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ssRNA can interact with dendritic cells (DCs) through binding to TLR7, inducing secretion of proinflammatory cytokines and type I IFN. Triggering TLR7 enhances cross-priming of CD8$^+$ T cells, which requires cross-presentation of exogenous Ag to DCs. However, how TLR triggering can affect Ag cross-presentation is still not clear. Using OVA as an Ag model, we observed that stimulation of TLR7 in DCs by polyuridylic acid (polyU), a synthetic ssRNA analog, generates a strong specific cytotoxic response in C57BL/6 mice. PolyU stimulate CD8α$^+$ DCs to cross-prime naive CD8$^+$ T cells in a type I IFN–dependent fashion. This enhanced cross-priming is accompanied by a higher density of $OVA_{256\text{-}264}$/H-2K^b complexes on CD8α$^+$ DCs treated with polyU, as well as by upregulation of costimulatory molecules and increased secretion of proinflammatory cytokines by DCs. Cross-priming of CD8$^+$ T cells by DCs treated with polyU requires proteasome and Ag translocation to cytosol through the Sec61 channel in DCs. The observed enhancement in OVA cross-presentation with polyU in DCs could be mediated by a limited Ag degradation in endophagosomal compartments and a higher permanence of OVA peptide/MHC class I complexes on DCs. These observations clearly reveal that key steps of Ag processing for cross-presentation can be modulated by TLR ligands, opening new avenues for understanding their mechanisms as adjuvants of the immune response. ***The Journal of Immunology*****, 2013, 190: 948–960.**

Cell-mediated immune responses characterized by the induction of cytotoxic CD8$^+$ T cells are crucial for therapeutic interventions in the context of tumor immunotherapy and for the induction of protective immunity against a variety of intracellular pathogens, such as the malaria parasite and HIV. Frequently, robust immunoprotection requires the generation of cytotoxic immune responses against tumor Ag or pathogens unable to infect APCs. The dendritic cell (DC) is the most specialized APC for the initiation of such responses, presenting exogenous Ag bound to MHC class I (MHC I) molecules to CD8$^+$ T cells (1, 2). This process, known as cross-presentation, is essential to cross-prime, that is, to activate naive cytotoxic CD8$^+$ T cells specific for Ag not expressed in APCs, and results in the triggering of antimicrobial and antitumor immunity. Through cross-presentation, DCs could ingest pathogens and infected, tumor, or dead cells and derive the antigenic peptides to the MHC I pathway, which could give rise to cross-priming or to cross-tolerance. Self Ags are also cross-presented, but rather than cross-priming, this normally results in deletion of autoreactive CTLs, a process termed cross-tolerance (3). DCs are the main Ag cross-presenting cell type in vivo (4). In vitro, however, other cell types (including macrophages, B cells, and granulocytes) can also cross-present Ag (5–7). However, it is unclear so far whether the intracellular pathways used by these cells are similar to those employed by DCs. Even in DCs, however, the intracellular compartments involved in cross-presentation are not fully defined. Several potential mechanisms of cross-presentation have emerged, but much work is required to reach a better understanding of the operating mechanisms.

It is now clear that TLR ligands not only stimulate transcription of cytokines and costimulatory molecules but they also signal an array of responses that affect the membrane vacuolar system, the cytoskeleton, and the machinery of protein translation and degradation (8). An aspect of cross-presentation that is currently being analyzed is whether it can be modified by triggering the TLR in DCs. It has been described that cross-priming could be enhanced

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; cDC, conventional dendritic cell; CM, culture medium; DC, dendritic cell; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate; ER, endoplasmic reticulum; ExoA, *Pseudomonas aeruginosa* exotoxin A; Flt3L, Flt3 ligand; IC-OVA, OVA forming immune complexes with rabbit anti-OVA IgG; IC-OVA-FITC, OVA coupled to FITC forming immune complexes with rabbit IgG anti-OVA; MFI, mean fluorescence intensity; MHC I, MHC class I; OVA beads, OVA covalently linked to synthetic polystyrene beads; OVA-FITC, OVA coupled to FITC; pDC, plasmacytoid dendritic cell; polyU, polyuridylic acid; polyU/DO, polyuridylic acid complexed with *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate; polyUs21, a 21-mer of polyuridylic acid.

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by microbial molecular patterns, in particular by TLR ligands (9, 10), whose best known effects include induction of DC maturation, upregulation of costimulatory molecule expression, and augmented Ag uptake and processing (11–17).

A set of TLRs, comprising TLR3, TLR7, TLR8, and TLR9, recognize nucleic acids derived from microorganisms as well as endogenous nucleic acids released in pathogenic contexts (18). Activation of these TLRs leads to the production of type I IFNs in addition to proinflammatory cytokines. Murine TLR7 and human TLR7/8 recognize ssRNA from viruses, as well as small purine analog compounds (imidazoquinolines). TLR7 also detects RNAs from bacteria such as group B *Streptococcus* in conventional DCs (cDCs) (19).

The TLR7 ligands resiquimod and imiquimod have been shown to only weakly enhance cross-priming of $CD8^+$ T cells (17, 20, 21). Recently, Rajagopal et al. (22) and Wei et al. (23) have demonstrated that other TLR7 ligands, such as a 21-mer of polyuridylic acid (polyUs21) and influenza-infected cell lines, respectively, can enhance cross-priming in DCs, generating strong cytotoxic immune responses. However, the molecular mechanisms underlying the stimulatory effect of TLR ligands on cross-presentation are not fully resolved.

In this study, we explore the use of the TLR7 ligand polyuridylic acid (polyU) as adjuvant to enhance the ability of DCs to cross-prime $CD8^+$ T cells and then to induce cytotoxic immune responses as well as the mechanisms implicated in this process. We report that polyU triggers TLR7 in $CD8\alpha^+$ cDCs to cross-prime $CD8^+$ T cells. This priming requires type I IFNs in an autocrine loop in $CD8\alpha^+$ cDCs. TLR7 triggering in DCs modulates the endophagosomal compartment and $t_{1/2}$ of the peptide/MHC I complex. The main steps of the MHC I processing pathway that exogenous Ag follows in DCs during cross-presentation are conserved after TLR7 triggering. Our results shed new light on the understanding of the mechanism involved in Ag cross-presentation and in cross-priming of $CD8^+$ T cells.

Materials and Methods

Mice and cell lines

Six- to 8-wk-old female C57BL/6 mice were provided by Fundación Facultad de Ciencias Veterinarias (Universidad Nacional de La Plata, La Plata, Argentina). OT-I mice, which express a transgenic TCR designed to recognize OVA residues 257–264 in the context of H-$2K^b$ (24), and OT-II mice, which express a transgenic TCR designed to recognize OVA residues 323–339 in the context of H-2 I-A^b (25) were provided by Dr. F.A. Goldbaum (Fundación Instituto Leloir, Buenos Aires, Argentina) and bred in our animal facility. IFN-$\alpha\beta R^{-/-}$ (IFNAR$^{-/-}$) mice in a C57BL/6 background (26) were provided by M. Albert (Institut Pasteur, Paris, France). MyD88$^{-/-}$ mice in a C57BL/6 background were purchased at The Jackson Laboratory (Bar Harbor, ME). Experiments involving animals were conducted with the approval of our Institutional Experimentation Animal Committee (authorization no. 15-07-62010). Our animal facility meets the terms of the *Guide to the Care and Use of Experimental Animals*, published by the Canadian Council on Animal Care, and has the assurance number A5802-01 delivered by the Office of Laboratory Animal Welfare (National Institutes of Health).

B16, a murine melanoma cell line transfected with the gene for the Flt3 ligand cytokine (B16-Flt3L), was generously provided by Dr. María Rosa Bono (Facultad de Ciencias, Universidad de Chile, Santiago, Chile). B3Z, a $CD8^+$ T cell hybridoma specific for $OVA_{257–264}$ epitope in the context of H-$2K^b$ (27), was a gift from Dr. N. Shastri (University of California, Berkeley, CA).

Ags, peptides, and TLR7 and TLR9 ligands

We used OVA grade V (Sigma-Aldrich, St Louis, MO) or chromatographically purified OVA (Worthington Biochemical, Lakewood, NJ) as Ag for the different assays. It was solubilized in PBS or with two forms of particulate Ag: either forming immune complexes with rabbit anti-OVA IgG (IC-OVA) or covalently linked to synthetic polystyrene beads (OVA beads). IC-OVA were formed by incubation of OVA/PBS at various concentrations with rabbit anti-OVA sera (Sigma-Aldrich). OVA beads were prepared as in Boisgérault et al. (28). Briefly, 0.5 mg/ml OVA was covalently coupled to 1 μm Polybead amino microspheres (Polysciences, Warrington, PA) after activation of beads with 8% glutaraldehyde. For endocytosis studies we employed OVA coupled to FITC (Molecular Probes/Invitrogen). The peptide corresponding to $OVA_{257–264}$ was synthetized by Facultad de Farmacia y Bioquímica (Laboratorio Nacional de Investigación y Servicios en Péptidos y Proteínas, Universidad de Buenos Aires, Buenos Aires, Argentina).

PolyU (Sigma-Aldrich) was used as TLR7 ligand, always complexed to *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP) liposomal transfection reagent (Roche Diagnostics, Indianapolis, IN), except in Supplemental Fig. 3, where protamine (Sigma-Aldrich) was used as a stabilizing agent. Endotoxin content in polyU preparations, determined by a standard *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD) was <1 endotoxin unit/ml. The synthetic oligodeoxynucleotides used were CpG1826 (CpG) (5′-TCCATGACGTTCCTGACGTT-′3) and IRS661 (5′-TGCTTGCAAGCTTGCA-′3). All oligodeoxynucleotides were synthesized with a nuclease-resistant phosphorothioate backbone and contained no LPS contaminants (Operon Technologies, Alameda, CA). PolyU treatment of DCs did not differentially affect the survival of any DC subset in particular.

Culture medium

Culture medium (CM) consisted of RPMI 1640 (Life Technologies Cell Culture Systems, Rockville, MD), supplemented with 1% L-alanyl-L-glutamine dipeptide (GlutaMAX I; Life Technologies Cell Culture Systems), 10% FCS (Natocor, Carlos Paz, Argentina), 5×10^{-5} M 2-ME (Sigma-Aldrich), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; PAA Laboratories, Pasching, Germany).

Flow cytometry

Cells were preincubated with anti-CD16/32 (clone 2.4G2) for 15 min at 4°C to block nonspecific binding of Abs to Fc receptors and then stained with fluorochrome-labeled Abs for 20 min at 4°C. Cells were washed twice and 7-aminoactinomycin D (7-AAD) was then added to exclude dead cells. Abs were specific to CD3 (145-2C11 or 17A2), CD4 (RM4-5 or H129.19), CD8α (53-6.7), CD8β (H35-17.2), CD11c (HL-3), CD86 (GL1), CD40 (HM40-3), CD80 (16-10A1), I-$A^{b,d,q}$/I-$E^{d,k}$ (M5/114.15.2), I-$A\beta^b$ (25-9-17), CD25 (PC61.5), B220 (RA3-6B2), PDCA-1 (129c), H-$2K^b$/$OVA_{257–264}$ complex (25-D1.16), and Vβ5.1, 5.2 TCR (MR9-4). A minimum of 1×10^5 events were acquired for each sample on a FACSCanto II cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star). Appropriate isotype controls were included. All Abs were obtained from Becton Dickinson Argentina (Buenos Aires, Argentina) or eBioscience (San Diego, CA).

