Vibrio vulnificus infection induces the maturation and activation of dendritic cells with inflammatory Th17-polarizing ability

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Abstract. *Vibrio vulnificus* (*V. vulnificus*) is a gram-negative bacterium, which causes life-threatening septicemia and gastroenteritis through the consumption of contaminated seafood or wound infection. In addition, *V. vulnificus* infection is known to stimulate the production of several pro-inflammatory cytokines, which are associated with inflammatory responses mediated predominantly by dendritic cells (DCs), functioning as antigen-presenting cells. The present study aimed to investigate whether *V. vulnificus* infection induced the maturation and activation of murine DCs, which have the ability to polarize T helper (Th) cells into Th17 cells. Dysregulated Th17 cell responses are known to cause tissue damage, promoting the penetration of pathogens; however, Th17 cells are also involved in host defense against infection. Infection with *V. vulnificus* significantly increased the expression of cell surface molecules, including CD40, CD80 and major histocompatibility complex class II, leading to the maturation and activation of DCs. In the present study, the analysis of the cytokine profiles of DCs upon infection with *V. vulnificus* revealed the preferential production of interleukin-1β (IL-1β) and IL-6, through which *V. vulnificus*-infected DCs induced the polarization of Th17 cells when naïve $CD4^+$ T cells were co-incubated. The reduction of Th17 cell generation through the use of anti-IL-6 neutralizing antibodies indicated that the Th17-polarizing capacity of *V. vulnificus* was predominantly dependent on DC-derived IL-6. The *in vivo* administration of *V. vulnificus*-infected DCs consistently increased the Th17 cell population in the lymph nodes of mice. Finally, the oral administration of *V. vulnificus* in mice also increased Th17 cell responses in the lamina propria of the small intestine. These results collectively demonstrated that *V. vulnificus* induced inflammatory Th17 cell responses via DCs, which may be associated with the immunopathological effects caused by *V. vulnificus* infection.

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Abbreviations: DC, dendritic cell; BMDC, bone-marrow-derived dendritic cell; MOI, multiplicity of infection

Key words: Vibrio vulnificus, dendritic cells, T helper 17, mouse, cytokine

Introduction

Vibrio vulnificus, a gram-negative bacterium, causes severe to life-threatening infections in susceptible individuals who have chronic liver disease following consumption of *V. vulnificus*-containing seafood or acquisition of wound infection. *V. vulnificus* infection can also cause several diseases, including primary septicemia, gastroenteritis and necrotizing fasciitis (1). Dendritic cells (DCs), which function as professional antigen presenting cells, are important in the link between innate and adaptive immune responses. The cytokines secreted by activated DCs are important factors determining the fate of $CD4^+$ T helper (Th) subsets. For example, interleukin-12 (IL-12), IL-4 and IL-6, in addition to transforming growth factor-β (TGF-β), are required for the maturation of Th1, Th2 and Th17 cells, respectively (2). IL-12 is released by *Mycobacterium tuberculosis*-infected DCs, polarizing naïve $CD4^+$ T cells to Th1 cells (3). In addition to the increase in Th1 cells by IL-12, the polarization of Th17 cells is induced by IL-6 derived from *Streptococcus pyogenes*-infected DCs (4).

Several effector Th cells are involved in inducing immune responses against infection. For example, Th1 and Th2 cells provide immune responses against intracellular bacterial infections and parasitic pathogens, respectively (5). Th1 cells, associated with cellular immune responses, are induced upon infection with several pathogens (6). Th17 cells are critical in extracellular bacterial and fungal pathogen infections (7,8). Th17 cells are induced by extracellular bacteria, which adhere to epithelial and mucosal barriers, including the intestine, lung and skin, and are involved in host defense against infection (9). For example, *Citrobacter rodentium* infection causes an increase in Th17 cells, inducing the production of IL-22

in Peyer's patches and colonic epithelial cells (10,11). Despite the importance of Th17 cells in protecting the host from bacterial infection, there are several studies that dysregulated Th17 cell responses can cause tissue damage, promoting the penetration of pathogens. For example, in the case of infection with pathogens, including *Borrelia burgdorferi*, *Helicobacter pylori* and *Candida albicans*, increased Th17 cells promote inflammation, resulting in tissue damage and exacerbated pathology (12-14). In addition, the significantly elevated levels of proinflammatory cytokines associated with excessive Th17 cells may contribute to sepsis, which is caused by dysregulated host immune responses against infection (15,16). Despite extensive studies investigating the induction of Th17 cells by certain extracellular bacteria, whether *V. vulnificus* infection increases the Th17 cell population in mice and whether the increased Th17 cell population is associated with sepsis remain to be elucidated.

The present study aimed to determine whether *V. vulnificus* infection modulated the responses of Th17 cells *in vitro* and *in vivo*. It was found that *V. vulnificus* infection activated DCs, leading to the upregulation of IL-17$^+$ CD4$^+$ cell populations through the DC-derived secretion of IL-6. Additionally, the oral administration of mice with *V. vulnificus* increased the numbers of Th17 cells in the lamina propria of the small intestine, suggesting that *V. vulnificus* may induce Th17 cell responses *in vitro* and *in vivo*.

Materials and methods

Mice. Female C57BL/6 mice were purchased from Orient Bio, Inc. (Seoul, Korea). All animals were used for experiments at 7-9 weeks of age (weight, 17-20 g). All mice were provided with *ad libitum* access to a standard laboratory chow diet (cat. no. 1314; Altromin Spezialfutter GmbH & Co. KG, Lage, Nordrhein-Westfalen, Germany) and water. The animals were housed in an SPF facility under a strict light cycle (lights on at 07:00 a.m. and off at 07:00 p.m.) at 22±1˚C and 52.5±2.5% relative humidity, and all animal experiments were ethically performed in accordance with the guidelines of the Korea University Institutional Animal Care and Use Committee (Seoul, Korea; approval no. KUIACUC-2015-244, 2016-170).

Bacterial strain and culture conditions. The *V. vulnificus* MO6-24/O wild-type (WT) used in the present study was obtained from Professor Sang Ho Choi of Seoul National University (Seoul, Korea) as described previously (17,18). For the infection experiments, the bacteria were grown overnight at 30˚C in Luria-Bertani medium supplemented with 2.0% NaCl (LBS medium) and the grown bacteria were incubated in fresh LBS medium at 30˚C, diluted to ~1.8x10^8 colony forming units (CFUs)/ml in LBS, centrifuged at room temperature for 3 min at 2,420 x g, and resuspended in antibiotic-free growth medium prior to infection of DCs, or in phosphate-buffered saline (PBS) prior to infection of mice.

