Expression of Human Endogenous Retrovirus HERV-K18 Superantigen Is Elevated in Juvenile Rheumatoid Arthritis

JOCELYN SICAT, NATALIE SUTKOWSKI, and BRIGITTE T. HUBER

ABSTRACT. ***Objective.*** To investigate the presence of a host-encoded superantigen as possible etiologic factor in pediatric rheumatic disease. We measured the expression and the ability of interferon-α (IFN-α) to induce the human endogenous retrovirus HERV-K18 superantigen in juvenile rheumatoid arthritis (JRA) and pediatric systemic lupus erythematosus (SLE).

Methods. Expression levels of HERV-K18 were measured in peripheral blood or synovial fluid mononuclear cells (SFMC) from 13 patients with JRA, 11 pediatric SLE patients, and 24 healthy controls, by semiquantitative reverse transcription-polymerase chain reaction, comparing 18S ribosomal transcripts as endogenous standard. IFN-α induction was tested by pretreatment of samples with 2000 U/ml.

Results. HERV-K18 expression was significantly elevated in peripheral blood from patients with JRA (mean ratio of HERV-K18 to 18S ribosomal transcripts 2.456, SD 2.122; p = 0.014), but not patients with SLE (mean 0.997, SD 0.579; p = 0.258), compared to controls (mean 0.749, SD 0.598). HERV-K18 transcripts were detected in SFMC of 7/7 JRA patients. IFN-α induced HERV-K18 strongly in JRA (mean fold induction = 8.934, SD 15.556) and controls (mean 8.270, SD 6.609), but weakly in SLE (mean 2.432, SD 2.219; p = 0.009). HERV-K18 levels were found to be independent of previously determined modifiers of expression, including Epstein-Barr virus infection, IFN-α levels, or the percentage of B cells in peripheral blood.

Conclusion. HERV-K18 superantigen levels were elevated in JRA patients, but not pediatric patients with SLE, suggesting a possible mechanism for autoimmunity in the former group by superantigen stimulation of autoreactive T cells. (J Rheumatol 2005;32:1821–31)

Key Indexing Terms:

JUVENILE RHEUMATOID ARTHRITIS — SUPERANTIGEN
PEDIATRIC SYSTEMIC LUPUS ERYTHEMATOSUS — HERV-K18
HUMAN ENDOGENOUS RETROVIRUS — AUTOIMMUNITY

Juvenile rheumatoid arthritis (JRA) and pediatric systemic lupus erythematosus (SLE) are chronic autoimmune diseases, whose etiologies are unknown, but are thought to be multifactorial. The prevailing view of autoimmunity entails a causal relationship between genetics and environment, with the immune response to infection playing a prominent role. It is thought that microbial pathogens may initiate autoimmune phenomena in genetically susceptible individuals, coincidentally activating immune cells that respond to self[1-3].

Superantigens are pathogen-derived proteins that provoke a strong T cell immune response. Superantigens are presented to T cells by MHC class II molecules on antigen-presenting cells. They bind outside of the peptide-binding groove of class II, forming a bridge between the MHC molecule and the Vß portion of the T cell receptor (TCRBV). In this way, superantigens are able to activate entire families of T cells bearing particular TCRBV chains[4,5]. Because of this potent immune stimulus, superantigens have been implicated in various autoimmune diseases, providing a possible mechanism for the activation of autoimmune T cells[6]; however, to date, studies pointing to their existence in human autoimmune disease have not convincingly resolved the issue. Recently, we reported that the human endogenous retrovirus (HERV) HERV-K18 has superantigen activity that can be induced by infection with the ubiquitous her-

From the Department of Rheumatology, Tufts-New England Medical Center, Boston, Massachusetts; Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina; and Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts, USA.

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J. Sicat, MD, Pediatric Rheumatologist, Department of Rheumatology, Tufts-New England Medical Center; N. Sutkowski, PhD, Assistant Professor of Microbiology and Immunology, Department of Microbiology and Immunology, Medical University of South Carolina; B.T. Huber, PhD, Professor of Pathology, Department of Pathology, Tufts University School of Medicine.

Address reprint requests to Dr. B.T. Huber, Department of Pathology, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111. E-mail: Brigitte.huber@tufts.edu

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pesvirus Epstein-Barr virus (EBV)[7] and by the cytokine interferon-α (IFN-α)[8], which is produced in response to viral infection. This led us to postulate that an exogenous pathogen, such as EBV or other viruses that elicit IFN-α secretion, might induce HERV-K18 in genetically susceptible individuals, resulting in autoimmune T cell activation.

HERV sequences make up to 8% of the human genome, with proviral copies integrated throughout the genome[9]. The HERV contain *gag*, *pol*, and *env* genes that are similar to exogenous retroviruses; however, most are defective, rendering the full-length proviruses replication-incompetent[10–13]. They are transmitted genetically in a Mendelian fashion[14]. There has been speculation that HERV are associated with pathogenesis, and studies implicating HERV in various diseases, such as insulin dependent diabetes mellitus[15,16], SLE[17,18], schizophrenia[19,20], multiple sclerosis[21], and cancer[22-25], have been reported. Unfortunately, many of these studies were designed so that they illustrate an increase in HERV activity, but not individual HERV. Studies using reverse transcription-polymerase chain reaction (RT-PCR) or Northern blotting almost invariably detect multiple HERV sequences, because HERV are highly homologous among each other. Similarly, others have described increased serum antibodies putatively specific for particular HERV proteins; however, these antibodies generally cross-react with other retroviral family members[26].

HERV-K18 is a member of the relatively large HERV-K family, which once used the amino acid lysine (K) as its primer-binding site during reverse transcription. There are estimated to be between 50 and 170 HERV-K proviruses, which are members of the C-type retrovirus family[10,12,27,28]. They have homology to the mouse mammary tumor viruses, the first group of viruses discovered to contain superantigens[29-31]. We have shown that the Env protein of HERV-K18 has superantigen activity, strongly activating T cells, with preference for the TCRBV13 T cell family[7].

