Modulation of the Gut Microbiota Alters the Tumor-Suppressive Efficacy of Tim-3 Pathway Blockade in a Bacterial Species- and Host Factor-Dependent Manner

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Research

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Abstract

Background T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3) is an immune checkpoint molecule and a potential target for anti-cancer therapy. Alterations in the tumor-suppressive efficacy of immunotherapy due to gut microbiota disturbance have been reported; however, the influence of gut microbiota on the efficacy of Tim-3 blockade is yet to be investigated. In this study, we examined whether gut microbiota manipulation altered the anti-tumor efficacy of Tim-3 blockade. The gut microbiota was manipulated by the administration of antibiotics and oral gavage of bacteria to mice.

Results Alterations in the gut microbiome were analyzed by 16S rRNA gene sequencing. Gut dysbiosis triggered by antibiotics attenuated the anti-tumor efficacy of Tim-3 blockade in both C57BL/6 and BALB/c mouse strains. Anti-tumor efficacy was restored via gut microbiota manipulation through oral gavage of fecal bacteria even as antibiotic administration continued. In the case of oral gavage of Enterococcus hirae or Lactobacillus johnsonii, the transferred bacterial species and host mouse strain were critical in determining the anti-tumor efficacy of Tim-3 blockade. Furthermore, oral bacterial gavage did not increase alpha diversity of the gut microbiota in antibiotics-treated mice but did alter microbiome composition, which was associated with restoration of anti-tumor efficacy of Tim-3 blockade.

Conclusions Our results highlight the importance of the gut microbiota in anti-cancer immunotherapy responsiveness and indicate that gut microbiota modulation may increase the efficacy of immunotherapy when concomitantly administered with antibiotics. The administered bacterial species and host factors should be considered so as to benefit from gut microbiota modulation.

Background

T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3) is an immunoregulatory protein encoded by the Hepatitis A virus cellular receptor 2 (Havcr2) gene and is an emerging target for cancer immunotherapy. It was discovered as a molecule that distinguished T helper type 1 (TH1) cells from T helper type 2 (TH2) cells [1]. However, it has been detected on the surface of exhausted CD8+ T cells and on certain innate cells such as natural killer (NK) cells, monocytes, and dendritic cells [2-4]. In T cells, Tim-3 delivers inhibitory signals when its cytoplasmic tail interacts with Lnc-tim3 instead of Bat3 [5]. Tim-3 is associated with the differentiation of T cells, leading to the formation of effector T cells rather than memory T cells and has also been linked to NK cell exhaustion [6,7]. Additionally, Tim-3 impedes nucleic acid-induced activation of dendritic cells, resulting in suppression of anti-tumor immunity [8]. Increased cellular expression of Tim-3 and inverse correlation of Tim-3 expression with cancer prognosis have been observed in various cancers including hepatocellular carcinoma, B cell lymphoma and pancreatic cancer [9-11]. Blocking Tim-3 signaling decreases tumor growth in mouse models [12, 13]. Furthermore, concomitant blockade of Tim-3 and PD-1 pathways enhances tumor suppression better than blocking either pathway alone [2, 14-16]. Several mechanisms are believed to underlie the tumor-suppressive effect of Tim-3 blockade, such as decrease in regulatory T cell frequencies, functional restoration of tumor-
infiltrated T cells, increased dendritic cell recruitment to the tumor tissue, and enhanced NK cell activity [7, 13, 16-19].

The efficacy of anti-cancer therapies is influenced by gut microbiota composition [20]. Certain enteric microbial enzymes directly modulate anti-cancer nucleoside analogues: for example, enhancement of fludarabine activity by purine nucleoside phosphorylase produced by *Escherichia coli* [21]. Germ-free mice and antibiotics-treated mice exhibit diminished response to chemotherapeutic drugs, including cyclophosphamide and oxaliplatin, because their therapeutic activities depend on gut microbiota-associated T cell immune response and reactive oxygen species (ROS) production [22]. Furthermore, response to immunotherapy targeting programmed cell death-1 (PD-1) or cytotoxic T-lymphocyte antigen-4 (CTLA-4) varies with gut microbiota composition in tumor bearing mice and patients [23-25]. Moreover, manipulation of gut microbiota by oral administration of *Enterococcus hirae* enhances the efficacy of cyclophosphamide in mice [26]. However, the relationship between efficacy of Tim-3 blockade and gut microbiota composition has not been investigated. In this study, we examined whether the gut microbiota modulation influences the efficacy of Tim-3 blockade in mouse tumor models.

