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Age-Dependent Myeloid Dendritic Cell Responses Mediate Resistance to La Crosse Virus-Induced Neurological Disease

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ABSTRACT

La Crosse virus (LACV) is the major cause of pediatric viral encephalitis in the United States; however, the mechanisms responsible for age-related susceptibility in the pediatric population are not well understood. Our current studies in a mouse model of LACV infection indicated that differences in myeloid dendritic cell (mDC) responses between weanling and adult mice accounted for susceptibility to LACV-induced neurological disease. We found that type I interferon (IFN) responses were significantly stronger in adult than in weanling mice. Production of these IFNs required both endosomal Toll-like receptors (TLRs) and cytoplasmic RIG-I-like receptors (RLRs). Surprisingly, IFN expression was not dependent on plasmacytoid DCs (pDCs) but rather was dependent on mDCs, which were found in greater number and induced stronger IFN responses in adults than in weanlings. Inhibition of these IFN responses in adults resulted in susceptibility to LACV-induced neurological disease, whereas postinfection treatment with type I IFN provided protection in young mice. These studies provide a definitive mechanism for age-related susceptibility to LACV encephalitis, where mDCs in young mice are insufficiently activated to control peripheral virus replication, thereby allowing virus to persist and eventually cause central nervous system (CNS) disease.

IMPORTANCE

La Crosse virus (LACV) is the primary cause of pediatric viral encephalitis in the United States. Although the virus infects both adults and children, over 80% of the reported neurological disease cases are in children. To understand why LACV causes neurological disease primarily in young animals, we used a mouse model where weanling mice, but not adult mice, develop neurological disease following virus infection. We found that an early immune response cell type, myeloid dendritic cells, was critical for protection in adult animals and that these cells were reduced in young animals. Activation of these cells during virus infection or after treatment with type I interferon in young animals provided protection from LACV. Thus, this study demonstrates a reason for susceptibility to LACV infection in young animals and shows that early therapeutic treatment in young animals can prevent neurological disease.

Pediatric populations are often more susceptible to virus infections than adults and develop more severe diseases, including virus-mediated neurological diseases (1–3). A more comprehensive understanding of differences underlying infection in pediatric populations may provide important interventions for treatment of virus infections of the central nervous system (CNS). One virus which induces disease primarily in the pediatric population is mosquito-borne La Crosse virus (LACV), genus *Orthobunyavirus*, family *Bunyaviridae*. LACV is a primary cause of pediatric viral encephalitis in the United States, and the expanding vector range makes LACV infections of significant concern for pediatric populations (2, 4–7). Over 80% of reported neurological disease cases caused by LACV occur in children under 16 and include clinical outcomes ranging from behavioral changes, cognitive defects, and seizures to coma or death (2, 6). In contrast, LACV infection in adults results in mild, febrile syndromes without significant neurological components.

Age disparity in neurological disease is also observed in mice infected with LACV. Infected weanling mice develop neurological disease with high CNS viral titers (8, 9). In contrast, adult mice do not generally show signs of neurological disease and have minimal titers of virus in the CNS (9–11). However, adult mice develop neurological disease when LACV is administered intracerebrally (i.c.), indicating that age-related resistance is due to either inhibition of viral replication in the periphery or inability of the virus to enter the CNS (8, 9). Low virus replication in peripheral tissues could be due to either a lack of virus-susceptible cells in adults or development of more effective innate and adaptive immune responses.

A critical immune response that limits virus replication is production of type I interferons (IFNs), including IFN-α and IFN-β. Type I IFNs are produced by both nonhematopoietic and hematopoietic cells, including myeloid dendritic cells (mDCs), although the primary producers are generally plasmacytoid dendritic cells (pDCs) (12). Triggering of the type I IFN response to virus infection relies on a signal cascade induced by pattern recognition receptor (PRR) detection of pathogens. Viral RNAs are recognized by endosomal Toll-like receptor 3 (TLR3) and TLR7 or by the cytoplasmic retinoic acid-inducible gene I (RIGI)-like receptors (RLRs) (13, 14). Activation of these PRRs results in signaling through interferon response factor 3 (IRF3) and IRF7, leading to induction of IFN-β and IFN-α (13–15). These type I IFNs

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bind to IFN receptor subunits IFNAR1 and IFNAR2, leading to upregulation of interferon-stimulatory genes (ISGs). ISGs are necessary for protection from a large number of viral infections (16–18).

Although the importance of type I IFN responses in adults has been well established, the ability of young animals to generate strong type I IFN responses and the influence of these responses on viral infections are not as clear. Some viruses, such as Sindbis virus (SINV), have strong type I IFN responses in young animals (19). However, recent studies with respiratory syncytial virus (RSV) and chikungunya virus (CHIKV) have indicated a less productive type I IFN response in children and young animals than that observed in adults (20, 21). Thus, the type I IFN response to virus infection in young animals may differ from that observed in adults, which could influence the pathogenesis of virus-induced disease.

Adult *Ifnar1*$^{-/-}$ mice are susceptible to LACV-induced neurological disease, indicating that type I IFN responses are necessary for resistance (10). However, whether type I IFN responses are deficient in young mice and whether these IFN responses are responsible for age-related differences in susceptibility have not been determined. In the present study, we found that young (3-week-old) mice had reduced type I IFN responses to LACV infection compared to those of adults (6 to 8 weeks old), despite having higher virus loads. Mechanistic studies indicated that mDC responses to endosomal TLR or cytoplasmic RLR stimulation were reduced in young animals. Additionally, activation of mDCs by targeted delivery of PRR ligands resulted in protection in young mice, suggesting a therapeutic role for early treatment in cases of pediatric encephalitic viral infections.

MATERIALS AND METHODS

Infection of mice with LACV. All animal studies were conducted under animal protocols RML2012-47 and RML2012-58, which were approved by the NIH/NIAID/RML Institutional Animal Care and Use Committee. *Irf3*$^{-/-}$ and *Irf7*$^{-/-}$ mice were kindly provided by Michael Diamond, Washington University (22). These mice as well as *Myd88*$^{-/-}$ mice and *Unc93b1* 3D mice (obtained from the Mutant Mouse Regional Resource Center) were maintained on a C57BL/6 background (23). *Mavs*$^{-/-}$ and *Tlr3*$^{-/-}$ mice were purchased from Jackson Laboratories on a C57BL/6, 129 mixed background. Although both mouse strains have similar susceptibility to LACV infection (unpublished observations), F_2 mice generated were used as an additional control. LACV human 1978 stock was a kind gift from Richard Bennett (NIAID, NIH) and has been previously described (24, 25). Mice were inoculated with 10^1, 10^3, or 10^5 PFU of LACV in phosphate-buffered saline (PBS) either intraperitoneally (i.p.) in a volume of 200 μl/mouse, intradermally (i.d.) on a shaved region of the back of each animal in a volume of 50 μl/mouse, or i.c. in a volume of 50 μl/mouse. Mice were observed daily for signs of neurological disease.

pDC depletion. Mice were depleted of pDCs by i.p. injection of 500 μg of anti-mPDCA-1 (Miltenyi Biotec) or IgG control (Jackson) 24 h prior to LACV infection. Depletion was confirmed by flow cytometry using PDCA1 and CD11c antibodies.

