Herpes simplex virus type-1 induces IFN-α production via Toll-like receptor 9-dependent and -independent pathways

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Type I IFN production in response to the DNA virus herpes simplex virus type-1 (HSV-1) is essential in controlling viral replication. We investigated whether plasmacytoid dendritic cells (pDC) were the major tissue source of IFN-α, and whether the production of IFN-α in response to HSV-1 depended on Toll-like receptor 9 (TLR9). Total spleen cells or bone marrow (BM) cells, or fractions thereof, including highly purified pDC, from WT, TLR9, and MyD88 knockout mice were stimulated with known ligands for TLR9 or active HSV-1. pDC freshly isolated from both spleen and BM were the major source of IFN-α in response to oligodeoxynucleotides containing CpG motifs, but in response to HSV-1 the majority of IFN-α was produced by other cell types. Moreover, IFN-α production by non-pDC was independent of TLR9. The tissue source determined whether pDC responded to HSV-1 in a strictly TLR9-dependent fashion. Freshly isolated BM pDC or pDC derived from culture of BM precursors with FMS-like tyrosine kinase-3 ligand, produced IFN-α in the absence of functional TLR9, whereas spleen pDC did not. Heat treatment of HSV-1 abolished maturation and IFN-α production from all TLR9-deficient DC but not WT DC. Thus pDC and non-pDC produce IFN-α in response to HSV-1 via both TLR9-independent and -dependent pathways.

The immune system recognizes pathogen-associated molecular patterns with the help of a broad panel of pattern recognition receptors, including the Toll-like receptors (TLRs) (1). Ligands for TLR include bacterial lipoproteins, double-stranded RNA, lipopolysaccharide, flagellin, imidazoquinolines, and bacterial DNA that are recognized by TLR2, -3, -4, -5, -7, and -9, respectively (2). Very recently, the natural ligand for murine TLR7 was identified as single-stranded RNA (3, 4). Lack of the adaptor molecule MyD88 completely abrogates any signal from TLR7 and TLR9. Thus, MyD88 is an essential adaptor molecule required for TLR7 and -9 signaling, whereas alternative adaptor molecules have been implicated for signaling via other TLRs (5).

Subsets of dendritic cells (DC) can be distinguished by origin, phenotype, and function (6). Plasmacytoid DC (pDC) are a subset of preDC that predominantly express TLR7 and -9 and have the ability to produce extremely high amounts of IFN-α in response to viruses, single-stranded RNA, or oligodeoxynucleotides containing CpG motifs (CpG-ODN) (7, 8). In humans, pDC are the only DC subset that express TLR9 and respond to CpG-ODN, whereas most murine DC subsets express TLR9 (9). pDC can be isolated *ex vivo* from various organs such as spleen, lymph nodes, blood, or bone marrow (BM) (10, 11). Furthermore, pDC and conventional DC (cDC) can be generated by culture of BM cells with FMS-like tyrosine kinase-3 ligand (FL) (12).

Microorganisms such as bacteria or fungi are recognized by multiple TLRs. A role for TLR2, -3, and -4 in the recognition of viruses has only recently been described (13–16). Like bacterial DNA, viral DNA is a ligand for TLR9 (17). Others have shown recently that pDC respond to herpes simplex virus (HSV) with IFN-α production in a TLR9- and MyD88-dependent manner (17, 18). However, previous reports suggested that other cell populations are able to respond to live HSV with IFN-α production (19). Indeed, our study shows that, apart from pDC, other cell types including cDC and macrophages produce IFN-α to HSV type 1 (HSV-1). This non-pDC IFN-α production is largely TLR9-independent and is responsible for the majority of total IFN-α produced by spleen or BM. The tissue source of the pDC is a determining factor in TLR9 dependence on the production of IFN-α and other cytokines by these cells. Highly purified pDC, purified directly from BM, or pDC generated with FL from BM precursors, can respond to HSV-1 with IFN-α production in the absence of TLR9, whereas spleen pDC depend entirely on TLR9 for activation via HSV-1. Thus, via TLR9-dependent and -independent mechanisms, pDC and non-pDC are important for the innate response to HSV-1.

Materials and Methods

Mice. C57BL/6J mice were purchased from Harlan Winkelmann (Borchen, Germany). TLR9 and MyD88 knockout (KO) mice were kindly provided by S. Akira (Osaka University, Osaka) and bred and backcrossed to C57BL/6 (eight backcrosses) in house. All animals were kept under specific pathogen-free conditions. All animal experiments were carried out according to German guidelines for animal care.

Reagents and Virus. Oligodeoxynucleotides were synthesized by TIB MOLBIOL (Berlin). CpG-ODN 2216 contains the sequence GGGGGACGATCGTCGGGGGG. The underlined poly(G) at the 5′ and 3′ ends were thioate stabilized. Resiquimod (R848) was a gift of G. Lipford (Coley Pharmaceutical, Langenfeld, Germany). HSV-1 (F strain) was originally obtained from B. Roizman (University of Chicago, Chicago), propagated on Vero cells, and isolated as described (20). In short, to harvest viral particles, the infected cells were scraped into medium, the suspension was frozen and thawed three times and sonicated, and the cell debris was removed by centrifugation. Viral particles were further purified by sucrose density gradient centrifugation, as described (21). Apart from HSV-1, disabled infectious single-cycle HSV-1 (HSV-1d), a mutated HSV-1 strain, was also used and was propagated and prepared as described (22). HSV-1d lacks the gene for glycoprotein H, needed for infection, and therefore this mutant virus performs only one cycle of infection (23). Heat treatment of HSV-1 (HSV-1-heat) was performed for 30 min at 56°C in a water bath. HSV-1 (KOS

