S100G expression and function in fibroblasts on colitis induction

Kazuhiro Ishiguro*, Osamu Watanabe, Masanao Nakamura, Takeshi Yamamura, Takafumi Ando, Hidemi Goto, Yoshiki Hirooka

Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya, Aichi, 466-8550, Japan

* Correspondence: Kazuhiro Ishiguro, Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya, Aichi, 466-8550, Japan. E-mail kio@med.nagoya-u.ac.jp, Fax. +81-52-744-2175, Tel. +81-52-744-2144.

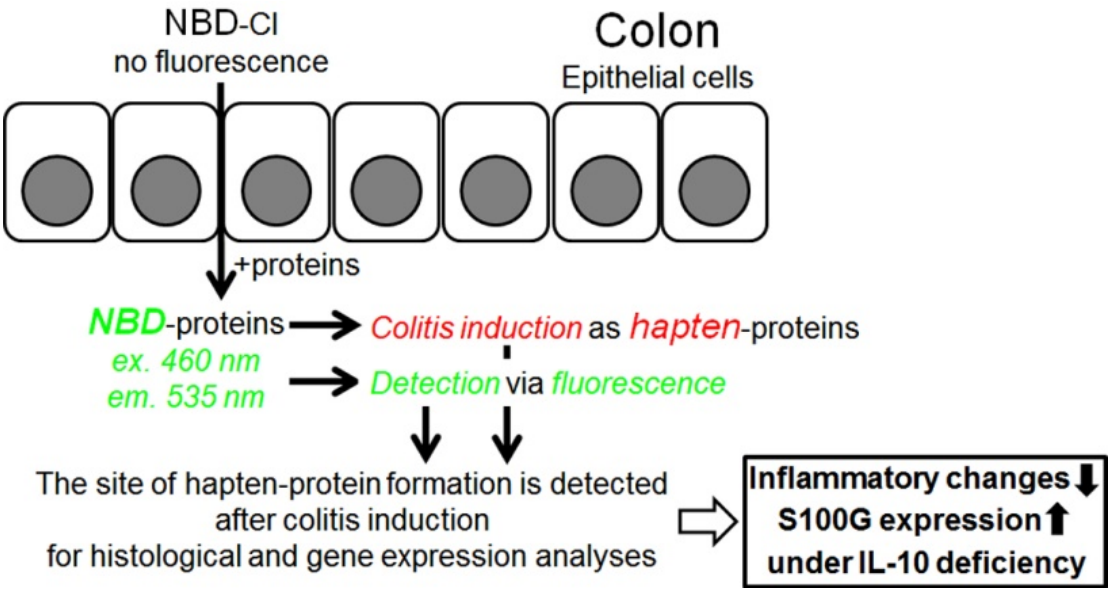
Abbreviations used

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hpf, high-power field; IBD, inflammatory bowel diseases; IDO1, indoleamine 2,3-dioxygenase 1; IL, interleukin; MCP-1, monocyte chemotactic protein-1; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole.

Abstract

Supplementation with interleukin (IL)-10, an important anti-inflammatory cytokine, has shown disappointing efficacy for inflammatory bowel diseases (IBD). IL-10 may down-regulate the expression of other anti-inflammatory mediators following colitis induction. We used a colitis model characterized by hapten-protein visualization, which indicates the site of hapten-protein formation after colitis induction for histological and gene expression analyses. Under IL-10 deficiency, following colitis induction inflammatory changes were reduced, and S100G expression was elevated. S100G was expressed in fibroblasts, and S100G expression was down-regulated by IL-10. S100G suppressed the production of monocyte chemotactic protein-1 (MCP-1) through the inhibition of NF-κB activation. Therefore, S100G, also known as Calbindin-D9k, may be an important anti-inflammatory mediator in fibroblasts following colitis induction, and down-regulation of S100G expression might be one reason for the insufficient performance of IL-10 supplementation.

