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TLR9 Contributes to the Recognition of EBV by Primary Monocytes and Plasmacytoid Dendritic Cells

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TLR9 plays an important role in innate defense against viruses by the detection of CpG motifs of foreign DNA within intracellular compartments. In this study, we evaluated the ability of EBV to promote monocyte and plasmacytoid dendritic cell (pDC) activation and cytokine release through TLR9 activation. We demonstrated that treatment of primary monocytes with EBV and with purified EBV DNA induced the release of IL-8 through TLR9. Activation of TLR9 by viral DNA requires endosomal maturation because pretreatment of monocytes with chloroquine strongly reduced IL-8 secretion. However, pretreatment of monocytes with siRNA directed against TLR2, with inhibitory ODN (iODN) or with a combination of both inhibitors strongly reduced the secretion of IL-8, providing evidence of a dual action of TLR2 and TLR9 in EBV recognition by monocytes. In contrast, production of MCP-1 and IL-10 in EBV-treated monocytes was mainly regulated through TLR2. Although EBV does not establish infection in pDCs, challenge with either live EBV particles or isolated EBV DNA was found to induce the release of IFN-α through TLR9, as supported by blockage of TLR9 activity with iODN or chloroquine. The role of TLR9 in the recognition of EBV by pDCs appears to be dominant, as confirmed by the marked inhibitory effect of iODN observed on the synthesis of IFN-α, IL-6, and IL-8 by pDCs. These results demonstrate that recognition of EBV by TLR9 is differently orchestrated in primary monocytes and pDCs to optimize viral recognition and antiviral response. *The Journal of Immunology*, **2010, 185: 3620–3631.**

EBV is known as the etiologic agent of infectious mononucleosis, a lymphoproliferative disorder generally observed in young adults, and is also associated with hematologic malignancies detected in immunocompromised patients. After primary infection, EBV establishes latency to ensure its life-long persistence in the host. Although the major target cells for EBV infection are B lymphocytes, many other cell types have been reported to be permissive of EBV (1–5). Because EBV has the potential to modulate the immune system by altering several cellular functions (6), a rapid detection of the virus is therefore essential to limit the spread of infection and to control the outgrowth of latently infected B cells.

TLRs represent the most common family of innate sensors characterized by their ability to recognize a broad range of pathogen-associated molecular patterns (PAMPs) and host associated-damage molecules. TLR engagement following viral stimulation results in a rapid production of inflammatory cytokines and antiviral mediators, such as type 1 IFN. Among those, cell surface TLR2 was reported to recognize several PAMPs conserved on virions from different members of human herpesviruses. In fact, CMV, herpes simplex virus (HSV) type 1 and type 2, and varicella zoster virus were all found to induce the release of proinflammatory cytokines in a TLR2-dependent manner (7–10). Recently we have demonstrated that stimulation of human monocytes with either inactivated or infectious EBV virions leads to the release of monocyte chemoattractant protein-1 (MCP-1) and to an increase of several cytokine mRNA levels, including IL-8, through the engagement of the cell surface TLR2 (11). After virus entry into the cell, viral particles are degraded in endosomal compartments, and their nucleic acid content is exposed to intracellular TLRs. Their involvement in antiviral immunity has been extensively studied, particularly in plasmacytoid dendritic cells (pDCs), which predominantly express TLR7 and TLR9. TLR9 is an important receptor for nucleic acid-containing unmethylated CpG motifs present in both bacterial and viral DNA (12, 13), whereas TLR7 senses single-stranded RNA (14).

Dendritic cells (DCs) and monocytes are important APCs linking the innate and the adaptive immunity; they are rapidly mobilized at sites of viral infection and produce a broad range of mediators contributing to the activation of the immune response. pDCs are known as an important actor of the antiviral immunity by their capacity to produce large amounts of type 1 IFN, and monocytes are recognized to produce several cytokines through a TLR-dependent pathway. Herpesviruses, such as HSV-1 and HSV-2, and murine CMV were reported to trigger expression of type 1 IFN by DCs through TLR9 signaling (13, 15–17). pDC activation of $CD3^+$ T cells to produce IFN-γ in response to EBV was also suggested to involve TLR9 (18).

Increasing evidence indicates that monocytes and DCs may have important implications in EBV pathogenesis. First, it was demonstrated that EBV has the capacity to infect monocytes and DCs (2, 19–23). In addition, infection of monocytes by EBV was reported to inhibit their development into DCs (24), a process that may affect normal regulation of EBV-host interactions because DCs are known to play a crucial role in the induction of primary T cell response against viruses, including EBV (25, 26). Monocytes were also suggested to contribute to the spread of EBV infection. In fact,

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Abbreviations used in this paper: CQ, chloroquine; DC, dendritic cell; EBV-GFP, EBV-tagged GFP; HSV, herpes simplex virus; iODN, inhibitory oligonucleotide; moi, multiplicity of infection; NS, not stimulated; NT, nontransfected; ODN, oligonucleotide; PAA, phosphonoacetic acid; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell.

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monocytic cells were found to facilitate EBV dissemination across the oral epithelium, causing lesions usually observed in HIV-infected patients (27). Therefore, we must consider that monocytes and DCs are likely to be involved during the course of EBV pathogenesis.

The interactions of EBV with the TLR system are largely unknown. In this study, we evaluated the role of TLR9 in EBV recognition by primary monocytes and pDCs. Our results demonstrate that TLR9 recognizes EBV and is orchestrated with other TLRs expressed in monocytes and pDCs to optimize cytokine production.

Materials and Methods

Isolation of primary monocytes, plasmacytoid DCs, and culture conditions

PBMCs were isolated from fresh heparinized blood of healthy donors. PBMCs were first separated over a lymphocyte separation density gradient (Wisent, Québec, Canada). Monocytes were separated from lymphocyte population by cell adherence on Petri dishes and further sorted by flow cytometry for a higher purity (~99%; BD FACSAria II, BD Biosciences, Ontario, Canada). Plasmacytoid DCs were purified using the Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs were incubated with a biotin-conjugated Ab mixture against non-pDC populations (e.g., B cells, T cells, NK cells, monocytes, myeloid DCs, and granulocytes). Subsequently, antibiotin-conjugated magnetic beads were added to the PBMC suspension. Magnetically labeled cells were retained within the column while untouched pDCs were collected in the flow-through, resulting in pure pDC population. pDCs carry distinctive marker pattern and are defined as $CD303^+$, $CD304^+$, $CD123^+$, $CD45Ra^+$, and $CD11c^-$ cells. A CD304 Ab was used to analyze pDC purity (~98%) in flow cytometry (Miltenyi). All primary cells were cultured in RPMI 1640 (Wisent) complemented with 10% heat-inactivated FBS (VWR, Ontario, Canada).

Virus purification and viral DNA isolation

Purified EBV was obtained from the productive EBV B cell lineage B95.8 as described previously (11). B95.8 cells were cultured and amplified in RPMI 1640 medium supplemented with 10% FBS and treated with PMA (20 ng/ml) to induce viral replication. When cellular mortality reached 80%, as determined with trypan blue dye exclusion procedure, supernatant was collected and filtered on a 0.45-μM size pore filter and ultracentrifuged. Viral titers were evaluated as described (11) and expressed as transforming units per milliliter. When indicated, UV-irradiated EBV virions were obtained by exposure of viral suspension under UV light during 30 min (265 nm) (28). EBV-tagged GFP (EBV-GFP) was obtained from culture of Akata-EBV-GFP positive cells stimulated with antihuman IgG (Sigma-Aldrich) as previously described (29, 30). EBV-GFP particles were purified from culture supernatants, as processed for EBV-B95.8 cells. Efficiency of EBV-GFP infection of primary human monocytes and pDCs was evaluated by flow cytometric analysis and compared with EBV-GFP–infected BJAB cells as positive control.

Viral dsDNA was purified from concentrated EBV-B95.8 suspension. First, virions were incubated for 7 h at 37°C in detergent solution (31) supplemented with 350 μg/ml proteinase K (Fisher Scientific, Whitby, Ontario, Canada). DNA was isolated by phenol-chloroform extraction and precipitated with sodium acetate 3.3 M at pH 5.5. DNA was resuspended in endotoxin-free water (Sigma-Aldrich) and visualized on agarose gel, and concentration was determined by spectrophotometry (260 nm).

