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Inhibitory oligodeoxynucleotides downregulate herpes simplex virus-induced plasmacytoid dendritic cell type I interferon production and modulate cell function

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KEYWORDS
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Summary Recognition of nucleic acids by TLR9 expressed by human plasmacytoid dendritic cells (PDC) plays a key role in the defense against viral infections. Upon microbial pathogen stimulation, PDC secrete large amounts of type I interferon and arbitrate thereby both innate and adaptive immune mechanisms. Unmethylated CpG motifs, which are an integral part of bacterial or viral DNA, are used *in vitro* and *in vivo* to activate the TLR9 pathway, whereas inhibitory oligodeoxynucleotide (iODN) are capable of depressing TLR9 signaling. In this study we demonstrate that TTAGGG motifs containing iODN efficiently block the TLR9 signaling in terms of herpes simplex virus (HSV)-induced type I interferon production by PDC. However, iODN, as well as control ODN, still promote PDC maturation with upregulated expression of costimulatory molecules, major histocompatibility complex molecules, and other signs for PDC maturation. Furthermore, iODN and control ODN incubated PDC demonstrate increased T-cell stimulatory functions. Coculture experiments with autologous T cells indicate that iODN-treated PDC induce more $CD4^+CD25^+Foxp3^+$ T regulatory cells from naive $CD4^+$ T cells and preincubation of HSV-stimulated PDC with iODN upregulated T cells' IFN-γ production. These data indicate that iODN, while blocking type I interferon production by PDC, modify PDC activation and maturation as well as T-cell priming and stimulation. Knowledge about the different functions of iODN on PDC elucidated might be crucial for immunotherapeutic strategies in which iODN motifs are used to prevent the interaction of CpG-DNA with TLR9 to calm down specific immunological responses, because our data indicate that iODN might not only have inhibitory functions but also be effective activators of immune cells.

Introduction

Human plasmacytoid dendritic cells (PDC), also known as interferon-producing cells [1–3], release high amounts of type I interferon (IFN) after pathogen challenge [3–5]. PDC selectively express Toll-like receptor (TLR) 7 and TLR9 [1,2] and are capable of sensing nucleic acids of microbial pathogens via pathogen recognition receptors, which are an integral part of the innate immune system. Upon stimulation with viral DNA, PDC secrete high amounts of IFN-α/β [6–8], which directly links PDC from the innate to the adaptive

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ABBREVIATIONS

BDCA	blood dendritic cell antigen
cpm	counts per minute
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
HSV	herpes simplex virus
IFN	interferon
IL	interleukin
iODN	inhibitory oligodeoxynucleotide
ISS	immunostimulatory oligonucleotide sequence
IRS	immunoregulatory oligonucleotide sequence
mAb	monoclonal antibodies
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MOI	multiplicity of infection
PBMC	peripheral blood mononuclear cells
PDC	plasmacytoid dendritic cells
RFI	relative fluorescence index
TLR	Toll-like receptor
UV	ultraviolet

immune system and inhibits viral replication by activation of effector cells such as NK cells, macrophages, and cytotoxic T cells [9]. Furthermore, type I IFN secreted by PDC modulates the maturation of bystander dendritic cells and promotes priming of $CD4^+$ T cells toward Th1 immune responses [10]. Following cytokine secretion, PDC differentiate into DC with high expression of costimulatory as well as surface molecules involved in antigen presentation [11–13]. Thus, PDC are regarded as potential therapeutic target cells in the treatment of autoimmune, neoplastic, or infectious diseases.

