Selective decrease in colonic CD56⁺ T and CD161⁺ T cells in the inflamed mucosa of patients with ulcerative colitis

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CONCLUSION: Selective reduction in the population of colonic mucosal NKR⁺ T cells may contribute to the development of intestinal inflammation in UC.

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Key words: Natural killer T cells; Ulcerative colitis; Interleukin-10

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Abstract

AIM: To investigate the role of local colonic mucosal NK receptor-positive T (NKR⁺ T) cells in the regulation of intestinal inflammation, we analyzed the population and function of these cells in ulcerative colitis (UC).

METHODS: Colonic mucosal tissues were obtained from colonoscopic biopsies of the descending colon from 96 patients with UC (51 endoscopically uninfamed, 45 inflamed) and 18 normal controls. Endoscopic appearance and histologic score at the biopsied site were determined by Matts' classification. A single cell suspension was prepared from each biopsy by collagenase digestion. Two NKR⁺ T cell subsets, CD56⁺ (CD56⁺CD3⁺) T cells and CD161⁺ (CD161⁺CD3⁺) T cells, were detected by flow cytometric analysis. Intracellular cytokine analysis for anti-inflammatory cytokine interleukin-10 (IL-10) was performed by *in vitro* stimulation with phorbol-myristate-acetate (PMA) and ionomycin.

RESULTS: CD56⁺ T cells and CD161⁺ T cells are present in the normal human colon and account for 6.7% and 21.3% of all mononuclear cells, respectively. The populations of both CD56⁺ T cells and CD161⁺ T cells were decreased significantly in the inflamed mucosa of UC. In contrast, the frequency of conventional T cells (CD56⁻CD3⁺ cells and CD161⁻CD3⁺ cells) was similar among the patient and control groups. The populations of NKR⁺ T cells were correlated inversely with the severity of inflammation, which was classified according to the endoscopic and histologic Matts' criteria. Interestingly, approximately 4% of mucosal NKR⁺ T cells expressing IL-10 were detected by *in vitro* stimulation with PMA and ionomycin.

INTRODUCTION

Human ulcerative colitis (UC) is a chronic relapsing disorder of ill-defined immunoregulatory dysfunction that leads to inflammation or ulceration of the intestinal tract^[1]. Increasing evidence suggests that dysregulation of mucosal T cells may play a key role in the pathogenesis of UC, which results in secretion of proinflammatory mediators, accumulation of inflammatory cells, and tissue damage^[2-4]. The pathogenesis of UC remains obscure, but it is now believed that dysfunction of immunoregulatory T cells is considered one of the mechanisms by which intestinal inflammation persists in UC^[5].

Human T cells that express natural killer (NK) markers, including CD56 and CD161, were originally discovered in the liver. They differentiate extrathymically and are considered immunoregulatory T cells^[6,7]. Hepatic CD56⁺ T cells and CD161⁺ T cells can rapidly produce Th1-type and Th2-type cytokines, suggesting that these cells have roles in the regulation of both innate and adoptive immune responses^[8-10]. They also have cytotoxic activity against some cancer cell lines^[11]. Several investigators reported that hepatic CD56⁺ T cells and CD161⁺ T cells are depleted significantly in liver with chronic hepatitis C virus infection^[12,13], suggesting that these cells may be involved in the development of hepatic inflammation.

These NK receptor-positive (NKR⁺) T cells include both conventional major-histocompatibility complex (MHC)-restricted T cells that recognize peptide antigens^[14]

and so-called NKT cells, which recognize glycolipid antigens presented by a non-classical antigen-presenting molecule, CD1^[15-17]. The latter cell type frequently expresses invariant T cell receptor α -chains (V α 24J α Q in humans and V α 14J α 18 in mice)^[9,18].

Recently it was reported that these NKR⁺ T cells reside in the human intestine^[19], and these populations are reduced in the colonic mucosa of patients with colorectal cancer^[20]. However, to date, there have been no reports of the roles of these cells in modulation of intestinal inflammation in UC. Here, we investigated the relation between these cell populations and the severity of colonic inflammation in patients with UC by assessing colonic mononuclear cells in biopsy specimens. We found that a selective decrease in the population of colonic NKR⁺ T cells may be involved in the progression of local colonic mucosal inflammation in UC.

