VISTA stimulation of VSIG4-positive macrophages strongly suppresses T cell proliferation via excessive nitric oxide production in sepsis

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ABSTRACT

Organ damage and immune deficiency are important problems in sepsis caused by an excessive immune response. There is controversy about the cause of immune suppression. In this study, we investigated the roles of macrophages that exhibit excessive activity on T cell immunity. Peritoneal macrophages from mice with CLP-induced sepsis migrated to different organs. In particular, VSIG4 positive macrophages appeared in the spleen 48 hr after CLP induction. When cocultured with splenic T cells, VSIG4(+) cells inhibited the proliferation of activated T cells through the release of nitric oxide compared to VSIG4(-) cells. Stimulation of VSIG4(+) cells with VISTA antibody increased the expression of several cytokine genes and the release of nitric oxide, but not phagocytosis, compared to those of hamster IgG-stimulated VSIG4(+) cells. When cocultured with splenic T cells, VISTA-stimulated VSIG4(+) cells induced excessive T cell suppression via more NO secretion compared to hamster IgG-stimulated VSIG4(+) cells. Taken together, the current study demonstrates that VSIG4(+) peritoneal macrophages play important roles in inducing immunosuppression and that VISTA acts as a costimulatory receptor in these cells. These data suggest that blocking the migration of VSIG4(+) cells might alleviate excessive immune activity and that blocking VISTA on VSIG4(+) macrophages might play a crucial role in the development of new therapies to prevent T cell suppression in sepsis.

Keywords: Sepsis, VSIG4, VISTA, T cell suppression, Nitric Oxide
INTRODUCTION

Sepsis is a major reason of death and the cause of high morbidity and mortality.\(^1\) The frequency of sepsis in the United States annually has been reported to be approximately 750,000 cases, and the mortality rate is also known to be more than 20%.\(^2\) Sepsis occurs when the initial immune response caused by excessive infection results in abnormal bacterial clearance, develops into a systemic infection, and causes multiorgan dysfunction.\(^3,4\) Sepsis often involves immunosuppression because it can induce extensive and severe apoptosis of immune cells in various animal experimental models and human studies.\(^5,6\) Increased lymphocyte apoptosis induces immunosuppression as the disease progresses, resulting in an impaired ability of the immune system to eliminate primary infection as well as common nosocomial infections.\(^7\) These changes result in higher mortality rates, but no effective treatments are currently available.

V-set immunoglobulin-domain-containing 4 (VSIG4) is a novel checkpoint regulator related to the B7 family member. VSIG4 is a 45 kDa transmembrane protein consisting of two extracellular Ig-like domains, a transmembrane domain and a short cytoplasmic domain.\(^8\) VSIG4 is mainly expressed in resting macrophages and suppresses the activation of macrophages in response to lipopolysaccharide, resulting in an important role in regulating macrophage-mediated inflammation.\(^9,10\) However, this molecule disappears on infiltrating macrophages.\(^11\) VSIG4 is also known as a complement receptor because it binds C3b and iC3b fragments. This complex internalizes to endosomes and VSIG4 is constitutively recycled to the cell surface.\(^12\) This receptor contributes significantly to phagocytosis of complement opsonized invading pathogens.\(^12,13\) VSIG4 is also known as a negative regulator of mouse and human and induces tolerance in T cells and NKT cells.\(^14\)

V-domain Ig suppressor of T cell activation (VISTA) is a cell surface molecule belonging to the B7/CD28 gene family and mainly expressed on the immune cells such as T cells and antigen-presenting cells (APCs).\(^15,16\) VISTA is also referred to as programmed death-1 homolog (PD-1H) and known as a coinhibitory molecule because it inhibits the activation of T cells.\(^17,18\) Wang et al. mentioned that VISTA-Fc fusion protein actively suppresses the activation of T cells and suggested that T cells and APCs have a unknown receptor for VISTA.\(^16\) VISTA on APCs could inhibit T cell activation when cocultured with APCs.\(^19\) Therefore, VISTA can act as an inhibitory ligand on APCs and suppress the activation of T cells via their unknown receptor.\(^16,20\) However, the exact function of VISTA on APCs in
Acute immune responses such as sepsis is unclear. Therapeutic drugs for treatment of sepsis, which target cytokine storms as shown in past studies, are known to be ineffective. Therefore, the major problem with treatment of sepsis is that there is no treatment target. The present study evaluated whether VSIG4-positive peritoneal macrophages can be targeted for treatment of sepsis and whether the surface antigen VISTA is available for cell activation or immune modulation in a CLP-induced sepsis model, focusing on the inhibition of T cell proliferation using a coculture system.

