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Malt1 inactivation attenuates experimental colitis through the regulation of Th17 and Th1/17 cells

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Abstract

Objective and design Protease activity of MALT lymphoma-translocation protein 1 (Malt1) plays an important role in the development of colitis, but the detailed mechanism has not been fully elucidated.

Method Effects of Malt1 protease on the activation of T cells and the development of experimental colitis was investigated using Malt1 protease-deficient (PD) mouse.

Results IL-2 production from $CD4^+$ T cells of Malt1 PD mice was decreased compared with that of wild-type (WT) mice. Intraperitoneal injection of anti-CD3 antibody into Malt1 PD mouse induced less productions of IL-17 in the plasma, as well as the colonic gene expression of IL-17A, compared with WT mice, whereas IFN-γ production was not impaired. In naïve T-cell transfer colitis model, Malt1 PD T cells induced less disease severity than WT T cells. Then, reduction in the populations of Th17 and Th1/17 cells was observed in the mesenteric lymph nodes of the recipient mice transferred with Malt1 PD T cells, whereas those of Th1 cells were not impaired. IL-17A expression in the colon was also decreased in the mouse receiving Malt1 PD T cells.

Conclusions Inactivation of Malt1 protease activity abrogates Th17 and Th1/17 cell activation, resulting in the amelioration of experimental colitis.

Keywords Malt1 · Experimental colitis · Th17 cells · Th1/17 cells · IL-17

Introduction

Patients with inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) suffer from chronic diarrhea associated with abdominal pain as well as a decrease in the quality of life. Currently, immunomodulating and immunosuppressive pharmacotherapies are the mainstay treatment for IBD but associated with debilitating side effects [1]. Anti-TNF-α monoclonal antibody (mAb) and anti-α4β7 mAb have been approved as second-line therapy alone or in combination with other drugs in the US and Europe when non-biologic pharmacotherapy alone fails to produce clinical remission [2–4]. While biologics are beneficial for most of patients with IBD, a small number of patients remain refractory and do not respond properly [5, 6]. Thus, new medications with novel mechanisms of action are crucial for the treatment of non-responder group of patients.

IL-23/Th17 axis has been found to play critical roles in the pathogenesis of IBD. Actually, anti-IL-23 mAb demonstrated significant efficacies in anti-TNF-α mAb naïve and

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refractory patients with CD without severe adverse events such as infection [7]. Since IL-23 induces the differentiation and activation of Th17 cells [8, 9], therapeutic approach targeting IL-23/Th17 axis is promising for the treatment with IBD. In addition, IL-17 and IFN-γ double-producing Th1/17 cells have been identified and also work as the pathogenic cells in several inflammatory diseases, such as multiple sclerosis, dry eye disease, and IBD [10–13]. Hence, Th17 and Th1/17 cells can be an attractive target for the treatment of IBD.

MALT lymphoma-translocation protein 1 (Malt1), or paracaspase, plays an important role in the activation of lymphocytes, including T cells [14]. Once immune receptors such as T-cell receptor (TCR) are stimulated, Malt1 and Bcl10 assemble with the caspase recruitment domain (CARD)-containing protein. Then, CARD–Bcl10–Malt1 (CBM) complex results in the NF-κB activation [15]. In addition to its scaffold function, Malt1 works as the cysteine protease, that catalytically processes regulatory proteins, such as RelB [16], cylindromatosis (CYLD) [17], and A20 (also known as TNFAIP3) on T cells [18]. Since these substrates are known as negative regulators of immune responses, Malt1-dependent processing of these proteins leads to T-cell activation. To clarify the role of Malt1 protease activity in the pathogenicity of the colitis, Malt1 protease-deficient (PD) mice were generated [19]. This study revealed that Malt1 protease activity is involved in the development of colitis induced by adoptive $CD4^+$ T-cell transfer from Malt1 PD mouse to immunodeficient mice; however, the effects of Malt1 protease activity on the activation and differentiation of Th17 and Th1/17 cells in the intestine have not been fully evaluated [19]. In the present study, we aimed to elucidate the role of Malt1 protease in the regulation of Th17 and Th1/17 cells in the intestine using Malt1 PD mouse.