Key words: interleukin-10; inflammatory bowel diseases; S100G; fibroblasts; monocyte chemotactic protein-1


NBD-Cl
no fluorescence
Colon
Epithelial cells
+proteins
NBD-proteins
ex. 460 nm
em. 535 nm
Colitis induction as hapten-proteins
Detection via fluorescence
The site of hapten-protein formation is detected
after colitis induction
for histological and gene expression analyses
Inflammatory changes ⬇
S100G expression ⬆
under IL-10 deficiency

1. Introduction

Inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, are chronic disorders of the gastrointestinal tract [1]. Although the precise pathogenesis of IBD still remains unclear, cytokines and chemokines are mediators of inflammation [2,3]. Tumor necrosis factor α (TNFα) is a critical pro-inflammatory cytokine, and anti-TNFα therapy has shown overwhelming efficacy for IBD [2]. Interleukin (IL)-10 is considered to be the most important anti-inflammatory cytokine [4-6]. IBD are associated with polymorphisms in IL-10 and IL-10 receptor genes [5], and chronic changes in inflammation are observed in IL-10-deficient mice [6]. However, IL-10 supplementation has shown disappointing results in clinical trials for IBD [4]. Marlow et al. suggested the following five potential explanations: 1) an insufficient dosage of IL-10, 2) the presence of different pathogeneses among IBD patients, 3) its prevention of only the establishment of IBD, 4) its failure to suppress all the pro-inflammatory mediators, and 5) IL-10's immunostimulatory effects, such as interferon γ (IFNγ) production [4]. We would like to propose another possibility, namely that IL-10 may down-regulate the expression of other anti-inflammatory mediators. IL-10-deficient mice gradually develop inflammatory changes from the proximal region of the colon, and the chronic inflammation reaches the distal region when the mice become 12-16 weeks old under specific pathogen-free conditions [6,7]. To investigate gene expression under IL-10 deficiency following colitis induction, we selected 8-week-old IL-10-deficient mice that did not show any signs of colitis, such as diarrhea or rectal prolapse, for colitis induction with 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl).

NBD-Cl-induced colitis is characterized by hapten-protein visualization [8] (Fig. 1). NBD-Cl binds to proteins through the substitution reaction of 4-Cl with amino or thiol groups of amino acids residues [9]. NBD-Cl alone shows no fluorescence, but NBD-proteins are fluorescent (excitation 460 nm, emission 535 nm), similar to fluorescein and GFP [9]. NBD-proteins also work as hapten-proteins; NBD-proteins are endocytosed by macrophages and are presented to T cells for T cell activation, leading to colitis induction [8]. Therefore, a NBD-Cl-enema treatment induces inflammation in the distal region of the colon, and the site of hapten-protein formation is detected via fluorescence after colitis induction for histological and gene expression analyses. Using this unique model, we investigated inflammatory changes and gene expression under IL-10 deficiency at the site of hapten-protein formation.

2. Materials and methods

2.1. Mice

We obtained BALB/c mice from Japan SLC (Shizuoka, Japan) and IL-10-deficient mice on the BALB/c background from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were kept in a 12-h light/dark cycle with controlled humidity (60-80%) and temperature (22 ± 1°C) under specific pathogen-free conditions. Food and water were freely available. Under the specific pathogen-free conditions, IL-10-deficient mice showed signs of colitis, such as diarrhea and rectal prolapse, at 12-16 weeks old as reported previously [6,7]. All animal experiments were performed according to the guidelines of the Institute for Laboratory Animal Research and with the approval of the ethics committee of Nagoya University.

2.2. Colitis induction

NBD-Cl (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in dimethyl sulfoxide to a concentration of 200 mg/ml for the stock solution, which was stored at -80°C. The NBD-Cl stock solution was diluted with ethanol and then with distilled water at a ratio of 1:100:100 to prepare a 50% ethanol solution containing 1 mg/ml NBD-Cl as the NBD-Cl enema.

For the experimental group, we selected 8-week-old male IL-10-deficient mice that did not show any signs of colitis, such as diarrhea or anal prolapse. Age-matched male wild-type mice were used as the controls. Colitis was induced with the NBD-Cl enema as described previously [8]. Briefly, we lightly anesthetized mice with isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and inserted a rubber catheter (2 mm outer diameter) fitted onto a 1 ml syringe via the anus. The tip was positioned 2 cm proximal to the anus. Then, 100 μl of the NBD-Cl enema was slowly administered to the mice through the catheter. The mice were kept in a head-down position for 30 s and then returned to their cages. Body weight was measured on days 0-2. The colon was dissected from the mice on day 2 for histological analysis and on day 1 for gene expression analysis, immunohistochemical staining and organ culture. We opened the colon longitudinally to remove stools by washing with phosphate-buffered saline, and obtained colon samples positive for NBD-proteins under fluorescence observation using the SZX-RFL2 fluorescence stereomicroscopy system (Olympus, Tokyo, Japan). Because NBD-proteins were detected in the portion of the colon 2-4 cm from the anus following the NBD-Cl-enema treatment as described previously [8], control samples were obtained at a similar portion of the colon dissected from mice that had not received the NBD-Cl-enema treatment.