Preparation of murine bone-marrow-derived dendritic cells (BMDCs). The BMDCs were generated using a method originally described by Inaba *et al* (19) with modification. For the generation of DCs, bone-marrow was isolated from the femurs and tibiae of mice, and flushed with RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with fetal bovine serum (FBS) (10%; Gibco; Thermo Fisher Scientific, Inc.), 2-ME (50 mM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), HEPES (10 mM; WelGENE, Daegu, Korea), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (both from Invitrogen; Thermo Fisher Scientific, Inc.), using a syringe equipped with a 26-gauge needle. Cell clusters were dissociated by gentle pipetting, and the cell suspension was filtered through a 70-μm cell strainer to remove debris. The red blood cells were lysed in a lysing solution containing 0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA, and 5x10^5 cells/ml of the bone marrow cells were seeded into Petri dishes containing 10 ml growth medium with GM-CSF (10 ng/ml; ProSpec, Rehovort, Israel). After 3 and 5 days of culture at 37˚C in a 5% CO_2 incubator, 5 ml of fresh medium containing 10 ng/ml of GM-CSF was added. On day 7, the loosely adherent DC aggregates were harvested, and 1.5-2x10^6 cells/ml were seeded in a 24-well plate for subsequent experiments.

In vitro infection protocol. The DCs were grown for 7 days in the growth medium at 37˚C in a 5% CO_2 incubator. On day 7, the cells were seeded onto a 24-well plate (1.5-2x10^6 cells/ml) in antibiotic-free growth medium. Prior to infection, the bacteria were centrifuged at room temperature for 3 min at 2,420 x g, resuspended and adjusted to 1.5-2x10^8 CFU/ml in antibiotic-free RPMI-1640 medium. The DCs were then treated with LPS (500 ng/ml) for 3-60 min or infected with *V. vulnificus* at various multiplicities of infection (MOI; the ratio of bacteria number to the number of BMDCs) for various infection durations. The timing and doses of *V. vulnificus* infection were determined based on the method described in previous studies on infected macrophages or epithelial cell lines, with minor modification to MOI 0-10 for 0-120 min (20-22). Following infection, the cells were washed twice with PBS and incubated for 20 h in antibiotic-containing growth medium at 37˚C under 5% CO_2.

In vitro polarization of CD4$^+$ T cells with DCs. The DCs were infected with *V. vulnificus* and post-incubated for 20 h. CD4$^+$ T cells were isolated from the lymph nodes of the C57BL/6 female mice by magnetic bead purification (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). In a co-culture system, naive CD4$^+$ T cells were mixed with *V. vulnificus*-infected DCs at a 5:1 ratio in the presence of anti-CD3ε (cat. no. 553057) and CD-28 (cat. no. 553294) monoclonal antibodies (mAbs) (BD Biosciences, San Diego, CA, USA). The two Abs were used at 1:1,000 dilution, at 4˚C for anti-CD3ε and 37˚C for anti-CD28. After 3 days, the cells were re-stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin for 6 h at 37˚C and 5% CO_2 in the presence of 1 μl/ml Golgiplug. After 6 h, the cells were harvested and stained with fluorescent antibodies for analysis using flow cytometry. In additional experiments, neutralizing mAbs against IL-1β (cat. no. 16-7012) and IL-6 (cat. no. 16-7061) (1 μg/ml; eBioscience, Inc., San Diego, CA, USA) were added to the DC-CD4$^+$ T cell co-cultures at 37˚C under 5% CO_2.

In vivo polarization of CD4$^+$ T cells with DCs. Naïve C57BL/6 mice were injected subcutaneously in each footpad with

100 μg of ovalbumin (OVA; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in 2 mg aluminum hydroxide dissolved in 100 μl of PBS. Following 7 days of immunization, the mice were injected subcutaneously with $1x10^6$ of the OVA (10 μg/ml)-pretreated DCs, which were either uninfected or were infected with *V. vulnificus* (MOI 5; 30 min). At day 7 post-injection, the mice were sacrificed and cells were isolated from draining lymph nodes of mice ($2x10^6$ cells/ml), which were cultured for 3 days in the presence of OVA (100 μg/ml) at 37˚C in a 5% CO_2 incubator. After 3 days, the cells were re-stimulated for 6 h with 50 ng/ml PMA and 1 μg/ml ionomycin at 37˚C and 5% CO_2 in the presence of 1 μl/ml Golgiplug. After 6 h, the cells were harvested and stained with fluorescent antibodies for analysis using flow cytometry.

In vivo infection protocol and lamina propria cell isolation. The mice were administered with 50 μg/ml rifampicin for 24 h to ablate normal flora. Food was removed from the cages of mice to empty their stomachs for 12 h prior to inoculation. Subsequently, 50 μl of 8.5% (w/v) $NaHCO_3$ was administered orally, immediately followed by 100 μl of bacterial suspension containing $1x10^7$ CFU of *V. vulnificus*. At day 2 post-infection, the mice were sacrificed and lamina propria cells were isolated from the small intestine, as described previously (23). Briefly, the small intestines from the mice were washed in cold PBS for feces clearance. Fat tissues and Peyer's patches were resected, and the intestines were cut longitudinally, followed by washing in cold PBS. The intestines were then cut into 2-3 cm segments and incubated for 15 min RPMI medium containing 1 mM EDTA with gentle stirring at 37˚C, followed by washing with warm PBS. The incubation was performed twice in medium containing EDTA. Subsequently, the tissues were cut finely and incubated for 30 min in RPMI containing collagenase D (0.1 mg/ml; Roche Diagnostics, Basel, Switzerland) at 37˚C with gentle stirring. The incubated supernatants were then collected and passed through a 70-μm cell strainer, and the unfractionated cells were centrifuged at 4˚C for 3 min at 400 x g. The lamina propria lymphocytes were isolated by gradient centrifugation with 40 and 85% Percoll gradient media (GE Healthcare Life Sciences, Little Chalfont, UK).

Flow cytometric analysis. Antibodies were used at 1:250 dilution for both surface and intracellular staining. The following mouse mAbs were used for flow cytometry: CD4-FITC (cat. no. 553047), CD11c-FITC (cat. no. 553801), interferon-γ (IFN-γ)-PE (cat. no. 554412), IL-4-PE (cat. no. 554435), CD40-PE (cat. no. 553791), CD80-PE (cat. no. 553769), I-Ab-PE (cat. no. 553552) (BD Biosciences), IL-17A-APC (cat. no. 17-7177), Forkhead Box p3 (Foxp3)-APC (cat. no. 17-5773) (eBioscience, Inc., San Diego, CA, USA) in pretitrated concentrations. For the intracellular detection of IFN-γ, IL-17A, IL-4 and Foxp3, the cells were re-stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin for 6 h at 37˚C and 5% CO_2 in the presence of 1 μl/ml Golgiplug. Subsequently, the cells were stained intracellularly following permeabilization of the cells using Cytofix/Cytoperm kits (BD Biosciences). The stained cells were detected using a flow cytometer (FACSCalibur or BD Accuri C6 Plus; BD Biosciences) gated on live $CD11c^+$ or $CD4^+$ cells. The data were analyzed using CellQuest software version 4.0.2 (BD Biosciences).

