or poly(I:C) using Lipofectamine2000. A) Cell-free supernatants were harvested at 24 h post-transfection and analysed for IFN-α/β in duplicates. Data are mean ± SD of one representative out of two independent experiments. A dashed line indicates maximum cut-off of assay. B) Cells were harvested, RNA isolated, and mRNA levels of IFN-β determined at 6 h post-transfection by quantitative real time-PCR. Data are means (± SD) of normalized ratios relative to control sample, using β-actin as internal reference. Representative data of three independent experiments are shown.

Type I IFN priming prior to dsDNA stimulation does not give rise to type I IFNs secretion in B-LCLs

Lastly, we examined whether the B-LCL JY respond to dsDNA when in a primed state. Type I IFNs induce expression of many ISGs via the JAK-STAT pathway, which acts downstream of the type I IFN receptor [1]. Upregulation of ISGs including PRRs such as RIG-I, MDA5, TLR3, cGAS, IFI16 and other signalling proteins limits viral replication [42,43]. Hence, type I IFN primes cells to detect and/or respond to PAMPs more vigorously. For example, human type I IFN-primed macrophages show enhanced responses to TLR stimuli in comparison to unprimed cells [44]. Therefore, we hypothesized that type I IFN-stimulated B cells might become responsive to dsDNA.

To this end, we pre-stimulated JY and THP1 cells with IFN-β for 24h prior to transfection with dsDNA or poly(I:C). Pre-stimulation of JY cells with IFN-β did not give rise to a dsDNA-

induced type I IFN response (**Figure 5A**, left panel). In contrast, enhanced IFN I levels were detected in IFN-β-primed THP1 cells upon dsDNA stimulation in comparison to unprimed cells (**Figure 5A**, right panel). Secretion of type I IFN following poly(I:C) stimulation was not altered by pre-stimulation with IFN-β in either of the cell lines (**Figure 5B**). In THP1 cells, IFN-β transcription appeared to be slightly increased in response to nucleic acids upon IFN pre-stimulation, but type I IFN treatment alone also elevated transcription (**Figure 5B**).

These data suggest that type I IFN-priming of the B-LCL JY did not enhance responsiveness to dsDNA. Overall, our data indicate that human B cells are unable to evoke type I IFN production in response to foreign dsDNA, and that this this is correlated with the absence of the adaptor protein STING in most of these cells. Curiously, despite the presence of STING, B cells appear to have a dysfunctional cytosolic DNA sensing pathway.

Discussion

In this study, we report that human B cells fail to secrete type I IFNs upon exposure to cytosolic dsDNA, although they produce the DNA sensors cGAS and IFI16. The EBV-negative B cell lines and primary B cells tested lacks the central adaptor protein STING that is required for signal transduction downstream of the cytosolic DNA sensors. B cell lines expressing STING did not secrete type I IFNs upon dsDNA or cGAMP stimulation.

Cytosolic DNA sensing plays a crucial role in host immune defense against invading DNA viruses as demonstrated by lethality in STING-knockout mice infected with HSV-1 [30]. The STING protein was originally identified in murine B cell lines as a molecule involved in apoptosis [45], but shortly thereafter, it was recognized as the central signalling hub for DNA sensing [11,12,13]. Murine B cell lines representing pre-B cells, immature, memory and plasma B cells were used to examine presence of STING [45]. Except for the plasma B cell line, STING was present in the different murine B cell lines, with the highest levels found in mature B cells. We examined human B lymphocytes, but only detected high levels of STING in B-LCLs, but not in other B cells. Thus, STING is present in murine, but not in most human B cells. This is interesting as murine and human STING exhibit a different activation profile in response to cyclic dinucleotides. Murine STING is potently triggered by bacterial 3'5'- and mammalian 2'3'-linked cyclic dinucleotides, while human STING is mainly activated by the mammalian 2'3'-cGAMP [41]. Hence, murine STING responds to a broader spectrum of ligands, which most likely dictates the role of this protein in innate immune activation. Therefore, one could speculate that a distinct expression profile in (immune) cells is evolutionarily favored due to differential sensitivity towards cyclic dinucleotide ligands.

