Targeting activation-induced cytidine deaminase prevents colon cancer development despite persistent colonic inflammation

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Nonstandard abbreviations used: AID, activation-induced cytidine deaminase; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; ISH, *in situ* hybridization; mAb, monoclonal antibody; NF, nuclear factor; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumor necrosis factor; WT, wild-type.

Grant Support: This work was supported by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science; a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare, Japan; a Research Grant of the Princess Takamatsu Cancer Research Fund.

Abstract

Inflammatory bowel disease (IBD) is an important etiologic factor in the development of colorectal cancer. However, the mechanism underlying carcinogenesis through chronic inflammation is still unknown. Activation-induced cytidine deaminase (AID) is induced by the inflammation and involved in various human carcinogenesis via its mutagenic activity. In the current study, we investigated whether the inflammation/AID axis plays an integral role in the development of colitis-associated cancers. Inflammation in the cecum was more severe than that in other colonic regions, and endogenous AID expression was enhanced most prominently in the inflamed cecal mucosa of interleukin (IL)-$10^{-/-}$ mice. Blockade of tumor necrosis factor (TNF)-α and IL-12 significantly suppressed AID expression. Although proinflammatory cytokine expression was comparable between IL-$10^{-/-}$AID$^{+/+}$ and IL-$10^{-/-}$AID$^{-/-}$ mice, sequencing analyses revealed a significantly lower incidence of somatic mutations in *Trp53* gene in the colonic mucosa of IL-$10^{-/-}$AID$^{-/-}$ than IL-$10^{-/-}$AID$^{+/+}$ mice. Colon cancers spontaneously developed in the cecum in 6 of 22 (27.2%) IL-$10^{-/-}$AID$^{+/+}$ mice. In contrast, none of the IL-$10^{-/-}$AID$^{-/-}$ mice developed cancers except only one case of neoplasia in the distal colon. These findings suggest that the proinflammatory cytokine-induced aberrant production of AID links colonic inflammation to an enhanced genetic susceptibility to oncogenic mutagenesis. Targeting AID could be a novel strategy to prevent colitis-associated colon carcinogenesis irrespective of ongoing colonic inflammation.

Key Words: AID, colitis-associated cancer, colonic inflammation, IL-$10^{-/-}$ mouse

Introduction

Chronic inflammation in epithelial tissues predisposes to the development of cancers (Mantovani *et al.,* 2008). For example, epidemiologic studies demonstrate that patients with chronic hepatitis caused by hepatitis virus infection and chronic gastritis caused by *Helicobacter pylori* infection leads to the development of hepatocellular carcinoma and gastric cancers, respectively (Chiba *et al.*, 2006; Ikeda *et al.*, 2007). Similarly, it is well recognized that the incidence of colorectal cancer is significantly higher in those with inflammatory bowel disease (IBD) than in the general population (Podolsky, 2002). Indeed, the cumulative risk of developing colorectal cancer for any patient with ulcerative colitis is estimated to be 1.6% at 10 years, 8.3% at 20 years, and 18.4% at 30 years from disease onset (Eaden *et al.*, 2001). The mechanisms of colon carcinogenesis in chronically inflamed tissue remain unclear, but it is reasonable to assume that multistep gene alterations required for malignant transformation occur in the constitutively inflamed colonic mucosa. Consistent with this idea, genetic alterations such as the *TP53* mutation appear to be an early event and are already present in colonic mucosa of patients with ulcerative colitis before cancer onset (Hussain *et al.*, 2000; Kern *et al.*, 1994; Leedham *et al.*, 2009; Yin *et al.*, 1993). Therefore, identifying the molecular pathway that links inflammation and genetic alterations in tumor-related genes is an important step to understand colitis-associated carcinogenesis.

Recently, we demonstrated that one of a human nucleotide-editing enzyme, activation-induced cytidine deaminase (AID), induces somatic mutations in several tumor-related genes, including *TP53*, in gastrointestinal epithelial cells (Endo *et al.*, 2007; Endo *et al.*, 2008; Komori *et al.*, 2008; Kou *et al.*, 2007; Matsumoto *et al.*, 2007). AID was originally identified as an inducer of somatic mutations and class switch recombination of immunoglobulin genes, which diversifies

the antibody production in B lymphocytes (Muramatsu *et al.*, 2000). Although AID expression is restricted to activated B cells under physiologic conditions, the inflammatory response can trigger aberrant AID expression in various epithelial organs. Stimulation of proinflammatory cytokines such as tumor necrosis factor (TNF)-α induces AID expression in hepatocytes, cholangiocytes, and gastric epithelial cells (Endo *et al.*, 2007; Komori *et al.*, 2008; Matsumoto *et al.*, 2007). More importantly, aberrant AID expression in these epithelial cells results in the generation of nucleotide alterations in tumor-related genes and possible malignant transformation of the AID-expressing cells. Consistent with this hypothesis, animal models with constitutive and ubiquitous AID expression revealed that AID expression in epithelial tissues results in the accumulation of genetic mutations in various genes, leading to the development of liver, lung and gastric cancers (Morisawa *et al.*, 2008; Takai *et al.*, 2009). These findings strongly suggest that AID nucleotide-editing activity is intimately involved in the pathogenesis of inflammation-associated carcinogenesis (Chiba and Marusawa, 2009).

Chronic production of various proinflammatory cytokines is thought to be responsible for tumor development and progression in colitis-associated cancers (Lin and Karin, 2007). One example of the oncogenic effect of proinflammatory cytokines on colonic cells is provided by the colon carcinogenesis model, in which TNF-α blockade reverses azoxymethane and dextran sodium sulfate-induced colonic mucosal damage and attenuates subsequent colon cancer development (Popivanova *et al.*, 2008). What is noteworthy is that aberrant AID expression is induced in colonic epithelial cells in response to TNF-α via the IκB kinase-dependent nuclear factor (NF)-κB signaling pathways (Endo *et al.*, 2008). Moreover, we showed that enhanced expression of endogenous AID protein is detectable in the inflamed colonic mucosa of patients with ulcerative colitis or Crohn's disease (Endo *et al.*, 2008). These findings suggest that the

inflammatory cytokine/AID axis may actually promote colon carcinogenesis by its genotoxic activity in the background of IBD. To clarify whether AID is a crucial mediator of the genetic alterations required for inflammation-mediated carcinogenesis, we investigated the impact of AID deficiency in the pathogenesis of colitis-associated colon cancer.

Results

AID gene expression in inflamed colonic mucosa of interleukin (IL)-10$^{-/-}$ mice

We first investigated endogenous AID expression in association with the degree of colonic inflammation in IL-10$^{-/-}$ mice, a representative model of human IBD. IL-10$^{-/-}$ mice were maintained under pathogen-free conditions and histologic analysis was performed using paraffin-embedded tissue sections from the cecum, the proximal colon, the distal colon and the small intestine. IL-10$^{-/-}$ mice spontaneously developed intestinal inflammation after 8 weeks of age, whereas no inflammatory change was observed in the colons of the wild-type (WT) mice (Figure 1a). In 52-week-old IL-10$^{-/-}$ mice, the histologic findings from the colonic tissue revealed epithelial hyperplasia, inflammatory cell infiltration, and goblet cell loss (Figure 1a). Inflammatory lesions occurred at greater severity in the cecum compared with the proximal colon, the distal colon and the small intestine.

Colonic mucosal inflammation in IL-10$^{-/-}$ mice is mediated by proinflammatory cytokines as well as an excessive Th1 T-cell response associated with increased inteferon (IFN)-γ and IL-12 secretion (Berg *et al.*, 1996; Davidson *et al.*, 1996). Consistent with previous studies, expression levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 were elevated in the inflamed cecal mucosa of the 52-week-old IL-10$^{-/-}$ mice (Figure 1b). Moreover, enhanced expression of Th1 cytokines, including IL-12 and IFN-γ, was observed in the 52-week-old IL-10$^{-/-}$ mice, but little expression of inflammatory cytokines was detected in 8-week-old IL-10$^{-/-}$ mice. In contrast, the expression levels of Th2 cytokines such as IL-4 and IL-13 did not differ between control and the IL-10$^{-/-}$ mice (Figure 1b).

We next examined whether aberrant AID expression appeared in association with colonic inflammation in IL-10$^{-/-}$ mice. Quantitative RT-PCR revealed a marked elevation of endogenous

AID expression in the cecal epithelial cells of IL-10$^{-/-}$ mice older than 20 weeks of age, whereas only trace amounts of AID expression in younger IL-10$^{-/-}$ mice and in WT mice (Figure 1c, Supplementary Figure 1). In the 20- and 52-week-old IL-10$^{-/-}$ mice, AID was strongly expressed in the cecal mucosa compared with the epithelium of the proximal and distal colon (Figure 1d).

To determine whether the increased AID expression derives from epithelial cells or infiltrating B lymphocytes, we examined the expression pattern of endogenous AID in the inflamed cecal mucosa of IL-10$^{-/-}$ mice using *in situ* hybridization (ISH). The specificity of the ISH results was confirmed by control staining performed on an intestinal lymphoid follicle containing mostly activated B cells or the tissues derived from the transgenic mice with constitutive AID expression (Figure 1e, Supplementary Figure 2). No AID expression was detected in the normal cecal mucosa, liver and kidney of WT mice, or the murine B lymphoma cells with the siRNA-mediated knock down of endogenous AID transcripts (Figure 1e, Supplementary Figure 2). In contrast, high AID expression was observed mainly in cytoplasm of both the cecal epithelium and lymphocytes in inflamed intestinal tissues (Figure 1e, Supplementary Figure 3).

These findings suggest that persistent inflammation in the cecum of the older IL-10$^{-/-}$ mice is closely associated with the enhanced production of various inflammatory cytokines, leading to the induction of aberrant AID expression in inflamed colonic mucosa.

Inhibition of TNF-α and IL-12 suppressed AID expression with the decrease of colonic inflammation in IL-10$^{-/-}$ mice.

