Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA

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Plasmacytoid dendritic cells (pDCs) play a critical role in antiviral immunity through their ability to produce large amounts of type I IFNs. Activation of pDCs upon viral infection has been shown to be dependent on MyD88 and mediated by Toll-like receptors (TLR) 7 and 9, which sense viral ssRNA and CpG DNA, respectively. In this study, we showed that murine pDC recognition of vaccinia virus (VV), a dsDNA virus, was MyD88-dependent but TLR9-independent. Using HEK293 cells transfected with murine TLR7 or TLR8 and a NF-κB luciferase reporter, we demonstrated that stimulation of TLR8-, but not TLR7-, transfected cells with either VV or VV DNA resulted in substantial NF-κB activation, and that siRNA-mediated knockdown of TLR8 expression in pDCs led to a complete ablation of VV-induced type I IFN production. We further identified that the VV genome was rich in poly(A)/T sequences, and synthetic poly(A) and poly T oligodeoxynucleotides were capable of activating pDCs in a TLR8-dependent manner. In vivo, TLR8-MyD88-dependent pDC activation played a critical role in innate immune control of VV infection. Collectively, our data are unique in demonstrating that TLR8 is required for sensing poly(A)/T-rich DNA in pDCs, and that murine TLR8 is functional in the context of a viral infection.

Plasmacytoid dendritic cells (pDCs) are specialized immune cells that play an important role in antiviral immunity (1). This role is in large part because of the ability of pDCs to rapidly produce large amounts of type I IFNs upon viral infection (2). Studies have shown that type I IFN production by pDCs in response to viral infection requires activation of Toll-like receptors (TLR) 7 and 9, which sense viral nucleic acids in the early endosomes (3). TLR7 recognizes specific sequences in guanosine- and uridine-rich ssRNA (4, 5), whereas TLR9 senses an unmethylated CpG motif in DNA (6, 7). TLR8 also plays a role in sensing viral ssRNA in humans (5, 8). Although TLR8 was initially thought to be nonfunctional in mice (5, 8), a recent study shows that murine TLR8 can be activated under certain circumstances (9). Induction of type I IFNs upon activation of TLR7, -8, and -9 is mediated by the common TLR adaptor, MyD88 (10).

Vaccinia virus (VV) is a member of the poxvirus family that includes smallpox (variola) virus, monkeypox virus, cowpox virus, and mousepox (ectromelia) virus (11). It is an enveloped, dsDNA virus with a genome measuring about 200 Kb. VV is the most studied member of the poxvirus family and is responsible for the successful elimination of smallpox worldwide in the late 1970s (12). However, mechanisms underlying the control of VV infection remain largely unknown. In a murine model of VV infection, we have recently shown that VV activates the TLR2-MyD88 pathway on conventional DCs (cDCs), leading to production of the proinflammatory cytokines IL-6, IL-1, and IL-12 (13). In addition, VV also stimulates cDCs to produce IFN-β in a TLR-independent manner (13). Given the importance of pDCs in antiviral immunity, it remains to be defined whether and how VV activates pDCs.

In this article, we showed that both VV and VV DNA were capable of activating pDCs, leading to MyD88-dependent secretion of type I IFNs. Unexpectedly, we found that pDC recognition of VV DNA was TLR9-independent. Using HEK293 cells transfected with a plasmid-expressing murine TLR7 or TLR8 and an NF-κB luciferase reporter construct, we showed that stimulation of TLR8-transfected cells with VV or VV DNA resulted in substantial NF-κB activation, whereas stimulation of TLR7-transfected cells did not. We further demonstrated that siRNA-mediated knockdown of TLR8 expression in murine pDCs essentially abolished type I IFN production by pDCs upon stimulation with VV or VV DNA. We next identified that the VV genome was rich in poly(A)/T sequences, and synthetic poly(A) and poly T oligodeoxynucleotides (ODNs) were capable of activating pDCs in a TLR8-dependent fashion. In vivo, TLR8-MyD88-dependent pDC activation and type I IFN production played a critical role in innate immune control of VV infection. Taken together, these data are unique in demonstrating that pDC sensing of VV DNA is mediated by TLR8 possibly through recognition of poly(A)/T-rich motifs, and that murine TLR8 is functional in the context of a viral infection.

Results

We first examined whether VV was capable of activating pDCs, as they play a critical role in antiviral immunity (1). pDCs, identified as $CD11c^{+}B220^{+}mPDCA\text{-}1^{+}$, were generated from bone marrow cells in the presence of Flt-3 ligand (Flt-3L) and purified by FACS sorting. These pDCs were then stimulated with VV, and the culture supernatants were assayed for the secretion of IFN-α and IFN-β. Indeed, pDCs stimulated with VV produced significant amounts of IFN-α (Fig. 1*A*) and IFN-β (Fig. 1*B*), and up-regulated the expression of CD86 (Fig. 1*C*), indicating that they were activated upon VV infection. We next investigated whether TLRs were involved in the induction of type I IFNs by pDCs upon VV infection. Because all TLR signaling in pDCs is mediated by MyD88 (1), pDCs deficient for MyD88 ($MyD88^{-/-}$) were tested for their ability to produce type I IFNs upon VV infection. The production of both IFN-α (Fig. 1*A*) and IFN-β (Fig. 1*B*) by $MyD88^{-/-}$ pDCs was abolished, demonstrating that the production of type I IFNs by pDCs in response to VV infection is TLR-mediated and dependent on MyD88. We further demonstrated that endogenous splenic pDCs also secreted type I IFNs in a MyD88-dependent fashion upon VV infection (Fig. 1*D*). However, in line with our previous finding (13), cDCs ($CD11c^{+}B220^{-}mPDCA\text{-}1^{-}$) activated with VV produced only lower levels of IFN-β in a MyD88-independent manner (Fig. 1*B*).

