one of the reasons humans are particularly susceptible to urinary tract infections is because the absence of TLR11 has removed a defense pathway with the unique ability to specifically recognize UPECs.

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Supporting Online Material

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Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8

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Double-stranded ribonucleic acid (dsRNA) serves as a danger signal associated with viral infection and leads to stimulation of innate immune cells. In contrast, the immunostimulatory potential of single-stranded RNA (ssRNA) is poorly understood and innate immune receptors for ssRNA are unknown. We report that guanosine (G)- and uridine (U)-rich ssRNA oligonucleotides derived from human immunodeficiency virus–1 (HIV-1) stimulate dendritic cells (DC) and macrophages to secrete interferon-α and proinflammatory, as well as regulatory, cytokines. By using Toll-like receptor (TLR)–deficient mice and genetic complementation, we show that murine TLR7 and human TLR8 mediate species-specific recognition of GU-rich ssRNA. These data suggest that ssRNA represents a physiological ligand for TLR7 and TLR8.

Dendritic cells (DC) are a heterogeneous cell population that bridge the innate and adaptive immune systems (*1*). In their immature form, DC take up and degrade pathogens or soluble antigens and subsequently channel degradation products such as peptides into the major histocompatibility complex (MHC) class I and class II pathways for presentation to T cells (*2*). Immature DC also express a variety of innate pattern recognition receptors (PRRs) including members of the TLR family (*3*). Recognition of TLR ligands induces MyD88-dependent signaling via the Toll–IL-1 receptor pathway, which activates DC and helps induce differentiation into professional antigen-presenting cells (APC) (*3*). In vertebrates, the TLR family is, so far, known to consist of 10 members (TLR1 to TLR10) (*3*), and of these TLR3 and TLR9 are known to recognize nucleic acid such as dsRNA and CpG motif–containing bacterial and viral DNA, respectively (*4*–*7*). On the basis of genomic structure and sequence similarities, it has been thought that TLR9 forms a subfamily with TLR7 and TLR8 (*8*, *9*). Although the natural ligands for TLR7 and TLR8 are not known, synthetic imidazoquinoline compounds and guanosine analogs with antiviral activities have recently been described to activate these receptors (*10*–*13*). The structural similarities of these compounds to nucleic acids prompted us to investigate whether ssRNA might activate APC via members of the TLR9 subfamily.

To define sequence requirements of immunostimulatory ssRNA, we first screened high concentrations of unmodified nucleosides for their potential to mimic ssRNA and to activate human peripheral blood mononuclear cells (PBMCs). A mixture of the nucleosides guanosine and uridine induced tumor necrosis factor–α (TNF-α) secretion, whereas other nucleoside combinations had no stimulatory effect [Fig. 1A and (*14*)]. This prompted us to synthesize phosphothioate-protected RNA oligonucleotides containing a GU-rich sequence from the U5 region of HIV-1 RNA (RNA40) and two derivatives, RNA41 and RNA42, in which all U or G nucleotides were replaced with adenosine, respectively (Fig. 1B). Because cationic lipids are known to facilitate the uptake of RNA by DC (*15*), we complexed the RNA to N-[1-(2,3-Dioleoyloxy)propyl]-N,N,Ntrimethylammoniummethylsulfate (DOTAP). RNA40 and RNA42 activated PBMCs to secrete TNF-α, interleukin-12p40 (IL-12p40), and interleukin-6 (IL-6) when complexed to DOTAP (Fig. 1C). In contrast, free RNA40, -41, or -42; RNAse-treated RNA; or corresponding DNA sequences did not lead to any cytokine production (Fig. 1C; fig. S1). Intracellular cytokine staining of PBMC cultures revealed that CD11c-positive myeloid cells were the main source of TNF-α when stimulated with RNA40 and RNA42 (Fig. 1D). Human PBMC also produced interferon-α (IFN-α) upon stimulation with RNA40 complexed to DOTAP (Fig. 1E). The production of IFN-α was similar in magnitude compared with the IFN-α inducing TLR9 ligand CpG-ODN 2216 (*16*). Within human PBMC, plasmacytoid DC (pDC) are the primary source of IFN-α, because IL-3 receptor [CD123, a marker for pDC (*17*)]–depleted PBMC cultures failed to produce IFN-α upon RNA stimulation. In contrast, the CD123-enriched fraction strongly responded to RNA40 when complexed to DOTAP. Similar data were obtained for BDCA-4 [another pDC-specific marker (*17*)]–depleted PBMC and enriched pDC, respectively (*14*). Interestingly, RNA42 failed to induce IFN-α, although other regulatory and inflammatory cytokines were readily induced (Fig. 1, C and E). Taken together, these data demonstrate

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that GU-rich ssRNA oligonucleotides derived from HIV-1 activate human myeloid cells as well as pDC and lead to the production of IFN-α, IL-6, TNF-α, and IL12p40. The production of IFN-α was strongly dependent on the GU composition of the RNA oligonucleotides, whereas U is a prerequisite for secretion of IL-6, TNF-α, and IL12p40.

