compared with UC or normal controls. According to the previous report, CD56^{bright} NK cells also are enriched at inflammatory sites, such as arthritis, infectious pleuritis, and bacterial peritonitis.[58] CD is regarded as a typical T helper type 1 response (Th1) disease driven by excessive IFN-γ production from dysregulated CD4 T cells infiltrating the inflamed tissue. However, given that NK cells constitute a considerable proportion of LPMCs or IELs (about 8%) and can highly produce IFN-γ, intestinal NK cells may contribute to the pathogenesis of CD. Overexpression of IFN-γ in CD may modulate intestinal NK cell differentiation because it was reported that this cytokine accelerated differentiation of human HSCs.[59]

In summary, we have identified c-kit$^+$ immune precursor cells in the human adult intestine for the first time. We also have shown that these cells are committed mainly to the NK cell lineage. Because this intestinal NK cell differentiation system may contribute to the pathophysiology of CD, further clarification of the role of intestinal NK cells will help to better understand the gut immune system and may lead to new therapeutic strategies against CD.

Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.05.017.

References

1. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 2000;404:193–197.
2. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 1997; 91:661–672.
3. Rodewald HR, Moingeon P, Lucich JL, Dosiou C, Lopez P, Reinherz EL. A population of early fetal thymocytes expressing Fc gamma RII/III contains precursors of T lymphocytes and natural killer cells. Cell 1992;69:139–150.
4. Carlyle JR, Michie AM, Furlonger C, Nakano T, Lenardo MJ, Paige CJ, Zuniga-Pflucker JC. Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. J Exp Med 1997;186:173–182.
5. Ikawa T, Kawamoto H, Fujimoto S, Katsura Y. Commitment of common T/Natural killer (NK) progenitors to unipotent T and NK progenitors in the murine fetal thymus revealed by a single progenitor assay. J Exp Med 1999;190:1617–1626.
6. Yoshida H, Honda K, Shinkura R, Adachi S, Nishikawa S, Maki K, Ikuta K, Nishikawa SI. IL-7 receptor alpha+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. Int Immunol 1999;11:643–655.
7. Finke D, Acha-Orbea H, Mattis A, Lipp M, Kraehenbuhl J. CD4+CD3- cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. Immunity 2002;17:363–373.
8. Kanamori Y, Ishimaru K, Nanno M, Maki K, Ikuta K, Nariuchi H, Ishikawa H. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit+ IL-7R+ Thy1+ lympho-hemopoietic progenitors develop. J Exp Med 1996;184:1449–1459.
9. Yoshida H, Naito A, Inoue J, Satoh M, Santee-Cooper SM, Ware CF, Togawa A, Nishikawa S, Nishikawa S. Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. Immunity 2002;17:823–833.
10. Saito H, Kanamori Y, Takemori T, Nariuchi H, Kubota E, Takahashi-Iwanaga H, Iwanaga T, Ishikawa H. Generation of intestinal T cells from progenitors residing in gut cryptopatches. Science 1998;280:275–278.
11. Suzuki K, Oida T, Hamada H, Hitotsumatsu O, Watanabe M, Hibi T, Yamamoto H, Kubota E, Kaminogawa S, Ishikawa H. Gut cryptopatches: direct evidence of extrathymic anatomical sites for intestinal T lymphopoiesis. Immunity 2000;13:691–702.
12. Eberl G, Littman DR. Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. Science 2004; 305:248–251.
13. Howie D, Spencer J, DeLord D, Pitzalis C, Wathen NC, Dogan A, Akbar A, MacDonald TT. Extrathymic T cell differentiation in the human intestine early in life. J Immunol 1998;161:5862–5872.
14. Gunther U, Holloway JA, Gordon JN, Knight A, Chance V, Hanley NA, Wilson DI, French R, Spencer J, Steer H, Anderson G, MacDonald TT. Phenotypic characterization of CD3-7+ cells in developing human intestine and an analysis of their ability to differentiate into T cells. J Immunol 2005;174:5414–5422.
15. Williams AM, Bland PW, Phillips AC, Turner S, Brooklyn T, Shaya G, Spicer RD, Probert CS. Intestinal alpha beta T cells differentiate and rearrange antigen receptor genes in situ in the human infant. J Immunol 2004;173:7190–7199.
16. Bas A, Hammarstrom SG, Hammarstrom ML. Extrathymic TCR gene rearrangement in human small intestine: identification of new splice forms of recombination activating gene-1 mRNA with selective tissue expression. J Immunol 2003;171:3359–3371.
17. Ashman LK, Cambareri AC, To LB, Levinsky RJ, Juttner CA. Expression of the YB5.B8 antigen (c-kit proto-oncogene product) in normal human bone marrow. Blood 1991;78:30–37.
18. Res P, Martinez-Caceres E, Cristina Jaleco A, Staal F, Noteboom E, Weijer K, Spits H. CD34+CD38dim cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. Blood 1996;87:5196–5206.
19. Sanchez MJ, Spits H, Lanier LL, Phillips JH. Human natural killer cell committed thymocytes and their relation to the T cell lineage. J Exp Med 1993;178:1857–1866.
20. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl 1989;170:2–6, 16–19.
21. Gower-Rousseau C, Salomez JL, Dupas JL, Marti R, Nuttens MC, Votte A, Lemahieu M, Lemaire B, Colombel JF, Cortot A. Incidence of inflammatory bowel disease in northern France (1988–1990). Gut 1994;35:1433–1438.
22. Bandeira A, Mota-Santos T, Itohara S, Degermann S, Heusser C, Tonegawa S, Coutinho A. Localization of gamma/delta T cells to the intestinal epithelium is independent of normal microbial colonization. J Exp Med 1990;172:239–244.
23. Ishikawa H, Li Y, Abeliovich A, Yamamoto S, Kaufmann SH, Tonegawa S. Cytotoxic and interferon gamma-producing activities of gamma delta T cells in the mouse intestinal epithelium are strain dependent. Proc Natl Acad Sci U S A 1993;90:8204–8208.
24. Eldridge JH, Kiyono H, Michalek SM, McGhee JR. Evidence for a mature B cell subpopulation in Peyer's patches of young adult xid mice. J Exp Med 1983;157:789–794.
25. Chang L, Gusewitch GA, Chritton DB, Folz JC, Lebeck LK, Nehlsen-Cannarella SL. Rapid flow cytometric assay for the assessment of natural killer cell activity. J Immunol Methods 1993;166:45–54.
26. Horie K, Fujita J, Takakura K, Kanzaki H, Suginami H, Iwai M, Nakayama H, Mori T. The expression of c-kit protein in human adult and fetal tissues. Hum Reprod 1993;8:1955–1962.
27. Feyerabend TB, Hausser H, Tietz A, Blum C, Hellman L, Straus AH, Takahashi HK, Morgan ES, Dvorak AM, Fehling HJ, Rodewald HR. Loss of histochemical identity in mast cells lacking carboxypeptidase A. Mol Cell Biol 2005;25:6199–6210.

28. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 1984;133:157–165.
29. Sanchez MJ, Muench MO, Roncarolo MG, Lanier LL, Phillips JH. Identification of a common T/natural killer cell progenitor in human fetal thymus. J Exp Med 1994;180:569–576.
30. Peiper SC, Ashmun RA, Look AT. Molecular cloning, expression, and chromosomal localization of a human gene encoding the CD33 myeloid differentiation antigen. Blood 1988;72:314–321.
31. Miller JS, Alley KA, McGlave P. Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor. Blood 1994;83:2594–2601.
32. Kim S, Iizuka K, Kang HS, Dokun A, French AR, Greco S, Yokoyama WM. In vivo developmental stages in murine natural killer cell maturation. Nat Immunol 2002;3:523–528.
33. Cepek KL, Parker CM, Madara JL, Brenner MB. Integrin alpha E beta 7 mediates adhesion of T lymphocytes to epithelial cells. J Immunol 1993;150:3459–3470.
34. Anderson MK, Hernandez-Hoyos G, Diamond RA, Rothenberg EV. Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. Development 1999;126:3131–3148.
35. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994;265:1573–1577.
36. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, Gruss P. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature 1999;397:702–706.
37. Iizuka K, Chaplin DD, Wang Y, Wu Q, Pegg LE, Yokoyama WM, Fu YX. Requirement for membrane lymphotoxin in natural killer cell development. Proc Natl Acad Sci U S A 1999;96:6336–6340.
38. Alimzhanov MB, Kuprash DV, Kosco-Vilbois MH, Luz A, Turetskaya RL, Tarakhovsky A, Rajewsky K, Nedospasov SA, Pfeffer K. Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. Proc Natl Acad Sci U S A 1997;94:9302–9307.
39. Hanna J, Gonen-Gross T, Fitchett J, Rowe T, Daniels M, Arnon TI, Gazit R, Joseph A, Schjetne KW, Steinle A, Porgador A, Mevorach D, Goldman-Wohl D, Yagel S, LaBarre MJ, Buckner JH, Mandelboim O. Novel APC-like properties of human NK cells directly regulate T cell activation. J Clin Invest 2004;114:1612–1623.
40. Colucci F, Caligiuri MA, Di Santo JP. What does it take to make a natural killer? Nat Rev Immunol 2003;3:413–425.
41. Pang G, Buret A, Batey RT, Chen QY, Couch L, Cripps A, Clancy R. Morphological, phenotypic and functional characteristics of a pure population of CD56+ CD16- CD3- large granular lymphocytes generated from human duodenal mucosa. Immunology 1993;79:498–505.
42. Leon F, Roldan E, Sanchez L, Camarero C, Bootello A, Roy G. Human small-intestinal epithelium contains functional natural killer lymphocytes. Gastroenterology 2003;125:345–356.
43. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 1986;136:4480–4486.
44. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, Carson WE, Caligiuri MA. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 2001;97:3146–3151.
45. Campbell JJ, Qin S, Unutmaz D, Soler D, Murphy KE, Hodge MR, Wu L, Butcher EC. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol 2001;166:6477–6482.
46. Robertson MJ. Role of chemokines in the biology of natural killer cells. J Leukoc Biol 2002;71:173–183.
47. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002; 347:417–429.
48. Di Santo JP. Natural killer cell developmental pathways: a question of balance. Annu Rev Immunol 2006;24:257–286.
49. Spits H, Blom B, Jaleco AC, Weijer K, Verschuren MC, van Dongen JJ, Heemskerk MH, Res PC. Early stages in the development of human T, natural killer and thymic dendritic cells. Immunol Rev 1998;165:75–86.
50. Oida T, Suzuki K, Nanno M, Kanamori Y, Saito H, Kubota E, Kato S, Itoh M, Kaminogawa S, Ishikawa H. Role of gut cryptopatches in early extrathymic maturation of intestinal intraepithelial T cells. J Immunol 2000;164:3616–3626.
51. Mebius RE, Rennert P, Weissman IL. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. Immunity 1997;7:493–504.
52. Yoshida H, Kawamoto H, Santee SM, Hashi H, Honda K, Nishikawa S, Ware CF, Katsura Y, Nishikawa SI. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. J Immunol 2001;167:2511–2521.
53. Fiocchi C, Tubbs RR, Youngman KR. Human intestinal mucosal mononuclear cells exhibit lymphokine-activated killer cell activity. Gastroenterology 1985;88:625–637.
54. Shibuya A, Nagayoshi K, Nakamura K, Nakauchi H. Lymphokine requirement for the generation of natural killer cells from CD34+ hematopoietic progenitor cells. Blood 1995;85:3538–3546.
55. Handgretinger R, Schafer HJ, Baur F, Frank D, Ottenlinger C, Buhring HJ, Niethammer D. Expression of an early myelopoietic antigen (CD33) on a subset of human umbilical cord blood-derived natural killer cells. Immunol Lett 1993;37:223–228.
56. Freud AG, Becknell B, Roychowdhury S, Mao HC, Ferketich AK, Nuovo GJ, Hughes TL, Marburger TB, Sung J, Baiocchi RA, Guimond M, Caligiuri MA. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. Immunity 2005;22:295–304.
57. Keilbaugh SA, Shin ME, Banchereau RF, McVay LD, Boyko N, Artis D, Cebra JJ, Wu GD. Activation of RegIIIbeta/gamma and interferon gamma expression in the intestinal tract of SCID mice: an innate response to bacterial colonisation of the gut. Gut 2005;54:623–629.
58. Dalbeth N, Gundle R, Davies RJ, Lee YC, McMichael AJ, Callan MF. CD56bright NK cells are enriched at inflammatory sites and can engage with monocytes in a reciprocal program of activation. J Immunol 2004;173:6418–6426.
59. Yang L, Dybedal I, Bryder D, Nilsson L, Sitnicka E, Sasaki Y, Jacobsen SE. IFN-gamma negatively modulates self-renewal of repopulating human hemopoietic stem cells. J Immunol 2005; 174:752–757.

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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 β1) (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 β7 (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 Cytofix/Cytoperm kit (BD Pharmingen), anti-granzyme A mAb (CB9; BD Pharmingen) and anti-perforin mAb (δG9; 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).

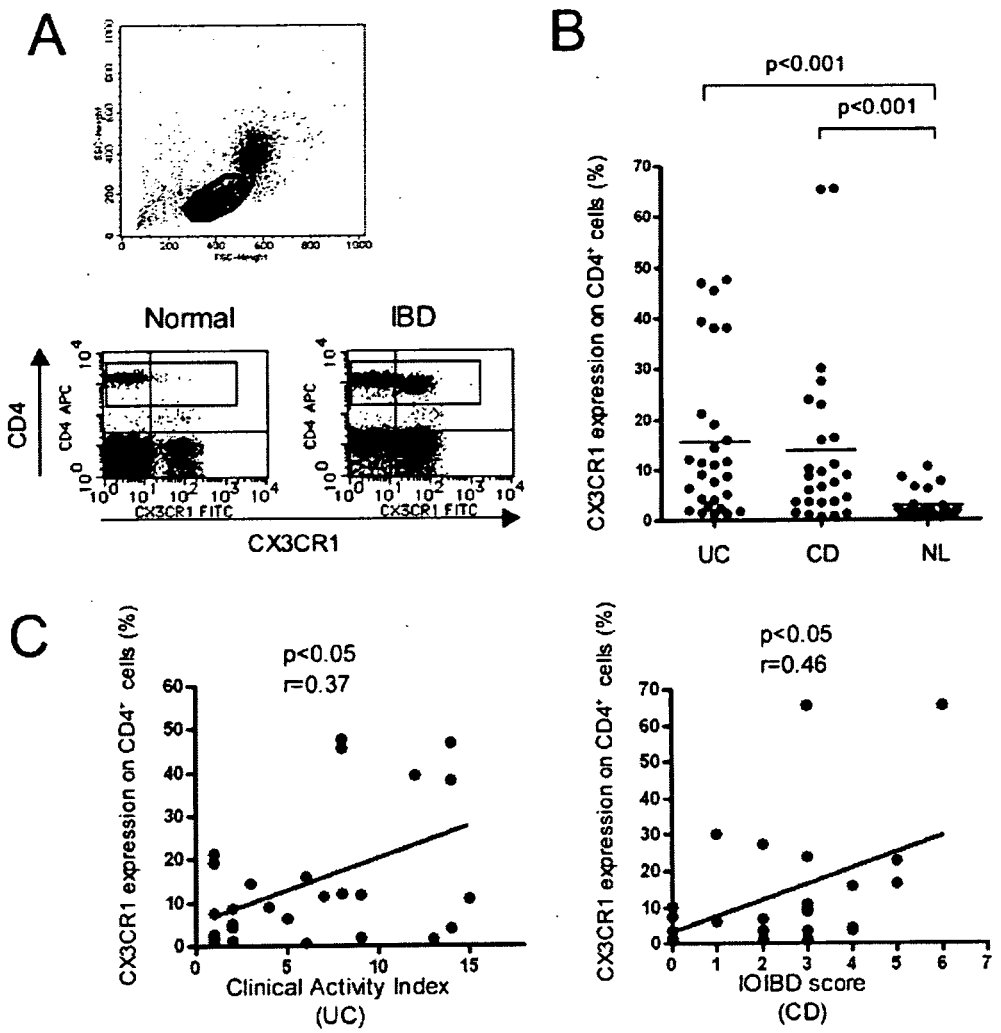

FIGURE 1. A: Representative results of the surface expression of CX3CR1 on PBMCs from patients with ulcerative colitis (UC) and normal controls by flow cytometry. Freshly isolated PBMCs were stained with anti-CX3CR1 mAb in combination with anti-CD4 mAb. Patterns are gated on lymphocytes using forward 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 μg/mL of immobilized anti-CD3 (UCHT1; BD Pharmingen) and 5 μg/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 μg/mL) (Sigma).

In migration assays using the Transwell system[19] (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-μm 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-μm-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 ± 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.