It has been postulated that the common herpesvirus EBV may be an instigating factor in various rheumatic diseases, but because infection with EBV is so widespread, with more than 90% of adults latently infected, it has been difficult to show a correlation[32-34]. Even so, there have been several reports finding multiple EBV copies in the inflamed joints of RA patients[32,33]. Because EBV transactivates the HERV-K18 superantigen, we were interested in determining whether patients with rheumatic disease might have enhanced EBV infection and correspondingly higher concentrations of HERV-K18. We therefore studied 2 autoimmune diseases with onset during childhood, JRA and pediatric SLE, to investigate the initiating factors of autoimmunity. We postulated that a disease correlation might be more apparent in younger patients. EBV infection generally occurs during childhood, is often asymptomatic or associated with mild flu-like symptoms, and goes unnoticed. Alternatively, primary EBV infection causes infectious mononucleosis, a self-limiting T cell proliferative disease, whose symptoms first caused us to look for an association between EBV and superantigens[35].

In addition, it has been reported that SLE patients have higher circulating levels of IFN-α, and we have shown that IFN-α also upregulates HERV-K18[8]. IFN-α is a type I interferon, predominantly produced by macrophages and dendritic cells, and its levels are increased during viral infection[36]. IFN-α induces MHC class I and class II molecules, augmenting macrophage antiviral function as well as potentiating antibody function and cytotoxic T lymphocyte and natural killer cell activity[37,38]. Recombinant IFN-α is now used as an antiviral/antitumor agent; interestingly, autoimmune manifestations, including thyroiditis, polyarthritis, arthralgias, and myalgias and autoantibody production, have been reported after IFN-α therapy[39]. In SLE patients it has been shown that serum IFN-α levels correlate with disease activity[40-42]. In addition, IFN-α causes dendritic cell differentiation[36], another trait that correlates with disease activity in SLE. Hence, we tested whether levels of IFN-α correlated with HERV-K18 expression levels in pediatric autoimmune patients. Further, we analyzed the extent to which recombinant IFN-α induces HERV-K18 in peripheral blood mononuclear cells (PBMC) from these patients *in vitro*.

Finally, this study is unique because we used a method of detection of HERV-K18 that ensures no cross-reactivity with other HERV sequences. It is based upon detection of retroviral readthrough transcripts, a general property of proviral transcription[43,44]. Our assay detects only HERV sequences that encode the chromosome 1 integration site adjacent to HERV-K18[11], guaranteeing specificity.

MATERIALS AND METHODS

Study population. Patients were recruited from the pediatric rheumatology clinic at Tufts-New England Medical Center in Boston. JRA and SLE patients were diagnosed according to the American College of Rheumatology criteria. Written informed consent, approved by the Human Investigative Research Committee at Tufts-New England Medical Center and Tufts University School of Medicine, was obtained from patients, healthy volunteers, and their parental guides.

Fifteen patients with JRA ages 4–18 years participated; 14 (93%) were female, one (7%) male; 7 (47%) had pauciarticular JRA, 4 (27%) polyarticular JRA, and 3 (20%) systemic JRA. Peripheral blood was obtained from 13 JRA patients, and synovial fluid mononuclear cells (SFMC) were obtained from 7 exhibiting acute exacerbation of the arthritis. Three patients (20%) received no medication during the study, 9 (53%) received nonsteroidal antiinflammatory drugs (NSAID), 4 (27%) methotrexate (MTX), and 2 (13%) prednisone.

Eleven patients with SLE ages 10–21 years, all female, participated in the study; 3 (27%) had nephritis, 3 (27%) had a SLE Disease Activity Index (SLEDAI) score > 4. Seven (64%) patients with SLE received prednisone < 0.5 mg/kg, mean dose 12 mg daily; 2 (18%) received prednisone 0.5–1.0 mg/kg, mean dose 25 mg daily; 4 (36%) received MTX, 2 (18%) cyclophosphamide, one (9%) mycophenolate, and one (9%) cyclosporin A.

Twenty-four healthy children ages 3–17 years, 13 (54%) girls and 11 (46%) boys, were recruited as controls. None had a history of autoimmune disorder. Age distribution of healthy male children was one 5-year-old, one 11-year-old, one 9-year-old, one 11-year-old, one 12-year-old, three 15-year-olds, one 16-year-old, and two 17-year-olds. Age distribution for

healthy female children was one 3-year-old, one 8-year-old, two 9-year-olds, two 10-year-olds, two 11-year-olds, three 16-year-olds, and two 17-year-olds.

Sample collection. Peripheral blood was collected by venipuncture from patients and healthy volunteers. Synovial fluid was collected from JRA patients requesting drainage of inflamed joints for relief. PBMC were isolated from 20 ml of peripheral blood, and SFMC cells were isolated from 5–20 ml synovial fluid by density gradient separation on Ficoll-Hypaque (Pharmacia). Serum samples were saved after the centrifugation step and stored at –80°C for ELISA. If < 5 ml synovial fluid was obtained, SFMC cells were isolated by centrifugation at 1600 rpm for 10 min at room temperature, then washed with 15 ml Dulbecco's phosphate buffered saline (Gibco).

Recombinant IFN-α stimulation. PBMC were resuspended at a density of 2 × 10^6 cells/ml in complete RPMI media (Invitrogen, Carlsbad, CA, USA) supplemented with fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), glutamine, HEPES, 2-mercaptoethanol, sodium pyruvate, and penicillin/streptomycin (all from Invitrogen). Cells were cultured with or without human recombinant IFN-α (Roferon, Roche Pharmaceuticals, Nutley, NJ, USA) 2000 U/ml, for 4 h in a 37°C, 5% CO_2 incubator.