Materials and methods

Animals

BALB/c genetic-background Malt1 PD mice were established at Takeda Pharmaceutical Company, Ltd (Kanagawa, Japan) as described previously [20]. Female Malt1 PD and wild-type (WT) mice were maintained under specific pathogen-free conditions and used for the study at 6–10 weeks age. BALB/c mice (female) were purchased from Charles River Japan (Japan). C.B-17/Icr-scid mice (SCID, female) were purchased from CLEA Japan, Inc (Japan). SCID mice were used as recipient mice and bred individually on paper chip. All procedures were performed in accordance with the standards for human care, and treatment of research animal was approved by Institutional Animal Care and Use Committee (IACUC) in Takeda Pharmaceutical Company, Ltd (Approval no. 4516, 4525, and 4531).

Stimulation of $CD4^+$ T cells

$CD4^+$ T cells were purified from splenocytes of Malt1 PD and WT mouse, as described previously [20]. Purification of $CD4^+$ T cells was performed using $CD4^+$ T cell isolation kit II (Miltenyi Biotec, USA), according to manufacturer's instructions. $CD4^+$ T cells were cultured in RPMI-1640 supplemented with 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS.

Isolated $CD4^+$ T cells from Malt1 PD and WT mouse were stimulated with phorbol 12-myristate 13-acetate (PMA) (Wako Pure Chemical, Japan) at 20 ng/mL and ionomycin (Wako Pure Chemical, Japan) at 250 ng/mL for 24 h. For TCR stimulation, anti-CD3/CD28 antibodies (Dynabeads Mouse T-activator CD3/CD28, purchased from Life Technologies, USA) were added as to a bead-to-cell ratio of 1:1, followed by incubating for 24 h. Concentration of IL-2 in the supernatant was determined by ELISA (R&D Systems, USA), according to manufacturer's manual.

Anti-CD3 antibody-induced in vivo cytokine release assay

Anti-CD3 mAb (145-2C11) (Bio X Cell, USA) and its isotype hamster IgG (BD Biosciences, USA) were intraperitoneally injected into Malt1 PD mice and WT mice at 5 μg/mouse. Three hours later, whole blood was obtained by cardiac puncture, followed by collecting plasma with heparin sodium solution (Ajinomoto, Japan). Concentrations of IL-2, IL-17 and IFN-γ in the plasma were measured by ELISA (all purchased from R&D Systems, USA), according to manufacturer's instructions. The colon of each mouse was surgically removed, rinsed with saline, and stored in RNAlater (Qiagen, Germany) at 4 °C. Quantitative reverse transcription-PCR (qRT-PCR) was conducted as mentioned below.

Experimental colitis

Experimental colitis was developed by an adoptive transfer of $CD4^+CD62L^+$ naïve T cells, as described previously [21]. In brief, total lymphoid cells were recovered from splenocytes of BALB/c, Malt1 PD and WT mice by lympholyte-M (CEDARLANE, Canada). Then, cell suspension was treated with HLB solution (IBL, Japan) to hemolyze. $CD4^+CD62L^+$ naïve T cells were isolated by MACS separation systems with $CD4^+CD62L^+$ T Cell Isolation Kit, II (Miltenyi Biotec, USA), according to the manufacturer's instructions. First, $CD4^+$ T cells were negatively selected by antibodies against CD8a (Ly-2), CD45R (B220), CD49b (DX5), CD11b (Mac-1), Ter-119, and TCRγ/δ, as well as antibodies against

CD25. Next, $CD4^+CD62L^+$ naïve T cells were positively isolated by antibodies against CD62L.

Naïve T cells prepared from Malt1 PD and WT mice were intravenously injected into SCID mice. Diarrhea score for stool consistency was graded under blind fashion on a scale of 1–4 as follows: 1, normal; 2, pasty and formed; 3, pasty and unformed; 4, diarrhea. Diarrhea and increase in colon weight were observed at 2 weeks and peaked at 3 weeks after transfer of naïve T cells from BALB/c mice into SCID mice (data not shown). SCID mice were sacrificed on day 21 under anesthesia. The colon of each mouse was surgically removed and rinsed with saline, and its weight was measured. The colon was stored in RNAlater at 4 °C, and qRT-PCR was conducted as mentioned below.

