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*J Immunol* 2008; 180:5799-5803; doi: 10.4049/jimmunol.180.9.5799

http://www.jimmunol.org/content/180/9/5799
CUTTING EDGE

Cutting Edge: Overlapping Functions of TLR7 and TLR9 for Innate Defense against a Herpesvirus Infection

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As initially demonstrated with murine cytomegalovirus (MCMV), plasmacytoid dendritic cells (pDCs) are the major source of IFN-α/β in response to a variety of viruses in vivo. However, contradictory results have been obtained pertaining to the mechanisms promoting IFN-α/β production by pDCs in response to MCMV. In this study we show that TLR7 and TLR9 exert redundant functions for IFN-α/β, IL-12p40, and TNF-α production by pDCs in vivo during MCMV infection. In contrast, we confirm that systemic production of IL-12p70 strictly depends on TLR9. The combined loss of TLR7 and TLR9 recapitulates critical features of the phenotype of MyD88-deficient mice, including a dramatic decrease in systemic IFN-α/β levels, an increase in viral load, and increased susceptibility to MCMV-induced mortality. This is the first demonstration of the implication of TLR7 in the recognition of a DNA virus. The Journal of Immunology, 2008, 180: 5799–5803.

In vertebrates, successful host defense against viral infections relies heavily on the early production of IFN-α/β, which promotes an antiviral state in adjacent noninfected cells as well as the activation of antiviral cytotoxic lymphocytes (1). IL-12 and TNF-α are also critical cytokines involved in antiviral defense. IL-12 stimulates the proliferation of T cells as well as the production of IFN-γ by both NK and T cells, whereas TNF-α takes part in the activation of cellular immunity and in the induction of apoptosis of infected cells. Plasmacytoid dendritic cells (pDCs)5 have been shown to be specialized in the production of high levels of IFN-α/β and TNF-α in response to a wide variety of viruses in both humans and mice (2).

The molecular mechanisms promoting the production of cytokines by pDCs in response to most viral infections are independent of productive viral replication within the pDC itself and rely on the detection of viral genomes from engulfed viral particles or apoptotic debris by TLRs in endosomes (2). pDCs express TLR7, which senses ssRNA, and TLR9, which senses unmethylated CpG DNA, but not TLR3, which senses dsRNA. Engagement of TLR7 or TLR9 drives the production of IFN-α/β and other cytokines in pDCs. In mice, but not humans, TLR7 and TLR9 are also expressed in conventional dendritic cells, although their engagement on these cells fails to induce significant levels of IFN-α (3). TLR2 and TLR4 have also been proposed to sense viral glycoproteins and to trigger pDC IFN-α/β production in response to certain enveloped viruses (4). Responses to TLR7 or TLR9 triggering depend on the signaling through the adaptor molecule MyD88, whereas the induction of IFN-α/β through TLR4 triggering depends on TRIF (Toll/IL-1R domain-containing adapter inducing IFN-β) (5).

During infection with murine cytomegalovirus (MCMV), pDCs account for the overwhelming majority of IFN-α/β production but also contribute significantly to IL-12 and TNF-α production (6, 7). There is a consensus that TLR9 signaling is critical for IL-12p70 production by pDCs and conventional dendritic cells in response to MCMV infection (8–10). In contrast, contradictory results have been published with regard to the mechanisms promoting pDC IFN-α/β production. The implication of TLR9 in this function is unclear, as different conclusions have been reached by the three independent studies published to date: 1) a complete dependency on TLR9 signaling for IFN-α/β production (10); 2) a partial, although strong, dependency on TLR9 signaling for IFN-α/β production (8); and 3) a lack of requirement for TLR9 for this function (9). MyD88-dependent signaling is strictly required for the in vivo production of IFN-α/β by pDCs early after the infection of immunocompetent animals (8, 9). This suggests that not only TLR9 but at least one

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Received for publication November 26, 2007. Accepted for publication March 10, 2008.

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1 This work was supported by an Action thématique et incitative sur programme Grant from the Centre National de la Recherche Scientifique (CNRS) to M.D., an Action thématique et incitative sur programme Grant from the CNRS to L.A., and a grant from the Association pour la Recherche sur le Cancer to M.D. N.Z. was supported by the Ministère de l’Enseignement Supérieur, de la Recherche et des Techniques. S.H.R. was supported by the Fondation pour la Recherche Médicale. S.T. received a CNRS postdoctoral fellowship. Centre d’Immunologie de Marseille-Luminy is supported by institutional grants from Institut National de la Santé et de la Recherche Médicale, CNRS, and the Université de la Méditerranée.

