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Cutting Edge: Cooperation of IPS-1- and TRIF-Dependent Pathways in Poly IC-Enhanced Antibody Production and Cytotoxic T Cell Responses

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Double-stranded RNA, polyriboinosinic-polyribocytidyl acid (poly IC), acts as an adjuvant that enhances adaptive immune responses. The recognition of poly IC is mediated by endosomal TLR3 and cytoplasmic RNA helicase melanoma differentiation-associated gene 5 (Mda5), which signals through the adaptors Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) and IFN-β promoter stimulator-1 (IPS-1), respectively. However, the contribution of these pathways to the adjuvant effects of poly IC remains unclear. In this study, we found that poly IC-enhanced, Ag-specific Ab production was severely decreased in IPS-1-deficient mice but not in TRIF-deficient mice. However, the double deficiency resulted in a complete loss of Ab production. Furthermore, Ag-specific CD8+ T cell expansion was reduced in both IPS-1-deficient and TRIF-deficient mice and entirely abrogated in the doubly deficient mice. Taken together, these results demonstrate that the adjuvant effects of poly IC require a cooperative activation of TLR and cytoplasmic RNA helicase pathways. The Journal of Immunology, 2008, 180: 683–687.

Initial detection of pathogens by the innate immune system provides the first line of defense against pathogens. Dendritic cells (DC)3 and macrophages express pattern recognition receptors that recognize pathogen-associated molecular patterns and trigger intracellular signaling pathways (1–3). They secrete cytokines such as proinflammatory cytokines and type I IFNs, up-regulate costimulatory molecules, present Ags, and develop Ag-specific adaptive immunity (4–6). Thus, activation of the innate immune system is a crucial element not only for the elimination of pathogens but also for the generation of the adaptive immune responses. In particular, type I IFN is shown to be critical for the production of Ab and CTL responses (7, 8). Therefore, ligands for pattern recognition receptors are considered as potent adjuvants that effectively enhance adaptive immune responses.

Polyriboinosinic-polyribocytidyl acid (poly IC), a synthetic double-stranded RNA polymer, induces the secretion of type I IFNs, proinflammatory cytokines, and the maturation of DC (6, 9). TLR3 recognizes poly IC in the endosome and initiates signaling through an adaptor, Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (10, 11). There is a TLR3-independent cytoplasmic pathway for poly IC recognition. Reticuloendothelial acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (Mda5) represent a family of RNA helicases that sense viral RNA in the cytoplasm (12–14). RIG-I recognizes the 5′-triphosphate end of RNA in various RNA viruses, whereas Mda5 recognizes poly IC (14–16). RIG-I and Mda5 signaling solely depend on an adaptor, IFN-β promoter stimulator 1 (IPS-1) (MAVS/Cardif/VISA) (17–22). Together, the host innate immune system employs at least two pathways for poly IC recognition. However, little is known about the contributions of these pathways to the innate immune responses against poly IC or their roles in modulating adaptive immune responses. In this study, we defined the roles of endosomal and cytoplasmic sensors in the adjuvant effects of poly IC by using TRIF- and IPS-1-deficient mice.

Materials and Methods

Mice and cells

IPS-1 and TRIF knockout (KO) mice were described (11, 21). Double KO (DKO) mice were generated by intercrossing IPS-1−/−;TRIF−/− mice. All mice were in 129/OlaXC57BL/6 (B6.129) background. GM-CSF-induced bone marrow DC (GM-DC) were prepared as described previously (21). Serum or cell culture supernatants were analyzed for cytokine levels by ELISA (21).

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3 Abbreviations used in this paper: DC, dendritic cell; DKO, double knockout; GM-DC, GM-CSF-induced bone marrow DC; IP-10, IFN-γ-inducible protein 10; IPS-1, IFN-β promoter stimulator 1; KO, knockout; Mda5, melanoma differentiation-associated gene 5; poly IC, polyriboinosinic-polyribocytidyl acid; RIG-I, retinoic acid-inducible gene I; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; WT, wild type.

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RT-PCR

After i.p., injection with poly IC (200 μg/mouse) (Amersham Biosciences), total RNA was isolated from spleen and RT-PCR was performed (21, 23).

Ab titer

Mice were immunized i.p., with OVA (10 μg/mouse) (Sigma-Aldrich) plus alum (Sigma-Aldrich) (125 μg/mouse) together with or without poly IC (100 μg/mouse) in 500 μl of PBS. Mice were again immunized after 2 wk. After 1 wk, OVA-specific Ab titer in the serum was measured by ELISA (24, 25).

Flow cytometry

DC were stimulated with poly IC (100 μg/ml) or transfected with poly IC (10 μg/ml) using Lipofectamine 2000 (Invitrogen Life Technologies) (21). Cell surface expression of CD86 was measured (21).