Semiquantitative RT-PCR for HERV-K18. Total RNA was prepared using Trizol reagent (Invitrogen). RNA was treated with DNAse I (Roche) according to manufacturer's protocol, then with phenol/chloroform to remove the enzyme. cDNA was prepared by random priming from 1 μg RNA for each sample in 20 μl reactions, using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. For each sample, a control reaction was simultaneously performed in the absence of reverse transcriptase to assay for possible DNA contamination. Polymerase chain reaction (PCR) sense primer was 5'-TCC GAA GAG ACA GTG ACA TCG A-3', directed against a HERV K18 *env*-specific sequence; PCR antisense primer was 5'-TGG CAA TGC TGG CTA TGT AAG T-3', which is directed against a chromosome 1q23.1-q24.1 (Accession no. AL121985) sequence located 127 bp downstream of the 3' viral LTR. The 50 μl PCR reaction consisted of Platinum Taq PCR Supermix (Invitrogen), 2.5 pM of each primer, and 0.5 μl of [^{32}P]α-dCTP (10 μCi/μl) and 2 μl of cDNA (1/10th volume). As an endogenous standard, primers specific for 18S rRNA were included in each reaction from the Ambion Gene Specific Relative RT-PCR kit (Ambion, Austin, TX, USA). Since the HERV-K18 readthrough transcripts (< 15% of total proviral transcripts) are extremely rare compared with the 18S rRNA transcripts, 18S Classic Competimers™ (Ambion) were added at a primer:competimer ratio of 1:20. PCR was performed on an Eppendorf Mastercycler, using a hot start of 4 min at 94°C, then 25 cycles of 30 s at 94°C, 1.5 min at 72°C, and 1 min at 55°C, followed by 7 min extension at 72°C, which yielded PCR products within a linear range. PCR products were separated on a 6% denaturing polyacrylamide gel. The gel was fixed in 10% glacial acetic acid/20% ethanol, for 30 min with shaking, then vacuum dried for 2 h at 80°C. HERV-K18 readthrough transcripts were quantified by phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA), and expression was measured after normalization against the 18S rRNA product.

ELISA. Human IFN-α ELISA (PBL Biomedical, Piscataway, NJ, USA) was performed according to the manufacturer's protocol on peripheral blood serum samples diluted 1:2. EBV viral capsid antigen (VCA) IgG ELISA (Zeus Scientific, Raritan, NJ, USA) was performed on peripheral blood serum samples according to the manufacturer's protocol.

Flow cytometry analysis. PBMC were stained for CD20 and CD3, using human CD20-FITC conjugate, and human CD3-PE conjugate, (both from BD Pharmingen, San Diego, CA, USA) according to manufacturer's protocol. At least 10,000 events were evaluated on a FACSCalibur (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis. The ratio of HERV-K18:18S transcripts was calculated by dividing the volume of the HERV-K18 band obtained after 6 h exposure on a phosphorimager, by the volume from the corresponding 18S band. Fold induction after IFN-α treatment was calculated by dividing the HERV-K18:18S ratio obtained with IFN-α treatment, by the ratio obtained without treatment. The arithmetic mean, standard deviation, and standard error were calculated from the ratios of HERV-K18:18S obtained from each group of patients or controls. Probability values were calculated using a 2-tailed distribution on the Student t test.

RESULTS

HERV-K18 superantigen transcripts are elevated in JRA, but not pediatric SLE patients. To measure the expression levels of the HERV-K18 superantigen in pediatric autoimmune patients, we previously set up a system for the detection of HERV-K18 readthrough transcripts[7]. Because up to 8% of the human genome is derived from HERV sequences[9], many of which are highly homologous, this method was devised to take advantage of the specificity created by using PCR primers that detect transcripts encoding the chromosomal insertion sequences adjacent to the HERV. For up to 15% of proviral transcripts, the RNA polymerase reads through the polyadenylation site in the 3' long terminal repeat, transcribing the adjacent integration sequences immediately downstream of the provirus[43,44]. Since HERV-K18 is located on chromosome 1q23.1-q24.1[11,45], we used a downstream primer specific for the chromosome 1 insertion site and an upstream primer specific for HERV-K18 *env* superantigen sequence. In this manner, only HERV-K18 transcripts are detected, and since < 15% are readthrough transcripts, we are detecting only a fraction of total HERV-K18 transcripts.

Total RNA was isolated from PBMC obtained from 13 JRA patients, 11 pediatric SLE patients, and 24 healthy controls. Semiquantitative RT-PCR analysis for HERV-K18 readthrough transcripts was performed on each sample. As an endogenous standard, primers specific for the 18S ribosomal subunit were included in each reaction, and results are presented in the form of a ratio of HERV-K18 transcripts to 18S ribosomal transcripts. The number of PCR cycles was limited to 25, which maintains the PCR products within the linear range[7]. To control for possible PCR or genomic DNA contamination, each reaction was performed in the presence or absence of reverse transcriptase. No PCR product was detected in any reaction lacking reverse transcriptase, indicating that DNA contamination was not a problem (data not shown). Representative RT-PCR results from JRA and SLE patients compared to healthy controls are depicted in Figure 1A. The HERV-K18 readthrough product runs at 1167 bp, while the 18S ribosomal product runs at 488 bp. As a positive control, we used DNA from cosmid clone 213, which contains the chromosome 1q23.1-q24.1 sequences[45]. The ratio of HERV-K18:18S was quantified by phosphorimager and is printed at the bottom of each lane. The results from all RT-PCR samples are summarized in Figure 1B, and the characteristics of each patient including subtype and duration of illness, medications, and the corresponding HERV-K18:18S ratio are listed in Table 1.

