

Reduced numbers and proapoptotic features of mucosal-associated invariant T cells as a characteristic finding in IBD patients

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ABSTRACT

Background Mucosal-associated invariant T (MAIT) cells are innate-like T cells involved in the homeostasis of mucosal immunity; however, their role in inflammatory bowel disease (IBD) is unclear.

Methods Flow cytometry was used to enumerate peripheral blood MAIT cells in 88 patients with ulcerative colitis (UC), 68 with Crohn's Disease (CD), and in 57 healthy controls. Immunohistochemistry identified MAIT cells in intestinal tissue samples from patients with UC (n=5) and CD (n=10), and in control colon (n=5) and small intestine (n=9) samples. In addition, expression of activated caspases by MAIT cells in the peripheral blood of 14 UC and 15 CD patients, and 16 healthy controls was examined.

Results Peripheral blood analysis revealed that IBD patients had significantly fewer MAIT cells than healthy controls ($p<0.0001$). The number of MAIT cells in the inflamed intestinal mucosae of UC and CD patients was also lower than that in control mucosae ($p=0.0079$ and 0.041 , respectively). The number of activated caspase-expressing MAIT cells in the peripheral blood of UC and CD patients was higher than that in healthy controls ($p=0.0061$ and 0.0075 , respectively), suggesting that the reduced MAIT cell numbers in IBD are associated with an increased level of apoptosis among these cells.

Conclusions The number of MAIT cells in the peripheral blood and inflamed mucosae of UC and CD patients was lower than that in non-IBD controls. Also, MAIT cells from IBD patients exhibited proapoptotic features. These data suggest the pathological involvement, and the potential for therapeutic manipulation, of these cells in IBD patients.

Keywords: inflammatory bowel disease, human, mucosal-associated invariant T cells

INTRODUCTION

Inflammatory bowel disease (IBD) comprises a group of disorders involving chronic inflammation of the digestive tract. It primarily includes ulcerative colitis (UC) and Crohn's disease (CD), and is clinically characterised by recurrent episodes of diarrhoea and abdominal pain. IBD is thought to result from dysregulated mucosal immunity directed against intraluminal antigens of bacterial origin in genetically predisposed individuals; however, the exact aetiology and pathogenesis of IBD remains unclear.

The initiation and perpetuation of IBD are thought to be governed by cytokine responses. In CD, interferon (IFN)- γ derived from T-helper (Th) 1 cells and interleukin (IL)-17 and IL-22 derived from Th17 cells are considered to play important roles in the initiation and propagation of mucosal inflammation.^{1 2} Although UC was first thought to be a Th2-mediated disorder, later studies showed that IL-5 and IL-13 derived from natural killer T (NKT) cells are the main pathologic factors.^{3 4}

NKT cells belong to a group of unconventional T-cell subpopulations that express invariant T-cell receptors (TCRs) restricted by monomorphic major histocompatibility complex (MHC) class I-like molecules; these cells respond to non-protein antigens.^{5 6} In addition to NKT cells, these unconventional T-cell populations also include another subset that express semi-invariant TCRs, and are restricted by the MHC class I-related

protein 1 (MR1), namely, mucosal-associated invariant T (MAIT) cells.⁷ While NKT cells are relatively abundant in mice compared to humans, the opposite is true for MAIT cells. MAIT cells are abundant in the human intestine, peripheral blood, and liver; indeed, they account for 1–8%, 1–8%, and 20% of the T lymphocytes in these organs, respectively.⁸⁻¹⁰ A murine model shows that MAIT cells require commensal flora for their expansion and are activated by MR1 molecules that present vitamin B metabolites, which are mainly produced by intestinal microbes.¹¹ Patients with X-linked inhibitor of apoptosis (XIAP) deficiency, who often present with haemorrhagic colitis resembling IBD, have reduced MAIT cell numbers in the peripheral blood.¹²⁻¹⁴ These lines of evidence suggest that MAIT cells play important roles in gut mucosal immunity. However, the association between MAIT cells and IBD remains unclear. Here, we examined MAIT cells in both the peripheral blood and intestinal mucosa of patients with IBD. The frequencies of the peripheral blood MAIT cells that expressed activated caspases and produced inflammatory cytokines were also determined. To the best of our knowledge, this is the first report to show that reduced numbers of intestinal and blood MAIT cells in IBD patients are associated with higher levels of activated caspase expression.

MATERIALS AND METHODS

Blood and intestinal tissue samples from IBD patients and controls

Blood samples were collected from 57 healthy volunteers and from 156 IBD patients who attended the Department of Gastroenterology and Hepatology at Kyoto University Hospital. Of the IBD group, 68 had CD and 88 had UC. The clinical disease activity in the patients with CD and UC was determined using the Crohn's Disease Activity Index (CDAI) and the Colitis Activity Index (CAI), respectively.¹⁵⁻¹⁷ Active disease was defined as CDAI ≥ 150 or CAI ≥ 4 ; remission was defined as CDAI < 150 or CAI < 4 .

Blood samples were also collected from two paediatric patients with XIAP deficiency; in both patients, the disease had been confirmed at the molecular level by the Department of Pediatrics, Faculty of Medicine, University of Toyama.

Intestinal tissue samples for immunohistochemistry were obtained from 15 IBD patients. Of these, ten were from CD patients who had undergone surgical resection. These samples were all from the small intestine. The other five patients had UC and all samples were biopsy specimens from the large intestinal mucosae. All 15 samples were taken from histologically inflamed mucosa. The diagnosis of CD and UC in these cases was based on clinical, endoscopic, radiological and histological parameters. Faecal bacterial culture did not yield any specific pathogens in any of the patients. For normal

controls, tissue judged by a pathologist at Kyoto University Hospital to be histologically normal was obtained from the small intestine (n=19) and colon (n=10) of non-IBD patients with gastrointestinal cancer who were surgically treated at Kyoto University Hospital. All intestinal samples used for immunohistochemistry were archived formalin-fixed, paraffin-embedded tissue specimens that were stored at the Institute of Pathology, Kyoto University Hospital. For flow cytometric analysis, intestinal tissue specimens were obtained from an additional 10 IBD patients (five with UC and five with CD) undergoing bowel resection and compared with tissue specimens from the small intestine or colon of non-IBD patients with gastrointestinal cancer who were surgically treated. All intestinal resection margins for the control mucosae were located at least 10 cm from the tumour.

All patients were recruited according to a protocol approved by the Institutional Review Board of Kyoto University Hospital and informed consent was obtained in accordance with the Declaration of Helsinki. All patients were informed that their blood and pathological specimens would be used for research purposes.

Preparation of intestinal lymphocytes

Lymphocytes were extracted from intestinal specimens as previously described, with some modifications^{18 19}. Briefly, dissected mucosae were incubated with calcium and magnesium-free Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO) containing 2.5% heat-inactivated foetal bovine serum and 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) to remove mucus. The mucosae were then treated with 0.02% collagenase type 3 (Worthington Biochemical, Freehold, NJ) for 60 min with constant shaking at 37°C. Digested tissue was dispersed over a 100-micron nylon mesh filter. Cell suspensions in 40% Percoll solution (GE Healthcare Japan, Tokyo) were layered onto 60% Percoll before density centrifugation at 780g for 20 min at room temperature. Intestinal lymphocytes were recovered from the layer interface.

Flow cytometry

Flow cytometry was performed according to standard protocols using the following antibodies: anti-TCRV α 7.2-allophycocyanin (APC; 3C10, BioLegend, San Diego, CA), anti-CD161-Brilliant Violet 421 (HP-3G10, BioLegend), anti-CD3-fluorescein isothiocyanate (FITC; SK7, eBioscience, San Diego, CA), anti-integrin β 7-PE (FIB504, BioLegend), anti-CD49d (integrin α 4)-PE/Cy7 (9F10, BioLegend), anti-CD314

(NKG2D)-PE (ON72, Beckman Coulter, France), and anti-BTLA (B and T lymphocyte attenuator) (J168-540, BD Biosciences, San Diego, CA) were used for surface staining, followed by the corresponding isotype controls (all from BD Biosciences); and anti-IL-10-PE (JES3-9D7, eBioscience), anti-IL-17A-PE (eBio64CAP17, eBioscience), anti-IL-18R α -PE (H44, eBioscience), anti-interferon (IFN)- γ -PE (4S.B3, eBioscience), anti-IL-22-PE (142928, R&D systems, Minneapolis, MN), and anti-tumour necrosis factor (TNF)- α -PE (6402, R&D systems) were used for intracellular staining, followed by the corresponding isotype controls (all from BD Biosciences). Peripheral blood MAIT cells were defined as CD161^{high}TCRV α 7.2⁺IL-18R α ⁺CD3^{+10⁻²⁰21}. Active caspases were detected using the FAM FLICA Caspase 3 & 7 Assay Kit (Immunochemistry Technologies, LLC, Bloomington, MN), according to the manufacturers protocol. Apoptotic cells were detected using PE Annexin V Apoptosis Detection Kit 1 (BD Biosciences), according to the manufacturer's protocol. Stained cells were analysed using a FACSVerse flow cytometer (BD Biosciences) equipped with FlowJo software (version 7.6.5; TreeStar, Ashland, OR).

Intracellular cytokine analysis

Peripheral blood mononuclear cells were seeded in 24-well culture plates at a density

of 5×10^5 cells per well and cultured with 50 ng/mL phorbol myristate acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and 500 ng/mL ionomycin (Sigma-Aldrich) for 4 h at 37°C in 5% CO₂. GolgiPlug (BD Biosciences) protein transport inhibitor was added after 2 h of culture at a concentration of 2 µM. After staining for cell surface antigens with anti-TCRVα7.2-APC, anti-CD161-Brilliant Violet 421, and anti-CD3-FITC, intracellular staining with anti-IFN-γ-PE, anti-TNF-α-PE, anti-IL-10-PE, anti-IL-17A-PE, and anti-IL-22-PE antibodies, or the corresponding isotype control antibodies was performed as described previously²².

Immunohistochemistry

Immunohistochemistry was performed using the Catalyzed Signal Amplification (CSA) System (DAKO, Carpinteria, CA), according to the manufacturer's instructions with minor modifications. In brief, 4 µm thick sections of formalin-fixed and paraffin-embedded tissues were deparaffinised and rehydrated. Antigen retrieval was performed by boiling in Target Retrieval Solution (pH 6.0; DakoCytomation) for 20 min. The tissue sections were then incubated in DAKO®Protein Block Serum-Free (DakoCytomation), followed by an overnight incubation with anti-TCRVα7.2 antibody (1:100 dilution) at 4°C. The signal was detected using FITC-labelled streptavidin

following enhancement with the CSA system (Vector Laboratories, Burlingame, CA). To allow double staining of the same section, the sections were then washed five times with PBS to remove any excess reagents used for the first staining. Subsequently, the sections were re-boiled in Target Retrieval Solution (pH 8.0) and incubated for 2 days at 4°C with antibodies against MR1, CD161, CD3 (1:50; BD Biosciences), or IL-18R α (1:50; eBioscience). Following enhancement with the CSA system, MR1, CD161, CD3 or IL-18R α signals were detected using PE-labelled streptavidin (Vector Laboratories, Burlingame, CA). Cell nuclei were counterstained with DAPI. Negative controls comprised sections treated according to the same protocol, but using an isotype-matched control primary antibody (DAKO). The triple-stained sections were analysed under a high magnification laser-scanning microscope (FSX100, BX50, and Olympus AX80TR, Olympus, Tokyo, Japan). The MAIT cells in the intestinal mucosae were considered to be TCRV α 7.2⁺CD161⁺ cells in the crypts. The number of intestinal MAIT cells in each sample was determined by counting the MAIT cells in 30 crypts. The numbers of small intestinal and colonic MAIT cells in tissue from patients with CD or UC were then compared with those in the controls.

Statistical analysis

To compare the IBD population with the controls, Student's t-test was used for continuous variables and the chi-square test was used for categorical variables. Pearson's correlation analysis was used to assess the correlation between two continuous variables. Multivariable linear regression models (adjusted for age) were used to evaluate the relationship between MAIT cell number or percentage and IBD status. All quantitative data were analysed using unpaired t-tests and Prism Version 4.0 software (GraphPad) or Microsoft Excel, as appropriate. Two-tailed *p*-values < 0.05 were considered significant.

RESULTS

Patients with IBD have a lower number and percentage of peripheral blood MAIT cells than healthy controls

The clinical characteristics of the study subjects are summarised in Table 1. Peripheral blood samples from 156 IBD patients (88 with UC and 68 with CD) and 57 age-matched healthy controls were assessed by flow cytometry to determine the absolute number of MAIT cells, defined as CD161^{high}TCRV α 7.2+IL-18R α ⁺CD3⁺, and the percentage of MAIT cells within the CD3⁺ cell population. IBD patients had a

significantly lower number and percentage of MAIT cells than controls ($p<0.0001$; Table 1 and Figure 1a and b). However, there was no significant difference in MAIT cell number or percentage between UC and CD patients (Figure 1c). Correlation analysis revealed that the number of MAIT cells in IBD patients fell with age (-0.27 cells/ μL per year; 95% confidence interval (CI), -0.45 to -0.089 ; $p=0.0033$). The percentage of MAIT cells in the peripheral blood also fell with age (-0.03% per year; 95% CI, -0.055 to -0.0052 ; $p=0.0097$; Figure, Supplemental Digital Content 1). Even after adjusting for age using multiple regression analysis, IBD patients still had a significantly lower median number (by 7 cells/ μL ; 95% CI, -13 to -1.5 ; $p=0.0135$) and percentage of MAIT cells (1.7% lower; 95% CI, -2.5 to -0.96 ; $p<0.0001$) than the healthy controls. Neither the MAIT cell number nor percentage was significantly affected by gender (Figure, Supplemental Digital Content 2) or treatment with medications (Figure, Supplemental Digital Content 3). There was no correlation between IBD severity and MAIT cell number (Figure, Supplemental Digital Content 4).

Patients with IBD have fewer intestinal MAIT cells than controls

To address whether the lower number of peripheral blood MAIT cells in IBD patients is related to intestinal inflammation, we next performed immunohistochemical analysis

of intestinal tissue (taken at the time of surgical resection or biopsy) using anti-TCRV α 7.2 and anti-CD161 antibodies. We found that MAIT cells were localised at the bottom of the crypts (Figure 2a and b). There were significantly fewer intestinal MAIT cells in inflamed mucosae samples from patients with active UC ($p=0.0079$) or CD ($p=0.041$; Figure 2c, Figure, Supplemental Digital Content 5a) than in histologically normal mucosae controls. However, patients with CD were significantly younger than the controls (41 ± 14 vs. 66 ± 13 years; $p=0.0048$). Moreover, the MAIT cell counts in the small intestine of CD patients decreased significantly with age (-0.45 cells/30 crypts per year; 95% CI, -0.78 to -0.11 ; $p=0.019$; Table 2). This is similar to the findings in peripheral blood. However, after making adjustments for age using multiple regression analysis, CD patients still had significantly fewer MAIT cells than control subjects (a mean of 26 fewer cells/30 crypts; 95% CI, -38 to -14 ; $p=0.0006$). The same tendency was observed in UC patients, who on average had 39 fewer cells/30 crypts (95% CI, -61 to -16 ; $p=0.012$) than the controls. The analysis revealed two subpopulations among the CD patient group: one with normal MAIT cell counts in the intestinal mucosa and one with significantly lower MAIT cell counts (Figure 2c).

To further examine whether MAIT cells were reduced specifically in inflamed mucosae, fresh samples obtained from patients undergoing surgical resection were

obtained and the percentage of MAIT cells in macroscopically inflamed and non-inflamed mucosae was compared using flow cytometry (Figure 3a and 3b). The percentage of intestinal MAIT cells (the percentage of $CD161^{high}TCRV\alpha7.2^{+}CD3^{+}$ cells among the $CD3^{+}$ cell population) in inflamed colon mucosae from UC patients was significantly lower than that in non-IBD control mucosae ($0.91\pm0.62\%$ vs. $3.2\pm2.7\%$, respectively; $p=0.044$). Similarly, the percentage of MAIT cells in inflamed small intestinal mucosae from CD patients ($1.35\pm0.53\%$) was significantly lower than in non-IBD control mucosae (4.93 ± 2.75 , $p=0.0093$; Figure 3b). There was no significant difference between the percentages in inflamed and non-inflamed mucosae from UC and CD patients (Figure 3b).

