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Role of MyD88 in Route-Dependent Susceptibility to Vesicular Stomatitis Virus Infection


TLRs are important components of the innate immune response. The role of the TLR signaling pathway in host defense against a natural viral infection has been largely unexplored. We found that mice lacking MyD88, an essential adaptor protein in TLR signaling pathway, were extremely sensitive to intranasal infection with vesicular stomatitis virus, and this susceptibility was dose dependent. We demonstrated that this increased susceptibility correlates with the impaired production of IFN-α and defective induction and maintenance of neutralizing Ab. These studies outline the important role of the TLR signaling pathway in nasal mucosa-respiratory tracts-neuroepithelium environment in the protection against microbial pathogen infections. We believe that these results explain how the route of infection, probably by virtue of activating different cell populations, can lead to entirely different outcomes of infection based on the underlying genetics of the host. The Journal of Immunology, 2007, 178: 5173–5181.

Mammalian hosts have evolutionarily developed the innate immune system and the adaptive immune system to combat invading pathogens. Innate responses are not only essential for limiting the systemic spread of invading pathogens but also provide critical signals for activation of the ensuing specific adaptive immune response (1, 2). The adaptive immune system consists of two arms, the humoral immune response, which produces neutralizing Abs, and the cellular immune response, through which pathogen-infected cells are eliminated by CTL or indirectly eliminated via the release of chemokines or cytokines from activated Th CD4+ T cells or CTL (1–5). A combination of innate and pathogen-specific adaptive immune responses is required for effective control of pathogens (1, 2, 4, 5).

TLRs are one of the important participants in the innate immune response (4–6). The mammalian TLR family is composed of ~12 germline-encoded type 1 transmembrane receptors which are related to the Drosophila Toll (6, 7). The engagement of all known TLRs and their ligands, with the exception of TLR3, activates the MyD88-IL-1R-associated kinase-TNFR-associated factor 6 signaling pathway, followed by nuclear translocation of NF-κB and activation of MAPKs such as JNK and p38, which induce the transcriptional regulation of the proinflammatory cytokines and effector cytokines (4, 6). MyD88 is also required for the IL-1 family cytokine (IL-1/IL-18)-induced signaling pathways (8). Cytokines produced as a result of the innate immune response lead to the activation of adaptive immune responses through the up-regulation of MHC class I and II molecules and costimulatory molecules on APCs, and direct activation and maturation of dendritic cells (DCs) and effector T cells (6, 7). The factors that regulate the development and polarization of either a Th1 or Th2 immune response are still not fully delineated (9). Increasing evidence has emerged from studies on TLR and MyD88-deficient mice, suggesting the importance of TLR-MyD88 signaling in host defense and the development of pathogen-specific adaptive immune responses (10–14).

We have previously demonstrated with a noncytopathic virus, lymphocytic choriomeningitis virus, that the TLR-MyD88 pathway plays a critical role in the activation of protective CD8+ T cells and control of the acute lymphocytic choriomeningitis virus infection (14). In the present study, we have evaluated the role of the TLR-MyD88 pathway in the protection of mice from a cytopathic virus infection, vesicular stomatitis virus (VSV). VSV is a member of the Vesiculovirus genus in the Rhabdoviridae family. There were two major reasons why VSV was used in this study. First, the immune responses to VSV have been well characterized (15, 16). VSV infection elicits the production of high levels of type 1 IFN (IFN-α/β) and activation of both CD4+ and CD8+ T cells. In addition, VSV infection initially induces a Th-independent IgM; this is followed by a lifelong Th-dependent IgG response (15). It has been demonstrated that the initial type 1 IFN, Th-independent neutralizing IgM Ab, CD4+ T cells, and Th-dependent neutralizing IgG Abs are all essential for the protection of mice against VSV infection (16, 17). Secondly, it has recently been shown in vitro in isolated cell populations that TLR7 and MyD88 participate in the recognition of VSV in the endosomal compartment and initiate an innate immune response, in particular, the production of type 1 (IFN-α) from plasmacytoid DCs (pDCs) (18). However, the involvement of the TLR-MyD88 pathway in the protection of mice from VSV-induced disease and VSV-induced adaptive immune responses is unknown.

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Abbreviations used in this paper: VSV, vesicular stomatitis virus; DC, dendritic cell; pDC, plasmacytoid DC; Flt3L, human recombinant fms-related tyrosine kinase 3 ligand; KO, knockout; WT, wild type.

