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

Claudia S. Wagner and Peter Cresswell

J Immunol 2012; 188:686-693; Prepublished online 9 December 2011; doi: 10.4049/jimmunol.1102214
http://www.jimmunol.org/content/188/2/686

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/12/09/jimmunol.1102214.DC1

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References
This article cites 62 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/188/2/686.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
TLR and Nucleotide-Binding Oligomerization Domain-like Receptor Signals Differentially Regulate Exogenous Antigen Presentation

Claudia S. Wagner and Peter Cresswell

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: 686–693.
trivial 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. PGN 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. Medzhitov (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, L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (penicillin/streptomycin [Pen/Strep]), 12 mM HEPES, nonessential amino acids (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 G. Belz (Walter and Eliza Hall Institute of Medical Research) and cultured described (41, 42). The HSV glycoprotein D (gD)290–302–specific MHC class II-restricted hybridoma HSV-2.2E2 was provided by A. Brooks (University of Melbourne) and cultured as HSV-2.2E2. The RR1/ICP6822–829–specific H-2Kb–restricted CTL clone 1D11 and corresponding stimulator cell line B6.T30 RR1322–829 were provided by R. Bonneau (Pennsylvania State University) and cultured as described (43). The BNZ CD8+ T cell hybridoma specific for OVA257–264 was a gift from N. Shastri (University of California, Berkeley) and kept in RPMI 1640 with 10% FBS, 2 mM L-glutamine, Pen/Strep, and 100 mg/ml gentamicin. HeLa cells and Vero cells were grown in DMEM with 10% FCS, L-glutamine, and 20 mg/ml gentamicin.

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 mg/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 mg/ml (except for Fig. 7), 10 mg/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 mg/ml (except for Fig. 7), 10 mg/ml α-g-glutamylmeso-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 Stratalinker 1800. HeLa-HSV was produced 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 in-
They were then cocultured with the H2-K<sup>b</sup>-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<sup>−/−</sup>, TRIF<sup>−/−</sup>, 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<sup>−/−</sup> and TRIF<sup>−/−</sup> 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<sup>−/−</sup>/TRIF<sup>−/−</sup> 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<sup>−/−</sup> DCs being slightly greater than in MyD88<sup>−/−</sup> 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).

**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 MyD88<sup>−/−</sup> 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 MyD88<sup>−/−</sup> 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. 5B). 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)-induced DCs showed a remarkable increase in IL-10 production, with Nod-activated cells producing less IL-10 than untreated DCs, whereas gDT presentation on MHC class II remained unaffected (Fig. 6A). Both concentrations of PGN (Nod) induced a comparable inhibition of 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 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 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 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 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 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).
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+Tol) (Supplemental Fig. 3A). However, the effect was not as profound as observed for HSV-gB 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 gB is a transmembrane protein does not appear to be relevant, because cross-presentation of RR1/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 inflammmasome 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 vivo 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.

Acknowledgments
We thank Noah Palm for helpful discussions and Nancy Dometios for manuscript preparation.

Disclosures
The authors have no financial conflicts of interest.

References