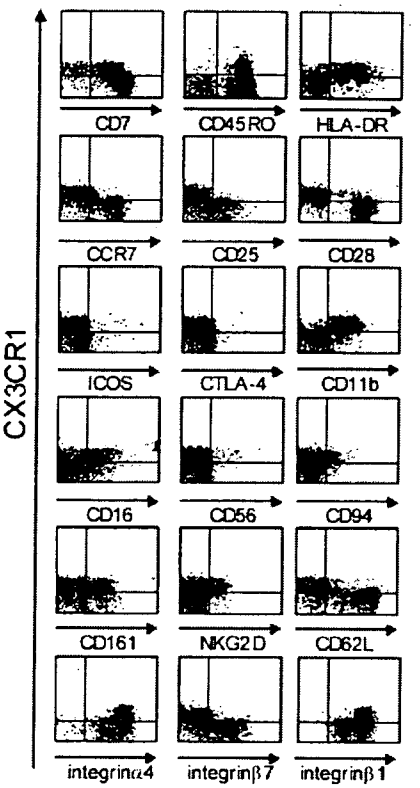

FIGURE 2. Representative results of expression of CX3CR1 and various functional antigens on $CD4^+$ T cells by flow cytometry. Freshly isolated PBMCs from a UC patient were stained with anti-CD4 mAb, anti-CX3CR1 mAb, and mAbs to various surface molecules. Patterns are gated on $CD4^+$ cells.

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 ± 2.9%) and CD (13.9 ± 3.4%) compared with controls (2.9 ± 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-

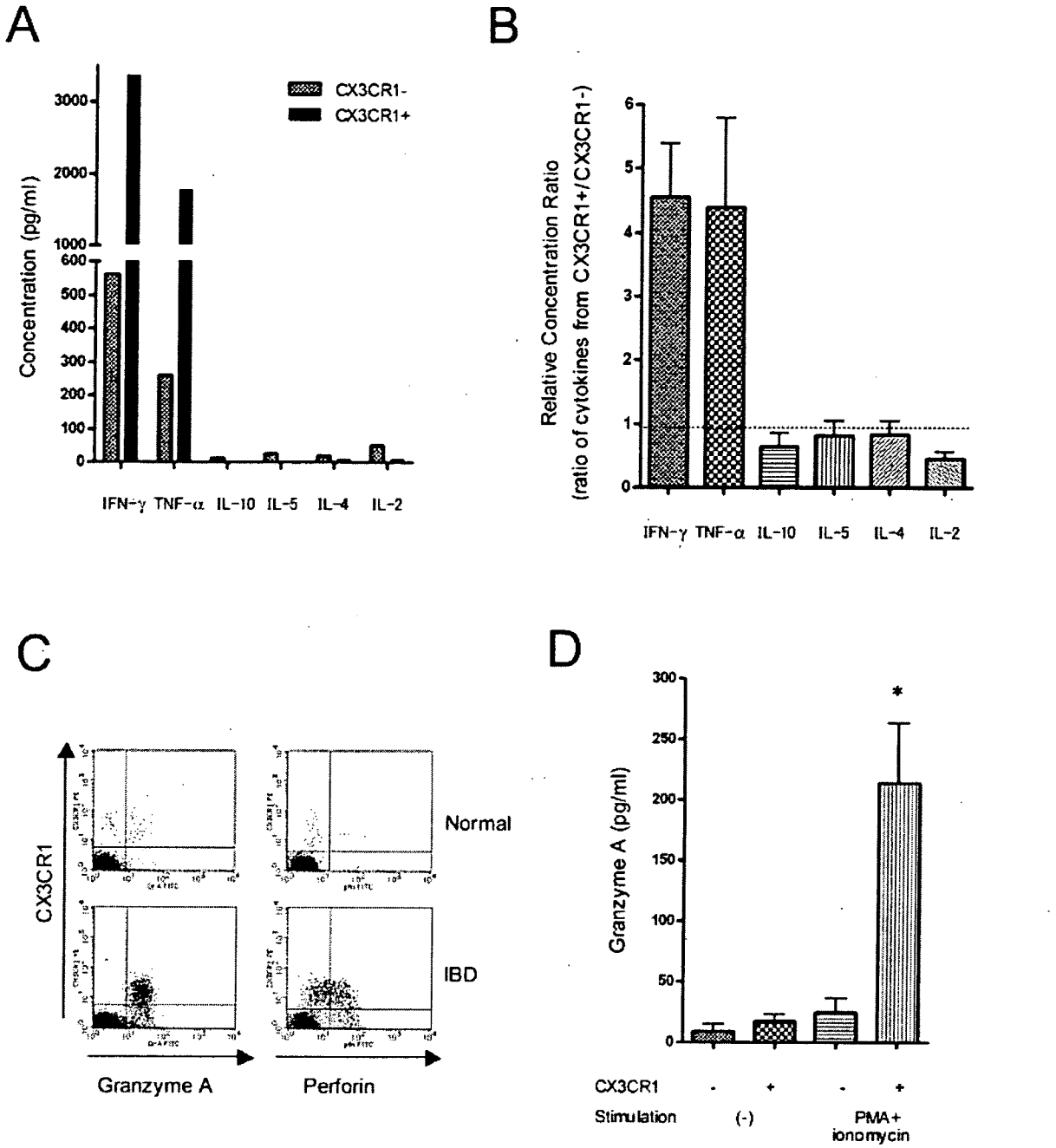

FIGURE 3. A: Cytokine production from CX3CR1$^+$CD4$^+$ cells. Sorted CX3CR1$^+$CD4$^+$ and CX3CR1$^-$CD4$^+$ cells were stimulated with immobilized anti-CD3 mAb and anti-CD28 mAb. After 48 hours supernatants were collected to measure concentration of IFN-γ, TNF-α, IL-10, IL-5, IL-4, IL-2 by Cytometric 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.

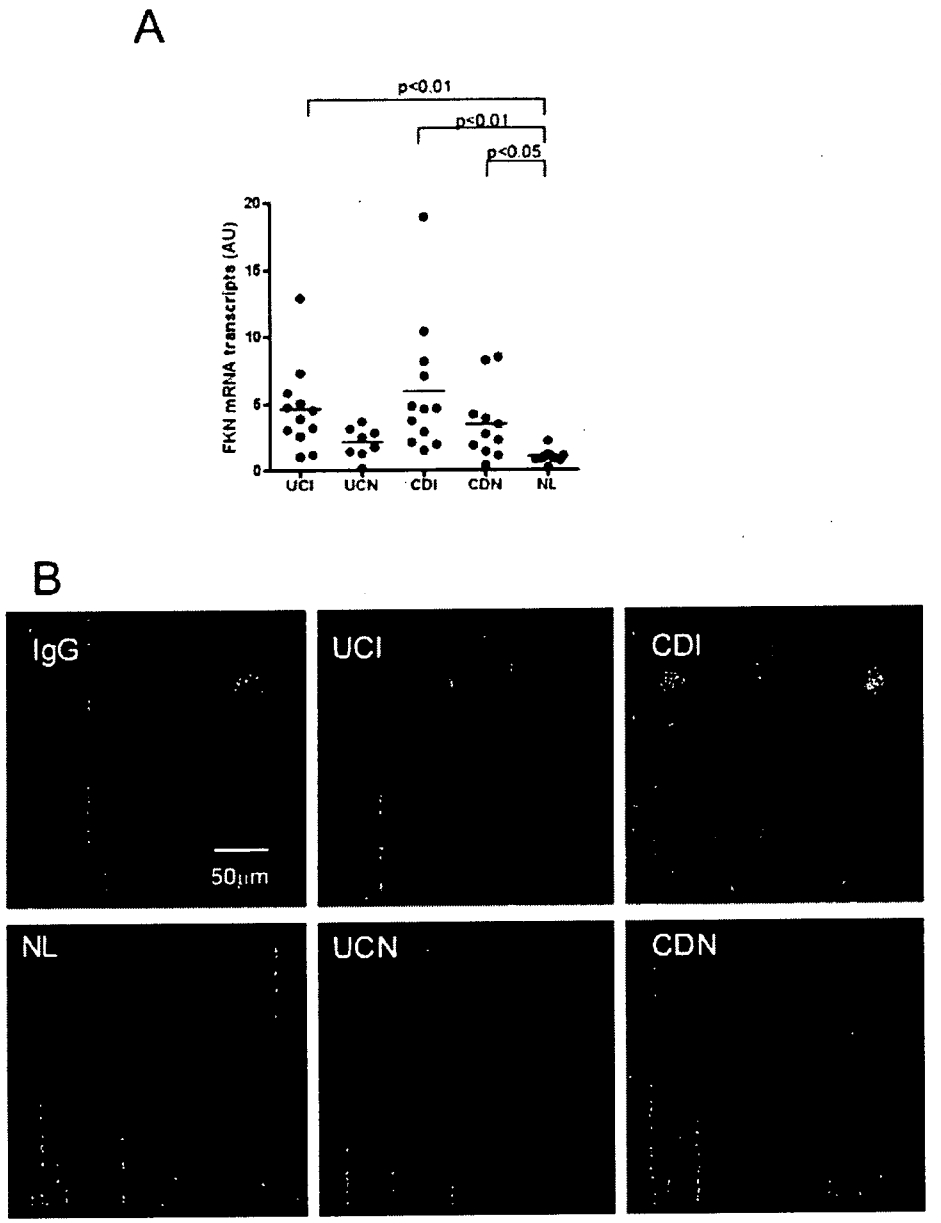

FIGURE 4. A: Relative fractalkine (FKN) mRNA expression in the colonic biopsy specimen from normal controls (NL, $n = 10$), patients with ulcerative colitis (UCI inflamed, $n = 12$; UCN noninflamed, $n = 8$), and patients with Crohn's disease (CDI inflamed, $n = 12$; CDN noninflamed, $n = 11$) were quantified 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 co-stimulatory 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 α4 and β1 but rarely expressed β7.

