Identification of novel gene signatures in patients with atopic dermatitis complicated by eczema herpeticum

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Background: A subset of patients with atopic dermatitis (AD) is prone to disseminated herpes simplex virus (HSV) infection (ie, atopic dermatitis with a history of eczema herpeticum [ADEH+]). Biomarkers that identify ADEH+ are lacking.
Objective: We sought to search for novel ADEH+ gene signatures in PBMCs.
Methods: An RNA-sequencing approach was applied to evaluate global transcriptional changes by using PBMCs from patients with ADEH+ and patients with atopic dermatitis without a history of eczema herpeticum (ADEH−). Candidate genes were confirmed by means of quantitative PCR or ELISA.
Results: PBMCs from patients with ADEH+ had distinct changes to the transcriptome when compared with those from patients with ADEH− after HSV-1 stimulation: 792 genes were differentially expressed at a false discovery rate of less than 0.05 (ANOVA), and 15 type I and type III interferon genes were among the top 20 most downregulated genes in patients with ADEH+. We further validated that IFN-α and IL-29 mRNA and protein levels were significantly decreased in HSV-1–stimulated PBMCs from patients with ADEH+ compared with those from patients with ADEH– and healthy subjects. Ingenuity Pathway Analysis demonstrated that the upstream regulators of type I and type III interferons, interferon regulatory factor (IRF) 3 and IRF7, were significantly inhibited in patients with ADEH+ based on the downregulation of their target genes. Furthermore, we found that gene expression of IRF3 and IRF7 was significantly decreased in HSV-1–stimulated PBMCs from patients with ADEH+.
Conclusions: PBMCs from patients with ADEH+ have a distinct immune response after HSV-1 exposure compared with those from patients with ADEH−. Inhibition of the IRF3 and IRF7 innate immune pathways in patients with ADEH+ might be an important mechanism for increased susceptibility to disseminated viral infection. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Atopic dermatitis, eczema herpeticum, herpes simplex virus, interferon regulatory factor 3, interferon regulatory factor 7, type I interferon, type III interferon*

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease worldwide, affecting up to 30% of children and 3% of adults in some countries.[1,2] A small subset (<3%) of patients with AD are susceptible to disseminated viral skin infections, including eczema vaccinatum. This has led to the Centers for Disease Control and Prevention recommendation that smallpox vaccination should be excluded in all patients with AD.[3] Therefore biomarkers that distinguish this subset of AD are needed because the majority of patients with AD would benefit from smallpox vaccination in the event of a bioterrorist attack.

Eczema herpeticum is a devastating complication occurring in patients with AD associated with disseminated herpes simplex virus (HSV) infection that can be life-threatening without effective treatment.[4] Previous studies have found that atopic dermatitis with a history of eczema herpeticum (ADEH+) is associated with genetic variants in a number of genes, including filaggrin *(FLG)*, IFN-α receptor 1 *(IFNAR1)*, IFN-γ receptor 1 *(IFNGR1)*, interferon regulatory factor 2 *(IRF2)*, and the T_H2-promoting cytokine thymic stromal lymphopoietin *(TSLP)*.[5-8] In addition, patients with ADEH+ often have increased *Staphylococcus aureus* skin colonization,[4] and the *S aureus* toxin α-toxin enhances viral loads in keratinocytes at sublytic concentrations.[9] These previous studies suggest that ADEH+ is a complex disease determined by genetic predisposition and environmental factors.[10-12] However, the exact pathogenic mechanisms leading to disseminated HSV infections in patients with AD remain elusive.

