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TLR and Nucleotide-Binding Oligomerization Domain-like Receptor Signals Differentially Regulate Exogenous Antigen Presentation

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The effect of dendritic cell (DC) maturation on MHC class II-restricted Ag presentation is well studied, but less is known about the effects of DC maturation on MHC class I-restricted cross-presentation. We investigated the ability of mature DCs to present Ags from cells infected with HSV-1. Pretreatment with pure LPS increased cross-presentation in a manner dependent on both MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN-β, whereas a similar dose of a less pure LPS preparation inhibited cross-presentation. The difference could not be attributed to differences in uptake or phenotypic maturation. The likely contaminant responsible for shutting down cross-presentation is peptidoglycan (PGN). Addition of PGN to pure LPS abrogated its ability to enhance cross-presentation. Direct activation of DCs with PGN inhibited cross-presentation through nucleotide-binding oligomerization domain-like receptor signaling. These results demonstrate that different maturation stimuli can have opposite impacts on the ability of DCs to cross-present viral Ags. The Journal of Immunology, 2012, 188: 000–000.

Dendritic cells (DCs) are specialized in the presentation of exogenous Ags by MHC class II and I molecules. The latter process is referred to as cross-presentation and, for most Ags, involves their transport into the cytosol and subsequent translocation of derived peptides into phagosomes, early endosomal compartments, or the endoplasmic reticulum for loading onto MHC class I molecules (1).

After activation with microbial products or inflammatory mediators, DCs undergo a maturation process that regulates key functions, including migration, Ag processing, and expression of costimulatory molecules, all required for efficient T cell priming (2, 3). Ag uptake is significantly reduced in mature DCs (4–10), and presentation of new Ags on MHC class II is impaired. Regulation of MHC class II presentation during maturation has been intensively studied and includes changes in proteolysis, redistribution of peptide-loaded MHC class II molecules to the cell surface, stabilization of surface complexes, and reduction of MHC class II synthesis (reviewed in Ref. 11). In contrast, MHC class I synthesis is increased during maturation (12, 13).

Much attention has been focused on the effects on cross-presentation of maturation stimuli given with or after Ag exposure (11, 14–16). How and to what extent activation prior to Ag exposure regulates cross-presentation is less clear. Cross-presentation was inhibited in vivo after systemic administration of TLR ligands (8) as well as after prolonged treatment of DCs with certain TLR ligands in vitro (8, 17, 18). Besides decreased Ag uptake (8, 17, 18), inhibition of Ag access to the cytosol has also been proposed to explain the inability of mature DCs to mediate cross-presentation (17), but the precise mechanism is not well characterized. A common rationale for the inhibition of cross-presentation upon maturation is that presentation needs to be restricted to Ags acquired during the initial stimulus (19). However, there are examples in which pretreatment of DCs with certain TLR ligands did not alter or even enhanced cross-presentation of subsequently acquired Ag (18, 20–25). This may have the benefit of allowing priming of CD8-positive T cells to pathogens during an ongoing infection. Continuous Ag presentation in mature DCs has been reported for MHC class II-restricted presentation in certain cases (24, 25).

TLR ligands are commonly chosen as activating stimuli for Ag presentation studies, reflecting the important role of TLR signaling in regulating DC maturation (26). TLRs are found on the cell surface and in endosomal compartments, sensing conserved structures of a broad range of microbial and viral ligands (27). MyD88 is an intracellular adaptor protein that couples all TLRs except TLR3 to signaling pathways that induce inflammatory cytokines. TLR4 is unique in that activation by this receptor involves sequential involvement of MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (28). The nucleotide-binding oligomerization domain (Nod), leucine-rich repeat-containing receptors (NLRs) are cytosolic pathogen sensors (29, 30). Two members of the NLR family, the Nod-like receptors 1 and 2 (Nod1, Nod2), recognize specific structures in peptidoglycan (PGN) (31), whereas TLR2 signaling in response to PGN is mediated by linked lipoteichoic acid and lipoproteins (32, 33). Signal transduction via Nod1 and Nod2 results in an NF-κB–mediated proinflammatory response (34). Besides bacterial recognition, Nod receptors are involved in many processes, including autophagy induction, an-
tiviral responses, and initiation of adaptive T cell responses (35, 36).

