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Gene Expression Profile of Herpesvirus-Infected T Cells Obtained Using Immunomicroarrays: Induction of Proinflammatory Mechanisms

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Herpesvirus infections can frequently lead to acute inflammation, yet the mechanisms regulating this event remain poorly understood. In order to determine some of the immunological mechanisms regulated by human herpesvirus infections, we studied the gene expression profile of lymphocytes infected with human herpesvirus 6 (HHV-6) by using a novel immunomicroarray. Our nylon-based immunomicroarray contained more than 1,150 immune response-related genes and was highly consistent between experiments. Experimentally, we found that independently of the HHV-6 strain used to infect T cells, multiple proinflammatory genes were increased and anti-inflammatory genes were decreased at the mRNA and protein levels. HHV-6 strains A and B increased expression of the genes for interleukin-18 (IL-18), the IL-2 receptor, members of the tumor necrosis factor alpha superfamily receptors, mitogen-activated protein kinase, and Janus kinase signaling proteins. As reported previously, CD4 protein levels were also increased significantly. Specific type 2 cytokines, including IL-10, its receptor, and IL-14, were downregulated by HHV-6 infection and, interestingly, amyloid precursor proteins and type 1 and 2 presenilins. Thus, T cells respond to HHV-6 infection by inducing a type 1 immune response that may play a significant role in the development and progression of diseases associated with HHV-6, including pediatric, hematologic, transplant, and neurologic disorders.

Human herpesvirus 6 (HHV-6) is a lymphotropic betaherpesvirus first isolated from the peripheral blood of immunocompromised patients with lymphoproliferative disorders (34). Infection with HHV-6 typically occurs before the age of 3 years, and primary infection can account for 10 to 40% of the hospitalizations of children in this age group. HHV-6 is a clinically relevant virus and is the causative agent of exanthem subitum, a pediatric fever and skin rash (roseola) (47) that can have serious and fatal complications (5). The seroprevalence of HHV-6 in the general population is greater than 90% (10, 15), its reactivation is common in immunocompromised individuals, and, like the closely related virus cytomegalovirus (CMV), it has been associated with organ, bone marrow, and peripheral blood cell transplantation failure and engraftment inhibition (for reviews, see references 1 and 48). HHV-6 infection may also enhance human immunodeficiency virus (HIV) replication and has been suggested to play a role in HIV/AIDS progression (13, 24).

In addition to its role in pediatric febrile illness and transplantation, HHV-6 infection has also been associated with central nervous system (CNS) complications, including neuroinflammation, febrile seizures, and encephalitis/encephalopathy (48). In immunocompetent adults, HHV-6 is considered a commensal virus of the CNS (8, 14). However, HHV-6 has been linked with the pathogenesis of two chronic progressive demyelinating diseases of the CNS, multiple sclerosis (MS) and progressive multifocal leukoencephalopathy (8). The findings in MS are based on immunological, molecular, and histological studies (2, 3, 8, 12, 17, 29, 35, 40, 42). Despite the association of HHV-6 with these clinical disorders, the pathological mechanisms and functional responses of cells infected with HHV-6 have yet to be defined. The virus/host interaction studies that are currently being done include analyses of viral variants, genetic susceptibility loci, and virus-specific immune responses (41).

Two major viral subgroups of HHV-6 have been defined, and they are designated variants A and B. Although there is significant DNA sequence homology between the two variants, each has distinctive genomic, antigenic, and biological properties (1, 6, 30). HHV-6B is found primarily in the peripheral blood, saliva, and lymph nodes of healthy individuals and has been detected in the serum of children with roseola (47). HHV-6A is detected less frequently than HHV-6B in healthy adults and appears primarily in the skin, brain, and cerebrospinal fluid. Little is known about the epidemiological and geographical distribution of the HHV-6A variant (13). A greater neurotropism of the HHV-6A variant has been suggested based on the detection of HHV-6A in the cerebrospinal fluid of children and adults (20). In addition, HHV-6A has

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been demonstrated in the CNSs of AIDS patients with areas of demyelination (8). Increased HHV-6A-specific immune responses and the detection of HHV-6A-specific DNA sequences in the serum, urine, and peripheral blood lymphocytes of MS patients support an involvement of the HHV-6A variant in this disorder (3, 23, 42).

Although herpesvirus infections can induce proinflammation and, in acute cases, cause neuroinflammation or become fatal (33, 48), relatively little is known about the basic biology of this betaherpesvirus and how it regulates the immune response. To determine immunologically related cellular responses to HHV-6 infection, the gene expression profile of HHV-6-infected T cells was evaluated by using a novel immunomicroarray system. This custom human immunomicroarray was composed almost entirely of known gene sequences that were selected from the human cDNA library from Research Genetics. The immunomicroarray consisted of interleukin ligands and receptors, chemokines, and cellular signaling molecules. An entire listing of the array can be found at www.grc.nia.nih.gov/branches/rrb/dna/dna.htm. We have also posted the raw data from these experiments and our analysis of HHV-induced changes in T-cell gene expression at www.sbrc.mb.ca/dnnd. It is demonstrated here that, independently of the HHV-6 variant studied, HHV-6 induces the gene expression and protein production of multiple proinflammatory molecules, in particular, IL-18 and CD4. In contrast, IL-10 and IL-14, specific chemokine receptors and members of the presenilin and amyloid beta processing pathway, were downregulated. HHV-6 variant-specific gene expression profiles were also identified. These results suggest that T cells infected with HHV-6 induce proinflammatory mechanisms and highlight the use of molecular profiling of virus-infected cells as a powerful tool with which to define novel mechanisms of virus/host cell interactions.

MATERIALS AND METHODS

Cell lines, HHV-6, and CPE. The human T-cell lymphoblast line SupT1 was used to propagate the HHV-6A variant (GS) and the HHV-6B variant (Z29). SupT1 cell culture conditions and input multiplicity of infection were previously determined for the HHV-6A and HHV-6B variants, and infected and uninfected cell cultures (5×10^5 cells/ml) were prepared as previously described (42). Approximately 7 days following passage of cells, cytopathic effects (CPE) were observed and fresh uninfected cells were added at an infected-uninfected cell ratio of 1:2. Infection of the SupT1 cell line by HHV-6 was confirmed by immunofluorescence assay (IFA; see below). Infected and uninfected cells were harvested 5 days after passage. Greater than 50% of the infected SupT1 cells exhibited CPE and were positive by IFA staining on the day of harvest. HHV-6-infected and uninfected cells (5×10^7) were washed with phosphate-buffered saline (PBS), pH 7.2; cells were centrifuged at $600 \times g$; and pellets were frozen at −80°C and used for total RNA isolation. Supernatants were also harvested after centrifugation at $600 \times g$ for 10 min.

