

- against p53 are associated with poor prognosis of colorectal cancer. *Br J Cancer*. 1995;72:637-641.
45. Angelopoulou K, Stratis M, Diamandis EP. Humoral immune response against p53 protein in patients with colorectal carcinoma. *Int J Cancer*. 1997;70:46-51.
 46. Schlichtholz B, Tredaniel J, Lubin R, et al. Analysis of p53 antibodies in sera of patients with lung carcinoma define immunodominant regions in the p53 protein. *Br J Cancer*. 1994;69:809-816.
 47. Trivers GE, Cawley HL, DeBenedetti VM, et al. Anti-p53 antibodies in sera of workers occupationally exposed to vinyl chloride. *J Natl Cancer Inst*. 1995;87:1400-1407.
 48. Trivers GE, De Benedetti VM, Cawley HL, et al. Anti-p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer. *Clin Cancer Res*. 1996;2:1767-1775.
 49. Cawley HM, Meltzer SJ, De Benedetti VM, et al. Anti-p53 antibodies in patients with Barrett's esophagus or esophageal carcinoma can predate cancer diagnosis. *Gastroenterology*. 1998;115:19-27.
 50. Ralhan R, Nath N, Agarwal S, et al. Circulating p53 antibodies as early markers of oral cancer: correlation with p53 alterations. *Clin Cancer Res*. 1998;4:2147-2152.
 51. Cioffi M, Riegler G, Vietri MT, et al. Serum p53 antibodies in patients affected with ulcerative colitis. *Inflamm Bowel Dis*. 2004;10:606-611.
 52. Hussain SP, Amstad P, Raja K, et al. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. *Cancer Res*. 2000;60:3333-3337.
 53. Shiota G, Ishida M, Noguchi N, et al. Circulating p53 antibody in patients with colorectal cancer. Relation to clinicopathological features and survival. *Dig Dis Sci*. 2000;45:122-128.

Exclusive Increase of CX3CR1⁺CD28⁻CD4⁺ T Cells in Inflammatory Bowel Disease and Their Recruitment as Intraepithelial Lymphocytes

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Background: CX3CL1/Fractalkine (FKN) has been reported to play important roles in various inflammatory diseases. We examined the role of FKN and its receptor CX3CR1 in T-cell migration in the inflammatory bowel diseases (IBDs), ulcerative colitis (UC) and Crohn's disease (CD).

Methods: CX3CR1 expression on peripheral CD4⁺ cells from normal controls (NL *n* = 24) and IBD patients (UC *n* = 28, CD *n* = 26) was examined using flow cytometry. CX3CR1⁺CD4⁺ T cells were further characterized for surface antigens, cytokine production, and cytotoxic granule release by flow cytometry and ELISA. FKN expression in 53 colonic biopsy specimens (UC *n* = 20, CD *n* = 23, NL *n* = 10) was analyzed by quantitative PCR and immunohistochemistry. Isolated lamina propria and intraepithelial lymphocytes were also analyzed by flow cytometry (UC *n* = 10, CD *n* = 10, NL *n* = 6).

Results: CX3CR1⁺CD4⁺ cells were increased in IBD while they were virtually absent in controls. Upregulation of CX3CR1 on CD4⁺ T cells was positively correlated with disease activity. These unique T cells expressed markers for both effector memory and cytotoxic cells. Interestingly, CX3CR1 was expressed on CD4⁺ T cells lacking CD28. CX3CR1⁺CD28⁻CD4⁺ cells produced more IFN- γ and TNF- α than CX3CR1⁻ counterparts and released cytotoxic granules. FKN mRNA was upregulated in inflamed colonic tissues and robust expression of FKN was immunohistochemically observed on epithelial cells. Although CX3CR1⁺ CD4⁺ cells could not be detected in the gut, CD28⁻CD4⁺ cells were found in IBD mainly as intraepithelial lymphocytes.

Conclusions: FKN/CX3CR1 may contribute to the pathogenesis of IBD through the emergence of unique CX3CR1⁺CD28⁻CD4⁺ T cells that can act both as proinflammatory and cytotoxic cells.

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Key Words: inflammatory bowel disease, fractalkine, CX3CR1, CD4, CD28

Crohn's disease (CD) and ulcerative colitis (UC) are 2 major forms of inflammatory bowel disease (IBD). Although the etiology of IBD remains unclear, accumulating evidence suggests that dysfunction of the mucosal immune system plays important roles in the pathogenesis of IBD.¹ Among a variety of inflammatory cells in the gut, mucosal CD4⁺ lymphocytes have been thought to play a central role both in the induction and persistence of chronic inflammation by producing proinflammatory cytokines.

Chemokines are chemotactic cytokines that regulate recruitment and/or activation of leukocytes. Based on the cysteine motifs and their cognate ligands, chemokines and chemokine receptors, respectively, are classified into C, CC, CXC, and CX3C. CX3CL1/fractalkine (FKN) is the CX3C chemokine and characteristically exists in both soluble and membrane-bound forms.² The membrane-bound form of FKN has a chemokine domain tethered to the cell surface by a long mucin-like stalk, followed by a single transmembrane domain. FKN has been reported to be expressed on stimulated endothelial cells and plays an important role in adhesion and migration of inflammatory cells. Its receptor CX3CR1 is expressed predominantly on NK cells, CD8⁺ T cells, and CD14⁺ monocytes but is virtually absent on CD4⁺ T cells.³

Thus, functional studies of the FKN/CX3CR1 system have so far been focused on the recruitment of monocytes and macrophages to vessel walls.⁴ In addition to atherosclerosis,⁵ FKN has been shown to be involved in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis,^{6–9} systemic sclerosis,¹⁰ bronchial asthma,¹¹ and atopic dermatitis.¹² More interestingly, CD4⁺ T cells that abnormally express CX3CR1 are found in peripheral blood from patients with rheumatoid arthritis^{7–9} and systemic sclerosis.¹⁰

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TABLE 1. Clinical Characteristics of Ulcerative Colitis (UC) and Crohn's Disease (CD) Patients from Obtained Blood Samples

	NL	UC	CD
No of patients	24	28	26
Sex (F/M)	7/17	8/20	9/17
Age (y) (mean, range)	38.0 (20–68)	42.6 (21–79)	34.2 (17–62)
Disease activity			
CAI (mean, range)	—	6.4 (1–15)	—
IOIBD (mean, range)	—	—	2.4 (0–6)
Medication			
5-ASA or SASP	—	27	22
Steroid	—	16	7
Azathioprine	—	5	9

NL, normal; 5-ASA, 5-aminosalicylic acid; SASP, salicylazosulphapyridine. Assessment of disease activity using clinical activity index (CAI) in UC and index of inflammatory bowel disease (IOIBD) in CD.

As for IBD, there have been only 3 reports describing FKN expression in intestinal epithelial cells^{13,14} and the role of CX3CR1 polymorphisms in CD.¹⁵ However, CX3CR1 expression on CD4⁺ T cells has not been investigated, although there have been several reports on other chemokine receptors.^{16,17}

In this study we focused on CX3CR1⁺CD4⁺ T cells to clarify the contributions of the FKN/CX3CR1 system to the pathogenesis of IBD. We found that CX3CR1⁺CD4⁺ T cells were increased in the peripheral blood of IBD, exclusively lacked the expression of CD28, and only in the inflamed mucosa of IBD did CD28⁻CD4⁺ T cells exist.

MATERIALS AND METHODS

Patients and Samples

UC and CD patients were diagnosed based on clinical, radiographic, endoscopic, and histological findings by established criteria. Peripheral blood samples were obtained from 24 normal controls (NL), 28 patients with UC, and 26 with CD. Clinical profiles of controls and patients are shown in Table 1. Fifty-three biopsy specimens (UC $n = 20$, CD $n = 23$, NL $n = 10$) were obtained endoscopically from inflamed and noninflamed areas of the colon for mucosal tissue samples. Samples of normal controls were taken from patients with colonic polyps and the absence of inflammation was histopathologically confirmed. For isolation of lamina propria and intraepithelial lymphocytes, surgical specimens were collected separately from the experiments above (NL, $n = 6$, M:F = 4:2, mean age = 55.7, UC, $n = 10$, M:F = 7:3, mean age = 33.2, CD, $n = 10$, M:F = 6:4, mean age = 34.0). As normal tissue samples, unaffected tissues in surgically resected specimens for colon cancers were obtained. Samples of IBD patients were obtained from inflamed area of each resected specimen.

Preparation of Peripheral Blood Mononuclear Cells (PBMCs), Lamina Propria Mononuclear Cells (LPMCs), and Intraepithelial Lymphocytes (IELs)

PBMCs were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway) from heparinized peripheral blood samples. For CD4⁺ PBMCs, cells were magnetically separated using MACS CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then sorted into CX3CR1-positive and -negative fractions by EPICS ALTRA (Beckman-Coulter, Fullerton, CA). LPMCs were isolated from surgically resected intestinal specimens using enzymatic techniques, as previously described.¹⁸ Briefly, dissected mucosa was incubated in calcium and magnesium-free Hanks' balanced salt solution (Sigma, St Louis, MO) containing 2.5% fetal bovine serum (BioSource, Camarillo, CA) and 1 mM dithiothreitol (Sigma). The mucosa was then incubated in medium containing 1 mM EDTA (Sigma) for 60 minutes at 37°C. During this treatment, intraepithelial lymphocytes (IELs) and epithelial cells were removed from the tissue. Then tissues that contained LPMCs were collected and incubated in medium containing 0.02% collagenase (Worthington Biochemical, Freehold, NJ). The fraction was pelleted and centrifuged over a 40%–60% Percoll solution (Amersham Biosciences, Piscataway, NJ) density gradient. For isolation of IELs, supernatants after EDTA treatment were washed, pelleted, then centrifuged.

Fluorescence-activated Cell Sorter (FACS) Analysis

Various antibodies were used for FACS analysis. Anti-CX3CR1 monoclonal antibodies (mAb) (2A9-1) were purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-CD4 (RPA-T4), anti-CD7 (M-T701), anti-CD8 (RPA-T8), anti-CD11b (ICRF44), anti-CD16 (3G8), anti-CD25 (M-A251), anti-CD28 (CD28.2), anti-CD29 (integrin $\beta 1$) (MAR4), anti-CD33 (HIM3-4), anti-CD45RO (UCHL1), anti-CD49d (9F10), anti-CD56 (B159), anti-CD62L (Dreg 56), anti-CD94 (HP-3D9), anti-CD152 (CTLA-4) (BNI3), anti-CD161 (DX12), anti-integrin $\beta 7$ (FIB504), anti-HLA-DR (G46-4), and isotype-matched control mAbs were purchased from BD Pharmingen (San Diego, CA). Anti-NKG2D (1D11) and anti-ICOS (C398.4A) mAbs were purchased from eBioscience (San Diego, CA). Anti-CCR7 mAb (FAB197p) was purchased from R&D (Minneapolis, MN). Cells were incubated with mAb for 20 minutes at 4°C and then rinsed. For intracellular staining for granzyme A and perforin, a BD Cytotfix/Cytoperm kit (BD Pharmingen), anti-granzyme A mAb (CB9; BD Pharmingen) and anti-perforin mAb (δ G9; BD Pharmingen) were used according to the manufacturer's instructions. Stained cells were analyzed by a FACSCalibur with CellQuest software (Becton Dickinson, San Jose, CA).