The field of cross-presentation is dominated by studies of the model Ag OVA, and the vast majority of maturation studies use OVA in soluble or immune-complexed forms. Bone marrow-derived DCs require an additional maturation stimulus during or shortly after Ag uptake to cross-present soluble OVA (15–17, 20). In this study, we have used apoptotic and necrotic HSV-1–infected cells as an Ag source, which have the advantage of providing sufficient intrinsic immunostimulatory signals for cross-presentation (37–40). Untreated and matured DCs were compared for their ability to take up and present HSV-derived Ags. We found that maturation induced by TLR2 and TLR4 signaling strongly inhibited subsequent MHC class II presentation, whereas cross-presentation was only inhibited after TLR2 triggering. PNG signaling through nod receptors inhibited cross-presentation but did not affect MHC class II presentation. Regulation of cross-presentation by intracellular innate immune receptors may reflect the involvement of critical cytosolic processes in this phenomenon.

**Materials and Methods**

**Mice**

C57BL/6 (B6) were obtained from The Jackson Laboratory (Bar Harbor, ME), MyD88 knockout (KO) mice, TRIF KO mice, and TRIF/MyD88 double-KO (DKO) mice were provided by R. M. Z. Mezhito (Yale University School of Medicine). Animals were housed and used according to Yale’s institutional guidelines.

**Cells**

Bone marrow-derived DCs were prepared from femurs/tibiae of mice between 6 and 12 wk of age and cultured for 5–7 d in 24-well plates in RPMI 1640 (Life Technologies, Rockville, MD) with 10% FCS (Thermo Fisher Scientific, Waltham, MA), 50 μM 2-ME, and 100 U/ml penicillin, 100 μg/ml streptomycin (penicillin/streptomycin [Pen/Strep]), 12 mM HEPES, nonessential amino acids (all Life Technologies), and 20 ng/ml GM-CSF (R&D Systems). Medium was replenished on day 3. The HSV-1 glycoprotein B (gB)498–505-specific MHC class I-restricted hybridoma HSV-2.3.2E2 was a generous gift from N. Shastri (University of California, San Diego, CA). Ultrapure PGN (insoluble and soluble sonicated form from *E. coli* K12; InvivoGen) was at 2 μg/ml (except for Fig. 7). 10 μg/ml γ-glutamyl-meso-diaminopimelic acid (iE-DAP), or muramyl dipeptide (MDP; InvivoGen).

**Maturation of DCs**

DCs were stimulated on day 5 or 6 of culture for 22 h or shorter times as indicated in supplemental figures. Stimuli were added to the medium in 24-well plates without disturbing DC clusters. Control cells were left untreated. Stimuli used were 0.1 and 1 μg/ml LPS (Escherichia coli 055:B5; catalog number L288; Sigma-Aldrich), ultrapure LPS (*E. coli* 0111:B4; InvivoGen, San Diego, CA), ultrapure PGN (insoluble and soluble sonicated form from *E. coli* K12; InvivoGen) at 2 μg/ml (except for Fig. 7), 10 μg/ml γ-glutamyl-meso-diaminopimelic acid (iE-DAP), or muramyl dipeptide (MDP; InvivoGen).

**Preparation of infected cells**

HeLa cells were infected with HSV-1 (F strain) at a multiplicity of infection of 5:1 for 1 h in culture medium. Cells were then washed and kept in medium containing 0.2 mM acyclovir (Calbiochem) for 10 h. Residual virus was UV inactivated with 200 mJ/cm² in a Stratallinker 1800. HeLa–HSV was cultured for an additional 6 h before use as apoptotic bodies. To produce necrotic bodies, HeLa-HSV were subjected to three rounds of freeze/thaw after residual virus inactivation and used without further incubation. Necrotic or apoptotic HeLa cells were resuspended to 2.5 × 10⁶/ml in DC medium. Plaque assays with both cell preparations confirmed that no infectious virus could be recovered from either. For direct infection of DCs, HSV was added at a multiplicity of infection of 3 to DCs in medium containing 10 μg/ml gentamicin instead of Pen/Strep. After 1 h of infection, DCs were washed three times and kept in acyclovir containing medium for 12 h before addition of T cells as described below.

**Ag presentation assays**

Matured or untreated DCs were harvested from 24 wells, washed, and fed with apoptotic or necrotic infected HeLa cells at a 4:1 (DC:HeLa) ratio for 5 to 6 h in 96-well round bottom plates. Subsequently, 1 × 10⁵ gB- or gD-specific hybridoma T cells were cocultured with 1 × 10⁴ Ag-loaded DCs for 20 h. For coculture with the RR1/ICP6-specific CTL, Vero cells were used instead of HeLa, and 5 × 10⁴ DCs were cocultured with 1 × 10⁷ CTL for 20 h. IL-2 in coculture supernatants was measured by ELISA according to the manufacturer's protocol (BD Biosciences).