In the preliminary experiment, naïve T cells from BALB/c mice were intravenously transferred into the SCID mice as to 1.6×10^3, 8.0×10^3, 4.0×10^4, 2.0×10^5, or 1.0×10^6 cells/mouse. The exacerbation of diarrhea and increase in colon weight were observed in a T-cell number-dependent manner (data not shown). Incidence rate of diarrhea at 3 weeks was as follows: 1.6×10^3 cells/mouse, 0/7; 8.0×10^3 cells/mouse, 6/8; 4×10^4 cells/mouse or higher, 8/8. In addition, decrease in body weight was observed at the dose of 4×10^4 cells/mouse (data not shown). According to these results, we conducted the following study under the condition of 4×10^4 cells/mouse.

Real-time qRT-PCR

Total RNA from the colon was isolated by RNeasy Mini Kit (Qiagen, Germany) and DNaseI (Qiagen, Germany), according to the manufacturer's instructions. High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) was used for cDNA synthesis. Quantitative PCR were performed on a ViiA 7 Real-Time PCR System (Life Technologies, USA), using TaqMan Fast Advanced Master Mix (Life Technologies, USA) with specific primers on TaqMan Gene Expression Assays (Life Technologies, USA) according to the manufacturer's manual. FAM-probed primers with following assay identification numbers were used: *Il17a*, Mm00439618_m1; *Ifng*, Mm00801778_m1. The data were normalized to β-actin (*Actb*) gene expression.

Intracellular cytokine staining of T cells in MLN

Single cell suspensions prepared from MLNs were stimulated with PMA (50 ng/mL, Wako, Japan) and ionomycin (1 μg/mL, Wako, Japan) in the presence of transport inhibitor containing monensin (BD Biosciences, USA), and incubated for 4 h in RPMI-1640 containing 10% of FBS. After blocking Fc receptor by anti-CD16/CD32 mAb (clone: 2.4G2, BD Biosciences, USA), cells were stained with PE-conjugated-anti-CD4 mAb (BioLegend, USA). Stained cells were fixed and permeabilized with fixation/permeabilization solution (BD Biosciences, USA). The intracellular cytokine staining was carried out using FITC-conjugated anti-IFN-γ mAb and Alexa Fluor 647-conjugated anti-IL-17A mAb (BioLegend, USA). Flow cytometry analysis was performed using BD Accuri C6 Flow Cytometer (BD Biosciences, USA). The population of Th17, Th1/17 and Th1 cells were defined as follows: Th17, IL-17^+ IFN-γ^- cells gated on $CD4^+$ cells; Th1/17, IL-17^+ IFN-γ^+ cells gated on $CD4^+$ cells; Th1, IL-17^- IFN-γ^+ cells gated on $CD4^+$ cells.

Statistical analysis

Data are presented as the mean ± SEM. Statistical analysis was performed using SAS System for Windows (SAS Institute). The difference between two groups was analyzed as follows. Statistical analysis was carried out using Student's *t* test when equality of variances was indicated by *F* test. When there were no equal differences, Aspin–Welch's *t* test was conducted. Wilcoxon test was performed for statistics of diarrhea score. A probability value of $p \le 0.05$ was considered statistically significant.

Results

IL-2 production from T cells isolated from Malt1 PD mouse

Effect of Malt1 protease activity on T-cell activation was investigated. $CD4^+$ T cells were stimulated with PMA/ionomycin or anti-CD3/CD28 antibodies. As shown in Fig. 1a, b, concentrations of IL-2 in the supernatants from Malt1 PD

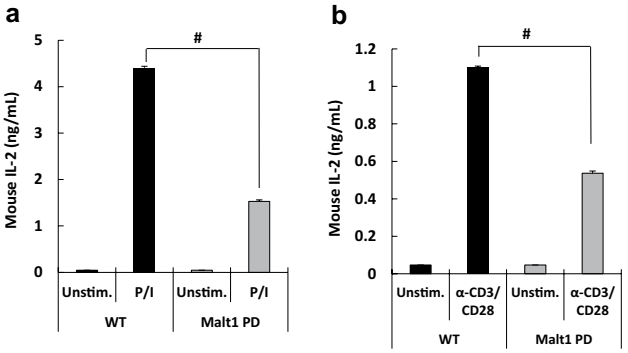

Fig. 1 Impaired IL-2 production from $CD4^+$ T cells isolated from Malt1 protease-deficient (PD) mice. Purified $CD4^+$ T cells from wild-type (WT) and Malt1 PD mice were stimulated with **a** PMA/ionomycin (P/I) or **b** anti-CD3/CD28 antibodies for 24 h. Concentrations of IL-2 in supernatants were measured by ELISA. Values are presented as the mean ± SEM of 3. $^{\#}p \le 0.05$ (Student's *t* test) versus WT cells

T cells were significantly lower than that from WT T cells in both stimulations.

