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MAJOR ARTICLE

Clinical Implications of the CMV-Specific T-Cell Response and Local or Systemic CMV Viral Replication in Patients With Moderate to Severe Ulcerative Colitis

Kyung Hwa Jung,[1,a] Jihun Kim,[2,a] Ho-Su Lee,[3,a] Jene Choi,[2] Se Jin Jang,[2] Jiwon Jung,[1] Min Jae Kim,[1] Yong Pil Chong,[1] Sang-Oh Lee,[1] Sang-Ho Choi,[1] Yang Soo Kim,[1] Jun Hee Woo,[1] Sang Hyoung Park,[4,5] Dong-Hoon Yang,[4] Byong Duk Ye,[4,5] Suk-Kyun Yang,[4,5,b] and Sung-Han Kim[1,b]

[1]Department of Infectious Diseases, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea, [2]Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea, [3]Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul, Republic of Korea, [4]Department of Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea, and [5]Inflammatory Bowel Disease Center, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

Background. The precise role of cytomegalovirus (CMV) in ulcerative colitis (UC) remains disputed. We evaluated the association of CMV-specific host immune responses and systemic or local viral replication with responses to systemic steroids in patients with moderate to severe UC.

Methods. Patients who were hospitalized for moderate to severe UC between April 2015 and June 2016 were enrolled. At baseline, all enrolled patients underwent CMV-specific enzyme-linked immunospot assays, quantitative polymerase chain reaction (qPCR) analysis of blood and colonic tissue for CMV viral load, histopathological testing for CMV in colonic tissue by hematoxylin and eosin staining, and immunohistochemical (IHC) analysis. Clinical responses to steroid therapy based on the Oxford index were assessed on day 3.

Results. Of the 80 patients evaluated, 28 (35.0%) had poor responses to steroid therapy on day 3 of intensive treatment. The presence of inclusion bodies (32.1%) and high-grade (≥3) positivity on IHC (50.0%), as well as colonic (mean 1440.4 copies/mg) and blood (mean, 3692.6 copies/mL) CMV viral load, were higher in steroid-refractory UC patients than the control group (13.5%, 1.9%, mean 429.2 copies/mg, and mean 231.2 copies/mL, respectively; $P = .046$, .009, .017, and .002, respectively). However, CMV-specific T-cell responses were not associated with steroid-refractory UC. Multivariate analysis revealed that a higher Mayo score (odds ratio [OR], 2.00; $P = .002$) and higher blood CMV viral load via qPCR analysis (OR, 3.58; $P = .044$) were independent risk factors for steroid-refractory UC.

Conclusions. In patients with moderate to severe UC, higher Mayo score and blood CMV expression determined by qPCR are independently associated with steroid refractoriness.

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Keywords. cytomegalovirus; enzyme-linked immunospot; T-cell response; ulcerative colitis.

Corticosteroids are the firstline of therapy for moderate to severe ulcerative colitis (UC); however, up to 30%–40% of patients fail to respond to treatment [1, 2]. In steroid-refractory colitis, the prevalence of cytomegalovirus (CMV) colitis appears to be particularly high [2]. Previous studies suggested that CMV infection should be suspected in patients with inflammatory bowel disease (IBD) who are refractory to steroid treatment. Current guidelines state that CMV infection should be excluded in patients with acute steroid-refractory colitis [2, 3].

However, the clinical importance of CMV colitis and its pathological contribution to the inflammatory process are still matters of debate. Whether CMV is an innocent bystander in inflamed intestinal tissue or increases the risk of colectomy is still unclear [3, 4]. Previous studies have reported that CMV infection might contribute to steroid refractoriness, megacolon, or exacerbation in UC patients [2]. However, other studies have shown that CMV infection is not associated with exacerbation of IBD [5, 6], because it resolved without antiviral therapy [7]. The causes of these conflicting results are not well understood.

Recently developed assays for investigating the CMV-specific T-cell response allow direct quantification of host immunity to CMV [8, 9]. Therefore, combined analysis of host immunity to CMV and quantitation of CMV replication in tissues, or at the systemic level in steroid-responsive or -unresponsive patients

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[a]K. H. J., J. K., and H.-S. L. contributed equally to this work.

[b]S.-K. Y. and S.-H. K. contributed equally to this work.

Correspondence: S.-H. Kim, MD, PhD, Department of Infectious Diseases, University of Ulsan College of Medicine, Asan Medical Center, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 05505, Korea (kimsunghanmd@hotmail.com).