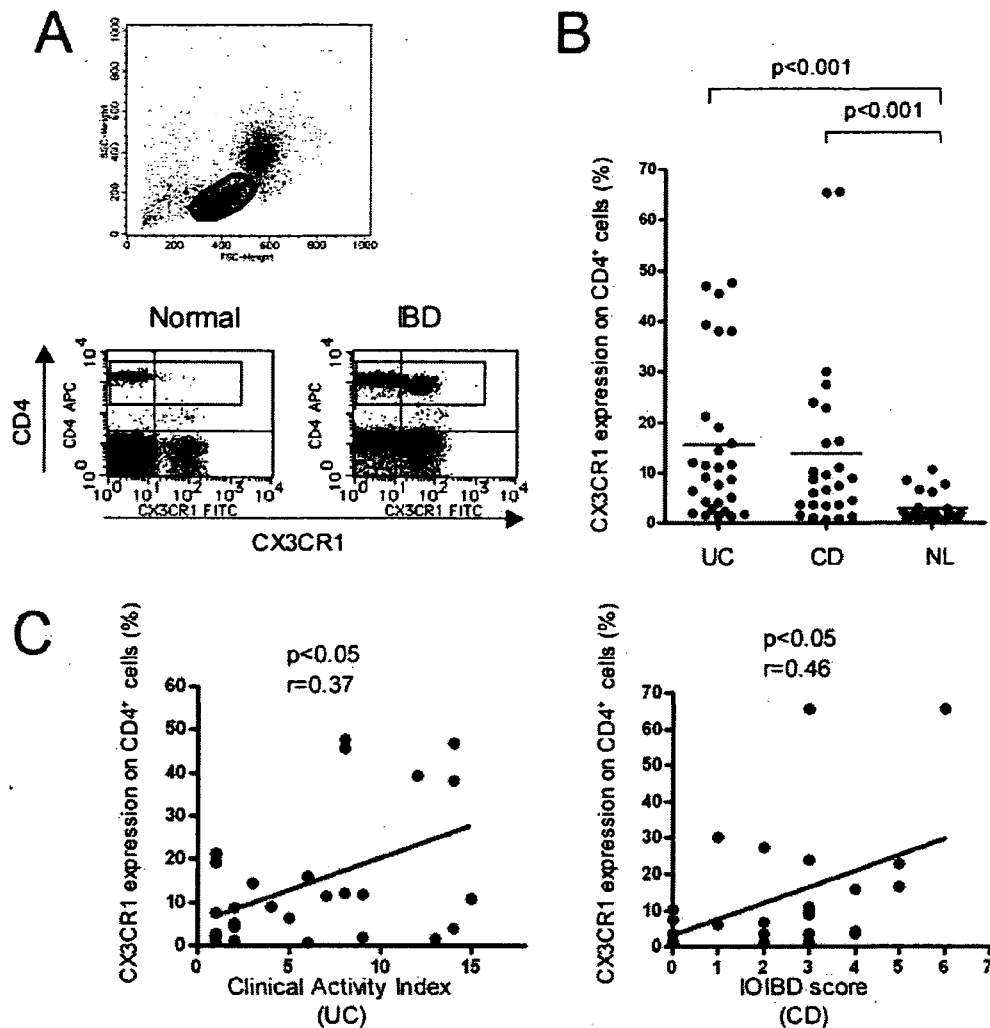


FIGURE 1. A: Representative results of the surface expression of CX3CR1 on PBMCs from patients with ulcerative colitis (UC) and normal controls by flow cytometry. Freshly isolated PBMCs were stained with anti-CX3CR1 mAb in combination with anti-CD4 mAb. Patterns are gated on lymphocytes using forward and side scatter. B: Percentage of CX3CR1-positive cells among total CD4⁺ cells. Freshly isolated PBMCs from patients with UC (*n* = 28), Crohn's disease (CD, *n* = 26), and normal controls (NL, *n* = 24) were analyzed by flow cytometry. The short bar indicates the mean value in each group. C: Correlation between the proportion of CX3CR1⁺CD4⁺ cells and disease activity. Correlation was analyzed by Spearman's correlation test.

Cell Culture and Stimulation

PBMCs were cultured at a concentration of 5×10^5 /mL in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA). For stimulation of PBMCs, 10 μ g/mL of immobilized anti-CD3 (UCHT1; BD Pharmingen) and 5 μ g/mL of anti-CD28 (CD28.2; BD Pharmingen) antibodies were used. In other experiments, cells were also stimulated with phorbol myristate acetate (PMA; 100 ng/mL) and ionomycin (1 μ g/mL) (Sigma).

In migration assays using the Transwell system¹⁹ (Corning, NY), soluble FKN was added in the lower chamber and peripheral blood mononuclear cells were added into the

upper chamber. These chambers were separated by a 5- μ m pore size membrane. Cells were allowed to migrate for 4 hours. Migrated cells in the lower compartments were then collected and counted by flow cytometry.

Enzyme-linked Immunosorbent Assay (ELISA) and Cytometric Bead Array (CBA)

Concentrations of FKN in sera and granzyme A in culture supernatants of sorted PBMCs were measured using specific ELISA (FKN: R&D, granzyme A: Bender MedSystems, Vienna, Austria). According to the manufacturer's instructions, the minimum detectable FKN and granzyme A concentrations were 0.63 ng/mL and 22.2 pg/mL, respec-

tively. To measure the concentrations of cytokines, BD Cytometric Bead Array (CBA) Human Th1/Th2 Kit (BD Pharmingen) was used according to the manufacturer's instructions.

Quantitative Reverse-transcription Real-time Polymerase Chain Reaction

Tissue samples were stabilized in RNA Later (Qiagen, Hilden, Germany) and kept at -20°C until homogenization. After homogenization using a QIA shredder (Qiagen), total RNA was extracted using an RNeasy Mini Kit (Qiagen). RNA was treated with Qiagen DNase (Qiagen) to remove any contaminating genomic DNA. Complementary DNA (cDNA) was synthesized using the Superscript first strand synthesis system for reverse transcription-PCR (Invitrogen), according to the manufacturer's instructions. Quantitative real-time PCR was performed using Taqman PCR master mix (Applied Biosystems, Foster City, CA) and VIC-labeled probe for β -actin with FAM-labeled probe for FKN obtained from Assays On Demand (Applied Biosystems), with DNA Engine Opticon 2 System (MJ Research, Waltham, MA).

Immunohistochemistry

Immunohistochemistry was conducted on OCT-embedded sections of frozen samples. Briefly, $6\text{-}\mu\text{m}$ -thick cryostat sections were fixed in 99% acetone for 10 minutes and then the samples were rehydrated 3 times in phosphate-buffered saline (PBS) for 5 minutes each time. Sections were treated with PBS-3% BSA for 30 minutes, and then incubated for 1 hour at room temperature with the following primary antibodies: rabbit anti-CX3CR1 antibody,⁸ rabbit antihuman FKN antibody (eBioscience), and normal rabbit immunoglobulin fraction as an isotype-matched control antibody. The samples were washed 3 times in PBS for 5 minutes each time and incubated for 1 hour at room temperature with Alexa Fluor 488 conjugated goat antirabbit IgG (Molecular Probes, Eugene, OR). After washing with PBS the sections were mounted with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA).

Statistical Analysis

Results are shown as mean and standard error (mean \pm SEM). The probability of significant differences was calculated using Mann-Whitney *U*-test and Dunn's multiple comparison test was used when needed. To assess the relationship between 2 parameters, Spearman's correlation coefficient test was used. A *P* value of <0.05 was considered significant.

Ethical Considerations

All experiments were approved by the local ethics committees. Informed consent was obtained from all patients before obtaining samples.

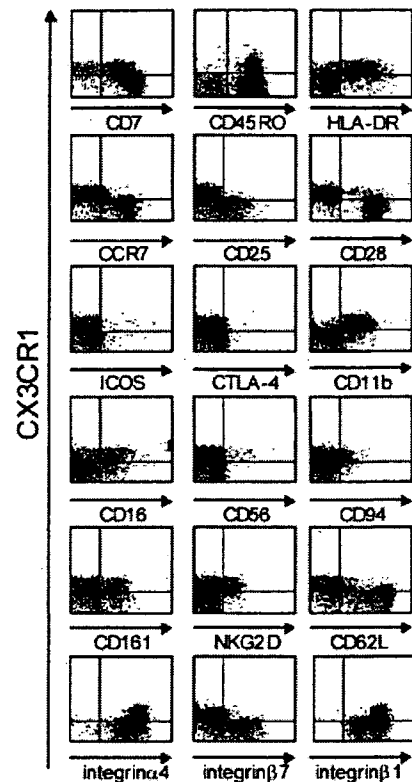


FIGURE 2. Representative results of expression of CX3CR1 and various functional antigens on CD4^+ T cells by flow cytometry. Freshly isolated PBMCs from a UC patient were stained with anti- CD4 mAb, anti-CX3CR1 mAb, and mAbs to various surface molecules. Patterns are gated on CD4^+ cells.

RESULTS

CX3CR1-positive CD4^+ T Cells Are Increased in Peripheral Blood of IBD Patients

We first performed FACS analysis to determine the expression of CX3CR1 on PBMC from normal controls and IBD patients. The majority of CD14^+ monocytes and more than one-third of CD8^+ T cells expressed CX3CR1 in normal controls, as previously reported,^{12,20,21} and the positive rates were not significantly different in the IBD samples (data not shown). However, CX3CR1-positive CD4^+ T cells were significantly increased in both UC ($15.7 \pm 2.9\%$) and CD ($13.9 \pm 3.4\%$) compared with controls ($2.9 \pm 0.6\%$) (Fig. 1A,B), although the other clinical factors including age and sex showed no significant differences among 3 groups. Further analysis showed that the positive rate among CD4^+ cells was significantly correlated with disease activity both in UC and CD (Fig. 1C). These findings indicated that CX3CR1 expression is increased in peripheral blood T cells of IBD patients especially in CD4^+ T cells of active IBD patients. More-