The primary goal of the current study was to determine whether patients with ADEH+ have identifiable defects in their immune system that dampen their ability to control HSV-1 infections. Because PBMCs contain all essential cells in the immune system to combat invading viruses, they are the most accessible sources for sampling to study human viral infection–related immune mechanisms. Indeed, previous studies have demonstrated that PBMCs are valuable sources for identification of abnormal immune pathways leading to serious HSV infections in human subjects.[13-15] RNA sequencing (RNA-seq) technology is an emerging and powerful global transcriptomic analysis approach that applies next-generation sequencing technology.[16,17] By deep sequencing the RNA of cells at a single nucleotide resolution, this approach provides unprecedented amounts of information of transcriptome dynamics that prior microarray methods could not achieve. This method can not only quantitate the abundance of expressed transcripts but also identify novel transcripts that are not yet in the public database. In addition, RNA-seq can also detect gene structure changes, including different

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Abbreviations used
AD: Atopic dermatitis
ADEH+: Atopic dermatitis with a history of eczema herpeticum
ADEH−: Atopic dermatitis without a history of eczema herpeticum
FDR: False discovery rate
HSE: Herpes simplex encephalitis
HSV-1: Herpes simplex virus 1
IPA: Ingenuity Pathway Analysis
IRF: Interferon regulatory factor
PCA: Principal component analysis
qPCR: Quantitative PCR
RNA-seq: RNA sequencing
RPKM: Reads per kilobase per million mapped reads
TLR: Toll-like receptor

splicing forms, small insertions and deletions, and single nucleotide polymorphisms. In the current study we applied this state-of-the-art technology to investigate global gene expression of PBMCs with or without HSV-1 exposure from patients with ADEH+ and patients with atopic dermatitis with a history of eczema herpeticum (ADEH−). We found that patients with ADEH+ had distinct transcriptional responses to HSV-1 exposure when compared with the responses of patients with ADEH− to the same virus.

METHODS

Subjects

Human subjects (age range, 6-65 years) included 20 nonatopic healthy subjects, 20 patients with ADEH−, and 20 patients with ADEH+. Each patient with ADEH+ was matched on age and sex with patients with ADEH− and nonatopic subjects to reduce confounding factors that might arise from age and sex. None of the patients with ADEH+ had acute HSV-1 infection at the time of participation, which was confirmed by physical examination by the clinical investigator and the measurement of anti–HSV-1 IgM serum titers. All patients with ADEH+ had positive serological results for anti–HSV-1 IgG. The detailed demographics information of human subjects is included in Table E1 in this article's Online Repository at www.jacionline.org. The institutional review board at National Jewish Health approved this study. All subjects provided written informed consent to participate in the study. We sent total RNA from sham- and HSV-1–stimulated PBMCs of 6 patients with ADEH− and 6 patients with ADEH+ with the best RNA quality and quantity for RNA-seq assays. However, the sequence results from 1 patient with ADEH+ had inadequate quality, and therefore the data from this subject could not be used. The potential genes of interest were replicated by means of quantitative PCR (qPCR) or ELISAs in the larger group of 60 study subjects, which included the 12 subjects used for the RNA-seq assay.

PBMC isolation and HSV-1 stimulation

Human PBMCs were isolated by using Ficoll-Hypaque density gradient centrifugation of heparinized venous blood from donors. Briefly, heparinized blood (≤25 mL per tube) was transferred to Ficoll tubes (Leucosep tubes [Greiner Bio-One North America, Monroe, NC] prepared with 15 mL of Ficoll-Hypaque) and centrifuged for 15 minutes at 2000 rpm. The white blood cells were washed 3 times with 1× PBS, and the cells were resuspended in RPMI 1640 containing 10% heat-inactivated FBS, 40 μmol/L L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 20 mmol/L HEPES buffer solution (GIBCO BRL Life Technologies). One million PBMCs in 200 μL of culture medium were stimulated with sham or HSV-1 (VR-733; ATCC, Manassas, Va) at a multiplicity of infection of 0.1 for 21 hours because our pilot experiments demonstrated that PBMCs stimulated at a multiplicity of infection of 0.1 of HSV-1 provided optimal cytokine induction (data not shown).

