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Cytosolic DNA-Activated Human Dendritic Cells Are Potent Activators of the Adaptive Immune Response

Katalin Kis-Toth,* Attila Szanto,† To-Ha Thai,*‡ and George C. Tsokos*†

Recent studies in cell lines and genetically engineered mice have demonstrated that cytosolic dsDNA could activate dendritic cells (DCs) to become effector APCs. Recognition of DNA might be a major factor in antimicrobial immune responses against cytosolic pathogens and also in human autoimmune diseases such as systemic lupus erythematosus. However, the role of cytosolic dsDNA in human DC activation and its effects on effector T and B cells are still elusive. In this study, we demonstrate that intracellular dsDNA is a potent activator of human monocyte-derived DCs as well as primary DCs. Activation by dsDNA depends on NF-kB activation, partially on the adaptor molecule IFN-promoter stimulator-1 and the novel cytosolic dsDNA receptor IFI16, but not on the previously recognized dsDNA sentinels absent in melanoma 2, DNA-dependent activator of IFN regulatory factor 3, RNA polymerase III, or high-mobility group boxes. More importantly, we report for the first time, to our knowledge, that human dsDNA-activated DCs, rather than LPS- or inflammatory cytokine mixture-activated DCs, represent the most potent inducers of naive CD4+ T cells to produce Th1-type cytokine production and generate CD4+ and CD8+ cytotoxic T cells. dsDNA-DCs, but not LPS- or mixture-activated DCs, induce B cells to produce complement-fixing IgG1 and IgG3 Abs. We propose that cytosolic dsDNA represents a novel, more effective approach to generate DCs to enhance vaccine effectiveness in reprogramming the adaptive immune system to eradicate infectious agents, autoimmunity, allergy, and cancer. The Journal of Immunology, 2011, 187: 1222–1234.

Dendritic cells (DCs) are central players in the initiation and regulation of effective immune responses against infectious agents. In addition, they are essential in the induction of tolerance and antitumor immunity and prevention of autoimmunity. DCs are located in nonlymphoid and peripheral lymphoid tissues, where they act as sentinels of environmental cues and orchestrate the interplay between the innate and adaptive immune system to provoke a successful response. Nonactivated, immature DCs (IDCs) specialize in Ag uptake, whereas activated mature DCs (MDCs) are professional APCs capable of activating T and B cells to become effector cells (1, 2). Ag uptake and activation of DCs are mediated through the interaction of cell-surface and intracellular receptors with Ags.

Several pattern-recognition molecules evolved to discriminate between foreign and self-Ags (3, 4), and several receptors have been described to identify foreign nucleic acids. Endosomal TLRs such as TLR3 and -7/8 can detect microbe-derived dsRNA and ssRNA, respectively (5). The RNA helicase domain-containing proteins RIG-I and Mda5 respond to negative-stranded viral RNA molecules present in the cytoplasm of infected cells (6). Although unmethylated CpG-rich DNA sequences found in certain microbes can be easily recognized by TLR9 in the endosome of the host (7), the presence of naked DNA in the cytosol, a danger signal independent of its microbial or self-origin, is of critical importance. The immunological detection of cytosolic naked dsDNA became evident during the past few years by demonstrating the presence of cytosolic DNA sensors that function independently of TLR9 and other TLRs (8–11) as well as from the RIG-I/Mda5 system (11). The cytoplasmic dsDNA induces many cell types to produce type I IFN, and this response requires signaling molecules such as the TNFR-associated factor family member-associated NF-kB activator-binding kinase-1 (TBK-1) and the inhibitor of NF-kB kinase (IKK) (12). Several candidate receptors have been identified recently to recognize such cytosolic DNA. For example, DNA-dependent activator of IFN-regulatory factor 3 (DAI) has been shown to associate with both TBK-1 and IFN regulatory factor (IRF) 3 (13). However, DAI-deficient cells and mice produce near wild-type amounts of type I IFN in response to cytosolic DNA (12), suggesting that other receptors are involved. The pyrin and HIN200 domain-containing protein absent in melanoma 2 (AIM2) has been identified as a cytoplasmic dsDNA receptor that induces the formation of a specific inflammasome and subsequent secretion of IL-1β and pyroptotic cell death (14–17). AIM2-deficient mice are defective in caspase-1 activation, IL-1β secretion, and cell death in response to cytosolic DNA or Francisella tularensis infection (18) and have a partial role in the sensing of Listeria monocytogenes (19, 20). However, AIM2 does not appear to mediate type I IFN response to dsDNA. IFI16, another pyrin and HIN200 domain-containing

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Abbreviations used in this article: AIM2, absent in melanoma 2; DAI, DNA-dependent activator of IFN regulatory factor 3; DC, dendritic cell; HMGB, high-mobility group box; IDC, immature dendritic cell; IKK, inhibitor of NF-kB kinase; IPS-1, IFN-promoter stimulator-1; IRF, IFN regulatory factor; MDC, mature dendritic cell; N.T., nontreated; poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; siRNA, small interfering RNA; STING, stimulator of IFN genes; TBK-1, TNFR-associated factor family member-associated NF-kB activator-binding kinase-1; TRAF6, TNFR-associated factor 6.