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with UC, would provide further insight into this complex issue. We therefore evaluated the association of the CMV-specific host immune response and systemic or local viral replication with the response to systemic steroids in patients with moderate to severe UC.

METHODS

Patient Selection

Patients aged >16 years with moderate to severe UC who were admitted to Asan Medical Center, Seoul, South Korea, a tertiary care teaching hospital, were prospectively enrolled between April 2015 and June 2016. The diagnosis of UC was based on clinical, endoscopic, and histological parameters [10, 11]. Disease activity was assessed using the Mayo score [11]. Moderate to severe UC was defined as a Mayo score of 6 to 12 with an endoscopic subscore of 2 or more. Only patients with left-sided or extensive colitis were enrolled. There were no exclusion criteria for disease duration or medication such as anti–tumor necrosis factor (TNF) agents. The study protocol was approved by the institutional review board of the Asan Medical Center, which confirmed that it adhered to the ethical principles of the Declaration of Helsinki (IRB number 2015-0129). This study was also registered at ClinicalTrials.gov (NCT 02439372), and informed consent was obtained from each patient.

Study Protocol

At baseline, samples from all enrolled patients were subjected to CMV-specific enzyme-linked immunospot (ELISPOT) assays, and quantitative blood CMV polymerase chain reaction (PCR), hematoxylin and eosin (H&E) staining of colonic tissue, immunohistochemistry (IHC) analysis, and CMV PCR were also performed to assess CMV expression. Endoscopic activity was assessed by expert endoscopists using the Mayo endoscopic subscore and the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) [12]. All enrolled patients with moderate to severe UC received intensive steroid treatment until clinical improvement was observed. The response to steroid therapy was assessed based on the Oxford index, which is the combination of C-reactive protein (CRP) >45 mg/L and stool frequency of 3–8 per day, or stool frequency >8 per day on day 3 [13]. Patients were classified as "poor responders" or "responders" to steroid treatment. We analyzed whether CMV infection was related to steroid responsiveness.

Laboratory Tests for CMV Infection

Real-time PCR was performed to assess CMV expression in the patients' blood. DNA was isolated from the blood using a NucliSens easyMAG Nucleic Acid Extraction System (bioMérieux, Lyon, France). CMV DNA was then quantified using the Qiagen Artus CMV RGQ MDx kit (Qiagen, Doncaster, Australia) on a Rotor-Gene Q platform (Qiagen,Germantown, MD, USA). A positive result for CMV was defined as >250 copies/mL [14].

We also performed CMV-specific ELISPOT assays (IFN-γ; T-track CMV, Lophius Bioscience, Regensburg, Germany) to evaluate the host response to CMV infection, as previously described [8, 9].

Tissues from macroscopically active mucosal lesions (rectum, sigmoid, or descending colon) were obtained during sigmoidoscopy. Using the colonic biopsy tissues, we first checked for histologic signs of CMV infection, which include cell enlargement, basophilic intranuclear inclusion bodies (surrounded by clear halos described as "owl eyes") [15, 16], thickened nuclear membranes, and granular intracytoplasmic inclusions. Next, we performed CMV IHC. Briefly, tissue sections were incubated with anti-CMV monoclonal antibody (clone CCH2^{+} DDG9, 1:50, Glostrup, Denmark). All staining procedures were performed using a BenchMark XT autostainer and the OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). We graded CMV immunostaining using a 4-tier grading system based on the number of cells showing positive nuclear CMV staining per 2-mm tissue. The criteria are as follows: (i) grade 0: negative; (ii) grade 1: 1–2; (iii) grade 2: 3–5; and (iv) grade 3: >5. All biopsy specimens were reviewed by an experienced gastrointestinal pathologist (Dr. Jihun Kim). To detect CMV in the colonoscopic biopsy tissue, DNA was extracted using a QIAamp DNA FFPE Tissue Kit. CMV DNA was amplified by PCR, as described previously [17], with slight modifications. A positive result for tissue CMV PCR was defined as >10 copies/mg [18].

Assessment of Outcomes and Definition of Terms

The primary outcomes were risk factors for poor response to steroid treatment in patients with moderate to severe UC. The response to steroid treatment was assessed on day 3 based on the Oxford index, as described above [13]. We categorized CMV gastrointestinal (GI) disease in the patients using the following criteria, which are based on the recent Infectious Diseases Society of America guidelines [19], with some modifications. Patients were classified as having "proven CMV GI disease" if they had lower GI symptoms, macroscopic mucosal lesions, and CMV expression in tissues, as determined by histopathology or immunohistochemistry analysis. Patients were classified as having "probable CMV disease" if they had lower GI symptoms and CMV expression in tissues, as determined by histopathology or immunohistochemistry analysis, without macroscopic mucosal lesions. Lastly, patients were classified as having "possible CMV colitis" if they only had CMV expression in tissues, as determined by PCR analysis.