MATERIALS AND METHODS

Study groups

Demographic features of the patients and history of drug therapy up to the time of colonoscopy are summarized in Table 1. The diagnosis of UC was based on established endoscopic and histopathologic criteria^[21]. Colonic mucosal tissues were obtained from colonoscopic biopsies of 96 patients with UC (45 inflamed, 51 uninfamed). The control group consisted of 18 patients. Ten of these control patients presented with a chief complaint of abdominal pain in which a histological diagnosis was not established. The remaining eight control patients were evaluated endoscopically for hematochezia and found to have solitary adenomatous polyps. All control colonic mucosal samples were taken from a histologically normal portion of the biopsied specimen at least 10 cm away from the involved sites with the polyps. The inflammatory activity of these areas was evaluated endoscopically and histologically according to Matts' criteria with some modifications^[22,23]. Inflamed and uninfamed areas were defined as grades 2-4 (and 5 for histologic Matts' criteria) and grade 1, respectively. All biopsy specimens were obtained from the descending colon. Standard 2.8 mm biopsy forceps (Olympus Optical, Tokyo, Japan) were used through all colonoscopes. All samples were obtained with informed consent in accordance with the Helsinki Declaration.

Isolation of lamina propria mononuclear cells

All specimens were weighed prior to isolation of colonic mononuclear cells (MNCs). Five biopsy samples for purification of MNCs and two biopsy samples for evaluation of histology were taken from the same region of each colon, and MNCs were purified as described previously^[24]. Briefly, specimens were digested with 150 U/mL of collagenase in RPMI medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS, Sigma), 100 mg/L of gentamicin (Gibco, Gaithersburg, MD), 500 μ g/L of penicillin, and 500 μ g/L of streptomycin (Gibco) at 37°C for 90 min. The cells were pelleted and washed with cold phosphate-buffered saline (PBS). Cells were resuspended in 44% isotonic Percoll (Sigma) underlaid with 66% isotonic Percoll and centrifuged for 20 min at 2200 r/min at room temperature. Cells at the interface were collected

Table 1 Demographic features of participating patients with UC

	Normal	Ulcerative colitis	
		Inflamed	Uninflamed
No. of patients (cases)	18	45	51
Sex (male/female)	11/7	31/14	35/16
Age (yr), mean $\pm$ SD	47.5 $\pm$ 20.1	35.3 $\pm$ 13.6	39.3 $\pm$ 14.1
Disease duration (yr)		6.5 $\pm$ 6.6	6.2 $\pm$ 5.5
Types of colitis			
Total	-	31	10
Left-sided	-	9	14
Proctitis	-	0	24
Others	-	5	3
Treatment			
Prednisolone (PSL) (no/yes)	-	15/30	29/22
Azathioprine (AZA) (no/yes)	-	31/14	42/9

and washed twice with cold PBS. Cell viability was determined by 0.1% trypan blue dye exclusion, and it was consistently > 90% in all of the patient groups.

Intestinal histology

Intestinal biopsy specimens were fixed immediately in 10% formalin in sodium phosphate buffer and sent to the Department of Pathology at Hiroshima University for processing. Biopsies were embedded in paraffin, and histological sections were stained with hematoxylin and eosin for evaluation. Inflammation was graded according to Matts' classification^[22]. The pathologist was blinded to cell surface analysis data.

Flow cytometric analysis

Cells were incubated with a saturating amount of FITC-conjugated anti-human CD3 (UCHT1) mAb and phycoerythrin (PE)-conjugated anti-CD56 (B159) mAb or anti-CD161 (DX12) mAb. All staining reagents were obtained from Becton Dickinson (BD, San Jose, CA, USA). After cells were washed twice with PBS, the stained cells were analyzed on a FACScan (BD), and data were processed with Cell Quest software (BD). The relative proportions of the lymphocyte subpopulations were determined as percentages of the total numbers of cells in a lymphogate defined by forward and side scatter properties. Non-viable cells were excluded by detection of propidium iodide uptake.

Stimulation of cells and staining for intracellular cytokines

Freshly isolated MNCs isolated from a patient with active UC were suspended in complete RPMI medium at a density of 1×10^6 cells/mL and stimulated for 6 h in 96-well plates (MicrotestTM 96, BD) at 37°C in 5% CO₂. Cells were stimulated with 50 ng/mL phorbol-myristate-acetate (PMA) plus 500 ng/mL ionomycin. As controls, unstimulated cells were treated similarly. Interleukin-10 (IL-10) production by NKT cells was examined by a combination of cell-surface and intracytoplasmic mAb staining for IL-10 (JES3-19F1, BD) with Cytofix/Cytoperm PlusTM (BD) and analyzed by flow cytometry.