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Abbreviations: M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte M-CSF; TLR, Toll-like receptor; DC, dendritic cells; pDC, plasmacytoid DC; cDC, conventional DC; FL, FMS-like tyrosine kinase-3 ligand; BM, bone marrow; KO, knockout; pfu, plaque-forming units; TNF-α, tumor necrosis factor α; HSV-1, herpes simplex virus type 1; CpG-ODN, oligodeoxynucleotides containing CpG motifs; HSV-1-heat, heat treatment of HSV-1; FL-DC, FL-dependent DC; GM-DC, GM-CSF-dependent DC.

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strain) was kindly provided by A. Krug (Washington University School of Medicine, St. Louis).

Cell Generation in Culture and Preparation from Lymphoid Organs. *In vitro*-generated FL-dependent DC (FL-DC) were generated and sorted essentially as described by us (24). In short, BM cells were cultured in the presence of human or murine recombinant FL for 8–9 days. Resulting cells were >90% $CD11c^+$, and 20–60% of cells displayed pDC phenotype ($CD11c^+CD45RA^{high}B220^{high}CD11b^{low}$). FL-DC were either used unseparated or sorted into pDC and cDC with a MoFlo instrument (DakoCytomation, Glostrup, Denmark). pDC were sorted as $CD11c^+CD45RA^{high}CD11b^{low}$ cells, and cDC were sorted as $CD11c^+CD45RA^{neg}CD11b^{high}$ cells.

In vitro-generated granulocyte/macrophage colony-stimulated factor (GM-CSF)-dependent DC (GM-DC) were generated by culturing BM cells in the presence of murine recombinant GM-CSF (R & D Systems), replacing half the volume of the culture with fresh GM-CSF-containing medium every second to third day. Cells were used after 7–9 days and contained 60–80% $CD11c^+$ cells.

Freshly isolated spleen cells and BM cells were either used unseparated or separated by using specific FITC- or phycoerythrin (PE)-labeled antibodies, as indicated in Figs. 2–5, followed by anti-FITC or PE beads (Miltenyi Biotec, Bergisch Gladbach, Germany) respectively, according to the manufacturer's recommendations. DC from spleen and BM were isolated by selecting $CD11c^+$ cells with magnetic beads, followed by staining and sorting of pDC ($CD11c^+CD45RA^{high}B220^{high}$) and cDC ($CD11c^+CD45RA^{neg}B220^{neg}$), by using a MoFlo instrument (DakoCytomation). Macrophages were generated by culturing BM cells in the presence of human recombinant M-CSF (R & D Systems) and replacing half the volume of the culture with fresh M-CSF-containing medium every third day. Cells were used after 7–9 days, and all stained positive for CD11b but were negative for CD11c and B220.

Stimulation of Cytokine Production. Cells were stimulated with R848 (1 μg/ml), CpG-2216 (1 μM), HSV-1, disabled infectious single-cycle HSV-1, or HSV-1-heat [F strain, 10^7 plaque-forming units (pfu)/ml or as indicated in Figs. 1–5] or HSV-1 (KOS strain, 10^6 pfu/ml). Stimulations were carried out in the presence or absence of IL-3 (10 ng/ml) and GM-CSF (10 ng/ml), as indicated, for 16–24 h.

After 16–24 h of stimulation, FL-DC were labeled with a combination of anti-CD11c-APC and anti-CD45RA-phycoerythrin together with FITC-labeled mAb (CD40, CD62L, CD69, or CD86; all from BD Biosciences, Heidelberg) and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cell culture supernatants were analyzed for IFN-α, IL-6, and tumor necrosis factor α (TNF-α) by ELISA, as described (4).

Results

***In Vitro*-Generated DC from TLR9 or MyD88-Deficient Mice Produce IFN-α in Response to HSV-1.** pDC have been described as the major source of IFN-α in response to CpG-ODN, and several viruses. pDC predominantly express TLR7 and -9 and respond to their corresponding natural or synthetic ligands, i.e., single-stranded RNA and imidazoquinolines for TLR7 and bacterial DNA or CpG-ODN for TLR9. We generated FL-DC, which contain pDC as well as cDC, from WT, TLR9 KO, and MyD88 KO mice and analyzed the response of these cells to HSV-1 or synthetic agonists for TLR9 or -7. The TLR9 agonist CpG-2216 induced IFN-α and IL-6 production from FL-DC of WT mice but, as described (25), not from FL-DC of TLR9 or MyD88 KO mice (Fig. 1*A*). The TLR7 agonist R848 induced IL-6 in FL-DC from WT and TLR9 KO mice but, as expected, not from MyD88 KO mice. As previously shown (4, 24), no detectable IFN-α was induced by R848 in FL-DC of WT or KO mice. In contrast, HSV-1 induced production of IFN-α and