Semi quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was obtained from the cells using TRIzol reagent and reverse transcribed into cDNA using a RocketScript™ Reverse Transcriptase kit (E-3162; Bioneer Corporation, Daejeon, Korea). PCR was conducted using 1 μl of each 5' and 3' primer, 1 μl of cDNA. dH_2O was then added to a final volume of 20 μl. PCR amplification of the cDNA was then performed using AccuPower® PCR PreMix (K-2016; Bioneer Corporation) with a thermal cycler (MJ Research, Inc., Watertown, MA, USA) or Bioneer Corporation (Bioneer Corporation). The sequences of the PCR primers used in the present study were as follows: Murine IL-1β forward, 5'-CTA AAGTATGGGCTGGACTG-3' and reverse, 5'-AGCTTCAAT GAAAGACCTCA-3'; murine IL-6 forward, 5'-TGAACAACG ATGATGCACTT-3' and reverse, 5'-CGTAGAGAACAACA TAAGTC-3'; murine IL-12p35 forward, 5'-TCAGCGTTCCA ACAGCCTC-3' and reverse, 5'-CGCAGAGTCTCGCCATT ATG-3'; murine IL-12p40 forward, 5'-TTATGCAAATTGT GAGCTTG-3' and reverse, 5'-AGCTTCTTCATGTCTCC AAA-3'; murine IL-23p19 forward, 5'-AGCGGGACATATG AATCTAC-3' and reverse, 5'-TAAGCTGTTGGCACTAA GGG-3'; murine TGF-β forward, 5'-TATAGCAACAATTC CTGGCG-3' and reverse, 5'-TCCTAAAGTCAATGTAC AGC-3'; murine IFN-γ forward, 5'-TGCATCTTGGCTTTG CAGCTCTTCCTCATGGC-3' and reverse, 5'-TGGACCTGT GGGTTGTTGACCTCAAACTTGGC-3'; murine IL-17A forward, 5'-CGCAAAAGTGAGCTCCAGAA-3' and reverse, 5'-TGAAAGTGAAGGGGCAGCTC-3'; murine β-actin forward, 5'-TGGAATCCTGTGGCATCCATGAAA-3' and reverse, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'. The temperature condition for PCR amplification was 95˚C for 5 min; followed by 28-36 cycles consisting of 95˚C for 30 sec, 55-61˚C for 30 sec, and 72˚C for 30 sec; plus a final cycle of 72˚C for 5 min. Following amplification, the products were separated on 1.5% (w/v) agarose gels and stained with StainingSTAR (DyneBio, Gyeonggi-do, Korea).

Cytokine assays. The DCs were infected with *V. vulnificus* and post-incubated in a 96-well plate ($1x10^5$ cells/ml) for 20 h. After 20 h, the cell supernatant was collected and the secreted levels of cytokines were measured. The quantities of IL-1β, IL-6, IL-12p40 and IL-12p70 in the culture supernatants were determined using Mouse ELISA Ready-Set-Go! kits (IL-1β, IL-12p40 and IL-12p70; eBioscience, Inc.) and a mouse IL-6 ELISA kit (BD Biosciences).

The cells isolated from the mesenteric lymph nodes, Peyer's patches and lamina propria of the PBS- or *V. vulnificus*-inoculated mice were seeded into a 96-well plate ($1x10^5$ cells/ml) for 48 h in the presence of anti-CD3ε (cat. no. 553057) and CD-28 (cat. no. 553294) mAbs (BD Biosciences). Both Abs were used at 1:1,000 dilution, at 4˚C for anti-CD3ε and 37˚C for anti-CD28. After 48 h, the cell supernatant was collected and the secreted levels of cytokines were measured. The quantities of IL-17A and IFN-γ in the culture supernatants were determined using a Mouse ELISA Ready-Set-Go! kit (IL-17A; eBioscience, Inc.) or sandwich ELISA with anti-mouse IFN-γ monoclonal antibody (clone HB170, 1:1,000 dilution) for plate coating and biotinylated secondary antibody (clone XMG1.2, 1:1,500 dilution). A standard curve was generated using recombinant IFN-γ (BD Biosciences).