**Methods**

**Cell culture**

B16 melanoma cells (American Type Culture Collection (ATCC), Manassas, VA, USA) and Chinese hamster ovary-K1 (CHO, ATCC) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, ThermoFisher, Carlsbad, CA, U.S.A.) containing 10% fetal bovine serum (Carpricorn Scientific, Ebsdorfergrund, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco BRL, Dublin, Ireland). CT-26 cells (provided by Dr. Kwon, Ajou University) were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (GIBCO) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. HEK-293 T cells (provided by Dr. Kwon, Ajou University) were cultured in FreeStyle™ 293 expression medium (Gibco).

**Construction of expression vectors for Tim-3 blocking molecules**

IgV domain of Tim-3 was amplified by PCR using primers (forward primer: 5’-CGG GGT ACC GAT TGG AAA ATG CTT ATG TGT TTG AG and reverse primer: 5’-GAA TTC TGC TTT GAT GTC TAA TTT CAG TTC) and plasmid pIRES2-EGFP-Tim3SVMhIg [19]. The Tim3 V-domain DNA segment was inserted into the pSecTag2C vector (ThermoFisher Scientific, Waltham, MA, USA) containing mouse immunoglobulin (mlgG2a) CH2CH3 with and without hinge region, and named pSecTag2C-Tim3Vdlg and pSecTag2C-Tim3Vmlg, respectively.
Western blotting for detection of Tim3VdIg protein

CHO cells or HEK-293 T cells were transfected with a Tim3 expression vector using Polyethylenimine (Polyscience, PA USA). After 2 days, culture supernatant was collected and loaded on the sodium dodecyl sulfate polyacrylamide gel for electrophoresis (PAGE) or non-denatured PAGE followed by transfer onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated with anti-mouse IgG Ab conjugated with horseradish peroxidase (ZYMED® Laboratories, Invitrogen, Carlsbad, CA, USA) and then developed using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK).

Production and purification of Tim3VdIg protein

HEK-293T cells (2x10⁶/mL) were transfected with pSecTag2C-Tim-3VdIg using polyethylenimine. After 7 days, the supernatant was harvested from the culture and then Tim3VdIg protein was purified using Protein A beads (GE Healthcare, Little Chalfont, UK).

Evaluation of tumor growth

Tumor growth experiments in mice were approved by the Institutional Animal Care and Use Committee, Ajou University Medical Center (IACUC protocol #2016-0003). Six-week-old male C57BL/6 and BALB/c mice were purchased form OrientBio (Gyeonggido, Korea). Mice were maintained in specific pathogen free conditions and in separate cages according to strain and treatment. Antibiotics mixture containing 900 mg/L for ampicillin (Gold Biotechnology, Saint Louis, MO, USA), 900 mg/L for neomycin (BioVision, Milpitas, CA, USA), 900 mg/L metronidazole (BioVision, Milpitas, CA, USA), and 300 mg/L for vancomycin (Gold Biotechnology, Saint Louis, MO, USA) was provided to mice via drinking water. Drinking water was replaced every third day. Three weeks later, all mice were challenged subcutaneously with 100 µL tumor cells (3x10⁶ cells/mL) which were B16 cells for C57LL/6 mice and CT-26 for BALB/c mice. Tim-3VdIg protein (60 µg/mouse) was intraperitoneally injected on every second day for 12 days after tumor challenge. Tumor progression was assessed every second day by determining tumor volume using the formula: tumor volume = 0.523 x tumor length x (tumor width)².