Clodronate liposome treatment. Mice were injected i.p. with 125 μl at −1 day postinfection (dpi) and 62 μl at 1 and 3 dpi of either clodronate encapsulated in liposomes (clodronate liposomes) or control liposomes (FormuMax Scientific). Mice were infected with 10^3 PFU of LACV i.p. and followed for disease. Peripheral blood mononuclear cells (PBMCs) and splenocytes were taken from 3 or 4 mice per group and analyzed by flow cytometry.

Cationic liposome treatment. Cationic liposomes (FormuMax Scientific) were combined with agonists for a final concentration of 100 μg/μl for poly(I·C) or 66 μg/μl for a combination of CL075 (InvivoGen tlrl-c75) and ssRNA40 (tlrl-lrna40; InvivoGen). Mice were inoculated i.p. with 100 μl of each complex at −1, 1, and 3 dpi with 10^3 PFU of LACV.

IFN-β treatment of mice. C57BL/6 mice were injected intravenously (i.v.) with 10^5 units/mouse of IFN-β (12410-1; PBL Assay Science) at days 1 and 3 postinfection with 10^3 PFU of LACV, i.p., and followed for signs of neurological disease.

Poly(I·C) treatment of mice. C57BL/6 mice were injected with 100 μg of poly(I·C) in PBS, which was composed of a mixture of noncomplexed poly(I·C) as well as low-molecular-weight poly(I·C) complexed to LyoVec (InvivoGen). At 4 h postinfection, blood was drawn for analysis by flow cytometry. Spleens were also removed at the same time and processed for RNA analysis.

Real-time PCR. Real-time PCR analysis of mRNA expression was completed as previously described (26). Primers used that were not previously described include LACVs.2-552F (ATTCTACCCGCTGACCATTG), LACVs.2-650R (GTGAGAGTGCCATAGCGTTG), Ifna4.1-451F (CTGCTGGCTGTGAGGACATA), Ifna4.1-565R (AGGAAGAGAGGGCTCTCCAG), Ifna1.1-108F (AGCCTTGACACTCCTGGTAC), Ifan1.1-22R (AGCCTTCTTGATCTGCTGGG), Ifna6.1-27F (GGTTTTGGTGGTGTTGAGCT), Ifna6.1-127R (GTACCAGGAGTGTCAAGGCT), Ifna9.2-286F (TCATCTGCTGCTTGGAATGC), Ifna9.2-395R (AGTTCCTTCATCCCGACCAG), Ifna11.2-95F (ACCTGTTCTCTAGGATGCGA), Ifna11.2-200R (TTAGGCAGGAAAGAGGGGTG), Ifna12.1-07F (CCTAGAGGGGAAGGTTCAGG), Ifna12.1-114R (AGCTCATCACTAGCAGGGTC), Ifne.2-298F (CCTTCAGCAGATCTTCACGC), Ifne.2-385R (TGACTCCACGTATTCCAGCT), Ifnk.1-238F (AAACGCCGTCTCCTATCGTA), and Ifnk.1-342R (CCCGATCTGATACGTTCCCA). All primers were subjected to BLAST analysis (NCBI) to ensure detection of only the specified gene and were tested on positive controls to ensure amplification of a single product. Data for each sample were calculated as the percent difference in threshold cycle (C_T) value ($\Delta C_T = C_T$ for glyceraldehyde-3-phosphate dehydrogenase [GAPDH] gene $- C_T$ for specified gene). Gene expression was plotted as the percentage of gene expression relative to that of the GAPDH gene for each sample or plotted as the fold change relative to results for mock-infected controls.

Flow cytometry. For analysis of activation of cell populations, cells were isolated and a single-cell suspension generated by homogenization through 70 μm filters. Red blood cells were removed using 2% dextran T500–PBS and/or lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 M EDTA). Cells were subsequently fixed in 2% paraformaldehyde and permeabilized with 0.1% saponin–2% bovine serum albumin (BSA) in PBS. Fc receptors were blocked using CD16/CD32 Fcγ III/II (BD Biosciences, clone 2.4G2). Cells were stained using a combination of the following antibodies: CD11c-phycoerythrin (PE)/Cy7 (BD Biosciences, clone HL3), F4/80-e450 (eBioscience, clone BM8), CD45-PE (BD Biosciences, clone 30-F11), CD80-fluorescein isothiocyanate (FITC) (BD Biosciences, clone 16-10A1), CD86-AF700 (BD Biosciences, clone GL1), CD4-APC/Cy7 (BD Biosciences, clone GK1.5), CD8-PE (BD Biosciences, clone 53-6.7), PDCA1-allophycocyanin (APC) (Miltenyi Biotech, clone JF05-1C2.4.1), IFN-β–FITC (R&D Systems, clone RMMB-1), and IFN-α–FITC (R&D Systems, clone RMMA-1). Data were acquired on an LSR II cytometer (BD) and analyzed using FCS Express software (De Novo). Gates were used to exclude cellular debris and doublets.

Plasma type I IFN levels. Plasma IFN-β and IFN-α levels were determined using the VeriKine mouse interferon beta enzyme-linked immunosorbent assay (ELISA) kit (PBL).

RESULTS

Age-dependent resistance to LACV in young C57BL/6 mice. Age-related resistance to LACV-induced neurological disease was first reported in outbred mice, where 3- and 22-day-old mice were susceptible but 30-day-old mice were resistant (27). To more carefully define age-related resistance, C57BL/6 mice ranging from 1 to 6 weeks of age were infected i.p. with 10^5 PFU of LACV. All mice infected at 3 weeks of age or younger, as well as the majority of