To clarify the role of TNF-α and IL-12 in the pathophysiology of colonic inflammation in association with aberrant AID expression in the IL-10$^{-/-}$ mice, the biologic activity of TNF-α and IL-12 was inhibited using the TNF antagonist etanercept and neutralizing IL-12p40 monoclonal

antibody (mAb), respectively (Liu *et al.*, 2006; Popivanova *et al.*, 2008; Watanabe *et al.*, 2006). First, we confirmed that both TNF-α and IL-12 expression were significantly upregulated in the cecum compared with the proximal and distal colon ($P<0.05$, Figure 2a). In agreement with established findings that TNF-α augments the expression of various cytokines and chemokines (Marra *et al.*, 1993; Popivanova *et al.*, 2008), etanercept treatment resulted in the suppression of a variety of proinflammatory cytokines and chemokines such as TNF-α, IL-12, IFNγ, IL-1β, IL6, and monocyte chemoattractant protein-1 (MCP-1) (Figure 2b, Supplementary Figure 4). In contrast, there was no apparent difference in the expression levels of IL-5, a Th2 cytokine, between the etanercept and control groups (Supplementary Figure 4). Histopathologic findings revealed that the cecal inflammation was substantially reduced in mice treated with etanercept compared with the control mice (Figure 2c, left and middle panels). Similar to etanercept, IL-12p40 mAb treatment effectively suppressed cecal inflammation in association with reduced levels of proinflammatory cytokines and chemokines other than IL-5 compared with the control mice (Figure 2b, Supplementary Figure 4).

NF-κB p65 is strongly activated in the inflamed colonic mucosa of IL-$10^{-/-}$ mice (Inoue *et al.*, 2009; Neurath *et al.*, 1996). To determine the effect of inhibition of the TNF-α- or IL-12-mediated signaling pathways on NF-κB activity, immunohistochemical staining of the cecal tissue from IL-$10^{-/-}$ mice was performed using the phospho-NF-κB p65 antibody. In the vehicle-treated IL-$10^{-/-}$ mice, phospho-NF-κB p65 was strongly positive in the nucleus of the epithelial cells, whereas NF-κB activation was markedly suppressed in the cecal mucosa of mice treated with the etanercept or IL-12p40 mAb (Figure 2c, right panels).

Endogenous AID expression is induced in response to TNF-α or IL-12 treatment and AID transcription is regulated via IκB kinase-dependent NF-κB signaling pathways in human colonic

cells (Endo *et al.*, 2008). Thus, we investigated AID expression levels when TNF-α or IL-12 activity was inhibited in the colonic tissue of IL-10$^{-/-}$ mice. Quantitative RT-PCR analyses showed that endogenous AID expression was markedly decreased in the cecal mucosa both in the etanercept group and the IL-12p40 mAb group compared with the control group (Figure 2d). These findings suggest that TNF-α and IL-12 play a critical role in not only intestinal inflammation but also the aberrant AID expression in the cecal mucosa of the IL-10$^{-/-}$ mice.

AID deficiency did not affect the expression levels of proinflammatory cytokines in colonic mucosa.

To gain insight into the role of AID expression in the inflamed colonic mucosa, we evaluated the effect of AID deficiency on the inflammatory response by crossing AID$^{-/-}$ mice with IL-10$^{-/-}$ mice (Figure 3a) and the cecal mucosa isolated from IL-10$^{-/-}$AID$^{-/-}$ mice were subjected to further analysis. Histologic findings revealed that mucosal inflammatory changes in IL-10$^{-/-}$AID$^{-/-}$ mice, including pronounced inflammatory infiltration and marked epithelial hyperplasia, were comparable with those of the IL-10$^{-/-}$AID$^{+/+}$ littermates (Figure 3b). In agreement with a previous study (Fagarasan *et al.*, 2002), hyperplasia of isolated lymphoid follicles developed in the cecum of 52-week-old AID$^{-/-}$ mice as well as in IL-10$^{-/-}$AID$^{-/-}$ mice (data not shown). RT-PCR analyses revealed only trace amounts of cytokine expression in WT or AID$^{-/-}$ mice (Figure 3c). In contrast, enhanced expression levels of various cytokines such as TNF-α, IL-12, IFNγ, IL-1β and IL-6 observed in the IL-10$^{-/-}$AID$^{-/-}$ mice were comparable with those in the IL-10$^{-/-}$AID$^{+/+}$ mice (Figure 3c). Quantitative RT-PCR analyses also showed markedly enhanced expression levels of MCP-1 in the cecum of IL-10$^{-/-}$AID$^{-/-}$mice to almost the same level as that in IL-10$^{-/-}$AID$^{+/+}$ mice (Figure 3d). Thus, the production levels of inflammatory cytokines and chemokines in the cecal

mucosa did not differ between IL-10$^{-/-}$AID$^{+/+}$ mice and of IL-10$^{-/-}$AID$^{-/-}$ mice.

Deficiency of endogenous AID resulted in the reduced frequencies of nucleotide alterations in the *Trp53* gene in IL-10$^{-/-}$ mice.

To clarify whether the AID upregulation induced by chronic colitis is genotoxic in colonic mucosa, we determined the nucleotide sequences of the *Trp53, Apc, Ctnnb1* and *Kras* genes, all of which are thought to be involved in human colorectal carcinogenesis (Fearon and Vogelstein, 1990). We first confirmed that the incidence of nucleotide alterations was less than 1.00 substitution per 10^4 nucleotides in these 4 tumor-related genes obtained from over 40 randomly picked clones from the normal cecal epithelial cells of WT mice, determined by conventional sequencing analyses with high-fidelity PCR amplification (data not shown). We then determined the sequences of the *Trp53, Apc*, *Ctnnb1* and *Kras* genes in 50 randomly selected clones amplified from the cecal epithelial cells of 3 IL-10$^{-/-}$AID$^{+/+}$ as well as 3 IL-10$^{-/-}$AID$^{-/-}$ mice (Representative data is shown in Table 1.). Nucleotide alterations had accumulated in the *Trp53* gene in the inflamed cecal mucosa of the IL-10$^{-/-}$AID$^{+/+}$ mice with a frequency of 2.19 substitutions per 10^4 nucleotides (Table 1). Although all the nucleotide changes determined in the *Trp53* gene were different in each clone, 9 of 12 (75%) alterations were accumulated in the regions corresponding to the DNA-binding motif of the human *TP53* gene (Figure 4). Among the nucleotide changes observed in the cecal epithelial cells of IL-10$^{-/-}$AID$^{+/+}$ mice, 7 (58.3%) of 12 genetic changes were single-base substitutions and 4 of these 7 alterations in the *Trp53* coding sequences resulted in amino acid replacements with potential functional consequences. In contrast, the mutation frequency of the *Trp53* gene in the cecal epithelial cells of IL-10$^{-/-}$AID$^{-/-}$ mice (0.71 substitutions per 10^4 nucleotides) was significantly lower than that of IL-10$^{-/-}$AID$^{+/+}$

mice (P<0.05, Table 1). On the other hand, the *Apc*, *Ctnnb1* and *Kras* genes did not have remarkable numbers of nucleotide alterations in the inflamed cecal mucosa of IL-10$^{-/-}$AID$^{+/+}$ mice, and thus the incidence of nucleotide changes in the *Apc*, *Ctnnb1* and *Kras* genes was not significantly different between IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$mice (Table 1). These findings suggest that the *Trp53* gene is a specific target gene in chronically inflamed cecal mucosa in IL-10$^{-/-}$ mice, and the accumulation of genetic changes in the *Trp53* gene of the inflamed colonic mucosa was due to AID activity.

The incidence of colon cancer was reduced in IL-10$^{-/-}$ mice in the absence of endogenous AID.

The findings that AID deficiency in IL-10$^{-/-}$ mice had no significant impact on the levels of colonic inflammation but reduced the frequencies of somatic mutations in the tumor-suppressor *Trp53* gene led us to speculate that the knockout of endogenous AID might reduce the incidence of colonic cancer development irrespective of ongoing colonic inflammation. Thus, we compared the neoplastic phenotype of the IL-10$^{-/-}$AID$^{+/+}$ mice with that of IL-10$^{-/-}$AID$^{-/-}$ mice. The frequency and spectrum of colonic tumors that developed in IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$AID$^{-/-}$ mice are summarized in Table 2. Dysplastic changes in the mucosa of the large intestine were equally observed in most of these mice. These dysplastic lesions more frequently developed in the cecum than in the proximal and distal colon. Interestingly, invasive adenocarcinomas were detected in 6 of 22 IL-10$^{-/-}$AID$^{+/+}$ mice and all the tumors characteristically developed from the dysplastic mucosa in the cecum (Table 2). Histopathologic analysis of colonic tumors revealed moderate- to poorly-differentiated adenocarcinomas and invasive tumor cells beyond the submucosa with strong β-catenin expression (Figure 5a,

Supplementary Figure 5). In contrast, IL-10$^{-/-}$AID$^{-/-}$ mouse developed no tumors in the inflamed colonic mucosa except only one tumor in the distal colon (Table 2). The colonic tumor that developed in the IL-10$^{-/-}$AID$^{-/-}$ mouse showed a trabecular pattern of growth within the submucosa, consistent with the morphologic appearance of well-differentiated adenocarcinoma (Figure 5b). These findings suggest that the upregulation of endogenous AID in the cecal mucosa driven by the inflammatory response contributes to the development of colonic cancers.

Discussion

The causal association between colonic inflammation and carcinogenesis is now well recognized (Eaden *et al.*, 2001; Podolsky, 2002). A recent genetic-linkage analysis of patients with IBD revealed that loss-of function mutations in genes encoding the IL-10 receptor proteins are associated with severe, early-onset enterocolitis, a finding that underscores the pivotal role of IL-10 in mediating the signals that control inflammation in the human gut (Glocker *et al.*, 2009). Consistent with the clinical finding, a mouse model with targeted disruption of the IL-10 gene invariably develops enterocolitis that eventually progresses to colon cancer under conventional housing conditions; this mouse model is thus extremely useful as a disease model of human IBD (Berg *et al.*, 1996; Kuhn *et al.*, 1993; Sturlan *et al.*, 2001). In the present study, expression of AID was most prominent in the inflamed cecal mucosa of IL-$10^{-/-}$ mice. Moreover, we demonstrated that a deficiency of endogenous AID reduces the incidence of both the accumulation of somatic mutations in the *Trp53* gene and the development of colon cancer in inflamed colonic mucosa. *In vitro*, we previously demonstrated that aberrant AID expression is induced in response to proinflammatory cytokine stimulation and colonic epithelial cells underlying chronic inflammation acquire the genetic mutations achieved by AID genotoxic activity (Endo *et al.*, 2008). Together, these findings suggest that inappropriate AID expression plays a pivotal role in the development of colorectal cancers via the accumulation of genetic alterations in the colonic mucosa of IBD.

