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Mucosal Polyinosinic-Polycytidylic Acid Improves Protection Elicited by Replicating Influenza Vaccines via Enhanced Dendritic Cell Function and T Cell Immunity

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Live-attenuated influenza vaccines (LAIVs) have the potential to generate CD8 T cell immunity that may limit the virulence of an antigenically shifted influenza strain in a population lacking protective Abs. However, current LAIVs exert limited T cell immunity restricted to the vaccine strains. One approach to improve LAIV-induced T cell responses is the use of specific adjuvants to enhance T cell priming by respiratory dendritic cells, but this hypothesis has not been addressed. In this study, we assessed the effect of the TLR3 ligand polyinosinic-polycytidylic acid (poly IC) on CD8 T cell immunity and protection elicited by LAIVs. Mucosal treatment with poly IC shortly after vaccination enhanced respiratory dendritic cell function, CD8 T cell formation, and production of neutralizing Abs. This adjuvant effect of poly IC was dependent on amplification of TLR3 signaling by non-hematopoietic radioresistant cells and enhanced mouse protection to homosubtypic, as well as heterosubtypic, virus challenge. Our findings indicate that mucosal TLR3 ligation may be used to improve CD8 T cell responses to replicating vaccines, which has implications for protection in the absence of pre-existing Ab immunity. The Journal of Immunology, 2014, 193: 1324–1332.

One of the major challenges in influenza vaccinology is to develop effective vaccines against a highly variable pathogen that causes seasonal epidemics that do not necessarily result in immunity to subsequent viral challenges (1). In addition, there is an urgent need to develop therapeutic and prophylactic strategies against putative pandemic influenza strains for which most of the human population lacks pre-existing Ab immunity. The development of live-attenuated influenza vaccines (LAIVs) has only partially addressed these issues. LAIVs have limited viral replication that allows processing of viral core proteins encoding broadly conserved T cell epitopes (2), thus having the potential to generate broad CD8 T cell–based protection. Although this has been consistently demonstrated in mouse models of infection (3, 4), LAIVs still induce suboptimal cross-reactivity against subtypes of influenza viruses different from the vaccine strains in humans (5). However, the question of whether novel strategies can be developed to increase CD8 T cell immunity induced by LAIVs, as well as whether these strategies could improve vaccine protection and cross-reactivity, has not been addressed.

The quantity and quality of vaccine-induced T cells are established during the innate phase of the immune response when migratory tissue-resident dendritic cells (DCs) encounter pathogen-derived Ags. Tissue DCs are myeloid cells that scan the skin and mucosal surfaces for Ags and have the ability to process these Ags, transport them to tissue-draining lymph nodes, and prime Ag-specific naive T cells (6). This process depends on DC maturation/activation, which requires signaling through various innate immune receptors, including TLRs. A substantial body of work indicates that TLR3+ respiratory DCs (rDCs) expressing CD103+ dominate the transport of influenza Ags to the lung-draining mediastinal lymph nodes (mLN), where they show an exceptional capacity for cross-priming of naive T cells (7, 8). Upon encountering their cognate Ag, naive T cells rapidly proliferate and become effector cells with cytotoxic and helper capacity. Most of these effector cells are eliminated afterwards during the T cell contraction phase (9). Roughty, 2–5% of effector T cells survive the contraction phase, giving rise to a small population of Ag-specific, tissue-resident, as well as circulating, memory T cells (10). These memory T cell populations are maintained in the host for many months postinfection and, in some instances, for the host’s lifetime (10).