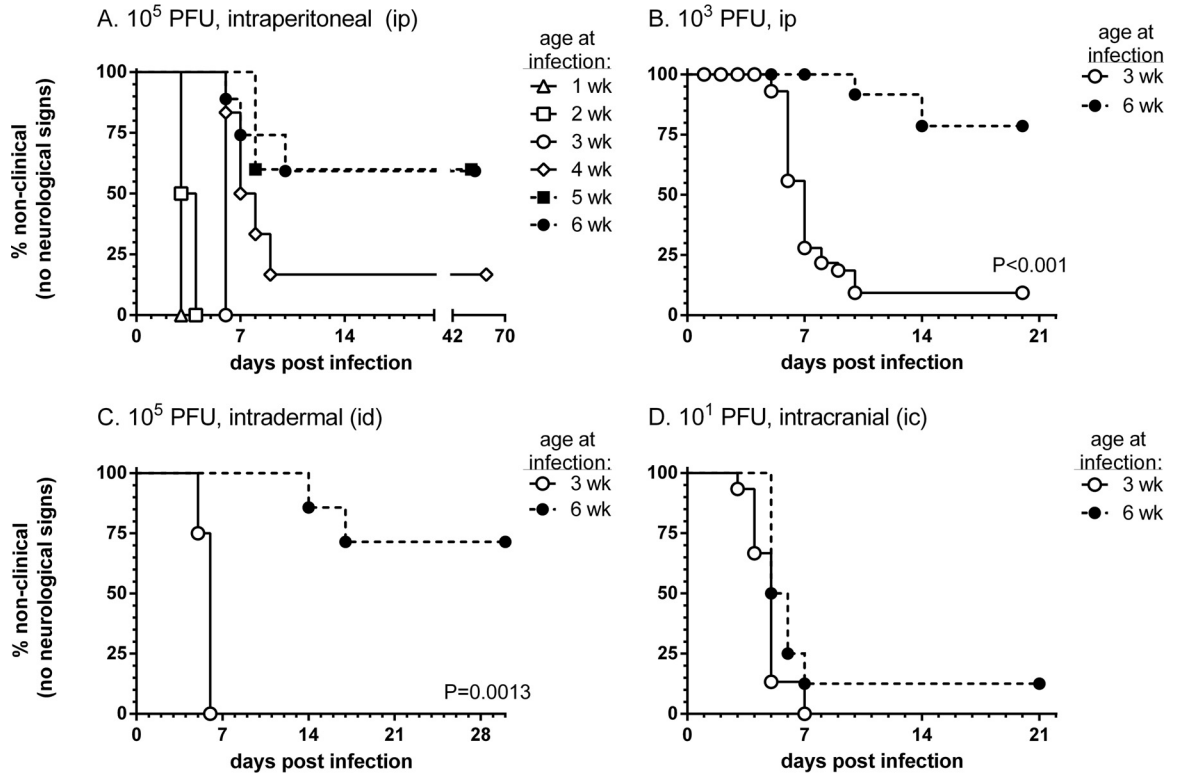

FIG 1 Age-related resistance to LACV infection is dependent on route of infection. (A) C57BL/6 mice at indicated ages were infected with 10^5 PFU of LACV by i.p. inoculation. Mice were then followed for clinical signs of disease, which included seizures, ataxia, or paralysis. Data are survival curves for 5 to 9 mice per age group. (B) Mice at 3 to 4 weeks or 6 to 8 weeks of age were infected with 10^3 PFU of LACV i.p. and followed for clinical disease. Data are survival curves for 30 mice for 3- to 4-week-old mice and 22 mice for 6- to 8-week-old mice. (C) Mice were inoculated with 10^5 PFU of LACV by i.d. inoculation and followed for disease. Data are survival curves for 4 mice for 3- to 4-week-old mice and 7 mice for 6- to 8-week-old mice. (D) Mice were inoculated with 10^1 PFU of LACV by i.c. inoculation and followed for disease. Data are survival curves for 15 mice for 3- to 4-week-old mice and 8 mice for 6- to 8-week-old mice. For all studies, statistical tests were completed using the Mantel-Cox test. Significant *P* values are indicated on the graphs.

4-week-old mice, developed neurological disease (Fig. 1A). However, fewer than 50% of 5- to 6-week-old mice developed disease, indicating age-related resistance. Dose comparison between young (3-week-old) and adult (6- to 8-week-old) mice indicated that a dose as low as 10^3 PFU was sufficient to induce disease in >90% of young mice (Fig. 1B). Comparable age resistance was also observed when mice were infected i.d. (Fig. 1C). However, similar to those in previous studies (8, 27), mice inoculated i.c. developed clinical disease regardless of age even at a very low inoculating dose (Fig. 1D). Thus, there is a clearly defined age-dependent resistance to peripheral infection by LACV with a 100-fold range of inoculating virus.

The peripheral type I IFN response to LACV differs between young and adult mice. In previous studies, deficiency in *Ifnar1* increased susceptibility to LACV in adult mice (10). Similarly, infection *of Irf3*$^{-/-}$, *Irf7*$^{-/-}$ or *Irf3*$^{-/-}$ *Irf7*$^{-/-}$ double-knockout (DKO) mice also resulted in significant disease in adults (Fig. 2A), indicating that type I IFN responses were essential for age-related resistance to LACV. To determine if type I IFN responses differed between age groups, we analyzed *Ifna4* and *Ifnb1* mRNA expression in spleens of infected mice. Surprisingly, no increase in *Ifna4* or *Ifnb1* mRNA was observed following LACV infection in young mice, despite the detection of viral RNA at 3 dpi (Fig. 2B). In contrast, both *Ifna4* and *Ifnb1* mRNAs were elevated in spleens from adult mice, despite lower levels of viral RNA (Fig. 2C). Real-time PCR analysis for other type I IFN genes in the spleen at 5 dpi resulted in the detection of *Ifna1* and *Ifn*κ, however, mRNA levels of these genes were not induced by LACV infection (Fig. 2D). Other type I IFN genes, including *Ifna2*, *Ifna6*, *Ifna9*, *Ifna11*, or *Ifna12* either were undetectable or were not consistently detected (data not shown). Analysis of plasma showed increased IFN-β protein from adults at 3 dpi, but not from young mice, while IFN-α protein was below the limits of detection in both groups (Fig. 2E, data not shown). Direct *ex vivo* or *in vitro* analysis of splenocytes or PBMCs by immunohistochemistry, fluorescence-activated cell sorting (FACS) or flow cytometry did not reveal a subpopulation of IFN-β- or IFN-α-producing cells in LACV-infected mice (data not shown). This suggests that the population of cells responsible for type I IFN responses is below the level of detection for these methods. Interleukin-6 (IL-6), IL-10, and IFN-γ were elevated in plasma from mice in both age groups compared to mock-infected controls, but no significant difference was observed between the two ages of infected mice (data not shown). Thus, young mice had significantly lower type I IFN responses to LACV infection than adults, despite higher viral RNA levels.

The type I IFN response is dependent on *Unc93b1* and *Mavs* but not *Myd88*. To better understand the generation of type I IFN responses in adults, we investigated the PRRs responsible for initiating type I IFN production. The primary PRRs known to recognize virus infection in mice are endosomal TLRs (TLR3, TLR7,

