Japanese Encephalitis Virus wild strain infection suppresses dendritic cells maturation and function, and causes the expansion of regulatory T cells

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Abstract

Background: Japanese encephalitis (JE) caused by Japanese encephalitis virus (JEV) accounts for acute illness and death. However, few studies have been conducted to unveil the potential pathogenesis mechanism of JEV. Dendritic cells (DCs) are the most prominent antigen-presenting cells (APCs) which induce dual humoral and cellular responses. Thus, the investigation of the interaction between JEV and DCs may be helpful for resolving the mechanism of viral escape from immune surveillance and JE pathogenesis.

Results: We examined the alterations of phenotype and function of DCs including bone marrow-derived DCs (bmDCs) in vitro and spleen-derived DCs (spDCs) in vivo due to JEV P3 wild strain infection. Our results showed that JEV P3 infected DCs in vitro and in vivo. The viral infection inhibited the expression of cell maturation surface markers (CD40, CD80 and CD83) and MHC Ⅰ, and impaired the ability of P3-infected DCs for activating allogeneic naive T cells. In addition, P3 infection suppressed the expression of interferon (IFN)-α and tumor necrosis factor (TNF)-α but enhanced the production of chemokine (C-C motif) ligand 2 (CCL2) and interleukin (IL)-10 of DCs. The infected DCs expanded the population of CD4+ Foxp3+ regulatory T cell (Treg).

Conclusion: JEV P3 infection of DCs impaired cell maturation and T cell activation, modulated cytokine productions and expanded regulatory T cells, suggesting a possible mechanism of JE development.

Background

JEV is a causative agent of JE which causes at least 50,000 clinical cases and about 10,000 deaths each year. It is a member of the mosquito-borne encephalitis complex of the Flaviviridae family and has recently been discovered in previously non-affected areas like Australia [1] and Pakistan [2]. The neurons in the central nervous system (CNS) are target cells of JEV. Studies show that a direct viral cytopathic response and both direct and indirect immunological responses can contribute to CNS degeneration through JEV-infected cell exclusion by macrophages and CTLs, secretion of cytokines and chemokines and activation of microglia [3-6]. However, few studies have investigated the mechanisms by which JEV evades the immune surveillance of the host and passes through the blood-brain barrier (BBB) to the CNS.

Dendritic cells (DCs) are the most prominent antigen-presenting cells (APCs) which induce dual humoral and cellular responses. While DCs also play unique role in inducing immune tolerance, avoiding immune surveillance and causing persistent infection. There are studies about the interaction between virus and DCs which showed that viral infection of DCs inhibited the cell maturation and impaired the cell function [7-9]. Human cytomegalovirus (HCMV) infection de-regulated the expression of surface MHC class I, CD40, CD80 and CD86 molecules on DCs. Furthermore, both T cell proliferation and cytotoxicity of T cells specific to an antigen presented by DCs were reduced via the release of soluble CD83 when DCs were infected with HCMV [8,10,11]. Likewise, human immunodeficiency virus (HIV) affected maturation of DCs within the thymus, which contributed to the loss of the naive T cell and
memory T cell population and even facilitated the dissemination of HIV [12].

Additionally, recent studies revealed that several viruses belonging to the *Flaviviridae* family, such as classical swine fever virus (CSFV), Dengue virus (DV) and Yellow fever virus (YFV), infected DCs and altered the cell phenotype and function [13-15]. Furthermore, Aleyas et al. [2009] recently reported that JEV Beijing strain replicated both in bmDCs and macrophages, and induced functional impairment of DCs through MyD88-dependent and independent pathways which subsequently led to poor CD4+ and CD8+ T cell responses [16]. Thus, the investigation of the interaction between virus and DCs is imperative for resolving the viral escape from immune surveillance and JE pathogenesis.