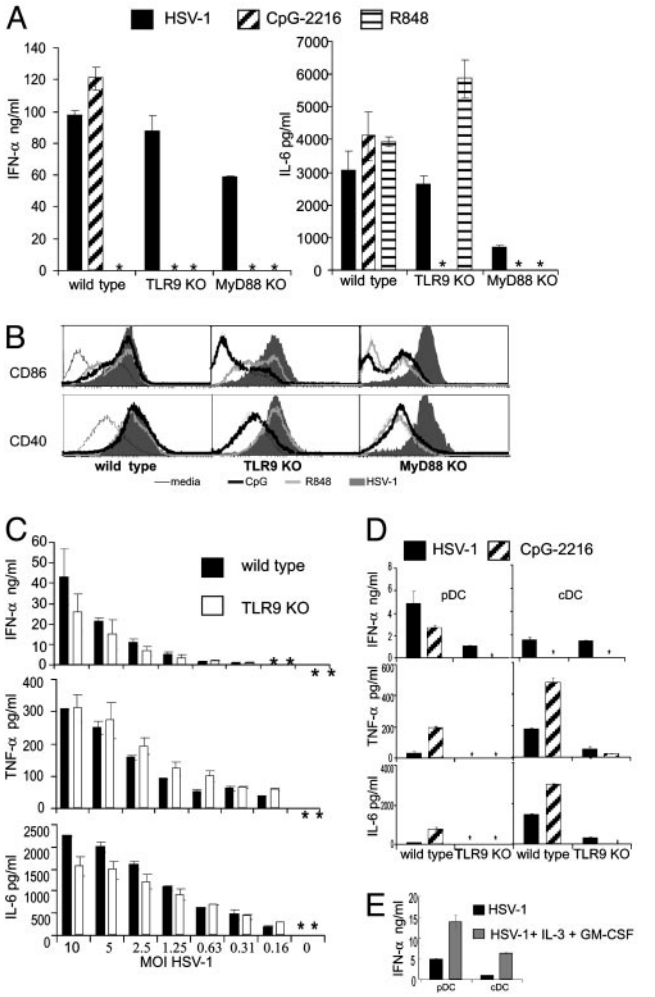

Fig. 1. FL-DC do not require TLR9 or MyD88 for activation in response to HSV-1. Total FL-DC (2.5×10^6 per ml) generated from WT, TLR9 KO, or MyD88 KO mice were stimulated with HSV-1 (10^7 pfu/ml), CpG-2216 (1 μM) or R848 (1 μg/ml). (*A*) The supernatants were removed and tested for IFN-α or IL-6 production by ELISA. (*B*) Cells were stained with antibodies directed to CD86 or CD40. (*C*) Total FL-DC (1×10^6 per ml) from WT or TLR9 KO mice were then stimulated for 22 h with a titration of HSV-1 from $1 \times 10^7 - 0.16 \times 10^6$ pfu/ml [10–0.16 multiplicity of infection (MOI)], and the supernatants were tested by ELISA for IFN-α, TNF-α, or IL-6 production. (*D*) Sorted pDC and cDC (2.5×10^5 per ml) purified from FL-DC of WT or TLR9 KO mice were stimulated with HSV-1 or CpG-2216. Supernatants were tested by ELISA for IFN-α, TNF-α, or IL-6 production. (*E*) Sorted pDC or cDC (5×10^5 per ml) from FL-DC of WT mice were stimulated with HSV-1 in the presence or absence of IL-3 and GM-CSF and the supernatants were tested by ELISA for IFN-α. One representative experiment of at least three is shown. Error bars represent the range of duplicate samples; asterisks indicate that cytokines were not detectable by ELISA.

IL-6 in FL-DC not only from WT but also from TLR9 and MyD88 KO mice. However, the levels of cytokines, particularly IL-6, were reduced in the MyD88 KO mice. DC maturation in response to HSV-1, as assessed by up-regulation of CD40 and CD86, was independent of TLR9 and MyD88, whereas DC maturation in response to R848 and CpG-2216 depended completely on MyD88, with CpG-2216 responses additionally depending on TLR9 (Fig. 1*B*). This suggested that the expression of neither TLR9 nor of MyD88, the essential adaptor molecule for TLR9 and -7, was required for activation of FL-DC by HSV-1.

To evaluate whether this effect of TLR9-independent IFN-α production was influenced by the concentration of virus, we titrated HSV-1 on FL-DC and analyzed the production of the cytokines

