Glutathione S-transferase theta 1 modulates intestinal inflammation via interleukin-22 mediated goblet cell differentiation

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Glutathione S-transferase theta 1 modulates intestinal inflammation via interleukin-22 mediated goblet cell differentiation

Directed by Professor Jae Hee Cheon

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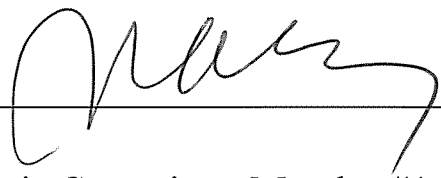
This certifies that the Master's Thesis of

Jae Hyeon Kim is approved

Thesis supervisor : Seung Won Kim



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Jae Hyeon Kim

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ABSTRACT

Glutathione S-transferase theta 1 modulates intestinal inflammation via interleukin-22 mediated goblet cell differentiation

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The glutathione S-transferases (GSTs) are a multigene family of enzymes importantly involved in detoxification, including reactive oxygen species (ROS). Furthermore, several members of GST superfamily were found to be partially associated with tyrosine kinases. In this study, I investigated the genetic association of glutathione S-transferase theta 1 (GSTT1) gene with susceptibility of intestinal Behçet's disease (intestinal BD) and elucidated its role in colitis development based on that not only oxidative stress but also cytokine receptor pathways might be key contributors to the pathogenesis of inflammatory bowel disease (IBD) and intestinal BD.

First, I found the ratio of a single nucleotide polymorphism (SNP) rs17856199 was observed to be significantly different in intestinal BD patients using genome-wide association study (GWAS). For phenotypic study on GSTT1 in colitis, I confirmed decreased expression of GSTT1 in tissues from IBD and intestinal BD patients than control patients, and verified the attenuation of acute colitis and goblet cell hyperplasia through gene transfer of Gstt1 to mouse colo-rectal or intra-peritoneal pathway to dextran sodium sulfate (DSS)-induced colitis model using wild-type mice with genes mixed in polyethyleneimine transfection solution-containing sterile PBS solution.

In order to determine the function of GSTT1 in colitis, I performed in vitro experiments using human colorectal adenocarcinoma cell line HT-29 and short-interfering RNA (siRNA) and found that genes essential for innate defense response such as *Mucin 2* (*MUC2)* or *Cathelicidin antimicrobial peptide* (*CAMP)*, are interestingly related with the existence of GSTT1. Another investigation using a knock-out cell line constructed by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system 9 (Cas9) gene knock-out system which is similar to rs17856199 mutation, diminished dimerization followed by the change dramatically connected to the insufficient phosphorylation of signal transducer and activator of transcription 3 (STAT3) and p38/mitogen-activated protein kinase (p38/MAPK) when stimulated by their common activator, recombinant human interleukin-22 (rhIL-22).

In conclusion, GSTT1 in intestinal epithelial cells contributes as a modulator of phosphorylation of STAT3 and p38/MAPK in goblet cells in response to IL-22 through their homo-dimerization and a specific SNP mutation rs17856199 is attributed to the chronic intestinal inflammation due to its insufficient dimerization and following decreased phosphorylation of STAT3 and p38/MAPK.

Keywords: glutathione S-transferase theta 1, intestinal Behçet's disease, inflammatory bowel disease, reactive oxygen species, interleukin 22, signal transducer and activator of transcription 3, p38/mitogen-activated protein kinase, goblet cell

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Ⅰ. INTRODUCTION

Behçet's disease (BD) is a rare chronic, inflammatory, multisystemic immunologic disorder which represents reoccurring ocular lesions, oral and genital ulcers, skin manifestations, arthritis, and vascular, neurological, and intestinal involvement.[1, 2] Multiple factors including undefined components of the environment and host genetic changes interact cooperatively to develop the disease.[1, 3] Intestinal BD (BD with intestinal involvement) is diagnosed when clinical manifestations which meet the diagnostic criteria for BD are accompanied by ulcer with typical shape found in the gastrointestinal tract.[2] Systemic BD is more frequently diagnosed in the area through the ancient silk-road extending from Far East Asia to the Middle East and the Mediterranean basin. Interestingly, intestinal BD is rarely diagnosed in Mediterranean BD patients (0–3%), while the frequency is relatively higher in BD patients in East Asia, including Korea and Japan (5–25%).[4] Currently, genome-wide association studies (GWASes) consistently

indicated that several variants which are more frequently observed in Turkish, Korean, Chinese and Japanese populations are associated with BD[1, 3, 5-7] Although numerous genetic factors that are suspected to attribute to the development of BD have been discovered, yet the exact mechanism or the correlation within the variants or with other environmental factors are remain unclear.

Inflammatory bowel disease (IBD) is a chronic and inflammatory disease that shares similar phenotypic (inflammation in the eyes, skin, and intestine), genetic risk factors such as MHC class I region, interleukin 10 (IL-10), and interleukin 23 receptor (IL23R),[8] and even the positive prognosis of tumor necrosis factor-alpha (TNF-α) blockade therapy. Additionally, considering its clinical phenotypes in intestinal manifestation and clinical courses of IBD, there may exist a common susceptibility factor between IBD and intestinal BD, though they are considered two distinct diseases. Thus, there may be genetic variants specific for intestinal BD but not for IBD however, has yet to be challenged to explore the risk factors of intestinal BD.

The involvement of the unbalanced oxidation-reduction ratio in the development of inflammatory diseases has been well documented. It has been shown that the recurrent cell injury and death, accelerated aging and age-related diseases are possibly caused by oxidative stress.[9] Since countless number of toxic agents and pathogens are introduced to the intestinal epithelia, their role as a barrier is considered as a key for the intestinal innate defense response. Surplus production of oxidative molecules can induce inflammation, injury, damage to the microtubule cytoskeleton, and cause death in intestinal epithelial cells (IEC) and cause the abnormality of the normal mucosal barrier fuction.[10, 11] Thus, it is now strongly suggested that oxidative stress might be a chief contributor to the pathogenesis of IBD.[11]

An enzyme family that is known to play an essential role within cells in conjugation

with and detoxification of oxidized molecules, glutathione S-transferases (GSTs), is discussed as a core environmental risk factor for the development of Crohn's disease.[12, 13] This ubiquitously synthesized enzyme family maintains their own expression levels highly in a few organs such as the liver, lung, gonads, and intestine, and uses their own electrophilic sulfhydryl groups to conjugate reduced glutathione and on the other hand to reduce a wide variety of substrates, which are useful in the detoxification of endogenous ROS. Production of oxidants and free radicals can also facilitate activation of signaling events that mediate expression of inflammatory genes as well as genes regulating cell division, differentiation, and apoptosis.[12] GSTs can be categorized into four main classes, GST alpha (GSTA), GST mu 1 (GSTM1), GST pi 1 (GSTP1) and GST theta 1 (GSTT1). Polymorphisms in GSTs can lead to a decreased enzyme function and an inadequate detoxification of ROS might modulate the susceptibility for IBD and considered to be a common pathogenic factor in IBD.

Of these members of GSTs, especially GSTT1 and GSTM1 are recently focused for the increased susceptibility of IBD, intestinal BD, and other diseases when at least one lost its function.[12, 14-17] This IBD-related susceptibility gene is located on chromosome 22q11.2 and is found to be homozygous null for nearly 10–20% of Caucasians and even higher in Asians (16–64%).[13] GSTT1-null genotype refers the entire gene deletion of the 8 kb of genomic sequence[18, 19] The GSTT1 gene is likely to be deleted, resulting in a null genotype, thus a gene deletion (GSTT1*0 allele) is responsible for a failed protein synthesis,[20] which might reduce the ability to detoxify damaging compounds. The homozygous GSTT1*0 genotype, $GSTT1^{null}$ leads to the absence of functional GSTT1 enzyme[13, 18] and may result in increased susceptibility to a number of diseases including IBD.[21-23]

Under an inflammatory condition, oxidative stress, DNA damage, growth factors or

cytokines can lead that IECs get into senescence or apoptosis through p38 mitogen-activated protein kinase (MAPK) pathway. However in previous reports, dimerization of normal functional GSTT1 enzymes in the presence of oxidative stress is known to activate p38/MAPK-activated protein kinase 2 (p38/MK2) heterodimer and to enhance phosphorylation cascade heading for apoptosis.[19] Recently, the dimerization was reported to occur at the R76-Y85 hydrogen bond and R76-D96 salt bond near glutathione (GSH)-binding cleft near phenylalaine 45 (F45).[24] One study revealed that the mutation on the dimerization domain could cause destabilization of GSTT1 in a deleterious ratio.[25] Further molecular study showed that two GSTT1s are attracted together and bind one oxidized GSH (GSSG) on each side. It has been also revealed that mutations with the null function mutation such as R76S destabilize homo-dimerization of GSTT1. Another single nucleotide polymorphism is known as rs17856199 (p.F45C).[26]

Previous GWASes have reported that a GSTT1 variant was associated with the development of BD or IBD and I found that the expression and distribution of GSTT1 were altered in the colonic mucosa of intestinal BD patients (Figure 1a). Therefore, I hypothesized that the null alleles of GSTT1 may increase the risk of intestinal BD due to either the less effective removal of toxic metabolites or the decreased homo-dimerization. I assessed the genetic polymorphisms in the GSTT1 in intestinal BD patients and normal controls in a Korean population, aiming to explore the relationship between the genotype and phenotype of GSTs. It was of interest that the expression of GSTT1 increases at the early time of mouse colitis. Increased expression of GSTT1 was also found in M2 macrophages. Based on these findings, I hypothesized that the GSTT1 is also involved in restoration, protection, and survival of IECs by the function in non-enterocytes. Finally, the present study showed a significant relationship between GSTT1 and the condition of oxidative stress or interleukin-22 (IL-22).

Ⅱ. MATERIALS AND METHODS

1. Study subjects and DNA extraction

A total of 368 BD patients of Korean descent, including 203 cases of intestinal BD and 165 cases of BD without intestinal involvement, were enrolled from the Behçet's Disease Clinic of Yonsei University College of Medicine, Severance Hospital, Seoul, Korea between June 2006 and August 2013. Intestinal BD was diagnosed according to established criteria based on colonoscopic features and clinical manifestations. Only patients who were finally classified as "definite" or "probable" types were included in this study. The Institutional Review Board of Severance Hospital, Yonsei University approved this study (IRB approval number: 4-2013-0805). All patients and controls provided written informed consent and all methods were performed in accordance with the relevant guidelines and regulations. Genomic DNA was extracted from whole blood samples, using the DNA blood maxi kit from Qiagen (Santa Clara, CA, USA).

2. Genotyping and validation study

Genotyping of rs17856199 was performed using TaqMan genotyping assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and recommended quality control measures.

3. In silico analysis of SNP functions

SIFT (Sorting Intolerant From Tolerant, http://siftdna.org/www/SIFT_dbSNP.html) were used to predict the possible impact of amino acid substitution on protein function, in which "Damaging" or "Probably/Possible damaging" were considered to predict an effect on protein function.

4. Network and pathway analysis

Biological pathways were analyzed using Ingenuity Pathway Analysis (IPA, ver 23814503, http://www.ingenuity.com/) to evaluate whether these sub-networks are biologically meaningful by comparing the IPA database. Enrichment of focus genes and functional categories was also evaluated in the IPA Knowledge Base. The network score or *P* value represents the significance of focus gene enrichment.

5. Immunohistochemical staining

For immunohistochemical analysis, formalin-fixed paraffin-embedded (FFPE) tissue sections (21 normal tissues obtained from cancer patients and 29 inflamed tissues from intestinal BD patients after surgical resection) were deparaffinized, washed with distilled water, and heated in 10 mM citrate buffer (pH 6.0) for 10 min for antigen retrieval. Sections were then washed with distilled water and treated with 3% hydrogen peroxide (H_2O_2) for 5 min. After being washed with Tris-buffered saline containing 0.1% Tween-20 (TBST), sections were blocked with 2.5% normal horse serum in TBST for 1 hr and then incubated with GSTT1 (1:100, Santa Cruz, Dallas, TX, USA) diluted in 1% normal horse serum at 4℃ overnight. After being washed, sections were incubated with biotin-conjugated IgG (1:500, Vector Laboratories, Burlingame, CA, USA) and then treated with reagents from a Vecta-Elite streptavidin-peroxidase kit (Vector Laboratories) with a benzidine substrate for color development. Sections were counterstained with diluted hematoxylin and examined by light microscopy (Olympus BX41; Olympus Optical, Tokyo, Japan). To quantify gene expression, randomly selected fields were studied for each sample at 100× magnification and scored ranging from 0 to 3.

6. Cell culture, treatments, and transfection

The HT-29 cell line (Korea Cell Line Bank, Seoul, South Korea) was maintained at 37℃ in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere of 5%

CO_2. Cells were incubated with lipopolysaccharide (LPS, 1 μg/mL), H_2O_2 (500 nM), TNF-α (40 ng/mL), or recombinant human IL-22 (rhIL-22, 100 ng/mL) for the indicated time. Knockdown of a specific gene was achieved by 12 hr transfection of short interfering RNA (siRNA) or non-targeting control (AccuTarget, Bioneer, Daejeon, South Korea) using Lipofectamine 2000 (Life Technologies, Rockville, MD, USA) into HT-29 cells. To assess inflammatory response, cell culture medium was replaced with medium containing LPS, H_2O_2 or rhIL-22 at 12 hr after transfection. All samples were harvested at 4 hr for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis, at 24 hr for immunostaining, and at 72 hr for ELISA after treatment. All transfections were replicated in triplicate.

7. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) for cell lines or RiboSpin™ (GeneAll, Daegu, South Korea) for tissue samples and 1–4 μg of RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. The cDNAs were mixed with SYBR Green master mix (Applied Biosystems) and pairs of primers (200 nmol of each primer, final concentration) in triplicate. PCR primers are listed in Table 1. Samples were amplified in a StepOne Plus real-time PCR system (Applied Biosystems) for 45–60 cycles using the following PCR variables: 95℃ for 30 sec, 56–61℃ for 30 sec, and 72℃ for 40 sec. Finally, quantitative analysis was performed using the relative standard curve method and the results are reported as the relative expression or fold change compared to the calibrator after normalization of the transcript level to endogenous control, *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) or *β-ACTIN* (*β-actin*).