MATERIALS AND METHODS

Mice
Female C57BL/6 mice at 8 week of age were obtained from Orient Bio (Seognam, Korea). The mice were supplied with standard rodent pellets and water, and the breeding room is maintained at a standard 12-h light/dark cycles. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Inje University College of Medicine (approval number: 2017-022).

Antibodies
FITC-conjugated CD3 (17A2), CD4 (GK1.5), B220 (RA3-6B2) and CD11b (M1/70) antibodies, FcR blocker (mouse CD16/CD32 2.4G2) antibody and APC-conjugated VSIG4 antibody (NLA14) were obtained from eBioscience (San Diego, CA). PE-conjugated VISTA antibody (MH5A) was purchased from BioLegend (San Diego, CA). The optimal cut-off for each marker was based on an appropriate isotype control antibody.

RT-PCR
Total RNA for cDNA synthesis was prepared using TRizol reagent from Invitrogen (Carlsbad, CA). Prepared RNA (2 μg) was reverse transcribed with Superscript II from Invitrogen following the method of the manufacturer’s instructions. The reverse transcribed cDNA (0.1 μg) was used as a PCR template. The mouse PCR primer sequences used are as follows: TNF-α forward, 5’-CCACACCGTCAGCCGATTTG-3’ and reverse, 5’-CACCATTCCCTTCACAGAGC-3’; IL-1β forward, 5’-CTCGTGCTGTGGAGTTATG-3’ and reverse, 5’-TTGAAGACAAAACC GCTTTTCCA-3’; IL-6 forward, 5’-TGGGAAATCGTGAAATGAG-3’ and reverse, 5’-GAAGGACTCTGGCTTTGTCT-3’;
COX2 forward, 5′-CCGTGGGAATGTATGAGCA-3′ and reverse, 5′-CCAGGTCCCTCGTTATGATCTG-3′; iNOS forward, 5′-TTTGCCACGGACGACGGAT-3′ and reverse, 5′-GCCACTGACACTTCGCACAAA-3′; Arg-1 forward, 5′-CTCCAAGCCAAAGTCCTTAGAG-3′ and reverse, 5′-AGGAGCTGTCATTAGGGACATC-3′; GAPDH forward, 5′-TTCACCACCATGGAGAAGGC-3′ and reverse, 5′-GGCATGGACTGTGGTCATGA-3′. PCR reaction was performed at 94 °C for 30 s, at 58 °C for 30 s, and at 72 °C for 1 min and amplified for 25–30 cycles according to target genes. PCR products were electrophoresis using a 1.2% agarose gel. The gels were stained with ethidium bromide and analysed using a UV transilluminator.

**Cecal ligation and puncture**

The cecal ligation and puncture (CLP) method as defined by Baker et al.21) was used for sepsis induction. Mice were anesthetized using the combination of ketamine (HUONS) and xylazine (Bayer Korea) intraperitoneally. The abdomen was disinfected with povidone iodine swab sticks, and a 1.5 cm midline abdominal incision was performed. The cecum was exposed and ligated with a 4-0 silk at 5 mm below the ileocecal valve, and then punctured the distal cecum with a 26-gauge needle twice for the sepsis induction. After the operation, the cecum was moved back to the original position and then abdomen and skin were sutured in layers. Sham mice were subjected to only abdominal incision and exposed for 5 minutes. All animals after operation received fluid resuscitation with 0.5 ml saline subcutaneously at the nuchal region. After surgery, pain control for mice was achieved by treatment with 0.05 mg/kg buprenorphine every 12 hr. The mice were returned to their cages with heating pads until full recovery.