In parallel, we analyzed RNA-induced cytokine production of purified murine pDC and bone marrow–derived macrophages. RNA40, but not RNA41 and RNA42, stimulated pDC to produce IFN-α, IL-6, IL-12p40, and TNF-α (Fig. 2). Murine macrophages responded to RNA40 stimulation with IL-6, IL-12p40, and TNF-α production; IFN-α was not detected (Fig. 2). We also tested 10- and 12-nucleotide oligomer GU-rich RNA oligonucleotides (RNA33, 5′-GUAGUGUGUG; RNA34, 5′-GUCUGUUGUGUG) for cytokine induction in human and murine immune cells. These unprotected RNA oligonucleotides containing one phosphothioate linkage at the 3′-end induced the same cytokine profile as RNA40 (*14*). Overall, these results imply that GU-rich ssRNA derived from HIV-1 stimulate human and murine immune cells. Interestingly, the ability of RNA42 to activate human but not murine cells implies a species specific difference in ssRNA recognition.

Because murine cells responded well to GU-rich ssRNA, we tested for the requirement of MyD88, and thus potential TLR involvement, in ssRNA-driven up-regulation of costimulatory molecules. RNA40 caused increased expression of CD40, CD69, and CD86 in DC when it was complexed to DOTAP (Fig. 3A). In contrast, MyD88-deficient DC were refractory to stimulation with ssRNA, and no CD40 up-regulation was observed (Fig. S2). TLR2/4 double-deficient DC, which served as control to rule out endotoxin and TLR2 ligand contamination of the stimulatory RNA, responded at a similar level to wild-type cells (fig. S2). RNA induced cytokine production in these deficient DC followed the pattern observed with expression of costimulatory molecules (*14*). Taken together, these results imply that GU-rich ssRNA stimulates immune cells via a TLR. To determine the TLR responsible for the observed effects, TLR-deficient mice were tested. Both TLR3- and TLR9-deficient DC were activated by GU-rich ssRNA, but failed to respond to poly (I:C) or CpG-DNA (Fig. 3B). This suggested the involvement of another TLR in ssRNA-driven activation. TLR7 deficient DC failed to up-regulate CD40 expression upon stimulation with RNA40 and the known TLR7 ligand R-848, whereas polyinosine-polycytidylic acid [poly (I:C)], CpG-DNA, or lipopolysaccharide (LPS)-driven stimulation were unaffected (Fig. 3B). Accordingly, TLR7-deficient DC failed to secrete TNF-α, IL-12p40, or IFN-α when stimulated with RNA40, but responded normally to CpG-DNA and poly (I:C) (Fig. 3C). In contrast, TLR8-deficient cells responded normally to stimulation with ssRNA (*18*). Overall, these data demonstrate that murine TLR7 recognizes GU-rich ssRNA oligonucleotides derived from HIV-1.

To identify the potential human TLR involved in ssRNA-mediated cell activation, we used genetic complementation. TLR-specific activation of RNA40, -41, and -42 were tested on human TLR2, -3, -7, -8 and -9–transfected human embryonic kidney (HEK) 293 cells (Fig. 4). TLR2, TLR3, and TLR9 transfectants responded to their cognate stimuli Pam3Cys, dsRNA [poly (I:C)], and CpG-DNA, respectively, but were not activated by RNA40, -41, or -42. Unexpectedly, human TLR7 transfectants were also refractory to RNA40, -41, and –42, although they responded to resiquimod (R-848). In contrast, human TLR8 strongly induced NF-κB activation upon stimulation with RNA40 and RNA42 (Fig. 4). This observation differs from the