Since there is no evidence for JEV infection of DCs *in vivo*, we investigated the alteration of phenotype and function of the JEV P3-infected DCs both *in vitro* and *in vivo*. Our results indicated that JEV P3 severely infected DCs *in vitro* and *in vivo*, and the infection with JEV impaired cell maturation and the capacity for T cell activation. In addition, our study also showed that the infection of DCs with P3 expanded the population of CD4+ Foxp3+ regulatory T cell (Treg) with immunosuppressive potential, suggesting that the virus-induced alteration of DCs is a likely cause of the immunosuppression found in JEV infection.

**Results**

**JEV P3 infection of DCs *in vitro* and *in vivo***
The purity of the bmDCs fraction from cell culture or infected mouse splenocytes was higher than 90% as determined by FACS analysis with surface molecules expression (CD11c). After JEV infection, a 467-bp specific RNA fragment of JEV was detected by RT-PCR (Figure 1A) and the E protein of the JEV was detected by Western blotting in DCs (Figure 1B). FACS results showed over 80% bmDCs and 90% spDCs were infected by JEV P3 (Figure 1C). Analysis by real-time PCR showed that DCs supported JEV replication and yielded infectious virus (Figure 1D). These results suggest that JEV infected DCs both *in vitro* and *in vivo*.

**P3 infection suppressed the maturation of DCs**

DCs present antigen to and activate T lymphocytes through up-regulating the expression of costimulatory and antigen presentation-associated molecules at the mature stage [17]. To examine whether the characteristics of immature DCs were altered by P3 infection, we tested the surface molecules of the infected DCs *in vitro* and *in vivo*. The expression of maturation surface markers, including CD40, CD80, CD83 and MHCII was up-regulated in UV-P3-stimulated, but not in P3-infected bmDCs and spDCs or mock-treated DCs (Figure 2), indicating that UV-P3 stimulation accelerated the maturity of DCs whereas P3 infection dramatically inhibited the cell maturation process.

**P3 infection modulated cytokine production of DCs**

In many cases, virus does not directly result in the destruction of host organism but instead causes indirect damage through the disordered release of cytokines [18]. In addition, imbalanced levels of cytokines may contribute to viral persistence and irreversible immunosuppression. Therefore, we examined the profiles of pro- and anti-inflammatory cytokines produced by P3-infected DCs *in vitro* and *in vivo*. Our results showed that P3 infection enhanced the releases of IL-10 and CCL2 of DCs but suppressed the production of IFN-α and TNF-α (Figure 3). And it was interesting to show that JEV which was inactivated by UV irradiation failed to induce the production of IL-10 and CCL2 but succeeded in inducing the expression of IFN-α and TNF-α. This indicates that the release of CCL2 and IL-10 from DCs was dependent on viral replication, while the production of IFN-α and TNF-α was independent on viral replication.

**DCs infected with P3 attenuated allostimulatory activities to T cells**

To test whether P3 infection will impair the ability of DCs to activate allogeneic naïve T cells, the direct effect of P3-infected DCs in activation of naïve T cells was analyzed by mixed lymphocyte reaction (MLR) and ELISPOT assay. In MLR, the allo-stimulative capability of P3-infected DCs was significantly suppressed by P3 infection compared to the UV-P3-stimulated group (*P* < 0.05). In addition, the viral infection blocked the LPS-induced allostimulatory activity of DCs (Figure 4A, B).

In ELISPOT assay detecting IFN-γ producing T cells, the number of spot forming units/10⁶ purified T cells was counted after twenty four hour incubation with differently treated bmDCs or spDCs. The results in vitro showed that P3-infected bmDCs activated 25 ± 9 spots/10⁶, while the UV-P3-stimulated bmDCs activated 68 ± 21 naïve T cells/10⁶. In vivo, P3-infected spDCs produced 52 ±12 spots/10⁶ whereas UV-P3-stimulated spDCs produced 107 ± 34 spots/10⁶. This was consistent with the result of MLR assay. P3 infection, *in vivo* or *in vitro*, significantly suppressed the ability of DCs to activate allogeneic naïve T cells in response to LPS treatment (Figure 5A, B and 5C). It implied that P3 infection played an important role in the dysfunction of DCs in activating allogeneic T cells.