Herpes simplex virus (HSV) triggers PDC type I IFN production with the help of TLR9 recognizing viral DNA *in vivo* and this can be mimicked *in vitro* using either ultraviolet (UV) inactivated HSV or purified genomic HSV DNA [6,8]. Mainly, two types of DNA sequence are able to modulate TLR9 signals in PDC: Immunostimulatory sequences (ISS), existing in most pathogen DNA, activate the TLR9 pathway, induce strong Th1 responses, and are therefore used as adjuvants in different immunotherapeutic approaches [14]. Inhibitory or immunoregulatory DNA sequences (IRS) [15] neutralize ISS activation via the TLR9 pathway. Inhibitory sequences exist in many species, such as viral DNA, mutated CpG sequences, and repeat TTAGGG motifs in mammalian telomeres [16–19]. The most potent inhibitory sequence, the 4 repeat (TTAGGG) sequence, is used in this study to modulate HSV-induced PDC TLR9 signaling activation [19]. The exact mode of action of these IRS is unclear so far. Therefore, further investigation of IRS and their influence on immune cells is indispensable to understand TLR9 modulation to target these pathways for the treatment of diseases with inappropriate TLR9 signaling. However, studies on the functional impact of inhibitory oligodeoxynucleotide so far available are mainly limited to TLR9 signaling in murine models, whereas studies on inhibitory oligodeoxynucleotide induced changes of human PDC are rare. Therefore, we investigated phenotypic and functional changes induced by iODN on human PDC.

Subjects and methods

Inhibitory oligodeoxynucleotide and UV inactivation of HSV

The inhibitory TTAGGG motif containing phosphorothioate ODN was obtained from Invitrogen (Carlsbad, CA, USA). The sequence of inhibitory oligodeoxynucleotide was 5′-TTT AGG GTT AGG GTT AGG GTT AGG G -3′ (25 mer). The sequence of phosphorothioate control ODN to TTAGGG containing iODN was 5′-TTC AAA TTC AAA TTC AAA TTC AAA-3′ (Sigma). To examine different iODN effects, iODN 2088 (5′-TCC TGG CGG GGA AGT-3′; Invitrogen) was also used in the study. The ODNs were dissolved in endotoxin-free water supplied by Invitrogen. HSV type 1 strain 17^+ was propagated on Vero cells in maintenance medium composed of Eagle's minimum essential medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom) with Earle's balanced salts. For virus stock preparation, Vero cells were infected with HSV at a multiplicity of infection (MOI) of 0.1. Cell culture supernatant was harvested 72 hours after inoculation and clarified by centrifugation at 1.931*g* for 10 minutes followed by 30 minutes of lasting centrifugation at 3.434*g*. Virus was inactivated in complete darkness by UV light at 254 nm. Samples were irradiated for 30 minutes in sterile petri dishes containing a magnetic stir bar stirring at low speed (thickness of virus suspension 1.5–2 mm, distance between virus suspension and mercury vapor lamp 15 cm). Determination of viral titer before and after UV radiation was performed by titration in microtiter plates using Vero cells starting with undiluted virus suspension. Additionally, supernatants of mock-infected Vero cells were prepared as controls. Inactivated HSV and mock control was stored at -80°C before use. All materials in medium with working concentration to stimulate PDC were analyzed for endotoxin and contained only very low endotoxin levels ($<$0.005 EU/ml; tested by Cambrex Bio Science Verviers, Belgium).

Isolation and stimulation of PDC

Peripheral blood mononuclear cells (PBMC) were isolated as described previously [20] from buffy coats of human healthy blood donors provided from the blood bank of the University of Bonn. PDC were isolated using a human BDCA-4 MicroBead isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as described before [20]. PDC were $>$90% $BDCA2^+CD123^+$ as measured by flow cytometry. PDC were diluted at 1 million cells/ml and seeded at 0.2 million cells/well into 96-well round-bottom plates in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, L-glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100 μg/ml), and recombinant IL-3 (10 ng/ml). PDC were divided into four groups: PDC incubated with inactivated Vero cell supernatant (control group), PDC stimulated with UvHSV (MOI = 1) (UvHSV group), PDC stimulated with 4 μM TTAGGG containing iODN (iODN group), and PDC stimulated with UvHSV (MOI = 1) combined with iODN(4 μM) (iODN + UvHSV group). Before the addition of UvHSV, PDC were preincubated for 40 minutes with iODN to allow full uptake of iODN as described elsewhere [21]. To examine different ODN stimulatory effects to PDC, a TTAGGG motif containing iODN (iODN), iODN 2088 (iODN 2088), and control ODN (Con ODN) was added to PDC at a final concentration of 4 μM; untreated PDC were left as blank control. All PDC were cultured for 18 hours at 37°C, 5% CO_2.