Statistical analysis

Data were analyzed with StatView software (Japanese version, Hulus, Tokyo, Japan) on a Macintosh Computer

Figure 1 Representative FACS profiles of human colonic mucosal NKT cells. Flow cytometric analysis of CD3 and CD56/CD161 expression on MNCs isolated from colonic samples showing normal mucosa (left), inflamed (Matts' grade 3b) UC mucosa (middle), and uninflamed (Matts' grade 1) UC mucosa (right). The numbers in the top right quadrants denote the percentages of CD56⁺ T and CD161⁺ T cells.

(Apple Computer, Cupertino, CA). Data are expressed as mean $\pm$ SD. Differences between groups were examined for statistical significance with Student *t* test after analysis of variances (ANOVA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Decreases in CD56⁺ T cells and CD161⁺ T cells in the inflamed colonic mucosa of patients with UC

Representative FACS patterns of NKR⁺ T cells are shown in Figure 1. Flow cytometric analysis of freshly isolated colonic LPLs revealed that the proportion of NKR⁺ T cells expressing CD56 was significantly lower in patients with active UC ($3.3\% \pm 1.7\%$, $P < 0.0001$; Figure 2A) in comparison with controls ($6.7\% \pm 3.2\%$) and patients with inactive UC ($7.5\% \pm 3.9\%$). Similarly, the proportion of NKR⁺ T cells expressing CD161 was significantly lower in patients with active UC ($13.4\% \pm 6.4\%$, $P < 0.0005$; Figure 2B) in comparison with controls ($21.3\% \pm 7.7\%$) and patients with inactive UC ($21.4\% \pm 1.5\%$). The proportion of CD3⁺CD56⁺ NK cells was also reduced significantly in colonic LPL from patients with active UC in comparison with controls and patients with inactive UC (data not shown). No significant differences in the proportions of CD56⁺CD3⁺ and CD161⁺CD3⁺ conventional T cells were observed among these groups (Figure 2C and D), suggesting that mucosal NKR⁺ T cells were selectively decreased in the inflamed UC mucosa. Furthermore, when we considered the inflamed mucosa by endoscopic and histologic classifications of Matts' grade, the populations of these NKR⁺ T cells decreased as the degree of inflammation increased (Figures 3 and 4A). In contrast, the proportions

of CD56⁺CD3⁺ and CD161⁺CD3⁺ conventional T cells were not influenced by the degree of histologic intestinal inflammation (Figure 4B). These results suggest that a relative decrease in the population of colonic NKR⁺ T cells may exacerbate intestinal inflammation.

Prednisolone and azathioprine therapies did not affect the percentage of colonic NKR⁺ T cells

The percentages of colonic NKR⁺ T cells were compared between UC patients treated with or without prednisolone (PSL). We separated the groups into patients with inflamed and uninflamed mucosa because inflammation affects the proportion of NKR⁺ T cells. PSL treatment did not influence the proportion of colonic NKR⁺ T cells (Figure 5A and B). PSL dose did not influence the population of NKR⁺ T cells (data not shown). Treatment with azathioprine (AZA) also did not affect the proportion of NKR⁺ T cells (Figure 5C and D).

In vitro stimulation with PMA and ionomycin results in IL-10 production by colonic NKR⁺ T cells

To further explore the functional role of NKR⁺ T cells, analysis of expression of the anti-inflammatory cytokine, IL-10 was performed. As shown in Figure 6, a subset of colonic NKR⁺ T cells from normal colonic mucosa produced intracellular IL-10 when stimulated *in vitro* with PMA⁺ ionomycin.

DISCUSSION

In the present study, we observed that the populations of CD56⁺ T cells and CD161⁺ T cells were decreased significantly in inflamed lesions on the colonic mucosa

Figure 2 Selective decreases in the proportions of CD56⁺CD3⁺ (A) and CD161⁺CD3⁺ (B) cells in inflamed colonic mucosa of patients with UC. Box plot graphical representation of the percentage of colonic NKR⁺ T cells from normal mucosa of non-UC patients ($n = 18$), uninflamed mucosa (endoscopic Matts' grade 1, $n = 51$), and inflamed mucosa (Matts' grades 2-4, $n = 45$). ^b $P < 0.0001$, ^d $P < 0.0005$; inflamed mucosa vs normal or uninflamed mucosa. (C, D) No relation was observed between the percentage of conventional T cells (CD56⁺CD3⁺ or CD161⁺CD3⁺ cells) and the degree of endoscopic inflammation.