Polyinosinic-polycytidylic acid (poly IC) is a synthetic mimic of dsRNA, a common subproduct of viral replication. Poly IC is recognized by both surface and cellular pattern-recognition receptors that include at least TLR3 and melanoma differentiation-associated protein 5 (11). Because of its ability to promote DC...
activation, poly IC has been used extensively as an adjuvant for inactivated, DC-targeted, DNA, and subunit vaccines (12). However, the putative use of poly IC to boost immune protection generated by LAIVs has not been investigated because, as a result of their ability to replicate in the host, LAIVs are believed to be intrinsically adjuvanted. In this study, we sought to determine whether poly IC, used as an adjuvant after mucosal administration of LAIV, could further potentiate rDC function and the generation of vaccine-specific CD8 T cells. We observed that poly IC enhanced the activation and migration of Ag-bearing TLR3+ CD103+ rDCs to the mLN.s, resulting in significant generation of influenza-specific CD8 T cells and neutralizing Abs. This, in turn, enhanced mouse survival after lethal viral challenge. Loss of TLR3 function in knockout mice abolished the adjuvant effect of poly IC, which was dependent on CD8 T cell immunity but not on neutralizing Abs. Finally, we demonstrate that poly IC–induced enhancement of CD8 T cell immunity requires amplification of TLR3 signaling by radioresistant nonhematopoietic cells. Our findings underscore the importance of CD8 T cell responses for LAIV-induced immune protection and provide the rationale for the use of TLR3 agonists to enhance influenza vaccine protection in a population lacking pre-existing Ab immunity.

**Materials and Methods**

**Mice, reagents, and viruses**

C57BL/6J and CD45.1+ coisogenic B6 mice were purchased from The Jackson Laboratory and bred at the Heinrich Pette Institute animal facility. TLR3–/– (Tlr3tm1Flv) and Cd207tm3Mal (TcraTcrb)1100Mjb/J, and Langerin–diphtheria toxin receptor (DTR) mice (B6.129S2-Cd207tm3Mal/J) were purchased from The Jackson Laboratory. All of the experiments described were performed with male mice between 8–10 wk of age, unless otherwise stated. Animal experiments were conducted according to the guidelines of the German animal protection law. All staff carrying out animal experiments passed training provided by the manufacturer. T cells were labeled with CFSE (eBioscience), as described elsewhere (14). A total of 2 × 10⁶ CFSE-labeled T cells was adoptively transferred into recipient mice via retro-orbital injection.

**Bone marrow chimeras**

Bone marrow chimera were generated as described previously (14). In brief, recipient mice were lethally irradiated (550 rad, 4 h apart by a cesium source) and reconstituted with 2 × 10⁶ bone marrow cells from coisogenic donor mice. Engraftment of donor cells was evaluated 4 wk after reconstitution in peripheral blood by flow cytometry, and the experiments were performed 6 wk after transplantation.

**Results**

**Poly IC enhances protection exerted by LAIVs**

Attenuation of influenza viruses by cold adaptation (i.e., restriction of viral replication to the upper respiratory tract without causing pulmonary pneumonia) is the basis of currently licensed LAIVs (15). To mimic vaccine formulations currently administered in humans, we used reverse genetics to engineer a reassortant cold-adapted influenza vaccine harboring the hemagglutinin (HA) and neuraminidase (NA) of PR8 on the background of the cold-adapted strain A/Ann Arbor/6/1960 (H2N2) (16). The recombinant H1N1 cold-adapted vaccine (CAV) grew in influenza-permissive cells at 33°C, but not at 37°C, confirming the temperature-restricted phenotype of the virus (Fig. 1A). Intranasal injection of C57BL/6J mice with up to 10⁶ PFU of CAV did not cause disease, in agreement with restriction of viral replication to the upper respiratory tract (Fig. 1B (17)). The efficacy of LAIVs administered via the mucosal route relies on the establishment of limited viral replication, which is necessary to trigger host immunity to the vaccine (15). We hypothesized that a timely intranasal administration of poly IC after the generation of sufficient Ag via viral replication could enhance vaccine-induced protection. To test this hypothesis, we vaccinated groups of mice with CAV alone, CAV plus poly IC administered simultaneously, and CAV plus poly IC administered 24 h after vaccination. A group of mock-vaccinated mice that received vehicle (PBS) was used as control. We challenged mice at 20 d postvaccination with a lethal dose of wild-type (WT) PR8 virus (100 × LD₅₀). Analysis of virus-induced morbidity and mortality indicated that poly IC administration 24 h after vaccination significantly improved vaccine protection. This group of mice showed reduced weight loss after lethal influenza challenge compared with mice vaccinated with CAV alone (Fig. 1C, 1D), as well as enhanced survival (Fig. 1E). Conversely, simultaneous administration of poly IC with CAV completely abrogated the

