Plasmacytoid Dendritic Cells in the Duodenum of Individuals Diagnosed with Myalgic Encephalomyelitis Are Uniquely Immunoreactive to Antibodies to Human Endogenous Retroviral Proteins

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Abstract. *Myalgic encephalomyelitis (ME) is a debilitating illness of unknown etiology characterized by neurocognitive dysfunction, inflammation, immune abnormalities and gastrointestinal distress. An increasing body of evidence suggests that disruptions in the gut may contribute to the induction of neuroinflammation. Therefore, reports of human endogenous retroviral (HERV) expression in association with neuroinflammatory diseases prompted us to investigate the gut of individuals with ME for the presence of HERV proteins. In eight out of 12 individuals with ME, immunoreactivity to HERV proteins was observed in duodenal biopsies. In contrast, no immunoreactivity was detected in any of the eight controls. Immunoreactivity to HERV Gag and Env proteins was uniquely co-localized in hematopoietic cells expressing the C-type lectin receptor CLEC4C (CD303/BDCA2), the co-stimulatory marker CD86 and the class II major histocompatibility complex HLA-DR, consistent with* plasmacytoid dendritic cells (pDCs). Although the significance of HERVs present in the pDCs of individuals with ME has yet to be determined, these data raise the possibility of an involvment of pDCs and HERVs in ME pathology. To our knowledge, this report describes the first direct association between pDCs and HERVs in human disease.*

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Myalgic encephalomyelitis (ME) is a debilitating disorder characterized by multi-systemic neuropathology, gastro-intestinal (GI) dysfunction, inflammation, and innate immune dysregulation (1). Immunological symptoms often include viral reactivation, cytokine and chemokine irregularities, and decreased natural killer (NK) cell function (2-7). Additionally, reports of individuals with ME-expressing autoantibodies (8, 9), and the successful treatment of ME with the B-cell-depleting drug rituximab (10, 11), suggest that a subset of these individuals may suffer from an uncharacterized antibody-mediated autoimmunity.

Little is known regarding the pathophysiology of ME; therefore, diseases with similar or overlapping symptoms often serve as useful guides when exploring new experimental concepts. For instance, autoimmune diseases such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE) have many symptoms that overlap with those of ME. Neurological manifestations often associated with ME (12), are analogous to the neuroinflammation and cognitive abnormalities associated with MS and SLE (13, 14). Additionally, GI aberrations, which are common to individuals with MS and SLE (15-17), are among the most frequent symptoms reported by those with ME (18, 19).

The gut-associated lymphoid tissue represents the largest immune compartment in the body. In fact, it has been estimated that more than 60% of all T-cells may reside within the small intestine (20), emphasizing the potential contribution of the gut to systemic immunity. Indeed, increases in serum bacterial by-products, particularly lipopolysaccharides resulting from bacterial translocation in the gut, are found to be associated with systemic immune activation in many diseases such as HIV/AIDS, inflammatory bowel disease (IBD), and acute graft-versus-host disease (21-23). Extraintestinal immune dysregulation originating within the gut is described in such diseases as HIV/AIDS and idiopathic lymphocytopenia (24, 21). Although, its contribution to neuroimmune disease in humans is largely unknown, recent studies using animal models support a connection between GI immunity and neuroinflammation. Lee and colleagues reported intestinal microbiota to significantly influence the balance between pro-inflammatory and anti-inflammatory immune responses during the induction of experimental autoimmune encephalomyelitis, an animal model for MS (25). While limitations always exist when using animal models to study human disease, these data clearly support the concept of neuroinflammation associated with alterations in the gut.

Emerging evidence supports a role for human endogenous retroviruses (HERVs) in the etiopathology of autoimmune diseases such as MS and SLE (26). HERVs are remnants of ancient retroviral infections that integrated into the germ line and are now transmitted vertically (27). Not including the long interspersed elements (LINE) and other retrotransposons, HERVs constitute approximately 8% of the human genome (28). Although HERV proteins are self-antigens and should not induce an immune response, HERV proteins and serum antibodies against HERVs have been associated with a number of autoimmune diseases, including MS and SLE (29-32). These associations have motivated researchers to search for a role of HERV proteins in human pathology. Although associations are often difficult to render into definitive causation, it is widely believed that the humoral immune response against HERV proteins leads to autoimmunity through a process of molecular mimicry (33). HERV proteins are also known to act as superantigens, which have the ability to cause polyclonal T-cell activation and massive cytokine production (34). In 2001, Perron and colleagues reported the potential immunopathogenic properties of the HERV-W envelope protein, as a major proinflammatory and superantigenic determinant associated with MS (35).

In summary, individuals with ME have a significant number of symptoms that are similar to those described in autoimmune diseases such as MS and SLE. Additionally, the expression of HERV proteins has been observed in the lymphoid tissue of individuals with autoimmune disease.

Finally, the gut represents the largest lymphoid compartment and is a significant site of ME-related pathology. For these reasons, we sought to determine if endogenous retroviral proteins could be detected in GI tissue of those with ME.