Figure 3 Relation between Matts' scores for endoscopy and the percentage of NKR⁺ T cells. Box plot graphical representation of the percentage of colonic NKR⁺ T cells in UC patients. The percentage of colonic NKR⁺ T cells was compared with the Matts' scores. "n" indicates the number of cases. ^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.0001$, vs Matts' 1.

in patients with UC. The proportions of these cells were inversely well correlated with the degree of endoscopic and histologic intestinal inflammation. In contrast, the percentages of conventional T cells were similar among our study groups. Our findings suggest that selective decreases in levels of colonic NKR⁺ T cells may contribute to the progression of intestinal inflammation.

CD56⁺ T cells and CD161⁺ T cells were originally identified in human liver. These cells have an extrathymic

origin and have properties of innate lymphocytes^[6,7]. These cells are uniquely capable of rapidly producing Th1 and Th2 cytokines upon stimulation^[8-10], indicating a broad role for these cells in the activation and regulation of multiple arms of the immune responses. Although the functions of these CD56⁺ T cells and CD161⁺ T cells are currently unknown, they express memory T cell phenotypes^[15] and homing chemokine receptors^[25], suggesting that they may be memory T cells. The proliferation and function of these

Figure 4 Relation between the histologic Matts' scores and the percentage of NKR⁺ T cells. Biopsy samples for evaluation of histology were taken from the same regions as samples for MNC purification were taken. The percentage of colonic NKR⁺ T cells was compared with the histologic Matts' score. Levels of CD56⁺ T cells (A) and CD161⁺ T cells (B) in the colon decreased significantly as a function of the severity of inflammation. "n" indicates the number of cases. ^b $P < 0.0001$, ^d $P < 0.005$ vs Matts' 1, ^a $P < 0.05$ vs Matts' 1 or Matts' 2. No relation was detected between the percentages of conventional T cells (CD56⁺CD3⁺ or CD161⁺CD3⁺ cells) in the colon and the severity of histological inflammation (C, D).

cells are regulated by various cytokines, including IL-15 and granulocyte-macrophage colony-stimulating factor^[26,27]. These NKR⁺ T cells include invariant NKT cells that recognize glycolipid antigens presented by the non-classical antigen-presenting molecule CD1^[15-17]. CD1d, which is an MHC Class I-like molecule, is expressed by human intestinal epithelial cells^[28,29]. Bendelac *et al* reported that V α 24 NKT cells recognize CD1d molecules and act as immunoregulatory cells by producing various cytokines^[30]. Blumberg *et al* showed that the CD1d molecule expressed by intestinal epithelial cells is functional because ligation with antibody against CD1d induces production of IL-10 by an intestinal epithelial cell line, T84^[31]. Therefore, the interaction between CD1d on intestinal epithelial cells and mucosal CD1d-restricted T cells may be important for maintaining intestinal homeostasis. Whether these NKR⁺ T cells are CD1d-restricted is currently under investigation.

The role of NKR⁺ T cells in the development of intestinal inflammation is not clear. A mouse study revealed that CD1d- α -galactosylceramide (α GalCer)-restricted NKT cells are critical for protection against the development of dextran sulfate sodium-induced colitis^[32]. The findings of another study suggested that IL-13-producing NKT cells are involved in the development of

oxazolone-induced colitis in mice^[33]. These data suggest that NKT cells are crucial for eliciting protective immunity against intestinal inflammation.

