Flaviviruses Induce Pro-inflammatory and Anti-inflammatory Cytokines from Murine Dendritic Cells through MyD88-dependent Pathway

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ABSTRACT

Background: The genus *Flavivirus* consists of many emerging arboviruses, including Dengue virus (DV), Japanese encephalitis virus (JEV) and West Nile virus (WNV). Effective preventive vaccines remain elusive for these diseases. Mice are being increasingly used as the animal model for vaccine studies. However, the pathogenic mechanisms of these viruses are not clearly understood. Here, we investigated the interaction of DV and JEV with murine bone marrow-derived dendritic cells (bmDC). **Methods:** ELISA and FACS analysis were employed to investigate cytokine production and phenotypic changes of DCs obtained from bone marrow following flavivirus infection. **Results:** We observed that these viruses altered the cytokine profile and phenotypic markers. Although both viruses belong to the same family, JEV-infected bmDC produced anti-inflammatory cytokine (IL-10) along with pro-inflammatory cytokines, whereas DV infection induced production of large amounts of pro-inflammatory cytokines (IL-6 and TNF-α) and no IL-10 from murine bmDCs. Both flaviviruses also up-regulated the expression of co-stimulatory molecules such as CD40, CD80 and CD86. JEV infection led to down-regulation of MHC II expression on infected bmDCs. We also found that cytokine production induced by JEV and DV is MyD88-dependent. This dependence was complete for DV, as cytokine production was completely abolished in the absence of MyD88. With regard to JEV, the absence of MyD88 led to a partial reduction in cytokine levels. **Conclusion:** Here, we demonstrate that MyD88 plays an important role in the pathogenesis of flaviviruses. Our study provides insight into the pathogenesis of JEV and DV in the murine model. **(Immune Network 2007;7(2):66-74)**

Key Words: Japanese encephalitis virus (JEV), Dengue virus (DV), bone marrow-derived dendritic cells (bmDCs), IL-10, IL-6, TNF-α, MyD88

Introduction

The genus *Flavivirus* comprises over 70 viruses, many of which are associated with severe human diseases. Of particular importance for public health are the mosquito-borne flaviviruses, such as yellow fever virus, Japanese encephalitis virus (JEV), West Nile virus (WNV), and Dengue virus (DV) (1). Flaviviral virions are composed of a lipid bilayer with two or more envelope proteins surrounding a nucleocapsid, which consists of single-stranded positive-sense genomic RNA associated with multiple copies of capsid proteins (1). Dengue fever occurs in tropical and subtropical regions and threatens an estimated 2.5 billion people. Growing urbanization, failure to control mosquito vector and increase in long-distance travel have contributed to the continuing spread and increase of the

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disease (2).

Dendritic cells (DCs), the most potent of the professional APCs, play a central role in the generation of primary T cell responses and the maintenance of immunity (3). Because of their importance in initiating antiviral responses, DCs represent an ideal target for viruses seeking to evade the immune system. Several viruses are known to target DCs and impair antiviral T cell responses (4-8). Ideal activation of naïve T cells depends on the presence of adequate co-stimulatory molecules on APCs and appropriate cytokines in the microenvironment. In human, DCs and Langerhans cells are the primary targets of DV infection (9). Similarly, the primary targets of JEV in human are peripheral blood monocytes (1). Mice are increasingly used as a model in flavivirus pathogenesis and vaccination studies. DV replicates in the brain, liver, spleen (10,11), skeletal muscle (12) and heart (13,11) of mice. JEV has also been shown to multiply in murine spleen (14). JEV has been shown to infect many immune cells including lymphocytes and macrophages (15).

In this study, we report the interaction of flaviviruses, namely JEV and DV, with primary murine DCs. Here, we show that murine DCs interact with these related viruses in completely different ways and respond differently to the viruses. Specifically, IL-10, an immunosuppressive cytokine, was produced by JEV-infected DCs, whereas DV invoked no such response. These results imply a novel potential immunosuppressive pathway by JEV. Moreover, we showed that DV-induced cytokine secretion from bmDC was entirely dependent on MyD88.

Materials and Methods

Mice. C57BL/6 mice were purchased from Damul Science, Inc. (Daejeon, Korea). Myd88$^{-/-}$ mice were a kind gift from the Immunomodulation Research Centre, University of Ulsan (Ulsan, Korea). The mice were housed in our pathogen-free facility and used at 6～9 weeks of age. All investigations adhere to the guidelines set by the Committee on the Care of Laboratory Animal Resources, Chonbuk National University. The animal facility of the Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Cells and viruses. Vero cells (ATCC, CCL 81) were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. Japanese encephalitis virus (JEV) Beijing-1 strain (Green cross Research Institute, Suwon, Korea) was propagated by mouse brain passage. Dengue virus (DV) type-2 was obtained from Dr. Young Seok Jang (Chonbuk National University, Jeonju, Korea) and propagated in Vero cells. Virus titer ($TCID_{50}$) was determined by cytopathic assays, and the virus was kept at $-70^{o}C$ until needed.