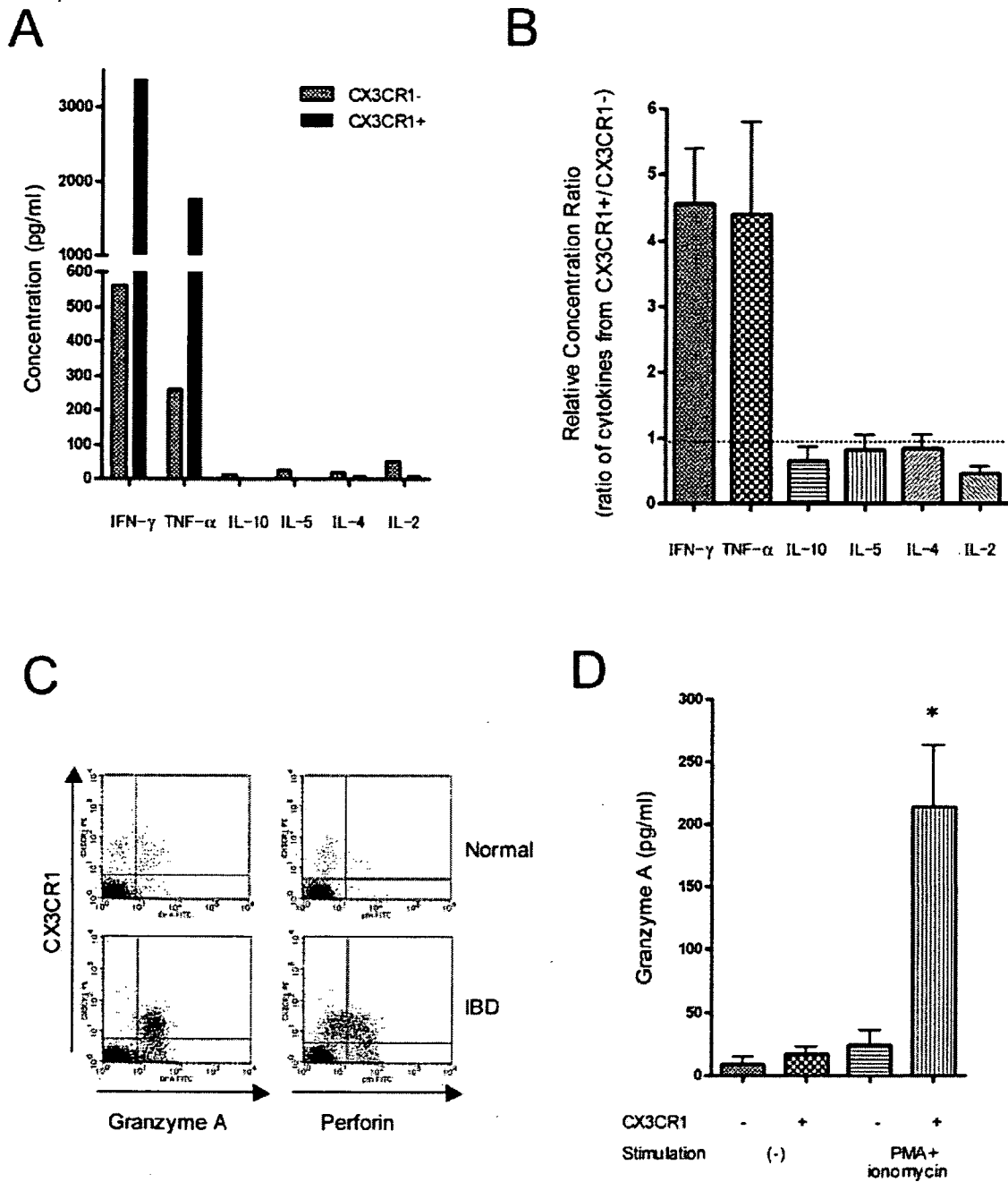


FIGURE 3. A: Cytokine production from CX3CR1⁺CD4⁺ cells. Sorted CX3CR1⁺CD4⁺ and CX3CR1⁻CD4⁺ cells were stimulated with immobilized anti-CD3 mAb and anti-CD28 mAb. After 48 hours supernatants were collected to measure concentration of IFN- γ , TNF- α , IL-10, IL-5, IL-4, IL-2 by Cytometric Beads Array. Representative data from a CD patient is shown. B: The ratio of cytokine concentration from CX3CR1⁺CD4⁺ cells to that of CX3CR1⁻CD4⁺ cells is shown. Cells were stimulated in the same manner as Figure 3A. Samples were obtained from patients with ulcerative colitis (UC, $n = 3$) and Crohn's disease (CD, $n = 3$) who had enough CX3CR1⁺CD4⁺ cells for sorting and further analysis. C: Granzyme A and perforin production by peripheral CD4⁺ T cells from a CD patient were analyzed by flow cytometry using anti-CD4 mAb, anti-CX3CR1 mAb, and anti-granzyme A mAb or anti-perforin mAb. Staining patterns are representative of 4 patients with IBD. D: Granzyme A released from CX3CR1⁺CD4⁺ was quantified by ELISA. After separation of CX3CR1⁺CD4⁺ from CX3CR1⁻CD4⁺ cells, 1×10^5 cells were cultured with PMA plus ionomycin. After 4 hours supernatants were collected to measure the concentration of granzyme A using a specific ELISA. Peripheral blood samples were obtained from patients with ulcerative colitis (UC, $n = 5$), and Crohn's disease (CD, $n = 3$). N.D., not detected. * $P < 0.05$ compared to CX3CR1⁻ cells without stimulation.

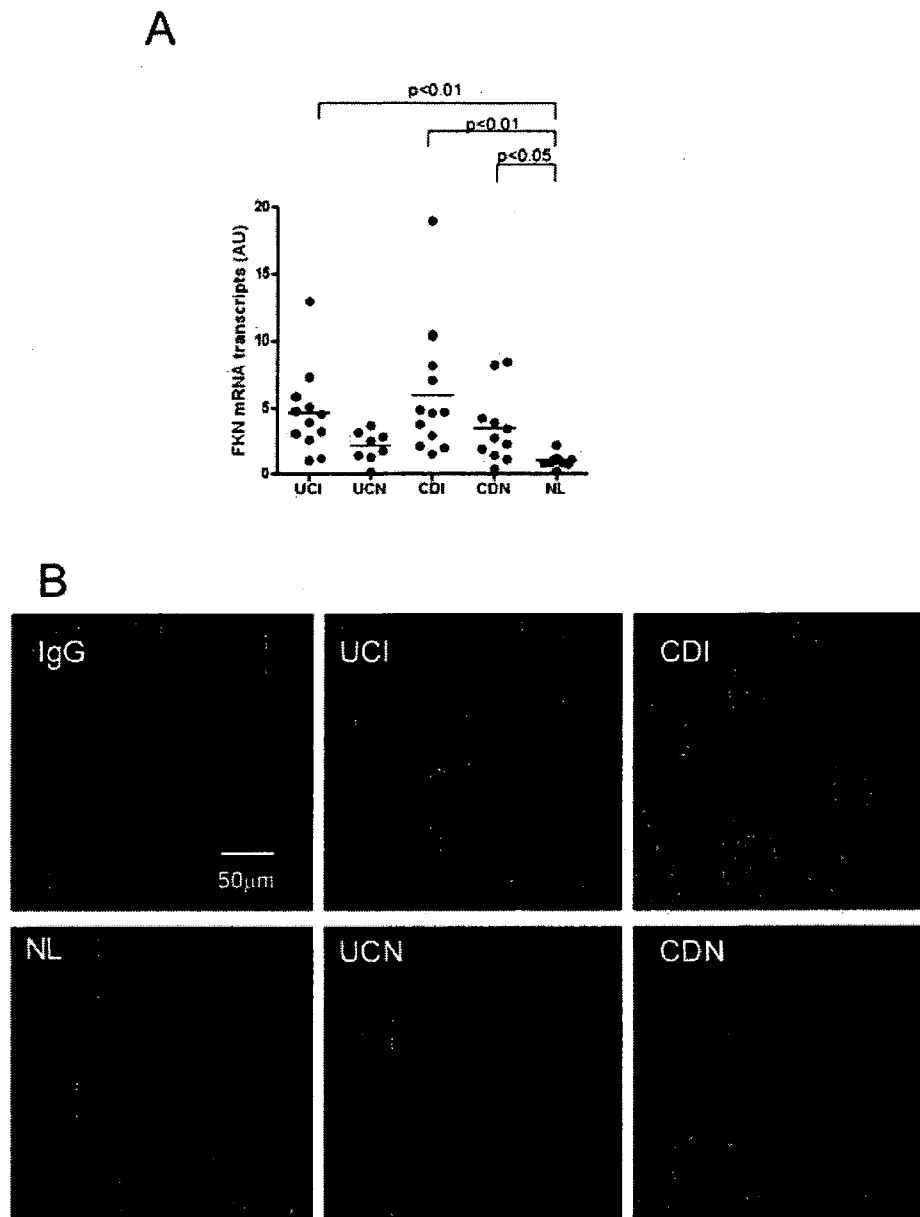


FIGURE 4. A: Relative fractalkine (FKN) mRNA expression in the colonic biopsy specimen from normal controls (NL, $n = 10$), patients with ulcerative colitis (UCI inflamed, $n = 12$; UCN noninflamed, $n = 8$), and patients with Crohn's disease (CDI inflamed, $n = 12$; CDN noninflamed, $n = 11$) were quantified by real-time reverse-transcription polymerase chain reaction. The short bar indicates the mean value in each group. Values were normalized to the housekeeping gene β -actin. B: Confocal imaging of FKN expression in colonic tissue. Tissue sections of normal control (NL), inflamed and noninflamed area of ulcerative colitis (UCI, UCN) and Crohn's disease (CDI, CDN) were processed for immunofluorescence staining with anti-FKN antibody.

over, to investigate the effect of treatment on CX3CR1 expression, we examined some patients suffering from active disease and with substantial CX3CR1⁺CD4⁺ T cells. CX3CR1⁺CD4⁺ T cells decreased significantly after clinical remission was achieved in the course of treatment (data not shown).

Characterization of Increasing CX3CR1⁺ CD4⁺ T Cells in IBD

To characterize the phenotype of increasing CX3CR1⁺CD4⁺ T cells in IBD patients, surface antigens were further examined using flow cytometry (Fig. 2). CX3CR1⁺CD4⁺ T cells always exhibited as the

CD45RO⁺CCR7⁻CD62L⁻ memory phenotype, and showed higher expressions of the cytotoxic lymphocyte markers, CD11b, CD56, CD94, CD161, and NKG2D than their CX3CR1-negative counterparts. Interestingly, nearly all of the CX3CR1⁺CD4⁺ T cells lacked CD28, an essential costimulatory molecule for CD4⁺ T cells. Conversely, all CD28⁻CD4⁺ T cells expressed CX3CR1, indicating that CD28 and CX3CR1 were alternately expressed on CD4⁺ T cells. We also examined integrin expression and found that CX3CR1⁺CD4⁺ T cells always expressed high levels of $\alpha 4$ and $\beta 1$ but rarely expressed $\beta 7$.