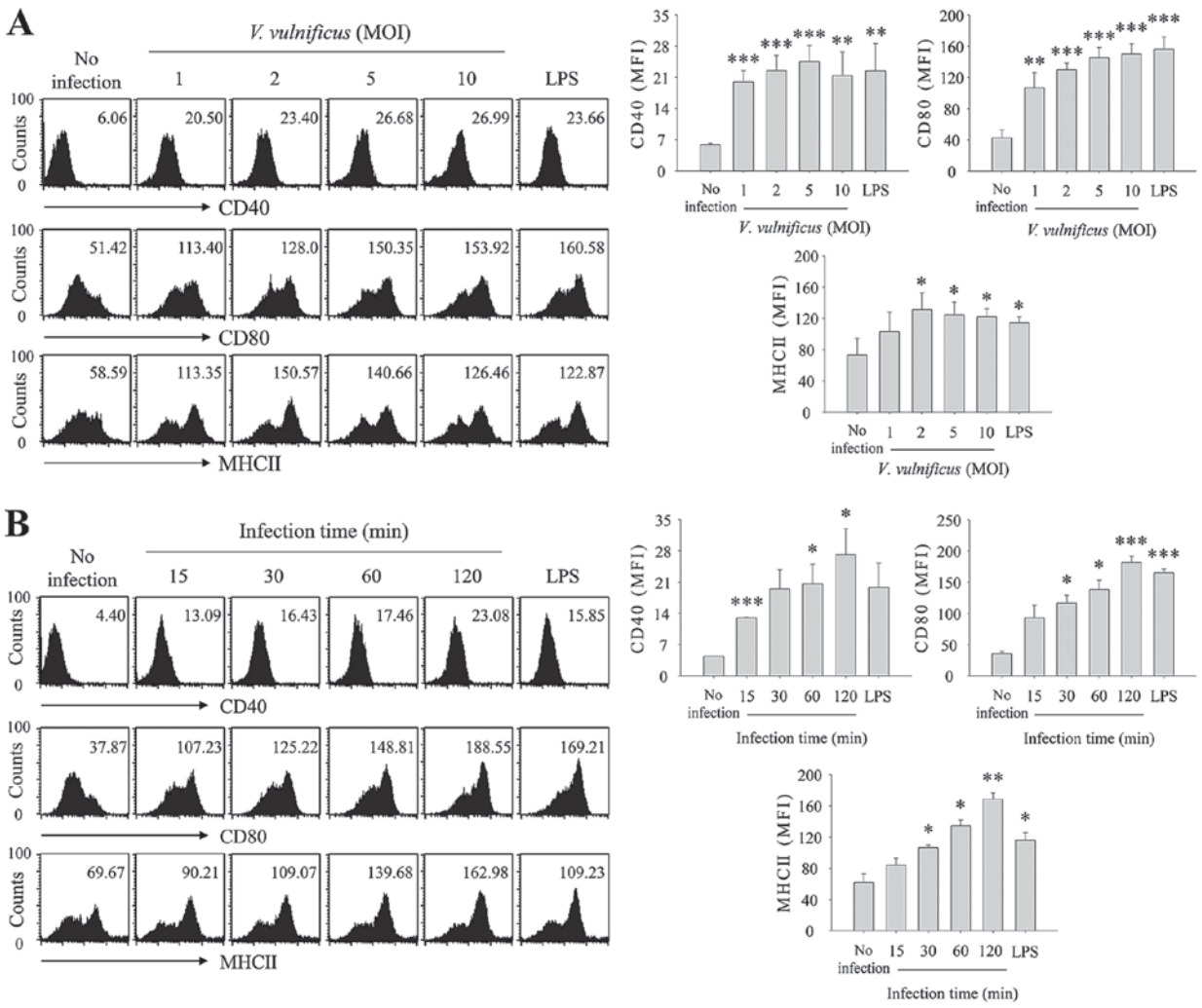

Figure 1. *V. vulnificus* infection induces the maturation of bone marrow DCs. DCs were either infected with *V. vulnificus* (A) at various MOIs (0, 1, 2, 5 and 10) or were treated with LPS (500 ng/ml) for (A) 30 min, or were infected with *V. vulnificus* (B) at an MOI of 1 for 0, 15, 30, 60 or 120 min or treated for 60 min with LPS (500 ng/ml). The cells were stained with antibodies targeting CD40, CD80 and MHC II for analysis using flow cytometry. The data shown are representative of three independent experiments, and represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. control (no infection); **P<0.01 vs. control (no infection); ***P<0.005 vs. control (no infection). *V. vulnificus*, *Vibrio vulnificus*; DCs, dendritic cells; MOI, multiplicity of infection; LPS, lipopolysaccharide.

Statistical analysis. All values are expressed as the mean ± standard deviation of at least three independent experiments. Student's t-test in SigmaPlot version 12.5 (Systat Software Inc., Chicago, IL, USA) was used to compare experimental groups with control groups. P<0.05 was considered to indicate a statistically significant difference.

Results

V. vulnificus infection induces the maturation and activation of BMDCs. To determine whether *V. vulnificus* infection affected the maturation and activation of DCs, the DCs were infected with *V. vulnificus* at an MOI of 0-10 for 30 min or at an MOI of 1 for 0-2 h, and the expression of cell surface molecules, CD40, CD80 and MHC II, were evaluated using flow cytometric analysis (Fig. 1). As shown in Fig. 1A, the expression levels of CD40, CD80 and MHC II were significantly increased in the *V. vulnificus*-infected DCs in an MOI-dependent manner. As the infection duration increased, the expression of cell surface markers also increased (Fig. 1B). These results showed that *V. vulnificus* infection induced the maturation and activation of DCs, and suggested that *V. vulnificus*-infected DCs may possess the ability to present antigen to naïve CD4^{+} T cells.

V. vulnificus infection increases the production of Th1/Th17-polarizing cytokines in DCs at mRNA and protein levels. DCs are known to provide three signals to naïve CD4^{+} T cells for differentiation into effector Th cells. In particular, as the third signal, IL-12p70, IL-1β and IL-6 are required for the polarization of Th1 and Th17 subsets. As shown in Fig. 1, *V. vulnificus* infection conferred DCs with the potential to differentiate naïve CD4^{+} T cells. To investigate the ability of *V. vulnificus*-infected DCs to provide CD4^{+} T cells with differentiation signals, the expression of Th cell polarization-associated cytokines in *V. vulnificus*-infected DCs were measured at mRNA and secretion levels. As shown in Fig. 2, infection of the DCs with *V. vulnificus* for 30 min at various MOIs increased the production of IL-1β, IL-6 and IL-12p35/40, which induced Th1 or Th17 cell differentiation in an MOI-dependent manner (Fig. 2A). Consistent with these