Inhibitory oligonucleotides and small interfering RNA sequences

Human-specific type A CpG ODN sequence 2216 (5′-ggG GGA CGA TCG TCg ggg gg-3′) and human type B CpG ODN 2006 (5′-tcg tcg ttt tgt cgt ttt gtc gtt-3′) were resuspended in endotoxin-free water and used as positive control for TLR9 activation at the indicated concentration (lowercase letters are phosphorothioate linkage and capital letters are phosphodiester linkage 3′ of the base). To prevent TLR9 and CpG colocalization in endosomal vesicles and to inhibit TLR9 activation by its agonist, we used the human ODN inhibitory sequence (5′-ttt agg gtt agg gtt agg gtt agg g-3′). Inhibitory ODN were also resuspended in endotoxin-free water and used at indicated concentrations in pretreatment during 30 min prior to stimulation with agonists. TLR7 antagonist IRS661 (5′-tgc ttg caa gct tga agc a-3′) (32) was used in pretreatment during 30 min prior to stimulation. All inhibitory oligonucleotide sequences were synthesized by Alpha DNA (Montreal, Québec, Canada). All antagonists had no effect on cell viability tested by trypan blue exclusion.

The predesigned siRNA against TLR2 used in this manuscript has the following sequence: 5′-GGC UUC UCU CUG UCU UGU GAC tt-3′ (sense strand) (Ambion, Austin, TX). Twenty-four hours after cells extraction, monocytes (10^6 cells/ml) were transfected with the indicated amount of siRNA against TLR2 using Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen Canada, Ontario, Canada). Monocyte stimulations were performed 36 h after transfection with EBV, CpG-2006, or PAM_3SCK_4 (Sigma-Aldrich). When indicated, cells were pretreated with 20 μM of chloroquine (CQ; Sigma-Aldrich), a known inhibitor of endosomal acidification, for 30 min prior to stimulation. To assess the potential of the siRNA to block specifically transcriptional activity of the TLR2 gene, nontransfected- and transfected-monocyte were lysed in TRIzol reagent (Invitrogen Canada) and total RNA was isolated according to the manufacturer's instructions. DNase-treated RNA was reverse transcribed using SuperScript reverse transcriptase (Invitrogen Canada) and PCR was performed to evaluate TLR2 expression using the specific primers: (forward 5′-GGC CAG CAA ATT ACC TGT GTG-3′, reverse 5′-CCA GGT AGG TCT TGG TGT TCA-3′. GAPDH expression (forward 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; reverse 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′) was used as internal control.

Cell treatment and measurement of cytokines

Freshly isolated monocytes and pDCs were stimulated as indicated with infectious EBV particles and UV-irradiated EBV particles or were transfected with purified viral DNA using Escort reagent according to manufacturer's instructions (Sigma-Aldrich). When indicated, viral DNA was digested with DNase (1 U/μg DNase of DNA, 40 μg/ml, 1 h at 37°C; Sigma-Aldrich) prior to stimulation. As positive TLR9 controls, monocytes were stimulated with type-B CpG-2006 (40 μg/ml), and pDCs were treated with type-A CpG-2216 (10 μg/ml), which was reported to be a potent inducer of type 1 IFN in pDCs (33). Treatment of pDCs with TLR7 agonist R837 (10 μg/ml) was used as control (InvivoGen, San Diego, CA). When indicated, cells were pretreated with CQ (20 μM), phosphonoacetic acid (PAA; 200 μg/ml), the TLR9 inhibitory ODN (5-fold concentration of DNA), or IRS661 (10 μg/ml; a TLR7 antagonist) for 30 min prior to stimulation. Cell-free supernatants from monocytes were harvested at indicated times after stimulation for IL-8 (Invitrogen Canada), MCP-1, and IL-10 (eBioscience, San Diego, CA) quantifications by ELISA. Culture supernatants from treated pDCs were tested for IL-6 (BioLegend, San Diego, CA), IL-8 (Invitrogen Canada), IL-12p70 (eBioscience), and IFN-α (PBL Interferon Source, Piscataway, NJ) determinations.

Flow cytometric analysis

TLR9 expression was evaluated using FITC-conjugated antihuman TLR9 (clone 5G5; Hycult Biotechnology, Uden, The Netherlands). Stimulated cells were fixed in paraformaldehyde 2% for 30 min and permeabilized with methanol 100% for 2 min before labeling with anti-TLR9 Ab. Expression levels of costimulatory proteins CD40 and CD86 on pDCs were determined using peridinin-chlorophyll-protein complex-cyanine (PerCP/Cy5.5)-conjugated antihuman CD40 (clone 5C3) and PE-conjugated antihuman CD86 (clone IT2.2; Biolegend). Cell samples (10,000 events) were analyzed using a BD LSR II flow cytometer system (BD Biosciences), unless otherwise indicated in the figure legend.

EBV infection of monocytes and pDCs was visualized using EBV-GFP particles produced from Akata cells (30). Monocytes were first pretreated or not with iODN (100 μg/ml, 1 h), CQ (20 μM, 1 h), or with anti-TLR2 Ab (clone TL2.5; 0.1 μg/ml, 15 min; Invivogen) prior to infection with EBV-GFP virions (multiplicity of infection [moi] of 1.0) for 48 h. Infection of pDCs with EBV-GFP (moi of 2.0) was performed for a period of 24, 48, or 72 h. BJAB cells were used as a positive control. Analyses were achieved using BD LSR II flow cytometer system (BD Biosciences) on a total of 10,000 cells.

Confocal microscopy

Intracellular trafficking of TLR9 and CD63 was performed using late endosome marker CD63 (mouse antihuman CD63, clone MEM-259; Biolegend) followed by a Cy3-conjugated antimouse IgG (Jackson Laboratories, Ontario, Canada). TLR9 labeling was done with a rat antihuman TLR9 (clone eB72-1665; eBioscience) detected by a Cy5-conjugated antirat IgG (Jackson Laboratories). Before labeling, cells were washed once with cold PBS and fixed with fresh paraformaldehyde 2% for 30 min. Cells were washed in PBS-BSA 1%-saponin 0.05% washing buffer and blocked in PBS-BSA 3%-saponin 0.05% for 20 min. Slides were mounted with Prolong Gold antifade reagent (Invitrogen Canada).

Confocal laser scanning microscopy was performed with a BX-61 microscope equipped with the Fluoview SV500 imaging software 4.3 (Olympus America, Melville, NY), using a 100× Plan-Apochromat oil-immersion ob-

jective (NA 1.35) and a 7× zoom ratio in the region of interest; 0.04 μm confocal z-series were acquired for each observation area and filtered by two-frame Kalman low-speed scans.

Statistical analysis

Data for secretion assays were analyzed by one-tailed ANOVA followed by Newman-Keuls post hoc test using PRISM3 software (GraphPad Software, La Jolla, CA). Bars represent SEM. Differences were considered significant at $p \leq 0.05$.

Results

EBV increases TLR9 expression level in primary monocytes

During the last decade, much attention has been directed on TLRs as key sensors of the innate response to detect viral components and to activate production of inflammatory cytokines. Interactions of EBV components with intracellular TLRs are not well documented. Because TLR9 is considered as an important sensor that recognizes viral CpG DNA following virus entry into the cell, we wanted first to assess whether EBV infection may modulate intracellular TLR9 expression in primary monocytes. Purified monocytes were stimulated with either EBV or with CpG-2006 as control. The results demonstrate that EBV stimulation induces an increase of TLR9 expression after 24 h of culture (Fig. 1*A*). In contrast, expression levels of TLR9 were downregulated in monocytes after CpG-2006 stimulation and were found to be unchanged in cell cultures composed of T and B lymphocytes after treatment with EBV or CpG-2006 (Fig. 1*B*). TLR9 expression level was also slightly increased in pDCs after 24 h of stimulation with EBV (Fig. 1*C*), but no variation was detected after CpG-2216 stimulation. Increased TLR9 expression induced by EBV was abolished by CQ treatment (data not shown). These observations suggest that depending on the cell type, TLR9 expression is differently modulated upon stimulation, as already suggested (34, 35).