Isolation of splenic DCs and $CD8^+$ T cells

Splenic DCs were isolated as described in Morón et al. (29). Briefly, spleens were perfused and treated for 45 min with 0.4 U/ml Liberase Blendzyme 2 and 100 U/ml DNase I (Roche Diagnostics). After inhibition of collagenase activity with 6 mM EDTA, spleens were dissociated and the single-cell suspensions were incubated with MACS-anti-CD11c (clone N418; Miltenyi Biotec, Bergisch Gladbach, Germany) in PBS containing 2.5 mM EDTA and 0.5% FCS (Natocor). After 20 min incubation at 6°C, cells were washed and $CD11c^+$ cells were selected on an LS MACS column (Miltenyi Biotec). Purity of $CD11c^+$ cells was always >70–75%. In some experiments, after MACS separation, cells were also labeled with anti-CD16/32 (2.4G2) and allophycocyanin- or PE-Cy7–labeled anti-CD11c (HL-3) and in other experiments with anti-CD16/32, anti-CD11c, anti-CD8α (53-6.7), anti-CD45R (B220, RA3-6B2), and anti-CD317 (PDCA-1, 129c). Cells were then further sorted out on a FACSAria IIu cell sorter (BD Biosciences). Sorted DCs and subpopulations were always >98% pure.

In some experiments, to obtain a large number of DCs, mice were injected s.c. with 10×10^6 murine B16-Flt3L, which renders a homogeneous expansion of all DC populations (30). Two weeks later, DCs were purified from spleen by sorting on a FACSAria IIu cell sorter.

$CD8^+$ T cells were isolated from OT-I mice by incubation of spleen cells with an anti–CD8β-chain mAb (which is only expressed in T cells and thymocytes) and further sorting on a FACSAria IIu. Sorted $CD8\beta^+$ cells were always >98% pure.

Immunizations

For CTL priming, mice were immunized i.v. with 2.5×10^9 OVA beads in combination with 100 μg polyU complexed with 60 μg DOTAP (polyU/DO), in HEPES-buffered saline (pH 7.4). As control, mice were immu-

nized with 2.5×10^9 OVA beads and 60 μg DOTAP, without polyU. The CTL assay was performed on day 7 after immunization. In some experiments, mice received 1–2 $\times 10^6$ splenic DCs preincubated during 90 min with 20 mg/ml OVA in combination with 20 μg/ml polyU/DO in RPMI 1640 without serum. In other experiments, mice were depleted of $CD4^+$ or $CD8^+$ T cells by i.p. injections of mAbs directed against either CD4 (GK1.5) or CD8 (53.6.7) on days −2, −1, 0, and 2 of immunization. An additional group of mice were i.p. injected with purified total IgG as control.

Abs and cytokines detection assays

Specific Abs against OVA were determined by ELISA. Briefly, 96-well flat-bottom plates (Greiner Bio One, Frickenhausen, Germany) were coated with OVA (1 μg/well) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were then blocked with 0.5% gelatin PBS and, after washing, the plates were incubated with plasma samples diluted in 0.05% Tween 20/0.5% gelatin PBS. For total and subclass IgG detection, plates were incubated with HRP-conjugated anti-mouse IgG, IgG1, and IgG2c (Sigma-Aldrich). Anti-mouse IgG2c (clone R19-15; Becton Dickinson Argentina) is a mAb which immunogen was pooled BALB/c and C57BL/6 mouse Ig and recognizes an epitope in the CH3 domain of mouse IgG_{2a}, with strong reactivity to the Igh-I[a] (IgG2a, BALB/c) allotype and weaker reactivity to Igh-I[b] (IgG2c, C57BL/6), and it does not react with other Ig isotypes. Thus, we employed this Ab to detect IgG2c in our C57BL/6 mice. Plates were examined on a microplate at 450 nm after incubation with a tetramethylbenzidine substrate reagent set (Becton Dickinson Argentina).

Levels of different cytokines were measured in culture supernatants and serum samples by standard double-Ab sandwich ELISA (Becton Dickinson Argentina) following instructions from the manufacturer. All assays were standardized with recombinant murine cytokines (Becton Dickinson Argentina). The Ab pairs used were as follows (listed by capture/biotynilated detection): IL-6, MP5-20F3/MP5-32C11; IL-12p40, C15.6/C17.8; IL-12p70, 9A5/C17.8; IFN-γ, R4-6A2/XMG1.2; IL-17, TC11-18H10/TC11-8H4; IL-5, TRFK5/TRFK4 (all from Becton Dickinson Argentina).

In vivo killing assay

Naive syngenic splenocytes were pulsed with 10 μg/ml $OVA_{257–264}$ and labeled with a high concentration (3 μM) of CFSE (Molecular Probes). A nonpulsed control population was labeled with a low concentration (0.5 μM) of CFSE. Then, $CFSE^{high}$- and $CFSE^{low}$-labeled cells were mixed in a 1:1 ratio (1×10^7 cells of each population) and injected i.v. into immunized mice. The number of $CFSE^+$ cells remaining in the spleen after 24 h was determined by flow cytometry. Cytotoxicity was expressed as the percentage of lysis, calculated from $[1 - (r_{control}/r_{immune})] \times 100$, where r is given by the expression of % $CFSE^{low}$/% $CFSE^{high}$ cells for nonimmune (control) and immune mice, respectively. At the same time, 1.25×10^6 splenocytes from immunized mice were incubated 72 h in the presence of 0.1 mg/ml OVA, 0.1 μg/ml $OVA_{257–264}$, or CM as control in 48-well culture microplates (Greiner Bio One) in a final volume of 0.5 ml CM. Supernatants were then collected and IFN-γ, IL-17, and IL-5 content was assayed by ELISA.

Kinetics of $OVA_{257–264}$/H-2K^b complex expression and stability on plasmatic cell membrane

Spleens of mice previously injected s.c. with B16-Flt3L cells were perfused and treated with Liberase Blendzyme 2 and DNase I. After inhibition of enzyme activity, spleens were dissociated and the single-cell suspensions were treated with RBC lysing buffer (Sigma-Aldrich). Splenocytes (2×10^5 cells/well) were then incubated with 1 mg/ml OVA alone or mixed with 20 μg/ml polyU/DO or DOTAP at 37°C for 4 h in culture microplates in a final volume of 0.2 ml CM. Splenocytes were then washed twice, resuspended in 0.2 ml CM, and labeled with an allophycocyanin-labeled Ab against $OVA_{257–264}$/H-2K^b complex (clone 25-D1.16) either before (OVA-MHC I complex stability experiment) or after (kinetics of Ag appearance experiment) chasing for the indicated times at 37°C. In the first case, at the end of the chasing time, cells were labeled with a PE-labeled Ab anti-mouse IgG that recognizes 25-D1.16 Ab present only on cell surface. In all cases, at the end of experiment cells were harvested and labeled with Abs against CD11c, CD8α, PDCA-1, and B220 and analyzed by flow cytometry. A similar experiment with only one chase time, performed using DCs from untreated mice (without Flt3L-secreting tumor) as control of tumor treatment, resulted in equivalent results.

T cell proliferation assay

T cell proliferation was assessed by coculturing splenic $CD8\beta^+$ cells from OT-I mice with splenic DCs. Splenic $CD8\beta^+$ cells from OT-I mice were previously stained with 5 μM CFSE in PBS with 5% FCS and then washed extensively. Purified DCs (1 or 2×10^5 cells/well) were incubated with the indicated amounts of OVA alone or mixed with 20 μg/ml polyU/DO or DOTAP at 37°C for 90 min in culture microplates in a final volume of 0.2 ml CM. DCs were then washed twice and CFSE-labeled OT-I $CD8^+$ T cells (2×10^5 cells/well) were added. After 72 h, the supernatants were collected for IFN-γ content assessment by ELISA and cultured cells were harvested, labeled with Abs against CD3 and CD25, and analyzed on a FACSCanto II flow cytometer. 7-AAD was added to samples before analysis to exclude dead cells. Proliferation was determined by the dilution of CFSE content in $CD3^+$ 7-AAD^- cells and is expressed as the percentage of cells under proliferation, that is, with a lower CFSE content than unstimulated cells at time 0 of culture.

Phagosomal protein degradation assay

A phagosomal protein degradation assay was performed following a protocol adapted from Savina et al. (31). Briefly, 10×10^6 purified spleen DCs were pulsed and chased for 20 min with 5×10^7 OVA beads in the presence of 20 μg/ml polyU/DO, DOTAP alone, or with CM. Cells were then disrupted in lysis buffer containing 50 mM Trizma base (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 10 μg/ml DNase I, and a mixture of protease inhibitors (Sigma-Aldrich) and centrifuged at 3000 rpm for 5 min at 4°C. Pellets were collected and stained with a rabbit polyclonal anti-OVA IgG and allophycocyanin-coupled anti-rabbit IgG in 96-well V-bottom microplates. Beads were then analyzed by flow cytometry to determine the residual OVA content on beads. At least 5×10^5 beads per sample were analyzed for accurate determination. Degradation arbitrary units were calculated as the ratio between the mean fluorescence intensity (MFI) at times 0 and 20 for normalization purpose.

Measurement of phagosomal pH changes

Modifications on the pH in phagosomes was adapted from Savina et al. (31). In brief, 1 μm Polybead amino microspheres (Polysciences) were covalently coupled with FITC (pH sensitive; Sigma-Aldrich) and Alexa 647 (pH insensitive; Molecular Probes) and suspended in PBS. Splenic DCs were pulsed in FCS-free medium with the coupled beads for 15 min at 37°C (pulse) and then extensively washed in cold PBS. The cells were resuspended in CM and incubated at 37°C (chase) for as long as indicated and immediately analyzed by flow cytometry, using a gating forward scatter/side scatter selective for cells that have phagocytosed one polystyrene bead. The ratio of the MFI emission between the two dyes was determined ($R_{Alexa\ 647/FITC}$). Alkalization arbitrary units were calculated as the ratio between $R_{Alexa\ 647/FITC}$ at chase time and basal $R_{Alexa\ 647/FITC}$ at time 0 for normalization purposes.