Materials and Methods

Clinical evaluation. Biopsies were acquired as existing de-identified surplus clinical diagnostic specimens, under an exemption to institutional review board (IRB) approval as determined by the University of Nevada, Reno Office of Human Research Protection. Therefore, patients' information was limited to a general pathological description by which the specimens were grouped prior to de-identification. ME specimens were from 12 individuals fulfilling both the Canadian consensus criteria of ME (36), as well as the less restrictive and overlapping Fukuda criteria for chronic fatigue syndrome (CFS) (37). Fecal microbial analysis established the presence of substantial disruption of gut microbiota composition, with occurrence of a higher load of microbes with higher pathogenic potential in all ME cases, particularly of the *Enterococcus*, *Streptococcus*, and *Prevotella* genera. Gastritis (mainly antritis) was present in all cases and routine histological examination showed a lympho-plasmatic infiltrate in the sub-mucosa in all specimens. All cases tested negative for *Helicobacter pylori* and all individuals with ME tested negatively for HIV. Controls were eight anonymous individuals without symptoms of ME who underwent routine gastroscopy for epigastric pain.

Tissues and preparation. Punch biopsies were obtained from the duodenum and stomach of ME cases and controls. Fresh tissues were fixed in 4% paraformaldehyde for 4 h at 4°C and cryoprotected with a 30% sucrose solution in phosphate-buffered saline (PBS).

Immunohistochemical analysis. Immunohistochemical staining was performed on 0.5-μm-thick tissue sections. Tissue slides were de-paraffinized with xylene and rehydrated through a graded alcohol series. Antigen retrieval was carried out by boiling slides in sodium citrate (0.01 M, pH 6.0) at 95°C for 10 min. The slides were next rinsed in PBS and incubated in cold methanol for 20 min at –20°C. Tissue sections were then incubated with serum (matching the host of the secondary antibody) to block non-specific staining (1 h at 37°C) and then incubated with the primary antibody overnight at 4°C in a humidified chamber. After washing 3 times with PBS containing 0.1% Tween 20, the sections were incubated with the secondary antibody for 1 h at 37°C. All cases and controls were analyzed for the presence of HERV and gamma-retroviral Env and Gag proteins. Isotype-matched controls or secondary-only controls were included with all experiments. A summary of each antibody and the concentration at which it was used are presented in Table I. Slides were examined using a Zeiss LSM 7000 scanning laser confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) and images were captured with the Zeiss Zen 2009 analysis software.

Statistical analysis. Statistical analysis of ME cases and controls for the presence of HERV proteins was performed using the Chi-square method. The non-parametric Mann Whitney method was used to analyze for the absolute differences in gut-associated plasmacytoid dendritic cells (pDCs).

Table I. *Antibodies and dilutions*.

Antibody	Source	Dilution used
Mouse anti-HERV-K-Gag IgG1 (clone 5i75) mAb	US Biological, Salem, MA, USA	1:500
Mouse anti-HERV-K18.1-Env IgG pAb	Ab Cam, Cambridge, MA, USA	1:500
Rabbit anti-HERV-R-Env IgG pAb	Ab Cam, Cambridge, MA, USA	1:500
Rabbit anti-HERV-FRD-Env IgG pAb	Ab Cam, Cambridge, MA, USA	1:500
Rat anti-SFFV-gp52 IgG1κ (clone 7C10) mAb[1]	S. Ruscetti, NCI, Frederick, MD, USA	1:500
Goat anti-MLV-Gag IgG pAb	S. Ruscetti, NCI, Frederick, MD, USA	1:500
FITC Mouse anti-Human CD303 (clone AC144) mAb	MiltenyBiotec, Auburn, CA, USA	1:500
Mouse anti-Human CD45 IgG1 (clone Ros220) mAb	Beckman Coulter, Brea, CA, USA	1:500
FITC Mouse anti-Human CD86 IgG1κ (clone 2231)	BD Pharmigen, San Jose, CA, USA	1:500
FITC Mouse anti-Human HLA-DR (clone AC122)	MiltenyBiotec, Auburn, CA, USA	1:500
Rat anti-HHV-8 ORF73 (clone LN-53) mAb	Advanced Bio., Columbia, MD, USA	1:1000
Alexa647 Rat IgG1κ (clone R3-34) mAb isotype control	BD Pharmigen, San Jose, CA, USA	1:500
Rat IgG1κ mAb isotype control	eBiosciences, San Diego, CA, USA	1:500
Mouse IgG1 isotype control	Ab Cam, Cambridge, MA, USA	1:500
APC Mouse IgG1κ isotype control	BioLegdend, San Diego, CA, USA	1:500
Rhodamine Donkey anti-Rat IgG pAb[2]	American Qualex, San Clemente, CA, USA	1:1000
FITC Donkey anti-Mouse IgG pAb[3]	Jackson Immuno., West Grove, PA, USA	1:1000
Dylight594 Donkey anti-Mouse IgG pAb[3]	Jackson Immuno., West Grove, PA, USA	1:500
Dylight488 Bovine anti-Goat IgG pAb[3]	Jackson Immuno., West Grove, PA, USA	1:500
Rhodamine Goat anti-Rabbit IgG pAb[2]	American Qualex, San Clemente, CA, USA	1:500
Normal goat serum	Invitrogen, Carlsbad, CA, USA	1:2000

[1]Conjugated to rhodamine by American Qualex; [2]Absorbed to cross-reactive species; [3]Minimum cross-reactivity reported by manufacturer; mAb (monoclonal antibody), pAb (polyclonal antibody).