Real-time quantitative PCR and analysis of HHV-6 DNA and RNA. The primer set used for amplification of the DNA encoding the major capsid protein of HHV-6 was 5′-CTGCAGCAAATACGTTCTAGCC-3′ (positions 102793 to 102772) and 5′-GTGACGAGCGTTATTCCTTCG-3′ (positions 102656 to 102676), yielding a product of 520 bp. The probe for HHV-6 was 5′-TGCCGC TCTGGATAATTTGGCTTTGTC-3′ (positions 102718 to 102744). Standard control was used as described previously (37). The primer set for β-actin as an internal calibration was 5′-CACACTGTGCCCATCTACGA-3′ (positions 2146 to 2165) and 5′-CTCAGTGAGGATCTTCATGAGGTAGT-3′ (positions 2250 to 2225). The probe for β-actin was 5′-ATGCCCTCC-CCCATGCCATCCTGC GT-3′ (positions 2171 to 2196). A standard curve for HHV-6 was generated by using serially 10-fold-diluted viral DNA (10^6 to 10^2 copies) that was purified from HHV-6B (Z29)-infected SupT1 cells. A standard curve for β-actin was generated following twofold serial dilution of genomic DNA purified from normal donor peripheral blood mononuclear cells (approximately 8×10^5 to 5×10^4 copies). Because individual peripheral blood mononuclear cells have two copies of β-actin, 1 ng of DNA contains approximately 333 copies of the gene for β-actin. Five hundred nanograms of sample DNA was used as the template and analyzed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Amplification of standard and sample DNAs was conducted in the same 96-well reaction plate (Applied Biosystems) by using 50°C for 2 min for activation of uracil-*N*-glycosylase, 95°C for 10 min in order to inactivate uracil-*N*-glycosylase, and 45 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and extension). All standards and samples were assayed in triplicate.

IFA. For the preparation of slides, infected cells showing a viral CPE were collected by centrifugation, washed twice with sterile PBS, and fixed on glass slides with acetone for 5 min at −20°C. Fixed slides were incubated with a 1:40 dilution of a mouse monoclonal antibody (MAb) against HHV-6 glycoprotein 116/64/54 (gp116/64/54; ABI, Columbia, Md.) for 30 min at 37°C. The slides were washed three times in PBS for 10 min and twice with distilled water and incubated with fluorescein-labeled goat anti-mouse immunoglobulin G serum (Sigma, St. Louis, Mo.) for 30 min at 37°C and washed as described above. Slides were stained with Evans blue at a 1:40 dilution, washed once with distilled water, and mounted with buffered glycerin. Uninfected SupT1 cells were used as negative controls.

NIA immunomicroarray. A defined set of 1,152 immune response-related cDNAs was PCR amplified and printed on nylon filters by using a GMS417 Microarrayer (Genetic Microsystems). Clones were selected from a commercially available (Research Genetics, Inc.) master set of approximately 15,000 human, verified-sequence, T1 phage-negative IMAGE Consortium clones. A small number of clones were acquired privately. The nylon membrane-based assays use a relatively small quantity of total RNA as a probe (100 to 1,000 ng) compared to fluorescent cDNA assays conducted using glass microarray slides. The National Institute on Aging (NIA) immunomicroarray includes cDNAs encoding immune response-specific cell differentiation antigens; cytokines and their receptors, including interferons, interleukins, and chemokines; apoptosis-related genes; structural and cytoskeletal genes; and signal transduction genes, including those for Janus kinase signaling protein (JAK), STATs, transcription factors, growth factors, and other cellular and metabolic molecules. An entire list of the genes printed on the immunomicroarray can be found at www.grc.nia.nih.gov/branches/rrb/dna/dna.htm. The raw data and Z ratios calculated in these experiments can be found at www.sbrc.mb.ca/dnnd.

RNA preparation and microarray analysis. Total RNA was prepared from infected SupT1 cells by using SNAP RNA isolation kits (Invitrogen), and RNA integrity was confirmed by 1.1% agarose formaldehyde gel electrophoresis. One microgram of total RNA was primed by using oligo (dT) oligonucleotide (Research Genetics), and the first-strand cDNA probe was labeled with α-^{33}P (3,000 Ci/mmol; ICN) in the presence of a final concentration of 1× first-strand cDNA buffer, 0.1 M dithiothreitol, 0.5 mM deoxynucleoside triphosphates minus dCTP (Pharmacia), RNase inhibitor (10 IU; Life Technologies), and 1 U of Superscript 2 reverse transcriptase (Life Technologies). The reaction was incubated for 70 min at 42°C, and RNA was removed by using a Biospin P30 spin column (Bio-Rad) following addition of 50 mM EDTA and 200 nM NaOH and incubation at 65°C for 30 min. Radioisotope incorporation was determined by counting a 1-μl sample, and 75 μl of each probe was denatured and added to an immunomicroarray that had been pretreated for 4 h at 50°C with microhybridization buffer (Research Genetics). Hybridization continued overnight at 50°C, and microarrays were washed in 1× SSC (1× SSC is 0.15 M sodium citrate plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 3×15 min at 50°C. Arrays were exposed to phosphorimaging sheets (Storm) for 48 h and analyzed with a Storm PhosphorImager (Molecular Dynamics). All genes produced a quantifiable signal following hybridization.

Gene expression was determined from microarray experiments by capturing the pixel density of each ^{33}P-labeled PCR product (gene) by using QuantOne Image Software (Bio-Rad) and exporting the resulting data to Microsoft Excel (Microsoft, Seattle, Wash.). Raw intensity data for each experiment was normalized by Z transformation. Briefly, first the raw pixel density data for each gene is $\log_{10}$ transformed and then Z scores are calculated. Z scores are calculated by subtracting the average gene intensity (calculated from the sum of all data points) from the log intensity value of each gene and dividing that result by the standard deviation of all of the measured intensities. Thus, Z scores transform raw intensity data into a more flexible data expression in which the value of each individual gene is reported as a function of its distance from the mean of all of the measured gene intensities and is expressed in units of standard deviation. This flexible normalization procedure facilitates comparisons between microarray experiments by adjusting for differences in hybridization intensity. Z scores