We revealed here that endogenous AID is upregulated in inflamed colonic mucosa of elder IL-$10^{-/-}$ mice and the degree of AID expression paralleled extent of colonic inflammation. This observation is consistent with the findings that AID protein expression was detected in the colonic epithelium of inflammatory lesions from patients with IBD (Endo *et al.*, 2008). Colonic

mucosal inflammation is usually mediated by either an excessive Th1 T-cell response associated with increased IFN-γ and IL-12 secretion, or an excessive Th2 T-cell response associated with increased IL-4, IL-5, and IL-13 secretion (Fuss *et al.*, 1996). We previously found that the proinflammatory cytokine TNF-α, the Th2 cytokines IL-4 and IL-13, and Th1 cytokine IL-12 enhanced aberrant AID expression in cultured colonic epithelial cells (Endo *et al.*, 2008). On the other hand, TNF-α expression is elevated in colonic tissues of IL-10$^{-/-}$ mice (Berg *et al.*, 1996), and colitis in IL-10$^{-/-}$ mice is predominantly mediated by Th1 type T cells with increased production of IL-12 (Berg *et al.*, 1996; Davidson *et al.*, 1996). Consistent with these previous findings, in the present study blockade of the activity of TNF-α or IL-12 suppressed AID expression in association with reduced production of various pro-inflammatory cytokines in the inflamed colonic mucosa of IL-10$^{-/-}$ mice. Thus, it is reasonable to assume that cytokine signalings, especially those mediated by TNF-α and IL-12, contribute to aberrant AID expression in the colonic cells of IL-10$^{-/-}$ mice.

A causal relationship between colonic inflammation and the accumulation of *TP53* tumor-suppressor gene mutations has been reported in human IBD (Hussain *et al.*, 2000; Kern *et al.*, 1994; Leedham *et al.*, 2009; Yin *et al.*, 1993). Alterations in the *TP53* gene, a late event in the pathogenesis of sporadic colorectal cancers, occur in dysplastic lesions with a background of ulcerative colitis (Holzmann *et al.*, 1998; Yin *et al.*, 1993) and are likely to proceed to dysplasia (Lashner *et al.*, 1999). Thus, the increased *TP53* mutation load in inflamed colonic epithelium of patients with IBD suggests that *TP53* mutations in noncancerous colon tissue of IBD patients specifically confer susceptibility to the development of colorectal cancers in an inflammatory microenvironment (Hussain *et al.*, 2000). In the present study, we found that high frequencies of nucleotide alterations had accumulated in *Trp53* gene mutation in inflamed mucosa of

IL-10$^{-/-}$AID$^{+/+}$ mice. In addition, we demonstrated that a deficiency of endogenous AID in inflamed colonic mucosa resulted in a significantly reduced occurrence of somatic mutations in the *Trp53* genes, while there was no significant accumulation of somatic mutations appeared in the *Apc*, *Ctnnb1* and *Kras* genes in the inflamed colonic mucosa of IL-10$^{-/-}$AID$^{+/+}$ mice compared with IL-10$^{-/-}$AID$^{-/-}$ mice. It is unclear why the *Trp53* gene was more sensitive to AID-mediated genotoxic activity than the *Apc, Ctnnb1* and *Kras* genes in colonic epithelial cells of IL-10$^{-/-}$ mice. The present findings, however, are consistent with a previous observation that target gene selection for AID-induced somatic mutations varies among tissues and target cells (Morisawa *et al.*, 2008) and AID expression in cultured human colonic epithelial cells preferentially targets the *TP53* gene *in vitro* (Endo *et al.*, 2008). On the other hand, alterations in the *APC* and *KRAS* genes are also detected in dysplastic lesions and cancer tissues that develop in human IBD (Redston *et al.*, 1995). Therefore, we assume that the mutations in *APC*, *CTNNB1*, and *KRAS* genes were also present, but that their frequencies were below the detection limits of the present study. Further comprehensive sequencing analyses are required to determine how the AID-mediated genotoxic effects achieve the target gene selection and whether IL-10$^{-/-}$ mice and human IBD share a similar process of mutational accumulation in tumor-related genes.

It is noteworthy that AID deficiency resulted in the reduced incidence of colitis-associated colon cancer development. AID deficiency caused the development of hyperplasia of isolated lymphoid follicles associated with an expansion of anaerobic flora in the small intestine (Fagarasan *et al.*, 2002; Suzuki *et al.*, 2004). We found no significant differences in the production levels of inflammatory cytokines in the colonic mucosa between the IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. This observation might be consistent with the previous findings that activated B cells were not the primary mediator of inflammatory response in the colon of IL-10$^{-/-}$

mice, as evidenced by their ability to transfer colitis to immunodeficient RAG2$^{-/-}$ mice (Davidson *et al.*, 1996). In contrast to the similar levels of colonic inflammatory activity, the incidence of colon cancers was significantly lower in IL-10$^{-/-}$AID$^{-/-}$ mice compared with the IL-10$^{-/-}$AID$^{+/+}$ mice harboring endogenous AID. It may be emphasized that expression levels of endogenous AID in the cecal mucosa was significantly higher than those of the remaining sites of the colon and all the colon cancers that developed in IL-10$^{-/-}$AID$^{+/+}$ mice were located at the cecum, while none of the IL-10$^{-/-}$AID$^{-/-}$ mice developed cancers in their cecum. Only one IL-10$^{-/-}$AID$^{-/-}$ mouse developed a tumor in the distal colon. Histologic examination indicated that this tumor had the morphologic appearance of well-differentiated adenocarcinoma located within the submucosa, while all the cancers developed in IL-10$^{-/-}$AID$^{+/+}$ mice invaded the muscularis propria or adventitia with the characteristics of moderate- to poorly-differentiated adenocarcinoma. Based on the above discussion, ectopic AID expression in the inflamed colonic mucosa is an indispensable factor for the development of colon cancers in IL-10$^{-/-}$ mice.

Recent studies revealed that AID is involved in regulating DNA methylation in certain systems (Bhutani *et al.*, 2010; Guo *et al.*, 2011; Rai *et al.*, 2008). Moreover, infiltrating leukocytes, including B cells, might modulate tumor cell properties via the production of certain chemokines or cytokines (Ammirante *et al.*, 2010). Therefore, further studies are necessary to examine the incidence of inflammation-associated cancers in mice in which AID is specifically deficient in the epithelial cells, and to clarify whether AID has a role in inflammation-associated tumorigenesis through the epigenetic modification of tumor-related genes.

In conclusion, we demonstrated that the proinflammatory cytokine TNF-α and the Th1 cytokine IL-12 are responsible for aberrant AID expression in the colonic mucosa of IL-10$^{-/-}$ mice with chronic inflammation. Aberrant AID expression in the inflamed colon is associated

with the accumulation of somatic mutations in tumor suppressor *Trp53* gene, and AID deficiency resulted in a reduced incidence of colitis-associated colon cancers. These findings may lead to a novel strategy for preventing carcinogenesis by targeting AID irrespective of the ongoing colonic inflammation in patients with IBD.

Materials and Methods

Animal experiments

The generation of AID$^{-/-}$ mice was described previously (Fagarasan *et al.*, 2001). IL-10$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) and AID$^{-/-}$ mice were crossed on a C57BL/6 background to generate IL-10$^{-/-}$AID$^{-/-}$ mice. All mice were maintained in a specific pathogen-free facility at Kyoto University Faculty of Medicine. Cecal and colonic epithelium was purified as follows; Cecum and colon were cut into 2.0cm long and incubated with 2mM EDTA in Hank's Balanced Salt Solution without calcium and magnesium for 10 minutes at room temperature. Then, the tissues were tumbled for 6 minutes and the mucosa was selectively stripped from the submucosa. The stripped mucosa was washed with phosphate buffered saline (PBS) for three times and the supernatant containing floating cells and debris were discarded. The obtained epithelial tissue samples and non-epithelial tissue samples were frozen in liquid nitrogen for nucleotide extraction. In some experiments, IL-10$^{-/-}$ mice were intraperitoneally injected with TNF antagonist etanercept and neutralizing antibody to murine IL-12p40 (Watanabe *et al.*, 2006). Accordingly, 40-week-old IL-10$^{-/-}$ mice were injected with etanercept at a dose of 3 mg/kg body over 5 days and killed on day 12. Other 40-week-old IL-10$^{-/-}$ mice were injected with IL-12p40 mAb at a dose of 0.5 mg/body on day 1 and day 8, and sacrificed at day 12. All animal experiments were approved by the Ethics Committee for Animal Experiments and performed under the Guidelines for Animal Experiments of Kyoto University.

Histopathologic and immunohistochemical analyses

The entire colon was removed and washed with PBS. The cecum, the proximal colon and the distal colon were dissected transversely and fixed in 4% (w/v) formaldehyde. The fixed tissue was embedded in paraffin and sectioned at 3 μm in a random manner. In particular, two types of

histologic preparations were sectioned from the tissues of the cecum. These samples were stained with hematoxylin and eosin and analyzed histologically in a blind fashion by three readers. Immunohistochemical staining was performed according to a previously described protocol (Toda *et al.*, 1999). The polyclonal antibodies for phospho-NF-κB p65 (Ser276) and β-catenin were purchased from Cell Signaling Technology (Danvers, MA) and BD Biosciences (Franklin Lakes, NJ), respectively.