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Our studies reveal that in response to intranasal infection of VSV, a natural route for VSV infection, MyD88 is essential for the induction of type 1 IFN, neutralizing Ab production, and protection of mice from lethal infection. In contrast, although i.v. infection, which is not a natural route for VSV infection, induced an impaired adaptive immune response in MyD88 knockout (KO) mice, MyD88 is not critical for i.v. VSV-induced type 1 IFN, as all MyD88 KO mice survived this challenge. This suggests that TLR and MyD88 may be expressed and regulated differently in different tissues and organs. Together, these studies identify the TLR-MyD88 pathway as a major component of protective antiviral immunity to VSV.

Materials and Methods

Mice

MyD88 KO mice were obtained from Dr. S. Akira (Osaka University, Osaka, Japan; Ref. 2). MyD88 KO mice were backcrossed to C57BL/6 mice for at least six generations. The genotypes of the mice were determined by PCR of tail DNA, and backcrossing was confirmed by satellite DNA analysis. Mice were bred and maintained under specific pathogen-free conditions. Age-matched C57BL/6 mice (wild-type (WT) control), and TCR-β-deficient mice were purchased from The Jackson Laboratory. RAG1 KO mice were obtained from Dr. K. L. Rock (University of Massachusetts Medical School, Worcester, MA). Animals were housed and experiments were performed in accordance with animal welfare guidelines.

Virus, virus detection, and experimental infection of mice

The VSV-Indiana (VSV-IND) serotype was used (19). Virus stocks were prepared on confluent BHK-21 monolayer cells infected at a low multiplicity of infection (0.01). Viral titers were determined by plaque assay on Vero cells (20). To verify VSV authenticity, two approaches were used. First, VSV-infected cells were stained positive with a mAb against VSV-GP (PSD4; Sigma-Aldrich); second, PCR and sequencing confirmed further that our VSV is an Indiana strain (our VSV strain shared 91–97% nucleotide identity with published VSV-Indiana strain sequence in GenBank within VSV-GP, M, and NP genes; and within NS gene, shared 92% identity with published VSV-Indiana, but only 54.7% identity with published VSV-NJ strain). As indicated, mice were either infected i.v. with 200 μl of 1–2 × 10^5 PFU of VSV or intranasally with VSV diluted in PBS buffer. For intranasal infection, mice were lightly anesthetized with Isoflurane and given 10 buffer. For intranasal infection, mice were lightly anesthetized with Isoflurane and given 10 buffer. For intranasal infection, mice were lightly anesthetized with Isoflurane and given 10 μl of various doses of VSV diluted with PBS. Mice were monitored daily, and the occurrence of hind limb paralysis and death was recorded. Virus titers of the VSV stock and VSV-infected mouse tissues were determined by plaque assay on Vero cells.

Viral peptides

The VSV-specific CD4 T cell epitope peptides used in this study were MHC class II-restricted p8 and p17 (21). Peptide was synthesized by the Tufts University peptide core facility and HPLC purified.

Determination of Ab responses to VSV

ELISAs were used to detect VSV-specific Ab responses. VSV or VSV-infected BHK-21 cell lysate was used to coat ELISA plates as described previously (3). Sera were prediluted 1/40 in PBS (pH 7.2) and then 2-fold diluted with 1% BSA (PBS, 1% BSA) and added to VSV-coated wells. Each sample was assessed in duplicate. The secondary Abs used were HRP-conjugated rat anti-mouse IgG and IgM. For specific IgG subclass analysis, ELISA was conducted using HRP-conjugated rat anti-mouse IgG1 and IgG2a. All these secondary Abs were purchased from BD Pharmingen and diluted at 1/1000 with PBS, 1% BSA. The substrate was tetramethylbenzidine. The OD was read at 450 nm. The results were expressed as percentage of survival per group to day 20 postintranasal infection. Data are representative of four experiments for both WT and MyD88 KO mice, two experiments for RAG1 KO mice and one for TCR-β KO mice. Age-matched groups of MyD88 KO and WT male mice were intranasally infected with the following doses of VSV: 5 × 10^2 PFU (n = 6 for both strains); 5 × 10^5 PFU (n = 6 for WT, n = 5 for MyD88 KO mice). The hind limb paralysis and death were recorded, and data were shown as percentage of survival per group up to day 14 postintranasal infection. Mice were sacrificed on day 9 postinfection for the analysis of the CD4+ T cell response and the virus titers. C. On day 9 postintranasal VSV (5 × 10^5 PFU) infection, the brains of both MyD88 KO and WT mice (n = 4 for both strain) were collected. The levels of VSV were determined by plaque assay. Results are shown as PFU per gram of tissue.

