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A Subset of Toll-Like Receptor Ligands Induces Cross-presentation by Bone Marrow-Derived Dendritic Cells

Sandip K. Datta,* Vanessa Redecke,* Kiley R. Prilliman,† Kenji Takabayashi,* Maripat Corr,* Thomas Tallant,‡ Joseph DiDonato,§ Roman Dziarski,§ Shizuo Akira,¶ Stephen P. Schoenberger,† and Eyal Raz‡*

Dendritic cells (DCs) are capable of cross-presenting exogenous Ag to CD8+ T cells. Detection of microbial products by Toll-like receptors (TLRs) leads to activation of DCs and subsequent orchestration of an adaptive immune response. We hypothesized that microbial TLR ligands could activate DCs to cross-present Ag to CTLs. Using DCs and CTLs in an in vitro cross-presentation system, we show that a subset of microbial TLR ligands, namely ligands of TLR3 (polyinosinic-cytidylic acid) and TLR9 (immunostimulatory CpG DNA), induces cross-presentation. In contrast to presentation of Ag to CD4+ T cells by immature DCs, TLR-induced cross-presentation is mediated by mature DCs, is independent of endosomal acidification, and relies on cytosolic Ag processing machinery. The Journal of Immunology, 2003, 170: 4102–4110.

Although the subsets of cells capable of cross-presentation have not been fully delineated, it appears that professional APCs, such as DCs and macrophages, possess this capability (2, 4, 6). DCs seem to be especially good candidates because of their potent ability to stimulate T cells after they endocytose Ags from their surroundings by macropinocytosis, phagocytosis, or receptor-mediated endocytosis (7). Indeed, recent evidence confirms the ability of DCs to cross-prime CD8+ T cells in vivo in mice (8, 9).

Because cross-presentation of self-Ags could lead to autoimmunity, exposure to microbial products, inflammatory cytokines, or other danger signals (10) probably serves as a cue for DCs to activate adaptive immunity, including activation of mechanisms to cross-prime CD8+ T cells. It appears that immature DCs constantly sample their environment without necessarily initiating an immune reaction until activation by a danger signal causes maturation, a state characterized by increased expression of surface MHC and costimulatory molecules, that results in decreased phagocytic ability but increased ability to stimulate T cells (7).

Pathogen-associated molecular patterns are conserved structural motifs derived from microbial products that activate innate immunity (11). The best characterized receptors involved in pathogen-associated molecular patterns recognition are the Toll-like receptors (TLRs) (12, 13). Recognition of TLR ligands by APCs induces cytokine production and surface costimulatory molecule expression, helping to shape an adaptive immune response. The ability of TLR-activated APCs to activate CD4+ T cells and shape a Th1-biased immune response has been well described (13). However, the role of TLRs in priming CD8+ T cell responses has not been thoroughly investigated.

CD8+ CTL responses have been successfully generated against a number of soluble injected Ags when mice have been immunized with the Ag in the presence of TLR9 ligands (14–17), immunostimulatory DNA sequences (ISS) structurally defined by CpG motifs found in bacterial DNA and its synthetic oligodeoxynucleotide analogs (ISS-ODN) (18). Furthermore, ISS-ODN induce in vivo CTL responses in the absence of CD4+ T cell help (16, 17), apparently replacing the CD40-CD40 ligand interaction between APCs and CD4+ T cells that is usually required before an APC can activate CD8+ T cells (19–21). These results suggest that ISS-ODN activate APCs in a manner that allows them to cross-present exogenous Ag to CD8+ T cells. However, direct proof of this mechanism has not yet been generated.

On the basis of these observations, we hypothesized that ISS-ODN and other microbial TLR ligands can directly activate DCs to cross-present Ag to CD8+ T cells in the absence of CD4+ T cell help. To investigate this hypothesis, we developed an in vitro system that demonstrated the ability of TLR ligands to enhance cross-presentation by DCs. Interestingly, despite the ability of all tested
microbial TLR ligands to induce DC maturation, only a subset of these ligands was able to induce cross-presentation. This in vitro system further revealed that TLR-induced cross-presentation occurs in mature DCs, is independent of endosomal acidification, and relies on cytosolic Ag processing machinery.