Anti-CD3 antibody-induced cytokine release in Malt1 PD mouse

Next, the effect of Malt1 protease activity on acute T-cell activation was evaluated in vivo in these strains. Intraperitoneal injection of anti-CD3 agonistic antibody into WT mouse elevated the concentrations of IL-2, IL-17 and IFN-γ in the plasma (Fig. 2a–c). The concentrations of IL-2 and IL-17 in Malt1 PD mice were significantly lower than that of WT mice (Fig. 2a, b). On the other hand, IFN-γ concentration in plasma was not significantly affected (Fig. 2c). In addition, the gene expression of IL-17A, but not IFN-γ, in the colon of Malt1 PD mouse was significantly lower than that of WT mouse (Fig. 2d, e).

Malt1 inactivation attenuates T-cell-mediated colitis

$CD4^+CD62L^+$ naïve T cells from Malt1 PD and WT mouse (4×10^4 cells/mouse) were intravenously transferred into the SCID mice. Three weeks later, diarrhea score, weight of colon and body weight gain were evaluated. In the mice transferred with naïve T cells from Malt1 PD mice, diarrhea score and colon weight were significantly lower than that from WT mice (Fig. 3a, b). In addition, Malt1 PD T-cell transfer restored the normal body weight gain compared with WT T-cell transfer (Fig. 3c).