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 ± 0.92AU) and 6-fold in CD (5.90 ± 1.42AU) 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-1β. 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 reported[22] (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 ± 0.05%, IELs, 1.3 ± 0.5%, $n = 6$). However, CD28$^-$CD4$^+$ cells could be detected in both LPMCs (UC, 1.01 ± 0.33%, $n = 10$, CD, 1.91 ± 0.44%, $n = 10$) and IELs (UC, 7.9 ± 3.2%, CD, 6.1 ± 2.4%,) isolated from patients with IBD and existed significantly more in IELs (7.0 ± 2.0%) than LPMCs (1.5 ± 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-

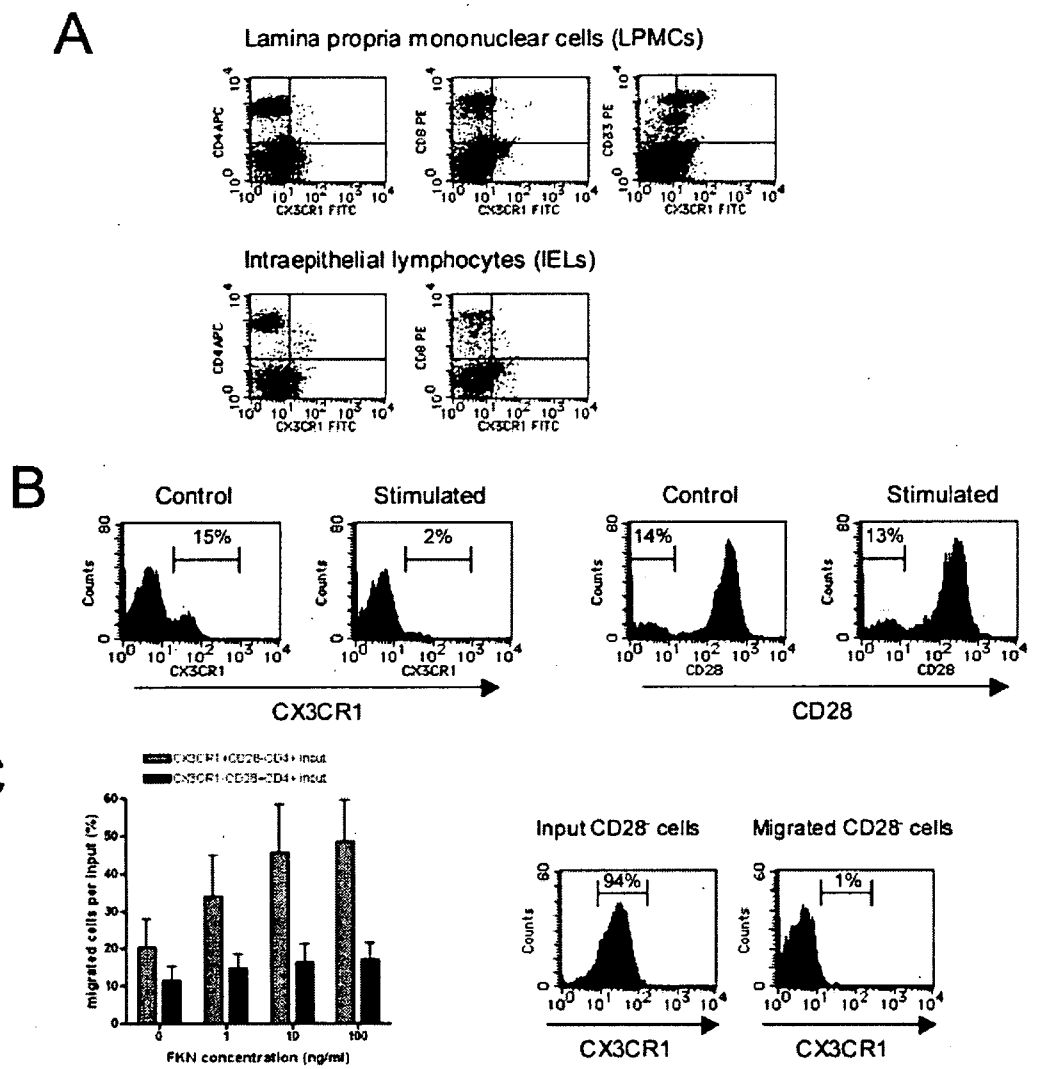

FIGURE 5. A: Flow cytometric analysis of the surface expression of CX3CR1 on lamina propria and intraepithelial lymphocytes by flow cytometry. Patterns are gated on lymphocytes for analysis of $CD4^+$ or $CD8^+$ cells using forward and side scatter. For CD33 staining, it is gated on macrophages. Representative 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.

tis[7–9] 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

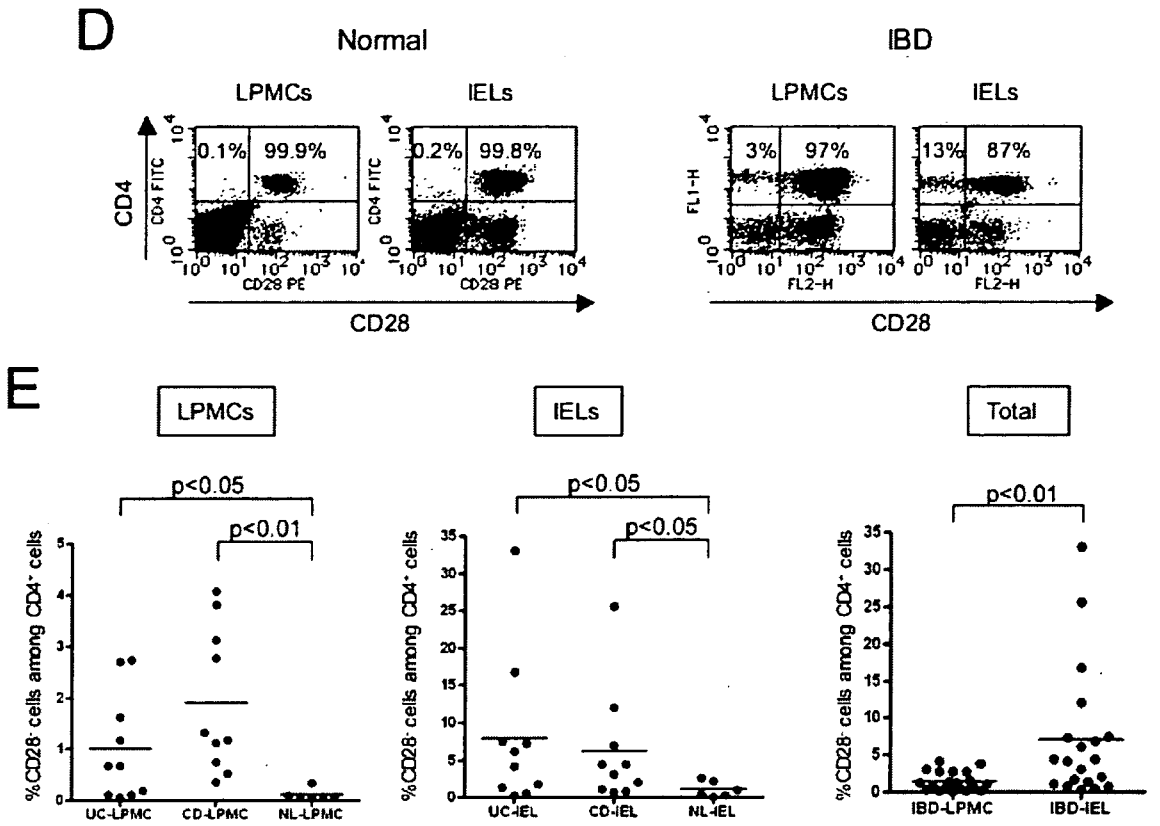

FIGURE 5. *(Continued)*

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 al[8] 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 al[13] 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