Neutralizing Ab assay

The neutralization activity of VSV-infected mouse serum was determined by a well-established assay (16). Briefly, sera were prediluted 1/200 in MEM, 2% FCS medium and heat inactivated for 30 min at 56°C. Fifty microliters of 2-fold serial dilutions was mixed with equal volume of VSV (500 PFU/ml) and incubated for 90 min at 37°C. Then the mixture was transferred onto confluent Vero cell monolayers in 96-well plates and incubated for 60 min at 37°C. The monolayers were overlaid with 100 μl of DMEM containing 1% methylcellulose. After incubation for 24 h at 37°C, the overlay was removed, and the monolayer was fixed with 4% formalin and stained with 0.5% crystal violet. The highest dilution of serum that moved every 3 days of culture, and fresh culture medium with GM-CSF was added. The neutralizing activity of VSV-infected mouse sera was determined by plaque assay on Vero cells (20). To verify VSV authenticity, two approaches were used. First, VSV-infected cells were stained positive with a mAb against VSV-GP (PSD4; Sigma-Aldrich); second, PCR and sequencing confirmed further that our VSV is an Indiana strain (our VSV strain shared 91–97% nucleotide identity with published VSV-Indiana strain sequence in GenBank within VSV-GP, M, and NP genes; and within NS gene, shared 92% identity with published VSV-Indiana, but only 54.7% identity with published VSV-NJ strain). As indicated, mice were either infected i.v. with 200 μl of 1–2 × 10^5 PFU of VSV or intranasally with VSV diluted in PBS buffer. For intranasal infection, mice were lightly anesthetized with Isoflurane and given 10 μl of various doses of VSV diluted with PBS. Mice were monitored daily, and the occurrence of hind limb paralysis and death was recorded. Virus titers of the VSV stock and VSV-infected mouse tissues were determined by plaque assay on Vero cells.

FIGURE 1. MyD88 KO mice were more susceptible to intranasal VSV infection. A. Age-matched groups of male mice (WT = 35, MyD88 KO mice = 33, RAG-1 KO mice = 8, TCR-β KO = 4) were intranasally infected as described in Materials and Methods. The hind limb paralysis and death were recorded, and data were shown as percentage of survival per group up to day 20 postintranasal infection. Data are representative of four experiments for both WT and MyD88 KO mice, two experiments for RAG1 KO mice and one for TCR-β KO mice. B. Age-matched groups of MyD88 KO and WT male mice were intranasally infected with the following doses of VSV: 5 × 10^2 PFU (n = 6 for both strains); 5 × 10^5 PFU (n = 6 for WT, n = 5 for MyD88 KO mice). The hind limb paralysis and death were recorded, and data were shown as percentage of survival per group up to day 14 postintranasal infection. Mice were sacrificed on day 9 postinfection for the analysis of the CD4+ T cell response and the virus titers. C. On day 9 postintranasal VSV (5 × 10^5 PFU) infection, the brains of both MyD88 KO and WT mice (n = 4 for both strain) were collected. The levels of VSV were determined by plaque assay. Results are shown as PFU per gram of tissue.

Preparation of GM-CSF or fms-related tyrosine kinase 3 ligand (Flt3L)-expanded bone marrow (BM) DCs

GM-CSF-stimulated BM conventional DCs were prepared according to a previously described method (22). Briefly, BM cells were seeded at 2 × 10^5/100-mm dish in 10 ml of RPMI 1640 containing 100 U/ml recombinant mouse GM-CSF (R&D Systems). One-half of the medium was removed every 3 days of culture, and fresh culture medium with GM-CSF was added.
Intranasal VSV infection induced much lower levels of type 1 IFNs in MyD88-deficient mice compared with WT control, but type 1 IFNs induction by i.v. VSV infection is MyD88 independent. A, MyD88-deficient and WT control mice were intranasally infected with 5 × 10^5 PFU of VSV. Serum samples were collected at different time points as indicated. The activity of type 1 IFN in sera was tested by an antiviral bioassay. The bioassay detects both IFN-α and IFN-β. Representative of two separate experiments (n = 6 for each group). B, Conventional DCs: recombinant mouse GM-CSF-derived BM DCs were challenged with different doses of VSV at 37°C for 24 h and type 1 IFN activity was measured by bioassay. D, Plasmacytoid DCs: Flt3L-expanded BM pDCs were challenged with VSV at a multiplicity of infection of 5 (5 × 10^5 PFU) for 40 h. The activity of type 1 IFNs in the supernatants was determined by bioassay. Results are representative of three separate experiments. *p < 0.05.

