

Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells

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Dendritic cells (DCs) express multiple Toll-like receptors (TLR) in distinct cellular locations. Herpes simplex viruses (HSV) have been reported to engage both the surface TLR2 and intracellular TLR9 in conventional DCs. However, the contributions of these TLRs in recognition of HSV and the induction of proinflammatory cytokines in DCs remain unclear. Here, we demonstrate that a rare population of HSV, both in laboratory strains and in primary clinical isolates from humans, has the capacity to activate TLR2. This virus population is recognized through both TLR2 and TLR9 for the induction of IL-6 and IL-12 secretion from bone marrow-derived DCs. Further, we describe a previously uncharacterized pathway of viral recognition in which TLR2 and TLR9 are engaged in sequence within the same DC. Live viral infection results in two additional agonists of TLR2 and TLR9. These results indicate that in cells that express multiple TLRs, pathogens that contain multiple pathogen-associated molecular patterns can be detected in an orchestrated sequence and suggest that the innate immune system in DCs is optimized to linking uptake and degradation of pathogens to microbial recognition.

cytokines | innate immunity | Toll-like receptor | viral infection | plasmacytoid dendritic cell

Innate recognition of viruses by the mammalian immune system is critical in providing both the immediate antiviral effects, mediated in large part by type I IFNs (1), and in inducing appropriate classes of adaptive immune responses required for clearing viral infection (2, 3). Distinct mechanisms are used to recognize viruses and the viral replication intermediates produced during viral infections. Whereas most cells use the retinoic acid inducible gene-1 to recognize certain ssRNA virus infections (4) and melanoma differentiation-associated gene 5 to recognize picornaviruses (5), plasmacytoid dendritic cells (pDCs) use exclusively the Toll-like receptors (TLRs) to recognize ssRNA and dsDNA viruses (4, 6). More recently, a TLR-independent cytosolic recognition of DNA has been shown to play a major role in the induction of type I IFNs (7, 8).

For innate recognition of herpes viruses, at least three pathways have been described. The first pathway involves the detection of viral genomic dsDNA by the TLR9. This pathway is used by the pDCs to recognize Herpes simplex virus (HSV)-1 (9, 10) and HSV-2 (10). TLR9-mediated recognition also represents the predominant pathways in non-pDCs, such as splenic CD11c⁺ DCs upon HSV-1 infection (9). Further, systemic inoculation of UV-irradiated HSV-2 results in IFN α production in a myeloid differentiation factor (MyD)88 and TLR9-dependent manner (10). Similarly, *in vivo* recognition of a beta herpes virus, murine cytomegalovirus, has been shown to depend on TLR9 and MyD88 (11, 12). The second type of TLR–herpesvirus interaction occurs when virions engage surface TLR2 on DCs and macrophages. Peritoneal macrophages (13) and microglial cells (14) secrete inflammatory cytokines in response to HSV-1 in a TLR2-dependent manner. The third type of viral recognition results in a MyD88-independent, IFN regulating factor (IRF)-7-dependent pathway. This third pathway has been demonstrated to play a major role during systemic infection with HSV-1, because IFN α secretion

and suppression of viral replication depended mostly on IRF7 and to a much lesser extent on MyD88 (6).

Although these three distinct pathways of herpesvirus recognition are known to exist, the relative contributions of these pathways in viral recognition vs. viral pathogenesis are unclear. The ability of HSV-1 to trigger TLR2 has been shown to be responsible for the exacerbation of neonatal herpes encephalitis (13), because neonatal mice deficient in TLR2 secreted less IL-6 and had a higher rate of survival compared with WT mice upon lethal HSV-1 challenge. Further, the ability of HSV-1 and HSV-2 to activate TLR2 has been postulated to play a role in the sepsis-like disease associated with human neonatal herpes infection (15). These studies suggested that inflammatory responses induced by TLR2 engagement by HSV leads to immunopathology in the infected host. However, these studies used laboratory strains of HSV, and the clinical and epidemiological relevance of TLR2-activating HSV-1 and HSV-2, hereby termed HSV-1_{TLR2}^{*} and HSV-2_{TLR2}^{*}, are unknown. Thus, in this study, we examined the occurrence of the HSV-1_{TLR2}^{*} and HSV-2_{TLR2}^{*} in both the laboratory strains and the human clinical isolates from a variety of tissues and mucosal secretions. We show that HSV_{TLR2}^{*} constituted a minor population of HSV found in either the laboratory or the clinical isolates. Further, by plaque purification of the HSV-1_{TLR2}^{*}, we demonstrate that these represent a minor subspecies amongst the non-TLR2 activating HSV-1, or HSV-1_{TLR2}[°].