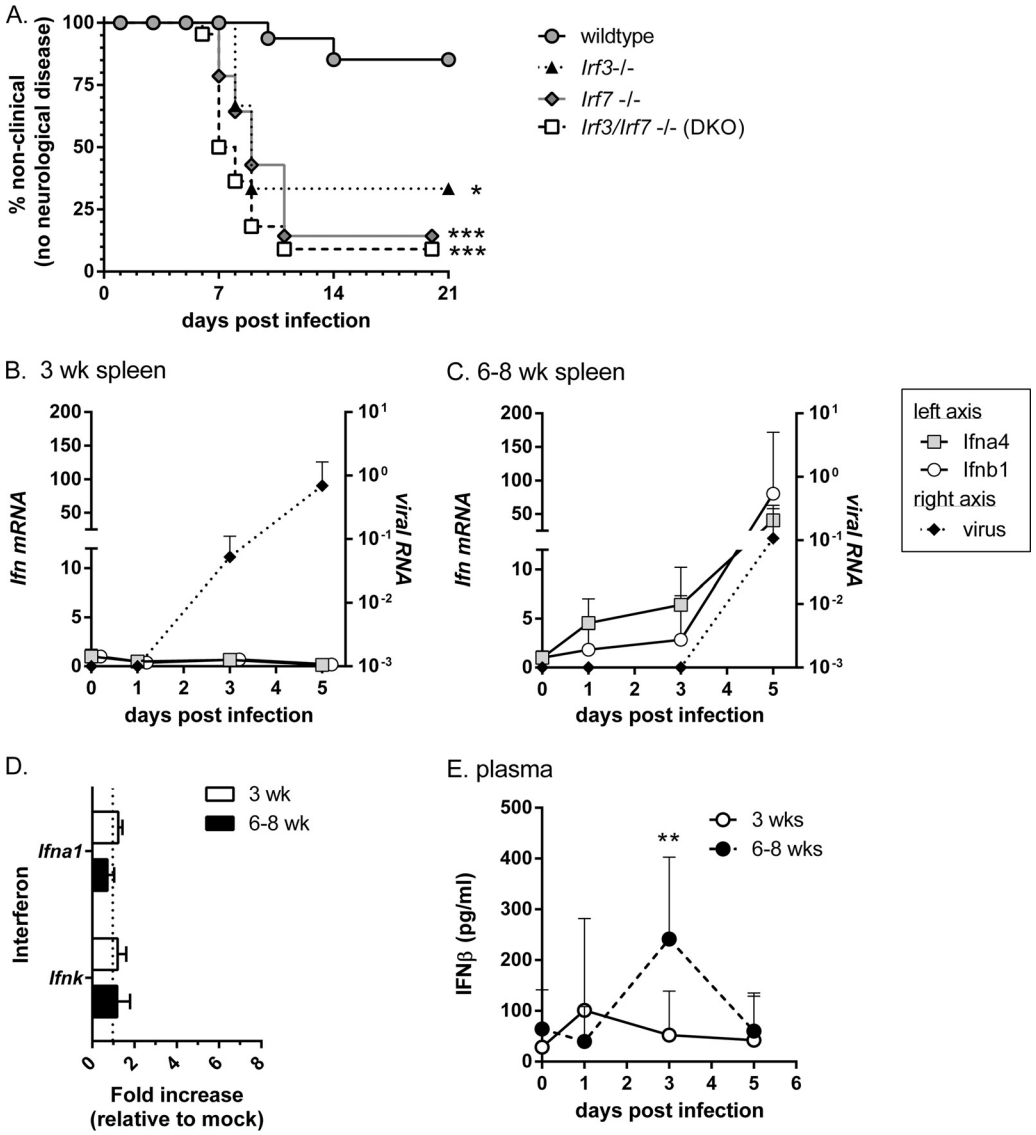

FIG 2 Type I IFN responses to LACV in spleen and blood are age dependent. (A) Six- to 8-week-old C57BL/6 (shown in Fig. 1), $Irf3^{-/-}$, $Irf7^{-/-}$, or DKO mice were infected with 10^3 PFU of LACV i.p. and followed for clinical disease. Data are from 22 wild-type (shown in Fig. 1B), 14 $Irf7^{-/-}$, 9 $Irf3^{-/-}$, and 22 DKO mice. Statistical analysis was completed using the log rank (Mantel-Cox) test. *, $P < 0.05$; ***, $P < 0.001$. (B and C) RNAs extracted from spleens of 3-week-old (B) or 6- to 8-week-old (C) wild-type mice at 1, 3, and 5 dpi with 10^3 PFU of LACV i.p. Data are plotted as fold change relative to the average for mock-infected controls for each age group and are the mean ± standard deviation (SD) for 3 to 7 mice per group per time point. Fold changes for *Ifna4* and *Ifnb1* mRNAs are plotted on the left axis, and virus RNA is plotted on the right axis. Day 0 represents mock-infected controls. (D) Fold expression of *Ifna1* and *Ifnk* mRNAs of spleens from mice at 5 dpi with10^3 PFU of LACV i.p. (E) Plasma collected from the above-described mice was analyzed for levels of IFN-β by ELISA. Data are the mean ± SD for 4 to 9 mice per time point per age group and are the combined results of multiple experiments. Day 0 represents mock-infected controls.

and TLR9) and cytoplasmic RLRs (RIG-I and MDA5) (28). To examine a role for RLRs, we infected mice deficient in *Mavs*, the downstream adaptor required for both RIG-I and MDA-5 responses. There was a significant increase in clinical disease in adult $Mavs^{-/-}$ mice compared to controls, indicating a role for RLRs in age-related resistance to LACV (Fig. 3A). This correlated with limited *Ifna4* or *Ifnb1* mRNA expression in $Mavs^{-/-}$ deficient mice following LACV infection (Fig. 3B and C).

To examine TLR-mediated responses, we utilized *Unc93b1* 3D mice, which cannot transport TLR3, TLR7, or TLR9 from the endoplasmic reticulum to endosomes, resulting in lack of function for these TLRs (29). We also used $Myd88^{-/-}$ mice, as MyD88 is the signaling adaptor protein for most TLRs, except TLR3. No significant difference in neurological disease development was observed between adult wild-type and $Myd88^{-/-}$ mice (Fig. 3A), demonstrating that MyD88 was not necessary for resistance to LACV-induced neurological disease. However, the incidence of neurological disease was significantly increased in adult *Unc93b1* 3D mice (Fig. 3A). This correlated with decreased IFN responses (Fig. 3B and C). Because TLR3 retains function in $Myd88^{-/-}$ mice but not *Unc93b1* 3D mice, these data suggest that TLR3 is necessary for protection from LACV infection. However, direct analysis of $Tlr3^{-/-}$ mice indicated that deficiency in TLR3 alone did not significantly increase susceptibility to LACV neurological disease