Since MAIT cell homeostasis is thought to be maintained by intestinal microbial antigens presented by MR1 molecules, we next examined MR1 expression by mucosal epithelial cells and mononuclear cells in the lamina propria of IBD ($n=10$) and control samples ($n=10$) by immunohistochemistry. There was no significant difference in MR1 expression between IBD patients and controls (Figure, Supplemental Digital Content 5b).

MAIT cells produce higher levels of IL-22 in the peripheral blood of UC patients

To assess the functional properties of MAIT cells in IBD patients, peripheral blood cells were subjected to intracellular cytokine staining after *ex vivo* stimulation with PMA and ionomycin. There was no difference in the percentages of IL-17- or IFN- γ -producing MAIT cells between IBD patients and healthy controls. However, blood MAIT cells from UC patients produced significantly higher levels of IL-22 ($3.2\pm 2.5\%$) than those from controls ($0.095\pm 0.019\%$, $p=0.026$; Figure 4). We also examined the expression of natural killer (NK) G2D and BTLA, the expression of which is increased in blood MAIT cells from IBD patients;²³ however, we found no significant differences between IBD patients and controls. (Figure, Supplemental Digital Content 6).

IBD patients have a higher percentage of activated caspase-expressing peripheral blood MAIT cells than healthy controls

MAIT cells display proapoptotic features, including increased levels of activated caspases, compared with conventional T cells. Moreover, MAIT cells are more prone to apoptosis and are present at lower levels in the peripheral blood of patients with XIAP deficiency.¹² To determine whether MAIT cells from IBD patients are more sensitive to

apoptosis, we compared the levels of activated caspases in peripheral blood MAIT cells from IBD patients and healthy controls. Peripheral blood from two patients with XIAP deficiency was also tested as positive controls. Patients with UC and CD had significantly higher percentages of activated caspase-expressing MAIT cells ($6.5 \pm 1.9\%$ vs. $6.0 \pm 1.5\%$, respectively) than the healthy controls ($3.9 \pm 1.6\%$; $p=0.0061$ and 0.0075 , respectively; Figure 5a and b and Table 3). There was no association between age and the percentage of blood MAIT cells expressing activated caspases (Figure, Supplemental Digital Content 7), suggesting that reduced percentage of MAIT cells in IBD patients is caused by mechanisms other than those that cause age-related declines in MAIT cell numbers. We also examined the percentage of early apoptotic blood MAIT cells by detecting Annexin V binding. IBD patients had a higher percentage of early apoptotic MAIT cells in the peripheral blood than healthy controls; however, the difference did not reach statistical significance (Figure, Supplemental Digital Content 8).

We next examined whether excessive apoptosis of gut-homing MAIT cells in the peripheral blood of IBD patients is associated with the reduced numbers of intestinal MAIT cells. The percentage of integrin $\alpha 4\beta 7^+$ MAIT cells in the peripheral blood of CD patients was significantly lower than that in healthy controls ($47 \pm 19\%$ vs. $81 \pm 16\%$,

respectively; $p=0.015$). The same trend was observed between UC patients and controls, although the difference was not statistically significant ($57\pm 23\%$ vs. $81\pm 16\%$, respectively; $p=0.087$) (Figure 6). Expression of activated caspases by integrin $\alpha 4\beta 7^+$ blood MAIT cells was marginally higher in UC and CD patients than in healthy controls ($4.07\pm 1.11\%$ and $5.8\pm 3.85\%$ vs. 2.56 ± 0.83 , respectively; $p=0.068$; Figure 6).

The percentage of Annexin V-positive early apoptotic MAIT cells was significantly higher in inflamed small intestinal mucosae from CD patients than in that of healthy control ($11\pm 4.4\%$ vs. 3.0 ± 0.6 , $p=0.019$; Figure 7). The percentage of MAIT cells in the inflamed mucosae of CD patients showing activated caspase expression was higher than that in controls, although the difference was not significant (Figure 7). There was no difference in the percentage of Annexin V-positive or activated caspase-expressing MAIT cells in colon mucosae from UC patients and controls (Figure 7).

Taken together, these data suggest that the numbers of circulating gut-homing MAIT cells were significantly lower, and those of apoptotic MAIT cells were significantly higher in the intestine of CD patients. Also, the number of gut-homing MAIT cells was marginally lower in the peripheral blood of UC patients; however, there was no increase in the number of apoptotic MAIT cells in their colon mucosae.

DISCUSSION

The present study shows that patients with UC and CD have significantly fewer intestinal and peripheral blood MAIT cells than non-IBD controls. The percentage of activated caspase-expressing MAIT cells in the peripheral blood of IBD patients was significantly higher than that in healthy controls, suggesting that MAIT cells from IBD patients are more susceptible to apoptosis, which may be the reason for the reduced number of these cells in IBD patients. To further support this idea, we showed that the number of gut-homing $\alpha 4\beta 7^+$ MAIT cells was reduced in the peripheral blood of IBD patients (Figure 6). The interaction of integrin $\alpha 4\beta 7$ (which is expressed on T cells) with its ligand, MAdCAM-1 (mucosal addressin cell adhesion molecule 1), is crucial for the trafficking of T cells to the intestinal mucosa³⁵. Expression of MAdCAM-1 is up-regulated in the lamina propria of patients with IBD³⁶, which may contribute to the transmural accumulation of inflammatory and immunoregulatory cells. Previous studies show that circulating and mucosal gut-homing lymphocyte populations are altered in IBD patients³⁷; therefore, integrin $\alpha 4\beta 7$ may also be involved in recruiting MAIT cells to the intestinal mucosa in IBD. The reduction in intestinal MAIT cell numbers might be attributed to reduced trafficking of MAIT cells to the intestinal mucosa due to increased apoptosis of gut-homing blood MAIT cells.

Cosgrove *et al.* reported that HIV-infected patients have fewer MAIT cells in both the peripheral blood and colon than controls.²⁵ In these patients, increased permeability of the gut results in bacterial translocation, which would be expected to result in increased presentation of bacterial antigens to MAIT cells. Since HIV-infected patients and IBD patients share increased intestinal permeability, which promotes microbial translocation and increased plasma endotoxin concentrations as common features,²⁶⁻³⁰ and *in vitro* activation of human peripheral blood mononuclear cells by paraformaldehyde-fixed *E. coli* induces apoptosis of MAIT cells in a dose- and MR1-dependent manner,²⁵ we can speculate that excessive induction of apoptosis after exposure to gut microbial antigens contributes to the reduction in MAIT cell numbers observed in IBD patients.

A reduction in the number of MAIT cells, along with their increased caspase activity, is a characteristic finding in XIAP-deficient patients.¹² XIAP is a physiologic inhibitor of caspases 3, 7 and 9,^{31 32} and MAIT cells intrinsically express higher levels of caspases than conventional T cells; therefore, XIAP deficiency results in a significant reduction in the number of peripheral blood MAIT cells.¹² Of note, XIAP-deficient patients often suffer from haemorrhagic colitis, which resembles IBD.^{13 14} Since IBD comprises a heterogeneous group of disorders,^{33 34} it is possible that a subpopulation of IBD patients share a mechanism of intestinal inflammation with XIAP-deficient patients.

In this regard, unravelling the pathogenesis of gut inflammation associated with XIAP deficiency may provide insights into the pathophysiology of IBD.

Recently, Serriari *et al.* reported that MAIT cells play an important role in the pathophysiological mechanisms underlying IBD.²³ As in the present study, they also showed reduced numbers of MAIT cells in the peripheral blood of IBD patients. However, a comparison between MAIT cells in inflamed and non-inflamed intestinal mucosae from the same CD patients showed that the percentage of MAIT cells among T cells was higher in inflamed tissue; however, we did not confirm this in the present study. Serriari *et al.* also reported that MAIT cells from CD patients produce significantly less IFN- γ ; again, this was not confirmed in the present study. Here, we defined intestinal MAIT cells as CD161⁺TCRV α 7.2⁺ as previously reported,³⁸ whereas Serriari *et al.* identified them as CD3⁺TCRV α 7.2⁺IL-18R α ⁺. Our flow cytometric analysis of mucosal lymphocytes showed that CD161⁺TCRV α 7.2⁺ cells expressed high levels of IL-18R α and CD3, and that CD3⁺TCRV α 7.2⁺IL-18R α ⁺ cells also expressed CD161 (Figure, Supplemental Digital Content 9a, 9b). Furthermore, our experiments involving immunohistochemical staining of serial sections showed that the CD161⁺TCRV α 7.2⁺, TCRV α 7.2⁺IL-18R α ⁺, and CD3⁺TCRV α 7.2⁺ cell populations were almost identical (Figure, Supplemental Digital Content 10). Collectively, these data

indicate that CD161⁺TCRV α 7.2⁺ cells are identical to CD3⁺TCRV α 7.2⁺IL-18R α ⁺ cells; thus, both we and Serriari *et al.* most likely examined the same intestinal MAIT cells.

The immunohistochemical analyses performed herein revealed that CD patients could be divided into two subgroups based on MAIT cell numbers in the intestinal mucosa: one group with normal numbers and the other with reduced numbers (Figure 2c). We do not have an explanation for this phenomenon; however, we speculate that it may reflect the heterogeneity of CD (*e.g.*, in terms of aetiology and stage of disease). In support of this, a previous study shows that CD is associated with various clusters of genetic susceptibility loci,³³ suggesting that it comprises distinct pathological subsets that cannot yet be classified. In addition, a study of a murine model of CD (2,4,6-trinitrobenzenesulphonic acid (TNBS)-colitis) revealed that Th1 and Th17 cytokine responses occur at different stages of the disease, which suggests that the intestinal inflammation in human CD could also be characterised by sequential cytokine responses and changes in MAIT cell numbers as the lesions mature. Therefore, it is possible that differences between study populations may have affected the MAIT cell percentage in the two studies. Further studies are warranted to determine whether MAIT cells are actually accumulated or depleted in the inflamed mucosae of IBD patients.

One important unresolved question is whether MAIT cells promote or inhibit the

inflammation in IBD. We found that MAIT cells from UC patients produce higher levels of IL-22, which protects against mucosal inflammation, which is consistent with previous studies.^{2 23} Human MAIT cells from patients with multiple sclerosis suppress the production of IFN- γ by conventional T cells *in vitro*.²² These results suggest that MAIT cells prevent rather than promote inflammatory responses. Because IL-22 deficiency is associated with severe colitis^{39 40}, a paucity of MAIT cells that produce IL-22 may contribute to gut inflammation in UC. Although few studies have undertaken functional assays of MAIT cells in patients with inflammatory diseases, the finding that MAIT cell numbers are reduced in the peripheral blood and intestinal mucosae of IBD patients implies that these cells are likely to play a protective role against mucosal inflammation.

Another interesting finding of the present study is that the percentage of apoptotic MAIT cells was not increased in the colon mucosae of UC patients, although it was increased in the peripheral blood; however, the percentage was increased in both the peripheral blood and intestinal mucosae of CD patients. We found that in general, in healthy controls, MAIT cells are more prone to apoptosis in the colon than in the small intestine (Figure, Supplemental Digital Content 11), suggesting that MAIT cell homeostasis differs in different parts of the bowel. These findings may provide a clue to

unravelling the complex pathogenesis of IBD.

We also examined the utility of MAIT cells as a biomarker for IBD. We found no significant relationship between the clinical activity of IBD and MAIT cell numbers; therefore, further studies are required to reveal the correlation between long-term changes in disease activity and MAIT cell numbers in each patient, and to determine whether MAIT cells can be used as a predictive biomarker for relapse or treatment response. MAIT cells expressing higher levels of activated caspases might also serve as an aid to IBD diagnosis.

In conclusion, the present study showed that MAIT cell numbers are lower in the peripheral blood and intestinal mucosa of IBD patients than in healthy controls. To the best of our knowledge, this is the first report to show that reduced numbers of intestinal and peripheral blood MAIT cells in IBD patients are associated with higher levels of activated caspase expression. Further studies are required to examine the involvement of MAIT cells in IBD pathophysiology, and to investigate the utility of manipulating these cells as a new therapeutic strategy in the future.

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FIGURE LEGENDS

Figure 1. Absolute numbers and percentages of MAIT cells in the peripheral blood of IBD patients.

Peripheral blood mononuclear cells from 57 healthy controls (HC) and 156 IBD patients were examined by flow cytometry. (a) Representative flow cytometry profiles. MAIT cells were defined as CD161^{high}TCRV α 7.2⁺IL-18R α ⁺CD3⁺ cells. The dot plots show staining with anti-CD161 and anti-TCRV α 7.2 antibodies after gating on CD3⁺IL-18R α ⁺ cells. (b) Comparison of absolute MAIT cell numbers and percentages relative to CD3⁺ lymphocyte numbers in the HC and IBD groups. The bars indicate the median number of MAIT cells. The Mann-Whitney U-test was used for the comparison. (c) Comparison of absolute MAIT cell numbers and percentages between UC (n=88) and CD (n=68) patients. The bars indicate the median number of MAIT cells. The Mann-Whitney U-test was used for the comparison.

Figure 2. Absolute numbers of MAIT cells in intestinal tissue samples from IBD patients.

Intestinal tissue samples from ten CD (all from inflamed lesions in the small intestine) and five UC (all from inflamed lesions in the colon) patients were subjected to immunohistochemical analysis. Control samples comprised normal small intestinal (n=19) or colonic (n=10) tissue resected from patients undergoing surgery for gastrointestinal cancer. (a) Representative immunohistochemistry images of the small intestine in a control subject stained with antibodies specific for TCRV α 7.2 (green) and CD161 (red). The arrows indicate TCRV α 7.2⁺CD161⁺ MAIT cells. Original magnification, $\times 400$. (b) Another representative immunohistochemical image showing the small intestine of a control subject stained with antibodies specific for TCRV α 7.2 (green) and CD161 (red). Original magnification, $\times 100$. (c) Comparison of the number of MAIT cells per 30 crypts in the intestine of UC (UC Pt) and CD (CD Pt) patients with that in control subjects. The UC vs. Control comparison involved colonic mucosae only (left). The CD vs. Control comparison involved small intestinal mucosae only (right). The bars indicate the median number of MAIT cells. The Mann-Whitney U-test was used for the comparison.

Figure 3. Percentage of MAIT cells in intestinal tissue samples from IBD patients.

Intestinal tissue samples from five patients with CD (all from the small intestine) and five patients with UC (all from the colon) were subjected to flow cytometry. Inflamed and non-inflamed intestinal mucosae were obtained from five CD patients and three UC patients. Also, inflamed colon mucosae were obtained from two additional UC patients. Control samples comprised normal small intestinal (n=7) or colonic (n=10) tissue resected from patients undergoing surgery for gastrointestinal cancer. (a) Representative flow cytometric profiles. Intestinal MAIT cells were defined as $CD161^{high}TCRV\alpha7.2^{+}CD3^{+}$. The dot plots show staining with anti-CD161 and anti-TCRV $\alpha7.2$ antibodies after gating on $CD3^{+}$ cells. (b) Comparison of the percentage of MAIT cells (percentage of $CD161^{high}TCRV\alpha7.2^{+}CD3^{+}$ cells among the $CD3^{+}$ cell population) in macroscopically inflamed and non-inflamed colon from UC patients and in colon samples from non-IBD controls (upper left). Comparison of the percentage of intestinal MAIT cell in macroscopically inflamed and non-inflamed small intestine from CD patients and small intestine from non-IBD controls (under left).

The bars indicate the median MAIT cell percentage. Statistical significance was determined using Kruskal-Wallis test followed by Mann-Whitney U-test with Bonferroni-Dunn-correction.

Comparison of inflamed and non-inflamed intestine in UC patients (upper right, n=3) and CD patients (under right, n=5). The Wilcoxon matched-pairs signed rank test was used for the comparison.

Figure 4. Cytokine production by peripheral blood MAIT cells and CD3⁺ cells from UC and CD patients and healthy controls.

Peripheral blood mononuclear cells from five UC and seven CD patients and from nine healthy controls (HC) were subjected to flow cytometry analysis for IFN- γ , IL-10, IL-17, and TNF α . Samples from four patients with UC, four with CD, and five HC were analysed for IL-22. MAIT cells were defined as CD161^{high}TCRV α 7.2⁺CD3⁺. Bars represent the median values.

Statistical significance was determined using Kruskal-Wallis test followed by Mann-Whitney U-test with Bonferroni-Dunn-correction.

Figure 5. Percentage of activated caspase-expressing MAIT cells in the peripheral blood of IBD patients.