**P3-infected DCs expanded Treg**

The immune response may be limited in magnitude and efficacy when the host with normal Treg function is infected with virus. We examined whether P3-infected
Figure 1 P3 infects DCs in vitro and in vivo. (A) The in vitro infected bmDCs and the spDCs from P3-challenged mice were harvested and analyzed with RT-PCR. Bands shown are 467-bp PCR products specific for JEV. (B) The bmDCs and spDCs were analyzed for E protein (JEV envelope protein) by separation of the proteins on a 10% SDS-PAGE gel followed by electrotransfer to NC membranes and incubation with monoclonal antibodies against E protein. (C) The bmDCs were harvested after 3 days infection and the spDCs were isolated from mice which had been challenged for 5 days. 1 × 10^6 bmDCs or spDCs were doubly stained with FITC-anti-E and PE-anti-CD11c and analyzed by FACS respectively. (D) The infected bmDCs and the spDCs from challenged mice were collected 3 times at day 1, 3 and 5, and a real-time PCR was performed to quantitatively detect RNA copies of JEV. Each point represents the mean ± SD determinants in triplicate.
DCs would modulate Treg differentiation. The test revealed that P3-infected bmDCs significantly enhanced the differentiation of Foxp3+ Treg in vitro which was consistent with the results in vivo (Figure 6A, B and 6C). However, the UV-P3-stimulated DCs did not alter the expansion of the Treg, as well as the mock-treated DCs.

Discussion
Most studies conducted to evaluate the pathogenesis of JEV infection have noted the interaction of the virus with macrophages, microglia and astrocytes, which are major contributors to the production of inflammatory cytokines and CNS degeneration [3,4,6]. In the present
study, we attempted to address the possible pathogenesis of JEV wild strain infection by testing the interaction of JEV and DCs in vivo and in vitro.

Carrasco et al., [2004] discovered that CSFV could infect and replicate in monocyte and myeloid-derived DCs [14]. Therefore, we hypothesized that JEV, which also belongs to the Flaviviridae family, may affect DCs to facilitate viral spread by escaping immune surveillance. Although Aleyas et al. [2009] recently reported JEV infection of DCs in vitro, whether JEV infects DCs in vivo remained unknown until now. Our research not only verified the results of Aleyas [16], but also investigated the JEV infection of DCs in vivo. Additionally, one of our preliminary experiments showed that when BALB/c mice were inoculated with C6FeK4N6-labeled P3-infected bmDCs or spDCs via intraperitoneal (i.p.), JEV and C6FeK4N6-labeled DCs were detected simultaneously in the brain of mice with severe symptoms of immunohistochemistry (unpublished data). It is likely that JEV could use DCs as a virus delivery vehicle as it moves through the CNS.

The impaired surface molecule expression of APCs may directly affect the process of antigen presentation and T cell activation. Thus, we analyzed the alteration of the surface-molecule expression of infected DCs in vitro and in vivo. The FACS analyses revealed an suppressed expression of surface molecules, such as CD40, CD80, CD83 and MHCI, on P3-infected DCs in vitro and in vivo, which is in accordance with Aleyas’s results [16]. While we also discovered that the antigen-presenting-associated molecules on bmDCs were significantly enhanced after JEV SA14-14-2 strain (a successful JEV live vaccine strain) infection [19]. This suggests the potential molecular mechanism of the immune escape of P3 and the high immunopotency of SA14-14-2.

Since we have verified that JEV infection impaired the expression of antigen presenting-molecules and co-stimulator molecules, whether this impairment of the crucial components on DCs would affect their capacity to activate CD4+ and CD8+ T cell directly is needed to be investigated[20,21]. Thus, we analyzed the capacity of the infected DCs for activating allogeneic T cells by MLR and ELISPOT assay. It was observed that the T cell activating ability of was dramatically impaired by P3-infection, but boosted by UV-P3 stimulation and SA14-14-2 infection. It has been reported that Hepatitis C virus (HCV), Ebola viruses and HIV escaped immune surveillance during acute or chronic infection because of the defect of APCs function for activating T cell [21-23]. Therefore it suggested that the impairment of activating of allogeneic naïve T cells of P3 infected DCs could be involved in the JE development.