Results

Detection of immunoreactive proteins in gut biopsies. Immunochemical analyses of 12 ME gut biopsies probed for viral antigens showed that eight samples of the duodenum were immunoreactive to antibodies raised against HERV proteins (Figure 1A-D). In contrast, no immunoreactivity was observed in any of the control duodenum samples (Figure 1E-H, *p*=0.003 by Chi-square). Additional analysis was conducted using two anti-gammaretroviral antibodies: goat polyclonal IgG antibody raised against the Gag protein of murine leukemia virus (Figure 2A) and a rat monoclonal IgG1κ antibody (clone 7C10) raised against the Env protein of spleen focus forming virus (Figure 2B). The observed immunoreactivity was reproducibly consistent with the previous anti-HERV results, suggesting that the anti-gammaretroviral antibodies were cross-reactive with the HERV antigen(s). Additionally, the immunoreactivity was observed to co-localize (Figure 2C) in cells with an eccentric nucleus and granular inclusions (Figure 2D). Consistent with previous observations using antibodies to HERVs, no immunoreactivity was observed in the control biopsies using either anti-gammaretroviral antibody (Figure 2E-G). Matched stomach biopsies collected from ME cases and controls were also analyzed, but were consistently non-reactive when probed with the same anti-HERV and anti-gammaretroviral antibodies (data not shown).

Specificity of the primary anti-gammaretroviral antibodies was established when their ability to detect Env and Gag proteins was ablated by pre-incubation with purified and heat-deactivated virus derived from 22Rv1 (Figure 3). In order to rule out non-specific binding of the secondary antibody, the rat monoclonal IgG1κ antibody to Env (7C10), was directly labeled with rhodamine and used to probe the tissue sections (Figure 4A). Potential non-specific binding of the secondary antibody was additionally excluded by using the rhodamine-conjugated donkey anti-rat IgG and the Alexa647-conjugated donkey anti-goat IgG secondary antibodies in the absence of the primary antibody (Figure 4B). Non-specific binding of the primary rat monoclonal antibody was further ruled out by the lack of immunoreactivity by using an irrelevant rat monoclonal antibody (rat anti-human herpes virus-8 UL84 IgG) and a rat IgG1κ isotype control (Figure 4C and D). Potential non-specific binding of the secondary antibody was excluded by replacing the primary antibody with normal goat serum (Figure 4E). Finally, to address the potential cross-reactivity of the secondary antibody with the incorrect primary antibody (for example, donkey anti-goat IgG secondary antibody cross-reacting with the rat IgG1κ primary antibody to Env), biopsies were probed with a primary antibody followed by incubation with the mismatched secondary antibody (Figure 4F and G).

Phenotype of the immunoreactive cells. In order to determine the phenotype of the immunoreactive cells, we first performed
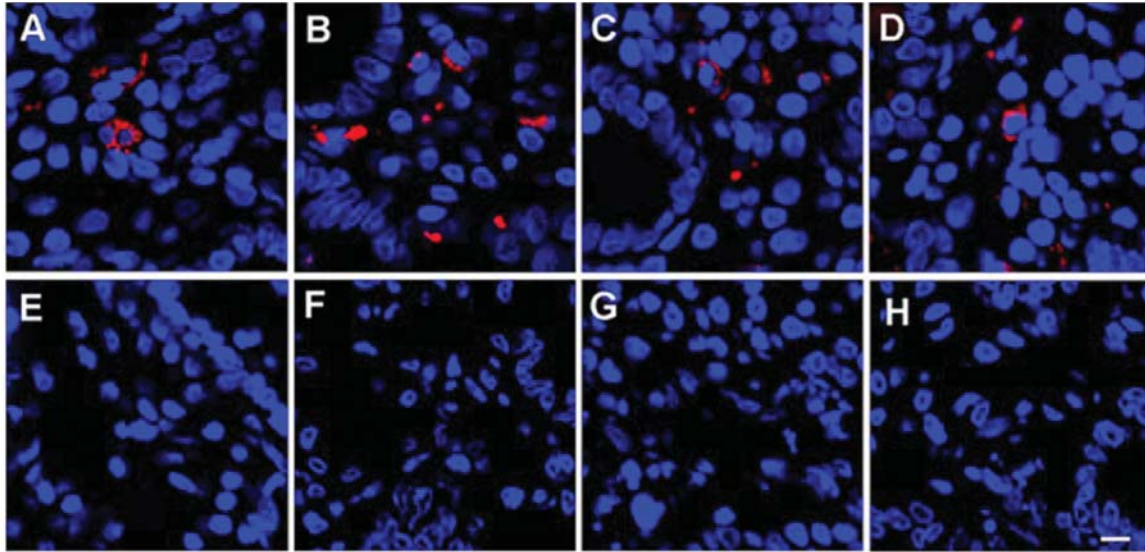
Figure 1. *Immunoreactivity to monoclonal (mAb) and polyclonal (pAb) antibodies against human endogenous retrovirus (HERV) in a duodenal biopsy of representative individuals. A: Immunoreactivity to anti-HERV-K Gag IgG1 mAb (Dylight594 donkey anti-mouse IgG secondary) in a duodenal biopsy of an ME case. B: Immunoreactivity to anti-HERV-K18.1 Env IgG pAb (Dylight594 donkey anti-mouse IgG secondary) in a duodenal biopsy of an ME case. C: Immunoreactivity to anti-HERV-FRD Env IgG pAb (rhodamine goat anti-rabbit IgG secondary in a duodenal biopsy of an ME case. D: Immunoreactivity to anti-HERV-R Env (rhodamine goat anti-rabbit IgG secondary) in a duodenal biopsy of an ME case. E: Immunoreactivity to anti-HERV-K Gag IgG1 mAb (Dylight594 donkey anti-mouse IgG secondary) in a duodenal biopsy of a control. F: Immunoreactivity to anti-HERV-K18.1 Env IgG pAb (Dylight594 donkey anti-mouse IgG secondary) in a duodenal biopsy of a control. G: Immunoreactivity to anti-HERV-FRD Env IgG pAb (rhodamine goat anti-rabbit IgG secondary) in a duodenal biopsy of a control. H: Immunoreactivity to anti-HERV-R Env (rhodamine goat anti-rabbit IgG secondary) in a duodenal biopsy of a control subject. (Bar represents 20 μm). TOPO3 was used to indicate nucleus localization in all images.*
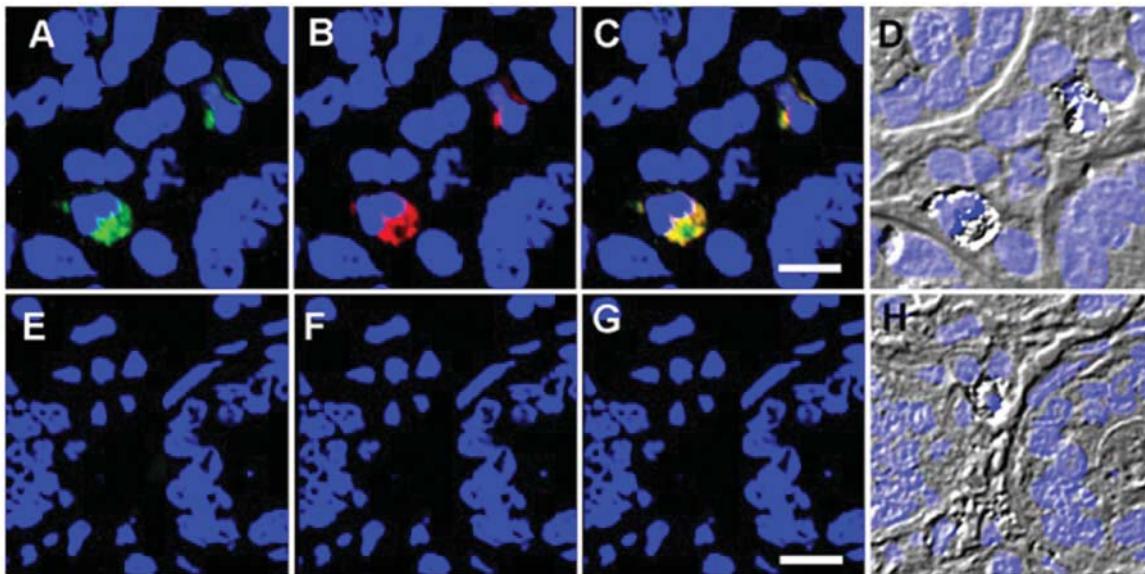