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protein, was also identified as an intracellular DNA sensor that mediates the induction of IFN-β in which stimulator of IFN genes (STING), a critical mediator (21), was recruited to IFN16 after DNA stimulation (22). Recently, RNA polymerase III was shown to efficiently trigger the production of type I IFN by transcribing microbial DNA templates into dsRNA containing 5′-triphosphate, which is a potent activator of RIG-I (23, 24). Another cytosolic nucleic acid sensor, LRRFIP1, could also mediate type I IFN production via a β-catenin–dependent pathway (25). Moreover, high-mobility group box (HMGB) proteins were reported also to function as universal sentinels for nucleic acids, modulating type I IFN as well as inflammatory cytokine production (26). Taken together, these observations suggest the existence of multiple cytosolic proteins with DNA sensor capability in various cell types; however, it still remains elusive how human APCs can recognize dsDNA.

DNA binding to the cytosolic receptors activates not only the RIG-I pathway but also the NF-kB transcriptional pathway to trigger the production of proinflammatory cytokines in addition to type I IFNs (4). The introduction of dsDNA, derived from either a pathogen or the host, into the cytoplasm of macrophages and DCs induces phenotypic and functional maturation by activating a set of co-stimulatory genes, cytokines, chemokines, and transcription factors (27–30), which are mediated by a TLR9-independent pathway (31–35). dsDNA activates IRF3 and the IFN-β promoters via both TBK-1 and IKKi, whereas NF-kB activation by dsDNA is independent of both TBK-1 and IKKi (11). Both pathways require the adaptor molecule IFN-promoter stimulator-1 (IPS-1) but not TLRs or RIG-I (11, 36). The TNFR-associated factor 6 (TRAF6), which is essential for the activation of NF-kB and the production of type I IFNs, mediates antiviral responses triggered by cytosolic viral DNA (37). The recognition of dsDNA is sequence independent and requires the DNA to be present in the cytoplasm of the target cells (27, 28). These studies are seminal to the understanding of the effects of cytosolic dsDNA on IFN production and response in cell lines and mouse gene knockout systems. However, how cytosolic dsDNA activates human DCs and what types of effector T and B cells these DCs generate remain undetermined.

In this study, we demonstrate that intracellular dsDNA is a potent activator of human monocyte-derived DCs, as well as of primary DCs. This activation is independent of the DNA sensors AIM2, DAI, RNA polymerase III, or HMGBs; however, it depends in part on the cytosolic IFI16 dsDNA receptor. We report that cytosolic dsDNA-activated human DCs, unlike LPS- or inflammatory cytokine mixture-activated DCs, can induce naive CD4+ T cells to produce more IL-2, IFN-γ, and granzyme, but not IL-4, and that these effector CD4+ T cells efficiently kill tumor cells in vitro cultures. In addition, we demonstrate that dsDNA-activated DCs generate cytotoxic CD8+ T cells that produce the effector proteins IFN-γ and granzyme. In the presence of T cells, dsDNA-activated DCs, but not LPS- or mixture-activated DCs, induce B cells to produce complement-fixing IgG1 and IgG3 but not the non–complement-fixing IgG2 and IgG4 Abs. Thus, synthetic cytosolic DNA represents a novel, more effective, and safer means of generating DCs to use in vaccines to orchestrate and reprogram the adaptive immune system to eradicate infectious agents, autoimmunity, allergy, and cancer.

Materials and Methods

Generation of monocyte-derived DCs and culturing

Human monocyte-derived DCs were generated from CD14+ blood monocytes isolated from PBMCs separated from buffy coats by Ficoll-Paque (Fisher Scientific, Pittsburgh, PA) gradient centrifugation (38) followed by positive selection with anti-CD14–coated magnetic beads (Miltenyi Biotec, Auburn, CA). Purified CD14+ monocytes (≥95%) were plated at 2 × 10^6 cell/ml concentration and cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin in the presence of 100 ng/ml IL-4 and 75 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) given on days 0 and 2. Activation of IDCs was induced on day 5 by an inflammatory medium containing 10 ng/ml TNF, 5 ng/ml IL-1β, 20 ng/ml IL-6, 75 ng/ml GM-CSF (PeproTech), and 1 µg/ml PGE2 (Sigma-Aldrich) or by LPS (500 ng/ml) for 24 h. For DC activation, poly(dI:dC)–poly(dI:dC) (poly[dA:dT]) (2.5 µg/ml) complexed with LyoVec transfection reagent (Invivogen, San Diego, CA) was used on 5-d IDCs for 24 h. In some experiments, the 5-d IDCs were pretreated with 5 µg/ml chloroquine, 2 µg/ml ML-60218, or 1 µg/ml MG132 for 1 h before the poly(dA:dT) treatment to inhibit the endosome acidification or RNA polymerase III or polymerase activity, respectively. The optimal inhibitor concentrations were titrated in preliminary experiments, and the viable cells of the treated cells was analyzed by flow cytometry.

Flow cytometry

The identification of DC and T cell activation was monitored by flow cytometric analysis using fluorochrome-conjugated anti-CD80, anti-CD83, anti-CD86 (DC, B cell), anti-CD19 (B cell), anti-CD3, anti-CD25, and anti-CD69 (T cell) Abs as compared with isotype-matched control Abs (BD Pharmingen, San Diego, CA). T cell proliferation was measured by CFSE staining (5 µM, 5 min incubation on 37˚C, washing twice with medium containing 10% FBS). Intracellular granzyme production of the T cell subsets was detected by staining of fluorochrome-conjugated anti-granzyme with anti-CD4 or anti-CD8 Abs in the CD3+ population. Fluorescence intensities were measured and analyzed by FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ). Data analysis was performed using the FlowJo Flow Cytometry Analysis software (Tree Star, Ashland, OR).