Antibodies. The following monoclonal antibodies were obtained from BD Pharmingen (San Jose, CA, USA): phycoerythrin (PE)-conjugated anti-mouse CD11c (clone M1/70), FITC-anti-mouse CD40 (clone 3/23), FITC-anti-mouse CD80 (clone 16-10A1), FITC-anti-mouse CD86 (clone GL1), and FITC-anti-mouse MHC II (clone 25-9-17). FITC-anti-mouse MHC I (clone 28-14-8) was obtained from eBiosciences (San Diego, CA, USA).

Preparation of bone marrow-derived dendritic cells (bmDC). Bone marrow-derived dendritic cells (bmDCs) were prepared as previously described (16) with some modification. Briefly, femurs and tibiae of C57BL/6 mice were removed from the surrounding muscle and connective tissue under sterile conditions. The bones were placed in a petri dish containing RPMI, their epiphyses were cut with scissors, and the marrow was flushed with RPMI 1640 medium using a 10 ml syringe and a needle. After one wash in RPMI 1640 medium, the cells were suspended, counted and diluted to the required concentration with complete RPMI 1640 medium. The bmDCs were seeded at a concentration of 1×10^{6} cells per ml and grown in complete RPMI 1640 medium containing GM-CSF (2 ng/ml) and IL-4 (10 ng/ml). On days 5 and 8, 50% of the culture supernatant was collected, centrifuged and re-suspended in complete RPMI-1640 medium containing cytokines and returned to the original culture flask. On day 10, the supernatants containing cells were admixed with the adherent cells, which were harvested by gentle scraping, and counted.

Virus infection. bmDCs prepared as above were seeded at 5×10^{5} cells per well in 24-well plate and infected with JEV (4×10^{5} $TCID_{50}$) or DV (10^{3} $TCID_{50}$). At different time points post-infection, the supernatant was collected, and cytokine levels in the supernatants were determined by conventional ELISA.

Flow cytometry. bmDCs were harvested 24 h post-infection by gentle scraping and subsequently washed twice with PBS containing 1% BSA and 0.05% NaN_3 (FACS buffer). Cells were resuspended in FACS buffer, divided into 5 equal parts, and incubated on ice for 30 min with adequate amounts of PE-anti-mouse CD11c antibody and appropriate FITC-labeled surface markers. Cells were then washed twice with FACS buffer and fixed with PBS containing 10% formaldehyde. Cells were subsequently resuspended in PBS and stored at 4°C until analysis. Labeled cells were analyzed using a FACSCalibur equipped with the CellQuest program (Becton-Dickinson, Mountain View, CA).

Cytokine ELISA. Sandwich ELISA was used to determine cytokine levels in the culture supernatant. The ELISA plates were coated with antibodies against IL-6, IL-12, IL-10 (Pharmingen: clones MP5-20F3, 9A5,

