CHC and pyoderma gangrenosum-like lesions was suspected.[12]

In this study, we analyzed sequentially various kinds of immune cells including activated $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, activated B cells, Th1 cells, Th2 cells, Th17 cells and $CD4^+$ $CD25^+$ interleukin (IL)-$7R^-$ Treg. Moreover, the detection of strand-specific HCV RNA was carried out because we previously reported that lymphotropic HCV could affect T-cell commitment and immunoglobulin hypermutation.[3,4,6,7,13,14]

METHODS

Patient

A CHC PATIENT with severe pyoderma gangrenosum-like lesions was enrolled in this study. The pyoderma gangrenosum-like lesions were diagnosed by the clinical phenotype and non-specific inflammation of skin lesions in the Division of Dermatology, Tohoku University Hospital. Although this patient was treated with steroid and skin transplantation, the skin condition was not improved. Therefore, we concluded that this patient needed antiviral treatment to remove the effects of HCV. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2006-194). Written informed consent was obtained from this patient. The patient was monitored for 3 years, and peripheral blood samples were obtained and assessed during PEG IFN/RBV treatment. At each assessment, the patient was evaluated for the serum levels of HCV RNA, blood chemistry and hematology. The liver histology was analyzed by the METAVIR score at the start of PEG IFN/RBV therapy.

Detection of IL-28B polymorphism

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMC) using an automated DNA isolation kit. Then, the polymorphism of IL-28B (rs8099917) was analyzed using real-time polymerase chain reaction (PCR; TaqMan SNP Genotyping Assay, Applied Biosystems, NY, USA). The detection of IL-28B polymorphism was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2010-323)

Isolation of PBMC, $CD4^+$ T cells and $CD19^+$ B cells, and flow cytometry analysis

Peripheral blood mononuclear cells were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation. PBMC were stained with CD3, CD4, CD8, CD19, CD25, CD40, CD86, IL-7R and HLA-DR antibodies (BD Pharmingen, San Jose, CA, USA) for 15 min on ice to analyze the $CD3^+$ $HLA\text{-}DR^+$ cells, $CD3^+$ $CD4^+$ $HLA\text{-}DR^+$ cells, $CD3^+$ $CD8^+$ $HLA\text{-}DR^+$ cells, $CD19^+$ $CD40^+$ cells, $CD19^+$ $CD86^+$ cells and $CD3^+$ $CD4^+$ $CD25^+$ IL-$7R^-$ Treg. Isotype-matched control antibodies were used for adjustment of the fluorescence intensity. The frequencies of the immune subsets were analyzed using FACS Canto-II (BD Biosciences, San Jose, CA, USA).

IFN-γ, IL-10 and IL-17A cytokine secretion assay

Peripheral blood mononuclear cells were stimulated with CD3- and CD28-coated beads (Miltenyi Biotec, Gladbach, Germany) for 12 h. Cells were washed by adding 2 mL of cold phosphate-buffered saline and resuspended in 90 μL of cold RPMI-1640 medium. After the addition of 10 μL of IL-10, IFN-γ or IL-17A Catch Reagent (Miltenyi Biotec), cells were incubated for 5 min on ice. Thereafter, the cells were diluted with 1 mL of warm medium (37°C) and further incubated in a closed tube for 45 min at 37°C under slow, continuous rotation. The cells were washed and IL-10- or IFN-γ-secreting cells were stained by adding 10 μL of IL-10, IFN-γ or IL-17A Detection Antibody (PE conjugated or APC conjugated; Miltenyi Biotec) together with anti-CD4-PerCP.

Quantification of IL-17A, IL-21 and IL-23

The amounts of IL-17A, IL-21 and IL-23 were quantified using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA). The serum samples were collected at sampling points and stored at −20°C. The ELISA procedure was performed according to the manufacturer's protocol. The IL-17A, IL-21 and IL-23 sample concentrations were calculated using a standard curve.

