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Accumulation of CCR5 + T cells around RANTES + granulomas in Crohn's disease: a pivotal site of Th1-shifted immune response?

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Immunological abnormalities are implicated in the pathogenesis of inflammatory bowel disease (IBD), that is, Crohn's disease and ulcerative colitis. In particular, Crohn's disease is considered to be a T helper type 1 (Th1)-shifted disease. Chemokines and their receptors are involved in various immune responses including Th1- and Th2 responses. In this study, we analyzed chemokines and their receptors by immunohistochemistry, using frozen sections derived from 33 patients with Crohn's disease and 24 with ulcerative colitis. In inflamed mucosa, small mononuclear cells predominantly expressed CCR5 and CXCR3, the receptors selectively expressed on Th1 cells, without significant differences between Crohn's disease and ulcerative colitis. We then focused on the noncaseating granulomas that are characteristic of Crohn's disease. Granuloma cells, observed in all the layers of intestinal tissues, were positive for RANTES/CCL5 protein along their cell membranes. Lymphocytes surrounding granulomas were mostly CCR5$^+$ and CXCR3$^+$ T cells with CD4$^+$ and CD8$^+$ cells at similar frequencies. Granuloma cells were positive for RANTES mRNA by *in situ* hybridization. By contrast, lymphoid aggregates in Crohn's disease and lymphoid follicles in the normal intestinal mucosa were characterized by abundant B cells, a predominance of CD4$^+$ T cells over CD8$^+$ T cells, and low frequencies of cells expressing CCR5 or CXCR3. Together with the notion that granuloma cells are possible antigen-presenting cells, our results suggest that the noncaseating granulomas could be one of the crucial sites of Th1-shifted immune responses in Crohn's disease.
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Crohn's disease and ulcerative colitis comprise inflammatory bowel disease (IBD), in which immunological abnormalities have been implicated in their pathogenesis.[1,2] Crohn's disease is characterized by a discontinuous spread of transmural inflammation, and formation of noncaseating granulomas and lymphoid aggregates. Ulcerative colitis shows continuous mucosal inflammation with ulcers, which are confined to the mucosa and submucosa, with abundant plasma cell responses. Importantly, Crohn's disease is regarded as T helper type 1 (Th1)-shifted disease.[1]

Chemokines are a group of small cytokines that play important roles in immune and inflammatory responses by recruiting various leukocytes into tissues.[3–5] The chemokine receptors are all seven transmembrane G protein-coupled receptors, which are also systematically named based on the class of chemokines that they interact with.[4] Recent studies have disclosed that CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 3 (CXCR3) are selectively expressed by Th1 cells that mediate cellular immunity and tissue-specific autoimmune disorders, while CCR4 and possibly CCR3 and CCR8 are positive in Th2 cells that promote humoral

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immunity and allergic responses.[6–8] CCR5 is the shared receptor for macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP-1β/CCL4, and regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5), while CXCR3 is the shared receptor of interferon-inducible protein-10 (IP10/CXCL10), monokine induced by IFN-γ (Mig/CXCL9), and interferon-inducible T-cell alpha chemoattractant (I-TAC/CXCL11), which are all commonly inducible by IFN-γ.[4,9]

In IBD, upregulation of chemokines such as interleukin-8 (IL-8/CXCL8),[10,11] monocyte chemoattractant protein-1 (MCP-1/CCL2), and RANTES has been reported.[12] However, no significant differences were noted between ulcerative colitis and Crohn's disease in terms of expression of chemokines[13] or CXCR3[14] as long as the mucosal inflammation is concerned. The present study was set up to examine the involvement of chemokines and their receptors in IBD from the standpoint of Th1 and Th2-immune responses. Here, we show that noncaseating granulomas in Crohn's disease, which are formed in all the layers of intestinal tissues, could be one of the potential sites of Th1-shifted immune responses and highlight their pathophysiologic significance.

Materials and methods

Immunohistochemistry

Patients' profiles are listed in Table 1. All the samples were obtained at surgical resection in Tohoku University Hospital (54 patients of IBD) and Osaka City University Hospital (three patients of IBD). As preoperative treatment, all 33 patients with Crohn's disease were treated with total parenteral nutrition. All 24 patients with ulcerative colitis were treated with corticosteroids. Fresh samples including all the layers of intestines were immediately fixed in 4% periodate-lysine paraformaldehyde (4% PLP) overnight at 4°C.[15,16] All the immunohistochemical procedures were performed using frozen sections prefixed in PLP.[15–17] The primary antibodies used are listed in Table 2. For the secondary antibody, Envision plus kit (DakoCytomation) was used with 3–3′ diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto, Japan) as a chromogen. For negative controls, the primary antibodies were replaced with negative control antibodies of the same isotype (DakoCytomation).