In situ hybridization

A digoxigenin-labeled RNA probe specific for murine AID was transcribed with digoxigenin-11-UTP according to the manufacturer's instructions (Roche, Basel, Switzerland) from a 1.7-kb cDNA amplified using the following primers: 5'-ATG GAC AGC CTT CTG GTG ATG AA-3' and 5'-CTT GTT CCC AAG GTC GCA AGG AAA GG-3'. Similarly, an RNA probe for murine villin1 was transcribed from a 1.6-kb cDNA amplified using the following primers: 5'- TGA ATG CCC AAC TCA AAG GCT CTC TC-3' and 5'-ACC TCA AAA GCC TTG GTG TTA TCA GC-3'. *In situ* hybridization was performed as described previously (Nakatani *et al.*, 2004). The alkaline phosphatase chromogen reaction was performed using Fast Red (Roche) as the substrate at room temperature for 48 hours. The sections were then washed with distilled water and mounted on glass slides in mounting medium.

Semiquantitative and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the tissues using QuickGene RNA Tissue Kit (Fuji, Tokyo, Japan). cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR amplification was performed using Takara Ex Taq DNA polymerase (Takara, Tokyo, Japan). The oligonucleotide primers for the semiquantitative RT-PCR are shown in Supplementary Table 1. Gene expression was quantified by quantitative real-time RT-PCR using LightCycler 480 System

II (Roche). The oligonucleotide primers for the quantitative RT-PCR are shown in Supplementary Table 2. To assess the quantity of isolated RNA as well as the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene 18S rRNA (Matsumoto *et al.*, 2007). For simplicity, ratios are represented as relative values compared with expression levels in lysate from control specimens.

Subcloning and sequencing analyses of tumor-related genes

The oligonucleotide primers for the amplification of the murine *Trp53, Apc, Ctnnb1* and *Kras* genes are shown in Supplementary Table 3. Amplification of targeted sequences was performed using high-fidelity Phusion Taq Polymerase (Finnzymes, Espoo, Finland), and the products were subcloned using pGEM-T Easy Vector Systems (Promega, Madison, WI). The resulting plasmids were subjected to sequence analysis using Applied Biosystems 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA).

Statistical analysis

Statistical analysis was performed using a Mann-Whitney *U* test, a chi-square test and Fisher's test. Differences were considered to be statistically significant if *P* values were less than 0.05.

Conflict of Interest

The authors are declared no conflict of interest.

Acknowledgements

We thank Dr. Keiichiro Suzuki and Dr. Tasuku Honjo for critical reading of this manuscript, and Dr. Masamichi Muramatsu for providing information of siRNA for AID. This work was supported by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science; a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare, Japan; and a Research Grant of the Princess Takamatsu Cancer Research Fund.

Figure legend

Figure 1. Endogenous AID expression in inflamed colonic mucosa of IL-10$^{-/-}$ mice. (a) Microscopic (hematoxylin and eosin (H&E) stain) images of the cecum of IL-10$^{-/-}$ mice and its WT littermate. The images of 8-week-old mice (i,iii) and 52-week-old mice (ii,iv) are shown. Scale bars are 200μm. (b) Representative results of RT-PCR for the expression of various proinflammatory cytokines in the cecal mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from cecal mucosa of 8- and 52-week-old IL-10$^{-/-}$ mice and their WT littermates. RT-PCR was performed using oligonucleotides specific for murine TNF-α, IL-12, IFN-γ, IL-1β, IL-6, IL-4, IL-13, and β-actin. (c) Time course changes of AID expression in the cecal mucosa of IL-10$^{-/-}$ mice. Total RNA was isolated from mucosa at the cecum of 8-, 12-, 20-, and 52-week-old IL-10$^{-/-}$ mice and WT littermates of 52-week-old IL-10$^{-/-}$ mice. Quantitative RT-PCR was performed using oligonucleotides specific for murine AID. *P<0.05 versus WT mice. (d) AID expression in various regions of colonic mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from mucosa at the cecum, and the proximal and distal colon of 52-week-old IL-10$^{-/-}$ mice and WT littermates. Quantitative RT-PCR was performed using oligonucleotides specific for murine AID. *P<0.05. (e) Representative images of AID expression in inflamed cecal mucosa determined by *in situ* hybridization assay. The images show the intestinal lymphoid follicle (left panels), the cecal mucosa of WT (middle panels) and IL-10$^{-/-}$ mice (right panels) stained with H&E (upper panels) or hybridized with the probe specific for the murine AID transcript (lower panels). Scale bars are 100μm.

Figure 2. Effects of TNF-α and IL-12 blockade on various cytokine expression, NF-κB

activation, and AID expression in the colonic mucosa of IL-10$^{-/-}$ mice. (a) TNF-α and IL-12 expression in various regions in colonic mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from mucosa at the cecum, and the proximal and distal colon of 52-week-old IL-10$^{-/-}$ mice and the cecum of the WT littermates. Quantitative RT-PCR was performed using oligonucleotides specific for murine TNF-α and IL-12. *P<0.05. (b) Changes of various cytokine expression under the inhibition of the biologic activity of TNF-α and IL-12. Total RNA was isolated from mucosa at the cecum of IL-10$^{-/-}$ mice treated with PBS, etanercept, and IL-12p40 mAb. Quantitative RT-PCR was performed using oligonucleotides specific for murine TNF-α and IL-12. Relative values of these cytokines are shown. *P<0.05 versus control mice. (c) Changes of histopathologic image and NF-κB activation induced by administration of etanercept and IL-12p40 mAb. Microscopic images (H&E stain) of the cecum of IL-10$^{-/-}$ mice treated with PBS (i,ii), etanercept (iv,v) and IL-12p40 mAb (vii,viii). Immunohistochemical staining for phospho-NF-κB p65 was performed using each sample including control group (iii), etanercept-treated group (vi), and IL-12p40 mAb-treated group (ix). Scale bars are 500μm. (d) Results of quantitative RT-PCR for AID expression in the cecum of IL-10$^{-/-}$ mice treated with PBS, etanercept, and IL-12p40 mAb. *P<0.05 versus control mice.

Figure 3. Effects of AID-deficiency in the colonic mucosa of IL-10$^{-/-}$ mice. (a) Results of quantitative RT-PCR for AID expression. Total RNA was extracted from the mucosa at the cecum of 52-week-old WT mice, AID$^{-/-}$ mice, IL-10$^{-/-}$ mice, and IL-10$^{-/-}$AID$^{-/-}$ mice. Quantitative RT-PCR was performed using oligonucleotides specific for murine AID. *P<0.05 versus WT mice. (b) Microscopic images (H&E stain) of the large intestine of 52-week-old IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$AID$^{-/-}$ mice. Upper panels (i,iii) are the images of the cecum and lower panels

(ii,iv) are the images of the distal colon. Scale bars are 200μm. (c) Representative results of semiquantitative RT-PCR for the expression of various proinflammatory cytokines in the cecum of 52-week-old WT, $AID^{-/-}$, $IL\text{-}10^{-/-}AID^{+/+}$, and $IL\text{-}10^{-/-}AID^{-/-}$ mice. RT-PCR analyses were performed by using specific primers for murine TNF-α, IL-12, IFN-γ, IL-1β, IL-6, IL-4, IL-13, and β-actin. (d) Results of quantitative RT-PCR for MCP-1 expression in the cecum of each genotype. **P*<0.05 versus WT mice.

Figure 4. Distribution of murine *Trp53* mutations found in the cecal mucosa of $IL\text{-}10^{-/-}AID^{+/+}$ mice and $IL\text{-}10^{-/-}AID^{-/-}$ mice. Murine p53 codon numbers are shown with equivalent human p53 domain structure with transactivation (TAD), DNA-binding and tetramerization (tetramer) domains and nuclear localization signal (NLS).

Figure 5. Colonic adenocarcinomas developed in $IL\text{-}10^{-/-}$ mice. (a) Microscopic images (H&E stain) of adenocarcinomas developed in the cecum of the $IL\text{-}10^{-/-}AID^{+/+}$ mice (i-vi). Scale bars are 500μm. (b) Microscopic images (H&E stain) of adenocarcinoma developed in the distal colon of the $IL\text{-}10^{-/-}AID^{-/-}$ mouse (i,ii). Scale bars are 500μm.

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Table 1. Gene mutation frequencies in inflamed cecal mucosa of the IL-$10^{-/-}$AID$^{+/+}$ mice and IL-$10^{-/-}$ AID$^{-/-}$ mice.

Gene	Genotype	Nucleotide alterations		*P***
		Number*	Frequency (/10^4)	
Trp53	AID (+/+)	12/54,787	2.19	<0.05
	AID (-/-)	5/70,380	0.71	
Apc	AID (+/+)	2/33,717	0.59	0.43
	AID (-/-)	1/42,762	0.23	
Ctnnb1	AID (+/+)	5/38,988	1.28	0.51
	AID (-/-)	2/36,037	0.55	
Kras	AID (+/+)	4/36,603	1.09	0.84
	AID(-/-)	3/31,955	0.94	

Representative results of mutation frequencies in *Trp53, Apc, Ctnnb1* and *Kras* genes in inflamed cecal mucosa of IL-$10^{-/-}$AID$^{+/+}$ and IL-$10^{-/-}$AID$^{-/-}$ mice are shown.

* Number: number of mutated nucleotides / number of total nucleotides examined.