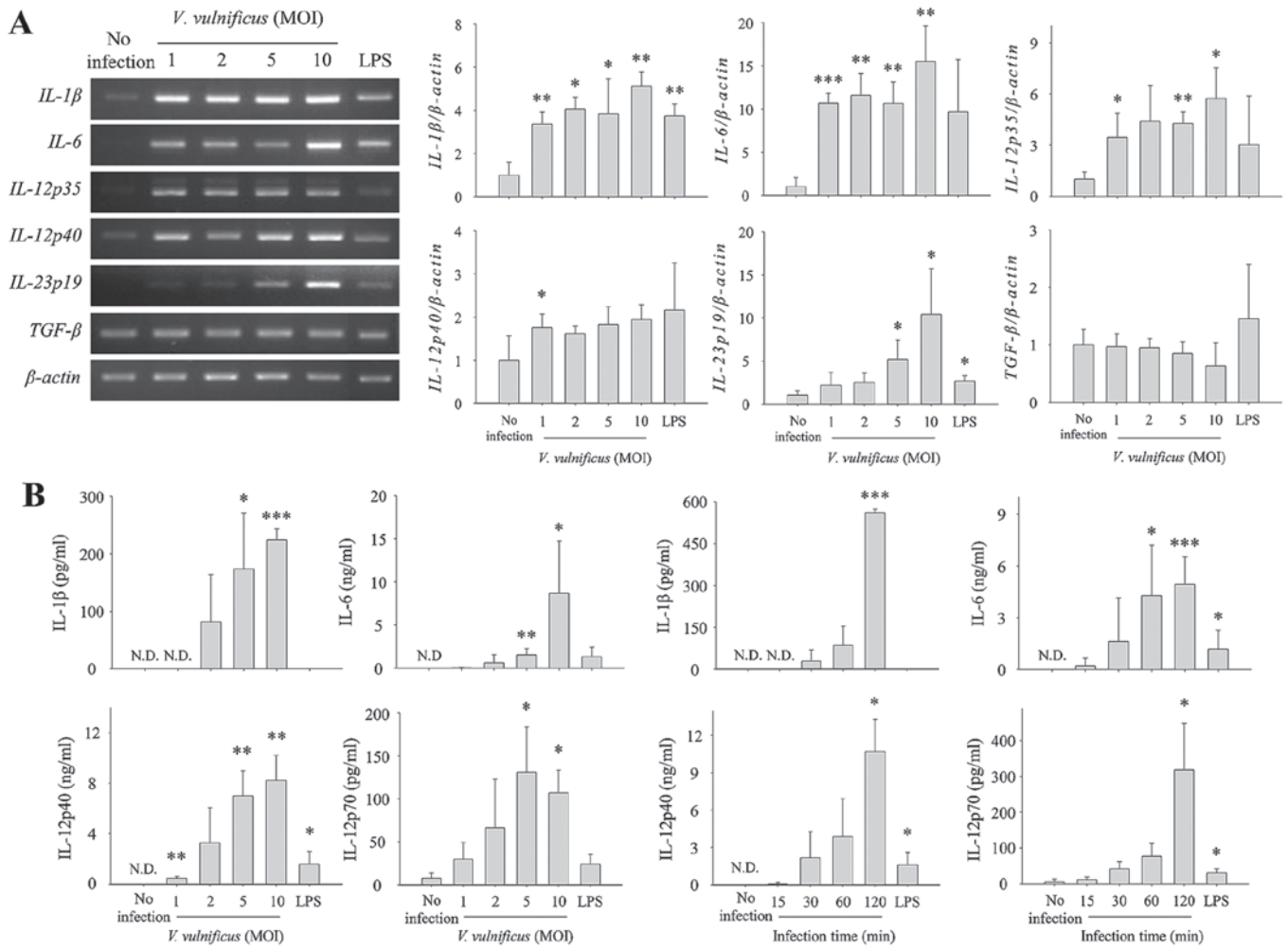

Figure 2. *V. vulnificus* infection induces the expression and secretion of Th1/Th17-polarizing cytokines in DCs. Total RNA was extracted from DCs infected with *V. vulnificus* at various MOIs for 30 min. (A) Expression levels of mRNAs of several Th-polarizing cytokines were determined using semi-quantitative reverse transcription-polymerase chain reaction analysis. The data shown are representative of three independent experiments and represent the mean ± standard deviation of three independent experiments. (B) DCs were infected for 30 min with *V. vulnificus* at various MOIs, or at an MOI of 1 for various incubation durations, or were treated for 60 min with LPS (500 ng/ml). The supernatants were collected, and the protein levels of IL-1β, IL-6, IL-12p40 and IL-12p70 were determined using ELISA kits. The data shown in (B) represent the means ± SD of three independent experiments. *P<0.05 vs. control (no infection); **P<0.01 vs. control (no infection); ***P<0.005 vs. control (no infection). *V. vulnificus*, *Vibrio vulnificus*; DCs, dendritic cells; Th, T helper; MOI, multiplicity of infection; LPS, lipopolysaccharide; IL, interleukin; TGF, transforming growth factor; ND, not detected.

results, the secretion levels of IL-1β, IL-6, IL-12p40 and IL-12p70, the active form of IL-12, significantly increased as the MOI and infection duration increased (Fig. 2B). These results suggested that the *V. vulnificus*-infected DCs induced the polarization of naïve CD4^{+} T cells into Th1 or Th17 cells by providing the appropriate signals.

V. vulnificus-infected DCs induce Th17 polarization predominantly through the secretion of IL-6. The above results demonstrated that the *V. vulnificus*-infected DCs acquired the ability to efficiently differentiate naïve CD4^{+} T cells into Th1 or Th17 cells. To determine whether *V. vulnificus* infection enabled DCs to induce the polarization of naive CD4^{+} T cells into Th1 or Th17 cells, the *V. vulnificus*-infected DCs were cocultured with lymph node-derived naïve CD4^{+} T cells at a ratio of 1:5 under T-cell receptor stimulation (1 μg/ml anti-CD3ε and 1 μg/ml CD28) for 3 days to assess the polarizing ability of DCs (Fig. 3). On day 3 of coculture, the populations of IFN-γ^{+} or IL-17^{+} CD4^{+} cells were measured using flow cytometry. As shown in Fig. 3, the *V. vulnificus*-infected DCs induced higher frequencies of IL-17^{+} CD4^{+} cells, compared with those of the uninfected DCs, in an MOI-dependent manner. By contrast, the population of IFN-γ^{+} CD4^{+} cells decreased upon *V. vulnificus* infection (Fig. 3), whereas the percentage of IL-4^{+} and Foxp3^{+} CD4^{+} cells remained unchanged upon *V. vulnificus* infection (data not shown).

To investigate the mechanism by which *V. vulnificus* infection mediates Th17 responses, IL-1β- and IL-6-neutralizing antibodies were added to cocultures of *V. vulnificus*-infected DCs and naïve CD4^{+} T cells. The results showed that the addition of IL-1β-neutralizing antibodies marginally reduced the frequencies of IL-17^{+} CD4^{+} cells, whereas the addition of IL-6-neutralizing antibodies markedly inhibited the increase in IL-17^{+} CD4^{+} cells (Fig. 4). As shown in Fig. 4C, the effects of neutralizing antibodies increased as the concentration increased. The addition of IL-6-neutralizing antibodies also increased the population of IFN-γ^{+} CD4^{+} cells in a concentration-dependent manner (data not shown). These results

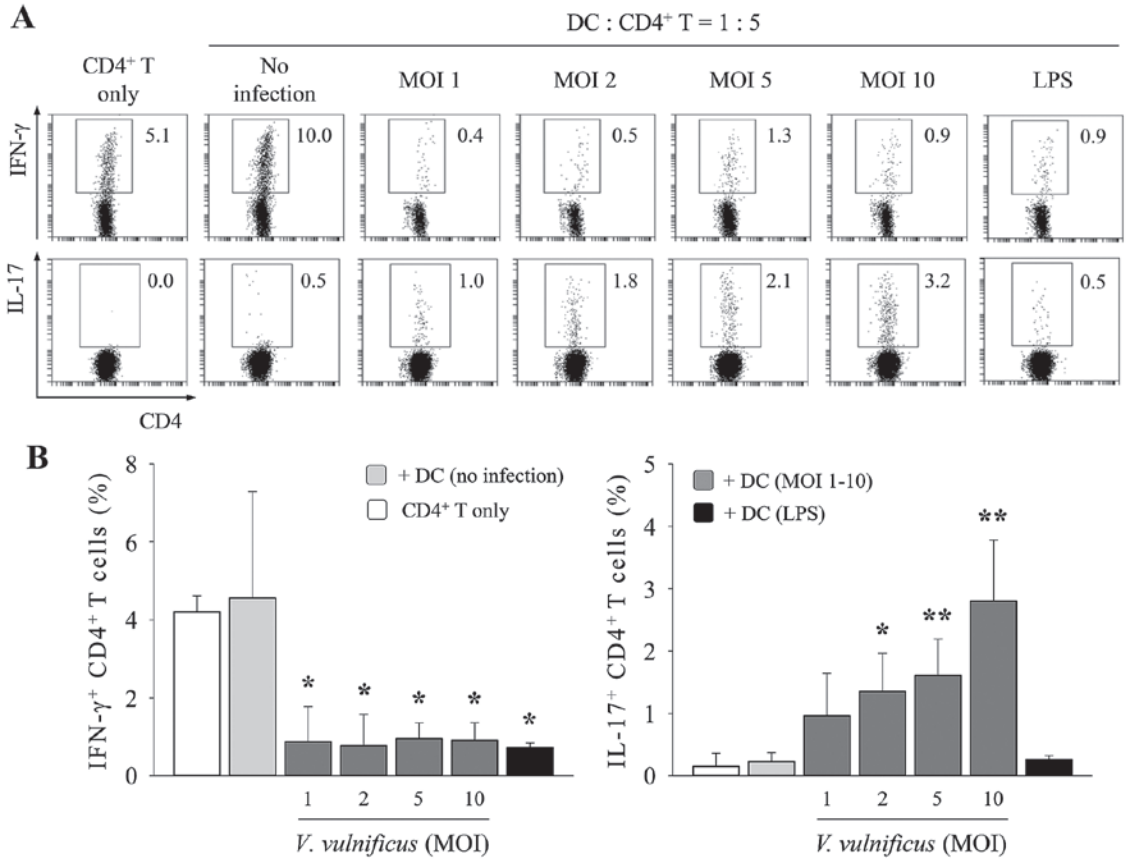

Figure 3. *V. vulnificus*-infected DCs induce T helper cell 17 polarization *in vitro*. DCs were infected with *V. vulnificus* at 0, 1, 2, 5 or 10 MOI or were treated with LPS (500 ng/ml) for 60 min. The DCs were then cocultured with naïve CD4+ T cells isolated from lymph nodes for 3 days in the presence of anti-CD3ε and anti-CD28 monoclonal antibodies prior to (A) flow cytometric analysis (data representative of three independent experiments) and (B) determination of the expression of CD4, IFN-γ and IL-17 (data presented as the mean ± standard deviation of three independent experiments). *P<0.05 vs. control (no infection); **P<0.01 vs. control (no infection). *V. vulnificus*, *Vibrio vulnificus*; DCs, dendritic cells; MOI, multiplicity of infection; LPS, lipopolysaccharide; IL-17, interleukin-17; IFN-γ, interferon-γ.

indicated that IL-6 secreted by the *V. vulnificus*-infected DCs was a critical factor, which significantly promoted the induction of IL-17-producing CD4+ T cells, and that IL-6 in particular may inhibit the differentiation of IFN-γ+ CD4+ cells, consistent with previous findings (24).