Rapid induction of TLR9 recruitment into late endosome following EBV stimulation

It was previously demonstrated that TLR9 is mainly expressed in the endoplasmic reticulum of resting pDCs (36). Following PAMP entry, a migration of TLR9 toward early endosomes is initiated, and a subsequent fusion of those vesicles with lysosomes promotes the formation of late endosomes and their maturation. We next determined whether EBV stimulation of monocytes and pDCs induces TLR9 redistribution in late vesicle compartments. To address this question, cells were treated with EBV, CpG-2006 (monocytes), or CpG-2216 (pDCs) and were stained for TLR9 and tetraspanin protein, also known as CD63, which is associated with late endosomes (37). Confocal microscopic analysis showed a rapid colocalization of TLR9 and CD63 proteins in monocytes, as soon as 15 min postinfection, indicating that TLR9 is redistributed in late endosomes after treatment with EBV (Fig. 2*A*). In resting pDCs, we observed that CD63 is expressed in cytosol (Fig. 2*B*) (36, 38). After 30 min of stimulation with EBV, redistribution of CD63 was observed and was maintained up to 120 min poststimulation. We also visualized TLR9 translocation in late endosomes after stimulation with EBV. Merged confocal images of pDCs stained for TLR9 and CD63 indicate that TLR9 colocalizes in late endosomes in pDCs treated with EBV. These results indicate that EBV (probably when EBV-DNA is internalized) initiates TLR9 redistribution into late compartments to activate TLR9 signaling.

EBV DNA is recognized by TLR9 in primary monocytes

Because we observed that EBV can modulate the cellular redistribution of TLR9, we wanted next to determine whether EBV interacts with TLR9 in monocytes. Treatment with class B CpG-2006 was performed as positive control, because this class of ODN was recognized as a potent TLR9 activator to induce cytokine secretion in different cell populations (39, 40). To confirm the activation of TLR9, cells were also pretreated with CQ, a known inhibitor of TLR9 activation. Purified human monocytes were stimulated with infectious EBV particles for 10 h, and IL-8 levels were assessed in cell-free supernatants by ELISA. IL-8 was chosen because we already observed that the IL-8 gene was activated in monocytes by EBV (11). The results indicate that infectious EBV induced a strong release of IL-8 from human monocytes (Fig. 3*A*). Similar results were obtained upon stimulation with UV-irradiated EBV (data not shown). However, when monocytes were pretreated with CQ to validate that maturation of endosomal vesicles is required for activation of TLR9, production of IL-8 in EBV-stimulated monocytes was partially inhibited by CQ, whereas induction of IL-8 by CpG was strongly impaired. To further confirm that EBV DNA is the ligand responsible for TLR9 activation, monocytes were stimulated with isolated viral DNA. Interestingly, stimulation of monocytes with EBV DNA also induced a strong release of IL-8 (Fig. 3*B*), but in this case CQ treatment significantly reduced IL-8 secretion, which further validates that EBV DNA-induced IL-8 secretion requires endosomal maturation. The effect of EBV DNA was specific; DNAse treatment suppressed its ability to stimulate IL-8 production, but had no effect on LPS-induced IL-8 production by monocytes. Overall, these results suggest that EBV DNA alone has the capacity to activate TLR9, but that other EBV-activated mechanisms may also participate to the production of IL-8 in monocytes.

Effect of TLR2 and TLR9 on the release of cytokines by monocytes stimulated with EBV

We have previously reported that recognition of EBV by TLR2 expressed on monocytes results in the activation of cytokines, including IL-8 and MCP-1 (11). Therefore, we considered that recognition of EBV by monocytes may engage a sequence of at least two TLRs, one expressed on the cell surface of monocytes and the other being intracellular. To corroborate the contribution of TLR2 and TLR9 in the production of proinflammatory cytokines by monocytes, we have suppressed functional activation of TLR2 with siRNA against TLR2 and the activation of TLR9 by treating monocytes with iODN, which specifically targets TLR9 prior to stimulation with EBV. Fig. 4*A* illustrates the knockdown efficiency of siRNA for TLR2. Purified monocytes were then treated with EBV or TLR2- and TLR9-specific agonists, and overnight supernatants were harvested for cytokine determinations. As shown in Fig. 4*B*, pretreatment of monocytes with siRNA or iODN alone reduced but did not entirely suppress the production of IL-8 induced by EBV. However, combined treatment with both inhibitors strongly reduced the capacity of EBV-stimulated monocytes to release IL-8. Interestingly, when supernatants were tested for MCP-1 production, treatment of monocytes with iODN alone did not affect MCP-1 release, although its suppressive effect was perceived against CpG-2006. In contrast, levels of MCP-1 were significantly reduced in supernatants from siTLR2-treated monocytes (Fig. 4*C*). We also evaluated whether release of IL-10, a known antiinflammatory cytokine, is regulated via TLR2 or TLR9 signaling in EBV-infected monocytes. Whereas relatively low levels of IL-10 were detected in the same culture of supernatants (Fig. 4*D*), such production was found to be significantly affected by blocking TLR2 with siRNA, indicating that TLR2 contributes to the secretion of IL-10 by monocytes in response to EBV. However, because production of IL-10 was not totally suppressed by siTLR2, this result may also suggest that other mechanisms activated by EBV could contribute to the release of IL-10. Our results indicate that TLR2 and TLR9 contribute

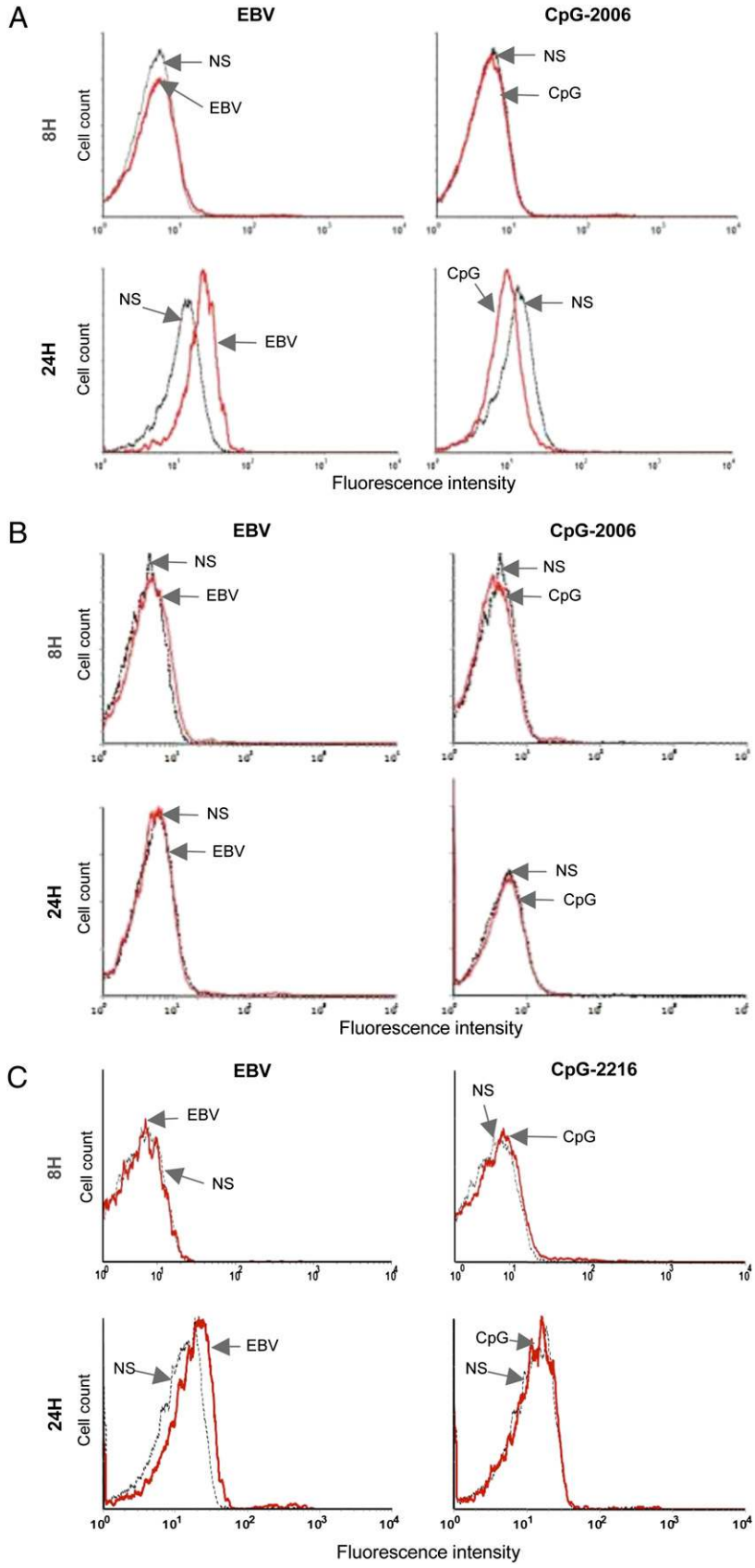

FIGURE 1. EBV modulates TLR9 expression. *A*, Enriched monocytes (10^6 cells/ml) and (*B*) isolated lymphocytes (10^6 cells/ml) were stimulated with CpG-2006 (40 μg/ml), and (*C*) freshly isolated pDCs (10^5 cells) were stimulated with CpG-22116 (10 μg/ml) or with EBV (moi 1.0) for the indicated times. Fixed and permeabilized cells were labeled with an FITC-conjugated antihuman TLR9 and expression levels were determined by flow cytometric analysis. Results displayed are one representative of three different donors. Black dotted lines indicate NS. Red full lines indicate cells stimulated with the indicated agonist. NS, not stimulated.