RNA isolation and sequencing

Total RNA was isolated from PBMCs by using RNeasy Mini Kits (Qiagen, Valencia, Calif), according to the manufacturer's guidelines. RNA concentration and purity were determined by using the Nano Drop-1000 Spectrophotometer and Agilent 2100 Bioanalyzer RNA Nano Chip (Agilent Technologies, Palo Alto, Calif). Library preparation and HiSeq sequencing were performed at the University of Colorado Denver Genomics and Microarray Core facility. A total of 200 to 1000 ng of total RNA was used to prepare the Illumina HiSeq libraries according to manufacturer's instructions for the TruSeq RNA kit (Illumina, San Diego, Calif). In brief, the poly-A–containing mRNA molecules were purified with poly-T oligo-attached magnetic beads. After purification, the mRNA was fragmented into small pieces by using divalent cations under increased temperatures. The cleaved RNA fragments were converted into first-strand cDNA by using reverse transcriptase and random primers. This was followed by second-strand cDNA synthesis with DNA polymerase I and RNase H. These cDNA fragments then went through an end-repair process, the addition of a single "A" base, and then ligation of the adapters. The products were then purified and enriched with PCR to create the final cDNA library. The cDNA libraries were validated on the Agilent 2100 Bioanalyzer with the DNA-1000 chip. After validation, the cDNA libraries were used for 2x100bp paired-end sequencing on the Illumina HiSeq2000 with a TruSeq SBS v3-HS Kit (Illumina). After sequencing, the samples were demultiplexed, and the indexed adapter sequences were trimmed with the CASAVA v1.8.2 Software (Illumina).

Sequence alignment and algorithm

The RNA-seq data were mapped and aligned with the computational pipeline of Bowtie and TopHat.[18] Each read generated by each sample was mapped to the *Homo sapiens* genome by using Bowtie. Subsequently, TopHat was used to analyze these mapped locations and assign them their gene of origin and to detect splice variants therein. After mapping and aligning, the resulting BAM files were imported into Partek Genomics Suite version 6.6 (www.partek.com) and converted into gene transcript levels as reads per kilobase of exon per million mapped reads (RPKM) by using a mixed-model approach (http://www.partek.com/Tutorials/microarray/User_Guides/RNASEQ.pdf). The RPKM reflects the molar concentration of a transcript in the starting sample by normalizing for gene length and for the total read number in the measurement. This allows for comparison of transcript levels both within and between experiments. The mapped and aligned files for this study, along with resulting transcript levels, have been deposited in the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/).

Quantitative real-time RT-PCR

Total RNA was reverse transcribed into cDNA with SuperScript III Reverse Transcriptase (Invitrogen, Grand Island, NY). Real-time RT-PCR was conducted on an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, Calif). Primers and probes for human *IFNA4* (Hs01681284_s1), *IFNA5* (Hs04186137_s1), *IL29* (Hs00601677_g1), *IRF3* (Hs01547283_m1), *IRF7* (Hs01014809_g1), and *18S* (Hs99999901_S1) were purchased from Applied Biosystems. The primer sequences for HSV-1 transcripts were prepared, as previously described.[19] Quantities of all target genes in test samples were normalized to the corresponding *18S* levels.

Measurement of IFN-α and IL-29

The concentration of IFN-α and IL-29 was measured by ELISA with the Human IFN-α multisubtype ELISA kit and the human IL-29 ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ), respectively. The experimental procedures were carried out according to the manufacturer's guidelines. The 96-well plates were analyzed on a DTX 880 Multimode detector (Beckman Coulter, Fullerton, Calif).

Statistical and bioinformatic analysis

The Partek Genomics Suite (www.partek.com) was used to calculate the normalized gene expression levels and run the statistical analysis on the