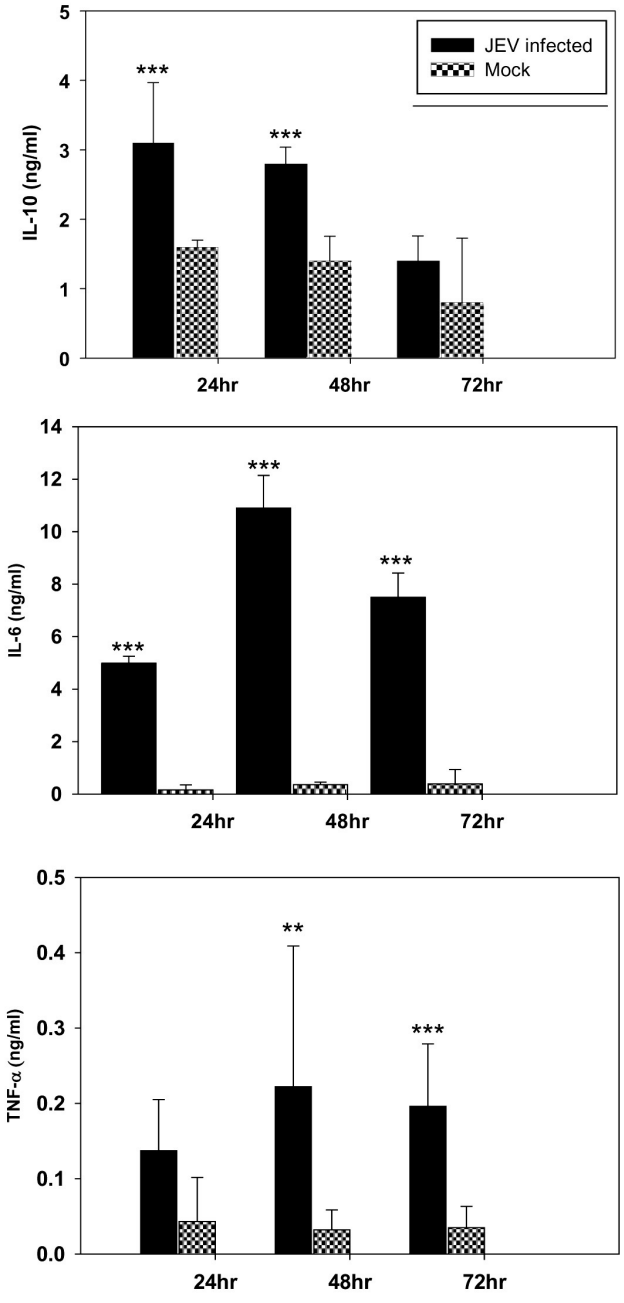

Figure 1. Cytokine levels in the supernatant of JEV-infected bmDCs at various time points. bmDCs were infected with JEV (5×10^4 $TCID_{50}$) and concentrations of IL-10, IL-6 and TNF-α in the supernatant at indicated time points were determined by cytokine ELISA. No IL-12 was detected. Bars represent mean and standard error. *Statistically significant between JEV and mock-infected bmDC ($p<0.05$). **Statistically significant between JEV and mock-infected bmDC ($p<0.01$). ***Statistically significant between JEV and mock-infected bmDC ($p<0.001$).

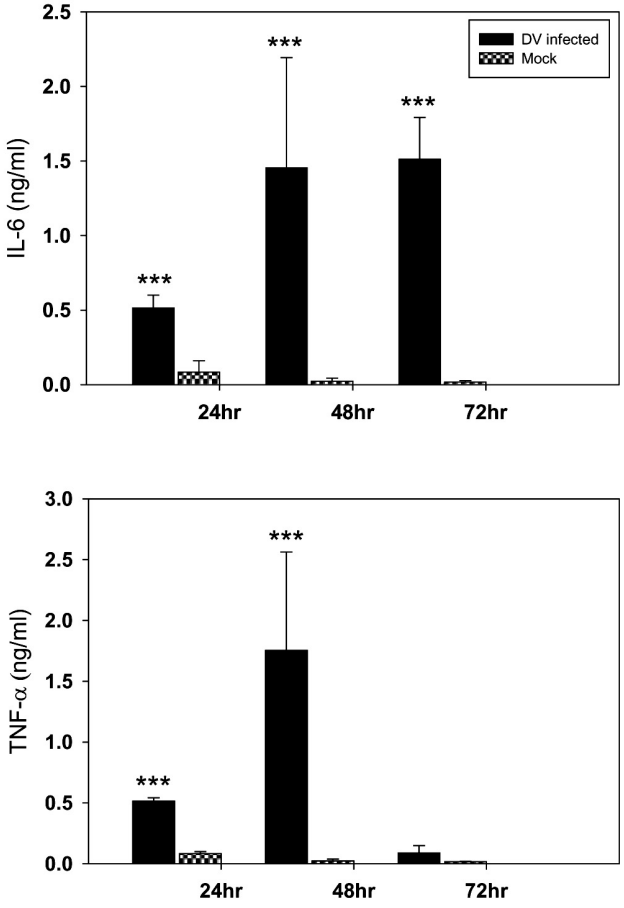

Figure 2. Cytokine levels in the supernatant of DV-infected bmDCs at various time points. bmDCs were infected with DV (10^3 $TCID_{50}$), and concentrations of IL-6 and TNF-α in the supernatant at indicated time points were determined by cytokine ELISA. No IL-10 or IL-12 was detected. Bars represent mean and standard error. *Statistically significant between DV and mock-infected bmDC ($p<0.05$). **Statistically significant between DV and mock-infected bmDC ($p<0.01$). ***Statistically significant between DV and mock-infected bmDC ($p<0.001$).