Real-time quantitative RT-PCR

Real-time PCR was performed as described previously (39). Total RNA was isolated from DCs by RNasey Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed at 37˚C for 120 min from 100 ng total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR for AIM2, DAI, IRF3, IRF7, IFN-β, HMG1B, HMG2B, HMG3B, IFI16, and IPS-1 genes were performed (Light Cycler 480; Roche) with 45 cycles at 94˚C for 15 s and 60˚C for 60 s using TaqMan assays (Applied Biosystems). All PCR reactions were run in triplicates with a control reaction containing no reverse transcription enzyme. The comparative cycle threshold method was used to quantify transcripts relative to the endogenous control gene 36B4. PCR arrays were performed using the Dendritic Cell–Ag Presenting Cell and the TLR Pathway PCR Arrays (SA Biosciences, Frederick, MD) following the manufacturer’s instructions. Reverse transcription was performed on 500 ng total RNA using the RT2 First Strand Kit (SA Biosciences). Quantitative real-time PCR were performed (Light Cycler 480; Roche) with 45 cycles at 94˚C for 15 s and 60˚C for 60 s. Fold changes were calculated for each gene using the manufacturer’s web-based PCR Array Data Analysis. The data was deposited at the Gene Expression Omnibus database (under accession number GSE29131; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29131).

ELISA

IL-6, TNF, IFN-γ, IL-2, IL-4, and IL-1β proteins were measured from cell supernatants using DuoSet human immunassay kits (R&D Systems, Minneapolis, MN); IFN-β cytokine was measured by VeriKine Human IFN-β ELISA Kit (PBL InterferonSource, Piscataway, NJ) following the manufacturer’s instructions. Total IgG levels from DC–T–B cell cocultures were measured by the Human IgG ELISA Kit (Immunology Consultants Lab, Newberg, OR), and the IgG subtypes were identified by the Human IgG Subclass Profile ELISA Kit (Invitrogen, Camarillo, CA). The OD of the wells was determined using a microplate reader set at 450 nm.

Migration

DCs were suspended in migration medium (0.5% BSA in RPMI 1640) at 10^6 cells/ml. Transmigration inserts (diameter 6.5 mm; pore size 5 µm) were obtained from Sigma-Aldrich. MIP3α (PeproTech) was diluted at 200 ng/ml in migration medium and added to the lower chambers in a final volume of 600 µl. DCs were added to the upper chamber in a final volume of 250 µl, and chemotaxis assays were conducted for 4 h in 5% CO2 at 37˚C. At the end of the assay, the inserts were discarded, and cells migrated to the lower chamber were collected. Migrated cell numbers were determined by flow cytometry.
were counted by using polystyrene standard beads (Sigma-Aldrich) by flow cytometry.

Transfection of small interfering RNA

A mix of three different constructs of AIM2, DAI, IRF3, IRF7, HMGB1, HMGB2, IFN16, and IPS-1 small interfering RNAs (siRNAs) and control siRNAs (Applied Biosystems) was transfected into 2×10^6 DC to a final concentration of 2.5 nM using the GenePulse X Cell electroporator and 0.4 cm cuvettes (Bio-Rad, Hercules, CA). After 4 additional d, the knockdown of the genes was tested by quantitative RT-PCR.

MLR

The monocyte-derived DCs were activated as mentioned above, and the MDCs were cocultured with allogeneic naive CD4^+ , CD8^+ , and total T cells or with B cells purified from peripheral blood using the Human Naive CD4^+ T cell Isolation Kit II (purity ≥98%), the Human Naive CD8^+ T cell Isolation Kit (purity ≥95%) (Miltenyi Biotec), the RosetteSep Human T cell Enrichment Cocktail (StemCell Technologies, Vancouver, Canada), or anti-CD19–coated magnetic beads (purity ≥98%) (Miltenyi Biotec), respectively, in 1:5 DC-T/B cell ratios for different time points. In some of the experiments, total T cells were replaced by soluble CD40L (25 μg/ml; PeproTech) to activate B cells. T and B cells were labeled with CFSE. T and B cell activation was monitored by cytokine production (IFN-γ; PeproTech) to activate B cells. T and B cells were labeled with CFSE. T and B cell activation was monitored by cytokine production (IFN-γ, IL-2, and IL-4), proliferation (CFSE staining), and cell-surface expression of activation molecules (CD25, CD69, and CD86).

In vitro tumor killing assay

Monocyte-derived DCs were differentiated and matured as mentioned before. Naive CD4^+ and CD8^+ T cells were purified using the above-mentioned magnetic cell separation method and cocultured with DC or the DNA-, LPS-, or inflammatory cytokine mixture-activated DCs and the EBV-specific Burkitt lymphoma cell line Oku-1 (latency type I) (40) in different target/killer ratios (from 1:10 to 1:50). After 4 d of coculture, fresh CFSE-labeled Oku-1 cells were added to the cultures. After an additional 4-h incubation, the cells were labeled with active caspase-3–specific Abs (BioLegend), and the percentages of active caspase-3–CFSE double-positive cells were measured by flow cytometry.