**Bioassay for type 1 IFN activity**

To determine the bioactivity of the type 1 IFNs in sera, a biological assay for protection against VSV was used as previously described (14, 24). Briefly, serum samples were preincubated 1/5 (culture supernatants) or 1/50 (serum) and exposed to UV light for 30 min to inactivate any potential live VSV in the samples. NCTC929 cells (provided by Dr. E. Szomolanyi-Tsuda, University of Massachusetts Medical School) were incubated with 2-fold diluted sera for 18–24 h at 37°C and were challenged with VSV. The protection of the NCTC929 cells was used as an index of type 1 IFN activity. The dilution of sera that resulted in a 50% reduction of VSV-induced cytopathic effect (inhibition of VSV-induced cytopathic effect in NCTC929 cells), and the level of type 1 IFN in individual mouse was shown (n = 5 per group) (A, *p = 0.087). B, MyD88 KO and WT control mice were infected with 1 × 10^6 PFU of VSV i.v. Sera were collected at different time points as indicated. The activity of type 1 IFN in sera was tested by an antiviral bioassay. Data are means ± SD of duplicate wells. Results were shown as units per milliliter. A comparison of levels of IFN-α between WT and MyD88-deficient mice revealed no significant differences (p > 0.05). Representative of two separate experiments (n = 6 for each group). C, Conventional DCs: recombinant mouse GM-CSF-derived BM DCs were challenged with different doses of VSV at 37°C for 24 h and type 1 IFN activity was measured by bioassay. D, Plasmacytoid DCs: Flt3L-expanded BM pDCs were challenged with VSV at a multiplicity of infection of 5 (5 × 10^5 PFU) for 40 h. The activity of type 1 IFNs in the supernatants was determined by bioassay. Results are representative of three separate experiments. *p < 0.05.
Ab use.

**Results**

**MyD88 KO mice were more susceptible to intranasal VSV infection**

VSV is able to access the brain following intranasal infection and cause encephalitis as well as meningitis, characterized by hind limb paralysis (3, 27, 28). MyD88, the important adaptor protein for the TLR signaling pathway, has been reported to be involved in VSV-induced IFN-α production. To evaluate whether MyD88 KO mice are more susceptible to VSV infection, MyD88 KO mice were infected by intranasal or i.v. administration of VSV. RAG-1 KO and TCR-β KO mice, which are deficient of both T and B cells (RAG-1 KO mice) or T cells (TCR-β KO mice), were included as controls.

Our results demonstrated that >50% (21 of 33) of MyD88 KO mice had hind limb paralysis and died after intranasal infection with 5 × 10^5 PFU of VSV (Fig. 1A), and furthermore, the intranasal VSV-induced paralysis and death in MyD88 KO mice was dose dependent (Fig. 1B). In addition, all RAG KO and TCR-β KO mice died after either intranasal or i.v. infection with VSV (Fig. 1A) (29). In contrast, very few (3 of 35) WT mice had paralysis and death after intranasal infection with various doses of VSV in at least three experiments (Fig. 1A and 1B). Interestingly, none of the MyD88 KO mice succumbed to i.v. VSV infection (with >40 MyD88 KO mice in at least four experiments; data not shown). Importantly, in one representative experiment, on day 9 postintranasal infection with 5 × 10^5 PFU of VSV, 3 of 4 MyD88 KO mice failed to clear VSV infection from the brains (Fig. 1C). In striking contrast, all 4 WT mice cleared VSV. In addition, on day 9 postintranasal infection with 5 × 10^5 PFU of VSV, the levels of VSV in the spleens, livers, and lungs of both MyD88 KO and WT mice were below the limit of detection (<50 PFU/ml). Thus, these results indicate that MyD88 has a major role in protection against VSV after an intranasal challenge.

The initial induction of type 1 IFN, neutralizing Th-independent IgM, and Th-dependent IgG Abs all contribute to the protection of mice from VSV-induced encephalitis. Our studies demonstrated that significantly more MyD88 KO mice were susceptible to intranasal VSV infection, but all MyD88 KO mice survived after i.v. VSV infection (data not shown and Ref. 18). These different outcomes prompted concern regarding tissue- or organ-specific involvement of MyD88 in the regulation of the protective innate and adaptive immunity against VSV infection.