Treg is a subset of CD4+ T-cell with regulatory properties. Previous studies on the role of Tregs in viral infections suggest that they suppresses antiviral effector T cell responses or local immune activation at the sites of viral replication [24,25], which may subsequently result in viral immune evasion and the establishment of chronic infections [26-28]. Our FACS results showed that P3 infection contributed to the differentiation of
Treg in vivo. The results also demonstrated the expansion of Treg population after the co-culture of P3-infected DCs and T cells. It suggested that JEV infection of DCs might influence the mode of T-cell differentiation. Thus, we assumed that induction and expansion of Treg cells by JEV-infected DCs may be associated with immunosuppression in JEV infection. It has previously been shown that immature DCs induced Treg cells are able to suppress other T-cell responses [29-33]. Furthermore, it has been demonstrated that the increased production of IL-10 played an important role in Treg responses which appeared to contribute to immune dysfunction, accounting for viral persistence and acute tissue damage. Therefore, the up-regulation of IL-10 in P3-infected DCs may partly contribute to the expansion of Treg. Based on these results, we suggest that P3 infection may have led to the expansion of Treg cell population in vivo, which could have been involved in the suppression of anti-JEV immune responses. In addition, it is essential to note that although CD25 is expressed on most regulatory T cells, it is not specific since it can also be expressed on activated CD4+ T cells.

Figure 4 Effects of P3 infection on DCs activation of naïve T cells by MLR. Mock-treated, P3-infected or UV-P3-stimulated DCs as well as differently treated spDCs were added in grade dose to 1 × 10⁵ allogeneic T cells at the indicated stimulator-responder ratios in triplicate, with (B) or without (A) LPS treatment for 20 h before the addition of 50 μl of CellTiter 96® AQueous One Solution Cell Proliferation Assay. The bmDCs, spDCs as well as T cells were served as spontaneous NADH/NADPH releases controls respectively. The presentation activities of differently treated bmDCs were measured as 100% (OD490D+C+T exp.-OD490D spont.-OD490T spont.)/(OD490T spont.). Results were expressed as the means ± SD of triplicates. *, P < 0.05.
Figure 5 IFN-γ producing T cells were detected by ELISPOT assay. P3-infected, UV-P3-stimulated or mock-treated DCs as well as differently treated spDCs were harvested and treated with Mitomycin C (Sigma-Aldrich, MO) at final concentration of 10 μg/ml for 1 h. The differently treated or mock DCs were seeded (1 × 10^4 per well) together with 1 × 10^5 per well T cells in triplicates for 20 h. LPS-stimulated DC/T cell co-cultures served as positive controls. One representative for IFN-γ spot forming unit (SFU) by ELISPOT assay was shown (A). The figure was representative of three independent experiments. Corrected data (SFU)/well were shown for bmDCs and spDCs activations for naïve T cells to expand and produce IFN-γ by ELISPOT assay (B, in vitro; C, in vivo). Results were expressed as the means ± SD of triplicates. *, P < 0.05.