Signaling via STING plays a central role in type I IFN induction following the recognition of the DNA virus, such as HSV-1 or KSHV [30,31]. Therefore, the absence of STING from

human B cells may render these cells more permissive for infection with the herpesviruses KSHV or EBV. Yet, human B cells are not devoid of functional DNA sensing. We have reported earlier that EBV genome activates the DNA sensor TLR9 in endosomes [26]. Moreover, IFI16 senses nuclear EBV and KSHV genomes resulting in subsequent formation of an IFI16 inflammasome and IL-1β maturation in B cells [28,29]. Thus, human B cells are likely to sense invading DNA viruses and initiate innate immune responses, yet they appear to fail to induce a type I IFN response upon cytoplasmic DNA exposure. It seems that STING may be expressed in human B cells under certain conditions suggested by high levels of the adaptor protein in the EBV-transformed B-LCLs. Since nine EBV latency proteins are present in B-LCLs, it remains to be determined whether they contribute to STING expression and/or type I IFN production.

Interestingly, B-LCLs or the 2A8-mSTING cell line were unable to mount a type I IFN response upon stimulation with dsDNA, despite presence of the proteins comprising the cytosolic DNA sensing pathway including STING. The B cell line JY has STING levels comparable to those observed in THP1 cells. JY cells carried the same STING variant as THP1 cells (data not shown), thus mutations interfering with its function could be excluded. Introduction of murine STING into the EBV-negative cell line 2A8 did not render these cells responsive to dsDNA or cGAMP stimulation, although murine STING can reconstitute human cells lacking STING [41]. Although we formally lack evidence that the construct coding for murine STING could functionally reconstitute STING in STING-deficient cells, our data imply that the cytoplasmic DNA sensing pathway is not functional in human B lymphocytes. A similar phenomenon has been reported for activated human T lymphocytes [38]. The T cells appeared to sense cytoplasmic DNA supporting formation of the STING signalosome including TBK1 recruitment, but still these cells failed to mount a type I IFN response [38]. In our study, we triggered STING-expressing B cells with the second messenger molecule cGAMP, which did not result in activation of the STING pathway and secretion of type I IFNs. This suggests that signal transduction was abrogated downstream of the STING protein, similar to the observations for T cells. We demonstrate that the B-LCLs 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 signalling involving the proteins TBK1 and IRF3 was supported, at least, in the B-LCLs. This implies, therefore, that STING failed to activate TBK1 and IRF3 in the B-LCLs.

It is unclear why poly(I:C) stimulation of the other B cells examined failed to induce production of type I IFNs. The signalling intermediates TBK1 and IRF3 are employed by the DNA and RNA sensing pathways. Western blot analysis (**Figure 3**) showed that primary B cells and the B cell clones had similar or even higher levels of the signalling molecules TBK1 and IRF3 in comparison to JY or THP1 cells. The latter two were responsive to poly(I:C). This suggests that all B lymphocytes examined were equipped with the molecules acting downstream of the adaptor proteins of the cytoplasmic nucleic acid sensors, but yet did not

respond. It remains to be determined whether human B cells have the cytoplasmic RNA sensors RIG-I and MDA5, as well as the adaptor protein MAVS.

Although B cells appear to have a dysfunctional cytosolic DNA sensing pathway, 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 [46,47]. cGAMP was found in virions of HIV, murine cytomegalovirus, and modified vaccinia virus Ankara suggesting that 1) these viruses trigger the DNA sensing pathway and 2) members of several viral families including herpesviruses, facilitate packaging of this cyclic dinucleotide into the virion. This viral particle-dependent cGAMP transfer triggered a type I IFN response in several cell types including the cell line THP1 and DCs derived from primary human monocytes [46,47]. Therefore, it would be interesting to examine whether virions of e.g. EBV or KSHV propagated in B lymphocytes contain cGAMP and whether this could stimulate other cell types infected by these viruses. Hence, triggering of cGAS in B cells could result in cGAMP production and packaging, and subsequent transfer to other cells by released virions. This may allow stimulation of STING prior to sensing of viral DNA released from the capsid.

Identification of several KSHV proteins, including the KSHV-specific K9/vIRF1 protein, interfering with the cGAS-STING pathway [31] argues that this pathway is involved in sensing of KSHV. The KSHV protein LANA inhibits cGAS and also the tegument protein ORF52 prevents cGAS-mediated cGAMP production *in vitro* and in infected cells [48,49]. The EBV homologue of ORF52, BLRF2, also prevented cGAMP formation in an *in vitro* assay, but whether the EBV protein EBNA1, the functional homologue of LANA, interferes with cGAS remains to be determined. Identification of viral proteins inhibiting cGAS function indicates that cGAS plays a role in detection of these viruses. As the cytoplasmic DNA sensing pathway appears to be dysfunctional in human B cells, it seems that viral inhibition of cGAS is not necessary in B cells, but required for infection of other cell types. For instance, KSHV infection of endothelial cells triggers activation of the cytosolic DNA sensing pathway [31].