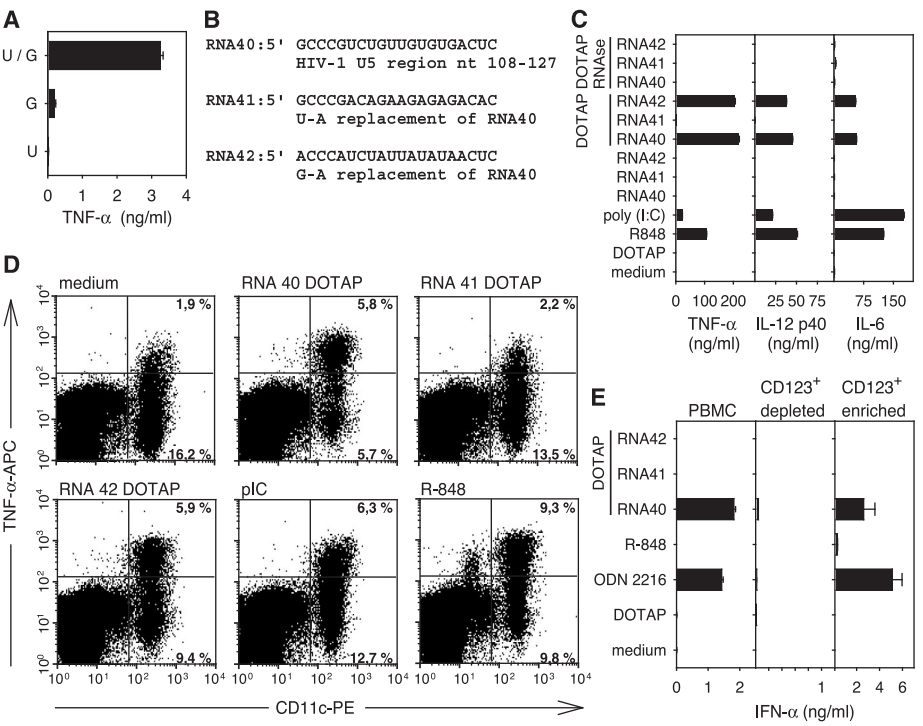

Fig. 1. Human PBMCs respond to nucleoside and RNA stimulation with cytokine production. (**A**) Human PBMCs were stimulated with 5 mM U, 0.25 mM G, or a U/G mixture and were tested for TNF-α secretion. (**B**) Sequence of synthetic RNA oligonucleotides. (**C**) Human PBMCs were stimulated with 1 μM R-848; 50 μg/ml poly (I:C); or 7.5 μg/ml RNA40, -41, or -42 free, complexed to DOTAP, or RNase-treated. (**D**) PBMCs were stimulated as in (C) and stained with CD11c-PE and intracellular produced TNF-α–APC. (**E**) PMBCs, CD123-depleted PMBCs, and CD123-enriched PBMCs were stimulated as in (C) and with 1 μM CpG-ODN 2216 and were tested for IFN-α secretion. One representative experiment of at least two independent experiments with two different donors is shown ($n = 2$, ± SD).

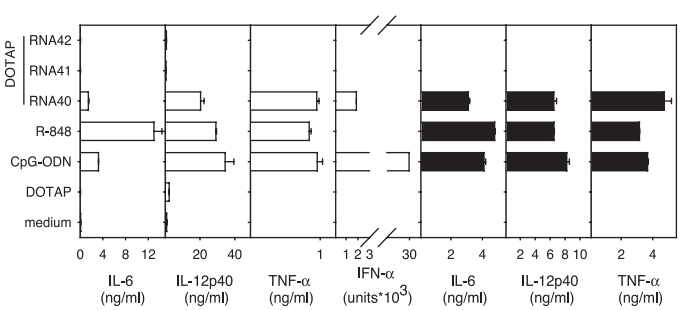

Fig. 2. RNA-induced cytokine expression on murine immune cells. Sorted FLT3 ligand induced murine plasmacytoid cells (pDCs) (white bars) or MCSF grown macrophages (black bars) were stimulated with 10 μM R-848; 1 μM CpG-DNA (ODN 1668 for macrophages and ODN 2216 for pDC); or 7.5 μg/ml RNA40, -41, or 42 complexed to DOTAP and tested for IFN-α, IL-6, IL-12p40, and TNF-α secretion. One representative experiment of at least two independent experiments is shown ($n = 2$, ± SD).