Materials and Methods

**Animals**

C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). TAPI-deficient mice and recombination activation gene-1-deficient mice on the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME) and then bred at our animal facilities. OT-I (OVA TCR, MHC I-restricted) mice on the C57BL/6 background, expressing a transgenic TCR that recognizes OVA-derived peptide in the context of MHC I (H-2Kb) (22), were a gift from M. Bevan and were subsequently bred at our animal facilities. Male OT-II (OVA TCR, MHC II-restricted) mice on the C57BL/6 background, expressing a transgenic TCR that recognizes OVA-derived peptide in the context of MHC II (I-Ab) (23), were a gift from W. Heath and were subsequently bred at our animal facilities. MyD88-deficient mice on the C57BL/6 background (24) were bred at our animal facilities. All animal procedures followed the University of California, San Diego animal care guidelines.

**B3Z T cell hybridoma assay**

The B3Z T cell hybridoma, containing la27 that is induced upon engagement of its TCR that recognizes OVA peptide (SIINFEKL), in the context of H2Kb (25), was a gift from N. Shastri. β2-Microglobulin, LPS (Sermad, Falmouth, MA), when necessary, to achieve endotoxin levels less than 1 pg/ml, and calf thymus DNA were purchased from Sigma. Single-stranded phosphorothioate oligonucleotides containing CpG motifs (5'-TGACTGTGACGTGGAGATGA-3'), methylated CpG motifs (the two cytosines in the underlined motif were methylated), and mutated CpG motifs (5'-TGACTTGGAGGTAGATAAG-3'), were purchased from Tri-Link Biotechnologie (San Diego, CA).

**Chicken OVA**

To minimize endotoxin and degradative peptide contamination, OVA that was chromatographically purified was purchased from Worthington Biochemicals (Lakewood, NJ).

**Endotoxin decontamination**

All reagents, including OVA and TLR ligands (except for LPS), were tested for endotoxin content by the LAL assay (BioWhittaker, Walkersville, MD) and treated with endotoxin removal resin (Seikagaku America, Falmouth, MA), when necessary, to achieve endotoxin levels ≤1 pg of endotoxin per μg of reagent. This resulted in experimental cultures containing endotoxin at a level below that needed to induce BMDC maturation (100 pg/ml; data not shown).

**RT-PCR**

The transcript levels for the TLRs in BMDCs were determined by RT-PCR. Total RNA was isolated from mouse BMDCs using Trizol (Invitrogen, Carlsbad, CA). The RNA was reverse transcribed to single-stranded cDNA using the Superscript First Strand System (Invitrogen) according to the manufacturer’s protocol. The cDNA was then used for semiquantitative analysis by PCR using primers specific for each TLR. The primer sequences used for PCR are as follows: mL-IR, sense 5'-ATG AAC TTT GAA CAA GGA GAA CCA CCA-3', antisense 5'-TTA GGA GAA CAC AAG TCT CAT GGT-3'; mLTLR2, sense 5'-CAT TGG GTG GAG AAC CTC ATG GTC CAG-3', antisense 5'-CTA GGA CTT TAT TGC ATC TGC CAG ATT-3'; mLTLR3, sense 5'-CGG ATT CTT GGT TCC AAG GAA ATA GAC-3', antisense 5'-TTA ATG TGC ATT CCA ACG AAG TCA ACG-3'; mLTLR4, sense 5'-TGC CTT CCC TAC TAC GAC GAT TTT ATT CTC-3', antisense 5'-TCA GGT CTT AGA TGG TGC TGT CAG CCA-3', antisense 5'-AGG AAG ACA GGGA CGA CAG CAA-3'; mLTLR5, sense 5'-ACC ACA GTC CAT GCC ATC AC-3', antisense 5'-TCC ACC ACC CGT TTG CTG TA-3'.

PCR was performed at 94°C for 30 s and 68°C for 1 min. After 18 cycles, 4 additional cycles were repeated in a stepwise manner, and PCR
products were analyzed after each step on a 1.5% TAE (40 mM Tris acetate and 1 mM EDTA)-agarose gel.

Flow cytometry
All flow cytometry data were acquired on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ) after staining of cells with the indicated Abs in PBS containing 0.5% BSA and 0.05% sodium azide according to standard protocols. Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

In vitro cross-presentation assay
BMDCs were incubated with appropriate stimuli (peptidoglycan, poly(I:C), LPS, flagellin, ISS) overnight. OVA was then added to the cells at indicated concentrations for 2–4 h. When Ag-processing inhibitors were used, the inhibitors (lactacystin, chloroquine, ammonium chloride; Sigma) were added to the cells 15 min before incubation with OVA for 2 h. CD8+ T cells from sex-matched OT-I mice were purified from splenocytes using anti-CD8 magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions. The purified CD8+ T cells were stained in PBS containing 1 μM CFSE (Molecular Probes, Eugene, OR). The nonadherent BMDCs were then washed and coincubated with an equal number of the CFSE-labeled CD8+ T cells in supplemented RPMI for 2 days. Flow cytometry was then done on the transgenic T cell population (gated on using a PE-labeled anti-Vß5.1, 5.2 TCR Ab (clone MR9-4) from BD PharMingen) to assess T cell proliferation, reflected by halving of CFSE fluorescence intensity in daughter cells produced with each round of proliferation. CD4+ T cell activation was assessed in similar fashion using magnetically isolated, CFSE-labeled CD4+ T cells from the spleens of male OT-II mice.