The HSV-1_{TLR2}^{*} in the clinical and laboratory viral isolates can engage both TLR2 and TLR9. However, the relative contributions of these two receptors in viral recognition and cytokine secretion in cells that express both of these receptors are unknown. TLR engagement leads to the activation of the IRF5 via MyD88 and tumor necrosis factor receptor-associated factor 6 (16). This TLR–MyD88–IRF5 axis is required for the production of proinflammatory cytokines, IL-6, IL-12, and TNF α (16). Given that TLR2 functions at the cell surface (17), whereas TLR9 functions in the endosomes (18, 19), if both receptors are involved in viral recognition, it is unknown whether TLR2 and TLR9 induce independent signals or whether the two TLRs synergize or antagonize to produce inflammatory cytokines. Here, we addressed the relative importance of TLR2 and TLR9 in herpesvirus recognition by classical DCs (cDCs) and pDCs.

Results

Only Certain Laboratory Strains of HSV Trigger TLR2 Activation. To understand the relative contributions of innate viral recognition

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Abbreviations: BM, bone marrow; cDC, classical dendritic cell; DN, dominant negative; HSV, Herpes simplex virus; IRF, interferon regulatory factor; MOI, multiplicity of infection; MOI_e, multiplicity of infection equivalent; MyD88, myeloid differentiation factor 88; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell; TK, thymidine kinase; TLR, Toll-like receptor; UL, unique long region.

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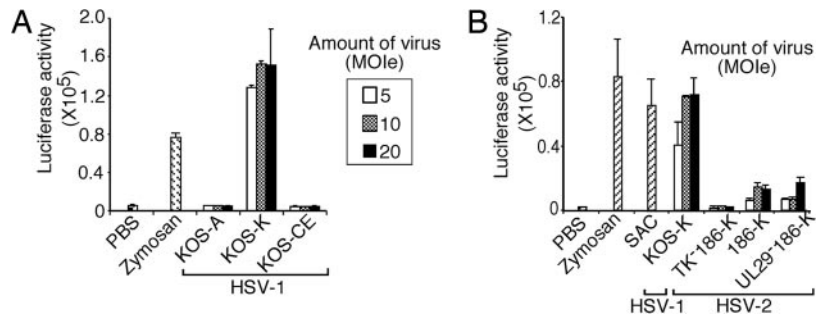


Fig. 1. Only certain laboratory strains of HSV trigger activation through TLR2. (A) HEK293T cells expressing TLR2, CD14, and NF κ B-driven firefly luciferase reporter gene (293T/luc/TLR2/CD14 cells) were cultured with the indicated MOI equivalents (MOIe) of UV-inactivated HSV-1 KOS strains from three different sources (A, ATCC; K, Knipe; CE, Cohen and Eisenberg) or 10 μ g/ml zymosan for 7 h, and luciferase activities were measured. (B) 293T/luc/TLR2/CD14 cells were cultured with the indicated MOIe of UV-inactivated HSV-1 or HSV-2 strain, zymosan (10 μ g/ml), or 0.02% SAC for 7 h, and luciferase activities were measured. The data are representative of six similar experiments.

through TLR2 and TLR9, we first examined the ability of a number of commonly used laboratory HSV strains to induce activation of NF κ B via TLR2. We investigated the ability of the purified HSV-1 and HSV-2 to induce luciferase reporter activity driven by the NF κ B promoter by using transiently transfected HEK293T cells. Because very similar results were obtained by using live or UV-inactivated viruses, these forms of viruses were used interchangeably in the luciferase reporter assays. As reported previously (13), HSV-1 KOS strain obtained from David Knipe's laboratory (KOS-K) induced strong activation of TLR2. In contrast, KOS purchased from American Type Culture Collection (KOS-A) or KOS obtained from the laboratory of Gary Cohen and Roselyn Eisenberg (KOS-CE) completely failed to trigger TLR2 activation even at high multiplicity of infection equivalent (MOIe) (Fig. 1A; see Table 1, which is published as supporting information on the PNAS web site). However, not all HSV strains obtained from the Knipe laboratory activated TLR2, as a thymidine kinase (TK) negative mutant, TK⁻ HSV-2 from the same laboratory (TK⁻186-K) failed to induce NF κ B activation via TLR2, whereas the WT 186 and unique long region 29 (UL29)-deficient 186 strains (186-K and UL29⁻186-K) stimulated low levels of TLR2 activation (Fig. 1B). No other TLR tested (TLR3, TLR4, TLR7, or TLR9) elicited NF κ B activation by KOS-K in this reporter cell line (data not shown). These observations prompted us to examine the basis for the difference between the HSV KOS that triggers TLR2 (HSV-1 KOS_{TLR2}^{*}) versus those that do not (HSV-1 KOS_{TLR2}^o).