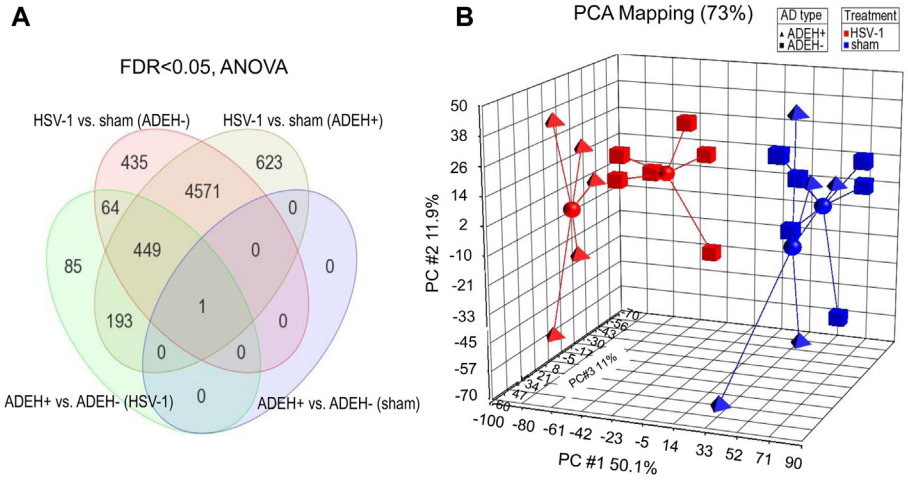


FIG 1. Expression profiles of PBMCs from patients with ADEH+ are distinct from those from patients with ADEH− after HSV-1 exposure. **A,** Venn diagram of genes significantly different (FDR < 0.05, ANOVA) in RNA-seq study between HSV-1 treatments and ADEH types. **B,** PCA analyses of the 4 experimental groups. HSV-1–stimulated patients with ADEH+, *Red triangles*; HSV-1–stimulated patients with ADEH−, *red squares*; sham-treated patients with ADEH+, *blue triangles*; and sham-treated patients with ADEH−, *blue squares*. A centroid connecting all associated members was added to the 4 different experimental groups in the PCA plot.

RNA-seq data. Partek's transcript algorithm is similar to that of Xing et al,[20] except Partek quantifies expression across the whole genome at the same time rather than each gene separately and accounts for the fragmentation step in RNA-seq by normalizing by transcript length (http://www.partek.com/Tutorials/microarray/User_Guides/RNASEQ.pdf). A 1-way ANOVA model was applied to the experimental groups and corrected for multiple testing at a false discovery rate (FDR) of less than 0.05. Genes that passed our statistical criteria were analyzed with the bioinformatics software Ingenuity Pathway Analysis (IPA; www.ingenuity.com). IPA uses the Fisher exact test to identify overrepresented connected biological units in a defined set of genes, which can include pathways, cellular functions, or even known targets of regulatory genes. In some cases a confidence score, or *z* score, can be made on the activity of the pathway or upstream regulator based on the expression pattern of the associated genes (http://pages.ingenuity.com/rs/ingenuity/images/0812%20upstream_regulator_analysis_whitepaper.pdf). The statistical analysis of real-time qPCR data was conducted with GraphPad Prism, version 5.03 (GraphPad Software, San Diego, Calif). Comparisons of expression levels were performed with ANOVA techniques and independent-samples *t* tests, as appropriate. Differences were considered significant at a *P* value of less than .05.

RESULTS

PBMCs from patients with ADEH+ have distinct expression profiles from those from patients with ADEH− in response to HSV

To more precisely investigate the global transcriptional changes of PBMCs' early response to HSV-1 between patients with ADEH+ and patients with ADEH−, we applied an RNA-seq approach to obtain gene expression profiles of PBMCs treated with and without HSV-1 for 21 hours. An average of 47 ± 11 million paired-end reads were obtained per sample, with 79% ± 4% of these sequences successfully aligned to the exon regions of the human genome (see Table E2 in this article's Online Repository at www.jacionline.org). A total of 19,463 transcripts were identified. We designated transcripts with RPKM values of greater than 5 and found at least 3 samples to be "present." By using this designation, 8,716 transcripts were considered "present." Only these transcripts were subjected to subsequent analyses.

We plotted the transcriptional data in a 3-dimensional space using principal component analysis (PCA) as implemented by the Partek Genomics Suite with a standard correlation method, observing clear separation of 2 main groups within the samples based on HSV-1 treatment (Fig 1, *B*). As evidence of the difference in expression profiles, we found 5837 and 5520 transcripts (more than half of all present genes) differentially expressed at an FDR of less than 0.05 (ANOVA) between HSV-1– and sham-treated PBMCs from patients with ADEH+ and patients with ADEH−, respectively (Fig 1, *A*). The majority of transcripts that changed because of HSV-1 treatment were shared between the 2 groups (5021 transcripts in common; Fig 1, *A*). We also observed spatial separation between the HSV-1–treated AD types in the PCA plot (Fig 1, *B*), with 792 genes different at an FDR of less than 0.05 (ANOVA, see Table E3 in this article's Online Repository at www.jacionline.org), whereas the sham-treated PBMCs from both types had similar expression profiles and tended to occupy the same 3-dimensional spaces in the PCA plot, yielding only 1 gene, glutathione-S-transferase mu 4 *(GSTM4)*, that was different between the 2 groups at an FDR of less than 0.05 (ANOVA; Fig 1, *A*, and see Table E4 in this article's Online Repository at www.jacionline.org).