Quantitative RT-PCR

$CD8\alpha^+$ cDCs, $CD8\alpha^-$ cDCs, and plasmacytoid DC (pDCs) were purified by sort and were stimulated with 20 μg/ml polyU/DO, DOTAP alone, 20 μg/ml polyU/DO plus 0.3 μM IRS661, or cultured in CM alone for 5 h. To analyze the *IFNα4* and *TLR7* gene expression, total RNA was extracted with TRIzol reagent from different populations of DCs (1×10^6 cells). Synthesis of cDNAs was primed with oligo(dT) followed by synthesis by Moloney murine leukemia virus reverse transcriptase (Promega). Quantitative RT-PCR was done with 15 ng cDNA with SYBR Green PCR core reagents. The primers used for quantitative RT-PCR were: *IFNα4*, forward, 5′-TGATGAGCTACTACTGGTCAGC-3′, reverse, 5′-GATCTCTTAGCACAAGGATGGC-3′; *TLR7*, forward, 5′-GGATCTGCCATCCAGCTTAC-3′, reverse, 5′-ATTAGGTGGCAAAGTGGTGG-3′; and *HPRT1*, forward, 5′-AAGCTTGCTGGTGAAAAGGA-3′, reverse, 5′-TCCAACAAAGTCTGGCCTGT-3′. The condition cycling used was 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing for 1 min at 60°C. To analyze the relative gene expression of *IFNa4* and *TLR7* data, the $2(-\Delta\Delta C_T)$ method was used as previously described (32) and was relativized to the expression of the housekeeping gene *HPRT1*.

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). Data analysis included one-way ANOVA followed by a Bonferroni posttest for multiple comparisons and the Student t test. All data were considered statistically significant for p values of <0.05.

Results

PolyU induces a $CD8^+$ T cell–mediated cytotoxic response in vivo

PolyU is a chain of uridine-rich synthetic ssRNA oligonucleotides known as a TLR7 ligand that induces IFN-α comparable to in-

fluenza RNA (33). Because cationic lipids are known to facilitate the uptake of RNA by DCs (34) and to protect it from RNases, polyU was complexed with DOTAP. The optimal ratio of polyU to DOTAP for complex formation was determined by dose titration (data not shown). It has been established that immunization with some TLR7 ligands, such as R848 (17), synthetic RNA oligonucleotides (35, 36), influenza-infected allogeneic cell lines (23), or polyUs21 (22), led to potent CTL responses. To evaluate whether polyU is capable of acting as adjuvant for CTL response, mice were i.v. immunized with OVA as a monitor Ag coated to polystyrene beads (OVA beads) plus polyU/DO. For CTL determination, an in vivo killing assay was performed 7 d after injection. Immunization with polyU/DO plus OVA beads led to a potent cytotoxic response, whereas the response induced by OVA beads plus DOTAP without polyU (DO plus OVA beads) was not significantly different from the one observed in saline-injected mice (Fig. 1A).

Th cell activity through CD40–CD40 ligand interactions or proinflammatory cytokines are required for in vivo generation of CTLs (37–40). Thus, we evaluated the participation of $CD4^+$ T cells in the activation of cytotoxic cells induced by polyU. With this purpose, we performed an in vivo depletion of either $CD4^+$ or $CD8^+$ T cells by injecting specific Abs to each of these molecules. This treatment led to $CD4^+$ or $CD8^+$ T cell depletion in the spleen (but not depletion in $CD4^+$ or $CD8\alpha^+CD11c^+$ cells; data not shown), whereas treatment with unrelated IgG had no observable effect (data not shown). On day 0, all mice were immunized with polyU/DO plus OVA beads. IgG-treated mice showed the same level of Ag-specific killing observed for nontreated animals (Fig. 1B). $CD4^+$ T cell–depleted mice showed a significant reduction in the level of Ag-specific killing compared with mice treated with IgG. Alternatively, $CD8^+$ T cell–depleted mice also showed a strong reduction in Ag-specific killing. These results show that both $CD8^+$ and $CD4^+$ T cells are required to observe OVA-specific cytotoxicity after immunization with polyU/DO plus OVA beads. In addition to Ag-specific killing, we also determined IFN-γ, IL-17, and IL-5 secretion in the supernatants of splenocytes of mice from the above-mentioned groups after being restimulated in vitro with whole OVA or $OVA_{257–264}$ during 72 h. We found that splenocytes from IgG-treated immunized mice secreted high levels of IFN-γ (Fig. 1C). In contrast, splenocytes from $CD4^+$ T cell–depleted mice failed to secrete IFN-γ when they were restimulated. As expected, splenocytes from $CD8^+$ T cell–depleted mice did not secrete IFN-γ upon restimulation with $OVA_{257–264}$, but they had a slight IFN-γ secretion upon restimulation with whole OVA, which could be produced by OVA-specific $CD4^+$ T cells present in the cell culture. Neither IL-5 nor IL-17 was detected in supernatants of restimulated splenocytes of any group (data not

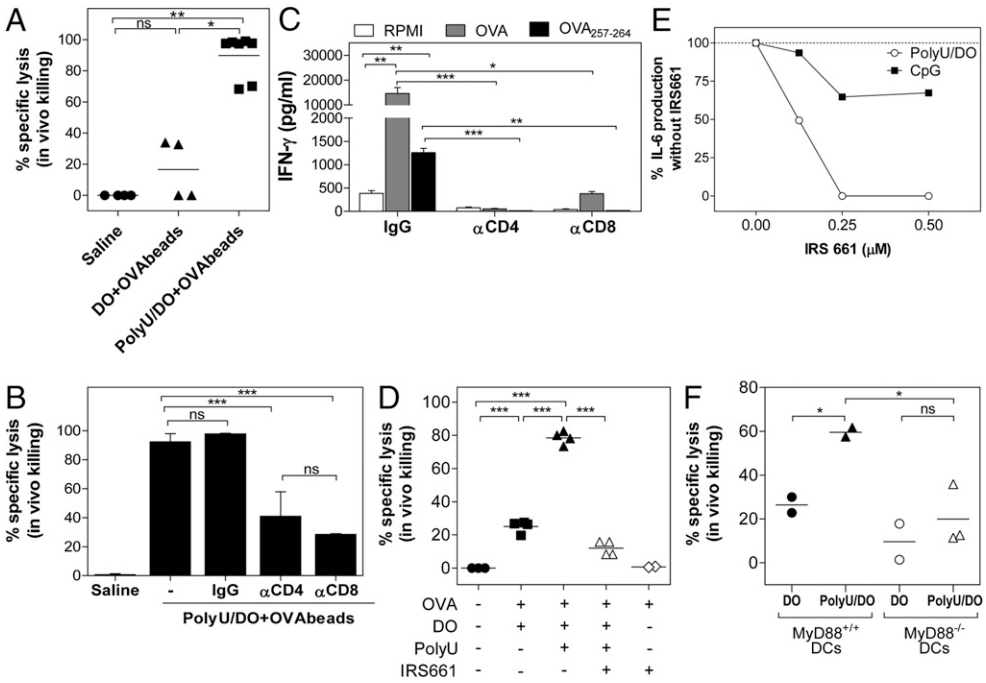

FIGURE 1. PolyU induces a CTL response in vivo in a TLR7-dependent fashion. (**A**) C57BL/6 mice were immunized by a single i.v. injection of saline or 2.5×10^9 OVA beads in 100 μg polyU complexed with 60 μg DOTAP (polyU/DO plus OVA beads) or in DOTAP alone (DO plus OVA beads). Seven days later, CTL was determined by an in vivo killing assay. Data show the percentage of specific in vivo killing of each individual mouse from three independent experiments, and the bars indicate the mean of each group. (**B** and **C**) CTL response (B) and in vitro secretion of IFN-γ (C) in mice depleted of $CD4^+$ or $CD8^+$ T cells. On day 0, mice were immunized with 2.5×10^9 OVA beads plus polyU/DO and 7 d after immunization CTL activity was assessed. IFN-γ was determined in culture supernatants of splenocytes, restimulated with OVA or $OVA_{257–264}$ during 72 h, and evaluated by ELISA. Data show the mean values ± SEM of two independent experiments ($n \geq 4$ mice/group). (**D**) Splenic DCs were incubated with 20 mg/ml OVA in 20 μg/ml polyU/DO (in the presence or absence of 0.3 μM IRS661) or in DOTAP or in RPMI 1640 for 90 min and then washed twice. DCs (1×10^6) were injected once by i.v. route into mice. Seven days later, CTL was determined by in vivo killing assay. Data show the percentage of specific in vivo killing of each individual mouse, and the bars indicate the mean of each group. (**E**) Inhibitory effect of IRS661 on IL-6 secretion. Splenic $CD11c^+$ cells from C57BL/6 mice were incubated for 48 h with either polyU/DO or CpG alone or in the presence of the indicated concentrations of IRS661. IL-6 production was measured by ELISA. To normalize the differential strength between polyU and CpG, IL-6 levels were plotted as a percentage of polyU or CpG without inhibitor. Data shown are representative of two experiments. (**F**) Splenic DCs from wild-type and $MyD88^{-/-}$ mice were incubated with 20 mg/ml OVA in 20 μg/ml polyU/DO or in DO alone and then washed twice. DCs (1×10^6) were injected once by i.v. route into mice. Seven days later, CTL was determined by in vivo killing assay. Data show the percentage of specific in vivo killing of each individual mouse, and the bars indicate the mean of each group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

shown). Mice immunized twice with polyU/DO plus OVA produced, in addition to IgG1, high titers of anti-OVA IgG2c (Supplemental Fig. 1), an isotype preferentially produced during Th1 responses. This result is consistent with the strong secretion of IFN-γ, clearly showing that polyU is modulating the immune response toward a Th1 phenotype. In conclusion, immunization of mice with polyU/DO plus OVA beads induces a cytotoxic response mediated by $CD8^+$ T cells and is dependent on $CD4^+$ T cell help.

Splenic DCs stimulated with polyU generate a strong anti-OVA cytotoxic response mediated by signaling through TLR7

Generation of MHC I–dependent cytotoxic responses requires the activation of naive $CD8^+$ T cells by recognition of MHC I–associated antigenic peptide on the APCs along with the encounter of costimulatory signals given by the APCs and/or $CD4^+$ T cells. DCs have been clearly recognized as being the only APC capable of stimulating naive T cells for CTL response (41). To determine the direct effect of polyU on the ability of DCs to elicit a CTL response, we transferred DCs preincubated with polyU/DO plus OVA to naive hosts and then measured the cytotoxic response against OVA in recipient mice. Seven days after DC transfer, mice that received polyU/DO plus OVA–preincubated DCs exhibited a stronger CTL response than did mice that received DO plus OVA–preincubated DCs (Fig. 1D). This result clearly confirms that polyU directly stimulates DCs to trigger CTL.

Several RNA receptors have been described in DCs and other cells, such as the RIG-I–like receptor family (42, 43). However, RIG-I–like receptors are localized in the cytoplasm of cells and recognize the genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediate of ssRNA viruses. In contrast, polyU is an analog of ssRNA that should be recognized by TLR7. Therefore, to confirm that polyU is targeting TLR7, we employed the oligodeoxynucleotide IRS661, which is a specific TLR7 inhibitor (44, 45). To verify that IRS661 effectively blocks DC stimulation by polyU, we measured IL-6 secretion by splenic DCs incubated with polyU/DO or CpG alone or in the presence of different concentrations of IRS661. Forty-eight hours later, we observed that IRS661 inhibited IL-6 secretion in DCs stimulated with polyU/DO but not in cells incubated with CpG (Fig. 1E). A similar lack of inhibition was observed upon stimulation with LPS (data not shown), indicating that IL-6 secretion induced by polyU/DO is mediated by TLR7 ligation in DCs.