HERV-K18 transcripts were significantly elevated in

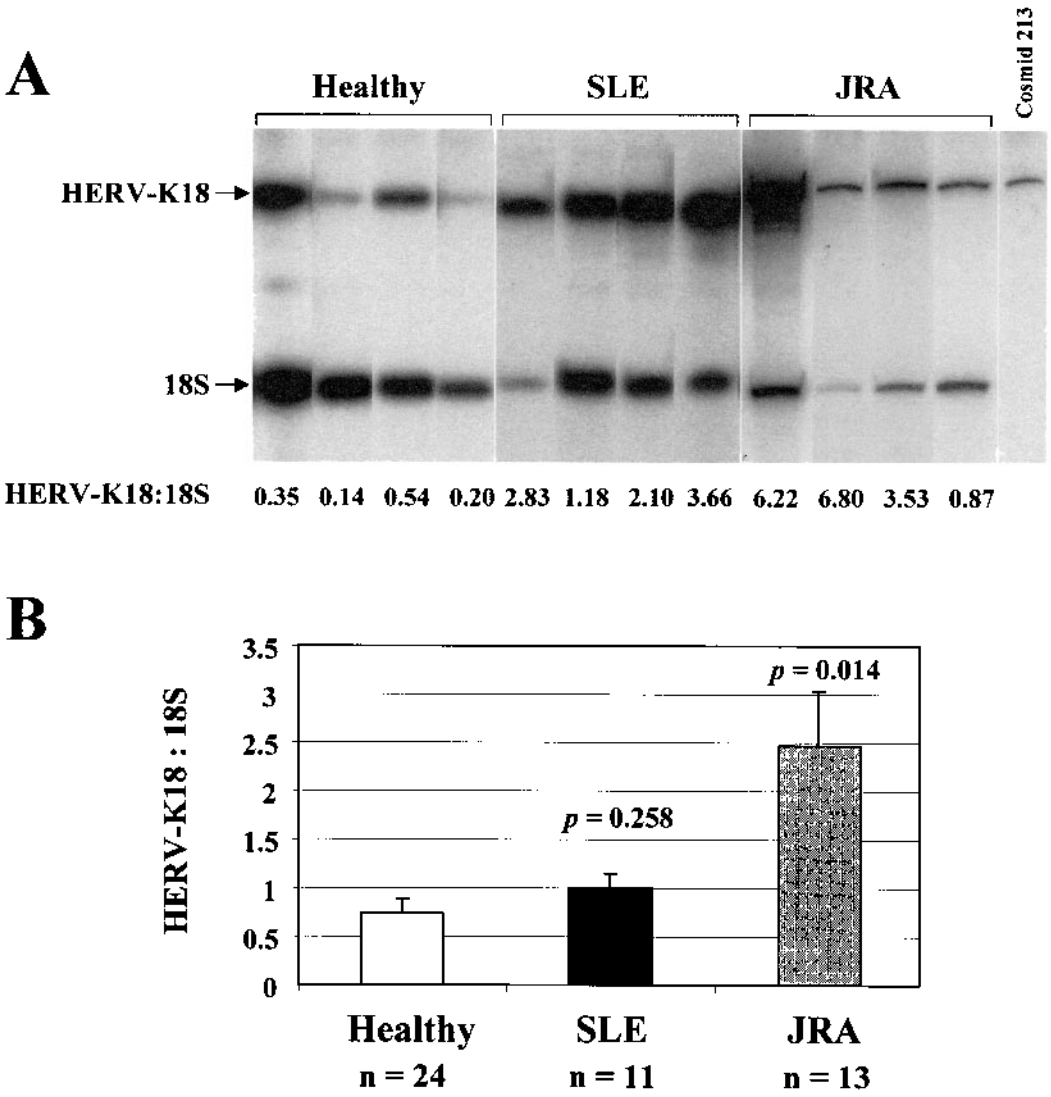

Figure 1. Peripheral blood levels of HERV-K18 are elevated in patients with JRA, but not pediatric SLE, compared with controls. A. The 1167 bp HERV-K18 readthrough transcript was separated from the 488 bp 18S ribosomal transcript by polyacrylamide electrophoresis. Results for 4 representative samples from each group are shown; arrows indicate HERV-K18 and 18S transcripts. As a positive control, cosmid clone 213 DNA derived from human chromosome 1q23.1-q24.1 was used for PCR template. Ratio of HERV-K18:18S transcripts was calculated by phosphorimager and is given under each lane. B. Ratio of HERV-K18:18S for each sample was calculated. The mean ratio for 24 controls (white bars), 11 pediatric SLE (black bars), and 13 JRA patients (gray bars) is shown. Significance values compared with controls are shown.

JRA patients (p = 0.014), but not in pediatric SLE patients (p = 0.258), compared with controls (Figure 1B). The mean ratio of HERV-K18:18S ribosomal transcripts in the peripheral blood of 13 JRA patients was 2.456 (SD 2.122) and for 11 SLE patients was 0.997 (SD 0.579) compared with 0.750 (SD 0.598) for 24 controls.

HERV-K18 superantigen transcripts are readily detected in JRA synovial fluid mononuclear cells. Paired samples of peripheral blood and SFMC were obtained from 5 JRA patients, and SFMC only were obtained from 2 additional patients (Table 1). HERV-K18 transcripts were detected in all (7/7) SFMC samples tested (Figure 2). In 2/5 of the paired patient samples tested, the ratios of HERV-K18:18S ribosomal transcripts were increased in SFMC compared with the peripheral blood. Figure 2A shows the RT-PCR analysis of the SFMC samples. Figure 2B depicts the results from paired SFMC and PBMC samples and the additional SFMC-only samples. These results prove that the HERV-K18 superantigen is not only expressed in the peripheral blood of JRA patients, but is also in the inflamed joints, lending credence to the hypothesis that superantigen activated T cells may contribute to pathogenesis in these patients.

Table 1. Patient disease characteristics and levels of HERV-K18 in peripheral blood (PB) and/or synovial fluid (SF) mononuclear cells of 15 patients with JRA and 11 with SLE.

JRA Patients	Subtype	Age, yrs	Sex	Disease Duration, yrs	Activity	Medication	Cytotoxics/ HERVK18:18S DMARD	PB	SF
1	Pauci	13	F	> 10	Remission	None		3.15	
2	Pauci	9	F	> 5	Remission	None		0.29	
3	Pauci	8	F	> 5	Knee flare	NSAID		1.90	8.42
4	Poly	10	F	3	Knee flare	NSAID	MTX	0.08	0.03
5	Poly	18	F	> 10	Neck/shoulder		MTX	2.50	
6	Pauci	15	F	> 5	Remission	None		2.54	
7	Pauci	10	F	> 5	Knee flare	NSAID		0.29	5.53
8	Poly	13	F	2	Wrist/hand	NSAID	MTX	3.53	
9	Systemic	7	F	3	Ankles/wrist		MTX	1.24	
10	Systemic	5	F	2	Flare	NSAID, Pred		0.87	
11	Pauci	4	F	3	Knee flare	NSAID		6.22	0.16
12	Pauci	12	F	> 10	Knee flare	NSAID		2.52	1.1
13	Systemic	14	M		Wrist	NSAID, Pred		6.80	
14	Poly	7	F	5	Knee flare	NSAID, Pred	MTX		0.54
15	Pauci	10	F	1	Knee flare	NSAID			0.54