Enzyme-Linked Double Immunohistochemistry (Performed in Representative Two Cases)

We performed enzyme-linked double immunohistochemistry for (a) CCR5 and CD4, and (b) CCR5 and CD8.[15–17] After the first step-immunohistochemistry with Vector blue (Vector Laboratories, Burlingame, CA, USA) as a chromogen

Table 1 Patients' profile

		Crohn's disease	*Ulcerative colitis*	*Control*
Number of patients		33	24	9
Age (years)	Mean (range)	31 (19–66)	36 (10–66)	54
Gender	M:F	21:12	11:13	5:4
Duration of disease (years)	Mean	9	9.6	—
Sampling sites	Ileum	15	—	4
	Colon and rectum	18	24	5

Types of Crohn's disease: ileitis, eight cases; ileitis+colitis, 17 cases; colitis, eight cases.

Table 2 List of antibodies used

Mouse monoclonal antibodies to:	*Clones*	*Isotypes*	*Sources*	*Working dilution (μg/ml)*
CD3	SK7	IgG1	BD (Franklin Lakes, NJ, USA)	0.02
CD4	SK3	IgG1	BD	0.02
CD8	SK1	IgG1	BD	0.03
CD19	HIB19	IgG1	BD	0.01
CD20	L27	IgG1	BD	0.25
CD68	EBM11	IgG	DakoCytomation (Glostrup, Denmark)	0.03
CXCR3	49801.111	IgG1	BD	0.04
CCR4	KM2160	IgG1	Kyowa Hakko[a], Tokyo, Japan	0.23
CCR5	2D7	IgG2a	BD-Pharmingen	0.5
CCR6	11A9	IgG1	BD-Pharmingen	0.17
MIP1-α (CCL3)	11A3	IgG2a	BD-Pharmingen	2
MIP1-β (CCL4)	24006.111	IgG2b	DakoCytomation	5
RANTES (CCL5)	VL1	IgG2b	Biosourse, Niveles, Belgium	1
IP10 (CXCL11)	33036.211	IgG1	DakoCytomation	5
MIG (CXCL9)	49106.11	IgG1	DakoCytomation	0.1

[a]Generous supply.

(blue), sections were washed with 0.2 M-glycine buffer (pH 2.2). The second step-immunohistochemistry was performed with 3-amino-9-ethylcarbazole (AEC, DakoCytomation) as a chromogen (reddish brown).

In Situ Hybridization (Performed in Representative Two Cases)

The coding region of RANTES was amplified from phytohemagglutinin-treated peripheral blood leukocyte cDNA by polymerase chain reaction (PCR). Digoxigenin-labeled sense and antisense riboprobes were generated from the 276-bp fragment of RANTES cDNA subcloned into pCR script SK+ by *in vitro* transcription as described previously.[17,18] The *in situ* hybridization procedures were also done as described previously.[17] The probe concentration was 1 μg/ml. The hybridized signals were visualized by alkaline phosphatase reaction (dark purple) as described in the manufacturer's manual (Boehringer Ingelheim, Germany)

Immunoelectron Microscopy (Performed in One Representative Case)

We adopted the pre-embedding, immunoperoxidase method using PLP-prefixed frozen sections as described previously.[16,18.]

Analyses on Granulomas and Morphometrical Analysis

Qualitative analyses on the chemokines and their receptors were performed in 75 granulomas, and the results were compared with those in 50 lymphoid aggregates observed in 13 cases of Crohn's disease (eight cases with colon and five with ileum), and eight lymphoid follicles observed in seven cases of normal tissue (five cases with colon and two with ileum).

Quantitative analyses of the chemokine receptors were performed using a $\times 400$ field. The average counts in four fields were used. First, we compared the number of immunoreactive cells in the mucosa. The number of cases was 24 for Crohn's disease, 12 for ulcerative colitis and nine for control tissue (five cases for colon and four for ileum). We next chose 20 granulomas and 29 lymphoid aggregates, and labeled them for CD4, CD8, and CCR5 by serial sections. The ratio of $CD8^+$ cells and that of $CCR5^+$ cells per total T cells (summation of $CD4^+$ and $CD8^+$ cells) was measured in each granuloma or lymphoid aggregate, and expressed by percentage. The differences between two groups were tested by Mann–Whitney's test, and differences among groups of more than three were tested by Kruskal–Wallis test and multiple comparison (SPSS 12.0, SPSS Japan, Tokyo, Japan).

