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Monocytes contribute to DNA sensing through the TBK1 signaling pathway in type 1 diabetes patients

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ABSTRACT

Background: The aberrant recognition of self-nucleic acids by the innate immune system contributes to the pathology of several autoimmune diseases. Although microbial DNA and, in certain instances, self-DNA that is released from damaged cells are primarily recognized by Toll-like receptor 9 (TLR9), recent evidence suggests that other cytosolic sequence-nonspecific DNA sensors contribute to DNA recognition. In this study, we focused on the sensing of microbial and host DNA in type 1 diabetes (T1D) patients.

Methods: Peripheral blood mononuclear cells (PBMCs) and monocytes from pediatric patients with T1D and from healthy donors were stimulated with microbial DNA (CpG) or with self-DNA (DNA contained within neutrophil extracellular traps, NETs). The production of cytokines was measured by flow cytometry and multiplex bead assays. The internalization of microbial DNA and its colocalization with STING was detected by image cytometry. Furthermore, the involvement of the TBK1 kinase was investigated by detecting its phosphorylation with phospho-flow cytometry or by using a TBK1 inhibition assay.

Results: We observed a prominent proinflammatory response in T1D PBMCs, especially pDCs and monocytes, to microbial DNA in comparison to that in controls. We further confirmed that monocytes could bind and internalize DNA and respond by releasing proinflammatory cytokines in a more pronounced manner in T1D patients than those in controls. Surprisingly, this cytokine production was not affected by TLR9 blockade, suggesting the involvement of intracellular receptors in DNA recognition. We further identified TBK1 and STING as two crucial molecules in the DNA-sensing pathway that were involved in CpG-DNA sensing by T1D cells. A similar DNA-sensing pathway that was dependent on intracellular DNA sensors and the STING-TBK1 interaction was employed in response to NETs, which were used to model self-DNA.

Conclusions: Here, we show that there were significant differences in DNA sensing in T1D patients compared to that in controls. We demonstrate that monocytes from T1D patients are able to sense microbial- and self-DNA, leading to proinflammatory cytokine secretion through the adaptor protein STING and the TBK1 kinase.

1. Introduction

Nucleic acids, which arise either as conserved patterns originating from microbial infection (PAMPs – pathogen associated molecular patterns) or as molecules derived from host cells that are released during cell damage or death (DAMPs - damage-associated molecular patterns), are recognized by innate immune cells via a set of pattern recognition receptors (PRRs). Microbial DNA is recognized mostly by Toll-like receptor 9 (TLR9), based on its unmethylated, CpG-rich contents [1]. TLR9 and other nucleic acid recognizing TLRs are located in endosomes, allowing for the discrimination between self and non-self nucleic acids. The activation of these receptors, which are expressed most prominently in plasmacytoid dendritic cells (pDCs), leads to the production of large amounts of type I IFNs (IFNα, β) [2]. While TLR9 is important for DNA sensing, TLR9 is not the only DNA recognizing sensor. A set of cytosolic sequence-nonspecific DNA sensors can

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recognize nucleic acids that reach the cytosolic compartment [3,4]. The group of intracellular receptors that is important for cytosolic ssDNA or dsDNA recognition consists of several members, including cyclic GMP–AMP synthase (cGAS) [5], gamma-interferon-inducible protein (IFI16) [6,7], and aspartate-glutamate-any amino acid-aspartate/histidine (DExD/H)-box helicase 36 (DHX36) [8,9]. These receptors bind DNA regardless of its sequence and signal through stimulator of interferon genes (STING or TMEM173), a central molecule in DNA sensing, or through the TLR adaptor molecule MyD88. Upon cytosolic DNA recognition, STING is activated and translocates from the endoplasmic reticulum (ER) membrane to the perinuclear vesicles of the Golgi apparatus. Subsequently, STING recruits and activates the downstream TANK-binding kinase 1 (TBK1). STING assembles with TBK1 and then initiates downstream signaling cascades that lead to the activation of the transcription factors interferon regulatory factor 3 and 7 (IRF 3 and 7) and NF-κB, which causes their translocation into the nucleus. In turn, IRFs and NF-κB induce the expression of genes encoding type I interferons and proinflammatory cytokines, respectively [5,10].

Self-nucleic acids that are released from damaged or dying cells in a complex with antibodies or antimicrobial peptides are aberrantly recognized by the innate immune system in a TLR9-dependent manner, thus contributing to the pathology of several autoimmune diseases with the excessive production of proinflammatory cytokines and interferons [11,12]. However, an increasing number of studies have suggested that the innate sensing of self-DNA is not limited only to endosomes and TLR9 but also to cytosolic pathways such as the above-described cGAS-STING and TBK1 pathways. These pathways are involved in the excessive immune responses in autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjögren syndrome [8]. A progressive interferon signature is increasingly recognized as a hallmark of these diseases, which has prompted studies using the inhibition of TBK1 and STING signaling as a potential novel treatment option in addition to the previously attempted and inefficient inhibition of TLR9 [13–15].

While the interferon-mediated inflammatory response to self-DNA recognition is well defined in the above-mentioned autoimmune diseases, there is limited knowledge about the role of self-DNA recognition and DNA intracellular receptors in type 1 diabetes (T1D). Diana et al. showed that self-DNA that is released upon physiological pancreatic beta cell death activates pDCs through TLR9 to induce type I IFN production, which consequently leads to the initiation of an autoimmune T cell response [16]. Interestingly, TLR9−/− NOD mice were protected against the development of T1D [17]. In addition, Xu et al. showed that in their T1D animal models intracellular TBK1/IKKε kinase inhibition enhances β-cell regeneration [18].

In this study, we show an aberrant immune response to microbial and self-DNA by T1D PBMCs and an unusual contribution of distinct cell populations to this recognition. The stimulation of T1D patient cells with microbial DNA triggered not only the expected IFNα production but also a robust release of proinflammatory cytokines. In addition to pDCs, monocytes were also found to bind, internalize and sense DNA via TLR9-dependent and TLR9-independent pathways, including the involvement of cytosolic receptors. DNA sensing in monocytes triggered the production of IFNα, IL-1β, TNFα and IL-6. Furthermore, we show, for the first time to the best of our knowledge, that TBK1 and STING, two molecules of the DNA-sensing pathway, are required for CpG-DNA sensing in T1D monocytes. Finally, we demonstrate that both TBK1 and STING also participate in the response of T1D monocytes to self-DNA-containing neutrophil extracellular traps (NETs) that were isolated from activated neutrophils.

2. Materials and methods

2.1. Patients and human blood samples

A cohort of 75 pediatric patients with T1D and 89 healthy donors was included in this study. The median age of the patients with T1D was 17.9 ± 2.6 years (range: 9.5–18.6 years), and the median age of the healthy donors was 25.8 ± 4.8 years (range: 10.2–32.1 years). All patients with T1D had been treated with insulin since disease onset and were T1D-specific autoantibody positive at onset. The median T1D duration was 7.8 ± 3.3 years (range: 1.1–15.2 years). The median of their last glycated hemoglobin (HbA_{1c}) was 61.8 ± 11.9 mmol/mol (7.8 ± 3.2%) (range: 39-81 mmol/mol, 5.7–9.6%). At the time of blood sampling, the patients were metabolically stable, and their actual glycaemia were between 4 and 12 mmol/L. None of the patients had signs of active infection, neoplasia or other comorbidities except for well-controlled celiac disease or autoimmune thyroiditis (11% of recruited patients with T1D). The healthy donors had a negative personal history of autoimmune diseases. Written informed consent was obtained from all the patients or the patients' parents/guardians in accordance with the Declaration of Helsinki, and the study was approved by the Ethics Committee of University Hospital Motol.