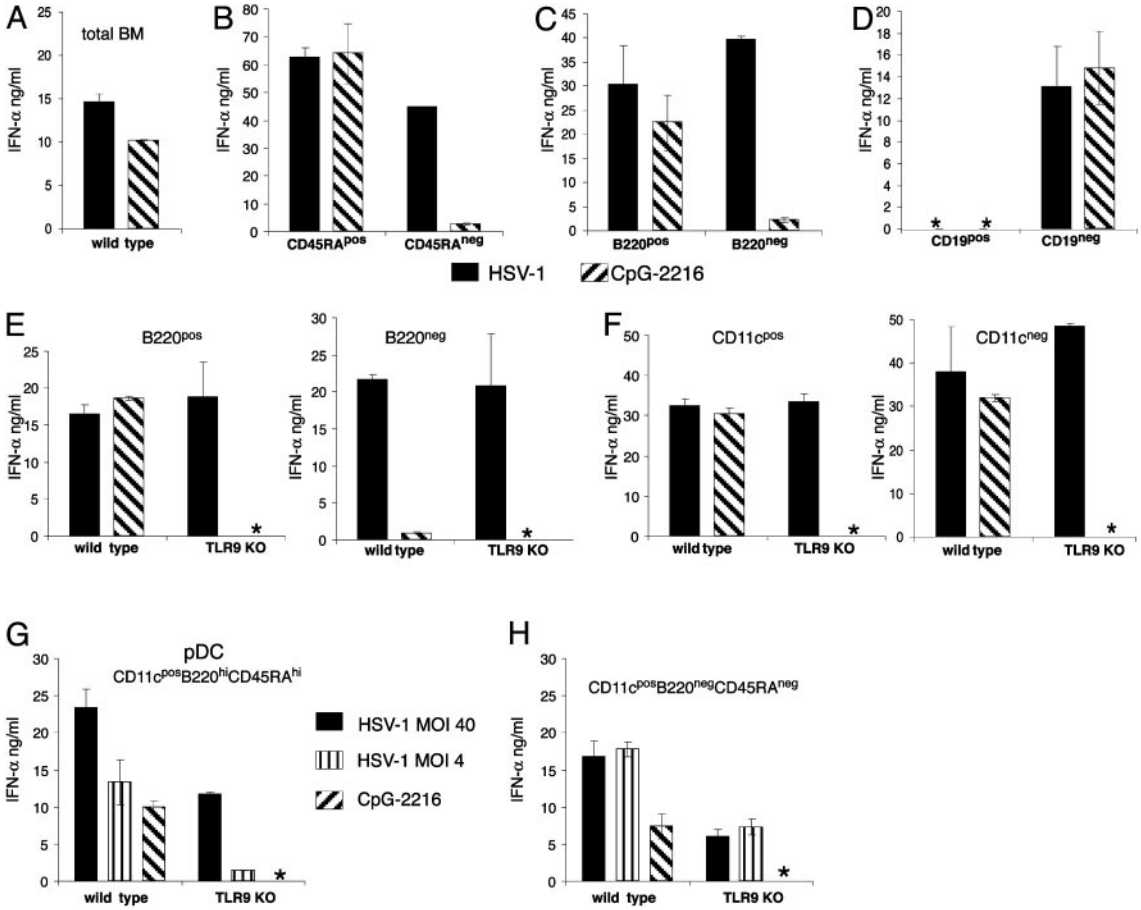

Fig. 2. Total, enriched, or highly purified CD11c$^+$ and CD11c$^-$ cells from BM are activated in response to HSV-1 to produce IFN-α, even in the absence of TLR9. WT total BM cells [2.5×10^6 per ml, in the presence of IL-3 and GM-CSF (*A*)] or CD45RA-enriched [2.5×10^6 per ml (*B*)], B220-enriched [2.5×10^6 per ml (*C*)], or CD19-enriched [1×10^6 per ml (*D*)] WT BM cells were stimulated with HSV-1or CpG-2216. B220-enriched (*E*) or CD11c-enriched (*F*) BM cells (2.5×10^6 per ml) from WT or TLR9 KO mice were stimulated with HSV-1 or CpG-2216 in the presence of IL-3 and GM-CSF. Sorted CD11c$^+$B220^{high}CD45RA^{high} pDC (*G*) or CD11c$^+$B220$^-$CD45RA$^-$ non-pDC (*H*) cells (2.5×10^5 per ml) were stimulated with either 10^7 pfu/ml [multiplicity of infection (MOI) 40] or 10^6 pfu/ml (MOI 4) of HSV-1 or CpG-2216, in the presence of IL-3 and GM-CSF. All supernatants were tested by ELISA for IFN-α production. One representative experiment of at least three is shown. Error bars represent the range of duplicate samples; asterisks indicate that production of IFN-α was below the detection limit of the ELISA.

IFN-α, TNF-α, and IL-6. As shown in Fig. 1*C*, at all virus concentrations tested, no significant differences in IFN-α, TNF-α, or IL-6 production between unseparated FL-DC from WT and TLR9-deficient mice were detected. Because the titration of HSV-1 on FL-DC revealed that higher virus titers promoted higher IFN-α production, we used 10^7 pfu/ml HSV-1 for the following experiments, unless otherwise specified.

cDC, as Well as pDC from FL-DC Cultures, Produce IFN-α in Response to HSV-1 in the Absence of TLR9. To identify the source of IFN-α within the mixed FL-DC, we separated pDC (CD11c$^+$CD45RA^{high}-CD11b^{low}) and cDC (CD11c$^+$CD45RA^{neg}CD11b^{high}) by cell sorting. Analysis of the sorted populations revealed a purity of at least 97%. In response to CpG-2216, pDC of WT mice produced IFN-α, TNF-α, and IL-6, whereas cDC produced only TNF-α and IL-6 but not IFN-α (Fig. 1*D*). As expected, no cytokines were induced by CpG-2216 in pDC or cDC from TLR9 KO mice. However, both pDC and cDC of WT mice produced IFN-α to HSV-1. In the absence of TLR9, the IFN-α production of pDC was reduced, indicating that recognition of HSV-1 by purified pDC was largely, but clearly not entirely, TLR9-dependent (Fig. 1*D*). In contrast, TLR9-deficient cDC produced similar amounts of IFN-α but reduced levels of TNF-α and IL-6 compared with WT cDC (Fig. 1*D*), indicating that production of TNFα and IL-6 by cDC was partly TLR9-dependent, but IFN-α production by cDC was independent, of TLR9.