A Minor Subspecies of the HSV-1 KOS_{TLR2}^{*} Activates TLR2. To investigate the molecular basis for the difference in HSV-1 KOS_{TLR2}^{*} and HSV-1 KOS_{TLR2}^o, we first prepared genomic DNA from the purified KOS-K and KOS-A and sequenced the whole genome. However, this analysis did not result in meaningful data as we observed sequences representing several subspecies of HSV from bulk cultures of KOS-K and KOS-A (unpublished observations). To obtain pure single clones of viruses, we carried out plaque purification of KOS-K and KOS-A. Sixty-four plaques were picked from the plates inoculated with KOS-K, and pools of 10 plaques were examined for the TLR2-agonist activity. To our surprise, not all pools of KOS-K clones stimulated TLR2 (Fig. 7A, which is published as supporting information on the PNAS web site). Next, some of the individual plaque isolates were examined for their ability to trigger TLR2 activation (Fig. 7B and C). Of the plaque isolates p11-p20, only p18 had the ability to stimulate TLR2. To compare the abilities of the KOS-K viral clones to trigger TLR2, plaque-purified viruses were propagated and purified, and various MOI of clones were tested for TLR2 activation. At all MOI tested, only the p63 of the plaque isolates p1 and p61-64 had the capacity to stimulate TLR2 activity (Fig. 7D). Thus, these results indicated that KOS-K consisted of a mixture of virus subspecies, most of which failed to activate TLR2. Thus, these results demonstrated that only certain laboratory isolates of KOS possess the ability to trigger TLR2 activation (Table 1), and that those strains consist of a mixture of viral subspecies, of which a minority activates TLR2 (Fig. 7).

Rare Occurrence of HSV_{TLR2}^{*} in Human Clinical Isolates. The observation that only certain laboratory isolates have the TLR2-activating capacity prompted us to examine the relevance of this type of activity in human clinical isolates. Thus, viruses were propagated from human clinical samples taken from a variety of mucosal and systemic sites from two different hospital sources (Table 2, which is published as supporting information on the PNAS web site). Because of the implications to neonatal herpes (13, 15), we included four samples from the neonates with infection involving the CNS and disseminated HSV-1. A preliminary screening of the primary clinical isolates from Yale New Haven Hospital found no TLR2-agonist activities (Fig. 8, which is published as supporting information on the PNAS web site). To extend this analysis, we examined 32 clinical isolates from various sources (Table 2). Vero cell lysates of certain clinical isolates showed strong activity (Gen A and CNS C), whereas others had modest activity (Ora E, CNS B, CNS D, and CNS F) (Fig. 8B and C). To confirm these findings with purified viruses, we propagated these clinical isolates and reexamined the purified virions for their TLR2-activating capacity. These analyses revealed that only two isolates (Gen A and CNS C) had the true capacity to activate TLR2 (Fig. 8D). To further examine whether clinical isolates that trigger TLR2 consists of a uniform population or a mixture of subspecies, several plaques were isolated from CNS C. Analysis of the purified plaque-isolated clones of CNS C revealed heterogeneity in the ability of individual clones to trigger TLR2 activation (Fig. 8E). These results indicated that a rare HSV-1 shed by the infected humans activated TLR2, and that such isolates consisted of a mixture of TLR2-activating and non-TLR2-activating subspecies.

HSV_{TLR2}^{*} Variants Are Recognized Through TLR2, TLR1, and TLR6 Independently of CD14. Thus far, we have demonstrated the ability of certain laboratory and clinical isolates of HSV to trigger NF κ B activation in HEK293T reporter cells transfected with TLR2. However, the importance of other TLRs that are known to pair with TLR2, such as TLR1 and TLR6 (17), and CD14, a coreceptor involved in TLR2 activation by CMV (20), in HSV-1_{TLR2}^{*} recognition is unknown. To determine the involvement of CD14, HEK293T cells were transfected with TLR2, CD14 or in combination, and the ability of the HSV-1_{TLR2}^{*} to induce NF κ B activation was assessed. It is known that HEK293T cells do not express functional levels of CD14 and require exogenous CD14 to respond to LPS (21). Thus, these results indicated that CD14 is not required for HSV-1_{TLR2}^{*} recognition (Fig. 2A). Next, we examined the requirement for TLR1 or TLR6 in TLR2-mediated recognition of HSV-1_{TLR2}^{*}. Because HEK293T cells express both TLR1 and TLR6 endogenously (22), we transfected TLR2-expressing HEK293T cells with the dominant negative (DN) TLR1 or DN TLR6. Expression of the DN TLR6, and to a lesser extent, DN TLR1, resulted in a reduction of NF κ B activation in cells triggered with HSV-1_{TLR2}^{*}. A similar reduction in the NF κ B activation was seen with zymosan (Fig. 2B), a known agonist of TLR2/TLR6 (17). These data suggested that the recognition of HSV-1_{TLR2}^{*} is mostly