CTL induction by DCs incubated with polyU/DO plus OVA was fully abrogated when DCs were coincubated with IRS661, showing that polyU acts as an adjuvant for CTLs through interaction with TLR7 in DCs (Fig. 1D). This observation was corroborated by the lack of CTL response in mice that received DCs from $MyD88^{-/-}$ mice incubated with polyU/DO plus OVA (Fig. 1F).

To study how polyU affects DCs in vivo, mice were i.v. injected with polyU/DO and 18 h later, the phenotypic maturation of splenic DCs was evaluated by flow cytometry. All DC subpopulations, that is, $CD8\alpha^+CD11c^+$ cells ($CD8\alpha^+$ cDCs), $CD8\alpha^-CD11c^+$ cells ($CD8\alpha^-$ cDCs), and $B220^+PDCA\text{-}1^+CD11c^{int}$ cells (pDCs) from mice injected with polyU/DO showed a significant increase in the expression of CD40 (data not shown) and CD86 (Supplemental Fig. 2A). In contrast, this effect was not observed in mice that received DOTAP or saline alone (Supplemental Fig. 2A). Furthermore, high levels of IL-12p70 were found in the sera of mice that received polyU/DO, whereas sera from mice that received only DOTAP did not contain more IL-12p70 than sera from control, saline-injected mice (Supplemental Fig. 2B). The augmented expression of CD40 (data not shown) and CD86 (Supplemental Fig. 2C) after polyU injection was fully abrogated in $MyD88^{-/-}$ mice. Moreover, pDCs upregulated the IFN-α4 mRNA transcription (Supplemental Fig. 2D). Taken together, these results indicate that polyU activates DCs in vivo through a MyD88-dependent mechanism.

PolyU stimulates DCs to cross-prime naive $CD8^+$ T cells

Because polyU promotes CTL responses through interaction with TLR7 on DCs, we evaluated in vitro the ability of polyU-stimulated DCs to activate naive $CD8^+$ T cells. Splenic DCs were incubated with polyU/DO plus OVA and cultured with CFSE-labeled $CD8^+$ T cells isolated from the spleen of OT-I mice. As controls, DCs were incubated with OVA alone or DO plus OVA. Proliferation of $CD8^+$ T cells was determined by the dilution of CFSE content in $CD3^+7\text{-}AAD^-$ cells and their activation by the expression of IL-2β–chain receptor (CD25). In the presence of DCs stimulated with polyU/DO plus OVA, a high percentage of $CD8^+$ T cells were proliferating (Fig. 2A, *top panel*) and had upregulated CD25 (Fig. 2A, *bottom panel*). Despite the fact that DOTAP alone was not capable of activating DCs, a minimal percentage of $CD8^+$ T cells was under proliferation and expressed CD25 when incubated in the presence of DO plus OVA. This could be due to an increase in the capture of OVA by DCs as a result of DOTAP treatment. DCs incubated with OVA alone neither activated $CD8^+$ T cell proliferation nor increased CD25 expression. Accordingly, $CD8^+$ T cells cultured with DCs incubated with polyU/DO plus OVA actively secreted IFN-γ, whereas those cultured with DCs incubated with OVA alone or plus DOTAP secreted low or negligible IFN-γ (depending on OVA concentration; Fig. 2B). Briefly, these results indicate that polyU stimulation licenses DCs to activate naive $CD8^+$ lymphocytes, demonstrating that polyU-induced signaling enhances cross-priming in DCs.

In order to confirm that the stimulatory effect of polyU on DCs in initiating a $CD8^+$ T cell response is mediated by TLR7 ligation, we abrogated TLR7 signaling by polyU in three different ways. First, we observed that DCs stimulated with polyU/DO in the presence of chloroquine, an endosomal maturation inhibitor that prevents endosome acidification, could not upregulate CD40 and CD86 expression (data not shown). This result confirms the requirement of functional endosomes for polyU stimulatory activity (33, 46). Second, DCs incubated with polyU/DO plus OVA plus IRS661 were unable to trigger either cell proliferation or IFN-γ secretion in $CD8^+$ T cells (Fig. 2C). Finally, DCs from $MyD88^{-/-}$ mice incubated with polyU/DO plus OVA induced a lower $CD8^+$ T cell proliferation and were unable to stimulate either secretion of IFN-γ (Fig. 2D) or upregulation of CD25 (data not shown). These results clearly show that $CD8^+$ T cell stimulation induced by DCs treated with polyU is mediated by TLR7 ligation.

PolyU stimulates $CD8\alpha^+$ cDCs to cross-prime naive $CD8^+$ T cells in a type I IFN–dependent fashion

We next proceeded to identify the DC subtype involved in cross-priming of $CD8^+$ T cells after TRL7 ligation with polyU. $CD8\alpha^+$ and $CD8\alpha^-$ cDCs and pDCs were purified by FACS and incubated with polyU/DO plus OVA with CFSE-labeled OT-I $CD8^+$ T cells. We found that only $CD8\alpha^+$ cDCs incubated with polyU/DO plus OVA were able to stimulate $CD8^+$ T cell proliferation, whereas pDCs and $CD8\alpha^+$ cDCs did not (Fig. 3A). This result would indicate that polyU directly stimulates $CD8\alpha^+$ cDCs to cross-prime $CD8^+$ T cells. However, that direct activation imposes the question about how $CD8\alpha^+$ cDCs can respond directly to polyU. Previous publications (47) showed by semiquantitative RT-PCR that $CD8\alpha^+$ cDCs do not express TLR7. Therefore, to address this very important question we determined the expression of TLR7 in all spleen DC subsets by quantitative RT-PCR. As expected, we

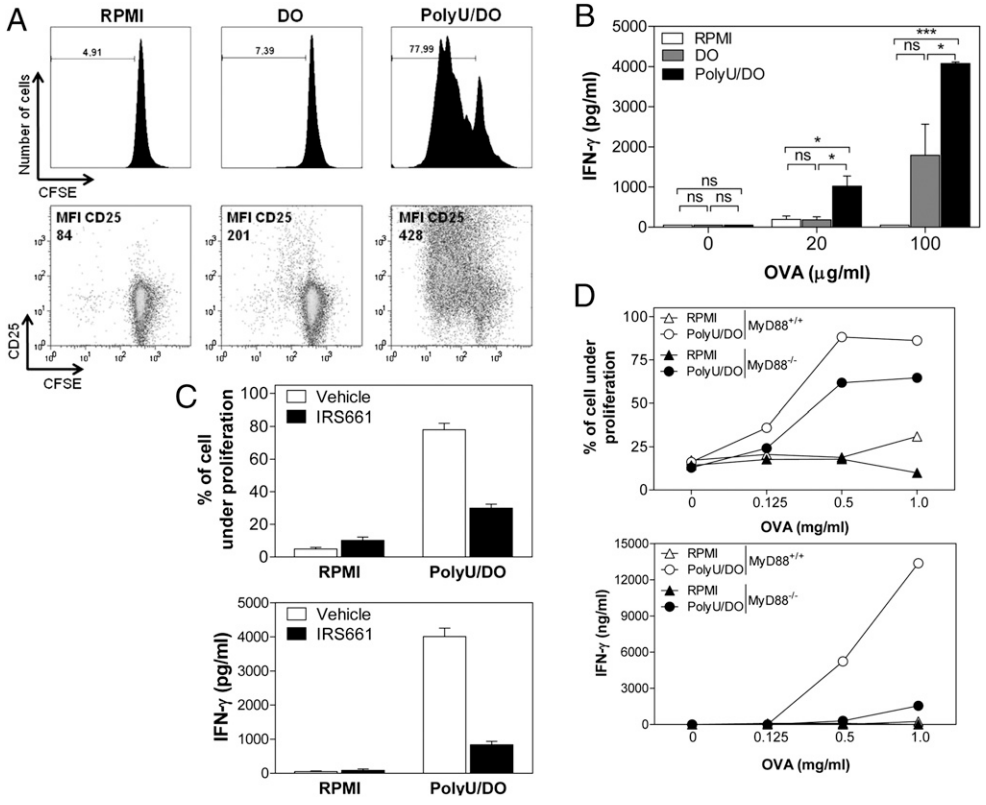

FIGURE 2. PolyU licenses DCs to cross-prime CD8$^+$ T cells in a TLR7-dependent fashion. Splenic DCs cells from C57BL/6 mice were incubated with 20 or 100 μg/ml OVA alone (RPMI) or mixed with 20 μg/ml polyU/DO or DOTAP alone (DO) for 120 min. DCs were then washed twice and cultured during 3 d with CFSE-labeled CD8β$^+$ cells isolated from spleens of OT-I mice. After culture, cells were labeled with anti-CD3, anti-CD25 Abs, and 7-AAD, and T cell proliferation and CD25 expression were analyzed by flow cytometry. (**A**) Representative T cell proliferation (*top*) and CD25 expression (*bottom*) are shown. Results correspond to experiments with 20 μg/ml OVA and are representative of three independent experiments. (**B**) IFN-γ content in supernatants from CD8β$^+$ OT-I cells cultured with DCs, assessed by ELISA. Data show the mean values ± SEM of triplicate cultures and are representative of three independent experiments. (**C**) T cell proliferation and IFN-γ content in supernatants from CD8β$^+$ OT-I cells cultured with DCs incubated with 100 μg/ml OVA alone (RPMI) or mixed with 20 μg/ml polyU/DO as above stated in the presence (IRS661) or absence (vehicle) of 0.3 μM IRS661. Data show the mean values ± SEM of triplicate cultures and are representative of two independent experiments. (**D**) T cell proliferation and IFN-γ content in supernatants from CD8β$^+$ OT-I cells cultured with DCs from wild-type and MyD88$^{-/-}$ mice incubated with OVA alone (RPMI) or mixed with polyU/DO. Data shown are representative of two independent experiments. *$p < 0.05$, ***$p < 0.001$.

found high levels of TLR7 mRNA in pDCs and CD8α$^-$ cDCs. However, we also detected a weaker but positive signal of TLR7 mRNA in CD8α$^+$ cDCs (Fig. 3B). This clearly suggests that CD8α$^+$ cDCs alone are not only able to provide the antigenic signal upon direct stimulation with polyU, but they also are endowed with the ability to give costimulatory signals to CD8$^+$ T cells. However, this last conclusion does not exclude that CD8α$^+$ cDCs can receive help from other cells.