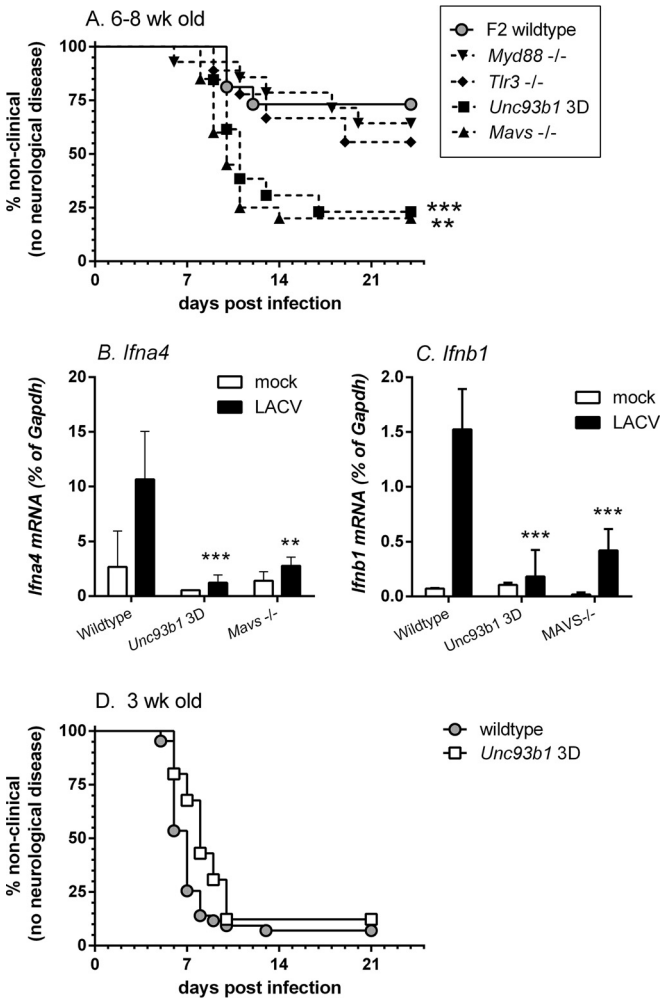

FIG 3 Resistance to LACV-induced neurological disease requires both endosomal and cytoplasmic PRRs. (A) Six- to 8-week-old F_2 wild-type, *Tlr3*$^{-/-}$, *Mavs*$^{-/-}$, *Myd88*$^{-/-}$, or *Unc93b1* 3D mice were infected with 10^3 PFU of LACV i.p. and followed for clinical signs of disease. Data are plotted as survival curves for 20 *Mavs*$^{-/-}$, 9 *Tlr3*$^{-/-}$, 13 *Unc93b1* 3D, 14 *Myd88*$^{-/-}$, and 16 F_2 mice. **, $P < 0.01$; ***, $P < 0.001$ (as determined by Mantel-Cox analysis). (B and C) Spleens from *Unc93b1* 3D, *Mavs*$^{-/-}$, and wild-type (C57BL/6) mice were removed at 5 dpi and analyzed for IFN mRNA by real-time PCR. Data are plotted as the mean ± SD for 4 to 5 mice per group and are the combined results from two experiments. Statistical analysis was completed using a two-way analysis of variance (ANOVA) with a posttest. **, $P < 0.01$; ***, $P < 0.001$. (D) Three-week-old wild-type or *Unc93b1* 3D mice were infected with 10^3 PFU of LACV i.p. and followed for clinical signs of disease. Data are plotted for 30 wild-type mice and 20 *Unc93b1* 3D mice. No statistical difference between groups was observed.

in adult mice (Fig. 3A). Thus, functional deficiency in all endosomal TLRs, but not individual TLRs (TLR3 or TLR7 and TLR9), abrogated resistance to LACV in adult mice.

Interestingly, young *Unc93b1* 3D mice did not significantly differ from controls in susceptibility to LACV infection (Fig. 3D). This is similar to a lack of an effect of *Mavs* deficiency on young mice (30). Thus, *Unc93b1* and *Mavs* are essential for protection in adults but do not substantially alter neurological disease in young mice.

Age-related resistance is associated with higher levels of mDCs. TLR7 and TLR9 are expressed at high levels on pDCs, while TLR3 is expressed on mDCs. While mDCs contribute to type I IFN production, pDCs are highly specialized and can produce up to 100-fold more IFN than other cell types. To examine which type I IFN-producing cells were necessary for age-related resistance, we directly targeted different innate immune cell types in adult mice. Treatment with anti-PDCA1 at −1 dpi resulted in depletion of pDCs, which was still observed at 5 dpi (Fig. 4A), just prior to disease onset in susceptible mice. However, treatment with anti-PDCA1 antibody did not significantly alter the incidence or onset of LACV-induced neurological disease in adult mice (Fig. 4B). Thus, pDCs were not necessary for age-related protection from LACV-induced neurological disease.

To determine whether other innate immune cell subsets, such as mDCs, were necessary for protection, we treated adult mice with clodronate liposomes. These liposome are taken up by phagocytizing cells, leading to cell death (31). Treatment of mice with clodronate liposomes increased the incidence of LACV-induced disease in adults (Fig. 4B). In contrast, treatment with control liposomes did not increase susceptibility to LACV-induced disease and instead resulted in a slight decrease in susceptibility (Fig. 4B). Analysis of cell populations from clodronate-treated mice showed an approximately 50% reduction in CD11c$^+$ mDCs as well as a reduction in F4/80$^+$ macrophages (Fig. 4C and D). In contrast, pDCs (PDCA1$^+$) and monocytes (CD11b$^+$, F4/80^{lo}, and CD11c$^-$) were increased (Fig. 4C and D). These data suggest that mDCs, but not pDCs or monocytes, may be responsible for protection from LACV infection in adults.

To determine if significant differences in these cell populations existed between age groups, we analyzed splenocytes by flow cytometry. Naive adult mice had significantly higher percentages of splenic mDCs than young mice, lower percentages of macrophages, and similar percentages of pDCs (Fig. 5A to C). LACV infection did not significantly alter the cell ratio for any of these cells in either age group (data not shown). Analysis of mDC subsets demonstrated similar ratios between the age groups (Fig. 5D and E), although the overall number of CD8$^+$ class II$^+$ mDCs was significantly higher in adults (Fig. 5F). No significant difference in TLR3 expression on mDCs was observed between the age groups (Fig. 5G). Thus, adults had higher levels of mDCs than young mice, which could contribute to the strong type I IFN responses observed in the older mice.

Increased type I IFN response to TLR3/RLR agonist stimulation in adult mice. mDCs respond to both TLR3 and RIG-I/MAVS stimulation (12). To directly determine if there were age-dependent differences in the response to these PRRs, we inoculated both age groups with poly(I·C), a ligand that stimulates both PRRs. At 4 h postinoculation, spleens from adults stimulated with poly(I·C) had 5- to 10-fold-higher responses in *Ifna4* and *Ifnb1* mRNA expression than those from young mice (Fig. 6A and B). In contrast, *Il1a* mRNA expression was comparable, indicating specificity of the IFN response (Fig. 6C). We also analyzed the percentage of innate immune cells expressing IFN-α by gating on viable PBMCs and selective markers to define each population. An increase in IFN-α-producing mDCs was observed in adults compared to young mice, indicating age-specific differences in mDC responses (Fig. 6E). This increase was not observed in macrophages or pDCs (Fig. 6D and F). IFN-α-positive cells in adult mice were confirmed to be mDCs based on CD11c expression and lack of CD11b or PDCA1 (Fig. 6G and data not shown). Thus, mDCs from young mice were less proficient than mDCs from adults at generating type I IFN responses in response to poly(I·C) stimulation.