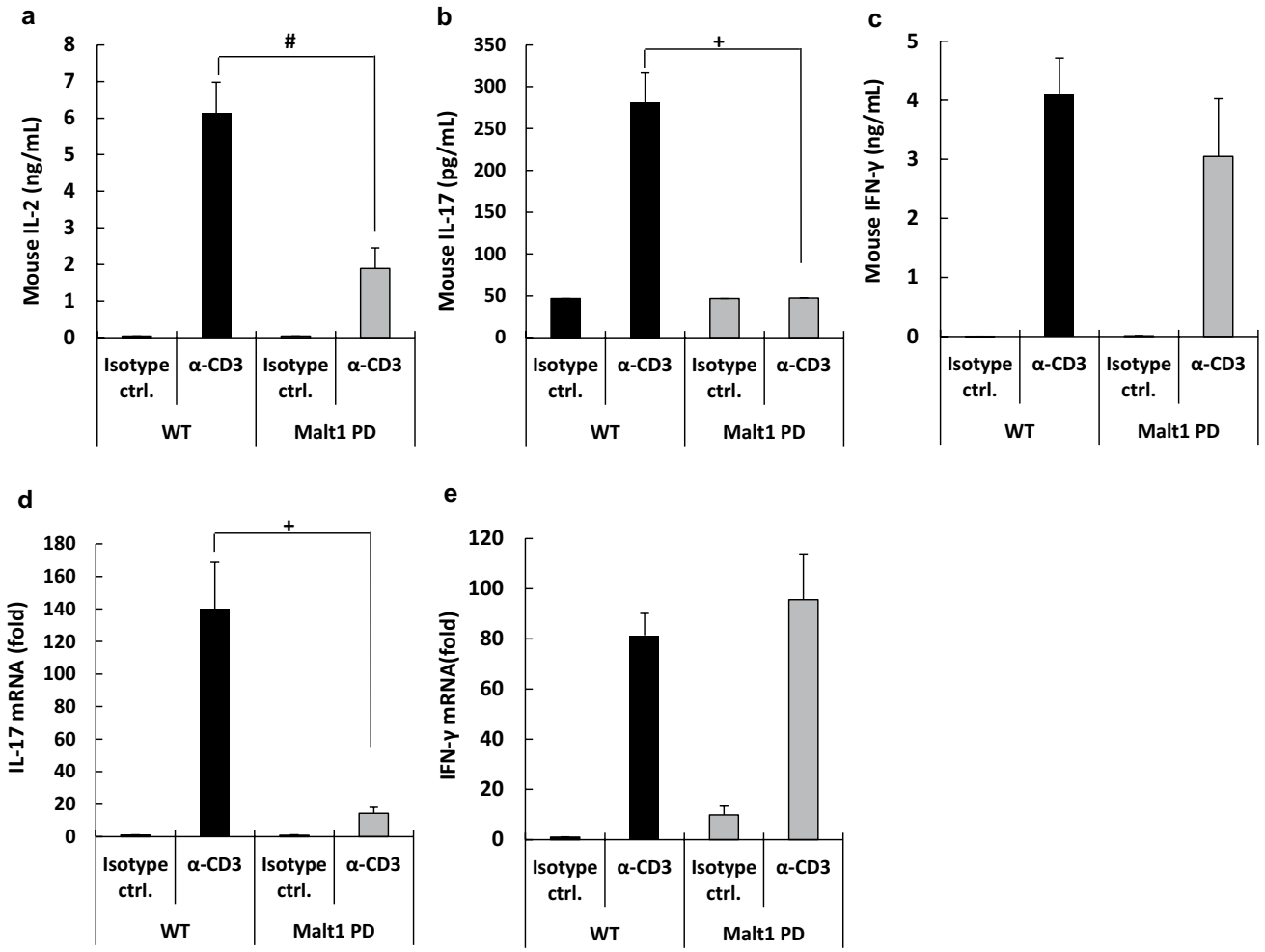

Fig. 2 In vivo impaired cytokine release in Malt1 protease-deficient (PD) mice. Anti-CD3 antibody was intraperitoneally injected into wild-type (WT) and Malt1 PD mice, followed by obtaining bloods 3 h later. Cytokine concentrations in the plasma were determined by ELISA. Colonic mRNA expressions were measured by quantitative reverse transcription-PCR (RT-PCR). **a** IL-2 concentration. **b** IL-17 concentration. **c** IFN-γ concentration. **d** IL-17A mRNA in the colon. **e** IFN-γ mRNA in the colon. Values are presented as the mean ± SEM of 5. $^{\#}p \leq 0.05$ (Student's *t* test) versus WT mice. $^{+}p \leq 0.05$ (Welch's *t* test) versus WT mice

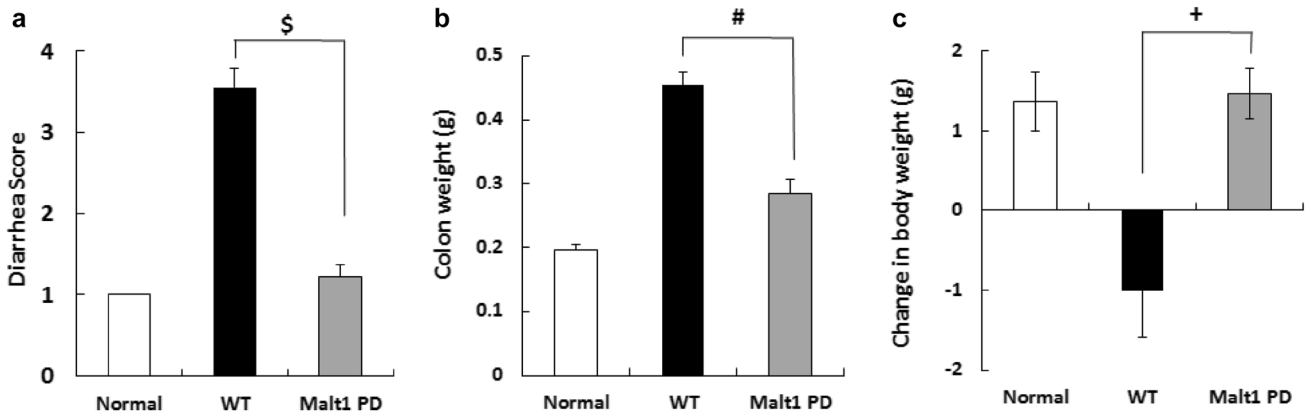

Fig. 3 Naïve T cells from Malt1 protease-deficient (PD) mice induced colitis with less severity. Naïve T cells (4×10^4 cells/mouse) isolated from Malt1 PD and wild-type (WT) mice were intravenously injected into the SCID mice, followed by harvesting the colon 3 weeks later. **a** Diarrhea score. **b** Colon weight. **c** Change in body weight. Values are presented as the mean ± SEM of 4 (normal), or 9 (WT and homozygous cell-transferred groups). $^{\$}p \le 0.05$ (Wilcoxon test), $^{\#}p \le 0.05$ (Student's *t* test), and $^{+}p \le 0.05$ (Welch's *t* test) versus WT group