Strand-specific intracellular HCV RNA detection

Strand-specific intracellular HCV RNA was detected using a recently established procedure that combines methods published elsewhere, with minor modification. Positive strand-specific and negative strand-specific HCV RNA were detected using a nested PCR method. Reactions were performed with 2 μL of 10 × reverse transcriptase (RT) buffer, 2 μL of 10 mmol/L magnesium chloride, 200 μmol/L each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxygua-

nosine triphosphate, 100 μmol/L of thymidine triphosphate (dTTP), 0.2 U of uracil-N glycosylase (UNG; Perkin Elmer [Fremont, CA, USA]/Applied Biosystems, Foster City, CA, USA), 5 U of rTth DNA polymerase; and 50 pmol of strand-specific HCV primers (position according to the 5′-untranslated region), nt −285 to −256 (ACTGTCTTCACGCAGAAAGCGTCTAGCCAT) and −43 to −14 (CGAGACCTCCCGGGGCACTCG CAAGCACCC), and template RNA. The RT mixture was incubated for 10 min at room temperature and then at 70°C for an additional 15 min. The cDNA product was subjected to the first PCR with 80 μL of PCR reaction buffer containing 50 pmol of HCV downstream strand-specific primer. The PCR amplification consisted of 5 min of 95°C, followed by 35 cycles and then 7-min extension at 72°C. For the second nested PCR, an aliquot (1/10) of the first PCR reaction mixture was re-amplified using 50 pmol of each of two primers, nt −276 to −247 (ACGCAGAAAGCGTCTAGCCATGGCGT TAGT) and nt −21 to −50 (TCCCGGGGCACTCGCAAG CACCCTATCAGG), which span the 255-base pair region nt −276 to −21 of HCV RNA, and Taq polymerase (Applied Biosystems). The reaction was run for 35 cycles. Semiquantification was achieved by serial four-fold dilution. The relative titer was expressed as the highest dilution giving a visible band of the appropriate internal control; semiquantification of β-actin mRNA was performed using the same RNA extracts.

Table 1 Biochemical, hematological, virological laboratory test

Laboratory test before the immunosuppressive therapy for pyoderma gangrenosum					
WBC	7600 uL	T-Bil	0.4 mg/dL	IgG	954 mg/dL
RBC	396 10^4/ul	D-Bil	0.1 mg/dL	IgA	259 mg/dL
Hb	12.4 g/dL	ALP	294 IU/L	IgM	439 mg/dL
HCT	37.2%	γ-GTP	52 IU/L		
MCV	94 fl	AST	36 IU/L	RF	746.2 U/L
MCH	31.4 pg	ALT	39 IU/L		
MCHC	33.4%	LDH	162 IU/L	C3	81 mg/dL
PLT	297 × 10^3/uL	ChE	304 IU/L	C4	4.3 mg/dL
				CH50	27.7/mL
PT-INR	0.97	TP	6.8 g/dL		
		ALB	3.8 g/dL	Cryo	+
Na	144 mEq/L				
K	4 mEq/L	HCV RNA	6.2 log copies/mL	ANA	<79
Cl	111 mEq/L	HCV Genotype	1b	M2	5(−)
Liver histology	METAVIR A2/F2				
IL-28B SNP (rs8099917)	T/T				

Sampling points	1	2	3	4
T-Bil (mg/dL)	0.4	0.5	0.4	0.5
D-Bil (mg/dL)	0.1	0.1	0.1	0.1
ALP (IU/L)	294	419	320	322
γ-GTP (IU/L)	52	45	28	18
AST (IU/L)	36	49	24	15
ALT (IU/L)	39	40	16	11
RF (U/L)	746.2	671.5	648.5	648.5
C3 (mg/dL)	81	88	82	95
C4 (mg/dL)	4.3	6.5	7.2	9.4
CH50 (/mL)	27.7	41.2	39.8	48.2

1. Before the immunosuppressive therapy for pyoderma gangrenosum.
2. Before the PEG IFN/RBV therapy for HCV eradication.
3. Just after the PEG IFN/RBV therapy for HCV eradication.
4. Six months after the PEG IFN/RBV therapy for HCV eradication (SVR).