**Intranasal VSV infection in MyD88 KO mice induced much lower levels of type 1 IFNs, whereas induction of IFN-α after i.v. VSV infection occurs independently of MyD88 in vivo**

Type 1 IFN is critical for the protection of mice from VSV infection (30). MyD88 has been shown to participate in type 1 IFN induction by pDC in response to ssRNA viruses including VSV (18). To evaluate the systemic role of MyD88 in the production of type 1 IFN, MyD88-deficient mice were infected intranasally or i.v. with VSV. Type 1 IFN activity in serum was assessed by bioassay (14, 24). Surprisingly, intranasal VSV infection did induce systemic type 1 IFNs in MyD88 KO mice, but their levels were much lower than those in WT mice (p = 0.087) (Fig. 2A). In contrast, i.v. VSV infection induced comparable levels of IFN-α in both MyD88-deficient and WT mice (Fig. 2B). To further dissect the possible involvement of MyD88 in VSV-induced type 1 IFN, we also examined type 1 IFN induction in GM-CSF-derived BM conventional DCs (CD11b^+ and CD11c^+) and Flt3L-expanded BM-derived pDC. In vitro production of type 1 IFNs in response to VSV by BM-derived conventional DCs was independent of MyD88 (Fig. 2C). However, consistent with published data (18), we found that VSV challenged Flt3L-expanded BM pDC produced type 1 IFN in a MyD88-dependent manner (Fig. 2D). MyD88 participates in the type 1 IFN response of pDC but not conventional DC after in vitro VSV challenge, but it does not appear to play a major role for MyD88 after i.v. VSV challenge.

Taken together, these studies demonstrated that despite a clear role for MyD88 in type 1 IFN production in response to intranasal VSV infection, MyD88 does not play a significant role in the total serum type 1 IFN response to i.v. VSV infection in vivo. We demonstrated that the involvement of MyD88 in VSV-induced type 1 IFN production is cell type dependent.

**MyD88 is critically involved in the regulation of the VSV-induced humoral immune response**

To better understand the involvement of MyD88 in the regulation of VSV-induced humoral immune response, mice were infected...
i.v. with VSV. VSV infection i.v. in WT mice induced IgM (data not shown) and IgG responses with an isotype bias of more IgG2a than IgG1, characteristic of a Th1-type immune response (Fig. 3, A and B). In contrast, MyD88-deficient mice showed much lower IgM levels on day 4 postintranasal or i.v. VSV infection (data not shown) and delayed isotype switching (Fig. 3, A and B). At the peak of the IgG response, MyD88-deficient mice produced more IgG1 subclass than IgG2a subclass anti-VSV Abs, a pattern consistent with a Th2 type immune response (Fig. 3, B and C). Thus, MyD88 affects the anti-VSV humoral immune response.

**MyD88 is required for the maintenance of neutralizing Ab after either intranasal or i.v. VSV challenge**

The neutralization activity of VSV-specific Abs plays a crucial role in the protection of mice from VSV-induced CNS disease. To further examine the bioactivity of these Abs, we determined whether Ab from WT or MyD88 KO mice could neutralize VSV. Intranasal VSV infection induced significant lower levels of neutralizing Ab in MyD88 KO mice than WT mice (Fig. 4A). In the case of i.v. VSV challenge, neutralizing Ab was detected in WT mice, increased from day 7 to day 20 postinfection, and then remained high thereafter (Fig. 4B). In contrast, neutralizing Abs in MyD88-deficient mice initially increased to a level comparable with that of WT on day 7, but this was followed by a rapid decline in titer. Thus, these data demonstrate that MyD88 is required for the maintenance of neutralizing antiviral Ab responses.

**FIGURE 4.** MyD88 KO mice have defective neutralizing Ab responses to VSV. Groups of age- and sex (male)-matched MyD88 KO and WT mice were infected intranasally (A) or i.v. (B) with $5 \times 10^5$ PFU or $1 \times 10^6$ PFU of VSV as described in Figs. 2 and 3. Serum samples were collected at different time points as indicated. The neutralizing Ab titers were measured by a neutralizing assay described in Materials and Methods.

**FIGURE 5.** MyD88 KO mice have impaired CD4$^+$ T cell response to VSV. Age-matched MyD88 KO and WT male mice ($n = 4$ for both strains) were intranasally infected with $5 \times 10^5$ PFU of VSV. On day 9 postinfection, splenocytes were restimulated with either VSV-specific CD4 epitope peptides or PMA plus ionomycin. The CD4$^+$ T cell response was quantified by intracellular staining for IFN-$\gamma$ (A) or TNF-$\alpha$ (B). Cells were gated on CD4$^+$ T cells. C and D, MyD88-deficient and WT control mice were infected i.v. with $2 \times 10^6$ PFU of VSV. Splenocytes isolated from VSV-infected day 7 MyD88-deficient and WT mice were seeded into 96-well plate at the density of $5 \times 10^5$ cells/well and stimulated with VSV-specific CD4 epitope peptides (p8 and p17) at a final concentration of $4 \mu$g/ml (C). In addition, splenocytes were stimulated with immobilized anti-CD3 (20 $\mu$g/ml) and soluble anti-CD28 (100 ng/ml; D). After incubation of 72 h, the levels of IFN-$\gamma$ in culture supernatants were determined by ELISA. Data are means ± SD of duplicate wells. Results are shown as picograms per milliliter. Results are representative of two separate experiments. *, $p < 0.05$. 

