Escherichia coli K1 Promotes the Ligation of CD47 with Thrombospondin-1 To Prevent the Maturation of Dendritic Cells in the Pathogenesis of Neonatal Meningitis

Rahul Mittal, Ignacio Gonzalez-Gomez and Nemani V. Prasadara

*J Immunol* 2010; 185:2998-3006; Prepublished online 30 July 2010; doi: 10.4049/jimmunol.1001296

http://www.jimmunol.org/content/185/5/2998

Supplementary Material http://www.jimmunol.org/content/suppl/2010/07/28/jimmunol.1001296.DC1

References This article cites 43 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/185/5/2998.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2010 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Escherichia coli K1 Promotes the Ligation of CD47 with Thrombospondin-1 To Prevent the Maturation of Dendritic Cells in the Pathogenesis of Neonatal Meningitis

Rahul Mittal,* Ignacio Gonzalez-Gomez,*‡ and Nemani V. Prasadarao*†,‡,§

Dendritic cells (DCs) are professional APCs providing a critical link between adaptive and innate immune responses. Our previous studies have shown that Escherichia coli K1 internalization of myeloid DCs suppressed the maturation of the cells for which outer membrane protein A (OmpA) expression is essential. In this study, we demonstrate that infection of DCs with OmpA+ E. coli significantly upregulates the expression of CD47, an integrin-associated protein, and its natural ligand thrombospondin 1 (TSP-1). Pretreatment of DCs with anti-CD47 blocking Ab or knocking down the expression of CD47 or TSP-1, but not signal regulatory protein α by small interfering RNA, abrogated the suppressive effect of E. coli K1. Ligation of CD47 with a mAb prevented the maturation and cytokine production by DCs upon stimulation with LPS similar to the inhibitory effect induced by OmpA+ E. coli. In agreement with the in vitro studies, suppression of CD47 or TSP-1 expression in newborn mice by a novel in vivo small interfering RNA technique protected the animals against bacterial meningitis. Reconstitution of CD47 knockout mice with CD47+ DCs renders the animals susceptible to meningitis by E. coli K1, substantiating the role of CD47 expression in DCs for the occurrence of meningitis. Our results demonstrate a role for CD47 for the first time in bacterial pathogenesis and may be a novel target for designing preventive approaches for E. coli K1 meningitis. The Journal of Immunology, 2010, 185: 2998–3006.

Bacterial meningitis is the most common and devastating infection of the CNS (1). Escherichia coli K1 is one of the most predominant pathogens causing neonatal septicemia and meningitis (2). Despite recent advances in antibiotic therapy and supportive care, bacterial sepsis and meningitis due to E. coli remain a serious disease with unacceptable rates of mortality and morbidity (3). The disease is fatal in 5–40% of infected neonates and causes neurologic sequelae in up to 30% of the survivors (4). A recent surge in antibiotic-resistant strains of E. coli K1 may increase the mortality rates significantly (5). A comprehensive understanding of the mechanisms involved at every step of the pathogenesis is vital to develop new methods of treatment and prevention. High degree of bacteremia is a prerequisite for the bacteria to cross the blood-brain barrier, indicating that the bacterium must evade host defense mechanisms and survive in blood or tissues (3). However, precise mechanisms by which E. coli K1 survives in host tissues despite the presence of potent immune cells like dendritic cells (DCs), neutrophils, macrophages, and T cells are still far from clear. DCs are professional APCs playing a crucial role in the induction and regulation of immune responses. Our previous studies have demonstrated that E. coli K1, expressing outer membrane protein A (OmpA), enters, survives, and replicates inside myeloid DCs, whereas OmpA+ E. coli was killed within a short period (6). Exposure of DCs to live OmpA+ E. coli K1 prevented DCs from progressing in their maturation process, as indicated by failure to upregulate costimulatory molecules, CD40, HLA-DR, and CD86. However, the mechanisms by which E. coli K1 makes DCs tolerogenic are still not clear.