ALT, alanine aminotransferase; Hb, hemoglobin; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

RESULTS

Clinical course of a CHC patient with pyoderma gangrenosum-like lesions

REPRESENTATIVE BIOCHEMICAL AND hematological analyses at several time points are shown (Table 1). The polymorphism of IL-28B (rs8099917) is of T/T major type. The patient (patient A) had genotype 1b and high HCV RNA titers (6.1 log copies/mL) before the immunosuppressive therapy for pyoderma gangrenosum-like lesions. Cryoglobulin could be detected at several time points. The rheumatoid factor was 746.2. The activity of the pyoderma gangrenosum-like lesions was high at this time point (Fig. 1a). Therefore, immunosuppressive therapy (cyclosporin) was started. During the immunosuppressive therapy, the activity of the pyoderma gangrenosum-like lesions did not improve. Then, we started the PEG IFN/RBV therapy to eradicate the HCV because the relevance of HCV to the pyoderma gangrenosum-like lesions was suspected. The titers of HCV RNA rapidly declined after the start of PEG IFN/RBV therapy. The patient achieved a sustained virological response (SVR) after the 48 weeks of treatment with PEG IFN/RBV. After the achievement of SVR, the pyoderma gangrenosum-like lesions rapidly improved without immunosuppressive therapy (Fig. 1a).

Strand-specific HCV RNA detection in lymphoid cells

Then, we carried out strand-specific nested PCR to detect the HCV RNA using a method previously published by

