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Influenza A Infection Enhances Cross-Priming of CD8+ T Cells to Cell-Associated Antigens in a TLR7- and Type I IFN-Dependent Fashion

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The initiation of antitumor immunity relies on dendritic cells (DCs) to cross-present cell-associated tumor Ag to CD8+ T cells (TCD8+) due to a lack of costimulatory molecules on tumor cells. Innate danger signals have been demonstrated to enhance cross-priming of TCD8+ to soluble as well as virally encoded Ags; however, their effect on enhancing TCD8+ cross-priming to cell genome-encoded Ags remains unknown. Furthermore, influenza A virus (IAV) has not been shown to enhance antitumor immunity. Using influenza-infected allogeneic cell lines, we show in this study that TCD8+ responses to cell-associated Ags can be dramatically enhanced due to enhanced TCD8+ expansion. This enhanced cross-priming in part involves TLR7- but not TLR3-mediated sensing of IAV and is entirely dependent on MyD88 and IFN signaling pathways. We also showed that the inflammasome-induced IL-1 and IFN-γ did not play a role in enhancing cross-priming in our system. We further demonstrated in our ex vivo system that CD8+ DCs are the only APCs able to prime TCR-transgenic TCD8+. Importantly, plasmacytoid DCs and CD8–DCs were both able to enhance such priming when provided in coculture. These observations suggest that IAV infection of tumor cells may facilitate improved cross-presentation of tumor Ags and may be used to augment clinical vaccine efficacy. The Journal of Immunology, 2010, 185: 6013–6022.

The activation of naive CD8+ T cells (TCD8+) requires at least two signals: the recognition of peptide–MHC class I complex by TCR, and the engagement of costimulatory molecules on T cells and mature dendritic cells (DCs) (1, 2). Due to the lack of such costimulatory molecules on the majority of somatic cells and tumor cells (3), antitumor immunity relies on cross-priming. This process requires professional APCs, such as DCs, to uptake exogenous tumor Ags and further process and present the contained antigenic epitopes onto their own MHC class I molecules, a process called cross-presentation, to activate naive TCD8+ (4, 5). The cross-presentation of cell-associated Ag by DCs is also important in immunity against viruses that do not directly infect DCs (6). Although the cross-presentation pathways are yet to be fully elucidated (7), there is a growing interest in identifying signals that may promote cross-priming to advance vaccine design.

Recently, studies have shown that the cross-priming of TCD8+ can be enhanced by signaling through TLRs expressed by DCs. TLRs recognize pathogen-associated molecular patterns, also called danger signals (8), such as pathogen-derived proteins, DNA, and RNA molecules (9, 10). The majority of the TLRs, such as TLR7 and TLR9, activate through the MyD88-dependent signaling pathway (11, 12). However, TLR3 signals through the TRIF-dependent signaling pathway (12). TLR signaling leads to cytokines that are crucial in both innate and adaptive immunity (10). How these cytokines coordinate immune response, especially subsequent activation and expansion of naive TCD8+, is yet to be fully understood (13). Although most TLRs are surface molecules, TLR3, -7, and -9 are found in the endosomal compartments (14), where they sense intracellular viral RNA (TLR3 and TLR7) and DNA (TLR9) (15–17).

Unlike viruses, most tumors do not stimulate robust TCD8+ responses due to the lack of innate danger signals (18). Recent studies have shown by conjugating polyinosinic:polycytidylic acid [poly(I:C)] (a TLR3 ligand) (19, 20), LPS (a TLR4 ligand) (19, 21), or CpG (a TLR9 ligand) (19, 20) with a soluble model Ag, chicken OVA, that cross-priming of naive OVA257–264-specific TCD8+ can be significantly enhanced. Unlike TLR3, -4, and -9, TLR7 (and TLR8 in humans) ligands, resiquimod or imiquimod, have been shown to only weakly enhance such priming of TCD8+ (20, 22). Similarly, Le Bon et al. (17) have shown that lymphocytic choriomeningitis virus infection, but not vaccinia virus infection, can enhance the cross-priming of OVA257–264-specific TCD8+ to soluble OVA. Such enhanced cross-priming was tightly associated with induction of IFN-α. As all of the above mentioned...
studies dealt with cross-presentation of soluble OVA, it remains unclear whether TLR stimulation will have a similar effect on T<sub>CD8</sub> immunity to cell-associated Ags.

Schulz et al. (15) showed that immunizing mice with Vero cells infected with suicidal Semelki Forest virus (SFV) encoding full-length OVA also enhanced cross-priming of OVA<sub>257–264</sub>-specific T<sub>CD8</sub>, in a TLR3-dependent manner. SFV is a negatively sensed single-stranded RNA (ssRNA) virus. Interestingly, the ssRNA-sensing TLR7 was not addressed in this system. Although technically a cell-associated Ag model, the virally encoded Ag could still be different from Ags encoded by the cellular genome. For example, viral factories, a subcellular-localized compartment where viruses conduct their protein synthesis and subsequent virion assembly, have been reported for both DNA and RNA viruses (23, 24).

Similar to SFV, influenza A virus (IAV) is an ssRNA virus. However, how IAV is sensed by the immune system remains unclear. Studies have shown the involvement of TLR3 (25) and TLR7 (16), as well as another dsRNA binding molecule RIG-I (26). Recently, studies have shown that IAV can interact with the inflammasome (27–29). To our knowledge, only TLR3 (15) has been shown to enhance cross-priming of cell-associated Ag in viral systems. Schulz et al. (15) proposed that IAV might not enhance cross-priming in the same manner as SFV due to the unique IAV nonstructural protein 1 (NS1). NS1 has been demonstrated to sequester ssRNA and prevent recognition by the immune system (30). However, Lund et al. (31) showed that IAV did stimulate plasmacytoid DCs (pDCs) through TLR7 to produce IFN-α, albeit significantly less than that stimulated by another ssRNA virus, vesicular stomatitis virus. We were therefore curious to know whether IAV has the potential to promote cross-priming of non-virally encoded, cell-associated Ag. To date, little is known of whether IAV has the potential to promote cross-priming of non-virally encoded, cell-associated Ag. For example, viral factories, a subcellular-localized compartment where viruses conduct their protein synthesis and subsequent virion assembly, have been reported for both DNA and RNA viruses (23, 24).