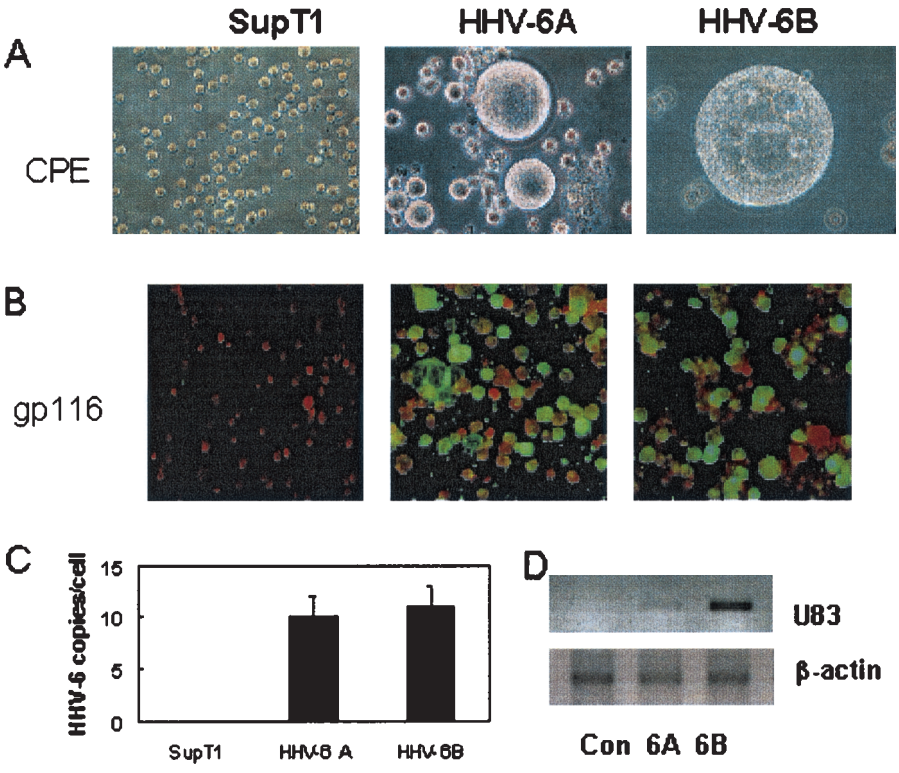

FIG. 1. SupT1 cells are highly infected with HHV-6A or HHV-6B. (A) CPE at 5 days following infection with HHV-6A or HHV-6B. Large, balloon-shaped cells were abundant (approximately 50% ± 7% of the total cell number) in SupT1 cultures infected with either HHV-6A or HHV-6B. HHV-6B induced a slightly greater individual CPE than did HHV-6A (magnification, ×32). (B) SupT1 cells were infected with HHV-6A or HHV-6B, and 5 days following infection, cells were fixed and stained with an HHV-6 anti-gp116/64/54 MAb. Fluorescence imaging shows that a high percentage (approximately 50% ± 5%) of SupT1 cells were infected with HHV-6A or HHV-6B (magnification, ×20). (C) TaqMan analysis determined that the HHV-6A and HHV-6B genomes were present at approximately 10 copies per T cell. (D) Semiquantitative reverse transcription-PCR determined that the HHV-6 U83 gene was expressed in SupT1 cells infected with either HHV-6A or HHV-6B. Because U83 is a late-expression gene, this result shows that HHV-6A or HHV-6B replication occurs in SupT1 cells. The data shown are representative of three independent infections. Con, control.

were exported and directly subjected to Cluster analysis (16) (Stanford University).

Gene expression differences between any two experiments are calculated by taking the difference between the observed gene Z scores. Z ratios express these differences in terms of their relationship to the standard deviation of the distribution of all of the observed gene changes. All of the calculated Z ratios for a given comparison can be rank ordered on this basis. Z ratios were determined for all of the individual genes in the appropriate comparisons; for example, a Z ratio for IL-18 would be calculated by determining the ratio of the IL-18 Z scores obtained from uninfected and HHV-6-infected SupT1 cells.

ELISA and immunoblot analysis. Cell culture supernatants were collected and centrifuged at 600 × *g* for 10 min. IL-18 and IL-10 protein levels were determined with an enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's (R&D Systems, Minneapolis, Minn.) suggestions. Western blot analysis for CD4 was performed by using approximately 5×10^6 HHV-6A-infected, HHV-6B-infected, and uninfected T cells that were harvested 5 days after passage by centrifugation at 600 × *g* for 10 min. Cell supernatants were discarded, and the remaining cell pellets were lysed in 500 μl of radioimmunoprecipitation assay buffer (50 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 50 mM Tris [pH 7.4], 1 mM EGTA, 10 mM Na orthovanadate, 10 mM NaF, and protease inhibitor cocktail [one tablet per 50 ml; Sigma]) for 10 min at 4°C. Each sample was centrifuged at 14,000 × *g* for 10 min at 4°C, and protein levels in cell lysates were determined (Pierce, Rockford, Ill.). Whole-cell lysates obtained from HHV-6-infected and uninfected cells were mixed with an equal amount of 2× sample buffer (0.125 M Tris, 4% sodium dodecyl sulfate, 20% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue, 10% 2-mercaptoethanol) and boiled for 5 min. Cell lysates (50 μg) were separated and resolved by using a 10% Tris-glycine gel (Invitrogen, Carlsbad, Calif.) and transferred to a 0.22-μm-pore-size nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), and Western analysis was performed with an anti-CD4 MAb (1:1,000; R&D Systems) or the housekeeping protein vinculin (1:1,000 dilution; Sigma). Proteins were visualized with horseradish peroxidase-conjugated anti-mouse immunoglobulin G antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) at a 1:30,000 dilution and subsequent detection by enhanced-chemiluminescence assay (Pierce). The Kodak (Rochester, N.Y.) Image Station 440 was used to estimate the net intensities of visualized bands.

Cluster analysis and statistics. Clustering of changes in gene expression was determined by using public domain Cluster based on pairwise average-linkage cluster analysis (16). Gene expression raw data, log values, and Z scores were averaged by using the mean ± the standard deviation. Comparisons between experiments were conducted by regression analysis. For all tests, statistical significance was considered to be at the $P < 0.05$ level (Instat2; GraphPad Software, San Diego, Calif.).