Results

Immunohistochemical Analysis of Chemokine Receptors in the Lamina Propria of Mucosa

Most of the small mononuclear cells in the lamina propria of noninflamed intestinal mucosa expressed $CCR5^+$ (Figure 1a) and $CXCR3^+$ (the same as in Yuan *et al*[14]), while $CCR4^+$ or $CCR6^+$ cells were rarely seen (not shown). The numbers of $CCR5^+$ cells in the lamina propria were moderately increased in Crohn's disease and ulcerative colitis (Figures 1a–c). Morphometric analysis revealed no significant differences in the numbers of $CCR5^+$

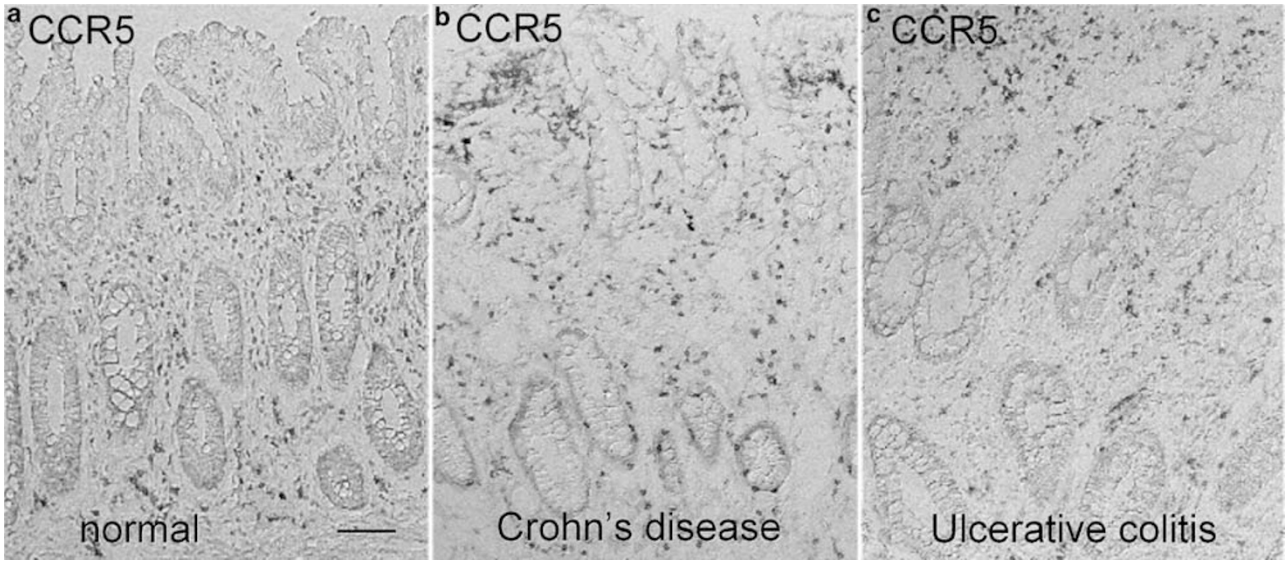

Figure 1 Immunohistochemistry for CCR5. Normal colon mucosa (**a**), mucosa of Crohn's disease (**b**), and mucosa of ulcerative colitis (**c**).

cells between Crohn's disease and ulcerative colitis, or between control and Crohn's disease or ulcerative colitis (Figure 2). The numbers of CXCR3$^+$, CCR4$^+$, and CCR6$^+$ cells in inflamed mucosa of the two IBDs did not significantly differ from those of control mucosa (data not shown).

Immunohistochemical Expression of Chemokines and Chemokine Receptors in Granulomas and Lymphoid Aggregates in Crohn's Disease

To search for disease-specific changes, we next focused our attention to the areas where lymphocytes were aggregated in Crohn's disease, that is, noncaseating granulomas (Figure 3a–e) and lymphoid aggregates (Figure 3f–j), both of which were present in all the layers of intestinal wall. The former are comprised of centrally located epithelioid cells and surrounding lymphocytes, whereas the latter are composed of dense aggregates of small lymphocytes with or without germinal centers. For comparison, we also analyzed lymphoid follicles in the normal intestinal mucosa. The number of lesions examined is described in the Materials and methods. Lymphoid aggregates and lymphoid follicles were characterized by an abundance of B cells that were positive for both CD19 (Figure 3g) and CD20. In contrast, granulomas were surrounded by T cells with CD4$^+$ and CD8$^+$ T cells at similar frequencies (Figure 3b, c) and they lacked B cells (photographs not shown, quantitative data in Figure 4a). T cells around granulomas were mostly positive for CCR5 and CXCR3 (Figure 3d, e), and granuloma cells also expressed CCR5 consistently (but not CXCR3). CCR4$^+$ cells were quite rare (not shown). On the other hand, T cells in lymphoid aggregates (Figure 3f) and lymphoid follicles (data not shown) showed a predominance of CD4$^+$ T cells over CD8$^+$ T cells (Figure 3h, i) and only limited numbers of cells expressed CCR5, CXCR3, or CCR4 (Figure 3j for CCR5). Morphometric analysis further revealed that T cells around granulomas were characterized by a higher ratio of CCR5$^+$ cells and CD8$^+$ T cells than those in the other two (Figure 4b, c).

