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COX-2 inhibition alters the phenotype of tumor-associated macrophages from M2 to M1 in *Apc$^{Min/+}$* mouse polyps

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Macrophages are a major component of tumor stroma. Tumor-associated macrophages (TAMs) show anti- (M1) or protumor (M2) functions depending on the cytokine milieu of the tumor microenvironment. Cyclooxygenase-2 (COX-2) is constitutively expressed in a variety of tumors including colorectal cancer. TAMs are known to be a major source of COX-2 in human and mice intestinal tumors. COX-2 inhibitor reduces the number and size of intestinal adenomas in familial adenomatous polyposis patients and *Apc$^{Min/+}$* mice. Although COX-2 inhibitor is thought to regulate cancer-related inflammation, its effect on TAM phenotype remains unknown. Here, we examined the effects of COX-2 inhibition on TAM phenotype and cytokine expression both *in vivo* and *in vitro*. Firstly, the selective COX-2 inhibitor celecoxib changed the TAM phenotype from M2 to M1, in proportion to the reduction in number of *Apc$^{Min/+}$* mouse polyps. Concomitantly, the expression of M1-related cytokine interfron (IFN)-γ was significantly upregulated by celecoxib, although the M2-related cytokines interleukin (IL)-4, IL-13 and IL-10 were not significantly altered. Secondly, IFN-γ treatment attenuated M2 phenotype of mouse peritoneal macrophages and oriented them to M1 even in the presence of M2-polarizing cytokines such as IL-4, IL-13 and IL-10. Thus, our results suggest that COX-2 inhibition alters TAM phenotype in an IFN-γ-dependent manner and subsequently may reduce intestinal tumor progression.

Introduction

Macrophages are a major component of the leukocyte infiltrates in various tumor stroma and macrophages that infiltrate tumors are called tumor-associated macrophages (TAMs) (1,2). TAMs have been recognized as a part of the inflammatory circuits that promote tumor progression (1,2). Since macrophages have functional plasticity and can change their activation status in response to the microenvironment (3,4), the concept of phenotypic heterogeneity of macrophages has been strengthened with the classification of M1 (classically activated) and M2 (alternatively activated) phenotypes in analogy with the Th1 and Th2 dichotomy (3,5,6). It is considered that when macrophages are exposed to lipopolysaccharides and interfron (IFN)-γ, they are polarized to the M1 phenotype and have antitumor functionality. When they are exposed to Th2 cytokines, such as interleukins (IL)-4, IL-13 and IL-10, they are polarized to the M2 phenotype and support cell proliferation and tumor growth (5,7). TAM is considered as a polarized M2 macrophage population in most mouse and human tumors (e.g. breast, prostate, glioma and lymphoma) (5,8).

Several studies have demonstrated that cyclooxygenase-2 (COX-2) is constitutively expressed in colorectal cancer, predominantly by stromal cells (9–18). Macrophages are one of the major sources of COX-2 in colorectal cancer (11,15–19). COX-2 promotes the progression of colorectal cancer through prostaglandin production and inhibition of COX-2 by traditional nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors reduces the number and size of adenomas in familial adenomatous polyposis patients and in *Apc* knockout mice (9,12–14,20–25). Although previous studies have shown that COX-2 inhibitors affect cytokine expression profiles and tumor microenvironments (26), the effect of COX-2 inhibition on TAM phenotype remains unclear.

To examine the effect of COX-2 inhibition on TAMs, we investigated the status of TAMs in *Apc$^{Min/+}$* mouse intestinal polyps and concomitant cytokine expression profiles with or without selective COX-2 inhibitors. In addition, we studied the effect of COX-2 inhibition and various cytokines on mouse peritoneal macrophages in terms of the M1/M2 phenotype.

Abbreviations: COX-2, cyclooxygenase-2; IFN, interfron; IL, interleukin; iNOS, inducible nitric oxide synthase; MR, mannose receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TAM, tumor-associated macrophage.

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Materials and methods

Mouse models

Apc$^{Min/+}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME), and 9- to 10-week-old male *Apc$^{Min/+}$* mice were treated with either drug-free chow or celecoxib-mixed chow (Pfizer, Groton, CT) for 8 weeks. Celecoxib were prepared in chow with a dose of 330, 66 or 6.6 μg/body wt g/day. After the mice were killed, their intestinal polyps were counted as described previously (27). All experiments were approved by the Animal Research Committee of Kyoto University and performed in accordance with the Japanese government regulations.

Histological analysis and immunofluorescence/immunohistochemistry stainings

For histological analysis, intestinal samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm thickness. Subsequently, sections were deparaffinized, rehydrated and stained with hematoxylin and eosin. For immunohistochemistry, sections were incubated with primary antibody (rat anti-mouse F4/80, 1:100; Abcam, Cambridge, MA) overnight and with the biotinylated secondary antibody followed by incubation with the avidin–biotin–peroxidase complex (Vector Labs, Southfield, MI). The sections were labeled with peroxidase and colored with diaminobenzidine substrate (Dako, Glostrup, Denmark). For immunofluorescence, intestinal samples were embedded in OCT compound (Sakura, Tokyo, Japan) and frozen at −80°C. These frozen samples were sectioned at 6 μm, fixed in methanol, washed with phosphate-buffered saline (PBS) and then incubated with the primary antibodies [rat anti-mouse F4/80 (1:100; Abcam), rabbit anti-mouse F4/80 (1:100; Abcam), rat anti-mouse mannose receptor (MR) (1:25; Hycult biotech, Uden, Netherlands), rabbit anti-mouse inducible nitric oxide synthase (iNOS) (1:100; Abcam), rabbit anti-mouse COX2 (1:100; Abcam), Alexa Fluor 488 conjugated hamster anti-mouse CD3ϵ (1:100; eBioscience, San Diego, CA), mouse anti-mouse Ncam (1:100; Abcam) or rat anti-mouse IFN-γ (1:400; eBioscience)] overnight at 4°C and washed again with PBS. Washed sections were treated with fluorescence-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 2 h.