In this study, we used simian virus 40 (SV40)-transformed cells (D2.SV, H-2<sup>b</sup>) (32) and OVA-transfected cells (Dap.OVA, H-2<sup>k</sup>) (33) to mimic tumor cell-associated Ag and to investigate the specific T<sub>CD8</sub> responses in C57BL/6 (B6) mice. D2.SV cells carry the SV40-derived large T Ag (T-Ag), whereas Dap.OVA cells carry the model Ag OVA, expressed as a membrane protein. D2.SV and Dap.OVA cells both lack the correct MHC class I molecules (H-2<sup>b</sup>) that are able to present H-2<sup>K</sup>-restricted T-Ag<sub>404–411</sub> and OVA<sub>257–264</sub> epitopes; thus, T<sub>CD8</sub> can only be activated by cross-presenting DCs from the host. We show in this study that IAV infection of D2.SV and Dap.OVA cells dramatically enhances cross-priming of T-Ag- and OVA-specific T<sub>CD8</sub>, in a TLR7-mediated, MyD88-, and IFN-α-dependent manner.

Materials and Methods
Mice
MyD88<sup>-/-</sup> and TLR7<sup>-/-</sup> mice were kindly provided by Professor Shizuo Akira (Osaka University, Osaka, Japan). TLR7<sup>-/-</sup> mice were obtained from Regeneron (Rensselaer, NY) and were originally described by Lund et al. (31). IL-1R<sup>-/-</sup> mice were originally acquired from Dr. M. Labow (Hoffmann-La Roche, Nutley, NJ) (34). IFN-αR<sup>1</sup> (35) and IFN-αR<sup>-/-</sup> (35) were bred, maintained, and supplied by the Peter MacCallum Cancer Centre (East Melbourne, Victoria, Australia). B6.129.S1-Ptgcs1a<sup>-/-</sup> mice were prepared from the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). All other mice were bred in house. Mice were housed in specific pathogen-free isolators. Experiments were performed with animals aged at 6–12 wk and were conducted under the auspices of the Austin Health Animal Ethics Committee and conformed to the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes.

Generation of TLR3<sup>−/−</sup> mice and PCR
Female TLR7<sup>−/−</sup>/ mice were crossed with male TLR3<sup>−/−</sup> mice. The male offspring (TLR7<sup>/−</sup>/TLR3<sup>/−</sup>) were backcrossed to female TLR7<sup>/−</sup>/ and the TLR7<sup>−/−</sup>/TLR3<sup>−/−</sup> genotype identified by PCR. Male TLR7<sup>−/−</sup>/ mice were then mated with female TLR7<sup>/−</sup>/, TLR7<sup>−/−</sup>/ and TLR7<sup>+/−</sup>/ mice, and the TLR7<sup>/−</sup>/TLR3<sup>−/−</sup> offspring were screened by PCR. Primers used for TLR3 screening were: TLR3A (5′-CCACGACCTGGTAAATTTGTTG-GCTG-3′), TLR3B (5′-TCCAGCAATTTGGCAAGTTATCGC3′), and TLR3C (5′-ATGCCCTTTCTACGTCTGACGAG-3′). A standard PCR was performed for 35 cycles. The annealing temperatures were 67˚C and 58˚C for TLR3 and TLR7 primers, respectively.

**Peptide and Abs**
IAV nucleoprotein (NP<sub>366–374</sub> ASNENMETM, restricted to H-2<sup>D</sup>, poly- merase 2 protein (PA<sub>224–233</sub> SSSLNFRAYV, H-2<sup>d</sup>), T-Ag<sub>404–411</sub> (SLENFRTGAYV, H-2<sup>d</sup>), and OVA<sub>257–264</sub> (SIINFEKL, H-2<sup>k</sup>) were procured and characterized by the Biologic Resource Branch, National Institute of Allergy and Infectious Diseases (gifts from Drs. J. Yewdell and J. Bennink, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). FITC-labeled anti–IFN-γ and CyChrome-labeled anti-CD8α were purchased from Becton Dickinson (North Ryde, Australia). For flow cytometry, Abs were used at 1/400 dilution in PBS supplemented with 1% FCS.

**Viruses**
Influenza A virus PR8 (Puerto Rico/8/34, H1N1) was grown in 10-d embryonic chicken eggs and used as infectious allantoic fluid.

**Cell line culture**
All cells were cultured in 5% CO<sub>2</sub>, 37˚C incubator. D2.SV and C57 SV cells were cultured in RPMI 1640 containing 10% FCS, 50 μM 2-ME, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (RF-10). The Dap.OVA cell line was a kind gift from Prof. Kenneth Rock (University of Massachusetts Medical School, Worcester, MA) (33) and was cultured in RF-10 supplemented with 1 mg/ml zeocin (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Cells were passaged and media were changed when cells became 80% confluent; the cells were cultured during the exponential growth phase. For the generation of D2.SV/PR8 and Dap.OVA/PR8, cells, D2.SV or Dap.OVA was incubated with 50 multiplicity of infection of PR8 in FCS-free, acidified RPMI 1640 for 1 h at 37˚C. Subsequently, 10 ml RF-10 was added and further incubated for 4 h. The infected cells were then washed three times with RF-10 to remove residual PR8. For the generation of D2.SV/ poly(I:C), D2.SV cells were transfected with poly(I:C) (2 mg/ml) using Lipofectamine 2000 (Invitrogen, Mulgrave, Victoria, Australia) for 6 h as per factory protocol. The cells were then washed three times with PBS. D2.SV/ PR8, Dap.OVA/PR8, and D2.SV/poly(I:C) were then irradiated at 10,000 rad immediately before use.

**Immunization**
Mice were immunized with either irradiated (100 Gy) D2.SV, Dap.OVA, D2.SV/PR8, Dap.OVA/PR8, or D2.SV/poly(I:C) at 5 × 10<sup>6</sup> cells/mouse (i.p.).

**In vitro activation of ex vivo Ag-specific T<sub>CD8</sub> and intracellular cytokine staining**
Peritoneal exudates and spleens were collected in RF-10. The Ag-specific T<sub>CD8</sub> were enumerated using intracellular cytokine staining (ICS) after being stimulated with 1 μM antigenic peptide (NP<sub>366–374</sub>, PA<sub>224–233</sub>, T-Ag<sub>404–411</sub>, and OVA<sub>257–264</sub>) in the presence of brefeldin A (36). For the generation of D2.SV/PR8 and Dap.OVA/PR8 cells, D2.SV or Dap.OVA was incubated with 50 multiplicity of infection of PR8 in FCS-free, acidified RPMI 1640 for 1 h at 37˚C. Subsequently, 10 ml RF-10 was added and further incubated for 4 h. The infected cells were then washed three times with RF-10 to remove residual PR8. For the generation of D2.SV/ poly(I:C), D2.SV cells were transfected with poly(I:C) (2 mg/ml) using Lipofectamine 2000 (Invitrogen, Mulgrave, Victoria, Australia) for 6 h as per factory protocol. The cells were then washed three times with PBS. D2.SV/ PR8, Dap.OVA/PR8, and D2.SV/poly(I:C) were then irradiated at 10,000 rad immediately before use.