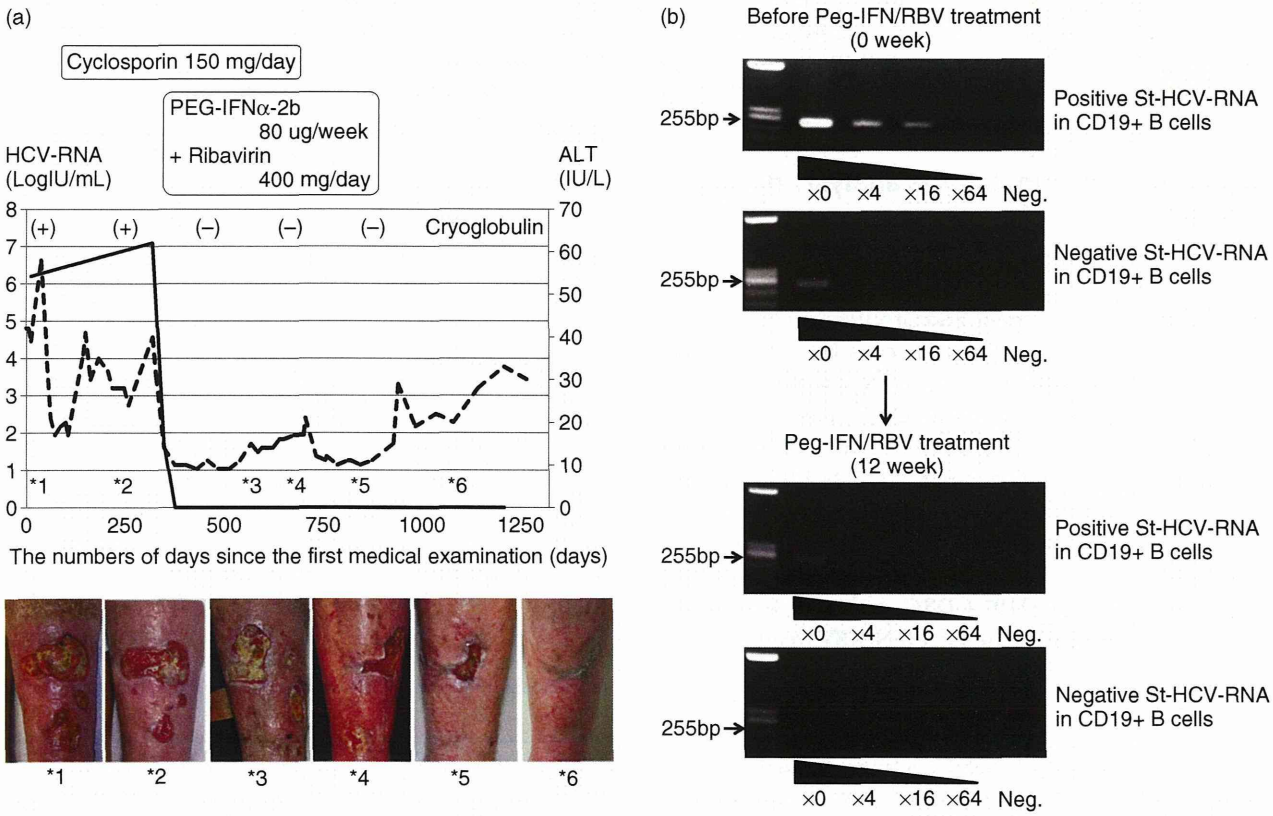


Figure 1 Clinical data and condition of the pyoderma gangrenosum-like lesions during the cyclosporin and/or pegylated interferon (PEG IFN)-α-2b/ribavirin (RBV) treatment. (a) Titers of hepatitis C virus (HCV) RNA and the level of alanine aminotransferase (ALT) are shown in the upper graph. This graph includes the positivity of cryoglobulin in the serum. Pictures of the same site of the pyoderma gangrenosum-like lesions are shown in the lower part of the figure. The numbers located at the lower part of the pictures and in the graph indicate the time points during treatment and follow up. (b) Representative polymerase chain reaction (PCR) bands that show the semiquantification of strand-specific HCV RNA detection in $CD19^+$ B cells are shown. The size of target PCR bands is 255 base pairs. For negative control, extracted HCV RNA was run in every reverse transcription PCR test without an upstream HCV primer. ——, HCV RNA; – – –, ALT.

Table 2 Detection of strand-specific HCV RNA in the $CD4^+$ T cells and $CD19^+$ B cells

	Time point	4 weeks before treatment	0 weeks	12 weeks	48 weeks
$CD4^+$ T cells	Positive St-HCV RNA	4	4	1	0
	Negative St-HCV RNA	1	1	0	0
$CD19^+$ B cells	Positive St-HCV RNA	16	16	1	0
	Negative St-HCV RNA	1	1	0	0

The titers of HCV RNA were expressed as the highest dilution giving a visible band of the correct size. The data of two independent studies had same results.
HCV, hepatitis C virus; St, strand.

our group. The detection of negative-strand HCV RNA indicates the existence of HCV RNA replication in the cells. The positive rate of negative strand lymphotropic HCV RNA among general CHC patients was approximately 10% (unpubl. data, Kondo *et al.*). We could detect positive and negative strand-specific HCV RNA in lymphoid cells ($CD4^+$ T cells and $CD19^+$ B cells) at two points before the PEG IFN/RBV treatment (Table 2) (Fig. 1b). After the eradication of HCV, we could not detect positive and negative strand-specific HCV RNA in the lymphoid cells or serum.