**Separation of peritoneal macrophages and VSIG4(+) or VSIG4(-) cells**

Total peritoneal immune cells were isolated from peritoneal cavity of normal mice. Mice were anesthetized using the combination of ketamine (HUONS) and xylazine (Bayer Korea) intraperitoneally and injected 5ml of ice cold PBS into the peritoneal cavity. After injection, the peritoneum was gently massaged to dislodge any attached cells into the solution. The suspended solution containing immune cells was collected from peritoneal cavity. CD11b(+) cells were positively separated from peritoneal immune cells using biotin-conjugated CD11b antibody and streptavidin cross-linked magnetic bead (the magnetic cell sorting (MACS)) according to the protocol provided by the manufacturer (Miltenyi Biotec, Bergisch Gladbach,
After first positive selection, CD11b(+) cells were treated with MutiSort Release Reagent (Miltenyi Biotec) following the method provided by the manufacturer to remove magnetic beads. VSIG4(+) cells (CD11b positive/VSIG4 positive cells) were enriched from purified CD11b positive cells using APC-conjugated VSIG4 antibody and anti-APC magnetic beads (Miltenyi Biotec). The cells were separated on MACS columns and yielded approximately >95% pure cells. VSIG4(-) cells (CD11b(+)/VSIG4(-) cells) were enriched from CD11b(+) cells without VSIG4(+) cells (Supplementary Fig. 2).

**In Vivo Image Analysis**

Peritoneal macrophages (CD11b(+) cells), VSIG4(-) and VSIG4(+) cells were stained with VivoTrack 680 (PerkinElmer) at RT for 15 min and washed 3 times with PBS. Stained cells were injected into peritoneal cavity of sham- or CLP-induced mice. All mice were anaesthetized with the combination of ketamine and xylazine intraperitoneally 48 h later, and the peritoneal cavity was washed with normal saline. The spleens were harvested individually and imaged by measuring the near infrared fluorescence (Excitation: 670±15; Emission: 700±15) using Xenogen IVIS™ and Living Image Software (Xenogen). Primary immune cells were isolated from the peritoneal cavity and spleen, and the fluorescence intensity of the cells was analysed using Alexa Fluor 700 in the flow cytometer.

**Confocal Analysis**

To confirm in vivo migration, spleens obtained from each mouse were freeze-dried using freezing medium and sectioned by a cryotome. Tissue slides were fixed with ice cold 10% formalin for 24 hours and stained with DAPI solution (BIO-RAD) to detect the nuclei positive cells. The tissue sections were analyzed using confocal laser microscopy (Nikon’s superior A1+).

**Flow Cytometry Analysis**

For the detection of VSIG4, VISTA and immune cell markers, cells were prepared from peritoneal cavity and spleen, and washed with FACS buffer from Sigma-Aldrich (St. Louis, MO). To prevent the nonspecific binding, cells were first reacted with FcR blocking antibody (2.4G2) at 4 °C for 10 min, and then stained with VSIG4 antibody or with matched pair of antibodies at 4 °C for 20 min. Prepared cells were analysed using BD FACSCanto II (BD Bioscience, Franklin Lakes, NJ), and FlowJo software was used for data analysis. Dead cells were removed using forward (FSC) and side scatter (SSC) gating and lived cells were only
analysed using the flow cytometer.

**Anti-VISTA Stimulation Assay**

Purified VSIG4(+) cells were stimulated with hamster IgG (5 μg/ml) or VISTA (5 μg/ml, MH5A; Biolegend) antibodies at various times. And then, cells were used for RT-PCR, phagocytosis and NO assay. These cells were also cocultured with splenic T cells stimulated with anti-CD3/CD28 for 72 hr and used for analysis of T cell proliferation and NO secretion.

**Phagocytosis Assay**

Phagocytosis assay was performed using purified VSIG4(+) cells isolated from C57BL/6 mice. Cells were incubated in serum-free RPMI containing 1 mg/ml FITC-dextran (Mr=2,000,000; Sigma) for 30 min at 37 °C, 5% CO2. And then, cells were washed with cold PBS three times to avoid a nonspecific reaction. Finally, cells were scraped from the culture plate and stained with APC-conjugated VSIG4 antibody, and analysed using the flow cytometer.