RESULTS

Variants HHV-6A and HHV-6B infect SupT1 cells with equal efficiency. The T-cell lymphoblast line SupT1 was infected with the HHV-6A (GS) and HHV-6B (Z29) variants to define virus-induced molecular profiles by using immunomicroarrays. Because the level of virus expression may influence molecular profiling of virus-infected cells, it was critical to demonstrate that comparable levels of infection with the HHV-6A and HHV-6B variants were attained. This was demonstrated in the following ways. (i) The percentage of large,

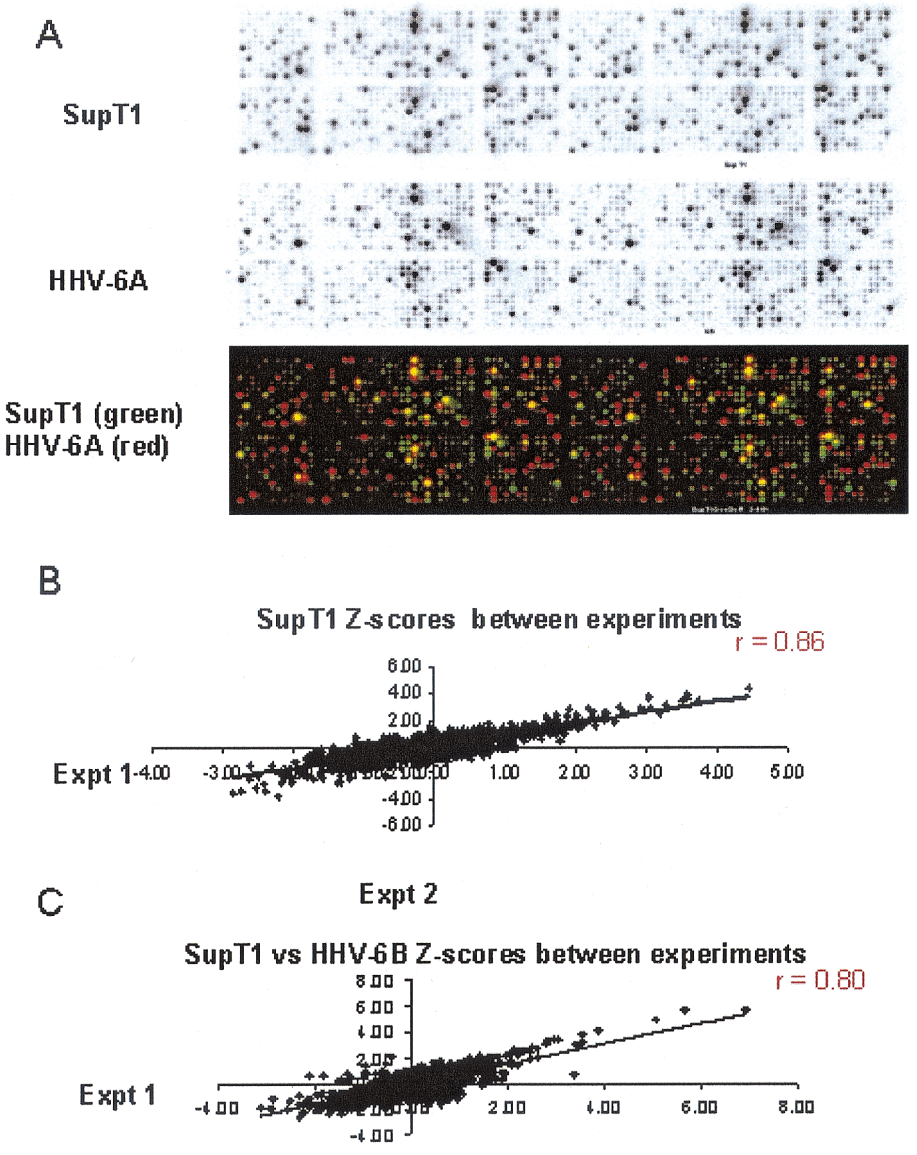

FIG. 2. Immunomicroarray analysis was consistent between experiments. (A) Representative radiolabeled nylon immunomicroarray showing hybridization patterns of mRNA populations from uninfected and HHV-6A-infected SupT1 cells and a false color overlay comparing changes in gene expression from uninfected cells (green channel) and SupT1 cells infected with HHV-6A (red channel). Yellow represents genes that were expressed at equal intensities. Each array contains two identical grids. (B and C) Regression analysis of Z scores from two independent experiments showing that the microarray hybridization patterns are highly similar. Z scores representing individual genes from each experiment were plotted, and the relationship (slope) between two independent experiments was calculated. A perfect relationship between experiments would equal a slope of 1. The average Pearson correlation for all experiments was $P = 0.83 \pm 0.10$.

balloon-shaped cells, as a measure of CPE observed in HHV-6A- and HHV-6B-infected cultures, was equivalent with each infection showing approximately 50% ± 7% CPE (11) (Fig. 1A). (ii) The percentage of cells expressing HHV-6 antigens, as defined by IFA with the gp116/54/64 MAb (Advanced Biotechnologies Inc., Columbia, Md.), which detects both variants of HHV-6, was similar (approximately 50% ± 5%; Fig. 1B). (iii) Quantitative TaqMan analysis of HHV-6 DNA confirmed equivalent HHV-6 viral loads in infected T cells (Fig. 1C). No HHV-6 DNA was amplified from uninfected T cells, while T cells infected with the HHV-6A and HHV-6B variants were infected at averages of 10 and 11 copies per cell, respectively (Fig. 1C). These results were obtained in samples from cell lines harvested for all microarray experiments (Fig. 1C). (iv) Finally, semiquantitative reverse transcription-PCR analysis of HHV-6-infected T cells showed that mRNA encoding a late-expression HHV-6 viral chemokine gene (U83) (49) was readily detected (Fig. 1D).

Consistency between immunomicroarray analyses. It was important to compare mRNA populations from uninfected and HHV-6-infected T cells to determine interassay variability. To correct for interassay variability (hybridization intensity), gene expression profiles were normalized for each microarray by determining Z scores. The Z score represents the average