Cell preparations

Mouse peritoneal macrophages were isolated from 8-week-old female C57BL/6J mice. Peritoneal cells were harvested by peritoneal lavage with 10 ml PBS. Cells were resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum, 100 mg/ml of penicillin, 100 mg/ml of streptomycin and 1.25 μg/ml of amphotericin B. 1×10^6 peritoneal cells were seeded into a 12-well dish and incubated for 2 h. Then, cells were washed in PBS and recultured in Dulbecco's modified Eagle's medium without fetal calf serum. To inhibit COX-2 activity, 10 μM of celecoxib was added to the culture medium with 20 ng/ml of mouse recombinant IL-4 (PeproTech, Rocky Hill, NJ) and 20 ng/ml of mouse recombinant IL-13 (PeproTech) or with 20 ng/ml of mouse recombinant IL-10 (R&D Systems, Minneapolis, MN). For the IFN-γ addition experiment, 20 ng/ml of mouse

recombinant IFN-γ (PeproTech) was added to the cells with 20 ng/ml of IL-4 and 20 ng/ml of IL-13 or with 20 ng/ml of IL-10. Cells were collected 4 h after stimulation.

RNA isolation and quantitative reverse transcription–polymerase chain reaction

We isolated RNA from $Apc^{Min/+}$ mice and mouse peritoneal macrophages using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions and then synthesized single-stranded complementary DNA from 1 μg of total RNA using Superscript III (Invitrogen). We performed quantitative polymerase chain reaction (PCR) using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Osaka, Japan). The amplification conditions included 10 s of denaturation at 95°C, 5 s of annealing at 57°C and 10 s of extension at 72°C for a total of 45 cycles. Quantitative reverse transcription (qRT)–PCR primers were as follows: mouse GAPDH-forward, AGGTCGGTGTGAACGGATTTG, mouse GAPDH-reverse, TGTAGACCATGTAGTTGAGGTCA; mouse Arginase 1-forward, TGGCTTGCGAGACGTAGAC, mouse Arginase 1-reverse, GCTCAGGTGAATCGGCCTTTT; mouse MR-forward, GCTGAATCCCAGAAATTCCGC, mouse MR-reverse, ATCACAGGCATACAGGGTGAC; mouse iNOS-forward, GTTCTCAGCCCAACAATACAAGA, mouse iNOS-reverse, GTGGACGGGTCGATGTCAC; mouse Ym1-forward, TTATCCTGAGTGACCCTTCTAAG, mouse Ym1-reverse, TCATTACCCTGATAGGCATAGG; mouse Trem2-forward, CTGGAACCGTCACCATCACTC, mouse Trem2-reverse, CGAAACTCGATGACTCCTCGG; mouse CXCL10-forward, CCAAGTGCTGCCGTCATTTTC, mouse CXCL10-reverse, GGCTCGCAGGGATGATTTCAA; mouse IFN-γ-forward, ATGAACGCTACACACTGCATC, mouse IFN-γ-reverse, CCATCCTTTTGCCAGTTCCTC; mouse IL-4-forward, GGTCTCAACCCCCAGCTAGT, mouse IL-4-reverse, GCCGATGATCTCTCTCAAGTGAT; mouse IL-10-forward, GCTCTTACTGACTGGCATGAG, mouse IL-10-reverse, CGCAGCTCTAGGAGCATGTG; mouse IL-13-forward, GGATATTGCATGGCCTCTGTAAC, mouse IL-13-reverse, AACAGTTGCTTTGTGTAGCTGA; mouse IL-12-forward, ACTCTGCGCCAGAAACCTC, mouse IL-12-reverse, CACCCTGTTGATGGTCACGAC; mouse CD4-forward, AGGTGATGGGACCTACCTCTC, mouse CD4-reverse, GGGGCCACCACTTGAACTAC; mouse CD8a-forward, CCGTTGACCCGCTTTCTGT, mouse CD8a-reverse, CGGCGTCCATTTTCTTTGGAA; mouse Nkg2d-forward, ACTCAGAGATGAGCAAATGCC, mouse Nkg2d-reverse, CAGGTTGACTGGTAGTTAGTGC.