Peripheral blood mononuclear cells from UC (n=14) patients, CD patients (n=15), healthy controls (HC, n=16), and patients with XIAP deficiency (n=2) were collected and activated caspase expression measured by FLICA. (a) Representative flow cytometric dot plots showing activated caspase expression in CD3⁺ or CD161^{high}TCRV α 7.2⁺CD3⁺ (MAIT) cells. (b) Comparison of the percentage of activated caspase-expressing MAIT cells (left) or CD3⁺ lymphocytes (right) among the UC, CD, and HC subjects. The bars represent the median values.

Statistical significance was determined using Kruskal-Wallis test followed by Mann-Whitney U-test with Bonferroni-Dunn-correction.

Figure 6. Expression of integrin $\alpha 4\beta 7$ on MAIT cells and the percentage of activated caspase-expressing integrin $\alpha 4\beta 7^+$ MAIT cells in the peripheral blood of IBD patients.

Peripheral blood mononuclear cells from 14 patients with IBD and six healthy controls (HC) were subjected to flow cytometry to examine the expression of integrin $\alpha 4\beta 7$ on MAIT (CD161^{high}TCRV α 7.2⁺CD3⁺) cells (left panel). Comparison of the percentage of activated caspase-expressing integrin $\alpha 4\beta 7^+$ MAIT cells between IBD patients (n=13) and HC (n=6) (right panel). The bars represent the median values. Comparisons were made using the Mann-Whitney U-test.

Figure 7. Percentage of activated caspase-expressing MAIT cells or Annexin V-positive MAIT cells in the intestinal mucosae of IBD patients.

Intestinal tissue samples from five CD patients and four UC patients were subjected to flow cytometry. Control samples comprised normal small intestinal (n=4) or colonic (n=7) tissue resected from patients undergoing surgery for gastrointestinal cancer. All samples were obtained from the same specimens, which were prepared for analysis of intestinal MAIT cell percentages.

Comparison of the percentage of activated caspase-expressing MAIT (CD161^{high}TCRV α 7.2⁺CD3⁺) cells in macroscopically inflamed and non-inflamed small intestine from CD patients and in small intestine from non-IBD controls (upper left). Comparison of the percentages of Annexin V-positive, 7AAD-negative MAIT cells between the three groups (upper right). Comparison of the percentage of activated caspase-expressing MAIT cells in macroscopically inflamed and non-inflamed colon from UC patients and colon from non-IBD controls (lower left). Comparison of the percentage of Annexin V-positive, 7AAD-negative MAIT cells between the three groups (lower right). The bars represent the median values.

Statistical significance was determined using Kruskal-Wallis test followed by Mann-Whitney U-test with Bonferroni-Dunn-correction.

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List of Supplemental Digital Content

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Kawai_Supplemental Digital Content 2.doc

Kawai_Supplemental Digital Content 3.doc

Kawai_Supplemental Digital Content 4.doc

Kawai_Supplemental Digital Content 5a and 5b.doc

Kawai_Supplemental Digital Content 6a and 6b.doc

Kawai_Supplemental Digital Content 7.doc

Kawai_Supplemental Digital Content 8.doc

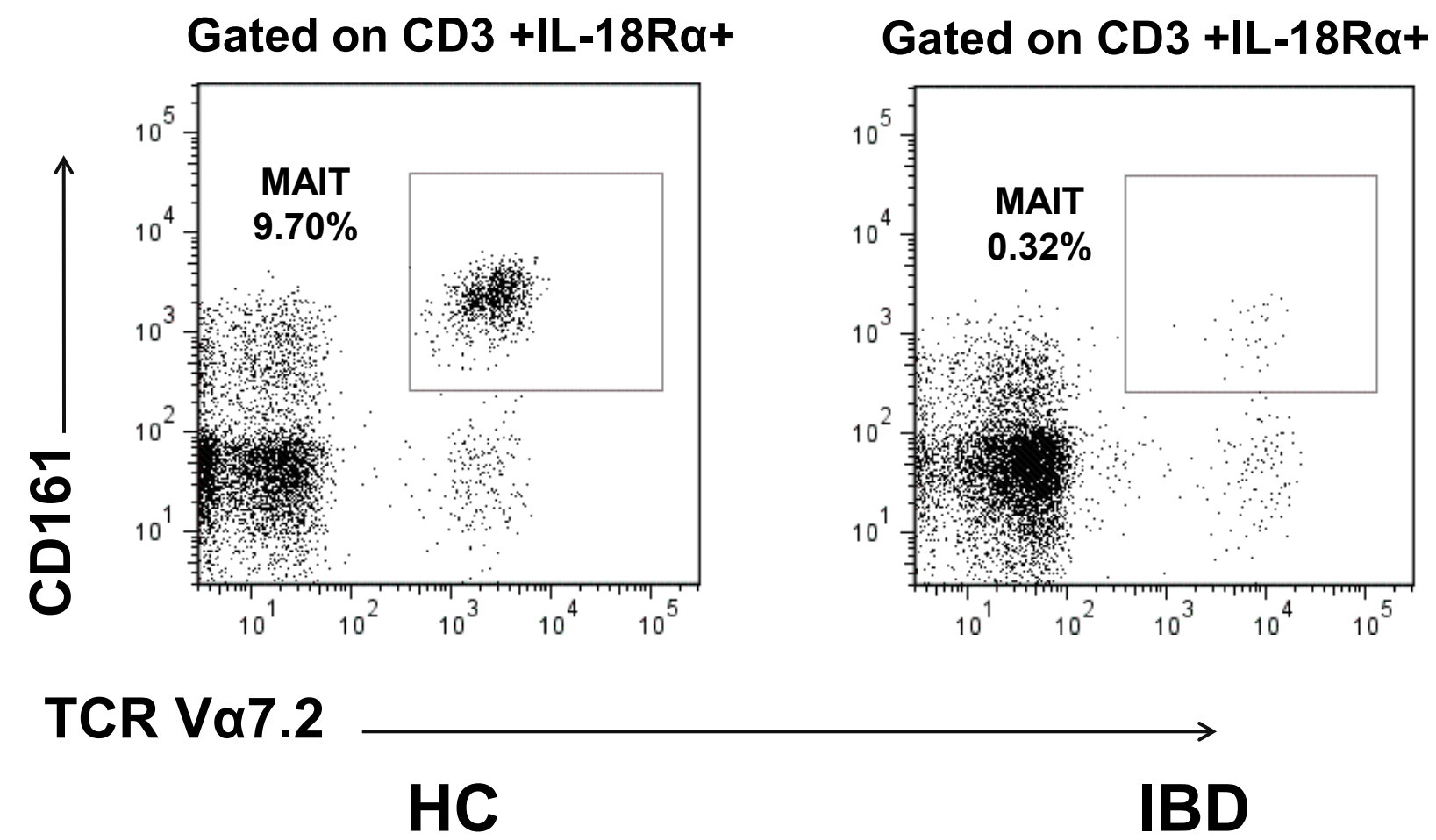
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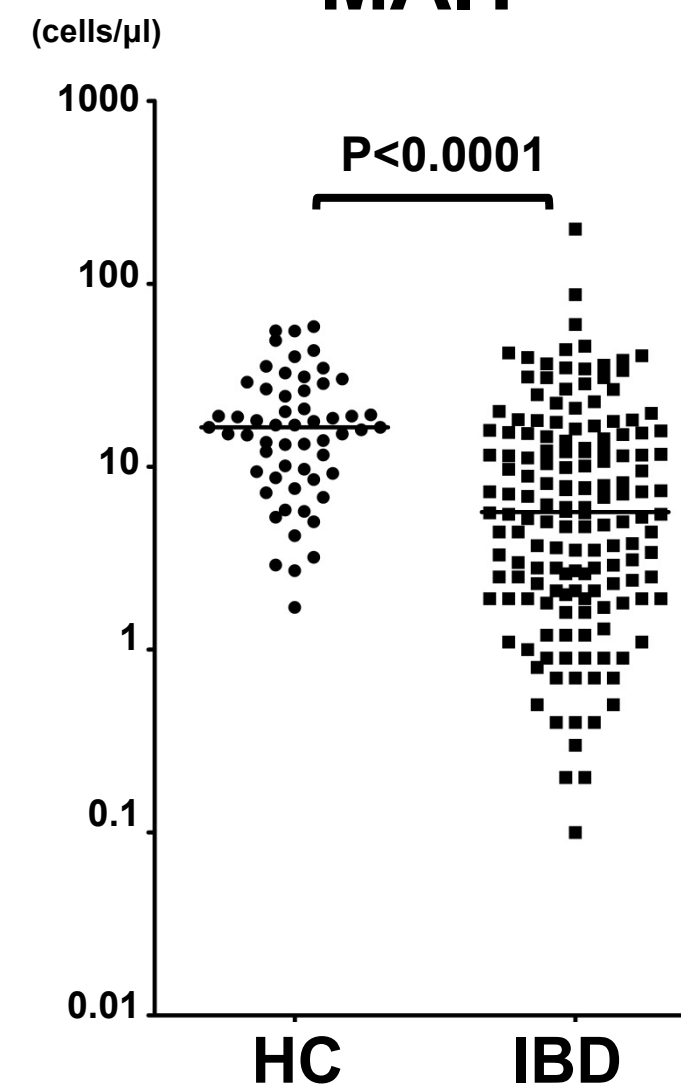
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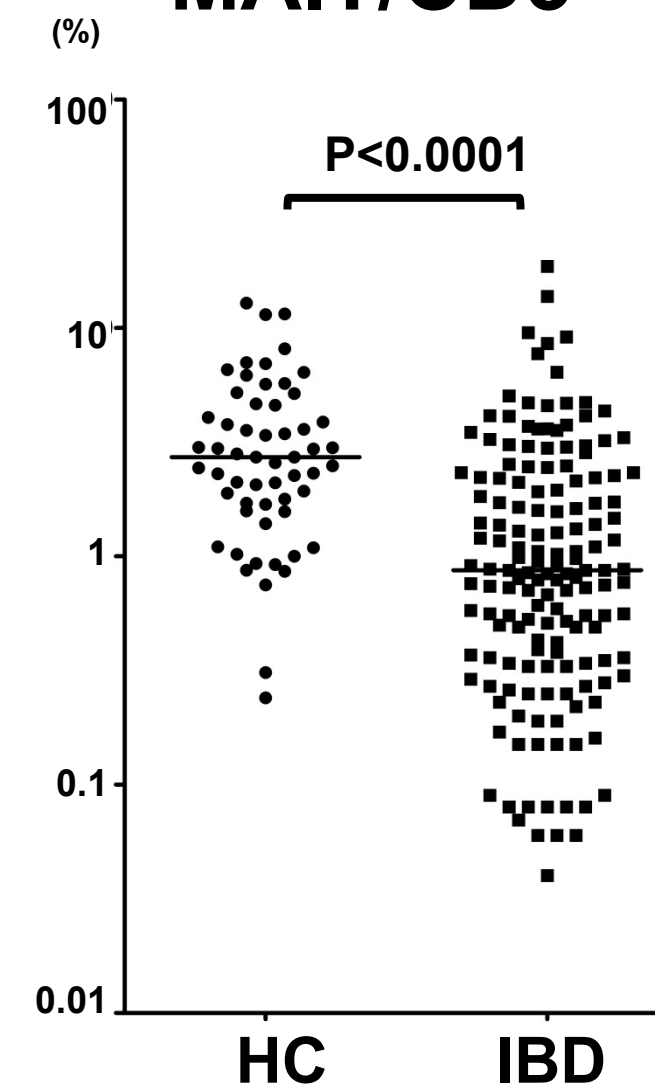
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1b