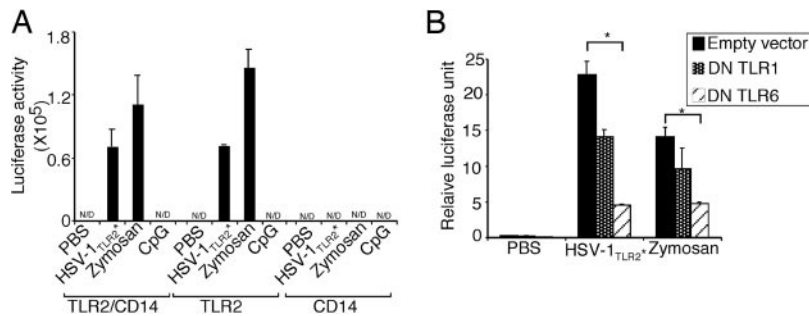


Fig. 2. HSV_{TLR2*} variants are recognized through TLR2, TLR1, and TLR6 independently of CD14. (A) HEK293T cells were transfected with TLR2 and/or CD14 plasmid. The cells were stimulated with 5 MOI of UV-inactivated HSV-1 KOS-K, 10 μ g/ml of zymosan, or 5 μ g/ml of CpG 1826 for 7 h. Luciferase activity was measured. (B) 293T/luc/TLR2/CD14 cells were transfected with 500 ng of DN TLR1 or DN TLR6 plasmid and *Renilla* luciferase plasmid. Twenty-four hours later, the cells were stimulated with 5 MOI of UV-inactivated HSV-1 KOS-K or zymosan for 7 h. Relative luciferase unit was calculated as a ratio of NF κ B-dependent firefly luciferase activity to NF κ B-independent *Renilla* luciferase activity. N/D, not detected. *, $P < 0.05$. The data are representative of three similar experiments.

mediated by TLR2 and TLR6, and to a lesser extent by TLR2/TLR1 complexes independent of CD14.

DCs Use both TLR2 and TLR9 to Recognize the HSV_{TLR2*} Variants. We thus far have demonstrated that a rare population of HSV-1 and HSV-2 can activate TLR2/TLR6 and TLR2/TLR1 independent of CD14. Key antigen presenting cell types, myeloid DCs, and macrophages, express TLR1, TLR2, TLR6, and TLR9, among other TLRs (3), and are known to respond to viruses including HSV-1 and HSV-2 (9, 13, 23–25). However, it remains controversial through which receptors DCs recognize HSV_{TLR2*}.

Several potential mechanisms can be postulated for HSV_{TLR2*} recognition by DCs (Fig. 3). The simplest is that in which HSV_{TLR2*} only activates TLR2 or TLR9 in DCs (Fig. 3A and B). Alternatively, HSV_{TLR2*} might activate both TLR2 and TLR9 in DCs. Within this scenario, there are at least two possibilities, the first is that HSV_{TLR2*} recognition occurs by two separate pathways upon engagement of TLR2 and TLR9 (Fig. 3C). This can occur in the same DC (as drawn), or in separate cells, depending on which receptor happens to be engaged in a given DC. The final possibility is that HSV_{TLR2*} is detected sequentially, first by TLR2 at the cell surface, and subsequently, via TLR9 in the endosome/lysosome compart-