Cytokine and CTL assays
Supernatant from CD8+ OT-I T cells after incubation with BMDCs for 2 days as described above was collected and assessed for IFN-γ production by routine ELISA techniques per the manufacturer’s instructions (BD PharMingen).

CTL activity of CD8+ OT-I T cells generated after incubation with BMDCs as described above was assessed by the JAM assay (30). Briefly, titrated numbers of effector OT-I cells were seeded in triplicate in 96-well round-bottom plates. EL4 target cells were added at a concentration of 1000 cells/well in the presence or absence of 0.1 μg/ml H-2Kb-restricted OVA peptide (SIINFEKL) and cultured for 5 h with [3H]thymidine. Spontaneous [3H] retention was determined by adding medium instead of effector cells. After 5 h of culture, cells were collected on glass fiber filters and the [3H] retained in live EL4 cells was measured in a beta-plate counter. Percentage specific lysis was calculated as follows: [(spontaneous cpm − experimental cpm) × 100]/spontaneous cpm.

Ag uptake assay
BMDCs were incubated for 2 h with 10 μg/ml FITC-labeled OVA (Molecular Probes, Eugene, OR), the minimal concentration detectable by flow cytometry. The cells were then washed, and uptake of OVA by CD11c+ BMDCs was determined by flow cytometry.

Results
Characterization of BMDC
Murine BMDCs were cultured as described in Materials and Methods and harvested on day 7. Flow cytometry after staining with the appropriate Abs revealed that 60–70% of the cells were CD11c+ dendritic cells (DCs) with characteristics similar to those previously reported: CD4−; CD8α−; CD11b+; CD14−; CD40−; CD54+; CD80+; CD86+; MHC I (H-2Kb)+; and MHC II (I-Aq)+. The CD11c+ population consisted mainly of Gr1+ granulocytes with virtually no contamination with CD3+ T cells, B220+ B cells, or NK1.1+ NK cells. Further purification of the cells for select studies using anti-CD11c magnetic beads and a MACS column, as described in Materials and Methods, resulted in a CD11c+ fraction that contained 98% CD11c+ cells and a CD11c− fraction that contained 95% CD11c− cells.

TLR ligands induce BMDC maturation
Different subpopulations of DCs have been reported to respond to different TLR ligands (31). Before testing the hypothesis that TLR ligands are able to induce cross-presentation by DCs, BMDCs were tested for their expression of TLRs that have known microbrial ligands, as well as for expression of IL-1β receptor, a TLR family member, using RT-PCR (Fig.1A). All of the tested TLRs could be detected, although expression levels varied as suggested by the number of PCR cycles needed to detect the PCR products.

To assess the ability of BMDCs to respond to various TLR ligands, the ability of TLR ligands to induce BMDC maturation was determined. The concentration of each TLR ligand was titrated, and the following concentrations needed to reliably induce BMDC maturation were determined: peptidoglycan (30 μg/ml; TLR2 ligand); poly(I:C) (10 μg/ml; TLR3 ligand); LPS (10 ng/ml; TLR4 ligand); flagellin (30 μg/ml; TLR5 ligand); and ISS-ODN (1 μg/ml; TLR7 ligand).

FIGURE 1. TLR ligands are able to mature BMDCs. A, The expression of the indicated TLR family members (and G3PDH as a loading control) in BMDCs was determined by reverse transcription followed by the indicated number of cycles of PCR. B, BMDCs were incubated for 24 h in the absence or presence of peptidoglycan (PGN, 30 μg/ml), poly(I:C) (P(I:C)), 10 μg/ml), LPS (10 ng/ml), flagellin (FLG, 30 μg/ml), or ISS-ODN (ISS, 1 μg/ml). Staining for surface markers of maturation (B7-1, B7-2, and CD40) on the CD11c+ population of treated (gray areas) or untreated (white areas) cells is shown. Results are representative of at least five independent experiments.
μg/ml; TLR9 ligand). After 24 h of incubation with these concentrations of TLR ligands, the maturation of CD11c+ BMDCs, as assessed by surface expression of B7-1 (CD80), B7-2 (CD86), and CD40 was determined by flow cytometry. All of the tested TLR ligands enhanced expression of these markers indicating maturation, although there was some variability in level of expression induced, and peptidoglycan and flagellin required high doses to induce maturation (Fig. 1B).