Table 1. List of primers used for qRT-PCR

Gene	Sequence (5'–3')
	Human
GSTT1	F: TCTTTTGCATAGAGACCATGACCAG R: CTCCCTACTCCAGTAACTCCCGACT
TNFA	Validated Primers (BioNeer)
MUC2	F: AGGATGACACCATCTACCTCACC R: GGTGTAGGCATCGCTCTTCTC
KLF4	F: CGGACATCAACGACGTGAG R: GACGCCTTCAGCACGAACT
CAMP	F: AGGATTGTGACTTCAAGAAGGACG R: GTTTATTTCTCAGAGCCCAGAAGC
IL8	F: CTCTTGGCAGCCTTCCTGATT R: TATGCACTGACATCTAAGTTCTTTAGCA
TLR4	F: CGGAGGCCATTATGCTATGT R: TCCCTTCCTCCTTTTCCCTA
BACTIN	F: CTCTTCCAGCCTTCCTTCCTG R: CAGCACTGTGTTGGCGTACAG
	Mouse
Gstt1	F: GTAGGTTAACATCCAGTTCTGC R: GGCACATGGCAGCATACGG
Muc2	F: GGTCCAGGGTCTGGATCACA R: GCTCAGCTCACTGCCATCTG
Klf4	F: AGAGGAGCCCAAGCCAAAGAGG R: CCACAGCCGTCCCAGTCACAGT
Cramp	F: GCACGCTGACACCACTACC R: CGGGCTATTCCCTGTCCAC
Tnfa	F: CAAAGGGAGAGTGGTCAGGT R: ATTGCACCTCAGGGAAGAGT
Il8	F: CAGAAACGTGACACTCTTCTCC R: CTGCACAATGCTCCACTGG
Il22	F: GGCCAGCCTTGCAGATAACA R: GCTGATGTGACAGGAGCTGA
bActin	F: AGTGTGACGTTGACATCCGT R: TGCTAGGAGCCAGAGCAGTA
Gapdh	Validated Primers (BioNeer)

F: forward primer, **R**: reverse primer

8. Mice, colitis model and administration

All wild-type C57BL/6 mice were purchased from OrientBio (Sungnam-si, Gyeonggi-do, South Korea). All mice were maintained under a strict 12:12 hr light:dark cycle and specific pathogen-free facility which is certified by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

For accomplishment of DSS-induced colitis induction, 8-week old mice were administered with filter-sterilized 2.5% (w/v) DSS (m.w 36,000–50,000; MP Biomedicals, Irvine, CA, USA)-supplemented drinking water for 7 days, followed by 2 days of autoclaved normal drinking water. The daily assessment was undergone to body weight and disease activity of each mice. Three factors (weight loss, stool consistency, and bleeding) were transformed to scores (0–4, for each factor) and the sum up score was considered as disease activity index (DAI; 0–12). Colons (from ilieo-cecal junction to the end) and blood were harvested at indicated time set for histology, IECs, RNA and protein extraction and serum glutathione/oxidative-glutathione (GSH/GSSG) ratio detection.

All experiments using animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC; Approval No: 2016-0275; Yonsei University Severance Hospital, Seoul, South Korea) and all methods were performed in accordance with the relevant guidelines and regulations.

9. Isolation of mouse colonic epithelial and lamina propria cells

Mice at 8–10 weeks were euthanized using CO_2 chamber and colons were harvested shortly. Isolation of colonic epithelium was operated following the previous protocol.[27] Briefly, harvested colons were cut, opened longitudinally, and sectioned in 5 mm length. Tissues were washed with iced cold 1× PBS four times and then incubated in pre-warmed 1× PBS supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) in shaking

incubator for 20 min at 37℃. Vigorous shaking or vortexing was performed after incubation and filtrated using 70-μm strainer. Flow-through was collected and washed with iced cold 1× PBS four times. Finally, harvested cells were resuspended in DMEM medium supplemented with 3% FBS without antibiotics and cultured until cells attached to the dishes. After attachment, dishes were washed gently and cells were transfected with GFP (green fluorescent protein)-containing vectors with or without *Gstt1* gene using polyethyleneimine (PEI).

To examine underlying lamina propria cells, tissues left upon the filter were digested with digestion solution supplemented with collagenase D (2 mg/mL), 1× DNase and 50 μM β-mercaptoethanol. Tissues were incubated in shaking incubator for 1 hr at 37℃ in digestion solution after vigorous shaking or vortexing. After incubation, the vortexed solution was filtrated with 100-μm strainer smashing yet undigested parts with syringe ends till no visible thing left. Adding 10 mL of 1× PBS washed the strainer and collected cells stuck on the filter. Flow-through was filtrated again using 100-μm strainer and cells were isolated by centrifugation. Cell pellets were resuspended in 40% Percoll thoroughly and 80% Percoll was put under very slowly to avoid mixing of two different gradient solutions. Cells were differentiated by centrifuging for 20 min at 600 g under brake-off state. After centrifuge, using transfer pipette, interphase was extracted and washed with 1× PBS. Pellets were resuspended in FACS buffer and flow cytometry was performed to examine the composition of GFP-positive cells.

10. Gene transfer model

To figure out the effect of GSTT1, GFP-tagged mouse GSTT1-containing cytomegalovirus (CMV) vector (pCMV-Gstt1-GFP, OriGene, Rockville, MD, USA) or pEGFP-N1 (pCMV-(AC)-EGFP-N1, AddGene, Cambridge, MA, USA) were transferred through intra-rectal (IR, 30 μg in 100 μL filter-sterilized PBS) or intra-peritoneal (IP, 100

μg in 1 mL filter-sterilized PBS) pathway to DSS-induced colitis model or control mice. For efficient gene transfer, transfection reagent PEI (1:1 (*w:v*, μg DNA:μL PEI) with vector DNA) were mixed with vectors. For IR injection of gene transfection solution, mice were anesthetized using Isoflurane (Ifrane liquid, MNS Korea, Goyang, Gyeonggi, South Korea) during the insertion of intra-rectal zonde and the administration. Mice were hung head-to-down position for 30 sec for the purpose of overall distribution of transfection solution.

11. Goblet cell counting

Colonic tissue sections were processed with PAS staining. Stained goblet cells were counted per crypt. Maximum 52 and minimum 20 fully conserved crypts on each section were examined and the average numbers were marked as a representative goblet cell counts of each section.

12. Bacterial culture and plasmid preparation

Competent *Escherichia coli* DH5α were mixed with pCMV vectors with or without Gstt1 gene and transformed giving heat shock. Cells were incubated on ice for 20 min and spread on Nutrient broth (BD Difco Beef Extract, BD Biosciences) agar plate, cultured in incubator overnight. After checking colonies forming, single colonies were moved to liquid broth and cultured for 8 hr in shaking incubator. 1 part out of 20 of broth was moved to larger scale of liquid broth culture flasks and incubated overnight in shaking incubator. Cells were isolated by centrifuging cultured broth and plasmids were prepared using QIAGEN Plasmid Giga kits (Qiagen), following the manufacturer's protocol. Obtained plasmid DNA was resolved in nuclease/endotoxin-free water and stored under -20℃.

13. CRISPR/Cas9 mediated genome editing

GSTT1 knock-out cells were generated using CRISPR/Cas9 technology. Guide RNAs (gRNA) were cloned in the pCRISPR-SG01 plasmid purchased from Genecopoeia (Rockville, MD, USA). Genome editing efficiency was evaluated using T7 Endonuclease I assay (ToolGen, Seoul, South Korea). GSTT1 knock-out clones were generated by single cell sorting of GFP-positive HT-29 cells co-transfected with pCRISPR-SG01 and a GFP plasmid (1:50 ratio) in 96-wells plates. After 2 weeks, the genotype of knock-out clones were verified by genomic PCR and Sanger DNA sequencing to detect the presence of out-of-frame insertions/deletions (indels) in all GSTT1.

14. SDS-PAGE and Western blotting

Protein in tissues or cells were obtained using Pierce RIPA buffer (Cat# 89900, Thermo Fisher Scientific) mixed with Halt Protease & Phosphatase Inhibitor Cocktail (100×, Cat# 78440, Thermo Fisher Scientific). Samples were quantified using bicinchoninic acid assay (BCA assay). Quantified samples were prepared with LDS sample buffer with or without β-mercaptoethanol (Sigma–Aldrich, MO, USA). Prepared samples were boiled at 58℃ for 10 min and briefly centrifuged.

Sodium dodecyl sulfate (SDS) – polyacrylamide gels divided with stacking (5%) and running (10–12%) parts were freshly made and prepared protein samples were loaded from 30 to 100 μg respectively and electrophoresis was performed in Tris-glycine SDS buffer (iNtRON Biotechnology, Sungnam, Gyeonggi, Korea). Differentiated proteins were transferred to PVDF membrane and it was blocked with TBST supplemented with 5% skimmed milk or 5% filtered-BSA. Primary antibodies (GSTT1-C_T, 1:1,000, Santa Cruz; GSTT1-N_T, 1:500, ABCAM; GFP, 1:1,000, Cell Signaling; B-ACTIN, 1:2,000, Santa Cruz; GAPDH, 1:2,000, Santa Cruz; phospho-p38, 1:1,000, Cell Signaling; phospho-c-Jun N-terminal kinases (JNK), 1:1,000, Santa Cruz; phospho-extracellular signal–regulated kinase (ERK, E-4), 1:1,000, Santa Cruz; phospho-signal transducer and

activator of transcription 3 (STAT3, 1:500, Santa Cruz) were diluted in 5% skimmed milk or 5% filtered-bovine serum albumin (BSA) and incubated overnight at 4℃, rocking. Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit, 1:2,500; anti-mouse, 1:2,500; anti-mouse IgG Fc, 1:2,500) diluted in 5% skimmed milk for 3 hr. Chemiluminescence was optimized by ECL (Supersignal West Pico Plus, Cat# 34577, Thermo Fisher Scientific) and signals were pictured using LAS 4000 mini (Fujifilm, Tokyo, Japan).

15. Flow cytometric analysis

Necrosis and apoptosis were examined by flow cytometry using Annexin V-FITC/PI staining kit (BD Biosciences, San Diego, CA, USA). GFP-positive cells in harvested mice colon were also counted by flow cytometry. Mice tissues were fully digested and total cell compositions were mixed together to be examined. The samples were analyzed with FACSverse flow cytometer (BD biosciences) with 30,000 events for necrotic-apoptotic cell counting assay or 100,000 events for mice colonic epithelium were collected and further analysis was performed under Flow Jo software

16. Statistical Analysis

The GSTT1 variant was selected based on a comparison between intestinal BD and BD without intestinal involvement using Nexus copy number version 7.0 (Biodiscovery, El Segundo, CA, USA). The association of SNP the with the disease or a subset of disease was analyzed by comparing minor allele frequencies between case and control groups. Hardy-Weinberg equilibrium (HWE) in controls were assessed by the Fisher exact test. Statistical significance was determined by chi-square test, using SAS 9.1.3 version (SAS institute Inc. Cary NC, USA). Logistic regression analysis was also used to obtain the odds ratio (OR), 95% confidence interval (CI) for OR, and corresponding *P* values between cases and controls regarding the selected SNPs under four alternative models

(dominant, recessive, codominant, and allelic). A Student's t-test and Kruskal-Wallis test were used to test for significant differences. $P < 0.05$ was considered significant between samples. A Mann-Whitney test was used to test for significant differences among immunostaining scores between groups with a $P < 0.05$ considered significant. The prognosis of intestinal BD (the cumulative probabilities of operation, admission, corticosteroid use, and immunosuppressant use after diagnosis) was analyzed using the Kaplan–Meier method, with differences determined using the log-rank test.

Ⅲ. RESULTS

1. Association of GSTT1 genetic polymorphism with susceptibility of intestinal Behçet's disease

For the case–control study, I enrolled 203 intestinal BD patients (mean age 41.8 years, S.D.±11.8), 165 BD patients without intestinal involvement (mean age 44.3 years, S.D.±12.3) and 391 healthy controls (mean age 47.2 years, S.D.±16.6). In this study, rs17856199 of GSTT1 is located in chromosome 22 (locus: 24381766). The distribution of the GSTT1 genotypes was in Hardy–Weinberg equilibrium in the healthy controls ($P > 0.05$). Table 2 shows the GSTT1 genotype (rs17856199) distribution in BD patients with intestinal involvement and without intestinal involvement in comparison with healthy controls. The frequency of the GSTT1-null genotype was significantly lower in BD patients ($P = 9.5 \times 10^{-4}$; OR 0.613; 95% CI 0.459–0.819), patients with intestinal BD ($P = 3.0 \times 10^{-4}$; OR 0.521; 95% CI 0.366–0.742), and BD without intestinal involvement ($P = 1.1 \times 10^{-1}$; OR 0.742; 95% CI 0.513–1.072) than in the healthy controls. Although no difference between BD with intestinal involvement and BD without intestinal involvement was observed ($P = 1.0 \times 10^{-1}$; OR 0.703; 95% CI 0.459-1.076, data not presented), the GSTT1-null genotype in intestinal BD patients was more rarely seen, suggesting that *GSTT1* is a new candidate gene of BD with intestinal involvement, though it requires further investigation. Interestingly, because reports showed that the variant of GSTT1 is significantly increased in BD or IBD patients in Caucasian and Asian population.

Ingenuity Pathway Analysis (IPA) based on the published data was used to identify the potential biological pathways of the genes responsible for disease susceptibility for the validated SNPs ($P < 0.05$). Notably, the pathway analyses showed that *GSTT1* has network with inflammatory molecules [TNF, peroxisome proliferator-activated receptor

alpha (PPARA), I-kappaB kinase-beta (IKBKB), NFκB inhibitor alpha (NFKBIA), inhibitor of nuclear factor kappa-B kinase subunit gamma (IKBKG), conserved helix-loop-helix ubiquitous kinase (CHUK)] and antioxidant enzymes [glutathione peroxidase, glutathione transferase, nuclear factor (erythroid-derived 2)-like 2 (NFE2L2)).

Table 2. Association between rs17856199 genotypes and Behçet's disease (BD)

Genotype	TT	null		
	n (%)	n (%)	a*P* value	OR (95% CI) †
Healthy control	201 (51.41)	190 (48.59)		
BD	233 (64.22)	135 (35.78)	9.5×10^{-4}	0.613 (0.459–0.819)
BD with intestinal involvement	136 (67.00)	67 (33.00)	3.0×10^{-4}	0.521 (0.366–0.742)
BD without intestinal involvement	97 (58.79)	68 (41.59)	1.1×10^{-1}	0.742 (0.513–1.072)

OR, odds ratio; 95% CI, 95% confidence interval; MAF, minor allele frequency. a*P* value: *P* value from logistic regression analysis adjusted for sex and age.