SLE Patients	Age, yrs	Sex	Duration,	SLEDAI Score	Medication	Cytotoxics/ HERVK18:18S DMARD	PB
1	11	F	> 5	14	Pred 25 mg	CSA/CYC	1.28
2	14	F	2	2	Pred 7.5 mg	MTX	1.09
3	10	F	3	4	Pred 20 mg		1.85
4	16	F		4	Pred 5 mg	MTX	1.80
5	14	F	5	2	Pred 30 mg	CYC	1.31
6	18	F	> 5	4	Pred 10 mg	MTX	1.32
7	21	F	5	0	None		0.28
8	16	F	10	4	Pred 8.75 mg	MTX	0.90
9	16	F	5	12	Pred 15 mg		0.43
10	15	F	< 1	8	None		0.27
11	13	F	2	4	Pred 15 mg		0.44

General patient characteristics including gender and age (in years), disease subtype, duration of disease (in years), disease activity or SLEDAI score at time of blood drawing, and medication used including cytotoxic agents or disease modifying anti-rheumatic drugs (DMARDs) as a separate category, are listed for 13 JRA patients and 11 pediatric SLE patients. Poly: polyarticular, Pauci: pauciarticular, Pred: prednisone (mg), MTX: methotrexate, CSA: cyclosporin A, CYC: cyclophosphamide, NSAID: nonsteroidal antiinflammatory drug.

IFN-α strongly induced HERV-K18 expression in PBMC from JRA patients and controls, but weakly in SLE samples in vitro. We previously reported that IFN-α transactivates HERV-K18 in the peripheral blood of healthy adults[8]. To determine whether IFN-α also transactivates the HERV-K18 superantigen in JRA and pediatric SLE patients, PBMC from each patient and from 13 controls were treated for 4 h with recombinant IFN-α, and then subjected to RT-PCR analysis. The results were compared with untreated samples from each subject to determine the extent of IFN-α induction of HERV-K18. Representative RT-PCR results from JRA and SLE patients compared to controls are shown in Figure 3A. The HERV-K18:18S ratio results from all RT-PCR samples are summarized in Figure 3B, and in Figure 3C we show the mean fold induction for each group. IFN-α significantly induced HERV-K18 in all groups tested. For controls, recombinant IFN-α induced HERV-K18 transcripts an average of 8.27-fold ($p < 0.002$, paired 2-tailed t test; Figures 3B and 3C). Similarly, in JRA patients, IFN-α also induced HERV-K18 expression, an average of 8.93-fold ($p = 0.0008$, paired 2-tailed t test; Figure 3B). The fold induction compared with healthy controls was not statistically different ($p = 0.889$ by heteroscedastic 2-tailed t test; Figure 3C); however, the absolute levels of HERV-K18 induction were significantly higher ($p = 0.004$; Figure 3B), because HERV-K18 was already significantly elevated in unstimulated JRA samples compared to controls (Figure 1B). In contrast, in SLE peripheral blood samples, IFN-α weakly induced HERV-K18 expression, an average of 2.43-fold (Figure 3B); this increase was still statistically significant ($p < 0.003$, paired 2-tailed t test). The fold induction was statistically decreased compared with controls ($p = 0.009$ by heteroscedastic 2-tailed t test; Figure 3C), although the absolute levels were not significantly different from those derived

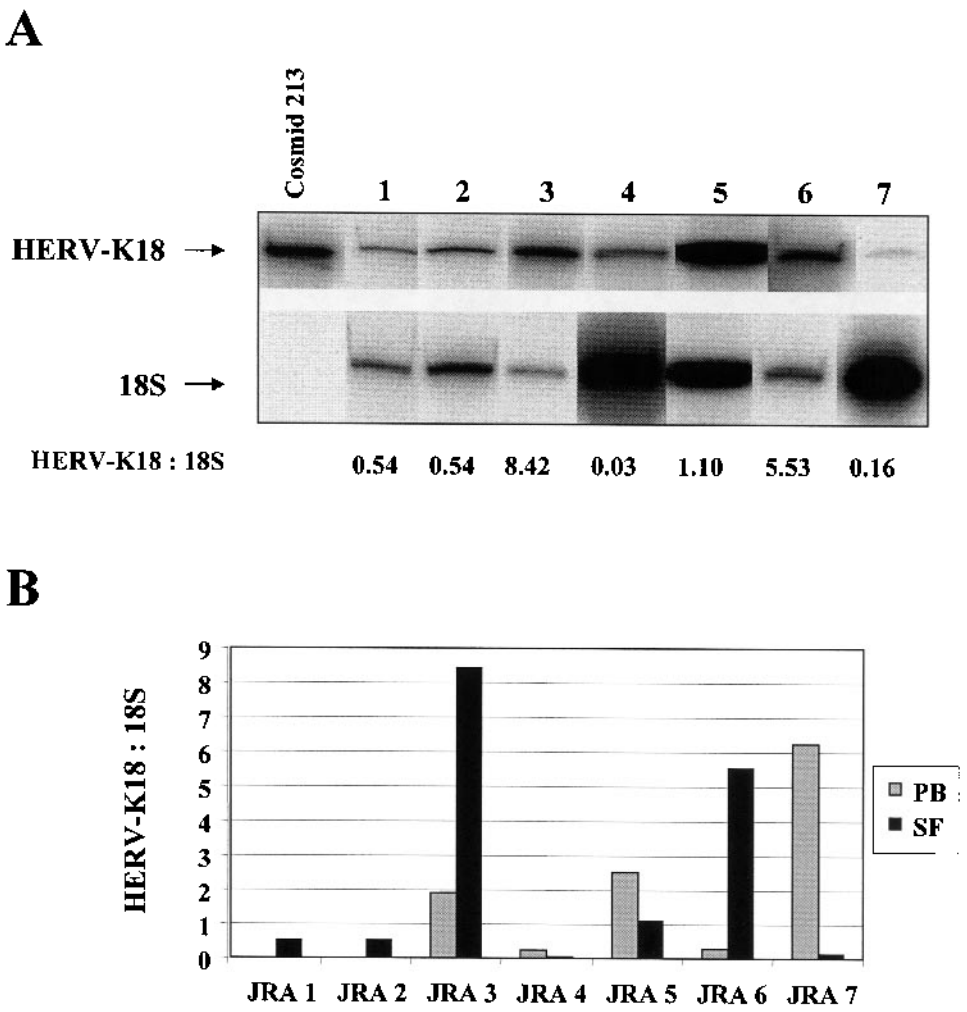

Figure 2. HERV-K18 is expressed in SFMC of JRA patients. A. Semiquantitative RT-PCR analysis from 7 JRA patient SFMC samples. The 1167 bp HERV-K18 readthrough transcript was separated from the 488 bp 18S ribosomal transcript by polyacrylamide electrophoresis; arrows indicate HERV-K18 and 18S transcripts. As a positive control, cosmid clone 213 DNA derived from human chromosome 1q23.1-q24.1 was used for PCR template. Ratio of HERV-K18:18S transcripts was calculated by phosphorimager and is given under each lane. B. Ratios of HERV-K18:18S for all synovial fluid tissue samples (SF) and paired peripheral blood samples (PB) are shown.

from IFN-α treated healthy PBMC (p = 0.081, Figure 3B). This poses an interesting question of why the SLE samples were relatively resistant to IFN-α induction.