Statistical analysis

Statistical comparisons were made using unpaired t test, and level of significance was set to 0.05 (labeled with an asterisk in figures). The statistical calculations compared experimental samples to the IDC or non-treated (N.T.) samples unless otherwise noted. For all experiments, the mean and SD are reported for at least n = 3, except in the case of the gene expression experiments, in which one representative data set out of at least three independent experiments is shown.

Results

Characteristics of cytosolic DNA-activated human monocyte-derived and primary DCs

To determine the capacity of cytosolic dsDNA to activate and mature human DCs, poly(dA:dT) was transfected into monocyte-derived DCs (henceforth DNA-DCs) by the Lyovec transfection reagent, and the level of activation was compared with two classical DC stimuli: LPS or an inflammatory cytokine (TNF, IL-1 β, IL-6, GM-CSF, and PGE_2) mixture. Poly(dA:dT) was as effective as LPS or the cytokine mixture in inducing DC activation as shown by CD80, CD83, and CD86 expression (Fig. 1A). Cytosolic DNA activated both CD1a^+ and CD1a^− subpopulations of monocyte-derived DCs (Fig. 1B), which have been shown to exhibit different functional properties (41), as well as primary CD11c^+ DCs, purified from human peripheral blood (Fig. 1C). The effect of poly(dA:dT) was dose dependent (Fig. 1D). Monocyte-derived DCs (Fig. 1E), as well as primary DCs (Fig. 1F), produced significant amounts of the proinflammatory cytokines IL-6 and TNF after poly(dA:dT) treatment. The migratory capacity of DNA-DCs toward the MDC chemokine MCP3-β was comparable to that induced by other stimuli (Fig. 1G).

The length of poly(dA:dT) did not dictate the magnitude of maturation of the DCs (Supplemental Fig. 1A, 1C). Besides synthetic DNA poly(dA:dT), natural DNA from different sources (human or foreign) was also able to activate DCs after transfection (Supplemental Fig. 1B, 1D). DNA treatment without transfection (data not shown) or with the Lyovec transfection reagent alone (Fig. 1D) did not activate DCs.

To identify and compare the spectrum of genes induced by cytosolic dsDNA, gene expression arrays focusing on DC-related genes and those implicated in APC functions or TLR pathways were performed. The heat maps showed similar expression profiles in DCs treated with poly(dA:dT), LPS, or the inflammatory cytokine mixture (Supplemental Fig. 2A, 2B). However, DNA- and LPS-DCs appeared to share more commonly regulated genes (Supplemental Fig. 2C, 2D), suggesting that these stimuli employ similar activation mechanisms and/or signaling pathways. Although most genes were induced similarly by all three stimuli, IFN-β expression was markedly upregulated in poly(dA:dT)-activated DCs (fold induction 189×) compared with LPS- (18×) or cytokine mixture-activated DCs (1.7×).

Taken together, the data demonstrate that synthetic or natural cytosolic dsDNA can activate/mature human DCs and suggest that during inflammatory processes such as viral infection or tissue injury, virus-derived or damaged cell-derived dsDNA once inside DCs may trigger their maturation to become full-fledged professional APCs.

DNA-DCs confer killing ability on CD4^+ and CD8^+ T cells

The above data clearly demonstrated the effectiveness of cytosolic DNA in producing activated, mature human DCs. Whether these DNA-DCs could function as professional APCs to invoke a fully functional cellular immunity has not been explored. To determine the type of effector functions that DNA-DCs would impart on CD4^+ and CD8^+ T cells, mixed leukocyte cultures using allogeneic total, naive CD4^+ or CD8^+ T cells from peripheral blood were performed. As shown in Fig. 2A, total allogeneic T cells proliferated slightly better when cocultured with DNA-DCs compared with IDCs. To dissect which subpopulation of T cells were most responsive to DNA-DC signals, total T cells were fractionated into naive CD4^+ or CD8^+ T cells using magnetic cell sorting. As shown in Fig. 2B, naive CD4^+ or CD8^+ T cells, as well as total T cells cocultured with DNA-DCs, displayed higher expression levels of the surface activation marker CD69 compared with LPS-DCs, inflammatory cytokine mixture-DCs, or IDCs. Naive CD4^+ or CD8^+ T cells as well as total T cells cocultured with DNA-DCs produced higher amounts of IL-2 and IFN-γ compared with LPS-DCs, mixture-DCs, or IDCs (Fig. 2C–E). Furthermore, DNA-DC-conditioned CD8^+ cells produced similar levels of granzyme compared with LPS-DCs, mixture-DCs, or IDCs, and total T cells produced the highest levels of granzyme when activated by DNA-DC (Fig. 2F). Surprisingly, a population of DNA-DC-conditioned CD4^+ T cells was also granzyme positive, suggesting that these T cells may be primed to perform killing functions (Fig. 2F).