2.3. Histological analysis

The colon samples were fixed overnight in 4% paraformaldehyde solution and embedded in paraffin to prepare sections (6 μm). After deparaffinization, sections were observed with the BZ-8000 fluorescence microscopy system (Keyence, Osaka, Japan) to confirm NBD-protein formation in the colon samples. Adjacent sections were stained with hematoxylin and eosin for histological evaluation of colitis. Colitis scores were determined by the following histological criteria [8]: 1) a low level of mononuclear cell infiltration with infiltration observed in < 50% of the high-power field (hpf, ×200) and no structural changes observed; 2) a low level of mononuclear cell infiltration, crypt distortion and no destruction of the epithelia; 3) a high level of mononuclear cell infiltration with infiltration observed in ≥ 50% of the hpf and no destruction of the epithelia; 4) the focal destruction of the epithelia covering < 1 hpf; 5) the destruction of the epithelia covering ≥ 1 hpf, and 6) the extensive destruction of the epithelia covering ≥ 2 hpf. All sections were blinded for scoring.

2.4. Gene expression analysis

RNA was isolated from the colon samples with an RNeasy Mini kit (QIAGEN, Hilden, Germany). Two-color microarray analysis was performed with a SurePrint G3 Mouse GE 8×60K microarray (Agilent Technologies, Santa Clara, CA, USA) by Hokkaido System Science (Hokkaido, Japan).

For Western blot analysis, the colon samples were lysed in lysis buffer (20 mM Tris-HCl pH8.0, 150 mM NaCl, 2 mM EDTA pH8.0, 1% Triton X-100, and 6.2 KIU/ml aprotinin). After centrifugation, protein concentrations in the supernatants were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA) to ensure equal amounts of total proteins in each lane for Western blot analysis using rabbit anti-S100G antibody (catalog number, sc-28532; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-indoleamine 2,3-dioxygenase 1 (IDO1) antibody (ABGENT, San Diego, CA, USA), or anti-tubulin α antibody (Thermo Scientific, Fremont, CA, USA).

2.5. Immunohistochemical staining

Sections were prepared as described in the section entitled *Histological analysis*. After deparaffinization, sections were incubated with anti-S100G antibody and then with biotinylated anti-rabbit IgG antibody. After reaction with avidin-biotin-peroxidase, staining was visualized with diaminobenzidine tetrahydrochloride-Ni^{3+}, Co^{2+} (Vector

Laboratories, Burlingame, CA, USA). L.A.B. solution was used to liberate antigens according to the manufacturer's instructions (Polysciences, Warrington, PA, USA).

2.6. Primary fibroblasts

Primary fibroblasts were obtained from BALB/c mouse embryos on day 13.5 of gestation as described previously [10]. After three passages, the cells were stocked in liquid nitrogen and used within five passages from the stocks. Unless otherwise specified, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 50 μg/ml streptomycin, and 125 ng/ml amphotericin B.

2.7. Organ culture

Organ culture was performed as described previously [8]. Briefly, the colon samples (1 cm in length) were placed on culture insert filters (0.4 μm pore size) in 6-well plates (BD Falcon, Franklin Lakes, NJ, USA) containing RPMI1640 with 5% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 10 μg/ml gentamicin, and 10 mM HEPES (600 μl in the upper chamber and 1400 μl in the lower chamber) and incubated under 5% CO_2 at 37°C for 24 h. The media in the lower chamber were used as the conditioned media.

Primary fibroblasts were seeded in 24-well plates (8 or 4×10^4/well). One day later, the cells were incubated in the conditioned media with 0-100 ng/ml IL-10 (Reprokine, Valley Cottage, NY, USA) for 6 h and then used for Western blot analysis or immunocytochemical staining.

2.8. Immunocytochemical staining

The cells were fixed for 5 min with 4% paraformaldehyde containing 0.1% Tween-20, incubated with anti-S100G antibody, and then reacted with anti-rabbit IgG antibody-Alexa568 (Molecular Probes, Eugene, OR, USA) and 250 ng/ml DAPI. Fluorescence was observed with the BZ-8000 fluorescence microscopy system.

2.9. S100G expression construct

Human S100G cDNA was obtained from Open Biosystems (Huntsville, AL, USA) and subcloned into the pCMV5-Flag vector.