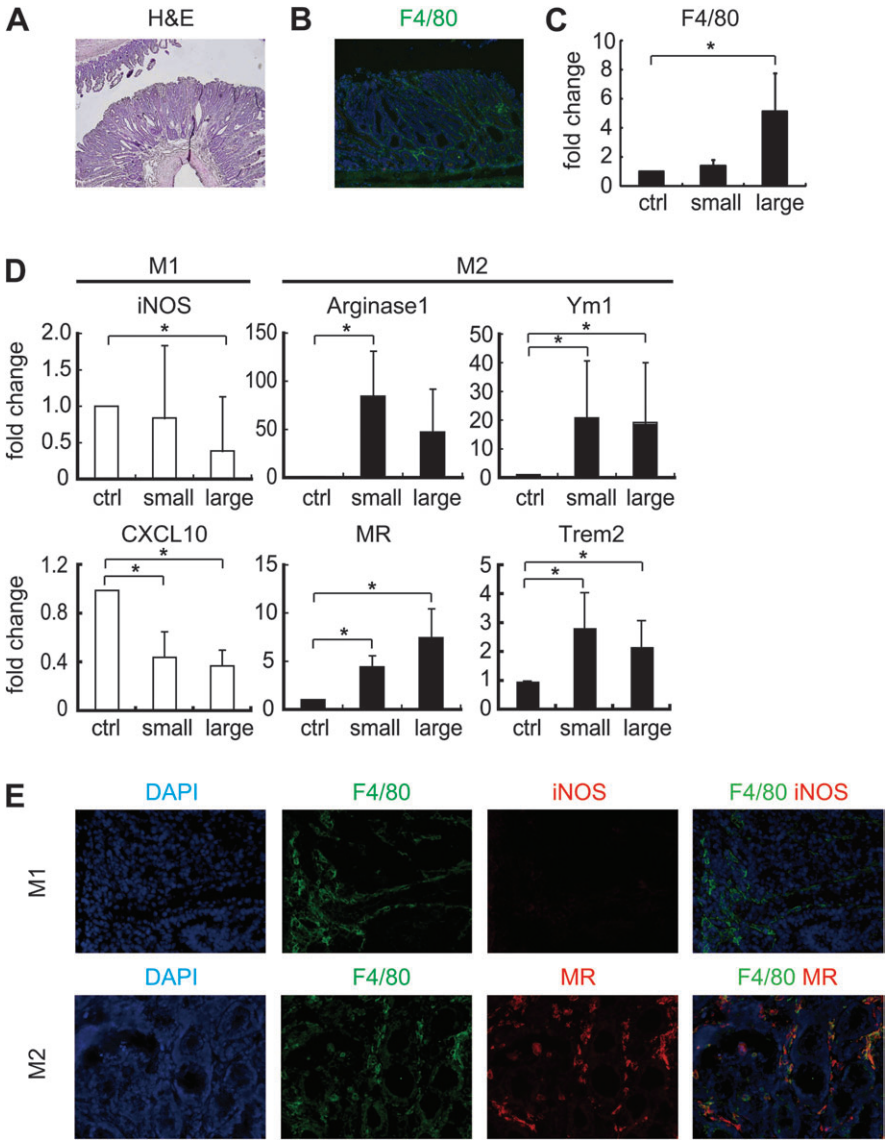

Fig. 1. TAMs infiltrating $Apc^{Min/+}$ mouse polyps were polarized to the M2 phenotype. (**A**) Representative hematoxylin and eosin staining of small intestinal polyp of $Apc^{Min/+}$ mouse. Original magnification, ×100. (**B**) $Apc^{Min/+}$ mouse polyp stroma was infiltrated with F4/80-positive TAMs (green). (**C**) qRT–PCR showed that expression of F4/80 was upregulated in large polyps. Data represent fold induction of mRNA expression compared with histologically normal mucosa (ctrl) ($n = 6$). $^{*}P < 0.05$. (**D**) The expression of M1 and M2 genes in small or large polyps was evaluated by qRT–PCR. In intestinal polyps, M1 markers (iNOS and CXCL10) were suppressed and M2 markers (Arginase1, MR, Ym1 and Trem2) were increased in a polyp size-dependent manner. Data represent fold induction of mRNA expression compared with histologically normal mucosa (ctrl) ($n = 8$). $^{*}P < 0.05$. (**E**) Polyps were double stained with F4/80 and iNOS (M1 marker, showing negative staining) or F4/80 and MR (M2 marker, showing positive colocalization). Original magnification, ×400.

Statistics

All values are presented as mean ± SD unless otherwise stated. Two-tailed Student's *t*-test was used for statistical analysis. A P value <0.05 was considered significant.

Results

Stroma of $Apc^{Min/+}$ mouse polyps was infiltrated by TAMs that were polarized to M2 phenotypes

We first investigated the status of TAMs in intestinal polyps of $Apc^{Min/+}$ mice. Here, we defined polyps with 1–2 mm in diameter as 'small' polyps and those >2 mm in diameter as 'large' polyps. Immunohistochemistry showed that polyp stroma was infiltrated by a massive number of F4/80-positive macrophages (Figure 1A and B). Although macrophages are a major stromal component in histologically normal mucosa of the small intestine, messenger RNA (mRNA) expression of F4/80 in polyps was increased in a size-dependent manner (Figure 1C). This finding was consistent with previous reports demonstrating massive infiltration of TAMs in mouse intestinal tumor stroma (28).

To evaluate the phenotype of TAMs in $Apc^{Min/+}$ mouse polyps, we next investigated mRNA expression of representative M1 and M2 genes by qRT–PCR (Figure 1D). mRNA of iNOS and C-X-C motif chemokine 10 (CXCL10), which are key effector molecules produced by M1 macrophages, were significantly lower in small and large polyps compared with histologically normal mucosa. In contrast, mRNA of arginase 1, Ym1, MR and triggering receptor expressed on myeloid cells 2 (Trem2), which are typical M2 markers, were higher in small and large polyps. Immunohistochemistry was consistent with these findings. F4/80- and iNOS-positive macrophages were scarcely detected in polyp stroma, but a number of F4/80- and MR-positive macrophages had infiltrated the stroma (Figure 1E). Taken together, TAMs infiltrating the small intestinal polyp stroma of $Apc^{Min/+}$ mice were polarized to M2 phenotypes.