**Flow cytometry**

Staining for cell-surface markers was performed with the following Abs and appropriate isotype controls (all BD Biosciences) for 20 min at 4˚C. Anti–H-2Kb (AF6-88.5), anti-I-A/I-E (M5/114.15.2), anti-CD40 (3/23), anti–I-A/I-E (M5/114.15.2), anti-CD70 (FR70), and all PE-conjugated and allophycoerythrin-conjugated anti-CD11c (HL3). Data was collected on an FACS-Calibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**Cytokine profile**

DCs were treated in the same manner as for Ag presentation assays to mimic cytokine levels in T–DC cocultures. After 22 h of maturation, DCs were washed extensively and plated in 96-well plates, either with or without the addition of HSV-infected HeLa cells. After 24 h, supernatants were harvested, and IL-2, IL-10, IL-12(p70), IFN-γ, and TNF-α were measured with the Bio-Plex Pro Mouse Cytokine Assay on a Bio-Plex System (Bio-Rad) according to the manufacturer’s instructions. Alternatively, supernatants of matured DCs cocultured with infected HeLa were used for transfer experiments and added to T–DC cocultures during Ag presentation assays with untreated DCs.

**Phagocytosis assays**

Untreated or matured DCs were allowed to phagocytose apoptotic HeLa cells labeled with the fluorescent membrane binding dye PKH26 (Sigma-Aldrich) for 4 h at a 4:1 (DC:HeLa) ratio in a total volume of 250 μl complete DC medium as was used for Ag presentation assays. As control for uptake, one sample with untreated DCs and PKH26-labeled HeLa cells was kept on ice during the 4-h uptake. Samples were then washed two times in cold PBS-1% FBS, labeled on ice with anti-CD11c allophycocyanin, and fixed in 2% paraformaldehyde after two additional washes. Alternatively, untreated or matured DCs were fed in 24-well plates with fixed bacteria (*E. coli* expressing DsRed) at a DC/bacteria ratio of 1:25. Samples were centrifuged for 1 min at 420 × g at the onset of a 25-min uptake. As a control in this experiment, one sample was pretreated for 20 min with 2.5 μg/ml cytolysin D, and the drug was also present during uptake of the *E. coli* DsRed. DCs were then harvested and washed three times over a cold 2-ml FBS cushion, stained with anti-CD11c, and fixed in 2% paraformaldehyde. Phagocytosis was assessed by flow cytometry, gating on CD11-positive cells.

**Statistical analysis**

Statistical analysis of ELISA data was done using ANOVA with Bonferroni’s post hoc test.

**Results**

LPS enhances cross-presentation while eliminating MHC class II-restricted presentation

There are conflicting data concerning the ability of mature DCs to take up, process, and present exogenous Ags. To determine whether DCs are capable of cross-presenting ligands from virally infected cells, we used HSV-1–specific T cell hybridomas as a readout, as they are less influenced by costimulation than primary T cells. DCs were matured for 22 h with 0.1 or 1 μg/ml LPS, either conventionally isolated or highly purified, or left untreated, and subsequently allowed to phagocytose apoptotic HSV-1–infected HeLa cells,
They were then cocultured with the H2-Kb–restricted HSV-1–specific hybridoma HSV-2.3.2E2, recognizing an epitope from gB (Fig. 1A), or assessed for their ability to present Ags on MHC class II using the HSV-1 gD-specific hybridoma F1 (Fig. 1B). DCs matured with 1 µg/ml dose of the less pure LPS lost the ability to cross-present, whereas the lower dose enhanced cross-presentation (Fig. 1A). The responses at the different doses could not be explained by a differential capacity to take up exogenous material (Fig. 1C). Although the DCs stimulated with either concentration of impure LPS exhibited decreased phagocytosis compared with untreated DCs, they were virtually identical in taking up PKH26-labeled apoptotic HeLa cells (Fig. 1C, upper panel) or E. coli expressing the fluorescent protein DsRed (Fig. 1C, lower panel). Notably, internalization was inhibited by cytochalasin D, indicating that the assay reflects genuine phagocytosis (Fig. 1C). The different response of DCs to high and low doses of LPS was not seen when the pure LPS was used. DCs pretreated with either dose of pure LPS cross-presented better than untreated DCs (Fig. 1A).