We next sought to determine which TLR was responsible for the production of type I IFNs by pDCs in response to VV infection. Among all TLRs characterized to date, only TLR7, -8, and -9 are known to mediate MyD88-dependent production of type I IFNs (14). Indeed, both pDCs and cDCs expressed TLR7, -8, and -9 (Fig. S1). Because the known ligands for TLR7 and TLR8 are ssRNA, and VV is a dsDNA virus, we first examined if VV or VV DNA activated TLR9 in pDCs to produce type I IFNs. pDCs generated from $TLR9^{-/-}$ mice secreted similar levels of IFN-α (Fig. 1*E*) and IFN-β (Fig. 1*F*) upon stimulation with VV or VV DNA compared

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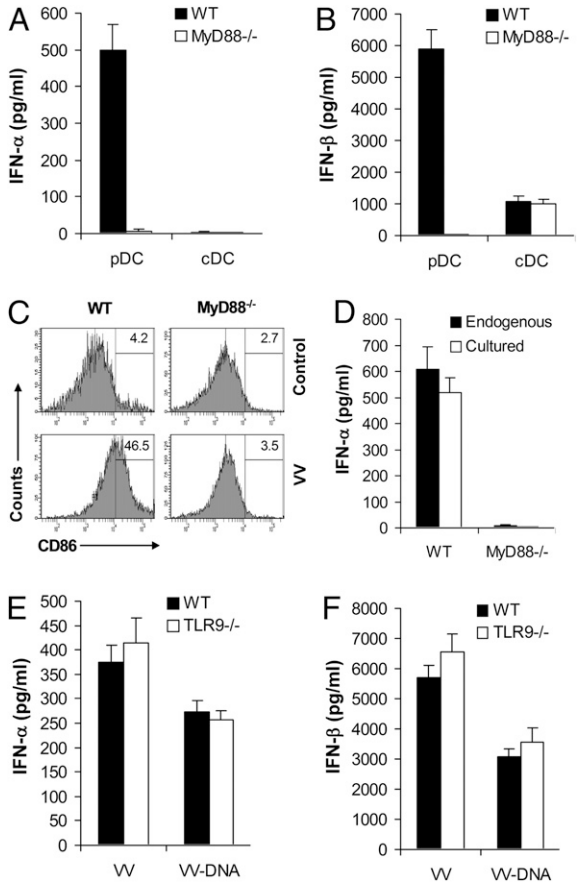

Fig. 1. MyD88-dependent, but TLR9-independent activation of pDCs by VV. (*A–C*) DCs were generated from bone marrow cells of WT or MyD88$^{-/-}$ C57BL/6 mice in the presence of Flt-3L for 9 days. Cells were stained with anti-CD11c and anti-B220, and sorted into pDC and cDC populations by flow cytometry. Purified cells were then stimulated with live VV at a multiplicity of infection (MOI) of 1 for 24 h. Culture supernatants were analyzed by ELISA for secretion of IFN-α (*A*) and IFN-β (*B*). In addition, pDCs were stained with anti-CD86 antibodies, and analyzed for CD86 expression. The percentages of pDCs expressing CD86 are indicated (*C*). (*D*) Endogenous splenic pDCs were isolated from C57BL/6 (WT) and MyD88$^{-/-}$ mice by flow cytometry sorting and stimulated with live VV at an MOI of 1 for 24 h. Culture supernatants were analyzed by ELISA for secretion of IFN-α in comparison with Flt-3L cultured pDCs. (*E* and *F*) WT or TLR9$^{-/-}$ pDCs were stimulated with live VV (MOI 1) or VV DNA (25 μg/mL) for 24 h. Culture supernatants were analyzed by ELISA for secretion of IFN-α (*E*) and IFN-β (*F*). Results are expressed as mean ± SD. Data shown is representative of three independent experiments.

with the WT controls, suggesting that TLR9 is not involved in induction of type I IFNs by VV DNA. Taken together, the above observations indicate that VV-induced production of type I IFNs by pDCs is MyD88-dependent, but TLR9-independent.