Th2 cytokines were predominant in the microenvironment of $Apc^{Min/+}$ mouse polyps

Since TAMs can alter their phenotype in response to the microenvironment in which they exist, we sought to investigate the factors that determine the M2 phenotype in TAMs in $Apc^{Min/+}$ mouse polyps. mRNA expression of IFN-γ and IL-12, which are responsible for driving Th1 responses and antitumor activity, were suppressed in polyps compared with histologically normal mucosa (Figure 2A). In contrast, mRNA of IL-4, IL-13 and IL-10, which induce M2 macrophage phenotype, were higher in polyps than in histologically normal mucosa (Figure 2B). Thus, the cytokine expression profile of $Apc^{Min/+}$ mouse polyps was consistent with the M2 TAM phenotype we observed.

COX-2 inhibitor reduced the size and number of polyps and altered TAM phenotype from M2 to M1 in $Apc^{Min/+}$ mice

Subsequently, we investigated the effect of COX-2 inhibition on TAM activation in $Apc^{Min/+}$ mouse polyps. Similar to previously reported studies (9), mRNA levels of COX-2 showed 2.01 ± 1.6 (mean ± SD)-fold elevation in small polyps as compared with histologically normal mucosa, and COX-2 protein was immunostained in polyp stroma cells, including F4/80-positive cells (Figure 3A).

To determine whether COX-2 inhibition affects TAM phenotype, we administered celecoxib, a widely used COX-2 selective inhibitor, to $Apc^{Min/+}$ mice for 8 weeks. We used 330 or 66 μg/body wt g/day of celecoxib based on previous reports (23,29). The number and size of small intestinal polyps were decreased in $Apc^{Min/+}$ mice treated with 330 or 66 μg of celecoxib (Figure 3B and C). We also used 6.6 μg of celecoxib, which is usual daily dose in human and could not find significant suppression of intestinal polyps in $Apc^{Min/+}$ mice (data not shown). As well as previous reports (10), we considered the required dose of COX-2 inhibitor would be different between mouse and human, and used 330 μg/body wt g/day of celecoxib in the following mouse studies. As described previously (10), large polyps almost disappeared in mice fed with celecoxib, we studied the phenotypes of

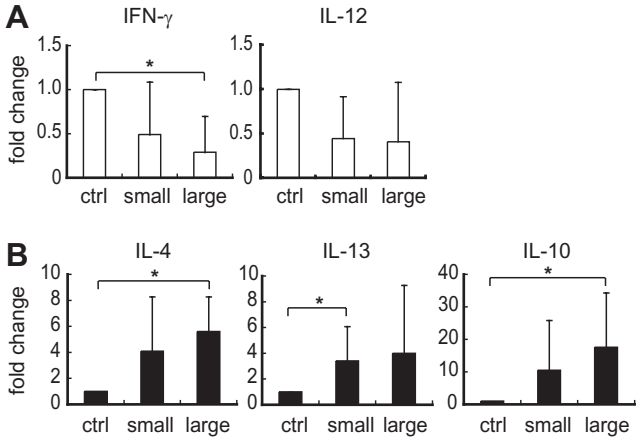

Fig. 2. $Apc^{Min/+}$ mouse polyps showed a Th2-predominant tumor microenvironment. (**A** and **B**) qRT–PCR analysis was performed for cytokine milieu in $Apc^{Min/+}$ mouse polyps. Histologically normal mucosa was used as control (ctrl) ($n = 6$). $^{*}P < 0.05$. (A) Th1 cytokines (IFN-γ and IL-12) were suppressed in $Apc^{Min/+}$ mouse polyps in a polyp size-dependent manner. (B) Th2 cytokines (IL-4, IL-13 and IL-10) were upregulated in $Apc^{Min/+}$ mouse polyps in a polyp size-dependent manner.

TAMs using the same size fraction of polyps 1–2 mm in diameter with or without celecoxib. As shown in Figure 3D, mRNA of the M1 genes iNOS and CXCL10 were restored with COX-2 inhibition. In contrast, mRNA of the M2 genes arginase 1, Ym1, MR and Trem2 were markedly downregulated in polyps and were almost similar to those in histologically normal mucosa with COX-2 inhibition. Thus, COX-2 inhibition skewed TAM phenotype from M2 to M1 in $Apc^{Min/+}$ mouse polyps.

COX-2 inhibition resulted in significant upregulation of IFN-γ in $Apc^{Min/+}$ mouse polyps

Based on the alteration of TAM phenotypes, we presumed that COX-2 inhibition may also alter Th1/2 cytokine levels in Apc^{Min} mouse polyps. Therefore, we investigated cytokine expression in the same size fraction of polyps (1–2 mm in diameter) with or without COX-2 inhibition. Among Th1 cytokines, mRNA expression of IFN-γ was significantly higher in polyps with COX-2 inhibition than in control polyps without COX-2 inhibition (Figure 4A). On the other hand, Th2 cytokines such as IL-4, IL-13 and IL-10 were suppressed by COX-2 inhibition. However, this suppression of Th2 cytokines was not statistically significant, and therefore the Th2 cytokines appeared to remain in Apc^{Min} mouse polyps even in the presence of COX-2 inhibitor. We next examined the source of IFN-γ in Apc^{Min} mouse polyps induced by COX-2 inhibition. qRT–PCR showed mRNA expression of CD4, CD8 and Nkg2d were enhanced by COX-2 inhibition, suggesting influx of T cells and natural killer cells was increased (Figure 4B). Immunofluorescence stainings demonstrated IFN-γ expression colocalized with both CD3ϵ-positive T cells and Ncam-positive natural killer cells in Apc^{Min} mouse polyps with COX-2 inhibition (Figure 4C). Collectively, these findings indicated that COX-2 inhibition altered cytokine profile by enhancing T cells and natural killer cells to produce IFN-γ and might skewed M2 TAMs to M1 phenotype in tumor microenvironment.