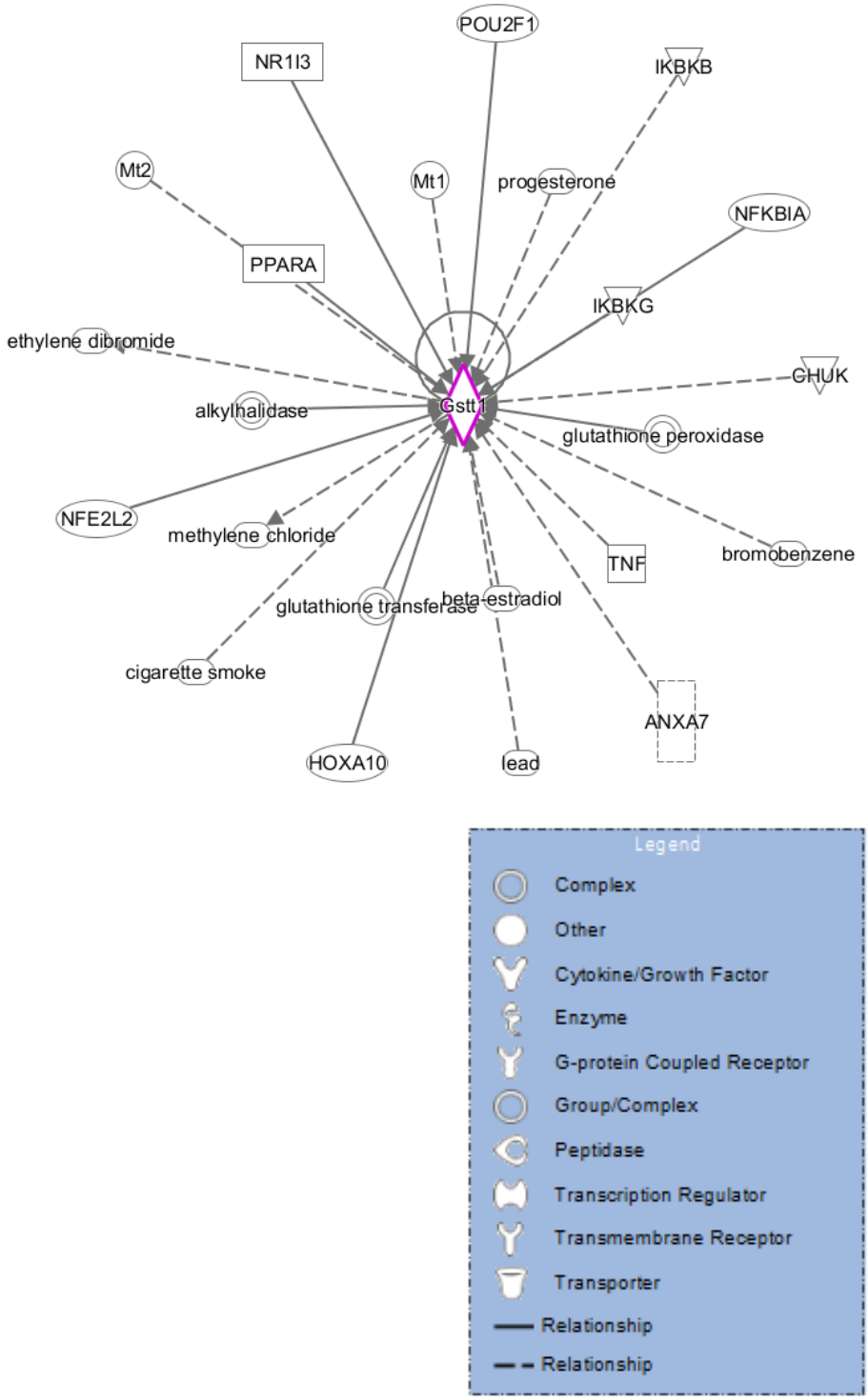


Figure 1. Network diagram of glutathione S-transferase theta 1. annexin A7, anxa7; conserved helix-loop-helix ubiquitous kinase, CHUK; homeobox protein Hox-A10, hoxa10; I-kappaB kinase-beta, IKBKB; inhibitor of nuclear factor kappa-B kinase subunit gamma, IKBKG; melatonin receptor 1, Mt1; melatonin receptor 1, Mt2; Nuclear factor (erythroid-derived 2)-like 2, NFE2L2; NFκB inhibitor alpha, NFKBIA; nuclear receptor subfamily 1, group I, member 3, NR1I3; peroxisome proliferator-activated receptor alpha, PPARA; POU class 2 homeobox 1, POU2F1; tumor necrosis factor, TNF;

2. Downregulation of GSTT1 in intestinal Behçet's disease patients

Next, I validated these associations using experimental functional studies to complement our genetic study results. In silico analysis, rs17856199 (p.F45C) in *GSTT1* on chromosome 22 was found to be highly deleterious and damaging to the protein structure in a previous study.[28] Followed by the data on RCSB protein data bank (PDB), GSTT1 protein affected by rs17856199 was found to be less stable due to the fact that it has less structural total energy (from -12,983.094 kcal/mol of native structure to -11,260.579 kcal/mol of rs17856199 structure) and further seems to require a higher force to be dimerized or interact with other molecules suggesting its increased RMSD values (Δ2.33Å).[29]

For further investigation, immunohistochemical analysis for GSTT1 was performed using colon tissues from intestinal BD or IBD patients and normal tissues from colorectal cancer patients after intestinal surgery. The protein expressions of GSTT1 were significantly decreased in the tissues of patients with IBD and intestinal BD, compared to control tissues especially in a level of epithelia (Figure 2a and b). Concordantly, the mRNA levels of *GSTT1* in the inflamed colon tissues of patients were lower than those of healthy controls, but its expression was opposed in DSS-induced colitis mice (Figure 2c and d), which inferring the possibility that the increased GSTT1 expression might have a protective role in colitis as a way of compensation for acute colitis but the genetic polymorphism and the suppressed expression of GSTT1 can be a critical factor of chronicity given to IBD or intestinal BD patients.

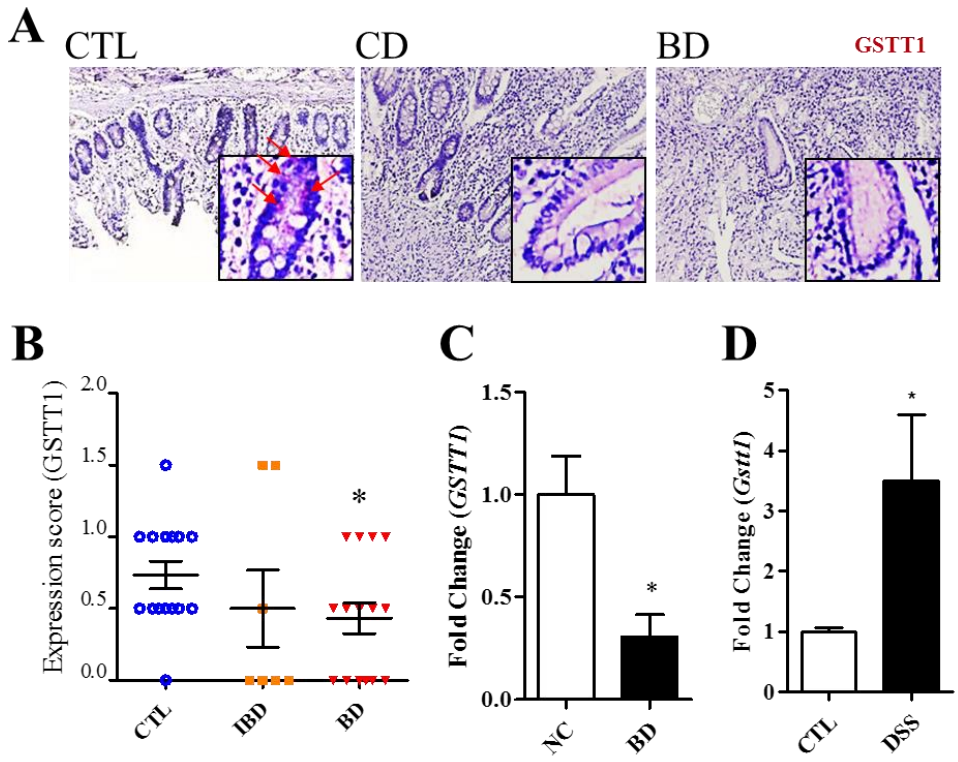


Figure 2. Gene expression in human and mouse colon tissues. (a) Immunohistochemistry of glutathione S-transferase theta 1 (GSTT1). (b) Expression scores for GSTT1 in (a). (c and d) mRNA levels in colon tissues. Transcript levels of *GSTT1* from human colon tissues from healthy controls or patients with BD (c), and *Gstt1* from mice (d) were quantified by real-time qRT-PCR. * $P < 0.05$ vs. CTL or NC. Data represent mean ± SEM; CTL (human; n = 7, mice; n = 3), BD (IHC or score; n = 8, mRNA; n = 4), CD (n = 7), NC (n = 4), DSS (n = 3). BD, Behçet's diseases; CD, Crohn's disease; NC, normal control; CTL, control; IBD, inflammatory bowel disease; DSS, dextran sodium sulfate.

3. Protective effect of GSTT1 administration in a murine colitis model

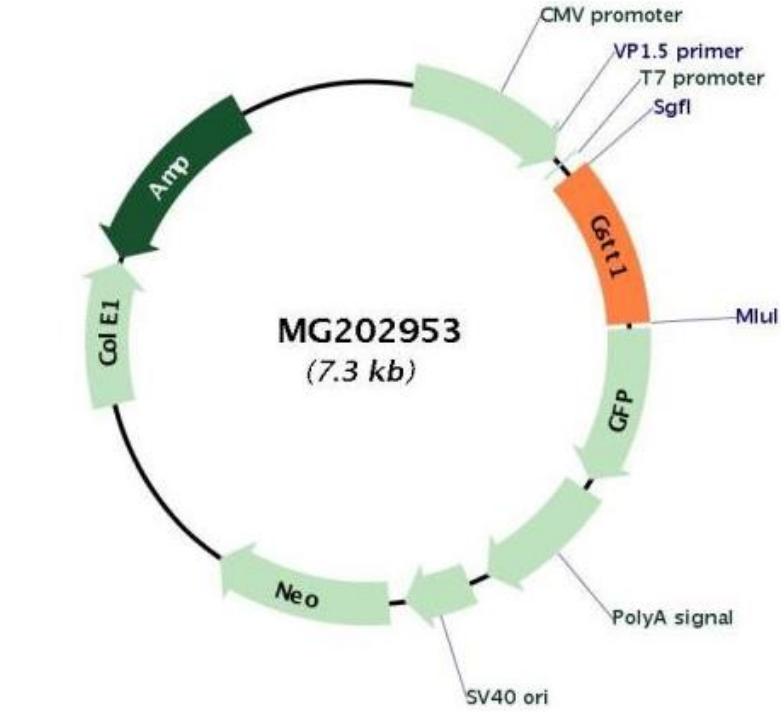
Since the results indicate that the expression of GSTT1 in inflammatory colon tissues was significantly decreased, by hypothesizing that GSTT1 helps maintaining gut homeostasis or enhances defense responses in the expression of epithelia, I examined whether additional expression of GSTT1 can make changes in colitis in a murine model. I tried a gene transfer of CMV vectors containing GFP-conjugated Gstt1 gene (pCMV-*Gstt1*-GFP; pGstt1; vector map: Figure 3) or enhanced GFP only (pCMV-(AC)-EGFP; pEGFP) in the mouse colonic epithelia directly by injecting PEI-mixed transfection solutions through intra-rectum. Since there have been few reports including direct intra-rectal vector gene transfer to colonic epithelia, before in vivo gene transfer, I checked an ex vivo transfection efficiency of pGstt1 or pEGFP to mouse IECs prepared from the mouse colon and confirmed a significant increase of Gstt1 or EGFP expression (Figure 2a).

Wild-type mice were provided with autoclaved drinking water supplemented with or without 2.5% DSS for 7 days to induce acute colitis. On days 1 and 5, freshly prepared and purified pGstt1 or pEGFP vectors were transferred to the colonic epithelia of mice by intra-rectal injection after mixing with PEI. In contrast to pEGFP-injected mice, administration of pGstt1 significantly attenuated DSS-induced colitis from day 9 as assessed by DAI or body weight change (Figure 2b). Also, harvested colons were even longer when pGstt was medicated with DSS administration than when pEGFP was treated, though histology was not significantly different between the groups (Figure 2c and d). Consequently, an assessment based on mRNA expression also showed significantly decreased *Tnfa* and *Il8* in the pGstt1 treated group (Figure 2e).

Interestingly, when mice had no induction of colitis, gene therapy of pGstt1 to mice exerted the higher number of goblet cells as tissues were treated with Periodic acid-Schiff

(PAS) staining, even though no significant difference in their histology compared to the pEGFP treated mice was detected (Figure 2f). However, there was no significant increase in a stem cell factor *Klf4* which is known to induce differentiation into goblet cells. Nevertheless, antimicrobial peptide gene *Cathelin-related antimicrobial peptide* (*Cramp)* and mucin gene *Muc2* which are known to be mainly expressed in goblet cells[30, 31] were considerably increased without the induction of colitis, suggesting a possible function of Gstt1 in terms of protection and homeostasis maintenance through regulation of goblet cell-derived protective genes (Figure 4g). Concordantly, the expression of a cytokine Il-22 that is known to induce the differentiation of the intestinal stem cells into goblet cells is found to be increased when GSTT1 was overexpressed. (Figure 4h).

This tendency of ameliorating colitis by Gstt1 gene therapy was also found when the vectors were medicated intra-peritoneally. pGstt1 treatment in mice peritoneal cavity yielded similar outcomes including lower DAI and body weight change and longer colon length (Figure 5a and b). A tendency of expressing additional goblet cells in the colon when pGstt1 was injected to mice in homeostatic condition was also observed though the histology did not show any sign of inflammation or infection (Figure 5c).