to the activation of IL-8 by EBV and that both receptors can be independently activated, leading to their additive contribution to produce IL-8 in monocytes. In contrast, TLR2 but not TLR9 seems to be involved in the secretion of MCP-1 and IL-10 induced in EBV-infected monocytes.

EBV triggers IFN-α secretion by human pDCs through TLR9

PDCs are recognized as potent producers of type 1 IFN after stimulation of TLR9. Because we observed that EBV can act on expression levels of TLR9, we next evaluated whether production

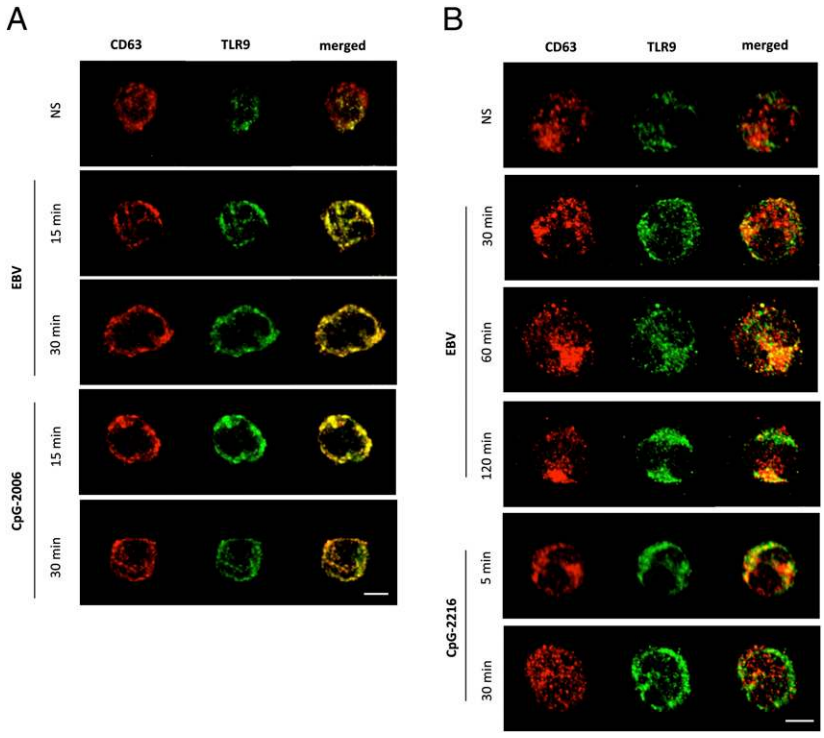

FIGURE 2. EBV induces TLR9 recruitment within late endosomal compartments in monocytes. *A*, Monocytes were treated with infectious EBV (moi = 1.0) or with CpG-2006 (40 μg/ml). *B*, pDCs were stimulated for the indicated times with EBV (moi = 1.0) or CpG-2216 (10 μg/ml). TLR9 expression and colocalization with late endosome were visualized by confocal microscopy using double labeling of cells with anti-TLR9 (FITC, green) and anti-CD63 (PE, red) Abs. Original magnification ×100. Scale bar, 5 μm. NS, not stimulated.

of IFN-α induced by EBV is dependent on TLR9 activation in pDCs. Purified pDCs were stimulated with infectious EBV for 24 h before determination of IFN-α levels. PDCs were also treated with either UV-irradiated EBV particles or PAA (a viral DNA polymerase inhibitor) to prevent viral replication. Stimulation of pDCs with infectious EBV induced the release of IFN-α, and cell pretreatment with CQ totally abolished this effect, indicating that endosomal acidification is essential in TLR9 engagement after viral uptake (Fig. 5*A*). Interestingly, pretreatment of pDCs with PAA or stimulation with UV-irradiated EBV particles did not significantly alter the release of IFN-α by pDCs, suggesting that viral replication would not be required for production of IFN-α (Fig. 5*A*). In addition, we observed that the use of iODN also reduces the secretion of IFN-α in cell cultures stimulated with live or inactivated EBV (Fig. 5*B*), supporting the notion that viral DNA may interact with TLR9.

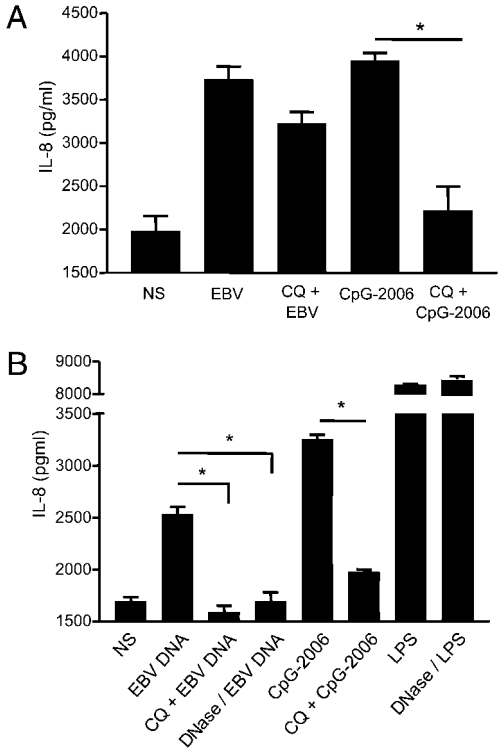

FIGURE 3. Primary human monocytes secrete IL-8 in response to EBV DNA through an endocytic-dependent pathway. Enriched human monocytes (10^6 cells/ml) were stimulated with (*A*) infectious EBV or with (*B*) purified EBV DNA (40 μg/ml), treated or not with DNase (40 μg/ml), LPS (1 μg/ml), or CpG-2006 (40 μg/ml) for 10 h, and supernatants were harvested for IL-8 determinations. When indicated, cells were pretreated with CQ (20 μM) for 30 min prior to stimulation. Results presented are one representative of three experiments. $*p \leq 0.01$, compared with respective control. NS, not stimulated.

To further confirm that EBV DNA is a ligand for TLR9 to promote IFNα secretion, pDCs were stimulated with purified EBV DNA instead of infectious particles. Cell cultures were pretreated with CQ to prevent endosome acidification and activation of TLR9 or with iODN to block activation of TLR9. We observed that pretreatment of pDCs with CQ strongly abrogated the production of IFN-α induced by EBV DNA (Fig. 5*C*), supporting the potential role of TLR9. Treatment of viral DNA with DNase clearly supports the ability of EBV DNA to induce IFN-α by pDCs. In addition, the use of iODN also drastically abolished the capacity of viral DNA to stimulate secretion of IFN-α by pDCs (Fig. 5*D*). Together, these data clearly indicate that EBV DNA is an activator of TLR9, a process requiring acidification of endosomes in pDCs.