COX-2 inhibition alone was not sufficient to alter the activation status of mouse peritoneal macrophages in the presence of IL-4, IL-13 and IL-10

In vivo studies raised a question whether up-regulation of Th1 cytokine IFN-γ by COX-2 inhibition could alter TAM phenotypes from M2 to M1 even in the presence of Th2 cytokines such as IL-4, IL-13 and IL-10. To answer this question, we examined the direct effect of these cytokines on freshly isolated mouse peritoneal macrophages in terms of M1/M2 phenotype.

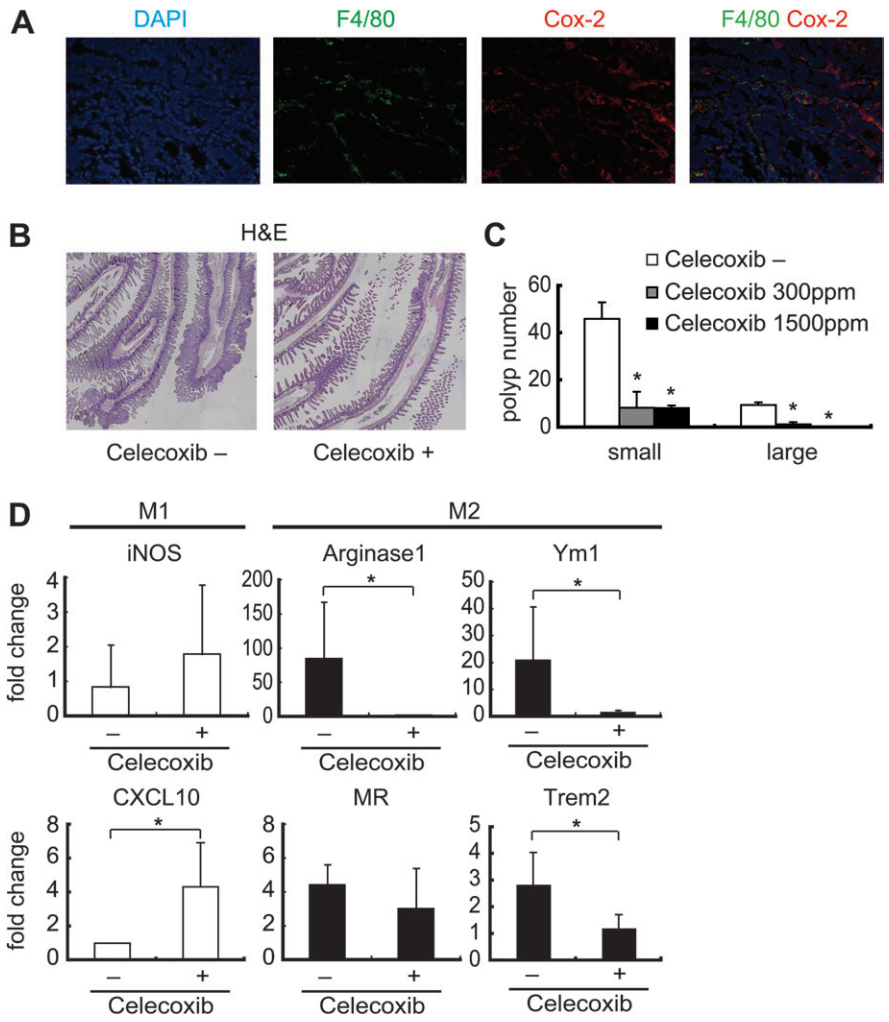

Fig. 3. COX-2 inhibitor altered TAM phenotypes from M2 to M1 in $Apc^{Min/+}$ mouse polyps. (**A**) Double staining with F4/80 (green) and COX-2 (red) showed that macrophages produced COX-2 in $Apc^{Min/+}$ mouse polyps. Original magnification, ×400. (**B**) Male $Apc^{Min/+}$ mouse were treated with either drug-free chow or 330 μg/body wt g/day celecoxib-mixed chow for 8 weeks ($n = 8$). Representative hematoxylin and eosin sections showed reduction of small intestinal polyps with celecoxib. Original magnification, ×40. (**C**) The number of small or large polyps of small intestine in control and 330 or 66 μg/body wt g/day celecoxib-treated cohorts was counted. (**D**) mRNA expression in polyps of control and celecoxib-treated cohorts was evaluated by qRT–PCR. M1 markers (iNOS and CXCL10) were upregulated and M2 markers (Arginase1, MR, Ym1 and Trem2) were downregulated. Data represent fold induction of mRNA expression compared with mRNA in control $Apc^{Min/+}$ mouse polyps ($n = 4$–8). $^{*}P < 0.05$.

As a previous report showed that macrophages were polarized to the M2 phenotype with IL-4 and IL-13 (4,30), we first administered 20 ng/ml of recombinant IL-4 and IL-13 to mouse macrophages. We found that the mRNA expression of the M1 marker iNOS, CXCL10 and IL-12 remained unchanged (Figure 5A). In contrast, the M2 marker MR, Trem2 and Ym1 mRNA were significantly upregulated. Thus, mouse peritoneal macrophages, though not completely, polarized to the M2 phenotype in the presence of IL-4 and IL-13, and therefore these cells seemed a model for TAMs.