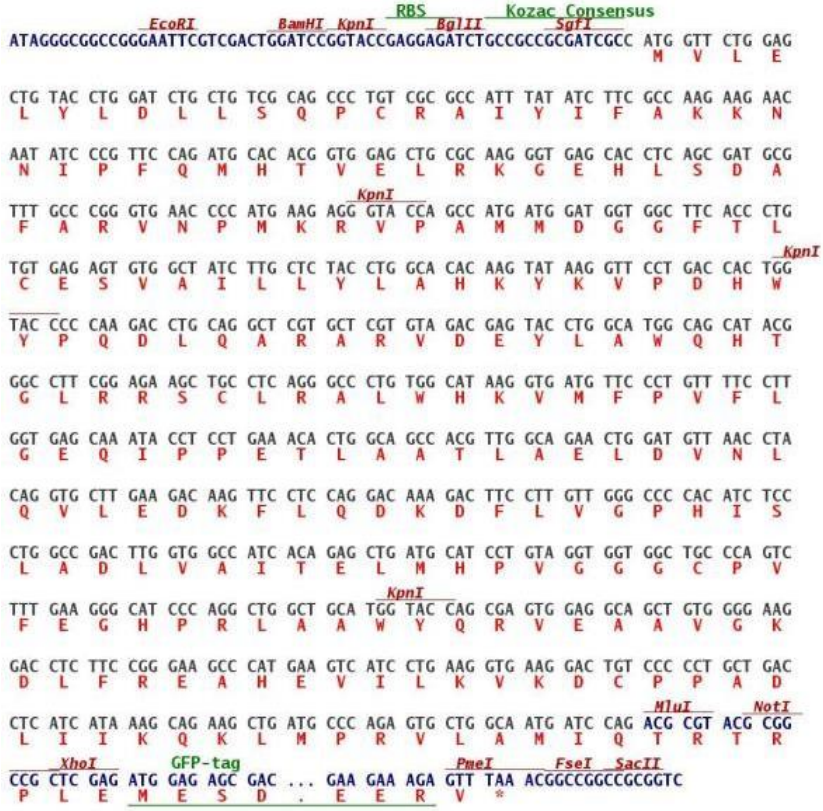
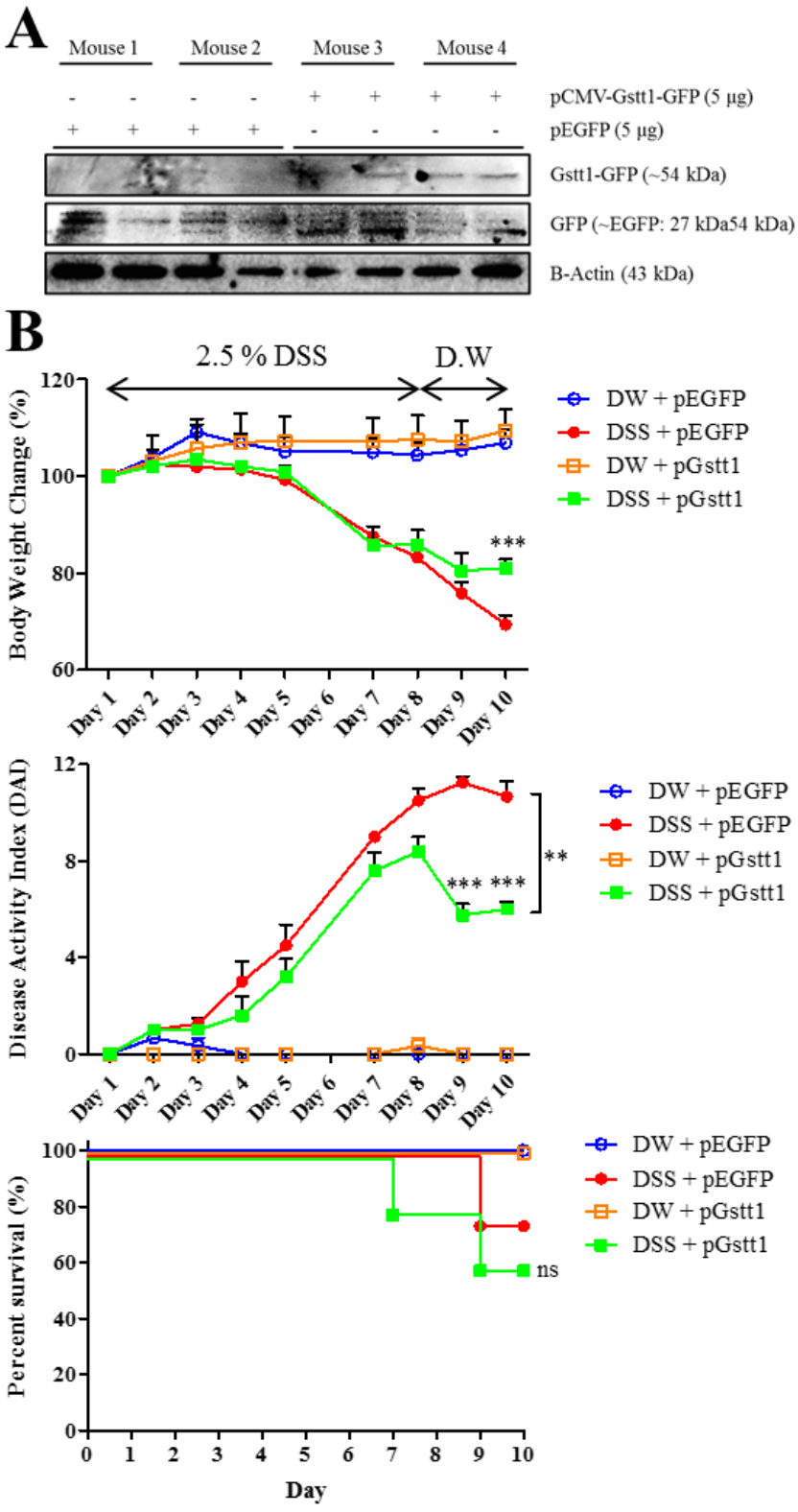
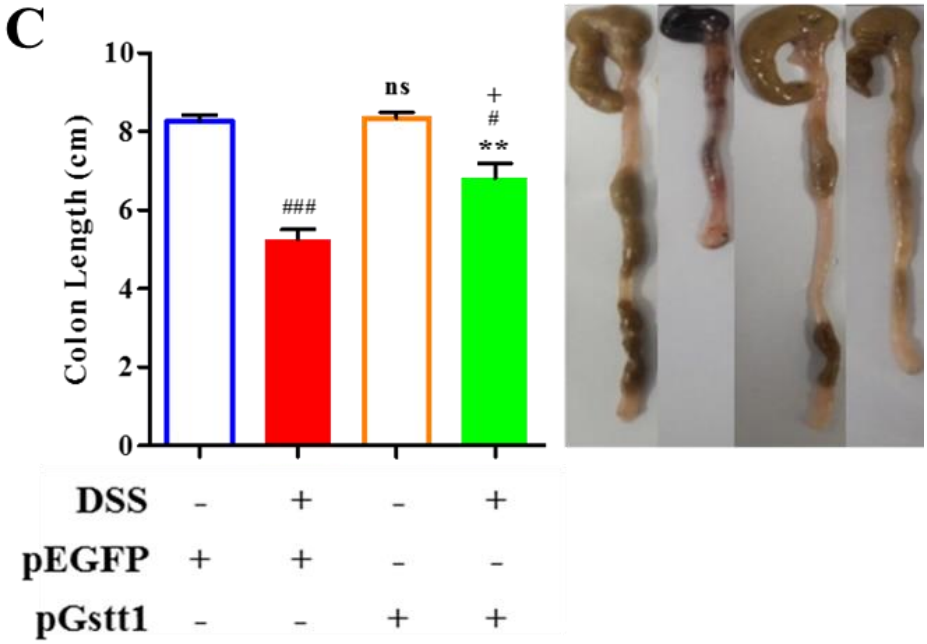
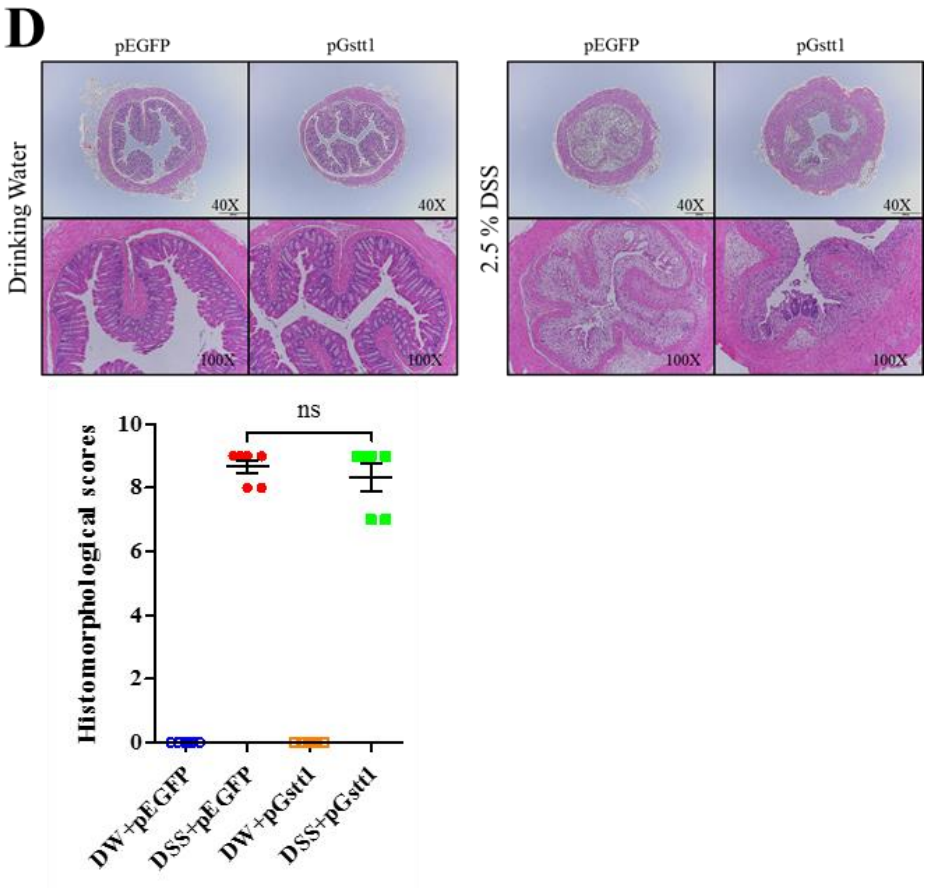
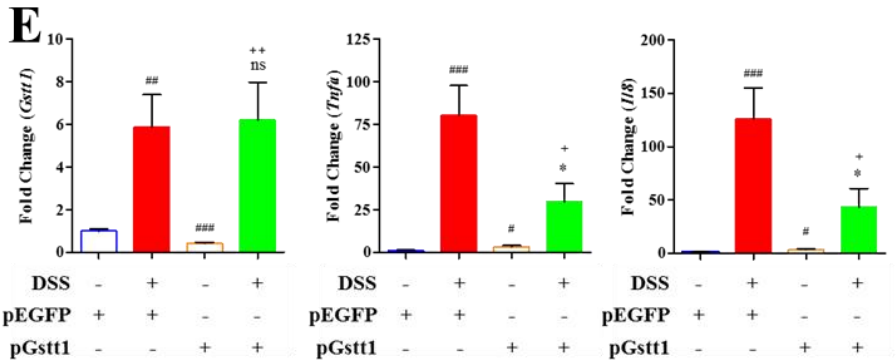


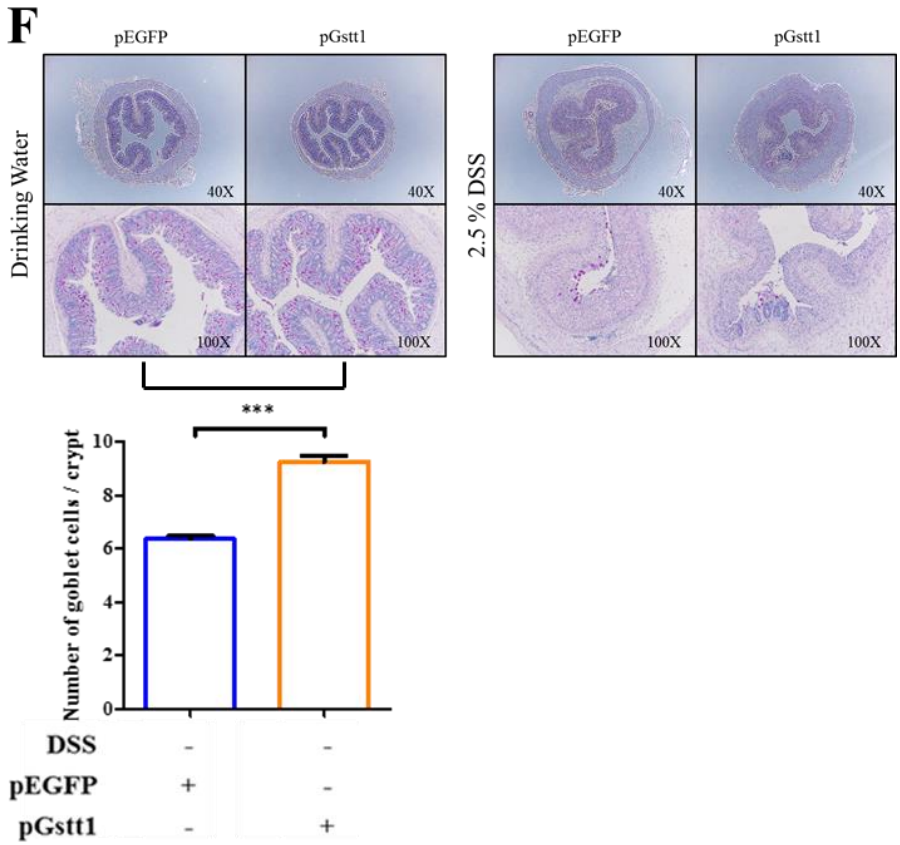
Figure 3. Vector map of pGstt1 (MG202953)