V. vulnificus-infected DCs induce the polarization of Th17 cells *in vivo*. As shown in Figs. 1-4, it was demonstrated that the *V. vulnificus*-infected DCs had the ability to polarize naïve CD4+ T cells into IL-17-producing cells *in vitro*. To confirm that *V. vulnificus* infection enables DCs to induce the polarization of Th17 cells, *V. vulnificus*-infected DCs were subcutaneously injected into OVA-immunized mice, and the IL-17+ CD4+ cell population was analyzed in isolated lymph node cells using flow cytometry. As shown in Fig. 5, the percentages of IL-17+ CD4+ cells significantly increased in the mice injected with *V. vulnificus*-infected DCs, whereas few IL-17+ CD4+ cells were detected in the mice injected with immature DCs. These results indicated that the *V. vulnificus*-infected DCs were capable of inducing Th17 responses *in vivo*.

Oral administration of V. vulnificus in mice increases the population of intestinal Th17 cells. The results of the present study showed that *V. vulnificus* infection promoted Th17 differentiation *in vitro* through antigen presentation and the secretion of Th17-polarizing cytokines by *V. vulnificus*-infected DCs. Therefore, to examine whether *V. vulnificus* infection directed the fate of CD4+ T cells toward Th17 cells *in vivo*, mice were orally administered with 10^7 CFUs of *V. vulnificus*. At 2 days post-infection, the mice were sacrificed and organs were retrieved for the analysis of Th cell populations. As shown in Fig. 6A, the mRNA expression of IFN-γ was higher in the Peyer's patches and the lamina propria of the *V. vulnificus*-infected mice, compared with that in the uninfected mice. The levels of IL-17 were also increased in the mesenteric lymph nodes, Peyer's patches and lamina propria of the *V. vulnificus*-infected mice. Flow cytometric analysis of the CD4+ T cells isolated from the small intestinal lamina propria revealed that IL-17+ CD4+ cells were also increased in *V. vulnificus*-infected mice, compared with uninfected mice (Fig. 6B). Elevated levels of IFN-γ and IL-17A in the *V. vulnificus*-infected mice were also confirmed by ELISA (Fig. 6C), indicating that infection through the ingestion of *V. vulnificus*-contaminated foods may induce an increase in Th17 cells.

Discussion

The present study demonstrated for the first time, to the best of our knowledge, that *V. vulnificus* infection induced Th17 responses by stimulating the maturation of DCs and