Recent studies have highlighted the relevance of type I IFNs for the activation of CD8$^+$ T cell responses, and in particular they have showed the crucial role for the adjuvant activity of TLR7 ligands (22, 48). Therefore, to determine whether type I IFNs are playing a role in the adjuvant capacity of polyU, we conducted a CD8$^+$ T cell proliferation assay using mice deficient in IFN-αβR (IFNAR$^{-/-}$). We found that DCs from IFNAR$^{-/-}$ mice incubated with polyU/DO plus OVA were unable to induce proliferation (Fig. 3C, *top panel*), upregulation of CD25 (Fig. 3C, *bottom panel*), and IFN-γ secretion (data not shown) in CD8$^+$ T cells, clearly indicating that type I IFNs are critical for cross-priming of CD8$^+$ T cells by DCs incubated with polyU. CD8α$^+$ cDCs alone activate CD8$^+$ T cells without an external source of type I IFNs (Fig. 3A). We then studied the ability of CD8α$^+$ cDCs purified from the spleen of IFNAR$^{-/-}$ mice to cross-prime CD8$^+$ T cells. We observed that CD8α$^+$ cDCs from IFNAR$^{-/-}$ mice incubated with polyU/DO plus OVA activated proliferation (Fig. 3D, *top panel*) but not CD25 upregulation (Fig. 3D, *bottom panel*) or IFN-γ secretion (data not shown) in CD8$^+$ T cells. The proliferative response observed with CD8α$^+$ cDCs from IFNAR$^{-/-}$ mice could be due to the well-known ability of DCs isolated after tissue disruption to proportionate antigenic stimulus (49). Collectively, these results indicate that CD8α$^+$ cDCs require type I IFNs to engage full differentiation of naive CD8$^+$ T cells into effector CD8$^+$ T cells and they suggest that the CD8α$^+$ cDC itself would be the source of type I IFNs.

DCs treated with polyU have an enhanced ability to present Ag in MHC I molecules

To elucidate the specific steps involved in the capability of polyU to enhance cross-priming by DCs, we studied the expression of specific $OVA_{257–264}$/H-2K^b complexes on the surface of polyU-stimulated DCs. Splenocytes were incubated with polyU/DO plus OVA or DO plus OVA or OVA alone to allow Ag internalization and processing and then chased for the times indicated. Once harvested, the splenic cells were labeled with the Ab 25-D1.16, which recognizes the $OVA_{257–264}$/H-2K^b complex, plus anti-CD11c, anti-B220, anti–PDCA-1, and anti-CD8α Abs to discriminate between DC subpopulations. At all of the chasing times evaluated, $OVA_{257–264}$/H-2K^b expression was higher in all DC subsets when they were stimulated with polyU/DO plus OVA as compared with DO plus OVA or with OVA alone (Fig. 4A). In all

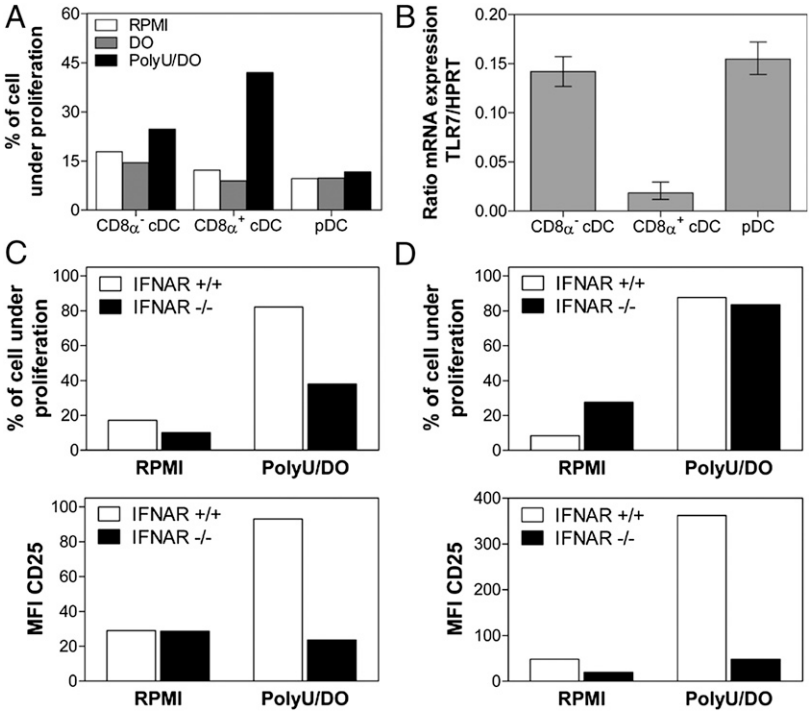

FIGURE 3. PolyU licenses CD8α$^+$ cDCs to cross-prime CD8$^+$ T cells in a type I IFN–dependent fashion. (**A**) Splenic CD8α$^-$ cDCs, CD8α$^+$ cDCs, and pDCs from C57BL/6 mice were incubated with 1 mg/ml OVA alone (RPMI) or mixed with 20 μg/ml polyU/DO or DOTAP (DO) for 120 min. DCs were then washed twice and cultured during 3 d with CFSE-labeled CD8β$^+$ cells isolated from spleens of OT-I mice. After culture, cells were labeled with anti-CD3 and anti-CD25 Abs and 7-AAD, and T cell proliferation and CD25 expression were analyzed by flow cytometry. (**B**) Total RNA of 1×10^6 CD8α$^+$ cDCs, CD8α$^-$ cDCs, and pDCs independently was extracted with TRIzol reagent and the mRNA for TLR7 was quantified by quantitative RT-PCR. (**C** and **D**) Whole (C) or CD8α$^+$ (D) splenic CD11c$^+$ cells from C57BL/6 or IFNAR$^{-/-}$ mice were incubated with 0.5 mg/ml ml OVA alone (RPMI) or mixed with 10 μg/ml polyU/DO for 120 min. DCs were then washed twice and cultured during 3 d with CFSE-labeled CD8β$^+$ cells isolated from spleens of OT-I mice. After culture, cells were labeled with anti-CD3 and anti-CD25 Abs and 7-AAD, and T cell proliferation (*top*) and CD25 expression (*bottom*) were analyzed by flow cytometry. Data shown are representative of two experiments.

cases, the signal of the 25-D1-16 Ab in DCs incubated with DO plus OVA or OVA alone was similar to that in untreated DCs (dotted line).

CD8α$^+$ cDCs have been shown in many experimental systems to cross-present Ags more efficiently than do CD8α$^-$ cDCs (50–53). Indeed, when the different subpopulations were analyzed, we

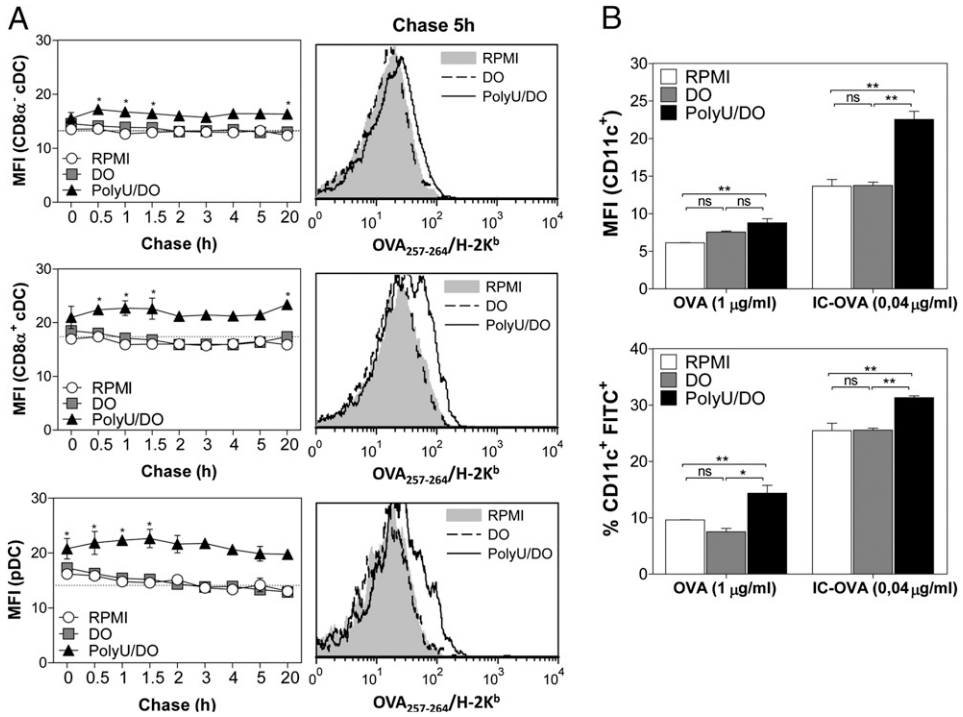

FIGURE 4. PolyU potentiates OVA cross-presentation in splenic DCs. (**A**) Spleens of mice previously injected s.c. with B16-Flt3L melanoma cells were incubated with 1 mg/ml OVA alone (RPMI) or mixed with 20 μg/ml polyU/DO or DOTAP (DO) at 37°C for 4 h. Splenocytes were then washed twice and incubated at 37°C for the indicated times. Once harvested, cells were labeled with Abs to identify DC subpopulations (CD8α$^-$ cDCs, CD8α$^+$ cDCs, and pDCs) and with an Ab that recognizes $OVA_{257–264}$/H-2K^b complexes and then analyzed by flow cytometry. *Left*, Kinetics of the MFI of the Ab anti-$OVA_{257–264}$/H-2K^b complex on DC surface. (As a guide, the dotted line represents the MFI of cells cultured without OVA.). *Right*, Representative overlaid histograms for each DC subpopulation are shown, corresponding to 5 h incubation after Ag wash. *$p < 0.05$ compared with basal. (**B**) Splenic cells from C57BL/6 mice were incubated with 1 μg/ml OVA-FITC or with IC-OVA-FITC (containing 0.04 μg/ml OVA) alone (RPMI) or in the presence of DOTAP (DO) or polyU/DO for 90 min at 37°C. Cells were then washed, stained with anti-CD11c, and analyzed by flow cytometry. Data are representative of two experiments and are expressed as the mean values ± SEM of the geometric MFI of the FITC channel in gated CD11c$^+$ cells and as the means ± SEM of the percentage of FITC$^+$ DCs in triplicate culture wells. *$p < 0.05$, **$p < 0.01$.

found that both CD8α$^+$ cDCs and pDCs showed higher levels of $OVA_{257–264}$/H-2K^b expression than did CD8α$^-$ cDCs. However, a different kinetics in $OVA_{257–264}$/H-2K^b expression was found between CD8α$^+$ cDCs and pDCs. Both curves showed a maximum value at 90 min chase, but in CD8α$^+$ cDCs the expression level was maintained for at least 20 h. In contrast, pDCs showed a reduction of $OVA_{257–264}$/H-2K^b expression after 5 h chase and remained positive but low throughout the duration of the study. The enhanced ability of DCs treated with polyU to present Ag in MHC I molecules was confirmed by an MHC I Ag presentation assay using the B3Z CD8$^+$ T cell hybridoma, specific for the MHC I (K^b)–restricted $OVA_{257–264}$ epitope. DCs were cultured in the presence of polyU with OVA preincubated with rabbit IgG to form IC-OVA (54), which enhances OVA uptake (data not shown). DCs pulsed with IC-OVA upon stimulation with polyU (55) efficiently presented the $OVA_{257–264}$ epitope to B3Z cells (Supplemental Fig. 3).