JES5-2A5, respectively) and TNF-α (eBioscience: clone 1F3F3D4) and incubated overnight at 4°C. The plates were washed three times with PBST and blocked with 3% non-fat dried milk for 2 h at 37°C. The culture supernatant and recombinant IL-6, IL-12, IL-10 and TNF-α protein standards (Pharmingen) were added to the plates, and the plates were incubated for 3 h at 37°C. After washing, biotinylated IL-6, IL-12, IL-10 (Pharmingen: clones MP5-32C11, C17.8, SXC-1, respectively) and TNF-α (eBioscience: clone XT3/XT22) antibodies were added, and the plates were further incubated overnight at 4°C. The plates were then washed and incubated with peroxidase-conjugated streptavidin (Southern Biotech, Birmingham, AL, USA) for 1 hr, followed by color development. The optical density was determined using an ELISA plate reader, and the cytokine concentrations were determined from the standard curve generated from the known standards incorporated in the plate.

Statistical analysis. Where specified, the data were analyzed for statistical significance using Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Cytokine profile of virus-infected bmDCs. To investigate the interaction of flaviviruses with murine DCs, we prepared DCs from murine bone marrow and determined the cytokine profiles after infection with JEV and DV. bmDCs were infected with JEV and levels of IL-6, IL-10, IL-12 and TNF-α in the supernatant were analyzed by ELISA 24, 48, and 72 h post-infection. JEV-infected bmDCs produced very high levels of the pro-inflammatory cytokines, IL-6 and TNF-α (Fig. 1). Surprisingly, the anti-inflammatory cytokine IL-10 was also produced as early as 24 h post-infection (Fig. 1). IL-12 was not detected at any time points (data not shown). The kinetics of pro-inflammatory cytokine and anti-inflammatory cytokine production had opposing patterns. Pro-inflammatory cytokines IL-6 and TNF-α reached peak levels by 48 h and declined thereafter. In contrast,