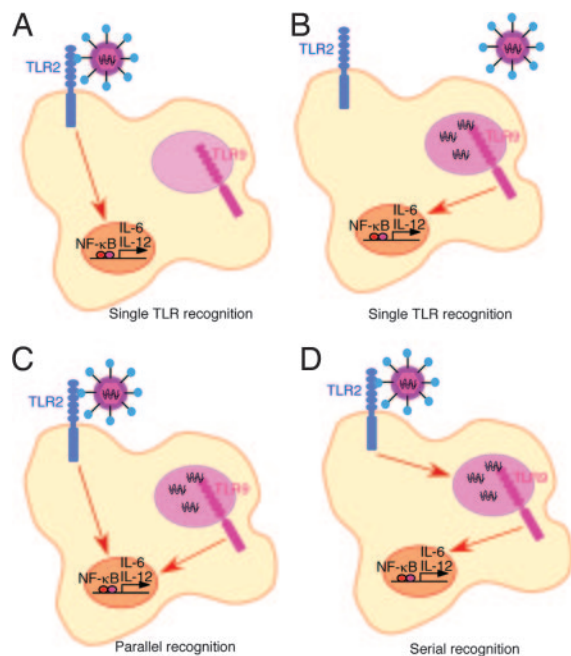


Fig. 3. Schematic of possible recognition mechanisms of HSV_{TLR2*} by DCs. (A and B) Single TLR-mediated recognition of HSV_{TLR2*} through either TLR2 (A) or TLR9 (B). (C) Parallel recognition of HSV_{TLR2*} via TLR2 and TLR9 in the same cells. (D) Serial recognition of HSV_{TLR2*}, first by the cell surface TLR2 and then by the intracellular TLR9.

ment in the same DC (Fig. 3D). In this scenario, TLR9 signal depends on the initial activation of the cell through TLR2.

To critically examine the relative roles of TLR2 and TLR9 in HSV recognition, bone marrow (BM) DCs from mice lacking TLR2, TLR9, TLR2 \times 9, or MyD88 were stimulated with either UV-inactivated or live HSV_{TLR2*} or HSV_{TLR2^o}, and the secretion of a representative proinflammatory cytokines IL-6 and IL-12 were measured (Fig. 4). Consistent with their ability to stimulate TLR2 activation in a reporter system (Fig. 1), UV-irradiated HSV_{TLR2*} induced robust IL-6 and IL-12 secretion that depended mostly on TLR2 and MyD88, whereas little IL-6 or IL-12 was secreted from DCs stimulated with UV-inactivated HSV_{TLR2^o} (Fig. 4A and D). However, to our surprise, IL-6 and IL-12 responses also were reduced significantly in the absence of TLR9 (Fig. 4A and D). To a control TLR agonist, LPS, TLR2^{-/-}, TLR9^{-/-}, or TLR2 \times 9^{-/-} BM DCs secreted WT levels of IL-6 (Fig. 4C) and IL-12 (Fig. 4F). These data indicated that DCs use both TLR2 and TLR9 to recognize HSV_{TLR2*}, but more importantly, a large fraction of the TLR2-dependent recognition of HSV_{TLR2*} also requires TLR9.

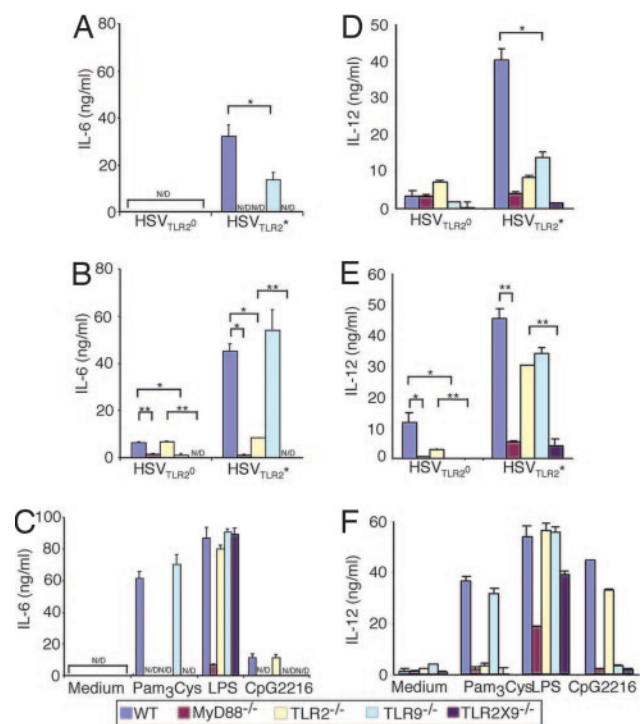


Fig. 4. DCs use both TLR2 and TLR9 to recognize the HSV_{TLR2*} variants. BM DCs from WT or the indicated knockout mice were cultured with 2 MOI of UV-inactivated (A and D), live HSV_{TLR2*} or HSV_{TLR2^o} (B and E), or known TLR agonists (C and F) for 24 h. Supernatants were analyzed for IL-6 (A–C) and IL-12 p40 (D–F) by ELISA. N/D, not detected. *, $P < 0.05$; **, $P < 0.01$. The data are representative of five similar experiments.