Quantitative RT-PCR

For quantitative RT-PCR analysis, RNA was isolated from the bronchoalveolar lavage fluid (BALF) using a QIAamp Viral RNA Mini Kit (QIAGEN), following the manufacturer’s instructions. Quantitative RT-PCR was performed using a 100-ng sample of RNA and SYBR Green (Roche) in an Applied Biosystems Prism 7900HT instrument, following the manufacturer’s instructions. The specific sequences of the primers used were AArbor NP forward: 5′-CCAGAAGTCACGTGGTTGCGA-3′ and AArbor NP reverse: 5′-GGCCAGTACCTGGTTCTCAG-3′. To calculate the relative index of gene expression, we used the 2⁻ΔΔCt method, as described elsewhere (12).
protective effect of the vaccine, and all of the mice challenged with WT virus died of the infection (Fig. 1C–E), in agreement with the ability of poly IC to induce an IFN-I–dependent antiviral state and prevent vaccine replication (18).

Poly IC improves rDC function and Ag transport to lymphoid tissues

We next hypothesized that the improved antiviral protection observed in the animals treated with poly IC postvaccination was due to greater ferrying of vaccine Ag from the lung to the mLNs, thereby enhancing the strength of vaccine-induced adaptive immunity. DCs are regarded as the main cell type capable of transporting peripheral Ags to lymphoid tissues for priming of naive T cells (6). Therefore, we next sought to evaluate rDC responses to intranasal vaccination. rDCs were detected in the lungs of vaccinated mice as CD11c+ MHC class IIhi Siglec-F^2 cells in the low side-scatter (Supplemental Fig. 1).

We (14) and other investigators (19) previously demonstrated that intranasal vaccination results in significantly increased numbers of CD11b^+ rDCs both in the lungs and the mLNs, likely as the result of infiltration and differentiation of activated blood-borne monocytes. However, only CD103^+ rDCs transported influenza Ags from the lung to the mLNs, as shown by intracellular staining of viral NP (data not shown), which was in agreement with previous reports (7, 8). To assess the effect of poly IC on antigenic transport by CD103^+ rDCs, we engineered a recombinant CAV expressing the OVA-derived immunodominant peptide SIINFEKL (CAV-SIINFEKL) inserted in the stalk of the viral NA. This vaccine efficiently induced the proliferation of CFSE-labeled naive TCR-transgenic OVA-specific CD8 T cells (OT-I) that had been adoptively transferred to mice shortly after mucosal vaccination, indicating effective immunity against the heterologous epitope (Fig. 2A). Thus, we quantified the arrival of SIINFEKL-bearing CD103^+ rDCs to the mLNs of mice that received CAV-SIINFEKL alone or in combination with poly IC or were treated with poly IC 24 h after vaccination. To this end, we used specific Abs to detect H-2b–SIINFEKL complexes on the surface of DCs by flow cytometry. Our results indicated that mucosal treatment with poly IC 24 h after vaccination with CAV-SIINFEKL significantly increased the arrival of peptide-bearing DCs to the mLNs compared with single vaccine administration; at day 2 postvaccination, 30% of total CD103^+ migratory DCs expressed SIINFEKL–H-2b complexes on their surface (Fig. 2B). Conversely, simultaneous administration of CAV and poly IC almost completely prevented the arrival of vaccine Ag to the mLNs, despite overt migration of CD103^+ rDCs (Fig. 2B, data not shown), suggesting that the poly IC–induced antiviral state abolished vaccine replication. To explore this possibility, we evaluated the RNA levels of vaccine-derived NP in the BALF of mice subjected to all of the vaccine regimens 48 h postvaccination. As shown in Fig. 2C, simultaneous CAV and poly IC significantly abrogated vaccine replication compared with CAV alone and CAV and poly