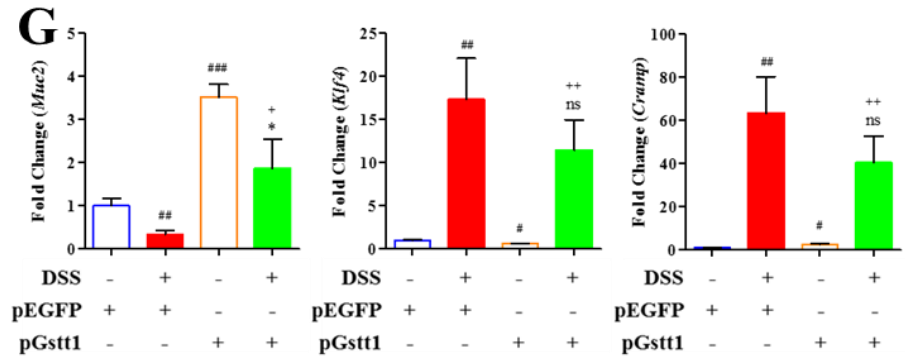












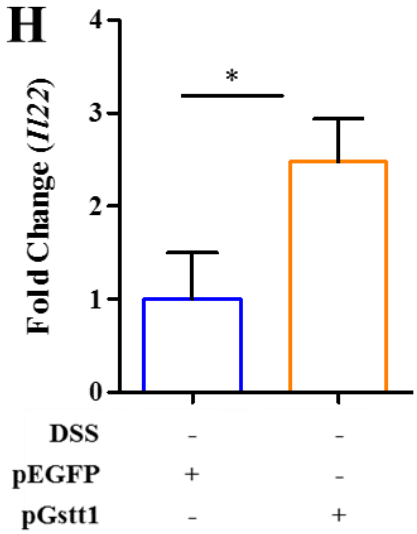
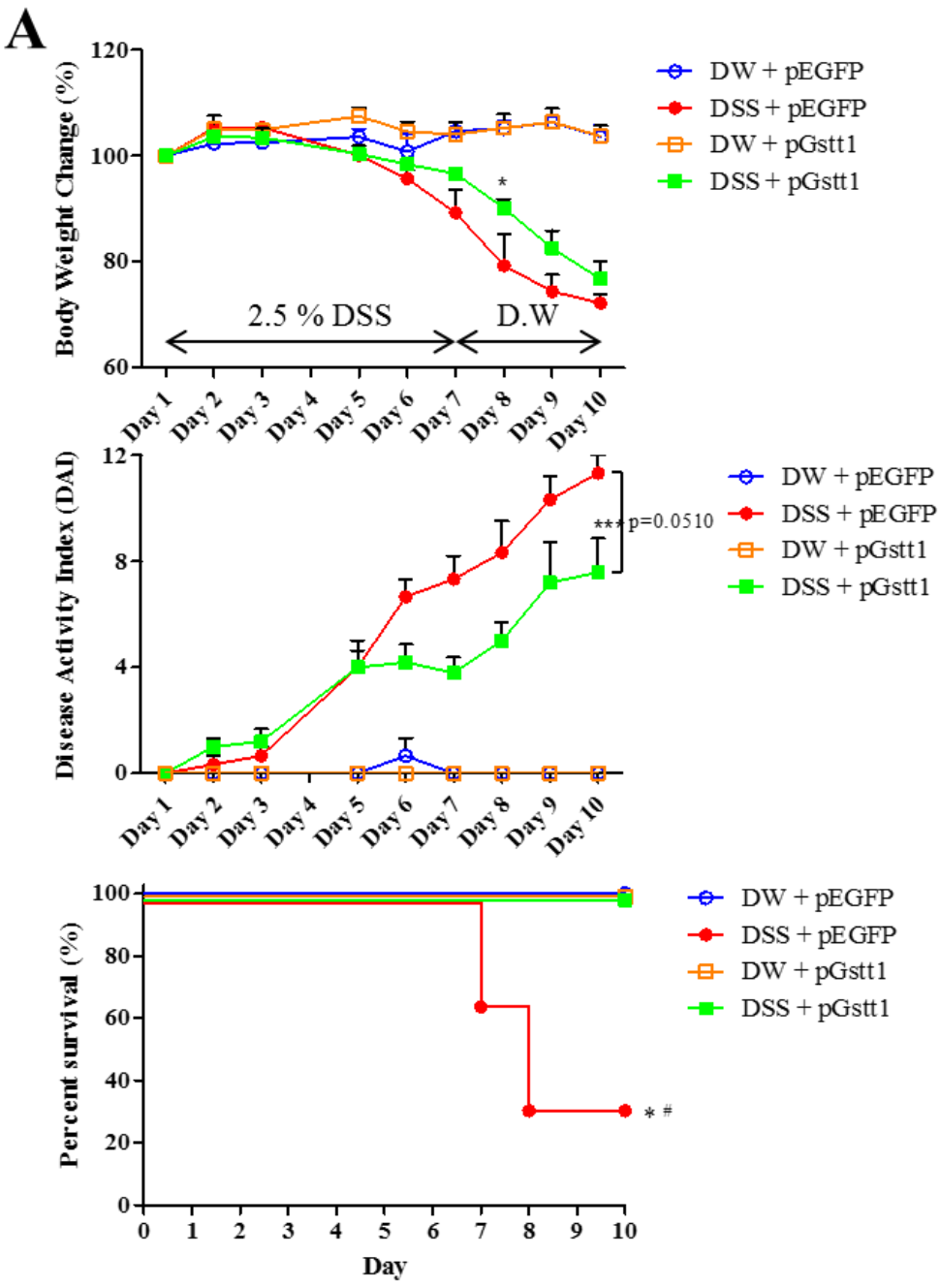


Figure 4. Attenuation of DSS-induced colitis by overexpression of glutathione S-transferase theta 1 in murine intestinal epithelial cells. (a) Protein analysis of glutathione S-transferase theta 1 (Gstt1) and green fluorescent protein (GFP) from mice colon prepared and transfected with Gstt1 containing or empty pCMV-GFP vectors. (b) Body weight change and disease activity index obtained from daily observation. (c) Length of colon of mice harvested from sacrificed mice at day 10. (d) Immunohistochemistry of colon sections obtained from sacrificed mice at day 10 and histological score. (e) Messenger RNA (mRNA) expression in the colons of colitis-induced mice. Expression levels are optimized by qRT-PCR. (f) PAS stained tissue sections pictured in 40× or 100× with the microscope and counted goblet cells. (g) mRNA expression in colon tissues of experiment mice. (h) mRNA expression of Il-22 in colon tissue of mice without DSS administration. ** $P < 0.01$, *** $P < 0.001$ vs. DSS + pEGFP, $^{\#}$ $P < 0.05$, $^{\#\#\#}$ $P < 0.001$ vs. Drinking Water + pEGFP, $^{+}$ $P < 0.05$ and $^{++}$ $P < 0.01$ vs Drinking Water + pGstt1. Data represent mean ± SEM.



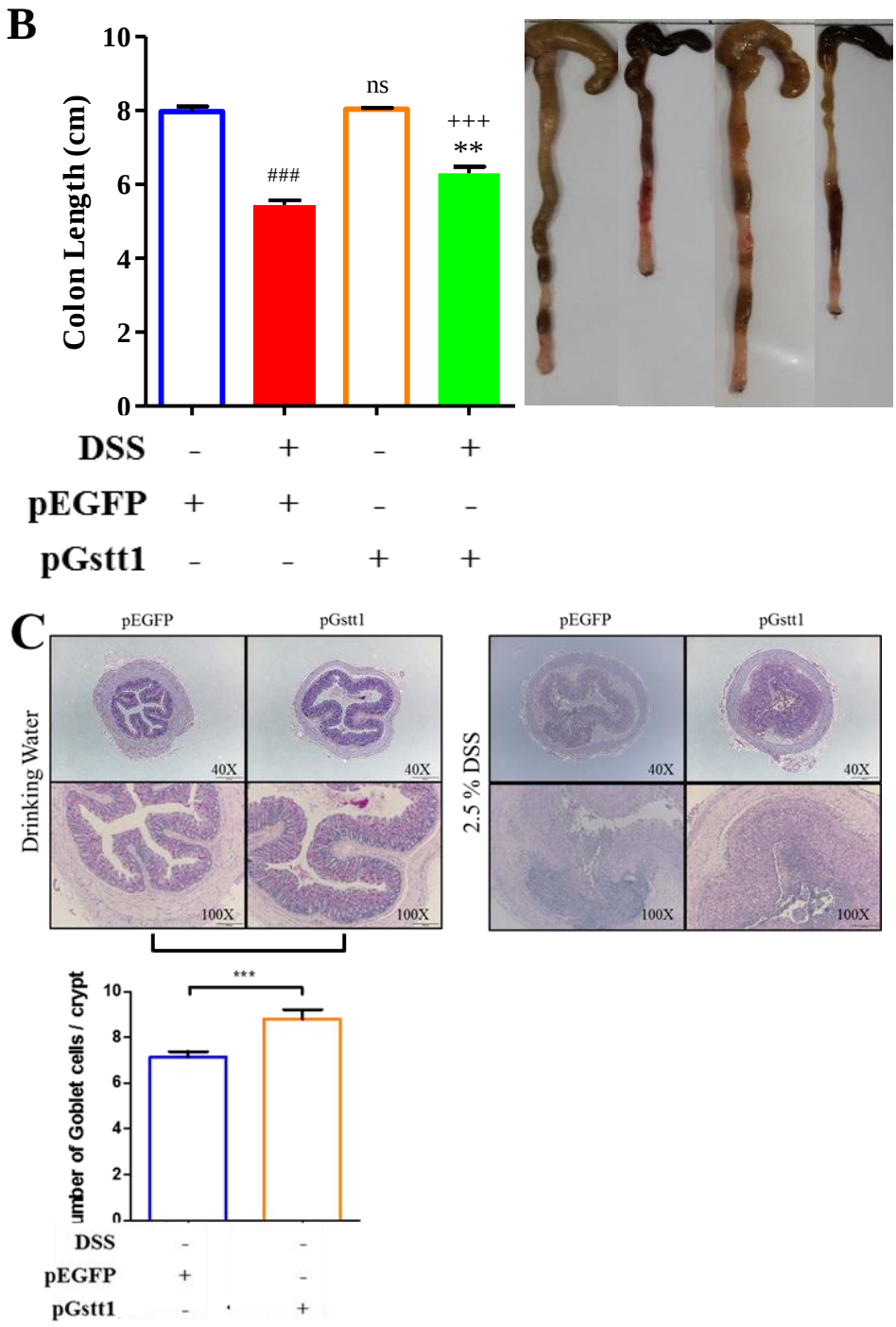


Figure 5. Attenuation of DSS-induced colitis by systemic overexpression of glutathione S-transferase theta 1 in mice. (a) Body weight change and disease activity

index obtained from daily observation. (b) Length of colon of mice harvested from sacrificed mice at day 10. (c) PAS stained tissue sections pictured in 40× or 100× with the microscope and counted goblet cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. DSS + pEGFP, $^{\#\#\#}$ $P < 0.001$ vs. Drinking Water + pEGFP, $^{+++}$ $P < 0.001$ vs Drinking Water + pGstt1. Data represent mean ± SEM.

4. Gene functional analysis using short interfering RNA gene knock-down system in human colorectal adenocarcinoma cell line HT-29

Since the data continuously show the clues that the function of GSTT1 in modulating inflammation is associated with the regulation of goblet cell activity, I further examined a functional analysis in vitro using human colorectal adenocarcinoma cell line HT-29 and a temporary gene knock-down system using siRNA. As I first assessed the GSTT1 silencing siRNA (siGSTT1), I found that the *GSTT1* increases ironically in relation to the concentration of siGSTT1 (Figure 6a). So I further observed that translated protein levels under a condition of *GSTT1* knock-down using siGSTT1 was considerably suppressed regardless of its increased mRNA level by performing western blotting (Figure 6b).

As far as known, the role of GSTT1 is not only concentrated but also largely associated with several pathways such as metabolism, tyrosine kinase or oxidation. I set three different treatment groups, ROS-treated, rhIL-22-treated, and LPS-treated in purpose of figuring out which is a major functional section that GSTT1 works in inflammatory condition. As a consistent result, GSTT1 seemed to be significantly related to the expression of mucin or anti-microbial peptides but not to pathogenic stimulus or cytokine expression. First as cells stimulated with high concentration of ROS (H_2O_2), more decreased *MUC2* transcription than scrambled-treated cells was observed. However the susceptibility against the stimulus was elevated with depleted GSTT1 function as seen in the expression of *TNFA* (Figure 3a). Similarly, stimulation of cells with rhIL–22, a cytokine that is known to increase the overall epithelial defense responses including enhancement of mucin and AMP production and goblet cell differentiation, up-regulated *MUC2* transcription, but GSTT1-silenced cells were not able to induce further expression of the gene (Figure 3b). Such result was connected to a considerably decreased *CAMP* expression, suggesting that the presence of GSTT1 is critical for colonic epithelial cells

producing mucin and secreting genes that construct and regulates homeostasis in the mucous layer.[32] However, there was no sign of association of GSTT1 with *KLF4* expression (Figure 7a and b), inferring that the observed hyperplasia of goblet cells in mice when administered with pGstt1 was obtained not by epithelial cells themselves but as a result of interaction with other cells underlying epithelium or intraepithelial cells which could stimulate epithelial stem cells through IL-22 to differentiate into goblet cells.[33]

In contrast to ROS– or rhIL-22–induced stimulation, LPS, pathogen from Gram-negative bacteria, did not make any spotlighting changes in accordance with blocked GSTT1 production. Although increased susceptibility against H_2O_2 was observed with siGSTT1 treatment, LPS treatment with diminished *GSTT1* expression did not cause the alteration of the induction of pro-inflammatory cytokines including *TNFA* or *IL8* secreted from HT-29 cells when stimulated with LPS (Figure 7c).

One interesting result was the altered expression of *GSTT1* after H_2O_2 or rhIL–22 treatment. Previously, no report has shown about the relationship with IL-22 and GSTT1, but it was found that not only oxidative stress but also a type of cytokines can change the GSTT1 expression to regulate the immune response in this study.

Conclusively, GSTT1 appears to play a role in producing Mucin 2 or CAMP in response to H_2O_2 or IL-22, but not in response to a pathogen like LPS. Furthermore, though epithelial cells are in a normal GSTT1 production state, it is insufficient itself for epithelial cells to differentiate into a professional secretory cell type, goblet cell, and it requires other types of cells.