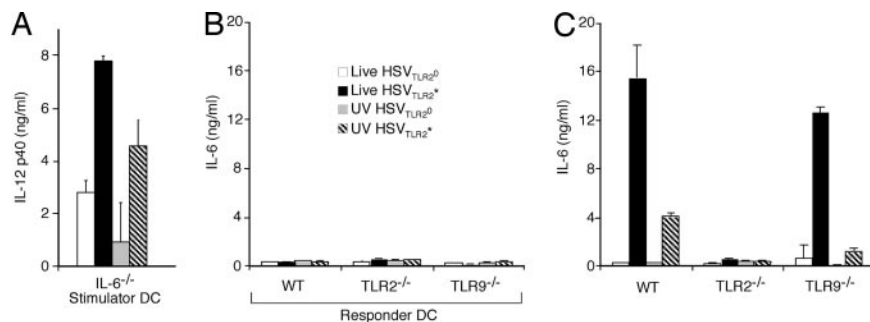


Fig. 5. Direct virus recognition, but not bystander stimulation, induces cytokine secretion from cDCs. IL-6^{-/-} BM DCs (stimulator) were incubated with 2 MOI of live or UV-inactivated HSV-1_{TLR2}^{*} or HSV-1_{TLR2}[°] for 24 h, and after thorough washing, they either were incubated for additional 24 h for assessment of IL-12 secretion (A) or exposed UV light to prevent *de novo* viral replication. (B) The stimulator DCs were cocultured with the indicated responder DCs. (C) In parallel, the responder DCs alone were incubated directly with live or UV-inactivated HSV-1_{TLR2}^{*} or HSV-1_{TLR2}[°] for 24 h, and IL-6 levels were measured. The data are representative of three similar experiments.

Thus, a sequential recognition of HSV_{TLR2}^{*} via TLR2 and TLR9 accounts for majority of the TLR2-mediated recognition of UV-irradiated HSV_{TLR2}^{*} virions (Fig. 3D), whereas the rest of the TLR2-dependent recognition is independent of TLR9 in DCs (Fig. 3A). Thus, UV-irradiated HSV_{TLR2}^{*} virions are detected by a combination of the serial TLR2 → TLR9 (Fig. 3D) and single TLR2 (Fig. 3A) recognition mechanisms in DCs.

Next, we examined the mechanism of DC recognition of live HSV_{TLR2}^{*} infection. In contrast to the UV-inactivated virions, live HSV_{TLR2}^{*} elicited levels of IL-6 (Fig. 4B) and IL-12 (Fig. 4E) from TLR9^{-/-} BM DC comparable with WT, an observation consistent with a previous study (23). These data suggested that a non-TLR9 MyD88-dependent signals induced by the replicating HSV_{TLR2}^{*} is sufficient to trigger maximum response in DCs. Moreover, TLR2^{-/-} BM DC secreted significant levels of cytokines, particularly IL-12, in response to live HSV_{TLR2}^{*} infection. To determine whether cytokine production from the TLR2^{-/-} DC depends on TLR9, BM DC from TLR2 × 9 double knockout mice were stimulated with the same viruses. The TLR2 × 9^{-/-} DCs secreted minimal to undetectable amounts of IL-6 or IL-12 upon infection with HSV_{TLR2}^{*}, similar to those observed from the MyD88^{-/-} BM DCs. These data indicated that the innate recognition of live HSV_{TLR2}^{*} can be largely accounted for by the combinations of signals from TLR2 and TLR9. Collectively, these data demonstrated that live HSV_{TLR2}^{*} infection of DCs engages two additional types of recognition pathways, TLR2-dependent TLR9-independent (Fig. 3A) and TLR2-independent TLR9-dependent (Fig. 3B), and that deficiency in either receptors can be compensated for by the other. In contrast to the HSV_{TLR2}^{*} infection of BM DCs with HSV_{TLR2}[°] resulted in low levels of cytokine secretion, mostly dependent on TLR9. Thus, HSV_{TLR2}[°] live infection is detected through the TLR2-independent TLR9-dependent pathway (Fig. 3B).