Dominant contribution of TLR9 in EBV-induced cytokine secretion by pDCs

Whereas production of IFN-α in EBV-stimulated pDCs seems to be regulated predominantly through TLR9, our results suggest that another mechanism may participate to the release of IFN-α. This hypothesis is supported by the fact that pretreatment of pDCs with iODN did not entirely abolish the secretion of IFN-α induced by EBV in contrast to a stimulation with purified viral DNA. Because pDCs predominantly expressed TLR7 and TLR9, this prompted us to evaluate the engagement of both TLRs in the production

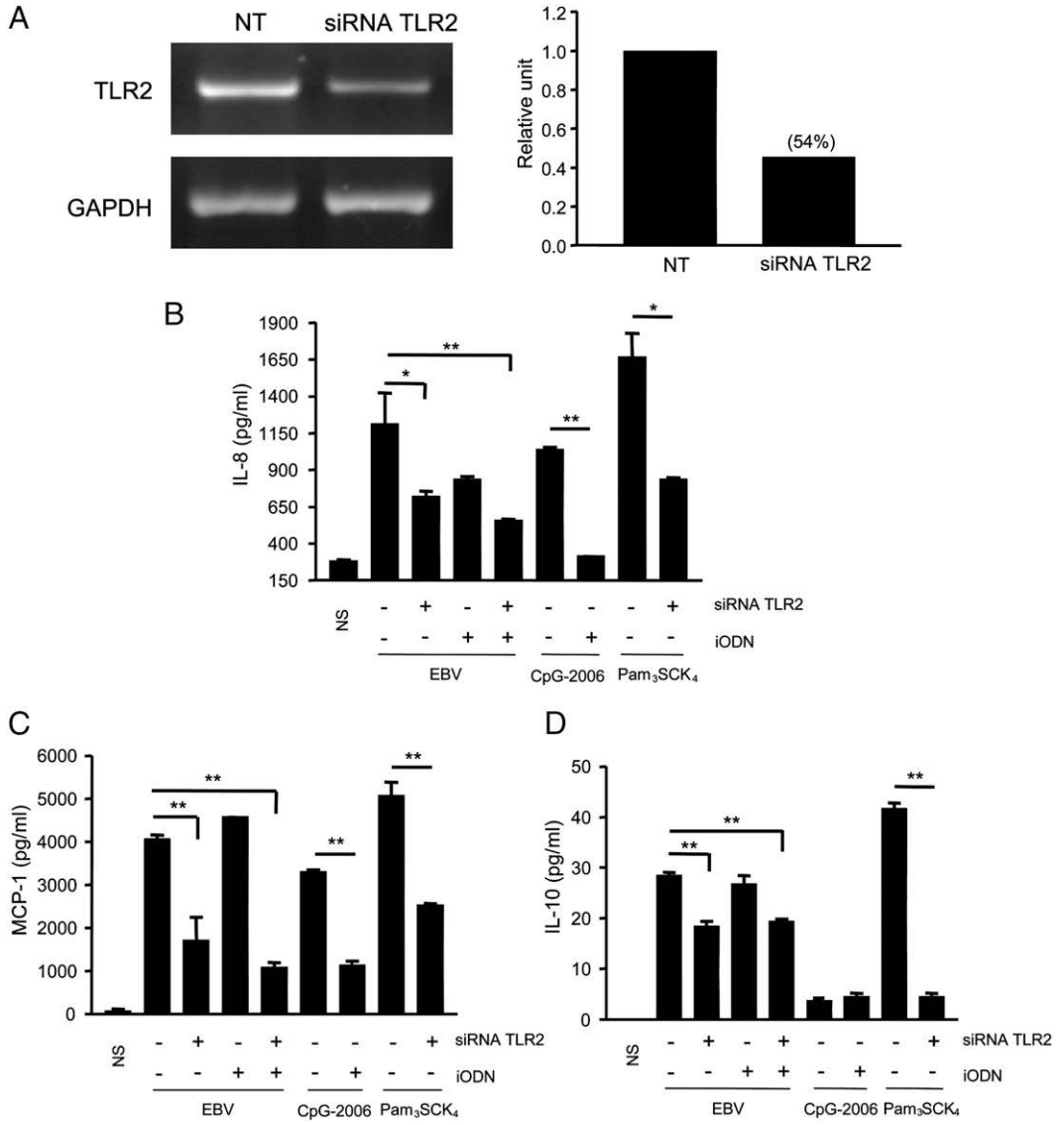

FIGURE 4. The distinctive contribution of TLR2 and TLR9 for cytokine release in EBV-stimulated monocytes. *A*, Purified monocytes (10^6 cells/ml, *left panel*) were transfected with siRNA directed against TLR2 (150 μM) with Lipofectamine 2000. Thirty-six hours after transfection, total RNA was extracted and RT-PCR was performed using specific primers for TLR2 and GAPDH. The *right panel* shows densitometric analysis normalized over GAPDH. The number in parentheses represents the percentage of inhibition. *B–D*, Human monocytes were pretreated with siRNA TLR2 or inhibitory ODN (100 μg/ml) for 30 min, or both, prior to further stimulation with infectious EBV, PAM_3SCK_4 (0.5 μg/ml), or CpG-2006 (40 μg/ml). Overnight supernatants were collected for IL-8 (*B*), MCP-1 (*C*), and IL-10 (*D*) level determinations. Results are representative of three different experiments. $*p \leq 0.05$; $**p \leq 0.01$, compared with samples treated with respective agonists. NS, not stimulated; NT, nontransfected.

of IFN-α by pDCs stimulated with EBV. Enriched pDCs were stimulated with live virus in the presence of either iODN or the TLR7 antagonist IRS661, or in the presence of both inhibitory oligonucleotides. The results clearly show that treatment with the inhibitory ODN significantly reduced the secretion of IFN-α by pDCs treated with EBV, whereas the use of TLR7 antagonist partially affected this synthesis. However, when both antagonists were added to cell cultures, production of IFN-α induced by EBV was dramatically impaired (Fig. 6*A*), suggesting that TLR7 contributes to the production of IFN-α in EBV-stimulated pDCs. We next investigated the respective contributions of TLR7 and TLR9 in the secretion of additional cytokines by pDCs upon stimulation with EBV. Blocking experiments with TLR7 and TLR9 antagonists showed that only TLR9 contributes to the production of IL-6 and IL-8 induced by EBV in pDCs, whereas TLR7 has no effect (Fig. 6*B*, 6*C*). pDCs did not produce a detectable amount of IL-12p70 upon stimulation with EBV (data not shown). Therefore, we can postulate that TLR9 and, to a lesser extent, TLR7 are engaged to induce IFN-α in pDCs, whereas TLR9 is likely the dominant receptor involved in IL-6 and IL-8 secretion in response to EBV.

Treatment of monocytes with inhibitory ODN or CQ alters EBV replication

In a previous report, we demonstrated that EBV can infect human primary monocytes (2). To monitor the infection of monocytes by EBV and to determine whether specific TLRs influence viral infection, we treated cell cultures with recombinant EBV expressing GFP and evaluated the number of positive cells by flow cytometry (30). As expected, BJAB cells (positive control) and primary monocytes have been successfully infected with EBV-GFP (Fig. 7). Pretreatment of monocytes with neutralizing anti-TLR2 Ab did not influence the number of infected monocytes, indicating that