**Preparation of CFSE-labeled OT-1 T<sub>CD8</sub>**
OT-1 T<sub>CD8</sub>, were prepared from lymph nodes (cervical and mesenteric) and/or spleens of OT-1 transgenic mice. Single-cell suspensions were incubated for 30 min with predetermined optimal concentrations of the following purified mAbs: anti-Mac-1 (M1/70), anti-F4/80 (F4/80), anti-erythrocyte (TER-119), anti-GR-1 (RB68CS), anti-I-A<sub>I</sub>-E (MS114), and...
anti-CD4 (GKL.5). The Ab-coated cells were then removed by incubation with sheep anti-rat IgG coupled Dynal beads (Invitrogen). Purity was normally >80%. For CFSE labeling, OT-I T<sub>CD8</sub><sup>+</sup> were resuspended in PBS containing 0.1% BSA (Sigma-Aldrich) and labeled with 2.5 μM CFSE (Invitrogen) for 10 min at 37°C. Cells were then washed twice in PBS.

**OT-I T<sub>CD8</sub>, proliferation assay**

A total of 5 x 10<sup>4</sup> T<sub>CD8</sub><sup>+</sup> from OT-1-Ly5.1 were adoptively transferred into naive B6.Ly5.2 mice (i.v.). Three days later, the mice were either left unimmunized or immunized with 5 x 10<sup>5</sup> Dap.OVA or Dap.OVA/PR8 (i.p.). Seven days postimmunization (dpi), immune organs were harvested, and V<sub>α</sub>2<sup>+</sup>, CD45.1<sup>+</sup> OT-I T<sub>CD8</sub><sup>+</sup> were collected by FACScount II (Becton Dickinson) and analyzed using FlowJo software (Tree Star, Ashland, OR).

**Cytokine bead array and ELISA**

Cytokine levels in the sera collected from immunized mice were determined for IL-6, IL-10, IL-12p70, MCP-1, IFN-γ, and TNF-α using a proinflammatory cytokine bead array kit (Becton Dickinson, Mountain View, CA) and 0.1% grade II bovine pancreatic DNase I (Boehringer Mannheim, Indianapolis, IN) for 20 min. To disrupt T cell–DC complexes, 10<sup>5</sup> of whole mouse spleen cells were incubated with 20 μg/ml anti-CD11c–PE–Cy7 and 5 μg/ml anti–pDC Ag-1–FITC for 20 min at 4°C. After incubation, cells were washed twice and analyzed on a MoFlo (DakoCytomation, Carpinteria, CA) into CD8<sup>+</sup> DCs (CD8<sup>+</sup>; CD11c<sup>+</sup> and PDCA-1<sup>+</sup>) and CD8<sup>+</sup> T<sub>CD8</sub><sup>+</sup>. The single-cell suspensions were then depleted of the following Ab–positive cells using sheep anti-rat IgG-coupled Dynal beads–CD3e (KT3; Invitrogen), anti-Thy-1 (T24/31.7), anti-CD19 (ID3), anti-Ly56 (IAb), and anti-erythrocyte (TER-119). For purification of splenic DCs into their different phenotypic subsets, DCs were labeled with anti–CD11c–PE (N418), anti-CD8–PE-Cy7 (53-6.7), and anti–pDC Ag-1 (PDCA-1)–FITC and sorted on a MoFlo (DakoCytomation, Carpinteria, CA) into CD8<sup>+</sup> DCs (CD8<sup>+</sup>; CD11c<sup>+</sup> and PDCA-1<sup>+</sup>), CD8<sup>+</sup> DCs (CD8<sup>+</sup>; CD11c<sup>+</sup> and PDCA-1<sup>+</sup>), and pDCs (CD8<sup>+</sup>; CD11c<sup>+</sup> and PDCA-1<sup>+</sup>).

**In vitro cross-priming assay**

Serial dilutions starting at 5 x 10<sup>4</sup> purified DC population cocultured in a U-bottomed 96-well plates (Costar, Cambridge, MA) with 5 x 10<sup>5</sup> Dap.OVA or Dap.OVA/PR8 cells and 5 x 10<sup>5</sup> CFSE-labeled OT-I T<sub>CD8</sub><sup>+</sup> for 20 min. To disrupt T cell–DC complexes, 0.1 M EDTA was added to the suspension and digested for a further 5 min. The single-cell suspensions were then depleted of the following Ab–positive cells using sheep anti-rat IgG-coupled Dynal beads–CD3e (KT3; Invitrogen), anti-Thy-1 (T24/31.7), anti-CD19 (ID3), anti-Ly56 (IAb), and anti-erythrocyte (TER-119). For purification of splenic DCs into their different phenotypic subsets, DCs were labeled with anti–CD11c–PE (N418), anti-CD8–PE-Cy7 (53-6.7), and anti–pDC Ag-1 (PDCA-1)–FITC and sorted on a MoFlo (DakoCytomation, Carpinteria, CA) into CD8<sup>+</sup> DCs (CD8<sup>+</sup>; CD11c<sup>+</sup> and PDCA-1<sup>+</sup>), CD8<sup>+</sup> DCs (CD8<sup>+</sup>; CD11c<sup>+</sup> and PDCA-1<sup>+</sup>), and pDCs (CD8<sup>+</sup>; CD11c<sup>+</sup> and PDCA-1<sup>+</sup>).