**Immunostimulatory DNA enhances cross-presentation by BMDCs**

We developed the DECOT system using dendritic cells and CD8+ T cells from OT-I mice to study cross-presentation in vitro. In this system, BMDCs from C57BL/6 mice were assayed for their ability to cross-present soluble OVA to CD8+ T cells from OT-I mice that express a transgenic TCR that recognizes OVA peptide (SIINFEKL) in the context of MHC I (H-2Kb). Because ISS-ODN induce Ag-specific CTLs in mice as previously mentioned, the BMDCs were incubated for varying lengths of time with varying concentrations of OVA in the presence or absence of ISS-ODN (1 μg/ml). Overnight incubation with ISS-ODN followed by 2–4 h of incubation with OVA resulted in maximal activation of the OT-I CD8+ T cells as measured by T cell proliferation indicated by successive rounds of decreasing CFSE fluorescence (Fig. 2A). As will be addressed later, BMDCs exposed to overnight coinoculation of OVA and ISS-ODN also showed enhancement of OT-I CD8+ T cell activation, but incubation with OVA for 4 h before overnight incubation with ISS-ODN did not enhance activation.

The amount of proliferation seen at a defined OVA concentration showed some variability between experiments, but ISS-treated BMDCs reliably cross-presented at an OVA concentration of 0.1 μg/ml whereas untreated BMDCs did not (Fig. 2A and Table 1). Untreated BMDCs did not reliably cross-present at an OVA concentration of 10 μg/ml (Fig. 2A). The amount of proliferation correlated with cytolytic activity (Fig. 2B) and IFN-γ production (Fig. 2C) by the CD8+ T cells, although IFN-γ production was not detected when <1 μg/ml OVA was used with ISS-treated BMDCs.

BMDCs had to be exposed to ISS-ODN for at least 6 h to demonstrate enhanced ability to activate CD8+ T cells (data not shown). Washing away of ISS-ODN before adding OVA did not affect the enhanced activation capabilities of ISS-treated cells (data not shown). Further purification of the BMDCs using anti-CD11c magnetic beads revealed that the CD11c-positive fraction retained ISS-induced cross-presentation ability, whereas the CD11c-negative fraction did not (data not shown). Using CD8+ T cells derived from OT-I mice on the recombination activation gene-1−/− background to eliminate effects of endogenous TCR expression produced results similar to those using OT-I mice on the C57BL/6 background (data not shown).

The ability of ISS-ODN to enhance BMDC cross-presentation was dependent on immunostimulatory CpG motifs because mutation or methylation of the CpG motif resulted in abrogation of the effect (Fig. 2D). Bacterial DNA derived from E. coli, which contains ISS, also had stimulatory activity, but mammalian DNA derived from calf thymus did not (Fig. 2D). DNase treatment of bacterial DNA abrogated the ability to enhance cross-presentation (data not shown). Compared with BMDCs, ISS-treated bone marrow-derived macrophages were much less efficient at cross-presentation, requiring 10–100 μg/ml OVA (Fig. 3).

**BMDC maturation enhances MHC I but not MHC II Ag presentation**

The ability of BMDCs to take up and cross-present Ag after exposure to a maturation factor such as ISS-ODN was surprising because the current paradigm suggests that immature DCs exhibit excellent Ag uptake abilities but poor T cell-stimulatory abilities, whereas mature DCs exhibit diminished uptake abilities but enhanced stimulatory abilities (7).
Interestingly, stimulation of OT-II CD4 T cells by ISS-ODN before incubation with OVA when compared with BMDCs treated with ISS-ODN alone or OVA (0.1 μg/ml) alone, as described in Fig. 2, are shown from 10 representative experiments.

To examine this issue further, BMDCs were treated with ISS-ODN (1 μg/ml) for 24, 48, or 72 h to ensure adequate maturation. Maturation was confirmed by assessing up-regulation of costimulatory molecules, and there were no significant differences in costimulatory molecule induction between the different time points (data not shown). The cells were then incubated with OVA (0.1 μg/ml) for 2 h. After washing, the BMDCs were cultured with CFSE-labeled OT-I CD8 T cells and assessed for cross-presentation ability by flow cytometry as previously described. All of the BMDCs treated with ISS-ODN showed retained cross-presentation ability despite maturation (Fig. 4A).