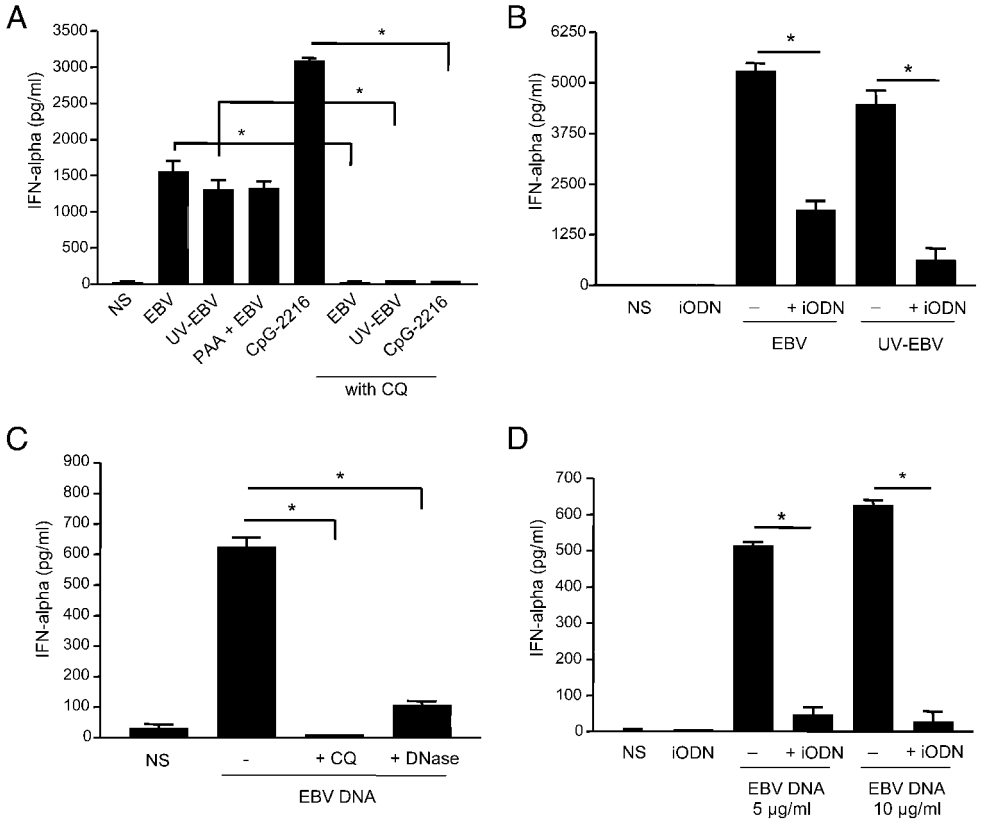

FIGURE 5. EBV induces the secretion of IFN-α in pDCs through TLR9. *A*, Human pDCs (2×10^5 cells/ml) were stimulated with infectious EBV, UV-irradiated EBV, or CpG-2216 (10 μg/ml). When indicated, cells were pretreated with PAA (200 μg/ml) and CQ (20 μM) 30 min prior to stimulation. *B*, Cells were stimulated as in *A*, but pretreated with iODN (50 μg/ml) 30 min prior to stimulation. *C*, Purified EBV DNA (10 μg/ml) treated or not with DNase was transfected with Escort reagent in pDCs. As indicated, samples were also pretreated with CQ (20 μM) for 30 min or (*D*) with iODN for 30 min. Overnight supernatants were tested for IFN-α levels by ELISA. Results shown are one representative of three different donors. $*p \leq 0.01$, compared with respective samples as indicated. NS, not stimulated.

whereas TLR2 recognizes EBV particles, it does not interfere with viral entry. Surprisingly, when monocytes were pretreated with iODN before EBV-GFP infection, the number of EBV-infected monocytes was found to decrease. In contrast, when we blocked endosomal maturation of monocytes with CQ, infectivity of monocytes was strongly increased, suggesting that this agent has completely abrogated all endosomal sensors susceptible to recognize EBV components and to block viral replication. These results are in line with a previous report showing that treatment of EBV-transformed lymphoblastoid cells with CQ can block EBNA1 processing and to decrease recognition of viral Ag by $CD4^+$T cells, a process that may affect immune response (41). We can thus postulate that TLR9 antagonists influence EBV infection of primary monocytes.

TLR7/9 inhibitors do not interfere with EBV-induced maturation of pDCs

It has been reported that EBV infects subclasses of DCs, such as skin resident Langerhans cells and follicular DCs (20, 21, 23). However, although we detected the presence of a viral genome in EBV-treated pDCs, we did not obtain convincing results supporting the ability of EBV to establish infection in these cells. In fact, pretreatment of pDCs with PAA did not significantly abrogate IFN-α synthesis induced by EBV, indicating that EBV replication is not required for induction of IFN-α (Fig. 5*A*). To determine whether this cell type is permissive of EBV infection, enriched pDCs were treated with EBV-GFP, and a number of EBV positive cells were evaluated by cytometric analysis. As shown in Fig. 8, EBV does not establish infection in pDCs. Because we observed that EBV treatment leads to an increase of TLR9 expression in pDCs, we next decided to evaluate the effects of EBV stimulation on pDC maturation by measuring the expression of costimulatory molecules CD40 and CD86 following EBV treatment. CD40, also named TNFR5, is one of the most costimulatory molecules upregulated following pDC stimulation, and CD86 is a ligand essential for dendritic-T cell interactions (42). Although no significant expression of CD40 and CD86 was detected in resting pDCs, treatment with EBV was found to induce upregulation of both molecules (Fig. 9*A*), indicating that EBV treatment induces maturation of pDCs. Similar effects were observed in pDCs stimulated with CpG-2216. In addition, pretreatment of pDCs with CQ strongly inhibits the effects of EBV on the expression levels of CD40 and CD86, suggesting a dependence of endosome acidification in this process. CQ does not seem to impact only on TLR9 activity, but also on essential functions, such as Ag processing, MHC class II presentation, and expression of CD40 as reported (43, 44). Our results are thus in perfect agreement with those observations. To further evaluate whether inhibitors of TLR7 and TLR9 have an effect on pDC maturation induced by EBV, cells were pretreated with TLR antagonists prior to being stimulated with EBV, and expression of CD40 and CD86 was determined by flow cytometric analysis. Our results indicate that the blockage of TLR7, TLR9, or both in pDCs has no effect on the expression levels of CD40 or CD86 induced by EBV (Fig. 9*B*). Pretreatment with iODN did not affect pDC maturation following CpG-2216 treatment, which is consistent with a previous

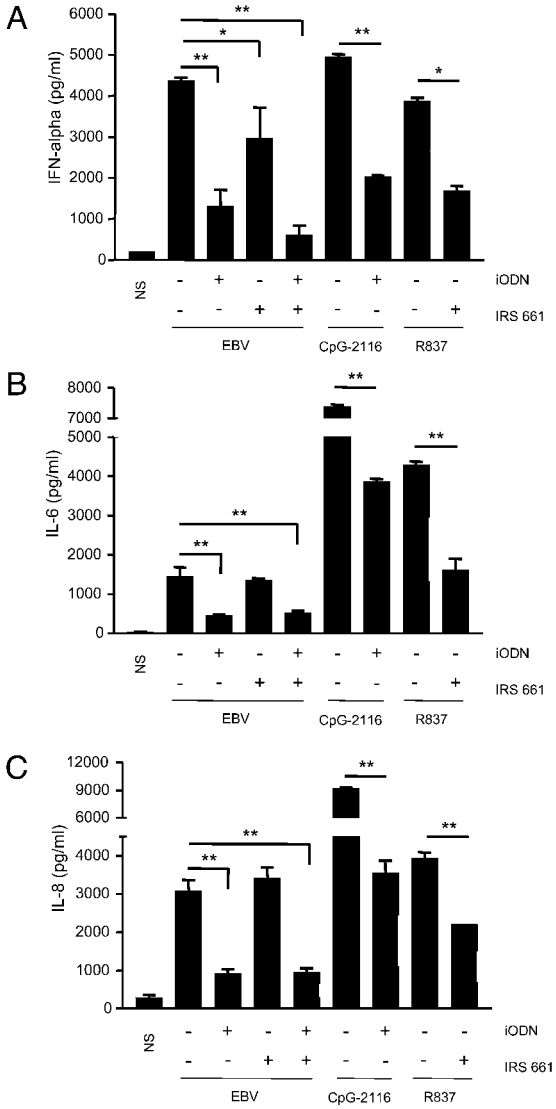

FIGURE 6. The dominant role of TLR9 in pDCs in response to EBV. Freshly isolated pDCs (2×10^5 cells/ml) were stimulated with infectious EBV (moi = 1.0), CpG-2216 (10 μg/ml) or with TLR7 agonist R837 (10 μg/ml) for 24 h. When indicated, cells cultures were pretreated with iODN (50 μg/ml), IRS661 (10 μg/ml), or a combination of both inhibitors for 30 min prior to stimulation with agonists. Supernatants were harvested and tested for IFN-α (*A*), IL-6 (*B*), and IL-8 (*C*) determinations. Results are one representative of three experiments. $*p \leq 0.05$; $**p \leq 0.01$, compared with treatment with respective agonist without inhibitors. NS, not stimulated.

report indicating that the ODN inhibitory sequence TTAGGG did not affect pDC maturation (45). In contrast, upregulated expression of CD40 and CD86 induced by TLR7 agonist was impaired after IRS661 treatment.