Production of Cytokines and Cytotoxic Granules in CX3CR1⁺CD4⁺ T Cells in IBD

Next, the cytokine production profile of CX3CR1⁺CD4⁺ T cells was compared with that of CX3CR1⁻CD4⁺ T cells after stimulation with anti-CD3 and anti-CD28 mAbs. When 6 cytokines were examined using CBA, CX3CR1⁺CD4⁺ T cells produced more IFN- γ and TNF- α , and less IL-10, IL-5, IL-4, and IL-2 than CX3CR1⁻CD4⁺ T cells in 3 UC and 3 CD patients (representative data [Fig. 3A] and the ratio of the production from CX3CR1⁺ to CX3CR1⁻ cells in 6 patients [Fig. 3B]). Because CX3CR1⁺CD4⁺ T cells expressed several cytotoxic lymphocyte markers, as shown in Figure 2, intracellular staining for cytotoxic granules was also performed to investigate whether these cells had cytotoxic activity in IBD patients. As shown in Figure 3C, intracellular staining revealed that CX3CR1⁺CD4⁺ T cells contained granzyme A and perforin in resting condition, while CX3CR1⁻CD4⁺ T cells did not. We then stimulated these cells to examine whether they were actually capable of releasing the cytotoxic granules in an activated state. As expected, only when CX3CR1⁺CD4⁺ T cells were stimulated could granzyme A (Fig. 3D) be detected in the culture supernatants. These results suggest that CX3CR1⁺CD4⁺ T cells can act both as proinflammatory cells and cytotoxic lymphocytes.

FKN Is Upregulated in the Inflamed Tissues of IBD

To investigate the expression of FKN, the ligand for CX3CR1, in the gut of IBD patients, we first examined mRNA expression using quantitative PCR. FKN mRNA expression was ≈ 5 -fold higher in inflamed tissues of UC (4.59 ± 0.92 AU) and 6-fold in CD (5.90 ± 1.42 AU) than in controls (Fig. 4A, $P < 0.05$). Furthermore, mRNA expression in inflamed tissues showed a tendency to be higher than that in noninflamed tissues in both UC and CD, although the difference was not statistically significant. Next, immunohistochemistry was performed to determine the localization of FKN in inflamed colonic tissues. FKN was strongly expressed in epithelial cells in the colonic tissues and the expression was stronger in the inflamed regions obtained from active UC and CD patients (Fig. 4B). No obvious FKN upregulation on endothelial cells was detected. Further, to

confirm the influence of inflammation on FKN expression, human colonic epithelial cell lines, HT29, HCT-15, and DLD-1, were cultured with proinflammatory cytokines, TNF- α or IL-1 β . As described previously,^{13,15} these cytokines clearly induced FKN mRNA expression in colonic cell lines (data not shown).

Exclusive Increase of CD28⁻CD4⁺ T Cells in IELs and LPMCs from Patients with IBD

To determine whether CD4⁺ T cells expressing CX3CR1 are also increased in inflamed tissues of IBD, LPMCs and IELs were stained for CX3CR1 and analyzed by flow cytometry (Fig. 5A). However, unexpectedly, CX3CR1 expression was not detected on either CD4⁺ or CD8⁺ T cells, while some of CD33⁺ macrophages expressed CX3CR1. This finding was irrespective of whether samples came from inflamed or noninflamed parts of resected specimens. Immunohistochemical analysis for CX3CR1 was also performed but lymphocytes were again negative for CX3CR1 (data not shown). Because it is known that lymphocytes infiltrating in mucosal tissues show an activated phenotype, we examined the possibility that CX3CR1 expression on T cells may be downregulated in the presence of inflammation. We then stimulated PBMCs from IBD patients and found that activated CD4⁺ T cells lost CX3CR1 expression within 6 hours, while CD28 expression was not affected by the stimulation of anti-CD3 mAb, as previously reported²² (Fig. 5B). Then we investigated CX3CR1 and CD28 expression after migration to FKN using a transwell system. As with CX3CR1⁺ T cells from healthy individuals,²³ CX3CR1⁺CD28⁻CD4⁺ T cells in patients with IBD were selectively attracted by FKN (Fig. 5C). Importantly, they again lost CX3CR1 expression after migration to FKN and remained negative for CD28. Combining these results, we have concluded that CX3CR1 expression on CD4⁺ T cells could be downregulated by either activation or migration. Thus, we next examined CD28 expression on mucosal CD4⁺ cells to identify CX3CR1⁺CD28⁻CD4⁺ mucosal T cells. We investigated both LPMCs and IELs, and found that CD28⁻CD4⁺ cells are extremely rare in normal controls (LPMCs, $0.15 \pm 0.05\%$, IELs, $1.3 \pm 0.5\%$, $n = 6$). However, CD28⁻CD4⁺ cells could be detected in both LPMCs (UC, $1.01 \pm 0.33\%$, $n = 10$, CD, $1.91 \pm 0.44\%$, $n = 10$) and IELs (UC, $7.9 \pm 3.2\%$, CD, $6.1 \pm 2.4\%$), isolated from patients with IBD and existed significantly more in IELs ($7.0 \pm 2.0\%$) than LPMCs ($1.5 \pm 0.3\%$) (Fig. 5D,E).

Taken together, these results suggest that FKN recruits CX3CR1⁺CD4⁺ T cells to the inflamed epithelium of IBD, where expression of CX3CR1 itself is downregulated.

DISCUSSION

We showed that CX3CR1 expression on peripheral CD4⁺ T cells was significantly upregulated in both UC and CD patients. This finding is consistent with previous reports on chronic inflammatory diseases such as rheumatoid arthri-

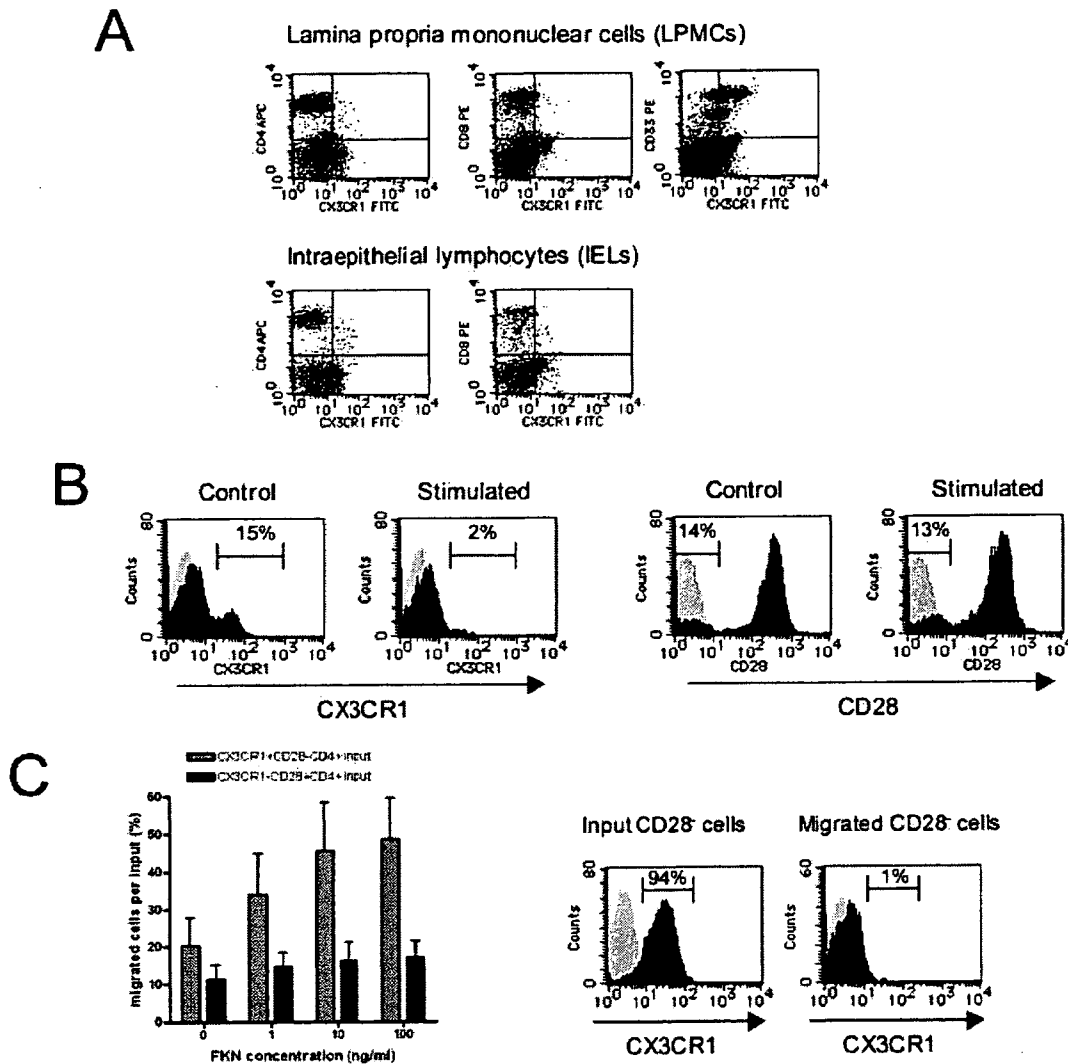


FIGURE 5. A: Flow cytometric analysis of the surface expression of CX3CR1 on lamina propria and intraepithelial lymphocytes by flow cytometry. Patterns are gated on lymphocytes for analysis of CD4⁺ or CD8⁺ cells using forward and side scatter. For CD33 staining, it is gated on macrophages. Representative results from 10 experiments are shown. B: CX3CR1 and CD28 expression on CD4⁺ cells after stimulation for 6 hours with immobilized anti-CD3 mAb and anti-CD28 mAb. Patterns are gated on CD4⁺ lymphocytes. C: Migration assays using a Transwell system. Numbers of the migrated cells into the lower chambers were counted by flow cytometry. The value is the percentage of migrated cells among input cells for each subset. Data are pooled from 4 independent experiments. Histogram patterns shown are gated on CD28⁻CD4⁺ lymphocytes and represent CX3CR1 expression on input and migrated cells (100 ng/mL of FKN). D: Representative flow cytometric analysis of the surface expression of CD28 on lamina propria and intraepithelial CD4⁺ T cells in normal control and CD. Patterns are gated on lymphocytes using forward and side scatter. Percentages of CD28⁻ and CD28⁺ cells among CD4⁺ cells are given. E: Percentage of CD28⁻CD4⁺ cells in LPMCs and IELs isolated from normal controls (NL, n = 6) and patients with IBD (UC, n = 10, CD, n = 10). The difference between LPMCs and IELs from all IBD samples was also assessed. The short bar indicates the mean value in each group.

tis⁷⁻⁹ and systemic sclerosis.¹⁰ As shown in Figure 1C, the increase of CD4⁺CX3CR1⁺ cells in IBD patients was positively correlated with disease activity. Moreover, CX3CR1⁺CD4⁺ T cells significantly decreased after they achieved remission. These findings are noteworthy because dysregulated CD4⁺ T cells have been considered to play a central role in the pathogenesis of IBD.