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**FIGURE 1.** Effect of mucosal administration of poly IC on CAV protection. (A) CAVs harboring HA and NA from the PR8 strain were rescued using reverse genetics protocols. Influenza-permissive MDCK cells were infected with CAV at a multiplicity of infection of 0.1 for 48 h. Cells were fixed and permeabilized, and influenza virus was detected by immunofluorescence using anti–NP-FITC Abs. The white circle indicates an area of viral replication. (B) Vaccine safety was assessed in C57BL6/J mice by intranasal administration of the indicated CAV doses, and mice were monitored daily for weight loss and clinical signs for up to 20 d. Results represent an average from at least three independent experiments. (C) Mice were vaccinated with 10^7 PFU of CAV in the presence of 20 μg of poly IC or poly IC administered 24 h after vaccination. Control mice received PBS instead of vaccine (mock vaccinated). At day 20 postvaccination, mice were challenged with 10^5 PFU of PR8 (100 × LD_50). Mouse weight was monitored daily, and mice were sacrificed when they reached the ethical end point of 25% loss of their initial weight as per animal approval guidelines. (D) Average body weight of mice in all vaccine groups after lethal PR8 challenge. Results represent animals pooled from three independent experiments. (E) Kaplan–Meier survival curve showing percentage of mice that survived to lethal influenza challenge in each conditions. Results represent animals pooled from three independent experiments. *p < 0.05, two-way ANOVA followed by Bonferroni posttest.
IC 24 h later. Of note, we observed significantly higher levels of vaccine RNA in the BALF of mice that received poly IC treatment 24 h postvaccination (Fig. 2C). Taken together, our findings indicate that, to elicit Ag-specific responses, CD103+ rDCs need to be activated and loaded with sufficient antigenic material in the periphery, which is dependent on vaccine replication.

Timely poly IC administration enhances vaccine-specific host immunity

We next assessed the effect of poly IC treatment on host immunity to LAIV. Because of the important role of CD103+ rDCs on CD8 T cell priming, we first evaluated whether poly IC treatment enhanced vaccine-generated CD8 T cell immunity. We vaccinated mice with CAV, CAV plus simultaneous poly IC, or CAV plus intranasal poly IC 24 h after vaccination. Twenty days later, mice were challenged with WT PR8, and we quantified influenza-specific T cells in the lungs before and after the T cell contraction phase (Fig. 1A). We observed that administration of poly IC 24 h postvaccination significantly increased the presence of CD8 T cells harboring TCRs specific for the H-2^d-restricted immunodominant peptide NP366–374, which were already detectable at day 3 postchallenge (Fig. 3B). Also, vaccination with CAV plus poly IC (24 h) resulted in improved maintenance of Ag-specific memory CD8 T cells after the contraction phase compared with CAV alone. The kinetics of NP-specific CD8 T cells in mice vaccinated with CAV plus simultaneous poly IC mimicked that of mock-vaccinated mice, which suggests a primary CD8 T cell response to viral challenge and, therefore, lack of pre-existing T cell memory (Fig. 3B, 3C). These data indicated that postvaccine administration of poly IC significantly increased the generation of Ag-specific CD8 T cells in the respiratory tract, presumably enhancing secondary responses to influenza virus at the natural portal of pathogen entry.

Based on the recently discovered link between the strength of the innate immune response and the magnitude of Ab production (20), we reasoned that poly IC treatment could also result in enhanced production of influenza-neutralizing Abs. To test this hypothesis, we collected serum samples from mice vaccinated with CAV alone, as well as with simultaneous or postvaccination administered poly IC, at day 20 after immunization. The presence of influenza-neutralizing Abs was determined in serum by focus reduction neutralization assay. Our results indicated that a single administration of CAV resulted in a modest neutralization titer (34.67 ± 13.26), which, as expected, was not increased by simultaneous administration of CAV and poly IC. However, postvaccine poly IC treatment resulted in 3-fold enhancement of influenza-neutralizing Abs in vaccinated mice (Fig. 3D). These results suggested that postvaccine poly IC treatment engaged both arms of the host immune response, likely as a result of improved induction of innate immunity and enhanced arrival of vaccine Ag to the lymphoid tissues.