Although NKT cells are known to have an anti-inflammatory role^[34], what is the mechanism by which these cells regulate the immune system? Sonoda *et al* showed that about 5% of NKT cells have the capacity to produce the immunosuppressive cytokine IL-10^[35]. We have shown here that some colonic mucosal NKR⁺ T cells can produce IL-10 when stimulated *in vitro*. IL-10 expression is also important for mucosal immunologic homeostasis. IL-10-deficient mice show enhanced production of colonic proinflammatory cytokines, including IFN- γ , and develop spontaneous chronic enterocolitis^[36,37]. The subset of regulatory T cells that produce IL-10 suppresses the development of experimental intestinal inflammation^[38]. Furthermore, mice with a macrophage/neutrophil-specific disruption of the Stat3 gene show impaired IL-10-mediated functions and develop chronic enterocolitis^[39]. These observations support the idea that mucosal immune homeostasis involves localized production of molecules that promote IL-10 expression by resident immunoregulatory cells in the mucosa. Therefore, decreased IL-10 production due

Figure 5 The percentage of colonic NKR⁺ T cells was not affected by treatment with PSL or AZA. The percentages of colonic NKR⁺ T cells were compared between untreated UC patients and those treated with PSL (A, B). The level of NKR⁺ T cells was compared between untreated UC patients and those treated with AZA (C, D). "n" indicates the number of cases.

Figure 6 Detection of IL-10-producing NKR⁺ T cells in the colonic mucosa. Flow cytometric analysis of IL-10 production by *ex vivo*-stimulated colonic MNCs isolated from a patient with active UC. FACSscan was gated on CD56⁺CD3⁺ cells. The numbers indicate the percentages of CD56⁺CD3⁺ cells producing cytokines relative to unstimulated control cells.

to depletion of NKR⁺ T cells in the colon of human UC as observed in the present study may result in insufficient inhibition of pathologic T cells and activated macrophages.

Further studies are needed to compare the percentage of IL-10-producing cells between normal and UC colons.

Several mechanisms may account for the decreased

proportions of NKR⁺ T cells in UC. First, the observed depletion of the local colonic NKT cell population may be the result of a continuous process of activation-induced cell death^[40]. Second possible mechanism may be the loss of surface NK markers. It was recently reported that NKT cells activated by glycolipid antigens down-regulate NK receptors^[41]. Third mechanism may be impaired recruitment of NKR⁺ T cells from the peripheral circulation. CD56⁺ T cells express chemokine receptors such as CCR5 or homing receptors such as $\alpha 4\beta 7$, a ligand for MAdCAM1 expressed specifically on the intestinal high endothelial venules^[25]. These issues are currently under investigation in our laboratory.

In conclusion, human colonic CD56⁺ T cells and CD161⁺ T cells are thought to play important roles as anti-inflammatory cells, and the decrease in the proportions of these cells in inflamed lesions of the colon may be one mechanism by which colonic inflammation progresses in UC.

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COMMENTS

Background

Human T cells that have natural killer markers are mainly located in the liver and considered immunoregulatory T cells. Recently it was reported that these natural killer receptor (NKR⁺) T cells reside in the human intestine. But the exact significance of these cells in the intestine is unknown. This study was aimed at investigating the changes and significance of these cells in human ulcerative colitis (UC).

Research frontiers

Previous studies have demonstrated the significance of NKR⁺ T cells in human colorectal cancer. However, the role of these cells in the chronic intestinal inflammation is undefined.

Innovations and breakthroughs

The populations of both CD56⁺ T cells and CD161⁺ T cells were decreased significantly in the inflamed mucosa of UC. The populations of these NKR⁺ T cells were correlated inversely with the severity of inflammation, which was classified according to the endoscopic and histologic Mats' criteria. In contrast, the frequency of conventional T cells (CD56⁺CD3⁺ cells and CD161⁺CD3⁺ cells) was similar among the patients with UC and healthy groups.

Applications

Selective reduction in the population of colonic mucosal NKR⁺ T cells may contribute to the development of intestinal inflammation in UC.

Terminology

CD56 and CD161 are known as natural killer cell surface antigens. CD56 was demonstrated to be a neural cell adhesion molecule-1 (NCAM-1). CD56⁺ T cells are believed to be major players in immunosurveillance and antitumor responses. CD161 is a human NKR-P1 family and is recognized as an analogue of murine NKR-P1C. Moreover, CD161⁺ T cells are also known to play an important role for antitumor immunity. CD161 is also expressed by invariant natural killer T cells that are restricted to CD1d molecule on antigen presenting cells.

Peer review

In this study, the authors demonstrate that the proportion of NKR⁺ T cells is selectively decreased in the colonic mucosa of UC patients and that this reduced

cell number correlates well with the severity of the disease. These cells are capable of producing an anti-inflammatory cytokine, IL-10. This work adds important information regarding cellular subsets that might be involved in the pathogenesis of UC.

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