MAIT

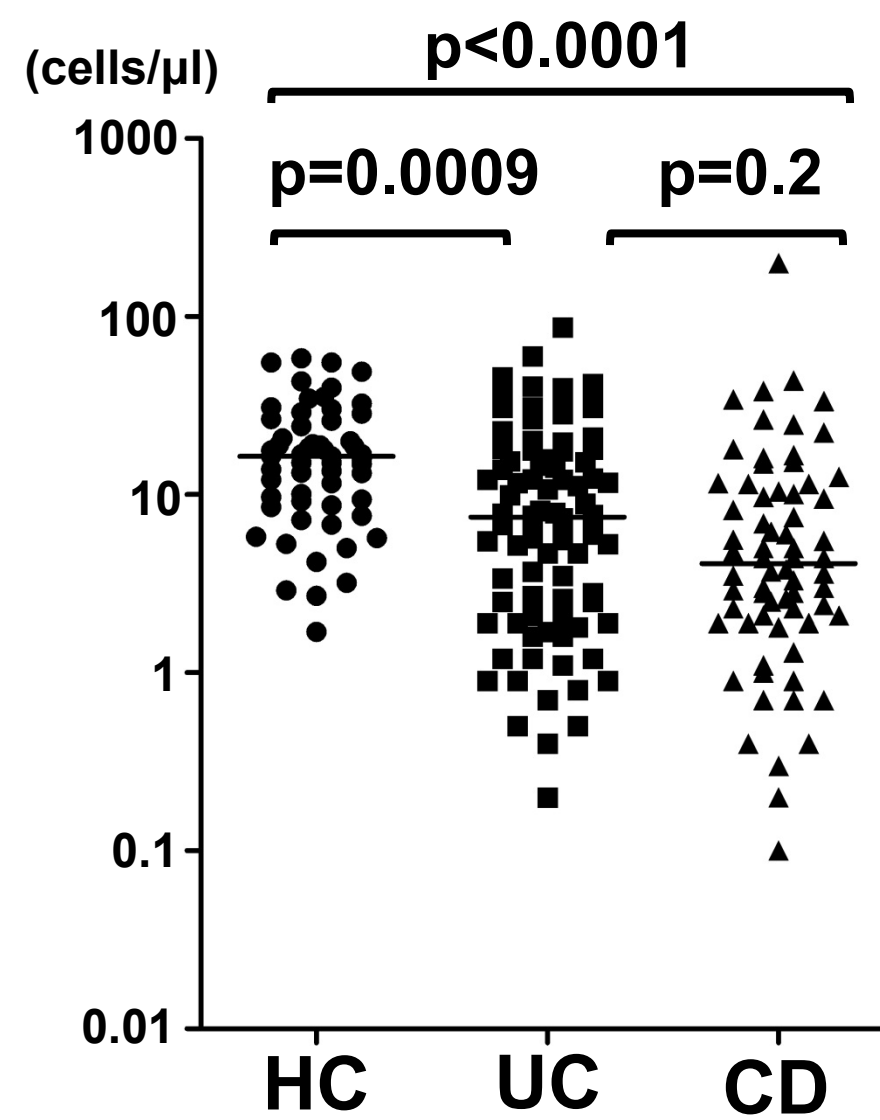


MAIT/CD3

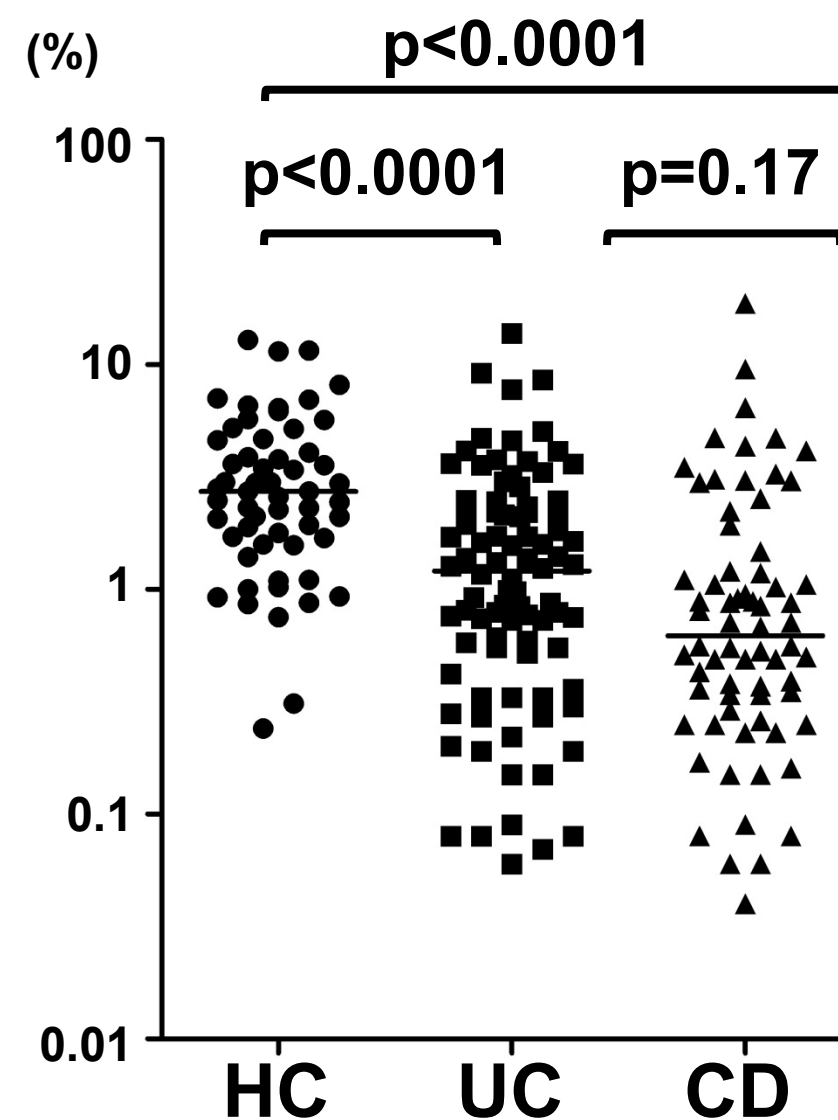


1c

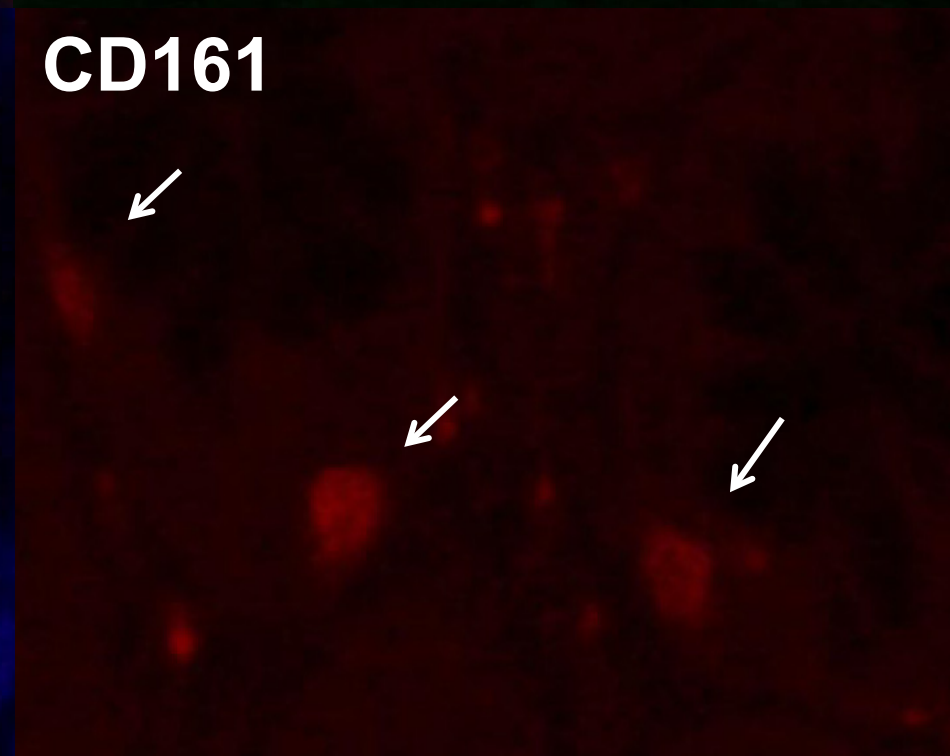
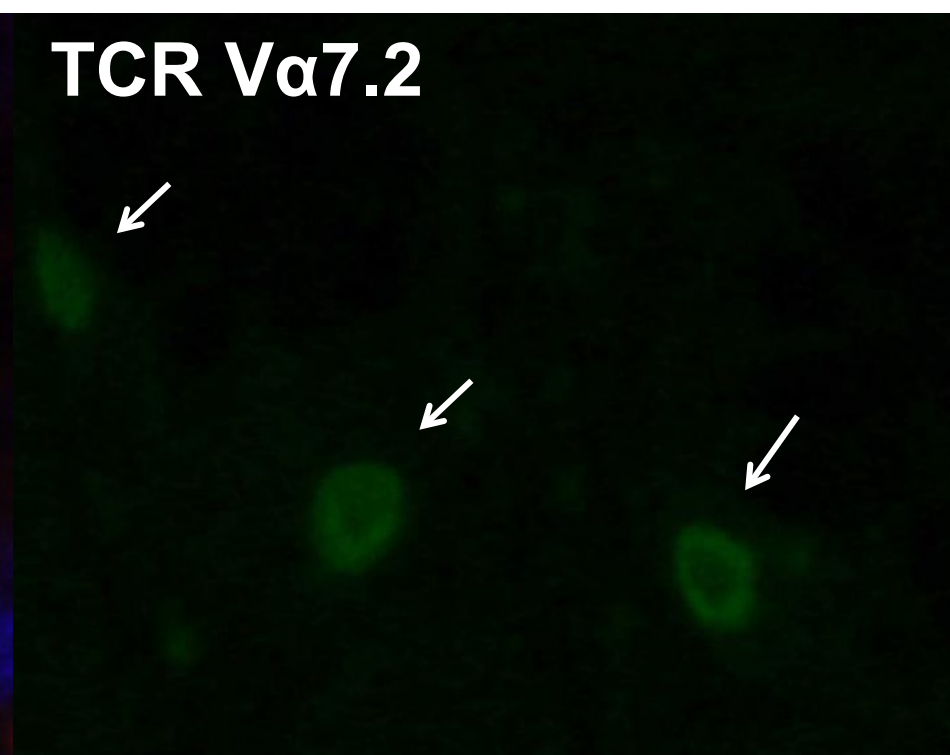
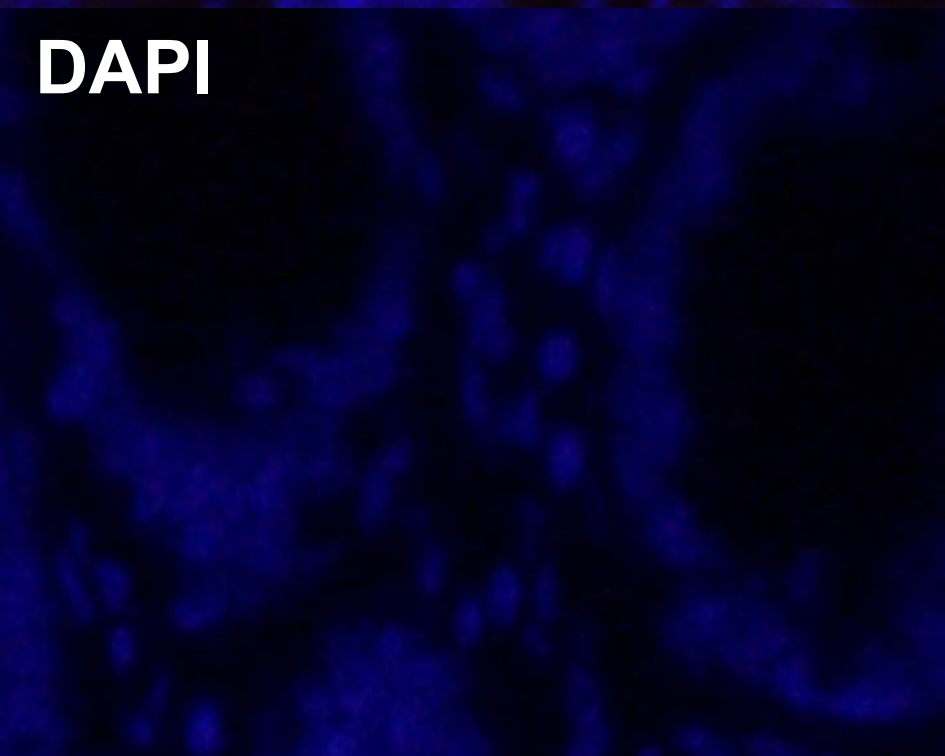
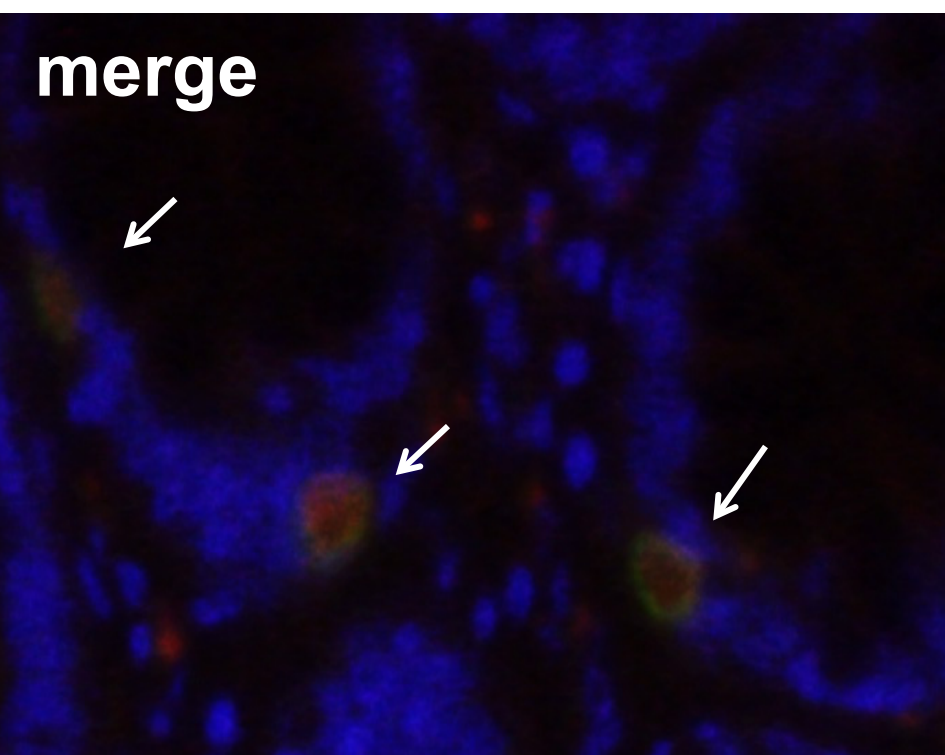
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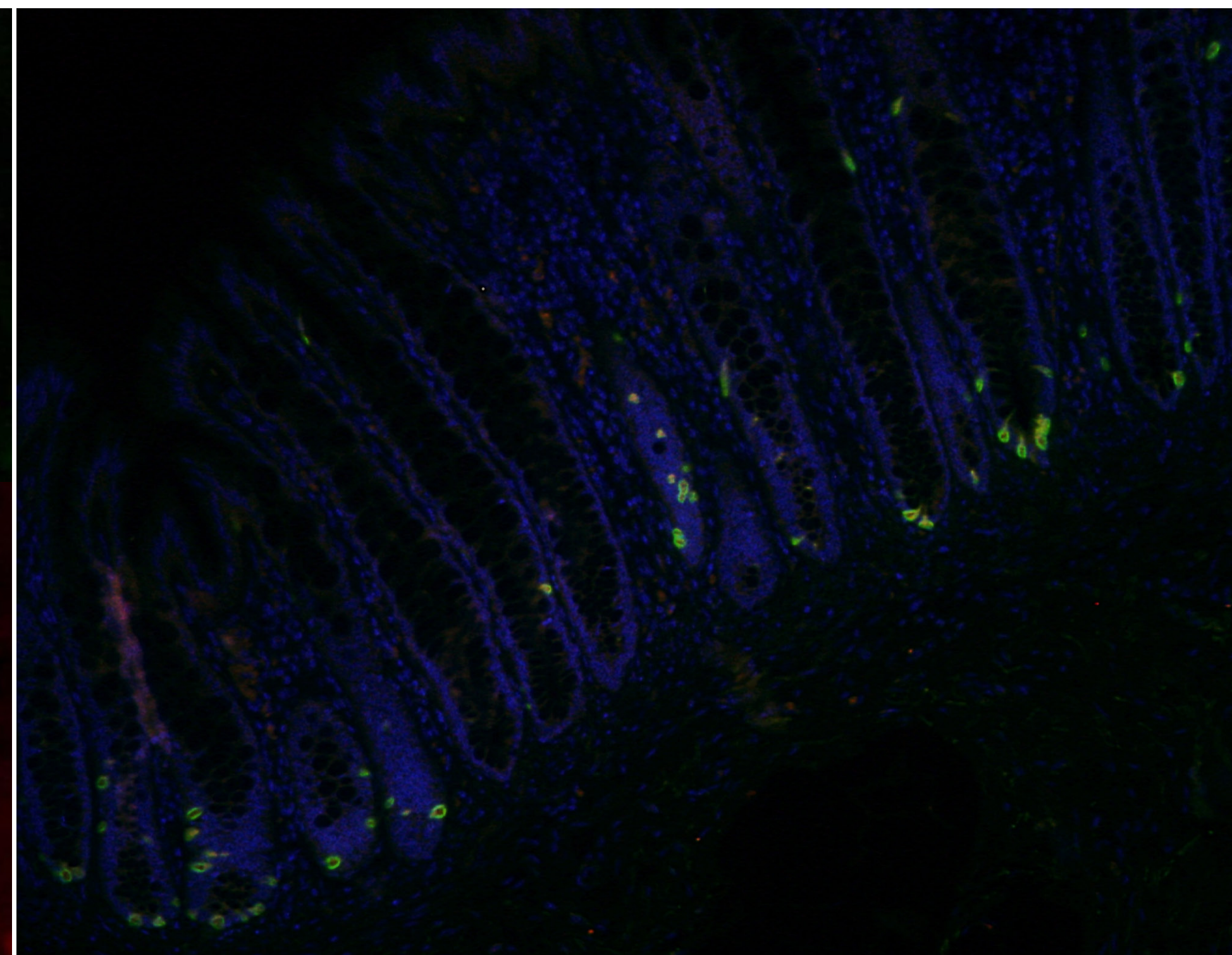
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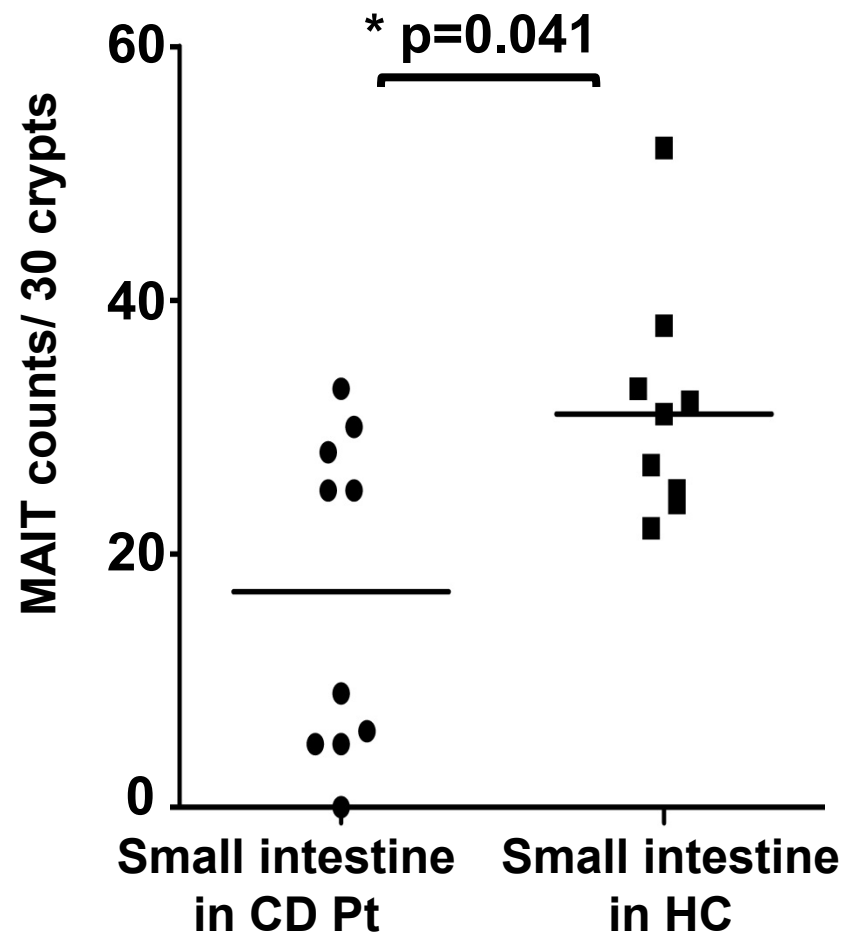
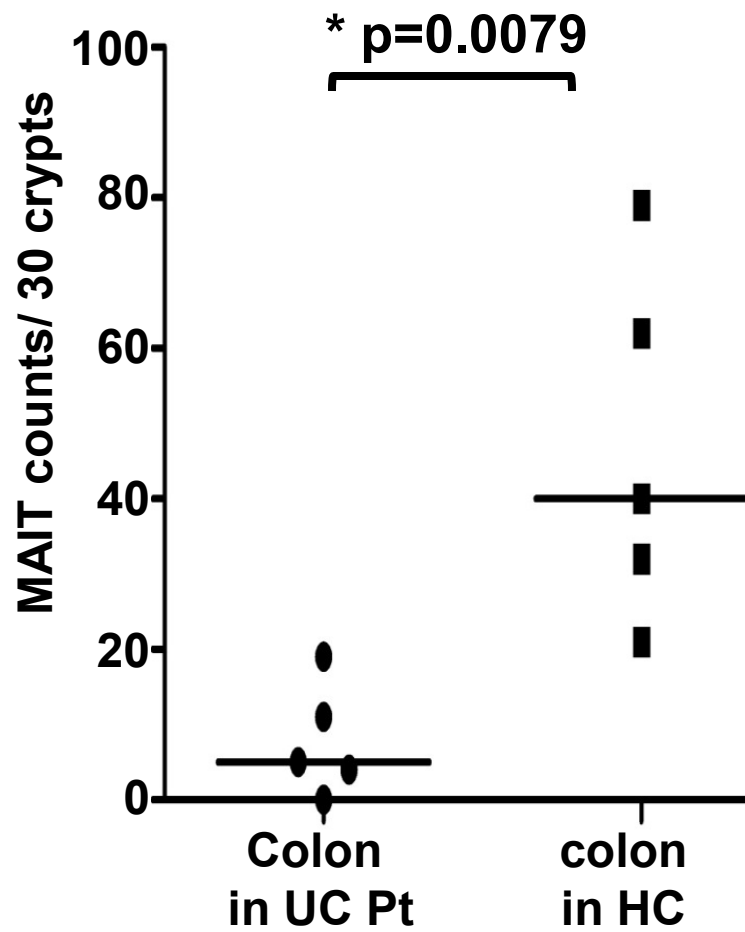
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2b