IL-3 and GM-CSF have been described to promote the production of IFN-α in response to HSV (26). We found that both pDC and cDC produced more IFN-α to HSV-1 in the combined presence of IL-3 and GM-CSF (Fig. 1*E*), and accordingly we then included both cytokines for many of the viral stimulations, as indicated in the legends to Figs. 1–5.

BM Cells, Including BM pDC, Produce IFN-α to HSV-1 in the Absence of TLR9. As we found that pDC and cDC from FL-cultured BM precursors produced IFN-α to HSV-1 independently of TLR9, we systematically investigated the situation in *ex vivo*-isolated BM cells. Unseparated BM cells produced IFN-α to HSV-1 and CpG-2216 (Fig. 2*A*). To define the IFN-α producers among the BM cells, pDC, known to highly express the surface antigens CD45RA and B220, were enriched from BM cells with antibodies directed against either CD45RA or B220. Stimulation with CpG-2216 revealed major IFN-α production was among the CD45RA$^+$- and B220$^+$- selected fractions, whereas the depleted fractions produced only small amounts (Fig. 2 *B* and *C*).

Importantly, either CD45RA- or B220-depleted cells produced large amounts of IFN-α to HSV-1 (Fig. 2 *B* and *C*). This suggested that cells displaying a different surface phenotype from classical pDC were able to produce IFN-α in response to HSV-1. Because

CD45RA and B220 are also expressed on B cells, we separated BM cells into cells that were positive for the B cell-specific antigen CD19 and cells that were negative for CD19 expression. Only $CD19^-$ cells produced IFN-α in response to HSV-1 and CpG-2216 (Fig. 2*D*). Thus B cells, a major cell type within BM, were not major producers of IFN-α to HSV-1.

The comparison of the IFN-α production of B220-selected cells from WT and TLR9 KO mice revealed that both the $B220^+$ fraction enriched for pDC and the $B220^-$ fraction depleted for pDC produced similar levels of IFN-α to HSV-1 independently of TLR9 (Fig. 2*E*).

Because our data with total BM cells and with B220- or CD45RA-fractionated BM cells did not support pDC as the sole IFN-α producers to HSV-1, we analyzed the response of BM cells, fractionated on the basis of their CD11c expression, to HSV-1 and CpG-2216. The $CD11c^+$ fraction of BM accounted for up to 3% of all BM cells. After the positive selection, 75–90% of the cells stained positive for CD11c. Among those, ≈40% expressed B220, thus resembling pDC (data not shown). CD11c-enriched as well as -depleted BM cells produced IFN-α to HSV-1 and also to CpG-2216 (Fig. 2*F*). Furthermore, IFN-α production to HSV-1 was fully functional in the absence of TLR9. Thus, BM DC and non-DC produced IFN-α in response to HSV-1 independently of TLR9.

To further define the freshly isolated BM $CD11c^+$ populations, we further separated the $CD11c^+$ fraction by cell sorting into highly purified pDC ($CD11c^+CD45RA^{high}B220^{high}$) and non-pDC ($CD11c^+CD45RA^{neg}B220^{neg}$) fractions. pDC (Fig. 2*G*) and $CD11c^+$ non-pDC (Fig. 2*H*) produced IFN-α to HSV-1 and CpG-2216 (Fig. 2 *G* and *H*). pDC produced more IFN-α than cDC, only at a high multiplicity of infection. This dependence on high viral input was even more pronounced for TLR9-independent IFN-α production by pDC; the lack of TLR9 had a greater effect on decreasing IFN-α production in response to low viral concentrations. In contrast, TLR9-independent IFN-α production by cDC was similar at both viral concentrations tested.

Thus, pDC, $CD11c^+$, non-pDC, and $CD19^-CD11c^-$ cells, all freshly isolated from BM, produce large amounts of IFN-α to HSV-1 via TLR9-independent as well as -dependent pathways.

Another strain of HSV-1, HSV-1 KOS strain [as used by Krug *et al.* (18)], also induced TLR9-dependent and -independent IFN-α production from $CD11c^+$ freshly isolated BM cells (Fig. 6, which is published as supporting information on the PNAS web site).

Splenocytes Produce IFN-α to HSV-1 in the Absence of TLR9. Next we extended our analysis of *ex vivo*-isolated cells to the spleen (see Fig. 7, which is published as supporting information on the PNAS web site). As was the case for BM, splenocytes produced high levels of IFN-α in response to HSV-1. $CD11c^+$-selected splenocytes produced IFN-α in response to HSV-1 in a TLR9-independent manner, but neither sorted pDC nor cDC produced IFN-α to HSV-1 independently of TLR9. Only the sorted pDC responded to the virus, and this was strictly TLR9-dependent recognition. Thus, as shown by Krug *et al.* (18), the IFN-α production of sorted splenic pDC in response to HSV-1 depended fully on TLR9. Although spleen pDC depended entirely on TLR9 for activation by HSV-1, our data indicated that, similar to BM, the total splenocyte response to HSV-1 was mainly TLR9-independent.