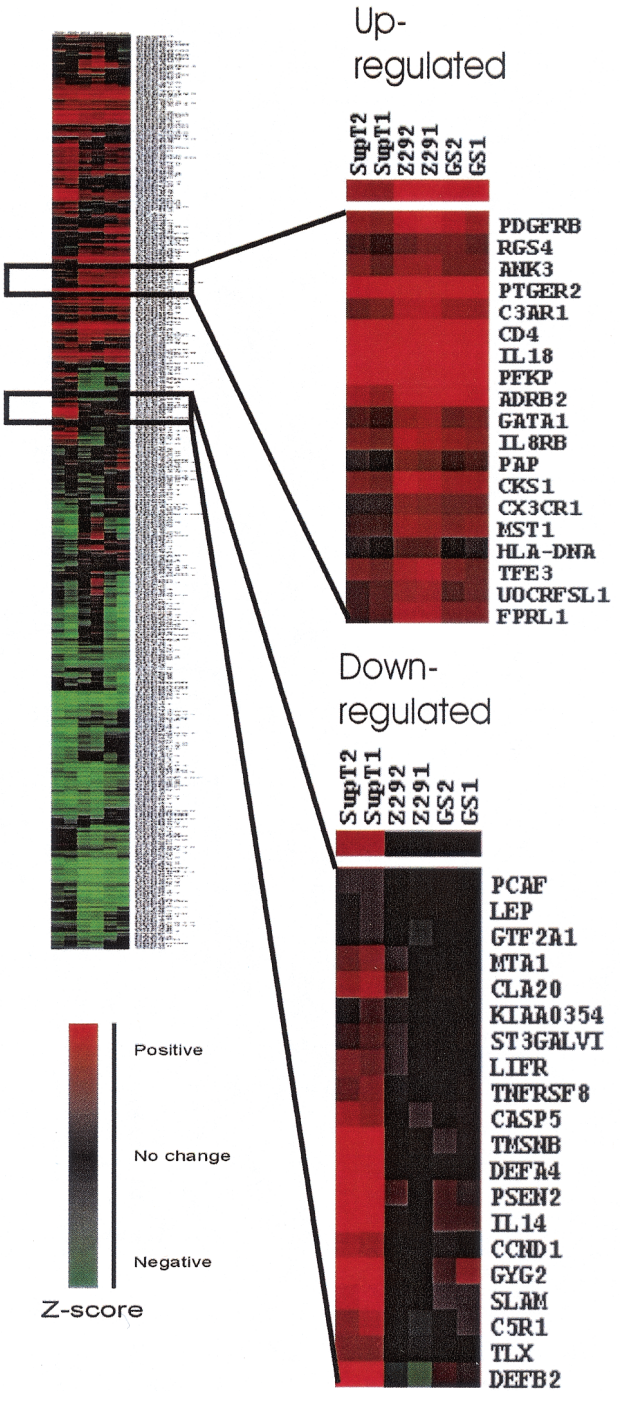

FIG. 3. Gene expression profiles were highly similar between independent experiments and experimental conditions clustered together. Z scores ranging from −5 to +5 were subjected to Cluster analysis, which determined that a wide array of gene expression profiles was present between uninfected and HHV-6-infected SupT1 cells. An example of genes that were upregulated or downregulated is enlarged and shown at the right. Importantly, experimental conditions also clustered together, demonstrating the reliability of microarray analysis.

change in gene expression from the mean hybridization value of each microarray (for a full description, see Materials and Methods). As shown in Fig. 2A, hybridization intensities between microarray experiments and gene expression profiles were highly consistent between experiments. Moreover, assignment of color channels for independent experimental conditions highlighted significant changes in gene expression (Fig. 2A). Results of regression analysis of Z scores of gene expression profiles from control experiments ranged from $r = 0.84$ to $r = 0.86$ with Z scores ranging from 5.5 to −3.9, ranged from $r = 0.79$ to $r = 0.85$ in T cells infected with variant A with Z scores ranging from 6.7 to −4.1, and ranged from $r = 0.76$ to $r = 0.84$ in T cells infected with variant B with Z scores ranging from 8.1 to −4.6. For all experiments, the Pearson correlation average was $P = 0.83$. An example of a Z score regression analysis from uninfected and HHV-6-infected T cells is shown in Fig. 2. These results demonstrate the reliability of immunomicroarray analysis from HHV-6-infected cells. In order to further examine microarray reliability, we conducted cluster analysis (16). Importantly, clustering of Z scores confirmed that similar experiments clustered together, indicating reliability in the microarray analysis of the gene expression profiles across experiments (Fig. 3).

HHV-6 infection induces multiple changes in gene expression. Immunomicroarray gene expression profiles were determined from HHV-6A- and HHV-6B-infected T cells and compared to that of uninfected cells. Z ratios were calculated by comparing Z scores from individual experiments. As shown in Table 1, a number of genes were increased or decreased at the mRNA level by at least 2 Z ratios in HHV-6-infected cells. For example, phosphofructokinase, which is important in energy metabolism; members of the JAK kinase signaling cascade; and HLA class 1 and 2 genes, which are important in antigen presentation, were all increased. Not shown are members of the apoptosis family that were highly induced independently of the HHV-6 variant used to infect SupT1 cells. In particular, apoptosis-associated tyrosine kinase (accession number ABI014541) was induced by 3.84 and 3.27 (Z ratio) in HHV-6B- and HHV-6A-infected T cells, respectively. In addition, caspases 3 and 8 were elevated independently of the variant used to infect T cells (not shown), suggesting that SupT1 cells undergo apoptosis during infection. Genes that were downregulated by at least 2 Z ratios during infection included members of the presenilin and amyloid beta precursor protein family and the recently identified measles receptor signaling lymphocytic activation molecule (SLAM; CDw150) (43; Table 1).

HHV-6 infection of SupT1 cells induces proinflammatory and decreases anti-inflammatory gene expression. Several proinflammatory genes showed increased mRNA levels, including IL-18, IL-2 receptor, mitogen-activated protein kinase family members, tumor necrosis factor (TNF) receptor superfamily members, and associated signaling molecules, including TRAF3 and CD4 (Table 2). Interestingly, the majority of the mRNA levels for proinflammatory interleukins, including IL-1, IL-8, and IL-12, their receptors, and gamma interferon (IFN-γ) were unchanged during HHV-6 infection, independently of the variant used (data not shown). Associated with increased expression in specific proinflammatory genes was the concurrent downregulation of the anti-inflammatory genes for IL-10 and its receptor, the IL-13 receptor, and IL-14. All of these genes are important in reducing proinflammatory cytokine synthesis (Table 2). Only reported here are genes whose expression profiles were modified by at least 1.5-fold. A full

TABLE 1. HHV-6 infection of SupT1 cells induces and inhibits multiple genes[a]

Gene type and Z ratio		Gene product	Description	Function(s)
HHV-6A/control	HHV-6B/control			
Up-regulated				
2.47	2.66	PFKP	Phosphofructokinase	Glycolytic pathway
1.55	2.62	PRKM1	Protein kinase	MAPS kinase 1 activation
2.78	2.46	WSX-1	Class 1 cytokine receptor	Proinflammation signaling
1.60	2.44	SSI-1	JAK binding protein	Tyrosine kinase signaling
1.73	1.91	BCL6	B-cell CLL/lymphoma 6	B-cell signaling, chemokine regulation
1.58	1.73	HLA-DQB1	MHC[b] class II, DQ beta 1	Antigen presentation
2.12	1.63	HLA-A	MHC class IA	Antigen presentation
Down-regulated				
−3.02	−3.41	RYK	RYKR-like tyrosine kinase	Tyrosine kinase
−1.83	−3.40	APPBP1	Amyloid precursor protein 1	Amyloid processing
−2.76	−3.34	ATF2	Activating transcription factor 4	Transcription regulation
−3.56	−3.23	MDM2	p53 binding protein	p53 regulation
−2.94	−2.92	APLP1	Amyloid beta precursor protein	Amyloid beta processing
−2.89	−2.76	PSEN2	Presenilin 2	ER[d] protein chaperone/amyloid processing
−2.27	−2.74	PSEN1	Presenilin 1	ER protein chaperone/amyloid processing
−2.98	−3.23	CXCR5	CXC chemokine receptor	Chemokine signaling
−1.66	−2.25	SLAM	Lymphocytic activation molecule	Lymphocyte activation/viral receptor
−2.13	−2.06	APLP2	Amyloid precursor protein 2	Amyloid beta processing