**Results**

**IAV injection enhanced cross-priming of T<sub>CD8</sub> response to cell-associated Ags**

D2.SV cells originate from SV40-transformed fibroblasts obtained from B10.D2 mice, and they continue to express T-Ag, a transforming, oncoprogenic protein. Four epitopes derived from the T-Ag have been mapped in B6 mice. T-Ag<sub>404-411</sub> is the most dominant, followed by T-Ag<sub>206-215</sub> (32, 38). We administered irradiated D2. SV cells i.p. into B6 mice. Seven dpi, peritoneal exudates and splenocytes were harvested, and Ag-specific T<sub>CD8</sub><sup>+</sup> responses were enumerated by standard ICS for IFN-γ. Approximately 1% of T-Ag<sub>206-215</sub>-specific T<sub>CD8</sub><sup>+</sup> were detected in the peritoneal cavity and spleen, whereas 5% and 1-2% of T-Ag<sub>404-411</sub>-specific T<sub>CD8</sub><sup>+</sup> were detected in the peritoneal cavity and spleen, respectively (Fig. 1A, 1B, open bars). We then explored the possibility of using IAV injection to increase cross-priming of the T-Ag-specific T<sub>CD8</sub><sup>+</sup>. D2.SV cells were first infected in vitro for 5 h by IAV strain PR8 (D2.SV/PR8) and then injected i.p. into naive B6 mice. Expectedly, D2.SV/PR8-immunized mice were able to elicit immunodominant, H-2D<sup>+</sup>-restricted NP<sub>366-374</sub>-specific and PA<sub>224-233</sub>-specific T<sub>CD8</sub><sup>+</sup> (39), generating ~5% and ~10% of IFN-γ<sup>+</sup> T<sub>CD8</sub><sup>+</sup> in the spleen (Fig. 1A) and peritoneal cavity (Fig. 1B), respectively. Interestingly, the number of cross-primed T-Ag<sub>404-411</sub>-specific T<sub>CD8</sub><sup>+</sup> was enhanced by 3- to 4-fold when mice were immunized with D2.SV/PR8, increasing the T-Ag<sub>404-411</sub>-specific T<sub>CD8</sub><sup>+</sup> response from 3-5 to 15-20% in the peritoneum and from 1-5% in the spleen (Fig. 1A, 1B). This enhancement was confirmed with the responses directed toward the SV40-transformed, syngeneic fibroblast cell line C57.SV, which naturally presents the H-2<sup>b</sup> epitopes from T-Ag (Fig. 1A, 1B). Approximately 3% of IFN-γ-producing, D2.SV allospecific T<sub>CD8</sub><sup>+</sup> were also detected. Notably, the magnitude of the allospecific response remained comparable between D2.SV and D2.SV/PR8 groups, indicating that PR8 infection specifically enhances cross-priming of tumor Ag-specific T<sub>CD8</sub><sup>+</sup>, but not allospecific T<sub>CD8</sub><sup>+</> (Fig. 1A, 1B).

To demonstrate that such enhanced cross-priming is not cell line- or MHC-dependent, we tested other SV40-transformed cell lines including SS.SV cells [H-2<sup>b</sup>, derived from B10.S mice (32)], and COS-7 cells, a xenogeneic kidney fibroblast cell line derived from the African green monkey (40). Both cell lines stimulated 3- to 4-fold more T-Ag<sub>404-411</sub>-specific T<sub>CD8</sub><sup>+</sup> when infected with PR8 compared with their uninfected counterparts (data not shown).

We also confirmed that this phenomenon was not a T-Ag–specific event by using the OVA system. Dap.OVA cells stably express transmembrane OVA (33). When B6 mice were immunized with Dap.OVA alone, the immunodominant OVA<sub>257-264</sub>-specific T<sub>CD8</sub><sup>+</sup> were barely detectable. However, immunizing B6 mice with IAV-infected Dap.OVA (Dap.OVA/PR8) significantly increased the response to OVA<sub>257-264</sub> by >10-fold in the spleen (Fig. 1C) and >4-fold in the peritoneal cavity (Fig. 1D).
To further understand the extent of enhanced T cell priming, we used TCR-transgenic T cells. Purified naive B6.Ly5.1 OT-I T\(_{\text{CD8+}}\) (5 × 10\(^3\)) were adoptively transferred into the B6 (Ly5.2) mice, and the mice were then immunized with either Dap.OVA or Dap.OVA/PR8. Seven days later, the numbers of CD45.1\(^+\) and V\(\alpha_2^+\) OT-I T\(_{\text{CD8+}}\) in the spleen, peritoneal cavity, and mediastinal lymph nodes of these mice were enumerated and compared with those recovered from mice receiving the same number of OT-I T\(_{\text{CD8+}}\) transferred without immunization. As shown in Fig. 2, expansion of OT-I T\(_{\text{CD8+}}\) was detected in the spleen of mice immunized with Dap.OVA. After Dap.OVA/PR8 immunization, OT-I T\(_{\text{CD8+}}\) expanded even further in the spleen (>7-fold OT-I T\(_{\text{CD8+}}\) expansion compared with the numbers from the Dap.OVA-immunized mice) and were readily detected in the peritoneal cavity and the mediastinal lymph nodes (Fig. 2).

Enhanced T\(_{\text{CD8+}}\) response followed similar expansion kinetics to that of IAV-specific T\(_{\text{CD8+}}\)

The dramatic increase in the number of T-Ag–specific and OVA-specific T\(_{\text{CD8+}}\), led us to consider the possibility that enhanced cross-priming may have resulted from prolonged T\(_{\text{CD8+}}\) proliferation. We therefore performed kinetics analyses in the spleen for IAV-specific and T-Ag–specific T\(_{\text{CD8+}}\). NP\(_{366–374}\) and PA\(_{224–233}\)–specific T\(_{\text{CD8+}}\) responses were quite small at 5 dpi, and they then peaked at 6 to 7 dpi and remained easily detectable until 12 dpi (Fig. 3A, 3B). This result is similar to the kinetics reported for IAV infection (41). The T-Ag\(_{206–215}\) and T-Ag\(_{404–411}\)–specific T\(_{\text{CD8+}}\) responses, from both D2.SV- and D2.SV/PR8-immunized groups, were easily detected at 5 dpi and peaked around the same time as the NP\(_{366–374}\) and PA\(_{224–233}\)–specific T\(_{\text{CD8+}}\) responses (Fig. 3C, 3D). Therefore, it is clear that the enhanced cross-priming that led to increased absolute Ag-specific T\(_{\text{CD8+}}\) numbers (Fig. 2) was not a result of prolonged proliferation that would be expected to delay the peak time of the response. Importantly, the enhanced cross-priming of T-Ag\(_{404–411}\)–specific T\(_{\text{CD8+}}\), resulted in proportionally increased memory precursors in the mice immunized with D2.SV/PR8 when compared with their D2.SV-immunized counterparts (Fig. 3E).

Enhanced T cell priming was not the result of increased T-Ag expression after PR8 infection

IAV uses host cellular machineries for the production of viral proteins. An increase in the quantity of Ag could lead to increased Ag uptake by cross-presenting DCs and therefore an enhanced Ag-specific T\(_{\text{CD8+}}\) response. To address this concern, protein levels of T-Ag and IAV NP were determined by Western blot of the D2.SV cells at 1, 3, 5, and 7 h post-PR8 infection (hpi) of the D2.SV cells. To increase the accuracy of the assessment, the supernatants were also serially diluted. Unsurprisingly, NP was not detected in the uninected cells, whereas T-Ag was readily detected. NP was only detected at 3 hpi, and the expression level increased as the infection proceeded, indicative of viral replication (Fig. 4). Contrary to our initial concern, the expression of T-Ag decreased significantly during the course of IAV infection, although it remained detectable at 7 hpi (Fig. 4). This is consistent with published studies showing reduced host mRNA levels after IAV infection (42), likely due to IAV’s ability to snatch 5’ caps from host mRNA onto viral mRNA, stabilizing viral mRNA and destabilizing host mRNA to thus bias viral protein production (43). From this piece of data, we ruled out the possibility of an increased T-Ag level in the infected cells and its potential contribution to the observed enhanced priming of T-Ag–specific T\(_{\text{CD8+}}\).