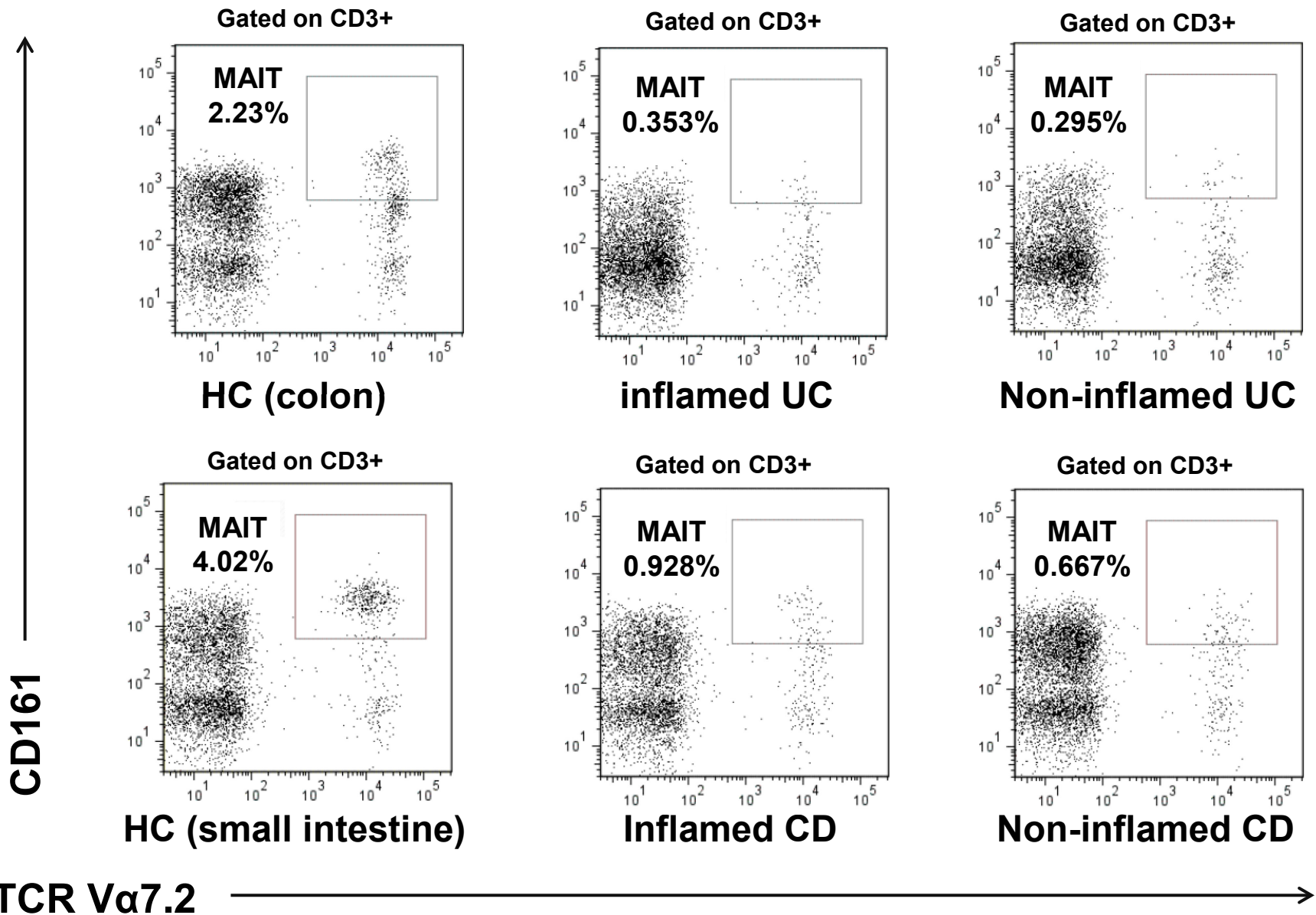


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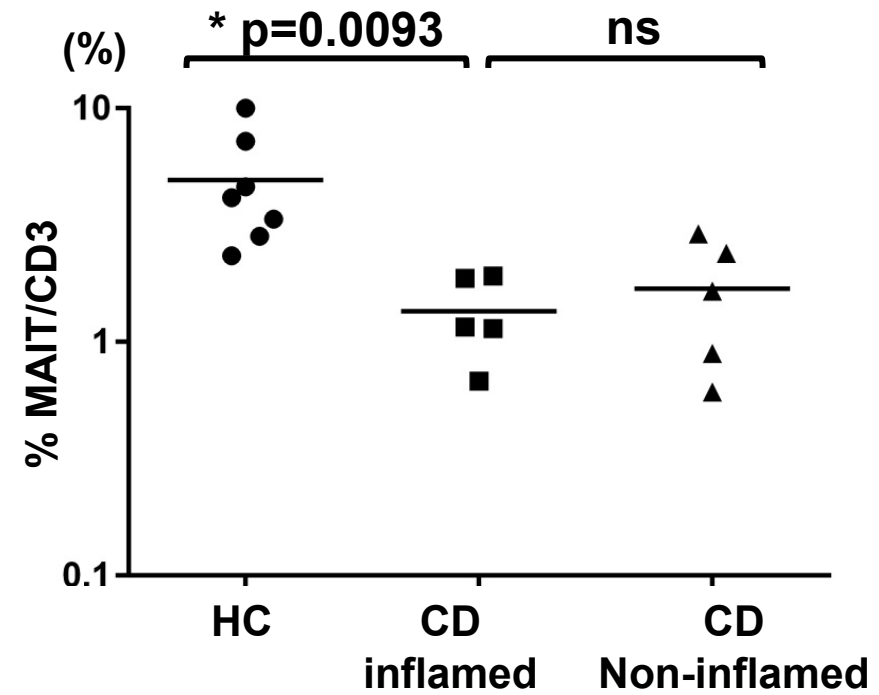
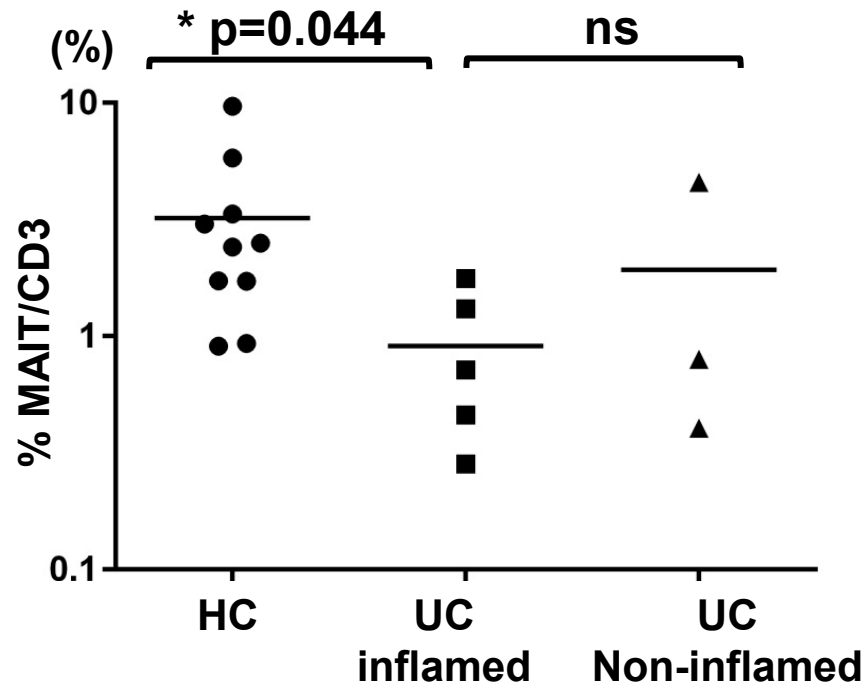


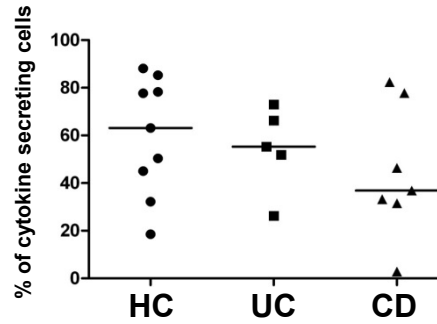
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Intestinal MAIT cells (CD161^{hi}TCR Vα7.2+CD3+)

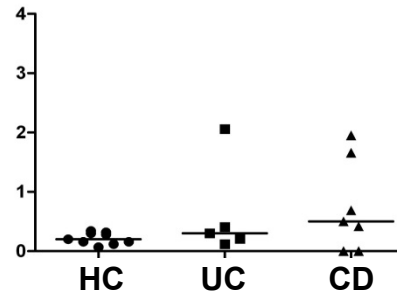


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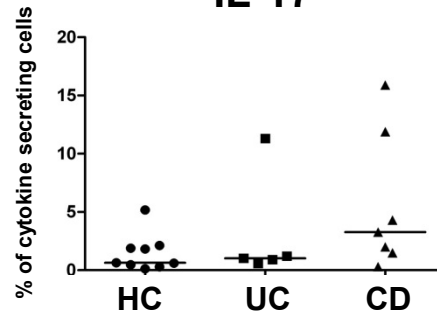
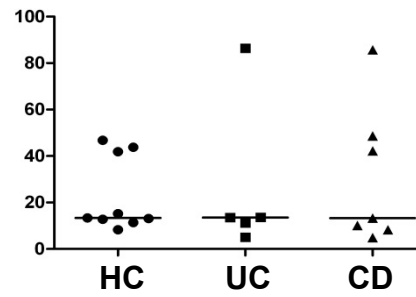


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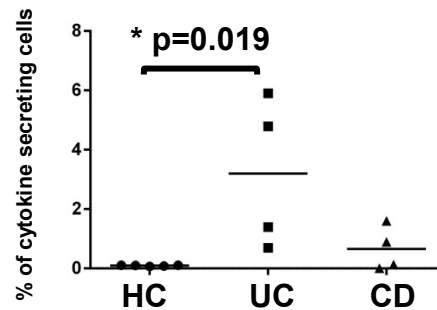
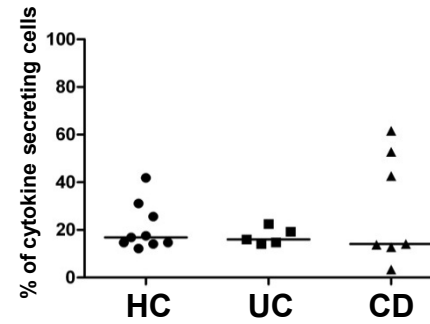
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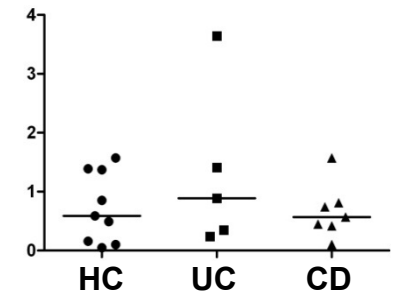
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TNF- α 

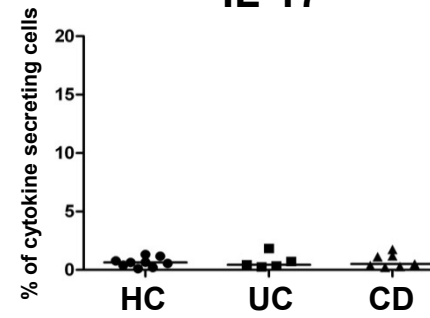
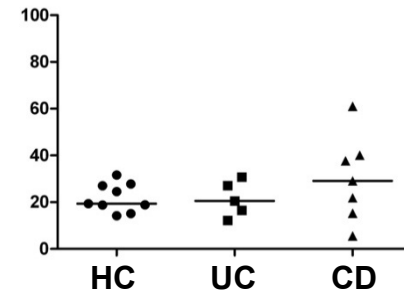
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IFN- γ 

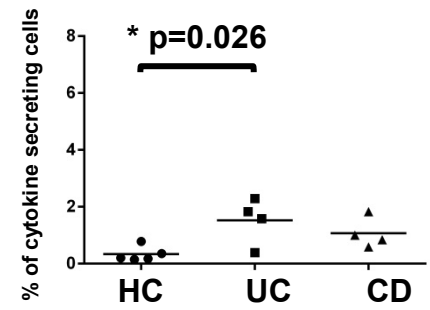
IL-10



IL-17

TNF- α 

IL-22



5a

**XIAP
deficiency**

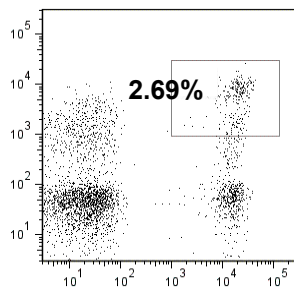
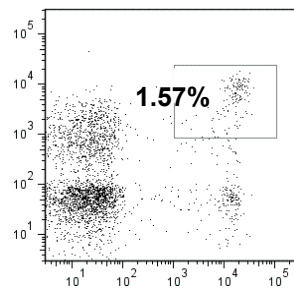
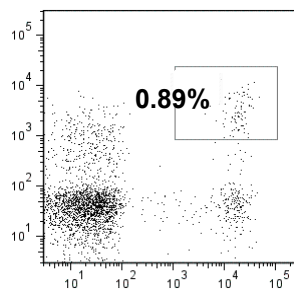
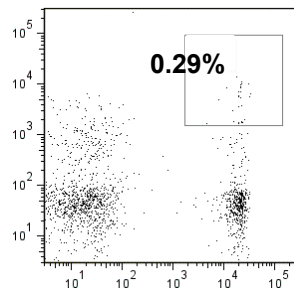
UC