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3 Abbreviations used in this paper: pDC, plasmacytoid dendritic cell; EGFP, enhanced GFP; MCMV, murine cytomegalovirus; WT, wild type.

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other MyD88-dependent receptor may trigger pDC activation for IFN-α/β production early after MCMV infection. Moreover, in MyD88-deficient animals the lack of a pDC response is partly compensated by a delayed and reduced production of IFN-α/β from an unknown cellular source (9). Thus, at later time points after infection cytoplasmic MyD88-independent receptors could contribute to induce the production of IFN-α/β by infected cells, especially in the absence of a pDC response. The picture is even more complex as the roles of TLR2 and TLR3 in IFN-α/β induction in response to MCMV infection are controversial (9–12).

The goal of this article was to thoroughly identify the pattern recognition receptors involved in the activation of pDC responses to MCMV infection in vivo. We confirm that systemic production of IL-12p70 strictly depends on TLR9. In contrast, we demonstrate that TLR7 functions can in part compensate TLR9 deficiency for IFN-α/β, IL-12p40, and TNF-α production by pDCs. Moreover, mice deficient for both TLR7 and TLR9 have significantly higher viral loads than TLR9−/−, TLR7−/−, and wild-type (WT) animals. All of the TLR7−/−/TLR9−/− double-deficient mice died from MCMV infectious doses better handled by TLR9−/− animals and appeared as sensitive to the infection as MyD88−/− animals. Overall, these data demonstrate the involvement of TLR7 in the sensing of and the defense against infection with a DNA virus.

**Materials and Methods**

**Mice and infection**

Pathogen-free C57BL/6 mice were purchased from Charles River Laboratories and bred at the Centre d’Immunologie de Marseille-Luminy, Marseille, France. MyD88−/− (B6.129P2-Myd88tm1Aki; Ref. 13), TLR2−/− (B6.129P2-Tlr2tm1Aki; Ref. 14), TLR4−/− (B6.129P2-Tlr4tm1Aki; Ref. 15), TLR7−/− (B6.129S-Thrlm1Gdy; Ref. 16), and TLR9−/− (B6.129P2-Tlr9tm1Gdy; Ref. 17), originally on 129 genetic background, were backcrossed to C57BL/6 for 10 generations before crosses to generate double-deficient (TLR7−/−/TLR9−/−; B6.129-Tlr7tm1Aki/Tlr9tm1Gdy) animals. All animals were housed under pathogen-free conditions at the Centre d’Immunologie de Marseille-Luminy and used for experiments between 6 and 12 wk of age. Infections were initiated at day 0 by i.p. injection of 5 × 104 or 105 PFU of Smith strain WT or RVG-vectored MCMV (where EGFP is enhanced GFP). Experiments were performed with a DNA virus.

**Sample collection, spleen preparation, and flow cytometry analysis**

After 36 h of infection, mice were anesthetized to collect blood and spleen. Spleen leukocyte suspensions were prepared, stained, and acquired on a FACSCalibur, FACSCanto I, or FACSCanto II flow cytometers (BD Bioscience) as described (6, 7).

**Quantification of cytokines in sera**

Cytokine levels in sera were measured with mouse IFN-α (PBL Biomedical Laboratories), mouse IL-12p70, and mouse TNF-α (R&D Systems) ELISA Kits. Limits of detection for these assays were 90 pg/ml for IFN-α, 20–60 pg/ml for IL-12p70, and 40–80 pg/ml for TNF-α, depending on individual experiments.

**MCMV plaque assay**

Three days after MCMV infection, spleens were harvested and mechanically disrupted in 1 ml of complete DMEM culture medium on ice using a Power Gen 125 tissue homogenizer (Fisher Scientific). Viral loads were measured in the spleen homogenates according to conventional plaque assay protocols using NIH-3T3 cells as described elsewhere (19).

**Statistical analysis**

Statistical analyses for the comparison of cytokine production were performed on Microsoft Excel 2003 with the Student’s t test. Statistical analyses for comparison of the death curves were determined with GraphPad PRISM 5 software by using the log rank test.

**Results**

Drastic decrease in serum IFN-α/β after MCMV infection in TLR7−/−/TLR9−/− mice

To determine the impact of various TLRs on the innate cytokine response to MCMV, we first measured the levels of IFN-α, IL-12p70, and TNF-α in the sera of a series of TLR-deficient mice at day 1.5 postinfection (Fig. 1). MyD88−/− mice were used as controls for the severe deficiency in early innate cytokine production in response to MCMV infection (8, 9). TLR7−/− mice did not show any significant decrease in serum cytokine levels as compared with WT mice (C57BL/6 animals). TLR9−/− mice exhibited significantly decreased serum levels of IL-12p70 and, to a lesser extent, TNF-α in response to MCMV infection. However, the IFN-α response of TLR9−/− animals remained high and did not significantly differ from that of WT mice. In contrast, TLR7−/−/TLR9−/− double-deficient mice showed a drastic decrease in IFN-α serum levels down to undetectable levels in three of five animals. As splenic pDCs are the major source of IFN-α/β after MCMV infection (6–8, 20) and significantly contribute to IL-12 and TNF-α production (6, 7), we next examined whether changes between the different mouse strains examined in absolute pDC numbers or in their activation could explain these results.