Malt1 inactivation abrogates the increase in Th17 and Th1/17 cells in the MLN

In the experimental colitis model, the populations of Th17, Th1/17 and Th1 cells on $CD4^+$ T cells in the MLNs were determined by flow cytometry analysis. As shown in Fig. 4a, the accumulations of Th17, Th1/17, and Th1 cells were observed in the MLN in the mice 3 weeks after transfer of naïve T cells of WT mice. The frequencies of Th17 and Th1/17 cells on $CD4^+$ T cells of the recipient mice transferred with Malt1 PD T cells were significantly lower than that of mice transferred WT T cells (Fig. 4b, c). On the other hand, the population of Th1 cells was almost similar between two groups (Fig. 4d).

Malt1 inactivation abrogates IL-17A expression in the colon

Finally, effects of Malt1 protease activity in the colonic gene expression of IL-17A and IFN-γ were investigated in the mouse of colitis. As shown in Fig. 5, tendency of the decrease in the colonic gene expression of IL-17A was observed in the mice transferred with Malt1 PD T cells compared with those of WT T cells with reduction rate of 84%. In contrast, a slight decrease in IFN-γ expression was observed in these mice.

Discussion

We figured out that Malt1 protease activity plays an important role in the production of IL-17 in the blood and colon upon TCR stimulation. In addition, Malt1 protease inactivation attenuates the development of experimental colitis with the regulation of the population of Th17 and Th1/17 cells.

As described previously, the Malt1 PD mouse expresses catalytically inactive Malt1, thereby affecting the processing of Malt1 substrates, such as RelB and CYLD [20]. First, we investigated whether Malt1 inactivation influences the T-cell activation in vitro and in vivo upon TCR stimulation using this strain. As shown in Fig. 1, IL-2 productions from $CD4^+$ T cells stimulated with PMA/ionomycin and anti-CD3/CD28 antibodies were significantly impaired in Malt1 PD T cells. Intraperitoneal injection of anti-CD3 mAb into the Malt1 PD mice induced less production of IL-2 in the plasma compared with WT mice (Fig. 2). Interestingly, IL-17 secretion in plasma was dramatically decreased in the Malt1 PD mice upon TCR stimulation (Fig. 2). On the other hand, plasma concentration of IFN-γ was not affected. In addition, the colonic gene expression of IL-17A, but not IFN-γ, in the Malt1 PD mice was also lower than WT mice. These results suggest that Malt1 protease activity regulates in vivo activation of innate IL-17-producing cells in the colon. Hence,