Figure 6 Effects of P3 infection on DCs-induced differentiation of regulatory T cells. 1 × 10^5 mock-, P3-, UV-P3- or LPS-treated bmDCs were incubated with 1 × 10^6 allogeneic naïve T cells for 5 days. T cells were purified and doubly labeled for CD4 and Foxp3, and assessed by FACS. The in vivo Treg in splenocytes were purified and examined by FACS from mice inoculated with 1 × 10^5 PFU P3 or identical UV-P3 i.p. for 5 days. Representative result was shown from three independent experiments (A). The percentage represented the ratio of CD4+ Foxp3+ cells in CD4+ T cells. P3-infected bmDCs elicited the Treg differentiation in vitro (B). After P3 infection or UV-P3 stimulation of mice i.p., Treg differentiation in vivo was analyzed immediately (C). Results were expressed as the means ± SD of triplicates. *, P < 0.05.
type I IFN is a key step in the innate immune response to virus infection of Th1 and Th2 [36-38]. In particular, secretion of IFN-I is extremely inhibited in those cell types in which IFN-I is constitutively expressed. On the contrary, the expression of IFN-I by pDCs, which generate the crucial signal adaptor IRF7, participates in the differentiation of regulatory T cells, which indicates that the functional impairment of viral infected DCs orchestrates the immunosuppression in response to the acute JE development.

Conclusion
Our data reveals that JEV P3 could infect mouse DCs in vitro and in vivo, and the infection affects the phenotype and function of DCs, including reducing expression of costimulatory molecules, modulating secretion of crucial cytokines, suppressing activation of T cells, and stimulating differentiation of regulatory T cells, which indicates that the functional impairment of viral infected DCs orchestrates the immunosuppression in response to the acute JE infection.

Methods
Reagents, virus and cells
The fluorescent antibodies, including CD11c-PE (N418), CD40-FITC (HM40-3), CD80-FITC (16-10A1), CD83-FITC (34-1-2S) and MHC I-FITC (Michel-17), recombinant mouse granulocyte-macrophage colony stimulating factor (rmGM-CSF) and IL-4 (rmIL-4) were purchased from eBioscience Inc. (San Diego, CA). The anti-E (JEV envelope protein) MAb was generated in our laboratory and purified with NAb™ Spin Kits (Thermo Scientific, USA) according to the manufacturer's instructions. JEV P3 strain was produced in BHK-21 which was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, MO) supplemented with 10% heated-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) of 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, MO) at 37°C with 5% CO2. And then the virus was titrated by plaque formation assay with BHK-21 cell line. JEV stock was treated with UV irradiation for 1 min (wavelength 253.7 nm, radiation intensity ≥ 60 μW/cm2, distance 30 cm).

Generation of bone marrow-derived DCs (bmDCs) and spleen-derived DCs (spDCs)
For generation of bmDCs from BALB/c mouse bone marrow cultures, the procedure of Inaba et al., [1992] was used.
was used with minor modifications [47]. Briefly, the bone marrow was flushed from femurs and tibias and subsequently depleted of erythrocytes with ammonium chloride. Cells were plated at 2 × 10⁶/ml in DCs media (RPMI 1640 supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 10 ng/ml of rmGM-CSF and rmIL-4). At day 2 and 4 of culturing, 50% of the supernatant was removed and replenished with fresh DCs media. At day 6, non-adherent cells were collected and transferred into a new dish. After a total of 7 to 9 days of culturing, bmDCs were harvested and purified with StemSep™ Mouse Dendritic Cell Enrichment Kit (StemCell, Vancouver, BC, Canada).

Four-week old BALB/c mice were infected with 1 × 10⁵ PFU of JEV P3 i.p., stimulated with identical quantity of UV-P3 or left mock-treated for 5 days. The splenocytes were obtained from P3-infected or UV-P3-stimulated or mock-treated mice. The spDCs were isolated from the splenocytes and purified with StemSepTM Mouse Dendritic Cell Enrichment Kit (StemCell, Vancouver, BC, Canada) according to the manufacturer’s guidelines. The purity of the bmDCs and spDCs fraction was higher than 90% as determined by FACS analysis of CD11c. Dendritic morphology was assessed by phase-contrast microscopy and viability was assessed by trypan blue exclusion.