Type I IFN stimulation of murine B cells was shown to augment B cell-receptor-dependent responses [50]. In human B cells, type I IFN enhances CpG-stimulated IgM production [51]. Hence, exposure of B cells to type I IFN appears to promote B cell functions such as antibody production that contribute to pathology of auto-immune diseases. 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 a type I IFN 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 signalling. In an attempt to unleash the cGAS-STING pathway in STING-expressing B cells, we pre-stimulated the cells with IFN-β prior to introduction of dsDNA. However, this did not result in induction or secretion of type I IFN in the B cells. Therefore,

further studies are needed to identify the underlying mechanism of frustrated type I IFN induction observed in T and B lymphocytes upon introduction of cytoplasmic DNA. Possibly, corroboration of such a mechanism may contribute to the development of autoimmunity or other inflammatory conditions upon DNA sensing of self-DNA.

Material and Methods

Isolation of primary human B cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation from buffy coats of healthy blood donors provided by Sanquin blood bank Amsterdam. Isolated PBMCs contained about 55% $CD3^+$, 15% $CD19^+$, 12% $CD16^+$, 7% $CD14^+$ cells and 11% of the isolated cells were negative for all these markers. The antibodies used for this analysis were the following: AlexaFluor700-conjugated anti-CD3 (BD Pharmingen, UCHT1), PE-conjugated anti-CD19 (BD Pharmingen, HIB19), FITC-conjugated anti-CD16 (BD Pharmingen, 3G8), and PE-Cy7-conjugated anti-CD14 (BD Pharmingen, M5E2).

Anonymized tonsil material was obtained from tonsillectomies. Tonsils were cut in small pieces and passed through a cell strainer. B cells from PBMCs and dispersed tonsils were positively isolated using anti-human CD19-coupled magnetic beads according to manufacturer's protocol (Invitrogen). The $CD19^-$ fraction left after B cell isolation was used as a control. Purity of isolated cells was determined by flow cytometry. For this, cells were stained using PE-conjugated anti-CD19 (BD Pharmingen, HIB19) and AlexaFluor700-conjugated anti-CD20 (Biolegends, 2H7) antibodies. In each experiment, purity of $CD19^+$ B cells was 95-99%, while $CD19^-$ cells contained less than 0,4% $CD19^+$ cells. Freshly isolated cells were used for experiments. Cells were resuspended in IMDM supplemented with 10% heat-inactivated FCS, 2 mM glutamax, 100 U/ml penicillin and 100 mg/ml streptomycin.

Cell lines

In this study, several transformed human B cell lines were included permitting long-term culture. The B cell lines were transformed either by EBV *in vitro* (i.e. B-lymphoblastoid cell lines (B-LCLs)) or *in vivo* (i.e. Burkitt's lymphoma-derived lines), or, alternatively, by B cell lymphoma protein 6 (BcL-6) and –xL transformation [34]. EBV-negative B cells originally isolated from BL-derived cell lines [35], were also included. An overview of all B lymphocytes used in this study is given **Table 1**.

The EBV-negative, antigen-specific B cell clones immortalized by introduction of Bcl-6 and Bcl-xL were cultured on murine CD40L-expressing feeder cells in the presence of recombinant mouse IL-21 as described elsewhere [34]. Prior to use in experiments, cells were carefully removed from the murine feeder cells and taken up in IMDM containing 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

The EBV-positive B-LCLs JY and MRJ, the BL-derived EBV-negative cell line 2A8, and

the monocytic 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 48h.