2.2. Isolation of PBMCs and monocytes

Peripheral blood mononuclear cells (PBMCs) from the patients and healthy controls were isolated using a Ficoll-Paque gradient (GE Healthcare, Chicago, Illinois, USA). Monocytes were sorted using an immunomagnetic EasySep human monocyte isolation negative selection kit according to the manufacturer's instructions (Stemcell technologies, Vancouver, Canada). The purity of monocytes was confirmed by FACS staining and was consistently > 90%. Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics (all from Thermo Fisher Scientific, Waltham, MA USA) as described previously [19].

Immunophenotyping of monocytes, DCs and the verification of monocyte purity was performed using Lin-FITC (CD3, CD19, CD20, and CD56), CD16-Alexa Fluor 700, CD11c-APC, CD14-PE-DyLight594 (Exbio, Prague, Czech Republic), HLA-DR-PerCP (BD Biosciences, San Jose, CA, USA), CD1c-BV510, CD141-BV421, and CD123-PE-Cy7 (Biolegend, San Diego, USA). Samples were analyzed on an Aria II (BD Biosciences, New Jersey, USA) and using FlowJo software (TreeStar, Ashland, OR, USA).

2.3. Monocytes and pDC phenotype

PBMCs were incubated overnight with or without 5 μg/ml CpG2216, synthetic oligonucleotide containing unmethylated CpG dinucleotides, which represent microbial DNA (CpG DNA) (InvivoGen, San Diego, CA, USA) , and the cell phenotype was determined using antibodies against lineage markers: (CD3, CD19, CD20, CD16, CD56) - FITC and CD14- PEDy594, CD11c-PB (both from Exbio), HLA-DR-PC7 and CD123-PerCP-Cy5.5 (both from BD Biosciences), CD83-PE (Beckman Coulter, Brea, USA); and CD86-A647 and CD40-A700 (both from BioLegend). Samples were acquired on a BD FACSAriaII and analyzed using FlowJo software.

2.4. Cytokine detection

For intracellular cytokine detection, PBMCs were cultured in a 24-well microtiter plate at a concentration of 1 × 10e6/well and stimulated using 5 μg/ml CpG DNA or 1 μg/ml lipopolysaccharides from *Escherichia coli* (LPS) (Sigma Aldrich, St. Louis, Missouri, USA) for 8 h, and 1 μl/ml Brefeldin A was added for the last 4 h of the incubation (BD Biosciences). Cytokine detection was performed according to a previously published protocol [20]. The data were collected using an Aria II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar). The levels of IL-1β, IL-6, TNFα and IFNα in the supernatants of the PBMCs and monocytes were determined 24 h after the addition of ligands using multiplex Luminex cytokine bead-based immunoassays (Merck Millipore, Bedford, MA). PBMCs and monocytes were cultured in a 96-well microtiter plate at a concentration of 1 × 10e6/well and

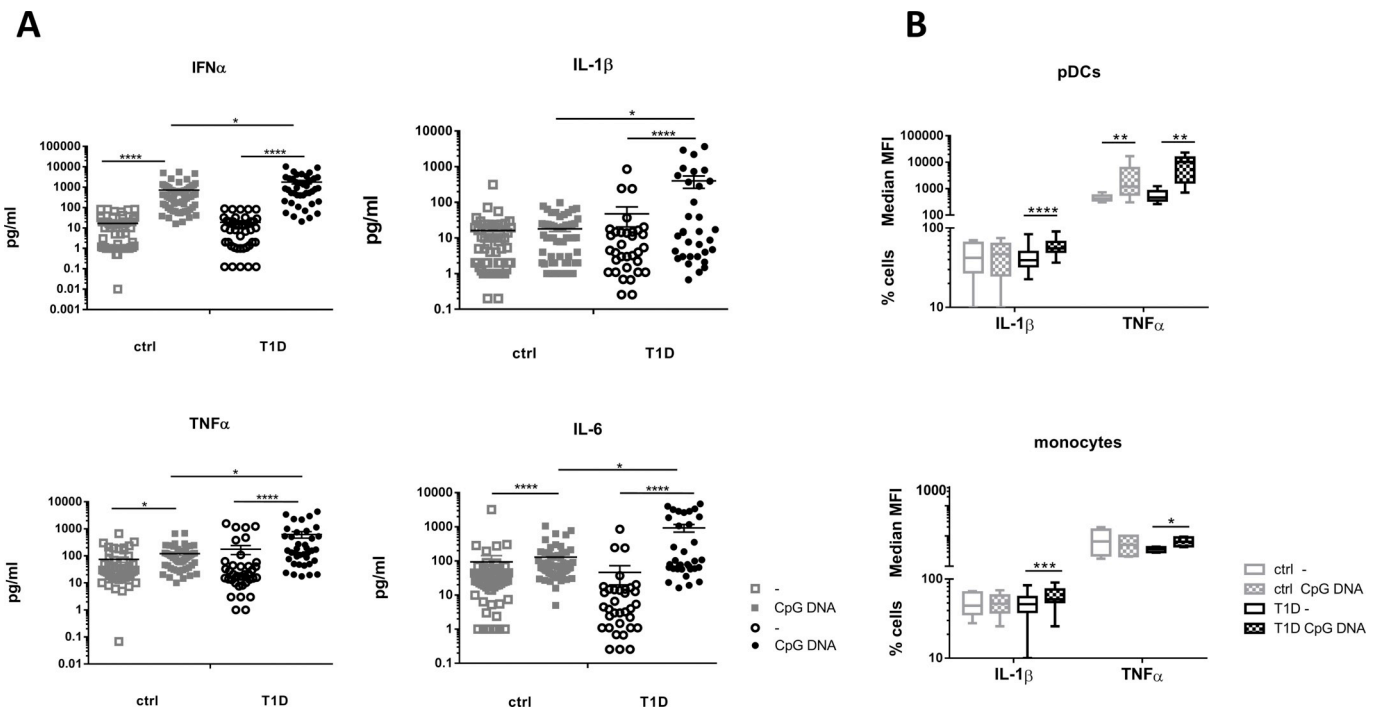

Fig. 1. CpG DNA causes a more robust proinflammatory response in monocytes and pDCs from T1D patients than from healthy donors. (A) Cytokine production that was measured by the Luminex analysis of the culture supernatants of PBMCs from T1D (n = 42) and healthy subjects (n = 50) that were stimulated for 24 h with CpG DNA. (B) Intracellular production of IL-1β and TNFα by the pDCs (n = 22) and monocytes (n = 17) of T1D patients after 8 h CpG-DNA stimulation. The results are shown as the mean ± SEM. Statistical analysis was performed using a two-tailed Wilcoxon paired *t*-test and an unpaired Mann-Whitney test. Values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant.

were stimulated with 5 μg/ml CpG DNA or 1000 ng/ml NET fragments. Data were collected using a Luminex-100 system (Luminex, Austin, TX). In some cases, cells were pretreated for half an hour before stimulation with 50 nM bafilomycin, an inhibitor of endosome acidification and TLR9 signaling (Sigma Aldrich), 1 μM BX795, an inhibitor of TBK1/IKKε and 1 μM MCC950, an inhibitor of NLRP3 (both from InvivoGen).

2.5. Internalization of CpG DNA

First, PBMCs were cultured in a 24-well plate as described above and were stimulated with 5 μg/ml FITC-labeled CpG DNA at the indicated times. HLA-DR negative populations were gated using antibodies against HLA-DR-PerCP, CD3-PC7, CD15-Alexa Fluor 700, CD56-APC, and HLA-DR positive cells were gated using HLA-DR-PerCP (BD Biosciences), CD19-Alexa 700 (Exbio) and CD14-PE-Dylight (Exbio) CD11c-APC, CD1c-BV510, CD141-BV421, and CD123-PE-Cy7 (Biolegend). For the verification of nonspecific extracellular CpG-DNA binding, the same experiment was performed at 4 °C. Data were collected on an Aria II and were analyzed using FlowJo software. Second, the analysis of CpG-DNA internalization was performed with an ImageStream multispectral quantitative imaging flow cytometer. To map the cellular localization of CpG DNA, cells were cultured with CpG DNA labeled with FITC for 4 h. Monocytes, pDCs, B cells and BDCA1$^+$ mDCs were distinguished with antibodies against CD19-Alexa Fluor 700, CD11c-APC, CD14-PE-DyLight594 (Exbio), HLA-DR-PerCP (BD Biosciences), CD123-PE-Cy7 and BDCA-1 Brilliant Violet (Biolegend). The ligand distribution in cells was quantified as the area features of FITC-CpG DNA intensity in the monocytes and pDCs.