GM-DC Produce IFN-α in Response to HSV-1 in the Absence of TLR9. FL culture-derived pDC and cDC and *ex vivo*-isolated pDC and cDC from BM but not spleen were able to produce IFN-α in response to HSV-1 in the absence of TLR9. As an alternative source of cDC, we tested how GM-CSF culture-derived DC responded to CpG-2216 and HSV-1. GM-DC responded to CpG-2216 with TNF-α and IL-6 production and the up-regulation of DC maturation markers but, as previously shown (3), not with IFN-α production (Fig. 3). Responsiveness to CpG-2216 depended fully on TLR9. It should be noted that low IL-6 production by TLR9 KO GM-DC

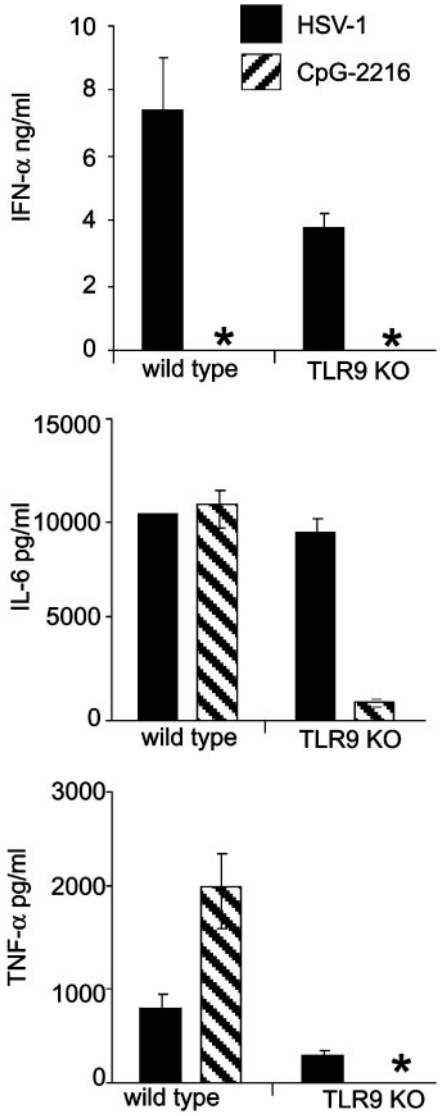

Fig. 3. GM-CSF-generated DC are activated in response to HSV-1 in the absence of TLR9. GM-DC cultures (2.5×10^6 cells per ml) from WT or TLR9 KO mice were stimulated with HSV-1 or CpG-2216 and the supernatants were tested by ELISA for the production of IFN-α, IL-6, or TNF-α. Error bars represent the range of duplicate samples; asterisks indicate that cytokines were not detectable by ELISA. Data are representative of one of three replicate experiments.

in response to CpG-2216 was the same level produced by these cells constitutively in the absence of stimulus (data not shown). In contrast, in the absence of TLR9, the same cells responded to HSV-1 with cytokine production, including IFN-α (Fig. 3), and DC maturation (data not shown). Additionally, GM-DC generated from MyD88-deficient mice showed robust IFN-α production and DC maturation to HSV-1 (data not shown) in accordance with our results by using FL-DC from MyD88-deficient mice (Fig. 1).

Macrophages also Produce IFN-α in Response to HSV-1. The results with fractionated BM cells suggested that cells other than DC were able to produce IFN-α in response to HSV-1. To evaluate the response of non-DC, we generated macrophages from cultures of BM cells stimulated with M-CSF. The resulting cells were negative for CD11c and B220 and thus contained neither cDC nor pDC (data not shown). Consistent with previous reports on the attenuation of CpG-ODN responsiveness by M-CSF, via down-regulation of TLR9 expression (27), the response of these macrophages to

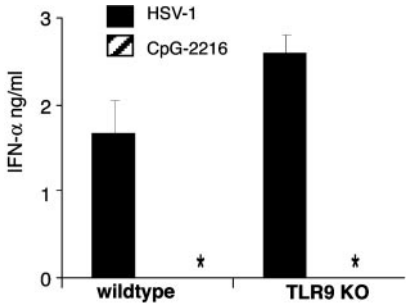

Fig. 4. M-CSF-generated macrophages are activated in response to HSV-1 in the absence of TLR9. M-CSF macrophage cultures (2.5×10^6 cells per ml) from WT or TLR9 KO mice were stimulated with HSV-1 or CpG-2216 and the supernatants were tested by ELISA for the production of IFN-α. Error bars represent the range of duplicate samples. Data are representative of one of three replicate experiments.

CpG-ODN was poor. Only minimal amounts of IL-6 (data not shown) were induced, and no IFN-α was detectable after CpG-2216 stimulation (Fig. 4). In contrast, HSV-1 induced both IFN-α and IL-6 production from these macrophages. Furthermore, the production of both cytokines was completely independent of TLR9 (Fig. 4 and data not shown).

The Response to Heat-Treated HSV-1 Depends Fully on TLR9. To gain insight into the mechanism behind the recognition of HSV-1, we modified the virus by heat treatment. Boiling of HSV-1 completely abrogated any WT FL-DC response to the virus, most likely due to denaturation of surface glycoproteins needed to support entry into the cells (data not shown). However, milder heat treatment (56°C for 30 min) resulted in a HSV-1 preparation that induced up-regulation of CD40 and down-regulation of CD62L on FL-DC of WT mice to the same extent as nontreated HSV-1 (Fig. 5*A*). In comparison with untreated HSV-1, heat-treated HSV-1 induced reduced but detectable levels of IFN-α and IL-6 production by FL-DC (Fig. 5*B*). In contrast, in the absence of TLR9, heat-treated HSV-1 did not induce detectable cytokines or DC maturation, whereas the response to untreated HSV-1 was still robust in the absence of TLR9.