The development of fully mature DCs is carefully controlled at its multiple differentiation steps, and DCs can be tolerized by simultaneous stimulation with endogenous products (7). This prevents the detrimental immune response to self and harmless indigenous flora (8). However, these differentiation control points can be exploited by pathogens to produce tolerogenic immature DCs (iDCs) (9). CD47 (integrin-associated protein) is one of the molecules that have been implicated in the tolerogenicity of immune cells (10). CD47 is a transmembrane glycoprotein, with a highly glycosylated extracellular IgG domain at its N terminus, a hydrophobic five-transmembrane–spanning domain, and a short cytoplasmic tail (11). It was first identified as a protein associated with vβ3 integrins in placenta and in neutrophil granulocytes, and later shown to have the capacity to regulate integrin function and leukocyte responses to arginine–glycine–aspartic acid–containing extracellular matrix proteins (12). CD47 has also been described to interact with the integrins Lfβ3 and 2β1 (13). Thrombospondin (TSP) is a natural ligand for CD47, which is a homotrimeric extracellular matrix protein that is produced not only by platelets, but also by monocytes, DCs, and macrophages (14, 15). Its expression is highly regulated by different hormones and cytokines, and is developmentally controlled (16). The levels of TSPs in body fluids and their distribution in tissues have been correlated with various pathological states (17). TSP-1 is transiently expressed at high concentrations in damaged and inflamed tissues. Signal regulatory protein α (SIRP-α) can also function as a ligand for CD47 (10, 18). SIRP-α is a type 1 transmembrane receptor of the Ig superfamily with one or three extracellular Ig
domains (alternative splicing) and an intracellular tail with two ITIMs (19). SIRP-α has been implicated in the suppression of anchorage-independent cell growth, mediation of macrophage multineululation, skeletal muscle differentiation, neuronal survival, and synaptogenesis (20, 21).

In this study, we demonstrate for the first time that E. coli K1 upregulates the expression of CD47 in DCs, making them tolerogenic so that it can evade immune defense mechanisms and survive in the host. Furthermore, suppression of CD47 expression by small interfering RNA (siRNA) in newborn mice protected the animals from E. coli K1-induced meningitis, and adaptive transfer of CD47+ DCs in CD47 knockdown (KD) mice renders them susceptible to infection, indicating that CD47 might be a novel target for the prevention of this deadly disease.

Materials and Methods

Bacterial strains

E44 (OmpA+ E. coli) is a spontaneous rifampicin-resistant mutant of E. coli K1 strain RS218 (serotype O18:K1:H7) that was isolated from the cerebrospinal fluid of a neonate with meningitis (6, 22). E91 (OmpA+ E. coli) is a tetracycline-resistant mutant of E44 lacking the entire OmpA gene (6, 22). The strains were grown in brain heart infusion media supplemented with appropriate antibiotics.

Generation of monocyte-derived DCs

DCs were generated from human PBMCs, as described previously (6). Briefly, monocytes were purified from PBMCs by positive selection using CD14 immunomagnetic beads (Miltenyi Biotec, Auburn, CA). CD14+ cells were then cultured in RPMI 1640 supplemented with 10% FCS, 2.4 mM L-glutamine (Invitrogen, Carlsbad, CA), 50 ng ml−1 human rGM-CSF, and 20 ng ml−1 human rIL-4 (PeproTech, Rocky Hill, NJ). The strains were used after 7 d of culture, and the phenotype was determined by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). iDCs were CD3−, CD14+, CD19−, CD83−, CD25−, and expressed low levels of HLA-DR, CD40, CD86, and CD1a. For stimulation experiments, DCs (5 × 105 ml−1) were infected with E. coli at a multiplicity of infection of 10 (cell to bacteria ratio 1:10) for 24 and 48 h. DCs were also treated with CD47 mAb (20 μg ml−1), CD47 blocking Ab, isotype-matched control Ab (20 μg ml−1), Neomarkers, Fremont, CA), SIRP-α Ab (20 μg ml−1; Abcam, Cambridge, MA), isotype-matched Ab (20 μg ml−1), or stimulated with LPS (10 ng ml−1; Sigma-Aldrich, St. Louis, MO). CD47 blocking Ab was observed to inhibit the interaction of CD47 with TSP-1, but not with SIRP-α (data not shown). In addition, DCs were transfected with human CD47 siRNA (Oligo ID HS1015900), TSP-1 siRNA (Oligo ID HSS10723), SIRP-α siRNA (Oligo ID HSS157282), or control siRNA (Invitrogen). To examine the bystander effects, DCs were infected with E44 or treated with CD47 mAb for 6 h, washed, and incubated with fresh DCs for 24 h (1:10 fresh:infected DCs). The DCs were then washed and stimulated with LPS for 24 and 48 h. In separate experiments, infected DCs were separated from fresh DCs by a Transwell.