Double-labeling immunohistochemistry demonstrated that granuloma cells were positive for CCR5 (central brown-area) and were surrounded by small round CCR5$^+$ cells that were either CD4$^+$ or CD8$^+$ T cells (Figure 5a, b; double-positive cells being shown as dark cells). Therefore, it was evident that noncaseating granulomas were characterized by T-cell populations that were distinct from those in lymphoid aggregates in Crohn's disease or lymphoid follicles in the normal intestinal tissues.

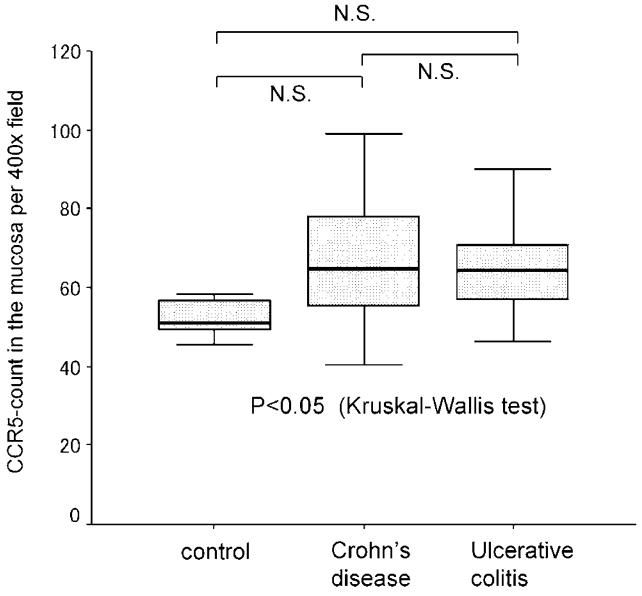

Figure 2 Quantitative analysis of CCR5$^+$ small round cells in the lamina propria of normal control colon mucosa, Crohn's disease, and UC mucosa. An overall difference was observed, but no statistically significant differences were noted between any two groups. As expressed by box-whisker plot, the central bar represents the median value, with 25th and 75th percentiles expressed by the box.

Immunohistochemical Analysis of Chemokines in Crohn's Disease

We next examined *in situ* expression of the ligands of CCR5, that is, MIP-1α, MIP-1β, and RANTES, in Crohn's disease. In 13 out of 25 granulomas (from eight cases), RANTES was clearly localized in granuloma cells (Figure 6a), particularly along the cell membrane (Figure 6b indicated by 'Gra'). *In situ* hybridization performed in two cases revealed that some granuloma cells clearly expressed RANTES mRNA (Figure 6c [signals] and d [negative control]). In all eight cases, a part of lymphocytes surrounding granulomas were also positive for the RANTES protein in the cytoplasm with a characteristic dotted pattern[17,19] (Figure 6b, arrows), and also positive for RANTES mRNA in the cytoplasm (Figure 6c, arrowheads). By contrast, lymphocytes in lymphoid aggregates did not stain for RANTES (data not shown). Granuloma cells did not stain for IP-10 or Mig, the ligands of CXCR3 (data not shown). We did not observe any clear staining of MIP-1α or MIP-1β in Crohn's disease (data not shown).

Immunoelectron Microscopy

To analyze the ultrastructural localization of RANTES protein in granuloma cells, we performed immunoelectron microscopy as described in Materials and methods. Granuloma cells were rich in mitochondria and microvillous projections on the cell surface.[20] We confirmed localization of RANTES along the plasma membrane as well as on the surface of microvillous projections of granuloma cells, in one representative case (Figure 7).