2.6. Gene expression

RNA isolation, reverse transcription and RT-PCR were performed according to a previously published protocol [21]. The sequences of the primer/probe sets are available upon request from the author.

2.7. TBK1 phosphorylation

PBMCs were stimulated with 5 μg/ml CpG DNA for 1, 2, and 4 h at 37 °C, and the intracellular TBK1 phosphoprotein was analyzed using BD phospho-flow staining (BD Bioscience) following the manufacturer's instructions. Monocytes and pDCs were discriminated as described previously, and the intracellular signaling was detected using an anti-phospho-TBK1-PE (Cell Signaling) antibody. The samples were acquired on an Aria II flow cytometer (BD Bioscience) and were analyzed with FlowJo software (TreeStar).

2.8. STING-CpG-DNA colocalization

To measure the interaction of the CpG ligand with the STING molecule, an ImageStream multispectral quantitative imaging flow cytometer was used. For the colocalization of CpG DNA with STING, PBMCs were cultured with 5 μg/ml FITC-labeled CpG DNA for 4 h. B cells, monocytes, pDCs and BDCA-1$^+$ mDCs with internalized FITC-CpG-DNA were distinguished with the gating strategy described above. Colocalization of STING and FITC-CpG-DNA was quantified as 'bright detail similarity' (BDS) using IDEAS software. The BDS median of each cell population was calculated, and this value was referred to as the colocalization score. Molecules were considered to be colocalized if the BDS was > 1.5, (where > 3.0 displayed a complete spatial overlap).

2.9. NET induction and cocultivation with PBMCs

NET formation was induced according a protocol adapted from Warnatsch et al. [22]. Shortly, the isolated neutrophils were plated in 6-well plates at a density of 10^6 cells/ml and stimulated with 50 nM phorbol myristate acetate (PMA) [23,24] (Cayman Chemicals, Ann Arbor, USA) for 3 h in 37 °C in 5% CO_2 to maintain physiological pH. The culture medium was then carefully removed; the NETs were washed with PBS to remove possible products of neutrophil activation or

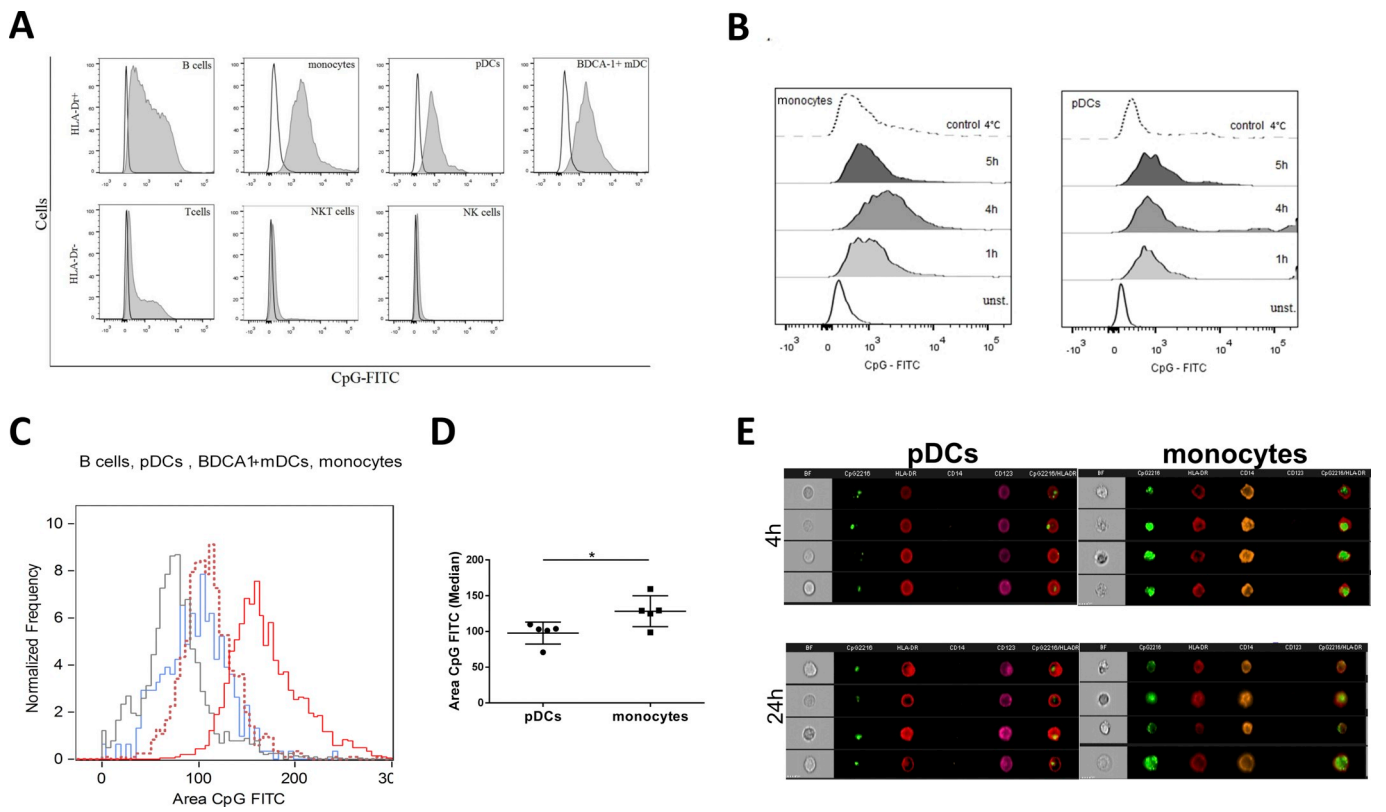

Fig. 2. CpG DNA shows a distinct uptake pattern in different cell types. (A) Representative histograms of CpG DNA internalization in HLA-DR negative (T-cells, NK cells and NKT cells) and HLA-DR positive cells (DCs, monocytes, and B cells) after 4 h of incubation with FITC-labeled-CpG DNA. (B) Uptake of CpG DNA at different time points (1, 4 and 5 h) by monocytes and pDCs. As a negative control, incubation at 4 °C was used. (C) Representative histogram of the intracellular distribution of CpG DNA, as measured by the area in B cells (gray), monocytes (red line), pDCs (blue) and mDCs (red dot) with ImageStream analysis. (D) Intracellular distribution of FITC-labeled-CpG DNA in pDCs and monocytes after 4 h of incubation. (E) Representative images of the gating of selected monocytes and pDCs with internalized CpG DNA after 4 h and 24 h of exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

degranulation and then partially digested by a restriction enzyme mix containing enzymes AluI, NdeI and PacI (all from ThermoFisher Scientific) at a concentration of 10 U/ml in complete media supplemented with 2% autologous serum. The digest was performed at 37 °C for 20 min, followed by centrifugation at 12000*g* for 10 min to remove cells and debris. The NET-DNA concentration was determined on a Nanodrop (ThermoFisher Scientific).

Next, 1000 ng/ml of NET fragments were added to 10 e6/ml PBMCs in culture, and the cytokine release was analyzed as described in 2.4.

2.10. mtDNA detection

Mitochondrial DNA content in NETs was analyzed according to a protocol that was previously published [25].

2.11. Statistical analysis

The results obtained from at least three independent experiments are given as the mean ± SEM. Not all patients were involved in all experiments due to the limited amount of blood that was available per sample. A two-tailed paired or unpaired Mann-Whitney or a *t*-test was used for the data analysis using GraphPad Prism 6. A p value ≤ 0,05(*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) were considered statistically significant.