Flow cytometry

DC maturation and activation were determined by flow cytometry to evaluate the expression of surface molecules (CD40, CD86, and HLA-DR) after staining with the appropriate FITC, PE, PE-CY5.5, or allophycocyanin mouse mAbs or mouse IgG isotype-matched Abs (eBioscience, San Diego, CA). CD47 surface expression was assessed using CD47 mAb or isotype-matched control Ab. Cells were first preincubated for 20 min with IgG blocking buffer to mask nonspecific binding sites and further incubated with the indicated Abs or isotype control Ab for 30 min at 4°C; the cells were washed three times with PBS containing 2% FBS, and subsequently fixed with BD Cytofix (BD Biosciences). Cells were then analyzed by four-color flow cytometry using FACSCalibur instrument (CellQuest Pro software; BD Biosciences). DCs form a distinct population when separated by side scatter parameters for which CD1a was used as a DC gating marker; this population formed the collection gate, and at least 10,000 events within this gate were collected for analysis.

Viability

The viability of DCs was examined by trypan blue exclusion as well as by annexin V-FITC apoptosis kit (BD Biosciences). In all culture conditions, a proportion of cells (ranging from 5 to 15%) was trypan blue or annexin V and/or propidium iodide positive. However, there was no significant difference observed in the proportion in cultures stimulated with medium, E. coli, LPS, or CD47 Ab-treated DCs.

Cytokine determination

TNF-α, IL-1β, IL-6, IL-12 p70, IL-10, and TGF-β production in cell culture supernatants of stimulated or unstimulated DCs collected after 24 and 48 h of incubation were carried out using Biosource (Invitrogen) ELISA kits, according to the manufacturer’s instructions. TSP-1 production was determined using ELISA kit from R&D Systems (Minneapolis, MN).

DC-induced naïve T cell activation

The ability of infected DCs to activate naïve T cells was assessed by allogeneic lymphoproliferation. DCs were infected with E. coli or pretreated with 2 μg ml−1 mAb, CD47 blocking Ab, isotype-matched control Ab, or transfection with CD47 siRNA or control siRNA. The DCs were then used to stimulate allogeneic naïve T cells. Briefly, DCs and T cells were added in the ratio of 1:300 in 96-well plates, cultured for 72 h, [3H] was added, and harvested for 18 h. The rate of incorporation of thymidine was assessed by a liquid scintillation counter, and the results were expressed as disintegration per minute.

Ag uptake assay

Identical aliquots of DCs were washed and resuspended (106 ml−1). An equal volume of BSA FITC or dextran FITC (50 μg ml−1 final concentration; Sigma-Aldrich) was then added, and the cells were incubated for 30 min. Ag uptake was stopped by extensive washing with cold PBS containing 1% BSA and 10 mM sodium azide before analysis with a FACSCalibur (BD Biosciences). The experiments performed at 4°C served as negative controls. To test the effect of CD47 ligation on phagocytosis, DCs were pretreated with CD47 mAb or isotype-matched control mAb (20 μg ml−1).

Newborn mouse model of meningitis

The animal studies were approved by the Institutional Animal Care and Use Committee of the Saban Research Institute at Children’s Hospital (Los Angeles, CA) and followed National Institutes of Health guidelines for the performance of animal experiments. Breeding pairs of C57BL/6 (CD47+/+) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Three-day-old mice were randomly divided into various groups and infected intranasally with 106 CFU/10 μl bacteria or pyrogen-free saline. Blood was collected from the tail or facial vein at different times of postinfection and plated on Luria-Bertani (LB) agar containing rifampicin. Cerebrospinal fluid samples were aseptically collected by cisternal puncture under anesthesia and directly inoculated into broth containing antibiotics in which the growth of E. coli was considered positive for meningitis. Mice were perfused intracardially with 0.9% saline to remove blood and contaminating intravascular leukocytes. Brains were aseptically harvested and homogenized in sterile PBS, and the bacterial counts were determined by plating 10-fold serial dilutions on rifampicin LB agar plates.

Suppression of CD47 or TSP-1 expression using Invivofectamine reagent

To suppress CD47 expression, newborn mice at day 1 were injected with mouse CD47 siRNA (Oligo ID MSS205576), TSP-1 siRNA (Oligo ID MSS217732), or control siRNA in Invivofectamine reagent (premixed, 10 nmol in 10 μl; Invitrogen, Carlsbad, CA). Invivofectamine is a proprietary liposomal nontoxic transfection reagent for in vivo RNA interference applications (Invitrogen). This reagent stabilizes siRNA in vivo and knocks down the expression of gene of interest. Mice were given four doses of CD47 or TSP-1 siRNA i.p., two doses before infection, one dose immediately after administration of bacteria, and final dose at 24 h postinfection. The KD of CD47 or TSP-1 in mice was confirmed in bone marrow-derived DCs (BMDCs) by flow cytometry. In addition, BMDCs were obtained from wild-type (WT) and CD47–/– mice at different postinfection time points, with total RNA isolated, and subjected to RT-PCR using primers sense, 5′-GGGGCGGAGATGTTGGCCCTT-3′ and antisense, 5′-CATTCTCC- CTTCCACACATTCC-3′ to evaluate the expression of CD47 mRNA. Mouse GAPDH was used as an internal control, using the following primer sequences: GAPDH sense, 5′-CATCACCATCTTTCAAGGAGC-3′ and GAPDH antisense, 5′-GAGGGGCCATCCCACAGTCTC-3′. Negative controls without primers were performed in parallel for every reaction for the primary and for the amplification of contaminating DNA. The amplified products were separated on a 1% agarose gel, stained with ethidium bromide, and photographed using gel-logic imaging system (Kodak, New Haven, CT).
Confocal microscopy