To test the role of COX-2 inhibition in macrophage phenotype, we administered 10 μM celecoxib to the medium together with 20 ng/ml of recombinant IL-4 and IL-13 (Figure 5A). mRNA expression of CXCL10 was upregulated. However, mRNA of iNOS and IL-12 were not altered significantly. Thus, at the cellular levels COX-2 inhibition alone was not sufficient to alter an M2 phenotype of macrophages in the presence of IL-4 and IL-13. Because IL-4 and IL-13 induce M2a or wound healing macrophages, and IL-10 induces M2c or regulatory macrophages (5,7), we investigated the effect of recombinant IL-10 on mouse macrophages. 20 ng/ml of IL-10 also upregulated mRNA of M2 markers, MR, Trem2 and Ym1 (Figure 5B). However, administration of COX-2 inhibitor together with 20 ng/ml of IL-10 failed to alter macrophages from M2 phenotype to M1. We tested another macrophage models using human monocyte cell line THP-1 and obtained similar results to mouse macrophages (data not shown). Collectively, these results indicated that COX-2 inhibition alone was not sufficient to alter macrophage activation status from M2 to M1 in the presence of Th2 cytokines.

M2-polarized mouse macrophages were oriented to the M1 phenotype by the addition of IFN-γ even in the presence of IL-4, IL-13 and IL-10

Based on the Apc^{Min} mouse polyp data, we sought to examine the direct effect of IFN-γ on the regulation of macrophage phenotypes. Several reports demonstrate that IFN-γ prevents tumor development and reverts the M2 phenotype of TAMs (31–33). Since IFN-γ appeared to be produced by T cells and natural killer cells in Apc^{Min} mouse polyps (Figure 4B and C) and mRNA of IFN-γ in mouse macrophages was not affected by COX-2 inhibition (data not shown), we added 2, 20 or 200 ng/ml of recombinant IFN-γ to mouse peritoneal macrophages. We found that 20 ng/ml of recombinant IFN-γ significantly elevated mRNA expression of iNOS, CXCL10 and IL-12 in macrophages that had been administered 20 ng/ml of recombinant IL-4 and IL-13 (Figure 6A). In contrast, IL-4 + IL-13-induced mRNA of MR, Trem2 and Ym1 were significantly suppressed by IFN-

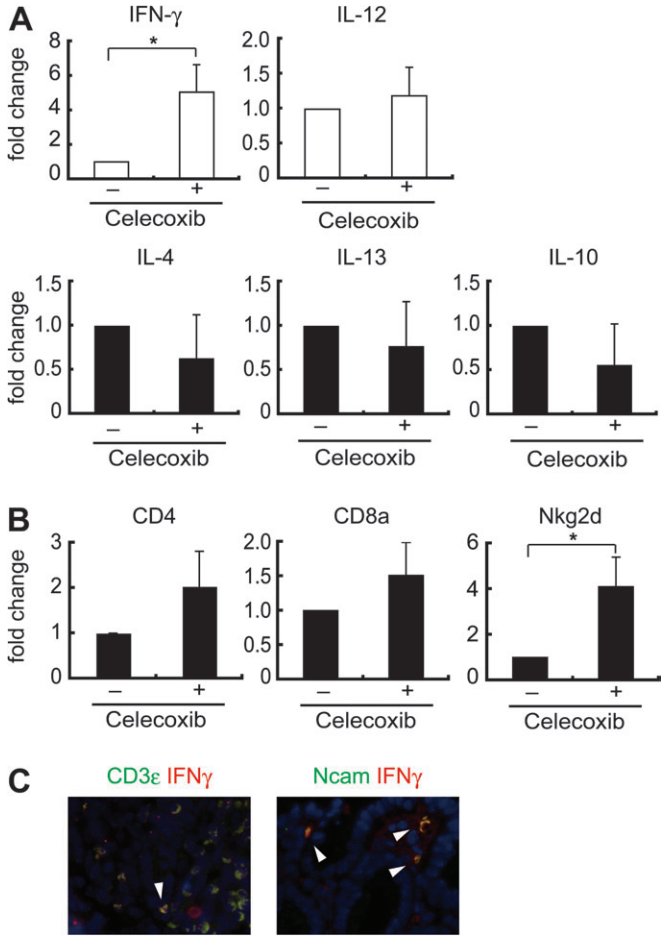

Fig. 4. Cytokine milieu in $Apc^{Min/+}$ mouse polyps showed upregulation of IFN-γ with celecoxib. (**A**) qRT–PCR analyses were performed for cytokine milieu in $Apc^{Min/+}$ mouse polyps treated with celecoxib. Data represent fold induction of mRNA expression compared with mRNA in control $Apc^{Min/+}$ mouse polyps ($n = 4$–8). $^{*}P < 0.05$ (upper). Among Th1 cytokines, the expression of IFN-γ was significantly upregulated by COX-2 inhibition (lower). Th2 cytokines (IL-4, IL-13 and IL-10) were suppressed, although not significantly, by COX-2 inhibition. (**B**) mRNA expression of CD4, CD8a and Nkg2d were elevated in 330 μg/body wt g/day celecoxib-treated $Apc^{Min/+}$ mouse polyps ($n = 4$–8) (**C**) IFN-γ expression (red) colocalized with both CD3 (left, green) and Ncam (right, green) in Apc^{Min} mouse polyps with COX-2 inhibition.