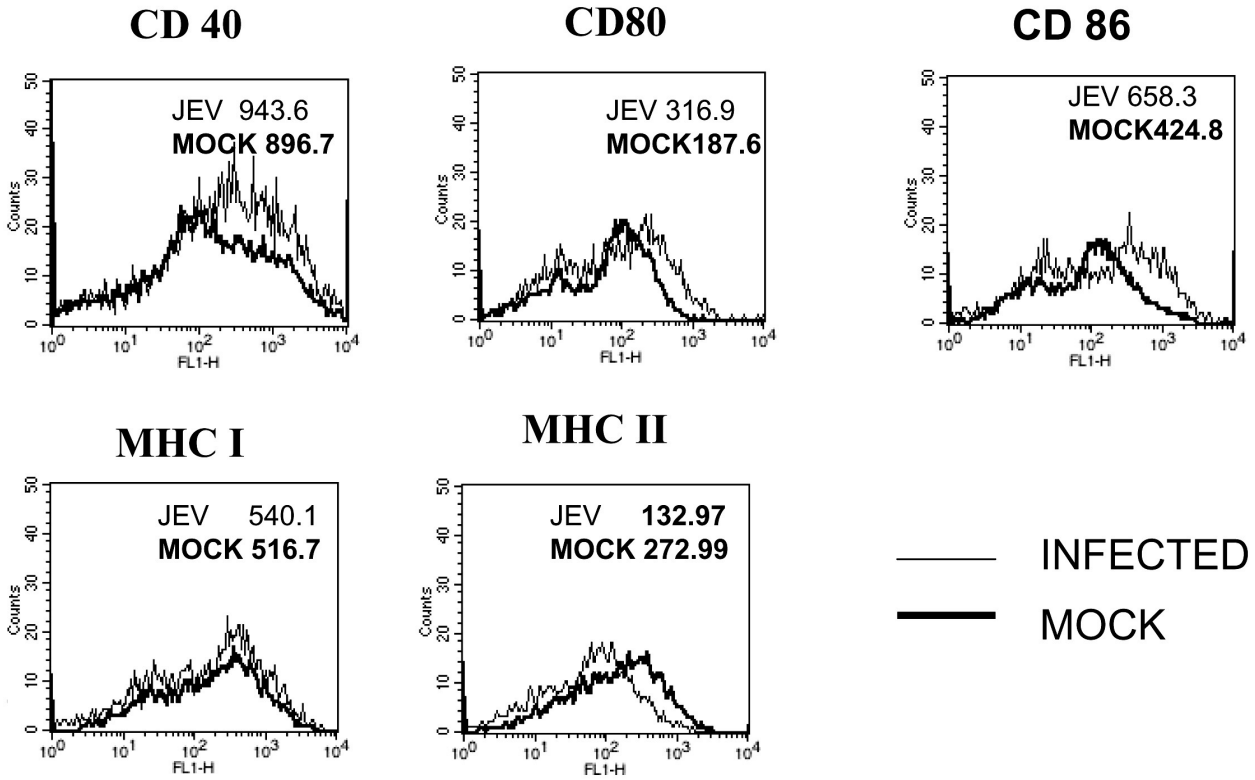

Figure 3. Phenotype of JEV-infected or mock-infected C57BL/6 bmDCs. bmDCs were infected with JEV (5×10^4 $TCID_{50}$). 24 h later, cells were harvested by gentle scraping. Phenotype of CD11c+ cells was determined by two-color FACS analysis. The narrow and bold lines denote JEV and mock-infected bmDC, respectively.

IL-10 levels were maximal at 24 h and declined to a level negligibly higher than background by 72 h. After JEV infection, very few bmDCs survived after 72 h. IL-10 levels in JEV-infected bmDCs varied widely between experiments, ranging from 1.5～4 ng/ml, but were always significantly above the background values. It is worth noting that JEV-infected bmDC produced the anti-inflammatory cytokine, IL-10.

Similarly, bmDCs were infected with DV and the levels of IL-6, IL-10, IL-12 and TNF-α were analyzed in the supernatant 24, 48, and 72 h post-infection. DV-infected bmDCs produced very high levels of pro-inflammatory cytokines, including IL-6 and TNF-α, at the indicated hours post-infection (Fig. 2). IL-6 reached a peak level 48 h post-infection and remained constant, whereas TNF-α attained higher level at 48 h post-infection and declined sharply thereafter. IL-12 and IL-10 were not detected at any time points. Unlike JEV, DV did not induce IL-10 production.

Phenotype of virus-infected bmDCs. Next, we investigated the levels of surface markers on virus-infected DCs, which are important indicators of DC activation and functional capability. Co-stimulatory molecules CD40, CD80 and CD86 are up-regulated after DC activation upon encountering a pathogen, which enables DCs to provide adequate co-stimulatory molecules. Activated DCs also express higher numbers of MHC molecules on the surface. JEV-infected bmDCs were harvested 24 h post-infection, stained with FITC-labeled anti-mouse CD40, CD80, CD86, MHC I and MHC II, and analyzed by flow cytometry. Among the surface markers, CD40, CD80, CD86 and MHC I were up-regulated on JEV-infected bmDCs, whereas MHC II surface expression was profoundly down-regulated (Fig. 3). In DV-infected bmDCs, CD40, CD80, CD86, MHC II and MHC I were up-regulated (Fig. 4). These data indicate that flaviviruses activate murine DCs, although in divergent ways.