[a] The Z ratios shown represent genes with the greatest changes in expression and are averages from two independent experiments performed in duplicate. The data were sorted based on high Z ratios comparing SupT1 cells infected with HHV-6B (Z29) or variant HHV-6A (GS) and uninfected cells (control). Genes with a variance between experiments of greater than 0.2 were not analyzed.
[b] MHC, major histocompatibility complex.
[c] MAP, mitogen-activated protein.
[d] ER, endoplasmic reticulum.

listing of the raw data, Z scores, and Z ratios of these experiments can be located at www.sbrc.mb.ca/dnnd.

HHV-6 variant-specific changes in gene expression. As variants HHV-6A and HHV-6B differ at the molecular, immunological, and tropic levels (9, 21), immunomicroarray analysis also demonstrated differential expression of several genes in T cells infected with either HHV-6A or HHV-6B (Table 3). When gene expression profiles were compared between the virus variants, HHV-6B-infected T cells had elevated levels of several proinflammatory molecules compared to HHV-6A-infected cells, including members of the TNF-α and lymphotoxin receptor superfamily, phospholipase C, and specific adhesion molecules, including CD28 relative to HHV-6A (Table 3). Compared to HHV-6B-infected T cells, HHV-6A-infected T cells elevated the expression of the gene for phospholipase D2 (2.48-fold [Z ratio] higher than in HHV-6B-infected SupT1 cells), NF-κB-inducing kinase (2.04-fold higher than in HHV-6A-infected cells), nitrogen oxide synthase (2.02-fold higher than in HHV-6B-infected cells), and JAK-1 (1.75-fold higher than in HHV-6A-infected cells).

Pro- and anti-inflammatory cytokine protein levels in HHV-6-infected SupT1 cells. In agreement with microarray analysis results, IL-18 protein levels were elevated significantly in supernatants from HHV-6A- and HHV-6B-infected T-cell cul-

TABLE 2. HHV-6 infection of SupT1 cells induces TH1-type gene expression profiles[a]

Gene type and Z ratio		Gene product	Description	Function/immune response
HHV-6A/control	HHV-6B/control			
Proinflammatory				
1.86	2.60	TRAF3	TNF receptor-associated factor	TNF signaling
2.47	2.55	CD4	CD4	MHC class 2 response
1.96	2.38	TNFRSF11A	TNF receptor superfamily	NFκB activation
2.25	2.30	MAPK11	Mitogen-activated protein kinase 11	Mitogen-activated signaling
3.12	2.20	SCYB11	Inducible cytokine subfamily B	CXC chemokine signaling
2.03	2.02	IL18	IL-18	IFN-γ activation
1.04	1.21	IL-2R	IL-2 receptor	Mitogen induction of T cells
Anti-inflammatory				
−2.95	−3.19	IL14	IL-14	Induction of B-cell proliferation
−1.51	−2.18	IL10	IL-10	Inhibition of cytokine synthesis
−0.34	−1.07	IL10R	IL-10 receptor	Affinity for IL-10
−1.26	−1.20	IL13	IL-13 receptor A	Inhibition of inflammatory cytokine synthesis

[a] The Z ratios shown represent averages from two independent experiments performed in duplicate. The data were sorted based on high Z ratios comparing SupT1 cells infected with HHV-6B (Z29) or variant HHV-6A (GS) and uninfected cells (control). Genes with a variance between experiments of greater than 0.2 were not analyzed.

TABLE 3. HHV-6 variant-dependent changes in gene expression[a]

Z ratio			Gene product	Description	Function(s)
HHV-6A/control	HHV-6B/control	HHV-6B/HHV-6A			
−0.70	1.67	2.37	MAPK4	Mitogen-activated protein kinase 4	Mitogen-activated signaling
−1.60	−0.01	1.59	PIGA	Phosphatidylinositol glycan	Phosphatidyl signaling
−0.62	0.93	1.55	PLAUR	Plasminogen activator, urokinase receptor	Plasminogen and urokinase activity
−0.88	0.66	1.54	LTBR	Lymphotoxin beta receptor	TNF-mediated proinflammation
−0.31	1.22	1.53	TNFRSF5	Tumor necrosis factor receptor superfamily	TNF-mediated proinflammation
−1.19	0.34	1.53	CD9	CD9 antigen	Cell adhesion and migration
−1.57	−0.07	1.50	PLCG2	Phospholipase C, gamma 2	IP_3 and intracellular calcium regulation
0.13	1.59	1.46	NFIC	nuclear factor I/C	CCAAT-binding transcription factor
−0.97	0.49	1.46	JUNB	*junB* proto-oncogene product	JunB signaling
−0.73	0.71	1.44	CD59	CD59 antigen	T-cell lymphocyte activation
−0.50	0.93	1.43	NFATC1	Nuclear factor of activated T-cells	T-cell transcription
−0.25	1.10	1.35	PTN	Pleiotrophin	Heparin binding growth factor 8
0.18	1.51	1.33	CD28	CD28	Adhesion molecule
−1.00	0.33	1.33	JUND	*junD* gene product	JunD signaling

[a] The Z ratios shown are averages from two independent experiments performed in duplicate. Column 3 (HHV-6B/HHV-6A) reports the Z ratio difference in gene expression between SupT1 cells infected with variant B and those infected with variant A. The data were sorted to show high levels of gene expression in SupT1 cells infected with variant B. Columns HHV-6A/control and HHV-6B/control report Z ratio changes in gene expression in SupT1 cells infected with GS and Z29, respectively. Genes with a variance between experiments of greater than 0.2 were not analyzed.