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**A**

**B**

**C**

**D**

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By guest on August 31, 2017
MyD88 plays a critical role in activation of VSV-specific CD4⁺ T cell response

CD4⁺ T cells are required for the protection of mice from either intranasal or i.v. VSV infection. To determine whether VSV-infected MyD88-deficient mice have defective CD4⁺ T cell response, the activity of CD4⁺ T cells in the spleens of VSV-infected mice was assessed by either ELISA or intracellular staining for the expression of IFN-γ. Splenocytes were taken from intranasal VSV-infected mice and stimulated in vitro with VSV-specific CD4 epitope peptides, p8 and p17 (21), or PMA plus ionomycin. The expression of IFN-γ or TNF-α was determined by intracellular staining. VSV infection in MyD88 KO mice induced a significantly lower level of VSV-specific CD4⁺ T cell response (Fig. 5A). In contrast, both WT and MyD88 KO CD4⁺ T cells responded equally well to the PMA plus ionomycin (Fig. 5B). Our studies showed MyD88 is also required for i.v. VSV-induced CD4⁺ T cell function. Although WT mice and MyD88 KO mice have comparable numbers of CD4⁺ T cells in their spleens 7 days after infection (data not shown), in the absence of MyD88, CD4⁺ T cells were impaired in their ability to produce IFN-γ in response to the restimulation with the VSV-specific CD4 epitope peptides (Fig. 5C), but they responded equally to anti-CD3 and anti-CD28 stimulation (Fig. 5D). Therefore, these studies demonstrated that MyD88 is required for the functional activation of CD4⁺ T cells in response to VSV challenge.

MyD88-deficient mice have defective IFN-γ and MCP-1 production in response to VSV infection

The initial induction of chemokines and cytokines could play a role in the determination of the adaptive immunity. To further study the possible contribution of MyD88 in the initial proinflammatory chemokine and cytokine response to VSV infection, we examined the initial cytokine levels (IFN-γ and MCP-1) in the peripheral blood of VSV-intranasally infected mice. MyD88-deficient mice produced significantly lower levels of MCP-1 (Fig. 6A) and IFN-γ (Fig. 6B) in the early stages of postintranasal VSV infection than did WT mice. We were unable to detect either IL-4 or IL-10 production from either serum of WT and MyD88 KO mice. VSV infection i.v. also induced impaired production of both MCP-1 and IFN-γ (data not shown).

Discussion

The TLR-MyD88 signaling pathway plays a critical role in the regulation of innate as well as adaptive immunity to protein Ags (6, 7, 31). In the present study, we have examined the contribution of the MyD88 signaling pathway to the protection of virus infection-induced pathogenesis, to in vivo production of type 1 IFN and to the production of neutralizing Abs in mice infected with a cytopathic virus, VSV. We found that VSV-induced mortality is both MyD88 and route dependent. Moreover, intranasal VSV infection in MyD88-deficient mice induced encephalitis in a dose-dependent manner. Second, MyD88 is involved in intranasally VSV-induced type 1 IFN production, which might account for the highly susceptible of MyD88 KO mice to intranasal VSV infection. Third, MyD88 is involved in the activation of the CD4⁺ T cells in response to VSV infection. Finally, MyD88 is essential for the initial IgM induction and for maintenance of the VSV-specific neutralizing Ab.

It has been demonstrated that several factors may contribute to the protection of mice from intranasal VSV-induced encephalitis, including initial type 1 IFN production, the early Th cell-independent IgM neutralizing Ab, and the later Th-dependent IgG neutralizing Abs (32). The essential role of the initial type 1 IFN in protection of mice from VSV infection has been clearly documented in IFN-α/βR KO mice (30). IFN-α/βR KO died within 3–6 days after i.v. VSV infection. The important role of CD4⁺ T cells and CD4⁺ T cell-dependent IgG-neutralizing Ab have been demonstrated in TCR-β KO mice (Ref. 3 and this study). TCR-β KO mice died on days 11–14 after i.v. VSV infection. The early Th-independent IgM-neutralizing Ab response, although not as important as the Th-dependent IgG response, plays a role in the initial restriction of VSV spreading to the brain (32). It has been well documented that intranasal inoculation of VSV initially leads to replication in olfactory receptor neurons, followed by the infection of CNS, which results in encephalitis and death within 6–10 days postintranasal infection (3, 28). Therefore, consistent with these observations, with their lack of T cell and long term protective neutralizing Ab (IgG) responses, all RAG-1 KO and TCR-β KO mice died.