Oral administration of bacteria to mice

Fecal bacteria stock was prepared by collecting feces from the large intestine of eight-week-old BALB/c and C57BL/6 mice under anaerobic condition. Feces were suspended in phosphate buffered saline (PBS) at concentration of 60 mg/mL and followed by centrifugation at 800 × g for 3 min. The supernatant was aliquoted for storage at -70°C until it was administered orally at an amount of 100 µL per mouse seven
times in total, once every third day starting seven days before tumor challenge to a mouse. *Lactobacillus johnsonii* (Korean Culture Center of Microorganisms) and *Enterococcus hirae* (Korean Culture Center of Microorganisms) were cultured at 37°C in Brain Heart Infusion media and De Man, Rogosa, and Sharpe (MRS) agar, respectively. The bacteria were cultured to an OD of 1.8 measured at 600 nm (corresponding to $10^9$ CFU/mL) and then aliquoted and cryopreserved in 15% glycerol. Each bacterial suspension (100 µL/head) was administered to a mouse seven times in total, every third day.

Collection of gut microbiota samples and bacterial DNA sequencing

Mice were sacrificed on eighth day after tumor cell challenge to collect cecal content, which was immediately frozen at -70°C for microbiome analysis. DNA was extracted from cecal samples using DNeasyPowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality was assessed by gel electrophoresis and fluorometry. Sequencing libraries were constructed according to the Illumina 16S Metagenomic Sequencing Library protocols using Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA) and the universal primer pair specific for the V3-V4 region of the 16S rRNA gene with Illumina adapter overhang sequences. The purified PCR product was quantified according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Paired-end sequencing was performed with Macrogen (Seoul, Korea) using the MiSeq™ platform (Illumina, San Diego, USA).

Sequence processing and taxonomic assignment

The FLASH (Fast Length Adjustment of SHort reads, 1.2.11) program was employed to merge paired-end reads [27]. Open reference operational taxonomy unit (OTU) picking was utilized using QIIME-UCLUST and NCBI databases.

Statistics

The statistical significance of differences in tumor growth between groups was analyzed using the Student's t-test or ANOVA with Bonferroni multiple comparison test. p< 0.05 indicated statistical significance. Significant differences in alpha diversity were computed using Kruskal-Wallis test with Dunn's multiple comparison test. Significant differences in beta diversity were computed using PERMANOVA and ANOSIM. Significant differences in dispersion were determined by permDISP.

**Results**
We previously reported the tumor-suppressive effect of Tim-3 blockade using Tim-3hIg fusion protein comprising of Tim-3 variable domain (V) and mucin domain linked to the Fc region of human IgG [19]. The Tim-3 V domain is sufficient to bind to its ligands. Furthermore, Tim-3 dimers may show greater stability in interacting with its ligand than Tim-3 monomers. Thus, the Tim3VdIg fusion protein, a dimer of two identical polypeptides consisting of Tim-3 V domain and mouse IgG hinge and Fc regions was produced. We first examined the expression of Tim3VdIg in the culture media of CHO cells that were transformed with the Tim3VdIg expression vector. As Tim3VdIg included the IgG hinge region (Cys-Pro-Pro-Cys-Lys-Cys-Cys-Pro) containing cysteine residues that formed disulfide bonds between two identical Tim3Vlg fusion proteins, it was detected approximately as a 110 kDa band in native gels and as a band of 55 kDa monomer in denatured gels (Fig. 1A). To show the dimer formation clearly, Tim3Vmlg that lacked the hinge region was compared in parallel. Both Tim3VdIg and Tim3Vmlg proteins were similar in size in denaturation condition but varied in native condition. Next, we assessed the purity of Tim3VdIg produced by HEK-293T cells (Fig. 1B). Tim3VdIg was detected as a single band in SDS-PAGE. We then assessed tumor-suppressive effect of purified Tim3VdIg in C57BL/6 which were inoculated with B16 melanoma cells. Mice treated with Tim3VdIg showcased significantly decreased tumor growth as compared to control mice injected with PBS (p<0.01).