Statistical Analysis

Data are expressed as median and interquartile range (IQR) or mean ± standard deviation. Continuous data were compared

using the Student *t* test or Mann-Whitney test, if the distribution was variable. Categorical data were described using contingency tables and a chi-square test or Fisher exact test. A univariate analysis was performed using a logistic regression to determine the independent risk factors associated with a poor response to steroid treatment. Subsequently, multiple logistic regression analysis was performed for variables with a *P* value <.2 in the univariate analysis, based on the backward Wald selection method. The results are reported as an odds ratio (OR) with 95% confidence interval (CI). A *P* value <.05 was considered significant. Calculations were performed using the SPSS for Windows software package, version 21.0 (IBM Co., Armonk, NY, USA).

RESULTS

Baseline Clinical Characteristics

During the study period, 103 patients were admitted. However, 23 patients (22%) were excluded for the following reasons: refusal to provide informed consent (n = 11), endoscopy performed before admission (n = 9), and an unclassified IBD type (n = 3). Finally, 80 patients were officially enrolled (Figure 1). The baseline demographic characteristics at admission are shown in Table 1.

Among these 80 patients, 28 (35.0%) were classified as poor responders to steroid therapy on day 3 based on the Oxford index. The remaining 52 patients (65%) were determined to be responders to steroid therapy (Figure 1). The median age of the poor responders (IQR) was 46 (34–57) years, and that of the responders was 39 (25–52) years. There was no statistical difference in disease duration at admission between the 2 groups. The median disease duration of the poor responders was 25.9 months, and that of the responders was 36.7 months (*P* = .824). Disease severity at admission was higher in the poor responders than the responders, with a mean Mayo score of 10.6 for the poor responders and 9.4 for the responders (*P* < .001).

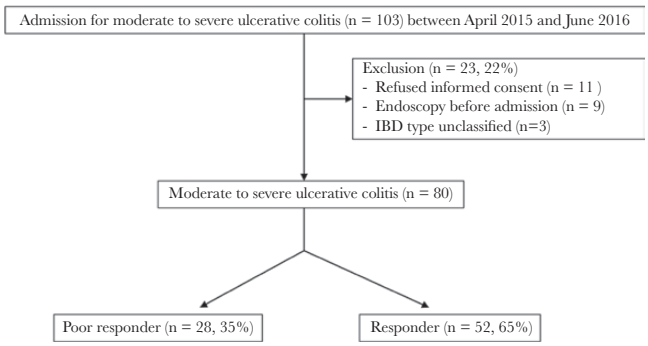

Figure 1. Flowchart of the study.

The Link Between Various Laboratory Parameters and Steroid Refractoriness

Of the 80 patients with moderate to severe UC evaluated in this study, 33 (41.3%) were shown to have CMV colitis, including 29 with proven CMV colitis and 4 with possible CMV colitis, based on the criteria described above. CMV colitis was more frequent in the poor responders to steroid therapy than in the responders (53.6% vs 34.6%); however, the difference was not statistically significant (*P* = .185). Proven CMV colitis was diagnosed in 14 of 28 (50%) poor steroid responders and in 15 of 52 (28.9%) steroid responders (*P* = .062). Possible CMV colitis was diagnosed in 1 of 28 (4%) poor steroid responders and in 3 of 52 (6%) steroid responders (*P* = .800).

We examined whether qualitative or quantitative evidence of CMV replication in colonic tissue or blood and CMV-specific T-cell responses were associated with steroid refractoriness. When we evaluated CMV expression in colonic tissue by PCR, we observed that viral load was higher in the poor responders (mean ± SD, 1440 ± 3637.8 copies/mg) than in the responders (mean ± SD, 429.2 ± 2056.9 copies/mg; *P* = .017). Inclusion bodies in colonic tissue, as assessed by H&E staining, were more frequent in the poor responders (32.1%, 9 of 28) than in the responders (13.5%, 7 of 52; *P* = .046). The overall positive expression of CMV by IHC analysis was higher in the poor responders (50.0%, 14 of 28) than in the responders (28.8%, 15 of 52); however, this was not statistically significant (*P* = .062). Among the IHC-positive patients, histologic grade 3 IHC was significantly higher in the poor responders (25.0%, 7 of 14) than in the responders (28.8%, 1 of 15; *P* = .009).