A presumable advantage of stronger CD8 T cell immunity is improved vaccine cross-reactivity to heterologous virus. The rationale for this is that T cell epitopes are primarily derived from internal viral proteins that undergo little host Ab pressure and, thus, are highly conserved among distantly related influenza strains (21). To test whether poly IC treatment improved vaccine-induced heterosubtypic immunity, we vaccinated mice with CAV; 24 h later we administered intranasal poly IC or vehicle control (PBS). Twenty days after vaccination, we challenged mice with recombinant X-31 virus, a recombinant influenza vaccine candidate harboring six internal segments from PR8 and the HA and NA of pandemic A/Hong Kong/2/68 (H3N2) (22). Analysis of weight loss in vaccinated mice showed that poly IC treatment significantly reduced morbidity associated with heterosubtypic X-31 infection (Fig. 3E), which indicated that poly IC improved early protection against homosubtypic viral challenge, as well as vaccine-induced heterosubtypic immunity.

The adjuvant effect of poly IC is dependent on TLR3

We next sought to determine the molecular mechanisms responsible for the adjuvant effect of poly IC. To do so, we first determined the effect of TLR3 function on vaccine protection. WT mice, as well as TLR3−/− mice, were vaccinated with CAV and treated with poly IC or PBS 24 h postvaccination. Twenty days later, mice were challenged with PR8, and morbidity and mortality were assessed. As expected, poly IC increased protection in WT mice and significantly reduced influenza-associated weight loss. However, loss of TLR3 function completely abolished the adjuvant effect of poly IC (Fig. 4A). Interestingly, in the absence of poly IC treatment, TLR3−/− mice showed significantly reduced morbidity compared with vaccinated WT mice, suggesting that TLR3 signaling may participate in the induction of an antiviral state in vaccinated mice and, thus, may prevent vaccine replication to

**FIGURE 2.** Poly IC enhances migration of Ag-bearing DCs. (A) Mice vaccinated with the indicated regimens were infused with 2 × 10^6 CFSE-labeled OT-1 T cells 3 h after vaccination. Four days after vaccination, OT-1 T cell proliferation was determined in the mLNs by analysis of CFSE dilution in the CD3+ CD4+ CD8+ T cell gate. Vaccination with CAV was used as a negative control, whereas CAV plus 2 μg of endotoxin-free OVA was used as a positive control. (B) Mice vaccinated with CAV-SIINFEKL alone or with CAV-SIINFEKL plus poly IC treatment or mice treated with poly IC 24 h after vaccination were sacrificed at day 3 postvaccination. Ag-bearing migratory CD103+ rDCs were identified as CD103^+ H-2^d–SIINFEKL+ cells. Data represent the average of three biological replicates. (C) Levels of vaccine-derived NP RNA were assessed in the BALF of vaccinated mice 48 h after vaccine administration using primers specific for the A/Ann Arbor/6/60 (H2N2) virus. PCR conditions and analysis are indicated in Materials and Methods. *p < 0.05, Student t test.
FIGURE 3. Effect of poly IC on vaccine-induced adaptive immunity. (A) Kinetics of total CD8 T cells in the lungs of mice subjected to the indicated vaccine regimens after viral challenge with $10^6$ PFU of PR8. Graphs represent the total number of CD8 T cells at the indicated time points in the T cell gate. At least five mice/time point are shown. (B) Mice vaccinated intranasally with the indicated regimens were challenged with $10^6$ PFU of PR8. Mice were sacrificed at days 3, 6, 10, or 25 postinfection, and NP$_{366-374}$ Ag–specific CD8$^+$ T cells were determined in the lung by multicolor flow cytometry. FITC-conjugated dextramers harboring the immunodominant peptide NP$_{366-374}$ were used to detect vaccine-specific CD8 T cells. Graphs depict the percentage of dextramer$^+$ cells in the CD8 T cell gate (CD3$^+$ CD4$^-$ CD8$^+$). (C) Representative plots illustrating the percentage of Ag-specific CD8 T cells in the lungs at days 3 and 25 postchallenge. (D) Mice vaccinated with the indicated regimens were bled at day 20 postvaccination. Preimmune mice (naive) are shown for baseline levels. Serum samples were used in a focus reduction neutralization assay, as described in Materials and Methods. Neutralization titers are represented as the reciprocal of the last dilution at which infection was completely blocked. (E) Mice were vaccinated with $10^3$ (Figure legend continues).
some extent. Strikingly, loss of TLR3 function did not prevent the ability of poly IC to enhance neutralizing Ab production (Fig. 4B), suggesting that the adjuvant effect of poly IC relies on its capacity to enhance the formation of vaccine-specific CD8 T cells, which was in agreement with previous findings (12).