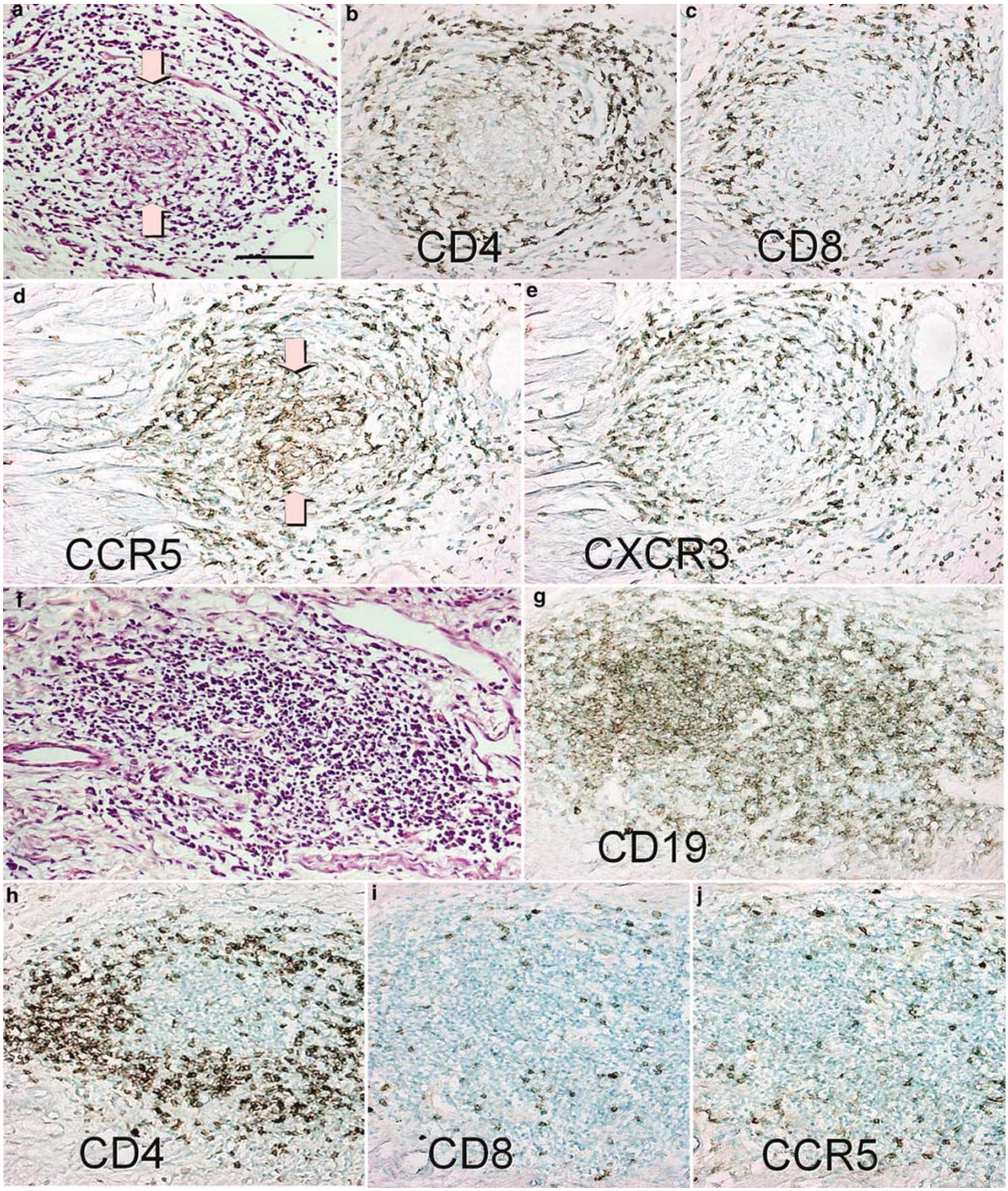

Figure 3 Immunostaining of granulomas (**a–e**) and lymphoid aggregates (**f–j**), both of which were located in deeper layers of the intestine (submucosa–subserosa) in Crohn's disease. Hematoxylin–eosin staining (**a**, **f**), and immunohistochemistry with signals in brown and counterstained with methyl green (**b–e**, **g–j**). Granulomas were composed of a collection of epithelioid cells (arrows in **a**), surrounded by small lymphocytes. Such lymphocytes were positive for CD4 (**b**) or CD8 (**c**) approximately at equal numbers, and they were consistently positive for CCR5 (**d**) and CXCR3 (**e**). Granuloma cells were also positive for CCR5 (**d**, arrows). Lymphoid aggregates were mainly composed of $CD19^+$ B cells (**g**), with moderate numbers of $CD4^+$ T cells (**h**) with scanty $CD8^+$ T cells (**i**) or $CCR5^+$ cells (**j**). Scale bar, 100 μm (**a–j**).