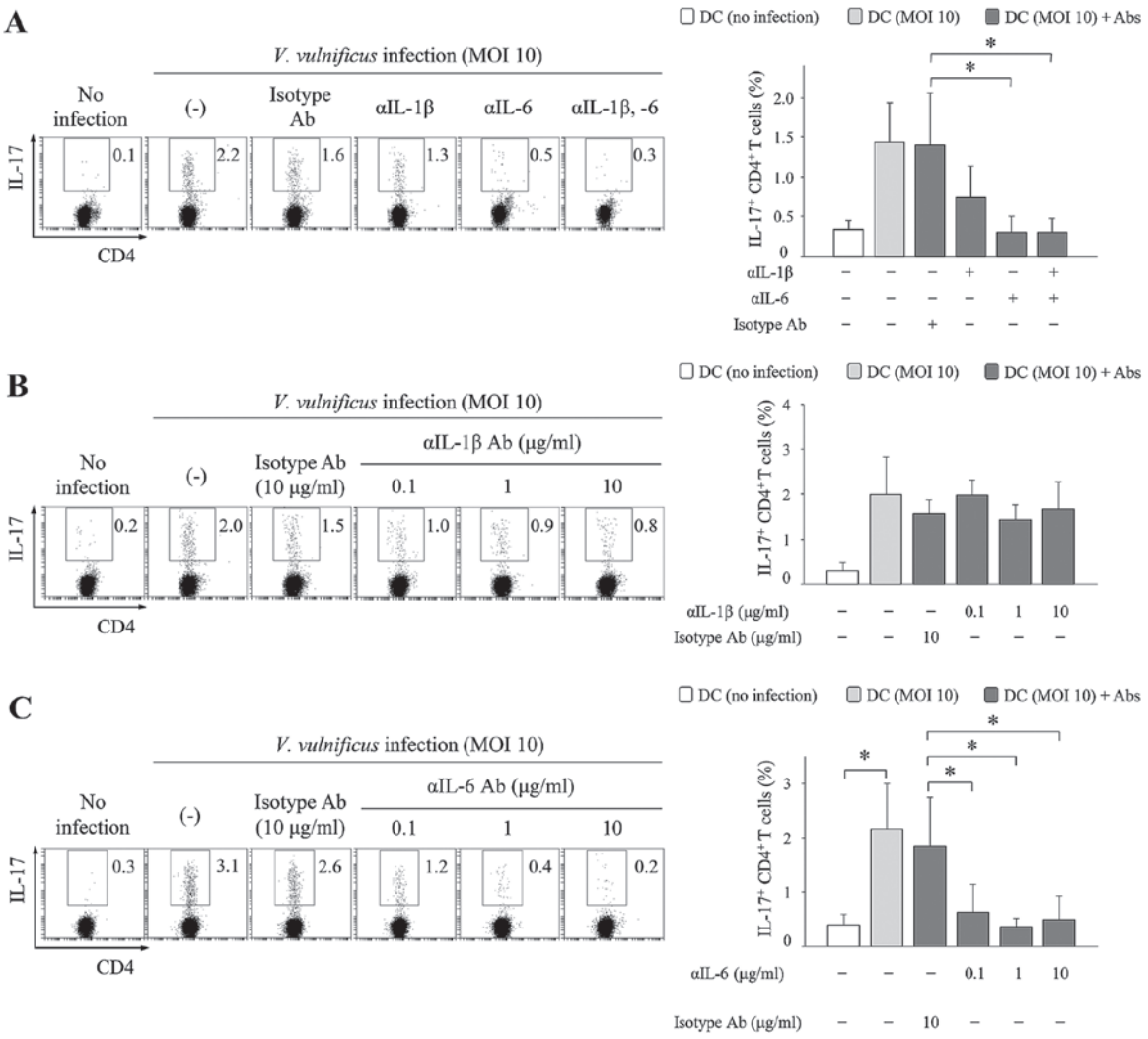

Figure 4. IL-6 is essential in *V. vulnificus* infection-induced T helper cell 17 polarization. DCs were infected with *V. vulnificus* at MOI of 1 for 60 min, following which the DCs were cocultured with naïve $CD4^+$ T cells isolated from lymph nodes for 3 days in the presence of anti-CD3ε and anti-CD28 monoclonal antibodies, and (A) anti-IL-1β (10 μg/ml), anti-IL-6 (10 μg/ml), or isotype antibodies (10 μg/ml), or (B) anti-IL-1β and isotype-specific antibodies (0.1, 1 or 10 μg/ml), or (C) anti-IL-6 and isotype-specific antibodies (0.1, 1 or 10 μg/ml) (C). Data shown are representative of three independent experiments and are presented as the mean ± standard deviation of three independent experiments. *P<0.05. *V. vulnificus*, *Vibrio vulnificus*; DCs dendritic cells; IL, interleukin; MOI, multiplicity of infection; Ab, antibody.

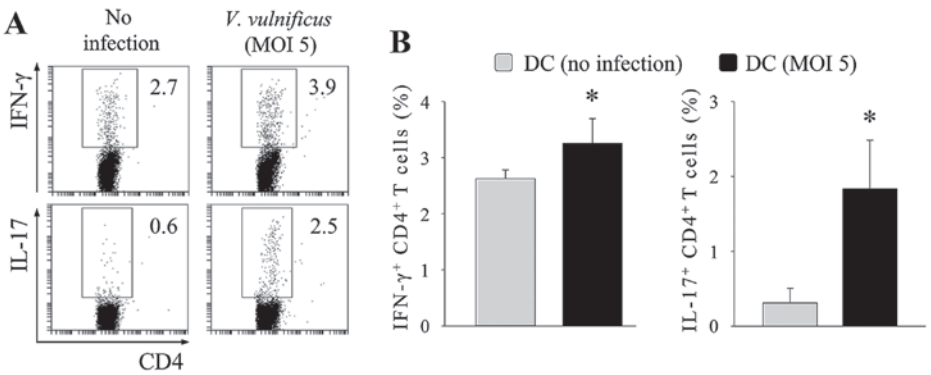

Figure 5. *V. vulnificus*-infected DCs induce T helper cell 17 polarization *in vivo*. The DCs were infected with *V. vulnificus* at 5 MOI for 30 min, and were then injected into ovalbumin-immunized mice. (A) Levels of IL-17 and IFN-γ were examined and (B) quantified. The data are representative of three independent experiments and presented as the mean ± standard deviation of three independent experiments. *P<0.05. *V. vulnificus*, *Vibrio vulnificus*; DCs, dendritic cells; MOI, multiplicity of infection; IL-17, interleukin-17; IFN-γ, interferon-γ.

the expression of Th17-polarizing cytokines in DCs *in vitro*. Additionally, the population of Th17 cells increased in the lamina propria of the small intestines in *V. vulnificus*-inoculated mice, compared with that in uninfected mice. *V. vulnificus*