Levels of type I and type III interferons are significantly reduced in patients with ADEH+

As shown in Fig 1, *B*, the transcription profiles of HSV-1–treated PBMCs from patients with ADEH+ were distinctly separated from those of patients with ADEH−. This suggested significantly different responses by patients with these 2 types of AD to HSV-1 stimulation. We then examined the 792 genes different between the HSV-1–treated cells from patients with ADEH− and those from patients with ADEH+ (see Table E3), given the manageable number for bioinformatic interpretation

TABLE I. Top 20 most downregulated genes in patients with ADEH+ compared with patients with ADEH− in response to HSV-1 stimulation

Ref-seq symbol	Entrez gene name	Gene symbol	*P* value	ADEH+ vs ADEH− (fold changes)
KCNE1L	KCNE1-like	*KCNE1L*	4.08E-03	−7.813
ANKRD1	Ankyrin repeat domain 1	*ANKRD1*	4.93E-05	−5.907
IFNA4	Interferon, alpha 4	*IFNA4*	8.46E-04	−4.333
IFNA5	Interferon, alpha 5	*IFNA5*	1.12E-03	−4.042
IFNA6	Interferon, alpha 6	*IFNA6*	1.92E-03	−3.846
IFNA17	Interferon, alpha 17	*IFNA17*	7.78E-04	−3.797
IFNA16	Interferon, alpha 16	*IFNA16*	1.15E-03	−3.651
IFNA8	Interferon, alpha 8	*IFNA8*	1.08E-03	−3.482
IFNA21	Interferon, alpha 21	*IFNA21*	7.49E-04	−3.417
IFNA7	Interferon, alpha 7	*IFNA7*	1.32E-03	−3.383
IFNA2	Interferon, alpha 2	*IFNA2*	5.35E-04	−3.202
IFNA14	Interferon, alpha 14	*IFNA14*	6.70E-04	−3.188
IFNA1/ IFNA13	Interferon, alpha 1	*IFNA13*	1.37E-03	−3.133
IFNA10	Interferon, alpha 10	*IFNA10*	2.74E-03	−3.065
IFNW1	Interferon, omega 1	*IFNW1*	6.74E-04	−3.036
IFNL1	Interferon, lambda 1	*IL29*	7.03E-04	−2.949
LRRN3	Leucine rich repeat neuronal 3	*LRRN3*	1.43E-03	−2.939
GSTM4	Glutathione-S-transferase mu 4	*GSTM4*	1.00E-03	−2.886
IFNB1	Interferon, beta 1, fibroblast	*IFNB1*	5.87E-04	−2.823
NMT2	N-myristoyltransferase 2	*NMT2*	4.13E-03	−2.033

and the obvious functional implications these genes might have in the differential response to virus in patients with ADEH+.

When we assessed the fold changes of gene expression levels between patients with ADEH− and patients with ADEH+, the most striking finding was that 15 different kinds of interferon genes, including type I and type III interferons, were among the top 20 most downregulated genes in patients with ADEH+ compared with patients with ADEH− (Table I). Because type I and type III interferons are the most important cytokines that protect host cells from viral infection by inducing "antiviral states,"[21,22] these genes became our first group of candidate genes to validate. Using samples from 20 healthy subjects, 20 patients with ADEH−, and 20 patients with ADEH+, with qPCR, we validated that *IFNA4* and *IFNA5* gene transcripts were significantly lower in HSV-1–stimulated PBMCs from patients with ADEH+ (Fig 2, *A* and *B*). To further validate whether this decreased gene transcription leads to reduced protein expression, we used an ELISA kit that measured 13 different kinds of type I interferons and confirmed that the protein levels of type I interferons were indeed significantly decreased in HSV-1–stimulated PBMC culture supernatants from patients with ADEH+ compared with those from healthy subjects and control subjects with ADEH− (Fig 2, *C*). In addition, using qPCR, we also confirmed that the type III interferon *IL29* gene transcription level was significantly downregulated in HSV-1–stimulated PBMCs from patients with ADEH+ compared with those from control subjects with ADEH− (Fig 3, *A*), and its protein level in culture supernatants was also significantly lower in patients with ADEH+ compared with that seen in healthy subjects and patients with ADEH− (Fig 3, *B*).