Discussion

APCs, such as monocytes and DCs, play a key role in host defense to elicit cellular and humoral responses. They participate in the recruitment of leukocytes and the initiation of adaptive immune response through the production of cytokines and chemokines, and they initiate and maintain immune response against pathogens, such as EBV. A rapid detection of EBV particles during primary infection and the detection of reactivated virus from latently infected cells are crucial for an efficient control of EBV infection. TLRs recognize several viral components in the extracellular environment and after virus entry into the cell. The role of TLRs in the

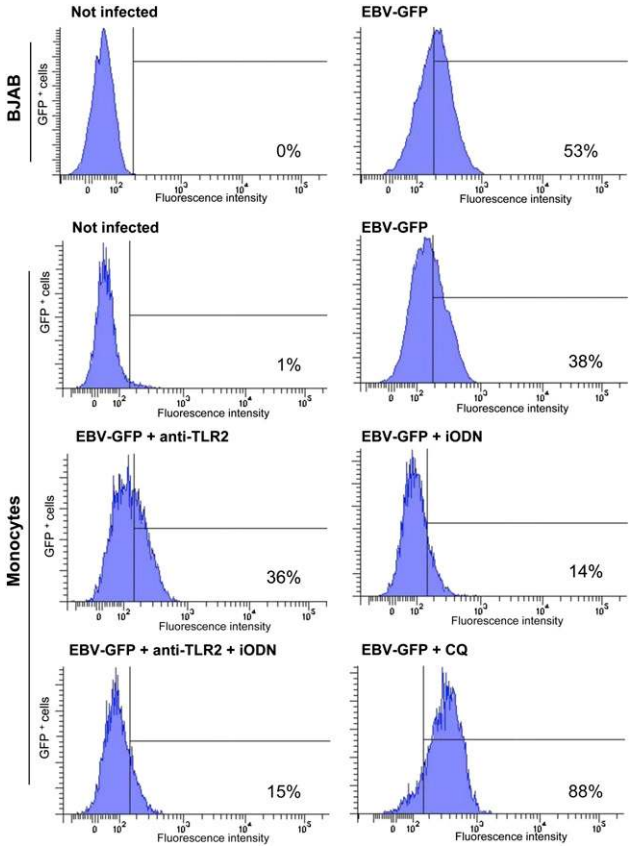

FIGURE 7. EBV infection of monocytes is influenced by pretreatment with TLR9 inhibitors. BJAB (10^5 cells) and monocytes (10^5 cells) were infected with EBV-GFP (moi = 0.5) for 24 and 48 h, respectively. When indicated, monocytes were pretreated with iODN (100 μg/ml), CQ (20 μM), or neutralizing Ab against TLR2 (0.1 μg/ml) prior to treatment with EBV-GFP. The percentage of EBV-GFP–positive cells was evaluated by flow cytometry. Results are one representative of three different experiments.

recognition of EBV by monocytes and DCs is not well known and remains to be investigated. In this study, we demonstrated that EBV triggers an inflammatory response in monocytes and pDCs through TLR9 activation. Interestingly, our results also indicate that based on the cell type involved, a second TLR may contribute with TLR9 to the induction of inflammatory cytokines. In fact, pretreatment of monocytes with CQ or with iODN before stimulation with live EBV particles partially reduced their capacity to produce IL-8, in contrast to monocytes stimulated with isolated EBV DNA in which treatment with CQ strongly blocked the IL-8 synthesis. These results confirm that the EBV genome is recognized by TLR9 via an endocytic pathway; they also suggest the potential contribution of another receptor in the induction of IL-8 secretion by human primary monocytes. Using siRNA targeting TLR2, we demonstrated that TLR2 participates in the production of IL-8 in response to EBV. We thus propose that innate recognition of EBV by monocytes could involve at least two pathways. First, EBV particles are recognized by the membrane surface TLR2. Second, following viral entry into the cells, viral DNA is subsequently recognized by TLR9. Such dual interactions then lead to a rapid production of IL-8 to initiate an effective immune response.

The involvement of a set of TLRs in the recognition of EBV is not surprising, because the dual contribution of TLRs in the recognition of herpesviruses has been previously suggested. Both TLR2 and TLR9 were reported to be engaged sequentially within classical DCs to detect HSV-1 (46). Using a double knockout

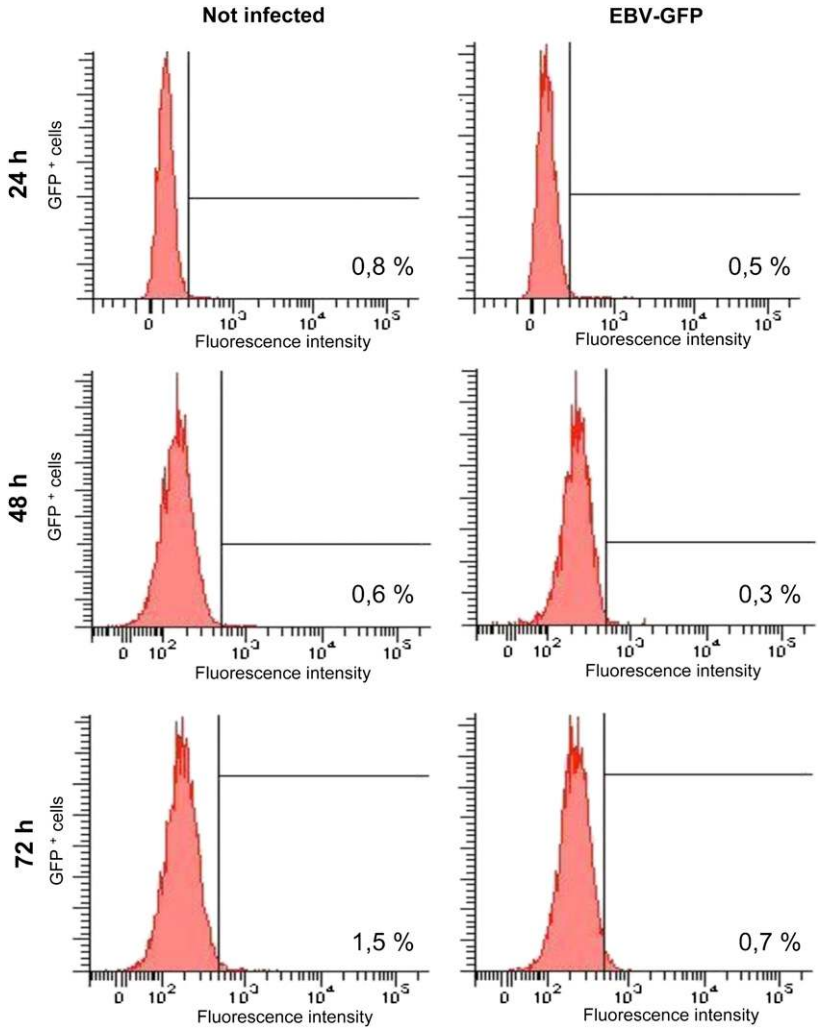

FIGURE 8. EBV does not establish infection in human plasmacytoid DCs. Freshly isolated pDCs (10^5 cells) were treated with EBV-GFP (moi = 2.0) for 24, 48, and 72 h and analyzed for GFP expression by flow cytometry. Results are representative of two different donors. Numbers indicate the percentage of positive cells.

murine model, another group demonstrated that TLR2 and TLR9 synergistically stimulate innate antiviral activities against HSV-2 infection and that expression of IFN-stimulated gene was dependent on both TLRs (47). Our results also indicate that production of MCP-1 and IL-10 by monocytes in response to EBV is mainly dependent on TLR2 rather than TLR9. Therefore, because monocytes express low levels of TLR9, it seems plausible that they have acquired the ability to use one TLR, in this case TLR2, to produce different cytokines to better respond to EBV before its entry into the cell. In this regard, the key role of TLR2 was recently reported by Barbalat et al. (48), who showed that the production of type 1 IFN by inflammatory monocytes was entirely TLR2-dependent in response to Vaccinia virus, highlighting the specificity of TLR2 for certain viral proteins and its ability to distinguish between viral and bacterial ligands.