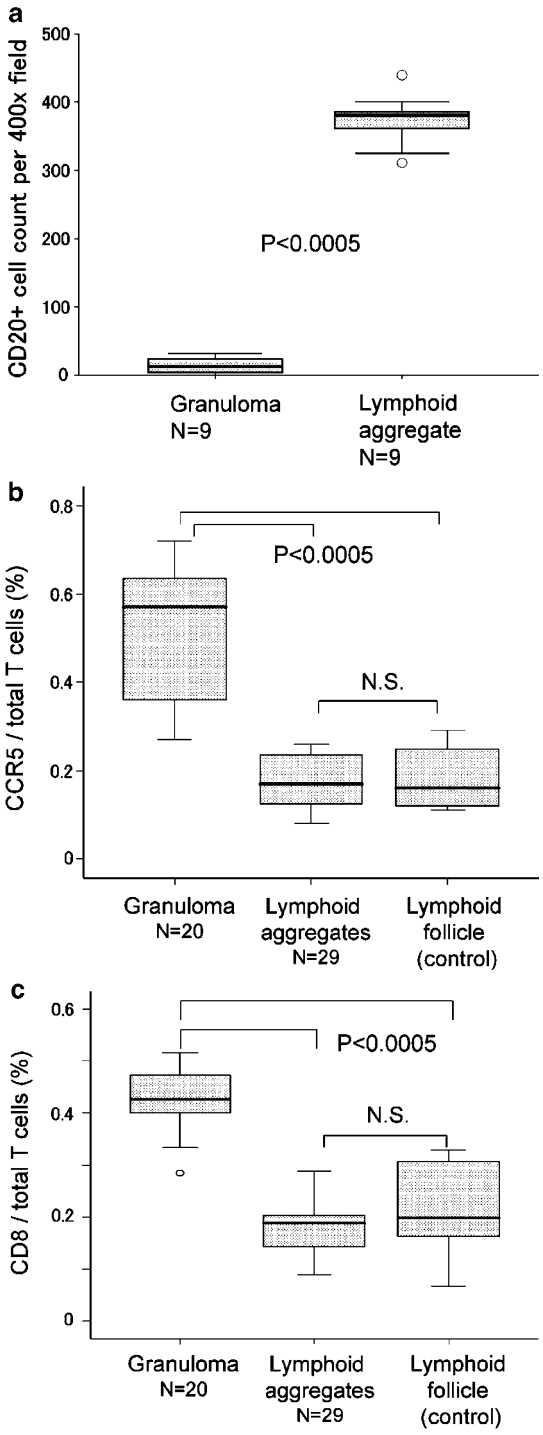

Figure 4 Box-whisker plots showing quantitative analysis between granuloma tissues, lymphoid aggregates and normal lymphoid follicles. $CD20^+$ B cells (**a**), ratio of $CCR5^+$ cells per total T cells (**b**), and ratio of $CD8^+$ T cells per total T cells (**c**). Granuloma tissues were characterized by a lack of B cells, and a higher ratio of $CCR5^+$ and $CD8^+$ cells per total T cells.

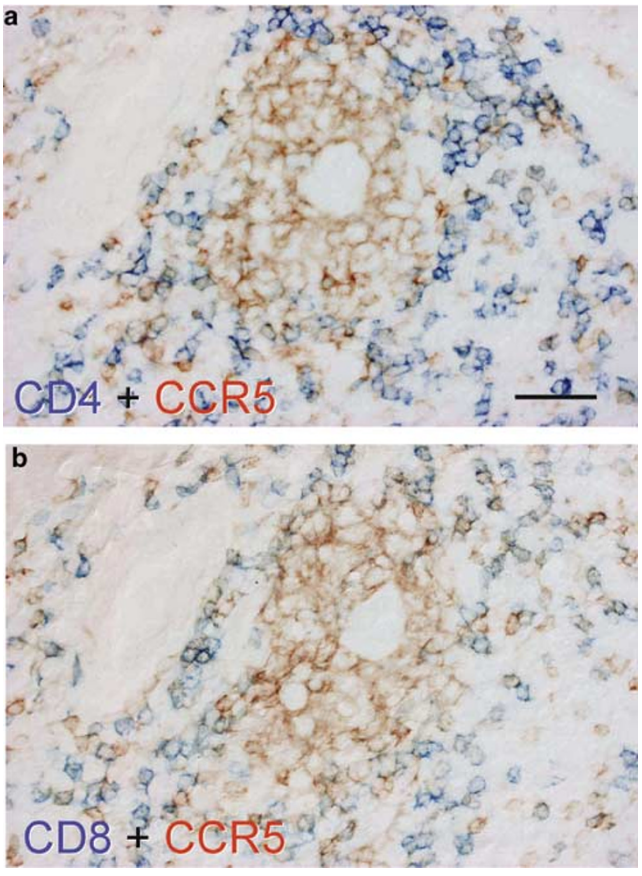

Figure 5 Double staining for CCR5-CD4 (**a**); and CCR5-CD8 (**b**) in granulomas in Crohn's disease. Lymphocyte markers are in blue and CCR5 in brown. Central brown areas represent $CCR5^+$ granuloma cells, and $CCR5^+$ lymphocytes surrounding them express either CD4 or CD8 (double-positive cells being shown as dark cells). No counterstaining. Scale bar, 20 μm (**a**, **b**).