To test the ability of DNA-DC–primed CD4^+ and CD8^+ T cells to kill target cells, the EBV^+ Burkitt’s lymphoma cell line Oku-1 (latency type I) was chosen as the target. Oku-1 cells were treated with mitomycin C to prevent overgrowth and then cocultured with T cells and DCs for 4 d at different target/killer ratios (from 1:10 to 1:50). To measure the killing capacity of DC-activated T cells, CFSE-labeled Oku-1 cells were added to the cocultures for an additional 4 h after the 4-d presensitization. To identify apoptotic target cells, flow cytometry was used to detect intracellular active caspase-3, which was processed from an inactive proenzyme form. Indeed, DNA-DC–primed CD8^+ T cells were more proficient in killing target cells compared with other MDCs or IDCs (Fig. 3A). Surprisingly, DNA-DC–primed CD4^+ T cells showed similar killing capacity compared with primed CD8^+ T cells. DNA-DCs
were slightly better at generating killer CD4+ T cells than LPS-DCs, mixture-DCs, or IDCs, especially when a 1:20 killer/target cell ratio was used (Fig. 3B). These findings support the notion that the quality and type of CD4+ T cell immunity generated were directly linked to the type of DCs they encountered during an immune response.

DNA-DCs enable T cells to help B cells generate a specific humoral response

Another function of APCs is to collaborate with T cells to induce an effective B cell humoral immunity. To test the efficiency of DNA-DCs to induce B cell responses, MLRs using allogeneic DCs cocultured with B and T cells were performed. Both B and T cells were obtained from the same donor. B cells proliferated well in the presence of DNA-DCs compared with IDCs (Fig. 3C) and expressed more CD86 on their surface (Fig. 3D). DNA-DCs, in collaboration with T cells, were capable of triggering B cells to produce higher levels of IgG compared with cocultures with IDCs or any other MDCs (Fig. 3E). Interestingly, soluble CD40L with DCs or DCs alone could not induce B cells to produce IgG (data not shown), suggesting that the effects of DNA-DCs were manifested mainly through the direct interaction among T cells, DNA-DCs, and B cells. Cytokines reportedly responsible for the Ab production did not appear to participate in the induction of IgG.
because neither IL-4 nor IL-21 cytokines were detected in the supernatants of these cocultures (data not shown). More importantly, IgG subclass profiling revealed that the main IgG subtypes produced by DNA-DC–activated B cells were the effector IgG1 and IgG3 isotypes, which are known potent activators of the complement system, compared with other DCs (Fig. 3F). The noncomplement-fixing IgG2 and IgG4 were not induced (Fig. 3F).

Thus, the type and quality of effector B cells generated during a humoral response depended on the quality and type of DCs they encountered. Again, they underscored the plasticity of DCs in shaping the adaptive immune system.

**DNA-DC–produced IFN-β is dispensable for dsDNA-induced DC activation**

The experiments above clearly showed that dsDNA can effectively activate human DCs and trigger potent adaptive immune system activation. Therefore, next we investigated the mechanism of this activation. Because the gene array experiments showed a significantly increased level of IFN-β expression in poly(dA:dT)-activated DCs compared with LPS- or cytokine mixture-activated DCs (Supplemental Fig. 2B), next we investigated the role of IFN-β in the maturation and function of DCs. First, the IFN-β production was confirmed at the gene (Fig. 4A) and protein (Fig. 4B) levels. The upregulation of IFN-β resulted in the induction of two IFN-regulated genes, AIM2 (Fig. 4C) and DAI (Fig. 4D).

Next, we asked how the autocrine-produced IFN-β affects DNA-DC functions, as type I IFNs are known to induce DC activation (42). To answer this question, cells were treated with different concentrations of type I IFN-α and -β or the type II IFN-γ (at 1 ng/ml to 100 ng/ml, data not shown). The low concentration of exogenous rIFN-β, comparable to the levels of IFN-β produced by DCs upon poly(dA:dT) treatment (Fig. 4B), was able to fully activate DCs, monitored by cell-surface molecule expression (Fig. 4E), proinflammatory cytokine production (Fig. 4F), and cell migration (Fig. 4G). In contrast, IFN-γ could not activate monocyte-derived DCs, and IFN-α activated the DCs moderately compared with IFN-β (Fig. 4E–G), even at the highest used concentration (data not shown). Both IFN-α and IFN-β upregulated mRNA expression of AIM2 (Fig. 4H) and DAI (Fig. 4I), but the efficiency of the upregulation was remarkably lower than upon poly(dA:dT) treatment, even at high (100 ng/ml) IFN concentration. The results indicated that cytosolic DNA skewed DCs toward IFN-β, but not IFN-α, (Supplemental Fig. 2B) production, thus the expression of the appropriate effector genes.
To evaluate whether IFN-β was the main trigger factor for DC activation by cytosolic DNA, the two molecules responsible for IFN signaling and the production of type I IFNs, IRF3 and IRF7, were silenced using siRNAs. The siRNAs were effective in specifically silencing both genes (Fig. 5A), and the expression of IFN-β was significantly downregulated by the specific siRNA treatment, especially in the case of the IRF3 and IRF7 double knockdown (Fig. 5B), showing that the IRF3/7 pathway is needed for the IFN-β production in DNA-activated DCs. In contrast, the activation of DCs by poly(dA:dT) was unaffected even when both IRF3 and IRF7 were knocked down simultaneously (Fig. 5C,5D) and had no effect on the ability of DCs to activate T cells (data not shown). Interestingly, the expression of AIM2 and DAI was only slightly lower than in case of the control siRNA treatment (Fig. 5E), suggesting that other mechanisms may also be responsible for the expression of these genes. Furthermore, blocking type I IFN receptor A with neutralizing Abs did not prevent the activation of DNA-DCs by poly(dA:dT) (Fig. 5F, 5G). Poly(dA:dT) could still induce the induction of IFN-β response genes DAI and AIM2 even in the presence of IFNRA neutralizing Abs; however, we could detect a slight decrease in the induction of these genes (Fig. 5H and data not shown).