Partial redundancy between TLR7 and TLR9 for innate cytokine production by pDCs during MCMV infection

We first measured absolute pDC numbers in the spleen of the different TLR-deficient mouse strains examined. A significant but rather small decrease in absolute numbers of splenic pDCs occurred in TLR7−/−/TLR9−/− animals as compared with
TLR7−/−, TLR9−/− and WT ones (Table I). This could make a contribution to the deficiency in IFN-α/β production observed in these animals but only to a small extent, such that additional functional impairment in the remaining pDCs must exist that is mostly responsible for this phenotype. Therefore, we next investigated at the single cell level, using flow cytometry staining of cytokines, the role of different TLRs in the promotion of pDC responses to MCMV infection. A drastic reduction in the proportion of pDCs producing IFN-α/β (Fig. 2, A and C), IL-12p40, or TNF-α (Fig. 2C) was observed in MyD88−/− animals as compared with WT controls that is in accordance with previous reports (8, 9) and consistent with the drastic decrease in the systemic induction of these cytokines in these animals as described above (Fig. 1). TLR2−/−, TLR4−/−, and TLR7−/− mice did not show any significant decrease in the ability of their pDCs to produce the cytokines (Fig. 2 and data not shown). In contrast, cytokine-expressing pDCs could still be clearly detected in TLR9−/− mice, although their frequency was significantly lower than that in WT mice (Fig. 2, B and C). Thus, these data confirm that TLR9 makes an important contribution to the activation of pDCs for innate cytokine production in response to MCMV infection but that other MyD88-dependent, TLR9-independent mechanisms also exist (8, 9). Strikingly, TLR7−/−TLR9−/− double-deficient mice showed a complete ablation in pDC innate cytokine production (Fig. 2, B and C) in a manner very similar to what was observed in MyD88−/− animals. These data thus demonstrate that TLR7 and TLR9 functions partly overlap to trigger pDC recognition of MCMV infection in vivo for subsequent production of innate cytokines and that no other TLR can compensate for these functions.

### Increased sensitivity of TLR7−/−TLR9−/− mice to MCMV-induced death

To determine the overall susceptibility of the TLR7−/−TLR9−/− double-deficient mice to MCMV challenge, their survival of various infectious doses was compared with that of WT controls, single TLR-deficient animals, or the highly susceptible MyD88−/− mice (8, 9) (Fig. 3). All of the MyD88−/− mice died within 5.5 days after infection whereas all WT animals survived. In contrast, TLR2−/−, TLR4−/−, and TLR7−/− mice did not show any significant decrease in their ability to survive MCMV challenge when compared with WT controls (data not shown and Fig. 3). As previously reported (9, 10), TLR9−/− mice were more susceptible to MCMV-induced mortality than WT mice (Fig. 3). Strikingly, TLR7−/−TLR9−/− mice showed an even more dramatic reduction in survival to the infection. Therefore, we next measured viral titers in the spleens of the different mouse strains examined at day 3 postinfection. Viral loads were comparable between TLR7−/−TLR9−/− and MyD88−/− mice and significantly higher than those of WT, TLR7−/−, or TLR9−/− animals. This was observed by two different techniques with two different viral strains: by plaque assay in total spleens from EGFP MCMV- or WT MCMV-infected mice (Table II) as well as by flow cytometry in isolated splenic leukocytes from EGFP MCMV-infected mice (data not shown). Thus, the high mortality of TLR7−/−TLR9−/− mice during MCMV infection correlated with enhanced replication of the virus in these animals. Overall, these data demonstrate that TLR7 or TLR9 are each able to confer a significant protection against MCMV in

### Table I. pDC percentages and absolute numbers in 36-h MCMV-infected mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage±</th>
<th>Absolute No. (×10⁶)±</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>1.2±0.5</td>
<td>7.1±2.6</td>
</tr>
<tr>
<td>TLR7−/−</td>
<td>0.9±0.2</td>
<td>5.4±1.7</td>
</tr>
<tr>
<td>TLR9−/−</td>
<td>0.9±0.1</td>
<td>5.5±1.0</td>
</tr>
<tr>
<td>TLR7−/−TLR9−/−</td>
<td>0.7±0.1</td>
<td>3.4±0.5</td>
</tr>
</tbody>
</table>