** *P* value is calculated using a chi-square test.

Table 2. Incidence of colonic tumors observed in IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$AID$^{-/-}$ mice

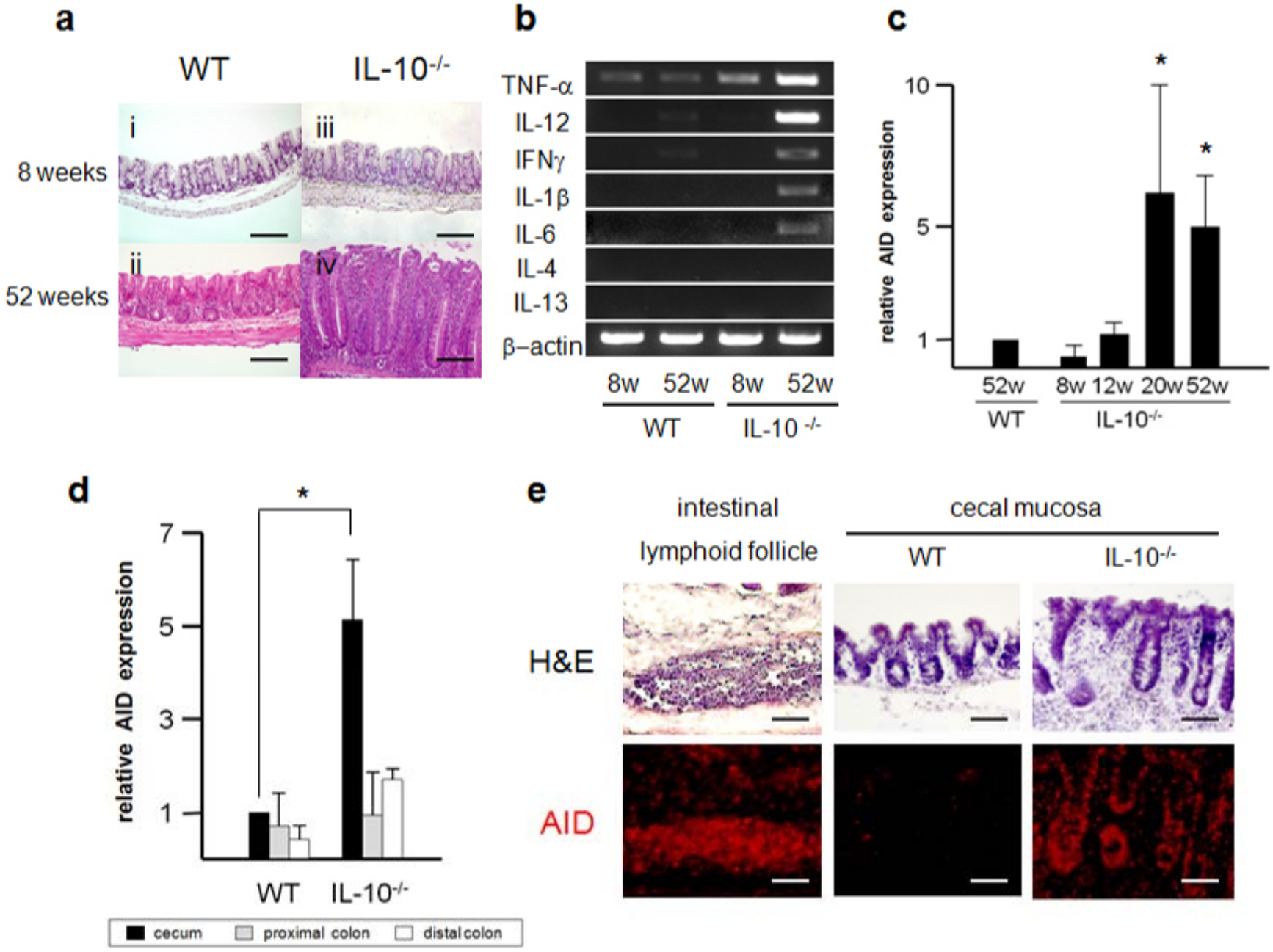
	IL-10$^{-/-}$AID$^{+/+}$ (n=22)		IL-10$^{-/-}$AID$^{-/-}$ (n=23)	
Mean age (weeks)	54.5		51.2	
Male / Female	13 / 9		13 / 10	
Tumor formation				
Adenoma	20 (90.9%)		21 (91.3%)	
Cecum	20	**	20	**
Proximal colon	1		1	
Distal colon	13		16	
Adenocarcinoma	6* (27.2%)		1* (4.3%)	
Cecum	6	**	0	**
Proximal colon	0		0	
Distal colon	0		1	

Values in parenthesis show percentages of animals that developed adenoma or adenocarcinoma.

* P<0.05 (P value is calculated using Fisher's test)

** Number of animals that developed adenoma or adenocarcinoma is shown.

Figure 1. Takai et al.



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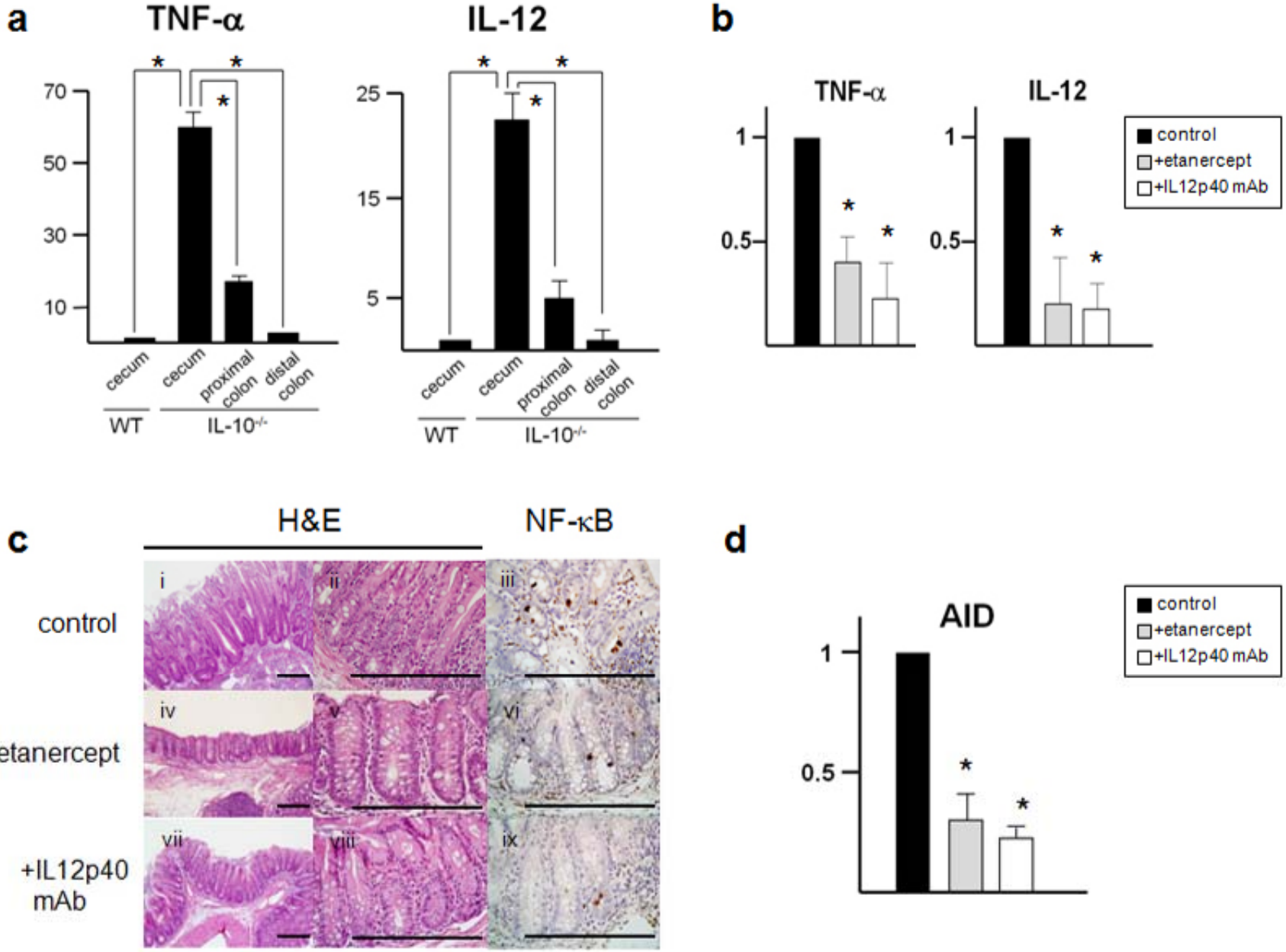

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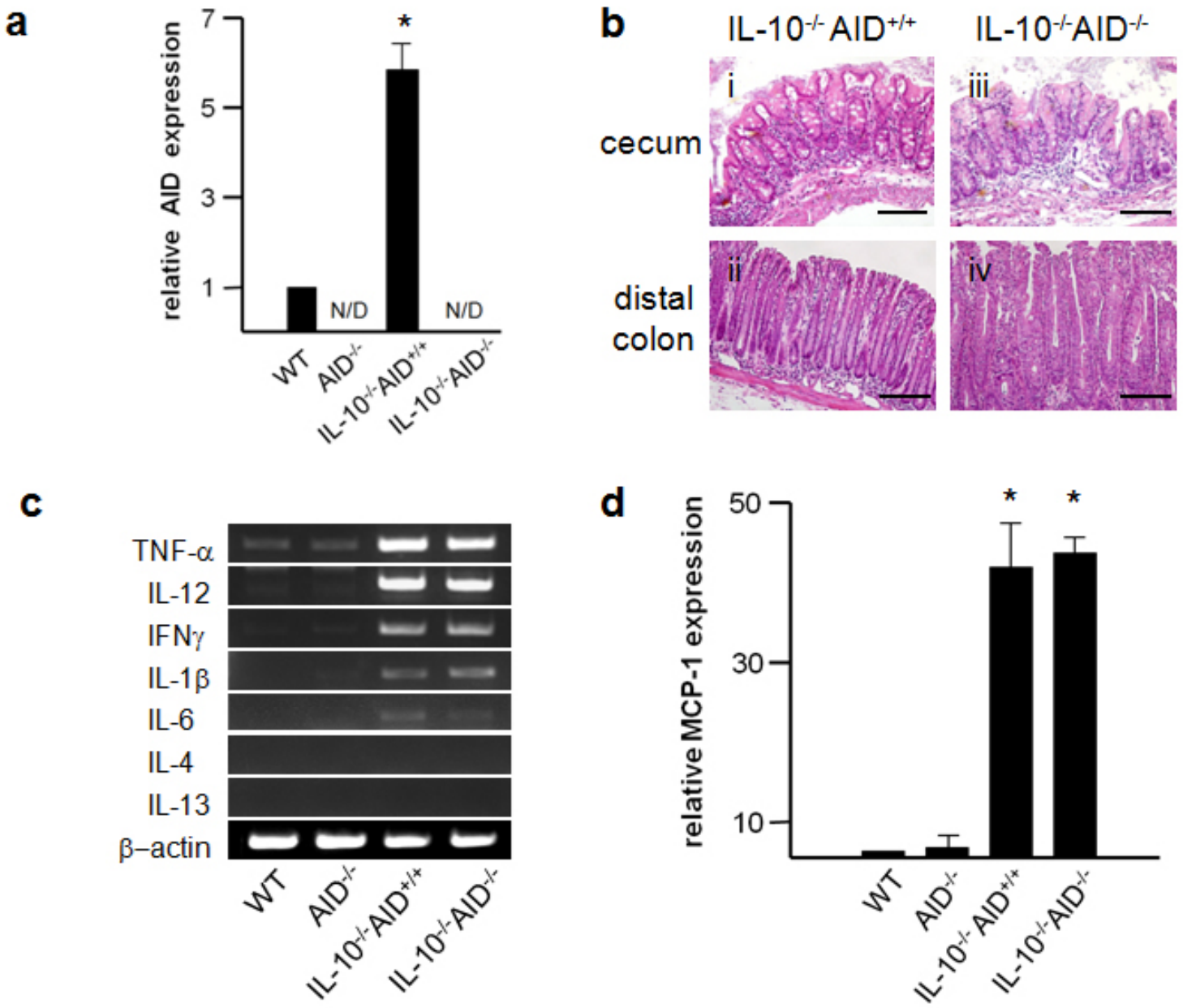