1. Podolsky DK. Inflammatory bowel disease. *N Engl J Med.* 2002;347: 417–429.
2. Bazan JF, Bacon KB, Hardiman G, et al. A new class of membrane-bound chemokine with a CX3C motif. *Nature.* 1997;385:640–644.
3. 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.
4. Ancuta P, Rao R, Moses A, et al. Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J Exp Med.* 2003;197:1701–1707.
5. Greaves DR, Channon KM. Inflammation and immune responses in atherosclerosis. *Trends Immunol.* 2002;23:535–541.
6. Blaschke S, Koziolek M, Schwarz A, et al. Proinflammatory role of fractalkine (CX3CL1) in rheumatoid arthritis. *J Rheumatol.* 2003;30: 1918–1927.
7. Ruth JH, Rottman JB, Katschke KJ Jr, et al. Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthritis Rheum.* 2001;44:2750–2760.
8. 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.
9. Sawai H, Park YW, Roberson J, et al. T cell costimulation by fractalkine-expressing synoviocytes in rheumatoid arthritis. *Arthritis Rheum.* 2005;52:1392–1401.
10. 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.
11. 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.
12. 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.
13. 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.
14. 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.
15. 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.
16. Connor SJ, Paraskevopoulos 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.
17. 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.
18. 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.
19. 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.
20. 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.
21. Damas JK, Boullier A, Waehre 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.
22. 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.
23. 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.
24. 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.
25. 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.
26. 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.
27. 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.
28. 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.
29. 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.
30. 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.
31. 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.
32. Nanki T, Urasaki Y, Imai T, et al. Inhibition of fractalkine ameliorates murine collagen-induced arthritis. *J Immunol.* 2004;173:7010–7016.
33. 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.
34. 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.

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Cytomegalovirus Is Frequently Reactivated and Disappears Without Antiviral Agents in Ulcerative Colitis Patients

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OBJECTIVE: The clinical significance of cytomegalovirus (CMV) reactivation complicating ulcerative colitis (UC) patients has been uncertain. It has therefore remained undetermined whether or not CMV reactivation should be treated in UC patients under immunosuppression. The aim of the study was to clarify the natural history of CMV reactivation in UC patients.

METHODS: Sixty-nine UC patients with moderate to severe activity were enrolled in the study. All of the patients were treated with prednisolone, and/or immunosuppressants such as cyclosporine A. We sequentially monitored CMV reactivation every 2 wk up until 8 wk using the CMV antigenemia (Ag) assay and plasma quantitative real-time polymerase chain reaction (PCR) assay for CMV.

RESULTS: Immunoglobulin (Ig) G for CMV was positive in 48 patients (69.6%) and negative in 21 patients (30.4%). CMV was reactivated in 25 patients out of the 48 seropositive patients (52.1%) during the study period. The CMV Ag and PCR values were low and none of the patients showed any evidence of CMV infection on biopsy specimens by hematoxylin and eosin staining. While gancylovir (GCV) was not used except in two patients, clinical outcomes including rates of remission and colectomy were not significantly different among the CMV reactivation-positive, -negative, and CMV IgG negative groups. Furthermore, CMV disappeared without GCV in most of the CMV reactivation-positive patients.

CONCLUSIONS: CMV is frequently reactivated in active UC patients; however, it disappears without antiviral agents. Therefore, antiviral therapies should not be necessary for most UC patients with only CMV reactivation as long as CMV Ag values are low.

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INTRODUCTION

Primary cytomegalovirus (CMV) infections are generally asymptomatic or minimally symptomatic in immunocompetent hosts and are followed by latent infection. Latent CMV infection could lead to active diseases via reactivation in immunosuppressive states, such as acquired immunodeficiency syndrome, organ transplant recipients, or patients treated with immunosuppressants. The main sites of CMV disease are the eyes, lungs, and intestines (1). A considerable number of reports have recently suggested that CMV infection could contribute to steroid resistance, megacolon, or exacerbation in ulcerative colitis (UC) patients (2–9). CMV infection was proven histologically in most of these studies, which suggests active CMV disease in the colon. Rapid, sensitive, and reliable methods, which can detect CMV reactivation before CMV disease becomes apparent, have been recently developed and applied to clinical use, especially in recipients of hematopoietic stem cell transplantation (HSCT) (10, 11). The CMV antigenemia (Ag) assay is one of the most widely used methods to detect CMV reactivation, and CMV Ag-based pre-emptive therapy has been shown to effectively prevent the occurrence of CMV diseases in HSCT recipients (12, 13). There have been only a few studies reporting that CMV reactivation has been assessed using CMV Ag in UC patients (14, 15). Wada *et al.* reported that the sensitivity of CMV Ag for CMV infection is higher than that of histological assessments in UC patients (15). Moreover, we have previously shown that the plasma quantitative real-time polymerase chain reaction (PCR) assay for CMV was more sensitive for detecting gastrointestinal CMV reactivation in HSCT recipients than CMV Ag (11). Thus, CMV Ag and the plasma real-time PCR assay for CMV could have a higher sensitivity for detecting CMV reactivation in UC patients compared with

histological detection methods. In addition to their higher sensitivity, they also have an advantage in that they can be repeated periodically, because both CMV Ag and the real-time PCR assay are blood examinations.

It is unknown whether CMV reactivation should be treated in UC patients during immunosuppressive therapy. It is therefore important to determine the natural history of CMV reactivation in UC patients. In this study, we aimed to determine (a) the prevalence and natural history of CMV reactivation in active UC patients, and (b) whether CMV reactivation would affect clinical outcomes. To achieve these goals, we sequentially monitored CMV reactivation using both CMV Ag and real-time PCR every 2 wk in active UC patients.

PATIENTS AND METHODS

Patient Selection

UC patients over the age of 15, who were admitted to our hospital, were eligible for this study. The diagnosis of UC was made by clinical, endoscopic, and histological findings according to standard criteria (16, 17). We selected patients with moderate to severe activity by the Truelove-Witts criteria (18). Patients with only proctitis were excluded. There were no inclusion/exclusion criteria for disease duration, medication, or complications. Colonoscopic examinations were performed for diagnosis and assessment of severity within 1 wk of admission. Biopsies were obtained for histological assessment. Fecal bacterial culture yielded no specific pathogens in all the patients. Disease activities were quantified and followed with the Lichtiger score (19) during the study period. The protocol was approved by the ethical committee of Keio University and informed consents were obtained.

Study Design

We first measured Immunoglobulin (Ig) M and IgG antibodies against CMV in all the patients. If both IgG and IgM antibodies against CMV were negative (CMV seronegative), we followed CMV IgG and IgM antibodies over 8 wk without further monitoring of CMV reactivation. If either IgG or IgM antibodies were positive, we monitored CMV reactivation every 2 wk until 8 wk with CMV Ag and the plasma CMV PCR assay. CMV reactivation was diagnosed when either CMV Ag or PCR was positive at least at one time point during the 8-wk study period. Based on each physician's decision, the patients were treated with conventional immunosuppressive therapies for UC, such as prednisolone (PSL), cyclosporine A (CsA), 6-mercaptopurine (6-MP), and apheresis (20, 21). In principle, CsA was administered to patients who did not respond to 1 wk of PSL therapy, and apheresis was indicated to patients who showed some improvement by PSL, but did not enter into remission.

CMV Ag Assay

The CMV Ag assay was performed according to previously reported methods (12). Briefly, EDTA-treated whole blood samples were fractionated by dextran sedimentation and lysis of erythrocytes. The granulocytes were then centrifuged to prepare a cytospin slide. The cells were then fixed with formaldehyde, sequentially immunostained with the monoclonal antibodies C10/C11 (Clonab CMV; Biotest, Dreieich, Germany), and reacted with goat alkaline phosphatase-labeled antimouse immunoglobulin (Yuka Medias Co, Ibaraki, Japan). One or more stained cells were considered to be CMV reactivation positive. The number of positive cells on a slide was counted. The examinations were performed in duplicate.

Plasma Real-Time PCR Assay for CMV

The real-time PCR assay for CMV was performed as previously reported (22). In brief, DNA extracted from 100 μL of plasma with a QIAmp Blood Mini Kit (Qiagen, Valencia, CA) was subjected to PCR. The sequences of PCR primers and the probe were selected from the US17 region of CMV AD169. The TaqMan probe selected between the primers was dual-labeled with 6-carboxyfluorescein and 6-carboxyteremethyl-rhodamine. The PCR reaction was performed using TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA). Quantification was carried out with a serially diluted standard ranging from 10 to 1×10^7 copies/well, and the gene copy numbers were then calculated with Sequence Detection System version 1.6.3 software (PE Biosystems). The minimum detection level was 20 copies/100 μL of plasma and values over this lower detection limit were considered to be CMV reactivation positive.