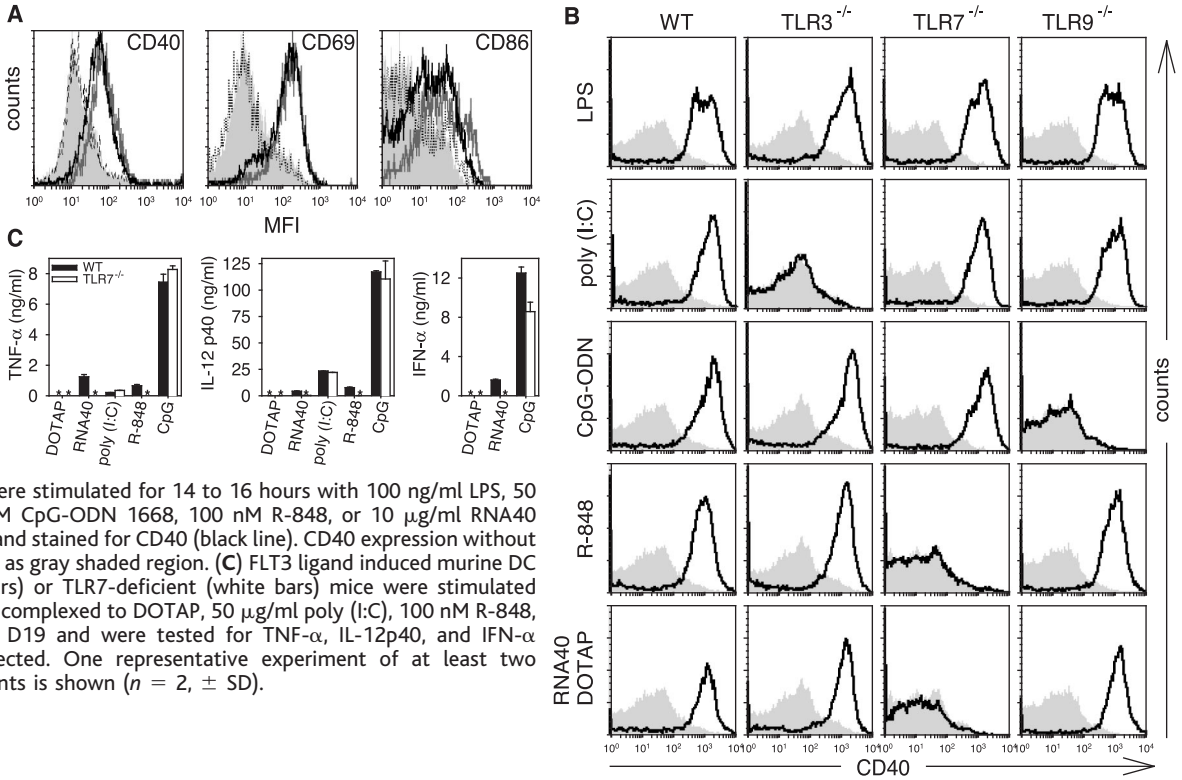

Fig. 3. Murine TLR7 recognizes GU-rich ssRNA. (**A**) FLT3 ligand–induced murine DC were stimulated for 14 to 16 hours with medium control (gray shaded region), 10 μg/ml RNA40 (dotted line), 10 μg/ml RNA40 complexed to DOTAP (black line), or 1 μM CpG-ODN 1668 (dark gray line) and were stained for CD40, CD69, or CD86. (**B**) FLT3 ligand induced murine DC from wild-type and TLR3-, TLR7-, and TLR9-deficient mice were stimulated for 14 to 16 hours with 100 ng/ml LPS, 50 μg/ml poly (I:C), 1 μM CpG-ODN 1668, 100 nM R-848, or 10 μg/ml RNA40 complexed to DOTAP and stained for CD40 (black line). CD40 expression without stimulation is depicted as gray shaded region. (**C**) FLT3 ligand induced murine DC of wild-type (black bars) or TLR7-deficient (white bars) mice were stimulated with 10 μg/ml RNA40 complexed to DOTAP, 50 μg/ml poly (I:C), 100 nM R-848, and 3 μM CpG-ODN D19 and were tested for TNF-α, IL-12p40, and IFN-α secretion. *, not detected. One representative experiment of at least two independent experiments is shown ($n = 2$, ± SD).

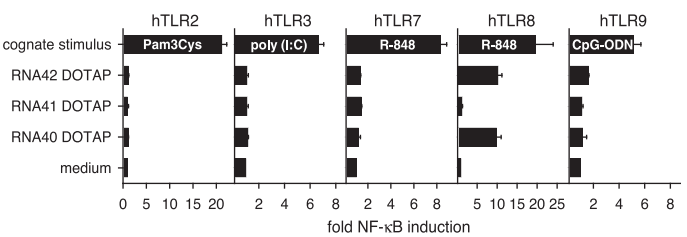

Fig. 4. Human TLR8 confers responsiveness to GU- and U-rich ssRNA. HEK 293 cells were transfected with human TLR2, TLR3, TLR7, TLR8, or TLR9 and a sixfold NF-κB luciferase reporter plasmid. Sixteen hours after transfection, cells were stimulated with 25 μg/ml RNA40, -41, or 42 complexed to DOTAP; 5 μg/ml Pam3Cys; 50 μg/ml poly (I:C); 10 μM R-848; or 1 μM CpG-ODN 1668. Values are given as -fold NF-κB activation compared with transfected, nonstimulated cells. One representative experiment of at least three independent experiments is shown ($n = 2$, ± SD).

results obtained with TLR7- and TLR8-deficient murine cells stimulated with ssRNA and support the previously observed species-specific difference between mouse and human TLR8 in response to R-848 (*10*, *11*). Because murine and human TLR7 transfectants failed to respond to RNA40 stimulation (Fig. 4) (*14*), we believe that TLR7 reconstitution in fibroblasts has some limitations. Therefore, it is possible that human TLR7 recognizes ssRNA in certain cell types, such as pDC, and leads to IFN-α production.