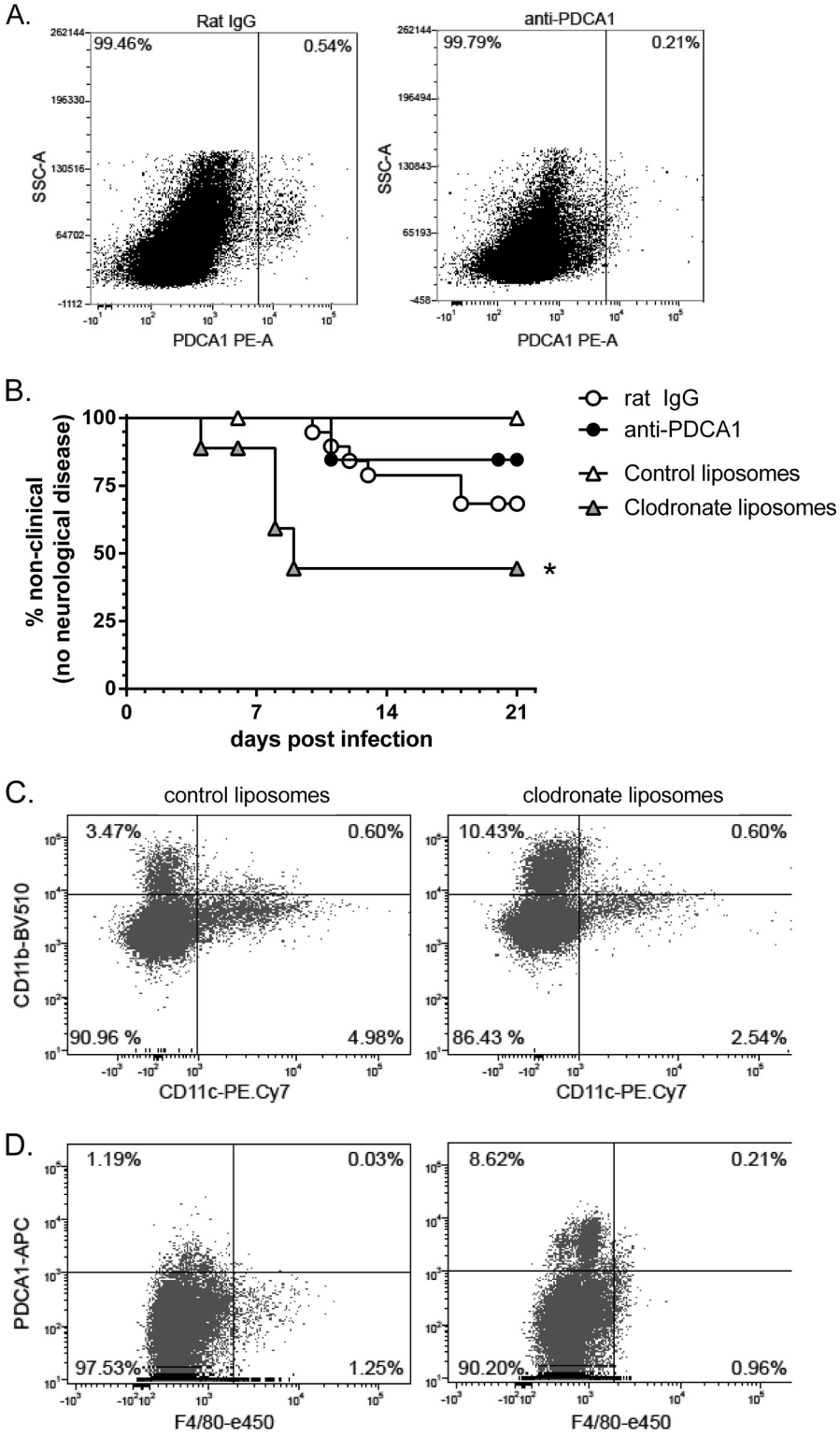

FIG 4 Reduction of mDCs, but not pDCs, increases susceptibility to LACV infection in 6- to 8-week-old mice. (A and B) Six-week-old wild-type mice were injected with either rat Ig or PDCA1 i.p. prior to infection with 10^3 PFU of LACV i.p. Mice were analyzed for pDC (A) or followed for signs of neurological disease (B). (A) PDCA1 staining (*x* axis) of splenocytes gated to remove debris and doublets. Cells were plotted for side scatter (granularity) and PDCA1. (B) Survival curve for mice treated with rat Ig or anti-PDCA1 as well as mice treated with control liposomes or clodronate liposomes at −1, 1, and 3 dpi with 10^3 PFU of LACV i.p. *, $P < 0.05$. Data are shown for 10 control liposome-treated, 9 clodronate liposome-treated, 19 control rat Ig-treated, and 13 anti-PDCA1-treated mice. Statistical significance of differences between rat Ig- and PDCA1-treated groups as well as control and clodronate liposome groups was determined using the log rank test. (C and D) At 5 dpi, splenocytes were removed from control and clodronate liposome-treated animals and analyzed by flow cytometry analysis. Gates were drawn to remove doublets and debris prior to analysis. Data are representative of 3 mice per group per replicate experiment.

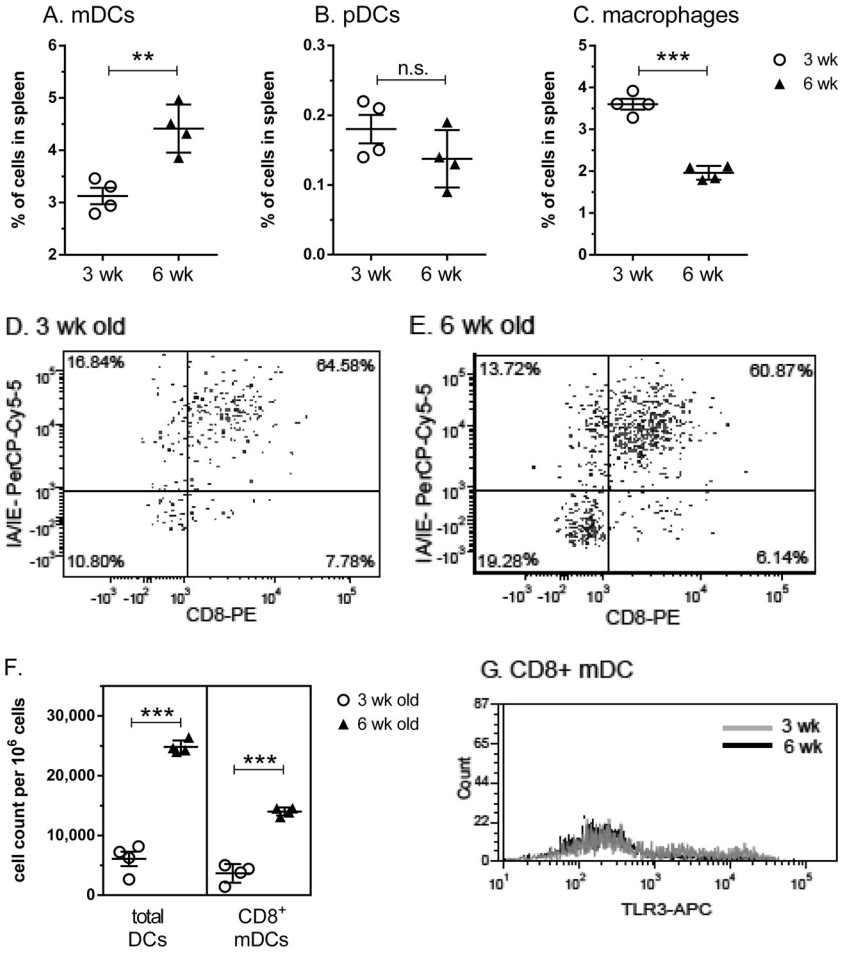

FIG 5 Higher levels of mDCs in spleens of adult mice than in those of young mice. (A to C) Splenocytes from 3- and 6-week-old mice were analyzed for F480$^+$ macrophages (A), CD11c$^+$ PDCA1$^-$ mDCs (B), and PDCA1$^+$ pDCs (C). Data are shown as percentage of total spleen population, with average and SD bars for 4 mice for each time point per group. Statistical analysis was completed with the Mann-Whitney test. (D and E) Splenocytes from 3- and 6-week-old mice were gated for CD11c$^+$ PDCA1$^-$ cells and then analyzed for CD8 and IA/IE. Data shown are representative of 4 mice per group. (F) Total numbers of CD11c$^+$ DCs or CD11c$^+$ CD8$^+$ MHC class II IA/IE$^+$ DCs per 10^6 splenocytes from 3- and 6-week-old mice. Data are shown as percentage of total spleen population, with average and SD bars for 4 mice for each time point per group. Statistical analysis was completed with the Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (G) CD8$^+$ mDCs from the experiments for panels D and E were analyzed for TLR3 expression by flow cytometry.