Similarly, we also observed that human pDCs produced high levels of IFN-α in response to EBV, and this production was markedly blocked by the addition of inhibitory ODN to the cell cultures. Curiously, treatment of pDCs with the TLR7 antagonist IRS661 partly reduced the synthesis of IFN-α induced by EBV, whereas the addition of both TLR9 and TLR7 antagonists to pDC cultures showed a stronger reduction of IFN-α release. Implication of TLR7 in the recognition of a DNA virus was previously suggested by Zucchini et al. (49), who presented the importance of TLR7 and TLR9 for pDCs in the control of infection caused by murine CMV. By using double-deficient mice for TLR7 and TLR9, they observed a complete inhibition of type 1 IFN production in pDCs and a dramatic reduction in survival in murin cytomegalovirus-infected deficient mice compared with the wild type control. The authors suggested that activation of TLR7 in mice was instead associated with engagement of apoptotic debris from infected cells, because pDCs were not permissive of infection by murin cytomegalovirus. We believe that a similar mechanism could be activated following in vitro treatment of pDCs with EBV. Whereas EBV does not sustain infection in pDCs, EBV challenge may gradually affect cell viability and initiate the release of self-RNA, which, in turn, could bind and activate TLR7. Recognition of EBV DNA by TLR9 in combination with the activation of TLR7 could thus potentiate the production of type 1 IFN via IRF7, which is highly expressed in pDCs (50). This scenario does not imply the production of IL-6 and IL-8, which appears to be solely regulated by TLR9. Therefore, TLR9 appears to play a key role in pDCs in response to EBV. Our results are consistent with a recent report showing that EBV-induced IFN-α production by pDCs is linked to TLR9 (51).

Blockade of TLR7 and TLR9 has no consequences on EBV-induced pDC maturation or viral entry. However, treatment of monocytes with the TLR9 antagonist iODN has dramatically reduced EBV infection, whereas treatment with CQ was found to increase the number of EBV-infected monocytes. Previous reports have demonstrated that ODNs with a phosphorothioate backbone exert a potent inhibitory activity on human CMV and HIV entry (52,

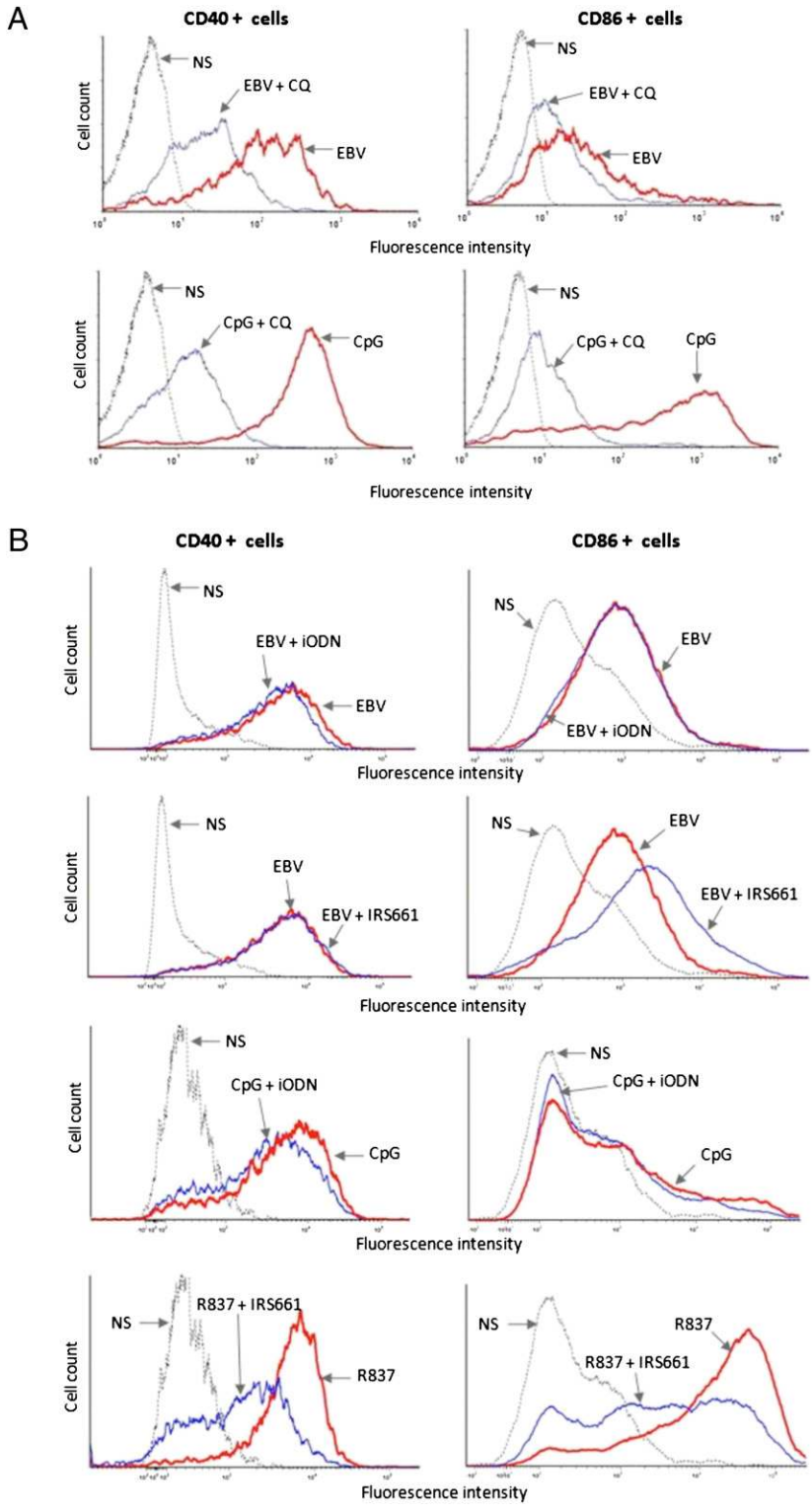

FIGURE 9. EBV stimulation induces maturation of pDCs independently of TLR7 and TLR9 signalization. *A*, Enriched pDCs (10^5 cells) were stimulated for 24 h with infectious EBV (moi = 1.0) or with ODN-2216 (10 μg/ml). Pretreatment with CQ (20 μM) was performed for 30 min prior to stimulation. After the incubation time, cells were incubated with PE-conjugated antihuman CD86 and PerCP/Cy5.5-conjugated antihuman CD40 and analyzed by flow cytometry (EPICS XL apparatus; Beckman Coulter, Fullerton, CA). Results shown are one representative of two different donors. *B*, PDCs (10^5 cells) were first pretreated with iODN (100 μg/ml), IRS661 (10 μg/ml), or both, for 30 min and subsequently stimulated with EBV (moi = 1.0), CpG-2216 (10 μg/ml), or R837 (10 μg/ml) for 24 h. CD40 and CD86 expression levels were determined using BD LSR II apparatus. Results shown in this figure are one representative of three different donors. Black dotted lines indicate NS. Red full lines indicate stimulated cells with the indicated agonist. Blue full lines indicate pretreatment with the respective antagonist prior to stimulation. NS, not stimulated.

53). It was suggested that such ODNs have the capacity to interact with proteins of the virus envelope to block fusion with cell membrane. It is thus plausible that the iODN we used in our study may interact with proteins of the EBV envelope, such as gp42 or gp350, and block virus entry. This aspect remains to be investigated. Regarding the treatment of monocytes with CQ, which resulted in an enhanced EBV infectivity, we believe that this effect could be the consequence of blocking endosomal maturation and thus the recognition of EBV nucleic acid by intracellular sensors.

Our study has described the involvement of TLR9 in the recognition of EBV by primary monocytes and pDCs. However, the contribution of TLR9 in the recognition of EBV and in the production of inflammatory mediators may differ depending on the cell type targeted. In monocytes, which express low levels of TLR9, production of cytokines in response to EBV requires TLR2 cooperation. In pDCs, such production of inflammatory mediators is mainly regulated by TLR9. Our observations agree with another report showing that TLR9 contributes differently to the immunity against MHV-68, depending on the location of infection (54). Such

recognition by a single cell type through multiple TLRs could then ensure an efficient innate response, but also may optimize detection of the virus once it has escaped recognition by membrane TLR or intracellular TLR following entry into the cytosol. In this regard, we cannot exclude the contribution of other TLRs or detectors expressed in different cells, such as RIG-1, which can also participate to EBV recognition as suggested previously (55). A rapid and efficient inflammatory response generated by the engagement of distinct sets of TLRs may certainly participate to the clearance of EBV during primo-infection and to control viral reactivation from latency. Understanding TLR implications during early and acute phases of EBV infection must therefore be investigated to further develop strategies to treat EBV infection, especially in immunosuppressed patients or in patients suffering from autoimmune diseases (56–58), in which EBV was suggested to exacerbate clinical symptoms.

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Disclosures

The authors have no financial conflicts of interest.

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