IAV-enhanced cross-priming was TLR7 but not TLR3 dependent

Because T-Ag expression was not elevated post PR8 infection, we examined the role of several described flu-sensing TLRs for their ability to facilitate enhanced cross-priming. TLR3 was reported to

FIGURE 2. PR8-infected Dap.OVA cells stimulated more OT-I T\(_{\text{CD8+}}\) expansion. Naive B6.Ly5.1 OT-I T\(_{\text{CD8+}}\) were purified as described in the Materials and Methods and i.v transferred into B6 mice at 5 × 10\(^3\) per mouse (i.v.). Three days later, recipient B6 mice were either left unimmunized (open bars) or immunized with either 5 × 10\(^5\) Dap.OVA (gray bars) or 5 × 10\(^5\) Dap.OVA/PR8 (black bars). Seven dpi, OT-I T\(_{\text{CD8+}}\) in various sites were enumerated, and the data were expressed as OT-I T\(_{\text{CD8+}}\) number per organ. This graph is plotted as a mean ± SEM of 10 mice (except for the unimmunized group, n = 1).

FIGURE 3. T\(_{\text{CD8+}}\) responded to D2.SV and D2.SV/PR8 with similar kinetics. B6 mice were immunized with either 5 × 10\(^5\) D2.SV or 5 × 10\(^6\) D2.SV/PR8. Splenocytes were harvested daily from immunized mice between 5 and 9 dpi, then again at 12 dpi. In A–D, NP\(_{366–374}\) and PA\(_{224–233}\), T-Ag\(_{206–215}\), and T-Ag\(_{404–411}\)–specific T\(_{\text{CD8+}}\) were enumerated by ICS as described in Fig. 1. In E, memory NP\(_{366–374}\) and T-Ag\(_{404–411}\)–specific T\(_{\text{CD8+}}\) in D2.SV- (open bars) and D2.SV/PR8-immunized (filled bars) mice were enumerated 30 dpi by ICS. These graphs are plotted as mean ± SEM of five animals.

FIGURE 4. PR8 infection decreased host cell protein synthesis. D2.SV cells were infected with PR8 in vitro and for various time intervals. The cells were washed and lysed by 1% Triton-X. Lysate supernatants were also serially diluted. Unsurprisingly, NP was not detected in the uninfected cells, whereas T-Ag was readily detected. NP was only detected at 3 hpi, and the expression level increased as the infection proceeded, indicative of viral replication (Fig. 4). Contrary to our initial concern, the expression of T-Ag decreased significantly during the course of IAV infection, although it remained detectable at 7 hpi (Fig. 4). This is consistent with published studies showing reduced host mRNA levels after IAV infection (42), likely due to IAV’s ability to snatch 5’ caps from host mRNA onto viral mRNA, stabilizing viral mRNA and destabilizing host mRNA to thus bias viral protein production (43). From this piece of data, we ruled out the possibility of an increased T-Ag level in the infected cells and its potential contribution to the observed enhanced priming of T-Ag–specific T\(_{\text{CD8+}}\).
enhance cross-priming in the SFV system, which is similar to IAV (15). As an intracellular dsRNA receptor, TLR3 may potentially recognize PR8 dsRNA made during viral genomic RNA replication from viral mRNA. However, the general role of TLR3 during viral infection was recently questioned (44). There are conflicting findings regarding the involvement of TLR3 during IAV infection: for example, TLR3−/− mice have been shown to have a survival advantage compared with their wild-type counterparts (25). To test the role of TLR3 in our system, we immunized wild-type and TLR3−/− mice with D2.SV, D2.SV/PR8, or D2.SV transfected with poly(I:C) [D2.SV/poly(I:C)], poly(I:C) is a synthetic ligand for TLR3 and had been shown to promote the cross-priming of OVA-specific T CD8+ when it is coimmunized with soluble OVA (19, 20). As shown in Fig. 5A, in the absence of TLR3, anti-IA V and anti-T-Ag T CD8+ responses were primed to a similar extent to those in the wild-type B6 mice. The TLR3 deficiency was confirmed by the lack of increased cross-priming stimulated by poly(I:C)-transfected D2.SV cells (Fig. 5A). As IAV has a segmented ssRNA genome, the involvement of TLR7 during viral infection was recently questioned (44). There are conflicting findings regarding the involvement of TLR3 during viral infection (10). Considering cytokines are conductors of the immune system, we investigated whether there was a difference between the cytokine profiles in the hosts following D2.SV or D2.SV/PR8 immunization. Sera were collected from D2.SV- or D2.SV/PR8-immunized mice at 12, 24, 72, and 144 h postimmunization, and six different proinflammatory cytokines were assessed. We found that IL-6, MCP-1, and TNF-α were expressed at higher levels when mice were immunized with D2.SV/PR8 cells (p < 0.05) (Fig. 6A–C). All of these cytokines peaked 12 hpi and tapered off by 72 hpi; MCP-1 levels were elevated markedly higher than the other cytokines tested (Fig. 6). IL-10 and IFN-γ were under the detection threshold (data not shown), and IL-12 was produced by each group at a similar level (Fig. 6D; p > 0.05). Because IFN-α and IFN-β were shown to be induced by IAV (16, 28), the levels of these cytokines were separately tested by ELISA. Indeed, both cytokines were expressed at higher levels in the sera of the D2.SV/PR8-immunized mice compared with the D2.SV-immunized mice (Fig. 6E).

**FIGURE 5.** PR8-enhanced cross-priming is TLR7 dependent, but TLR3 independent. B6 mice (A–C, open and filled bars), TLR3−/− mice (A, striped and crossed bars), TLR7−/− mice (B, striped and crossed bars), and TLR3/TLR7−/− mice (C, striped and crossed bars) were immunized with 5 × 10^6 D2.SV (white bars), 5 × 10^6 D2.SV/PR8 (black bars), or 5 × 10^6 D2.SV/poly(I:C) (A–C, gray bars) cells (i.p.). Seven dpi, peritoneal cells and spleen were harvested, and Ag-specific T CD8+ were enumerated as described in Fig. 1. This graph is plotted as a mean ± SEM of three mice and is representative of three separate experiments.