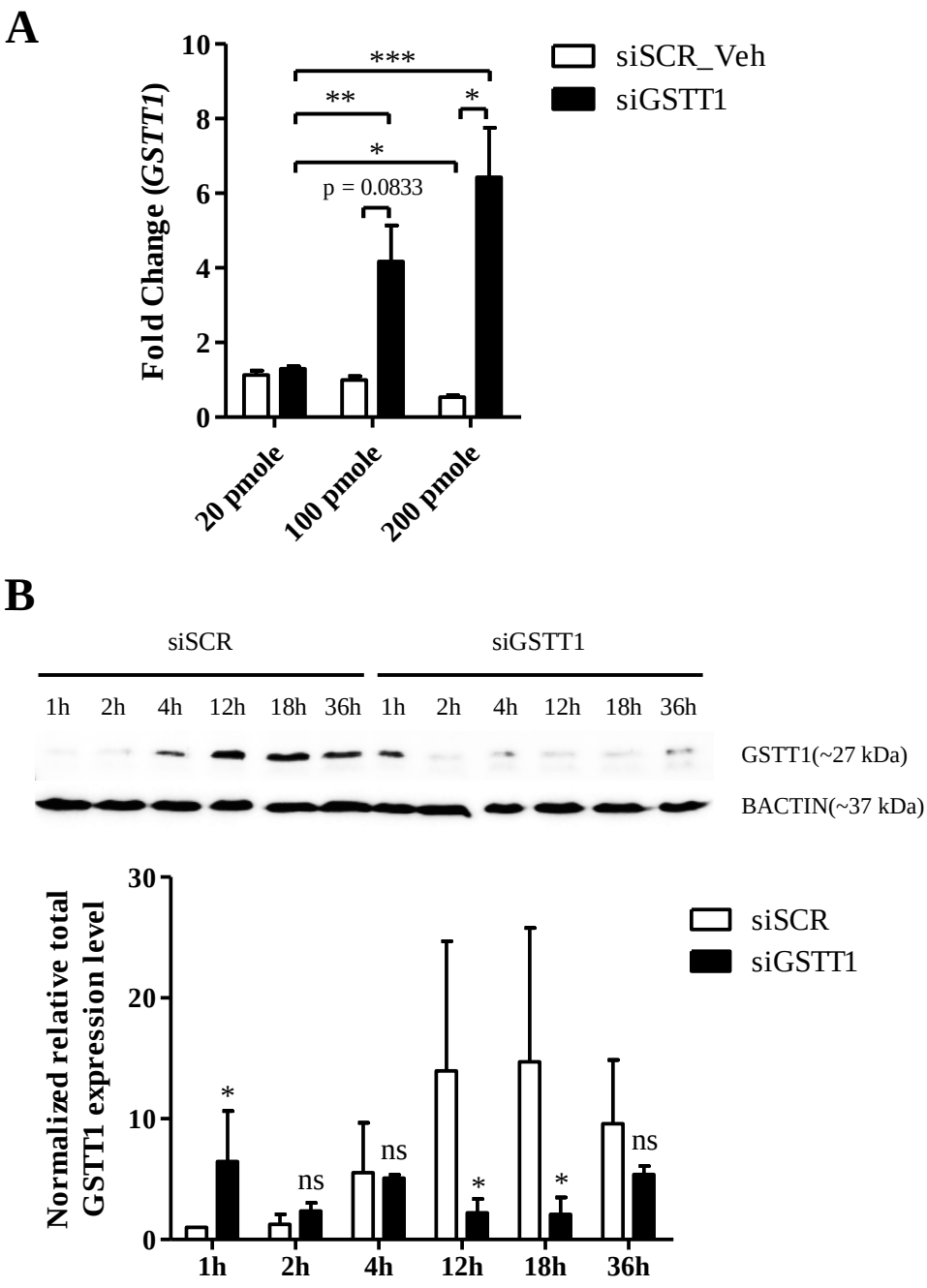
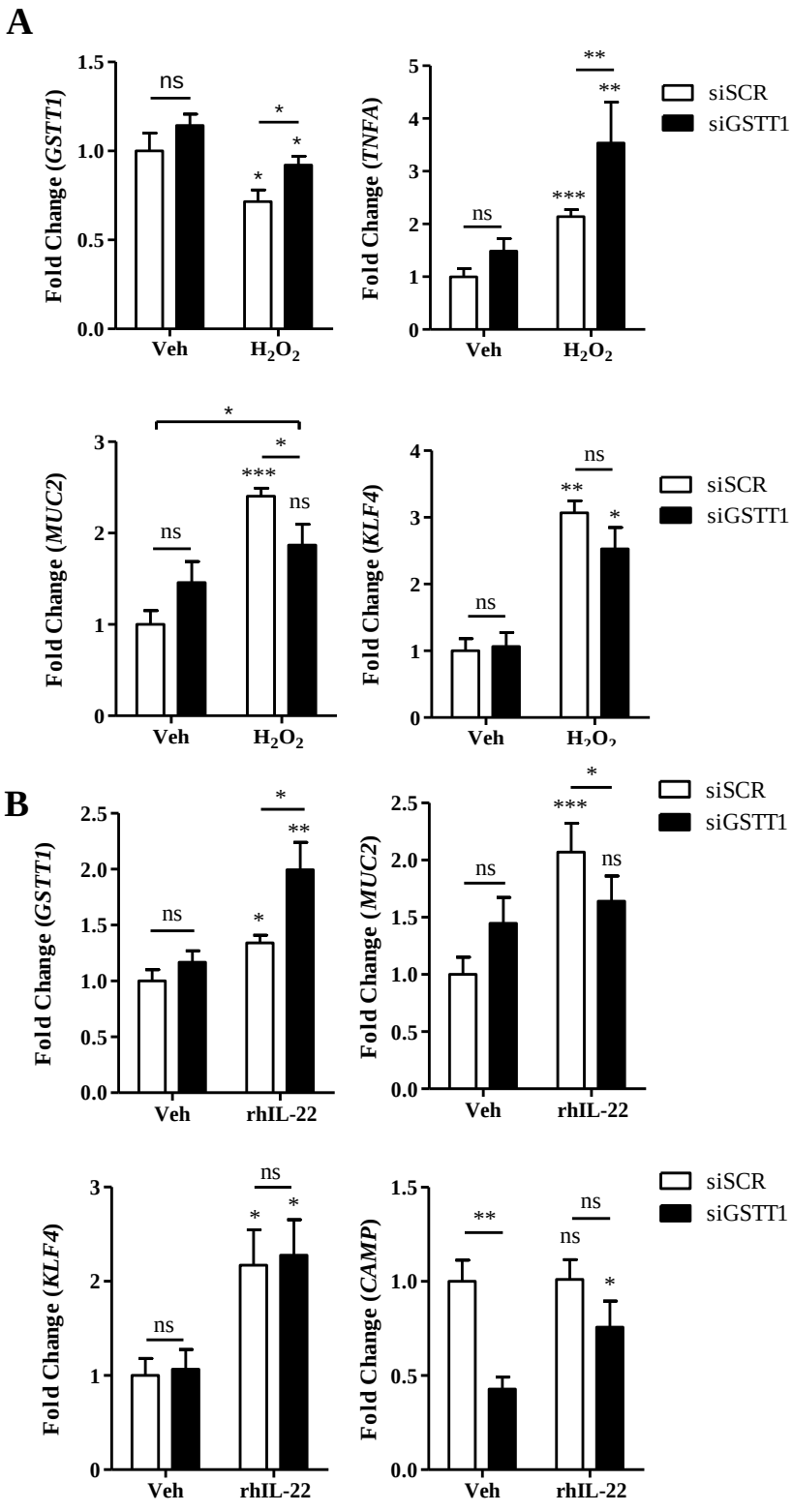


Figure 6. Confirmation of efficacy of short-interfering RNA targetingglutathione S-transferase theta 1 in human colorectal adenocarcinoma cell line HT-29. (a) Differentiated *GSTT1* level in reaction to siGSTT1 or siSCR treatment observed using

qRT-PCR. (b) GSTT1 protein expression level after stimulated with rhIL-22 when transfected with siGSTT1 or siSCR optimized by western blotting and densitometry. All measurement were normalized to the expression of β-ACTIN. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data represent mean ± SEM.



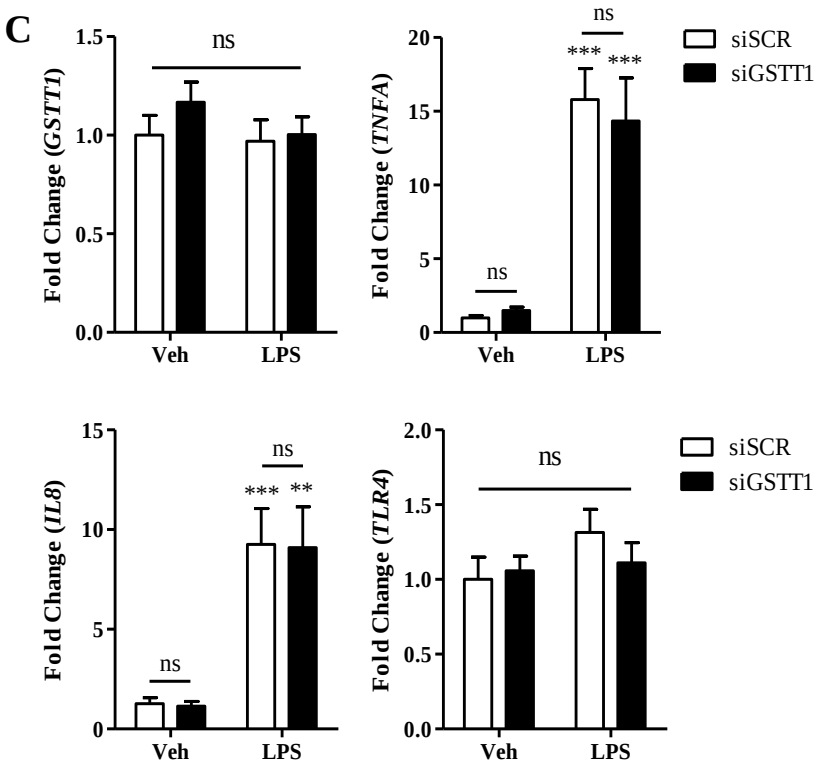


Figure 7. Altered response in human colorectal adenocarcinoma cell line HT-29 in response to H2O2 or rhIL-22 but not to lipopolysaccharide after siRNA-induced glutathione S-transferase theta 1 knock-down. mRNA expression observed by qRT-PCR after (a) H_2O_2, (b) rhIL-22 or (c) LPS treatment with or without siGSTT1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for Veh. Data represent mean ± SEM**.**

5. Gene functional analysis using CRISPR/Cas9 gene knock-out system in human colorectal adenocarcinoma cell line HT-29

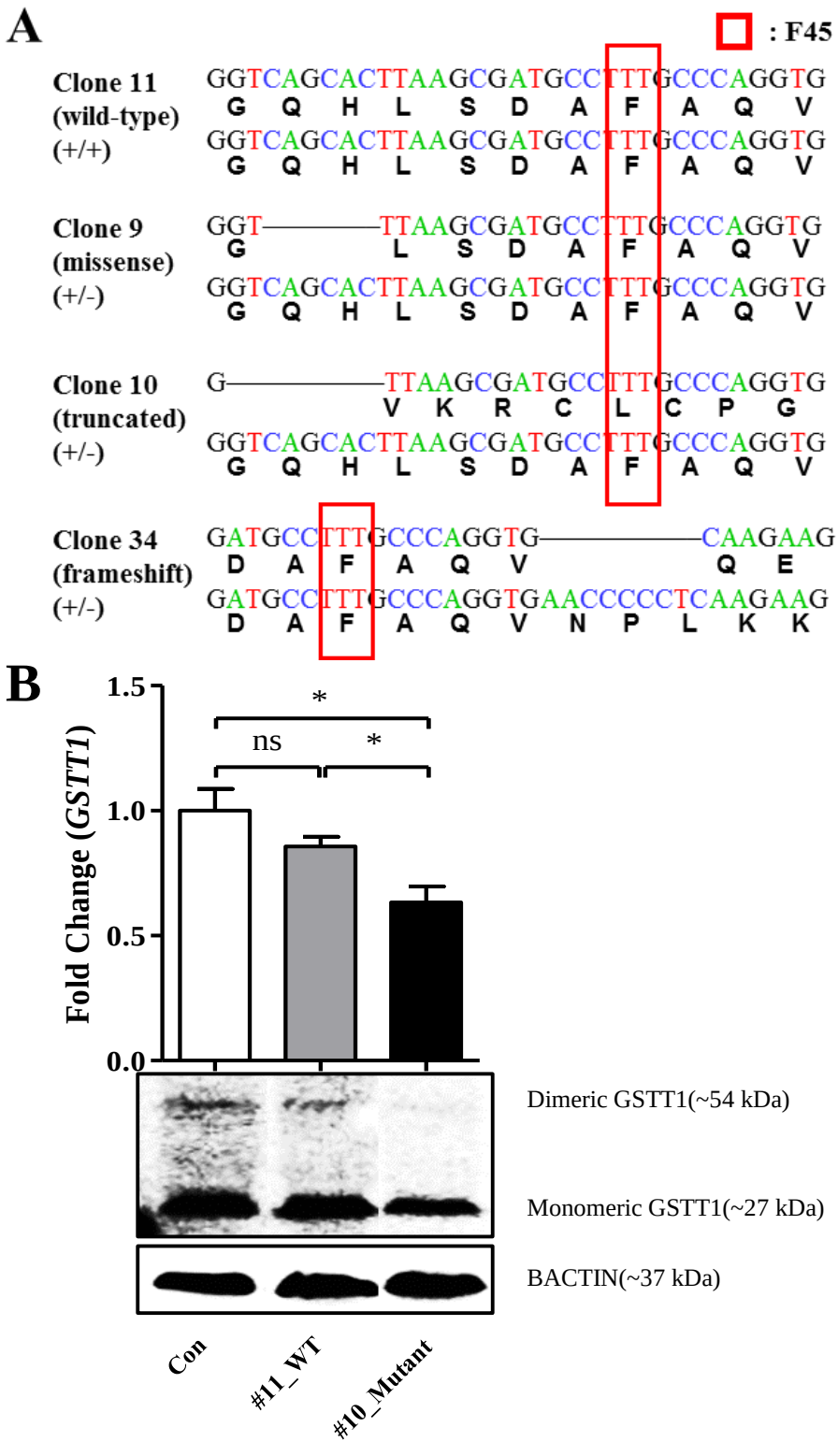
In order to figure out the underlying mechanism how GSTT1 regulates the immune response in the intestinal epithelia, I created GSTT1-mutated HT-29 cell lines using CRISPR/Cas9 technology similar to GSTT1-null genotype, rs17856199. I found three different hetero-type mutant cell lines and one wild-type cell line which were confirmed by gene sequencing (Figure 8a). With limitations accompanied with CRISPR/Cas9 knock-out system per se that non-homologous end joining did not create the exact sequence of guide RNA, no identical sequence I initially intended to produce was obtained, so I chose the most similar mutation of GSTT1 to rs17856199 (F45C), #10_mutant cell line ($GSTT1^{+/-}$).

First, I confirmed GSTT1 expression level using qRT-PCR and western blotting (Figure 8b). The mRNA *GSTT1* levels was slightly declined as one allele was mutated. Concordantly, GSTT1 protein translation was decreased more dramatically, the homo-dimer formation of GSTT1 was nearly completely diminished by single allele knock-out. Further examination through densitometry confirmed the depleted dimer formation (Figure 8c).

To figure out the phenotypic change caused by GSTT1 mutation with no F45 amino acid, I performed following experiments. First, I assessed cell death assay induced by ROS (H_2O_2), and found that GSTT1 mutant cells were more susceptible to H_2O_2 and likely to go through apoptotic phase due to the high concentration of ROS (Figure 9a), even though there was no significant difference within normal states in their morphology.

As I found in this study, GSTT1 expression was increased by interleukin-22; however, nonetheless the elevated expression of the monomeric GSTT1 in response to rhIL-22 was observed, the homo-dimerization of GSTT1 in mutant cells was still barely detected

(Figure 9b). Densitometry analysis also confirmed the eliminated GSTT1 dimerization (Figure 9b). As a result of the inhibited dimer-formation, in vitro phosphorylation analysis showed considerably down-regulated STAT3 phosphorylation and slightly decreased p38/MAPK phosphorylation (Figure 9c). Since STAT3 phosphorylation cascade and p38/MAPK confer a main signaling pathway in IECs induced by IL-22 and crucial for expressing MUC2 or AMPs such as CAMP, as hypothesized, the depletion of GSTT1 can disrupt gut homeostasis or innate defense responses in the intestinal epithelial cell line.