Our studies demonstrated that in response to VSV infection, MyD88-deficient mice had impaired induction of type 1 IFN, significantly delayed or deficient IgM Ab responses, and impaired CD4⁺ T cell responses, an initial comparable but rapidly decreased neutralizing Ab, and >50% of MyD88 KO mice had the hind limb paralysis and died after intranasal challenge with a virus that was not lethal to WT mice. We demonstrated that the TLR essential adaptor protein, MyD88, is required for the protective immunity to intranasal VSV infection by both regulating the production of type 1 IFN and the activation of CD4⁺ T cells and regulating the production of neutralizing Abs (early Th-independent IgM and later Th-dependent IgG response).

It has been reported that neither i.v. nor i.p. infection with VSV causes morbidity in immunocompetent mice and that VSV does not replicate to detectable levels. However, if VSV is given intranasally, virus can gain access to the brain and survival depends on the host’s natural resistance (28, 33), i.e., the production of type 1 IFN-γ and MCP-1.
IFN as well as the levels of IgM- and IgG-neutralizing Abs. The initial impaired induction of type I IFNs together with impaired induction of neutralizing IgM and IgG Abs in intranasal VSV-infected MyD88 KO mice may be relevant to this failure of MyD88 KO to control VSV spread into brain and cause encephalitis (Ref. 33 and Fig. 1 and Table I).

How the MyD88 signaling is involved in the regulation of VSV-induced type 1 IFN production, the activation of both CD4+ T cells and B cells, as well as the induction and maintenance of the neutralizing Ab is currently not clear.

The mechanism responsible for VSV-induced type 1 IFN is complex. Several distinctive key molecules and signaling pathways have been reported to be involved in the VSV-induced type 1 IFN production, such as TLR7 and MyD88 (18), TLR4/CD14 (34), protein kinase R (33), RIG-1 (35), IRF7 (36), TANK-binding kinase 1 and IκB kinase-ε (37), and recently TNFR-associated factor 3 (38). These studies suggested that VSV might interact with multiple molecules including TLRs and through both MyD88-dependent and –independent mechanisms to activate the innate as well as adaptive immunity (38, 39).

With respect to the involvement of TLR signaling in the induction of type 1 IFN, Lund et al. (18) first reported that VSV interacts with TLR7 to initiate a MyD88-dependent type 1 IFN. However, we have demonstrated in the present study that the involvement of MyD88 in VSV-induced in vitro type 1 IFN is cell type dependent. Most importantly, our in vivo experiments revealed that i.v. VSV-induced type 1 IFN was MyD88 independent, and interestingly, intranasal VSV-induced type 1 IFN was MyD88 dependent. Many cell types besides pDC are capable of production of type 1 IFN (36, 40). Like Lund et al. (18), we found that Flt3L-derived pDC produce type 1 IFN in response to VSV via MyD88-dependent pathways. Yet we also found that GM-CSF-derived BM conventional DCs produce type 1 IFNs in response to VSV largely independent of MyD88. Interestingly, a recent study has suggested that pDC are not the major type 1 IFN producer in response to VSV (41). Lund et al. used ELISA to quantify the levels of type 1 IFN. In this study, we predominantly used the bioassay to measure the activities of type 1 IFN. The bioassay is a well-defined classical and sensitive assay for the determination of the bioactivity of all types/subspecies of type 1 IFN (24, 42), whereas ELISA may not be able to detect all subtypes of type 1 IFN. The IFN-α activity in VSV-infected MyD88-deficient and WT murine sera could be completely blocked by a neutralizing Ab specific for mouse IFN-α, which verified the specificity of the type 1 IFN detection bioassay. Moreover, the bioactivity of type 1 IFN produced from BM pDC (Fig. 2D) is comparable with the protein levels measured by ELISA (data not shown and Ref. 3). We used a natural VSV-Indiana strain, whereas Lund et al. used a recombinant VSV strain. Different strains/isolates of VSV could have different properties (43), including induction of type 1 IFN and replication in different type of murine cells.