MHC class II presentation was shut off independently of the LPS source or dose (Fig. 1B). The inhibition of MHC class II presentation was faster than that of cross-presentation and could already be detected after 2 h of LPS pretreatment (Supplemental Fig. 1A, 1B). Both LPS concentrations induced a strong mature phenotype, and both sources resulted in the same degree of phenotypic maturation (Fig. 1D, Supplemental Fig. 1C) and induced comparable reduction in phagocytosis (see below). Similar results were obtained using freeze-thawed infected HeLa cells, mimicking the process of necrotic death (data not shown).

MyD88 and TRIF signaling pathways are both involved in regulation of Ag presentation during LPS-induced maturation

LPS can signal through two different pathways. The MyD88-dependent pathway is initiated after LPS binding to surface TLR4, whereas a second pathway involves TRIF signaling from the endosome (28). To investigate which pathway is involved in the regulation of cross-presentation, we prepared DCs from MyD88\textsuperscript{-/-}, TRIF\textsuperscript{-/-}, or DKO mice and compared their ability to present HSV-1 Ags after 22 h of maturation induced by the less pure LPS. The enhancement of cross-presentation that was seen in wild-type DCs pretreated with 0.1 µg/ml LPS was clearly attenuated in both MyD88\textsuperscript{-/-} and TRIF\textsuperscript{-/-} DCs, and in the DKO cells, the enhancement was eliminated (Fig. 2A). Unexpectedly, however, we found that high-dose (1 µg/ml) LPS pretreatment abolished cross-presentation even in the MyD88\textsuperscript{-/-}/TRIF\textsuperscript{-/-} DCs, despite the complete absence of phenotypic maturation (Fig. 2C). Loss of either MyD88 or TRIF reduced phenotypic maturation after LPS treatment, with the reduction in the TRIF\textsuperscript{-/-} DCs being slightly greater than in the MyD88\textsuperscript{-/-} DC (Fig. 2C). Elimination of either adaptor protein alleviated to some extent the LPS-induced block in MHC class II presentation (Fig. 2B). As expected, MHC class II presentation by DKO DCs treated with either dose of LPS was essentially identical to that of untreated DCs (Fig. 2B).

**FIGURE 1.** Pure LPS enhances gB cross-presentation. DCs were activated for 22 h with 0.1 or 1 µg/ml LPS (Sigma-Aldrich) or pure LPS (InvivoGen), fed apoptotic HSV-1–infected HeLa cells for 5 h, and finally cocultured with the MHC class I-restricted gB-specific hybridoma (A) or the MHC class II-restricted gD-specific hybridoma (B). IL-2 was measured by ELISA after 20 h. One representative of three experiments is shown. Error bars depict SD. C, Phagocytic capacity of LPS-matured DCs. DCs were matured with the indicated amounts of LPS (Sigma-Aldrich) for 22 h or left untreated. DCs were then fed for 4 h with PKH26-labeled infected apoptotic HeLa cells (upper panel) or fixed E. coli-expressing DsRed (lower panel). As controls, DCs were left on ice or treated with cytochalasin D. Samples were gated on CD11c\textsuperscript{+} cells. Numbers indicate percent uptake. One representative of three experiments is shown. D, DCs prepared as in A and B were labeled with anti-CD11c and anti-H2K\textsuperscript{b} (MHC I), anti-I-A/-I-E (MHC II), anti-CD86, anti-CD40, or anti-CD70 and analyzed by flow cytometry gating on CD11c\textsuperscript{+} cells. Black histograms represent untreated DCs, DCs treated with 0.1 µg/ml LPS (Sigma-Aldrich) are in orange, 1 µg/ml LPS (Sigma-Aldrich) in red, 0.1 µg/ml LPS (InvivoGen) in light blue, and 1 µg/ml LPS (InvivoGen) in dark blue. *p < 0.05, **p < 0.01, ***p < 0.001.

**PGN both blocks cross-presentation and overrides its LPS-induced enhancement**

A possible explanation for the observation in Fig. 2A that cross-presentation was eliminated in a MyD88/TRIF-independent manner at the higher concentration of the less pure LPS is that the preparation was contaminated with a component that inhibits cross-presentation independently of TLR signaling. LPS preparations are often contaminated with lipoproteins and/or PGNs. To determine whether PGN could inhibit cross-presentation and/or override the LPS-mediated enhancement of gB cross-presentation, DCs were matured for 22 h in the presence of either stimulus or with a combination of both. Subsequently, DCs were tested for the ability to take up and process HSV-1 Ags for cross-presentation. DCs matured with 2 µg/ml PGN alone completely lost the ability to cross-present (Fig. 3). PGN also eliminated cross-presentation when added together with either 0.1 µg/ml of the less pure or 1 µg/ml of the pure form LPS, which, when added alone, enhanced cross-presentation as before.
PGN-mediated signaling through Nod receptors can inhibit cross-presentation