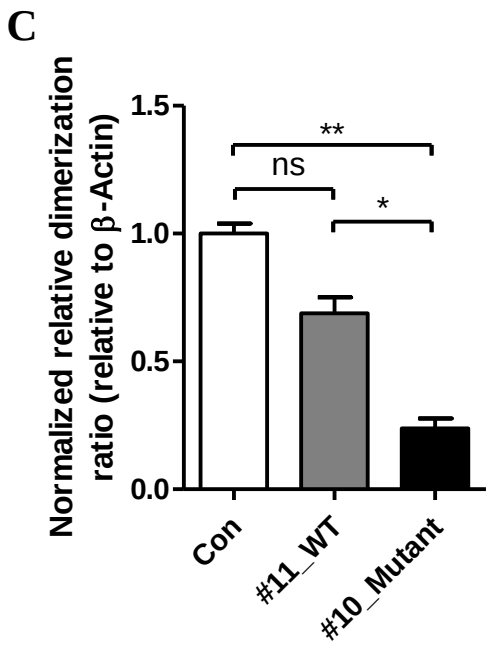
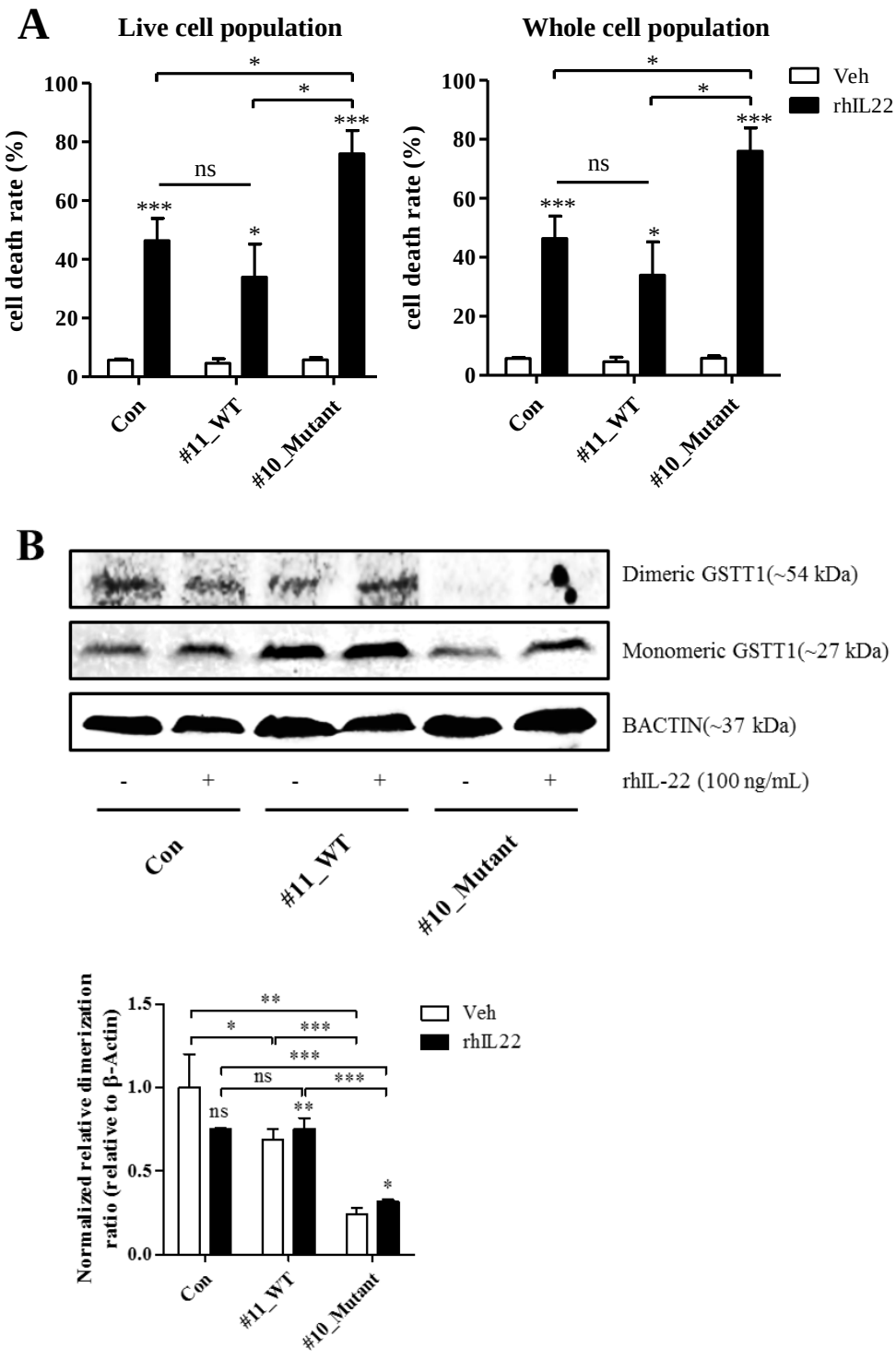


Figure 8. Glutathione S-transferase theta 1 knock-out cell lines generated using CRISPR/Cas9. (a) Schematic representation of GSTT1 locus deletion. Cells were simultaneously co-transfected with CRISPR/Cas9 plasmids targeting near the site with variation. By genomic DNA sequencing analysis of the 5'UTR-3'UTR PCR product, all GSTT1$^{-/-}$ clones were determined to be homozygous in which all mutated alleles were re-ligated exactly in correspondence with the two predicted double-strand breaks. Allelic GSTT1 deletions were identified by PCR amplification using a forward primer in the 5'UTR and a reverse primer in the 3'UTR. In this example, 3 full GSTT1$^{-/-}$ clones (red rectangles) were identified by the absence of both PCR amplification products in the 5'UTR and in the 3'UTR. (b) mRNA and protein expression of GSTT1 in knock-out cell lines. (c) Densitometry analysis of GSTT1 protein expression. * $P < 0.05$, ** $P < 0.01$. Data represent mean ± SEM.



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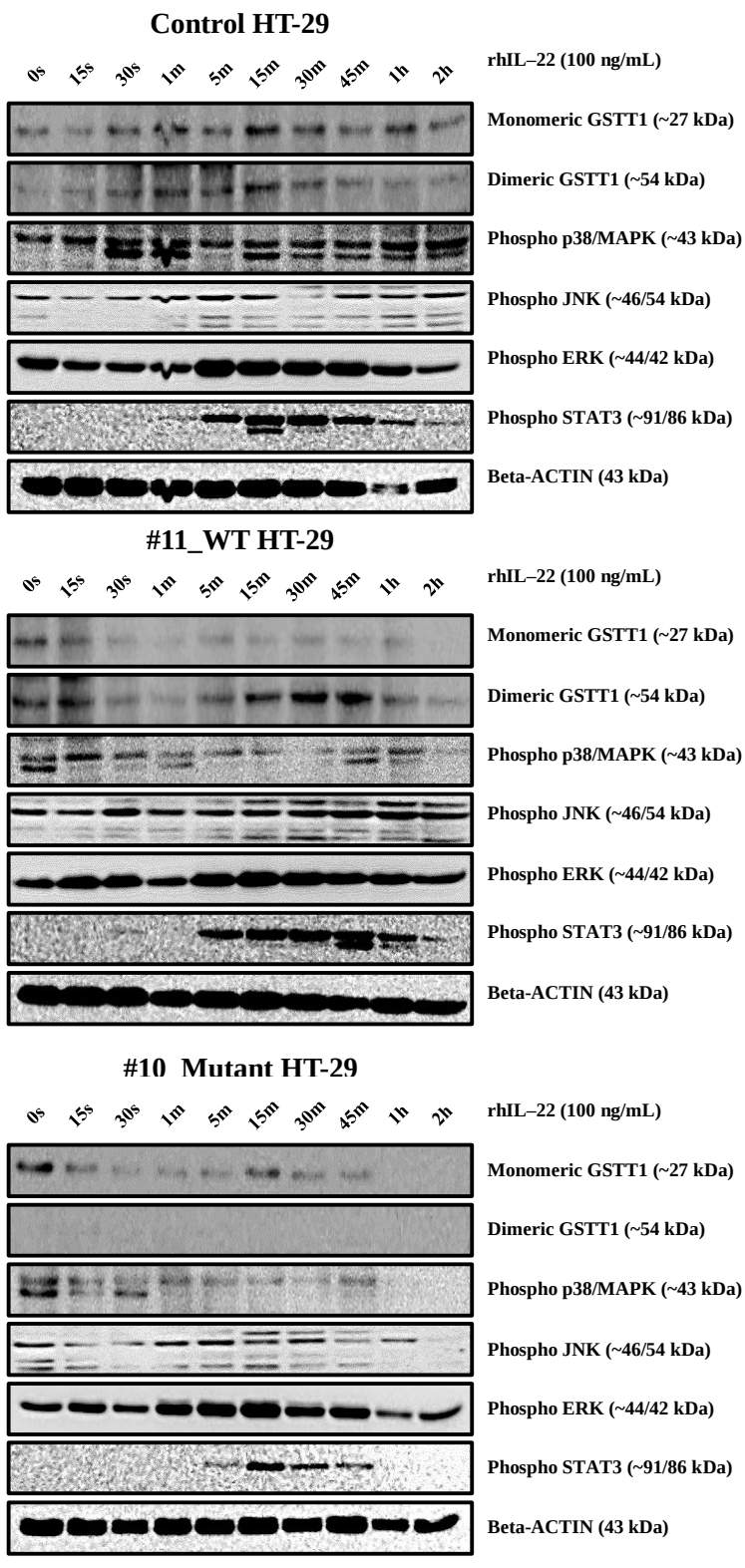

Figure 9. Phenotypic change in glutathione S-transferase theta 1 knock-out human colorectal adenocarcinoma cell line HT-29. (a) ROS-induced cell death assay using Annexin V/PI stained cell lines were analyzed using flow cytometry (b) Protein expression change in reaction of rhIL-22 and densitometry analysis. (c) Phosphorylation analysis of normal, wild-type or mutant cell lines. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for Veh. Data represent mean ± SEM.

6. Effects of GSTT1 on epithelial defense response and chemokine secretion

Followed by the results, goblet cells are suspected as a main functional type of enterocytes associated with GSTT1 in colitis and even under homeostasis. Thus I observed the expression of the genes associated with epithelial defense responses under stimulation in vitro with GSTT1-mutated cells.

As TNF-α is considered as one of the most crucial cytokines that modulate overall inflammation and its duration, I hypothesized that an inflamed condition in vitro was obtained by treating rhTNF-α to control, wild-type and mutant HT-29 cells. Examination of genes expressed in goblet cells revealed an altered expression of protective genes such as *MUC2* from GSTT1-mutant cells (Figure 10). Furthermore, the expression of *TNFA* in mutant cells has not exceeded that of wild-type or control cells. However, chemokines such as (*C-X-C motif*) *ligand 1 (CXCL1*) or *C-C motif ligand 20* (*CCL20*) showed a tendency of being less transcribed though TNF signal induces them through non-canonical pathway.[34]

To summarize, GSTT1 appears to be a regulator of epithelial immune response not only enclosed to primary defense responses or gut homeostasis such as *MUC2* or *CAMP* expression but also pathways related to chemoattraction of neutrophils.

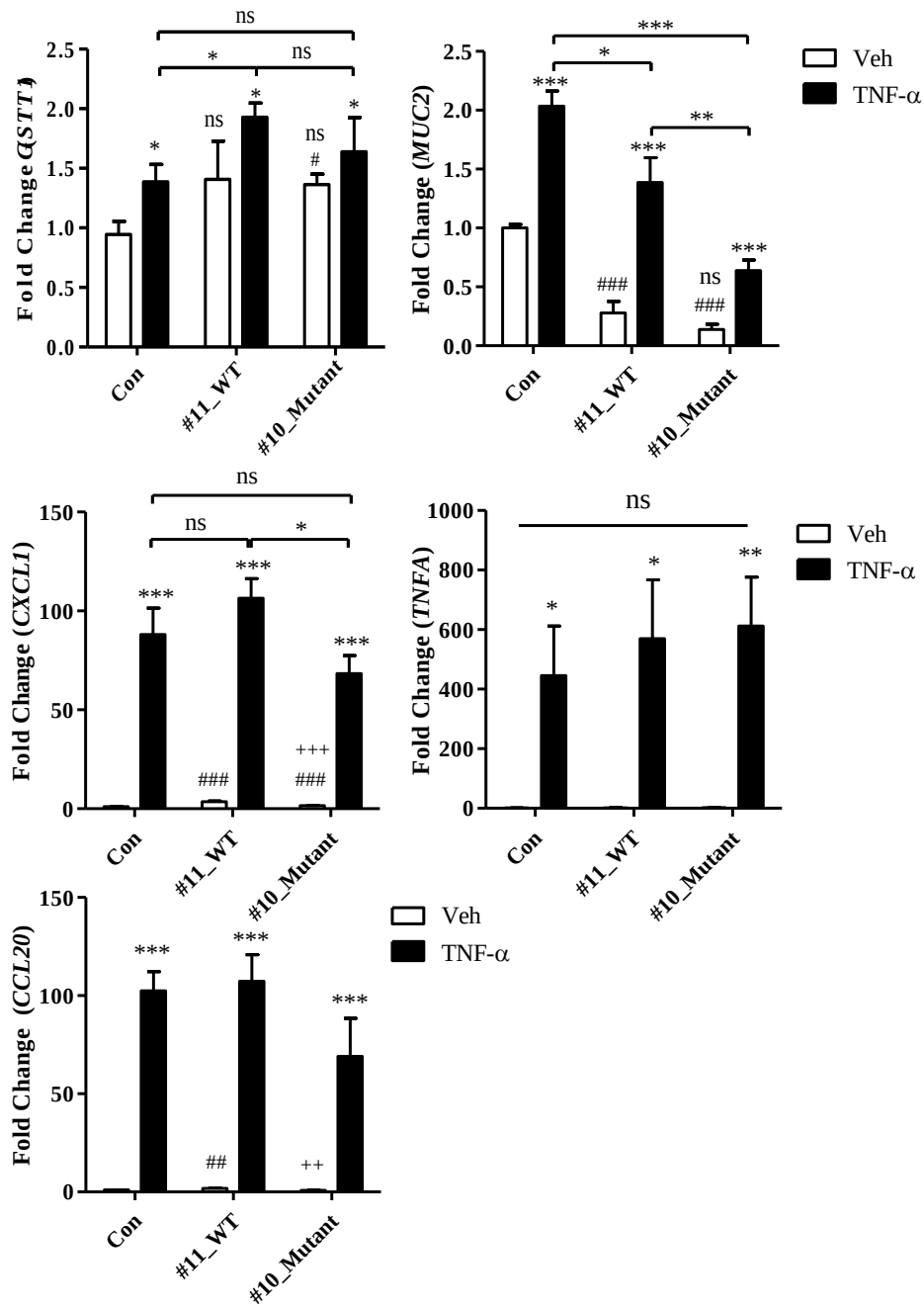

Figure 10. Altered gene expression in GSTT1-mutant HT-29 cells. mRNA expression from control, wild-type and mutant HT-29 cell lines observed using qRT-PCR under treatment of rhIL–22. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Veh, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ vs. Con_Veh, $^{++}$ $P < 0.01$, $^{+++}$ $P < 0.001$ vs. #11_WT_Veh. Data represent mean ± SEM.

IV. DISCUSSION

Although studies conducted in Asia showed a larger association between the GSTT1-null genotype and IBD than European studies, all studies revealed an increased risk with the only variation in the magnitude. Based on the published data, Korean and Japanese populations have a higher frequency of the null genotype of GSTT1. Overall, the frequency of GSTT1-null genotype showed distinct differences in Caucasians and Asians. Interestingly, the null alleles of GSTs were associated with ulcerative colitis (UC) development in China[35] and also had a positive relationship with the possibility of UC developing colorectal cancer[21]. GSTs are highly expressed in the liver, gonads, lung and colon and detoxify endogenous toxic metabolites, such as peroxidized lipids. The underlying contribution of genetic variations of *GSTT1* to intestinal BD has not yet been explored. I identified intestinal BD-specific associations of *GSTT1*. An association between genetic variation and the alteration of gene expression was also represented by altered expression levels of GSTT1 in IHC and qRT-PCR. Conversely, I found the increased *GSTT1* expression in DSS-induced colitis mice colon inferring its protective role in colitis or an existence of compensatory response related to GSTT1. Thus I hypothesized that the reduced expression of *GSTT1* caused chronicity which is shown in IBD or intestinal BD patients differentially from healthy individuals.