We also examined the top upregulated genes revealed by using the RNA-seq assay. We found that *IFNG* is the most upregulated gene (see Table E5 in this article's Online Repository at www.jacionline.org) in this short-term culture. However, upregulation of *IFNG* was not replicated when we included the remainder of the subjects in our study cohort (see Fig E1 in this article's Online Repository at www.jacionline.org).

Because part of our population of healthy control subjects and patients with ADEH− did not have positive serological anti–HSV-1 IgG results, to exclude the confounding effect of anti–HSV-1 IgG antibody, we reanalyzed the data that only included subjects who were anti–HSV-1 IgG positive. The results demonstrated that the gene expression of type I and type III interferons was still significantly downregulated in patients with ADEH+ compared with that seen in patients with ADEH− or healthy control subjects (see Fig E2 in this article's Online Repository at www.jacionline.org). We further confirmed the sources of type I and type III interferons were predominantly produced by innate immune cells or antigen-presenting cells but not T cells and natural killer cells (see Fig E3 in this article's Online Repository at www.jacionline.org). These data demonstrated that innate immune responses were significantly impaired in patients with ADEH+.

Upstream regulators of type I and type III interferons were downregulated in patients with ADEH+

To identify the important regulators involved in type I and type III interferon expression, we used IPA software to compute the probability of obtaining a set of target genes for a given protein, micro-RNA, chemical, or drug. Because much is known about the effect these master regulators have on their target molecules, IPA also produces a confidence score, or *z* score, on the activity of these molecules based on the direction of expression of the target genes. This analysis revealed that 5 IRF genes (Fig 4, *A*), *IRF1*, *IRF3*, *IRF7*, *IRF8*, and *IRF9*, were predicted to be inhibited in PBMCs from patients with ADEH+ on HSV-1 stimulation. Among them, *IRF3*, *IRF7*, and *IRF9* had statistical significance, with *z* scores of less than −1.0. The gene with the most statistically overrepresented list of target genes ($P = 1.2 \times 10^{-16}$, Fisher exact test) and the lowest inhibition score (*z* score = −3.6) was *IRF7*, with 30 target genes included in our list of 792 genes different between samples from patients with ADEH+ and samples from patients with ADEH− after exposure to virus, with all but 6 of these target molecules suggesting decreased activity of *IRF7* (Fig 4, *B*). *IRF3* stands as the second regulator in terms of affected target genes.

Both IRF3 and IRF7 are important transcription factors involved in many viral recognition signaling pathways,[23-25] such as Toll-like receptor (TLR) 3, TLR7/8, and TLR9; the cytoplasmic RNA sensors retinoic acid–inducible gene 1 (RIGI) and melanoma differentiation-associated protein 5 (MDA5); and cytoplasmic DNA sensors.[26,27] Their activation led to the production of type I and type III interferons. Using qPCR, we investigated *IRF3* and *IRF7* gene expression in PBMCs from patients with ADEH+ compared with that seen in patients with ADEH− and healthy subjects. We found that *IRF3* expression was significantly lower in patients with ADEH+ on HSV-1 stimulation compared with that seen in healthy subjects and patients with ADEH−. *IRF7* was significantly induced by