The MyD88-dependent, TLR9-independent sensing of VV by pDCs suggested that the TLR responsible for pDC activation would likely be TLR7 or TLR8, as both of them are expressed in murine pDCs (Fig. S1). To address this question, we transfected HEK293 cells with a plasmid-encoding murine TLR7 or TLR8, in combination with an NF-κB luciferase reporter construct. The transfected cells were subsequently stimulated with VV or VV DNA, and cell lysates were assayed for luciferase activity. Stimulation of the TLR7-transfected cells with a synthetic TLR7 ligand, CL087, yielded high levels of luciferase activity compared with the NF-κB luciferase reporter construct-only control (Fig. 2*A*). However, no significant levels of luciferase activity were produced by TLR7-transfected cells stimulated with VV, VV DNA, or a TLR8 ligand, CL075 (Fig. 2*A*). In contrast, stimulation of TLR8-transduced cells with VV, VV DNA, or CL075 resulted in high levels of luciferase activity (Fig. 2*A*). These results suggest that TLR8, but not TLR7, can be activated by VV or VV DNA in this system.

We next tested whether murine TLR8 was also responsible for sensing of VV or VV DNA by pDCs. We evaluated the effect of small interfering RNA (siRNA)-mediated knockdown of TLR8 in pDCs on the induction of type I IFNs by VV or VVDNA. pDCs were transduced by a GFP-containing lentiviral construct encoding siRNA specific for murine TLR8 or a control siRNA, and the transduced GFP^{+} pDCs were sorted and assayed for the expression of TLR8 by RT-PCR. No expression of TLR8 was detected in pDCs transduced with two different siRNA constructs specific to TLR8, compared with the control siRNA-transduced or untransduced pDCs (Fig. 2*B*), thus confirming the knockdown of TLR8 in pDCs. The pDCs were then stimulated with VV or VV DNA and assayed for IFN-α secretion. The production of IFN-α was essentially abolished in pDCs transduced with TLR8-specific siRNAs, compared with the control siRNA (Fig. 2*C*). We further demonstrated that the TLR8-knockdown pDCs failed to produce IFN-α upon stimulation with VV-DNA or the TLR8 ligand, CL075, but retained the ability to secret IFN-α in response to the TLR7 ligand, CL087, or the TLR9 ligand, CpG (Fig. 2*D*), confirming the specificity of TLR8 knock-down by siRNA. We also found that pDCs produced little proinflammatory cytokines IL-1 (Fig. S2*A*), IL-6 (Fig. S2*B*), and IL-12 (Fig. S2*C*) upon VV stimulation. Collectively, these results indicate that pDC sensing of VV

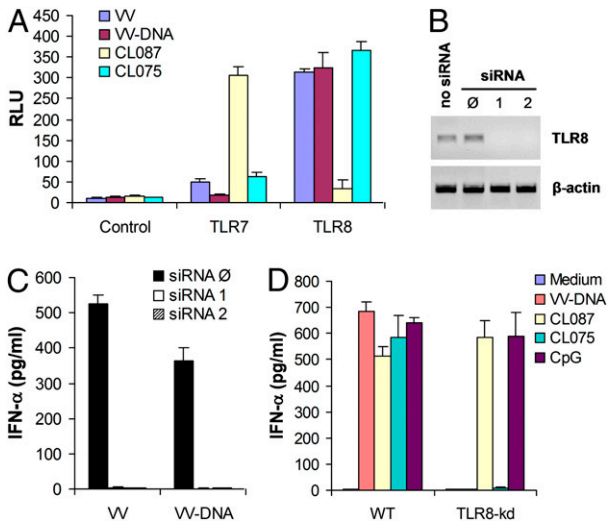

Fig. 2. VV or VV DNA activates pDCs via TLR8. (*A*) HEK293 cells were transfected with a plasmid encoding either murine TLR7 or TLR8 and an NF-κB-Luciferase reporter construct. Forty-eight hours after transfection, cells were stimulated with live VV (MOI 1), VV DNA (25 μg/mL), TLR7 ligand, CL087 (0.1 μg/mL), or TLR8 ligand, CL075 (0.1 μg/mL) for 12 h. Cells were then lysed and NFκB activation was analyzed by a luminometer. Data are presented as mean relative luciferase units ± SD (*B* and *C*) pDCs generated from bone marrow cells were transduced with two different lentiviral constructs encoding siRNA specific for murine TLR8, pNL-SIN-GFP-siRNA1 (siRNA1) or pNL-SIN-GFP-siRNA2 (siRNA2), or a control virus pNL-SIN-GFP without siRNA (siRNAØ), or left untransduced (no siRNA). RNA was purified from the transduced GFP+ pDCs and subjected to TLR8 expression by RT-PCR (*B*). Sorted EGFP^{+} pDCs were stimulated with live VV (MOI 1) or VV DNA (25 μg/mL) for 24 h and the culture supernatants were analyzed by ELISA for secretion of IFN-α (*C*). (*D*) WT or TLR8-knockdown (TLR8-kd) pDCs were stimulated with VV DNA (25 μg/mL), TLR7 ligand, CL087 (0.1 μg/mL), TLR8 ligand, CL075 (0.1 μg/mL) or TLR9 ligand, CpG (1 nM) for 24 h, or left unstimulated (medium). Culture supernatants were analyzed by ELISA for secretion of IFN-α. Results are expressed as mean ± SD. Data shown is representative of three independent experiments.

DNA is mediated by TLR8, leading to production of type I IFNs. This finding is in contrast to our previous observation on cDCs, which sense nonnucleic acid components of VV via the TLR2-MyD88 pathway, leading to secretion of proinflammatory cytokines IL-1, IL-6, and IL-12 (13). Indeed, we further showed that cDC production of proinflammatory cytokines was independent of TLR8 in response to VV stimulation (Fig. S2).