Figure 2. *Immunoreactivity to rat anti-SFFV-gp-52 Env and goat anti-MLV Gag (gamma-retroviral) antibodies. A: Immunoreactivity to goat anti-Gag IgG pAb (Dylight488 bovine anti-goat IgG pAb secondary) in a duodenal biopsy of an ME case. B: Immunoreactivity to rat anti-Env IgG1κ mAb (rhodamine donkey anti-rat IgG pAb secondary) in a duodenal biopsy of an ME case. C: Overlay of images A and B (Bar represents 20 μm). D: Morphology of the immunoreactive cells of an ME case. E: Immunoreactivity to goat anti-Gag IgG pAb (Dylight488 bovine anti-goat IgG pAb secondary) in a duodenal biopsy of a control. F: Immunoreactivity to rat anti-Env IgG1κ mAb (rhodamine donkey anti-rat IgG pAb secondary) in a duodenal biopsy of a control. G: Overlay of images E and F (Bar represents 20 μm). H: Morphology of the immunoreactive cells of a control. TOPO3 was used to indicate nucleus localization in all images.*
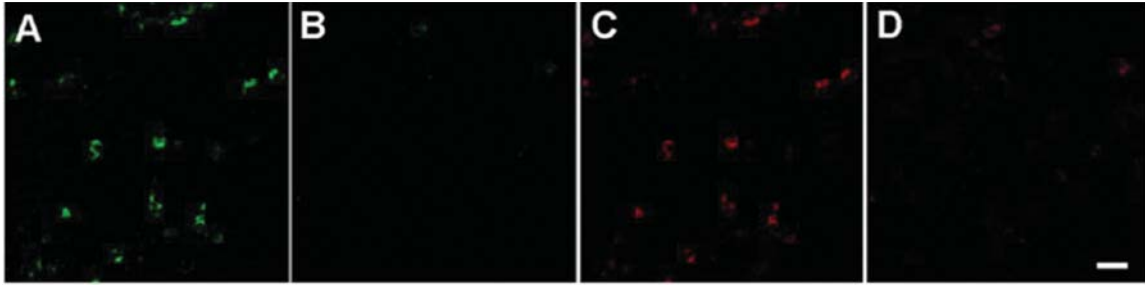
Figure 3. *Specificity of the rat anti-SFFV-gp-52 Env and goat anti-MLV Gag antibodies established by antibody depletion. Antibodies were pre-incubated (1 h, 25°C, constant shaking) with purified and heat-inactivated retrovirus derived from 22Rv1, prior to probing duodenal biopsy sections. A: Duodenal biopsies were probed with polyclonal goat antibodies to MLV Gag. B: Duodenal biopsies were probed with polyclonal goat antibodies against MLV Gag pre-incubated with heat-inactivated 22Rv1. C: Duodenal biopsies were probed with monoclonal rat antibody against SFFV-gp-52 Env. D: Duodenal biopsies were probed with monoclonal rat antibody against SFFV-gp-52 Env, pre-incubated with heat-inactivated 22Rv1 (Bar represents 20 μm).*
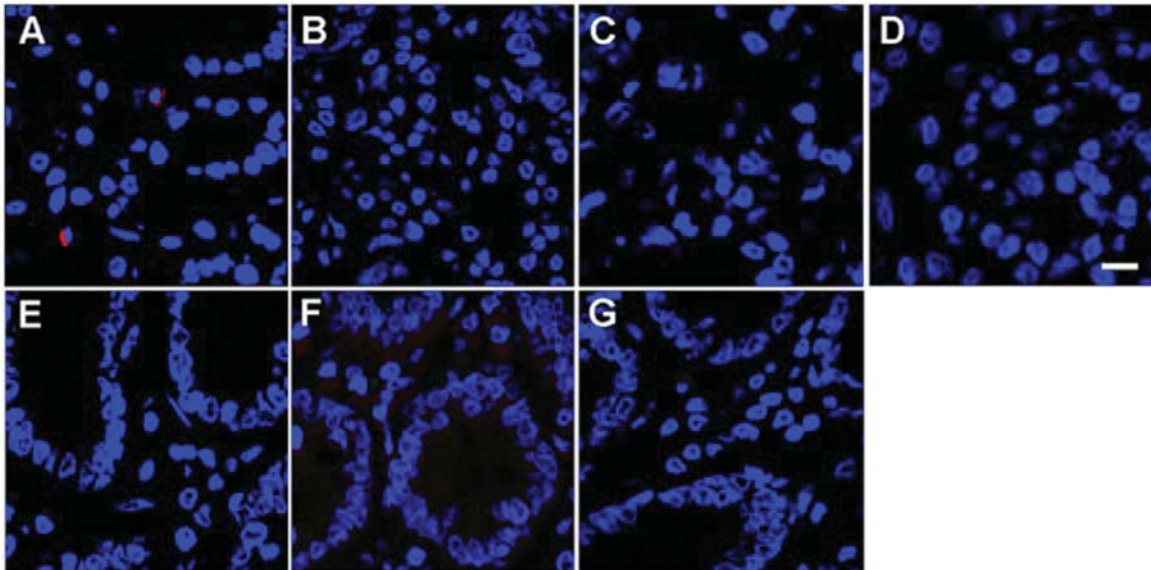