Table 1 B cells used in this study

Cells	Source	Immortalization	EBV status	References
Primary CD19⁺ B cells	Tonsil	no	unknown	
Primary CD19⁺ B cells	PBMC	no	unknown	
B cell clone (tet or flu)	PBMC	Yes, Bcl-6, Bcl-xL	-	[34]
B-LCL (JY or MRJ)	PBMC	Yes, EBV *(in vitro)*	+	[36,37]
2A8	BL	Yes, EBV *(in vivo)*	-	[35]

Replication-deficient lentivirus and transduction

Third generation SIN lentiviruses were generated in 293T cells transfected with the lentivirus 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 cells were exposed to with lentivirus-containing supernatant to transfer the genes encoding murine STING and the GFP marker. To enhance transduction efficiency, cells were spin inoculated (1000x*g*, 33° C, 2h) in the presence of 4 ug/ml polybrene. GFP⁺ 2A8 cells were FACS-sorted. A pure GFP⁺ population of cells was propagated and used for experiments.

Reagents

For stimulation of the cytoplasmic DNA pathway, we used dsDNA (HSV60mer is derived from the HSV-1 genome (nucleotides 144,107-144,166 [38]), or 2',3'-cGAMP (Sigma-Aldrich). Unconjugated or fluorescein (FAM)-labeled DNA oligos were mixed and 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 used. For stimulation of TLR9, 10 µg/ml CpG DNA (ODN 2006, Invivogen) was added to culture medium and incubated with cells for 24h. The cytoplasmic RNA pathway was triggered by polyinosinic-polycytidylic acid (poly(I:C), Invivogen). Where indicated, cells were treated with 1000 U/ml IFN-β (pbl assay science) added to culture medium 24h prior to transfection.

Transfections and transfection efficiencies

Cells were transfected with dsDNA or poly(I:C) using Lipofectamine2000 (Invitrogen). DNA or poly(I:C) were mixed at a ratio of 1 µg stimulant per 1ul lipofectamine2000. Complexes were allowed to form for 20 min before adding it to the cells. In general, $0.3x10^6$ 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 indicated time points, but typically 6h or 24h post-transfection. Transfection efficiencies were determined by exposing cells to cells with FAM-labeled dsDNA/lipofectamine, and assessing fluorescence by flow cytometry after overnight incubation.

Flow cytometry

To determine cell surface levels of indicated markers, cells were stained with antibodies of indicated specificity. Cell were washed in PBS supplemented with 0,5% BSA, and 0,02% sodium azide. Cells were antibody-stained with the following antibodies: mouse α-HLA-DR-PE (L243, BD Biosciences), and anti-CD86-PE (BD Pharmingen). Stained cells were washed, fixed, and subjected to a LSR II flow cytometer (BD Biosciences). Flow cytometry data were analysed using FlowJo (Treestar).

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.

Type I IFN Bioassay

IFN-α/β levels in cell-free culture supernatants were determined using the HEK Blue IFN-α/β reporter cell line (Invivogen), which in response to type I IFN stimulation produces the secreted embryonic alkaline phophatase (SEAP). Supernatants were plated with 5x104 reporter cells in 100 µl in a 96 well plate. Cells were incubated for Colorimetric analyses were performed at 620-655 nm using a plate reader.

Quantitative real-time PCR

RNA was isolated from stimulated cells at indicated time points by using the High Pure RNA Isolation Kit (Roche) according to manufacturer's protocol. Levels of mRNA were assessed in duplicates by real-time PCR using the TaqMan RNA-to-CT 1-step kit (Applied Biosystems). The following TaqMan probes were used: hIFN-β (Hs01077958_s1), hISG54 (IFIT2) (Hs01922738_s1), hTNF-α (Hs01113624_g1), and hβ-actin (Hs99999903_m1). Duplicates with CT values > 1 apart were excluded from analysis. Expression levels were

normalized to β-actin using the 2-ΔΔCT method, and data are presented as fold induction (±SD) over lipofectamine2000-treated control samples.

Western blot analysis

Total cell lysates were generated using RIPA lysis buffer (ThermoFisher) supplemented with 0,2% SDS, protease inhibitor mix (Roche), 50 U/ml Benzonase (Sigma-Aldrich), and 50 mM sodium fluoride (phosphatase inhibitor). NP40 lysis mix (0.5% NP40 (Igepal-CA630), 50 mM Tris HCl (pH 7.5), 150 mM NaCl) containing protease inhibitor mix (Roche) was used to generate post-nuclear lysates as described elsewhere [39]. Lysates were denatured using Laemmli sample buffer containing 20 mM DTT. Proteins were separate on Bio-Rad premade Criterion Tris-HCl gels (4-20% gradient) or on handcast gels (10%) and transferred on 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-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 an ImageQuant LAS 4000 mini Luminescent Image Analyzer (GE Healthcare Life Sciences) or films.

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