What then is the potential sequence motifs in VV DNA required for activation of TLR8? Because poly T ODN has been shown to enhance human TLR8 responsiveness to small molecule ligands (15, 16), we first examined the VV genome for the A/T and G/C compositions. Indeed, ≈67% of the VV genome is composed of A/T sequences (Fig. 3*A*), indicating the VV genome is rich in A/T sequences. This is in contrast to only 44% of the human adenovirus type 5 (Ad5) (Fig. 3*A*) and 30% of the herpes simplex virus type 2 (HSV-2) genomes. It has been shown that both Ad5 and HSV-2 activate TLR9 in pDCs via their CpG motif (17, 18). Indeed, we showed here that activation of pDCs by Ad5 DNA was dependent on TLR9, but not TLR8 (Fig. 3*B*). We further found that about 45% of the VV genome is composed of A/T-rich islands, defined as 10 base-pair stretches with five or more A/T, compared to only about 1% of G/C-rich islands (Fig. 3*C*). These results suggest that poly(A) or poly T DNA might contribute to the activation of TLR8. To test this theory, poly A10, poly T10, poly C10, and poly G10 ODNs were generated and used to stimulate WT or TLR8-knockdown pDCs, and the culture supernatants were analyzed for secretion of IFN-α. Stimulation of WT or TLR9$^{-/-}$, but not TLR8-knockdown, pDCs with poly A10 or poly T10 led to high levels of IFN-α production (Fig. 3*D*). However, no significant levels of IFN-α were secreted when WT or TLR9$^{-/-}$ pDCs were stimulated with poly C10 or poly G10 (Fig. 3*D*). These results suggest that poly(A)- and poly T-rich DNA are responsible for the activation of murine pDCs via TLR8.

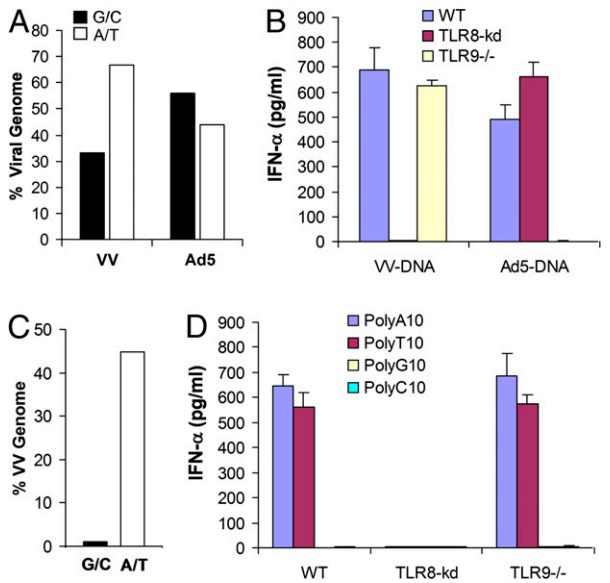

Fig. 3. The poly(A) and poly T motifs are responsible for activation of TLR8 on pDCs. (*A*) The complete genomes of VV and human adenovirus type 5 (Ad5) were analyzed for A/T and G/C contents and expressed as a percentage using pDRAW32 software. (*B*) WT, TLR8-knockdown (TLR8-kd), or TLR9$^{-/-}$ pDCs were stimulated with VV DNA (25 μg/mL) or Ad5 DNA (5 μg/mL) for 24 h and culture supernatants were assayed for IFN-α secretion. (*C*) The VV genome was analyzed for A/T- or G/C-rich islands, defined as 10 base-pair stretches with at least 5 A/T or G/C. (*D*) WT, TLR8-knockdown (TLR8-kd), or TLR9$^{-/-}$ pDCs were stimulated with PolyG10, PolyC10, PolyA10, or PolyT10 (all at 5 μg/mL) ODNs for 24 h. Culture supernatants were analyzed by ELISA for secretion of IFN-α. Data shown is representative of three independent experiments.

We next investigated the functional significance of TLR8-dependent activation of pDCs by VV in vivo. WT or MyD88$^{-/-}$ mice were injected with 1×10^7 pfu of VV intravenously. Forty-eight hours later, serum was assayed for IFN-α secretion, and the spleen was harvested for determination of the viral titers by the plaque assay. Sera from VV-infected WT mice were found to contain significant levels of IFN-α (Fig. 4*A*), as well as proinflammatory cytokines IL-1 (Fig. S3*A*), IL-6 (Fig. S3*B*), and IL-12 (Fig. S3*C*). In contrast, the production of IFN-α was greatly diminished in VV-infected, MyD88$^{-/-}$ mice (Fig. 4*A*). This finding was accompanied by a significant ($P < 0.001$) (Fig. 4*B*) increase in viral titers in the spleen of MyD88$^{-/-}$ mice. Because the production of proinflammatory cytokines is dependent on the TLR2-MyD88 pathway (13), the levels of these cytokines were also greatly diminished in VV-infected, MyD88$^{-/-}$ mice (Fig. S3). Adoptive transfer of WT pDCs into MyD88$^{-/-}$ mice resulted in restoration of the secretion of IFN-α (Fig. 4*A*), but not proinflammatory cytokines (Fig. S3), leading to reduction of viral titers in the spleen (Fig. 4*B*). However, transfer of TLR8-knockdown pDCs into MyD88$^{-/-}$ mice did not promote IFN-α secretion or reduction of viral load (Fig. 4). These results suggest that TLR8-MyD88-dependent pDC activation and secretion of type I IFNs are critical for innate immune control of VV infection in vivo, whereas TLR2-MyD88-dependent secretion of proinflammatory cytokines by cDCs is dispensable in this process.