Oral administration of antibiotics to mice attenuates tumor-suppressive effect of Tim-3 blockade

To investigate the impact of gut microbiota modulation on the tumor-suppressive efficacy of Tim3VdIg, we examined tumor growth in mice administered with Tim3VdIg concomitantly with or without antibiotics, based on a report of gut microbiota disturbance by antibiotic treatment [28]. A mixture of ampicillin, neomycin, metronidazole, and vancomycin was given to mice via drinking water from three weeks before tumor challenge until the end of the experiment. Tumor growth was monitored in C57BL/6 and BALB/c mice after injection of B16 melanoma cells and CT-26 colon cancer cells, respectively (Fig. 1D to F). Given that immunotherapeutic efficacy may vary with age, we included 8-week-old (Fig. 1D) and 1-year-old C57BL/6 mice (Fig. 1E). Significant tumor suppression by Tim3VdIg treatment was observed in both 8 week old and 1 year old C57BL/6 and 8 week old BALB/c mice compared to their controls starting from day 12 (Fig. 1D and F) or 14 (Fig. 1E) (P<0.001); however, significant suppression of tumor growth by Tim3VdIg was not observed in mice treated with antibiotics except for 1 year old C57BL/6 on day 14. These results indicate that Tim3VdIg may exert tumor-suppressive effect in subjects varying in genetic background, tumor type and age, but not in subjects treated with antibiotics.

Oral administration of fecal bacteria or Enterococcus hirae restores tumor-suppressive effect of Tim-3 blockade in mice treated with antibiotics
We next analyzed the influence of gut microbiota modulation on the efficacy of Tim3VdIg to ascertain whether attenuation of the tumor-suppressive effect of Tim3VdIg in mice treated with antibiotics was a result of disturbance of gut microbiota (Fig. 2). Gut microbiota was modulated by feeding mice with fecal bacteria that were prepared from normal mouse feces, Enterococcus hirae or Lactobacillus johnsonii once every third day to antibiotics-treated mice. The tumor-suppressive effect of Tim3VdIg was consistently lost in antibiotics-treated mice. However, Tim3VdIg-induced tumor suppression was partially but significantly restored in antibiotics-treated C57BL/6 mice by oral transfer of fecal bacteria or E. hirae (Fig. 2A). In the case of C57BL/6 mice fed with L. johnsonii, tumor suppression was partially restored on days 8 and 10 (the suppression percentage was approximately 70% and 60% on days 8 and 10, respectively). However, tumor suppression was not maintained thereafter (the suppression percentage declined to the level of the group treated with antibiotics alone without bacterial feeding) (Fig. 2B). In the BALB/c group, the tumor-suppressive effect of Tim3VdIg was partially restored after oral gavage with fecal bacteria but not with E. hirae or L. johnsonii (Fig. 2C and D). These results indicate that gut microbiota modulation may affect the tumor-suppressive effect of Tim-3 blockade.

**Gut microbiota composition varies with antibiotic treatment, bacterial gavage and mouse strain**

To verify changes in gut microbiota of mice administered with antibiotics and bacteria, we analyzed microbiome of the cecum harvested on eighth day after tumor challenge. At that time point, antibiotics treatment had been continued and oral gavage of bacteria was performed five times in total. Read count ranged from 69012 to 106016 per sample. Rarefaction measurement of 12311 reads per sample shows sufficiently covered diverse microbiome (Fig. 3A). We compared alpha diversity, representing complexity of the microbiome within a sample using Chao1 and Shannon methods (Fig. 3B to E). As expected, alpha diversity was higher in groups not treated with antibiotics than in groups treated with antibiotics, although the statistical significance was not found in all comparisons. This may be due to the small sample size. Although bacterial feeding restored the tumor-suppressive efficacy of Tim3VdIg in antibiotics-treated mice, it did not increase alpha diversity significantly.