HERV-K18 levels in peripheral blood were independent of serum IFN-α levels, EBV infection, and percentage of circulating B cells. Because it has been reported that circulating IFN-α levels are increased in SLE patients[40-42], we investigated whether blood concentrations of IFN-α correlated with levels of HERV-K18 expression in JRA and pediatric SLE patients compared to controls. Serum from peripheral blood samples was tested for IFN-α using a commercial ELISA. As can be seen in Figure 4A, there was no obvious correlation between the level of IFN-α in the peripheral blood and the ratio of HERV-K18:18S.

Since we have shown that EBV infection can transactivate HERV-K18[7], we were interested in testing whether the level of HERV-K18 expression in the pediatric autoimmune patients correlated with EBV seropositivity. We therefore tested the serum from 12 JRA and 10 SLE patients and 23 healthy controls for the presence of IgG specific for EBV viral capsid antigen (a lytic protein), using a commercial ELISA. As can be seen in Figure 4B, there was no correlation between high HERV-K18:18S ratio and EBV infection, neither for patients nor for the healthy controls.

Finally, we previously showed that HERV-K18 is preferentially induced in B cells[8], but not in T cell populations. Because SLE patients may have a disproportionate number of B cells compared with healthy subjects, and because fol-

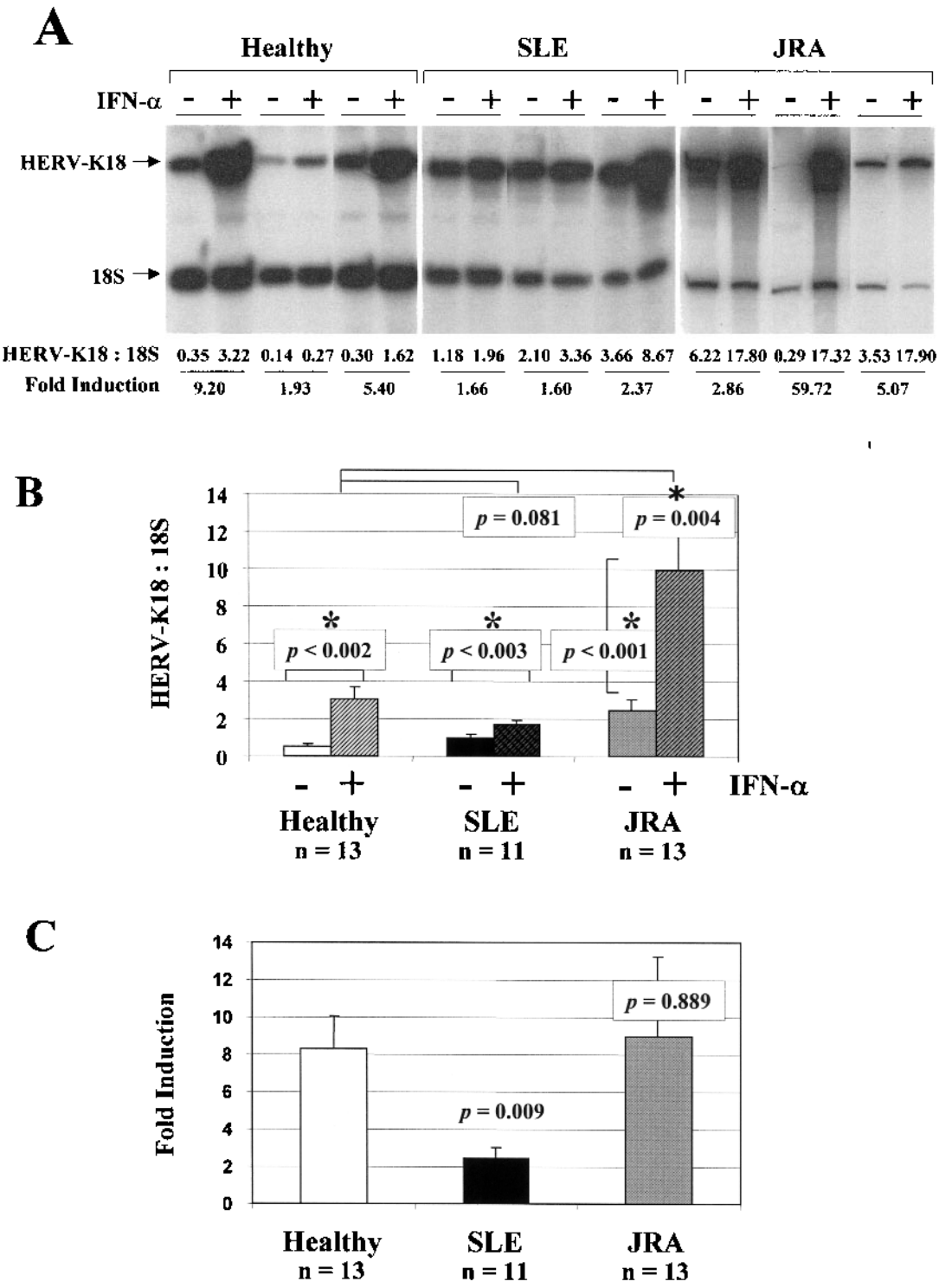

Figure 3. IFN-α induced HERV-K18 strongly in JRA patients and controls, but weakly in pediatric SLE patients. A. The 1167 bp HERV-K18 readthrough transcript was separated from the 488 bp 18S ribosomal transcript by polyacrylamide electrophoresis; arrows indicate HERV-K18 and 18S transcripts. Results for 3 representative samples from each group are shown, in the presence (+) or absence (–) of IFN-α. Ratio of HERV-K18:18S transcripts was calculated by phosphorimager and is given under each lane, and the fold induction of HERV-K18 after IFN-α treatment is shown below. B. Ratio of HERV-K18:18S is given for each sample in the presence (+) or absence (–) of IFN-α. The mean ratio for 13 healthy controls, 11 pediatric SLE, and 13 JRA patients is illustrated. *Significance values compared with controls after IFN-α treatment. C. The fold induction of HERV-K18 after IFN-α treatment was calculated for each sample. The mean induction for 13 healthy controls, 11 pediatric SLE, and 13 JRA patients is shown. Significance values compared with controls are shown.