To further investigate the effect of PGN on cross-presentation, we compared highly purified PGN, detected solely through Nod receptors, termed PGN (Nod), with PGN that still contains TLR2-stimulating activity [PGN (TLR2+Nod)]. Cross-presentation was abrogated when DC maturation was induced with either PGN preparation prior to Ag uptake (Fig. 4A, left panel). A time course for PGN pretreatment showed a progressive inhibition, with clearly decreased cross-presentation after 12 h and almost complete inhibition after 18 h (Supplemental Fig. 1D). That activation by the Nod signaling pathway is capable of inhibiting cross-presentation was confirmed by using DCs from MyD882/2 mice, in which the TLR2 signal induced by the PGN(TLR2+Nod) preparation would be absent (27) (Fig. 4A, right panel). This experiment does not, of course, exclude the possibility that TLR2 signaling by PGN might also be capable of inhibiting cross-presentation.

We also examined the effects of these PGN preparations on MHC class II-restricted presentation. In wild-type DCs, this was dramatically reduced by PGN (TLR2+Nod), whereas there was hardly any effect when the PGN preparation only capable of Nod triggering was used (Fig. 4B, left panel). Although still showing a statistically significant difference to untreated DCs, MHC class II presentation by PGN (TLR2+Nod) was largely restored in the MyD882/2 DCs (Fig. 4B, right panel), arguing that TLR2 signaling is mainly responsible for inhibition of MHC class II presentation. Consistent with this, in wild-type cells PGN (TLR2+Nod) treatment induced greater phenotypic maturation, including increased upregulation of MHC class II cell surface expression, than did PGN (Nod) (Fig. 4C).
Differences in Ag uptake could not explain the results. PGN (TLR2+Nod) reduced uptake to a similar extent as LPS, and PGN (Nod)-treated DCs were comparable to untreated DCs in the phagocytosis of apoptotic HeLa cells (Fig. 5A). PGN-treated DCs did not have a general defect in MHC class I presentation as direct presentation after DC infection with HSV-1 was not compromised (Fig. 6B). Neither was the ability to stimulate T cells after pulsing with the antigenic peptide gB498–505 (Fig. 5C). These experiments also rule out a possible role for a loss of a costimulatory signal or induction of an inhibitory molecule by the DCs that might affect hybridoma stimulation. Secreted cytokines are also unlikely to be responsible for the observed inhibition of cross-presentation (Fig. 5D, Supplemental Fig. 2). The cytokine profile of DCs (IFN-γ, TNF-α, IL-2, IL-10, and IL-12) was assessed under conditions mimicking the situation in T cell cocultures. PGN (Nod)-matured DCs induced slightly more TNF-α and IL-12 than untreated DCs, but overall cytokine levels were low (Supplemental Fig. 2). DCs cultured with infected HeLa cells showed a remarkable increase in IL-10 production, with Nod-activated cells producing comparable levels and TLR-stimulated DCs producing less IL-10 than untreated (UT) (Supplemental Fig. 2). To directly test whether secreted cytokines interfered with the outcome of cross-presentation, these supernatants were added to DC–T cocultures during Ag presentation assays (Fig. 5D). Supernatant of PGN-stimulated DCs had no influence, whereas supernatant of LPS-treated DCs enhanced cross-presentation to some extent, although not reaching statistical significance.

**Nod1 and Nod2 signaling interferes with cross-presentation**

Nod1 and Nod2 recognize different structural motifs in PGN. Nod1 recognizes the dipeptide iE-DAP, found in PGN of all Gram-negative and only certain Gram-positive bacteria (44, 45). Nod2 has a broader detection range, recognizing MDP that is found in PGN from both Gram-positive and Gram-negative bacteria (46, 47). To confirm that PGN regulated cross-presentation through Nod signaling, DCs were pretreated with synthetic iE-DAP or MDP. Both abrogated the ability to cross-present HSV-1–gB (Fig. 6A). Similar to pure PGN, iE-DAP signaling had little effect on MHC class II presentation of HSV-gD (Fig. 6B). Treatment with MDP, however, also reduced MHC class II presentation to a greater extent (Fig. 6B) and induced stronger phenotypic maturation than pure PGN or iE-DAP (Fig. 6C). No differences in cell viability were observed after treatment with any of these stimuli as assessed by flow cytometry (data not shown).