Thus, dsDNA can activate DCs even if the autocrine production of IFN-β is inhibited, suggesting that IFN-β is dispensable for the activation of DCs by cytosolic dsDNA. However, we could not exclude the possibility that it may contribute to the maturation of DCs at least by inducing some of its target genes.

Cytosolic DNA activation of DCs is independent of AIM2, DAI, and the endosome or RNA polymerase III pathways but depends in part on IFI16

The induction of AIM2 and DAI in MDCs after cytosolic DNA treatment (Fig. 4C, 4D) appears to be, at least partially, indirect and due to the autocrine effect of the secreted IFN-β by the MDCs. Yet, it is not clear whether the induced AIM2 and DAI proteins function as cytosolic DNA sensors in DNA-DCs. To answer this question, specific siRNAs were used to downregulate the expression of these two molecules. Surprisingly, silencing of
AIM2 or DAI did not abrogate the activation of DCs by cytosolic DNA, even when both genes were knocked down simultaneously (Supplemental Fig. 3A–C) and had no effect on the ability of DCs to activate T cells (data not shown). However both AIM2 and DAI siRNAs were effective in knocking down these genes (Supplemental Fig. 4D). To further show that the siRNA knockdown was specific, the production of IL-1β was monitored in activated DCs. As shown in Supplemental Fig. 3E, the production of IL-1β.

**FIGURE 4.** dsDNA-activated DCs produce IFN-β and mature upon type I IFN treatment. Gene expression (A) and protein secretion (B) of IFN-β upon different activation of monocyte-derived and primary DCs. AIM2 (C) and DAI gene (D) expression upon different activation of monocyte-derived and primary DCs. E, DCs were treated with type I (α and β) and type II (γ) IFNs, and the levels of activation were compared by CD80, CD83, and CD86 surface expression. Dashed line: isotype control; thin line: immature DC; thick line: activated DC. IL-6 and TNF production (F) and migratory capacity (G) of the IFN-activated DCs compared with poly(dA:dT) activation. AIM2 (H) and DAI (I) gene expression in IFN-activated DCs compared with poly(dA:dT) activation. n = 3 independent experiments. *p < 0.05.
was reduced when AIM2 was downregulated, showing its role in forming an inflammasome responsible for processing IL-1β (14–17). The data imply that AIM2 and DAI do not function as intracellular sensors responsible for the activation of human DCs by cytosolic DNA in this case.

A recent study suggested that the HMGB proteins function as universal sentinels for nucleic acids, modulating type I IFN as well as inflammatory cytokine production by DNA or RNA (26). However, it is not known whether activated human monocyte-derived DCs express HMGB proteins and whether they serve as DNA sensors. In this study, we show that DCs express HMGB1 and HMGB2 but no detectable amount of HMGB3. Moreover, the expression levels of HMGB2 were significantly upregulated after treatment with poly(dA:dT) or IFN-β (Supplemental Fig. 4A).

FIGURE 5. The type I IFN pathway is not required for DC activation by cytosolic dsDNA. A, Effectiveness of IRF3- and IRF7-specific knockdowns. B, IFN-β gene expression in siRNA-treated DNA-DCs. C, CD80, CD83, and CD86 surface expression of the siRNA-treated, DNA-activated DCs. Dashed line: isotype control; thin line: DNA-DC treated with control siRNA; thick line: DNA-DC treated with IRF3- and IRF7-specific siRNAs. D, IL-6 and TNF production by siRNA-treated DNA-DCs. E, AIM2 and DAI gene expression in siRNA-treated DNA-DCs. F, CD80, CD83, and CD86 surface expression of the IFN receptor neutralizing Ab-treated, DNA-activated DCs. G, IL-6 and TNF production in control or neutralizing Ab-treated DNA-DCs.

n = 3 independent experiments.
However, in human DCs, HMGB1 and HMGB2 did not appear to serve as cytosolic DNA sensors and regulate MDC functions including activation, cytokine production, migration (Supplemental Fig. 4C–E), and T cell activation (data not shown) using specific siRNA treatments (Supplemental Fig. 4B). Similarly, the inhibition of the endosome formation by chloroquine, thus endosomal TLR activation, and of the RNA polymerase III nucleic acid-sensing pathway with the specific inhibitor ML-60218 did not appear to affect human DC activation by cytosolic DNA and DNA-DC functions (Supplemental Fig. 4C–E) or the capacity of DCs to activate T cells (data not shown).

*IFI16*, the newly characterized IFN-inducible gene and cytosolic DNA sensor (22), was upregulated in DCs treated with poly(dA:dT) but not in LPS- or inflammatory cytokine mixture-activated DCs (Fig. 6A). Silencing *IFI16* in DNA-DCs by specific siRNA was efficient (Fig. 6B), and the production of IFN-β by IFI16-silenced DCs was downregulated (Fig. 6C). Inhibition of IFI16 associated with slight inhibition of the upregulation of cell-surface activation molecules CD80, CD83, and CD86 (Fig. 6D); however, the production of proinflammatory cytokines by these DCs was not inhibited (Fig. 6E), and the T cell activation provided by the IFI16-deficient DCs was only slightly downregulated (Fig. 6F).