2.10. Monocyte chemotactic protein-1 (MCP-1) production

Primary fibroblasts were seeded in 24-well plates (4×10^4/well). One day later, the cells were transfected with 1.2 μg Flag vector or Flag-S100G expression construct with 3 μl of FuGENE HD transfection reagent according to the manufacturer's instructions (Promega, Fitchburg, WI, USA). Twenty-four hours later, the cells were stimulated for additional 6 h with 200 ng/ml recombinant mouse S100A8 (Flarebio, College Park, MD, USA) in DMEM containing 0.1% FBS. MCP-1 concentrations in the media were determined with an ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.11. Reporter assay

Primary fibroblasts were seeded in 24-well plates (4×10^4/well). One day later, the cells were transfected with 0.4 μg NF-κB-dependent *Photinus* luciferase reporter, 20 ng phRL-TK *Renilla* luciferase construct, and 0.8 μg Flag vector or Flag-S100G expression construct with 3 μl of FuGENE HD transfection reagent. Twenty-four hours later, the cells were stimulated for additional 6 h with 200 ng/ml S100A8 in DMEM containing 0.1% FBS to determine their luciferase activities with the dual-luciferase assay system (Promega). NF-κB-dependent reporter activity was assessed by normalization of *Photinus* luciferase activity to *Renilla* luciferase activity.

2.12. Statistical analysis

The data are presented as the means ± s.d. We used the chi-square test, Student's *t*-test or Mann-Whitney *U* test for statistical analysis between the two groups and considered a *P* value < 0.05 to be statistically significant.

.

3. Results

3.1. NBD-Cl-induced colitis in IL-10-deficient mice

We administered the NBD-Cl enema to wild-type and IL-10-deficient mice to compare their colitis induction. Severe diarrhea was not observed in IL-10-deficient mice unlike in wild-type mice (Table 1). IL-10-deficient mice displayed much less body weight reduction than wild-type mice (Fig. 2A). Inflammatory cell infiltration and crypt-epithelial destruction were not remarkable in the histological analysis of IL-10-deficient mice compared with wild-type mice, although hapten-proteins were formed in the colonic mucosa of these mice (Fig. 2B and 2C).

3.2. S100G expression and function

To compare gene expression at the mRNA level following colitis induction, we obtained the colon samples positive for hapten-protein formation under fluorescence observation from wild-type and IL-10-deficient mice treated with the NBD-Cl enema, and we isolated their RNA for two-color microarray analysis. The microarray analysis indicated prominent elevation of IDO1 and S100G mRNA expression under IL-10 deficiency (Table 2 and Supplementary data).

To determine IDO1 and S100G protein expression, we performed Western blot analysis. S100G expression was induced by the NBD-Cl-enema treatment, and the induction of S100G expression was elevated in IL-10-deficient mice compared with wild-type mice (Fig. 3A, first panel). However, we observed only minimal elevation of IDO1 protein expression in IL-10-deficient mice following the NBD-Cl-enema treatment (Fig. 3A, second panel).

To identify the S100G-expressing cells, we performed immunohistochemical staining. S100G was expressed in fusiform or spindle-shaped cells existing mainly in the submucosal layer (Fig. 3B). The characteristic shape and localization of these cells suggested fibroblasts [11]. We confirmed that S100G was expressed in primary fibroblasts cultured in the media conditioned with the colon samples from IL-10-deficient mice treated with the NBD-Cl enema, and the expression of S100G was down-regulated by IL-10 (Fig. 3C). Immunocytochemical staining showed the localization of S100G in the cytoplasmic region of primary fibroblasts (Fig. 3D).

To identify a possible function for S100G, we transfected the S100G expression construct into primary fibroblasts, stimulated the cells with S100A8 and evaluated the production of MCP-1, a critical chemokine for inflammatory reactions. We targeted MCP-1 because the induction of MCP-1 expression was reduced in the microarray analysis under IL-10 deficiency after colitis induction (Supplementary data, chemokine

C-C motif ligand 2 is identical to MCP-1). The induction of MCP-1 production was suppressed by the expression of S100G (Fig. 3E and 3F). Using a NF-κB-dependent reporter assay, we also observed that S100A8-induced NF-κB activation was inhibited by S100G expression (Fig. 3G).

4. Discussion

The NBD-Cl-induced colitis model allows us to perform histological and gene expression analyses after colitis induction at the site of hapten-protein formation [8]. In this unique model, we observed reduced inflammatory changes in IL-10-deficient mice, suggesting that expression of anti-inflammatory molecules is up-regulated under IL-10 deficiency. Expression of S100G was elevated after colitis induction in IL-10-deficient mice at both the mRNA and protein levels.