Animal colitis model was used to assess the role of GSTT1 in intestinal inflammation through overexpression of Gstt1 in the mice colon. As a result, enhancing Gstt1 expression in mice colon decreased *Tnfa* or *Il8*, but did not appear to attenuate colitis as found in histological scoring, though body weight was less changed and DAI score was lower on the day of sacrifice. However, I found the remarkably increased number of goblet cells in mice when administered with pGstt1 than when treated with pEGFP medicated mice. Concordantly, increased expression of *Muc2* and an upstream cytokine

which induces the intestinal stem cell differentiation into goblet cells, *IL-22* was detected.[33] However, the stem cell factor that makes the differentiation of goblet cells, *Klf4* was observed to be slightly decreased.[36] This confusing result can be explained as follows. These results were observed at the 5th day from the latest Gstt1 administration and a week-long life cycle of epithelial cells could remain the excessive number of goblet cells at the mid-to-top area of crypts on the contrary to the less counted number in deeper area of crypts on the day of detection. This hypothesis requires more examination and confirmation later. Furthermore since the source of *IL-22* is absolutely not epithelial cells but other immune cells, another systemic gene transfer animal experiment was performed and similar tendency of amelioration of colitis in DSS-induced colitis model mice was observed with pGstt1 medication, accompanied by significantly increased number of goblet cells again, indicating an unknown role of GSTT1 in immune cells to enhance goblet cell differentiation through IL-22 induction.

In order to figure out the way how GSTT1 relates in inflammation in the intestinal epithelia, I assessed in vitro assays using siRNA-induced knock-down system or CRISPR/Cas-9-mediated gene mutation study in human colorectal adenocarcinoma cell line HT-29. Since there can be several pathways that GSTT1 is involved in, I partitioned the stimulants off into three groups, LPS as a pathogen, hydrogen peroxide as a ROS, and rhIL-22 as an anti-inflammatory cytokine. In GSTT1 knock-down condition led by transfection of siRNA specifically targeting GSTT1 mRNA, stimulation with LPS did not show any change compared to the phenotype of control cells. However, the depletion of GSTT1 was strongly related to epithelial protective gene expressions such as *MUC2* or *CAMP* when treated with H_2O_2 or rhIL-22 by diminishing normal expression showed in siSCR treated cells. The insufficient expression of GSTT1 can block the general responses such as a transcription of innate response genes in reaction to H_2O_2 or rhIL-22. However, no alteration of *KLF4* expression was observed with or without GSTT1

suppression, on the contrary to my expectation. Thus a hypothesis can be exerted that the increased population of goblet cells is not by intestinal epithelium itself, but due to other immune cell types which can secrete IL-22, or induce the production of IL-22. This hypothesis will be worth to be assessed for further knowledge of the function of GSTT1 in intestinal inflammation independent of intestinal epithelium.

To support the results in my gene knock-down study, I constructed CRISPR/Cas9-induced GSTT1-mutant cell lines. Within three mutant cell lines and one wild-type cell line, the mutation site F45, a SNP site of interest was changed in only one cell line, #10, but not the exact SNP was generated. With examination using the mutant cell line and the wild-type cells, I detected several phenotypic changes. First, hypersensitivity and increased susceptibility against H_2O_2 was observed as seen in Annexin V-FITC/PI staining assay. Single strand mutated GSTT1-mutant cells were observed to be easier to go through the apoptotic state. Another study figured out that depleted functional GSTT1 diminished dimer-formation of GSTT1 and such phosphorylated molecules affected by GSTT1, STAT3 and p38/MAPK[37-42] were inhibited from being activated to neutral states as seen in control or wild-type cells. This can be connected to increased expression of *Muc2* and *Cramp* in my mice study, explaining that these are induced by the increased expression of IL-22 in the colon tissue.

Lastly, since TNF-α is a well-known and critical modulator of intestinal inflammation as far as known and an increased *TNFA* expression with the high concentration of H_2O_2 was found in vitro, I observed a broad range of responses which epithelial cells can express when exposed to rhTNF-α. Similarly, as TNF-α has the ability to induce STAT3 or p38/MAPK phosphorylation non-canonically, decreased *MUC2* expression was observed, accompanied with less induction of *CXCL1*, a chemokine which recruits neutrophils.[43] There was a study presenting the epithelium-specific knock-out of CXCL1

inhibited recruiting of neutrophils causing delayed remission from DSS-induced colitis model mice.[44] Thus less infiltration of neutrophils incurred from decreased expression of *CXCL1* can bring chronicity to colitis when orchestrated with diminished reactivity against TNF-α to transcribe protective genes after GSTT1 null functional mutation.

This is the first study to elucidate the specific genetic polymorphisms of GSTT1 contributing to the susceptibility to intestinal involvement in BD. GWAS results operated in this research separating intestinal BD to BD without intestinal involvement enabled a more comprehensive analysis of disease specificity and provide insight into the common and different pathogenic mechanisms for intestinal BD and IBD suggesting new therapeutic strategies, although further larger multi-center replicated studies and additional functional experiments are required to confirm our findings.

To sum up, through a series of studies on SNP null mutation of GSTT1 which was found to be a critical susceptibility gene based upon GWAS is intestinal BD development, I identified the immune-protective function of GSTT1 in intestinal epithelium that induce MUC2 expression when accompanied by sufficient homo-dimerization of themselves and resultant modulation of STAT3 or p38/MAPK phosphorylation and showing further possibility that the attribution of GSTT1 in immune cells to enhance IL-22 production thus driving goblet cell differentiation. (Figure 11.)

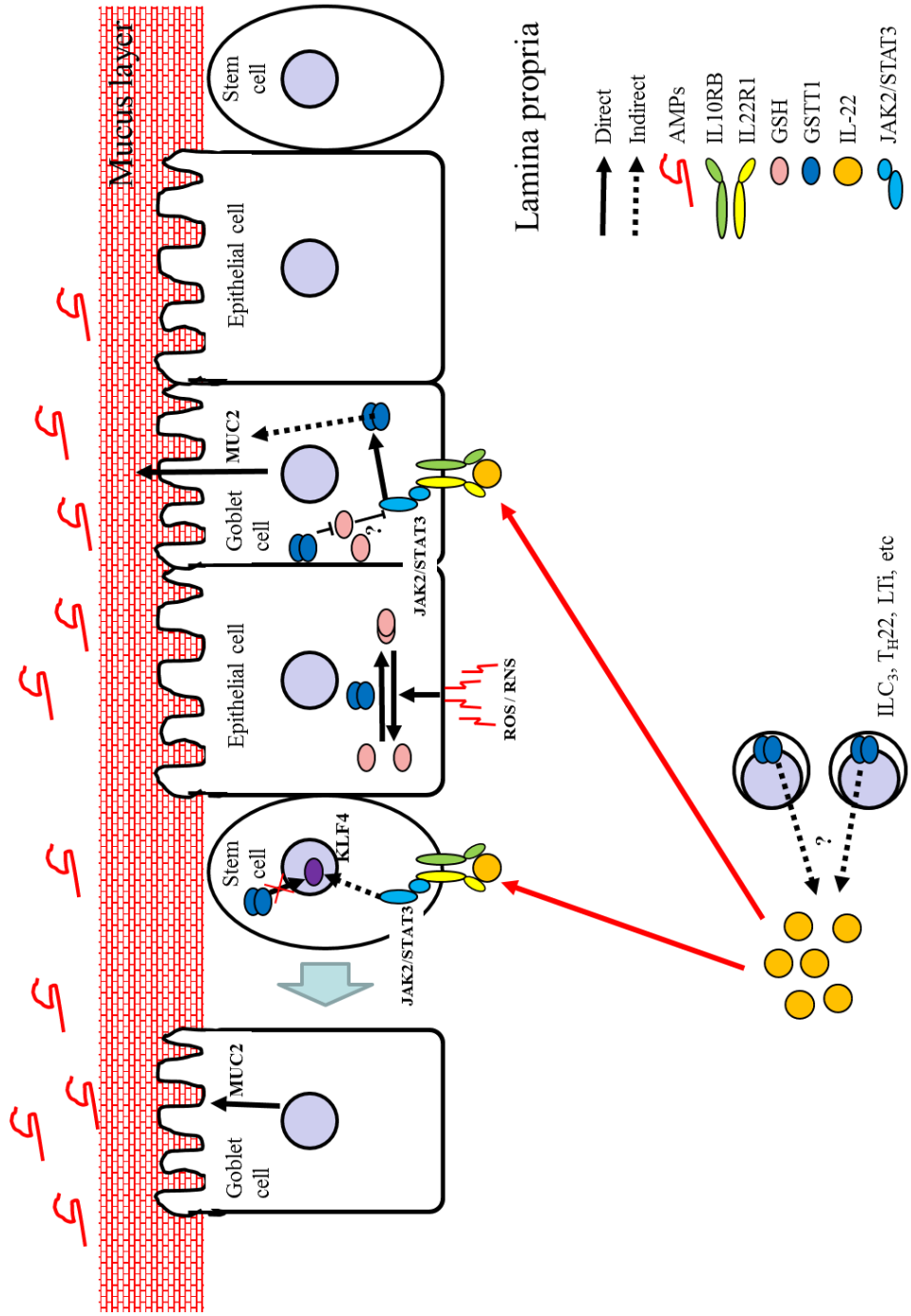

Figure 11. The expected roles of glutathione S-transferase theta 1 in the intestinal mucosa.

Ⅴ. CONCLUSION

In our GWAS exerted different frequency of single nucleotide polymorphism locus, rs17856199 upon glutathione S-transferase theta 1 (GSTT1), in Behçet's disease (BD) patients especially in individuals with intestinal involvement. I assessed a line of experiments confirming significantly decreased GSTT1 expression in the tissue of intestinal BD patients compared to normal controls, in opposite to mice study results in dextran sodium sulfate (DSS)-induced colitis model. In order to figure out the function of GSTT1 in intestinal inflammation, I performed a gene transfer assay to DSS-induced colitis model mice and found the increased number of goblet cells and the elevated *Mucin protein 2* (*Muc2*) expression with concordance of an *Interleukin-22* (*Il22*) expression. However, confusing result with *Krüppel-like factor 4* (*Klf4*) expression needs to be further assessed for the validation of the exact function of GSTT1 in colitis. Functional analysis of GSTT1 was conducted in vitro using short interfering RNA (siRNA)-induced gene knock-down analysis using human colorectal adenocarcinoma cell line HT-29. GSTT1 was found to be related only H_2O_2 or recombinant human IL-22 (rhIL-22) but not with lipopolysaccharide in accordance of intestinal inflammation. In the knock-down study, GSTT1 showed a strong positive correlation with *MUC2* or *Cathelicidin antimicrobial peptide* (*CAMP*) which are mainly expressed in goblet cells. In accordance to no alteration of *KLF4* with or without *GSTT1* interference, goblet cell hyperplasia detected in mice study was not a result of intestinal epithelial cell (IEC)-dependent overexpression of GSTT1, but due to the action of other immune cells. Using CRISPR/Cas9-mediated single strand-mutated HT-29 cell line, I detected increased susceptibility against H_2O_2 to undergo apoptotic state, which can partially explain the chronic inflammation due to cell death in inflammatory bowel disease (IBD) or intestinal BD patients. Through phosphorylation assay, I found two related phosphorylation signals, signal transducer and activator of transcription 3 (STAT3) and p38/mitogen-activated

protein kinase (p38/MAPK) were less activated in cells with the mutated GSTT1 expression which are both major downstreams of IL-22 in IECs. Considering these data, my hypothesis that GSTT1 have a function in maintaining gut homeostasis and producing defense response genes were demonstrated. At last, broad screening of responses against tumor necrosis factor-alpha (TNF-α) which is the most critical cytokine in IBD or intestinal BD was performed upon GSTT1-mutated cells and concordantly, diminished expression of *MUC2* was observed and further alteration of *(C-X-C motif) ligand 1 CXCL1* which is fundamental for neutrophil infiltration was detected.

In conclusion, GSTT1 regulates goblet cell-induced protective genes or chemokines through modulation of phosphorylation rate of STAT3 or p38/MAPK under the stimulus of IL-22 or TNF-α, and the loss of function through SNP, rs17856199 expresses less reactivity to IL-22 and causes chronicity in patients including IBD or intestinal BD.

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ABSTRACT (IN KOREAN)

Interleukin-22 에 의한 술잔세포 분화 유도를 통한 glutathione S-transferase theta 1 의 장 내 염증 조절 역할

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Glutathione S-transferases (GSTs)는 활성산소를 포함한 화학물질을 해독하는 주요한 다유전자 효소군이다. 또한, 이 효소군의 여러 일원들은 일부 tyrosine 인산화 신호전달과정에 관여하는 것으로 확인되었다. 본 논문에서는 glutathione S-transferase theta 1 (GSTT1)의 특정 돌연변이와 베체트 장염 사이의 유전적 연관성 연구를 진행하였고, 대장 염증에서 이 유전자가 갖는 역할을 활성산소 및 신호전달체계와 관련하여 연구를 진행하였다.

먼저 유전자 전장분석(genome-Wide Association Study)을 통해 베체트 장염 환자에게서 GSTT1의 돌연변이인 rs17856199의 발견이 유의하게 다른 것을 확인하였다. 대장염증에서 GSTT1 역할 규명 연구를 위해 베체트 장염 환자와 염증성 장질환 환자 장 조직에서 대조군에 비해 GSTT1 발현이 유의하게 감소되었음을 확인하였고, dextran sodium sulfate에 의한 장염 유도모델 동물에서 polyethyleneimine을 이용해 대장에 직접 혹은 전신에 GSTT1 유전자를 전달하여 과 발현 유도 시 급성대장염증 완화 및 술잔세포(goblet cell) 분화현상을 확인하였다.

In vitro 상에서 short interfering RNA (siRNA)를 이용한 유전자 발현억제를 유도, 술잔세포의 역할과 관련이 깊은 *MUC2*, *CAMP* 등의 mRNA 발현과 GSTT1이 높은 관련성이 있음을 확인하였고, CRISPR/Cas9 유전자 제거 기술을 통한 rs17856199 돌연변이와 유사한 아미노산 제거 유전자 모델을 만들어 해당 돌연변이 발생을 통해 GSTT1 이합체 형성이 억제되고, 그 결과 Signal Transducer and Activator of Transcription 3 (STAT3)와 p38/Mitogen-Activated Protein Kinase (p38/MAPK) 인산화를 감소시켜 재조합 interleukin 22 (rhIL-22)에 의한 항염증효과가 감소하는 결과를 확인하였다.

이 연구는 GSTT1이 IL-22에 의해 작동하는 술잔세포의 정상적인 방어기작을 그 수용체의 하위 신호전달물질인 STAT3와 p38/MAPK의 인산화를 통해 조절하는 주요한 조절인자로서 작용하고 있으며, 해당 유전자에서 발견되는 rs17856199 돌연변이는 GSTT1 이합체 구성 감소에 의한 염증완화 관련 전사인자들의 인산화 및 활성화 감소로 장내 염증의 만성화에 기여할 수 있음을 확인하는 최초의 연구이다.

핵심되는 말: glutathione S-transferase theta 1, 베체트장염, 염증성장질환, CRISPR/Cas9, 활성산소, interleukin-22, signal transducer and activator of transcription 3, p38/mitogen-activated protein kinase, 술잔세포