**Cross-presentation of HSV-RRI/ICP6 is also affected by PGN-induced maturation**

To test whether PGN (Nod)-mediated effects are specific for gB, we assessed cross-presentation for another HSV protein, HSV-RRI/ICP6, which encodes a cytosolic ribonucleotide reductase. Pretreatment with 2 μg/ml PGN (Nod) significantly reduced responses, and 10 μg/ml resulted in strong inhibition (Fig. 7A), whereas gDT presentation on MHC class II remained unaffected (Fig. 7B). Both concentrations of PGN (Nod) induced a comparable level of mild phenotypic maturation (Supplemental Fig. 3B).

**Discussion**

Presentation of exogenous Ags is differently regulated for MHC classes I and II during maturation. Our results demonstrate that, depending on the nature of the activation stimulus, cross-presentation can be either enhanced or inhibited in a manner independent of regulation of Ag uptake. In addition to plasma membrane and endosomal TLRs, cytosolic receptors can contribute to regulation of cross-presentation.
A common explanation for downregulation of cross-presentation during DC maturation is inhibition or decrease in Ag uptake (8, 17, 18). However, more recent literature suggests that certain forms of endocytosis are still functional in mature DCs (22, 24, 25). In our hands, mature DCs could still take up apoptotic cells or bacteria, albeit in reduced amounts compared with untreated DCs. By comparing less pure with highly purified LPS, we defined conditions that induced the same strong phenotypic maturation and decreased uptake to a similar degree, as well as eliminating MHC class II-restricted Ag presentation, but had opposite effects on cross-presentation. A change in Ag uptake was thus ruled out as the major determining factor in the difference in the ability to cross-present. We have also been unable to observe any changes in phagosomal acidification upon PGN treatment that could have explained the block in cross-presentation (data not shown).

Both enhancement of cross-presentation and downregulation of MHC class II-restricted presentation by prior LPS treatment of DCs were codependent on MyD88 and TRIF, consistent with the involvement of TLR4 (28). Based on the increased response toward

**FIGURE 5.** Other functions of PGN treated DCs are normal. A, Uptake of PKH26 labeled apoptotic cells. DCs were left untreated or matured for 22 h with the indicated stimuli, then fed infected apoptotic PKH26 labeled HeLa cells for 4 h and analyzed by flow cytometry as in Fig. 1C. Percent uptake in CD11c+ cells was normalized to uptake in CD11c+ UT DCs. Numbers after stimuli indicate concentration in micrograms per milliliter. Summary of three experiments; error bars depict SD. B, Direct presentation on MHC class I. UT or DCs matured for 22 h were directly infected with HSV overnight and cocultured with the gB-specific hybridoma. Numbers after stimuli indicate concentration in micrograms per milliliter. C, UT or DCs matured for 22 h with the indicated stimuli (PGN, 2 μg/ml; pure LPS, 0.1 μg/ml) were pulsed with no gB498–505 peptide, 0.5, or 5 nM peptide for 1 h, washed, and cocultured with the gB-specific hybridoma. D, Effect of cytokine release by PGN-matured DCs. DCs were matured for 22 h or left untreated, washed extensively, and recultured with HeLa-HSV for supernatant collection for additional 20 h. Supernatants were added to DC–T cocultures during Ag presentation assays. For B–D, one representative of two experiments is shown. *p < 0.05, **p < 0.01.

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Both enhancement of cross-presentation and downregulation of MHC class II-restricted presentation by prior LPS treatment of DCs were codependent on MyD88 and TRIF, consistent with the involvement of TLR4 (28). Based on the increased response toward

**FIGURE 6.** Nod receptor signaling interferes with cross-presentation. DCs were activated for 22 h with PGN, iE-DAP, or MDP, then fed apoptotic HSV-infected or uninfected HeLa for 5 h, and finally cocultured with the MHC class I-restricted gB-specific hybridoma (A) or the MHC class II-restricted gD-specific hybridoma (B). IL-2 was measured by ELISA after 20 h. One representative of three experiments is shown. Error bars depict SD. C, DCs prepared as in A and B were labeled with anti-CD11c and anti–I-A/I-E (MHC II), anti-CD86, anti-CD40, or anti-CD70 and analyzed by flow cytometry, with gating on CD11c+ cells. Shaded histograms represent untreated, black lines DAP-treated, and gray lines MDP-treated DCs. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 7.** PGN inhibits cross-presentation of a second HSV Ag. DCs were activated for 22 h with the indicated stimuli (numbers give concentration in micrograms per milliliter) and then fed apoptotic HSV-infected Vero cells (A) or HSV-infected HeLa cells (B) as described in the Materials and Methods section. The RR1822–829-expressing stimulator cell line (Stim) was used as positive control in A. DCs were subsequently cocultured with an MHC class I-restricted RR1/ICP6-specific T cell line (A) or the MHC class II restricted gD-specific hybridoma (B). IL2 was measured by ELISA; error bars show SD. One experiment of at least two is shown. **p < 0.01, ***p < 0.001.
directly infected or peptide-pulsed matured DCs compared with UT DCs, it is likely that at least some of the increase in cross-presentation observed with LPS-treated DCs was due to costimulation. However, costimulation could not explain inhibition of cross-presentation by the different PGN preparations or less pure LPS. Phenotypic maturation is inversely correlated with the ability to present newly acquired Ags on MHC class II, but in our experiments, there was no correlation between the degree of phenotypic maturation and the ability to cross-present Ags on MHC class I.