Cytokine production was MyD88-dependent. Pathogens are

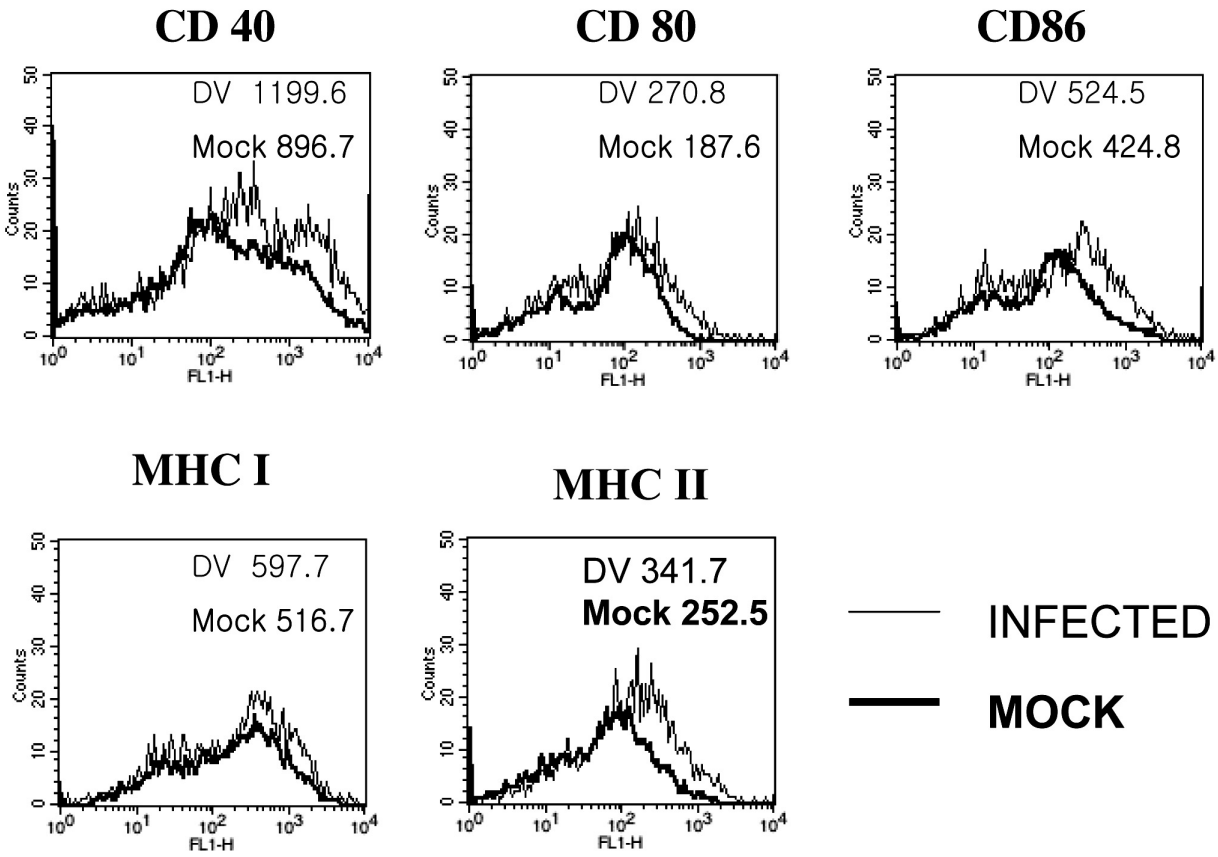

Figure 4. Phenotype of DV-infected or mock-infected C57BL/6 bmDCs. bmDCs were infected with dengue virus (10^3 $TCID_{50}$). 24 h later, cells were harvested by gentle scraping. Phenotype of CD11c+ cells was determined by two-color FACS analysis. The narrow and bold lines denote DV and mock-infected bmDC, respectively.

recognized by the mammalian immune system by innate receptors. Toll-like receptors (TLRs) are an important class of innate receptors. Several viruses, such as HSV and WNV, are recognized by TLRs (17,18). In order to assess the involvement of TLRs in recognition of JEV and DV, we generated bmDCs from MyD88 knock-out (MyD88 KO) mice. MyD88 is a pan-TLR adaptor molecule. It acts as an adaptor molecule in signal transduction from all TLRs, except TLR3. JEV infection of MyD88-deficient bmDCs led to production of the pro-inflammatory cytokines, IL-6 and TNF-α and of the anti-inflammatory cytokine, IL-10, but cytokine levels were less than that in wild-type bmDCs (Fig. 5). Surprisingly, DV infection failed to elicit any cytokines from MyD88-deficient