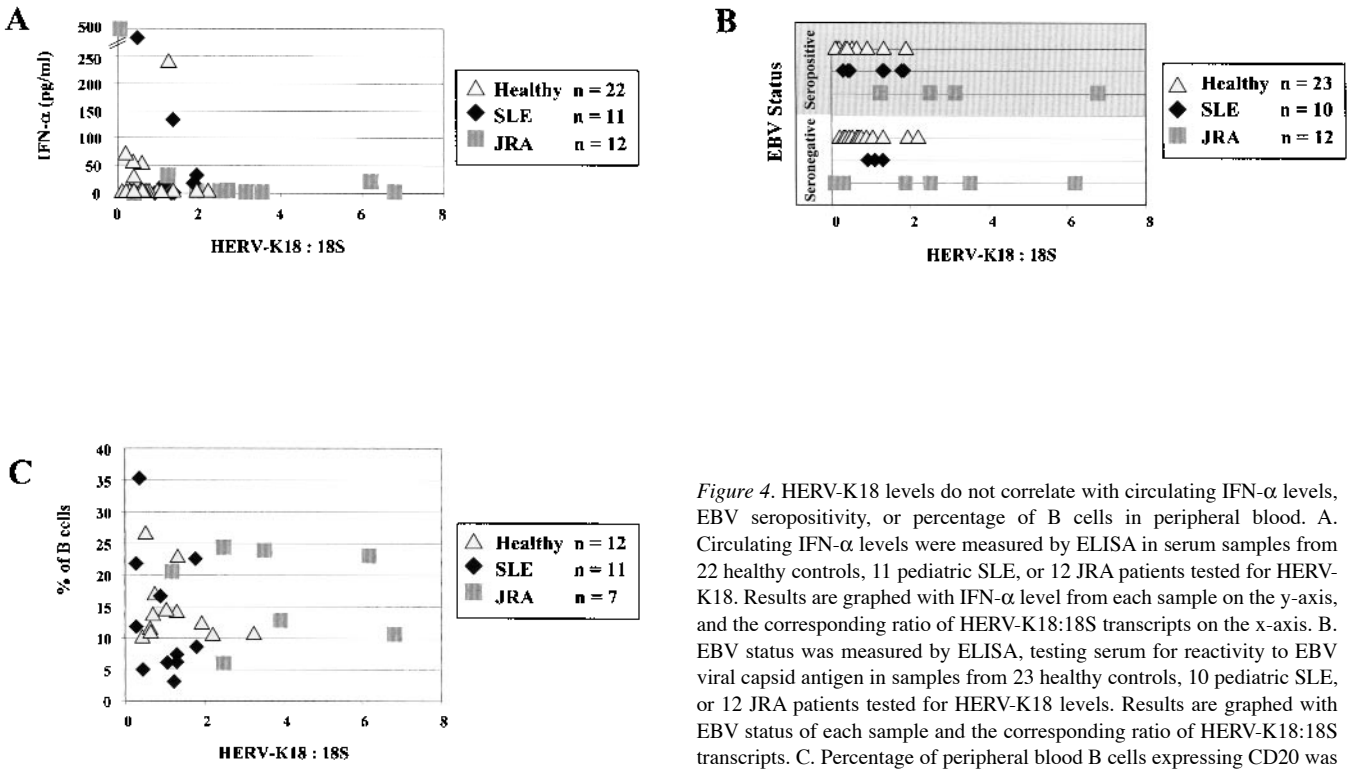

Figure 4. HERV-K18 levels do not correlate with circulating IFN-α levels, EBV seropositivity, or percentage of B cells in peripheral blood. A. Circulating IFN-α levels were measured by ELISA in serum samples from 22 healthy controls, 11 pediatric SLE, or 12 JRA patients tested for HERV-K18. Results are graphed with IFN-α level from each sample on the y-axis, and the corresponding ratio of HERV-K18:18S transcripts on the x-axis. B. EBV status was measured by ELISA, testing serum for reactivity to EBV viral capsid antigen in samples from 23 healthy controls, 10 pediatric SLE, or 12 JRA patients tested for HERV-K18 levels. Results are graphed with EBV status of each sample and the corresponding ratio of HERV-K18:18S transcripts. C. Percentage of peripheral blood B cells expressing CD20 was quantified by flow cytometry for 12 healthy controls, 11 pediatric SLE, or 7 JRA patients tested for HERV-K18 levels. Results are graphed with percentage of B cells from each sample and the corresponding ratio of HERV-K18:18S transcripts.

licle-like structures containing B cells have been identified in the inflamed joints of RA patients[46,47], we set out to test whether the percentage of B cells in the patient and control samples correlated with HERV-K18 expression. PBMC samples were therefore stained with Mab specific for CD20, a pan-B cell marker, and CD3, representative of the T cell lineage. Samples were analyzed by flow cytometry to quantify the percentage of B cells, and the values were compared with the ratios of HERV-K18:18S ribosome. As can be seen in Figure 4C, there was no evident correlation between the percentage of B cells in each sample and HERV-K18 expression. Similarly, the percentage of CD3 cells was not statistically different among the groups of patients and controls (data not shown). It should be noted that not all analyses could be performed for every sample due to insufficient quantities of blood drawn from some patients and controls who did not consent to a second blood draw. In summary, we could not account for the unusually high levels of HERV-K18 found in JRA patients by showing a correlation with known modifiers of HERV-K18 expression, including circulating IFN-α levels, EBV infection, and percentage of B cells. It is likely that no single factor is responsible for the elevated superantigen levels in these autoimmune patients, but instead, multiple factors contribute.