We next analyzed beta diversity using Bray-Curtis distance (Fig. 4). Principal coordinate analysis revealed the differences between samples treated with Tim3VdIg (indicated as B_T for C57BL/6 and C_T for BALB/c) and the samples treated with both Tim3VdIg and antibiotics (indicated as B_T/A for C57BL/6 and C_T/A for BALB/c). B_T and B_T/A were separated along PC2 whereas C_T and C_T/A were separated along PC1. The C57BL/6 groups administered with Tim3VdIg, antibiotics and bacteria (indicated as B_T/A/F for transfer of fecal bacteria and B_T/A/E for transfer of E. hirae) were separated from B_T and B_T/A along PC1 as well as PC2. The BALB/c samples from mice treated with Tim3VdIg, antibiotics and fecal bacteria (indicated as C_T/A/F) were separated from C_T and C_T/A along PC1, as well as from the samples from mice treated with Tim3VdIg, antibiotics and E. hirae (indicated as C_T/A/E) along PC1 and PC2. Notably, B_T/A/F, B_T/A/E and C_T/A/F, the groups showing restoration of anti-tumor efficacy of Tim-3VdIg, were clustered. Furthermore, C_T/A/E, in which tumor suppression was
not restored, was proximal to B_T/A. These results demonstrated clustering of groups according to whether tumor suppression was observed or not.

We next compared the microbiome composition at the phylum, class, and order levels (Fig. 5). One major bacterial population belonging to *Firmicutes* (phylum) *Clostridia* (class) *Clostridiales* (order) was observed in the B_T group, whereas in the C_T group, two major populations belonging to *Bacteroidetes* (phylum) *Bacteroidia* (class) *Bacteroidales* (order) and *Firmicutes* *Clostridia* *Clostridiales* were observed. In the B_T/A group of C57BL/6, and in all other groups of BALB/c (C_T/A, C_T/A/F and C_T/A/E), *Proteobacteria Gammaproteobacteria Enterobacterales* was predominant (except in C_T/A, in which *Verrucomicrobia Verrucomicrobiae Verrucomicrobiales* was most frequent). In B_T/A/F and B_T/A/E, there were two dominant populations, namely, *Proteobacteria Gammaproteobacteria Enterobacterales* and *Firmicutes Bacilli Lactobacillales*. These results indicated that antibiotics treatment altered microbiome drastically and that feeding of antibiotic-treated mice with bacteria did not fully reconstitute gut microbiota even though it restored the tumor-suppressive effect of Tim3VdIg.

We then analyzed the difference in microbiota composition at the species level between mouse group of the same strain to find species that account for the effect of microbiota modulation on the efficacy of Tim3VdIg (Table 1 and 2). The abundance of 45 and 41 bacterial species significantly varied in C57BL/6 and BALB/c groups, respectively. Two species *Beduini massiliensis*, and *Propionispira paucivorans* were potentially linked with the negative effect on anti-tumor efficacy of Tim3VdIg in C57BL/6 as they were observed in the T/A group but not in the T, T/A/F and T/A/E groups. The increased abundance of *Proteus alimentorum* and *Akkermansia muciniphila* in T/A/E or T/A/F relative to T/A indicated their potential role in the anti-tumor effect of Tim3VdIg in C57BL/6. In BALB/c mice, we could not identify any bacterial species whose modulation may be associated with the tumor-suppressive efficacy of Tim3VdIg. Finally, we compared the microbiota composition between Tim3VdIg-treated C57BL/6 and BALB/c mice, as the feeding with *E. hirae* enhanced tumor-suppressive efficacy of Tim3VdIg in C57BL/6 mice but not in BALB/c mice (Table 3). Among the 117 bacterial species, 10 species were significantly more abundant in C57BL/6 mice and 16 species in BALB/c mice, whereas 91 species were similarly abundant in these mouse strains. Taken together, these results demonstrate that gut microbiota varies with mouse strain, antibiotic treatment and oral transfer of bacteria, and that gut microbiota modulation affects tumor-suppressive efficacy of Tim-3 blockade.

**Discussion**

The Tim-3 pathway is a promising cancer immunotherapy candidate. Thus, identification of factors affecting its anti-tumor efficacy is significant. In this study, the influence of gut microbiota on tumor growth in mice injected with Tim3VdIg, a Tim-3 pathway blocking molecule, was examined and three novel findings were observed. Firstly, the anti-tumor effect of Tim3VdIg dissipated in mice treated with antibiotics. All these mice showcased gut dysbiosis. Secondly, oral transfer of bacteria restored the anti-tumor efficacy of Tim3VdIg in antibiotics-treated mice, even though their gut microbiota varied in
composition compared to mice not treated with antibiotics. Thirdly, the restorative effect of bacterial transfer on anti-tumor efficacy of Tim3Vdlg varied with bacterial species and mouse strain.