As further evidence that ISS-matured BMDCs take up Ag and present it on MHC I, BMDCs incubated with OVA (0.1 μg/ml) for 2–4 h before overnight incubation with ISS-ODN were unable to cross-present to OT-I CD8 T cells (Fig. 4B). Consistent with prior experiments, BMDCs treated overnight with ISS-ODN before incubation with OVA were able to cross-present, and BMDCs cocultured overnight with ISS-ODN and OVA showed an intermediate ability to cross-present (Fig. 4B). In contrast, the capacity to stimulate CFSE-labeled CD4 T cells from OT-II mice, which express a transgenic TCR that recognizes OVA peptide in the context of MHC II (I-A^K), was diminished in BMDCs pretreated with ISS-ODN before incubation with OVA when compared with BMDCs incubated with OVA concurrently with or before ISS-ODN treatment (Fig. 4C). Interestingly, stimulation of OT-II CD4 T cells required BMDCs treated with higher OVA concentrations (10 μg/ml) than that required for OT-I CD8 T cell stimulation (0.1 μg/ml). The inhibitory effect of ISS-ODN pretreatment on CD4 T cell activation could be overcome by using higher OVA concentrations (data not shown).

Microbial TLR ligands have differential abilities to induce cross-presentation

Using the DECOT system, the ability of other microbial TLR ligands to induce cross-presentation by BMDCs was assessed.

When used at concentrations described above to induce BMDC maturation (Fig. 1), the various TLR ligands showed differing abilities to enhance cross-presentation by BMDCs (Fig. 5A). Only the TLR3 ligand (poly(I:C)) and TLR9 ligand (ISS-ODN), showed an ability to induce cross-presentation at the treatment-dependent OVA concentration (0.1 μg/ml). Overnight coincubation of OVA with each of the TLR ligands also gave similar results (data not shown). As shown previously (Fig. 2), higher OVA concentrations (10 μg/ml) induced cross-presentation even in the absence of treatment with TLR ligands. The concentration of the other TLR ligands in the DECOT system was titrated to ensure that dose-dependent effects were not missed. LPS concentrations ranging from 1 ng/ml to 1 μg/ml and peptidoglycan and flagellin concentrations between 1 and 30 μg/ml also showed no induction of cross-presentation (data not shown).

Table 1. Cross-presentation by ISS-treated BMDCs

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* The percentage of CFSE-labeled OT-I CD8 T cells undergoing at least one division after incubation with DCs treated with ISS-ODN (1 μg/ml) plus OVA (0.1 μg/ml), or OVA (0.1 μg/ml) alone, as described in Fig. 2, are shown from 10 representative experiments.

![FIGURE 3.](image-url) Macrophages are inefficient at cross-presentation. Bone marrow-derived macrophages treated with the indicated concentration of OVA after incubation with or without ISS-ODN (1 μg/ml) were assessed for their ability to induce OT-I CD8 T cell proliferation. Results are representative of three independent experiments.

![FIGURE 4.](image-url) ISS-induced maturation of BMDCs enhances MHC I but not MHC II Ag presentation. A, BMDCs were treated for 24, 48, or 72 h with or without ISS-ODN (1 μg/ml) and then incubated with OVA (0.1 μg/ml) for 2 h. The ability of these BMDCs to induce OT-I CD8 T cell proliferation was assessed by flow cytometry as described in Fig. 2. B and C, BMDCs were incubated with OVA for 2 h (OVA), with OVA for 2 h, and then with ISS overnight (OVA→ISS), with OVA and ISS overnight (OVA+ISS), or with ISS overnight followed by OVA for 2 h (ISS→OVA). The BMDCs were then washed and cocultured for 2 days with CFSE-labeled CD8 T cells from the spleens of OT-I mice (B) or CFSE-labeled CD4 T cells from the spleens of OT-II mice (C), and the proliferation of the T cells was assessed by flow cytometry. ISS-ODN was used at 1 μg/ml, and OVA was used at 0.1 μg/ml in B and 10 μg/ml in C. Results are representative of two independent experiments.
TLR ligand-induced BMDC maturation reduces Ag uptake

Maturation of DCs has been shown to down-regulate endocytosis (7, 32). The differential ability of TLR ligands to induce cross-presentation could be explained by differences in their abilities to induce maturation, resulting in either potently matured DCs that take up insufficient amounts of Ag to allow appropriate presentation or less matured DCs that are able to take up sufficient amounts of Ag. To test this possibility, BMDCs were treated with the various TLR ligands overnight and then incubated with FITC-labeled OVA for 2 h. Uptake of OVA by CD11c<sup>+</sup> BMDCs was then assessed by flow cytometry. Each of the TLR ligands reduced Ag uptake, although to varying degrees (Fig. 5B). Compared with the other TLR ligands, ISS and poly(I:C) actually tended to reduce Ag uptake more potently than the other TLR ligands, suggesting that their unique ability to induce cross-presentation was not due to higher Ag uptake but rather due to induction of other mechanisms required for cross-presentation.

