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Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA

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Interferons (IFNs) are critical for protection from viral infection, but the pathways linking virus recognition to IFN induction remain poorly understood. Plasmacytoid dendritic cells produce vast amounts of IFN-α in response to the wild-type influenza virus. Here, we show that this requires endosomal recognition of influenza genomic RNA and signaling by means of Toll-like receptor 7 (TLR7) and MyD88. Single-stranded RNA (ssRNA) molecules of nonviral origin also induce TLR7-dependent production of inflammatory cytokines. These results identify ssRNA as a ligand for TLR7 and suggest that cells of the innate immune system sense endosomal ssRNA to detect infection by RNA viruses.

Influenza epidemics kill up to half a million people worldwide every year and impose a substantial burden on the global economy (*1*). Yet, the influenza virus is immunogenic, triggering protective antibody and cytotoxic T lymphocyte responses in most healthy adults, and vaccines composed of the inactivated virus without adjuvant induce antibody responses. Thus, intrinsic components of the virus presumably activate the innate immune system, but little is known about their identity or the pathways involved in their recognition. One of the earliest responses to influenza and other viruses is the production of type I IFNs, critical cytokines that establish an antiviral state and bridge the innate and adaptive immune systems (*2*). Conventional dendritic cells (DC) produce high levels of IFN-α in response to cytosolic double-stranded RNA (dsRNA) made during viral replication (*3*). However, influenza suppresses this response by means of the NS1 viral protein, which sequesters dsRNA (*3*). In contrast, human and mouse plasmacytoid dendritic cells (PDC) appear resistant to NS1, given that they produce high levels of IFN-α after infection with wild-type influenza (*4–9*). This observation, and evidence that they also respond to the inactivated nonreplicating virus (*10*), suggests that PDC possess a dsRNA-independent pathway for recognizing influenza.

To identify this pathway, we first purified plasmacytoid CD11c^{low} Ly6C^{+} DC from mouse spleen and confirmed their ability to respond directly to the influenza virus (*11*). Both the live influenza and the virus that had been inactivated by 56°C heat exposure induced the production of high levels of IFN-α (Fig. 1A). In contrast, only low levels of the cytokines were detected in response to the virus that had been inactivated at 65°C, which further denatures hemagglutinin and prevents cell attachment (*12*) (Fig. 1A). Equivalent results were obtained with Flt3L expanded cells from bone marrow (Fig. 1B) (*13*). Although these cultures contained both conventional (CD11b^{+} B220^{-}) and plasmacytoid (CD11b^{-} B220^{+}) DC, costaining of