These unique CD4⁺ T cells also express cytotoxic lymphocyte makers such as CD11b, CD56, CD161 and possess intracellular cytotoxic granules, granzyme A and perforin. After stimulation, they have a tendency to produce proinflammatory cytokines compared to their counterparts, typical CX3CR1⁻CD4⁺ T cells. Moreover, only CX3CR1⁺ cells among CD4⁺ cells actually could

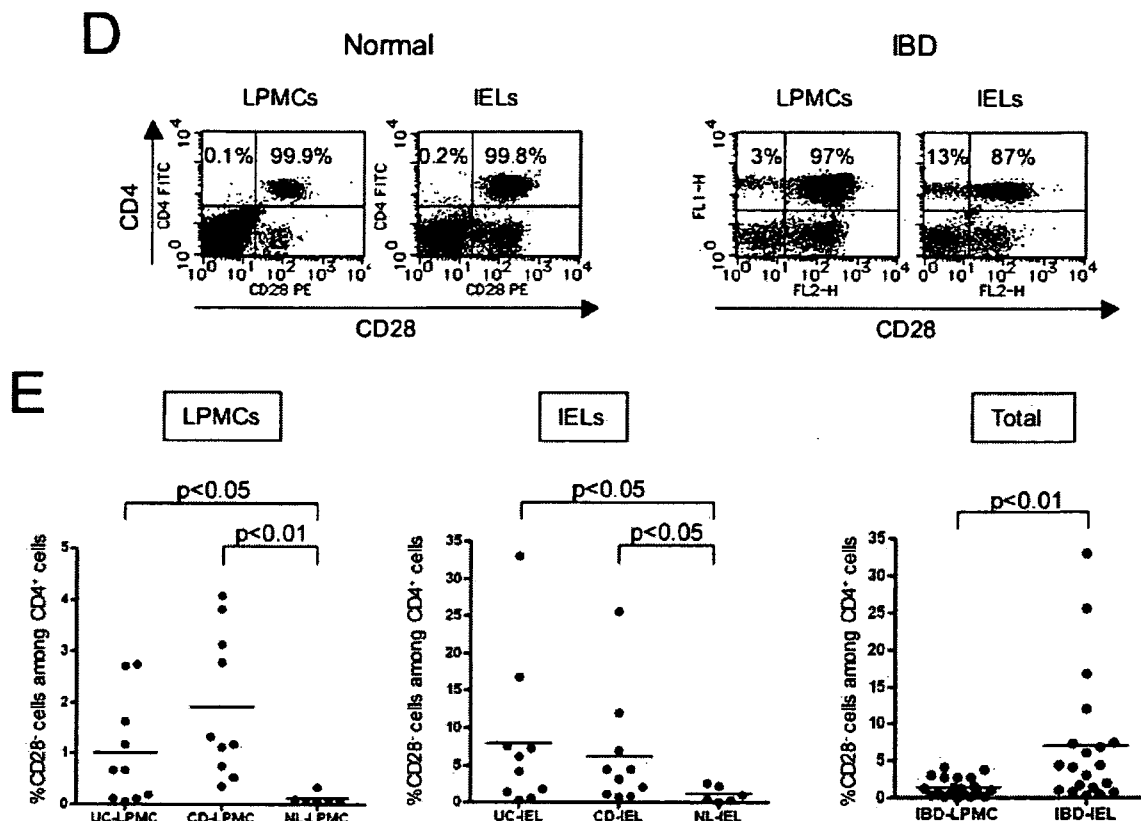


FIGURE 5. (Continued)

release cytotoxic granules. These data suggest that CX3CR1⁺CD4⁺ T cells have dual functions as Th1 effector and cytotoxic T cells.

Another unique feature of CX3CR1⁺CD4⁺ T cells is that they lack the CD28 molecule, which is an essential costimulatory molecule for CD4⁺ T cell activation. Previously, Nanki et al⁸ reported that CX3CR1⁺CD4⁺ T cells could not be found in inflamed synovium from patients with rheumatoid arthritis because of the downregulation of CX3CR1, and we also failed to detect CX3CR1⁺CD4⁺ T cells in the gut of IBD patients. Therefore, we examined CD28 expression on CD4⁺ cells instead. As expected, we could demonstrate that CD28⁻CD4⁺ mucosal T cells were found only in the gut of patients with IBD, especially in IELs, while we failed to detect CX3CR1 expression. This result is highly intriguing. First, the existence of CD28⁻CD4⁺ T cells has recently been reported in several chronic inflammatory diseases such as rheumatoid arthritis,²⁴⁻²⁶ Wegener's granulomatosis,²⁷ multiple sclerosis,²⁸ and also UC.²⁹ As reported, these CD28⁻CD4⁺ T cells have cytotoxic activity and in some cases are autoreactive CD4⁺ T cells, which suggests their pathogenic role in chronic inflammation. Second, there have been reports concerning the cytotoxic activity of IELs

obtained from IBD patients.^{30,31} Although these reports did not mention CX3CR1 expression, CX3CR1⁺CD4⁺ T cells in IBD are likely to be overlapped with these cytotoxic CD28⁻CD4⁺ populations. Finally, although they did not mention CD4⁺ IELs, Muehlhoefer et al¹³ reported that FKN produced from epithelial cells is important in the retention of CD8⁺ IELs. Combining our data with these reports, it might imply that a fraction of IELs was derived from circulating CX3CR1⁺CD28⁻CD4⁺ T cells especially in an inflammatory condition, which suggests the pathogenic role for the FKN/CX3CR1 system in IBD.

Inhibition of the FKN-CX3CR1 interaction ameliorated disease activity in animal models for chronic inflammatory diseases such as murine collagen-induced arthritis,³² glomerulonephritis,³³ and experimental autoimmune myositis.³⁴ Thus, it is important to examine whether anti-FKN antibody is also effective in murine IBD models, as inhibition of FKN-CX3CR1 could be a new therapeutic approach for IBD treatment.

In summary, our data indicate that CX3CR1⁺CD4⁺ T cells, which have dual functions as proinflammatory and cytotoxic T cells, are increased in peripheral blood and infiltrate to inflamed epithelial cells overexpressing FKN, contributing to the pathogenesis of IBD.

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REFERENCES

- Podolsky DK. Inflammatory bowel disease. *N Engl J Med*. 2002;347:417–429.
- Bazan JF, Bacon KB, Hardiman G, et al. A new class of membrane-bound chemokine with a CX3C motif. *Nature*. 1997;385:640–644.
- Goda S, Imai T, Yoshie O, et al. CX3C-chemokine, fractalkine-enhanced adhesion of THP-1 cells to endothelial cells through integrin-dependent and -independent mechanisms. *J Immunol*. 2000;164:4313–4320.
- Ancuta P, Rao R, Moses A, et al. Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J Exp Med*. 2003;197:1701–1707.
- Greaves DR, Channon KM. Inflammation and immune responses in atherosclerosis. *Trends Immunol*. 2002;23:535–541.
- Blaschke S, Koziolok M, Schwarz A, et al. Proinflammatory role of fractalkine (CX3CL1) in rheumatoid arthritis. *J Rheumatol*. 2003;30:1918–1927.
- Ruth JH, Rottman JB, Katschke KJ Jr, et al. Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthritis Rheum*. 2001;44:2750–2760.
- Nanki T, Imai T, Nagasaka K, et al. Migration of CX3CR1-positive T cells producing type 1 cytokines and cytotoxic molecules into the synovium of patients with rheumatoid arthritis. *Arthritis Rheum*. 2002;46:2878–2883.
- Sawai H, Park YW, Roberson J, et al. T cell costimulation by fractalkine-expressing synovocytes in rheumatoid arthritis. *Arthritis Rheum*. 2005;52:1392–1401.
- Hasegawa M, Sato S, Echigo T, et al. Up regulated expression of fractalkine/CX3CL1 and CX3CR1 in patients with systemic sclerosis. *Ann Rheum Dis*. 2005;64:21–28.
- Rimaniol AC, Till SJ, Garcia G, et al. The CX3C chemokine fractalkine in allergic asthma and rhinitis. *J Allergy Clin Immunol*. 2003;112:1139–1146.
- Echigo T, Hasegawa M, Shimada Y, et al. Expression of fractalkine and its receptor, CX3CR1, in atopic dermatitis: possible contribution to skin inflammation. *J Allergy Clin Immunol*. 2004;113:940–948.
- Muehlhoefer A, Saubermann LJ, Gu X, et al. Fractalkine is an epithelial and endothelial cell-derived chemoattractant for intraepithelial lymphocytes in the small intestinal mucosa. *J Immunol*. 2000;164:3368–3376.
- Lucas AD, Chadwick N, Warren BF, et al. The transmembrane form of the CX3CL1 chemokine fractalkine is expressed predominantly by epithelial cells in vivo. *Am J Pathol*. 2001;158:855–866.
- Brand S, Hofbauer K, Dambacher J, et al. Increased expression of the chemokine fractalkine in Crohn's disease and association of the fractalkine receptor T280M polymorphism with a fibrostenosing disease phenotype. *Am J Gastroenterol*. 2006;101:99–106.
- Connor SJ, Parakevopoulos N, Newman R, et al. CCR2 expressing CD4+ T lymphocytes are preferentially recruited to the ileum in Crohn's disease. *Gut*. 2004;53:1287–1294.
- Papadakis KA, Landers C, Prehn J, et al. CC chemokine receptor 9 expression defines a subset of peripheral blood lymphocytes with mucosal T cell phenotype and Th1 or T-regulatory 1 cytokine profile. *J Immunol*. 2003;171:159–165.
- Kanai T, Watanabe M, Okazawa A, et al. Interleukin 18 is a potent proliferative factor for intestinal mucosal lymphocytes in Crohn's disease. *Gastroenterology*. 2000;119:1514–1523.
- Carr MW, Roth SJ, Luther E, et al. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A*. 1994;91:3652–3656.
- Imai T, Hieshima K, Haskell C, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell*. 1997;91:521–530.
- Damas JK, Boullier A, Wachre T, et al. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, is elevated in coronary artery disease and is reduced during statin therapy. *Arterioscler Thromb Vasc Biol*. 2005;25:2567–2572.
- Warrington KJ, Vallejo AN, Weyand CM, et al. CD28 loss in senescent CD4+ T cells: reversal by interleukin-12 stimulation. *Blood*. 2003;101:3543–3549.
- Foussat A, Coulomb-L'Hermine A, Gosling J, et al. Fractalkine receptor expression by T lymphocyte subpopulations and in vivo production of fractalkine in human. *Eur J Immunol*. 2000;30:87–97.
- Warrington KJ, Takemura S, Goronzy JJ, et al. CD4+, CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. *Arthritis Rheum*. 2001;44:13–20.
- Namekawa T, Snyder MR, Yen JH, et al. Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis. *J Immunol*. 2000;165:1138–1145.
- Schmidt D, Goronzy JJ, Weyand CM. CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest*. 1996;97:2027–2037.
- Komocsi A, Lamprecht P, Csernok E, et al. Peripheral blood and granuloma CD4(+)CD28(-) T cells are a major source of interferon-gamma and tumor necrosis factor-alpha in Wegener's granulomatosis. *Am J Pathol*. 2002;160:1717–1724.
- Markovic-Plese S, Cortese I, Wandinger KP, et al. CD4+CD28- costimulation-independent T cells in multiple sclerosis. *J Clin Invest*. 2001;108:1185–1194.
- Yeung MM, Melgar S, Baranov V, et al. Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR-gammadelta expression. *Gut*. 2000;47:215–227.
- Melgar S, Hammarstrom S, Oberg A, et al. Cytolytic capabilities of lamina propria and intraepithelial lymphocytes in normal and chronically inflamed human intestine. *Scand J Immunol*. 2004;60:167–177.
- Nussler NC, Stange B, Hoffman RA, et al. Enhanced cytolytic activity of intestinal intraepithelial lymphocytes in patients with Crohn's disease. *Langenbecks Arch Surg*. 2000;385:218–224.
- Nanki T, Urasaki Y, Imai T, et al. Inhibition of fractalkine ameliorates murine collagen-induced arthritis. *J Immunol*. 2004;173:7010–7016.
- Inoue A, Hasegawa H, Kohno M, et al. Antagonist of fractalkine (CX3CL1) delays the initiation and ameliorates the progression of lupus nephritis in MRL/lpr mice. *Arthritis Rheum*. 2005;52:1522–1533.
- Suzuki F, Nanki T, Imai T, et al. Inhibition of CX3CL1 (fractalkine) improves experimental autoimmune myositis in SJL/J mice. *J Immunol*. 2005;175:6987–6996.