JEV P3 infection of DCs

The immature bmDCs were infected with P3 at an MOI of 1. After 1 h of infection in incomplete medium (DCs media without FBS), cells were washed thoroughly three times and cultured in DCs medium. In some instances, the infected bmDCs were cultured for up to 5 days and on each day cell supernatants were collected and measured for viral RNA quantity. Similarly, the spDCs were harvested from mouse splenocytes every other day thrice after challenge with 10⁵ PFU of JEV per mouse i.p. to detect the viral load in spDCs. Relative levels of viral load in P3-infected bmDCs or spDCs were determined by conducting quantitative real-time PCR analysis by ABI prism 7500 Sequence Detection System (Applied Biosystems) reverse transcription of total RNA isolated from infected samples. Thermal cycling conditions were 2 min at 50°C, 10 min at 94°C, 40 cycles of 15 s at 94°C and then 1 min at 60°C. Gene expression was measured by relative quantity and normalized to β-actin expression by the subtraction of Ct’s to provide ΔCt values. After 3 days culture, cells were harvested and used to detect the viral production by RT-PCR and Western blotting and the samples were subjected to PCR. The consensus primers 5’-GCTCTGAAAGGCACACCC-3’ (primer1) and 5’-CTGAAAGGCATCATCAACAC-3’ (primer2) were used to amplify the 467-bp DNA products which were specific for JEV. For Western blotting analysis, cells were collected after 3 days infection and the total proteins were separated by 10% SDS-PAGE. Separated proteins were electroblotted onto a nitrocellulose membrane. The nonspecific antibody-binding sites were blocked with 1% bovine serum albumin (BSA) in TBS-T buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween-20), and then membranes reacted with anti-E MAb. The resulting blot was treated with peroxidase-conjugated goat anti-mouse IgG (Southern-Biotech, USA). 3, 3-Diaminobenzidine tetrahydrochloride (DAB) was used as substrate for membrane development. The in vitro bmDCs were harvested after 3 days infection and the in vivo spDCs were isolated from mice which had been challenged for 5 days. 1 × 10⁵ bmDCs or spDCs were doubly stained with 1.0 μg FITC-anti-E and 1.0 μg PE-anti-CD11c and analyzed by FACS respectively.

Phenotypic analysis

After 3 days in vitro infection or 5 days post inoculation, as described in the JEV P3 infection of DCs, the expression of maturation markers of bmDCs and spDCs were determined by FACS on a FACSCalibur (Beckton-Dickinson [BD], San Jose, CA). 1 × 10⁵ bmDCs or spDCs were stained with surface marker antibodies including CD11c, CD40, CD80, CD83 and MHCI, or isotype controls at 4°C for 30 min as per manufacturer’s guidelines (eBioscience Inc., San Diego, CA). After washing three times with PBS containing 1% FBS, DCs were phenotypically analyzed by FACS.

Analysis of cytokine production

The cytokine releases (IFN-α, TNF-α, CCL2 and IL-10) from P3-infected, UV-P3-stimulated or mock-treated bmDCs or spDCs from differently treated mice were measured by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience Inc., San Diego, CA) in accordance with the manufacturer’s guidelines. LPS or poly (IC) served as positive agonist. The concentrations of cytokines in the samples were accessed from the standard curves.

T cells activation capacity of P3-infected DCs (MLR and ELISPOT assay)

Mixed lymphocyte reactions (MLR) were performed by co-incubation of 1 × 10⁵, 2 × 10⁵ or 1 × 10⁶ P3-infected, UV-P3-stimulated or mock-treated, bmDCs or spDCs from differently treated mice with or without 1 μg/ml LPS treatment and 1 × 10⁵ allogeneic naive T cell per well in 96-well plates (Costar, Cambridge, MA). The mock-treated, P3-infected, UV-P3-stimulated, bmDCs and spDCs or T cells served as spontaneous NADH/ NADPH release controls respectively. After 3 days of incubation in a humidified chamber at 37°C in 5% CO₂,
50 μl of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was added to each well for 30 min at RT, and then 50 μl of stop solution (10% SDS) was added. The absorbance at 490 nm was recorded by ELISA reader (AD340; Beckman Coulter, Fullerton, CA, USA). The activities for activating T cells of differently treated bmDCs were measured as 100% (OD490DC+T exp.-OD490DC spont.-OD490T spont.)/(OD490T spont.).