Figure 4. *Evaluation of the specificity and potential cross-reactivity of the antibodies used. A: Duodenal biopsies were probed with rhodomine-conjugated anti-SFFV-gp-52 Env IgG1κ monoclonal antibody (mAb) (1:500). B: Duodenal biopsies were probed with rhodamine-conjugated donkey anti-rat IgG polyclonal antibody (pAb) (1:1000) and Dylight488-conjugated bovine anti-goat IgG pAb (1:500). C: Duodenal biopsies were probed with rat anti-HHV8 ORF73 mAb (1:1,000), followed by incubation with rhodamine-conjugated donkey anti-rat IgG pAb (1:1,000). D: Duodenal biopsies were probed with rat IgG1κ mAb isotype control (1:500), followed by incubation with rhodamine-conjugated donkey anti-rat IgG pAb (1:1,000) (Bar represents 20 μm). E: Duodenal biopsies were probed with normal goat serum (1:2,000), followed by incubation with Dylight488-conjugated bovine anti-goat IgG pAb (1:500). F: Duodenal biopsies were probed with rat anti-SFFV-gp-52 Env IgG1κ mAb, followed by incubation with Dylight488-conjugated bovine anti-goat IgG pAb (1:500). G: Duodenal biopsies were probed with goat anti-MLV-Gag IgG pAb followed by incubation with rhodamin-conjugated donkey anti-rat IgG pAb (1:1,000). TOPO3 was used to indicate nucleus localization in all images.*