To further confirm the significance of TLR8-dependent pDC activation in innate immune control of VV in vivo, we first depleted WT mice of pDCs using anti-Ly6G/C Ab as described (19, 20), and then reconstituted the pDC-depleted mice with TLR7-knockdown, TLR8-knockdown, or TLR9$^{-/-}$ pDCs and examined for their ability to restore innate immune control of VV infection in vivo. Previous studies have shown that administration of anti-Ly6G/C can efficiently deplete pDCs (19, 20). Indeed, we found that injecting mice twice with Ly6G/C Ab depleted >92% of pDCs in vivo (Fig. S4). pDC depletion abrogated IFN-α secretion in response to VV infection (Fig. 5*A*) and resulted in a significant increase in viral titers and viral DNA in the spleen (Fig. 5 *B* and *C*). Previous studies have shown that the liver is also a target organ for VV upon i.v. infection (21). We thus also examined viral titer and DNA in the liver and obtained similar results (Fig. 5 *D* and *E*). These results further illustrate the importance of pDCs in VV control in vivo. Reconstitution of pDC-depleted mice with TLR7-knockdown or TLR9$^{-/-}$ pDCs restored IFN-α secretion (Fig. 5*A*) and resulted in a significant reduction of viral titers and viral DNA in the target organs (Fig. 5 *B*–*E*). However, transfer of TLR8-knockdown pDCs into pDC-depleted mice failed to elicit the production of IFN-α (Fig. 5*A*) or a reduction of viral DNA or viral

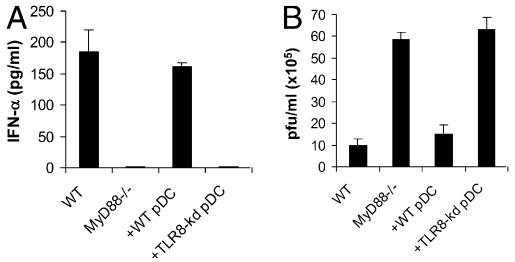

Fig. 4. WT, but not TLR8-knockdown, pDCs restore VV clearance in MyD88$^{-/-}$ mice. MyD88$^{-/-}$ mice were reconstituted with WT or TLR8-knockdown (TLR8-kd) pDCs, and infected with 1×10^7 pfu of live VV, intravenously. WT and MyD88$^{-/-}$ mice were also infected with VV and used as controls. Forty-eight hours after infection, serum was analyzed by ELISA for secretion of IFN-α (*A*), and the spleen was evaluated for viral titers by the plaque forming assay (*B*). Results are expressed as mean ± SD ($n = 5$). Data shown is representative of two independent experiments.

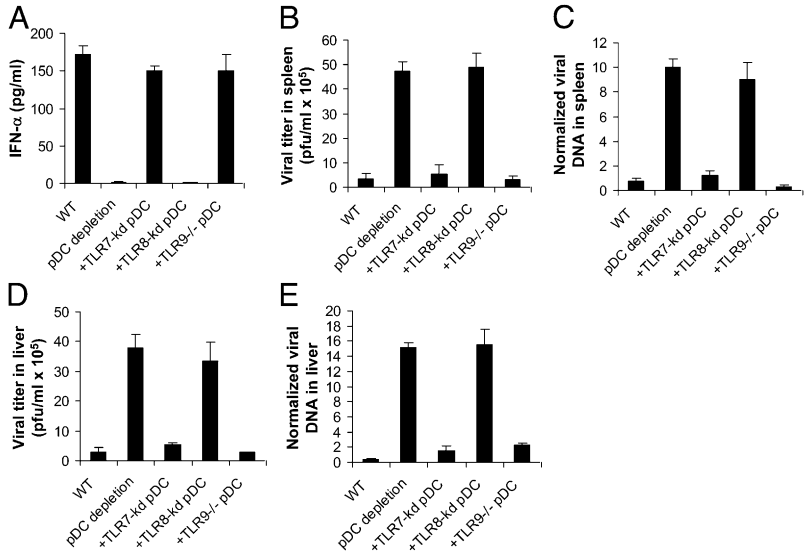

Fig. 5. TLR8-knowdown pDCs fail to restore VV clearance in pDC-depleted mice. WT mice were depleted of pDCs with anti-Ly6G/C on days −3 and −1, and reconstituted on day 0 with TLR7-knockdown (TLR7-kd), TLR8-knockdown (TLR8-kd) or TLR9$^{-/-}$ pDCs, or left unreconstituted (pDC depletion). These mice were subsequently infected with 1×10^7 pfu of live VV, intravenously. WT mice were also infected with VV and used as a positive control. Forty-eight hours after infection, serum was analyzed by ELISA for secretion of IFN-α (*A*), and the spleen (*B*, *C*) and the liver (*D*, *E*) were evaluated for viral titers by the plaque-forming assay (*B*, *D*) and viral DNA by real-time quantitative PCR (*C*, *E*). Results are expressed as mean ± SD ($n = 5$). Data shown is representative of two independent experiments.

titers in target organs (Fig. 5 *B–E*). Taken together, these data further support the conclusion that TLR8-dependent pDC activation is crucial to innate immune control of VV infection in vivo.