The adjuvant effect of poly IC requires amplification by radioresistant cells

To determine the cellular compartments responsible for the amplification of TLR3 signaling in vivo, we next engineered bone marrow chimeric mice in which loss of TLR3 function was targeted to specific cell types. First, we reconstituted irradiated coisogenic CD45.1 mice with a 3:1 mix of Langerin-DTR/WT bone marrow (Lang-WT mice) or a 3:1 mix of Langerin-DTR/TLR3−/− bone marrow (Lang-TLR3−/−). In this model, intranasal administration of DT depleted lung-resident DTR-expressing langerin+ TLR3+ CD103+ DCs, allowing us to study vaccine immunity in the presence of either WT or TLR3−/− CD103+ rDCs (Supplemental Fig. 2). We used the X-31 candidate vaccine strain to compare vaccine-induced T cell immunity using commercially available dextrans that harbor immunodominant peptides derived from the PR8 strain (22). Mice were vaccinated with X-31 and treated with poly IC or PBS (mock) 24 h later. Twenty days postinfection, we compared the generation of CD8 T cells specific for the NP366–374 epitope between DT-treated Lang-WT and Lang-TLR3−/− mice. Our results indicated that loss of TLR3 function in CD103+ rDCs did not prevent the enhancement of virus-specific CD8 T cell formation in both chimeras after poly IC treatment, strongly suggesting that the adjuvant effect of poly IC required signal amplification by radioresistant stromal cells (Fig. 5B). To test this possibility, we vaccinated WT and TLR3−/− mice with X-31 and again evaluated the generation of NP-specific CD8 T cells in the lung 20 d later. Loss of TLR3 function in both the hematopoietic and stromal compartments completely abolished the effect of poly IC in the generation of CD8 T cells (Fig. 5C), suggesting that amplification of TLR3 signaling by radioresistant stromal cells in the lung is necessary for the adjuvant effect of poly IC. This hypothesis was further confirmed when we engineered reverse chimeras in which loss of TLR3 signaling was restricted to the radioresistant compartment (TLR3−/−w). In these mice, poly IC treatment after vaccination did not result in greater generation of memory CD8 T cells in the lung, confirming the requirement of radioresistant cells for amplification of TLR3 signaling (Fig. 5D).

Discussion

With the notable exception of the polio and yellow fever vaccines, most, if not all, existing prophylactic vaccines exert their protective effect by enhancing Ab responses, including inactivated influenza vaccines (23). However, to protect the human population against pandemic, as well as antigenically shifted, influenza strains, research efforts are needed to develop vaccines that elicit cross-reactive CD8 T cell memory (24), in addition to humoral responses. A number of previous studies demonstrated that poly IC as an adjuvant for inactivated and subunit-based influenza vaccines improves Ab responses, as well as vaccine-specific T cell immunity (12, 25, 26). Unfortunately, even in the presence of adjuvants, nonreplicating influenza vaccines generate very little T cell response (15, 27). Alternatively, simultaneous administration of experimental LAIVs with α-galactosylceramide or chitosan resulted in enhanced protection against influenza challenge (28, 29), but the involvement of memory T cell responses in vaccine protection was not assessed in those studies. In this study,
we sought to determine whether mucosal administration of poly IC could be used to boost T cell immunity elicited by LAIVs via local activation of respiratory DCs, as well as the generation of broadly conserved T cell epitopes by viral replication (2). We observed that poly IC administered shortly after vaccination enhanced the activation and migratory ability of CD103+ rDCs, the main cross-presenting DC population in the periphery (8). Enhanced rDC function resulted in greater transport of vaccine Ag to the lymphoid tissue and increased generation of vaccine-specific CD8 T cells. Our findings are consistent with previous studies demonstrating that optimization of the amount of Ag that tissue DCs carry to the lymph nodes is essential for proper CD8 T cell responses because peptides that are bound to MHC class I dissociate quickly (30); this is especially limiting in vaccination
condition because of the low overall production of vaccine-derived Ag (31). Of note, poly IC treatment enhanced CD8 T cell formation, as well as increased the production of influenza-neutralizing Abs. The combination of cross-reactive CD8 T cells and neutralizing Abs was previously proposed by other investigators as a surrogate of adjuvant strength based upon the idea that it reflects enhanced clonal expansion of naïve T cell pools (32). Remarkably, despite the fact that coadministration of poly IC and LAIV triggered DC migration, the low amounts of influenza Ag on migratory DCs prevented sufficient T cell priming. Simultaneous poly IC and LAIV treatment prevented LAIV replication, likely as a result of poly IC’s ability to induce an IFN-I–dependent antiviral state (18). Surprisingly, poly IC administered 24 h postvaccination significantly enhanced vaccine replication. We hypothesized that this may be due to inflammation-dependent protection of viral replication (33, 34), a common phenomenon in the upper respiratory tract that is especially relevant in the context of influenza and bacterial confections (35). Research is underway in our laboratory to elucidate the molecular mechanisms responsible for this effect of poly IC. Therefore, it is possible that the enhanced transport of vaccine Ag from the lungs to the mLNs observed in the LAIV and poly IC (24-h) group may be due to enhanced DC function, as well as to greater generation of vaccine Ag. In any case, our findings highlight the requirement for sufficient vaccine replication in the upper respiratory tract for the generation of influenza T cell epitopes derived from the viral core proteins (36, 37). In this regard, it is tempting to speculate that the strong induction of type I IFNs observed in humans vaccinated with LAIV in comparison, for example, with individuals vaccinated with YF17D may prevent sufficient accumulation of influenza Ag by migratory DCs and, thus, may preclude optimal T cell immunity (27).