3. Results

3.1. CpG DNA causes a more robust proinflammatory response in monocytes and pDCs from T1D patients than in healthy donors

PBMCs from both T1D patients and healthy donors produced large amounts of IFNα, as well as the proinflammatory cytokines TNFα and IL-6, after CpG-DNA stimulation. The production of these cytokines was accompanied by the release of IL-1β. Importantly, the cytokine release was more pronounced in T1D patients than in healthy controls (Fig. 1A). Intracellular cytokine staining flow cytometry was used to ascertain which cell populations contributed to cytokine production after CpG-DNA stimulation. Both pDCs and monocytes of T1D patients contributed to IL-1β and TNFα production (Fig. 1B), unlike the BDCA-1^+ mDCs cells (data not shown). On the other hand, only pDCs, and not monocytes, from healthy donor PBMCs responded to CpG-DNA stimulation with excessive TNF-α production (Fig. 1B), as demonstrated by the flow cytometry analysis.

Additionally, the proinflammatory response to CpG DNA was also reflected by the increased expression of the maturation markers CD86, HLA-DR (Supplementary Fig. 1A), CD83 and CD40 (data not shown) on the surface of monocytes and pDCs. The increased expression of surface markers was more prominent on T1D cells, especially on monocytes, than on healthy donor cells, but the surface markers were present in healthy donors as well.

3.2. CpG DNA shows distinct uptake patterns in different cell types

To clarify which cell types contributed to CpG DNA recognition, we cultivated patient PBMCs with fluorescently-labeled CpG DNA

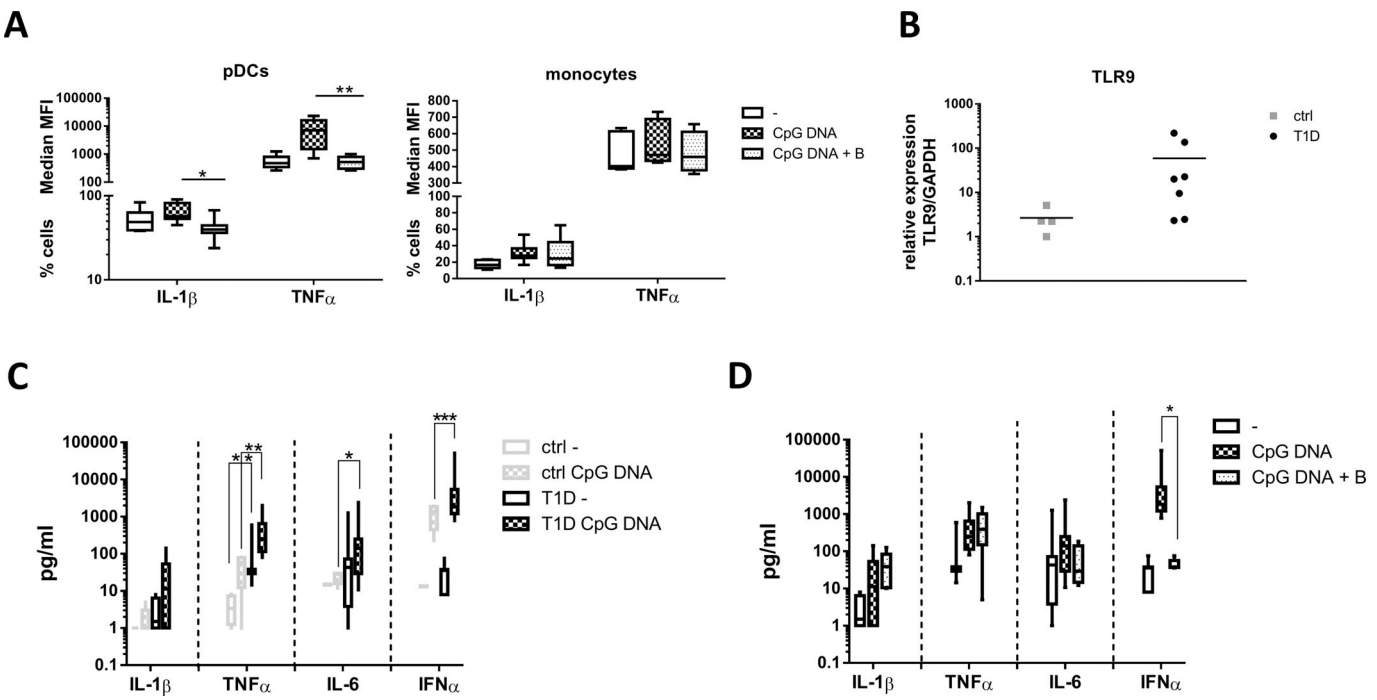

Fig. 3. In T1D monocytes, CpG DNA stimulates both TLR9-dependent and TLR9-independent pathways. (A) Intracellular production of IL-1β and TNFα by the pDCs (n = 4) and monocytes (n = 9) of T1D patients after 8 h CpG-DNA stimulation with or without bafilomycin pretreatment, as assessed by flow cytometry. (B) TLR9 relative expression in monocytes, as analyzed by RT-PCR. (C) Cytokine production in the cell supernatants of isolated monocytes from children with T1D (n = 8) and from healthy controls (n = 4) after 24 h CpG DNA stimulation. (D) Cytokine production in the cell supernatants of isolated monocytes that were incubated 24 h with CpG DNA with or without bafilomycin pretreatment in T1D patients (n = 6). The results are shown as the mean ± SEM. Statistical analysis was performed using a two-tailed Wilcoxon paired *t*-test and an unpaired Mann-Whitney test. Values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant.

(Fig. 2A). The CpG DNA was found to be efficiently internalized by all antigen presenting cells (B cells, monocytes, pDCs and BDCA-1$^+$ mDCs) in contrast to T cells, NKT cells and NK cells. The uptake kinetics showed a similar pattern in pDCs and monocytes, with the majority of nucleic acids being processed within the first hour of stimulation (Fig. 2B). To exclude the possibility of artificial, extracellular CpG DNA binding, the cells were incubated with the ligand at 4 °C, which completely abolished its uptake (Fig. 2B). Further, we observed a focused, localized CpG DNA signal in B cells, mDCs and pDCs. In pDCs, the signal was usually observed in one to three foci inside the cells, suggesting an endosomal localization. In contrast, a diffuse signal pattern was detected in monocytes, implying a broader cytoplasmic distribution of CpG DNA (Fig. 2C–E).

3.3. CpG DNA stimulates both TLR9-dependent and TLR-independent pathways in T1D monocytes

The identification of monocytes and pDCs as a major source of proinflammatory cytokines upon CpG stimulation raised the question of which signaling pathways were involved in CpG DNA sensing in these cells. To test whether DNA sensing occurred in the endolysosomal compartment (where TLRs that recognize nucleic acid are localized), PBMCs were cultivated in the presence of bafilomycin, an inhibitor of endolysosomal acidification and of TLR signaling [26]. As shown in Fig. 3A, the intracellular detection of IL-1β and TNFα in CpG-stimulated pDCs revealed that the production of these cytokines was completely inhibited after bafilomycin treatment, which suggested that the CpG DNA signaling in pDCs is TLR9-dependent. In contrast, such an inhibition was not detected in monocytes, which implied that TLR9-independent signal transduction was occurring.

To examine the ability of monocytes to sense CpG DNA without interference or assistance from other cell types, we tested isolated monocytic cultures for basal TLR9 expression and for cytokine production after CpG DNA treatment. T1D monocytes expressed significantly elevated levels of TLR9 (Fig. 3B) and displayed increased proinflammatory responses to CpG DNA, as demonstrated by increased production of TNFα, IL-6 and IFNα, compared to those of healthy controls (Fig. 3C), thus verifying that monocytes were not reliant on paracrine stimulation with cytokines that were produced by other cell types. To validate the involvement of TLR9 in CpG DNA-induced cytokine production, we treated isolated T1D monocytes with bafilomycin, thus inhibiting TLR signaling. The bafilomycin treatment efficiently blocked only IFNα production, confirming the role of TLR9 in CpG-induced IFNα release, but the treatment failed to block IL-1β, TNFα and IL-6 production, or it even enhanced the production of these cytokines, suggesting once again that other cytosolic DNA receptors might regulate the proinflammatory cytokine response in T1D monocytes upon CpG-DNA sensing (Fig. 3D).