PRR agonist-containing liposomes increase protection against LACV infection in young mice. The above results suggest that protection from LACV infection is dependent on IFN production by mDCs and that young mice are unable to generate this response. To determine if we could induce protection in young mice, we treated mice with cationic liposomes containing PRR agonists, which were previously shown to stimulate dendritic cells (32). Greater than 50% of the CD11c$^+$ PDCA1$^-$ mDCs took up fluorescently labeled liposomes (Fig. 7A and B), indicating that the target population phagocytizes liposomes. Poly(I·C) was used to stimulate TLR3/RIG-I responses, and a combination of ssRNA40 and CL075 was used to stimulate TLR7 responses. Liposomes were administered at −1, 1, and 3 dpi. Treatment with liposomes containing either set of agonists induced protection in greater than 50% of young mice, while liposome alone resulted in 20% survival (Fig. 7C). Thus, stimulation of innate immune responses using PRR-containing liposomes was sufficient to induce protection in young mice. To further examine the ability of the type I IFN response to provide protection in young mice, we administered IFN-β at 1 and 3 dpi. Interestingly, these two injections of IFN-β were sufficient to induce protection against LACV infection in 50% of the infected animals (Fig. 7C). This indicates that therapeutic activation of peripheral innate immune responses during the early stages of virus infection may be protective in suppressing the development of neurological disease.

DISCUSSION

In the current study, we determined that mDCs have an important role in resistance to LACV infection and that this response is limited in young mice, resulting in increased susceptibility to neurological disease. We found that peripheral IFN responses to LACV were significantly reduced in young mice compared to adults. These IFN responses in adults were dependent on RLRs and endosomal TLRs. Inhibition of RLR or endosomal TLR signaling pathways or reduction of mDCs in adults increased their susceptibility to LACV infection. The requirement for endosomal TLRs appears to be complex, since Unc93b1 deficiency resulted in increased susceptibility to LACV infection, while deficiency in TLR3 or MyD88 did not increase susceptibility (Fig. 3A). This suggests that the requirement for endosomal TLRs in mediating age-re-

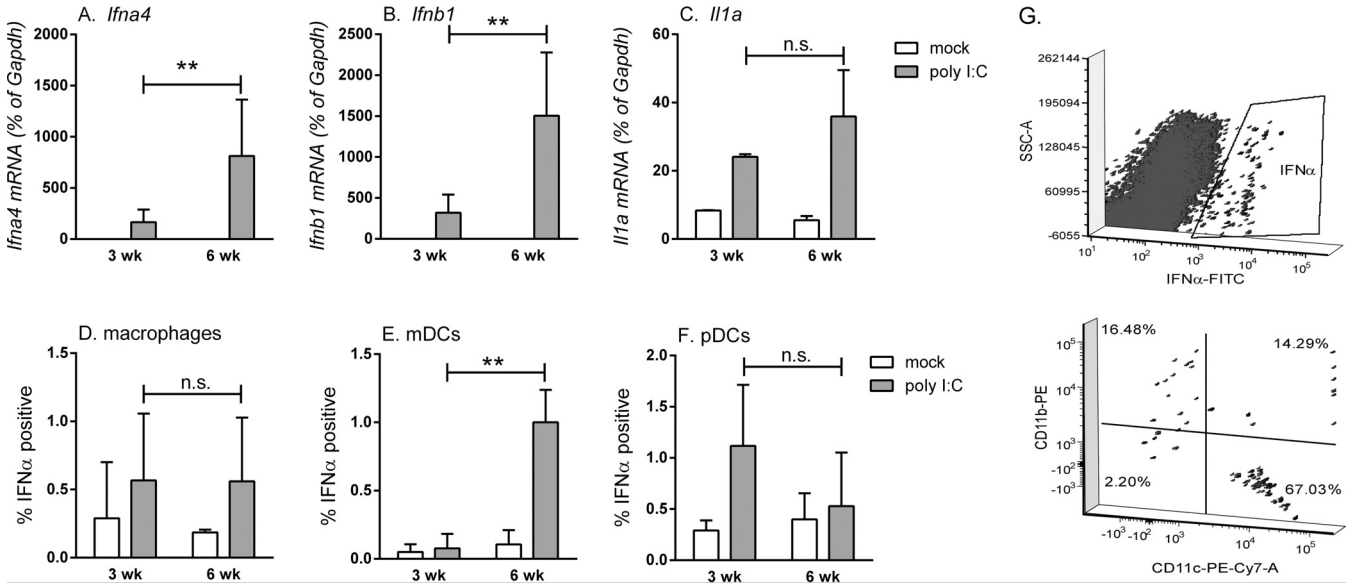

FIG 6 Poly(I·C) induces a stronger response in mDCs of adult mice than in those of young mice. Three- and 6-week-old mice were injected intravenously with poly(I·C). (A to C) At 4 h postinoculation, spleens were removed and processed for RNA analysis by real-time PCR for *Ifna4*, *Ifnb1*, and *Il1a* mRNA expression. (D to F) PBMCs were removed and analyzed for cellular expression of IFN-α by flow cytometry. Gates were drawn to exclude cellular debris and doublets. Cells were then gated for F4/80$^+$ macrophages (D), CD11c$^+$ PDCA1$^-$ mDCs (E), or PDCA1$^+$ pDCs (F). These cell populations were then analyzed for IFN-α, and the percentage of cells positive for IFN was calculated. Data were analyzed by two-way ANOVA. **, ($P < 0.01$). (G) A gate was drawn on the whole cell population for just the IFN-α-positive cells The IFN-α positive cells were then analyzed for expression of CD11c and CD11b (shown) as well as PDCA1 and F4/80 (data not shown). The majority of cells were CD11c$^+$, CD11b$^-$, F4/80$^-$, and PDCA1$^-$.

lated resistance can be provided by either the MyD88-dependent TLR7/TLR8/TLR9 pathways or the MyD88-independent TLR3 pathway. However, neither pathway is sufficient for protection in the absence of RLR signaling, since adult mice deficient in MAVS were susceptible to LACV-induced neurological disease. Thus, the regulation of the type I IFN response to LACV is multifaceted, requiring activation of both endosomal and cytoplasmic PRRs.