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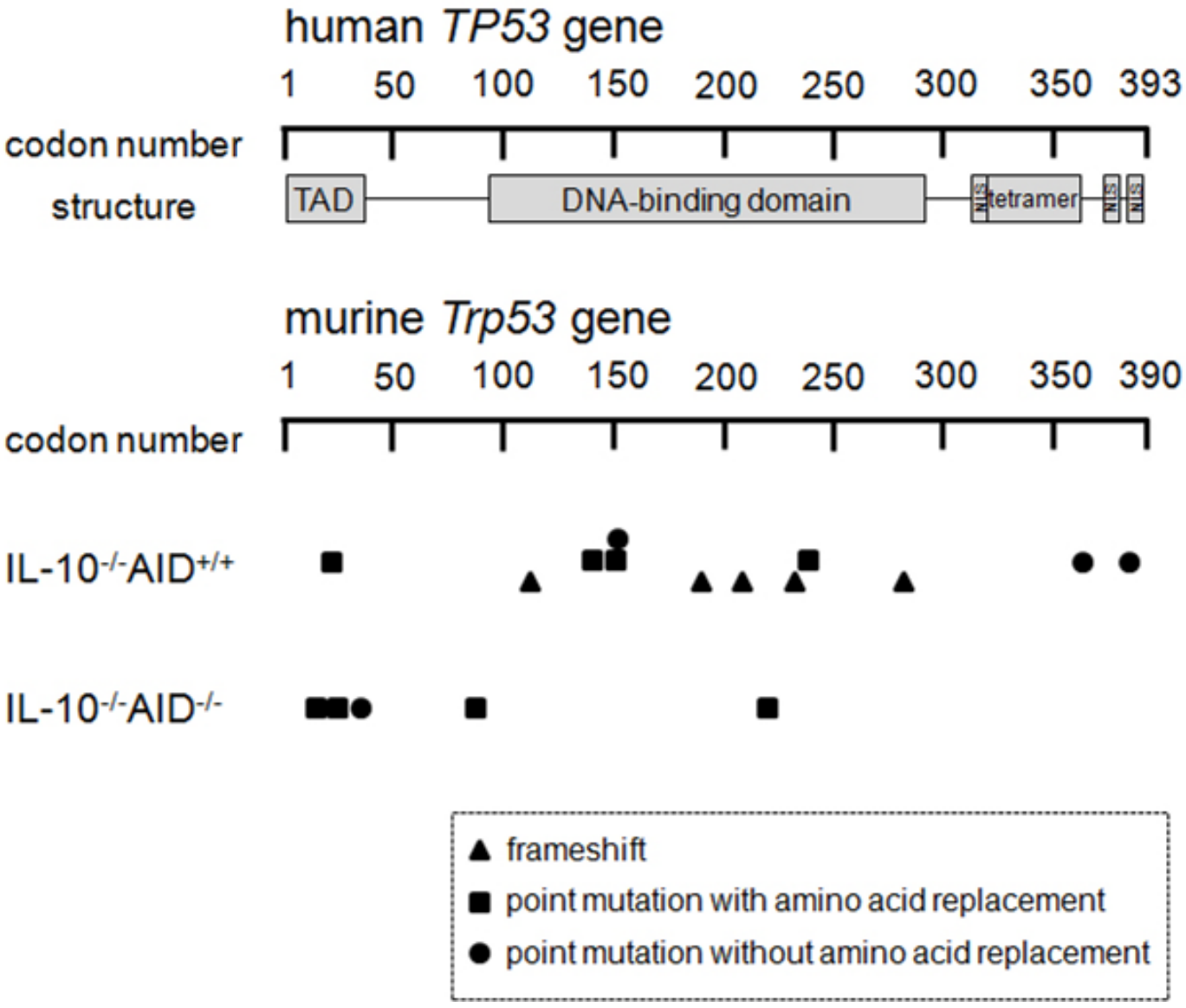

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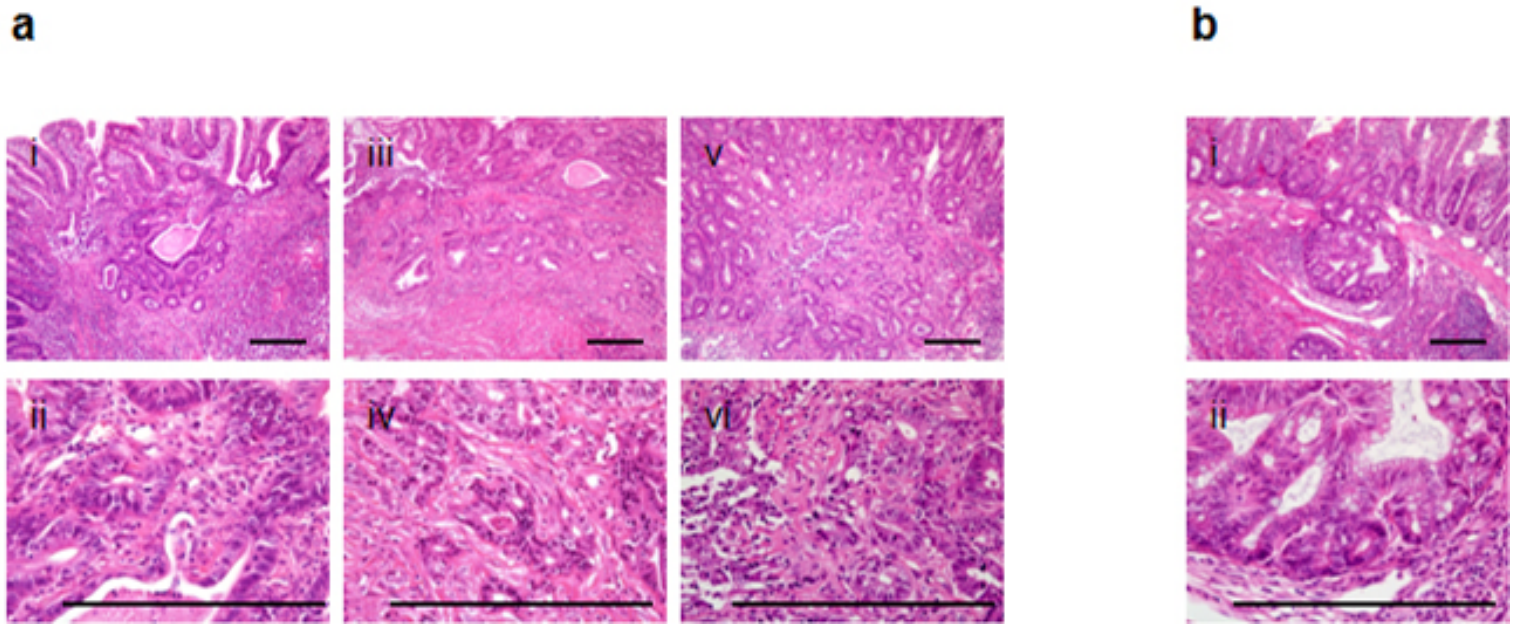


Supplementary Materials and Methods

RNA interference

Stealth siRNA specific for AID (5'- AAA GGA UGC GCC GAA GUU GUC UGG U -3') was obtained from Invitrogen (Carlsbad, CA). The Stealth siRNA was introduced into CH12F3-2 cells using Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD).

Supplementary Figure legend

Supplementary Figure 1. Representative results of RT-PCR in the cecal epithelium and non-epithelium of WT and IL-10$^{-/-}$ mice. RT-PCR was performed using oligonucleotides specific for murine villin, CD19, AID and β-actin. Villin and CD19 were specifically detected in the cecal epithelial cells (E) and non-epithelial tissues (N), respectively. These results indicate that those fractions selectively comprised colonic epithelial cells and lymphocytes, respectively. A considerable level of endogenous AID was found in both colonic epithelial cells and non-epithelial cells in IL-10$^{-/-}$ mice, indicating that colonic epithelial cells derived from the inflamed cecum of the IL-10$^{-/-}$ mice exhibit a high expression of AID. E, epithelium; N, non-epithelium.