P3-infected, UV-P3-stimulated or mock-treated bmDCs or spDCs from differently treated mice were harvested and treated with Mitomycin C (Sigma-Aldrich, MO) at final concentration of 10 μg/ml for 1 h and washed twice before assessment with enzyme-linked immunospot assay with Mouse IFN-γ ELISPOT Kit (eBioscience Inc., San Diego, CA). PVDF-membrane-bottomed 96-well plates (Millipore) were coated with 10 μg/ml of mAb on IFN-γ in carbonate coating buffer. The treated or mock bmDCs were seeded in triplicates (1 × 103 per well) together with 1 × 105 per well T cells. LPS (lipopolysaccharide, Sigma-Aldrich, MO)-stimulated DC/T cell co-cultures were used as controls. After incubation for 20 h, cells were discarded and the plates were washed in PBS-0.05% Tween and incubated with biotinylated anti-IFN-γ mAb (1:1000). After washing, plates were incubated with HRP-Avidin, washed and incubated with AEC solution (Sigma-Aldrich, MO). The staining was stopped by rinsing with water and a red spot was counted as single spot forming unit (SFU). After rewash, the cytokine-producing cells were visualized with substrate in accordance with the manufacturer’s guidelines and counted with an automated ELISPOT reader (AID). The spot-forming T cell number was calculated as following: No.DC+T-No.DC.

**T cell isolation and Treg differentiation**

T cells from splenocytes of BALB/c mice were enriched by StemSep™ Mouse T Cell Enrichment Kit (StemCell, Vancouver, BC, Canada) in accordance with the manufacturer’s guidelines. Purified T cells were cultured in RPMI 1640 supplemented with 5% FBS, 1 × nonessential amino acids, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 500 mM 2-ME, 100 μg/ml streptomycin and 100 U/ml penicillin.

To assess the impact of JEV infection on Treg cell differentiation in vivo, 1 × 105, P3-infected, UV-P3-stimulated, LPS- or mock-treated bmDCs were added to 1 × 106 allogeneic naïve T cells in 12-well flat-bottom plates (Costar, Cambridge, MA) in triplicate. After 5 days of co-culture, in vitro Treg cells (CD4+ and Foxp3+) were isolated (StemCell, Vancouver, BC, Canada) and stained with Mouse Regulatory T Cell Staining Kit (eBioscience Inc., San Diego, CA) in accordance with the manufacturer’s instructions and analyzed by FACS. The in vivo Treg in splenocytes were purified and conducted on FACS from mice challenged with 105 PFU P3 or inoculated with identical UV-P3 for 5 days or from mock-treated mice.

**Statistical analysis**

Statistical analysis was performed using the Student’s t-test. Means were considered significantly different at P < 0.05.

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**Authors’ contributions**

SC, YL and JY carried out most of the experiments and wrote the manuscript. XY, LC and XL participated part of experiments. HC and SC conceived of the study, participated in its design and coordination, and revised the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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10. Se'nech'al B, Borouch AM, Reagan JL, Hart DNU, Young JW. Infection of mature monocyte-derived dendritic cells with human cytomegalovirus inhibits stimulation of T-cell proliferation via the release of soluble CD83. Blood 2004, 103:4207-4215.

11. Beck K, Meier-König U, Weidemann M, Nern C, Hufert FT. Human cytomegalovirus impairs dendritic cell function: a novel mechanism of human cytomegalovirus immune escape. Eur J Immunol 2003, 33:1528-1538.

12. Knight SC, Patterson S. Bone marrow-derived dendritic cells, infection with human immunodeficient immunodeficiency virus, and immunopathology. Arnu Rev Immunol 1997, 15:599-615.

13. Ho LJ, Wang JJ, Shiao MF, Kao CL, Chang DM, Han SW, Lai HJ. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. J Immunol 2001, 166:1499-1506.