**T cell Proliferation Assay**

T cells were prepared from the normal splenocytes using mouse T cell positive isolation kit (CD90.2 microbeads; Miltenyi Biotec Inc.) following the method provided by the manufacturer. The purity of cells was measured at >95% CD3+ cells using the flow cytometer. Purified T cells were stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) following the method provided by the manufacturer and used as the responder cells. VSIG4(+) cells were obtained from mouse peritoneal CD11b(+) cells using the magnetic cell sorting (MACS) and used as the stimulator cells. Responder T cells (2x10^5) were mixed stimulator cells in a 1:1/2 (1x10^5) or 1:1/4 (5x10^4) ratios in a 96-well round bottom plate and reacted with anti-CD3 (5 μg/ml, and clone 145-2C11; eBioscience) and anti-CD28 (2.5 μg/ml, and clone 37.51; eBioscience) antibody combination for 72 hr. In some experiments, the L-NMMA (NO inhibitor, Calbiochem), NAC (ROS inhibitor, Sigma) and nor-NOHA (Arginase inhibitor, ENZO) were added to a final concentration of 0.5 mM in culture media. After 72 hr of culture, the cell proliferation was estimated using the flow cytometer measuring CFSE fluorescence. The percent of CFSE labeled cells and replication index were calculated using FlowJo software.

**Measurement of NO**
Nitric Oxide (NO) production was measured following the protocol provided by Sigma. In brief, culture supernatants (100 μL) were reacted with an equal volume of Griess reagent from Sigma-Aldrich (St. Louis, MO) and incubated at room temperature for 15 min, and then measured at 540-570 nm (SpectraMax M2e). The levels of NO were measured the absorbance of culture supernatants compared to that of a standard.

**Statistical analysis**

Student's t test was performed wherever applicable. Data were exhibited as mean values ± S.E.M. of the executed number of experiments. The statistical significance was determined using a one-way or a two-way ANOVA for unpaired observations. The p values were defined as less than 0.05.

**RESULTS**

**Peritoneal VSIG4-positive cells migrate into the spleen after CLP**

According to our recent results in Cho et al.\(^{22}\), peritoneal VSIG4(+) cells migrate to the thymus and induce the apoptosis of thymocytes after CLP induction. Because VSIG4 is mainly expressed in peritoneal macrophages and VSIG4(+) macrophages induce T cell suppression\(^{14}\), VSIG4 expression was studied using FACS analysis of primary murine immune cells in the peritoneal cavity. VSIG4(+) cells were observed in control peritoneal macrophages (approximately one-third of all macrophages), but these cells disappeared after CLP induction (Fig. 1a). As we presumed that VSIG4(+) cells might have migrated from the peritoneal cavity to another organ(s) after CLP induction, immune cells from the spleen in sham-operated and CLP-induced mice were analysed. VSIG4(+) cells in the spleen after CLP induction gradually increased compared to those in the sham-operated mice, but these cells showed low levels of VSIG4 expression (Fig. 1b). Peritoneal VSIG4(+) cells showed F4/80 positive, but Gr-1 negative phenotypes (Fig. 1c). As an effective method to trace VSIG4(+) cells, we employed VivoTrack because it barely affects the viability and migration of immune cells. Purified peritoneal cells were first isolated from wild-type mice and stained with VivoTrack. The stained cells were injected into the peritoneal cavity of the sham-operated or CLP-treated mice, and then, the mice were analysed using FACS at 48 hr. For detailed quantitative analysis, peritoneal and splenic cells isolated from both the sham-operated or CLP-treated mice were analysed using FACS (Fig. 1d). Compared to those in the sham-
operated mice, VivoTrack-stained cells were increased more than 20-fold in the spleen of the CLP-treated mice (Fig. 1e).