Bone marrow was harvested from WT and CD47 KD and infected mice, and differentiated to DCs in vitro. The cells were then incubated with IgG blocking buffer to prevent nonspecific binding. Cells were then fixed and permeabilized with BD Cytofix and Cytoperm kit (BD Biosciences), washed, and incubated with 5 μg ml⁻¹ CD47 Ab (eBioscience), followed by Alexa Fluor 568 Ab (Invitrogen). Cells were then allowed to adhere to poly(lysine)-coated slides for 10 min (Paul Marienfeld, Lauda-Königshofen, Germany) and mounted in an antifade Vectashield solution (Vector Laboratories, Burlingame, CA). The cells were viewed with a Zeiss (Oberkochen, Germany) 710 microscope with Plan-apochromat oil immersion objective lenses. The TIFF images were assembled using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Adoptive transfer of DCs

Bone marrow cells from tibias and femurs were flushed with RPMI 1640 supplemented by 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2-ME, 1 mM essential amino acids, and 1 mM sodium pyruvate. After RBC lysis, cells were cultured in the presence of GM-CSF (30 ng ml⁻¹) and IL-4 (10 ng ml⁻¹; Pepro-Tech). Cells were harvested on day 6, and the purity was examined by flow cytometry using CD11c as the marker. Fresh BMDCs (5 x 10⁶

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** *E. coli* K1 upregulates the expression of CD47 and its natural ligand TSP-1 in DCs to prevent maturation. DCs were treated with OmpA⁺ *E. coli* (E44), OmpA⁻ *E. coli* (E91), LPS, or left untreated for 24 and 48 h, and the expression of CD47 was examined by flow cytometry (A). In addition, TSP-1 production was determined in the supernatants by ELISA (B). In separate experiments, DCs were incubated with CD47 mAb (CD47 treat, 20 μg ml⁻¹) and then stimulated with LPS for 24 and 48 h. DCs were also treated with 20 μg ml⁻¹ CD47 blocking Ab (CD47 block Ab + E44), SIRP-α blocking Ab (SIRP-α block Ab + E44), or control siRNA to CD47 (CD47 siRNA + E44), SIRP-α (SIRP-α siRNA + E44), TSP-1 (TSP-1 siRNA + E44), or control siRNA, and then infected with bacteria. DCs from all of these treatments were then washed separately; stained with Abs to CD40 (C), HLA-DR (D), and CD86 (E); fixed; and analyzed by flow cytometry. The data represent geometric mean fluorescence intensity of logarithmic data after subtraction of isotype-matched controls. The Ag presentation capacity of DCs to naive T cells was assessed in MLR, as described in Materials and Methods (F). The error bars represent SDs from the means of triplicate samples. The results are representative of five independent experiments with similar results. The inhibition of cell surface marker expression or Ag presentation was significantly reduced in comparison with LPS-treated DCs. *p* < 0.001 by two-tailed Student t test.
cells) were adoptively transferred i.p. into mice before infecting with *E. coli*.

**Histopathology**

Brain tissue samples were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Sections (4–5 μm) were cut using Leica microtome and stained with H&E (23, 24).

**Statistical analysis**

Statistical significance was determined by ANOVA, Fischer test, and paired, two-tailed Student t test. Values of *p* < 0.05 are considered statistically significant.