* Mean percentage of pDC within splenocytes ± SD.
* Mean absolute number of pDC in total spleen ± SD.
* p < 0.05 as compared with all other mouse strains.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Analysis of pDC cytokine production by flow cytometry. C57BL/6 (n = 5) and MyD88−/− (n = 2) mice (A and C) and C57BL/6 (n = 3), TLR7−/− (n = 5), TLR9−/− (n = 5), and double-deficient TLR7−/−TLR9−/− (n = 5) mice (B and C) were used to analyze the impact of these molecules on pDC responses against MCMV. Intracellular stainings for IFN-α/β, IL-12p40, or TNF-α and membrane staining for the pDC marker 120G8 were performed on total splenic leukocytes. A and B, Analysis by flow cytometry of IFN-α/β expression in total splenic leukocytes. Numbers in dot plots represent the percentages of cells that produce IFN-α/β (boxed dots) within pDCs (120G8HIGH). C, Bar graphs represent the mean ± SD for the percentages of pDCs that produce cytokines for one representative experiment of six. *, p < 0.05; **, p < 0.01.
The measurement of viral load in spleen 72 h after MCMV infection is shown in Table II. Mice infected with MCMV showed a significant increase in viral load compared to uninfected controls. The viral load was highest in mice infected with high-dose virus inoculum, and the difference was statistically significant (p < 0.01). Mice infected with low-dose virus inoculum showed a lower viral load, but the difference was not significant (NS).

To determine the molecular pathways promoting the production of IFN-α/β, TNF-α, or IL-12 during MCMV infection in vivo, we analyzed cytokine serum levels, pDC cytokine production, viral loads, and the ability to survive infection in mice deficient in MyD88 in various TLRs or in combinations thereof. Our results reveal overlapping functions of TLR7 and TLR9 for resistance to MCMV infection. This is due to their shared ability to promote the sensing of MCMV infection and the subsequent production of IFN-α/β and TNF-α by pDCs. Our demonstration that TLR7 or TLR9 are each able to confer significant protection against MCMV in vivo and that impairment of the functions of both of these receptors is required to dramatically increase susceptibility to infection-induced death explains the much more dramatic susceptibility to MCMV infection of MyD88−/− animals when compared with TLR9−/− ones (8, 9). Additional candidates that could contribute to the MyD88-dependent resistance to MCMV infection include IL-18 and IL-1. A role of IL-18 is unlikely, because IL-18−/− animals are as resistant as WT controls (19, 21). In contrast, a role of IL-1 cannot be formally excluded, although IL-1R−/− animals harbor normal pDC functions and systemic levels of IL-12, IFN-α, and TNF-α (data not shown).

Our study describes the involvement of TLR7 in the recognition of a DNA virus infection. Interestingly, the cytoplasmic receptor for uncapped 5′-phosphorylated RNA, RIG-I (retinoic acid-inducible gene I), has been recently demonstrated to trigger IFN-α/β responses in cells infected by EBV in vitro (22), further supporting a possible role for RNA sensing in the detection of and the response to DNA virus infection. In infected cells, viral mRNAs locate into the cytosol and are therefore accessible to RIG-I. In pDCs infected with vesicular stomatitis virus, it has been recently demonstrated that viral cytosolic RNAs could access TLR7 in the endosomes due to the constitutive autophagic activity that occurs in this cell type (23). This mechanism can be excluded in our model, because pDCs are not infected with MCMV (6, 24) and in vitro activation of pDCs by the virus is strictly dependent on TLR9 for IL-12, IL-18, and IL-1. A role of IL-18 is unlikely, because IL-18−/− animals are as resistant as WT controls (19, 21). In contrast, a role of IL-1 cannot be formally excluded, although IL-1R−/− animals harbor normal pDC functions and systemic levels of IL-12, IFN-α, and TNF-α (data not shown).

In conclusion, our data demonstrate overlapping functions of TLR7 and TLR9 in vivo for pDC activation by, and innate defense against, a herpesvirus. This redundancy may have been selected during evolution to increase the advantage of the host to survive the infection. These results uncover a new aspect in immune sensing of infections by DNA viruses that may help to develop new therapeutic strategies to fight these pathogens.
Acknowledgments
We thank the staff of the animal care facilities and the flow cytometry core facility of the Centre d’Immunologie de Marseille-Luminy for excellent assistance.

Disclosures
The authors have no financial interest of interest.

References