an immunohistochemical analysis using the pan-lymphocyte marker CD45 (Figure 5A) in combination with the rhodamine-conjugated rat IgG1κ monoclonal antibody against Env (Figure 5B). Consistent with a hematopoietic lineage, only cells expressing CD45 proved immunoreactive with the antibody against Env (Figure 5C). We next sought to determine if the immunoreactive cells were pDCs. pDCs are professional antigen-presenting cells (APCs) that express CD45 and exclusively express the surface marker CD303 (38-40). Our analysis revealed that CD303 expression (Figure 5E) and immunoreactivity to the rhodamine-conjugated rat monoclonal antibody to Env (Figure 5F) uniquely co-localized in the same cells (Figure 5G), suggesting that the observed immunoreactivity was specific for pDCs. In order
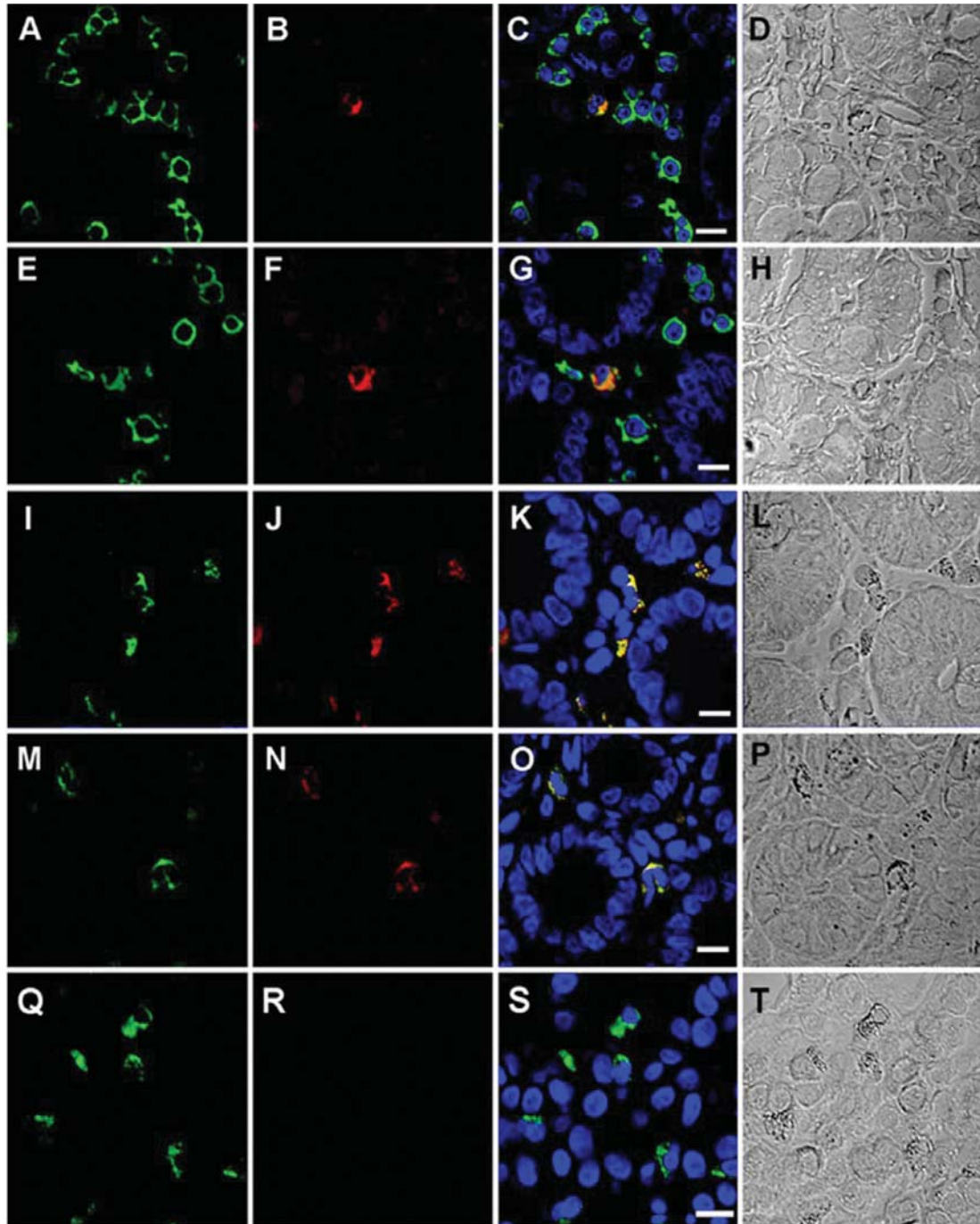
181

Figure 5. *Phenotype of duodenal-associated cells immunoreactive to rat monoclonal antibody to SFFV-gp-52 Env. A: Expression of CD45 (FITC) in a duodenal biopsy of an ME case. B: Immunoreactivity of rhodamine-conjugated monoclonal rat antibody to SFFV-gp-52 Env in duodenal biopsy of an ME case. C: Merged images A and B, (Bar represents 20 μm). D: Morphology of the immunoreactive cells. E: Expression of CD303 (FITC) in a duodenal biopsy of an ME case. F: Immunoreactivity of rhodamine-conjugated monoclonal rat antibody to SFFV-gp-52 Env in a duodenal biopsy of an ME case. G: Merged images E and F (Bar represents 20 μm). H: Morphology of the immunoreactive cells. I: Expression of CD86 (FITC) in duodenal a biopsy of an ME case. J: Immunoreactivity of a rhodamine-conjugated monoclonal rat antibody to SFFV-gp-52 Env in a duodenal biopsy of an ME case. K: Merged images I and J, (Bar represents 20 μm). L: Morphology of the immunoreactive cells. M: Expression of HLA-DR (FITC) in a duodenal biopsy of an ME case. N: Immunoreactivity of rhodamine-conjugated monoclonal rat antibody to SFFV-gp-52 Env in duodenal biopsy of an ME case. O: Merged images M and N, (Bar represents 20 μm). P: Morphology of the immunoreactive cells. Q: Expression of CD303 (FITC) in a duodenal biopsy of an ME case. R: Immunoreactivity of APC-conjugated mouse IgG1 isotype control in duodenal biopsy of an ME case. S: Merged images Q and R (Bar represents 20 μm). T: Morphology of the immunoreactive cells. TOPO3 was used to a indicate nucleus localization in images C, G, K, O and S.*