S100G is a member of the S100 calcium binding protein family and is also known as Calbindin-D9K. Previous papers have reported that S100G is expressed in epithelial cells of the small intestine, kidney and uterus [12,13]. We identified fibroblasts as a novel source of S100G expression. The expression of S100G is induced by media conditioned with the colon samples from IL-10-deficient mice treated with the NBD-Cl enema, suggesting that fibroblasts react to soluble factors produced after colitis induction. To date, we have used cytokines (IL-1β, IL-4, IL-6, IL-12, TNFα and IFNγ), alarmins (S100A8 and IL-33) and growth factors (basic FGF and PDGF) at concentrations up to 200 ng/ml to stimulate primary fibroblasts, but S100G expression was not induced by any of these molecules. The soluble factors necessary to induce fibroblasts to express S100G still remain unknown. On the other hand, the induction of S100G expression is down-regulated by IL-10. This finding is consistent with the up-regulation of S100G expression in IL-10-deficient mice.

The function of S100G has been uncertain. Initially, S100G was proposed to be involved in calcium absorption because S100G is expressed in epithelial cells of the small intestine and the protein's structure contains two Ca^{2+}-binding EF-hands [14]. However, the serum calcium level is not affected in S100G-deficient mice [12,13]. The present study has revealed that S100G expression is dramatically elevated in IL-10-deficient mice treated with the NBD-Cl enema, in which the manifestations of colitis are reduced in spite of IL-10 deficiency. These findings suggest that S100G is a novel anti-inflammatory molecule expressed after colitis induction. Indeed, in S100A8-stimulated fibroblasts, the transient expression of S100G suppresses the production of MCP-1, which recruits monocytes and memory T cells to inflammatory sites [15,16]. S100A8 is released from many types of cells, such as granulocytes and epithelial cells, upon tissue damage and is categorized as one of alarmins because of its involvement in inflammatory reactions, including colitis, via toll-like receptor 4 [17,18]. The activation of toll-like receptor 4 releases Ca^{2+} from intracellular stores to the cytoplasm, where S100G is localized, and then Ca^{2+} entry participates in the activation of NF-κB, a critical transcription factor for MCP-1 expression [19], via

Ca^{2+}/calmodulin-dependent kinase II [20,21]. S100G may sequester Ca^{2+} with its Ca^{2+}-binding EF-hands in the cytoplasm of fibroblasts, resulting in the inhibition of S100A8-induced NF-κB activation. To date, spontaneous induction of colitis has not been reported in S100G-deficient mice, which is consistent with our observation that S100G expression is induced in the colon after the NBD-Cl-enema treatment. To further confirm an anti-inflammatory role for S100G *in vivo*, the NBD-Cl-induced colitis model should be applied to mice deficient in S100G and IL-10. It is difficult for antibodies to go into the cytoplasm of living fibroblasts, where S100G is localized, so that anti-S100G antibodies are not useful to validate *in vivo* function by blocking endogenous S100G in fibroblasts.

In conclusion, S100G is expressed in fibroblasts following colitis induction, and the expression of S100G is down-regulated by IL-10. S100G can function as an anti-inflammatory molecule by suppressing S100A8-induced MCP-1 production through the inhibition of NF-κB activation. The down-regulated expression of S100G might be one of the reasons for the insufficient performance of IL-10 supplementation as a treatment for IBD.

Acknowledgements
We thank Ms. Moriyama for her technical supports. This work was supported by grants from the Japan Society for the Promotion of Science (to K.I. and T.A.).

Conflict of interest
All authors declare that they have no conflicts of interest.

References

[1] G. Bouma, W. Strober, The immunological and genetic basis of inflammatory bowel disease, Nat Rev Immunol 3 (2003) 521-533.

[2] W. Strober, I.J. Fuss, Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases, Gastroenterology 140 (2011) 1756-1767.

[3] S. Thomas, D.C. Baumgart, Targeting leukocyte migration and adhesion in Crohn's disease and ulcerative colitis, Inflammopharmacology 20 (2012) 1-18.

[4] G.J. Marlow, D. van Gent, L.R. Ferguson, Why interleukin-10 supplementation does not work in Crohn's disease patients, World J Gastroenterol 19 (2013) 3931-3941.