We also measured CMV replication in the blood using qPCR. CMV was significantly higher in steroid-refractory UC (mean ± SD, 3692.6 ± 7823.2 copies/mL) than in the control samples (mean ± SD, 231.2 ± 1036.1 copies/mL; *P* = .002).

ELISPOT assays with stimulation by IE-1 and pp65 demonstrated that CMV-specific T-cell responses were slightly lower in the poor responders; however, this effect was not statistically significant. The IE-1-specific ELISPOT (mean ± SD) was 7.9 ± 15.7 spot-forming units (sfu)/250 000 cells in the poor responders and 27.7 ± 64.7 sfu/250 000 cells in the steroid responders (*P* = .200). The results of the pp65-specific ELISPOT (mean ± SD) were 84.9 ± 116.6 sfu/250 000 cells in the poor responders and 104.4 ± 131.8 sfu/250 000 cells in the responders (*P* = .642).

The multivariate analysis (Table 2) demonstrated that a higher Mayo score (OR, 2.00; 95% CI, 1.28–3.14; *P* = .002) and higher CMV expression in the blood, as assessed by qPCR (OR, 3.58; 95% CI, 1.03–12.34; *P* = .044), are independent risk factors for steroid-refractory UC. Neither proven nor possible CMV colitis, as determined using the above-mentioned criteria, was an independent risk factor for steroid-refractory UC. However, there was a strong trend for proven CMV colitis as a risk factor for steroid-refractory UC based on univariate analysis (OR, 2.47; 94% CI, 0.95–6.40; *P* = .063).

Table 1. Demographic and Clinical Characteristics of the Study Participants

Patient Characteristic	Total (n = 80)	Poor Responder (n = 28)	Responder (n = 52)	*P*
Male, No. (%)	47 (58.8)	17 (60.7)	30 (57.7)	.793
Age, median (IQR), y	41 (26–54)	46 (34–57)	39 (25–52)	.098
Disease duration before admission, median (IQR), mo	50.3 (7.4–74.6)	25.9 (0.1–213.5)	36.7 (0.1–227.5)	.824
Disease extent at admission, No. (%)				
Left-sided colitis	17 (21.3)	2 (7.1)	15 (28.8)	.024
Extensive colitis	63 (78.8)	26 (92.9)	37 (71.2)	.024
Mayo score, mean ± SD	9.9 ± 1.4	10.6 ± 1.1	9.4 ± 1.4	<.001
UCEIS score, mean ± SD	6.2 ± 1.1	6.6 ± 0.8	5.9 ± 1.2	.007
Hemoglobin, mean ± SD, g/dL	11.7 ± 2.4	11.1 ± 2.6	12.1 ± 2.3	.103
Albumin, mean ± SD, g/dL	3.0 ± 0.66	2.7 ± 0.69	3.2 ± 0.58	.002
C-reactive protein, mean ± SD, mg/L	48.0 ± 52.1	67.2 ± 52.4	37.7 ± 49.4	.002
Recent exposure to medication, No. (%)				
Anti-TNF agents[a]	12 (15.0)	5 (17.9)	7 (13.5)	.744
Thiopurines[b]	24 (30.0)	13 (46.4)	11 (21.2)	.019
Steroids[a]	42 (52.5)	20 (71.4)	22 (42.3)	.013
Clostridium difficile infection, No. (%)				
Negative	67 (83.8)	23 (82.1)	44 (84.6)	.778
Positive[c]	9 (11.3)	4 (14.3)	5 (9.6)	.534
Not checked	4 (5.0)	1 (3.6)	3 (5.8)	.672
CMV colitis,[d] No. (%)	33 (41.3)	15 (53.6)	18 (34.6)	.185
Proven	29 (36.3)	14 (50)	15 (28.8)	.062
Possible	4 (5.0)	1 (4)	3 (6)	.800
Colonic tissue CMV PCR, mean ± SD, copies/mg	783.1 ± 2736.7	1440.4 ± 3637.8	429.2 ± 2056.9	.017
Colonic tissue CMV PCR (>10 copies/mg), No. (%)	29 (36.3)	14 (50.0)	15 (28.8)	.060
IB on H&E staining, No. (%)	16 (20.0)	9 (32.1)	7 (13.5)	.046
Immunohistochemistry, No. (%)	29 (36.2)	14 (50.0)	15 (28.8)	.062
Grade 1	15/29 (51.7)	6/14 (42.9)	9/15 (60.0)	.355
Grade 2	6/29 (20.7)	1/14 (7.1)	5/15 (33.3)	.081
Grade 3	8/29 (27.6)	7/14 (50.0)	1/15 (6.7)	.009
Inclusion body on H&E staining or immunohistochemistry (%)	29 (36.3)	14 (50.0)	15 (28.8)	.062
IB/IHC/colonic tissue PCR, No. (%)				
All positive	16 (20.0)	9 (32.1)	7 (13.5)	.046
Quantitative blood CMV PCR, mean ± SD, copies/mL	1497.7 ± 4994.8	3692.6 ± 7823.2	231.2 ± 1036.1	.002
IE1-specific ELISPOT, mean ± SD, sfu/250 000 cells	20.8 ± 53.7	7.9 ± 15.7	27.7 ± 64.7	.200
pp65-specific ELISPOT, mean ± SD, sfu/250 000 cells	97.6 ± 126.3	84.9 ± 116.6	104.4 ± 131.8	.642