Our results define single-stranded GU-rich RNA as a natural ligand for murine TLR7 and human TLR8. Because TLR7 and TLR8 signaling is dependent on endosomal acidification like TLR9 signaling (*13*, *19*), we hypothesize that TLR7 and TLR8 recognize ssRNA in endosomal or lysosomal compartments and lead to secretion of inflammatory and regulatory cytokines. In case of viral infection, ssRNA possibly reaches the endosome through receptor-mediated uptake of viral particle or by fusion events of budding virus. Synthetic ssRNA complexed to cationic lipids may mimic viral particles, and the cationic lipids promote uptake of RNA (*15*) as well as protection from degradation by abundant extracellular RNases. Because GU sequences are found in viral as well as endogenous RNA, TLR7 and TLR8 may not discriminate between "foreign" and "self" RNA unless differences in GU frequency or RNA base modification exist. We therefore suggest that "foreign" or "self" ssRNA acts as "danger signal," depending on its compartmentalization. Accordingly, autoantibodies against RNA, which are one characteristic of autoimmune diseases such as systemic lupus erythematosus (SLE) (*20*), could mediate uptake of "self"-RNA and lead to TLR-driven stimulation of self-reactive B cells. This idea fits the recent finding that chromatin-antibody complexes induce stimulation of self-reactive B cells through TLR9 (*21*, *22*).

Furthermore, the data presented here may also shed light on the mechanisms of how transfection of DC with total tumor RNA or messenger RNA (mRNA) encoding specific tumor antigens elicits strong cytotoxic T cell (CTL) responses and tumor immunity (*15*, *23*). For example, lipid carrier–mediated transfection of DC with RNA encoding specific antigen not only is effective in gene expression but causes maturation of immature DC into mature APC (*24*, *25*). This maturation step is possibly driven via TLR7 or TLR8 that recognize GU-rich ssRNA sequences in mRNA or ribosomal RNA (rRNA). Additionally, the use of synthetic GU- or U-rich RNA oligonucleotides may be fundamental to the generation of powerful new adjuvants for vaccination and immunotherapy.

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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

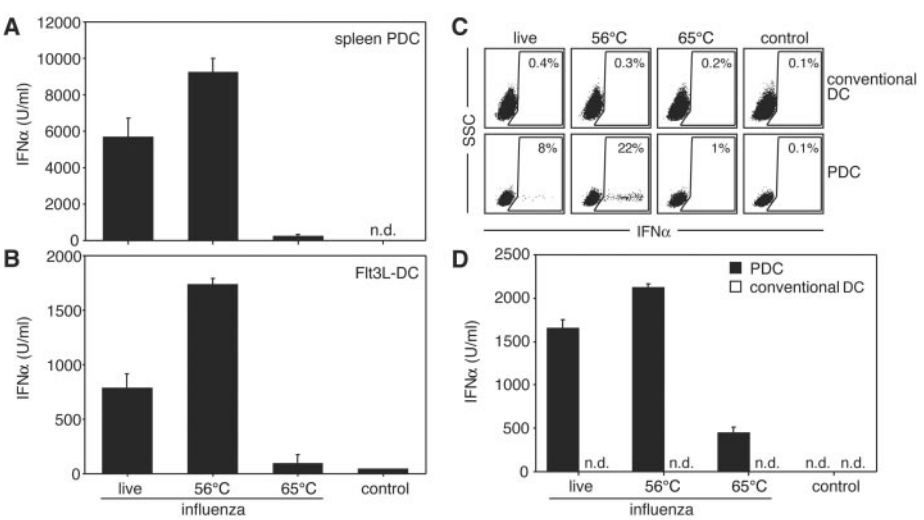

Fig. 1. PDC produce high levels of IFN-α in response to live or inactivated influenza virus. (**A**) Purified BALB/c spleen PDC were cultured with live or heat-inactivated influenza virus or in medium alone (control). (**B**) Bulk cultures of C57BL/6 Flt3L DC were treated as in (A). (**C**) Intracellular 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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