Supplementary Figure 2. Representative images of AID expression in control specimens determined by *in situ* hybridization assay. (a) These images show the intestinal lymphoid follicle, liver, and kidney of wild-type mice, and liver of the transgenic mice with constitutive AID expression (AID tg mouse) stained with hematoxylin and eosin (upper panels) or hybridized with the probe specific for the murine AID transcript (lower panels). Endogenous AID expression was detected at the germinal center of the intestinal lymphoid follicle, while no signal was detectable in the liver and kidney of wild-type mice. On the other hand, AID transcripts were abundantly expressed in the liver of the transgenic mice with constitutive AID expression (AID tg mouse). (b) These images show CH12F3-2 cells, which are the murine B lymphoma cells with abundant endogenous AID expression, hybridized with the probe for the murine AID transcript. AID transcripts were abundantly expressed in CH12F3-2 cells (left panels). AID signals

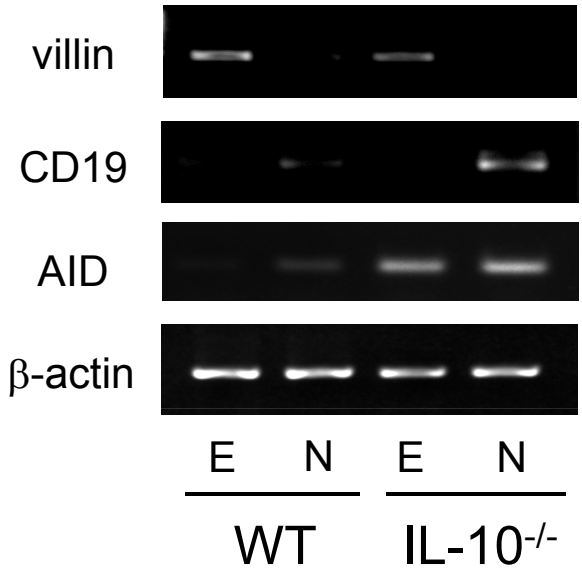
substantially decreased when endogenous AID expression was knocked down by introducing the siRNA specific for AID transcripts (right panels). Scale bars are 500μm.

Supplementary Figure 3. Representative images of AID and villin1 expression in inflamed cecal mucosa determined by *in situ* hybridization assay. The images show the cecal mucosa of wild-type (left panels) and IL-$10^{-/-}$ mice (right panels) stained with hematoxylin and eosin (upper panels) or hybridized with the probe specific for the murine AID transcript (middle panels) and the murine villin1 transcript (lower panels). Arrows indicate intestinal lymphoid follicle in which AID transcripts are abundantly expressed. Scale bars are 200μm.

Supplementary Figure 4. Changes of various cytokine and chemokine expression in the colonic mucosa of IL-$10^{-/-}$ mice under the inhibition of the biologic activity of TNF-α and IL-12. Total RNA was isolated from mucosa at the cecum of IL-$10^{-/-}$ mice treated with PBS, etanercept, and IL-12p40 mAb. Quantitative RT-PCR was performed using oligonucleotides specific for murine IFN-γ, IL-1β, IL-6, MCP-1 and IL-5. Relative values of these cytokines are shown. *P<0.05 versus control mice.

Supplementary Figure 5. β-Catenin expression in colonic adenocarcinomas that developed in IL-$10^{-/-}$ mice. Immunohistochemical staining was performed for β-catenin of cecal adenocarcinoma in IL-$10^{-/-}$AID$^{+/+}$ mice and adenocarcinoma in the distal colon of IL-$10^{-/-}$AID$^{-/-}$ mice. β-Catenin was strongly expressed in the cecal adenocarcinomas of IL-$10^{-/-}$AID$^{+/+}$ mice, while no nuclear staining for β-catenin protein was detectable in the adenocarcinoma developed in the distal colon of IL-$10^{-/-}$AID$^{-/-}$ mice. Scale bars are 100μm.

Supplementary Figure 1. Takai et al.



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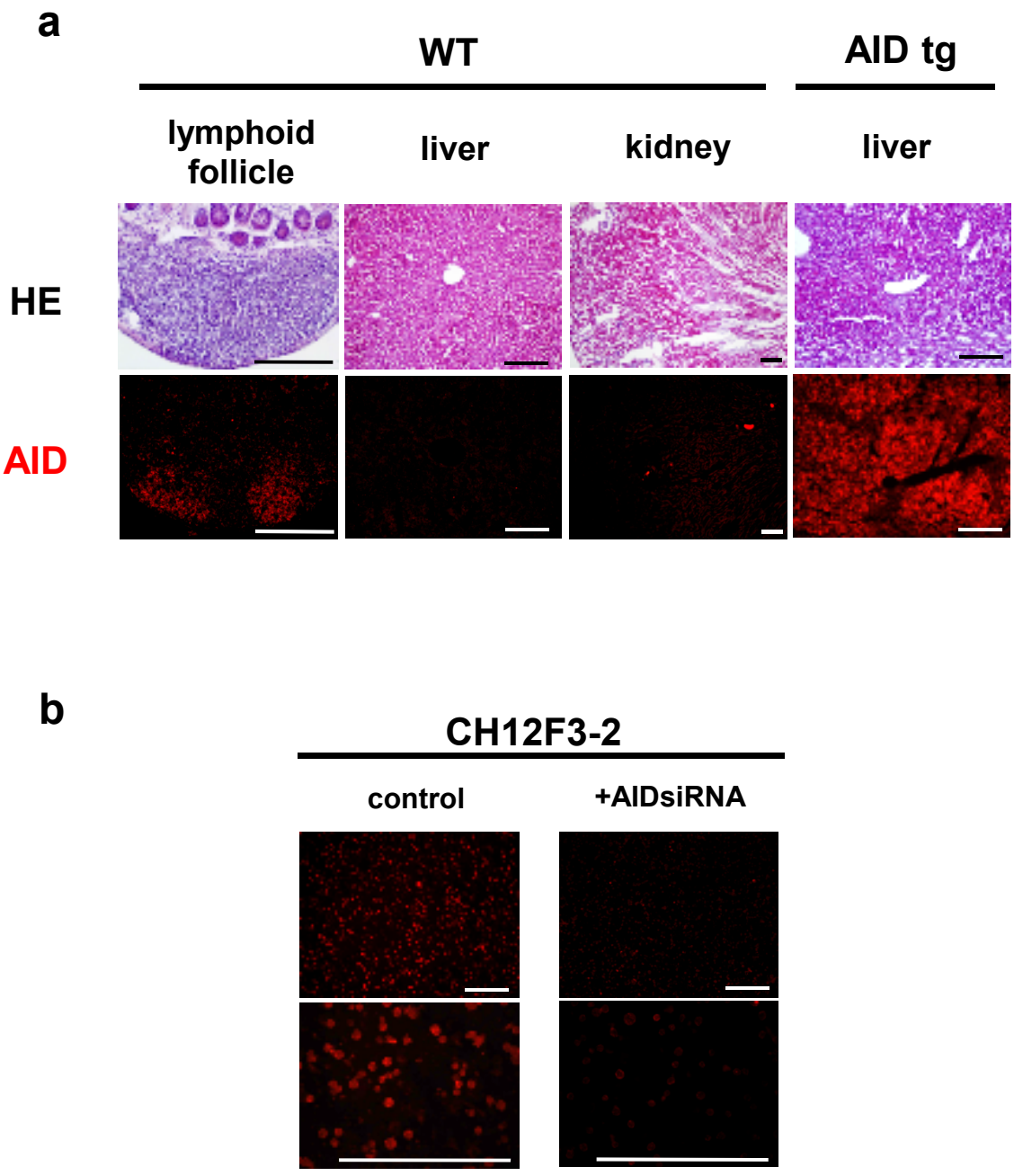

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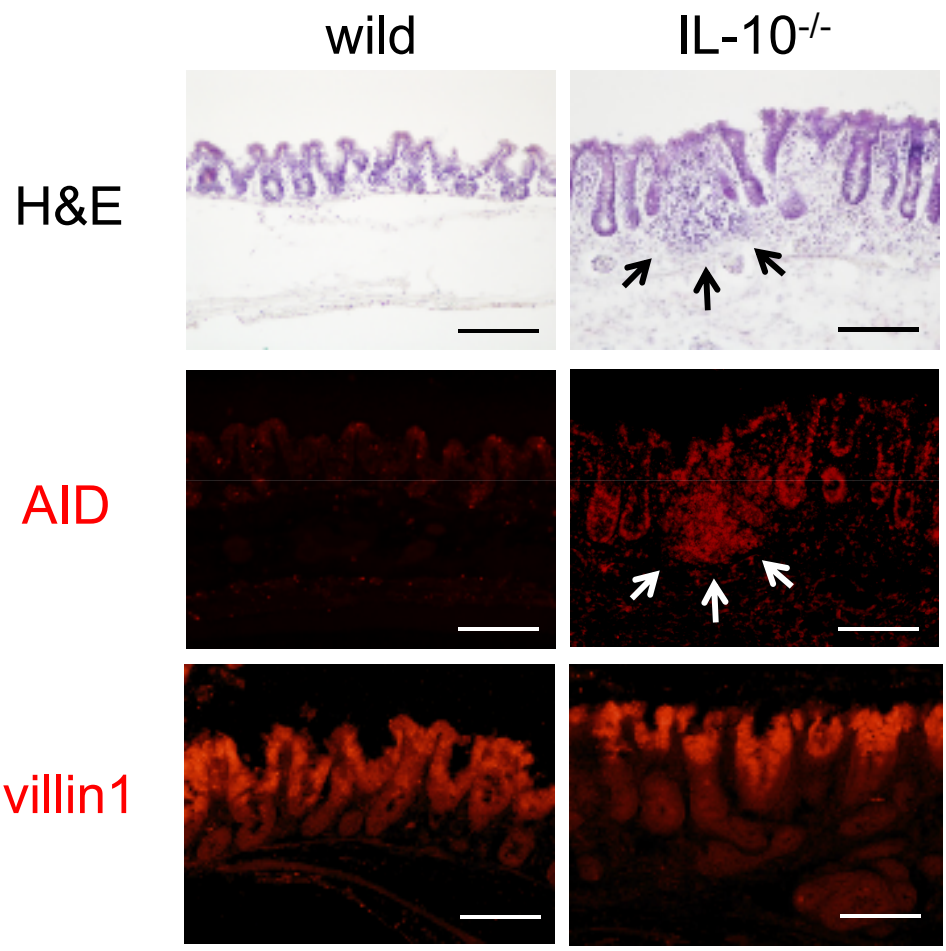