**Results**

_E. coli_ K1 upregulates the expression of CD47 and its ligand TSP-1 to prevent the maturation of DCs

Our previous studies have shown that OmpA⁺ *E. coli* prevents the maturation of DCs, whereas OmpA⁻ *E. coli* induces the maturation of DCs (6). Because CD47 has been implicated in the tolerogenicity of DCs, we examined the expression of CD47 and its ligand TSP-1 in DCs upon infection with *E. coli*. The expression of CD47 and TSP-1 in DCs infected with OmpA⁺ *E. coli* (E44) was significantly higher compared with OmpA⁻ *E. coli*-infected or LPS-treated DCs at 24 and 48 h postinfection (*p* < 0.001; Fig. 1A, 1B). TSP-1 levels increased from 33 ng ml⁻¹ at 24 h postinfection to 87 ng ml⁻¹ by 48 h postinfection in OmpA⁺ *E. coli*-infected DCs. Similar to OmpA⁺ *E. coli*-infected DCs, engagement of CD47 on DCs with a CD47 mAb exhibited significant inhibition in the expression of maturation markers despite treatment with LPS (*p* < 0.001), indicating that ligation of CD47 is sufficient to suppress the maturation of DCs. To examine whether preventing the interaction of CD47 with TSP-1 modulates DC maturation, DCs were treated with CD47 blocking Ab or introduced siRNA to CD47 or TSP-1 and then stimulated with *E. coli*. Blocking of CD47 to bind to TSP-1 or knocking down CD47 or TSP-1 expression led to maturation of _E. coli_-infected DCs, as evident by increased expression of CD40, HLA-DR, and CD86 (Fig. 1C–E), indicating that the interaction of CD47 and its ligand TSP-1 is playing a major role in the tolerogenicity of DCs induced by _E. coli_ K1. Anti–SIRP-α blocking Ab or control Ab-treated or SIRP-α or control siRNA-transfected DCs showed no increase in the expression of maturation markers, ruling out the possibility that SIRP-α, another ligand of CD47, plays a role in this process. Because DCs play a crucial role in Ag presentation to T cells, we examined whether upregulation of CD47 and TSP-1 affects the Ag-presenting capacity of DCs in MLR (25). DCs pretreated with CD47 mAb or infected with OmpA⁺ *E. coli* failed to present Ag, as indicated by the inability of T cells to proliferate in MLR (Fig. 1F). In contrast, blocking or knocking down CD47 or TSP-1, but not SIRP-α, led to proliferation of T cells. These results suggest that _E. coli_ prevents the maturation and Ag-presenting capacity of DCs by promoting the ligation of CD47 to its natural ligand TSP-1, but another ligand SIRP-α plays a minimal role in this process.

**CD47 interaction with TSP-1 prevents cytokine production by DCs**

Upon stimulation, DCs release several cytokines and chemokines that subsequently recruit more DCs and other immune cells to the site of infection (26). In contrast, our previous studies demonstrate that OmpA⁺ _E. coli_ suppressed the production of proinflammatory...
cytokines in DCs (6). Therefore, we examined the effect of CD47 mAb and blocking Ab on the cytokine profile of DCs by ELISA upon infection with E. coli. As expected, CD47 ligation with CD47 mAb on DCs and then stimulation with LPS for 24 and 48 h failed to produce TNF-α, IL-1β, IL-6, and IL-12 (Fig. 2). In contrast, treatment of DCs with blocking CD47 Ab abrogated OmpA⁺ E. coli-induced suppressive effect on the production of cytokines, whereas SIRP-α blocking or control Ab did not show any effect. Similarly, KD of CD47 or TSP-1 expression by siRNA, but not SIRP-α or control siRNA, also triggered the production of cytokines by DCs despite infection with OmpA⁺ E. coli. These data indicate that CD47 is involved in E. coli-induced suppressive effect on the production of proinflammatory cytokines by DCs.

CD47-induced tolerogenicity in DCs exerts contact-dependent bystander effects on other DCs without altering the phagocytic activity

To determine whether CD47-mediated suppressive effects on DCs have bystander effects on other DCs, fresh DCs were incubated with OmpA⁺ E. coli-infected or CD47 mAb-treated DCs (1:10 ratio), and then stimulated with LPS. No increase in the upregulation of CD40, HLA-DR, and CD86 was observed in the DCs (Fig. 3A–C). In addition, these DCs also fail to present Ag to T cells, as indicated by the inability of T cells to proliferate in MLR (Fig. 3D). To examine whether these bystander effects are due to the soluble factors secreted by infected DCs, the experiments were performed in which infected DCs were separated from uninfected DCs by a Transwell and then stimulated with LPS. Interestingly, the DCs in the bottom chamber showed enhanced expression of maturation markers, indicating that direct contact is required for the bystander effect by infected DCs. The production of IL-10 and TGF-β was significantly higher in the culture supernatants of uninfected and infected DCs, which were reduced to basal levels, by separating them using a Transwell (Fig. 3E,3F). iDCs are professional phagocytic cells that have a strong capacity to ingest macromolecules. Because we observed that E. coli-induced CD47 interferes with the maturation of DCs and prevents cytokine production, we next determined the effect of CD47 expression on the phagocytic capability of DCs. First, DCs were incubated with CD47 mAb, OmpA⁺ E. coli (E44), or left untreated, and the uptake of dextran and BSA coupled to a fluorochrome was then examined by flow cytometry. There was no difference in phago-
cytosis of dextran or BSA in CD47 mAb-treated or OmpA* E. coli-treated DCs compared with untreated DCs (Fig. 4). These results demonstrate that CD47 expression exhibits bystander effects by direct contact with other DCs and does not interfere with the phagocytic ability of DCs.