DISCUSSION

We show that expression of a host-encoded superantigen, HERV-K18 Env, is significantly elevated in the peripheral blood of patients with JRA, but not pediatric SLE patients, compared with samples from healthy controls. We have previously shown that this superantigen strongly activates peripheral blood T cells[7,35], and have postulated that it may contribute to autoimmune disease by activating autoreactive T cells. While we saw no striking differences in T cell numbers in peripheral blood in the tested groups, it is likely that a more detailed spectrotypic analysis might be required to detect any differences. Further, we have shown that the HERV-K18 superantigen was expressed in inflamed joints of 7/7 JRA subjects tested, and in 2/5 patients the expression was at an even higher level than was found in the peripheral blood. While our sample number was small, the results suggest that the HERV-K18 superantigen may be associated with disease exacerbation in JRA.

In addition, we observed that IFN-α transactivates HERV-K18 strongly in JRA patients and healthy children, but transactivates HERV-K18 weakly in pediatric lupus patients. This difference was highly significant ($p = 0.009$), although the finding was unexpected and its interpretation is subject to debate. Interestingly, the elevated levels of

HERV-K18 in the JRA patients were found to be independent of previously reported modifiers of transactivation, including EBV infection, circulating IFN-α levels, and the percentage of B cells. This suggests that expression of the HERV-K18 superantigen is a complicated issue and likely is not dependent upon a single factor, but instead may result from a combination of events.

It is well known that steroid treatment interferes with the host immune response to viruses. Patients treated chronically with steroids often respond poorly to viral infection. In various animal models, it has been shown that steroid treatment affects expression of certain mouse endogenous retroviruses[48,49]; however, there have been no studies of the effects of cytotoxic agents on either human or murine endogenous retroviruses. Because of our small sample numbers, it was not possible to accurately determine whether different drug regimens used to treat the JRA and SLE patients affected HERV-K18 expression. We did, however, note several trends. As shown in Table 1, all but 2 of the SLE patients were undergoing prednisone therapy, and interestingly, those 2 patients, who were not taking any drugs, had the lowest levels of superantigen expression. In contrast, of the 3 JRA patients taking no medication, only one had normal HERV-K18 levels. Since only 2 JRA patients were taking prednisone, steroid use could not account for the generally higher levels of HERV-K18 seen in this group. It is possible, however, that the steroid use downregulated HERV-K18 transcription in the lupus patients, as has recently been reported for mouse mammary tumor virus (MMTV). Steroid treatment was shown to upregulate transcription originating from the MMTV long terminal repeat promoters, but paradoxically to downregulate MMTV superantigen transcription[49]. On the other hand, in our sample of JRA patients, there was no obvious correlation with any of the medications that would explain the high levels of HERV-K18 expression in this group.

It has been reported that SLE patients have higher circulating levels of IFN-α[40-42]. This trend was evident in our small group of patients: 5/11 (45.4%) SLE patients tested had detectable serum levels (> 10 pg/ml), while 3/13 (23.1%) JRA patients had detectable levels compared with 5/22 (22.7%) controls; however, we did not see drastically higher levels of IFN-α in the SLE group (Figure 4A). It has recently been reported that prednisone treatment inhibits plasmacytoid dendritic cell production of IFN-α[50]. Since these cells are the predominant IFN-α producers in humans[42], and because the majority of our lupus patients were receiving prednisone, this might account for the relatively lower levels of circulating IFN-α that we observed in comparison with reports from other studies.

Alternatively, since IFN-α levels correlate with levels of anti-dsDNA antibody in SLE, the observed levels might be due to the relatively low disease activity of these patients, as indicated by SLEDAI score (Table 1)[51-54]. There was no correlation in any of the groups between circulating levels of IFN-α and HERV-K18 superantigen expression. On the other hand, *in vitro* induction of HERV-K18 using rIFN-α (Roferon) was significantly different among the groups. In PBMC samples from JRA patients and controls, HERV-K18 was induced on average more than 8-fold after IFN-α treatment (Figure 3C). In contrast, pediatric SLE PBMC were relatively resistant to IFN-α treatment. The average induction for each SLE sample was only 2.43-fold, much reduced in comparison, but still a statistically significant increase. It is possible that negative feedback or a desensitization effect was active in the SLE patients in response to recurrently increased IFN-α levels. An intrinsic defect in SLE lymphocyte responsiveness to IFN-α is another possibility. Alternatively, prednisone may interfere with IFN-α responsiveness in patients with SLE. Prednisone acts on multiple cell types, thus it might directly affect transcription of HERV-K18 in B cells (we are currently testing this possibility), or it could also affect the responding T cell population, where it is known to mediate cytokine responses and nuclear factor-κB binding[55] and cause apoptosis of activated T cells[56,57]. Additionally, prednisone might act on other cells that regulate IFN-α production, as is the case with plasmacytoid dendritic cells[50]. The JRA samples instead showed a pronounced response to IFN-α (Figure 3B), with absolute levels of HERV-K18 sharply increased compared with controls treated with IFN-α ($p = 0.004$), paralleling the significant increase in untreated JRA samples compared to controls (Figure 1B). While we previously reported that IFN-α preferentially upregulates HERV-K18 in B cells[8], the percentage of B cells in each sample did not correlate with HERV-K18 and we could not account for the differences seen in IFN-α induction (Figure 4C). Instead, it was the response of the cells to IFN-α, but not the quantity of cells, that influenced superantigen expression. Because of the important role of IFN-α in SLE, it has recently been proposed that blocking IFN-α production might have benefit in patients with lupus, and efforts to inhibit plasmacytoid dendritic cell production of IFN-α through the blood dendritic cell antigen pathway are being investigated[58,59]. It will be interesting to examine the effects of such treatments on HERV-K18 expression.

To date, this is the first study demonstrating the presence of a host-encoded superantigen in rheumatic disease. Although our sample number was small, the high expression levels of the HERV-K18 superantigen in the blood and SFMC of patients with JRA is striking. It has been hypothesized for years that JRA may have an infectious etiology. Exogenous pathogens have been considered, but this is the first evidence indicating that an endogenous "pathogen" may play a role in activating autoimmune T cells. We conclude that further investigations are warranted, involving larger numbers of subjects, comparing untreated patients with glucocorticoid treated patients, and controlled with

another inflammatory condition like asthma, with repeated testing during various timepoints in the course of disease.

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