Tim-3 is an immune checkpoint molecule, such as PD-1 and CTLA-4. These molecules are targets of cancer immunotherapy. In line with our results, the impact of gut microbiota on the efficacy of PD-1 and CTLA-4 pathway blockade has been reported [23, 24]. In both mice and humans, PD-1 or CTLA-4 blockade has been associated with gut microbiota composition [24, 29]. The presence of Bifidobacterium and Bacteroides species in mouse gut microbiota has been correlated with tumor suppression by PD-1 and CTLA-4 blockade, respectively [29, 30]. In patients with non-small cell lung carcinoma or renal cell carcinoma, clinical responsiveness to PD-1 blockade has been associated with Akkermansia muciniphila and T cell response against this species [31]. Notably, A. muciniphila also showcased positive correlation with the tumor-suppressive effect of Tim3Vdlg in C57BL/6 mice in our study (Table 1). Along with A. muciniphila, Proteus alimentorum was also associated with good response to Tim3Vdlg in C57BL/6 mice (Table 1). However, our study was limited by the low sample number for microbiome analysis in the case of BALB/c mice. Thus, no bacterial species was found to be significantly correlated with the tumor-suppressive effect of Tim-3Vdl in this strain. Nevertheless, our results clearly demonstrate the influence of gut microbiota on the therapeutic efficacy of Tim-3 blockade.

Although the mice were continuously treated with antibiotics, oral gavage of bacteria successfully restored the efficacy of Tim3Vdlg. Many studies have reported that administration of antibiotics causes transient gut dysbiosis, with the duration of dysbiosis depending on the duration of antibiotics administration and frequency of antibiotics courses, as well as bacterial coverage of the antibiotics [28, 32]. However, it was difficult to find a report regarding microbiota recovery by concomitant oral administration of bacteria during a course of antibiotics. Cancer patients may have to consume antibiotics either just before or during their immunotherapy as a treatment for concomitant infection. They may also be administered as a preventive measure for medical procedures such as cystoscopy in bladder cancer patients, in order to monitor recurrence of a tumor. We evaluated the influence of oral administration of bacteria on the efficacy of Tim3Vdlg in mice concomitantly being administered antibiotics and discovered the favorable effect of bacterial administration on the efficacy of Tim3Vdlg. Although neither gut microbiota diversity or composition was fully recovered in mice by oral bacterial administration (Fig. 3 and 4), alterations in gut microbiota composition were observed (Fig. 4 and 5). Furthermore, clustering of mouse groups according to tumor suppression and oral bacterial administration during the principal coordinate analysis of the microbiome suggests that presence or absence of principal bacterial species may affect the efficacy of Tim3Vdlg more than the microbiome alpha diversity does. A recent study reported that 28% of patients with a tumor took antibiotics within 60 days before or 30 days after the first treatment of PD-1 inhibitor, and their outcomes were poor compared to patients who did not take antibiotics[31]. Our results indicate that concomitant modulation of gut microbiota with anti-tumor immunotherapy may be beneficial in such cases.