Discussion

In this study, we found that recognition of VV DNA by pDCs was MyD88-dependent, but TLR9-independent, leading to production of type I IFNs. We identified that pDC sensing of VV DNA was mediated by TLR8 possibly through recognition of poly(A)/T-rich motifs. We further demonstrated that TLR8-MyD88-dependent pDC activation and secretion of type I IFNs were crucial to the control of VV infection in vivo.

Previous studies have shown that type I IFN production by pDCs in response to a viral infection is linked to endosomal TLR7, which senses guanosine- and uridine-rich ssRNA (4, 5), and TLR9, which recognizes unmethylated CpG DNA (6, 7). Although TLR8, another enodosomal TLR, has been shown to sense viral ssRNA in humans (5, 8), it was thought that TLR8 was nonfunctional in mice (5, 8). A recent study has indicated, however, that murine TLR8 can be activated by a combination of imidazoquinoline TLR8 ligands and poly T ODNs (9). Our findings that poly(A)/T-rich VV DNA alone is sufficient to activate murine pDCs via TLR8 not only further extend this observation, but more importantly, are unique in demonstrating that TLR8 is required for sensing poly (A)/T-rich DNA in pDCs and that murine TLR8 is functional in the context of a viral infection. It remains unclear whether TLR8 senses poly(A)/T DNA directly. Thus, future work is required to determine direct binding of poly(A)/T DNA to TLR8.

Using bulk, unsorted, Flt-3L-cultured DCs, a recent study indicated that the production of IFN-α by these DCs upon infection with modified VV Ankara (MVA) was TLR9-independent, but partially dependent on MyD88 (22). Because bulk Flt-3L-cultured DCs contain both pDC and cDC populations (18), our finding that pDC recognition of VV DNA is dependent on the TLR8-MyD88 pathway, leading to production of both IFN-α and IFN-β, whereas cDC sensing of VV DNA is TLR-independent, resulting in secretion of only IFN-β, may provide explanation for their observation. In fact, our data may also help explain the observed TLR7- and TLR9-independent, but MyD88-dependent pDC activation in response to some other viral infections (1). On the other hand, a recent report showed that pDC recognition of ectromelia virus (mousepox), but not MVA, was dependent on TLR9 (23). It is not clear why pDC recognition of VV/MVA vs. mousepox virus is mediated through different TLRs. This result may reflect differences between VV/MVA and mousepox virus in terms of their genetic compositions (i.e., A/T islands, CpG contents) and endosomal trafficking pathways, as TLR8 and TLR9 are localized to late and early endosomes, respectively.

Our results suggest that although TLR2-MyD88-dependent activation of cDCs upon VV infection leads to production of significant amounts of proinflammatory cytokines, this pathway in innate immune clearance of VV infection is dispensable. However, cDCs can also secret IFN-β, albeit at lower levels, independent of TLR upon VV infection (13). Thus, the requirement of TLR8-MyD88-dependent pDC activation and secretion of type I IFNs in innate immune control of VV infection in vivo suggests that the low levels of IFN-β produced by non-pDCs might not be sufficient for the control of VV in vivo. Alternatively, pDCs could exert type I IFN-independent anti-viral mechanisms. Future studies will be needed to delineate the underlying mechanisms.

The observation that TLR8-dependent pDC activation is critical for innate immune control of VV infection in vivo suggests that different pathogens have evolved to adopt different mechanisms to effectively activate the innate immune system. Although our results establish a role for TLR8 in pDC recognition of VV in mice, human pDCs only express TLR7 and TLR9 (24). However, human TLR8 is widely expressed in non-pDCs, such as cDCs and monocytes (24). At present, it is not clear whether TLR8-mediated recognition of VV by nonpDCs is of importance in VV control in humans or whether human pDCs use alternative TLRs in recognition of VV DNA. Thus, it will be important to address these questions in future studies.

In conclusion, our study reveals a previously unappreciated role for murine TLR8 in pDC activation, possibly through recognition of poly(A)/T-rich motifs in response to VV infection, and suggests a unique strategy of poly(A) and poly T ODN-mediated pDC activation for the efficient viral control in vivo.

Methods

Mice. C57BL/6 mice were obtained from The Jackson Laboratory. MyD88$^{-/-}$ and TLR9$^{-/-}$ mice on C57BL/6 background were kindly provided by S. Akira

(Osaka University, Osaka, Japan). Groups of 6- to 10-week-old mice were selected for this study. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of Duke University.