CD

HC

CD161

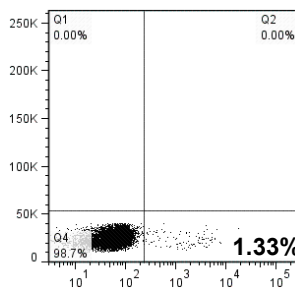
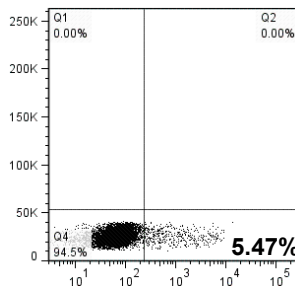
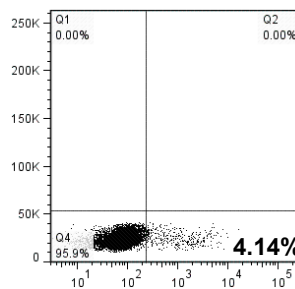
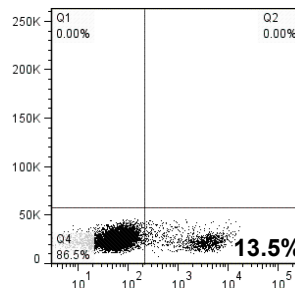
MAIT/CD3



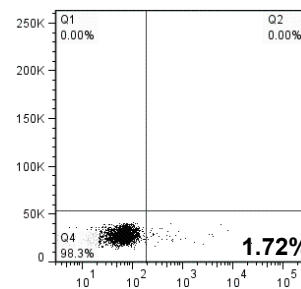
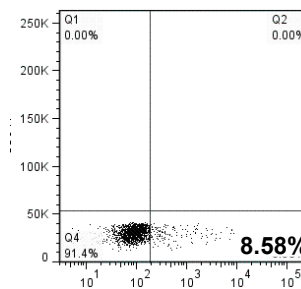
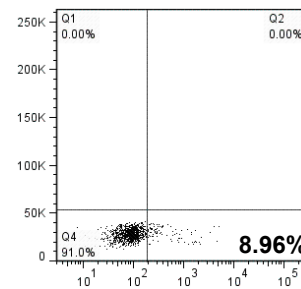
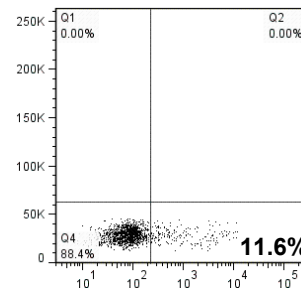
TCR Va7.2

SSC

gated on CD3+



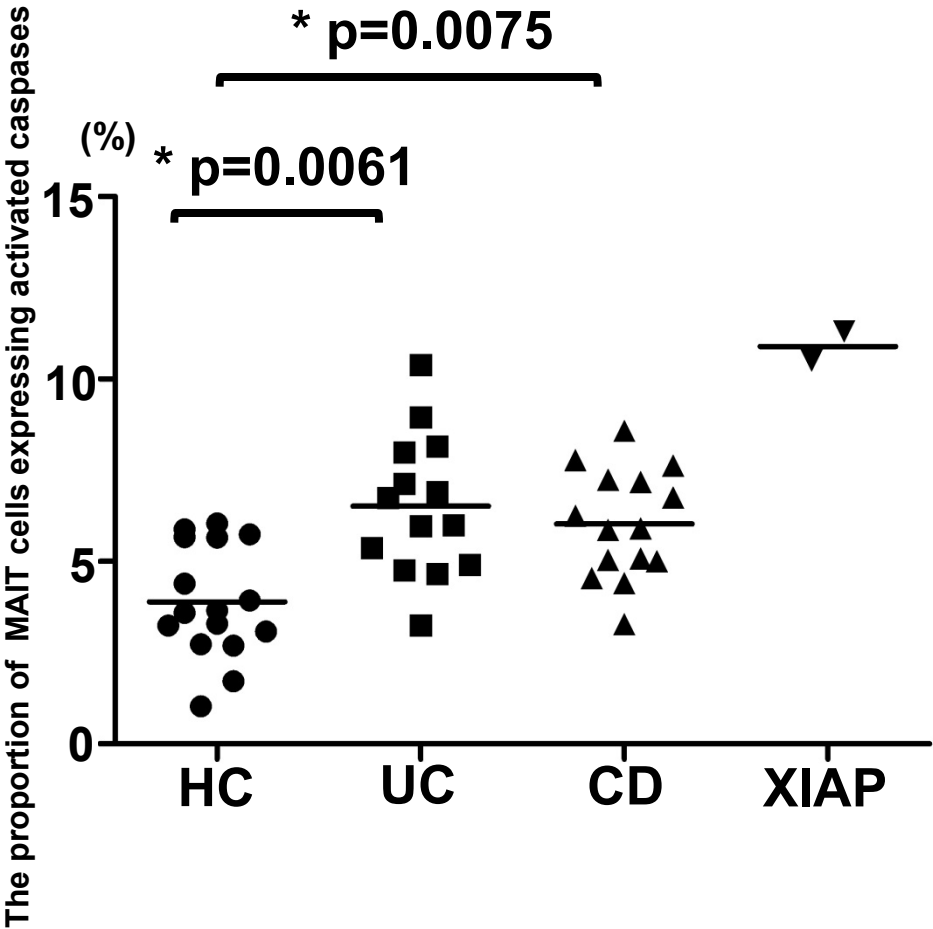
gated on MAIT



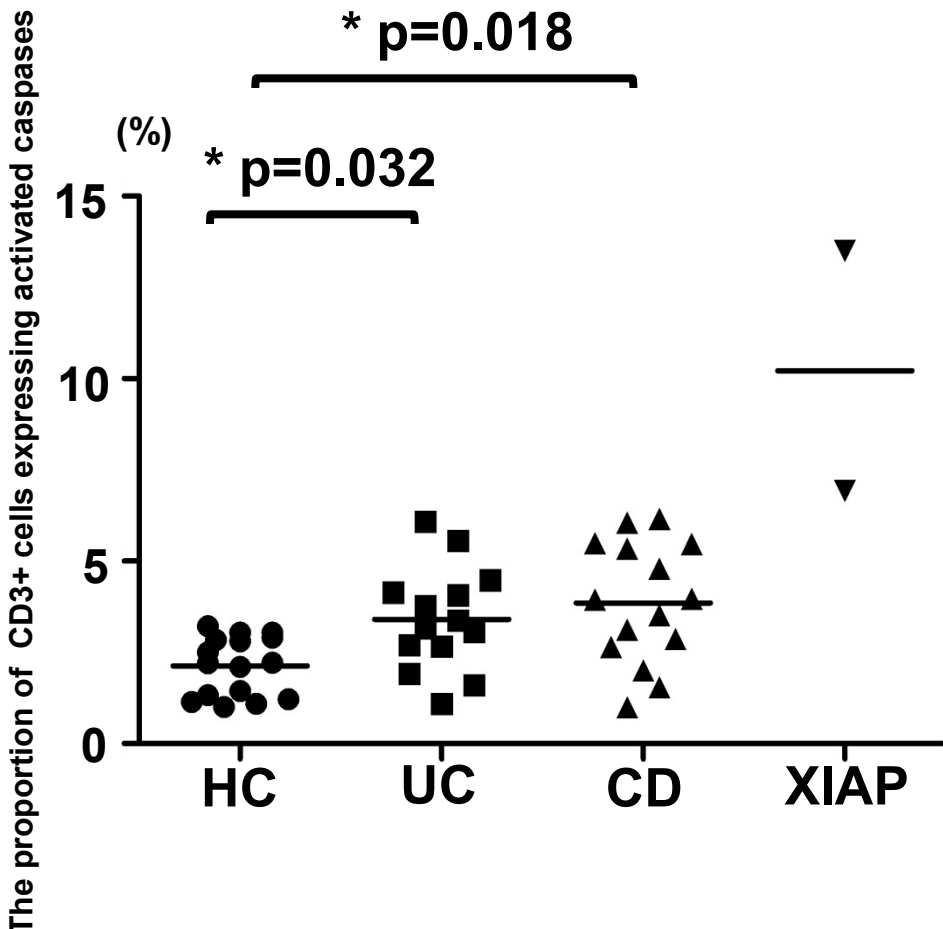
FLICA

5b

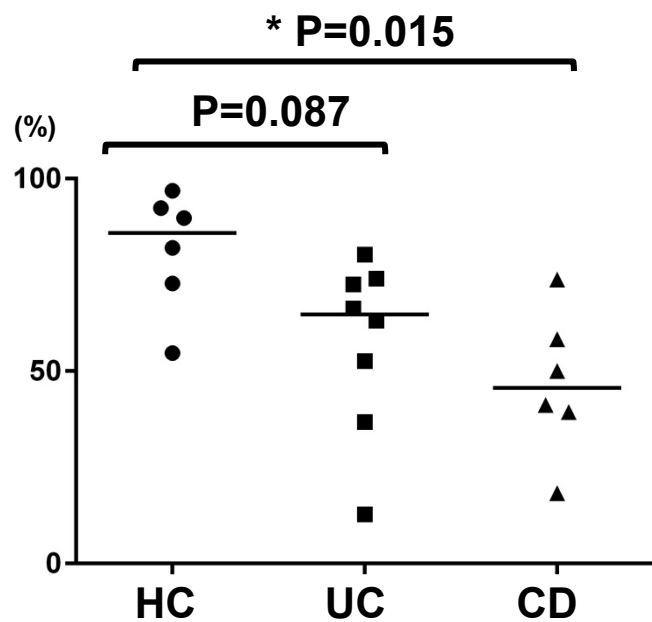
MAIT cells



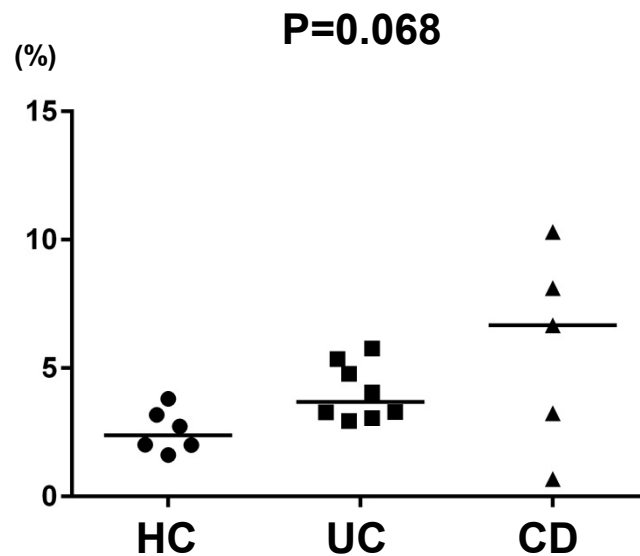
CD3+ lymphocytes



Expression of integrin $\alpha 4\beta 7$
on MAIT cells

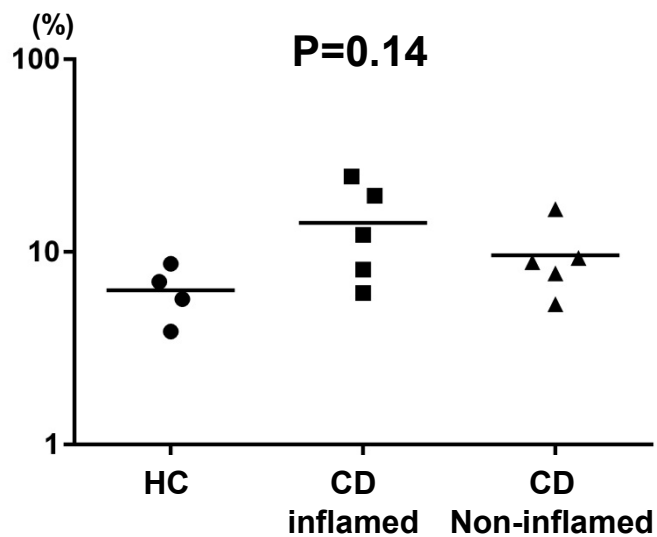


Expression of integrin $\alpha 4\beta 7$
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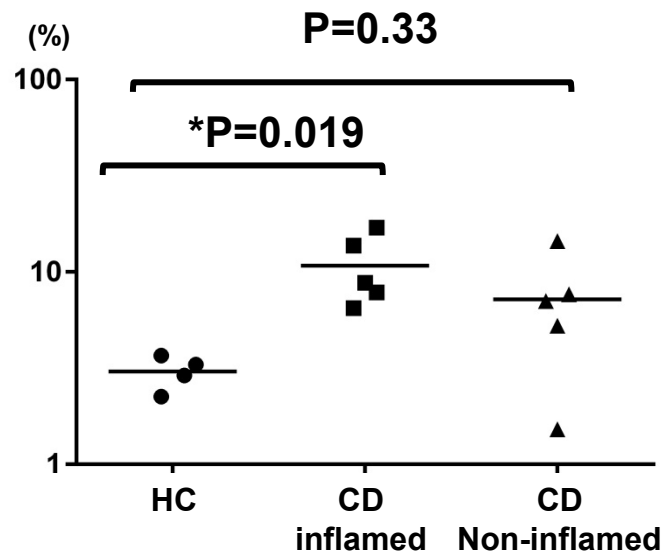


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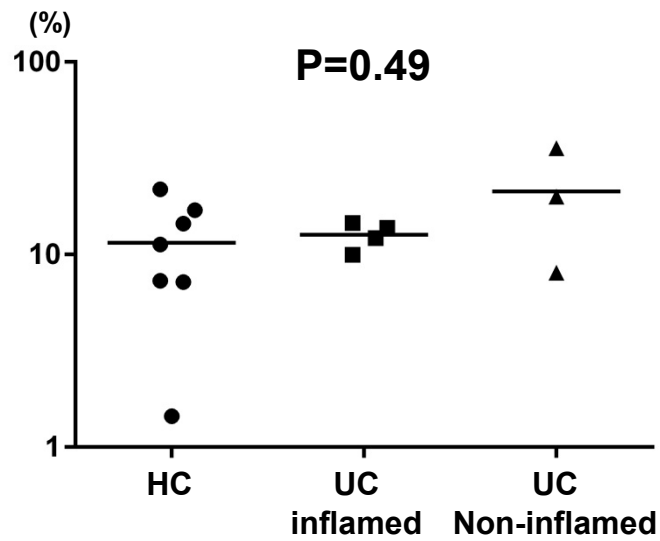
The proportion of MAIT cells expressing activated caspase