Although VivoTrack-stained VSIG4(+) cells were shown to be present in the spleen after CLP induction, there remained a question if they were either VSIG4(+) or VSIG4(-) cells, because VSIG4 low-expressed cells were found in the spleen. In order to answer the question, we purified the VSIG4(+) and VSIG4(-) cells separately from the peritoneal CD11b(+) cells and repeated In Vivo Image System (IVIS) using them. The purified VSIG4(+) and VSIG4(-) cells were stained with VivoTrack and injected into the peritoneal cavity of CLP mice. After 48 hr, the VivoTrack signal was only detected in the spleen of the VSIG4(+) cell-injected mice than that of VSIG4(-) cell-injected mice (Fig. 2a). When splenocytes were further analysed, the number of VivoTrack-stained CD11b positive cells was increased in the VSIG4(+) cell-injected mice than that of V4(-) cell-injected mice (V4(+)/V4(-): 5.1 fold (3.6/0.703) (Fig. 2b). As shown in Fig. 2c, VivoTrack-stained VSIG4(+) cells in spleen also exhibited F4/80 positive and Gr-1 negative phenotypes. Furthermore, these cells showed VTK positive and VISTA positive phenotypes. The above results strongly suggested that VSIG4(+) cells in the peritoneal cavity migrate from the peritoneum to the spleen upon CLP induction in mice. Given the presence of VivoTrack-stained VSIG4(+) cells in spleen after CLP induction, we next examined whether these cells are colocalized with splenic immune cells using confocal microscopy. Interestingly, we found that VivoTrack-stained VSIG4(+) cells exist between the splenic immune cells in the white pulp (Fig. 2d). Taken together, these results suggested strongly that peritoneal VSIG4(+) cells migrate to the spleen after CLP induction and may affect the activation of splenic immune cells such as T cells after CLP induction.

**VSIG4-positive cells inhibit T cell proliferation via NO secretion**

In our Fig. 1d and 2b data, some VivoTrack-stained VSIG4(+) cells were found in the spleen. Therefore, we assumed that VSIG4(+) cells may migrate to the spleen and that these cells affect splenic immune cells. When anti-CD3/CD28-activated splenic T cells were cocultured with purified VSIG4(+) cells, T cell proliferation was specifically inhibited at ratios of 1:1/2 or 1:1/4 (T cell:VSIG4(+) cells), while VSIG4 negative cells showed some suppression of T cell proliferation only at a ratio of 1:1/2 (Fig. 3a). Because VSIG4(+) cells
suppress the T cell proliferation and the IL-2 production in vitro\(^4\), we next investigated the exact mechanism by which VSIG4(+) cells suppress T cells. When the concentrations of nitric oxide (NO) were measured in coculture supernatants, VSIG4(+) cells secreted more NO than VSIG4(-) cells (Fig. 3b). Furthermore, the suppression of T cells by VSIG4(+) cells was ameliorated by treatment with an NO inhibitor but not an ROS or arginase inhibitor (Fig. 3c). These results indicate that VSIG4(+) macrophages may inhibit splenic T cell proliferation via NO release.

**Functional study of VISTA expressed on VSIG4-positive cells**

VISTA on APCs can suppress T cell activation.\(^9\) To confirm this result, we next estimated the expression of VISTA on peritoneal macrophages. As shown in Fig. 4a, VISTA was expressed in approximately two-thirds of peritoneal macrophages and most VSIG4-expressing peritoneal macrophages (Fig. 4a). After CLP induction, there was little change in the expression of VISTA on splenic CD4, CD8 T cells and B cells. On the contrary, the number of CD11b positive cells expressing VISTA increased significantly in proportion to the increase in CD11b positive cells in spleen with CLP mice compared to that of sham mice (Supplementary Fig. 1). To fine out the function of VISTA, purified VSIG4(+) cells were stimulated with an agonistic VISTA antibody. VISTA engagement on VSIG4(+) cells further increased the basal level expression of TNF-\(\alpha\) and IL-1\(\beta\), but not iNOS, COX2 and Arginase-1, compared to that of hamster IgG-treated cells (Fig. 4b). However, VISTA stimulation did not affect the phagocytosis of VSIG4(+) cells using dextran substrate (Fig. 4c). Because NO secreted by macrophages suppresses T cell proliferation, we next examined the role of VISTA in the production of NO. When VSIG4(+) cells were stimulated with IFN-\(\gamma\), the production of NO increased in a time-dependent manner compared to that of hamster IgG-treated cells. Surprisingly, when IFN-\(\gamma\)-treated cells were stimulated with an agonistic VISTA antibody, NO production was increased 2-fold compared with that after hamster IgG stimulation (Fig. 4d).