Recovery of Tim3Vdlg efficacy by oral administration of bacteria relied on transferred bacterial species and the host strain. Transfer of E. hirae but not L. johnsonii stably upregulated the efficacy of Tim-3
blockade in C57BL/6 mice. Similar to our results, *E. hirae* but not *L. johnsonii* improved the efficiency of cyclophosphamide in C57BL/6 mice pretreated with antibiotics [26]. However, we should mention that *L. johnsonii* is deficient in mice that are more susceptible to cancers and can activate NK cells. It can also lower the kynurenine-tryptophan ratio, which is associated with immune suppression in healthy humans [33-35]. *E. hirae* crosses the small intestinal epithelial barrier, migrates to peripheral lymphoid organs, and upregulates the cytotoxic/regulatory T cell ratio in tumor tissues [26]. Additionally, lipoteichoic acid from *E. hirae* elicits release of several cytokines including tumor necrosis factor-α in mice [36]. Furthermore, *E. hirae*-specific T cell responses are correlated with favorable outcomes in cancer patients [37]. Contrary to data obtained from C57BL/6 mice, BALB/c mice did not benefit from administration of *E. hirae*. Notably, administration of their own fecal bacteria induced a greater restoration of the anti-tumor efficacy of Tim3VdIg in C57BL/6 mice than in BALB/c mice (70-80% vs 40-50% suppression). The cause of this variation is unknown. It may be attributed to variations in intestinal response to transferred bacteria, immunologic response, and gut microbiota composition between C57BL/6 and BALB/c mice. Differential gene expression in the intestine of BALB/c after probiotic treatment compared to that in C57LL/6 [38], propensity towards TH1- and TH2- dominant response in C57BL/6 and BALB/c, respectively [39, 40], and our results showing difference in gut microbiome between C57BL/6 and BALB/c support these hypotheses (Table 3). These host factors may interact to determine the impact of gut microbiota modulation on the tumor-suppressive activity of Tim-3 blockade.

**Conclusions**

Our results emphasize the critical role of gut microbiota in cancer immunotherapy involving Tim-3 pathway blockade and indicate the beneficial effect of gut microbiota modulation on tumor suppression even during the continuous antibiotics administration. Further research will help ascertain the appropriate modulation of gut microbiota via appropriate bacterial selection, taking host factors into consideration.

**List Of Abbreviations**

CHO, Chinese hamster ovary-K1; CTLA-4, Cytotoxic T-lymphocyte antigen 4; 

*Havcr2*, Hepatitis A virus cellular receptor 2; NK, natural killer; 

PAGE, polyacrylamide gel for electrophoresis; PBS, phosphate buffered saline; 

PD-1, programmed cell death-1; ROS, Reactive oxygen species; 

TH1, T helper type 1; TH2, T helper type 2; 

Tim-3, T cell immunoglobulin and mucin domain-containing protein-3

**Declarations**
Ethics approval

This study was approved by the Institutional Animal Care and Use Committee, Ajou University Medical Center (IACUC protocol #2016-0003).

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors have no conflict of interest to declare.

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Authors’ Contributions

B Lee, J Lee, M-Y Woo and MJ Lee performed all experiments and statistical analysis of all data and produced figures. K Kim critically reviewed the manuscript. S Park conceived the study, designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Tables provided as Supplementary Files
Antibiotic administration hinders the tumor-suppressive effect of Tim3VdIg in two different tumor mouse models. (A) Tim3VdIg is expressed as a dimer linked with disulfide bonds between identical polypeptides consisting of Tim-3 V domain and mouse IgG2a hinge-Fc domain. The culture supernatants of untransfected CHO cells (Un), and CHO cells transfected with pSecTag2C (Con), pSecTag2C-Tim3VdIg or pSecTag2C-Tim3VmIg were analyzed using Western blotting in both native and denatured conditions. Tim3VmIg is devoid of the IgG2a hinge region of Tim3VdIg. (B) Tim3VdIg purified from culture media of transfected HEK-293F cells was examined using SDS-PAGE (Left) and western blotting (Right). (C) Tumor
growth in mice injected with PBS (Con, n=4) or Tim3VdIg (60 µg/head, n=4) five times, once every second day after B16 melanoma challenge (3 x 105). Tumor growth in 8-week (D) or 1-year (E) old C57BL/6 mice injected with B16 cells (3 x 105). Tumor growth in 8-week-old BALB/c mice (F) injected with CT-26 cells (3 x 105). Tim3VdIg was injected five times, once every second day after tumor challenge to the mouse in two groups, one of which was orally administered with antibiotics (T/A) and the other was not (T). Control group (Con) was treated with PBS. Data are represented as mean ± standard deviation. (n = 3 to 8 per group). **P<0.01, ***P<0.001 vs Con.