Thus, HSV-1-heat led to the loss of TLR9-independent recognition of HSV-1 by FL-DC. To confirm these results with *ex vivo*-isolated cells, we tested the recognition of heat-treated and untreated HSV-1 by total BM cells (data not shown) and $CD11c^+$-selected BM cells from both WT and TLR9-deficient mice (Fig. 5*C*). In accordance with the data generated with FL-DC, the cytokine production of these cells to heat-treated HSV-1 but not untreated HSV-1 was completely abrogated in the absence of TLR9, suggesting that immune recognition of heat-treated HSV-1 depends exclusively on the presence of TLR9.

Discussion

In this study, we analyzed the response of various innate immune cells to CpG-2216 and HSV-1. We found that pDC ($CD11c^+CD45RA^+B220^+$), either derived from cultured FL-treated BM cells or purified *ex vivo* from spleen, were the major source of IFN-α in response to CpG-2216 stimulation. In *ex vivo* BM cells, the IFN-α response to CpG-2216 was also mainly confined to $CD45RA^+$ and $B220^+$ cells, but both $CD11c^+$ and $CD11c^-$ cells produced IFN-α to CpG-2216. From the data of Fig. 2, it is likely that these $CD11c^-$ cells producing IFN-α in response to CpG-ODN are $CD45RA^+$ $B220^+$. $CD45RA^+$ pDC precursors, not yet expressing the full panel of pDC markers, and with a high capacity to produce IFN-α, have been identified in human BM (28). We hypothesize that the murine equivalent of these pDC precursors is contained within the $CD11c^-$ fraction.

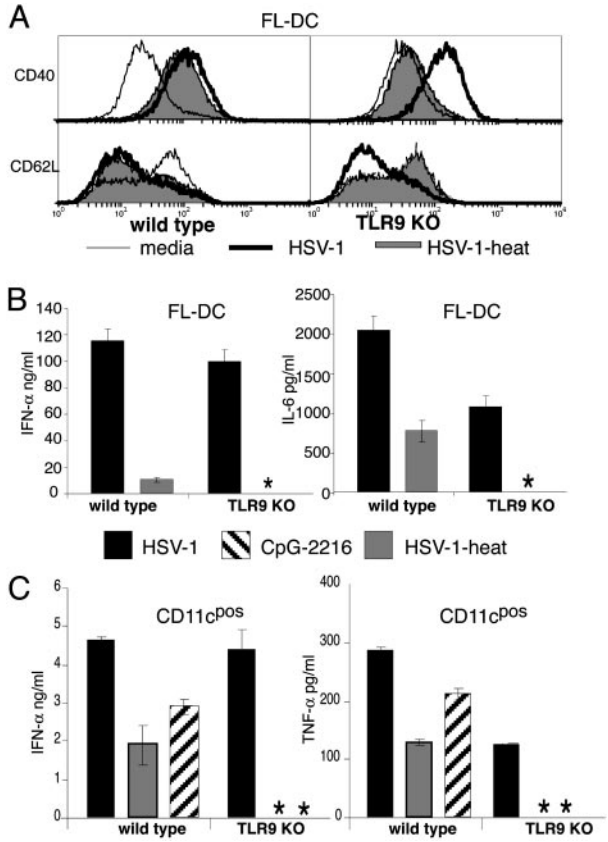

Fig. 5. FL-DC and $CD11c^+$ BM cells are not activated by heat-treated HSV-1 in the absence of TLR9. (*A*) FL-DC (2.5×10^6 per ml) generated from WT or TLR9 KO mice were stimulated with HSV-1 (10^7 pfu/ml) that was either left untreated or heated to 56°C for 30 min (HSV-1-heat). Cells were stained with antibodies directed to CD40 or CD62-L. Open black histograms represent HSV-1-stimulated cells, shaded histograms represent HSV-1-heat stimulated cells, and open gray histograms represent cells in media only. (*B*) Supernatants were tested by ELISA for the production of IFN-α or IL-6. (*C*) Magnetic cell-sorting-enriched $CD11c^+$ cells (2×10^5 per ml) purified directly from BM were stimulated with HSV-1, HSV-1-heat, or CpG-2216. Supernatants were tested by ELISA for production of IFN-α or TNF-α. Error bars represent the range of duplicate samples; asterisks indicate that cytokine production was below the detection limits of the ELISA. Data are representative of one of at least three replicate experiments.

As expected, all responses to CpG-2216 depended completely on TLR9 and MyD88, with the absence of either of these molecules leading to abrogation of pDC maturation and cytokine production in response to this TLR9 agonist.

pDC were also potent producers of IFN-α in response to HSV-1. As shown by Krug *et al.* (18), the spleen pDC response to HSV-1 depended completely on TLR9. However, BM pDC, whether isolated *ex vivo* or generated in culture from BM precursors, still responded to HSV-1 with reduced, although significant, IFN-α production, in the absence of TLR9 or MyD88. Thus, the BM pDC response to HSV-1 was composed of both TLR9-dependent and -independent recognition events. Because the cytokine response, particularly IL-6, but not maturation of MyD88-deficient FL-DC was reduced relative to WT and TLR9-deficient mice, it is possible that MyD88-dependent priming events (possibly through IL-1, IL-18, or other TLR) are important during the culture of the FL-DC for optimal cytokine production.