**Functional study using an agonistic VISTA antibody**

VSIG4(+) cells migrated from the peritoneal cavity to the spleen after CLP induction (Fig. 1a-1d), and most VSIG4(+) cells expressed VISTA on their surface (Fig. 4a); thus, we next...
investigated the roles of VISTA expressed on VSIG4(+) cells. When activated splenic T cells were cocultured with VSIG4(+) cells, VISTA-stimulated VSIG4(+) cells showed greater suppression of the proliferation of T cells than hamster IgG-stimulated VSIG4(+) cells (ratio 1:1/8) (Fig. 5a). VISTA antibody barely affected activated T cells. Next, we tested the concentrations of NO in coculture supernatants, and the results showed that VISTA-stimulated VSIG4(+) cells secreted more NO than hamster IgG-stimulated VSIG4(+) cells (approximately 2-fold in 1:1/8 ratio) (Fig. 5b). Furthermore, the inhibition of T cell proliferation by VISTA-stimulated VSIG4(+) cells was almost completely recovered by treatment with an NO inhibitor (Fig. 5c). These results indicate that VISTA stimulation expressed on VSIG4(+) macrophages inhibits T cell proliferation mainly via increased NO release.

DISCUSSION

The main problem with treatment of sepsis is that there is currently no treatment target because drugs to control cytokine storms are ineffective. Therefore, the study aimed to search for new targets in treatment of sepsis. In our preliminary study, we found that peritoneal macrophages moved to different organs after sepsis induction, and the fluorescence intensities of the thymus were detected with an in vivo imaging system. However, the fluorescence intensities of the spleen were barely detected (data not shown). We presumed that the spleen was larger than the number of migrating cells. Therefore, we analysed VivoTrack-stained cells in the spleen using the flow cytometer and found that peritoneal macrophages migrated to the spleen after CLP induction (Fig. 1c and 1d).

The function of T cells is inhibited and immune suppression occurs during the course of sepsis. In this study, we further elucidated the immune cell migration and T cell suppression mechanisms that occur as the disease progresses. The CLP-induced mouse model of sepsis mimics the characteristics of clinical sepsis by enteric bacteria. Therefore, the CLP model is commonly used for the induction of sepsis. When we first induced CLP in mice in the peritoneal cavity, thymic atrophy occurred in all mice, as indicated by a decrease in cell number, and thymocytes, especially double-positive cells, underwent apoptosis in a time-dependent manner (data not shown). Several studies indicate that the excessive expression of proinflammatory cytokines can induce the apoptosis of thymic T cells during
sepsis. In addition, the overwhelming production of cytokines is involved in multiple organ dysfunction and death in the context of sepsis. However, the exact mechanism is still unclear. In this study, we revealed that VSIG4(+) cells in the peritoneal cavity migrated to the spleen after induction of sepsis and inhibited the activity of T cells.

Many studies indicate a possible role for VSIG4 in protecting against inflammatory diseases including microbial infection. However, there has been no study of the effect of VSIG4(+) cells on splenic T cells. Many researchers have suggested that NO is increased in sepsis and plays an important role in morbidity and mortality. NO suppresses the function of lymphocytes in the small intestine epithelium. Vogt et al. also reported that VSIG4(+) cells suppress the proliferation of T cells and the production of IL-2 in vitro, and established that VSIG4 is an inhibitory signaling molecule. Nevertheless, the mechanism by which NO mediates the suppression of T cells in sepsis is uncertain, and the exact mechanisms by which VSIG4(+) cells suppress T cells are still undefined. Our data showed that when activated splenic T cells were cocultured with VSIG4(+) cells, T cell proliferation was specifically inhibited compared to that with VSIG4(-) cells (Fig. 3a). Furthermore, as shown in Fig. 5a, VISTA engagement on VSIG4(+) cells further suppressed T cell proliferation (Fig. 5a) and increased NO production compared to that of hamster IgG-stimulated VSIG4(+) cells (Fig. 5b). These results indicate that VSIG4(+) macrophages inhibit the proliferation of T cells mainly via NO release and that VISTA induces enhanced activity in macrophages, resulting in suppression of T cell proliferation.

The function of VISTA in sepsis is still unknown. Files et al. noted that VISTA expressed on APCs and Tregs can act as a ligand and that the VISTA ligand represents the extrinsic regulation of conventional T cells by interacting with an unknown receptor, resulting in suppression of T cell activation. In this study, we used two commercially available types of VISTA antibodies. One type is blocking antibodies, and the other type is for stimulation. The VISTA blocking antibody was not useful in the experiment, and the stimulatory antibody showed an effect on macrophages but not T cells. In our experiment, the VISTA stimulation antibody increased gene expression in macrophages and played a role in increasing macrophage activity (Fig. 4b, 4d). Therefore, we believe that VISTA expressed in macrophages functions as a positive receptor and that the detailed function of the receptor should be determined through further research.