Tetramer assay

Mice were immunized i.p. with OVA (2 μg/mouse) plus alum (25 μg/mouse) together with or without poly IC (50 μg/mouse). Mice were again immunized after 1 wk. One wk later, spleen cells were prepared and cultured with or without OVA class I peptide (5 μg/ml) for 1 wk and incubated with H-2Kb tetramer specific to OVA peptide (SIINFEKL) (MBL International), PE-Cy5-conjugated TCRβ, allophycocyanin-conjugated CD8, and anti-CD16/32 (Fc block) (BD Biosciences). Cells were then fixed and analyzed with FACS Calibur (24, 25). The background of activated T cells was also checked. Spleen cells were incubated with anti-CD3 plus anti-CD28 Abs or Con A for 3 days to stain with OVA or an irrelevant peptide tetramer, respectively.

IFN-γ production and CTL assay

Spleen cells were prepared from the same mice as used in Ab titer experiments. Cells plated on 96-well plates were stimulated with OVA class I peptide. After 72 h, culture supernatants were analyzed for IFN-γ levels by ELISA (24, 25). For CTL assay, cells plated on 24-well plates were stimulated with class I peptide (5 μg/ml) for 1 wk and used as effector cells. EL4 cells pulsed with the same peptide were incubated with effector cells and lactate dehydrogenase production was measured (24, 25).

Results and Discussion

We initially analyzed innate immune responses against poly IC of IPS-1 KO, TRIF KO, and DKO mice. DKO mice were born normally and the composition of immune cells was normal as tested (data not shown). We measured serum cytokines after i.p., injection of poly IC. Production of IFN-α, IFN-β, IL-6, and IL-12p40 was induced to the maximum level at 4 h in wild-type (WT) mice (Fig. 1A). IPS-1 KO mice showed severe defects in IFN-α and -β production, whereas TRIF KO mice produced comparable amounts of these IFNs with WT mice. IL-6 production was markedly reduced in IPS-1 KO but modestly reduced in TRIF KO mice. By contrast, IL-12p40 production was entirely dependent on TRIF but not on IPS-1. In DKO mice, the production was totally abolished. Altogether, cytokine production in sera is differentially controlled by IPS-1– and TRIF–pathways.

We next analyzed the induction of cytokine mRNAs in spleen. Poly IC injection into WT mice resulted in the augmented expression of IFN-α, IFN-β, IFN-γ-inducible protein 10 (IP-10), RANTES, IL-6, IL-12p40, and IFN-γ (Fig. 1B). The induction of IFN-α, IFN-β, IL-6, and IFN-γ mRNA was abrogated and IP-10 and RANTES mRNA induction was markedly decreased in IPS-1 KO cells. In TRIF KO cells, the induction of all genes except for IP-10 was decreased. Overall, IPS-1 KO cells displayed more severe impairment than TRIF KO cells. Spleen cells derived from DKO mice exhibited a complete abrogation in induction of these genes (Fig. 1B).

![Graphs showing immune responses](http://www.jimmunol.org/)

**FIGURE 1.** Innate immune responses to poly IC. A, WT, IPS-1 KO, TRIF KO, and DKO mice (n = 3) were i.p. injected with poly IC (200 μg/mouse). The levels of IFN-β, IFN-α, IL-12p40, and IL-6 were measured in serum by ELISA. B, Mice were i.p. injected with poly IC. After 2 and 6 h, total RNA was prepared from spleen and analyzed for the expression of the indicated genes by RT-PCR. Similar results were obtained from two independent experiments. C, DC were stimulated with poly IC. After 24 h, the concentration of IFN-β and IL-12p40 in the culture supernatant was measured by ELISA. +, not detected. D, DC were stimulated or transfected with poly IC (pIC). After 24 h, the surface expression of CD86 was analyzed. med, Medium; tfx, transfection.
We also determined cytokine production by cultured DC. IFN-β production after poly IC stimulation was abrogated in IPS-1 KO DC and decreased in TRIF KO DC (Fig. 1C). IL-12p40 production was severely impaired in both IPS-1 KO and TRIF KO DC. The production of IFN-β and IL-12p40 was completely diminished in DKO DC. We also analyzed CD86 surface expression on DC. CD86 surface expression after poly IC stimulation was severely impaired in IPS-1 KO DC, whereas it was modestly reduced in TRIF KO DC (Fig. 1D). DKO DC exhibited a complete loss of CD86 expression. We also examined CD86 expression after poly IC transfection. It was suggested that poly IC transfection preferentially activates the cytoplasmic pathway. Consistently, the expression of CD86 was decreased in IPS-1 KO DC but normal in TRIF KO DC. In DKO, the induction was entirely abrogated. The response of DKO-derived DC to CpG DNA, which functions through the TLR9-MyD88 pathway, was normal in terms of cytokine induction and CD86 expression (data not shown).

We next examined the roles of the IPS-1 and TRIF pathways in the adjuvant effects of poly IC. We immunized mice with OVA in alum together with or without poly IC and then measured Ag-specific Ab production. We found that the immunization of WT mice with OVA in alum was insufficient to increase the titer of IgG, whereas the addition of poly IC resulted in increased titer (Fig. 2). In IPS-1 KO and TRIF KO mice, total IgG titer was reduced ~80 and 60%, respectively, of that in WT mice (Fig. 2). The titer of IgG1 in IPS-1 KO mice was reduced ~60% of that in WT mice whereas the titer was comparable between WT and TRIF KO mice. IgG2a production was severely reduced in IPS-1 KO mice whereas it was reduced by nearly half in TRIF KO mice. There was no increase in the titer in DKO mice (Fig. 2), indicating that the activation of both the IPS-1 pathway and the TRIF pathway is required for robust Ab responses.