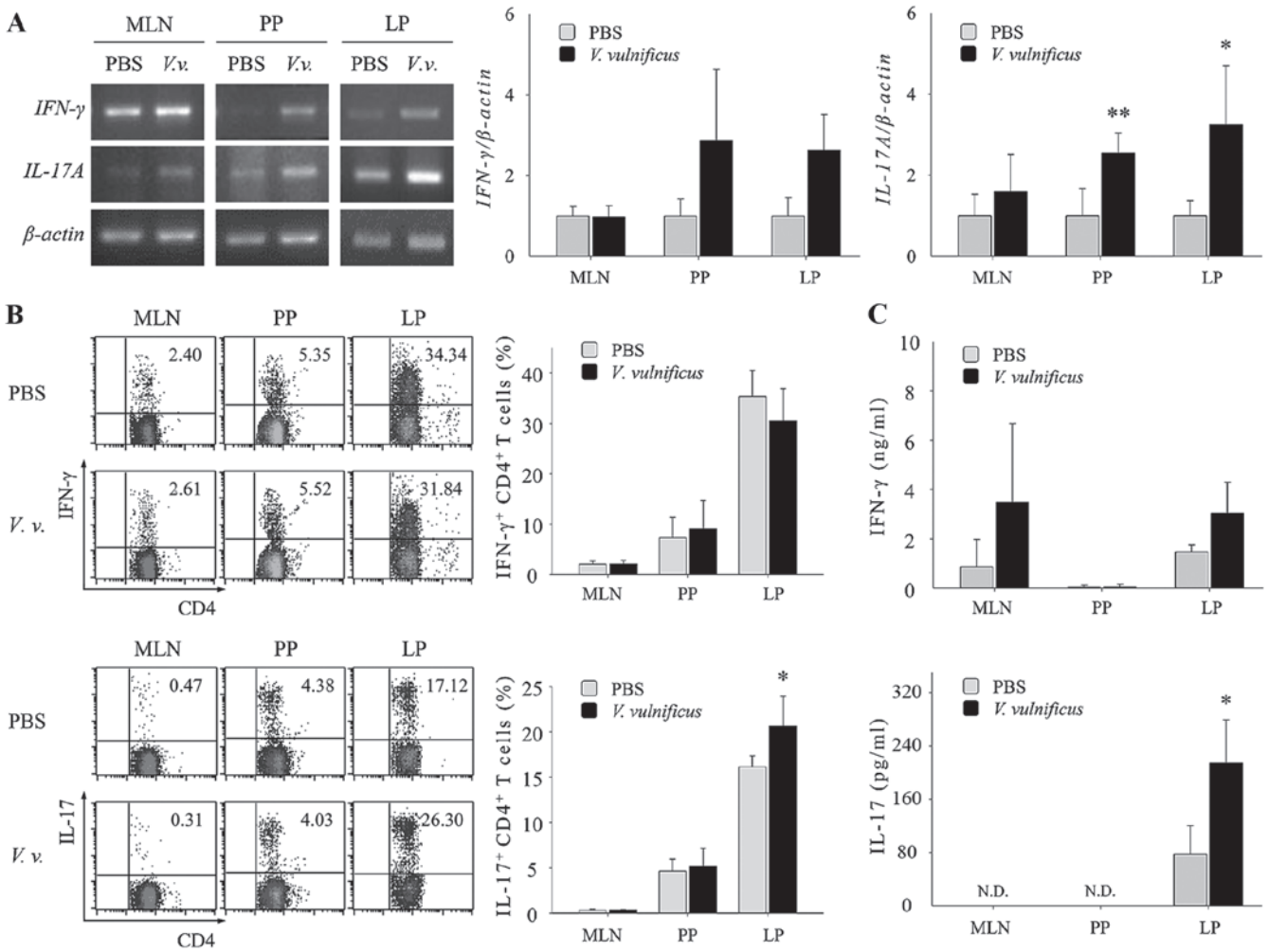

Figure 6. *V. vulnificus* infection in mice increases the population of the Th17 subset in the small intestines. (A) Mice were orally administered with *V. vulnificus* ($1x10^7$ CFU/mouse). After 2 days, the mice were sacrificed and total RNA was extracted from the MLN, PP and LP of uninfected mice or the mice infected with *V. vulnificus*. Semi-quantitative reverse transcription-polymerase chain reaction analysis was performed to determine the mRNA expression levels of Th1/Th17-associated genes. (B) Flow cytometric analysis of the expression of CD4, IFN-γ and IL-17 in the MLN, PP and LP of uninfected mice or mice infected with *V. vulnificus*. The data shown are presented as the mean ± standard deviation of three independent experiments. (C) Cells isolated from the MLN, PP and LP were cultured for 48 h, and supernatants were collected. The protein levels of IFN-γ and IL-17A were determined using ELISA kits. *P<0.05 vs. control (PBS group); **P<0.01 vs. control (PBS group). Th, T helper cell; IL-17, interleukin-17; IFN-γ, interferon-γ; V.v, *Vibrio vulnificus*; MLN, mesenteric lymph nodes; PP, Peyer's patches; LP lamina propria; ND, not detected.

is a gram-negative bacterium, which causes several diseases, including gastroenteritis and sepsis, via the ingestion of contaminated food or wound infection (1). Although the innate immune response against *V. vulnificus* infection has been investigated, few studies have described the adaptive immune response against *V. vulnificus* infection. Therefore, in the present study, whether the DC-mediated Th cell response occurs upon *V. vulnificus* infection was investigated.

The mechanism by which *V. vulnificus* infection increases Th17 cell responses remains to be fully elucidated. However, the findings of the present study suggested that the population of Th17 cells was increased by the secretion of IL-1β and IL-6 from *V. vulnificus*-infected DCs. *V. vulnificus* infection was shown to significantly induce the maturation and activation of DCs, which secreted the Th17-polarizing cytokines IL-1β and IL-6. Inhibiting IL-6 with neutralizing anti-IL-6 mAb significantly decreased the Th17 cell responses induced by *V. vulnificus* infection. In addition, the oral administration of *V. vulnificus* increased Th17 cell populations in mice, indicating that *V. vulnificus* induced Th17 cell responses, predominantly via DC-derived IL-6.

The effects of *V. vulnificus* infection on Th1 responses *in vitro* and *in vivo* are likely to be different. In a coculture system of *V. vulnificus*-infected DCs and $CD4^+$ T cells, the Th1 cell population was significantly decreased upon *V. vulnificus* infection, despite the observation that *V. vulnificus* increased the expression of IL-12, a Th1-polarizing cytokine, in the DCs. The decrease in the Th1 cell population by *V. vulnificus* infection was recovered to the level observed in the uninfected control by inhibiting IL-6 with neutralizing anti-IL-6 monoclonal antibodies (data not shown), indicating that the *V. vulnificus*-mediated inhibition of the Th1 cell population may be due to the secretion of IL-6 from DCs. A previous study showed that Th1 differentiation is negatively regulated by IL-6, a Th17-polarizing cytokine, in the presence of anti-CD3ε/28 stimulation (24). Of note, the oral administration of *V. vulnificus* in mice did not decrease Th1 cell responses. This discrepancy between *V. vulnificus*-induced Th1 effects

in vitro and *in vivo* may be due to the different environments and conditions encountered during exposure of *V. vulnificus* to host cells. In a coculture system of *V. vulnificus*-infected DCs with $CD4^+$ T cells, the infected DCs was in contact with $CD4^+$ T cells only, and the secreted factors were restricted to the small space within the coculture wells; by contrast, in the whole body, *V. vulnificus* is likely to come into contact with several types of immune cell, including DCs, with secreted factors rapidly dispersed throughout the body.

Several studies have demonstrated that Th17 cells can be induced by a variety of bacterial infections. Induced Th17 cells have a positive or a negative effect on the host defense mechanism against infection. In terms of the positive effects of Th17 cells, increased Th17 cells provide protection to the host by assisting in the clearance of pathogens. For example, in the Peyer's patches of *C. rodentium*-infected mice, the population of Th17 cells is increased, promoting the production of IL-22, which is crucial in directly inducing the Reg family of antimicrobial peptides, including RegIIIβ and RegIIIγ, in colonic epithelial cells (10,11). However, if Th17 cell responses are inappropriately regulated in infections, dysregulated Th17 cell responses promote tissue damage, exacerbate inflammation and lead to disease progression (13,14,25). Similarly, excessive Th17 cells responses induced by pathogen infection, which are initially designed to be critical in the clearance of pathogens, are known to be important in sepsis. The neutralization of IL-17 improves sepsis by suppressing plasma proinflammatory cytokines, including tumor necrosis factor-α, IL-1β and IL-6. In addition, in LPS-stimulated peripheral blood mononuclear cells from patients with sepsis, the absence of IL-17 results in increased production of IL-10 and decreased production of IL-12 (16,26). Whether the role of Th17 cells in infections is protective or detrimental may depend on the type of pathogen and the condition of infection.

Adaptive immune responses against infection with other *Vibrio* species, including *V. cholera*, have also been examined. *V. cholera* O395 is known to secrete outer membrane vesicles (OMVs), which are internalized into intestinal epithelial cells. These epithelial cells are stimulated by the internalized OMVs and then activate DCs, resulting in upregulation of the expression of costimulatory molecules and promoting the release of Th17-polarizing cytokines; these activated DCs promote the polarization of $CD4^+$ T cells into inflammatory Th2/Th17 cells (27). Similarly, in the present study, the numbers of Th17 cells were increased upon *V. vulnificus* infection. However, whether structural components of *V. vulnificus* and/or secreted factors from *V. vulnificus* infection are involved in the induction of Th17 cell responses remains to be elucidated.

According to several studies, the upregulation of certain virulence factor-associated genes depends on the contact of host cells with pathogens (22,28). The expression of several virulence factors of *V. vulnificus* is significantly increased upon contact with host cells (29-31). Furthermore, *V. vulnificus* has been reported to activate the host innate immune response by the binding of lipoprotein and flagellin to toll-like receptor 2 or 5 (32,33). Therefore, induced virulence factors and the detrimental function of Th17 cells may be involved in aggravating the pathogenesis caused by *V. vulnificus* by impeding the defensive role of Th17 cells.

In conclusion, the present study demonstrated that DCs were activated upon *V. vulnificus* infection, resulting in an induction of the Th17 response by producing Th17-polarizing cytokines, including IL-1β and IL-6. The oral administration of *V. vulnificus* in mice also induced an increase in the numbers of intestinal Th17 cells. Taken together, the results suggested that an understanding of the adaptive immune responses against *V. vulnificus* infection may facilitate the development of prophylactic and therapeutic agents against diseases caused by *V. vulnificus*. In addition to the results obtained in the present study, elucidating the roles of *V. vulnificus* virulence factors in the pathogenesis of *V. vulnificus* and the host immune response is crucial in future investigations on *V. vulnificus*.

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