Abbreviations: anti-TNF, anti–tumor necrosis factor; CMV, cytomegalovirus colitis; ELISPOT, enzyme-linked immune absorbent spot; H&E, hematoxylin and eosin; IB, inclusion body; IHC, immunohistochemistry; IQR, interquartile range; PCR, polymerase chain reaction; sfu, spot-forming unit; UCEIS, Ulcerative Colitis Endoscopic Index Of Severity.

[a]Within the last 2 months.

[b]Within the last month.

[c]Positive *C. difficile* infection was defined as *C. difficile* toxin-positive in enzyme-linked fluorescent assay or PCR or culture positive.

[d]CMV colitis was defined as proven CMV colitis and/or possible CMV colitis based on the criteria defined in the "Methods."

DISCUSSION

Several studies indicate that CMV infection is associated with, or causes, refractory colitis and/or steroid refractoriness [2, 20–24]. However, some studies have contradicted these findings [5, 6]. The varying results of these studies could be attributed to the small sample sizes and heterogeneity in the study designs. Another possible source of bias is likely the variety of techniques used to characterize CMV infection and disease. In addition, the broad spectrum of investigated CMV markers was not evaluated in the previous studies [22, 23]. Owing to these limitations, no definite conclusions can be drawn from the available data.

To resolve these issues, we used both blood-based and tissue-based tests to identify CMV. First, we performed qPCR to evaluate CMV in the blood to diagnose CMV viremia. Second, we measured CMV in colonic tissue using qPCR. To avoid observer bias, a single experienced pathologist graded the CMV IHC results on a scale from 0 to 3. We also used cutting edge technology to identify the host response to CMV infection, including evaluating CMV-specific T-cell responses. We then comprehensively analyzed all the variables to identify which

Table 2. Univariate and Multivariate Analyses of the Risk Factors Linked to Poor Steroid Response in Patients with Moderate to Severe UC

	Univariate Analysis		Multivariate Analysis[b]	
Characteristics	Unadjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
Age	1.03 (0.99–1.06)	.21		
Mayo score	6.93 (2.38–20.16)	<.001	2.00 (1.28–3.14)	.002
General laboratory test results				
Albumin, g/dL	0.25 (0.11–0.61)	.02		
C-reactive protein, mg/L	1.11 (1.02–1.22)	.02		
Recent use of steroids	3.41 (1.27–9.15)	.015		
Laboratory tests for CMV infection				
Quantitative blood CMV PCR	1.68 (1.21–2.32)	.002	3.58 (1.03–12.34)	.044
Colonic tissue CMV PCR (>10 copies/mg)	2.47 (0.95–6.40)	.063		
CMV colitis[a]	1.89 (0.74–4.81)	.183		
Proven	2.47 (0.95–6.40)	.063		
Possible	0.73 (0.62–8.55)	.798		

Abbreviations: CI, confidence interval; CMV, cytomegalovirus colitis; OR, odds ratio; PCR, polymerase chain reaction.

[a]CMV colitis was defined as proven CMV colitis and/or possible CMV colitis.s based on the criteria defined in the "Methods."