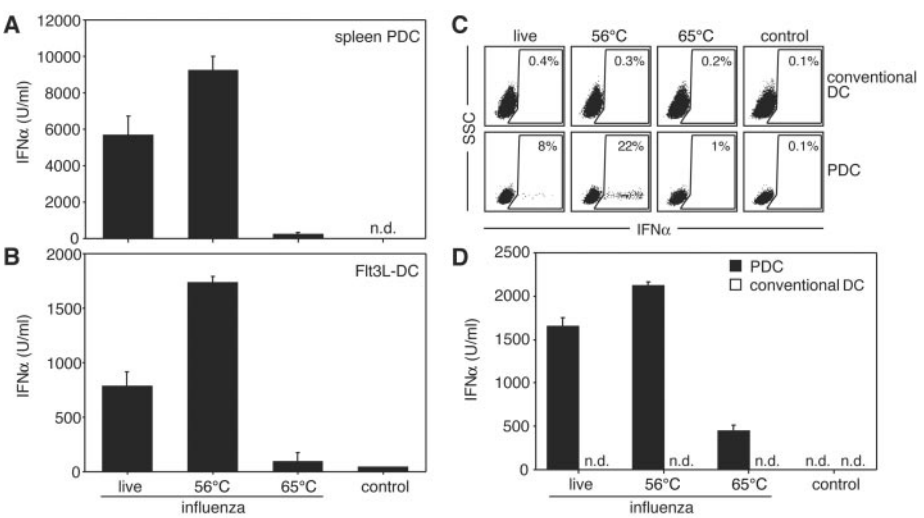

Fig. 1. PDC produce high levels of IFN-α in response to live or inactivated influenza virus. (**A**) Purified BALB/c spleen PDC were cultured with live or heat-inactivated influenza virus or in medium alone (control). (**B**) Bulk cultures of C57BL/6 Flt3L DC were treated as in (A). (**C**) Intracellular IFN-α staining of bulk cultures of C57BL/6 Flt3L DC treated with live or heat-inactivated influenza virus. The profiles shown correspond to gated CD11b^{+} B220^{-} (conventional) or CD11b^{-} B220^{+} (plasmacytoid) DC, as indicated. SSC, side scatter. (**D**) B220-enriched (PDC) and B220-depleted (conventional DC) cell fractions from Flt3L DC cultures were cultured with live or heat-inactivated influenza virus, as in (A). Data in (A), (B), and (D) represent IFN-α levels in supernatants, measured by enzyme-linked immunosorbent assay (ELISA) after overnight culture, and are the average of triplicate samples ± 1 SD. Results are representative of at least three independent experiments. n.d., not detectable.

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cells for IFN-α, CD11b, and B220, as well as sorting experiments, revealed that only PDC produced IFN-α (Fig. 1, C and D).

After the attachment of the viral particle to sialic acids at the cell surface, influenza is internalized into an endocytic compartment in which the low pH triggers a conformational change in hemagglutinin, allowing viral fusion and the release of the nucleocapsid into the cytosol (*14*). To determine whether innate recognition of the influenza virus similarly requires endosomal acidification, bulk Flt3L cultures were treated with the virus in the presence of chloroquine, a lysosomotropic agent. Chloroquine completely abrogated IFN-α induction by influenza or by CpG-containing DNA (CpG), an alternative PDC stimulus (Fig. 2A) (*15*). The inhibition did not appear to be due to drug toxicity, because chloroquine had no measurable effect on PDC viability (*16*). These results suggest that influenza recognition by PDC might occur in an endosomal compartment.

TLRs have emerged as a major class of pattern-recognition receptors controlling innate responses (*17*). To assess TLR involvement in influenza recognition by PDC, we examined the dependence of the IFN-α response on the TLR signaling adaptor, MyD88. Flt3L cultures of MyD88-deficient cells were completely unable to synthesize IFN-α in response to the live virus, the 56°C inactivated virus, or CpG (Fig. 2B). However, MyD88$^{-/-}$ PDC produced IFN-α upon infection with the ΔNS1 influenza mutant, showing that they were not intrinsically impaired in their ability to produce IFN-α (fig. S1). These results demonstrate that MyD88 is essential for PDC recognition of wild-type live or inactivated influenza.

The dependence of virally induced IFN-α production on MyD88 strongly suggested that PDC recognition of influenza was mediated by a TLR. TLRs 2, 3, 4, and 9 have all been implicated in innate viral recognition (*17*), and ligand recognition by TLR3 and TLR9 is known to occur in endosomal compartments (*18*, *19*). However, TLR3$^{-/-}$ and TLR9$^{-/-}$ Flt3L DC, as well as Flt3L DC from C3H/HeJ TLR4 mutant mice, showed normal IFN-α responses to influenza (*16*). Because responses to some TLR7 and TLR8 agonists also require endosomal acidification (*20*, *21*), we next tested whether TLR7 might be involved. PDC purified from TLR7$^{-/-}$ Flt3L DC bulk cultures produced only background levels of IFN-α in response to influenza but showed normal responses to CpG, a non-TLR7 agonist (Fig. 2C). IL-12 p40 induction and IL-6 induction by influenza were also abrogated in bulk cultures of TLR7$^{-/-}$ Flt3L DC (fig. S2, A and B). These results identify TLR7 as a critical receptor for murine PDC responses to both live and inactivated wild-type influenza.