14. Carrasco CP, Rogden RC, Vincent IE, Balmelli C, Ceppi M, Bauhofer O, Täcke V, Hjertner B, McNelly F, van Gennip HG, McCullough KC, Summerfield A. Interaction of classical swine fever virus with dendritic cells. J Gen Virol 2004, 85:1633-1641.

15. Barba-Spaeth G, Longman RS, Albert ML, Rice CM. Live attenuated yellow fever 17D infects human DCs and allows for presentation of endogenous and reconstituent T cell epitopes. J Exp Med 2005, 202:1179-1188.

16. Aleya AG, George JA, Han YW, Rahman MM, Kim SJ, Han SB, Kim BS, Kim K. Eo SK. Functional modulation of dendritic cells and macrophages by Japanese Encephalitis virus through MyD88 adaptor molecule-dependent and -independent pathways. J Immunol 2009, 183:2462-2474.

17. Banerjee A, Steinman RM. Dendritic cells and the control of immunity. Nat Immunol 1998, 9:245-252.

18. Quaglìorelli V, Wispelwyk L, Long WJ, Scheld WM. Reconstituent human interleukin-1 induces meningoitis and blood-brain barrier injury in the rat: characterization and comparison of tissue necrosis factors. J Clin Invest 1991, 87:1360-1366.

19. Li YM, Ye J, Yang XH, Xu M, Chen L, Mei L, Zhu JH, Liu XQ, Chen HC, Cao SB. Japanese encephalitis virus induces cells maturation and triggers T cells activation. Vaccine 2011, 29:153-160.

20. Salio M, Cellà M, Suter M, Lanazzacchia A. Inhibition of dendritic cell maturation by herpes simplex virus. Eur J Immunol 1999, 29:3245-3253.

21. Kanto T, Hayashi N, Takehara T, Tatsuni T, Kuzushita N, Ito A, Sasaki Y, Kasahara A, Hori M. Impaired allostochasticity capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol 1999, 162:5584-5591.

22. Donaghy H, Gazzard B, Gocht F, Patterson S. Dysfunction and infection of freshly isolated blood myeloid and plasmacytoid dendritic cells in patients infected with HIV-1. Blood. 2003, 101:4505-4511.

23. Mahanty S, Hutchinson K, Agarwal S, Mclaren M, Rollin PE, Pulendran B. Cutting edge: impairment of dendritic cells and adaptive immunity by Ebola and Lassa viruses. J Immunol 2003, 170:2879-2881.

24. Ishiwata M, Messer RJ, Patterson KE, Stromnes IM, Iwashiro M, Sakaguchi S, Ottenhoff TH. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ T cells. J Immunol 2005, 174:5071-5079.

25. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 12 and the immune response, disease and therapy. Immunol Today 1994, 15:460-463.

26. Carrasco CP, Rigden RC, Vincent IE, Balmelli C, Ceppi M, Bauhofer O, Täcke V, Hjertner B, McNelly F, van Gennip HG, McCullough KC, Summerfield A. Interaction of classical swine fever virus with dendritic cells. J Gen Virol 2004, 85:1633-1641.

27. Cao Talmor M, Ravetch JV, Kayo Inaba, Steinman RM: Regulatory T cells activation. J Immunol 2000, 163:293-303.

28. Macatonia SE, Hensken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O’Garra A. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol 1995, 154:5071-5079.

29. Trinchieri G, Scott P. The role of interleukin 12 in the immune response, disease and therapy. Immunol Today 1994, 15:460-463.

30. Chang TH, Liao CL, Lin YL: Flavivirus induces interferon-beta gene expression through a pathway involving IRF5-dependent IRF-3 and HIV-1-dependent NF-kB activation. Microbes and Infection 2008, 8:1517-1521.

31. Silva MC, Guerrero-Plata A, Gaffo FD, Garafalo RP, Mason PW. Differential activation of human Monocyte-derived and plasmacytoid dendritic cells by West Nile Virus generated in different host cells. J Virol 2007, 81:40-48.