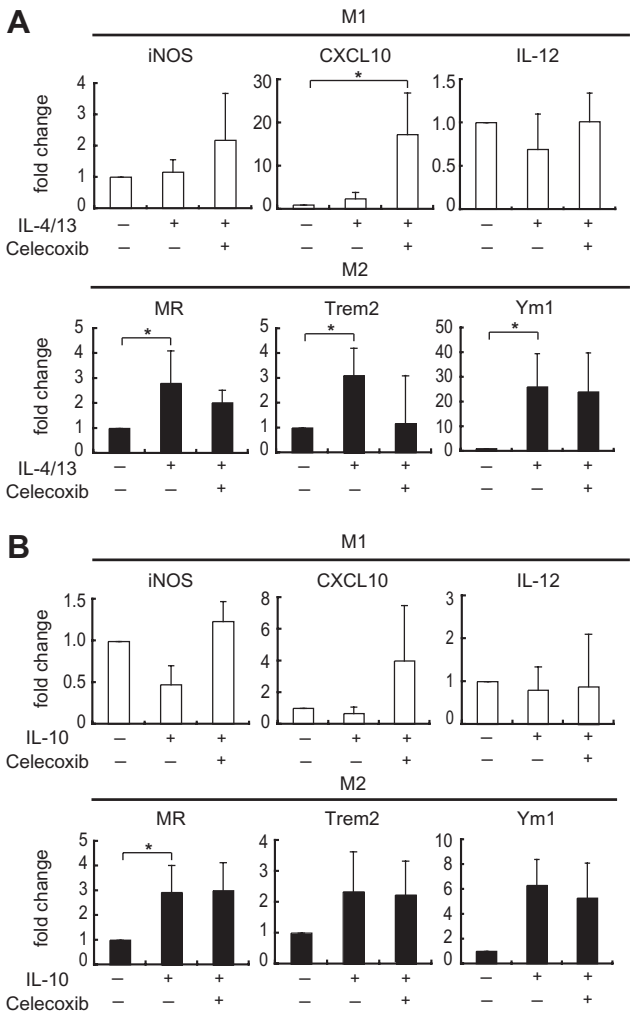

Fig. 5. COX-2 inhibition alone was not sufficient to regulate the activation status of mouse peritoneal macrophages in the presence of IL-4, IL-13 and IL-10. (**A** and **B**) mRNA expression of M1 and M2 genes was evaluated by qRT–PCR in freshly isolated mouse peritoneal macrophages. (A) When treated with 20 ng/ml of recombinant IL-4 and IL-13 for 4 h, mRNA of MR, Trem2 and Ym1 were upregulated, suggesting polarization to the M2 phenotype. Administration of 10 μM celecoxib for 4 h upregulated CXCL10. However, other markers were not altered siginificantly. $^{*}P < 0.05$. (B) When treated with 20 ng/ml of recombinant IL-10 for 4 h, mRNA of MR, Trem2 and Ym1 were upregulated, suggesting that macrophages were oriented to the M2 as well as IL-4 + IL-13. Administration of 10 μM celecoxib for 4 h altered M1/M2 markers in part (iNOS and CXCL10) without significant alterations. $^{*}P < 0.05$.

γ. Similar outcomes were obtained when the experiment was performed with 2 or 200 ng/ml of IFN-γ (data not shown). These results indicate that IFN-γ has sufficient capacity to alter the activation status of macrophages from M2 to M1 even in the presence of IL-4 and IL-13. We also confirmed the crucial role of IFN-γ on mouse macrophages in the presence of IL-10. Treatment with 20 ng/ml of IFN-γ upregulated M1 markers (iNOS, CXCL10 and IL-12) and downregulated M2 markers (MR, Trem2 and Ym1) again in mouse macrophages in the presence of 20 ng/ml of IL-10 (Figure 6B).

Discussion

In the present study, we demonstrated that TAMs in $Apc^{Min/+}$ mouse polyps possessed alternatively activated (M2) phenotypes and that the cytokine milieu of the polyps was Th2 predominant. Our *in vivo* study revealed that COX-2 inhibition altered TAM phenotypes from M2 to M1 in association with the increase of IFN-γ in the polyps, and our *in vitro* study showed that IFN-γ played a key role in the alteration of macrophage phenotypes even in the presence of the Th2 cytokines IL-4, IL-13 and IL-10. These data suggest that COX-2 inhibition alters TAM phenotype in an IFN-γ-dependent manner.

To the best of our knowledge, this study is the first direct demonstration that TAM phenotype is polarized to M2 in $Apc^{Min/+}$ mouse intestinal polyps (18). Due to the functional plasticity of macrophages, TAMs are strongly influenced by tumor microenvironment (e.g. Th1/Th2 balance) and are considered to be alternatively activated in most tumors (4,8,34–38). TAMs, especially M2 TAMs, promote tumor progression in a number of experimental models (8, 34–39). However, the significance of TAMs in gut tumorigenesis has been unclear, with some reports showing a correlation between increased number of TAMs and good prognosis, which is in contrast to other malignancies (40,41). To date, few experimental reports have directly demonstrated the role of TAMs in intestinal tumorigenesis in this context. Our study using $Apc^{Min/+}$ mouse supports the possibility