We initially had hypothesized that pDC were the major producers of IFN-α in response to HSV-1, because these natural IFN-producing cells have been shown to be the primary source of IFN-α in response to another herpes virus (murine cytomega-

lovirus) *in vivo* (29). This was clearly not the case, because cDC from FL-DC cultures, non-pDC splenocytes, CD45RA$^-$ or B220$^-$ BM cells, CD11c$^+$CD45RA$^-$B220$^-$ BM cells, GM-DC, and M-CSF-generated macrophages were all able to produce IFN-α (as well as other cytokines) in response to HSV-1. Given that CD11c depletion of spleen and BM hardly altered the total production of IFN-α in response to HSV-1, it follows that although on a per-cell basis the pDC are potent IFN-α producers, the total spleen and BM response to HSV-1 *in vivo* probably only marginally depends on pDC-derived IFN-α. All of these other cell types still responded vigorously to HSV-1 in the absence of TLR9. Although CD11c$^+$ splenocytes selected with magnetic cell-sorting beads produced IFN-α in response to HSV, highly purified CD11c$^+$ cDC or pDC did not. Reasons for the "lost" TLR9-independent response have not been elucidated but might include a requirement for cell–cell interactions or cytokine crosstalk between CD11c$^+$ pDC and CD11c$^+$ non-pDC. In addition, because the CD11c$^+$ splenocytes were an "enriched population," it is conceivable that another, non-CD11c$^+$ cell, absent in the highly purified sorted CD11c$^+$ populations, interacted with CD11c$^+$ cells to induce a synergistic increase in TLR9-independent IFN-α production. Thus HSV-1 responses that include cellular activation and IFN-α production are induced in many different cell types apart from pDC, and in all cases, these responses are composed of TLR9-independent and -dependent events.

The HSV-1F strain was used for most of these studies, but comparative analyses using the HSV-1 KOS strain indicated that the TLR9-dependent and -independent elements of HSV recognition were not limited to a single strain or single laboratory preparation of virus.

To gain insight into the mode of recognition of HSV-1, we tested cellular responses to heat-treated virus. In all cells tested, total FL-DC cells, total *ex vivo* BM cells, and CD11c$^+$ *ex vivo* BM cells, the TLR9-independent but not -dependent response was abrogated by heating the virus to 56°C. These results suggest that HSV-1 is recognized by at least two independent mechanisms, a heat-stable stimulus recognized via a TLR9-dependent pathway and a heat-labile stimulus independent of TLR9 and MyD88. The heat-stable TLR9-dependent stimulus is most likely viral DNA. Indeed, high concentrations of isolated HSV DNA have been shown to be stimulatory (17, 30). The TLR9-independent mechanism of HSV-1 recognition is not fully elucidated, but two different mechanisms could be envisaged. Previously, recombinant glycoprotein D, known to be important for cellular entry of HSV-1, was shown to induce IFN-α production in human peripheral blood mononuclear cells (31). A proteinous stimulus could explain the observed heat sensitivity of the TLR9- and MyD88-independent pathway, potentially disrupting the glycoprotein D interaction with its cell surface receptor(s). The reduced WT response to heat-inactivated HSV is potentially due to a block in this TLR9-independent pathway, as well as a reduced TLR9-dependent response, because less HSV is internalized (possibly via glycoprotein D viral interactions). Another mechanism could be stimulation via RNA intermediates, produced during HSV-1 replication. Single-stranded RNA could be excluded, because it depends in mice on TLR7 that signals via MyD88, and we found that HSV-1 induced IFN-α and DC maturation in the absence of MyD88. However, recently it was shown that the double-stranded RNA analogue poly(I:C) induced high levels of IFN-α in GM-DC after transfection, via a double-stranded RNA-activated protein kinase-dependent mechanism (32). We could confirm the IFN-α production in response to transfected poly(I:C) by GM-DC and extended it further to FL-DC (data not shown). Loss of TLR9-independent recognition with heat-treated HSV-1 could then be explained by the inactivity of the virus that prevents formation of RNA intermediates.

In agreement with two recent studies (17, 18) focusing on the pDC response to HSV, we have shown that pDC can recognize HSV-1 via TLR9-dependent mechanisms. We have extended these studies by showing that, depending on the tissue source, pDC can additionally recognize HSV-1 via TLR9-independent mechanisms. This TLR9-independent recognition of HSV-1 by BM-pDC was not observed in the study of Lund *et al*. (17), and this could possibly be explained by the fact that different viruses were used; we used active HSV-1, whereas Lund *et al.* (17) used active HSV-2 mutant lacking thymidine kinase, UV-inactivated HSV-1, or UV-inactivated HSV-2 mutant lacking thymidine kinase.

Overall, our data indicate that HSV-1 recognition via TLR9-independent and -dependent pathways, leading to IFN-α production and cellular activation, is a common event among many cell types, including pDC. This TLR9-independent IFN-α production by pDC and other more numerous cell types, including cDC or macrophages, might explain the lack of difference between WT and TLR9 KO mice in the two *in vivo* HSV-1 infection models presented by Krug *et al*. (18). Although pDC are probably the most potent producers of IFN-α in response to this virus on a per-cell basis, the total IFN-α produced by pDC is probably only a fraction of the IFN-α induced by the concurrent activation of several cell types *in vivo*.

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