Statistical Analysis

The Kruskal-Wallis test was used to analyze the variance on each factor among CMV reactivation-positive and negative groups, and CMV seronegative groups. If the difference was considered significant by the Kruskal-Wallis test, the Mann-Whitney U test with Bonferroni correction was applied for multiple comparisons. A P value <0.05 was considered significant.

RESULTS

Baseline Clinical Features of the Patients

A total of 69 patients were enrolled in this study. Based on the status of the CMV-IgG antibody and CMV reactivation, we classified the patients into the three following groups: (a) CMV IgG positive and CMV reactivation positive patients were in the CMV reactivation positive group, (b) CMV IgG positive and CMV reactivation negative patients were in the CMV reactivation negative group, and (c) CMV IgG negative patients were in the CMV IgG negative group. The CMV IgG negative group was regarded as a control in which CMV could never contribute to the exacerbation of UC. The clinical characteristics at 0 wk in the three groups are shown in Table 1. The CMV IgG negative group was significantly younger than the other groups and the disease duration of the CMV reactivation positive group was significantly shorter compared with that of the CMV reactivation negative group.

Table 1. Baseline Clinical Characteristics of the Patients

	[a]CMV Reactivation (+) (N = 25)	[b]CMV Reactivation (−) (N = 23)	[c]CMV IgG (−) (N = 21)	a *vs* b	a *vs* c
Age (yr)*	40.0 (15–73)	33.0 (19–64)	28.0 (15–45)	n.s.	$P < 0.05$
Sex (M/F)	12/13	17/6	11/10	n.s.	n.s.
Disease duration (yr)*	1.9 (0.1–15.5)	7.4 (0.1–21.1)	2.4 (0.1–22.7)	$P < 0.05$	n.s.
Extent of the disease					
Pancolitis	19 (76.0%)	18 (78.3%)	15 (71.4%)	n.s.	n.s.
Left	6 (24.0%)	5 (21.7%)	6 (28.6%)		
Lichtiger score*	13.0 (7.0–19.0)	12.0 (5.0–20.0)	13.0 (5.0–20.0)	n.s.	n.s.
Medication					
Amount of PSL within previous 4 wk* (mg)	610 (0–1,740)	475 (0–1,180)	670 (0–1,640)	n.s.	n.s.
Mesalamine/Sulfasalazine	23 (92.0%)	22 (95.7%)	22 (100.0%)	n.s.	n.s.
6-MP	2 (8.0%)	0 (0.0%)	2 (9.5%)	n.s.	n.s.
Apheresis†	6 (24.0%)	5 (21.7%)	7 (33.3%)	n.s.	n.s.

*Values are median (range).
†Apheresis includes granulocytapheresis and leuckocytapheresis. CMV = cytomegalovirus; n.s. = not significant; PSL = prednisolone; 6-MP = 6-mercaptopurine.

Besides these differences, the three groups were similar in baseline clinical features, including sex, disease activity, extent, and medications.

CMV Seropositivity and Reactivation

We first measured IgG and IgM antibodies against CMV in all the patients. None of the patients were positive for the CMV-IgM antibody. The CMV-IgG antibody was negative in 21 patients (30.4%) and positive in 48 patients (69.6%). The CMV-IgG and IgM antibodies remained negative over 8 wk in all 21 seronegative patients. In 48 seropositive patients, CMV reactivation was diagnosed when either CMV Ag (one or more positive cells) or CMV PCR (≥20 copies/100 μL) was positive at least at one time point during the 8-wk study period. In total, 25 patients had CMV reactivation (52.1% in the seropositive patients) (Table 2). CMV reactivation was diagnosed by Ag (+)/PCR (+) in 15 patients, Ag (−)/PCR (+) in 8 patients, and Ag (+)/PCR (−) in 2 patients (Table 2). None of the patients showed any evidence of CMV infection on biopsy specimens by H&E staining. These results showed that active UC patients frequently experienced CMV reactivation.

The Level of CMV Reactivation

Because it has been reported that the positive cell number of CMV Ag parallels the risk of developing CMV disease (10, 12, 13), we showed the maximum values of CMV Ag during the study period (Table 3). In 17 patients positive for CMV Ag, the maximum value was 1 in approximately half of the patients (8/17, 47.1%). While 10 or more positive cells indicates a higher risk for developing CMV disease in HSCT and organ transplantation recipients (10, 23), only one patient showed over 10 positive cells. However, the patient's symptoms improved with PSL, and CMV reactivation turned out to be negative without antiviral agents.

We have previously reported that the real-time PCR assay is useful for diagnosis of gastrointestinal CMV disease in HSCT recipients (11). The previous study demonstrated that the maximum values of CMV PCR ranged from 3.0×10^3 to 7.0×10^5 copies/100 μL (median 2.4×10^4 copies/100 μL) in HSCT recipients with gastrointestinal CMV disease. In contrast, the maximum values of CMV PCR ranged from 1.0×10^2 to 4.6×10^3 copies/100 μL (median 3.8×10^2 copies/100 μL) in UC patients in this study. Thus, the values of CMV PCR in our study were much lower than those of patients with gastrointestinal CMV disease.

Treatment and Outcome

Treatment and outcome at 8 wk are summarized in Table 4. While the amount of PSL during the study period was not different among the three groups, CsA was administered more frequently in the CMV reactivation positive group compared with the CMV reactivation negative group (13/25 [52.0%] *vs* 3/23 [13.0%], $P < 0.05$). However, the rate of CsA therapy was not significantly different between the CMV reactivation positive and the CMV IgG negative groups. Of 13 CMV reactivation positive patients administered CsA, CMV reactivation was first detected after CsA administration in five

Table 2. Prevelance of CMV Reactivation Among CMV IgG Positive Patients (N = 48)

CMV Ag	CMV PCR	N	%
−	−	23	47.9
+	+	15	31.3
−	+	8	16.7
+	−	2	4.2

CMV = cytomegalovirus; UC = ulcerative colitis; Ag = antigenemia; PCR = polymerase chain reaction.

Table 3. Maximum Value of CMV Ag

No. of Positive Cells (/slide)	N	%
1	8	47.1
2	1	5.9
3	1	5.9
4	3	17.6
5	2	11.8
9	1	5.9
14	1	5.9

Table 4. Treatment and Outcome

	[a]CMV Reactivation (+)* (N = 25)	[b]CMV Reactivation (−) (N = 23)	[c]CMV IgG (−) (N = 21)	a *vs* b	a *vs* c
Therapies					
CsA	13 (52.0%)	3 (13.0%)	7 (33.3%)	$P < 0.05$	n.s.
FK 506	1 (4.0%)	5 (21.7%)	0 (0.0%)	n.s.	n.s.
Amount of PSL during the study† (mg)	1,289 (489–2,700)	1,265 (45–2,470)	1,594 (43–3,680)	n.s.	n.s.
Apheresis‡	10 (40.0%)	9 (39.1%)	10 (47.6%)	n.s.	n.s.
6-MP	11 (44.0%)	10 (43.5%)	9 (42.9%)	n.s.	n.s.
Outcome					
Remission at 8 wk	18 (72.0%)	15 (65.2%)	13 (61.2%)	n.s.	n.s.
Colectomy	4 (16.0%)	1 (4.3%)	4 (19.0%)	n.s.	n.s.

*Two patients were treated with ganclylovir.
†Values are median (range).
‡Apheresis includes granulocytapheresis and leuckocytapheresis.
Remission is defined as a Lichtiger score below 4.
CMV = cytomegalovirus; CsA = cyclosporine A; n.s. = not significant; PSL = prednisolone; 6-MP = 6-mercaptopurine.

patients (38.4%). The remaining eight patients (61.5%) were already positive for CMV reactivation before CsA administration. While antiviral agents were not administered except for two patients described below, the rates of remission (as defined by a Lichtiger score of 4 or less) and colectomy were not significantly different among the three groups. Importantly, even in the CMV reactivation positive group, most of the patients (18/25, 72%) entered remission with conventional immunosuppressive therapies without administration of antiviral agents. In four patients requiring an operation in the CMV reactivation positive group, two patients showed positive only for CMV-PCR at low levels and it turned out to be negative without antiviral agents before the operation. The remaining two patients were treated with gancylovir (GCV) at 5 mg/kg twice a day for 2 wk, because they showed a progressive clinical course and their CMV Ag values increased: one patient's values increased from 0 to 3, and the other increased from 1 to 4. Immunohistochemical staining for CMV was negative in both of the patients before GCV administration. Although CMV disappeared with the agent, the disease activity remained high and an operation could not be avoided. We were not able to find histological evidence of CMV infection on the patients' surgical specimens. These results suggest that CMV reactivation has little effect on the clinical course of UC patients.