Because some TLR7 ligands have been characterized as ribonucleoside analogs (*20*, *21*), we focused on viral genomic ssRNA as the putative influenza ligand for TLR7. Substantial levels of IFN-α, IL-6, and IL-12 p40 were induced by purified influenza RNA condensed with polyethylenimine (PEI) to protect it from degradation (Fig. 3A) (*16*); PEI alone did not induce IFN-α (fig. S3A). Again, PDC were specifically responsible for producing IFN in response to PEI-condensed influenza RNA, as determined by IFN-α staining (fig. S4). We conclude that viral genomic ssRNA can substitute for intact influenza in triggering IFN-α production by PDC [supporting online material (SOM) text S1].

To determine whether the viral origin of the RNA was critical, we tested the response of bulk Flt3L cultures to synthetic RNAs. Polyuridylic acid [poly(U)] condensed with PEI or encapsulated in liposomes induced comparable levels of IFN-α to influenza RNA/PEI complexes (Fig. 3, B and C). Polyadenylic acid [poly(A)], polycytidylic acid [poly(C)], polyguanylic acid [poly(G)], and polyinosinic acid [poly(I)] each failed to induce IFN-α or other cytokines at any dose tested (Fig. 3B) (*16*). In vitro synthesized RNA [encoding green fluorescent protein (GFP)] also elicited high levels of IFN-α from Flt3L cultures when condensed with PEI (Fig. 3D). This response was unaffected by the presence or absence of a poly(A) tail or by the inclusion of a 7-methylguanosine cap analog during RNA synthesis (Fig. 3D). All RNAs also induced IL-6 and IL-12 p40 production from bulk Flt3L cultures (fig. S2, C and D). However, as was the case for influenza RNA, only PDC and not conventional DC produced IFN-α (fig. S4). Inhibition of the IFN-α response was seen at high doses of RNA, possibly because of a combination of carrier toxicity (fig. S3B) as well as a requirement for oligomerization of the RNA receptor (SOM text S2). Mouse spleen mRNA and some short ssRNA oligonucleotides (of the type used to make short interfering dsRNA) also induced IFN-α production (Fig. 3E) (*16*).

It remained possible that influenza RNA elicits IFN-α production by PDC but was not the component responsible for TLR7-dependent responses to the intact virus. Two lines of evidence suggested that this is not the case. First, as for the intact virus, the response to viral RNA was abrogated by chloroquine (*16*). Second, IFN-α, IL-12 p40, and IL-6 responses to influenza RNA were completely lost in Flt3L cultures from TLR7$^{-/-}$ mice, whereas the response to CpG was unaffected (Fig. 3F and fig. S2, C and D). Similarly, all responses to the nonviral RNAs were completely abrogated by TLR7 deficiency (Fig. 3F and fig. S2, C and D). These results demonstrate that innate responses to ssRNA are critically dependent on TLR7 and strongly suggest that the influenza-derived TLR7 ligand is the viral genome.

Here, we used IFN-α production by PDC as the primary readout for dissecting pathways mediating innate activation by influenza. We found that the ssRNA viral genome likely accounts for PDC activation by live and inactivated influenza, and we show that, in mouse, this viral RNA is recognized exclusively by TLR7, one of a prominent family of innate receptors (*17*). Nevertheless, influenza RNA may also be recognized by TLR8 in other species, given that mouse TLR8 is thought to be nonfunctional (*22*). In addition, influenza infection generates dsRNA, another potential stimulus for innate activation. PDC produce more IFN-α in response to ΔNS1 than to the wild-type influenza virus, and infection with the mutant rescues IFN-α production by MyD88$^{-/-}$ PDC (fig. S1). These observations imply that PDC share with conventional DC the ability to sense cytosolic dsRNA that is not sequestered