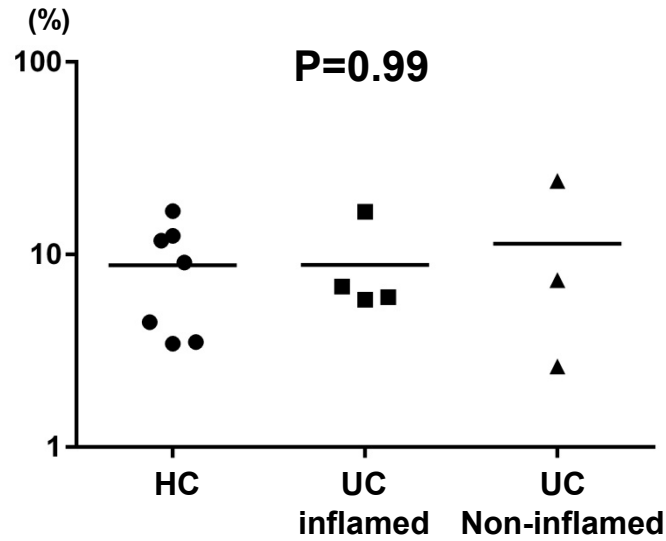
The proportion of Annexin V+ MAIT cells



The proportion of MAIT cells expressing activated caspase

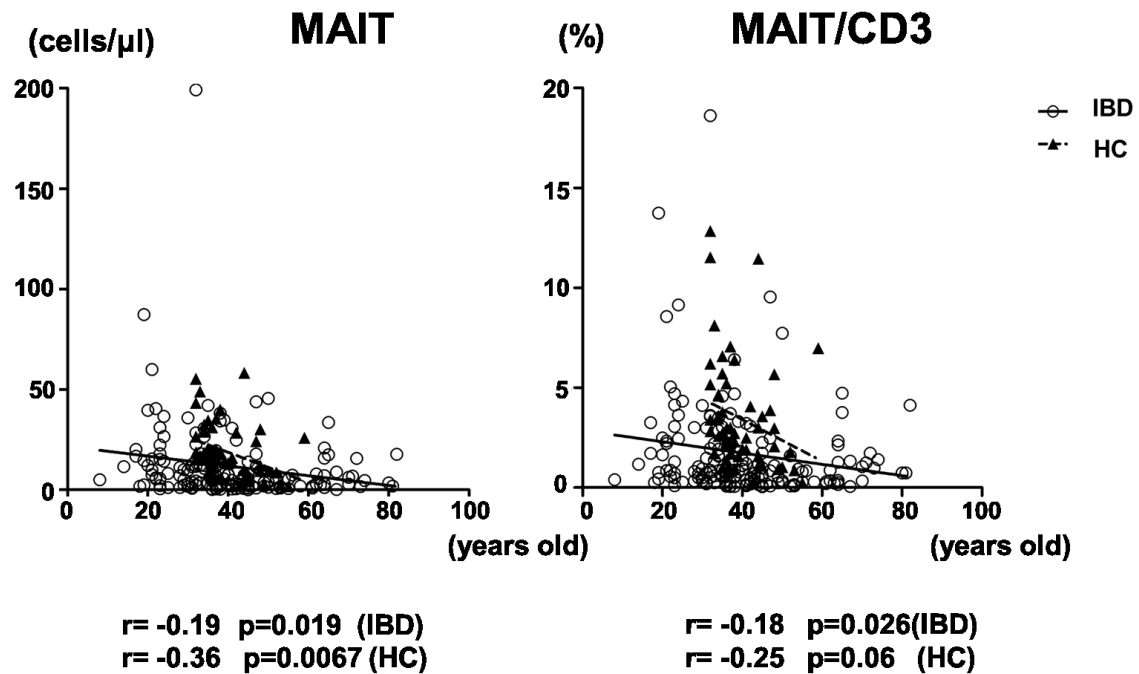


The proportion of Annexin V+ MAIT cells



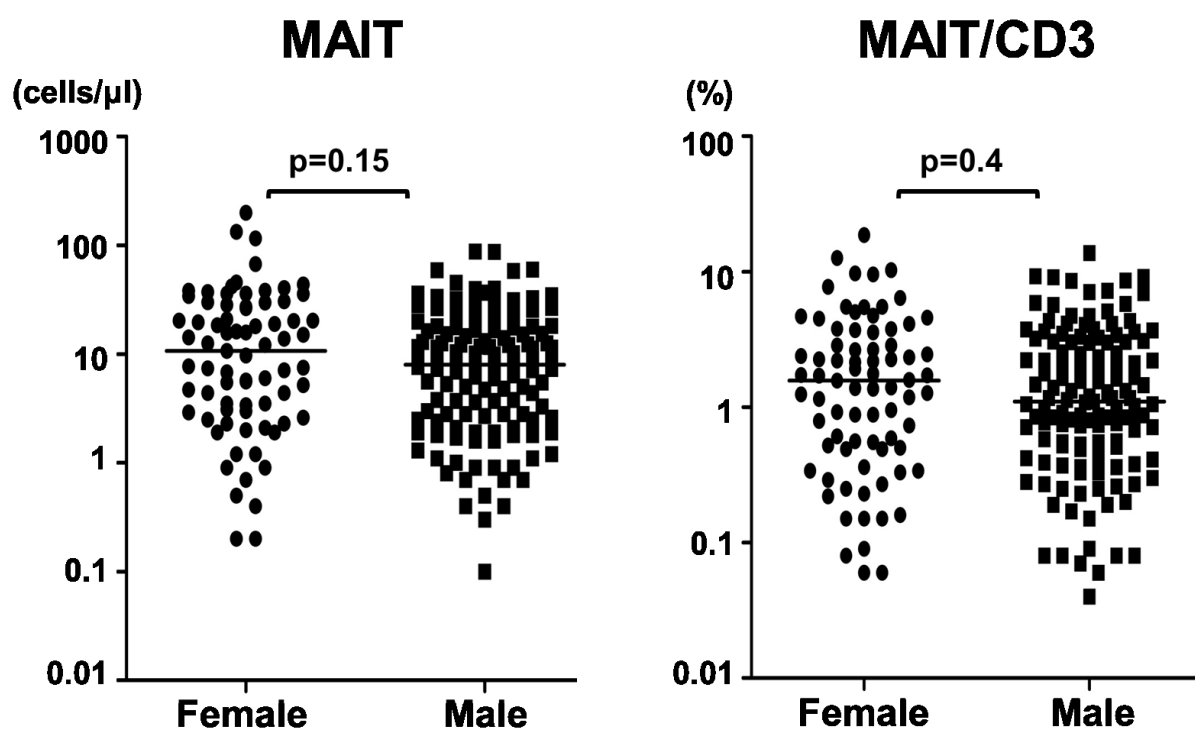
Supplemental Digital Content 1. Associations between age and MAIT-cell number and percentage in patients with IBD (n=156) and in healthy controls (n=57)

Correlation between MAIT-cell number or percentage with age. Open circle, IBD; closed triangle, HC. The linear regression lines for IBD and HC are indicated by solid and dashed lines, respectively. The correlations were analysed using the Pearson correlation.



Supplemental Digital Content 2. Associations between gender and MAIT-cell number and percentage in patients with IBD (n=156) and in healthy controls (n=57).

All 213 study subjects who provided peripheral blood were grouped according to whether they were female (n=81) or male (n=132). The bars indicate the median number of MAIT cells. The Mann-Whitney U-test was used for the comparison.

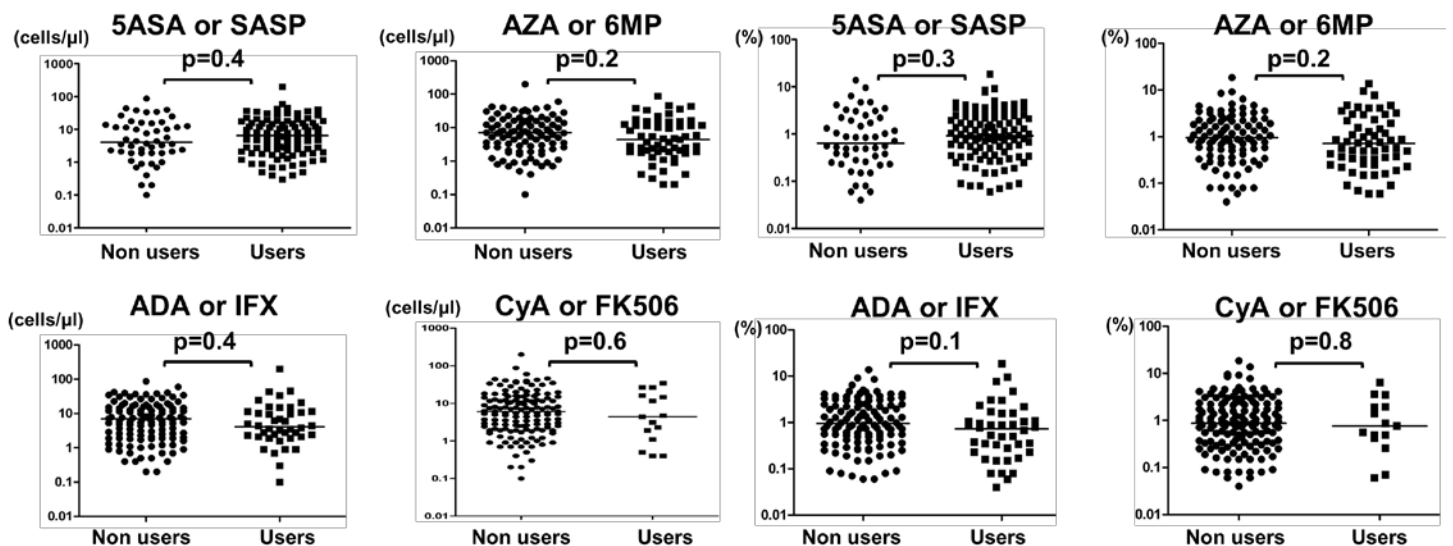


Supplemental Digital Content 3. Association between medication and MAIT-cell number and percentage in patients with IBD (n=156).

The IBD group was divided into those who had or had not received medications (Users and Non-users, respectively). The bars indicate the median number of MAIT cells. The Mann-Whitney U-test was used for the comparisons. 5ASA, 5-aminosalicylate; SASP, salazosulfapyridine; AZA, azathioprine; 6MP, 6-mercaptopurine; ADA, adalimumab; IFX, infliximab; CyA, cyclosporine A; FK506, tacrolimus.

MAIT

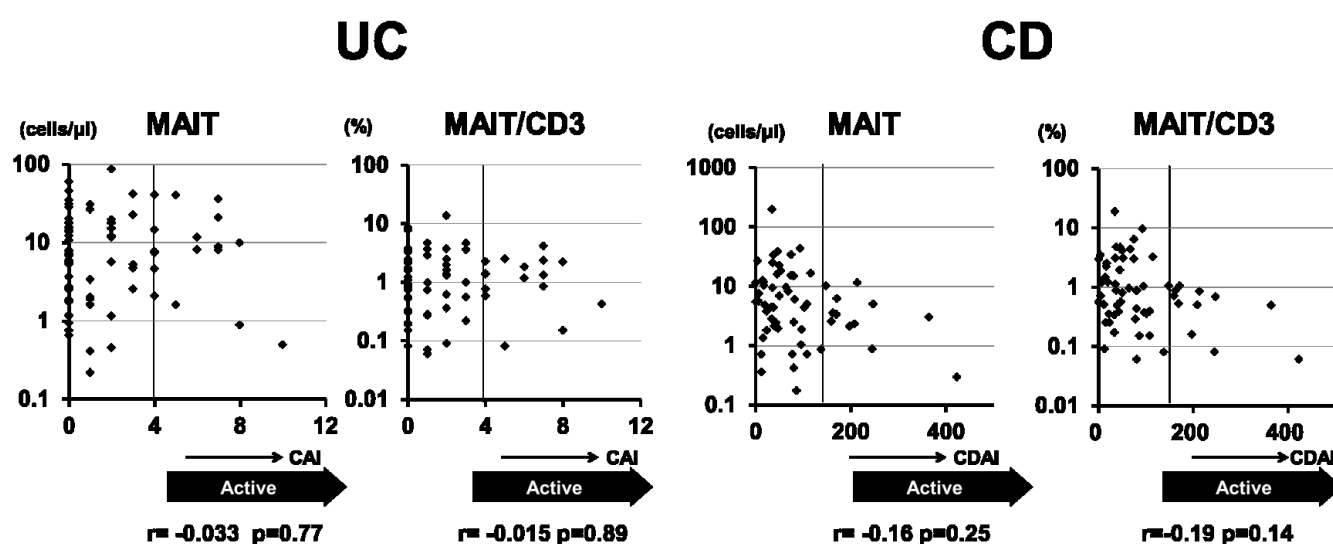
MAIT/CD3



Supplemental Digital Content 4. Associations between clinical activity and MAIT-cell number and percentage in patients with UC (n=88) and CD (n=68).

Correlation between the absolute number of MAIT cells or percentage and the clinical activity of UC (left) and CD (right). CAI, clinical activity index; CDAI, Crohn's disease activity index. A CAI of ≥ 4 was considered to indicate clinically active UC (n=17).

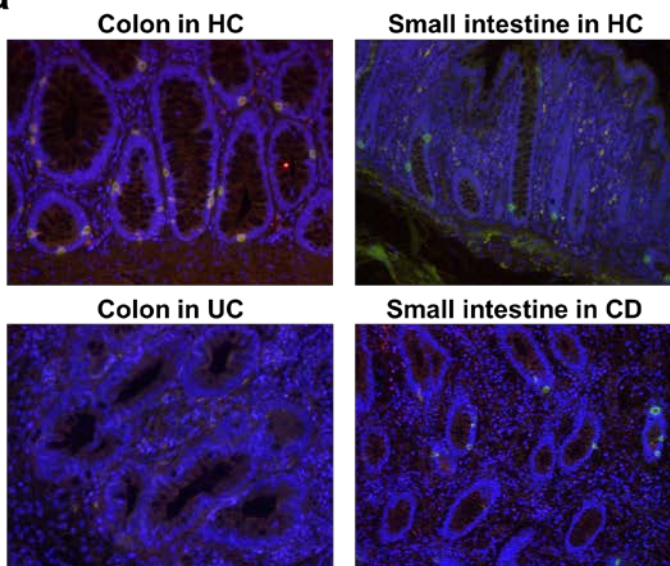
A CDAI of ≥ 150 was considered to indicate clinically active CD (n=11). The correlations were analysed using the Pearson correlation.



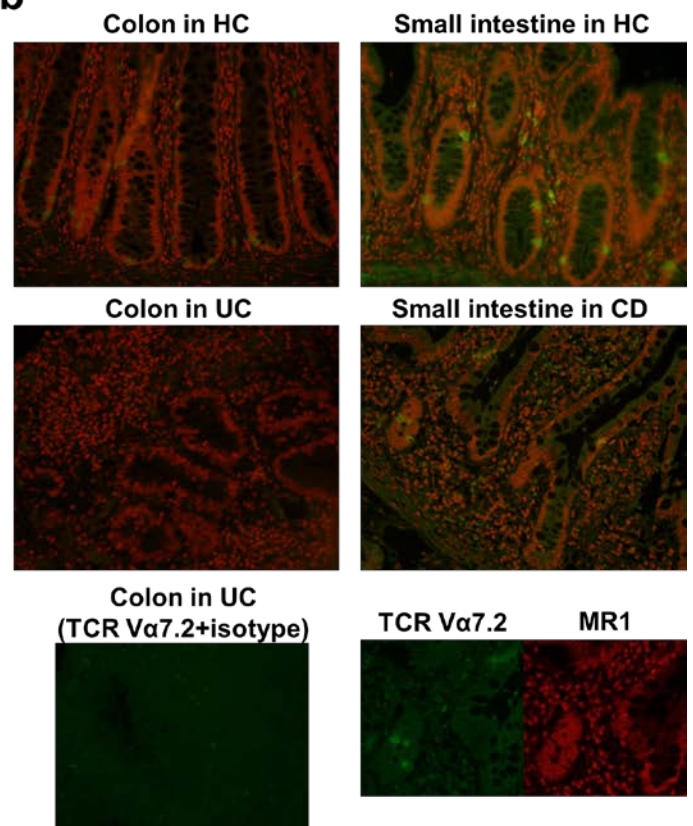
Supplemental Digital Content 5a and 5b. Immunohistochemical analysis of MAIT-cell number and percentage in intestinal tissue samples from patients with UC (n=5), CD (n=10) and controls.

All UC samples were from the colon, while all CD samples were from the small intestine. The control samples consisted of normal small intestinal (n=9) or colonic (n=5) tissue resected from patients undergoing surgery for gastrointestinal cancer. (5a) Representative immunohistochemistry images showing the MAIT cells (CD161⁺TCRV α 7.2⁺) in the colonic mucosae of a control subject (upper left), the ulcerative colonic mucosae of a patient with UC (lower left), the small intestinal mucosae of a control subject (upper right), and the injured small intestine mucosae of a patient with CD (lower right). Original magnification, $\times 100$. (5b) Representative image of the small intestine in a control subject after immunohistochemical staining with antibodies specific for TCRV α 7.2 (green) and MR1 (orange). Original magnification, $\times 100$.

S5a



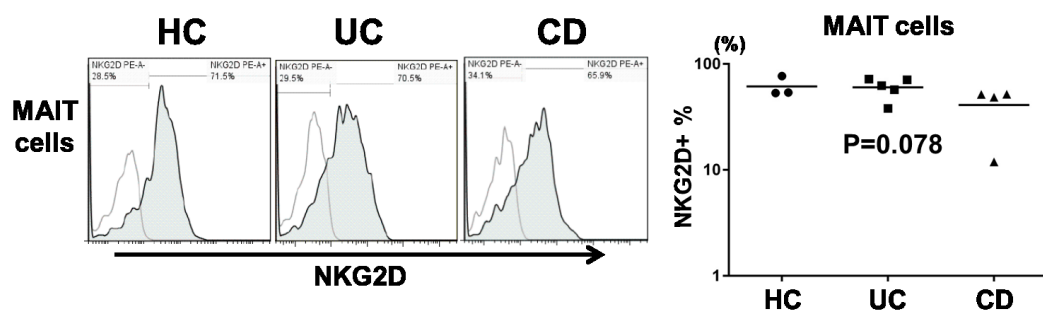
S5b



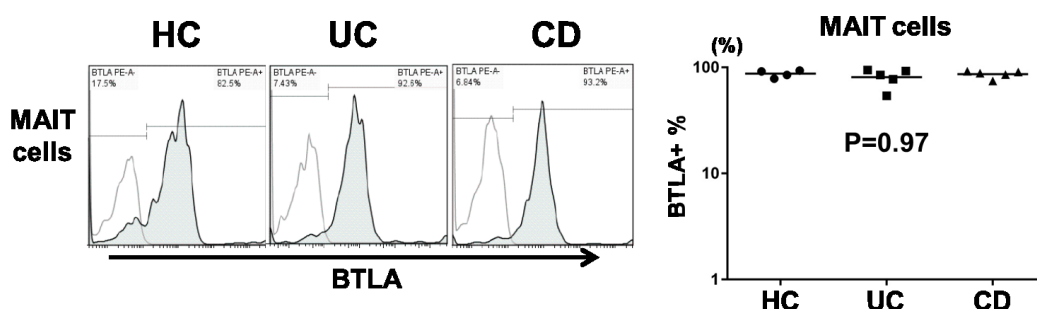
Supplemental Digital Content 6a 6b Natural killer (NK)G2D and BTLA expression on MAIT cells in the peripheral blood from IBD patients and healthy controls.