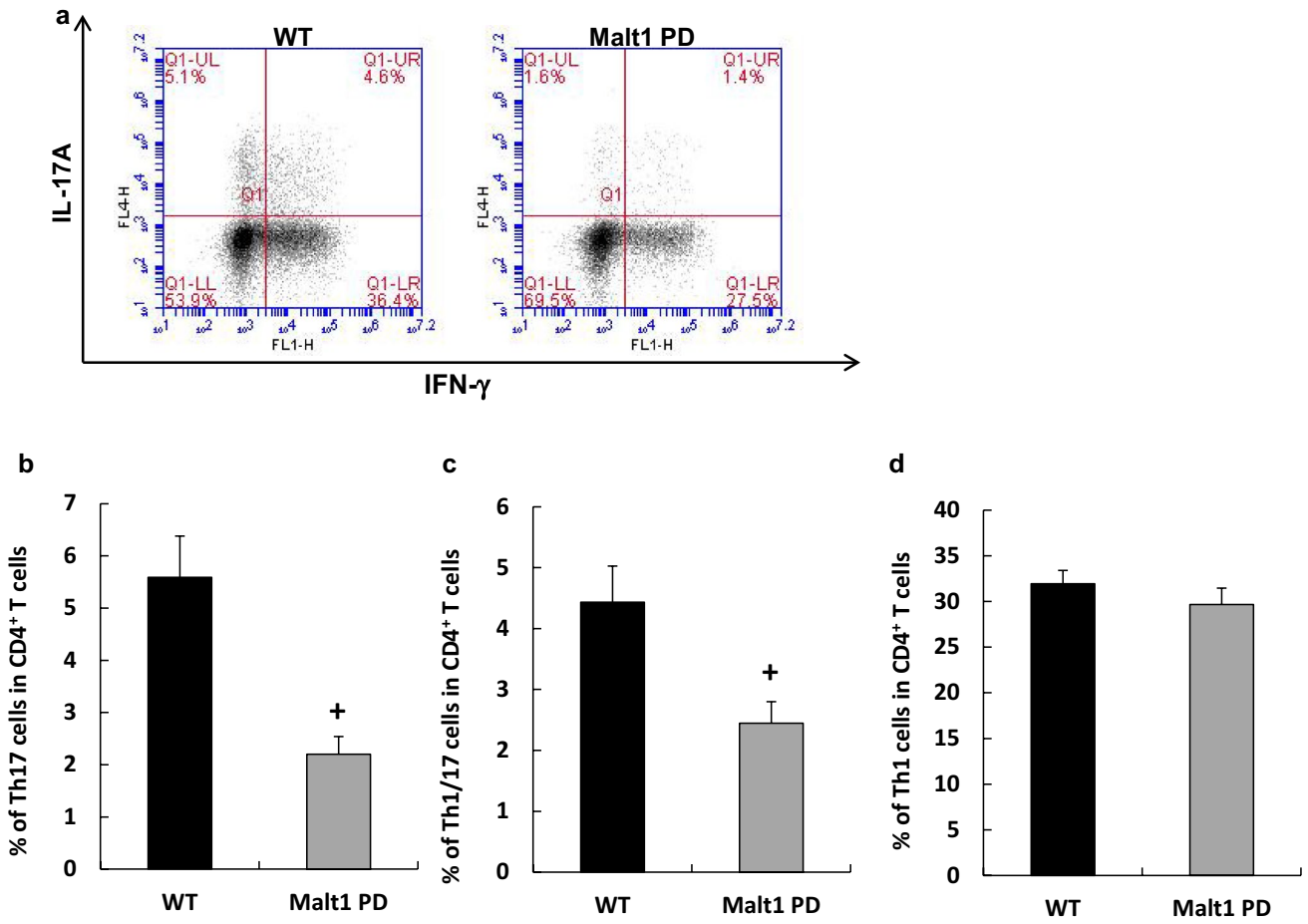

Fig. 4 The population of $CD4^+$ Th cells from mesenteric lymph nodes of the mouse receiving Malt1 protease-deficient (PD) and wild-type (WT) T cells. **a** The population of Th17, Th1/17 and Th1 cells gated on $CD4^+$ T cells were determined by flow cytometry. **b** The percentage of Th17 cells. **c** The percentage of Th1/17 cells. **d** The percentage of Th1 cells. Data are presented as the mean ± SEM of 9 (WT and Malt1 PD groups). $^+p \leq 0.05$ (Welch's *t* test) versus WT group

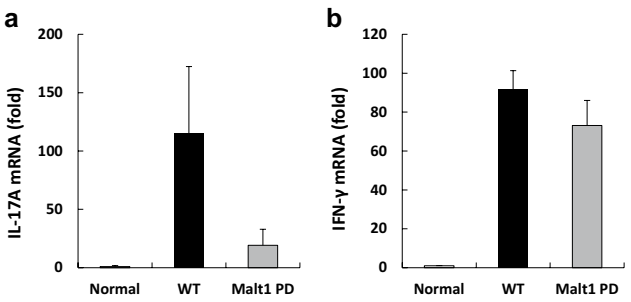

Fig. 5 Colonic cytokine expression in the mice receiving Malt1 protease-deficient (PD) and wild-type (WT) T cells. Colonic mRNA expressions were determined by quantitative RT-PCR. **a** IL-17A mRNA. **b** IFN-γ mRNA. Values are presented as the mean ± SEM of 3 (normal), or 9 (WT and Malt1 PD T-cell groups). $^+p \leq 0.05$ (Welch's *t* test) versus WT group

Malt1 protease activity may contribute to the initiation of acute inflammation upon TCR stimulation in the colon as well as peripheral blood through the regulation of IL-17-producing cells.