Recent studies have revealed that TLR-MYD88 pathways are involved in the activation of CD4+ T cell and humoral immune responses to model Ags (7, 44, 45). Our study demonstrates that, in response to either intranasal or i.v. VSV infection, MyD88-deficient mice had a negligible IgM response and delayed isotype switching. Analysis of the IgG isotypes revealed that VSV infection induced more IgG1 than IgG2a in MyD88-deficient mice, characteristic of a Th2 type immune response, whereas VSV infection in WT mice induced more IgG2a than IgG1 which is characteristic of a Th1-type immune response. Thus, based on analysis of Ab switching and formation of IgG subclasses, our study demonstrates that the MyD88 signaling is required for the regulation of the balance of Th1- and Th2-type immune responses and in the absence of MyD88, the immune response to VSV infection is skewed toward a Th2-type immune response. This has physiological significance because the neutralization activity of VSV-specific Ab in MyD88-deficient mice decreased rapidly after infection, while neutralizing titers in WT mice remained stable over time. Moreover, although the initial levels of total IgG Abs in both WT and MyD88-deficient mice were comparable, the neutralization activity and concentration of the Abs in WT mice were much higher than those in MyD88-deficient mice after day 7 post either i.v. or intranasal infection with VSV (Fig. 4). In addition, the similar physiological role of MyD88 in the regulation of Ab responses to microbes has also been demonstrated in Borrelia burgdorferi-infected MyD88 KO mice (46, 47). B. burgdorferi infection in MyD88 KO mice induced comparable levels of total IgG Ab but induced significantly lower levels of IgG2a isotype Ab in MyD88 KO mice than in WT mice.

It has been suggested that several factors may be involved in the regulation of the polarization of naive CD4+ T cells. These include the dose of Ag, the type and activation status of APC, the costimulatory molecules, and the local cytokine environment (31, 48). It has been proposed that IL-12, together with IFN-γ, positively regulates the Th1 polarization of naive CD4+ T cells through up-regulation of IL-12Rβ2 and IL-18R expression, whereas IL-4 negatively regulates IL-18R expression to polarize naive CD4+ T cell toward Th2 development (49). VSV infection induces a normal Th1 type immune response in IL-12-deficient mice, and IL-12 is not required for the protection of mice from VSV infection (50). IFN-γ has been found to promote Ab response switching to IgG2a isotype cells (3, 51, 52). Thus, the initial MyD88-dependent production of chemokines, including IFN-γ production in response to VSV infection, may affect the activation and differentiation of the naive CD4+ T cells as well as the activation of the B cells and the production of neutralizing antiviral Abs (3, 51, 52).

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Route of Infection</th>
<th>IFN-αβ Response</th>
<th>CD4 T Cell Response</th>
<th>Neutralizing Ab</th>
<th>Mortality (%)</th>
<th>p vs WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyD88</td>
<td>i.n.</td>
<td>Low</td>
<td>None</td>
<td>Low</td>
<td>77</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MyD88</td>
<td>i.v.</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>WT</td>
<td>i.n.</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>WT</td>
<td>i.v.</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>0</td>
<td>NS</td>
</tr>
</tbody>
</table>

a Both intranasal (i.n.) and i.v. VSV infections in WT mice induce normal levels of type 1 IFN as well as neutralizing Ab. In marked contrast, intranasal VSV infection in MyD88 KO mice induces both impaired type 1 IFN and CD4 T cell response as well as neutralizing Ab responses, which result in >50% of the MyD88 KO death (p < 0.005). Alternatively, i.v. VSV infection in MyD88 KO mice induces a normal level of type 1 IFN response but an impaired neutralizing Ab; all mice survive.

Table I. Neutralizing Ab production is MyD88 dependent, but the mortality is both MyD88 and route dependent.
The MyD88 adaptor protein is also required for IL-1 and IL-18 signaling pathways (53), and IL-1 and IL-18 have been proposed to contribute to the balance of Th1 and Th2 type immune responses (54, 55). However, our study (unpublished observations) and the study of Hodges et al. (56) have demonstrated that IL-18 does not contribute to intranasal VSV-induced neuropathogenesis. A role for IL-1 in the defective antiviral response of MyD88 KO mice cannot be ruled out. We are conducting studies to address that question.

In conclusion, by using VSV as a model, we have demonstrated that the MyD88 signaling pathway plays an important role in the regulation of innate immunity (type 1 IFN) as well as adaptive immunity (CD4+ T cells and neutralizing Abs) to viral infection. Significantly more MyD88-deficient mice succumbed to intranasal VSV infection (Fig. 1 and Table I). These findings indicate that VSV inoculated via different routes may interact with different types of cells to make type 1 IFN, and these studies further suggest that MyD88 is more critically involved in type 1 IFN induction from cells in nasal-respiratory tract-neuroepithelium environment after intranasal VSV infection. These studies provide further evidence that the TLR-MyD88 pathway is critically involved in the regulation of the protective antiviral immune response. They also emphasize the importance of different routes of infection on activation of innate immunity and its consequences in terms of viral pathogenesis.

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Disclosures
The authors have no financial conflict of interest.

References