In summary, we have shown for the first time that in a CLP-induced mouse model of
sepsis, VSIG4-positive peritoneal macrophages actively migrate to the spleen and suppress T cell proliferation. These effects are thought to result in immunosuppression as the disease progresses. Thus, this is a novel finding in the field of sepsis, and controlling VSIG4-positive macrophages via the VISTA molecule may be an effective method for preventing immune suppression by sepsis.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2015R1D1A1A01057446).

Conflict of Interest

The author declares no conflict of interest.

Supplementary materials

The online version of this article contains supplementary materials.
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Fig. 1. Analysis of peritoneal cells and splenocytes after CLP.
(a) Total murine peritoneal cells were isolated from sham-operated and CLP-induced mice 48 hr. Immune cells were stained with B220 or VSIG4 and CD11b antibodies and analysed using
the flow cytometer. (b) CLP was induced in mice, and after 24 or 48 hr, immune cells were isolated from the peritoneal cavity and spleen. Prepared cells were stained with VSIG4 and CD11b antibodies and analysed using the flow cytometer. Cells from the sham-operated mice were used as controls. (c) Peritoneal cells were isolated from peritoneal cavity of normal mice. Cells were stained with VSIG4/CD11b, VSIG4/Gr-1 or VSIG4/F4/80 antibodies. Stained cells were analysed by flow cytometry. (d) Peritoneal macrophages (CD11b(+) cells) were stained with VivoTrack 680 (PerkinElmer) and the stained cells (2 × 10⁶ cells/mouse) were injected into the peritoneal cavities of Sham and CLP-induced mice. After 48 hr, the mice were anesthetized using the mixture of ketamine and xylazine, and the peritoneal cavity was washed with normal saline. Primary immune cells were isolated from the peritoneal cavity and spleen, and the fluorescence intensity of the cells was gated and analysed using the flow cytometer. The data represents the average value of triplicates. (e) The number of VivoTrack-positive cells from the peritoneal cavity and spleen as shown in (d) was counted using the flow cytometer. Data indicates the mean ± S.E.M. obtained from three separate experiments. *p<0.05, **p<0.01, Student’s t-Test. VTK indicates the activity of VivoTrack.

(Color figure can be accessed in the online version.)
Fig. 2. Migration Assay using VSIG4(+) and VSIG4(-) cells in CLP-induced mice. (a) Purified VSIG4(+) and VSIG4(+) cells were stained with VivoTrack 680, and the stained cells (2 × 10^6 cells/mouse) were injected into the peritoneal cavities of mice after CLP.
induction. After 48 hr, the mice were euthanized, and the peritoneal cavity was washed with PBS three times. Then, spleens were isolated from mice and fluorescence intensities were detected with an In Vivo Imaging System (IVIS Lumina; Perkin Elmer). (b) After IVIS analysis, splenocytes were purified and stained with CD11b antibody, and then analysed using flow cytometry by gating only lived cells. VSIG4(-) and VSIG4(+) indicates splenocytes prepared from VTK-stained VSIG4 positive or negative cell-injected mice. The number of VivoTrack-positive cells was counted using the flow cytometer. Data indicates the mean ± S.E.M. obtained from three separate experiments. *p<0.05, Student’s t-Test. (c) Purified VSIG4(+) and VSIG4(-) cells were stained with VivoTrack 680 (PerkinElmer) and stained cells (2 × 10^6 cells/mouse) were injected into peritoneal cavity of CLP-induced mice. After 48 hr, the mice were euthanized and spleens were isolated from mice. The tissue sections were made from the frozen spleen block and the tissue slides were stained with DAPI solution. Confocal fluorescence images show VivoTrack-labeled cells (red) and total nuclei stained cells with DAPI (blue). Merged image shows overlap (arrowheads) of VivoTrack-stained cells with DAPI. (d) Purified VSIG4(+) and VSIG4(-) were stained with VivoTrack 680 (PerkinElmer) and stained cells (2 × 10^6 cells/mouse) were injected into peritoneal cavity of CLP-induced mice. After 48 hr, the mice were euthanized and spleens were isolated. Splenocytes were stained with VSIG4, Gr-1, F4/80 or VISTA antibodies. Stained cells were analysed by flow cytometry. The data represents the average value of triplicates. VTK indicates the activity of VivoTrack.