3.4. CpG DNA colocalizes with the signaling molecule STING and induces the expression of DNA sensor genes in T1D

Based on our data that supported the participation of TLR9-independent CpG DNA signaling, we investigated other intracellular DNA sensors by assessing the colocalization of CpG DNA with STING, a central adaptor protein for most DNA-sensing pathways, in T1D PBMC subpopulations. A significantly higher colocalization, respective BDS score and cell percentage with colocalized molecules was detected in T1D monocytes compared to those in pDCs and B cells (Fig. 4A and B). As documented in the representative images in Fig. 4C, STING formed aggregates and colocalized with DNA upon CpG-DNA stimulation in monocytes. In pDCs and B cells, STING was more dispersed throughout the cytoplasm, and the colocalization was less pronounced. In mDCs, the interaction between STING and CpG DNA was weak (Supplementary Fig. 1C).

Furthermore, the enhanced STING and CpG DNA colocalization was

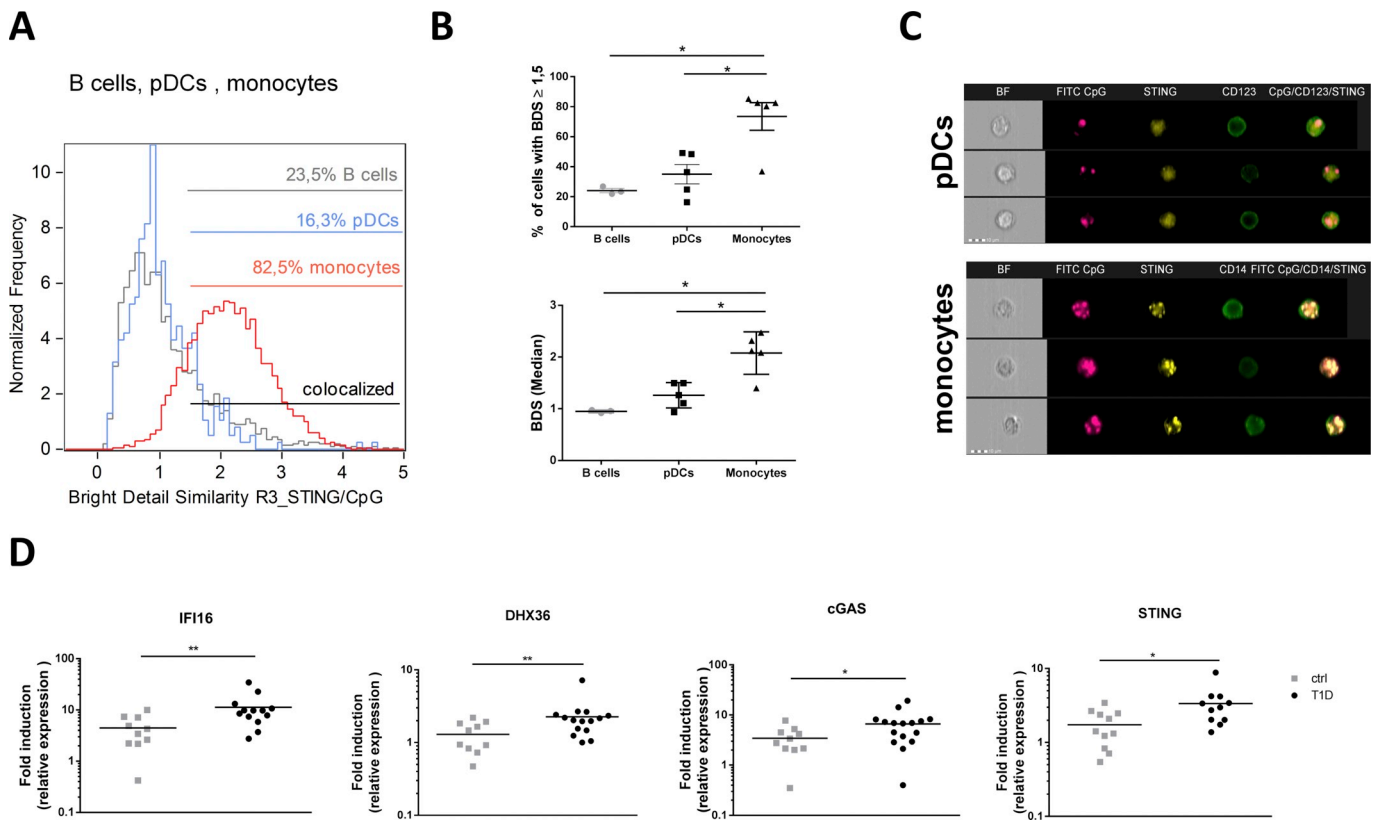

Fig. 4. CpG DNA colocalizes with STING and induces the expression of DNA sensor genes in T1D. (A) Representative histogram of the bright detail similarity (BDS) of CpG/STING in monocytes (red), pDCs (blue) and B cells (gray). Colocalization was quantified as the percentage of cells with a bright detail similarity (BDS) > 1.5. BDS is a feature of the IDEAS software that compares the bright detail image detail of two images to quantify the colocalization. Cells were stimulated for 4 h with FITC-labeled CpG, fixed, stained and analyzed by ImageStream (B) Percentage of cells with a (BDS) > 1.5 and with a BDS score attained in particular subsets. (C) Representative images of STING/FITC-CpG DNA in pDCs and monocytes. STING forms aggregates and co-localizes with CpG DNA in monocytes. (D) Relative expression of DNA sensors in T1D and healthy PBMCs after 7 h of CpG DNA stimulation. The results are expressed as the fold change between the CpG DNA-stimulated and unstimulated cells. The results are shown as the mean ± SEM. Statistical analysis was performed using a two-tailed Wilcoxon paired *t*-test and an unpaired Mann-Whitney test. Values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

accompanied by significantly higher levels of gene expression of other cytosolic DNA receptors, such as IFI16, DHX36 and cGAS, as well as the adaptor protein STING, in T1D PBMCs compared to those in PBMCs from healthy controls (Fig. 4D).

3.5. TBK1 is essential for DNA sensing in T1D monocytes and PBMCs

Our observation that STING is required for the cytoplasmic sensing of CpG DNA in monocytes led us to analyze the activation of TBK1, a cytosolic kinase that mediates STING-dependent downstream signaling. Phospho-flow cytometry indicated that there was an activation of TBK1 due to phosphorylation after CpG DNA stimulation in both monocytes and pDCs (Fig. 5A). To verify the involvement of TBK1, we treated PBMCs (Fig. 5B, D, and Supplementary Fig. 1D) and isolated monocytes (Fig. 5C) with a TBK1 inhibitor, BX795, before CpG-DNA and control lipopolysaccharide (LPS) stimulation and analyzed cytokine production. In T1D PBMCs and isolated T1D monocytes, CpG DNA-induced IL-6, TNFα, IFNα and IFNβ were strongly reduced after TBK1 inhibition compared to those of the controls. In healthy controls, the TBK1 inhibitor failed to inhibit CpG-DNA-induced IFN-β production in PBMCs; among the other cytokines, we observed a similar trend as in T1D cells (data not shown). The use of BX795 inhibition in LPS-stimulated cells confirmed its specificity (Supplementary Fig. 1D). The production of IL-1β seemed to be independent of TBK1 and TLR9. Since the recruitment of another AIM2-dependent pathway in the production of after nucleic acid sensing has previously been reported by Zhang et al. [27], we hypothesized that other intracellular sensors or inflammasomes, such as AIM2 or NLRP3, may have been involved. Although IL-1β production was dependent on potassium efflux, NLRP3 participation in the production was excluded, as NLRP3 inhibitor failed to significantly inhibit IL-1β production in T1D PBMCs (Data in Brief).