**CD47 or TSP-1 KD animals are resistant to E. coli K1 meningitis**

To determine whether CD47 plays a role in the pathogenesis of neonatal meningitis induced by *E. coli* K1, mice were injected with CD47 siRNA using Invivofectamine to suppress the expression of CD47. To confirm the KD of CD47, bone marrow was isolated from CD47 KD animals, differentiated to DCs in vitro, and examined for the expression of CD47 by flow cytometry. As shown in Supplemental Fig. 1A and Fig. 5A, the surface and intracellular expression of CD47 in BMDCs was completely absent in transfected mice, whereas control siRNA did not affect the expression. BMDCs from transfectant and infected mice revealed that CD47 expression was suppressed even after seventh day postinfection, as evidenced by RT-PCR (Supplemental Fig. 1B). In contrast, BMDCs from WT-infected mice showed significant increase in CD47 transcription compared with control uninfected mice. Similar results were also observed with confocal microscopy of BMDCs from WT and transfectant and infected mice, confirming the suppression of CD47 in siRNA-injected animals (Supplemental Fig. 1C). Similarly, there was no expression of TSP-1 in BMDCs from TSP-1 KD mice (Supplemental Fig. 1D, 1E). CD47 or TSP-1 KD mice were then infected intranasally with 10^7 CFU of *E. coli*, and various parameters of the infection were studied, as described previously (23, 24). Interestingly, CD47 or TSP-1 KD mice could survive beyond 7 d, whereas 100% WT animals succumbed by 96 h postinfection (Fig. 5B). The survival of CD47 or TSP-1 KD mice was similar even at higher doses of infection (10^8 CFU) compared with WT mice, which succumbed to infection within 72 h (Fig. 5C). We then examined the bacterial burden in the blood of infected animals at different postinfection time intervals. Bacteria were demonstrable in the blood of WT and control siRNA-treated animals as early as 2 h postinfection (Fig. 5D). Bacteremia levels increased with the increase in postinfection time, showing a bacterial count of 7.89 log CFU ml^-1 at 72 h postinfection. In contrast, the bacterial burden initially increased by 24 h in CD47 or TSP-1 KD mice to a log CFU ml^-1 of 4.19 and 3.81, respectively, after which bacteria were cleared from circulation to undetectable levels by 168 h postinfection. Cerebrospinal fluid cultures were positive in 94 and 100% of WT and control siRNA-infected mice, respectively, whereas bacteria were not detected in the cerebrospinal fluid of CD47 or TSP-1 KD mice (Fig. 5E). On par with these findings, brain tissues of WT or control siRNA-treated mice showed high bacterial counts, whereas no bacteria were found in the brains of CD47 or TSP-1 KD mice (Fig. 5F). Histopathological examination of the brain tissues of WT mice infected with *E. coli* revealed severe brain damage (Fig. 5G). Acute inflammation was observed in the meninges and the cortical area. Abscess in certain areas of brain parenchyma and apoptosis of neurons was seen in the dentate gyrus and Ammon’s horn in the hippocampus of the brains. In contrast, there were no signs of damage in the brains of CD47 or TSP-1 KD-infected mice. These studies demonstrate that CD47 and TSP-1 expression is pivotal for the onset of meningitis in newborn mice by *E. coli* K1.