Taken together, the data indicate that, in humans, activation of DCs by dsDNA is independent of the previously recognized dsDNA sentinels AIM2, DAI, RNA polymerase III, or HMGBs. In contrast, IFI16, the novel cytosolic dsDNA receptor, may play a role in DC activation; however, knocking down this molecule did not significantly affect DC capability to activate T cells. Because the exact role of the IFI16 molecule as an exclusive dsDNA sensor in DCs has not been definitely proven, we cannot exclude the existence of a yet-to-be-identified cytosolic DNA sensor(s) in human DCs.

**Cytosolic DNA activation of monocyte-derived DCs depends on the proteosome and IPS-1 pathways**

Previously published data have shown that signaling and transcription factors such as STING, TRAF6, IPS-1, TBK-1, and NF-kB mediated the genetic reprogramming of DCs activated by dsDNA. To identify which signaling and transcriptional pathways

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**FIGURE 6.** Cytosolic DNA activation of DCs partly depends on the IFI16 sensor. A, *IFI16* gene expression upon DC maturation with different activation stimuli. B, Effectiveness of *IFI16*-specific knockdown. C, IFN-β gene expression by siRNA-treated DCs measured after 4 h of poly(dA:dT) activation by quantitative PCR. D, CD80, CD83, and CD86 surface expression of the siRNA-treated, DNA-activated DCs. Dashed line: isotype control; gray thin line: IDC; black thin line: DNA-DC treated with control siRNA; thick line: DNA-DC treated with *IFI16*-specific siRNA. E, IL-6 and TNF production by siRNA-treated DNA-DCs. F, Total allogeneic T cells were cocultured with IDCs or siRNA-treated, poly(dA:dT)-activated DCs, and IL-2 and IFN-γ cytokine production by T cells was measured by ELISA. *n* = 3 independent experiments.
participated in human DC activation by cytosolic DNA, specific siRNAs against STING, TRAF6, TBK-1, and IPS-1 were used to disrupt the respective pathways. Effective silencing of IPS-1 (Fig. 7A) resulted in the downregulation of the cell-surface molecules CD83 and CD86 (Fig. 7B), a slight downregulation of IL-6, and a significant inhibition of TNF production (Fig. 7C), as well as the migratory capacity of the DCs (Fig. 7D) compared with the control siRNA-treated cells. The T cell activation capacity of IPS-1–silenced DCs was not affected (data not shown). It was not clear whether STING, TRAF6, and TBK-1 regulated cytosolic DNA signaling in human monocyte-derived DCs, because the knockdown siRNAs were not effective (data not shown).

**FIGURE 7.** Cytosolic DNA activation of DCs depends on the IPS-1 and proteosome pathways. A, Effectiveness of IPS-1–specific knockdown. B, CD80, CD83, and CD86 surface expression of the siRNA-treated, DNA-activated DCs. Dashed line: isotype control; thin line: DNA-DC treated with control siRNA; thick line: DNA-DC treated with IPS-1–specific siRNA. C, IL-6 and TNF production by siRNA-treated DNA-DCs. D, Migratory capacity of siRNA-treated DNA-DCs. E, CD80, CD83, and CD86 surface expression of the proteosome inhibitor MG132-treated DCs. Dashed line: isotype control; gray thin line: IDC; black thin line: DNA-DC treated with DMSO; thick line: DNA-DC treated with MG132. IL-6 and TNF production (F) and migratory capacity (G) of MG132-treated DNA-DCs. H, cytokine production by total T cells cocultured with MG132-treated DNA-DCs. n = 3 independent experiments. *p < 0.05.
To inhibit the NF-κB pathway, cell-permeable proteosome inhibitor MG132 was used at a concentration that did not affect cell viability. Treatment of DNA-DCs with the inhibitor resulted in a complete inhibition of DC activation and function monitored by cell-surface molecule expression (Fig. 7E), proinflammatory cytokine production (Fig. 7F), migration (Fig. 7G), and total T cell activation (Fig. 7H). The data suggest that the activation of human monocyte-derived DCs by cytosolic dsDNA required the IPS-1 pathway and a functional NF-κB pathway.

Discussion

At present, there is an emerging interest in understanding the mechanisms by which the innate immune system is able to detect nucleic acids as danger signals. Much of the efforts focus on identifying DNA receptors and pathways leading to cell activation, especially in cells with Ag-presenting capacity. Recent studies in cell lines and mouse knockout systems have elegantly demonstrated that cytosolic dsDNA activates APCs by interacting with unknown intracellular nucleic acid sensor(s) (11, 27, 34, 36, 43). In humans, myeloid DC activation by cytosolic DNA has been observed but only partly characterized (35, 44).

Our results clearly indicate that naturally derived or synthetic cytosolic DNAs induce activation/maturation of human monocyte-derived DCs as well as of primary CD11c+ blood DCs. Activation of DCs by dsDNA resulted in the induction of DC-related genes similar to those present in LPS-DCs and inflammatory mixture-DCs. DNA-DCs and LPS-DCs appeared to share commonly regulated genes, suggesting that these stimuli employed similar activation mechanisms and/or signaling pathways. Although most genes were induced at comparable levels by all three stimuli, IFN-β expression was markedly upregulated in DNA-DCs. In turn, the induced IFN-β, but not IFN-α or IFN-γ, cooperated with cytosolic DNA to mediate the full spectrum of DC activation and function including CD86 upregulation, proinflammatory cytokine production, and the acquisition of migratory capacity. Interestingly, IRF3/IRF7-deficient DCs could be fully activated by cytosolic DNA. Thus, the signaling/transcription pathway(s) by which cytosolic DNA mediated the production of IFN-β was distinct from those that cooperated with cytosolic DNA to induce the expression of IFN-β response genes and function. Furthermore, the data suggested that IFN-β is important but not required for DC activation/maturation by cytosolic DNA.