3.6. T1D PBMCs and monocytes respond to neutrophil extracellular traps in a TBK1-dependent manner

To investigate the mechanisms involved in self-DNA sensing, we isolated neutrophil extracellular traps (NETs) to use as a model of self-DNA. These structures contain self-DNA in addition to antimicrobial peptides and histones. Previously, we reported that T1D NETs are characterized by having more DNA than those from healthy controls and inducing distinct proinflammatory reactivity in T1D PBMCs (Parackova, 2019 under review). The clearly observed differences in the composition and amount of the DNA content between T1D and healthy NETs prompted us to examine the mitochondrial DNA (mtDNA) presence in the DNA content of NETs, as both nuclear and mtDNA were previously found among the DNA of NETs [28]. We found that T1D NETs contained significantly higher amounts of mtDNA (Fig. 6A) compared to that in healthy control NETs.

Human mtDNA is structurally similar to bacterial DNA and is enriched in unmethylated CpG dinucleotide motifs, which exhibit strong immunostimulatory effects [29]. Thus, we hypothesized that the mtDNA enrichment observed in T1D NETs would induce a similar

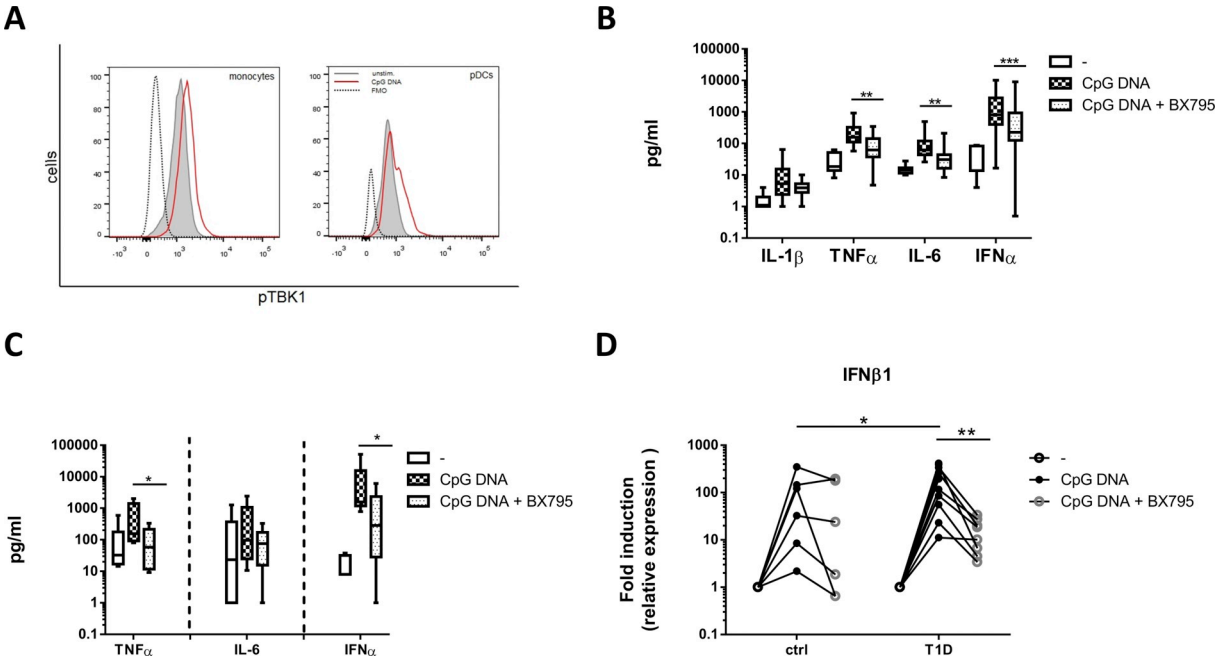

Fig. 5. TBK1 is essential for DNA sensing in T1D monocytes and PBMCs. (A) Representative histograms of the TBK1 phosphorylation in monocytes and pDCs after 4 h CpG DNA stimulation, as determined by phospho-flow cytometry. (B) Cytokine production by PBMCs after 24 h CpG-DNA stimulation with or without the TBK1 inhibitor BX795 pretreatment in T1D patients, as analyzed by Luminex assays. (C) Cytokine production by monocytes after 24 h CpG-DNA stimulation with or without the TBK1 inhibitor BX795 pretreatment in T1D patients, as analyzed by Luminex assays (n = 6). (D) Relative expression of IFNβ after 17 h CpG-DNA stimulation with or without the 1 μM BX795 pretreatment in T1D and control PBMCs. The results are expressed as the fold change (CpG-DNA stimulated/unstimulated cells). Statistical analysis was performed using a two-tailed Wilcoxon paired *t*-test and unpaired Mann-Whitney test and *t*-test. Values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant.

proinflammatory response as documented upon microbial-DNA (CpG DNA) stimulation in T1D monocytes. Therefore, by performing inhibition experiments with bafilomycin and BX765, we examined which signaling pathways orchestrate NET-induced proinflammatory cytokine production in T1D monocytes. With the use of intracellular detection (Fig. 6B) and Luminex assays (Fig. 6C), we demonstrated that NET-induced IL-1β and TNFα release from monocytes and PBMCs was preserved after bafilomycin treatment. A similar pattern was observed for CpG-DNA sensing, where the intracellular detection of TNFα and IL-1β in monocytes also preserved cytokine production after bafilomycin treatment (Fig. 3A). Together, these data implied the cytosolic sensing of NET-DNA in monocytes. Indeed, TBK1 blockade with the BX795 inhibitor reduced the release of all proinflammatory cytokines in T1D patients compared to healthy controls, with the exception of the IFNα cytokine (Fig. 6D). The response to NET stimulation was also accompanied by elevated STING expression, which was slightly higher in T1D PBMCs upon NET treatment (Fig. 6E) compared to that in healthy controls. Together, these findings indicate that TBK1 is also a key molecule in self-DNA sensing in T1D patients.

4. Discussion

Discrimination between self versus non-self during nucleic acid recognition is a crucial mechanism to deter invaders without inducing autoimmunity [30]. The endosomal localization of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), is an essential protective system that reduces the response to self-DNA. Other mechanisms involve the different expression of PRRs in distinct immune cells, including different signaling pathways. An important example is the restricted expression of TLR9 in plasmacytoid dendritic cells, which, upon microbial DNA sensing, induces high levels of type I IFNs. Recent work has indicated that disrupted nucleic acid sensing is involved in many autoimmune diseases. Even the excessive production of type I IFNs, such as IFNα and IFNβ, and increased IFN signaling induced by aberrant DNA recognition was shown to play a pathogenic role in various autoimmune diseases, including T1D [31–34]. Although pDCs are the main producers of type I IFNs [8,35], other cell populations, such as monocytes and B cells, were reported to participate in the exaggerated IFN signaling in systemic lupus erythematosus, Sjögren syndrome and T1D [36–38].

In the present study, we show for the first time that in T1D patients, not only pDCs but also monocytes play an important role in the type I IFN signature. We demonstrate that T1D monocytes express higher levels of TLR9 compared to those in healthy controls, which might contribute to the augmented DNA sensing and subsequent IFNα overproduction in T1D patients. The fact that not only pDCs and B cells but also monocytes are capable of DNA sensing, TLR9 expression [39,40] and IFNα production through the TLR9-independent pathway [3,41] has been previously reported. Additionally, the increased levels of TLR9 in monocytes have already been shown to orchestrate enhanced responsiveness to TLR9 agonists in patients with active rheumatoid arthritis [42]. Here, we show that monocytes also play a substantial role in DNA sensing in T1D patients; thus, we have extended the list of autoimmune diseases with an aberrant response to DNA-based ligands that results in type I IFN overproduction.