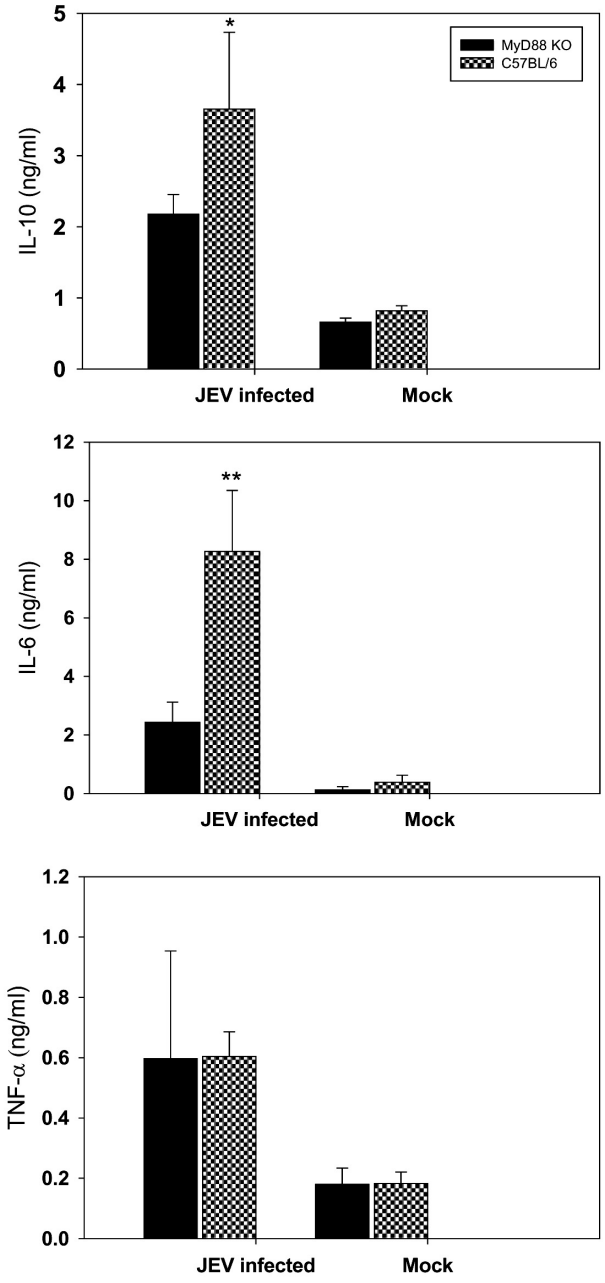

Figure 5. Cytokine levels in the supernatant of JEV-infected bmDCs prepared from MyD88−/− mice and wild-type mice at various time points. bmDCs were infected with JEV (5×10^4 $TCID_{50}$), and IL-10, IL-6 and TNF-α concentrations in the supernatant 24 hr post-infection were determined by cytokine ELISA. The bars represent mean and standard error. *Statistically significant between JEV and mock-infected bmDC ($p<0.05$). **Statistically significant between JEV and mock-infected bmDC ($p<0.01$). ***Statistically significant between JEV and mock-infected bmDC ($p<0.001$).

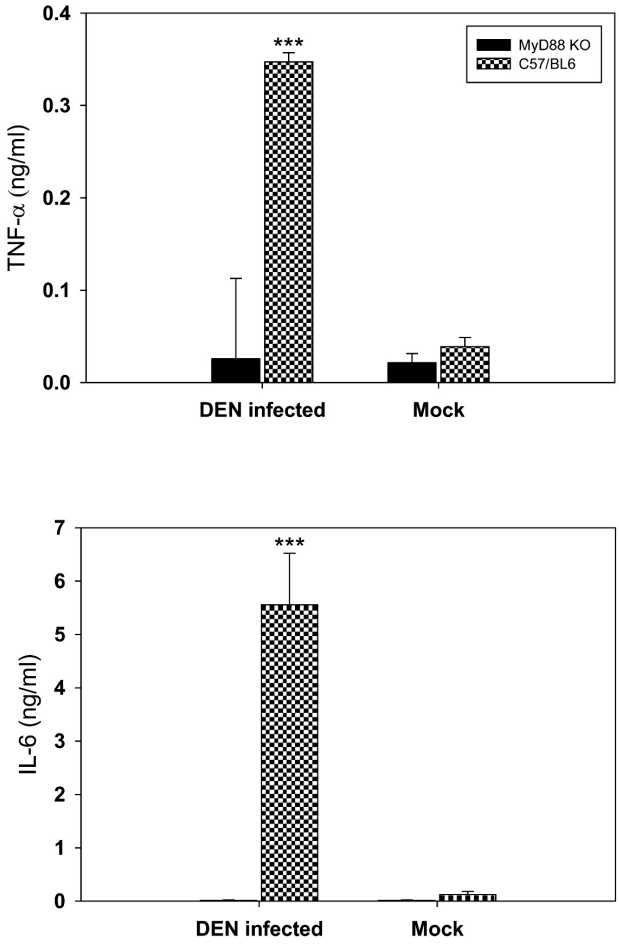

Figure 6. Cytokine levels in the supernatant of Dengue-infected bmDCs prepared from MyD88−/− mice and wild-type mice at various time points. bmDCs (5×10^5) were infected with DV (10^3 $TCID_{50}$), and concentrations of IL-6 and TNF-α in the supernatant 24 hr post-infection were determined by cytokine ELISA. The bars represent average and standard error. *Statistically significant between DV and mock-infected bmDC ($p<0.05$). **Statistically significant between DV and mock-infected bmDC ($p<0.01$). ***Statistically significant between DV and mock-infected bmDC ($p<0.001$).

bmDCs (Fig. 6). These data indicate that, even though JEV and DV belong to same family, both viruses have divergent pathways of interaction with host cells.

Discussion

In this study, we showed that JEV and DV interacted with murine hosts in different ways. The activation of DCs by DV is contingent on the presence of MyD88, a pan-TLR adaptor molecule (Fig. 6). Moreover, JEV induces IL-10 from murine DCs along with the pro-inflammatory cytokines, IL-6 and TNF-α (Fig. 1), indicating a previously unrecognized, possible immune suppression mechanism for this virus.