Sequential immunological analysis during PEG IFN/RBV treatment

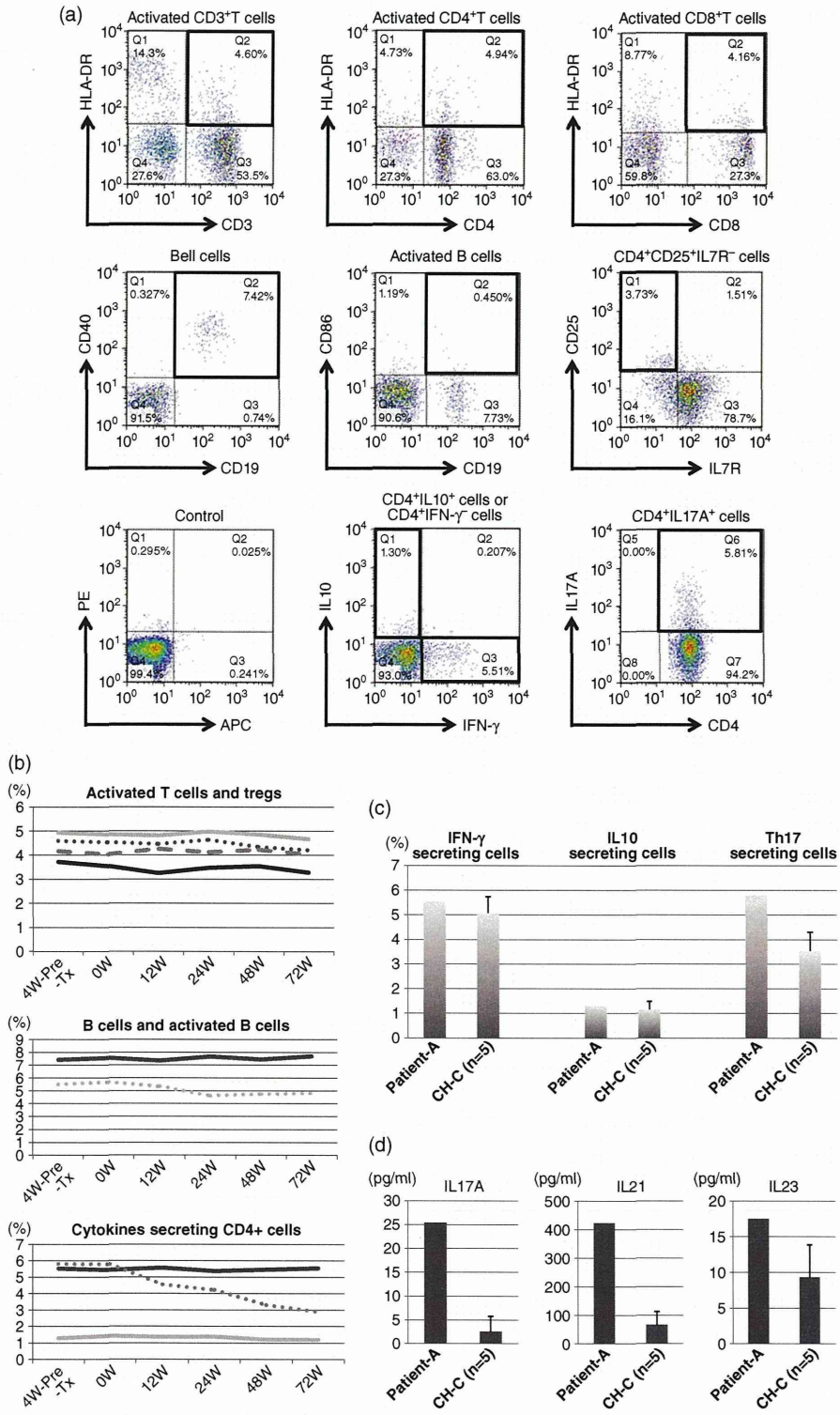
We analyzed various kinds of lymphoid cells, because we previously reported that lymphotropic HCV could affect the Th commitment and proliferation.

The frequencies of activated T cells ($HLA\text{-}DR^+$ $CD3^+$, $CD4^+$ or $CD8^+$ T cells), Th1 cells (IFN-γ-secreting $CD4^+$ T cells), Th2 cells (IL-10-secreting $CD4^+$ cells), Treg ($CD4^+$ $CD25^{high}$ $IL\text{-}7R^-$ cells) and $CD19^+$ $CD40^+$ B cells were not remarkably changed during the PEG IFN/RBV treatment (Fig. 2a,b). On the other hand, the frequency of Th17 cells (IL-17A-secreting $CD4^+$ T cells) was remarkably decreased after the PEG IFN/RBV treatment (Fig. 2b). The frequency of $CD19^+$ $CD86^+$ activated B cells was slightly decreased after the PEG IFN/RBV treatment. The frequency of Th17 cells in patient A was remarkably high in comparison to those in CHC patients ($n = 5$) without extrahepatic immune-related diseases (Fig. 2c). The time point of PBMC sampling was before the PEG IFN/RBV treatment (Fig. 2c). All five CHC patients included in this study had high viral loads (>5 log copies/mL), IL-28B T/T, genotype 1b HCV RNA. All CHC patients were male. Moreover, we analyzed the amounts of Th17-related cytokines (IL-17A, IL-21 and IL-23) using ELISA. Patient A had remarkably higher amounts of these cytokines (IL-17A, IL-21 and IL-23) in comparison to the control CHC patients (Fig. 2d). Some of the control patients had lower than measurable amounts of these cytokines (Fig. 2d). Therefore, we used reference amounts of cytokines calculated using a standard curve.