Figure 2

Bacterial gavage restores Tim3VdIg efficacy in antibiotics-treated mice depending on bacterial species and mouse strain. Tumor growth in C57BL/6 (A) and BALB/c (C) injected with B16 melanoma cells and CT-26 cells, respectively. Control group (Con, 8 and 4 heads for C57BL/6 and BALB/c, respectively) was injected with PBS and other groups were injected with Tim3VdIg five times, once every second day after tumor challenge. Antibiotics were administered orally. Bacteria were administered orally to the mice two times before and three times after tumor challenge. T: Tim3VdIg treatment alone (9 and 10 heads for C57BL/6 and BALB/c, respectively). T/A: Tim3VdIg and antibiotic treatment (10 and 9 heads for C57BL/6 and BALB/c, respectively). T/A/F: Treatment with Tim3VdIg, antibiotics and fecal bacteria (10 and 9 heads for C57BL/6 and BALB/c, respectively). T/A/E: Treatment with Tim3VdIg, antibiotics and Enterococcus hirae (7 and 6 heads for C57BL/6 and BALB/c, respectively). T/A/L: Treatment with Tim3VdIg, antibiotics and Lactobacillus johnsonii (7 and 6 heads for C57BL/6 and BALB/c, respectively). Tumor suppression (C and D) was calculated as (1 – tumor volume of each mouse relative to mean tumor volume of control group) x 100. Data represent mean ± standard deviation of two independent experiments. * P<0.05, **P<0.01, ***P<0.001 vs Con or T/A.
Figure 3

Figure 3 not provided

Figure 4
Bacterial oral administration does not increase the alpha diversity of the microbiome in antibiotics-treated mice. Microbiome was analyzed by 16S rDNA V3V4 sequencing using cecal content obtained from mice on the 8th day after tumor challenge. (A) Rarefaction measure after adjusting the read number of each sample by subsampling. (B-E) Alpha diversity determined by Chao1 or Shannon method. Each symbol represents each sample. C_T and B_T: BALB/c and C57BL/6 treated with Tim3VdIg alone, respectively; C_T/A and B_T/A: BALB/c and C57BL/6 treated with Tim3VdIg and antibiotics; C_T/A/F and B_T/A/F: BALB/c and C57BL/6 treated with Tim3VdIg, antibiotics and fecal bacteria, C_T/A/E and B_T/A/E: BALB/c and C57BL/6 treated with Tim3VdIg, antibiotics and Enterococcus hirae. Data represent mean ± standard deviation. * P<0.05, **P<0.01, ***P<0.001.

Bray-Curtis analysis

Figure 5

Beta diversity separates samples according to treatment type and mouse strain. Principal coordinate analysis of microbiome samples using Bray-Curtis distance. Microbiome was analyzed in cecal content obtained from mice on the 8th day after tumor challenge and the indicated treatment. Each symbol represents each sample. C_T and B_T: BALB/c and C57BL/6 treated with Tim3VdIg alone, respectively; C_T/A and B_T/A: BALB/c and C57BL/6 treated with Tim3VdIg and antibiotics; C_T/A/F and B_T/A/F: BALB/c and C57BL/6 treated with Tim3VdIg, antibiotics and fecal bacteria, C_T/A/E and B_T/A/E: BALB/c and C57BL/6 treated with Tim3VdIg, antibiotics and Enterococcus hirae. P = 0.001 via PERMANOVA, p = 0.013 via permDISP, R=0.813, P=0.001 via ANOSIM.
Figure 6

Relative bacterial abundance across mouse strains and treatments. Relative bacterial abundance at the phylum level (A), class level (B) and order level (C). Each bar represents each sample. T: treatment with Tim3VdIg alone; T/A: treatment with Tim3VdIg and antibiotics; T/A/F: treatment with Tim3VdIg, antibiotics and fecal bacteria; T/A/E: treatment with Tim3VdIg, antibiotics and Enterococcus hirae.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- Table2.docx
- Table3.docx
- Taxonomyabundancecount.xlsx
- OTUBLAST.xlsx
- otusrepfasta.txt