TLR ligand-induced cross-presentation is dependent on MyD88 and TLR

All TLRs are known to trigger similar signaling events through the adaptor protein, MyD88. However, a subset of cellular effects induced by LPS and poly(I:C), including costimulatory molecule up-regulation, are known to use MyD88-independent pathways (33–35). To evaluate the role of MyD88 in TLR-induced cross-presentation, the ability of MyD88<sup>−/−</sup> BMDCs to cross-present in the DECOT system was compared with wild-type controls. ISS- and poly(I:C)-induced cross-presentation was found to be MyD88-dependent (Fig. 5C). As expected, use of BMDCs from TLR9<sup>−/−</sup> and TLR2<sup>−/−</sup> mice showed that ISS-induced cross-presentation was dependent on TLR9 but not TLR2 (Fig. 5D).

TLR-induced cross-presentation by DCs involves intracellular Ag processing

The ability of only a subset of TLR ligands to induce cross-presentation suggests that, in addition to up-regulating costimulatory molecules needed for adequate DC-T cell interaction, these TLR ligands uniquely affect Ag-processing mechanisms involved in presentation of exogenous Ag. To assess the need for intracellular processing of OVA, the ability of fixed BMDCs to stimulate CD8<sup>+</sup> T cell proliferation was assessed. Fixation stabilizes cell surface MHC on APCs, resulting in enhanced peptide-binding capacity but inability to process proteins (36, 37). Because primary T cells such as OT-I cells are not efficiently stimulated by fixed APCs due to lack of appropriate cell contact- and cytokine-induced signals (38, 39), B3Z T cell hybridomas, which are less dependent on these signals and contain a lacZ reporter gene that is induced upon recognition of H2K<sup>b</sup>-restricted OVA peptide (25), were used. Use of anti-CD28 as a costimulatory signal increased sensitivity of the B3Z system when fixed APCs were used, but it was still less sensitive than the DECOT system and therefore required higher OVA concentrations but showed a similar pattern of ISS-induced cross-presentation (Fig. 6A). BMDCs fixed before exposure to OVA were unable to process OVA protein to prime B3Z cells but had enhanced ability to prime B3Z cells when directly loaded with SIINFEKL peptide, confirming the requirement for intracellular processing.

FIGURE 5. TLR ligands have differential abilities to induce cross-presentation. A, BMDCs from C57BL/6 mice were treated overnight with medium alone (media), peptidoglycan (PGN, 30 μg/ml), poly(I:C) (P(I:C), 10 μg/ml), LPS (10 ng/ml), flagellin (FLG, 30 μg/ml), or ISS-ODN (ISS, 1 μg/ml). They were then treated with OVA (0.1 μg/ml) for 2 h before culturing with OT-I CD8<sup>+</sup> T cells and assessing proliferation by flow cytometry as described in Fig. 2. Results are representative of at least three independent experiments. B, BMDCs treated overnight with the indicated TLR ligands as in A were then incubated with FITC-labeled OVA (10 μg/ml) for 2 h, and uptake of the OVA by CD11c<sup>+</sup> cells was assessed by flow cytometry. C, BMDCs from wild-type (WT) and MyD88<sup>−/−</sup> mice were incubated overnight in the presence of medium, ISS, or PI(1:C) and then treated with OVA (0.1 μg/ml) or SIINFEKL peptide (10 pM) for 2 h. OT-I CD8<sup>+</sup> T cells were then added, and proliferation was assessed as described in Fig. 2. D, BMDCs from wild-type, TLR9<sup>−/−</sup>, or TLR2<sup>−/−</sup> mice were incubated in the presence of ISS overnight and then treated with OVA (0.1 μg/ml) for 2 h. Proliferation of OT-I CD8<sup>+</sup> T cells was then assessed as described in Fig. 2. Results are representative of two independent experiments.
processing of OVA in ISS-induced cross-presentation. Furthermore, this need for intracellular processing made it unlikely that peptide contamination of the chromatographically purified OVA preparation was responsible for functionally significant cell surface MHC I loading. As expected, BMDCs fixed after incubation with OVA retained the ability to prime B3Z cells (data not shown).