Interestingly, IFNα was not the only cytokine produced by T1D cell cultures in response to CpG-DNA stimulation. PBMCs and especially monocytes also produced other proinflammatory cytokines, such as IL-1β, IL-6 and TNFα. This production was significantly higher in T1D cells than in healthy controls. Moreover, the proinflammatory response to CpG DNA was accompanied by pDCs and monocytes with a more mature phenotype in T1D patients than in healthy controls. A similar proinflammatory response that accompanied IFN release after CpG DNA (CpG2216) stimulation was previously reported [43]. By analyzing the intracellular distribution pattern of CpG DNA in various cell types, we established that while all antigen presenting cells can bind and internalize DNA, different patterns appear in distinct cell types. pDCs, mDCs and B cells internalized DNA that was concentrated at a few well-

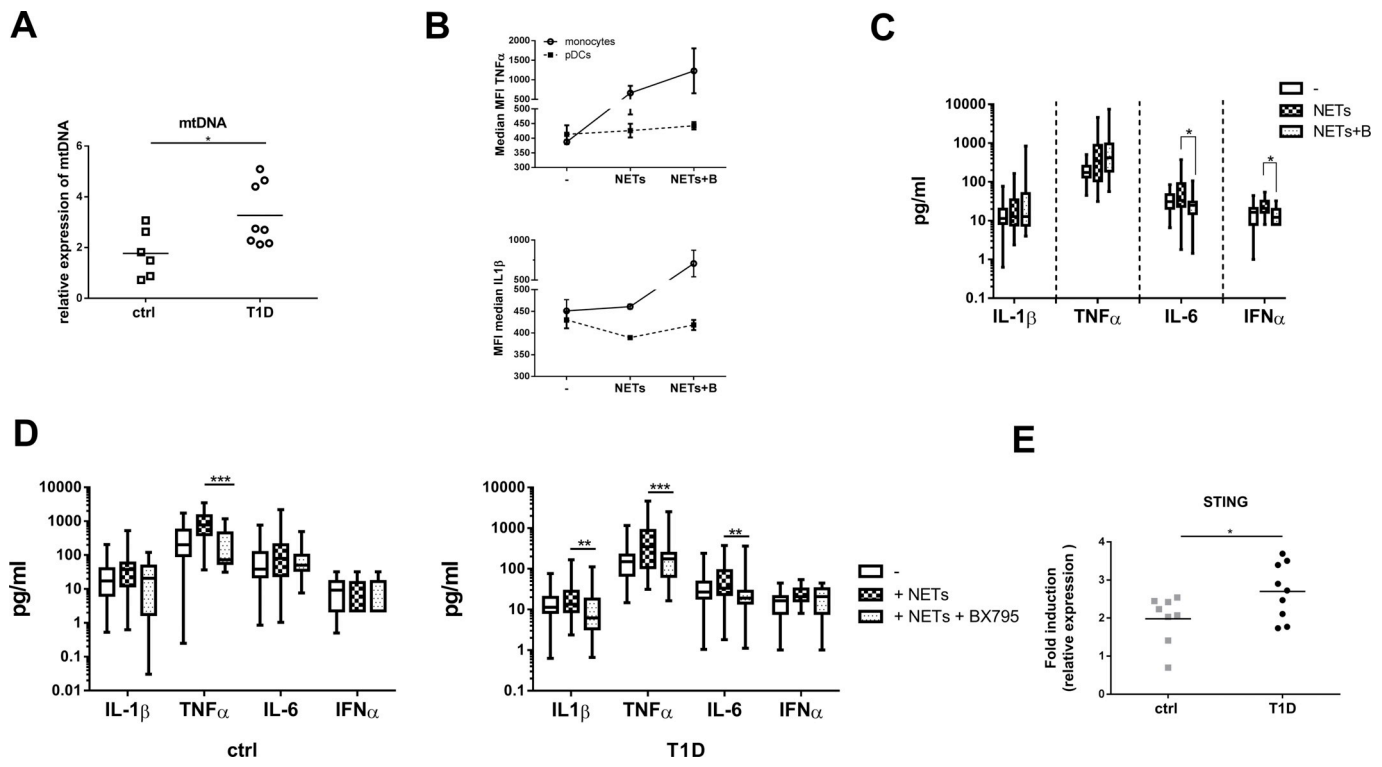

Fig. 6. PBMCs and monocytes from children with T1D that responded to neutrophil extracellular traps in a TBK1-dependent manner. (A) Level of mitochondrial DNA (mtDNA) in NETs in T1D patients and healthy controls. (B) Intracellular production of cytokines by monocytes and pDCs stimulated with 1000 ng/ml NETs for 12 h with or without bafilomycin pretreatment, as analyzed by flow cytometry (n = 4). (C) Cytokine production by PBMCs stimulated with 1000 ng/ml NETs for 24h with or without pretreatment with bafilomycin (n = 14), as measured by Luminex assays. . (D) Cytokine production by the PBMCs of T1D patients or healthy subjects after stimulation with 1000 ng/ml NETs for 24 h with or without the TBK1 inhibitor BX795 pretreatment in T1D patients (n = 12) and in healthy (n = 9) controls, as measured by Luminex assays. (E) Relative gene expression of STING in T1D PBMCs and in healthy PBMCs after 16 h of NET stimulation. The results are expressed as the fold change between NET-stimulated and unstimulated cells. Statistical analysis was performed using a two-tailed Wilcoxon paired *t*-test and an unpaired Mann-Whitney test. Values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant.

defined foci, most likely in the endosomes. On the other hand, monocytes internalized DNA that was distributed with a more disperse cytoplasmic pattern. Our data are in agreement with previously documented variance in cytoplasmic distribution by pDCs and monocytes reported by Chamilos et al. [3], who demonstrated that in monocytes, the DNA-protein complexes were able to escape the endosomal vesicles and enter the cytosol. Our data showing the different localization of CpG DNA in distinct cell types, together with the preserved production of CpG DNA-induced IL-1β, TNFα and IL-6 production upon bafilomycin treatment in monocytes, suggests the involvement of different DNA binding patterns within these cell populations, such as endosomal TLR9 in pDCs and other cytoplasmic sensors in monocytes.

The existence of numerous intracellular DNA receptors in innate immune cells [44–46] and their role in the pathogenesis of various autoimmune diseases, such as SLE or Sjögren syndrome, have been previously reported [14,47]. In our experiments, the involvement of TLR9-independent DNA sensing was implied by the undisturbed IL-1β, TNFα and IL-6 production by T1D monocytes in response to CpG DNA after targeted TLR9 inhibition. In fact, significantly higher expression of the non-TLR9 DNA sensors cGAS, STING, IFI16 and DHX36 was noted in T1D cells compared to those in healthy controls after stimulation. To our knowledge, only one study to date has reported a cytosolic DNA sensor (IFI16) that was associated with T1D susceptibility [48]. Our results illustrate the strikingly different reactivity of T1D cells to CpG DNA, demonstrating an enhanced response, which is likely due to abnormal increases in non-TLR9-mediated DNA sensing (Fig. 7).

To further support this hypothesis, we expanded on our results by studying STING, which is a crucial molecule at the intersection of the signaling cascades that are induced by DNA sensors and also pathogen recognition receptor itself [49,50]. The CpG/STING colocalization studies, particularly in monocytes, implied that the cytosolic receptors played a prominent role in DNA sensing in T1D. Upon activation, STING recruits another important part of the STING signaling cascade, TBK1. The participation of both molecules in DNA sensing in monocytes was previously reported by Chamilos et al. in healthy monocytes [3]. Similarly, we noted that TBK1 was phosphorylated in both pDCs and monocytes in T1D patients upon CpG-DNA stimulation. Additionally, TBK1 blockade with BX795 resulted in the strong inhibition of the CpG-induced production of proinflammatory cytokines, especially type I IFNs. Such a reduction in IFNs might have been coorchestrated by the simultaneous inhibition of autocrine IFN stimulation because BX795 also inhibits IKKε. a TBK1 structural homologue that is involved in downstream IFN signaling [51]. The importance of TBK1 for IFN production is well established, but it has only recently been implicated in the NF-kB pathway, which generally leads to a proinflammatory response [10,15,27,52]. Remarkably, not only was TBK1 inhibition shown to be beneficial in some autoimmune diseases [14,15,53], but a recent study also suggested that inhibiting TBK1 might promote the regeneration of pancreatic β-cells [18]. Our results are therefore consistent with the currently available knowledge on TBK1 engagement in inflammatory responses in T1D.