[5] K.R. Engelhardt, B. Grimbacher, IL-10 in humans: lessons from the gut, IL-10/IL-10 receptor deficiencies, and IL-10 polymorphisms, Curr Top Microbiol Immunol 380 (2014) 1-18.

[6] D.J. Berg, N. Davidson, R. Kuhn, W. Muller, S. Menon, G. Holland, et al., Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses, J Clin Invest 98 (1996) 1010-1020.

[7] H. Tamagawa, T. Hiroi, T. Mizushima, T. Ito, H. Matsuda, H. Kiyono, Therapeutic effects of roxithromycin in interleukin-10-deficient colitis, Inflamm Bowel Dis 13 (2007) 547-556.

[8] K. Ishiguro, T. Ando, O. Maeda, O. Watanabe, H. Goto, Novel mouse model of colitis characterized by hapten-protein visualization, Biotechniques 49 (2010) 641-648.

[9] P.B. Ghosh, M.W. Whitehouse, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole: a new fluorigenic reagent for amino acids and other amines, Biochem J 108 (1968) 155-156.

[10] K. Ishiguro, K. Kadomatsu, T. Kojima, H. Muramatsu, S. Tsuzuki, E. Nakamura, et al., Syndecan-4 deficiency impairs focal adhesion formation only under restricted conditions, Journal of Biological Chemistry 275 (2000) 5249-5252.

[11] R. Kalluri, M. Zeisberg, Fibroblasts in cancer, Nat Rev Cancer 6 (2006) 392-401.

[12] G.S. Lee, K.Y. Lee, K.C. Choi, Y.H. Ryu, S.G. Paik, G.T. Oh, et al., Phenotype of a calbindin-D9k gene knockout is compensated for by the

induction of other calcium transporter genes in a mouse model, J Bone Miner Res 22 (2007) 1968-1978.

[13] G.D. Kutuzova, S. Akhter, S. Christakos, J. Vanhooke, C. Kimmel-Jehan, H.F. Deluca, Calbindin D(9k) knockout mice are indistinguishable from wild-type mice in phenotype and serum calcium level, Proc Natl Acad Sci U S A 103 (2006) 12377-12381.

[14] I. Marenholz, C.W. Heizmann, G. Fritz, S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature), Biochem Biophys Res Commun 322 (2004) 1111-1122.

[15] E. Melgarejo, M.A. Medina, F. Sanchez-Jimenez, J.L. Urdiales, Monocyte chemoattractant protein-1: a key mediator in inflammatory processes, Int J Biochem Cell Biol 41 (2009) 998-1001.

[16] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant protein-1 (MCP-1): an overview, J Interferon Cytokine Res 29 (2009) 313-326.

[17] J.M. Ehrchen, C. Sunderkotter, D. Foell, T. Vogl, J. Roth, The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer, J Leukoc Biol 86 (2009) 557-566.

[18] J.K. Chan, J. Roth, J.J. Oppenheim, K.J. Tracey, T. Vogl, M. Feldmann, et al., Alarmins: awaiting a clinical response, J Clin Invest 122 (2012) 2711-2719.

[19] D. Ping, G.H. Boekhoudt, E.M. Rogers, J.M. Boss, Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene, J Immunol 162 (1999) 727-734.

[20] K. Ishiguro, T. Green, J. Rapley, H. Wachtel, C. Giallourakis, A. Landry, et al., Ca2+/calmodulin-dependent protein kinase II is a modulator of CARMA1-mediated NF-kappaB activation, Mol Cell Biol 26 (2006) 5497-5508.

[21] E. Shumilina, S.M. Huber, F. Lang, Ca2+ signaling in the regulation of dendritic cell functions, Am J Physiol Cell Physiol 300 (2011) C1205-1214.

Table 1. Diarrhea observed within 2 days following the NBD-Cl-enema treatment

	n	No	Mild	Severe
Wild-type mice	6	1	1	4
IL-10-deficient mice	6	4	2	0

Apparent discharges of loose stool were defined as severe diarrhea, and traces of loose stool around the anus were defined as mild diarrhea. $P = 0.0466$ for the chi-square test.

Table 2. Top three most elevated genes under IL-10 deficiency at 24 h after the NBD-Cl-enema treatment

Gene name	Accession	Description	IL-10-deficient / wild-type
Ido1	**NM_008324**	**indoleamine 2,3-dioxygenase 1**	**197.094**
S100g	**NM_009789**	**S100G**	**113.622**
Abcg5	NM_031884	ATP-binding cassette, sub-family G, member 5	48.5962

RNA was isolated from the colon samples positive for hapten-protein formation, and cDNA was synthesized with Cy3 (wild-type mouse) and Cy5 (IL-10-deficient mouse) labeling for two-color microarray analysis. IL-10-deficient / wild-type, the ratio of Cy5 signal intensity to Cy3 signal intensity.