3. 腸管免疫研究の最前線

—腸管の恒常性維持と炎症性腸疾患におけるその破綻—

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key words inflammatory bowel disease, intestinal macrophage, dendritic cell, interleukin-10, interleukin-12, interleukin-23, Paneth cell, α -defensin, intestinal epithelial cell, NF- κ B

動 向

潰瘍性大腸炎 ulcerative colitis (UC) とクローン病 Crohn's disease (CD) に二分される炎症性腸疾患 inflammatory bowel disease (IBD) はいまだ原因不明の疾患であるが、近年の研究から腸内細菌に対する自然免疫応答の制御異常が病態に深く関与していることが明らかとなってきた。

腸管免疫分野の研究では Toll like receptor family (TLRs) の発見以来、菌体成分に対する自然免疫応答の研究が注目されるようになった。さらに細胞内 pathogen 認識分子である NOD2/CARD15 が CD の疾患関連遺伝子として同定され^{1,2)}、IBD 病態研究がこれまでの獲得免疫中心から自然免疫研究あるいは両者の cross talk へとシフトしてきている。

腸管は常在腸内細菌や病原体、食餌抗原に常に曝露されている臓器であり身体の内にながら常に外界と接している特殊な環境下にある。腸管粘膜の面積はテニスコート 1.5 面分にも及び、そこには多くの免疫担当細胞が集簇している。このなかでマクロファージ macrophage (M ϕ) と樹状細胞 dendritic cell (DC) は抗原認識、お

よび異物処理といった自然免疫を司どる key player である。さらに腸管には腸上皮細胞が構造的、機能的バリアーとして存在し外来抗原から宿主を守っている。

近年、腸管上皮細胞や腸管 M ϕ 、DC が病原体の侵入を防ぐだけでなく、腸管免疫の恒常性維持にも重要であることが解明され、その破綻が IBD 発症に関与している可能性が指摘されてきている。

A. 腸管マクロファージによる恒常性維持

M ϕ は細菌などの外来抗原に対する自然免疫の主な担当細胞であり感染防御において重要な役割を果たしている。M ϕ は生体のあらゆる場所に存在しているが、それらは均一な集団ではなく、M ϕ は局在する組織により、クッパー細胞 (肝)、肺胞 M ϕ (肺)、マクログリア (脳) など異なる名称でよばれている。これらの M ϕ は、形態、機能、表面抗原の発現などの点で異なり、M ϕ 特有の抗原を取り込むスカベンジャーとしての役割だけでなく、各組織において固有の働きを担っていると考えられる。このような M ϕ の多様性は、生体の防御とホメオスターシスの維持に重要な役

割を果たしていると考えられる。

顆粒球マクロファージコロニー刺激因子 (GM-CSF) により分化誘導される GM-M ϕ は強い抗原提示能を示し、また炎症性サイトカイン IL-12, IL-23 を高産生することで Th1, Th17 型の獲得免疫応答誘導に寄与している炎症性 M ϕ であると考えられる。一方で、マクロファージコロニー刺激因子 (M-CSF) により分化誘導される M-M ϕ は IL-12 や IL-23 を全く産生せず、反対に炎症抑制性サイトカインである IL-10 を高産生し抗炎症的に働く抑制性 M ϕ であると考えられる³⁾。一般的に、M ϕ は細菌などの外来抗原に対する自然免疫の主な担当細胞であり外界からの侵入者に対して貪食処理および免疫反応を誘導することで生体を守っている。しかしながら、消化管は病原体や食餌抗原などの外来抗原に恒常的に曝露されている。そのため、多くの外来抗原に対して過剰な免疫反応を誘導するのは好ましくなく、むしろ恒常性を保つため過剰な免疫反応を制御するシステムが存在すると考えられる。

マウス正常腸管では M-CSF が優位に発現し、M-CSF 欠損マウスである *op/op* マウスでは腸管 M ϕ の分化が障害されているという事実からも、正常腸管局所は炎症抑制性である M-M ϕ の分化の場であると考えられる^{4,5)}。マウス腸管 M ϕ は腸内細菌抗原刺激に対し TNF α や IL-6 などの急性反応性のサイトカインは産生するものの、決して Th-1, Th-17 型免疫応答を引きこす IL-12 や IL-23 を産生せず、むしろ抑制性サイトカインである IL-10 を高産生する抑制性の M ϕ である^{5,6)}。また Smythies らはヒトの腸管 M ϕ は細菌に対し貪食能を保ったままサイトカイン産生に関しては低応答となっていることを報告している⁷⁾。さらに腸管 M ϕ が炎症抑制的に働いていることを裏付ける報告として腸管 M ϕ 欠損マウスでは DSS 誘導腸炎が増悪することが明らかとなっている⁸⁾。このように正常な腸管 M ϕ は腸内細菌に対し抑制

性の免疫反応を誘導し、腸管免疫恒常性の維持にかかわっていると考えられる。

B. 異常な M ϕ や DC からの IL-23 が IBD を引き起こす？

最近になり、新しい IL-12 ファミリーサイトカインである IL-23 が Th-1/Th-17 誘導性サイトカインとして炎症性疾患の病態に関与している可能性が注目されている。実際に CD の腸管 M ϕ からは IL-12 のみならず、IL-23 も高産生されるという報告もあり、CD の病態において腸管 M ϕ から誘導される IL-12/IL-23 の役割が注目されている^{9,10)}。IBD モデル動物の1つである IL-10 遺伝子欠損 knockout (KO) マウスは Th-1 型の慢性腸炎を自然発症するモデルである。IL-10 KO マウスの M ϕ や DC はナイーブ T 細胞やメモリー T 細胞の Th-1 反応を誘導する。また、腸管 M ϕ を選択的に除去することで腸炎の発症が抑制されることから、本モデルにおいて M ϕ は炎症の主体を担っていると考えられる¹⁴⁾。さらに IL-12 と IL-23 の共通のサブユニットである IL-12/23p40 とのダブルノックアウトマウスや IL-12/23p40 サブユニット抗体治療により IL-10 KO マウスの腸炎発症が劇的に抑制されることから、CD と同様に、M ϕ からの IL-12/23 産生が、本モデルの病態形成の鍵となっていると考えられる¹¹⁾。Hirotsu らは正常マウスと IL-10 KO マウスの腸管 M ϕ の遺伝子発現を比較し、正常マウス腸管 M ϕ においては IL-10 により誘導される I κ BNS が NF- κ B の DNA binding activity を変化させる (p50/p50 のホモダイマーの結合を誘導する) ことで LPS 誘導性 IL-6 産生に対して抑制的に働いていることを報告している⁶⁾。また、Kobayashi らは骨髄系細胞選択的に IL-10 の下流シグナル分子である STAT3 を欠損したマウスで TLR4 を介した LPS 刺激によって誘導される IL-12p40 依存

性に腸炎を発症することを報告している¹²⁾。一方、筆者らがIL-10KOマウスを用いて細菌刺激に対する腸管Mφの機能について検討を行った結果、IL-10KOマウスでは、本来抑制的に働くべきM-Mφおよび腸管Mφにおいて腸内細菌である*Escherichia coli*や*Enterococcus faecalis*加熱死菌抗原刺激により過剰なIL-12p70, IL-23の産生が認められることが明らかになった。興味深いことに活性型IL-12p70についてはLPSなどのPAMPSでは誘導されず、また貪食作用を抑制するとIL-12p70産生は抑制されることからMφによる細菌貪食処理後の細胞内メカニズムが重要であると推測された⁵⁾。以上の報告からIL-10KO腸炎モデルマウスでは腸管Mφの抑制性機能が破綻していることがわかる。さらに筆者らは単球がM-CSFでMφに分化誘導される過程で産生される内因性IL-10が抑制性Mφ（腸管Mφも含めて）の機能

的成熟に必要であることを明らかにした⁵⁾。つまり、通常では腸管Mφの分化段階で内因性IL-10によりMφによるIL-12誘導機構（つまりはTh-1優位な獲得免疫の誘導）は負に制御されているが、IL-10欠損下では抑制性Mφの正常な分化が障害されIL-12の抑制機構が破綻し、その結果、腸内細菌認識により過剰なIL-12が産生され、Th-1優位な腸炎を引き起こすと考えられる。

ヒトCDでは病理組織学的特徴として非乾酪性肉芽腫（granuloma）の存在があるが、マウス腸炎モデルでこれを再現することはこれまで困難であった。MizoguchiらはマウスモデルにおいてMφマーカーであるF4/80を発現する未分化なCD11c陽性DCがIL-23を産生しgranuloma形成に働いていることを報告した¹³⁾。筆者らはヒトCDにおいてやはりDCマーカーの一部を発現する特殊なMφ細胞集団が増加し、かつ腸内細菌刺