**FIGURE 6.** Concentration of proinflammatory cytokine profile in the sera of D2.SV- or D2.SV/PR8-immunized mice. B6 mice were immunized with D2.SV (open boxes and white bars) or D2.SV/PR8 (filled boxes and black bars). Sera were collected from naive mice and immunized mice were drawn at 12, 24, 72, and 144 hpi. Cytometric bead array assay was performed with the sera, detecting for proinflammatory cytokines IL-6, IL-10, IL-12p70, MCP-1, IFN-γ, and TNF-α (A–D). All cytokine levels peaked at 12 hpi. Standard sandwich ELISA was also performed with the 12 hpi sample, detecting for IL-1β and IFN-α (E). This graph is plotted as a mean ± SEM of three mice and is representative of two separate experiments.
These data prompted us to investigate the possible source of above-detected cytokine/chemokine. Thus, candidate local cell populations at the site of immunization during the peak (12 hpi) of proinflammatory cytokine/chemokine production were enumerated. From peritoneal exudates, we found only small numbers of macrophages/monocytes (CD11b<sup>F4/80<sup>+</sup></sup>) were recruited when mice were immunized with D2.SV and pDCs were undetectable (Fig. 7A, 7B). However, in D2.SV/PR8-immunized mice, an increase in the recruitment of macrophages/monocytes to the peritoneum as well as a drastic increase in pDC number from virtually zero to ∼100,000 cells was observed (Fig. 7A, 7B). This latter observation was also represented in the spleen, where the pDC number increased by ∼2.5-fold in the presence of IAV (Fig. 7C).

**Signaling pathways involved in the IAV-enhanced cross-priming**

MyD88 is an adaptor molecule that is responsible for the activation of transcription factors such as NF-κB for the production of many chemokines and proinflammatory cytokines (10). The MyD88 signaling pathway is involved in signaling by all TLRs, except for TLR3. MyD88 has also been shown to be important for MyD88 signaling pathway is involved in signaling by all TLRs, activation of transcription factors such as NF-

**FIGURE 7.** Differential cell recruitment into the peritoneum cavity and the spleen of D2.SV- and D2.SV/PR8-immunized mice. B6 mice were immunized with 5 × 10<sup>6</sup> D2.SV or D2.SV/PR8 cells i.p. Twelve hpi, peritoneal exudates and spleen were harvested and macrophages/monocytes (M<sub>ø</sub>) (CD11b<sup>F4/80</sup>)<sup>+</sup>, pDCs (CD11c<sup>hi</sup>, I-A<sup>E</sup>, and PDCA-1<sup>+</sup>), CD8α<sup>+</sup> DCs (CD11c<sup>+</sup>, I-A<sup>E</sup>, and CD8α<sup>+</sup>), and CD103<sup>+</sup> DCs (CD11c<sup>+</sup>, I-2A<sup>E</sup>, and CD103<sup>+</sup>) in the peritoneum (A, B) and spleen (C) were identified by surface markers and counted by flow cytometry. A, Dot plots of Mø, pDCs, and CD103<sup>+</sup> DCs 12 hpi in the peritoneal cavity. B and C, Cell numbers are expressed as mean ± SEM of n = 3 mice. *p < 0.05; **p < 0.001 (Student t test). n.d., not detected.

**FIGURE 8.** PR8-assisted cross-priming requires MyD88 and type I IFN signaling. A, H-2<sup>b</sup> B6 mice (filled bars) or MyD88<sup>−/−</sup> mice (striped and crossed bars) were immunized with 5 × 10<sup>6</sup> D2.SV (white bars), D2.SV/PR8 (black bars), or D2.SV/poly(I:C) (gray bars) cells (i.p.). B, H-2<sup>b</sup> B6 mice (white and black bars), H-2<sup>b</sup> IL-1R<sup>−/−</sup> mice (dotted bars and checked bars), IFN-γR<sup>−/−</sup> mice (thin and thick horizontal striped bars), and IFN-αR<sup>−/−</sup> mice (striped and crossed bars) were immunized with 5 × 10<sup>6</sup> D2.SV (white, dotted, thin horizontal, and striped bars) or D2.SV/PR8 (black, checked, thick horizontal and crossed bars) cells (i.p.). Seven dpi, peritoneal cells and spleens were harvested. Peritoneal and splenic cells were restimulated for 2 h and then continued for 4 more h in the presence of brefeldin A with 1 μM peptide (H-2D<sup>b</sup>-restricted NP<sub>366-374</sub> or H-2K<sup>b</sup>-restricted T-Ag<sub>404-411</sub>) in 96-well U-bottom plates containing 10<sup>5</sup> peritoneal or splenic cells well per IFN-γ production by Ag-specific T<sub>CD8</sub>, was measured by ICS. This graph is plotted as mean ± SEM of three mice and is representative of three separate experiments.
Finally, IFN-α has been shown to enhance both direct priming of lymphocytic choriomeningitis virus-specific T<sub>CD8+</sub> as well as cross-priming of T<sub>CD8+</sub> to soluble OVA by promoting survival of activated T cells (49). However, its role in promoting cross-priming of cell-associated Ag is yet to be demonstrated. It is known that IFN-α is produced after TLR7 stimulation, which requires the adaptor molecule MyD88. Thus, we conducted the same cross-priming experiments in the IFN-αR1<sup>−/−</sup> mice. Surprisingly, the results mirrored those obtained from the MyD88<sup>−/−</sup> mice (i.e., although the PR8-specific T<sub>CD8+</sub> response was not influenced, the PR8-facilitated and enhanced cross-priming of the T-Ag<sub>404–411</sub>-specific T<sub>CD8+</sub> response was eliminated). Taken together, these observations indicate that the IAV-enhanced cross-priming of the T<sub>CD8+</sub> response to cell-associated Ag totally depends on IFN-αR signaling.