Fig. 1. Illustration of NBD-Cl-induced colitis. Unlike NBD-Cl, NBD-proteins fluoresce, so that NBD-protein formation can be detected in the colonic mucosa under fluorescence observation. NBD-proteins also work as hapten-proteins; NBD-proteins are endocytosed by macrophages and are presented to T cells for T cell activation, leading to colitis induction.

Fig. 2. Body weight reduction and histological analysis following the NBD-Cl-enema treatment. (A) Wild-type (IL-10 +/+) and IL-10-deficient (IL-10 -/-) mice were treated with the NBD-Cl enema on day 0. Body weight was measured on days 0-2 to determine body weight (BW) reduction. *P* values were calculated for Student's *t*-test at each time point (n = 6). (B and C) On day 2, colon samples were obtained to prepare histological sections. Adjacent sections were used for hematoxylin-eosin staining to determine the colitis score (B) and for fluorescence observation to confirm hapten-protein formation (C). The *P* value was calculated for the Mann-Whitney *U* test (n = 6). Scale bar, 100 μm.

Fig. 3. S100G expression and function. (A) Wild-type and IL-10-deficient mice were treated with the NBD-Cl enema on day 0. On day 1, colon samples were obtained to determine S100G and IDO1 expression at the protein level. Control samples were obtained from mice that had not received the NBD-Cl-enema treatment. (B) IL-10-deficient mice were treated with the NBD-Cl enema on day 0. On day 1, colon samples were obtained for hematoxylin-eosin and immunohistochemical staining. The indicated area is magnified. Scale bar, 100 μm. (C) Primary fibroblasts were cultured in the media conditioned with the colon samples from IL-10-deficient mice with or without the NBD-Cl-enema treatment. IL-10 was added at concentrations of 0-100 ng/ml. Six hours later, the cells were lysed for Western blot analysis. (D) Primary fibroblasts were cultured in the media conditioned with the colon samples from IL-10-deficient mice treated with the NBD-Cl enema. Six hours later, the cells were fixed for fluorescence immunocytochemical staining. Scale bar, 10 μm. (E and F) The Flag vector or Flag-S100G construct was transfected into primary fibroblasts. Twenty-four hours later, the cells were lysed for Western blot analysis (E) or stimulated with S100A8 for additional 6 h to induce MCP-1 production (F). MCP-1 concentrations in the media were determined with an ELISA. The *P* value was calculated for Student's *t*-test (n = 3). (G) NF-κB-dependent *Photinus* luciferase reporter, phRL-TK *Renilla* luciferase construct, and the Flag vector or Flag-S100G construct were transfected into primary fibroblasts. Twenty-four hours later, the cells were stimulated with S100A8 for

additional 6 h to induce NF-κB activation.

Fig. 1 Ishiguro et al.