DCs treated with polyU have an enhanced ability to internalize Ag

To elucidate the mechanisms underlying the increased OVA cross-presentation in polyU-stimulated DCs, we first evaluated the capture of soluble and FcR-mediated endocytosis of OVA. We incubated splenic cells with soluble OVA coupled to FITC (OVA-FITC) or OVA-FITC forming immune complexes with rabbit IgG anti-OVA (IC-OVA-FITC) in the presence of polyU/DO, DOTAP alone, or medium. Cells were then washed, stained with anti-CD11c, and analyzed by flow cytometry. A clear increase in the FITC signal was observed when DCs were stimulated with polyU/DO as compared with the basal condition for IC-OVA-FITC and, to a lesser extent, for OVA-FITC (Fig. 4B). This effect was due to an increase in both the amount of internalized OVA per DC (Fig. 4B, *top panel*) and the number of cells that internalize OVA (Fig. 4B, *bottom panel*).

Priming of CD8$^+$ T cells by polyU requires Ag translocation to the cytosol and proteasome in DCs

As for many other exogenous Ags, cross-presentation of OVA by DCs involves degradation of OVA fragments by the proteasome (56, 57). To determine whether the proteasome is still required for OVA degradation upon polyU stimulation in DCs, we repeated the CD8$^+$ T cell proliferation assay preincubating DCs with polyU/DO plus OVA and lactacystin, a specific proteasome inhibitor (58, 59). Cell proliferation (Fig. 5A) and IFN-γ secretion (Fig. 5B) were severely reduced when DCs were stimulated with polyU/DO plus OVA in the presence of lactacystin. These results demonstrate that the cross-presentation of OVA by polyU-stimulated DCs still requires proteasome and that polyU stimulation does not change the cytosolic pathway of OVA processing, that is, OVA is endocytosed and then translocated to the cytoplasm where it is degraded by the proteasome.

Sec61 channel is the major pathway for retrotranslocation of misfolded endoplasmic reticulum (ER) proteins for subsequent proteolytic degradation by proteasome in the cytosol (60) and it has been involved in the process that facilitates the translocation of internalized proteins into the cytosol of DCs (61), allowing cross-presentation. As showed in Fig. 5C, OVA cross-presentation by polyU/DO plus OVA–stimulated DCs was absolutely inhibited by coincubation of DCs with *Pseudomonas aeruginosa* exotoxin A (ExoA), an alleged inhibitor of Sec61 channel, (61) by knocking down the Sec61 complex (57). As control, we performed a cell proliferation assay using CD4$^+$ T cells from OT-II mice plus DCs preincubated with polyU/DO plus OVA and ExoA. Under these experimental conditions, CD4$^+$ T proliferation was unaffected by ExoA, showing that ExoA blocks a specific step in the MHC I Ag processing pathway (data not shown). Collectively, these results strongly support a role for the Sec61 complex in MHC I cross-presentation of OVA by polyU-stimulated DCs.

PolyU regulates Ag degradation in endophagosomal compartments and MHC I stability on DCs

It has been demonstrated that pH in phagosomes and endosomes is less acidic in DCs than in other phagocytes, such as macrophages and neutrophils (62–64). Acidic pH, with the consequent activation of endosomal proteases, could degrade endocytosed Ag until antigenic epitopes are destroyed (65). We therefore hypothesized that polyU/DO plus OVA–stimulated DCs may either have higher or equal pH values than nonstimulated DCs. To determine the modulation of pH in DC phagosomes by polyU, DCs were allowed to phagocytose polystyrene beads bearing a mixture of pH-sensitive (FITC) and -insensitive (Alexa 647) dyes alone or mixed with polyU/DO or polyU/DO plus IRS661. Changes in phagosomal pH were measured by flow cytometry using a procedure adapted from Savina et al. (63) and it was expressed as arbitrary units of alkalinisation. As shown in Fig. 6A, phagosomes in polyU/DO–stimulated DCs have a significantly higher alkalinisation level than that of nonstimulated DCs (RPMI 1640). DOTAP-incubated DCs had a similar result than nonstimulated DCs (data not shown). When IRS661 was added together with

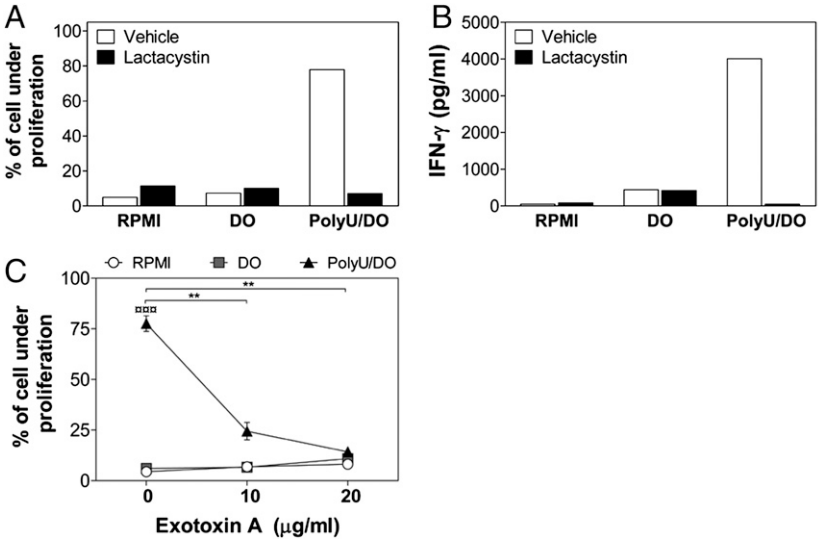

FIGURE 5. Priming of CD8$^+$ T cells by polyU requires Ag translocation to cytosol and proteasome in DCs. Splenic DCs from C57BL/6 mice were incubated with 100 μg/ml OVA alone (RPMI) or plus polyU/DO or plus DOTAP (DO) in the presence or absence (vehicle) of 5 μg/ml lactacystin (**A**, **B**) or 10 or 20 μg/ml ExoA (**C**) for 120 min. DCs were then washed twice and cultured during 3 d with CFSE-labeled CD8β$^+$ cells isolated from spleens of OT-I mice. (A and C) T cell proliferation was analyzed by flow cytometry after labeling cells with an anti-CD3 Ab and 7-AAD. (B) IFN-γ content in supernatants from CD8β$^+$ OT-I cells cultured with DCs as assessed by ELISA. Data in (A) and (B) are representative of two independent experiments. Results in (C) show the mean values ± SEM of triplicate cultures and are representative of two independent experiments. $^{**}p < 0.01$ for comparison between polyU/DO groups, $^{□□□}p < 0.001$ with respect to RPMI 1640 and DOTAP groups without ExoA.

polyU/DO, the values observed were similar to those in nonstimulated DCs. After 30 min chase, all groups decreased their pH value toward a similar, more acidic value. This behavior was similar in both CD8α$^-$ and CD8α$^+$ cDCs (Supplemental Fig. 4).

We next evaluated the functional consequences of such differences in phagosomal pH in terms of Ag degradation. To follow Ag degradation selectively in phagosomes, a quantitative cytofluorometric assay for phagosomal degradation described by Savina et al. (63) was performed. Purified DCs were incubated with OVA beads in the presence of polyU/DO and then washed and chased for 20 min. DCs were then lysed and the amount of OVA remaining on the beads was quantified by flow cytometry using a polyclonal anti–OVA-specific IgG. Ag degradation was expressed as an arbitrary degradation value, where a lower fluorescence signal is consistent with higher OVA degradation and then with a higher degradation value. As shown in Fig. 6B, a marked increase in the degradation value was observed in nonstimulated DCs after 20 min pulse and 20 min chase. A similar result was found in DOTAP-incubated DCs. In contrast, polyU/DO–stimulated DCs showed low degradation until 60 min chase (data not shown). As control, we added a mixture of protease inhibitors to untreated DCs and no degradation was observed (Fig. 6B).

Taken together, these results demonstrate that polyU promotes Ag preservation through alkaline pH and lower Ag degradation in DCs for a short period of time. This could account for the higher OVA cross-presentation observed in TLR7-stimulated DCs.

PolyU regulates Ag/MHC I stability on DCs

Previous reports indicate that inflammatory stimuli can modulate the expression of peptide/H-2K^b complexes by extending the half-life of MHC I molecules expressed on the surface of mature DCs (49, 66, 67). We therefore hypothesized whether polyU/DO plus OVA–stimulated DCs may have more stable OVA$_{257–264}$/H-2K^b complexes on their cell surface throughout the coincubation period, thus increasing the Ag availability on the DC surface and allowing more TCRs on CD8$^+$ T cells to be triggered. To examine this possibility, splenic DCs were incubated with OVA$_{257–264}$ peptide alone or mixed with polyU/DO or DOTAP at 37˚C for 90 min. OVA$_{257–264}$ peptide was used instead of whole OVA to avoid interference from the differences in Ag capture and processing observed between stimulated and unstimulated DCs. Cells were then labeled with the 25-D1.16 mouse IgG1 Ab, washed, and chased at 37˚C for the indicated times. Once harvested, the cells were labeled with anti-mouse IgG1 to detect the amount of anti-OVA$_{257–264}$/H-2K^b complexes remaining on the surface of DC subpopulations after chase. Treatment of DCs with polyU/DO resulted in a greater persistence of OVA$_{257–264}$/H-2K^b expression on the surface of these cells (Fig. 6C). However, when DC subsets were analyzed, we found that both CD8α$^+$ cDCs and pDCs incubated with polyU showed higher levels of fluorescence intensity for all times assayed than did CD8α$^-$ cDCs and also compared with incubation with DOTAP or medium only. These results