Previous studies underscored the effect of poly IC on maintenance of memory T cells, and this effect has been attributed to type I IFN–dependent upregulation of IL-15, TLR3 ligation, and up-regulation of proinflammatory cytokines, such as RANTES and IP-10, at early points after poly IC administration (38, 39). Our results indicated that TLR3 function was required for the adjuvant effect of poly IC but was dispensable for neutralizing Ab production. These results strongly suggest that the increase in vaccine protection elicited by poly IC is dependent on its capacity to generate higher levels of CD8 T cells and not of neutralizing Abs. These findings are in agreement with previous reports indicating that CD8 T cells alone can generate protective immunity to secondary influenza challenge in the absence of Abs (40).

Finally, using bone marrow chimeras in which loss of TLR3 function was targeted to specific cell compartments, we identified radioreistant nonhematopoietic cells as the primary cell type responsible for amplification of TLR3 signaling. These results presumably reflect the requirement for TLR3 signal amplification for efficient T cell priming (26, 41), as well as for maintenance of Ag-specific memory T cells (42). These observations are also consistent with the requirement of IFN-I signaling for the generation of CD8 T cells (38, 43). It is also very likely that the observed effects of poly IC in the induction of DC activation and CD8 T cell immunity require signal amplification by additional cellular sensors, such as melanoma differentiation-associated protein 5 and RIG-I (44, 45); this is the topic of current investigations in our laboratory.

Our study highlights that a timely mucosal poly IC treatment increases CA V-induced CD8 T cell immunity, which plays a major protective role after influenza infection. To induce efficient CD8 T cell immunity, CA V must replicate in the upper respiratory tract, produce sufficient antigenic material, and allow rDCs to load these Ags and transport them to the lymphoid tissue for T cell priming. The future design of novel adjuvants targeted to coordinate vaccine replication with DC migration could improve T cell memory and protection elicited by mucosal influenza vaccines.

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Disclosures
The authors have no financial conflicts of interest.

References


**Suppl. Figure 1. Gating strategy respiratory DCs.** DCs in the lungs were gated as CD11c^+ MHC class II^hi^SiglecF^- on the low side scatter as shown above. Cells were acquired in a FACS Canto II instrument.
Suppl. Figure 2. Efficiency of the depletion of Langerin$^+$ cells in the lung. Fifty ng of DT was administered intranasally in 50 ml of PBS. Percentage of depletion is shown in CD11c$^+$ MHC class II$^{hi}$CD103$^+$ cells in the lung at day 3 post treatment.