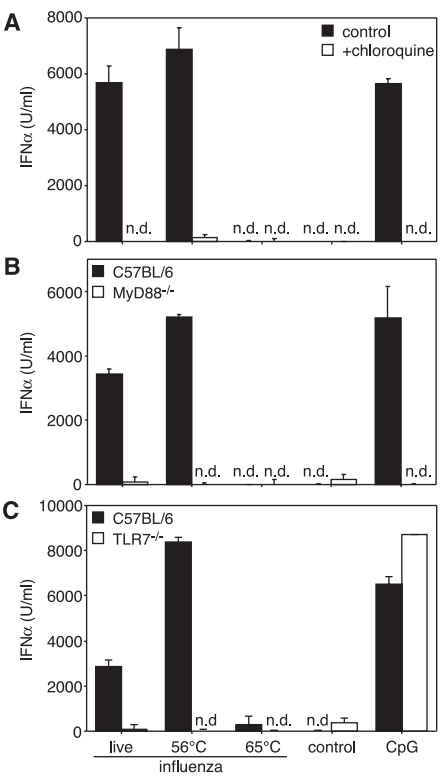

Fig. 2. Induction of IFN-α by influenza virus is chloroquine-sensitive and dependent on MyD88 and TLR7. (**A**) Bulk cultures of C57BL/6 Flt3L DC were cultured with live or heat-inactivated influenza virus, with CpG, or in medium alone (control) in the presence or absence of chloroquine (10 μM), as indicated. (**B**) Bulk cultures of C57BL/6 or MyD88$^{-/-}$ Flt3L DC were cultured overnight with live or heat-inactivated influenza virus, with CpG or in medium alone. (**C**) B220-enriched PDC fractions from C57BL/6 or TLR7$^{-/-}$ Flt3L DC were cultured with influenza virus, CpG, or no stimulus. Data in (A) to (C) represent IFN-α levels in supernatants measured by ELISA after overnight culture (average of triplicate samples ± 1 SD). Data are representative of at least three independent experiments. n.d., not detectable.

by viral proteins (*3*). Conversely, it appears that at least some conventional DC share with PDC the ability to sense ssRNA by means of TLR7, on the basis of our finding that $CD11b^+$ $B220^-$ DC contribute to the IL-12 p40 and IL-6 response in bulk Flt3L cultures (*16*). However, conventional DC lack the ability to produce high levels of IFN-α in response to TLR signals, which is a unique property of PDC (*23*).

Our data lead us to propose a model in which, during infection, some influenza particles are degraded by endosomal proteases, exposing the viral genome and allowing TLR7 signaling, which is known to occur in endosomes (*20*, *21*). Thus, PDC and probably other cells of the innate immune system exploit the necessity of influenza to enter acidified compartments in order to detect the viral presence before replication has begun. In addition, in immune individuals, the antibody-coated virus may be directly targeted to endosomal compartments by means of Fc receptors, leading to innate activation by non-infectious viral particles (*24*). This model is reminiscent of that proposed for the recognition of herpes simplex virus 1 (HSV-1) and HSV-2, whose unmethylated DNA genome is detected in endocytic compartments by TLR9 (*15*, *25*), suggesting that the immune system uses similar strategies for detecting ssRNA and DNA viruses. Notably, TLR3, a receptor for dsRNA, also localizes to endosomes (*19*), such that endosomal sensing of viral nucleic acids could also apply to recognition of reoviruses bearing dsRNA genomes.

Although we have not excluded the possibility that TLR7 has a preference for a particular RNA motif, the fact that it mediates responses to poly(U) suggests that this motif is very simple and likely to be present in nonviral RNAs. Consistent with the latter, we found that mouse mRNA (*16*) and a wide range of synthetic RNAs can be immunostimulatory (Fig. 3). If TLR7 can recognize both self- and viral RNA, how does it distinguish the presence of an RNA virus? The answer may lie in the compartmentalization of RNA in uninfected organisms. The presence of extracellular ribonucleases in interstitial fluids ensures that little if any self-ssRNA ever reaches the endosomal compartments of antigen-presenting cells. A possible exception may be undegraded cellular RNA in apoptotic bodies, but, in this respect, it is notable that $CD8\alpha^+$ DC, the major murine DC subset involved in the uptake of dying cells (*26*, *27*), lacks expression of TLR7 (*28*). Thus, TLR7 and possibly TLR8 may differ from other pattern recognition receptors in detecting the abnormal localization of ligands rather than structures or motifs absent from the host. These considerations suggest that endosomal delivery of ssRNA could be exploited as an adjuvant for vaccination and immunotherapy.