tures (Fig. 4C). IL-18 mRNA levels were increased by approximately 2 Z ratios, independently of the HHV-6 variant analyzed; however, IL-18 protein levels were approximately fivefold higher in supernatants from SupT1 cell cultures infected with HHV-6B than in those infected with HHV-6A. Because IL-18 levels were elevated significantly in supernatants from infected cells, we chose to analyze other proinflammatory cytokines, including IFN-γ and IL-6, although their mRNA levels did not change during HHV-6 infection. Although IFN-γ protein levels did not change during HHV-6 infection, IL-6 levels were elevated significantly. T cells infected with HHV-6A had 14 ± 8 pg/ml ($P < 0.01$), and T cells infected with HHV-6B had 10 ± 2 pg/ml ($P < 0.01$). IL-6 was not detected in uninfected cells. We further examined the TH1-type response of T cells infected with HHV-6 by examining the levels of the anti-inflammatory cytokine IL-10, a cytokine that was downregulated by HHV-6 infection (Table 2). In agreement with the microarray data, IL-10 protein levels decreased significantly in supernatants from T cells infected with HHV-6A or HHV-6B (Fig. 4D). Although IL-10 mRNA was detected in all of the samples and IL-10 protein levels were readily detectable in uninfected cell culture supernatants, IL-10 protein could not be detected in supernatants from T cells infected with HHV-6. These results further indicate that HHV-6 infection induces proinflammatory mechanisms and, importantly, confirm the reliability of using these immunomicroarrays to determine functional changes in T cells infected with HHV-6.

DISCUSSION

Herpesvirus infections frequently activate an extensive immune response that, in acute cases, can be fatal (13, 33). However, the biological events that regulate the development of proinflammatory responses to herpesvirus infection remain poorly understood. This report focuses on HHV-6, a virus that has been associated with inflammatory disorders in pediatric and transplant patients and, more recently, with chronic progressive neurologic disease (39, 48). To further understand the mechanisms by which HHV-6 regulates a variety of immune mechanisms, a microarray analysis was performed on a large panel of genes involved with the human immune response. The NIA immunomicroarray was designed to include a wide assortment of pro- and anti-inflammatory cytokines, chemokines, and their receptors, as well as specific signaling molecules, including tyrosine kinases, protein kinase C, phospholipase C, and dephosphorylating phosphatases. The use of a focused array comprising known gene sets allowed a targeted survey of virus-specific immune responses in human T cells infected with HHV-6. We chose to study the immortalized SupT1 cell line because these cells were adapted previously to culture HHV-6A and HHV-6B, and thus, we could compare the HHV-6 variant-specific actions within one cell line. Primary human T cells would also provide an excellent model; however, multiplicity of infection would vary between T-cell populations and changes in gene expression between clinical specimens would further complicate microarray analysis.

The use of our novel immunomicroarray was a valid and reproducible approach, as demonstrated by a number of experimental observations. The intra- and interassay variability was remarkably consistent from RNAs obtained from independent HHV-6-infected and uninfected T cells (Fig. 2). Altered expression of several genes identified by this approach (Table 1) was consistent with known properties of T cells infected with HHV-6. For example, one of the highest Z ratios for both HHV-6A- and HHV-6B-infected T cells (Z ratio = 2.5; Table 2) was for CD4 mRNA. In agreement with these results, a significant increase in CD4 protein levels was reported previously for HHV-6-infected T cells (26) and confirmed in this study by Western analysis (Fig. 4A). The function of HHV-6-induced upregulation of CD4 remains unclear because HHV-6 uses CD46 as its primary receptor (36). Several genes were found to be downregulated by HHV-6 infection, according to immunomicroarray analysis (Table 1). Although it was not the focus of this study, it is of interest that SLAM was downregulated significantly by HHV-6 (Z ratio = −2.25; Table 1).