Vaccinia Virus and VV DNA isolation. The Western Reserve strain of VV was purchased from American Type Culture Collection (ATCC). VV was grown in TK-143B cells (ATCC) and purified, and the titer was determined by plaque assay on TK-143B cells and stored at –80 °C until use as described (13). Isolation of VV DNA was performed by incubating live VV with 1% SDS and 200 μg/mL Proteinase-K for 4 h at 37 °C. After incubation, phenol chloroform extraction followed by sodium acetate precipitation was performed to purify the VV DNA. DNA concentration was determined using a spectrophotometer.

DC Culture. pDCs and cDCs were generated as described (18). Briefly, bone marrow cells were harvested from femurs and tibiae of mice and cultured in the presence of 100 ng/mL of Flt-3L (R & D Systems). After 9 d of culture, DCs were harvested, stained, and sorted into pDC ($CD11c^+B220^+$mPDCA-1^+) and cDC ($CD11c^+B220^-$mPDCA-1^-) populations. Purified pDCs and cDCs were stimulated with various agents at a density of 1×10^6 cells/mL.

Isolation of Endogenous Splenic pDCs. $CD11c^+$ $B220^+$ mPDCA-1^+ pDCs were purified from the spleen by flow cytometry sorting. Sorted pDCs were stimulated at a density of 1×10^6/mL.

Oligodeoxynucleotides. Phosphorothioate-stabilized Poly A10 (5′-A*AAA-A*A*A*A*A* A-3′), Poly C10 (5′-C*CCCC*C*C*C*C*C-3′), Poly G10 (5′-G*G-GGG*G*G*G*G*G-3′), Poly T10 (5′-T*TTTT*T*T*T*T*T-3′), and CpG (5′-G*G*G*G*G*A*C*G*A*T*C*G*T*C*G*G*G*G*G*G-3′) ODNs were synthesized by Integrated DNA Technologies.

Antibodies and Flow Cytomtery. FITC-conjugated anti-B220, PE-conjugated anti-CD80, PE-conjugated anti-CD86, Streptavidin-conjugated PE-Cy5, and biotin-conjugated anti-CD11c were purchased from BD Biosciences. PE-conjugated anti-PDCA-1 was obtained from Miltenyi Biotec. FACS Canto (BD Biosciences) was used for flow cytometry event collection, which was analyzed using FACS DiVA software (BD Biosciences).

Cytokine Measurements by ELISA. Production of cytokines by DCs in response to various stimuli was detected by ELISA kits according to manufacturer's standard protocols. IFN-α and IFN-β kits were obtained from PBL Biomedical Laboratories. IL-1 and IL-12 kits were obtained from Pierce Endogen. IL-6 kits were obtained from BD Biosciences.

NF-κB-Luciferase Assay. HEK293 cells (CRL-1573; ATCC) were seeded in six-well plates at 5×10^5 cell/well and cotransfected with 1 μg of murine TLR7 (pUNO-mTLR7, Invivogen) or murine TLR8 (pUNO-mTLR8, Invivogen), along with 0.1 μg of NF-κB-luciferase reporter (pNF-κB-Luc, Clontech) with lipofectamine transfection reagent (Invitrogen) following the manufacturer's instructions. Forty-eight hours after transfection, cells were then stimulated with live VV, VV DNA, TLR7 agonist CL087, or TLR8 agonist CL075 (Invivogen). NF-B activation was determined by lysing the transfected HEK293 cells with reporter lysis buffer (Clontech), and the lysate was assayed for luciferase activity using an LMax Luminometer (Molecular Devices). NF-κB activation is directly proportional to relative luciferase units.

RT-PCR. Total RNA was isolated from flow cytometry sorted pDCs using TRIzol reagent according to manufacturer's instructions. One-step semiquantitative RT-PCR was performed using template RNA and primers specific for murine TLR7 (forward 5′-GGTATGCCGCCAAATCTAAA-3′, reverse 5′-TTGCAAAGAA-AGCGATTGTG-3′), TLR8 (forward 5′-CAAACAACAGCACCCAAAT G-3′, reverse 5′-GGGGGCACATAGAAAAGGTT-3′), TLR9 (forward 5′-GCAAGCTCAACCTG-TCC TTC-3′, reverse 5′-TAGAAGCAGGGGTGCTCAGT-3′), and β-actin (forward 5′-AGCCATGTACGT AGCCATCC-3′, reverse 5′-CTCTCAGCTGTGGTGGTGAA-3′).