Time-Course Change of CMV Reactivation

We analyzed the time-course change of CMV reactivation in the CMV reactivation positive patients (Table 5). As shown in Table 2, CMV reactivation was observed in a total of 25 patients during the study period, it was positive in 16 patients at 0 wk. It was notable that PSL administration had already been begun in all of these 16 patients before the study, and all of the eight patients who had not been administered PSL at 0 wk were negative for CMV reactivation (data not shown). The number of CMV reactivation positive patients was highest at 2 wk, which may reflect the highest rate of CsA therapy. Although we treated the patients with immunosuppressive therapies for UC regardless of the results of CMV reactivation, CMV reactivation was gradually resolved without antiviral agents. The resolution of CMV reactivation paralleled the decline of PSL. While two patients remained positive for CMV reactivation at 8 wk, they were negative at 10 wk (data not shown). We started 6-MP as a maintenance therapy for some patients; however, it did not appear to affect CMV reactivation during the study period. Thus, CMV reactivation could be resolved without antiviral agents in most of the UC patients treated conventionally with PSL and other immunosuppressants.

DISCUSSION

CMV Ag and PCR are widely used to monitor CMV reactivation in HSCT patients. CMV Ag-guided pre-emptive therapy has been shown to be effective in preventing CMV disease in HSCT recipients (12, 13). In HSCT and organ transplantation recipients, 10 or more positive cells indicate a higher risk of developing CMV disease (10, 23). While the clinical significance of CMV reactivation in HSCT recipients has been established, it is unknown whether CMV reactivation

Table 5. Time-Course Change of CMV Reactivation Among CMV Reactivation Positive Patients

Wk (N)*	0 (25)	2 (24)	4 (23)	6 (21)	8 (21)
Positive for CMV reactivation (N)	16 (64.0%)	19 (79.2%)	11 (47.8%)	4 (19.1%)	2 (9.5%)
Dose of PSL (mg)†	30 (0–80)	20 (0–80)	20 (0–60)	15 (7–40)	10 (6–30)
CsA (N)	0	12	4	1	1
6-MP (N)	2	3	10	10	10

*Numbers are decreased because the patients subject to an operation are eliminated.
†Values are median (range).
CMV = cytomegalovirus; PSL = prednisolone; CsA = cyclosporine A; 6-MP = 6-mercaptopurine.

could affect clinical course of UC patients. We conducted the present study to clarify the natural history of CMV reactivation in active UC patients. Distinguishing characteristics of this study are described as follows: (a) we monitored CMV reactivation sequentially and prospectively, (b) we used both CMV Ag and the real-time PCR assay, and (c) we evaluated a large number of UC patients. There are only a few reports that have evaluated CMV reactivation with CMV Ag in UC patients (14, 15). Furthermore, to our knowledge, this is the first prospective study to monitor CMV reactivation with plasma CMV PCR in a large number of UC patients. By monitoring CMV reactivation sequentially and prospectively, we were able to reveal the time-course kinetics of CMV reactivation in UC patients for the first time. The most important finding of this study is that CMV is frequently reactivated in active UC patients; however, it disappears without antiviral agents.

Some reports have suggested the clinical significance of CMV infection in UC patients. Berk *et al.* reviewed 16 reported cases of inflammatory bowel disease with histologically proven CMV infection of the colon and showed that CMV infection was associated with a poor prognosis such as a high mortality rate (seven of 16), toxic megacolon (five of 16), and requirement of colectomy (10 of 16) (2). Cottone *et al.* have reported that histological analysis by H&E and immunohistochemical staining of rectal biopsies revealed CMV positive cells in seven (36%) of 19 patients who did not respond to IV steroids among 62 patients with severe colitis, including 55 with UC and seven with Crohn's disease (6). Kambham *et al.* have demonstrated similar data in that CMV infection was histologically detected in 10 of 40 (25%) patients with steroid refractory UC, but not in patients with nonrefractory UC (9). In addition to these studies, some other studies have raised the possibility that CMV infection could play a role in steroid resistance (5, 24). However, it has not been determined whether CMV reactivation could affect clinical course of UC. We showed that clinical outcomes including colectomy and remission did not differ regardless of CMV reactivation. Furthermore, these outcomes of CMV reactivation positive patients were not different from those of the CMV seronegative patients. These results suggest that the clinical significance of CMV reactivation may be limited in UC patients.

Only few data are available concerning the necessity of antiviral agents for CMV infection complicated with UC. Vega *et al.* demonstrated that five of six UC cases with histologically proven CMV infection responded to GCV (5). Cottone *et al.* reported similar data in UC patients with histologically proven CMV infection. de Saussure *et al.* reported that one out of three UC cases with CMV infection, who was positive for CMV on biopsy specimens, benefited from antiviral therapy (14). Wada *et al.* reported that clinical course was distinctly improved by the administration of GCV in eight of 12 UC patients (66.7%) positive for CMV Ag (15). They used CMV Ag to assess CMV infection, which is similar to our study, but CMV infection was also detected histologically in some of their cases. These data suggest that antiviral therapy may be beneficial for UC patients with histologically proven CMV infection. On the other hand, it has not been reported whether early intervention at the reactivation stage is necessary to prevent the progression to active CMV disease in UC patients under immunosuppression. We demonstrated that CMV reactivation could be resolved without antiviral agents and that most of the CMV reactivation positive patients responded to conventional immunosuppressive therapies. Given that GCV has many serious adverse effects, such as bone marrow suppression, renal toxicity, or irreversible damage of spermatogenesis, our data suggest that it would not be necessary to treat all of the UC patients who become positive for CMV Ag or PCR. Before starting this study, we experienced two steroid-refractory UC patients treated with GCV. Their symptoms unquestionably improved with GCV, even though they did not enter remission. Immunohistochemical staining for CMV was positive in one patient and negative in the other patient. The two patients showed high values of CMV Ag (34 and 13). It should be noted that our patients in the present study had low CMV Ag and PCR values. High values of CMV Ag may be an indicator for antiviral treatment regardless of histology. This is consistent with a report that patients with symptomatic CMV diseases after liver transplantation show higher CMV Ag values compared to asymptomatic patients (25). Further studies are needed to answer this issue.

We speculate that immunosuppression by PSL or CsA affected CMV reactivation. As our hospital is a referral center, we were not able to start following CMV reactivation before PSL administration in all of the patients. However, all of the eight patients who had not been administered PSL at 0 wk were negative for CMV reactivation at that time. In contrast, PSL administration had already been started in all of the 16 patients who were positive for CMV reactivation at 0 wk. In addition, CMV reactivation decreased as the dose of PSL was decreased. CMV was also reactivated after CsA administration in five patients. On the other hand, as approximately half of the patients receiving PSL did not experience CMV reactivation, it is possible that other factors also contributed to CMV reactivation. It is reported that proinflammatory cytokines such as tumor necrosis factor α can reactivate CMV (26). Proinflammatory cytokine profiling might affect CMV reactivation in addition to PSL. In contrast to PSL, it appeared that 6-MP did not affect CMV reactivation during the study period. However, it remains to be determined whether long-term use of 6-MP could affect CMV reactivation.

CsA was administered in the CMV reactivation positive patients more frequently compared with the CMV reactivation negative group. Because CsA therapy is applied to steroid-refractory patients (19), this result could indicate that CMV might contribute to steroid resistance. However, this would be unlikely, because (a) in the 13 patients treated with CsA in the CMV reactivation positive group, five patients (38.4%) turned out to be CMV reactivation positive after CsA administration, and (b) the rate of CsA therapy was not statistically different between the CMV reactivation positive and the CMV-IgG negative groups. Steroid refractory patients

tend to be administered more steroids (9), and indeed, the 13 patients requiring CsA therapy in the CMV reactivation positive group were administered more PSL (median 800 mg [range 196–1,710 mg]) within the previous 4 wk before the study than those in the CMV reactivation negative group (Table 1; median 475 mg [range 0–1,180 mg], $P < 0.05$). Thus, the patients requiring CsA might be susceptible to CMV reactivation, which biased them toward the CMV reactivation positive group in the CMV IgG positive patients.