IL-10 is an anti-inflammatory cytokine. It has recently been reported that viruses induce IL-10 from DCs, thereby preventing development of effective protective immunity. IL-10 induction by LCMV has been shown to lead to persistent infection and development of inadequate adaptive immune response (19,20). JEV has also been reported to cause persistent infection in mice (21). Conceivably, induction of IL-10 from murine DCs might indicate possible pathogenesis of JEV infection. It has previously been reported that DV infection of human monocyte-derived DCs led to IL-10 production. The infected DCs exhibited reduced ability to prime CD8 T cells. Moreover, the same study indicated that neutralization of IL-10 by a neutralizing antibody restored T cell proliferation (22). Therefore, induction of IL-10 from DCs may be an immune evasion mechanism used by flaviviruses. Apart from suppression of APCs and T cells, IL-10 is an important cytokine in the induction of Th2-type responses (23). Virus-induced IL-10 is also implicated in polarizing the T cell response to Th0-type (24). We speculate that these opposing cytokine milieu synergize for an optimal immune evasion mechanism. Further investigation will provide a definitive mechanism for immune suppression by JEV through IL-10 induction.

In humans, DV infection is characterized by very high levels of pro-inflammatory cytokines in the blood of affected individuals (25). DV-infected mice also have high levels of cytokine production (2). In this study, we showed that DV virus induced IL-6 and TNF-α from DCs. Our results indicate a possible source of pro-inflammatory cytokine, which induces immunopathology, in DV infections. DV infection of human DCs has been shown to induce the pro-inflammatory cytokines, IL-6 and TNF-α, along with IL-10. However, in the murine system, no detectable IL-10 was produced. Dengue infection of murine bmDCs preferentially induces high levels of IL-6 (Fig. 2). IL-6 is a pro-inflammatory cytokine produced by many cell types. It plays an important role in T cell activation. Nevertheless, excessive amounts of IL-6 have been shown to inhibit T cell response through a TGF-β-dependent mechanism (26).

Our study indicates that expression of cell surface proteins involved in antigen presentation (MHC II) is reduced in bmDCs infected with JEV (Fig. 3). The expression of these molecules is associated with DC maturation, an essential step for generation of immune responses. The defect in antigen presentation through the class II pathway likely has a profound effect on priming and development of a T helper immune response leading to deficiency in CD4 T cell responses which in turn impairs the ability of a host to resolve viral infection, despite an intact CD8 response (27). LCMV strain Cl-13, which causes a chronic infection in mice, down-regulates class II antigen presentation machinery in splenic DCs leading to development of impaired immune response which fails to clear the infection (28). Our study shows that flaviviruses modulate expression of co-stimulatory molecules on infected bmDCs.

In this paper, we showed that pro-inflammatory cytokine induction by dengue virus was entirely dependent upon MyD88, an adaptor molecule involved in TLR signaling. Previous reports have described that JEV is recognized by RIG-1, a non-TLR innate receptor (29,30). However, cytokine production by JEV was also reduced in the absence of MyD88, indicating a role for MyD88 in virus detection. The TLRs play an essential role in the initiation of innate immunity. In mammals, the TLR family is composed of at least 11～12 members and each TLR acts as a primary sensor of conserved microbial components and drives the induction of specific biological responses. Many viruses are recognized by TLRs or other innate immune receptors. For example, HSV is recognized by TLR2 and TLR9 (31). TLR3, which is a MyD88-independent TLR, is involved in the recognition of viral components, such as dsRNA from viruses and is involved in WNV pathogenesis. TLR7 and TLR8 are vital for the recognition of ssRNA viruses such as influenza (32).

Furthermore, TLR9, which recognizes unmethylated CpG motifs in DNA, is involved in defense against mouse cytomegalovirus infection and HSV. TLR engagement leads to activation of NF-kB, c-Jun N-terminal kinase (JNK), p38, phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) signal transduction pathways and to production of pro-inflammatory chemokines and cytokines. DV and JEV are single-stranded RNA viruses, and RNA intermediates formed during replication or interaction of viral proteins with signaling molecules might induce cytokine production from bmDCs. Further studies are needed to elucidate the clear pathway. Our studies conclusively indicate that MyD88 is an essential factor for innate immune responses against flaviviruses in mice.

In summary, this study demonstrates that flaviviruses, namely JEV and DV, interact with murine DCs and modulate their function in separate ways. JEV induced a strong IL-10 response, along with the cytokines IL-6 and TNF-α, from bmDCs; this induction might be critical in the microenvironment of T cell-DC interaction. We also suggest DCs as a possible source for the pathogenic pro-inflammatory cytokine surge seen in DV infection. Moreover, we observed for the first time that induction of pro-inflammatory cytokines by DV from murine bmDCs is dependent on MyD88. This study provides insights into the pathogenesis and host-virus interaction of JEV and DV in the murine model.

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