Unusually high amounts of LPS have often been used experimentally for DC stimulation. Concentrations have ranged up to 10–20 µg/ml (17, 21, 23) even though LPS can mediate effects at nanogram/milliliter levels (20, 48). In the experiments reported in this study, inhibition of cross-presentation by 1 µg/ml LPS occurred only when using the less pure LPS, and it is possible that impurities could have contributed to the inhibitory effects of high-dose LPS described in the literature (17, 21). Although we cannot be certain of the exact nature of the impurity in LPS responsible for eliminating cross-presentation PGN is a likely candidate. In analyzing the problem, we unexpectedly found that PGN signaling via cytosolic Nod receptors can inhibit cross-presentation of HSV-1 Ags without affecting MHC class II presentation.

Under physiological conditions, DCs often meet a combination of TLR and NLR ligands. It is thus not surprising that cross talk occurs not only between different TLRs but also between TLR and Nod receptors as seen in this study. Synergistic or antagonistic effects may occur (49). Nod ligands injected into mice concomitant with OVA resulted in enhanced cross priming, which was in part due to upregulation of costimulatory molecules (50). TLR triggering through signals delivered together with antigenic material during phagocytosis or endocytosis enhances cross-presentation and cross priming (16, 51, 52). This is somewhat different from our in vitro experiments, which examined how maturation prior to Ag encounter modulates the ability to cross-present. We clearly saw that Nod ligation abrogated cross-presentation of HSV Ags even in the presence of an LPS signal that would otherwise enhance it. Cross-presentation of vaccinia virus-expressed OVA (VV-OVA) was also decreased by DC pretreatment with either PGN (Nod) or PGN (TLR2+Nod) (Supplemental Fig. 3A). However, the effect was not as profound as observed for HSV-γb or HSV-ICP6 (Figs. 4, 7). This may either argue for an additional role of signaling events triggered by HSV-infected cells not present in VV-infected cells, or it may be explained by the nature of the OVA Ag. The fact that OVA is soluble and expressed in the cytosol, whereas γb is a transmembrane protein does not appear to be relevant, because cross-presentation of RRI/ICP6, also a cytosolic soluble molecule, is inhibited by PGN (Nod) (Fig. 7). Depending on the delivery form, OVA is predominantly cross-presented through either the vacuolar or the cytosolic pathway (53). Although VV-OVA is thought to follow the cytosolic route for cross-presentation (54 and data not shown), a certain degree of endosomal loading that is not affected by maturation could still take place. The high Ag expression generated by VV-driven OVA compared with naturally expressed HSV Ags may also play a role.

In the 1970s, MDP was identified as the minimal active component in Freud’s adjuvant (55). Nod2 triggering by MDP induces autophagy in human DCs, influencing MHC class II Ag presentation (56). MDP has also been reported to activate Nalp3, another member of the NLR family, leading to inflammasome formation and IL-1β release (57, 58), although this MDP-mediated effect is controversial (59). In our study, treatment of DCs with purified PGN did not affect MHC class II presentation, whereas decreased MHC class II presentation was observed with synthetic MDP. Perhaps we were unable to achieve sufficiently high cytosolic concentrations of MDP to observe this. How PGN degradation products reach the cytosol to meet their respective receptors is not fully clarified (49), similar to the situation regarding the mechanism for translocation of phagosomal Ags for cross-presentation (1). Nevertheless, cross-presentation was highly susceptible to signaling mediated by cytosolic Nod receptors. Pretreatment with pure PGN as well as both synthetic ligands for Nod1 and Nod2 impaired cross-presentation.