**Adaptive transfer of DCs lacking CD47 provides protection against E. coli K1 meningitis**

To examine whether replenishing the CD47+ DCs in CD47 KD mice renders the animals susceptible to *E. coli* infection, adoptive transfer experiments were performed. Mice were divided into four groups, as follows: 1) CD47+/+ recipient/CD47+/+ donor DCs; 2) CD47+/− recipient/CD47 KD donor DCs; 3) CD47 KD recipient/CD47+/− donor DCs; and 4) CD47 KD recipient/CD47 KD donor DCs. There was a significant enhancement in the survival of mice that received CD47+ DCs compared with animals that received CD47− DCs (Fig. 6A). In contrast, CD47 KD mice that received CD47+ DCs died within 96 h compared with CD47 KD animals that received CD47− DCs. Cerebrospinal fluid cultures were negative in mice that received CD47− DCs, whereas 89% of CD47+/− animals that received CD47+ DCs showed positive cerebrospinal fluid cultures (Fig. 6B). Interestingly, all of the cerebrospinal fluid cultures were positive in CD47 KD mice that received CD47+ DCs, whereas CD47 KD animals that received CD47− DCs showed negative cerebrospinal fluid cultures. CD47 KD mice that received CD47+ DCs showed high levels of bacteremia compared with mice that received CD47− DCs (Fig. 6C). In the former group, the bacterial counts in the blood increased up to 72 h postinfection time, whereas in the latter group, the bacterial burden was increased until 24 h postinfection, after which bacteria started clearing from circulation. In CD47 KD mice that received CD47+ DCs, blood bacterial load increased from 1.69 log CFU ml^-1 at 2 h postinfection to 7.87 log CFU ml^-1 at 72 h postinfection. In contrast, CD47 KD animals that received CD47− DCs showed no bacteria by 168 h postinfection. CD47+/− animals that received CD47+ DCs showed a high bacterial load of CFU.
FIGURE 5. CD47 KD mice are resistant to \textit{E. coli} K1 meningitis. Newborn mice were injected with CD47 siRNA in Invivofectamine reagent to knock down the expression of CD47, as described in Materials and Methods. To confirm the depletion of CD47, bone marrow cells were isolated from CD47 KD animals, differentiated to DCs in vitro, permeabilized, and examined for the presence of CD47 inside the cells by flow cytometry. The intracellular expression of CD47 in BMDCs was completely absent in transfected mice (A). Lack of CD47 or TSP-1 improved survival of mice infected intranasally with $10^3$ (B) or $10^5$ (C) CFU of \textit{E. coli}. Blood was collected from CD47 or TSP-1 KD mice, control siRNA-injected or CD47$^{+/+}$ (WT) mice infected with \textit{E. coli} at various postinfection times, and plated on antibiotic containing LB agar to determine the bacteremia levels (D). In addition, cerebrospinal fluid from infected animals was collected by cisternal puncture and cultured in antibiotic containing LB to assess the occurrence of meningitis (E). Brains harvested from the infected animals were homogenized and plated on antibiotic-containing LB agar plates (F). CD47 or TSP-1 KD mice showed no bacteria in cerebrospinal fluid and brain cultures compared with WT or control siRNA-injected mice. Brain sections from infected mice were subjected to histopathological examination after H&E staining (original magnification $\times$20) (G). Abscess formation (indicated by yellow arrow) was conspicuous in the parenchyma in brains of WT mice. In addition, apoptosis of neurons (indicated by yellow arrows) was observed in the Ammon’s horn in the hippocampus. Cortex and meninges showed severe inflammation (black arrow) and apoptosis of cells (yellow arrow). None of these changes were seen in the brains of CD47 or TSP-1 KD mice. The results are representative of six independent experiments with 18 animals per group. Data represent mean $\pm$ SD. *p $<$ 0.001 by two-tailed Student t test.

Discussion

DCs represent the pacemaker of the immune system and play a vital role in the induction and regulation of immune responses (27–29). DCs are present in a so-called immature state (iDCs), and are unable to stimulate T cells and elicit immune responses (30). However, whole bacteria or bacterial products induce several phenotypic and functional changes in iDCs, a process known as DC maturation, leading to the development of fully competent Ag-presenting and costimulatory cells (31–33). Our previous studies demonstrate that \textit{E. coli} K1 expressing OmpA prevents the maturation of DCs by suppressing the production of proinflammatory cytokines (6). The present study demonstrated that \textit{E. coli} K1 that causes meningitis in neonates manipulates the expression of CD47 on the surface of DCs and triggers the production of its natural ligand TSP-1 to prevent the maturation process. Although some studies have shown that ligation of CD47 by a mAb inhibits cytokine production and maturation of DCs (34), no studies have been reported to date by a bacterium modulating CD47 expression. Ligation of CD47 by TSP-1 or mAb has two major effects on the response of iDCs to \textit{E. coli} K1. First, it prevents the production of proinflammatory cytokines, and second, it inhibits DC maturation and Ag presentation to T cells. In agreement with these speculations, we demonstrated that treatment of DCs with CD47 blocking Ab abrogates the suppressive effects induced by \textit{E. coli} K1, as shown by the upregulation of MHC II class Ag, costimulatory molecules, and Ag-presenting capability of the cells. In contrast, \textit{E. coli} O157:H7 downregulated the expression of CD47 in platelets, which subsequently induced thrombocytopenia in animals (35). Of note, CD47-mediated suppressive effects of DCs also
FIGURE 6. DC reconstitution experiments demonstrate that CD47 expression in DCs contributes to the pathogenesis of E. coli K1 meningitis. Reconstruction of CD47+/+ mice with CD47− DCs enhanced survival (A) and showed no occurrence of meningitis (B) compared with CD47+/+ mice with CD47+ DCs. In contrast, CD47 KD mice reconstituted with CD47+ DCs succumbed to infection within 96 h postinfection and exhibited positive cerebrospinal fluid cultures compared with CD47 KD mice with CD47+ DCs. CD47+/+ mice replenished with CD47+ DCs showed lower bacteremia (C) and brain bacterial load (D) compared with CD47+/+ mice with CD47+ DCs. The results are representative of five independent experiments with 18 animals per group. Data represent mean ± SD. *p < 0.001 by two-tailed Student t test.