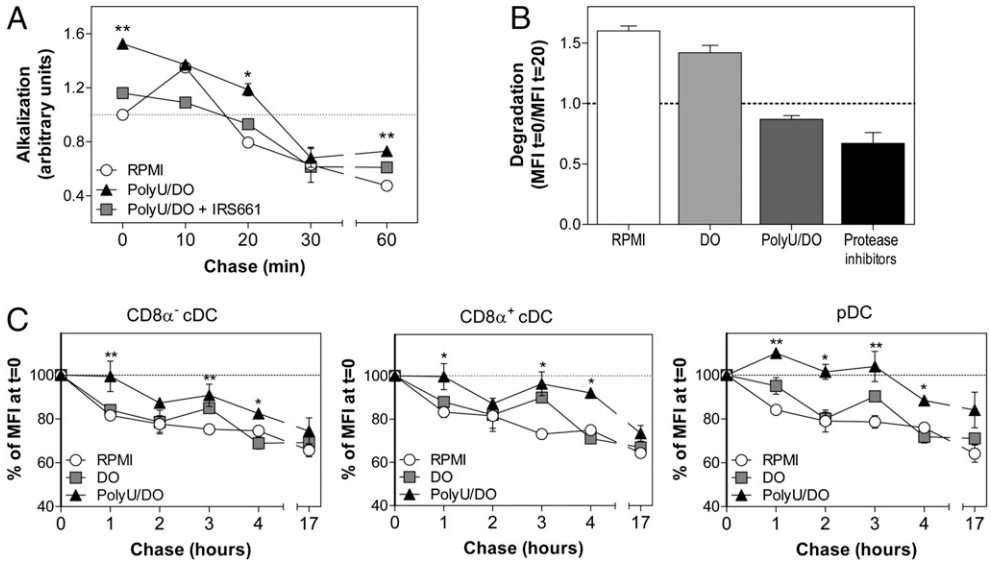

FIGURE 6. PolyU regulates Ag degradation in endophagosomal compartments and stability of MHC I/peptide complexes on DCs. (**A**) Kinetics of endophagosomal pH in polyU/DO–stimulated DCs. Splenic DCs (1×10^6) from C57BL/6 mice were incubated with 9×10^7 polystyrene beads bearing FITC and Alexa 647 for 15 min alone (RPMI) or in the presence of 20 μg/ml polyU/DO alone (polyU/DO) or plus 0.3 μM IRS661 (polyU/DO plus IRS661) and then extensively washed and incubated for as long as indicated. At the end of incubation, cells were analyzed by flow cytometry. For normalization purposes, data from three separate experiments were combined and expressed with an alkalization ratio, which is the ratio between $R_{\text{Alexa 647/FITC}}$ at the respective chase time and $R_{\text{Alexa 647/FITC}}$ at time 0, where $R_{\text{Alexa 647/FITC}}$ is the ratio between MFI for Alexa 647 and MFI for FITC of each sample. As a guide, the dotted line represents the $R_{\text{Alexa 647/FITC}}$ at time 0. $^*p < 0.05$, $^{**}p < 0.01$ with respect to RPMI 1640. (**B**) Quantification of OVA degradation in phagosomes from polyU/DO–stimulated DCs. Splenic DCs (10×10^6) from C57BL/6 mice were incubated with 50×10^6 OVA beads for 2 h alone (RPMI) or plus DOTAP (DO) or in the presence of 20 μg/ml polyU/DO or mixed with a mixture of protease inhibitors, and afterward extensively washed and incubated for 20 min. DCs were then lysed and free beads were labeled with a polyclonal Ab against OVA and analyzed by flow cytometry. For normalization purposes, data from three separate experiments were combined and expressed as the ratio between the MFI at time 0 and the MFI at chase time. As a guide, the dotted line represents that ratio at time 0. (**C**) Spleen cells from mice previously injected s.c. with B16-FLt3L melanoma cells were incubated with 0.1 μg/ml OVA$_{257–264}$ alone (RPMI) or mixed with 20 μg/ml polyU/DO or DOTAP alone (DO) at 37˚C for 90 min. Splenocytes were then labeled with a mAb specific for the OVA$_{257–264}$/H-2K^b complex, washed, and incubated at 37˚C for as long as indicated. Once harvested, cells were labeled with PE-anti-IgG to detect the remainder of OVA$_{257–264}$/H-2K^b complexes on the cell surface and with Abs to identify DC subpopulations (CD8α$^-$ cDCs, CD8α$^+$ cDCs, and pDCs). Cells were then analyzed by flow cytometry. For normalization purposes between different DC subpopulations, data from each group are expressed as the ratio, in percentage, between the MFI_{PE} at each time and the MFI_{PE} at time 0. As a guide, the dotted line represents the ratio at time 0 (100%). Data are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ with respect to RPMI 1640 conditions in each case.

correlated with those observed in the kinetics of $OVA_{257–264}$/H-$2K^b$ appearance in DC subsets (Fig. 4A).

Discussion

Cross-presentation can be enhanced by TLR ligands such as LPS (a TLR4 ligand) (14, 67, 68), CpG (a TLR9 ligand) (11, 12, 17, 20, 67), or polyinosinic-polycytidylic acid (a TLR3 ligand) (12, 15, 20, 67). Most recently, it has been demonstrated that stimulation of DCs with TLR7 ligands such as R848 (17), synthetic RNA oligonucleotides (35, 36), influenza-infected allogeneic cell lines (23), or polyUs21 (22) enhances cross-priming of $CD8^+$ T cells. However, to our knowledge, no one had addressed how TLR7 ligands can affect the mechanisms of Ag cross-presentation in DCs. In this study, we demonstrate that polyU, a synthetic TLR7 ligand, stimulates OVA cross-priming by enhancing OVA cross-presentation in $CD8\alpha^+$ cDCs. After polyU stimulation, splenic DCs cross-prime naive $CD8^+$ T cells, leading to their differentiation into functional cytotoxic cells in vivo. This in vivo cross-priming required $CD4^+$ Th1 cooperation cells for the induction of a correct CTL response.

The "classical" view of TLR ligand–mediated adjuvanticity on a T cell response is based in large part on providing stimulation through mediators collectively grouped in signals 2 (i.e., costimulatory molecules) and 3 (polarizing cytokines). Activation of DCs by polyU not only supplied a strong signal 2 and 3 to $CD8^+$ T cells, but it also clearly provided a stronger signal 1, because polyU increased OVA presentation to $CD8^+$ T cells by changing the Ag density on the DC surface resulting from upregulation of MHC I Ag processing and presentation.

In mice, DCs can be classified in several subsets by the mean of different surface markers (69). Three subpopulations have been recognized in murine spleen: $CD8\alpha^+$ cDCs, $CD8\alpha^-$ cDCs, and pDCs. The functional relevance of DC heterogeneity is still not fully understood, but each subpopulation expresses different sets of receptors, including TLRs, produce different cytokines and chemokines, and present Ags with variable efficiency to different types of T lymphocytes (69). Among DC subsets, many different experimental systems have revealed that $CD8\alpha^+$ cDCs are the most efficient cell cross-presenting Ags to $CD8^+$ T cells (29, 50–53). We have observed that after incubation of all DC subpopulations together with polyU/DO plus OVA, all showed $OVA_{256-264}$/H-$2K^b$ complexes on the cell surface, particularly in $CD8\alpha^+$ cDCs and, strikingly, in pDCs. However, upon TLR7 stimulation of sorted DCs subsets with polyU, only $CD8\alpha^+$ cDCs were able to sustain $CD8^+$ T cell proliferation, CD25 expression, and IFN-γ secretion, whereas $CD8\alpha^-$ cDCs and pDCs were absolutely incapable of such priming. Therefore, in this study we demonstrate that $CD8\alpha^+$ cDCs were the DC population responsible for cross-priming of $CD8^+$ T cells without strictly requiring additional help of any other cell. This result clearly suggests that $CD8\alpha^+$ cDCs were not only able to provide the antigenic signal but also were endowed with signals 2 and 3 to allow in vitro cross-priming of $CD8^+$ T cells. However, this direct activation imposes the question about how $CD8\alpha^+$ cDCs can respond directly to polyU if, according to other studies (47, 70), this population does not express TLR7. We addressed this point by quantitative RT-PCR for TLR7 in all DC subsets in the spleen of C57BL/6 mice. We found that $CD8\alpha^+$ cDCs have low but consistent expression of TLR7 mRNA despite showing huge differences with the expression levels observed in pDCs and $CD8\alpha^-$ cDCs. This low but positive expression of TLR7 transcript is observable in a previous report by Doxsee et al. (70), where they showed by quantitative RT-PCR that both $CD8\alpha^+$ and $CD8\alpha^-$ DCs express TLR7 mRNA, although the latter express 2- to 5-fold more TLR7 than do $CD8\alpha^+$ DCs. Edwards et al. (47) reported no expression of TLR7 mRNA in $CD8\alpha^+$ DCs by using both semiquantitative and quantitative RT-PCR assays, which correlated with lack of IL-12p40 secretion upon R848 stimulation. Nevertheless, a faint mRNA signal was recognizable in both types of PCR assays. The discrepancy with our results could be explained by the type of TLR7 ligand employed, as we have observed that polyU is a much stronger stimulant of IL12p40 secretion in DCs than several imidazoquinoline compounds (data not shown).

Collectively, these results seem to indicate that cross-priming of $CD8^+$ T cells observed upon stimulation with polyU is mediated by $CD8\alpha^+$ cDCs. Thus, the effect of TLR7 on the cross-presenting $CD8\alpha^+$ cDCs would be direct rather than indirect, without excluding that other DC populations can contribute in vivo to the modulation of cross-priming through interaction with $CD8\alpha^+$ cDCs or with $CD8^+$ T cells.

Type I IFNs are induced primarily during viral infections and have been shown to promote NK cell, Th1 cell, and, in particular, CTL responses through stimulation of Ag cross-priming (71) and DC maturation (72–75). It has been reported that type I IFNs are crucial factors regulating the accumulation of DCs in lymph nodes and their maturation into activated APCs during a TLR7-driven response (76). They are also required for intratumoral accumulation of the $CD8\alpha^+$ cDC subset (48) and for their ability to develop antitumoral immunity (75). In this study, we have observed that type I IFNs are strictly required to license $CD8\alpha^+$ cDCs to cross-prime $CD8^+$ T cells, at least in in vitro conditions. Abolishment of cross-priming abilities in $IFNAR^{-/-}$ $CD8\alpha^+$ cDCs suggests that this subset itself would produce the type I IFNs that act in an autocrine loop. Gautier et al. (77) previously showed the need of an autocrine/paracrine loop of type I IFNs for bioactive IL-12p70 secretion by myeloid DCs. Interestingly, Diamond et al. (75) showed that, as opposed to $CD8\alpha^+$ cDCs from wild-type mice, $CD8\alpha^+$ cDCs isolated from $IFNAR^{-/-}$ mice and cultured with irradiated OVA-loaded MHC I–deficient splenocytes induced a poor proliferation of OT-I T cells, indicating that type I IFNs act directly on $CD8\alpha^+$ cDCs to enhance cross-presentation. However, the source of type I IFNs was not identified in that study. Certainly, under in vivo conditions other sources of type I IFNs, acting in a bystander fashion, are expected to be more relevant than $CD8\alpha^+$ cDCs themselves. Indeed, Wei et al. (23) have reported that pDCs and $CD8\alpha^-$ cDCs assist $CD8\alpha^+$ cDCs in cross-priming of Ag-specific $CD8^+$ T cell responses to cell-associated Ags. According to these authors, the enhancement provided by pDCs was likely due to type I IFN production as a result of TLR7 engagement. Moreover, it has been observed by Fuertes et al. (48) that IFN-β induction is comparable between wild-type and $Batf3^{-/-}$ mice (lacking $CD8\alpha^+$ and $CD103^+$ DCs). Considering that when total DCs were incubated with polyU/DO plus OVA, all DC subsets showed $OVA_{257–264}$/H-$2K^b$ complexes upon polyU stimulation, type I IFNs could be participating by stimulating Ag cross-presentation. Another alternative is that type I IFNs can help DCs to upregulate costimulatory molecules. We have observed that after injection of polyU, all DC subsets from wild-type mice upregulated costimulatory molecules (Supplemental Fig. 2) whereas DCs from $IFNAR^{-/-}$ mice were unable to upregulate them (data not shown).