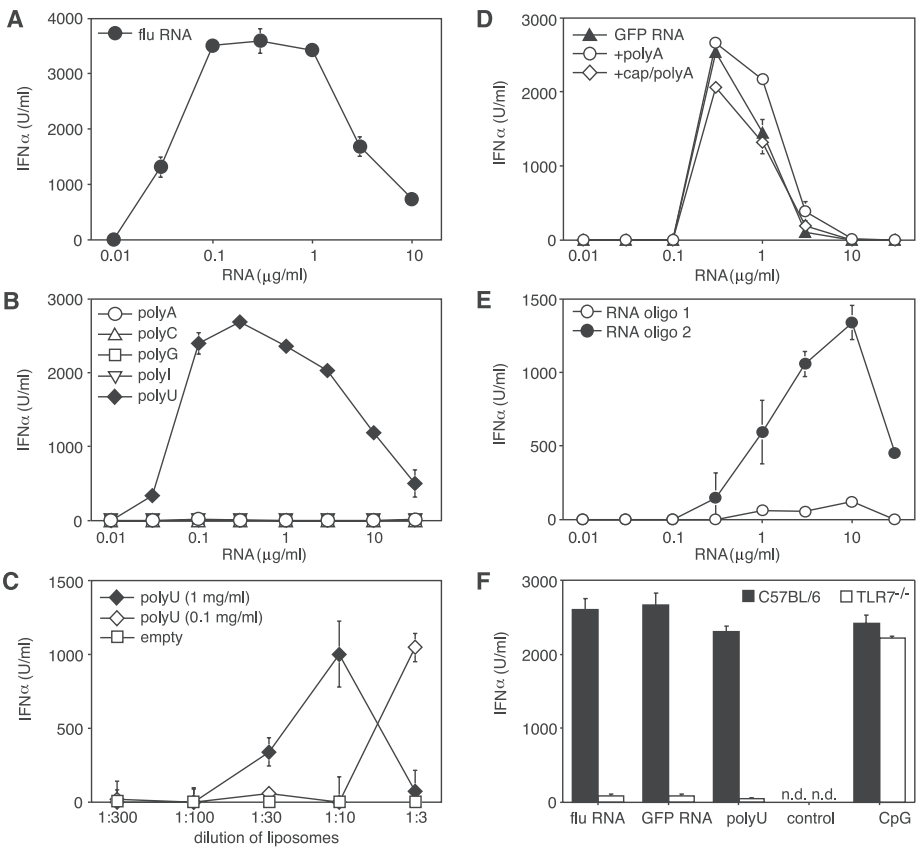

Fig. 3. Influenza genomic RNA and synthetic ssRNAs elicit high levels of IFN-α from PDC by means of TLR7. Bulk cultures of C57BL/6 Flt3L DC were stimulated with different doses of (**A**) influenza RNA/PEI complexes (flu RNA); (**B**) poly(A), poly(C), poly(G), poly(I), or poly(U) as complexes with PEI; (**C**) liposomes prepared in the absence or in the presence of the indicated concentrations of poly(U); (**D**) complexes of PEI with GFP RNA with or without a poly(A) tail and synthesized in the presence or absence of cap analog, as indicated; or (**E**) two different complexes of PEI and ssRNA oligonucleotides. (**F**) Bulk cultures of C57BL/6 or $TLR7^{-/-}$ Flt3L DC were treated with PEI complexes of influenza RNA, GFP RNA [lacking a poly(A) tail], or poly(U) RNA (all RNAs at 0.3 μg/ml). Control cells were cultured in medium alone or with CpG. In all panels, data represent the average IFN-α levels in supernatants measured by ELISA after overnight culture (triplicate samples ± 1 SD). Data are representative of at least two independent experiments.

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