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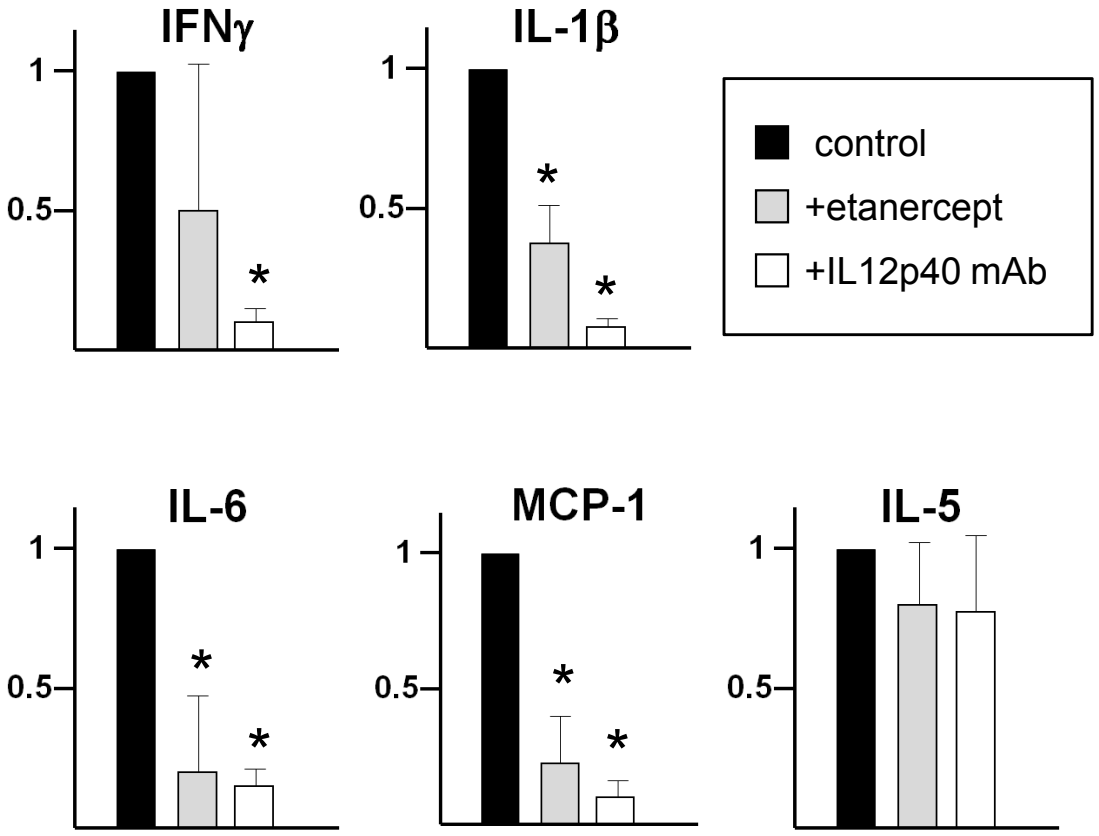

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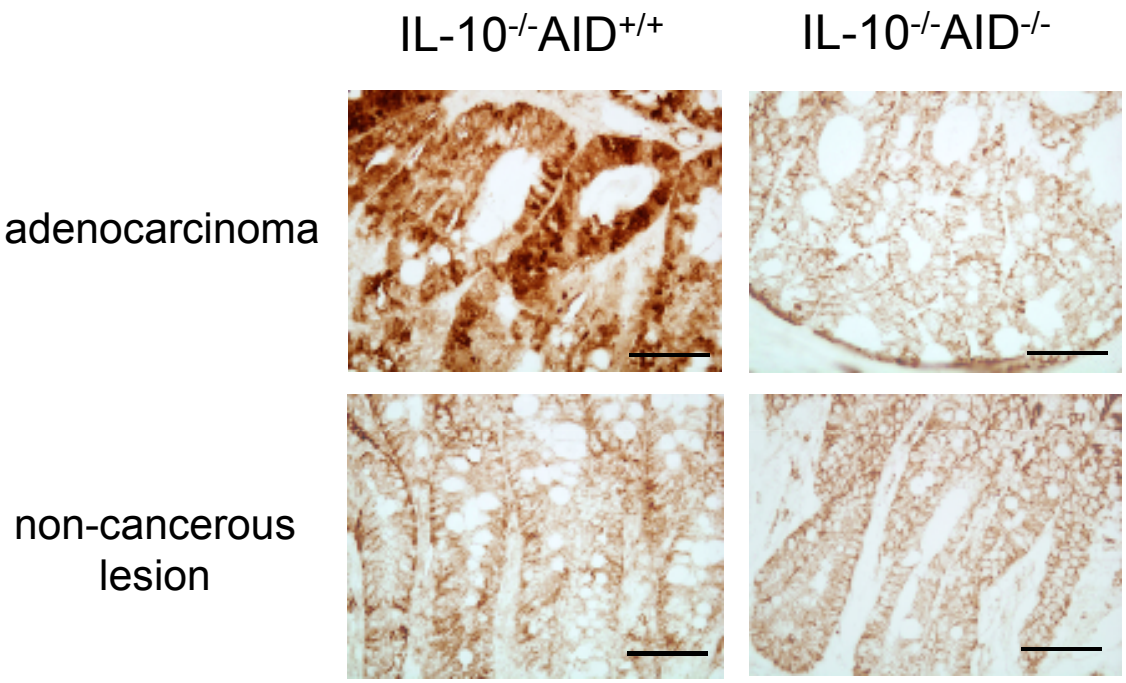

Supplementary Table 1. Primer sequences used for semiquantitative RT-PCR amplification

Primer	Nucleotide sequence
TNF-α-298S	5’-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3’
TNF-α-651AS	5’-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3’
IL-12-544S	5’-TGTGAAGCACCAAATTACTCCG-3’
IL-12-995AS	5’-TTCTTGCGCTGGATTCGAAC-3’
IFN-γ-101S	5’-CTGAGACAATGAACGCTACACACTG-3’
IFN-γ-556AS	5’-GCTTCCTGAGGCTGGATTCC-3’
IL-1β-161S	5’- GAAGGGCTGCTTCCAAACCT-3’
IL-1β-616AS	5’- TTCTTTCCTTTGAGGCCCAAG-3’
IL-6-95S	5’- ACCACGGCCTTCCCTACTTC-3’
IL-6-551AS	5’- GGAGAGCATTGGAAATTGGG-3’
IL-4-56S	5’-TGTCTCTCGTCACTGACGGC-3’
IL-4-522AS	5’-GGTGGCTCAGTACTACGAGTAATCC-3’
IL-13-29S	5’-GTTCTACAGCTCCCTGGTTCTCTC-3’
IL-13-511AS	5’-AGGAAAATGAGTCCACAGCTGAG-3’
Villin-356S	5’-GAGGTTCAAGGCAACGAGAG-3’
Villin-828AS	5’-AGCTTGAGTGCAGCCTTAGC-3’
CD19-463S	5’-CCACAGGTCCACTTCTGG-3’
CD19-875AS	5’-AGAGCCACACTGCTGAC-3’
β-actin-S	5’-GTGGGCCGCTCTAGGCACCAA-3’
β-actin-AS	5’-CTCTTTGATGTCACGCACGAT-3’

Supplementary Table 2. Primer sequences used for quantitative RT-PCR amplification

Primer	Nucleotide sequence
AID-93S	5’-CGTGGTGAAGAGGAGAGATAGTG-3’
AID-203AS	5’-CAGTCTGAGATGTAGCGTAGGAA-3’
TNF-α-181S	5’- GACGTGGAACTGGCAGAAGAG-3’
TNF-α-325AS	5’- CGATCACCCCGAAGTTCAGTAG-3’
IL-12-419S	5’- GCCAGTACACCTGCCACAAAG-3’
IL-12-566AS	5’- CCGGAGTAATTTGGTGCTTCAC-3’
IFN-γ-133S	5’- GCTTTGCAGCTCTTCCTCATG-3’
IFN-γ-282AS	5’- TTGCCAGTTCCTCCAGATATCC-3’
IL-1β-316S	5’- CCTTCCAGGATGAGGACATGAG-3’
IL-1β-462AS	5’- GTTGTTCATCTCGGAGCCTGTAG-3’
IL-6-57S	5’- TCCATCCAGTTGCCTTCTTG-3’
IL-6-221AS	5’- CCACGATTTCCCAGAGAACA-3’
MCP-1-193S	5’-CAGGTCCCTATGGTGCCAAT-3’
MCP-1-343AS	5’-TCTCGGTTCTTGACGGTTATCA-3’
IL-5-113S	5’- CCCATGAGCACAGTGGTGAA-3’
IL-5-237AS	5’- TCTCCAATGCATAGCTGGTGAT-3’
18S rRNA-S	5’-TAGAGTGTTCAAAGCAGGCCC-3’
18S rRNA-AS	5’-CCAACAAAATAGAACCGCGGT-3’

Supplementary Table 3. Primer sequences used for sequencing analyses

Primer	Nucleotide sequence
Apc-1852S	5’-CTCACTTACCGGAGCCAGAC-3’
Apc-2703AS	5’-GTCGTCCTGGGAGGTATGAA -3’
Apc-3735S	5’-TACTTGCAAAGTCCCCTCCA -3’
Apc-5021AS	5’-CTGACCCCATCTCCAGTAGC -3’
Ctnnb1-S	5’-CTGTTCTACGCCATCACGAC-3’
Ctnnb1-AS	5’-ATGGTGGGTGCAGGAGTTTA-3’
Kras-S	5’-AGGCCTGCTGAAAATGACTG -3’
Kras-AS	5’-ACACCTTGTCCTTGACTTCTTC-3’
Trp53-S	5’-ATGACTGCCATGGAGGAGTCA -3’
Trp53-AS	5’-AGAGGCAGTCAGTCTGAGTCA -3’