Then, we investigated the involvement of Malt1 protease activity in the development of experimental colitis that was induced by intravenous adoptive transfer of naïve $CD4^+CD62L^+$ T cells into the SCID mice. Malt1 PD T cells induced less severity of colitis in parameters of diarrhea score, colon weight gain and body weight loss than WT T cells (Fig. 3). In the separate preliminary study, severe colitis with mucosal thickening accompanied by goblet cell destruction and infiltration of inflammatory cells was observed in the all mice receiving naïve T cells from WT mice; however, in the mice transferring T cells isolated from Malt1 PD mice, only a slight mucosal hypertrophy and cellular infiltration were induced, and no mouse showed moderate and severe colitis colons (Supplemental Figure). Thus, Malt1 protease activity was closely associated with

the development of T-cell-mediated colitis as reported by Jaworski et al. [19].

To elucidate how Malt1 protease regulates the development of colitis, we evaluated the population of $CD4^+$ T cells in MLN harvested from the recipient mice. As shown in Fig. 4, the frequency of Th17 cells in the MLN from the mice receiving Malt1 PD T cells was significantly lower than that receiving WT T cells, whereas that of Th1 cells was not affected. In addition, a trend of numerical decrease in IL-17A gene expression in the colon of mice transferring Malt1 PD T cells was observed, while the expression of IFN-γ was slightly decreased (Fig. 5). These results suggest that inactivation of Malt1 protease activity affects the differentiation and activation of Th17 cells in the colon, resulting in attenuating the induction of colonic inflammation. In another report, decreased Th1 cell population in the MLN was observed when Malt1 PD T cells were transferred to immunodeficient mice, but there were no data about cell ratio of Th1 and Th17 population [19]. Thus, our finding indicated that Malt1 protease activity may have key role to regulate Th1 and Th17 cell balance at the onset of colitis. In Fig. 4, we used IL-17A as an activation marker of Th17 cells in the colon. We do not consider that IL-17A itself largely contributes to the pathology of colitis, since IL-17A is reported to show a protective role in the epithelium of intestine [22]. Th17 cells secrete IL-17A as well as other inflammatory cytokines such as IL-17F and granulocyte macrophage colony-stimulating factor [23, 24]. Malt1 protease activity might accelerate the colonic inflammation via activation and differentiation of Th17 cells, not only through the production of IL-17A.

Interestingly, the population of Th1/17 cells was also significantly reduced in the MLN from the mouse receiving Malt1 PD T cells compared with WT T cells (Fig. 4). Since Th1/17 cells are considered to be differentiated from Th17 cells [12], Malt1 protease activity might indirectly affect the population of Th1/17 cells. The slight decrease in IFN-γ gene expression in the colon was possibly due to the regulation of Th1/17 cells (Fig. 5b). As Th1/17 cells are suggested to contribute to the pathophysiology of IBD, Malt1 inactivation would be beneficial to IBD patients through the inhibition of Th1/17 cell activation as well as Th17 cells [11, 12].

Our study for the first time revealed that Malt1 protease specifically regulates Th17 and Th1/17 cells in the experimental colitis, and plays an important role in the induction of colonic inflammation. Interestingly, naïve $CD4^+CD62L^+$ T-cell transfer colitis model described in this study was refractory to anti-TNF-α mAb (Igaki et al. in submission). Therefore, we expect that Malt1 protease inhibition might be efficacious to anti-TNF-α therapy-refractory patients, though additional experiments are necessary. In addition, Uo and colleagues suggest that FcγR pathway is involved in the pathophysiology of UC [25]. Our previous study revealed that Malt1 inactivation prevents in vitro- and in vivo FcγR-mediated activation of innate immune cells [20]. Thus, Malt1 protease blockade would provide the powerful option for the treatment of IBD with the novel mechanism by suppressing both Th17 and Th1/17 cell activation and FcγR pathway.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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