In conclusion, we have shown that while CMV is frequently reactivated in active UC patients, it disappears without antiviral agents. Furthermore, most of the CMV reactivation positive patients entered remission with conventional immunosuppressive therapy, such as PSL and CsA. These results suggest that clinical significance of CMV reactivation is limited in UC patients, and antiviral therapy should not be necessary for most CMV reactivation positive UC patients even during immunosuppression, as long as CMV Ag values are low. However, further studies are needed to clarify the necessity of antiviral therapy for UC patients with high values of CMV Ag or histologically proven CMV infection.

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STUDY HIGHLIGHTS

What Is Current Knowledge

- Cytomegalovirus (CMV) infection may exacerbate ulcerative colitis (UC).
- However, it is unknown whether CMV reactivation should be treated in UC patients under immunosuppression.

What Is New Here

- Approximately half of active UC patients experienced CMV reactivation during immunosuppressive therapy.
- Despite treating with prednisolone and other immunosuppressants such as cyclosporine A, most CMV reactivation was resolved without antiviral agents in UC patients. Antiviral therapies should not be necessary for most UC patients with only CMV reactivation.

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REFERENCES

1. Goodgame RW. Gastrointestinal cytomegalovirus disease. Ann Intern Med 1993;119:924–35.
2. Berk T, Gordon SJ, Choi HY, et al. Cytomegalovirus infection of the colon: A possible role in exacerbations of inflammatory bowel disease. Am J Gastroenterol 1985;80:355–60.
3. Orloff JJ, Saito R, Lasky S, et al. Toxic megacolon in cytomegalovirus colitis. Am J Gastroenterol 1989;84:794–7.
4. Loftus EV Jr, Alexander GL, Carpenter HA. Cytomegalovirus as an exacerbating factor in ulcerative colitis. J Clin Gastroenterol 1994;19:306–9.
5. Vega R, Bertran X, Menacho M, et al. Cytomegalovirus infection in patients with inflammatory bowel disease. Am J Gastroenterol 1999;94:1053–6.
6. Cottone M, Pietrosi G, Martorana G, et al. Prevalence of cytomegalovirus infection in severe refractory ulcerative and Crohn's colitis. Am J Gastroenterol 2001;96:773–5.
7. Papadakis KA, Tung JK, Binder SW, et al. Outcome of cytomegalovirus infections in patients with inflammatory bowel disease. Am J Gastroenterol 2001;96:2137–42.
8. Streetz KL, Buhr T, Wedemeyer H, et al. Acute CMV-colitis in a patient with a history of ulcerative colitis. Scand J Gastroenterol 2003;38:119–22.
9. Kambham N, Vij R, Cartwright CA, et al. Cytomegalovirus infection in steroid-refractory ulcerative colitis: A case-control study. Am J Surg Pathol 2004;28:365–73.
10. Gondo H, Minematsu T, Harada M, et al. Cytomegalovirus (CMV) antigenaemia for rapid diagnosis and monitoring of CMV-associated disease after bone marrow transplantation. Br J Haematol 1994;86:130–7.
11. Mori T, Mori S, Kanda Y, et al. Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 2004;33:431–4.
12. Mori T, Okamoto S, Matsuoka S, et al. Risk-adapted preemptive therapy for cytomegalovirus disease in patients undergoing allogeneic bone marrow transplantation. Bone Marrow Transplant 2000;25:765–9.
13. Kanda Y, Mineishi S, Saito T, et al. Pre-emptive therapy against cytomegalovirus (CMV) disease guided by CMV antigenemia assay after allogeneic hematopoietic stem cell transplantation: a single-center experience in Japan. Bone Marrow Transplant 2001;27:437–44.
14. de Saussure P, Lavergne-Slove A, Mazeron MC, et al. A prospective assessment of cytomegalovirus infection in active inflammatory bowel disease. Aliment Pharmacol Ther 2004;20:1323–7.
15. Wada Y, Matsui T, Matake H, et al. Intractable ulcerative colitis caused by cytomegalovirus infection: A prospective study on prevalence, diagnosis, and treatment. Dis Colon Rectum 2003;46:S59–65.
16. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl 1989;170:2–6; discussion 16–9.
17. Gower-Rousseau C, Salomez JL, Dupas JL, et al. Incidence of inflammatory bowel disease in northern France (1988–1990). Gut 1994;35:1433–8.
18. Truelove SC, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. BMJ 1955;2:1041–8.
19. Lichtiger S, Present DH, Kornbluth A, et al. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. N Engl J Med 1994;330:1841–5.
20. Naganuma M, Funakoshi S, Sakuraba A, et al. Granulocytapheresis is useful as an alternative therapy in patients with

steroid-refractory or -dependent ulcerative colitis. Inflamm Bowel Dis 2004;10:251–7.
21. Sawada K, Kusugami K, Suzuki Y, et al. Leukocytapheresis in ulcerative colitis: Results of a multicenter double-blind prospective case-control study with sham apheresis as placebo treatment. Am J Gastroenterol 2005;100:1362–9.
22. Mori T, Okamoto S, Watanabe R, et al. Dose-adjusted preemptive therapy for cytomegalovirus disease based on real-time polymerase chain reaction after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 2002;29:777–82.
23. Bernabeu-Wittel M, Pachon-Ibanez J, Cisneros JM, et al. Quantitative pp65 antigenemia in the diagnosis of cytomegalovirus disease: Prospective assessment in a cohort of solid organ transplant recipients. J Infect 2005;51:188–94.
24. Takahashi Y, Tange T. Prevalence of cytomegalovirus infection in inflammatory bowel disease patients. Dis Colon Rectum 2004;47:722–6.
25. Van Den Berg AP, Klompmaker IJ, Haagsma EB, et al. Antigenemia in the diagnosis and monitoring of active cytomegalovirus infection after liver transplantation. J Infect Dis 1991;164:265–70.
26. Simon CO, Seckert CK, Dreis D, et al. Role for tumor necrosis factor alpha in murine cytomegalovirus transcriptional reactivation in latently infected lungs. J Virol 2005;79:326–40.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ORIGINAL PAPER

Acid-suppression Therapy Offers Varied Laryngopharyngeal and Esophageal Symptom Relief in Laryngopharyngeal Reflux Patients

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Abstract It is widely accepted that laryngopharyngeal reflux requires more aggressive and prolonged therapy than gastro-esophageal reflux disease. Otolaryngologists often observe that laryngopharyngeal symptoms, such as throat clearing, hoarseness, cough, and globus pharyngeus, are slower to resolve than esophageal symptoms, such as heartburn and regurgitation. The aim of this was to provide empirical evidence to support this observation and to carry out a detailed investigation of the differences between these symptoms. Forty-five patients with laryngopharyngeal and esophageal symptoms received acid-suppression therapy that involved the continuous administration of a proton-pump inhibitor for up to 6 months. We investigated the differences in response to acid-suppression therapy between patients suffering from laryngopharyngeal and esophageal symptoms, respectively, who received upper gastrointestinal endoscopy and were assayed for serum *Helicobacter pylori* antibodies. The significance of the rate of symptom improvement was estimated by Kaplan–Meier analysis and the logrank test. Laryngopharyngeal symptoms improved significantly more slowly than esophageal symptoms following acid-suppression therapy (49.8 vs. 78.3%, 60 days after the start of acid suppression; $P = 0.003$). These differences were observed both in patients with erosive esophagitis ($P = 0.008$) and in *H. pylori*-seronegative patients ($P = 0.001$).

Keywords Acid-suppression therapy · Gastresophageal reflux disease · Laryngopharyngeal reflux · Symptom relief

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Introduction

Laryngopharyngeal reflux (LPR) is caused by the retrograde flow of gastric contents to the larynx and hypopharynx, where they come into contact with the tissues of the upper aerodigestive tract. Up to 10% of patients under the care of an otolaryngologist [1] and more than 50% of patients suffering from hoarseness have been diagnosed with reflux-related disease [2]. Laryngopharyngeal reflux has also been suggested as a major cause of laryngeal inflammation and presents with a variety of symptoms that differ from classic gastro-esophageal reflux disease (GERD) [3]. An international survey of American Bronchoesophagological Association members revealed that the most common LPR symptoms were throat clearing (98%), persistent cough (97%), globus pharyngeus (95%) and hoarseness (95%) [4]. The failure to appreciate LPR as a separate disorder from GERD has caused major diagnostic uncertainty in the past. Koufman was the first to clearly distinguish LPR from GERD, noting that among a combined series of 899 patients, only 20% of LPR patients complained of heartburn compared with 83% of GERD patients [1].

Mainstream LPR management is based on acid-suppression therapy using proton-pump inhibitors (PPIs) [5]. Unlike GERD patients, the response of LPR patients to PPI therapy has been described as unpredictable [6], and it is sometimes necessary for treatment to be more aggressive