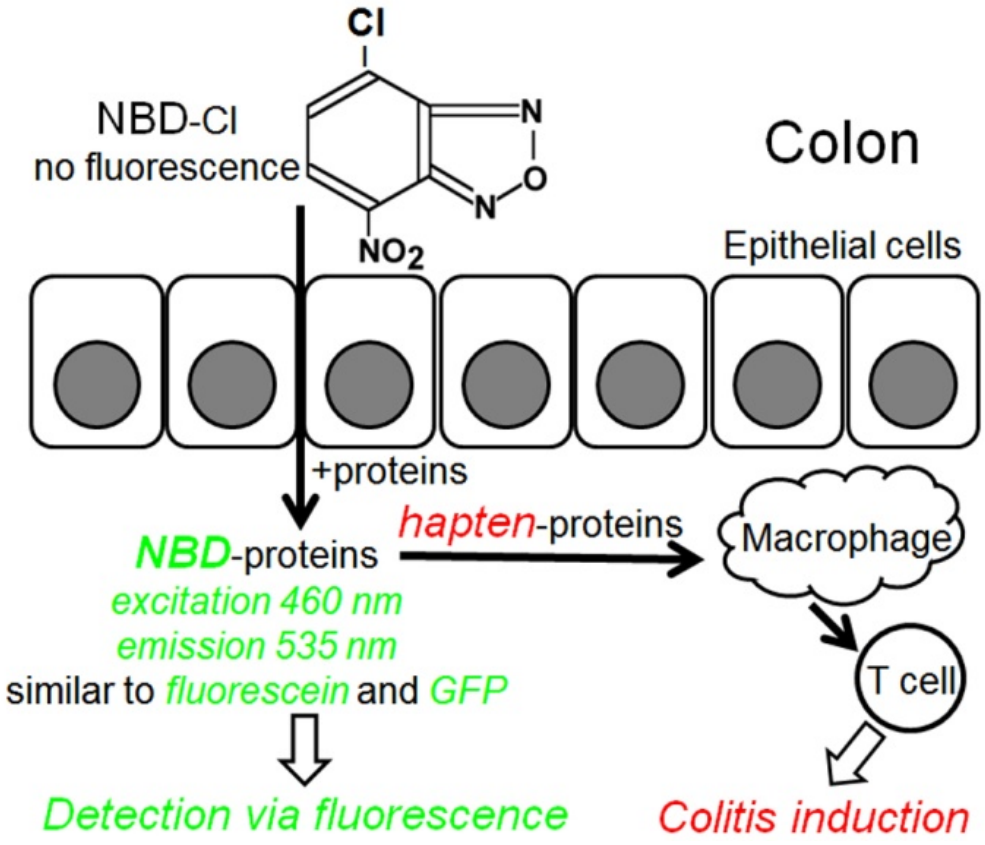


Fig. 2 Ishiguro et al.

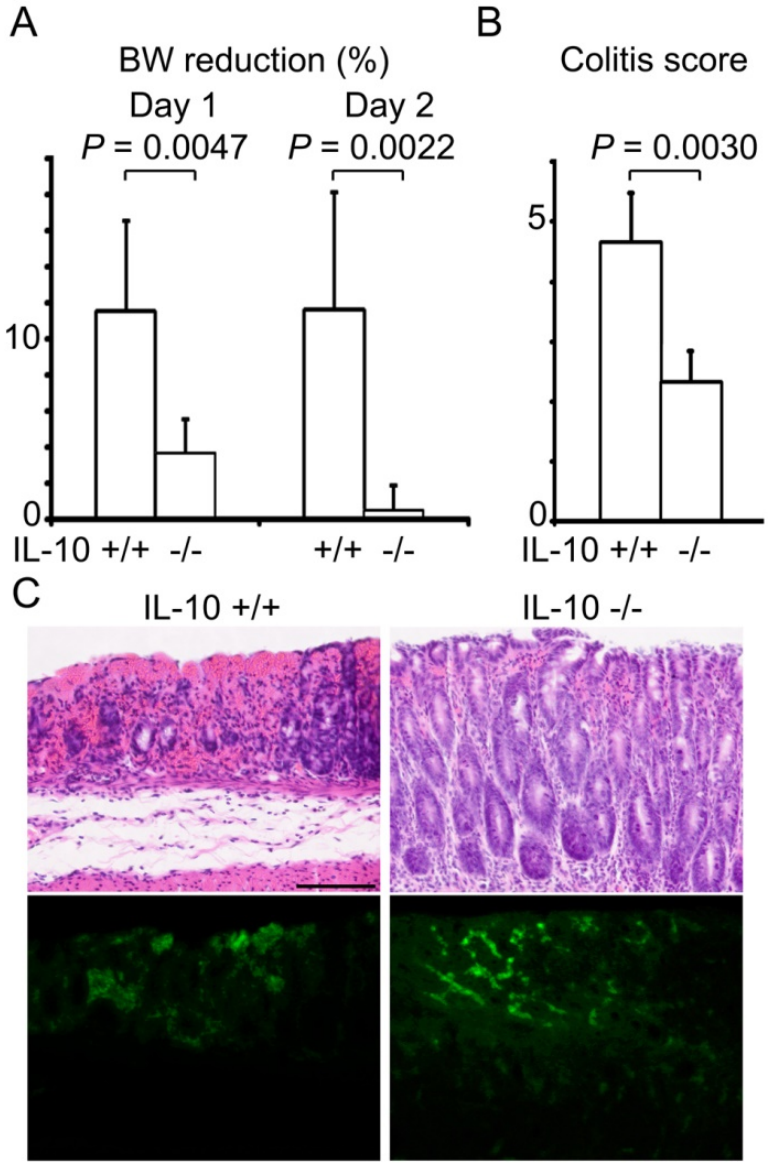


Fig. 3 Ishiguro et al.