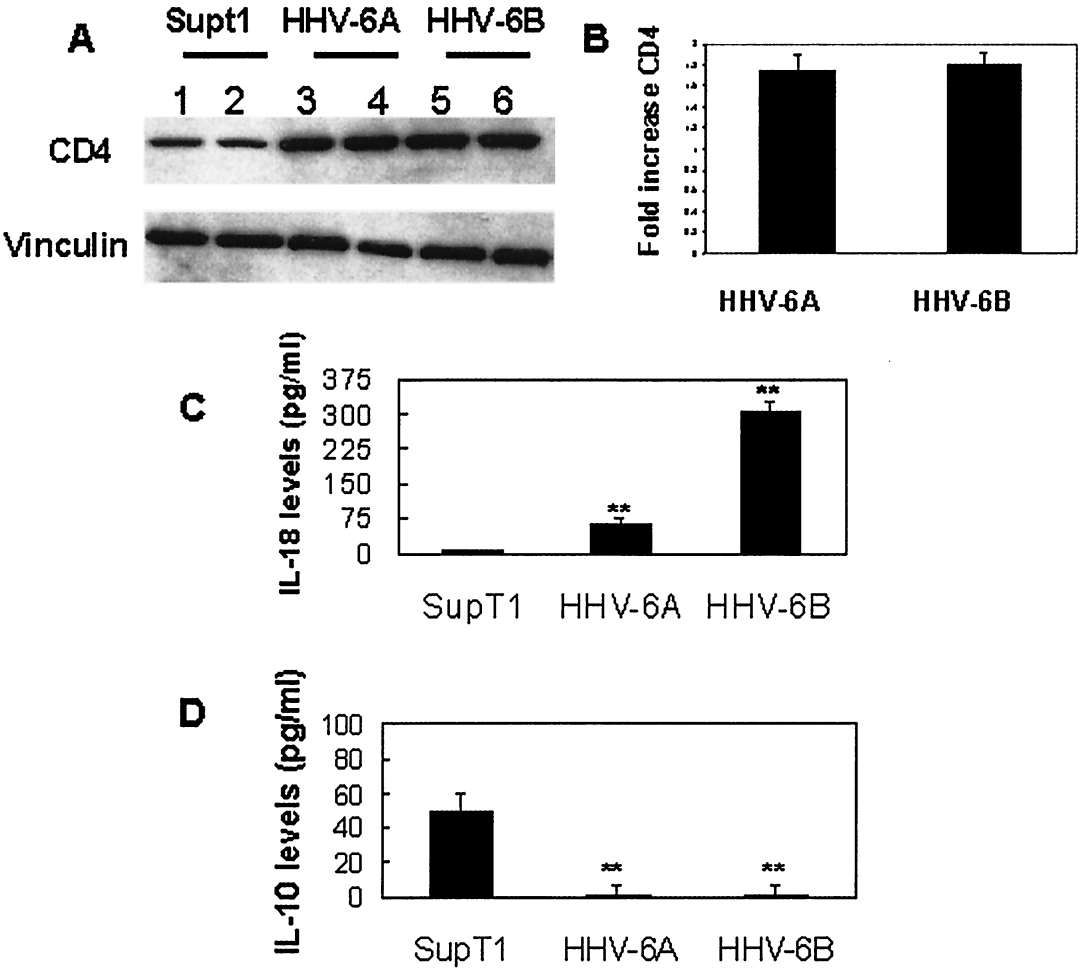

FIG. 4. Protein levels mirror mRNA levels in uninfected and HHV-6-infected SupT1 cells. (A) Whole-cell lysates were collected from the uninfected and infected cells used for both microarray experiments. Immunoblot analysis for CD4 and the housekeeping protein vinculin was performed on uninfected (lanes 1 and 2), HHV-6A-infected (lanes 3 and 4), and HHV-6B-infected (lanes 5 and 6) whole-cell lysates. Lanes 1, 3, and 5 represent data from cells harvested for the first microarray analysis, while lanes 2, 4, and 6 represent data from cells harvested for the second microarray experiment. (B) CD4 expression was increased 1.74-fold ± 0.24-fold and 1.87-fold ± 0.17-fold in cells infected with the HHV-6A and HHV-6B variants, respectively, compared to that in uninfected SupT1 cells, as shown by densitometry. IL-18 (C) and IL-10 (D) protein levels present in cell culture supernatants were analyzed by ELISA. IL-18 protein levels increased and IL-10 protein levels decreased during HHV-6A or HHV-6B infection, which is in agreement with observed changes in mRNA expression. The data shown are representative of two independent experiments. **, $P < 0.001$.

SLAM was identified recently as the coreceptor for measles virus (43), which, along with HHV-6, also uses CD46 as an entry receptor. CD46 was originally defined as a receptor for HHV-6 through the observation that CD46 is markedly downregulated during the course of HHV-6 infection (36). Therefore, the downregulation of SLAM, as identified by immunomicroarray analysis, suggests a possible role for SLAM as a coreceptor for HHV-6.

Of particular interest to this study is the observation that a number of proinflammatory genes were upregulated by HHV-6 infection while several anti-inflammatory genes were downregulated (Table 2). The recently described lymphokine IL-18 was identified by the immunomicroarray as upregulated in cultures infected by both variants of HHV-6 (Table1). IL-18, which was first identified as an IFN-γ-inducing agent in mice with endotoxic shock (28), increases IFN-γ production independently of IL-12 and stimulates the production of TH1-type cells (4). It is possible that IL-18 elevation is an important part of innate immunity, as IL-18 levels are also elevated in macrophages infected with influenza A virus or Sendai virus (31). In support of the observed increase in IL-18 mRNA as identified by immunomicroarray assay, IL-18 protein levels were demonstrated to be increased in supernatants of T cells infected with HHV-6 (Fig. 4C). In T cells infected with the HHV-6B variant Z29, the expression of IL-18 was increased approximately fivefold compared with that in uninfected T cells (Fig. 4C). This confirms the validity of the immunomicroarray data and suggests an important role for IL-18 in HHV-6-mediated inflammation. An important caveat of these studies is that microarray analysis of our in vitro HHV-6 model, albeit highly reproducible, could not differentiate between direct and indirect actions of HHV-6. This is particularly

evident for cytokine molecules that are released from T cells, including IL-18. It is possible that most of IL-18 is released from uninfected cells and, thus, the production of this proinflammatory cytokine results from an indirect mechanism of HHV-6. In contrast, it is possible that the majority of IL-18 is released from infected cells. Regardless, HHV-6 infection of T cells elevates the gene for IL-18 and other proinflammatory genes that would affect the local cellular environment during HHV-6 replication. Finally, although the infection efficiency was approximately 50%, the microarray analysis may not have identified genes that were modestly affected (a Z ratio change of <1.5).

As HHV-6 is currently under investigation as a possible etiologic agent in MS (13), it is of interest that IL-18 is expressed in demyelinating lesions of MS brains (7) and that levels of caspase 1, the regulatory enzyme of pro-IL-18, are elevated in the peripheral blood of MS patients (18, 19). These studies suggest that activation of the IL-18 pathway may be involved in the development of this demyelinating disease. Therefore, the increase in IL-18 expression regulated by HHV-6 as demonstrated by immunomicroarray analysis may be clinically relevant in this autoimmune disorder. HHV-6 has been associated with other diseases that target the immune system and have pathological implications for the CNS, including HIV (24). Specifically, HHV-6 infection enhances HIV replication in vitro (27) and may break the latency of HIV (24). Because IL-18 stimulates HIV type 1 replication in human monocytic cells (38), it would be of considerable interest to know if an HHV-6-mediated increase in IL-18 regulates HIV replication.

The finding that IL-18 was increased in T cells infected with HHV-6 suggested that other proinflammatory signaling molecules may also be increased. Indeed, immunomicroarray analysis demonstrated that HHV-6-infected T cells have significant increases in the gene expression of multiple signaling molecules associated with a proinflammatory response, including the IL-2 receptor, mitogen-activated protein kinase, phosphofructokinase, TNF receptor-associated proteins, JAK binding proteins, and small inducible cytokines (Table 1). Activation of proinflammatory signaling events and specific proinflammatory molecules are frequent features of viral infections (22, 25, 32, 46). It is of interest that IL-18 reduces the levels of IL-10 (44). High levels of IL-18 may explain the observed decrease in IL-10 levels in this study (Table 2 and Fig. 4D). IL-10 is an anti-inflammatory cytokine that inhibits the production of several proinflammatory molecules, including IL-1 and IL-12, and blocks the production of chemokines, including IL-8 and MIP-1α (45). Because IL-18 and IL-10 play important pro- and anti-inflammatory roles, respectively, these results suggest that HHV-6 infection of T cells could promote a type 1 proinflammatory cytokine response.

This study has demonstrated that pro and anti-inflammatory gene expression in T cells is altered during HHV-6 infection. Use of a defined immunomicroarray is a strategy that allows the targeted "mining" of information obtained from the analysis of changes in gene expression within complete cellular mRNA populations. Importantly, the upregulation of IL-18 mRNA was confirmed by protein analysis of infected cell culture supernatants. Likewise, the downregulation of IL-10 suggests that IL-18 and IL-10 work in concert as part of a virus-induced cytokine-mediated mechanism involved in the pathogenesis of diseases associated with HHV-6. As the list of HHV-6-associated diseases expands, including pediatric, hematologic, transplant, and neurologic disorders, mechanisms of virus-mediated pathogenesis need to be characterized. The use of targeted immunomicroarray analysis will be an important tool in the identification of genes and gene sets involved in the host immune response to viral infections.

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