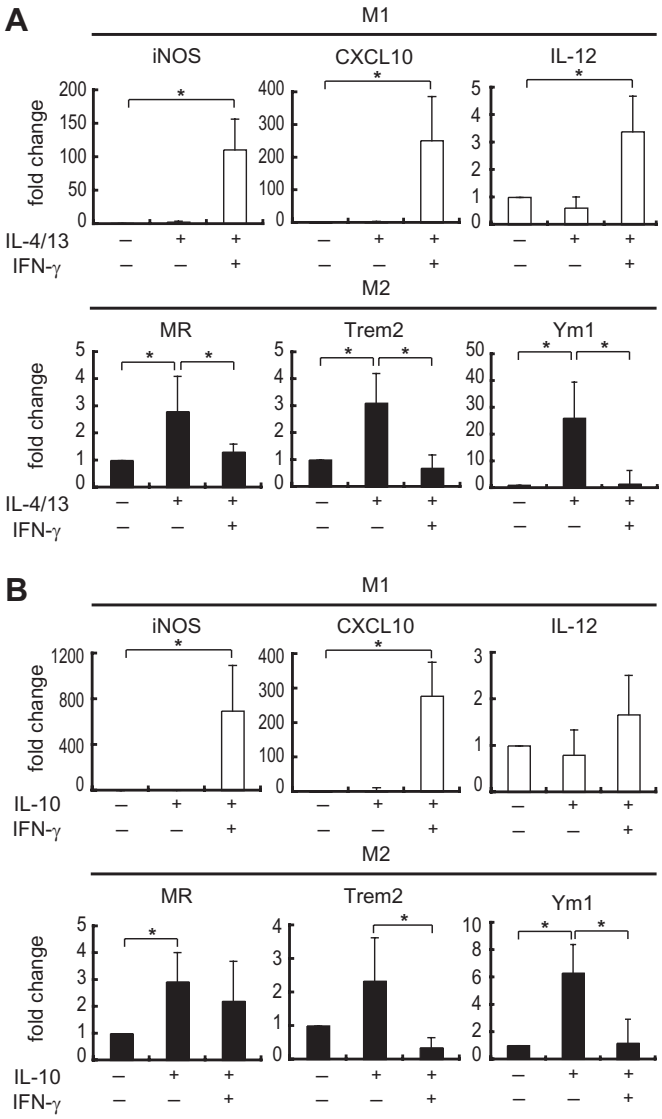

Fig. 6. IFN-γ had sufficient capacity to alter the phenotype of mouse peritoneal macrophages from M2 to M1 even in the presence of IL-4, IL-13 and IL-10. (**A** and **B**) mRNA expression of M1 and M2 genes was evaluated by qRT–PCR in freshly isolated mouse peritoneal macrophages. (**A**) Together with 20 ng/ml of IL-4 and IL-13, 20 ng/ml of IFN-γ was added to M2-polarized macrophages. qRT–PCR showed significant upregulation of all M1 markers and downregulation of all M2 markers by administration of IFN-γ, even in the presence of IL-4 and IL-13. Representative data from at least four independent experiments are shown. $^{*}P < 0.05$. (B) Together with 20 ng/ml of IL-10, 20 ng/ml of IFN-γ was added to M2-polarized macrophages. qRT–PCR showed upregulation of M1 markers and downregulation of all M2 markers by administration of IFN-γ, even in the presence of IL-10. Representative data from at least four independent experiments are shown. $^{*}P < 0.05$.

that accumulating M2 TAMs play a role in intestinal tumor progression that is similar to that in other organs.

Subsequently, we investigated the effect of COX-2 inhibition on TAM activation in $Apc^{Min/+}$ mouse polyps. As previously reported (9,10,23), COX-2 inhibitor reduced intestinal polyps in $Apc^{Min/+}$ mice. Although it is generally accepted that COX-2 inhibition induces tumor reduction partly through the regulation of cancer-related immune responses (20–23), few papers have directly demonstrated the effect of COX-2 inhibition on TAM phenotypes. In the present study, we showed that COX-2 inhibition skewed M2 TAMs in $Apc^{Min/+}$ mouse polyps to M1 with enhanced IFN-γ production.

Although IFN-γ is known to be one of the key inducers of M1 polarization (4,5), the superiority between Th1 and Th2 cytokines to regulate the activation of TAM remains to be elucidated. Therefore, to determine this, we used mouse peritoneal macrophages and demonstrated that COX-2 inhibition alone was not sufficient to alter M2 markers of macrophages in the presence of IL-4, IL-13 and IL-10. These data suggest that additional factors, secreted from other cell types, are required to alter TAM phenotypes in $Apc^{Min/+}$ mouse polyps, particularly in response to administration of a COX-2 inhibitor. Notably, we observed that the administration of IFN-γ altered macrophage phenotypes even in the presence of IL-4, IL-13 and IL-10. Taken together with our data in $Apc^{Min/+}$ mice, COX-2 inhibition appeared to change TAM phenotypes not directly but through IFN-γ induction in T cells and natural killer cells. These results are consistent with a pivotal role for IFN-γ in altering the tumor microenvironments (33,42,43). Therefore, it can be suggested that the skewing of TAM phenotypes by IFN-γ contributes to COX-2–dependent reduction of $Apc^{Min/+}$ mouse polyps.

In conclusion, we demonstrated that COX-2 inhibition altered TAM phenotypes, possibly in an IFN-γ-dependent manner, in the polyps of $Apc^{Min/+}$ mouse. The study provides a new insight into the regulation of TAM phenotypes, and bridges the immune network with the antitumor properties of COX-2 inhibitor. These findings may support the development of novel therapeutic strategies in colorectal cancer patients through the skewing of TAM phenotypes.

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