**TLR-induced cross-presentation by DCs involves cytosolic Ag processing**

Published results suggest several possible pathways for the routing of exogenous Ag onto MHC I, including escape of endosomal Ag to the cytosol for further processing, association of endosomal Ag with recycling MHC I molecules in the endosome, and regurgitation of endosomal Ag into the extracellular space for association with MHC I molecules at the cell surface (2, 40). If cytosolic processing is required, TAP, which shuttles peptides from the cytosol into the endoplasmic reticulum, will likely be necessary for cross-presentation. To test the requirement for TAP in TLR-dependent cross-presentation by BMDCs, BMDCs from TAP1−/− mice were used in the DECOT assay. TAP1−/− BMDCs treated with OVA (0.1 μg/ml) after overnight incubation with ISS-ODN or poly(I:C) were unable to cross-present, suggesting a TAP-dependent process (Fig. 6B). However, TAP1−/− cells are known to express less surface MHC I than wild-type cells due to insufficient peptide transport into the endoplasmic reticulum for MHC I loading, and this decreased MHC I expression may render these cells intrinsically unable to stimulate CD8+ T cells. TAP1−/− BMDCs loaded with SIINFEKL peptide, however, were able to stimulate CD8+ T cells (Fig. 6B), demonstrating that TAP-dependent cross-presentation is TAP dependent. Treatment of BMDCs with lactacystin (10 μM), a proteasome inhibitor, before incubation with OVA also inhibited TLR-dependent cross-presentation in the DECOT assay, consistent with cytosolic Ag processing (data not shown).

The above results suggest that TLR-induced cross-presentation involves escape of Ag from the endosomal pathway to the cytosol. To further define the step at which Ag escapes from the endosomal pathway, the requirement for endosomal acidification was assessed. After treatment with ISS-ODN or poly(I:C) overnight, BMDCs were incubated with OVA (0.1 μg/ml) for 2 h in the absence or presence of the endosomal acidification inhibitors chloroquine (100 μM) or ammonium chloride (50 mM). The inhibitors 100 mM), or ISS-ODN followed by peptide. The BMDCs were then washed and incubated overnight with B3Z T cell hybridomas in the presence of anti-CD28 Ab, β-Galactosidase activity of the B3Z T cells was then assessed with a colorimetric assay as described in Materials and Methods. Mean OD595 of duplicate samples is shown. Error bars, SEM. B, The ability of wild-type (WT) or TAP1−/− BMDCs treated with medium, ISS-ODN (1 μg/ml), or poly(I:C) (10 μg/ml) overnight followed by OVA (0.1 μg/ml) for 2 h or treated with SIINFEKL peptide (10 μM) to induce OT-I CD8+ T cell proliferation was assessed by flow cytometry as described in Fig. 2. C, BMDCs were incubated overnight in the absence or presence of ISS-ODN (1 μg/ml) or poly(I:C) (10 μg/ml) and then incubated for 2 h with OVA (0.1 μg/ml) in the absence (neg) or presence of chloroquine (CQ) or ammonium chloride (NH4Cl). The ability of these BMDCs to induce proliferating CFSE-labeled OT-II CD4+ T cells was assessed by flow cytometry. Results are representative of three independent experiments.
were not used during incubation with ISS-ODN or poly(I:C) because their specific effect on TLR-induced Ag processing was of interest and they inhibit ISS-ODN activity (41–43). Neither of these endosomal acidification inhibitors inhibited TLR-induced cross-presentation (Fig. 6C), suggesting that Ag escapes early in the endosomal pathway, before acidification. In contrast, these inhibitors did successfully inhibit activation of OT-II CD4+ T cells by BMDCs incubated with OVA (Fig. 6D), verifying that the inhibitors were used at effective concentrations.

Discussion

The DECOT system presented in this paper is a useful tool for studying the mechanisms involved in cross-presentation. It conveniently uses cultured BMDCs and spleen-derived OT-I CD8+ T cells. Importantly, TLR-dependent cross-presentation is a TAP-dependent process, correlating with in vivo data that shows that CTL responses in mice immunized with ISS-based vaccines are also dependent on TAP because wild-type mice reconstituted with TAP−/− bone marrow failed to develop ISS-induced CTL responses (44). This correlation with in vivo data supports the physiological relevance of the TLR-dependent cross-presentation system.