**pDCs and CD8<sup>+</sup> DCs assist CD8<sup>+</sup> DCs in cross-priming of Ag-specific T<sub>CD8+</sub>.**

The best-characterized cross-presenting APC to date is the CD8<sup>+</sup> DCs (50, 51). However, it is unclear whether other DC subsets or cytokines could further influence their cross-priming capacity. In addition, it is possible other DC subsets, during viral infection or in the presence of TLR stimulation, are also able to cross-prime T<sub>CD8+</sub>. Thus, we decided to investigate the DC subsets and their role in our cell-associated Ag system. pDCs are well equipped with abundant TLR7 (52) and are best known for their ability to produce large quantities of type I IFN upon viral stimulation (53). As no in vivo DC depletion model is available for pDCs and CD8<sup>+</sup> DCs, and considering our attempts at in vivo pDC depletion using Ab were not satisfactory (data not shown), we used an in vitro cross-presentation system to investigate the involvement of different DC subsets. We sorted DCs from naïve B6 spleen cells into CD8<sup>+</sup> DCs, CD8<sup>−</sup> DCs, and pDCs as described in the Materials and Methods. These DCs were then incubated with irradiated Dap.OVA or Dap.OVA/PR8 to allow Ag capture and cross-presentation of OVA<sub>257–264</sub> to CFSE-labeled, naïve OT-I T<sub>CD8+</sub>. As shown in Fig. 9A and 9B, minimal proliferation was observed for naïve OT-I T<sub>CD8+</sub> that were cocultivated with CD8<sup>+</sup> DCs and Dap.OVA, and no proliferation was observed for OT-I T<sub>CD8+</sub> incubated with pDC and CD8<sup>−</sup> DCs and Dap.OVA cells. However, the OT-I T<sub>CD8+</sub> extensively proliferated when the CD8<sup>−</sup> DCs were incubated with Dap.OVA/PR8 cells. Again, neither the CD8<sup>−</sup> DCs, nor the pDCs stimulated OT-I T<sub>CD8+</sub> proliferation when incubated with Dap.OVA/PR8. Interestingly, when either of these DC subsets was mixed with CD8<sup>+</sup> DCs and incubated with Dap.OVA/PR8, the ability of CD8<sup>−</sup> DCs to cross-present OVA<sub>257–264</sub> to OT-I T<sub>CD8+</sub> was significantly enhanced (Fig. 9A, 9C). Furthermore, such enhanced cross-priming was observed over the entire DC titration range, indicating that it was not a cell number but rather a cell-type related effect. Importantly, neither of these DC subsets enhanced the cross-presentation ability of CD8<sup>+</sup> DCs in the absence of PR8 (data not shown).

**Discussion**

Cross-priming is critical for efficient antitumor immunity. In this study, we demonstrate that TLR7, MyD88, and IFN signaling enhance cross-priming. To date, there is little documentation on enhancing cross-priming of T<sub>CD8+</sub> to Ags that are encoded by cellular genome using TLR ligands. Studies that have demonstrated enhanced cross-priming through TLR signaling involved soluble or virally introduced Ags (15). Virus-encoded Ags may vary in behavior from Ags that are expressed constitutively in a host cell (23, 24). Although these earlier studies assist in understanding some aspects of the intricate cross-priming system, how to boost cross-priming of cell-genome-encoded Ag, such as tumor Ags, needs to be urgently addressed. In this study, we show that cross-priming of T<sub>CD8+</sub> to Ags constitutively expressed by allogeneic D2.SV and Dap.OVA cells was enhanced by IAV infection of these source Ag-carrying cells. It is now well established that IAV RNA can be recognized by TLR expressed by DCs, and this recognition leads to DC maturation. The IAV ssRNA genome has been shown to be sensed by TLR7 (16). However, this sensing does not enhance the IAV-specific T<sub>CD8+</sub> responses (Fig. 5B) (54). Similarly, TLR7 has not been reported to enhance cross-priming when stimulated by its ligand. In fact, resiquimod, a synthetic ligand for TLR7, has been tested together with other TLR ligands and shown to be unable to enhance the cross-priming of OVA<sub>257–264</sub>-specific T<sub>CD8+</sub> when soluble OVA was provided (20). To our surprise, we demonstrated in this study that the PR8-facilitated enhancement of cross-priming is largely governed by TLR7, not TLR3, suggesting that viral ssRNA, rather than viral dsRNA, is being sensed by the host immune system. These results are consistent with the hypothesis that IAV NS1 can sequester the IAV dsRNA during viral infection.

**FIGURE 9.** Cross-priming of cell-associated Ag is enhanced in vitro in the presence of PR8. Naïve spleens were harvested, and DCs were negatively enriched as described in Materials and Methods, stained with anti-PDCA-1–FITC, anti–CD11c-PE, and anti-CD8–PE-Cy7, and subsequently sorted into CD8<sup>+</sup> DCs, CD8<sup>−</sup> DCs, and pDCs. Meanwhile, naïve OT-I T<sub>CD8+</sub> were also negatively enriched and then labeled with CFSE. The individual sorted DC subset (B) or a mixture of the two DC subsets (C) were then incubated with 2.5 × 10<sup>5</sup> irradiated Dap.OVA or Dap.OVA/PR8 and 2.5 × 10<sup>5</sup> CFSE-labeled naïve OT-I T<sub>CD8+</sub>. A, Representative FACS plots of OT-I T<sub>CD8+</sub>, CFSE dilution (proliferation) for B (2.5 × 10<sup>5</sup> DCs) and C (5 × 10<sup>5</sup> DCs). The calculation of the proliferated OT-I T<sub>CD8+</sub> was described in Materials and Methods. In B, individual DC subsets were serially diluted and tested for their ability to cross-prime OT-I T<sub>CD8+</sub> in the presence of Dap.OVA or Dap.OVA/PR8. C, 1:1 mixed DC subsets were serially diluted and tested for their combined ability to cross-prime OT-I T<sub>CD8+</sub> in the presence of Dap.OVA or Dap.OVA/PR8.
RNA replication, rendering them less visible to the immune cells (15). Nevertheless, IAV dsRNA has been reported more recently to be sensed by the RNA helicase RIG-I (26), indicating that the sequestering by NS1 is not absolute. Considering RIG-I signals through IPS-1 (55) and not the adaptor molecule MyD88, which we have demonstrated to be responsible for the entire enhanced cross-priming, its potential role in cross-priming is yet to be established.

The complete dependence of cross-priming on the adaptor protein MyD88 was only partially accounted for by the TLR7-dependent response. This necessitates the involvement of additional MyD88-dependent signaling pathway(s). Accordingly, we investigated the role of the established MyD88-dependent inflammasome-mediated response (47). This pathway has been a primary focus area of investigation in recent years and there is evidence to indicate that IAV interacts with the inflammasome (27–29). Activation of the inflammasome leads to caspase-1 activation, which converts the inactive pro–IL-1β and pro–IL-18 to their active forms. The active IL-1β binds to IL-1βR to influence many aspects of the immune response, such as infiltration of neutrophils and induction of Th1 and CTL response (28, 47). Although there is convincing evidence that the activation of the inflammasome can enhance cross-priming of soluble OVA (46), and increased IL-1β was detected in mice immunized with D2.SV/PR8 (Fig. 6E), we found that the PR8-enhanced cross-priming was not affected in the IL-1R−/− mice in which IL-1β signaling was deficient. In contrast to the reported decrease in the anti-IAV cellular response in the IL-1R−/− mice (28), the IAV-specific TCD8+ responses remained unchanged in our experiments (Fig. 8B). This difference may be due to the fact that we immunized mice with cell lines infected with PR8 via an i.p. route rather than PR8 viral particles via an i.n. route.