Discussion

Previous studies have demonstrated no significant differences between Crohn's disease and ulcerative colitis in terms of expression of chemokines and chemokine receptor CXCR3, so long as mucosal inflammation is concerned.[13,14] We further obtained the same results concerning CCR5 in the present study. As a novelty, the present study has showed that noncaseating granulomas, specifically observed in Crohn's disease, express both protein and mRNA for RANTES, and that they are consistently surrounded by T cells expressing CCR5 and CXCR3. This Th1-type expression of the chemokine receptors suggests that granulomas could be one of the sites of Th1-shifted immune responses.[4–7]

Immunoelectron microscopy clearly demonstrated RANTES along the cell membrane of granuloma cells. This localization pattern may be explained as follows. Most chemokines have a strong affinity to glycosaminoglycans, including heparan sulfate. This is important for the association of chemokines with the cell surface.[21–23] Thus, it is likely that the RANTES we observed on the surface of granuloma cells were first secreted by granuloma cells and bound to glycosaminoglycans on their cell surface. RANTES secreted by

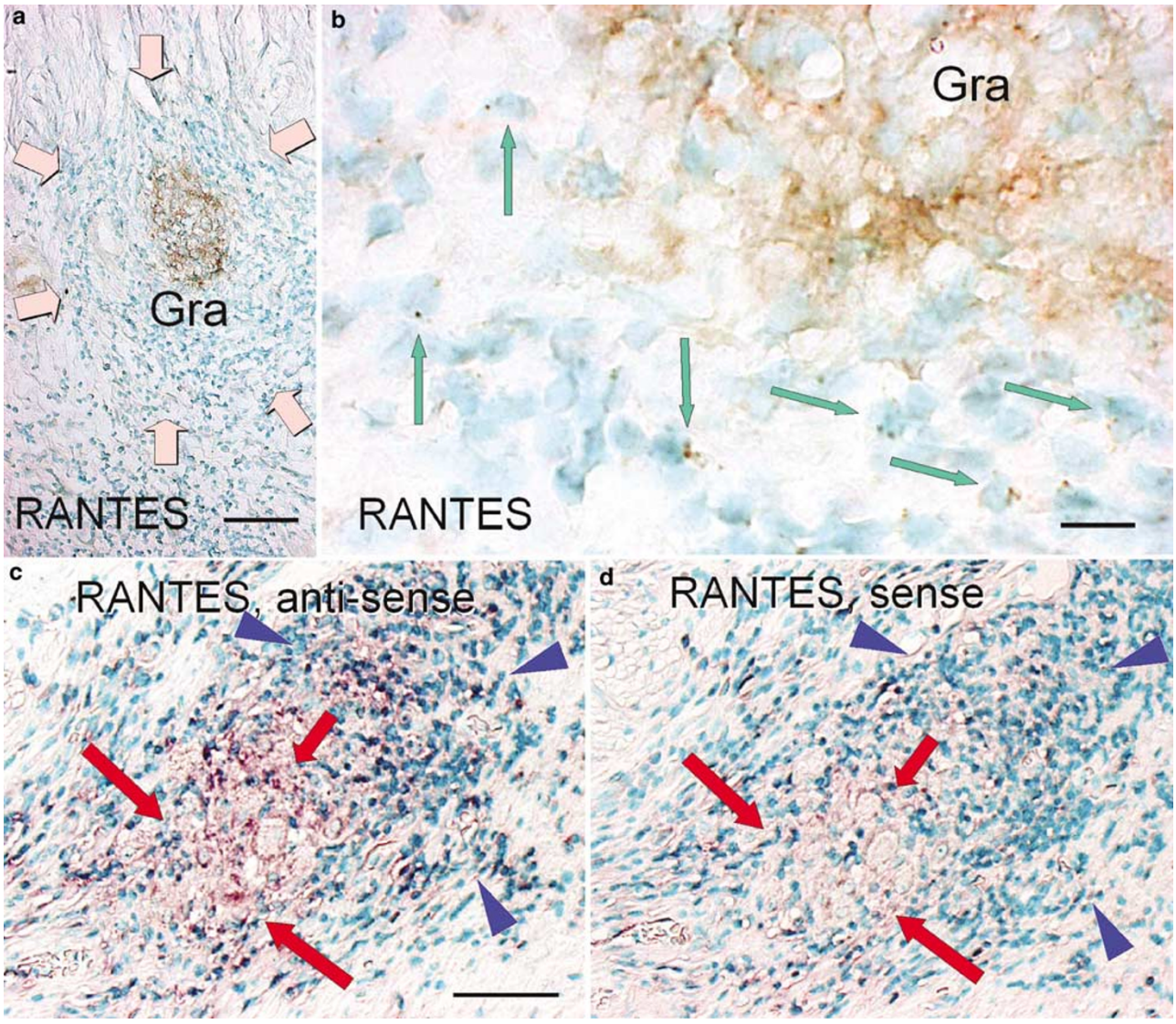

Figure 6 RANTES expression in granuloma cells in Crohn's disease. Immunohistochemistry at lower magnification (**a**) and oil-immersion figure (**b**). 'Gra' in (**a**) indicates RANTES$^+$ granuloma cells (brown), and arrows in (**a**) indicate lymphocytes surrounding granulomas. 'Gra' in (**b**) indicates higher magnification of RANTES$^+$ granuloma cells, and arrows in (**b**) indicate lymphocytes containing RANTES$^+$ granules. *In situ* hybridization with antisense (**c**) and sense (**d**) probes. Arrows in (**c**) and (**d**) indicate a granuloma. Some of granuloma cells show dark purple signals in the cytoplasm (**c**). Arrowheads in (**c**) indicate positive signals in some lymphocytes surrounding granuloma. Counter-stained with methyl green (**a–d**). Scale bar, 50 μm in (**a**), (**c**), and (**d**), and 10 μm in (**b**).

granuloma cells would also attract CCR5$^+$ T cells toward them. Lymphocytes surrounding granulomas were mostly CCR5$^+$ and CXCR3$^+$ and approximately 50% of them were CD8$^+$ with some of them also carrying RANTES$^+$ granules. Swanson *et al*[24] have reported that memory CD8$^+$ T cells constitutively contain cytoplasmic RANTES mRNA but produce RANTES protein only upon TCR-stimulation. Given that granuloma cells could be regarded as antigen-presenting cells,[20] RANTES$^+$ T cells surrounding granuloma cells, we observed here could be memory T cells that have been stimulated by granuloma cells. Our previous observation of close contacts between microvillous projections of granuloma cells and surrounding lymphocytes could support this notion.