[b]The following variables were included in the multivariate analysis: Mayo score, albumin, C-reactive protein, recent use of steroids, quantitative blood CMV PCR, colonic tissue CMV PCR, and CMV colitis (proven and possible).

viral and host risk factors for CMV infection were associated with steroid refractoriness. We found that only higher blood CMV expression, as determined by qPCR, was independently associated with poor steroid response in patients with moderate to severe UC. Therefore, our data suggest that increased CMV replication in colonic tissue and decreased CMV-specific T-cell responses may not be associated with steroid refractoriness in moderate to severe UC but may merely reflect disease severity.

We initially thought that if CMV-specific T-cell responses and systemic or local viral replication were evaluated together, it would be possible to predict the host immunomodulatory or immunopathological effects of CMV infection as well as the response to steroid treatment in UC patients with CMV colitis. However, we found that host immune responses were not independently related to a poor response to steroid treatment. However, systemic viral replication was associated with a poor response to steroid treatment. Recent studies have indicated that measuring CMV-specific T cells may provide important information about CMV infection and help predict the risk of developing CMV disease, especially in immunocompromised patients, such as those with hemato-oncologic malignancies or transplant recipients [8, 9]. To the best of our knowledge, few studies have used CMV-specific T-cell response assays to evaluate CMV infection in IBD patients. Nowacki et al. showed that CMV-specific granzyme B and perforin-producing CD8 T cells in blood were elevated in IBD patients. In addition, active long-lasting IBD exhibited increased CMV expression, although CMV DNA could not be detected in the blood by qPCR analysis during flares. However, their study compared IBD patients with healthy controls and failed to establish whether CMV reactivation was a cause or consequence of IBD or whether increased CMV-specific T-cell activity was directly associated with CMV colitis [25]. In the present study, we performed CMV-specific ELISPOT to determine whether the IE-1 protein and pp65 were associated with steroid-refractory UC; however, we did not obtain any evidence that the decreased T-cell response to CMV was associated with a poor response to steroid treatment in patients with moderate to severe UC. This discrepancy between our study and that of Nowacki et al. may be due to the difference in the level of immunosuppression between hemato-oncologic malignancy patients/transplant recipients and UC patients.

Previous studies have indicated that higher CMV load is associated with a poor clinical outcome in patients with IBD [20–22]. Recent studies also showed that positive CMV DNA expression in colonic tissue via PCR is associated with a longer time to steroid-free UC remission, with an increased rate and earlier need for proctocolectomy [23, 24]. In the current study, colonic tissue CMV viral load was not independently associated with a poor steroid response in moderate to severe UC. We assumed that expression of CMV DNA in colonic tissue could not distinguish active CMV colitis from CMV colonization or past CMV infection. Instead, high CMV expression in the blood via qPCR was independently associated with a poor response to steroid therapy, which is consistent with a previous study reporting that CMV antigenemia was associated with subsequent colectomy [14]. CMV infection or reactivation may induce persistent immunological dysfunction, which not only plays a key role in the aggravation of UC but is also related to steroid refractoriness [26]. We therefore speculate that evidence for systemic CMV infection or reactivation, such as CMV viremia, may reflect this pathophysiologic sequence. We suggest that, of the various laboratory parameters, simple and noninvasive quantitative CMV PCR using blood samples could represent the most useful strategy to investigate CMV infection in patients with moderate to severe UC.

There were, however, several limitations to this study. First, the seropositivity of CMV is high in Korea; thus, caution needs to be exercised while applying our findings to other populations. Second, although both pp65 and IE-1 are considered to play an important role in targeting T cells in CMV infections [9], the optimal target antigens for achieving a protective T-cell response in CMV infection are not known. Therefore, further studies are needed before applying our findings to other target antigens. Third, we measured CMV-specific cell-mediated immunity only at the time of steroid treatment. Thus, we could not assess whether CMV-specific immune responses were already affected before steroid therapy or in mild to moderate UC. Cell-mediated immunity is not static, and dynamic changes in CMV cell-mediated immunity may depend on the severity of UC or steroid treatment. Fourth, some host and viral risk factors for CMV were statistically significant in the univariate but not in the multivariate analysis. This may be attributed to the small sample size and heterogeneity of disease severity, which may have reduced the statistical power. In addition, although a trend was observed in the proven CMV colitis patients with steroid refractory moderate to severe UC, the small sample size is a barrier to identifying any association between CMV colitis and response to steroid treatment in these patients.

In conclusion, in patients with moderate to severe UC, higher disease severity and positive blood CMV expression by qPCR rather than high local CMV replication or low systemic CMV-specific T-cell response appear to be independently associated with steroid refractoriness.

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