In contrast, the activation of the NF-κB pathway was absolutely required for the acquisition of the full spectrum of DNA-DC function such as CD86 upregulation, proinflammatory cytokine production, migration, and T cell activation. In contrast, the adaptor protein IPS-1 was necessary for the induction of CD83 and CD86, TNF production, and migration of DC by cytosolic DNA but it was not required for the activation of T cells by DNA-DCs. Thus, in human DCs, the interaction of cytosolic DNA with the receptor activated the NF-κB pathway, in part via the IPS-1 adaptor molecule, which then mediated the induction of all of the functional characteristics of mature APCs.

Systemic elimination of the known nucleic acid sensors by siRNA knockdown or by specific inhibition showed that AIM2 and DAI (13–17) did not abrogate the activation of DCs by cytosolic DNA. Similarly, the inhibition of the endosome formation by chloroquine, thus endosomal TLR activation (5), and of the RNA polymerase III nucleic acid sensing pathway with ML-60218 (23, 24) did not appear to affect human DC activation by cytosolic DNA and DNA-DC functions. A recent study had suggested that the HMGB proteins function as universal sentinels for nucleic acids and modulate type I IFN as well as inflammatory cytokine production by DNA or RNA (26). However, it was not known if human monocyte-derived DCs expressed HMGB proteins and if they served as DNA sensors. Indeed, DCs were found to express HMGB1 and HMGB2 but not HMGB3. Moreover, the expression levels of HMGB2 were significantly upregulated after treatment with poly(dA:dT) or IFN-β. However, in humans, HMGB1 and HMGB2 did not appear to serve as cytosolic DNA sensors and to regulate DC functions including activation, cytokine production, migration, and T cell activation.

The newly characterized cytoplasmic DNA sensor IFI16 (22) was exclusively upregulated in DCs treated with poly(dA:dT) but not in LPS- or inflammatory cytokine mixture-activated DCs. IFI16-deficient DCs produced less IFN-β and cell-surface activation molecules showing the functional effectiveness of the gene knockdown. However, the production of proinflammatory cytokines (IL-6 and TNF) by these DCs was not inhibited, and the T cell activation provided by the IFI16-deficient DCs was only slightly downregulated. In contrast, IFI16 deficiency resulted in a significant downregulation of IL-6 and TNF gene expression in mouse RAW264.7 cells and embryonic fibroblasts (22). The difference between our results and those of Unterholzner et al. (22) may represent the difference between humans and mice and/or the type of DNA used [a 70 bp-long vaccinia virus DNA transfection and infection with HSV-1 versus poly(dA:dT)].

Our data showed that IFI16 played a minor role in human DC activation by dsDNA and in the subsequent activation of the adaptive immune system, thus we could not exclude the existence of a yet-to-be-identified cytosolic DNA sensor(s).

In humans, the increasing number of DC subsets residing in unique niches exhibiting distinct activation and maturation states suggested that, depending on the nature of the extrinsic or intrinsic signals, each subset of DCs should induce a specific adaptive immune response. This hypothesis was further supported by our results showing that in MLCs, dsDNA-activated DCs, but not LPS- or inflammatory cytokine mixture-activated DCs, induce naive CD4+ T cells to produce more IL-2, granzyme, and IFN-γ but not IL-4, and these effector CD4+ T cells efficiently kill tumor cells in vitro cultures. The existence of CD4+ cytotoxic T cells, capable of killing tumor and virally infected cells, had been documented in the mouse and cell lines (45–47) as well as in humans (48–50). However, this was the first demonstration, to our knowledge, that human DCs could be conditioned by cytosolic DNA to endow in vitro-primed naive CD4+ T cells with killing capacity. In addition, in the presence of T cells, dsDNA-activated DCs, but not LPS- or mixture-activated DCs, activated B cells to produce complement-fixing IgG1 and IgG3 but not non–complement-fixing IgG2 and IgG4 Abs. This finding extended the knowledge on how DCs were able to enhance plasma cell differentiation (51–53). DNA-DCs could also generate CD8+ cytotoxic T cells that produced the effector proteins IFN-γ and granzyme.

Interestingly, the activation/maturation of DCs by cytosolic DNA was dose dependent, suggesting that the amount of cytosolic nucleic acids released by intracellular viruses or parasites dictated the functional outcome of DNA-activated DCs. These data further emphasized the functional plasticity nature of human DCs and their monocyte precursors imparted by different stimuli.

In recent years, the recognition that DCs play a pivotal role in the initiation and regulation of the adaptive immunity and the realization that adjuvants acted primarily as DC activators lead to the development of preventive and therapeutic vaccines using DCs (54). Despite important breakthroughs that murine studies had contributed to our understanding of DC biology, subtle but highly relevant differences between the human and mouse immune systems have been identified (55, 56). Therefore, to successfully generate effective human DC vaccines, complete understanding of
Disclosures

The authors have no financial conflicts of interest.

References