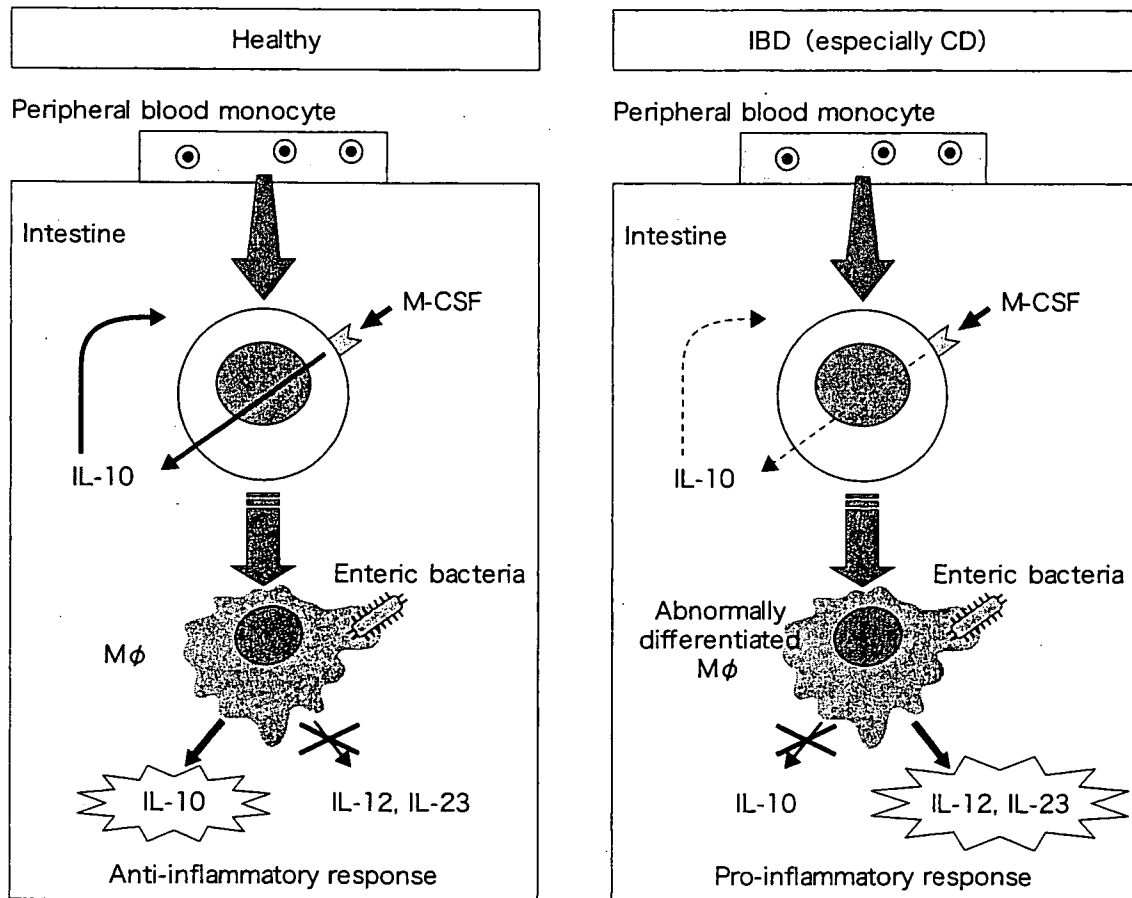


図1 IBDにおける腸管マクロファージの機能異常

激に対してIL-23の過剰産生を引き起こし、異常なTh-1サイクル形成に寄与していることを突き止めた(論文投稿中)(図1)。すなわちCDにおいて腸管MφおよびDCは何らかの原因によりその免疫制御機能を失い、その結果、腸内細菌に対する過剰な免疫反応、IL-12/23産生に起因するTh-1/Th-17型獲得免疫反応の増強を引き起こしている可能性がある。

C. Paneth細胞と α -defensinの機能異常はCrohn病の原因か?

Paneth細胞は小腸陰窩底部に存在し抗菌ペプチドである α -defensinを管腔側に分泌し病原体から生態を守っている特殊な腸管上皮細胞である。このPaneth細胞にCD疾患関連遺伝子であるNOD2が特異的に発現していることがすでに明らかとなっている^{14,15}。Wehkampらは回腸病変を有するCD患者の腸管抽出物では抗菌活性が低下していること、さらに特にPaneth細胞での α -defensinの発現がdefensin familyの中で特異的に低下していることを報告した¹⁶。また、TanabeらはPaneth細胞で蓄えられている α -defensin, proHD-5が蛋白分解酵素の作用からのがれるためにはs-s結合による立体構造が重要であり、CD患者ではこのs-s結合が欠損した不安定な還元型proHD-5が存在していることを報告している¹⁷。一方Shiらはmature formの α -defensinが分泌できないMMP-7欠損マウスではdextran sulfate sodium誘発腸炎における感受性が高いこと、 α -defensinはIL-1 β の転写後調節に関与しその分泌を抑制していることを報告している¹⁸。このようにPaneth細胞から分泌される α -defensinの産生および機能異常がCDの病態に関与している可能性が強く示唆されている。

D. 腸管上皮細胞の腸管免疫恒常性維持における役割

腸管上皮細胞は病原体を含む外来抗原に対する構造的なバリアーであるとともに抗菌ペプチドの産生、IL-8産生による好中球のリクルートなど機能的防御機構としても重要である。これまで腸管上皮細胞の腸管免疫恒常性維持における役割についてはあまり明らかとされてこなかったが、NenchiらとZaphらの2つのグループから腸管上皮細胞におけるNF- κ Bシグナルが腸管免疫恒常性維持に重要であるという知見が報告された^{19,20}。Nenchiらは腸管上皮細胞から特異的にNEMO (IKK γ) をノックアウトしたマウスが大腸炎を自然発症することを報告した。このマウスでは腸管上皮細胞のアポトーシス亢進しており腸管上皮のintegrityが低下している。さらに腸管上皮細胞からのdefensinの産生が低下している。その結果、腸内細菌のtranslocationを引き起こし初期には樹状細胞や好中球を主体とした自然免疫担当細胞が集簇し、最終的にT細胞を中心とした全大腸炎が引き起こされる。NEMOとMyD 88のダブルノックアウトおよびNEMOとTNF受容体Iのダブルノックアウトマウスでは大腸炎が発症しない。すなわちNEMOノックアウトマウスにおける慢性大腸炎の発症には免疫担当細胞のTLRsシグナル系と腸管上皮細胞のTNF誘導性アポトーシスが必須であると考えられる¹⁹。しかしながらNEMOノックアウトマウスではNEMOの発現は小腸と大腸の両方で欠損しているにもかかわらず大腸のみがターゲットとなる。多くのマウスモデルがそうであるようにこの大腸の易罹患性については腸内細菌との関係を含めてさらなる研究が必要であろう。一方、Zaphらは腸管上皮細胞におけるIKK β の重要性について*Trichuris*感染マウスを用いて証明した。*Trichuris*感染マウスは人の線虫感染モデルであり*Trichuris*特異的な

Th-2型CD4T細胞が感染抵抗性に必須である。上皮細胞のIKK β を欠損したマウスでは上皮細胞からのNF- κ B依存性thymic stromal lymphopoietin (TSLP)の発現が低下している。このためDC上のTSLP受容体からの抑制刺激が入らずDC由来のIL-12/23が増加しTh-1免疫応答が誘導され腸炎が発症する²⁰⁾。またRimoldiらは腸管上皮細胞由来TSLPは樹状細胞をIL-10産生IL-12非産生型へ誘導し結果としてTh-1型免疫応答へのシフトを抑制していること、さらにヒトCDでは腸管上皮細胞でのTSLP発現が低下し調節機構が破綻している可能性があることを報告している²¹⁾。

以上の報告はいずれも腸管上皮細胞特異的にNF- κ Bシグナルやその下流の遺伝子発現を変化させるだけで腸管免疫の恒常性が破綻し、引き続き自然免疫や獲得免疫系に異常を引き起こした結果、慢性炎症が誘導されうることを示している。ヒトの炎症性腸疾患の病態を考える上で腸管上皮細胞と免疫担当細胞とのcross talkは新たな視点として注目されよう。

むすび

腸管免疫の恒常性維持における自然免疫系の役割とその破綻によるIBD発症メカニズムについて最近の知見を中心に概説した。腸管免疫機構と腸内細菌との繊細なバランスの上に腸管の恒常性は保たれている。このメカニズムはおそらくは二重、三重にもはりめぐらされており、その破綻が炎症性腸疾患を引き起こすと考えられる。今後はマウスモデル中心の解析をいかにヒトIBDの病態解明へ応用するかが課題である。また、IBD疾患関連遺伝子には腸管免疫恒常性維持に関与するものが多く、これらの遺伝子がどのように病態に関与しているかも今後解明すべき課題であろう。

文献

- 1) Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001; 411: 599-603.
- 2) Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001; 411: 603-6.
- 3) Akagawa KS. Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Int J Hematol*. 2002; 76: 27-34.
- 4) Cecchini MG, Dominguez MG, Mocci S, et al. Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development*. 1994; 120: 1357-72.
- 5) Kamada N, Hisamatsu T, Okamoto S, et al. Abnormally differentiated subsets of intestinal macrophage play a key role in Th1-dominant chronic colitis through excess production of IL-12 and IL-23 in response to bacteria. *J Immunol*. 2005; 175: 6900-8.
- 6) Hirotsu T, Lee PY, Kuwata H, et al. The nuclear IkappaB protein IkappaBNS selectively inhibits lipopolysaccharide-induced IL-6 production in macrophages of the colonic lamina propria. *J Immunol*. 2005; 174: 3650-7.
- 7) Smythies LE, Sellers M, Clements RH, et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest*. 2005; 115: 66-75.
- 8) Qualls JE, Kaplan AM, van Rooijen N, et al. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol*. 2006; 80: 802-15.
- 9) Fuss IJ, Becker C, Yang Z, et al. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12p40 monoclonal antibody. *Inflamm Bowel Dis*. 2006; 12: 9-15.
- 10) Yen D, Cheung J, Scheerens H, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest*. 2006; 116: 1310-6.

- 11) Davidson NJ, Hudak SA, Lesley RE, et al. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol.* 1998; 161: 3143-9.
- 12) Kobayashi M, Kweon MN, Kuwata H, et al. Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J Clin Invest.* 2003; 111: 1297-308.
- 13) Mizoguchi A, Ogawa A, Takedatsu H, et al. Dependence of intestinal granuloma formation on unique myeloid DC-like cells. *J Clin Invest.* 2007; 117: 605-15.
- 14) Lala S, Ogura Y, Osborne C, et al. Crohn's disease and the NOD2 gene: a role for Paneth cells. *Gastroenterology.* 2003; 125: 47-57.
- 15) Ogura Y, Lala S, Xin W, et al. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut.* 2003; 52: 1591-7.
- 16) Wehkamp J, Salzman NH, Porter E, et al. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci USA.* 2005; 102: 18129-34.
- 17) Tanabe H, Ayabe T, Maemoto A, et al. Denatured human alpha-defensin attenuates the bactericidal activity and the stability against enzymatic digestion. *Biochem Biophys Res Commun.* 2007; 358: 349-55.
- 18) Shi J, Aono S, Lu W, et al. A Novel role for defensins in intestinal homeostasis: Regulation of IL-1beta secretion. *J Immunol.* 2007; 179: 1245-53.
- 19) Nenci A, Becker C, Wullaert A, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature.* 2007; 446: 557-61.
- 20) Zaph C, Troy AE, Taylor BC, et al. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature.* 2007; 446: 552-6.
- 21) Rimoldi M, Chieppa M, Salucci V, et al. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol.* 2005; 6: 507-14.