DISCUSSION

IT HAS BEEN reported that various kinds of extrahepatic disease could be induced by persistent infection of HCV.[15,16] Among the extrahepatic diseases, cryoglobulin and autoimmune-related diseases including pyoderma gangrenosum were most frequently detected in CHC patients.[12,17,18] The relationship between cryoglobulin and Th17 cells has been studied in our group (unpubl. data, Kondo *et al.*).[19] It has been reported that

Figure 2 Immunological analysis during pegylated interferon (PEG IFN)-α-2b/ribavirin (RBV) treatment in patient A. (a) Representative dot plots indicating various kinds of immune cell subsets and cytokine-secreting cells are shown. Bold boxes indicate the target subsets in the quadrant of dot plots. (b) Sequential immunological data are shown. The frequencies of various kinds of immune cell subsets are shown. (c) The frequencies of cytokine-secreting cells were compared between patient A and chronic hepatitis C (CHC) without extrahepatic diseases ($n = 5$). (d) Amounts of interleukin (IL)-17A, IL-21 and IL-23 are shown in the bar graph. Some of the control patients had lower than measurable amounts of these cytokines. Therefore, we used reference amounts of cytokines calculated using a standard curve. Error bars indicate standard deviation. • • • •, $CD3^+$ $HLA\text{-}DR^+/CD3^+$ ×100; ▬, $CD4^+$ $HLA\text{-}DR^+/CD4^+$ ×100; ▬ ▬, $CD8^+$ $HLA\text{-}DR^+/CD8^+$ ×100; ▬▬, $CD4^+$ $CD25^{high}$ $IL\text{-}7R^-/CD4^+$ ×100; ▬▬, $CD19^+$ $CD40^+$ B cells/lymphocytes ×100; ◦ ◦ ◦ ◦, $CD19^+$ $CD86^+$ B cells/$CD19^+$ ×100; ▬▬, $CD4^+$ $IFN\text{-}\gamma^+$ cells/$CD4^+$ ×100; ▬, $CD4^+$ $IL\text{-}10^+$ cells/$CD4^+$ ×100; ◦ ◦ ◦ ◦, $CD4^+$ $IL\text{-}17A^+$ cells/$CD4^+$ ×100.



Th17 cells were associated with the immunopathogenesis of autoimmune diseases.[20–22] Previously, we reported that lymphotropic HCV could affect the commitment of helper T cells, T-cell proliferation, and immunoglobulin-hypermutation that may affect the dysregulation of the immune system.[3,4,6,13,19] The biological significance of lymphotropic HCV could be its contribution to not only the mechanism of HCV-persistent infection but also the induction of extrahepatic diseases including pyoderma gangrenosum, because cryoglobulin and the dysregulation of T cells may be involved in the pathogenesis of pyoderma gangrenosum. In patient A, the frequency of Th17 cells was remarkably decreased after the eradication of HCV. During the immunosuppressive therapy, the titers of HCV RNA slightly increased. Therefore, the modulation of the immune system directly induced by HCV had an important role in the induction of pyoderma gangrenosum-like lesions. After the eradication of HCV, the pyoderma gangrenosum-like lesions completely recovered without immunosuppressive therapy. These observations suggest that the eradication of HCV is a significant treatment option for extrahepatic diseases with dysregulation of the immune system in CHC patients. However, we could not analyze the basal level of the immune response without immunosuppressive therapy due to the lack of PBMC samples. We could not exclude the contribution of other immune cells in addition to Th17 cells to the induction of pyoderma gangrenosum-like lesions, because the administration of cyclosporin could suppress the T-cell response. The frequencies of IFN-γ- and IL-10-secreting cells in patient A were comparable to those of the CHC patients without immunosuppressive therapy. Therefore, the immune response in patient A might have been strongly activated in comparison to the CHC patients without autoimmune-related diseases.

In conclusion, we sequentially examined the existence of lymphotropic HCV and the immune response during PEG IFN/RBV treatment for difficult-to-treat pyoderma gangrenosum-like lesions. The results suggest the relevance of the frequency of Th17 cells to the activity of pyoderma gangrenosum-like lesions in CHC patients. Moreover, the existence of lymphotropic HCV may be involved in this phenomenon.

ACKNOWLEDGMENT

THIS WORK WAS supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sport, Science, and Technology of Japan (Y. K., no. 23790861).

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