Direct Stimulation of DCs by Viruses Is Required to Induce Cytokine Secretion. Thus far, our data suggested that a sequential recognition of HSV_{TLR2}^{*} occurs within the same DC through TLR2 followed by TLR9. However, these data also are consistent with the possibility of a positive cross-talk between the responder DCs (recognizing the virus via TLR2) and bystander DCs (activated in a TLR9-dependent manner). To examine the latter possibility, we established a DC-DC coculture system in which the stimulator DCs are treated with HSV_{TLR2}^{*} or HSV_{TLR2}[°] before addition to the uninfected responder DCs. To ensure that the readout cytokine (IL-6) from the responders are not contaminated by that from the stimulators, we used IL-6^{-/-} DCs as the stimulator cells. Thus, IL-6^{-/-} DCs were incubated with either live or UV-inactivated HSV_{TLR2}^{*} or HSV_{TLR2}[°] for 24 h, washed thoroughly, UV-irradiated and then were added to the responder DCs. To confirm that the stimulator cells were fully activated by the viral treatment, IL-12p40 secretion from the stimulator cells was measured after the respective treatments (Fig. 5A). These data indicated that the IL-6^{-/-} stimulator DCs responded to the virus infection and secreted cytokine in a manner similar to the WT DCs (Fig. 4). To

assess the ability of the stimulator DCs to activate bystander DCs, IL-6 secretion from the responder DC supernatants was measured after 24 h of coculture with the stimulator DCs. Remarkably, the bystander responders failed to secrete IL-6 in response to the virus-infected stimulator cells (Fig. 5B), despite their intact ability to secrete IL-6 after direct virus infection (Fig. 5C). Therefore, these data indicated that bystander activation of the uninfected DCs by other HSV-infected DCs was not observed and that direct recognition of the virus is required to activate DCs to produce inflammatory cytokines.

pDCs Use TLR9 to Recognize both HSV_{TLR2}^{*} and HSV_{TLR2}[°]. Thus far, the dual receptor-mediated recognition of HSV_{TLR2}^{*} was shown for cDCs in response to live and UV-inactivated viruses. However, IFN α was not detectable from cDCs infected with any of these viruses (data not shown). It is known that pDCs respond to HSV-1 and HSV-2 via TLR9 and secrete robust levels of type I IFNs (9, 10). In an effort to understand innate recognition of HSV_{TLR2}^{*} and HSV_{TLR2}[°] by pDCs, BM pDCs purified from mice lacking TLR2, TLR9, or both were stimulated with either live or UV-inactivated HSV_{TLR2}^{*} or HSV_{TLR2}[°], and IFN α was measured. In contrast to the cDCs, pDCs recognized both HSV_{TLR2}^{*} and HSV_{TLR2}[°] via TLR9 and not TLR2 resulting in comparable IFN α secretion in response to these viruses (Fig. 6). Further, pDCs failed to respond to either TLR2 or TLR4 agonists, whereas they responded to CpG in a TLR9-dependent manner (Fig. 6C). Thus, unlike cDCs, pDCs recognize both HSV_{TLR2}^{*} and HSV_{TLR2}[°] via TLR9, regardless of

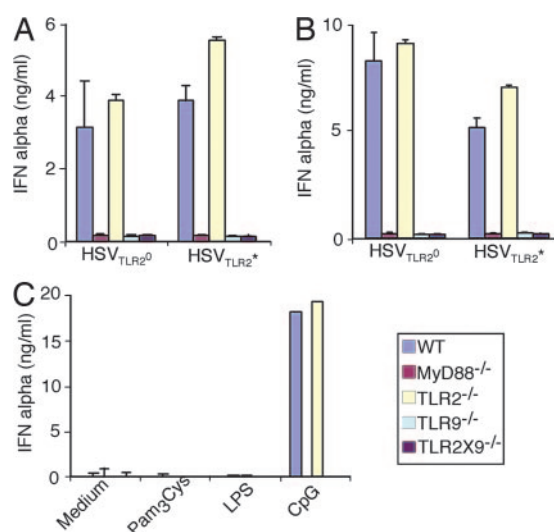


Fig. 6. pDCs secretion of IFN α depends on TLR9 in response to both HSV_{TLR2}^{*} and HSV_{TLR2}[°]. BM pDCs from the indicated mice were purified and stimulated with UV-irradiated (A) or live (B) HSV_{TLR2}^{*} or HSV_{TLR2}[°] at MOI of 2, or with the indicated TLR agonists (C). Supernatants were analyzed for IFN α by ELISA. The data are representative of three similar experiments.