特集I 消化管における免疫と疾患

クローン病の発症と 樹状細胞様マクロファージ からのIL-23*

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Key Words : Crohn's disease, dendritic cell, macrophage, IL-23

はじめに

通常、免疫装置は外界からの侵入者に対して免疫反応を誘導することで外来抗原に対しすみやかに反応、処理することで生体を守っている。しかしながら、常に食餌抗原や腸内細菌に曝されている腸管粘膜では、それらの抗原に対して過剰な免疫反応を誘導するのは好ましくなく、むしろ恒常性を保つため過剰な免疫反応を抑制していると考えられる。近年、この腸管の低反応性を説明する機序として腸管におけるマクロファージや樹状細胞の特殊性が明らかとなってきた。

自然免疫、獲得免疫の担い手 “樹状細胞”

樹状細胞は自然免疫と獲得免疫を架橋する上でもっとも重要な免疫担当細胞の一つであると考えられている。樹状細胞は外来もしくは自己抗原を補足し、ナイーブT細胞に対し提示する。ナイーブT細胞は樹状細胞からの抗原提示を受けた後にT helper(Th)1やTh2, Th17といった異なる免疫誘導を行うメモリーT細胞へと分化

を遂げる。興味深いことに、樹状細胞は前述のヘルパーT細胞の分化誘導を介した獲得免疫反応の誘導だけではなく、それらの免疫反応に対し抑制的に働く制御性T細胞(Treg)の分化も誘導する。このように、樹状細胞は免疫反応の正の制御および負の制御の両面において中心的な役割を担っている。すなわち、樹状細胞は、自己および非自己抗原に対する免疫反応および免疫寛容を決定しており、樹状細胞による免疫制御の乱れは、炎症性疾患や自己免疫性疾患などの引き金になると考えられる。

腸管は病原体や食餌抗原などの外来抗原に恒常的に曝露されている。マクロファージや樹状細胞などの抗原提示細胞は通常、外界からの侵入者に対して免疫反応を誘導することで外来抗原に対しすみやかに反応、処理することで生体を守っている。しかしながら、常に食餌抗原や腸内細菌に曝されている腸管粘膜では、それらの抗原に対して過剰な免疫反応を誘導するのは好ましくなく、むしろ恒常性を保つため過剰な免疫反応を抑制的に制御する機構が存在すると考えられる。近年、この腸管の免疫寛容を説明する機構として、腸管特異的な抑制性マクロファージや樹状細胞サブセットが見出された¹⁾²⁾。なかでも、腸管特異的な抑制性樹状細胞についての報告は最近のトピックであり、腸管恒常性維持

* Role of IL-23 producing dendritic cell-like macrophage in the development of Crohn's disease.

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機構および腸管特異的炎症性疾患発症機構の解明において非常に重要な知見であると考えられる。

腸管樹状細胞の特異性

では、腸管特異的な抑制性樹状細胞サブセットはどのように誘導されるのであろうか？ Rimoldiらは、腸内細菌刺激により腸管上皮細胞から産生されるTSLPが樹状細胞をIL-10高産生かつIL-12低産生型へと誘導することを報告した³⁾。これは、腸管上皮細胞が単なる物理的なバリアーとして働いているのではなく、腸管特異的免疫誘導において、腸管上皮細胞と腸管粘膜樹状細胞の相互作用が重要な役割を担っていることを示唆している。また、最近の研究で、腸管樹状細胞機能におけるレチノイン酸の重要性が明らかになってきた。Iwataらは、ビタミンAからレチノイン酸への代謝酵素であるレチナール脱水素酵素(RALDH)が腸管樹状細胞で特異的に発現していることを報告した⁴⁾。さらに、RALDH陽性の腸管樹状細胞から産生されるレチノイン酸は抗原提示反応時にT細胞に対し、腸管へのホーミングにかかわる分子である $\alpha 4\beta 7$ インテグリンやCCR9の発現を誘導することが明らかになった⁴⁾。このように腸管樹状細胞はレチノイン酸生成を介して腸管指向性のT細胞分化に重要であることが示唆された。しかしながら、T細胞の腸管への移行を亢進することは、腸管樹状細胞の免疫抑制作用を説明する上で矛盾するように思われる。最近になって、その疑問の答えとなる報告が相次いでなされた。それらの報告により、レチノイン酸はT細胞に対し腸管へのホーミングマーカーの発現誘導を行うだけでなく、炎症性免疫反応であるTh17型免疫の抑制作用、さらにFoxp3⁺制御性T細胞誘導において重要な役割を果たしていることが明らかになった^{5)~8)}。すなわち、腸管樹状細胞はレチノイン酸を介して腸管指向性Foxp3⁺制御性T細胞を優位に誘導することで、腸管の免疫反応を負に制御していることが示唆された。また、レチノイン酸はT細胞分化に作用するのみならず樹状細胞自身にも作用し、腸管局在にかかわるインテグリン $\beta 7$ やCCR9, TGF- β などの腸管粘膜樹状細胞特異的分子の発現を誘導することも報告されている⁹⁾。同様にわれわれ

は、レチノイン酸がTh1型免疫抑制的な樹状細胞の分化を誘導することを見出した(論文投稿中)。興味深いことに、腸管では樹状細胞のみならず腸管上皮細胞もRALDHを発現している¹⁰⁾。また、RALDHやレチノイン酸受容体であるRAR発現誘導にはレチノイン酸自身がかかわっていることから¹¹⁾、腸管特異的なRALDH発現樹状細胞の誘導には腸管上皮細胞との相互作用が関与している可能性がある。

クローン病における免疫異常と樹状細胞の役割

一方で、樹状細胞による免疫寛容誘導機構の破綻は、過剰な獲得免疫反応の誘導を介して慢性の炎症性疾患発症の引き金になると考えられる。炎症性腸疾患(inflammatory bowel disease; IBD)は慢性の腸管炎症を主体とする難治性炎症性疾患であり、大きく潰瘍性大腸炎とクローン病の2疾患に区別される。潰瘍性大腸炎、クローン病ともにその病因はいまだ明らかにはなっていないが、自然免疫、獲得免疫のいずれもが発症に関与していると考えられ、その担い手として、樹状細胞の病態への関与が注目されている¹²⁾。これまでの研究により、クローン病ではIFN- γ を主体とするTh1型獲得免疫反応が病態の形成において重要な役割を果たしていることが報告されている。実際に、クローン病腸管局所ではIFN- γ 産生や、そのmaster regulatorであるT-betの発現が亢進していることが明らかとなっている¹³⁾¹⁴⁾。では、クローン病におけるTh1型獲得免疫誘導において樹状細胞はどのような役割を担っているのであろうか？クローン病腸管樹状細胞はこれまでに、クローン病の腸管局所において、樹状細胞からのIL-12, IL-23, IL-27などのサイトカインが亢進していることが報告されてきた¹⁵⁾。なかでも、IL-12はTh1型免疫反応の誘導に深くかかわっているサイトカインであり、Th1型の免疫反応を主体とするクローン病において必須のサイトカインであると考えられてきた。しかしながら、最近になり、新たなヘルパーT細胞サブセットであるTh17が発見されたことで、事態は大きく変わる事となった。IL-23は当初、メモリーT細胞に作用しTh1細胞からのIFN- γ 産生を誘導す

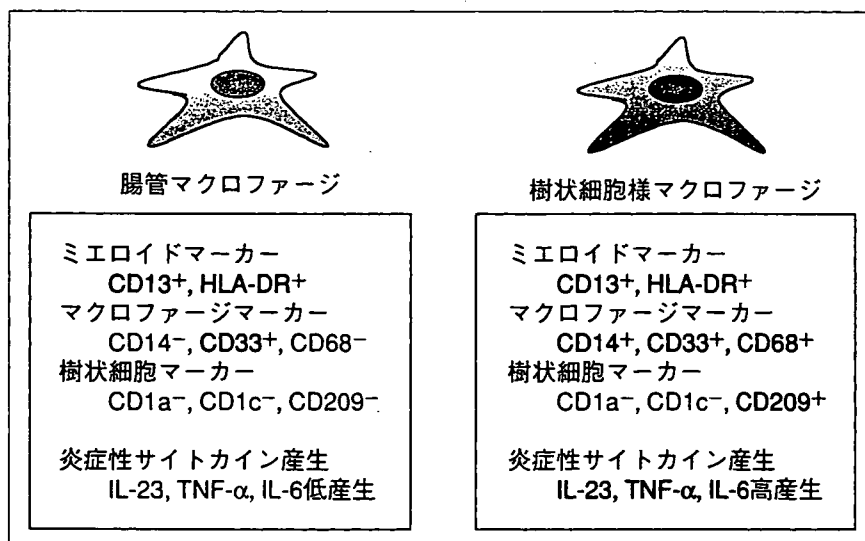


図1 腸管特異的樹状細胞様マクロファージ

腸管マクロファージは通常マクロファージや樹状細胞マーカーの発現を欠き、炎症性サイトカインの産生も低い抑制性のフェノタイプを示す。クローン病腸管粘膜では樹状細胞とマクロファージのマーカーを共発現し、IL-23を高産生するミエロイド系細胞サブセットが増加している。

るIL-12様サイトカインとして同定されたが、最近の研究でIL-23はむしろTh17型免疫反応にかかわっていることが明らかになった¹⁶⁾。Th17はROR γ t依存性に誘導されるヘルパーT細胞サブセットであり、IL-17やIL-22, IL-26, CCL20などのサイトカインやケモカインを産生する炎症性サブセットである。マウスではTh17細胞はTGF- β とIL-6の共刺激により誘導され、その誘導にIL-23は必要としない¹⁷⁾。マウスにおいてIL-23はTh17細胞の活性化、生存などにかかわると考えられている。ヒトではマウスと同様にIL-23はTh17細胞の活性化に関与するという報告がなされている一方で、IL-23はIL-1 β と協調的に働き、Th17細胞の分化誘導にかかわるという報告もある¹⁸⁾。さらに、これまでIFN- γ /Th1型の免疫異常を主体とすると考えられていた疾患の多くが、実はIL-23/Th17型の免疫反応がより重要であることが報告された^{19)~21)}。実際に、IBDにおいても、腸炎モデルマウスでIL-12p35をノックアウトしたものは腸炎発症に差異が認められないのに対し、IL-23 p19をノックアウトすることで腸炎の発症が抑制されることが報告された²²⁾。この結果は、IBDモデルマウスにおいてもIL-12よりむしろIL-23が病態形成にかかわっていることを示唆している。実際に、最近ヒトIBD患者でIL-23受容体の変異

が発見され、ヒトIBDにおけるIL-23の関与を裏づけるものである²³⁾。

では、IL-23/Th17がクローン病においても病態の本質を担っているのであろうか？ たしかに、クローン病、および潰瘍性大腸炎患者の腸管粘膜局所ではマウス腸炎モデルと同様にIL-17の発現は亢進していることが報告されている²⁴⁾。しかしながら、興味深いことにIL-17の中和抗体の投与はこれらの腸炎モデルにおいて有効性を示さない、もしくは悪化させるという報告もされている²²⁾²⁵⁾。これらの結果は、腸炎モデルにおいてIL-17が必須の病原因子ではないことを示唆している。すなわち、IL-23はIL-17以外の因子(Th1細胞の活性化やTh17細胞からのIL-17以外の炎症性サイトカインなど)を介して病態の形成にかかわっていることが考えられる。事実、細菌認識にかかわる分子であるNOD2の変異を有するクローン病患者において、細菌抗原刺激による樹状細胞からのIL-1産生、および付随するTh17細胞からのIL-17産生誘導能が低下することが報告された²⁶⁾。

IL-23産生樹状細胞のクローン病病態への関与

では、クローン病においてIL-23はどのようにその病態に関与しているのであろうか？ われわ