(Color figure can be accessed in the online version.)
Fig. 3. VSIG4-positive cells suppress T cell proliferation via NO secretion. (a) T cells were prepared from normal spleens using the T cell isolation kit. VSIG4(+) and VSIG4(-) cells were isolated from the peritoneal immune cells of normal mice using VSIG4 antibody and MACS MicroBeads. CFSE-labeled T cells were cocultured at different ratios (1:1/2 or...
1:1/4) with VSIG4(+) and VSIG4(-) cells and reacted with CD3 and CD28 antibodies. After 72 hr, cells were analysed using the flow cytometer by gating only lived cells. Cell proliferation was analysed using the flow cytometer. (b) The concentrations of NO were measured in the culture supernatants prepared in (a). Data indicates the mean ± S.E.M. obtained from three separate experiments. **p<0.01, One-way ANOVA. (c) CFSE-stained activated T cells were cocultured with VSIG4(+) cells with or without an NO inhibitor, an ROS inhibitor, or an arginase inhibitor. After 72 hr, cells were analysed using the flow cytometer by gating only lived cells. Cell proliferation was analysed using the flow cytometer. NO indicates nitric oxide, ROS indicates reactive oxygen species, and Arg indicates arginase.
Fig. 4. Effects of VISTA stimulation on peritoneal macrophages

(a) Total murine peritoneal cells were isolated from normal mice. Prepared cells were stained with VISTA and CD11b antibodies or VISTA and VSIG4 antibodies and analysed using the
flow cytometer by gating only lived cells. (b) VSIG4(+) cells were purified using VSIG4 antibody and MACS MicroBeads. Prepared cells were stimulated with hamster IgG (5 μg/ml) or VISTA (5 μg/ml) antibodies. Cells were harvested after 24 hr, and the expression levels of various genes were analysed by RT-PCR. (c) VSIG4(+) cells were reacted with hamster IgG (5 μg/ml) or VISTA (5 μg/ml) antibodies and cultured with dextran for the indicated times. Phagocytosis was estimated using the flow cytometer by gating only lived cells. The percent of VSIG4/dextran-positive cells was counted using the flow cytometer. Data indicates the mean ± S.E.M. obtained from three separate experiments. (d) Prepared VSIG4(+) cells were stimulated with hamster IgG (5 μg/ml) or VISTA (5 μg/ml) antibodies and IFN-γ (100 ng/ml) for 24 and 48 hr. The concentrations of NO were measured in the culture supernatants. Data indicates the mean ± S.E.M. obtained from three separate experiments. **p<0.01, Two-way ANOVA. ISO indicates isotype control, NT indicates no treatment, h-IgG indicates hamster IgG antibody and a-VISTA indicates VISTA antibody.
Fig. 5. Functional study using an agonistic VISTA antibody

(a) T cells were prepared from normal spleens using the T cell isolation kit. VSIG4(+) cells were isolated from the peritoneal immune cells of normal mice using VSIG4 antibody and
MACS MicroBeads. CFSE-labeled T cells were cocultured at different ratios (1:1/4 and 1:1/8) with hamster IgG- or VISTA-stimulated VSIG4(+) cells and treated with CD3 and CD28 antibodies. After 72 hr, cells analysed using the flow cytometer by gating only lived cells. Cell proliferation was analysed using the flow cytometer. (b) The concentrations of NO were estimated in the culture supernatants (48 hr) prepared in (a). Data indicates the mean ± S.E.M. obtained from three separate experiments. *p<0.05, Two-way ANOVA. (c) CFSE-stained activated T cells were cocultured with hamster IgG- or VISTA-stimulated VSIG4(+) cells (1:1/4 ratio) with or without an NO inhibitor. After 72 hr, cell proliferation was assessed using flow cytometry by gating only lived cells. NO indicates nitric oxide.