Table II. *Quantitative analysis of plasmacytoid dendritic cells (pDCs). Five microscopic fields were randomly chosen and used to count total pDCs in the duodenum of 8 controls (top) and 8 cases (bottom). No Env-positive pDCs were observed in any controls. Cases are reported as a ratio of Env-positive pDCs to total pDCs.*

Field	Controls								Group	Standard
	1	2	3	4	5	6	7	8		
1	7	10	6	8	7	12	7	8		
2	6	13	1	9	6	13	6	9		
3	4	11	4	10	5	10	3	12		
4	0	13	7	13	0	11	0	13		
5	9	10	9	0	3	15	0	10		
Average	5.2	11.4	5.4	8	4.2	12.2	3.2	10.4	7.5	3.5
	Cases									
	1	2	3	4	5	6	7	8		
1	26/76	17/35	19/48	10/30	18/28	12/25	18/25	10/16		
2	16/50	17/47	16/28	11/29	12/25	15/45	20/55	11/17		
3	18/52	12/25	12/40	12/31	10/18	19/40	30/56	14/30		
4	25/40	17/26	16/45	12/29	8/19	16/41	19/51	10/19		
5	26/52	13/30	16/40	12/36	15/20	21/50	13/27	12/27		
Average	22.2/54	15.2/32.6	15.8/40.2	11.4/31	12.6/22	16.6/40.2	20/42.8	11.4/21.8	15.7/35.6	3.9/10.9

to provide further evidence that the immunoreactive cells were APCs, immunohistochemical analysis was performed using the co-stimulatory marker CD86 and the major histocompatibility (MHC) class II antigen HLA-DR, both of which are expressed by APCs, including pDCs (41, 42). Again, our analysis confirmed that expression of CD86 (Figure 5I) and immunoreactivity to the rhodamine-conjugated rat monoclonal antibody against Env (Figure 5J) co-localized in the same cell (Figure 5K). Likewise, expression of HLA-DR (Figure 5M) and immunoreactivity to the rat monoclonal antibody to Env (Figure 5N) also co-localized in the same cells (Figure 5O). Lastly, the possibility of non-specific binding of the antibodies to CD86 and HLA-DR was addressed by the inclusion of matched isotype controls (Figures 5R). When taken together; these observations support the premise that the observed anti-HERV immunoreactivity is uniquely present in pDCs.

Quantitative analysis of pDCs. Finally, in order to evaluate if the relative number of duodenum-associated pDCs differed between ME cases and controls, we analyzed five random microscopic fields of view for eight individuals from each group (Table II). We also determined the frequency at which the pDCs of ME cases were immunoreactive to the HERV antibodies. ME cases were found to have approximately 4.7-times as many pDCs per field, when compared to the controls (35.6±10.9 *vs.* 7.5±3.5, respectively, $p<0.001$ by Mann Whitney). Additionally, we observed that approximately 44% (15.7/35.6) of the duodenum-associated pDCs in the eight ME cases were immunoreactive to antibodies against HERVs.

Discussion

In this study, we have shown that gut biopsies from 8 out of 12 individuals with ME displayed immunoreactivity consistent with the presence of HERV proteins. However, the same immunoreactivity was not observed in the biopsies of the controls. Additionally, we have shown that the immunoreactivity was observed in cells with a phenotype that is consistent with pDCs. These observations suggest that the presence of the HERV protein in pDCs may be associated with a pathological manifestation in at least a subset of individuals with ME.

Our detection of proteins that react with monoclonal antibodies to HERVs is consistent with HERV expression. Nevertheless, it could be argued that an exogenous retroviral infection may potentially account for the observed results. The present study was conducted using surplus clinical biopsies; therefore, the proper matched specimens were not available to conduct a rigorous transcriptional analysis on all cases. This issue was compounded by the de-identified nature of the specimens, thus preventing the collection of additional biological material. Nonetheless, cryopreserved lymphocytes and preserved RNA from a duodenal biopsy were available from two HERV-positive ME cases. Therefore, we performed unbiased next-generation sequencing (NGS) on both RNA derived from the duodenum and purified pDCs from these individuals. Multiple contigs of known HERV genes were observed; however, no open reading frames were identified that could account for an infectious retrovirus (unpublished data). We also attempted

to identify an infectious murine leukemia virus (MLV)-related virus by co-culturing lymphocytes and purified pDCs using the DERSE indicator cell line (43). The DERSE indicator cell line (a kind gift from Dr. Vineet KewalRamani) is derived from the prostate cancer cell line LNCaP and stably transfected with an MLV vector containing the green fluorescent protein (GFP) gene in reverse orientation. Only after rescue and transfer to new cells through reverse transcriptase and integrase enzymatic activity can the GFP be detected. In previous experiments, the sensitivity of this assay was established by detecting infectious MLV-related virus derived from two individual 22Rv1 cells per culture (unpublished data). In spite of using this sensitive detection method, we were unable to identify an infectious MLV-related retrovirus. Although we cannot definitively rule out the possibility that our observations were the result of an infectious retrovirus, our results using HERV-specific monoclonal and polyclonal antibodies, as well as NGS and co-culture methods, strongly argue against this.