Finally, we examined whether the same mechanisms that are involved in microbial CpG DNA recognition are also involved in self-DNA recognition in T1D patients. Self-DNA is an integral part of neutrophil extracellular traps (NETs), web-like structures that are released by activated neutrophils. Aside from their role in defense against infections, NETs have also attracted substantial attention in the field of autoimmune disease, as they represent a potent source of autoantigens and endogenous danger molecules, including DNA [54–56]. Previously, we reported that the T1D NET composition significantly differed from that

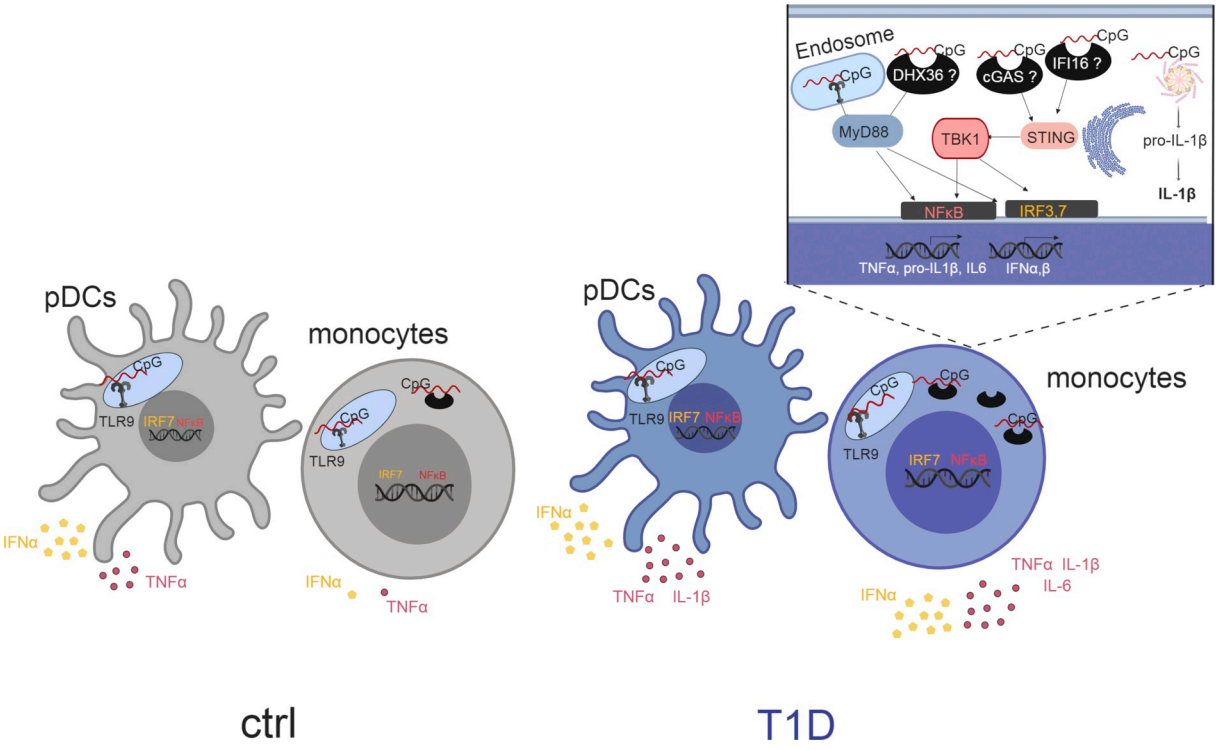

Fig. 7. Specific differences in DNA sensing in the context of T1D. CpG DNA induced more robust proinflammatory and IFN responses in T1D monocytes compared with those of healthy subjects. As a result of CpG-DNA sensing, the TLR9 and intracellular receptor pathways are activated. This signaling leads to STING translocation, TBK1 phosphorylation and the increased expression of intracellular receptors, such as IFI16, cGAS and DHX36, by T1D monocytes. This upregulation of intracellular receptors is in accordance with the elevated mtDNA contained in neutrophil extracellular traps (NETs), which could lead to rapid cytokine responses by T1D monocytes that are exposed to NETs. For the sake of simplicity, only the signaling molecules referred to in this study are shown.

of control NETs, which was mirrored by the distinct proinflammatory reactivity of T1D cells to NETs (Parackova, 2019 under review). Here, in the context of DNA sensing and its importance for T1D pathogenesis, we showed that T1D NETs contained more mtDNA, which had potential consequences for altered DNA sensing in T1D cells. Mitochondrial DNA, which is essentially of prokaryotic origin, is mostly unmethylated, has immunostimulatory activity [29] and is known to activate both STING [36,57] and TLR9. Thus, we proposed that NET-derived DNA might significantly contribute to interferon- and cytokine-mediated inflammation in T1D. Indeed, within the scope of our experiments, we demonstrated that T1D NETs were capable of inducing the secretion of proinflammatory cytokines by T1D PBMCs. In this inflammatory response, in addition to monocytes, myeloid BDCA-1+ mDCs were involved (data not shown), whereas pDCs did not participate. The role of mDCs in NET-stimulated responses has already been studied in our previous work (Parackova, 2019 under review). Furthermore, in accordance with these results, we observed the increased expression of STING after the stimulation of PBMCs with NETs, which was slightly higher in T1D cells than in controls. To summarize our data, we described for the first time to the best of our knowledge, an essential role of TBK1 in NET-induced proinflammatory cytokine production in children with T1D.

Another intriguing hypothesis concerning the factors that contribute to the abnormal cell activation and ongoing inflammation in T1D is the role of imbalanced NET release and NET degradation. The self-DNA that is released from NETs has an increased susceptibility to oxidation because of its mitochondrial origin, which could result in greater stability and resistance against DNases [58]. The NET DNA stability and potency for the activation of pDCs is also enhanced due to the formation of complexes with other proteins that are normally present at NETs, as was already shown in SLE patients [59]. Altogether, NETs may represent an additional and potentially more persistent source of self-DNA that potentiates the proinflammatory bias that is associated with T1D.

5. Conclusion

In conclusion, this work represents the first ever complex study on the specifics of DNA sensing in T1D patients. Our findings demonstrate that both pDCs and monocytes from T1D patients sense DNA and that DNA-induced activation results in an abnormally high inflammatory response. We show that in T1D, pDCs and monocytes engage TLR9 during DNA sensing, but monocytes also rely heavily on cytoplasmic DNA sensors. These sensors trigger the STING and TBK1 signaling pathways, resulting in the increased secretion of inflammatory cytokines and IFNs, thus contributing to the chronic inflammation underlying the T1D immunopathology. We further show that T1D NETs from T1D patients are characterized by increased mitochondrial DNA content, which, similarly to CpG DNA, engages TBK1 and STING molecules with subsequent inflammatory and IFN responses. Altogether, based on our data, we propose that aberrant DNA sensing significantly contributes to T1D pathogenesis. However, further research is warranted to build upon the currently available data.

Author contribution

IZ and ZP designed the study and experiments, performed experiments, analyzed the data, interpreted the results and wrote the manuscript.

JK, NV and PV performed the experiments and interpreted the results.

ZS, LP and SP provided patient information and biological material and reviewed the manuscript.

LPJ reviewed the manuscript.

A.S. designed the study and reviewed and edited the manuscript.

Conflicts of interest

None of the authors have declared any financial or commercial conflicts of interest.

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We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All of the authors have approved the manuscript and agree with its submission to the Journal of Autoimmunity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.06.005.

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