[20] Furthermore, the release of RANTES from surrounding lymphocytes may further recruit CCR5$^+$ T cells in a self-augmenting manner, a probable common feature of Th1-shifted inflammatory diseases.[17,19]

In contrast to CCR5, CXCR3 may not play a major role in the recruitment of lymphocytes toward granuloma cells as judged by the absence of its ligands, IP-10 and MIG, in granuloma cells or surrounding lymphocytes.

Taub *et al*[25,26] have reported that RANTES induces cytotoxic T lymphocyte (CTL)-specific cytolytic responses, proliferation of T-cell clones, production of IL-2, and enhancement of CD25 expression. Furthermore, macrophage-derived dendritic cells

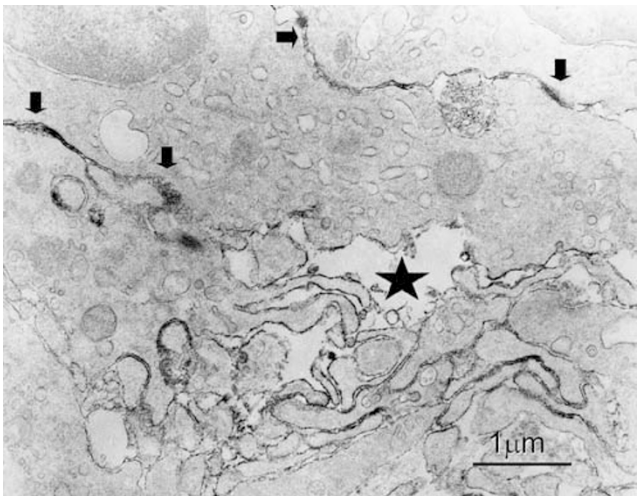

Figure 7 Immunoelectron microscopy for RANTES in granuloma cells in Crohn's disease. Positive signals were expressed by black color (osmificated DAB). Note clear localization of signals for RANTES protein along the plasma membrane (arrows), and along the membranes of microvillous projections (asterisk).

have a strong Th1-polarizing potential through the production of RANTES.[27] Taken together, our observation of RANTES-production by granuloma cells is consistent with the notion that granuloma cells could stimulate T cells,[20] particularly Th1-shifted memory T cells. This notion is consistent with a previous immunohistochemical analysis showing an increased expression of interleukin-12 and interferon γ in granuloma cells and lymphocytes surrounding them.[28] An interesting role of RANTES was reported in trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis, a model of Crohn's disease: RANTES was markedly upregulated during its chronic phase with upregulation of CCR5 in macrophages, which was effectively treated with Met-RANTES, an antagonist of RANTES.[29]

In conclusion, our findings raise the possibility that the granulomas in Crohn's disease could be one of the sites of ongoing Th1-shifted immune response through the interaction of RANTES and CCR5. Therefore, the granulomas may be crucial for the pathogenesis of Crohn's disease.

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