Construction of Lentiviral Vectors Encoding siRNA for Murine TLR7 and TLR8. The lentiviral construct, pNL-SIN-GFP (25), was a generous gift from B. Cullen (Duke University, Durham, NC). Murine TLR8 siRNA constructs were generated in two PCR steps. The first PCR used genomic DNA from HEK293 cells to generate a fragment containing an Xba I restriction site, the RNA polymerase III-dependent ^{1}H promoter, the TLR8-specific siRNA "sense" strand sequence, and the hinge-region sequence. The primers used in this initial PCR are ^{1}H forward primer (5′- TGGCAGGAAGATGGCTGTGA-3′) and TLR8 siRNA1-Sense (5′- ACTGACAGGAAGACTA CCCAGTTTACAAATCCGGGGATCTGTGGTCTCA-TACAGAACTTATAAGATTCCC-3′) or TLR8 siRNA2-Sense (5′-CTGACAGGAA-GAGCTTCGTTCTCTCAAGAAGGGGGATCTGTGGTCTCA TACAGAACTTATAA-GATTCCC-3′). The second PCR used the first PCR product as the template to add the TLR8 siRNA "antisense" strand sequence, terminal sequence, and a Cla I restriction site. The primers used in the second PCR are ^{1}H forward primer and TLR8 siRNA1-Antisense (5′-GCGCATCGATAGCTGGCAAGGAAAAAGGATTTG-TAAACTGGGTAGTTACTGACAGGAAG ACTA-3′) or TLR8 siRNA2-Antisense (5′-GCGCATCGATAGCTGGCAAGGAAAAACTTCTTGA GAGAACGAAGCTTACTG-ACAGGAAGAGCT-3′). Similarly, primers for generating TLR7 siRNA are: Sense (5′ACTGACAGGAAGACAAAAGTACCTGAGAGATTTTGGGGATCTGTGGTCTCA TACAGAACTTATAAGATTCCC-3′) and Antisense (5′- CGCATCGATAGCTGGC-AAGGAAAA AAATCTCTCAGGTACTTTTGTTGACAGGAAGACAA-3′). The TLR8 and TLR7 siRNA expression cassettes were then digested with Xba I and Cla I and subcloned into pNL-SIN-GFP to generate pNL-SIN-GFP-TLR8 siRNA1 (or -2). Insertion and sequence were confirmed by restriction enzyme digestion and DNA sequencing.

Lentivirus-Mediated siRNA Knock-Down. Lentiviruses were produced by cotransfecting plasmids pNL-SIN-GFP-TLR8 or TLR7 siRNA (16 μg), pcRev (800 ng), pcTat (800 ng), and pHIT/G (400 ng) into human 293T cells. The viral supernatants were then used to infect day 2 Flt-3L DC cultures in the presence of 8 μg/mL polybrene. After 24 h, the lentivirus-containing media was replaced with DC media and Flt-3L. This procedure was repeated on day 4 of DC culture. GFP-expressing pDCs and cDCs were isolated on day 9 by FACS. Empty vector controls were generated by performing the above procedure with empty plasmid, pNL-SIN-GFP.

Viral Genome Analysis. Vaccinia virus (Accession No. NC_006998), Ad5 (Accession No. AC_000008), and HSV-2 (Accession No. NC_001798) were analyzed by the DNA analysis software pDRAW32 (AcaClone). Whole genomes were analyzed for G/C and A/T content and expressed as a percentage. To determine the frequency of G/C- or A/T-rich sequences, the VV genome was analyzed for instances where 10 consecutive base pairs included 5 or more G/C or A/T (defined henceforth as an "island"). The percentage of the VV genome that exists in G/C-rich or A/T-rich islands is calculated by dividing the total number of base pairs that exist in such islands by the total number of base pairs in the VV genome.

Reconstitution of pDCs in Vivo. WT and $MyD88^{-/-}$ pDCs were generated from bone marrow cells in the presence of Flt-3L and purified by FACS sorting as described above. Next, 1×10^6 sorted pDCs were adoptively transferred into $MyD88^{-/-}$ hosts intravenously, followed by infection with 1×10^7 pfu of VV intravenously. Serum, spleen, and liver were harvested at 48 h postinfection for analysis.

In other experiments, reconstitution of pDCs was performed in WT mice depleted of pDCs. Briefly, 150 μg of anti-Ly6G/C (clone RB6-8C5, a generous gift of D. Cain and G. Kelsoe, Duke University) (26) was administered i.v. to WT mice on days −3 and −1 to deplete pDCs. On day 0, 5×10^5 sorted TLR7-knockdown, TLR8-knockdown, or $TLR9^{-/-}$ pDCs were adoptively transferred into pDC-depleted hosts intravenously, followed by infection with 1×10^7 pfu live VV intravenously. Serum, spleen, and liver were harvested at 48 h postinfection for analysis.

VV DNA Quantitative Real-Time PCR. Total genomic DNA was isolated from the spleen and the liver tissues as described (27). Real-time quantitative PCR was used to measure the amount of VV genomic DNA in the spleen and liver using primers located in the VV *A33R* gene. Amounts of VV DNA were normalized to β-actin gene within each sample. The sequences of the forward and reverse primers for A33R, were 5′-TATTACTGACGCCGCTGTTG-3′ and 5′-GTGTTGATGATTCC GCAGTG-3′, respectively. Normalized value for VV DNA in each sample was calculated as the relative quantity of vaccinia viral DNA divided by the relative quantity of β-actin gene.

VV Titer Assay. VV titers in the liver and the spleen were measured by plaque-forming assay as described (13). Briefly, mice were killed 2 d after infection, and the organs were harvested and stored at −80 °C. Organs from individual mice were homogenized and freeze-thawed three times. Serial dilutions were performed on confluent TK-143B cells, and viral titers were then determined 2 d later by crystal violet staining.

Statistical Analysis. Results are expressed as mean ± SD. Differences between groups were examined for statistical significance using a Student *t* test.

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