MyD88 signaling leads to the translocation of transcription factors, such as NF-κB, into the nucleus of immune cells and subsequently initiation of the production of proinflammatory cytokines and chemokines such as IL-6, IL-12, MCP-1, TNF-α, and type I IFN. We have shown that there is an increase in the above-mentioned cytokines in the sera of D2.SV/PR8-immunized mice, which most likely results in the recruitment of multiple immune cell types at the site of immunization. Coadministration of IFN-α (a subtype of type I IFN) with soluble OVA has been shown to enhance the cross-priming of soluble Ag. IAV has been shown to induce the production of type I IFN (16). Therefore, it is not surprising that IFN-α is induced at a higher level in D2.SV/PR8-immunized mice compared with those immunized with the uninfected D2.SV. Using IFN-αR1−/− mice, we found that type I IFN signaling through its receptor is required for PR8 to enhance cross-priming of cell-associated Ag. Whether type I IFN is working on CD8+ DCs or TCD8+, directly in our system is currently unclear, but there is published evidence that type I IFN can lead to the maturation of DCs (56) as well as directly support the survival of Ag-specific TCD8+. In our system, we observed a dramatic increase in the numbers of Ag-specific TCD8+, in all the sites investigated during the first 7 dpi with IAV-infected allocells (Fig. 2), and the overall TCD8+ response kinetics was not altered (Fig. 3). Therefore, it appears most likely that the enhanced cross-priming is a result of increased priming of more naive precursors and/or increased expansion during this time from individual primed TCD8+.

pDCs are known to express TLR7 (52), and following TLR ligation, they produce large amounts of type I IFN. We have shown in vitro that pDCs do not cross-prime OT-1 TCD8+, but instead enhance the cross-priming of these TCD8+ through CD8+ DCs (Fig. 9). Interestingly, CD8+ DCs can also enhance the cross-priming of Ag-specific TCD8+, which has not previously been documented. The enhancement provided by pDCs is likely due to the type I IFN production as a result of the engagement of TLR7 with IAV ssRNA. The observation that CD8+ DCs can enhance cross-priming is intriguing. This is a heterogeneous DC population that can be further classified into CD4+ and CD4− DCs. Most of these DC subsets have been reported to be able to produce type I IFN upon activation, albeit not at the large quantity produced by pDCs (52, 56, 57). It is well established that CD8+ DCs express TLR3, but lack TLR7, whereas pDCs express TLR7, but lack TLR3, and CD8− DCs also express TLR7 (52). Thus, it is envisaged that CD8− DCs may also sense IAV ssRNA through TLR7 and make type I IFN, which in turn enhances cross-priming. However, we cannot exclude the possibility that CD8− DCs may be able to cross-present Ag to already primed CD8+ T cells and drive increased proliferation. Unexpectedly, CD8+ DCs alone are more effectively able to cross-prime in the presence of PR8. As our CD8+ DCs were ∼85% pure, it is therefore possible that there was residual contamination from either CD8− DCs or pDCs (or both), as shown in Fig. 9C, and that the enhanced priming may not require large numbers of such DCs.

From our in vivo experiments performed in TLR3−/−, TLR7−/−, and MyD88−/− mice, we showed that the MyD88-dependent, IAV-enhanced cross-priming of T-Ag–specific TCD8+ response had two components: the TLR7-dependent and TLR7-indepen- dent. For the latter, because CD8+ DCs lack TLR7, we postulate that PR8 may influence CD8− DCs, or other DC subsets, directly in a TLR7-independent but MyD88-dependent manner. Murine CMV (MCMV), although a dsDNA virus, was reported to be recognized by TLR3 as a possible result of bidirectional transcription from the MCMV genome (58); thus, it is possible for IAV to be recognized by other TLRs rather than TLR7 and/or TLR3. Furthermore, there is evidence that other host proteins, rather than TLRs, might be involved in viral recognition. For instance, IAV has been reported to interact with CD14 molecules expressed on murine macrophages, and the interaction is required for subsequent cytokine and chemokine production (59). Compton et al. (60) showed that CD14 was able to enhance MCMV recognition mediated by TLR2. CD14 does not have a signaling domain; it is highly likely that it forms partnerships with other proteins, such as TLRs, to influence cellular function. Clearly, more investigation needs to be conducted to confirm these observations and to further illustrate other potential IAV-sensing mechanisms involved in our observations.

We also showed that the total cross-priming of TCD8+ to T-Ag appears to have two distinct components that likely use different cross-presentation pathways. First, the baseline cross-priming component of the T-Ag−specific TCD8+ response achieved during immunization with D2.SV alone. This response does not seem to be dependent on TLRs, although we have not formally assessed that possibility using MyD88 and TRIF double-deficient mice (61). The response is clearly independent of MyD88, type I IFN, and inflammasome-induced IL-1 signaling, as this was intact in all gene-targeted mice investigated in this study. Second, the enhanced cross-priming component that was clearly elicited by IAV infection of the Ag-carrying allogeneic cells. We have shown that similar enhanced cross-priming can be achieved by transfecting these cells with a TLR3 ligand, poly(I:C) (Figs. 5A, 5B, 8A). This phenomenon seems general, as it was also observed in our Dap.OVA and Dap.OVA/PR8 system (data not shown). There are two major cross-presentation pathways previously reported: the phagosome-to-cytoplasm pathway, which is TAP- and proteasome-dependent and was involved in most cross-presentation studies reported to date, and the vacuolar pathway, which is largely TAP- and proteasome-
independent and responsible for some particulated Ags. Ags derived from viral-like particles and some soluble Ags (5). Using SV40 T-Ag transfected allogeneic cell lines and cultured DCs, Kammerer et al. (62) demonstrated that the cross-presentation of T-Ag–derived epitope was largely TAP-independent, which may account for the baseline cross-priming in our system. In contrast, it has been shown that TLR activation not only stimulates DC membrane ruffling and phagosome maturation, but also specifically causes an F-actin-rich structure inside DCs, called the podosome, to cluster, which enhances DC’s migration capacity (63, 64). It is therefore possible that the enhanced cross-priming is largely mediated by the phagosome-to-cytosol pathway. Alternatively, it remains possible that the two portions of the cross-priming might be induced by different APCs, such as DCs and macrophages (5).

Although cross-priming of TCD8+ specific to cell genome-encoded T-Ag and OVA was enhanced by IAV infection through TLR7-mediated sensing and its MyD88, IFN-α signaling pathways, IAV-specific TCD8+ responses were not influenced in either the absence of TLR7 or IFN-αR1. This may indicate that either PR8-specific TCD8+ responses are largely dependent on direct-priming due to either direct or secondary IAV infection of DCs (65, 66), or the IAV-derived source Ags are located in a subcellular compartment that is different from where T-Ag and OVA locate. As little difference was observed for the IAV-specific TCD8+ response in the above-mentioned gene-deficient mice, and knowing that TLR7-mediated sensing and its MyD88, IFN-α-induced type I interferon signaling due to cross-presentation of MyD88-deficient dendritic cells. J. Immunol. 166: 5688–5694.