We have extended our results by analyzing which steps in Ag processing in DCs could be modified by polyU to allow the enhancement of $CD8^+$ T cell cross-priming. Ag capture is the first step required for Ag presentation and in this study we provide evidence that splenic DCs stimulated with polyU improved their intrinsic capacity to capture soluble and particulate Ags. Mannose and FcR-mediated endocytosis of OVA and phagocytosis of OVA

particles seem to share the same general pathways of Ag processing for MHC I presentation, requiring transport of Ag from early endosomes or phagosomes to the cytoplasmatic proteasome, followed by TAP-mediated transport of Ag-derived peptides to MHC I–containing organelles (78–81). Macrophages and neutrophils exhibit a well-developed capacity for lysosomal proteolysis. After internalization of proteins, pH in phagosomes and endosomes drops very rapidly to values <5 and remains acidic for several hours, allowing a strong activation of the lysosomal proteases (65). Conversely, DCs degrade internalized proteins at a much lower rate than both macrophages and neutrophils (82, 83), and pH in phagosomes and endosomes is less acidic in DCs than in macrophages (62–64). A role has been established for low proteolysis and high pH in cross-presentation, in which limiting the endophagosomal proteolytic capacity serves to preserve Ags from complete degradation (63–65). These Ags could then be loaded on MHC I molecules for Ag cross-presentation. However, the role of TLRs in this particular feature of DCs has not been addressed yet. In this study, we demonstrated that both $CD8\alpha^+$ and $CD8\alpha^-$ cDCs have, at short times after stimulation with polyU, a more alkaline phagosomal pH compared with untreated cells, taking a longer time to drop pH values to those found in untreated cells. This slowed acidification in endophagosomal organelles would prevent excessive Ag degradation. This assumption was corroborated in this study by the diminished OVA degradation observed at short periods of time in polyU-stimulated DCs. The ability of DCs to avoid rapid degradation of internalized Ags may contribute to their capacity to cross-present exogenous Ags on MHC I by favoring more Ags reaching the cytosol than it would be expected in unstimulated DCs or, even more, in macropages (83). How TLR stimulation is related to alkalization of pH in phagosomes is still not very well understood.

OVA cross-presentation is still proteasome-dependent in polyU-treated DCs, suggesting that Ag escapes from the phagosome into the cytosol for proteasome digestion. It has been proposed that one possibility for delivering proteins into the cytosol might be through a membrane pore, using some protein complexes responsible for transporting misfolded proteins out of the ER, such as Sec61 (61, 84), Der1, and others (85). For example, the Sec61 channel has been involved in the process that facilitates the translocation of internalized proteins into the cytosol of DCs (61) to allow cross-presentation. In addition to ER-derived protein channels, other hypotheses of how peptides or proteins travel from the phagosome to the cytoplasm for cross-presentation have been proposed, including rupture of the phagolysosomal membrane (85), by which peptides might traverse the lipid bilayer and gain access to the cytosol without the need for a conventional energy-dependent protein channel. However, the inhibition of $CD8^+$ T cell proliferation observed when DCs were incubated in the presence of polyU plus ExoA strongly supports the participation of Sec61 translocon in polyU-stimulated cross-presentation and clearly excludes leakage of OVA by phagolysosomal membrane rupture as the primary mechanism. In this way, Sec61 could be the channel through which OVA or some OVA fragments would be allowed to reach the cytosol for proteasome digestion and then to regain access to an MHC I–containing organelle in polyU-activated DCs, presumably by TAP molecules. TAP is present in OVA-containing ER-phagosome vesicles (78, 80, 81) and endosomes (79), reimporting antigenic peptides into these organelles, which then travel from endosomes to the DC surface. Recruitment of TAP to early endosomes is dependent on MyD88 (79). Thus, relocation of TAP to early endosomes by a MyD88-dependent mechanism allows Ag entry for subsequent cross-presentation. Because TLR7 signals through MyD88, polyU could be also participating by relocation of TAP to endophagocytic organelles, a hypothesis that remains to be confirmed.

PolyU also increased the expression and the stability of $OVA_{257–264}$/H-$2K^b$ complexes on the DC surface. This finding differs from previous data of CpG-mediated cross-presentation enhancement (86), which is at least in part mediated by an increased total MHC I surface expression, controlled by augmenting the half-life and then the stability of total MHC I mRNA and not by an enhanced stability of $OVA_{257–264}$/H-$2K^b$ complexes, as previously reported for LPS (49).

PolyU-mediated enhancement of $OVA_{257–264}$/H-$2K^b$ stability is clearly related to the capacity of DC subsets to cross-present Ag, with $CD8\alpha^+$ cDCs and pDCs showing higher complex expression than for $CD8\alpha^-$ cDCs. These observations suggest that $CD8\alpha^-$ cDCs and pDCs possess a machinery to deliver exogenous Ags to the MHC I pathway; contrary to $CD8\alpha^+$ cDCs, however, the machinery in pDCs does not allow cross-priming of $CD8^+$ T cells under the conditions assayed in our model with polyU. We have shown elsewhere that pDCs stimulated with R848, an imidazoquinoline derivative that is a TLR7 ligand, can effectively trigger a strong CTL response (17). We have also demonstrated that $CD8\alpha^-$ cDCs can cross-prime a naive $CD8^+$ T response in vivo (29) through TLR9 signaling (our unpublished results).

Finally, nucleic acid–based ligands of TLRs have been shown to induce Th1 type immune responses (87). However, there are different reports showing that immunization with TLR7 ligands induce preferably the isotype IgG1, which is associated with a Th2 phenotype (36, 88, 89). Because of this contradiction, it was important to determine the humoral response induced by immunization with polyU. The results indicated that polyU/DO plus OVA induced IgG1 and IgG2c Abs, with the latter being an isotype preferentially produced during Th1 responses. This is in accordance with IFN-γ secretion and the lack of IL-5 production by T cells in polyU/DO plus OVA. A possible explanation for this discrepancy may be the difference between the TLR7 ligands used.

In summary, our data provide evidence that polyU is a potent adjuvant for CTL induction, as polyU/DO is capable of initiating cytotoxic immune responses mediated by $CD8^+$ T cells and the generation of Th1 responses. This effect is mediated by enhancing signals 1, 2, and 3 in $CD8\alpha^+$ cDCs in a type I IFN– and TLR7-dependent fashion. Enhancement of Ag presentation by MHC I molecules in DCs is mediated by Ag preservation in endophagosomal organelles that would allow a higher input of Ag into the proteasome machinery and a higher Ag permanence on the DC surface associated to MHC I molecules. In this way, polyU can be considered as an interesting adjuvant candidate for future tumor immunotherapy and for vaccination against pathogens.

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Disclosures

The authors have no financial conflicts of interest.

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1

Humoral response in mice immunized with OVA and polyU

C57BL/6 mice were immunized on days 0 and 21 with an i.v. injection of 60 µg OVA plus 100 µg of polyU complexed with 60 µg DOTAP (polyU/DO) or in DO alone. As control, some mice were immunized i.v. with saline or polyU/DO without OVA or s.c. with aluminum hydroxide mixed with 60 µg OVA (AlumOVA). One week after the second immunization, OVA-specific **A)** IgG, **B)** IgG_1, and **C)** IgG_{2c} titers were determined in serial dilutions of sera by ELISA. Data show the mean values of individual mice (n≥4) ± SEM of two independent experiments. (**p<0.01, ***p<0.001 compared to normal and polyU/DO, ¤¤ p<0.01, ¤¤¤ p<0.001, ns, not significant vs saline).

Figure S2

PolyU stimulates in vivo DC maturation in a MyD88-dependent fashion

A B and C) C57BL/6 mice were injected i.v. with saline solution or 100 µg polyU complexed with 60 µg DOTAP (polyU/DO) (A-C) or with DOTAP alone (A,B). 18 h later, **A)** splenic cells were labeled with antibodies to identify DC subpopulations and for CD86 and analyzed by flow cytometry. **B)** Also sera were collected and assayed for IL-12p70 by ELISA. Data show the mean ± SD with n≥4. **C)** Comparison of CD86 expression between wild-type and $MyD88^{-/-}$ mice. **D)** $1x10^6$ $CD8\alpha^+$ cDCs, $CD8\alpha^-$ cDCs and pDCs were purified and incubated with 20 µg/ml polyU/DO alone or plus 0.3 µM IRS661,

DOTAP alone or only with medium for 5 h. Total RNA was extracted with TRIZOL reagent and the mRNA for *IFN-α4* was quantified by qRT-PCR.

Figure S3

Antigen presentation assay using a T cell hybridoma as reporter

Splenic DCs (0.5, 1 or $2x10^5$ cells/well) were pulsed with 0.1µg/ml $OVA_{256-264}$ alone or 3 mg/ml OVA mixed with 20 µg/ml polyU complexed with protamine, protamine alone or medium only at 37 °C for 4 h in 96-well culture microplates. Then, cells were washed twice and incubated overnight at 37 °C with 10^5 B3Z cells/well, an $OVA_{257-264}$-specific $CD8^+$ T cell hybridoma. The stimulation of B3Z cells was monitored by colorimetric bulk determination of β-galactosidase activity in PBS-washed B3Z cells incubated during 4 h with 0.15 mM chlorophenolred-β-D-galactopyranoside (CPRG, Roche Diagnostics Corporation) in 100 mM β2mercaptoethanol, 9 mM $MgCl_2$ and 0.125 % detergent NP40 (IGEPAL CA 630, Sigma-Aldrich) in PBS. Results are representative of two independent experiments.

Figure S4

Kinetics of endophagosomal pH in polyU/DO-stimulated CD8α+ and CD8α- DCs

$1x10^6$ splenic $CD11c^+$ cells from C57BL/6 mice were incubated with $9x10^7$ polystyrene beads bearing FITC and Alexa647 for 15 min alone (RPMI) or in the presence of 20 µg/ml polyU/DO alone (polyU/DO) or plus 0.3 µM IRS661 (polyU/DO+IRS661) and then

extensively washed and incubated for as long as indicated. At the end of incubation, cells were labeled for CD11c and CD8α and analyzed by flow cytometry. For normalization purpose, data from three separate experiments were combined and expressed with an alkalization ratio which is the ratio between $R_{Alexa647/FITC}$ at the respective chase-time and $R_{Alexa647/FITC}$ at time 0, where $R_{Alexa647/FITC}$ is the ratio between MFI for Alexa647 and MFI for FITC of each sample. As a guide, the dotted line represents the $R_{Alexa647/FITC}$ at time 0 without stimulation. (*$p<0.05$, **$p<0.01$ with respect to basal conditions).