The mechanism by which exogenous Ag is routed to the MHC I pathway remains an intriguing aspect of cross-presentation. As mentioned previously, both endosomal and cytosolic pathways of Ag processing in cross-presentation have been documented (2, 45). The current data show that TLR-induced cross-presentation involves cytosolic handling of Ag that is dependent on TAP and proteasomal processing. Endosomal acidification is not required, suggesting that escape of the Ag to the cytosol occurs early in the endosomal pathway, before acidification. This is in contrast to the requirement for endosomal acidification seen with FcR-mediated cross-presentation of immune complexes (46). However, in that study, endosomal acidification was apparently primarily needed for disassembly of immune complex aggregates, because shuttling of dextrans to the cytosol in the same study did not require acidification and, interestingly, occurred in a size-dependent manner without detectable leakage of other endosomal contents into the cytosol. Those findings are consistent with the current study which suggests that certain TLR ligands promote shuttling of Ag from early in the endosomal pathway to the cytosol for processing by the MHC I pathway. The molecular mechanisms involved in this Ag escape from endosome to cytosol remain elusive and will require further investigation.

The unexpected dichotomy between CD4+ and CD8+ T cell activation seen after maturation of BMDCs also supports the shuttling of Ag from the endosomal pathway, required for MHC II processing, to the cytosol for processing by the MHC I pathway. Consistent with prior reports showing the enhanced phagocytic but poor stimulatory ability of immature DCs and the poor phagocytic but improved stimulatory ability of mature DCs (7) maturation of DCs with ISS-ODN before incubation with Ag resulted in decreased ability to stimulate CD4+ T cells. In contrast, maturation of DCs after incubation with Ag resulted in increased ability to stimulate CD4+ T cells. Surprisingly, the exact opposite effects were seen for CD8+ T cell activation. Enhanced cross-presentation was seen when DCs were matured with ISS-ODN before incubation with Ag, and decreased cross-presentation was seen when ISS-ODN was added after incubation with Ag. The cross-presentation ability of DCs pretreated with ISS-ODN correlates with in vivo data showing that mice preprimed with ISS-ODN up to 2 wk before Ag administration developed CTL responses (14).

The ability of certain microbial TLR ligands to induce cross-presentation of soluble Ags identifies a cross-presentation trigger distinct from other stimuli such as apoptotic cells (47) and immune complexes (46). The TLR-mediated signal appears to replace the requirement for CD4+ T cell help through CD40-CD40L interactions that has been reported to be necessary for cross-presentation (48, 49). The unique ability of TLR9 and TLR3 ligands to induce cross-presentation is an intriguing puzzle. All of the TLRs use a canonical signaling pathway involving MyD88, but alternative and complementary pathways have been proposed (13). Although MyD88 is required for ISS- and poly(I:C)-induced cross-presentation, it seems probable that complementary pathways are activated by ISS-ODN and poly(I:C) that are not activated by the other TLR ligands. These complementary pathways likely activate MHC I-related Ag-processing machinery. Intriguingly, ISS-ODN and poly(I:C) are potent inducers of IFN-αβ (50–52). Diminished CTL activity after ISS-based immunization in IFN-αβ receptor-deficient mice (44, 53) and our preliminary data in vitro showing that cross-presentation is diminished in BMDCs from IFN-αβ receptor-deficient mice (data not shown) suggest that IFN-αβ plays a role in inducing cross-presentation.

Although the differential effects of microbial products on cross-presentation may be a useful tool to dissect the differences in molecular mechanisms between the TLRs, microbial products are encountered in combination during a natural infection. What is the significance of the observed differential effects in host-microbe interactions? A potential answer is that some microbial products, such as DNA and RNA, are typically encountered intracellularly, either during intracellular infection or after ingestion of microbes. In contrast, products such as LPS, flagellin, and peptidoglycan are predominantly encountered in the extracellular milieu. Because CD8+ T cells are primarily required for control of intracellular infections, the immune system may have evolved to elicit CTL activity when triggered by products encountered in the intracellular milieu. Indeed, recognition of ISS-ODN requires internalization and endosomal acidification, leading to the assumption that TLR9 functions in an intracellular compartment (43, 54). This assumption is consistent with the correlation between intracellular recognition of microbial products and the induction of cross-presentation pathways.

The exact mechanisms that lead to TLR-induced cross-presentation are yet to be delineated. TLR activation likely leads to multiple effects in DCs that promote cross-presentation. TLR ligands up-regulate costimulatory molecules and induce cytokine secretion, both of which play a role in T cell activation (55). In addition, TLR ligands such as ISS-ODN induce components of the Ag-processing machinery, including TAP and MHC molecules (44) and, as suggested in this study, reroute endocytosed Ag to the cytosol. Further investigation of the abilities of microbial TLR ligands to induce cross-presentation will lead to a better understanding of host-pathogen interactions and uncover vaccination strategies that elicit improved cell-mediated immunity compared with currently used vaccines.

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References