Data are representative of expression of NKG2D (6a) or BTLA(6b) on MAIT(CD161^{high}TCRV α 7.2⁺CD3⁺) cells among UC patients, CD patients, and healthy controls (HC)(left).The shaded histograms represent the staining of NKG2D (6a) or BTLA (6b); the empty profiles represent the staining with isotype-matched control. Percentages of NKG2D–positive (6a) and BTLA-positive (6b) MAIT cells among three groups (6a: UC n=5, CD n=4, HC n=3; 6b: UC n=5, CD n=5, HC n=4) were compared (right). The bars indicate the median values. P-values of the Kruskal-Wallis test are shown.

S6a

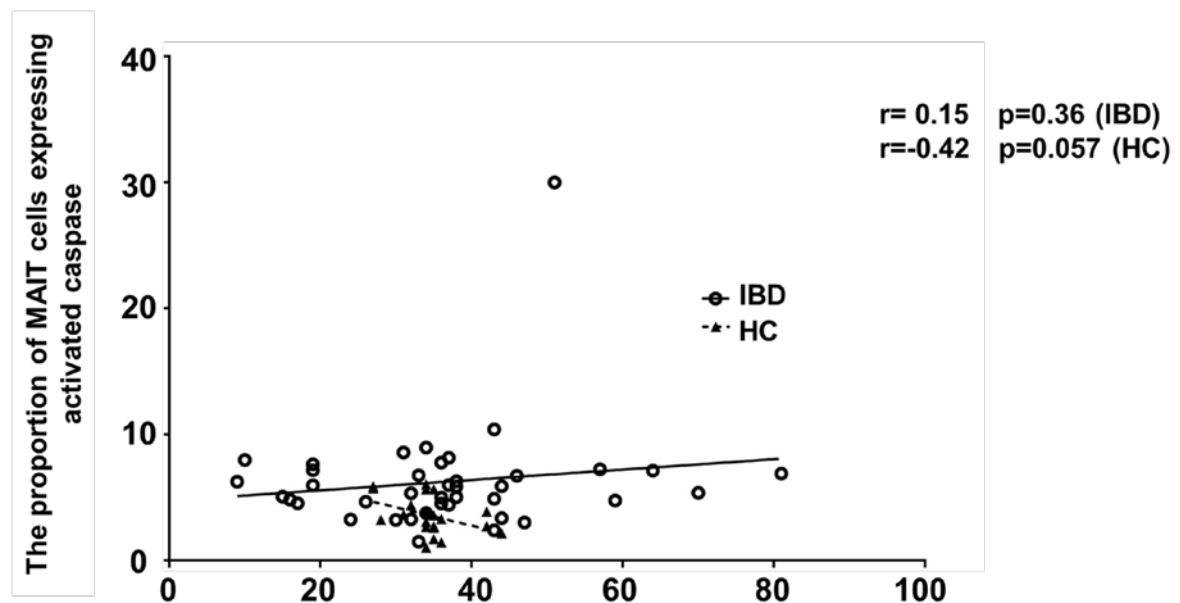


S6b



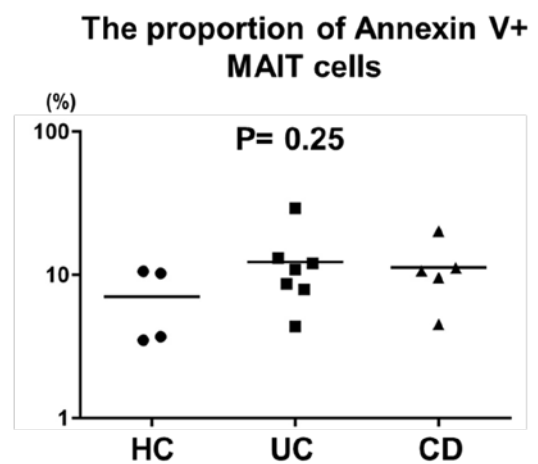
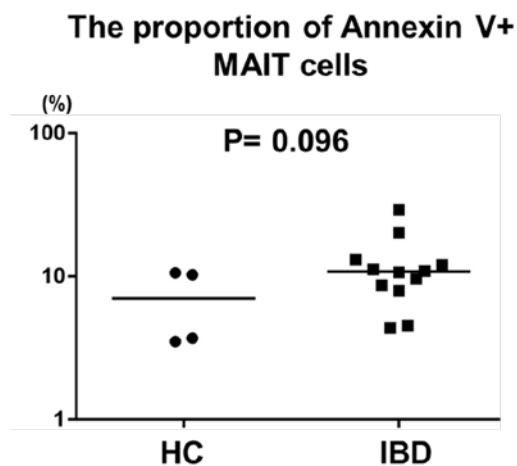
Supplemental Digital Content 7. Associations between age and the proportion of MAIT cells expressing activated caspase in patients with IBD (n=40) and in healthy controls (n=21)

Correlation between age and the proportion of MAIT cells expressing activated caspase. Open circle, IBD; closed triangle, HC. The linear regression lines for IBD and HC are indicated by solid and dashed lines, respectively. The correlations were analysed using the Pearson correlation.



Supplemental Digital Content 8. The proportion of Annexin V positive MAIT cells in the peripheral blood of patients with IBD

Peripheral blood mononuclear cells from patients with UC (n=7), patients with CD (n=5), and healthy controls (HC, n=4) were collected. Percentages of annexin V-positive, 7AAD-negative MAIT cells were analysed. Comparison between IBD and HC (left). The bars indicate the median number of MAIT cells. The Mann-Whitney U-test was used for the comparison. Comparison between UC and CD patients, and HC (right). The bars indicate the median values. P-values of the Kruskal-Wallis test are shown.



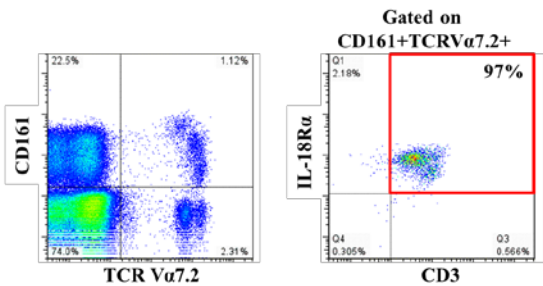
Supplemental Digital Content 9a and 9b. Flow cytometric analysis of mucosal lymphocytes from IBD patients and non-IBD controls.

Dot plots depicting the expression of IL-18R α (y-axis) and CD3 (x-axis) among CD161⁺TCRV α 7.2⁺ cells (9a), and the expression of CD161 (x-axis) among IL-18R α ⁺CD3⁺TCRV α 7.2⁺ cells (9b) in mucosal lymphocytes from the colon of an UC patient, the small intestine of a CD patient, and histologically normal colon and small intestine.

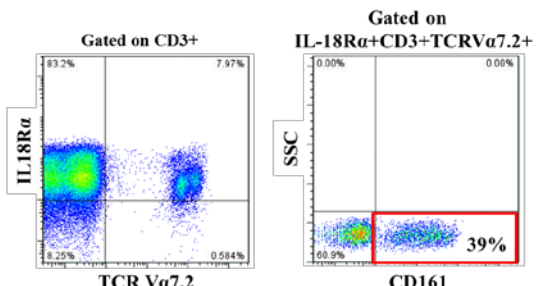
S9a

S9b

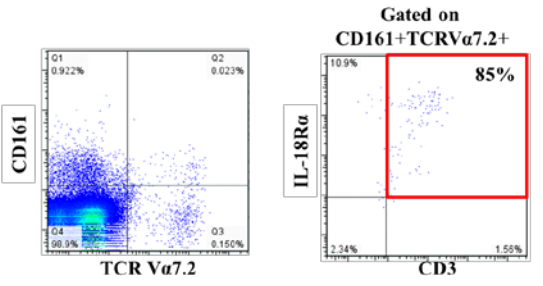
Colon
in HC



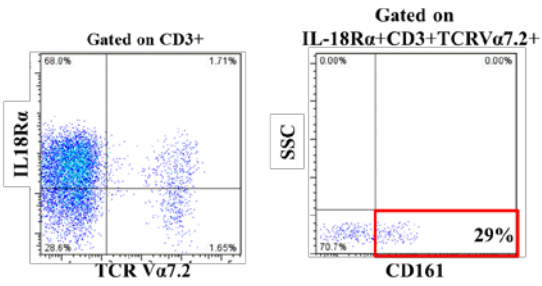
Colon
in HC



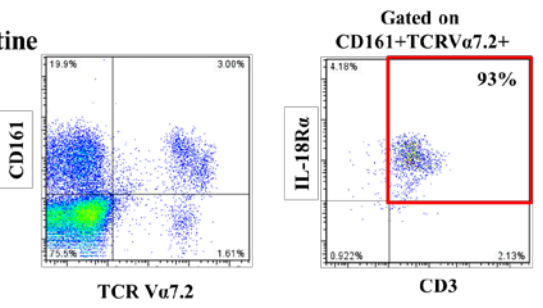
Colon
in UC



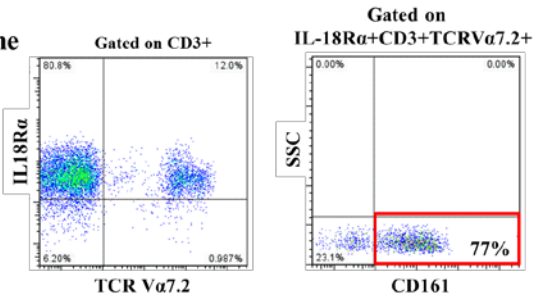
Colon
in UC



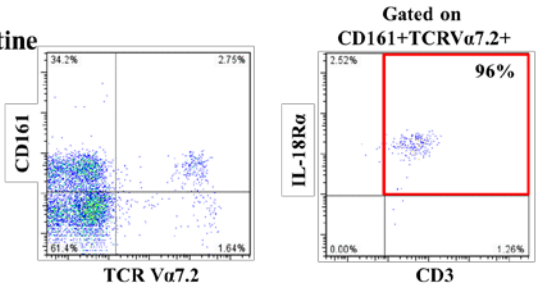
Small intestine
in HC



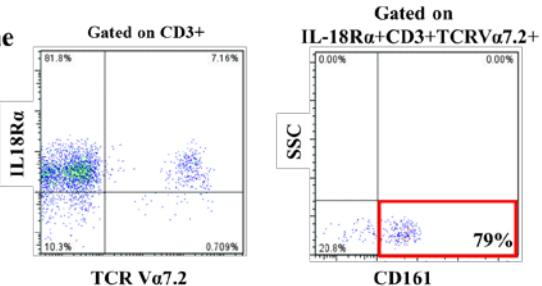
Small intestine
in HC



Small intestine
in CD

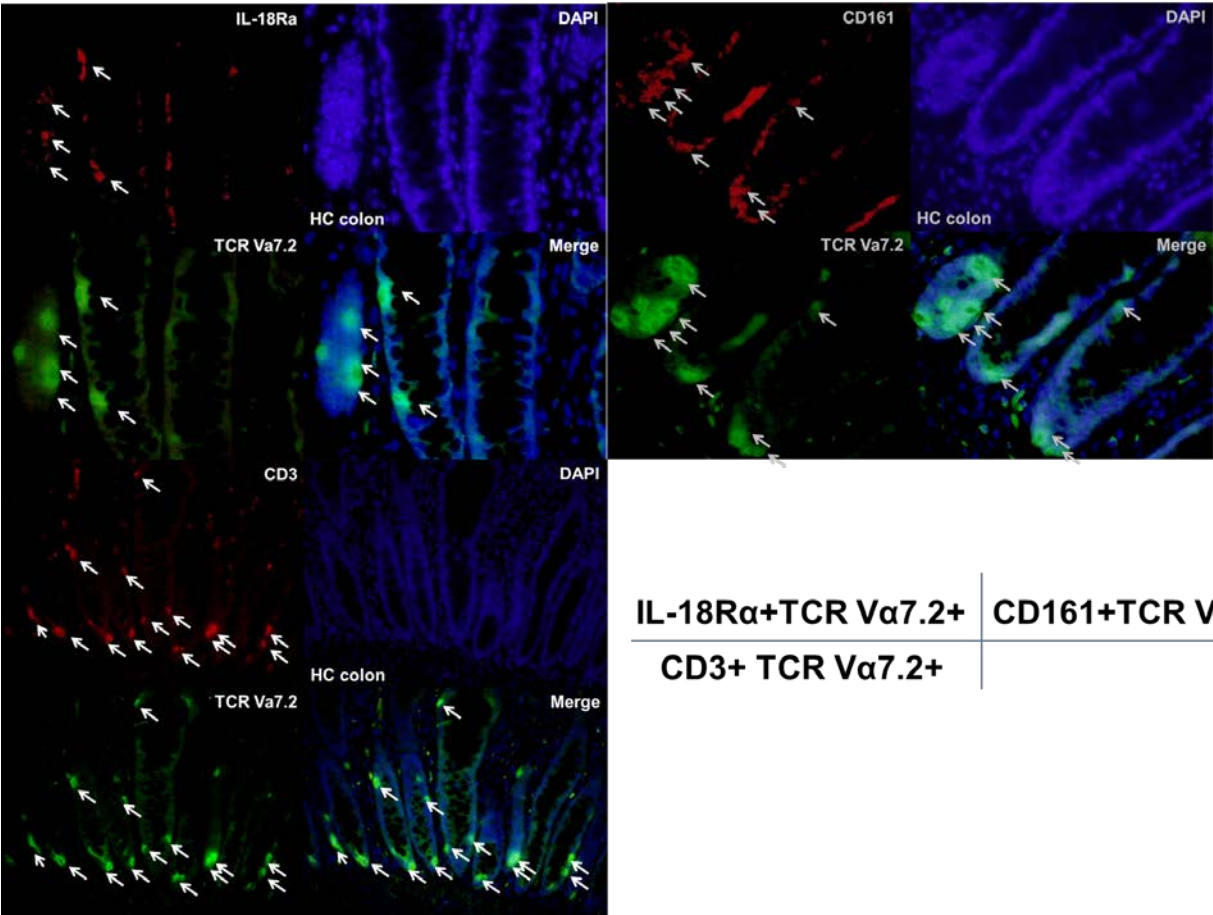


small intestine
in CD



Supplemental Digital Content 10. Immunohistochemical double staining of serial sections of normal control colon samples.

Histologically normal control colon samples were obtained from non-IBD patients undergoing surgery for colon cancer. To verify whether CD161⁺TCRVα7.2⁺ cells and IL-18Rα⁺CD3⁺TCRVα7.2⁺ cells were identical, serial sections were subjected to immunohistochemical analysis with antibodies specific for TCRVα7.2 (green) and CD161 (red; upper right), TCRVα7.2 (green) and IL-18Rα (red; upper left), and TCRVα7.2 (green) and CD3 (red; lower left). The arrows indicate double positive cells. Original magnification, ×100.



Supplemental Digital Content 11. The proportion of activated caspase-expressing MAIT cells or Annexin V positive MAIT cells in the small intestine and colon of non-IBD control.

Intestinal tissue samples consisted of normal small intestinal (n=4) and colonic (n=7) tissue of control were subjected to flow cytometry. All samples were obtained from the same specimens which were prepared for analysis of the intestinal MAIT-cell frequencies (Figure 3 and 7). Comparison of percentage of activated caspase-expressing MAIT (CD161^{high}TCRV α 7.2⁺CD3⁺) cells (left) in non-IBD control small intestine and colon. Comparison of percentage of annexin V-positive, 7AAD-negative MAIT cells (right) between these groups. The bars indicate the median number of MAIT cells. The Mann-Whitney U-test was used for the comparison.

The proportion of Annexin V+ MAIT cells

The proportion of MAIT cells expressing activated caspase