The requirement for both cytoplasmic RLR and endosomal TLR pathways suggests that these pathways function in a nonredundant manner for the generation of protective IFN responses. These pathways independently generate an IFN response. Thus, their codependency in this model may be due to a high level of IFN that is needed to control virus replication and protect against virus inhibition of IFN responses. LACV encodes a nonstructural small (NSs) protein that inhibits type I IFN production by degrading RNA polymerase II subunit RPB1, thereby inhibiting transcription of type I IFN genes (33). The ability of the immune response to control virus infection may depend on the early production of enough IFN to control virus replication and prevent the shutdown of the IFN response. The early production of IFN may require both endosomal and cytoplasmic PRR pathways to generate sufficient levels of IFN for protection. The absence of either pathway may tilt the balance between IFN production and virus replication, resulting in age-related susceptibility to virus-induced neurological disease.

The inability to mount a strong type I IFN response through these pathways explains the susceptibility of young mice to LACV-induced neurological disease. Enhancing the DC-mediated IFN response in young mice by administration of either PRR ligands or IFN-β resulted in protection against LACV-induced disease (Fig. 7). This induction of the IFN response can be mediated by stimulating either DC population, as using liposomes containing TLR7 ligands to stimulate pDCs was as protective as using poly(I·C), which stimulates mDCs.

The current study found age-dependent mDC responses to poly(I·C) stimulation in mice (Fig. 6). Interestingly, stimulation of human DCs with poly(I·C) also shows age-dependent responsiveness. DCs from adults produced stronger type I IFN responses following poly(I·C) stimulation than DCs from 1- to 2-year-old children (34). This was not observed with other TLR ligands (34). The lack of responses to poly(I·C) stimulation in DCs from children correlates with a recent study showing attenuation of type I IFN responses to RSV in children up to 5 years of age that was directly linked to RIG-I (20). Thus, the susceptibility of infants and young children to virus infections may be strongly dependent on RIG-I-mediated detection of virus infection.

Both pDCs and mDCs are substantially lower in neonates than in adult mice, with an approximately 600-fold increase in splenic DCs from birth to 6 weeks of age (35). However, pDCs expand rapidly within a few days of birth, while mDCs take longer to reach adult numbers (12, 35–37). Our current studies indicate that mDCs in young mice up to 3 weeks of age are less responsive than those in adult mDCs and that this difference affects susceptibility to virus infection. Although we were unable to directly detect *in vivo* type I IFN production by mDCs during LACV infection, we did find significant differences in the ability of mDCs from young and adult mice to generate a strong type I IFN response to poly(I·C) (Fig. 6). This suggests that young animals may be particularly sensitive to virus infections that require mDC activation for protection.

The lack of detectable IFN-α- or IFN-β producing cells in the periphery after LACV infection in this study may be due to the ability of NSs to inhibit type I IFN production (38), thereby lowering the amount of IFN produced to amounts undetectable by

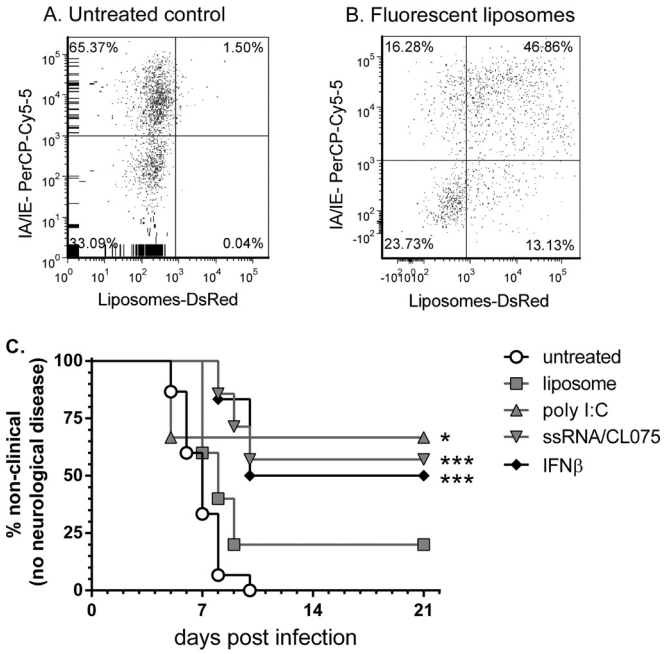

FIG 7 Treatment with agonist-containing liposomes reduces the incidence of neurological disease in 3-week-old mice. (A and B) C57BL/6 mice at 3 weeks of age were either untreated (A) or injected with fluorescent liposomes i.p. to determine uptake by different cell populations (B). Splenocytes were gated for $CD11c^+$ $PDCA1^-$ mDCs, $PDCA1^+$ pDCs, or $F4/80^+$ macrophages. Gated cells were then analyzed for liposome uptake (*x* axis) and class II expression (IA/IE) (*y* axis). The percentages of cells positive for liposomes were 45 to 50% for mDCs, 10 to 15% for pDCs (data not shown), and greater than 60% for F4/80-positive cells (data not shown). (C) Survival curve for 3-week-old mice treated with poly(I·C)-containing liposomes or ssRNA/CL075-containing liposomes at −1,1, and 3 dpi with 10^3 PFU of LACV, infected i.p. An additional group received 10^5 U of IFN-β at 1 and 3 dpi. Data are plotted as a survival curve for 6 to 15 mice per group. Statistical analysis was completed with the log rank test, with comparison of treatment groups to untreated controls. *, $P < 0.05$; ***, $P < 0.001$.

cellular assays. The detection of *Ifna4* and *Ifnb1* mRNAs but not protein-producing cells is most likely due to the ability to amplify the transcript for detection using real-time PCR. NSs suppression of type I IFN responses combined with the limited ability of young mice to produce type I IFNs may allow LACV to overwhelm peripheral innate immune responses, resulting in high virus titers and infection of the CNS.

The generation of a peripheral type I IFN response in this study did not correlate with high virus titers (Fig. 2). This contrasts with similar studies done with SINV, where type I IFN responses in younger mice were elevated and correlated with higher type I IFN responses (19). This disparity may be due to the cell types activated or the PRRs stimulated by the two viruses. Possibly, SINV induces strong activation of pDCs, resulting in high levels of type I IFNs. Interestingly, the generation of high levels of type I IFN was not protective during SINV infection in young mice and instead may contribute to pathology (19). This also contrasts with LACV infection, where activation of cells with PRR ligands induced protection in young animals.

The current studies suggest that the ability of the innate immune response to produce type I IFN responses to virus infection develops as the animal matures. Activation of DCs during LACV infection appears to be a key mediator of protective type I IFN responses in the periphery. Using other means to increase type I IFN responses early during virus infection, through either administration of PRR ligands or administration of IFN-β itself, resulted in protection of 3-week-old mice (Fig. 7). The ability of CL075/ssRNA to induce protection in greater than 50% of young mice suggests that induction of the pDC response in these mice is sufficient to control virus infection. The lack of protection by this cell type during LACV infection may simply be due to a lack of pDC activation by LACV. These data indicate that early augmentation of the IFN response to LACV, rather than relying on just the host response to generate type I IFNs, may be an important mechanism to limit LACV encephalitis.

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