Why should a cytosolic receptor be involved in regulating cross-presentation of exogenous Ags? Nod receptors have been shown to be important for immune responses against multiple bacteria (reviewed in Refs. 35, 36), including bacteria that directly gain access to the cytosol such as Listeria monocytogenes (60, 61) or Shigella flexneri (62). There is evidence that Nod receptor activation occurs in vicinity to cellular membranes. Close proximity to endosomal and phagosomal membranes positions Nod receptors at sites that potentially have the highest concentration of translocated ligands liberated during degradation of bacterial cell wall components in the respective organelle or ligands that are directly injected via bacterial secretion systems (29, 30). In terms of Ag presentation, it may make sense that a DC invaded by a bacterium ceases to shuttle exogenous Ags into the cytosol for the cross-presentation–dependent MHC class I pathway. Ags already in the cytosol could be presented via the classical MHC class I route, which is still functional in activated DCs (8, 17). Unlike for MHC class II presentation, most protein Ags or Ag fragments must first be transported for further processing into the cytosol for cross-presentation to occur. Nod receptor triggering may thus act as a feedback loop that signals that sufficient foreign material is present in the cytosol to induce CD8-positive T cell priming. The precise mechanism by which the inhibition of cross-presentation occurs remains to be determined, but a potentially exciting explanation is that Ag translocation from the phagosome into the cytosol is shut down, either by inhibiting translocation directly or by eliminating the translocation machinery from the phagosomal membrane.

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References


**Supplementary figure legend**

**Figure S1** Effect of LPS induced maturation on antigen presentation. DCs were activated with 0.1 or 1 μg/ml LPS for the indicated times, subsequently fed apoptotic HSV-1-infected HeLa cells for 5h and then co-cultured with (A) the MHC class I-restricted gB-specific hybridoma or (B) the MHC class II-restricted gD-specific hybridoma. IL-2 was measured by ELISA after 20h. Data from 6 different experiments were normalized to responses of untreated DCs (UT). Error bars depict SD. (C) DCs used in antigen presentation assays in (A) and (B) were labeled with anti-CD11c and either anti-CD40, anti-CD86, anti-I-A/I-E (MHC II) or anti-H2Kb (MHC I) and analyzed by flow cytometry gating on CD11c+ cells. Shaded histograms represent untreated DCs, gray lines DCs treated with 0.1 μg/ml LPS, black lines DCs treated with 1 μg/ml LPS. (D) Time course of PGN mediated inhibition. DCs were activated with 2 μg/ml PGN (Nod) for the indicated times, subsequently fed apoptotic HSV-1-infected HeLa cells for 5h and then co-cultured with the MHC class I-restricted gB-specific hybridoma. IL-2 was measured by ELISA after 20h. Error bars depict SD, ** p<0.01, *** p<0.001. One representative of 2 experiments is shown.

**Figure S2** The cytokine profile of untreated or matured DCs was assessed for the indicated analytes. DCs were matured for 22h, washed, and re-cultured with or without HSV infected HeLa cells for another 20h prior to supernatant collection. Supernatants from 3 independent experiments was used. Error bars depict SD, * p<0.05, ** p<0.01.

**Figure S3** The effect PGN induced maturation on cross-presentation of vaccinia-expressed OVA (A) Vero cells were infected with VV-OVA at a MOI of 5 and chased for 7h before virus inactivation by UV irradiation. As a control for complete inactivation, VV-OVA-infected Vero were added to H2Kb-expressing 293T cells instead of DCs, which did not activate the B3Z hybridoma (data not shown). DCs were matured with the indicated stimuli for 22h (numbers indicate concentration in μg/ml), fed apoptotic VV-OVA-infected Vero cells (DC:Vero ratio 4:1) for 5h, washed and fixed in 1% PFA for 10min. Fixation was stopped with 200mM glycine in PBS, pH 7.4. After 2 washes, 1x10^5 B3Z cells were added to 0.5x10^5 DCs for 18h. (B) Stimulated or untreated DCs were labeled with anti-CD11c and either anti-H2Kb (MHC I), anti-I-A/I-E (MHC II), anti-CD86, anti-CD40 or anti-CD70 and analyzed by flow cytometry gating on CD11c+ cells.
Supplementary Figure S2
Supplementary Figure S3

A

IL2 Pgf/ml

LT PGN (Nod) 10 PGN (Nod) 30 PGN (TLR2+ Nod) 2 DC no OVA W-Vero no DC

B

% of Max

FL2-H: MHCII FL2-H: MHC I

% of Max

FL2-H: CD40 FL2-H: CD70 FL2-H: CD86

*  **  ***