TLR2^{-/-} × TLR9^{-/-} mice crossed to homozygosity. WT control littermates (F₂ generations from 129/svJ × C57BL/6) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Viruses. HSV-1 KOS (KOS-K), HSV-2 186Syn (186-K), HSV-2 186TKΔKpn (TK⁻186-K) (36), and HSV-2 5BLacZ (UL29⁻186-K) (37) were kind gifts of David Knipe (Harvard Medical School, Boston, MA). HSV-1 KOS (KOS-CE) was provided by Gary H. Cohen and Roselyn J. Eisenberg (University of Pennsylvania, Philadelphia, PA). HSV-1 KOS (KOS-S) was provided by Stephen Straus (National Institute of Allergy and Infectious Diseases/National Institutes of Health, Bethesda, MD). HSV-1 clinical isolates (Table 2) were generous gifts from Yale Clinical Virology Laboratory (Marie Landry, Department of Laboratory Medicine) and from University of Pennsylvania as described (38). HSV-1 KOS (KOS-A) and HSV-2 G (HSV-2 G-A) were purchased from American Type Culture Collection. UL-24⁻KOS (UL24⁻KOS-C) and TK⁻KOS (TK⁻KOS-C) were provided by Donald Coen (Harvard Medical School, Boston, MA). All viral strains were propagated and assayed on Vero cells (39), except UL29⁻186-K, which were propagated on UL-5 and UL29-expressing Vero cell line, V529 (40). In some cases, the virus was UV-inactivated by exposure to 1 J/cm² UV light with a Stratallinker UV Cross-linker (Stratagene, La Jolla, CA) as described (41).

Luciferase Assay. HEK293T cells and HEK293T cells expressing NFκB-driven firefly luciferase reporter gene, TLR2, and CD14 (293T/luc/TLR2/CD14) were generous gifts of Ruslan Medzhitov (Yale University). 293T/luc/TLR2/CD14 cells or HEK293T cells transiently transfected with TLR2 and/or CD14 plasmid were incubated with virus, TLR2 ligands [10 μg/ml zymosan (Molecular Probes, Eugene, OR), 0.02% inactivated *Staphylococcus aureus*, Cowan's strain (Calbiochem, San Diego, CA), or 200 ng/ml Pam₃Cys₄ (InvivoGen, San Diego, CA)], TLR4 ligand (10 μg/ml LPS (List Biological Laboratories, Campbell, CA), or TLR9 ligands [5 μg/ml phosphodiester CpG 1826; TCCATGACGTTCTT-GACGTT or CpG 2216; ggGGGACGATCGTCgggggG (synthesized by Invitrogen, Carlsbad, CA)] for 7 h. Cells were lysed and luciferase activity was measured by using Luciferase assay system (Promega, Madison, WI). For measuring relative luciferase activity, 293T/luc/TLR2/CD14 cells were transfected with dominant neg-

ative DN TLR1 or DN TLR6 plasmid (42) and *Renilla* luciferase plasmid and luciferase activity was measured by a Dual-Glo luciferase assay system (Promega).

In Vitro Differentiation and Stimulation of BM DCs. BM DCs were prepared by the modified protocol as described (43). BM DCs were incubated with the indicated MOI of virus or TLR ligands at 37°C for 24 h. Unless otherwise indicated, KOS-K plaque 63 (p63) and KOS-K plaque 62 (p62) were used as representative HSV-1 KO-S_{TLR2}* and HSV-1 KOS_{TLR2}^o, respectively. Culture supernatant was collected and IL-6 and IL-12 levels were measured by ELISA with Abs from eBioscience (San Diego, CA) according to manufacturer's instructions. The IFNα levels were measured by ELISA as described (10).

Purification of BM pDCs. BM was isolated from femurs and tibia, depleted of erythrocytes with ACK lysis buffer (BM cell). The pDCs were purified by using anti-mPDCA-1 microbeads (Miltenyi Biotec Auburn, CA) according to manufacturer's instructions. To further enrich for pDCs, the procedure was repeated twice. The resulting populations of cells were >89% pure by flow cytometric analyses (data not shown).

Bystander DC Stimulation. BM DCs from IL-6^{-/-} (stimulator) or WT, TLR2^{-/-}, and TLR9^{-/-} (responder) mice were prepared as described above. Stimulator DCs were incubated with 2 MOI of live or UV-inactivated HSV-1_{TLR2}* or HSV-1_{TLR2}^o for 24 h, washed three times in PBS, and were either incubated for additional 24 h or exposed to 1 J/cm² UV light to prevent *de novo* viral replication. These stimulator DCs (5 × 10⁵ cell per well) were cocultured with responder DCs (5 × 10⁵ cell per well) for 24 h, and IL-6 levels in the supernatants were measured by ELISA. Another group of responder DCs were incubated directly with live or UV-inactivated HSV-1_{TLR2}* or HSV-1_{TLR2}^o for 24 h.

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