A number of immunological observations have been described in relation to ME; however, the data presented here, to our knowledge, represent the first report of an association of pDCs with this disease. While the expression of endogenous retroviral proteins in the pDCs of ME cases does not intrinsically explain pathology, the observation that the immunoreactive proteins are only observed in pDCs is supportive of this concept. This supposition is further supported by our previous report of the dysregulation of inflammatory cytokines in a cohort of ME cases (44). One subset of this cohort was characterized by elevated interleukin-8 (CXCL8), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interferon gamma-induced protein-10 (CXCL10), macrophage inflammatory proteins-alpha and beta (CCL3 and CCL4) and depressed interferon-alpha (IFN-α) production, all of which are produced in significant quantities by pDCs (45). The production of type-I IFN by pDCs is a result of the engagement of Toll-like receptors (TLR) 7 and 9 in early endosomes (46), whereas inflammatory cytokine production occurs as a result of their engagement in late endosomes (47). A deviation in the spatiotemporal regulation of pDCs favoring TLR-activation in late endosomes over early endosomes would be consistent with our previous observations of cytokine dysregulation in those with ME. Similar abnormalities in the pDC function and distribution has also been reported in individuals with IBD. Brumgard *et al*. observed that isolated pDCs from patients with flaring Crohn's disease and ulcerative colitis overproduce inflammatory cytokines while underproducing type-I IFN in response to the synthetic TLR-9 agonist ODN 2216 (48). Additionally, they reported on a significantly higher frequency of pDC in the inflamed colonic mucosa of patients with IBD. These data suggest that our observations in subjects with ME may not be unique to this disease but may, in fact, be common to diseases characterized by chronic inflammation.

A potential dysregulation of pDCs may suggest an explanation for a number of other clinical observations associated with ME. As stated previously, pDCs are most remarkable for their ability to produce copious amounts of type-I IFN. In 1978, Trinchieri and colleagues indirectly identified pDCs by their ability to activate natural killer (NK) cell-mediated cytotoxicity through the production of IFN-α (49) and several subsequent studies have expanded upon the importance of type-I IFN in modulating NK cell function (50). Decreased NK cell activity is a commonly reported observation associated with ME (51); therefore, an aberrant pDC response leading to a decrease in IFN-α would be consistent with dysregulation of NK cells. Inflammatory cytokine and chemokine abnormalities have also been reported in association with ME by other researchers. For instance, Natelson *et al*. reported elevated levels of CXCL8 and IL-10 in the spinal fluid of individuals with influenza-like onset of ME (52, 53). Moreover, Vernon *et al*. and others reported that CXCL8 gene transcription was elevated in those with ME (52, 53). Finally, Chao *et al*. reported IL-6 to be up-regulated in subsets of ME cases (54).

In our previous report, the expression of inflammatory cytokines was more prevalent in a subset of individuals characterized by a gamma-delta T-cell clonality (44). A similar clonality has been observed in autoimmune diseases characterized by the expression of HERVs, such as rheumatoid arthritis and MS (55, 56). Additionally, the expansion of gamma-delta T-cells in response to the expression of endogenous gamma retroviruses has been reported in animal models (57). Some HERV proteins act as superantigens (34), promoting the expansion of T-cell populations and, therefore, the observation of gamma-delta T-cell clonality associated with ME, as well as other diseases, may be the result of HERV superantigen stimulation.

Although the Canadian consensus criteria for ME and the Fukuda criteria for CFS do not include symptoms of autoimmunity, the recent study by Fluge *et al*. supports the notion that at least a subset of individuals with ME may have an autoimmune element associated with their disease (11). Autoimmune diseases, such as SLE, MS and rheumatoid arthritis, have several symptoms that overlap with those of ME and all have been associated with the pDC dysfunction. Moreover, the same autoimmune diseases are also reported to be associated with the expression of HERVs, although a physical connection between HERVs and pDCs has not yet been reported. As Stoye points out in his recent review (58), the role of HERVs in autoimmunity remains an unproven hypothesis; however, an increasing number of studies suggest that HERVs may have the capacity to contribute to disease pathology (59, 60). It is noteworthy that work conducted in the laboratory of Dr. Bridget Huber showed that HERV-K18 expression could be induced by herpes viruses such as Epstein-Barr virus and human herpes virus 6 (HHV-6) (34,

61). Consistent with that work and with the data presented here, both of these viruses have been observed in the duodenum of individuals with ME (62).

The expression of HERV proteins in autoimmune diseases such as SLE, MS and Sjögren's syndrome, is also evident by reports of antibodies to retroviral proteins in those who are not found to be infected with an exogenous retrovirus (26). If the expression of HERV proteins in pDCs is found to be associated with these autoimmune diseases, it may help explain the presence of such antibodies. Inflammation is known to increase HERV expression (63, 64); therefore, if pDC-associated inflammation drives the expression of endogenous retroviruses, it is also conceivable that dysregulated expression of other proteins in pDCs may occur. Consequently, the antigen-presenting abilities of pDCs may contribute to the production of auto-reactive antibodies, as is observed in ME (65, 66, 8, 67-69). The etiopathology associated with autoimmune diseases, HERV expression, and pDCs is clearly not a simple relationship. Nevertheless, the prospect of an involvement of HERVs and pDCs in ME pathology provides an opportunity for more directed studies into the immune dysregulation associated with this disease.

The expression of HERV proteins in pDCs may lead to ME-related pathology. Conversely, their expression might merely be the result of the inflammation associated with the disease, or perhaps a combination of both. Nevertheless, the presence of these proteins in the pDCs of individuals with ME but not in controls does support an involvement of pDCs in ME. The results presented in this report, if confirmed and extended by other studies, may provide a greater understanding over the etiopathology of ME.

Acknowledgements

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