exert bystander effects on other DCs, which did not mature by having infected DCs next to them. This bystander suppression may be attributed to secreted anti-inflammatory cytokines, IL-10 and TGF-β. However, the separation of infected DCs from uninfected DCs using Transwell and then stimulating uninfected DCs with LPS led to maturation of DCs due to suppression of these cytokines. This indicates that a direct contact is required for the infected DCs to exert a bystander effect on other DCs. It is tempting to speculate that this cell to cell interaction could be due to exosomes released by DCs. Exosomes are small (50–90 nm) membrane vesicles of endocytic origin that are released into the extracellular environment on fusion of multivesicular bodies with the plasma membrane. Exosome-mediated genetic transfer has been demonstrated to be an important communication mechanism in DCs (36, 37). This cell to cell-mediated tolerogenicity in DCs may help bacteria in evading the potent immune responses. The cytokine profile of DCs exerts an important quality control on the immune response. The proinflammatory cytokines, especially IL-12 produced by DCs, induce an innate immune response against invading pathogens. We observed that E. coli induced CD47 expression, and its interaction with TSP-1 inhibited the production of IL-12, which was increased upon treating the cells with anti-CD47 Ab. This contrasts with the data obtained by Demeure et al. (34), as inclusion of anti-CD47 Ab did not affect the IL-12 production. However, our studies corroborate with the findings of other investigators who also observed that engagement of CD47 on monocytes by TSP-1 downregulates IL-12 production (38, 39).

The iDCs are highly endocytic and thus efficient at Ag capture (40). iDCs play an important role in the clearance of apoptotic and necrotic cells from the body (41). However, maturation of DCs is accompanied with a marked reduction in the endocytic capacity (42). Whereas iDCs actively carry out macroinocytosis and phagocytosis, these activities are barely detectable in mature DCs (43). In agreement, E. coli or CD47 mAb-treated DCs, despite being immature, still retained higher capacity to endocytose FITC dextran or BSA. These data provide evidence that CD47 does not interfere with the phagocytic capacity of DCs and is involved in the functional immaturity of DCs. A compelling observation of our studies is that suppression of CD47 or TSP-1 in infected DCs leads to lack of sufficient bacteremia required for the onset of meningitis, as CD47− or TSP-1–suppressed animals efficiently killed the circulating E. coli by 168 h postinfection. The data also suggest that manipulation of DCs and survival inside DCs is an important pathogenic event during the onset of meningitis by E. coli. However, CD47 is expressed by several cells, and therefore, the survival of the bacteria could be due to lack of CD47 on other cell types, such as macrophages. Nonetheless, our data on adoptive transfer of CD47+/+ DCs to CD47 KD mice, which abrogates the protective effect, confirmed the role of CD47 expression in DCs for the onset of meningitis.

In summary, the current study identified a novel mechanism by which E. coli K1 makes DCs tolerogenic by promoting the ligation of CD47 with its natural ligand TSP-1, which is likely to have important consequences for the establishment of antibacterial immunity. The ability of E. coli K1 to inhibit the maturation of DCs may be biologically and clinically relevant to defeat the immune responses. Targeting CD47 and its ligand TSP-1 might help in restoring maturation of DCs in response to E. coli K1, leading to efficient Ag presentation and thus promoting clearance of bacteria. Our results identify a novel target to design preventive strategies for E. coli K1-induced meningitis in neonates.

Disclosures
The authors have no financial conflicts of interest.
References


MODULATION OF CD47 EXPRESSION BY E. coli K1