We next determined Ag-specific CD8+ T cells. After immunization with OVA in alum with or without poly IC, spleen cells were cultured in the presence of class I peptide. Tetramer-positive, OVA-specific CD8+ T cells in WT mice immunized with OVA in alum was detected at a similar frequency as those in unimmunized mice (2.96 vs 1.98%) (Fig. 3A). The frequency was increased when immunized with OVA in alum plus poly IC (16.93%). In IPS-1 KO and TRIF KO mice, the frequency was decreased by nearly half (8.54 and 8.91%, respectively) of that of WT mice, and DKO mice did not show expansion of tetramer-positive CD8+ T cells (2.93%) (Fig. 3A). We have obtained similar results when we used freshly isolated spleen cells. The frequency was considerably increased from 0.12% (immunized with OVA in alum) to 0.72% (immunized with OVA in alum with poly IC) in WT mice, whereas it was not increased in DKO (0.09 vs 0.12%) (data not shown). We next analyzed Ag-specific T cell activation. After immunization, spleen cells were cultured with class I peptide and IFN-γ levels was measured. IFN-γ production was considerably reduced in both IPS-1 KO and TRIF KO mice compared with WT mice, and the production was totally abolished in DKO mice (Fig. 3B). Moreover, the IFN-γ production and the frequency of tetramer-positive CD8+ T cells in DKO mice after immunization with OVA in alum plus CpG DNA were the same as those in WT mice (data not shown). Finally, we examined CTL responses. CTL of WT mice after immunization with OVA in alum plus poly IC displayed a strong cytotoxicity against target cells, whereas the cytotoxicity was completely abrogated in DKO mice (Fig. 3C). The cytotoxicity in IPS-1 KO and TRIF KO mice was the similar to that in WT mice (Fig. 3C).

In this study we provided evidence that the adjuvant effects of poly IC require both the IPS-1 pathway and the TRIF pathway. IPS-1 KO mice had severe defects in poly IC-enhanced Ab production whereas TRIF KO mice displayed modestly reduced production. However, IPS-1 and TRIF double-deficiency resulted in a complete loss of Ab production. It was reported that signaling through the IFN receptor α is indispensable for poly IC-enhanced production of Ab (26). Consistently, the level of serum type I IFN after poly IC challenge was severely impaired in IPS-1 KO mice, but not in TRIF KO mice. In addition, DC and spleen cells derived from IPS-1 KO mice exhibited dramatic decreases in type I IFN induction. However, IPS-1 KO spleen cells could induce a small amount of IFN-inducible IP-10 and RANTES, suggesting that IPS-1 KO mice are still capable of inducing a small amount of IFN-inducible genes that may be sufficient to trigger low levels of Ab production.

The expansion of Ag-specific CD8+ T cells and the production of IFN-γ from CD8+ T cells were similarly decreased in both IPS-1 KO and TRIF KO mice. It was reported that IFN-γ production from CD8+ T cell during viral infection was also dependent on type I IFN (27). However, TRIF KO mice displayed normal production of type I IFN in the serum, although the induction was modestly decreased in DC and spleen. Of note, poly IC-induced IL-12p40 production in the serum was diminished in TRIF KO mice. Because IL-12 is important in IFN-γ production from CD8+ T cells, the impairment in TRIF KO mice might be due to defective IL-12p40 production rather than type I IFN. It should be determined which cell types

FIGURE 2. Ag-specific Ab production. Mice (n = 3) were immunized with OVA in alum (indicated by minus sign) or OVA in alum plus poly IC (indicated by plus sign). Titer of total IgG, IgG1, and IgG2a specific to OVA in the serum was measured by ELISA. ND, Not detected. Mean ± SE of two independent experiments (**, p < 0.05 vs WT control) is shown.
produce IL-12p40 in vivo through the TRIF pathway in the future.

Notably, cytokine production by GM-DC derived from IPS-1 KO mice was severely impaired in the absence of transfection. In addition, TRIF KO GM-DC also showed reduced cytokines. Thus, it is likely that poly IC incorporated by GM-DC can be accessible to both endosomal TLR3 and cytoplasmic Mda5, which activate respective signaling pathways to robustly induce cytokines. Alternatively, GM-DC may contain functionally distinct subsets of DC that express either TLR3 or Mda5, and these subsets of DC may cooperatively function together to initiate the immune responses. Indeed, it is shown that TLR3 expression is confined to CD8α- DC that promote cross priming whereas Mda5 is inducibly expressed in most types of cells, including immune and nonimmune cells (28, 29). In either case, activation of both pathways is required for the adjuvant effects of poly IC, because all of the responses examined were completely abolished in DKO mice. Altogether, our results indicated that a combinational recognition of poly IC through endosomal and cytoplasmic receptors is essential for the robust induction of innate and adaptive immune responses.

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Disclosures
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