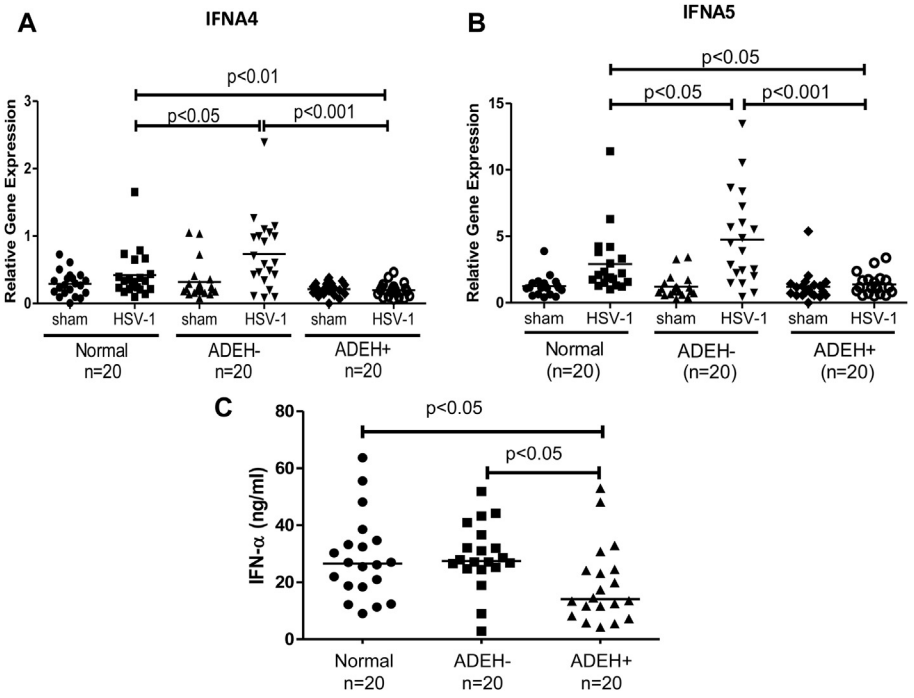


FIG 2. Induction of IFN-α by HSV-1 is significantly decreased in patients with ADEH+. **A** and **B,** *IFNA4* (Fig 2, *A*) and *IFNA5* (Fig 2, *B*) mRNA levels were evaluated by using real-time qPCR in sham- and HSV-1–treated PBMCs from healthy subjects (n = 20), patients with ADEH− (n = 20), and patients with ADEH+ (n = 20). **C,** IFN-α protein levels were evaluated by means of ELISA in culture supernatants from HSV-1–stimulated PBMCs from healthy subjects (n = 20), patients with ADEH− (n = 20), and patients with ADEH+ (n = 20).

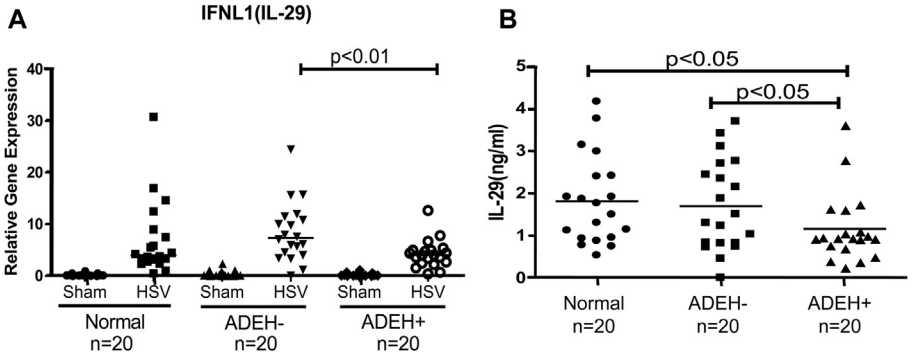


FIG 3. Induction of the type III interferon IL-29 by HSV-1 is significantly decreased in patients with ADEH+. **A,** *IL29* mRNA levels were evaluated by using real-time qPCR in sham- and HSV-1–treated PBMCs from healthy subjects (n = 20), patients with ADEH− (n = 20), and patients with ADEH+ (n = 20). **B,** IL-29 protein levels in HSV-1–stimulated PBMCs from healthy subjects (n = 20), patients with ADEH− (n = 20), and patients with ADEH+ (n = 20) were evaluated by using ELISA.

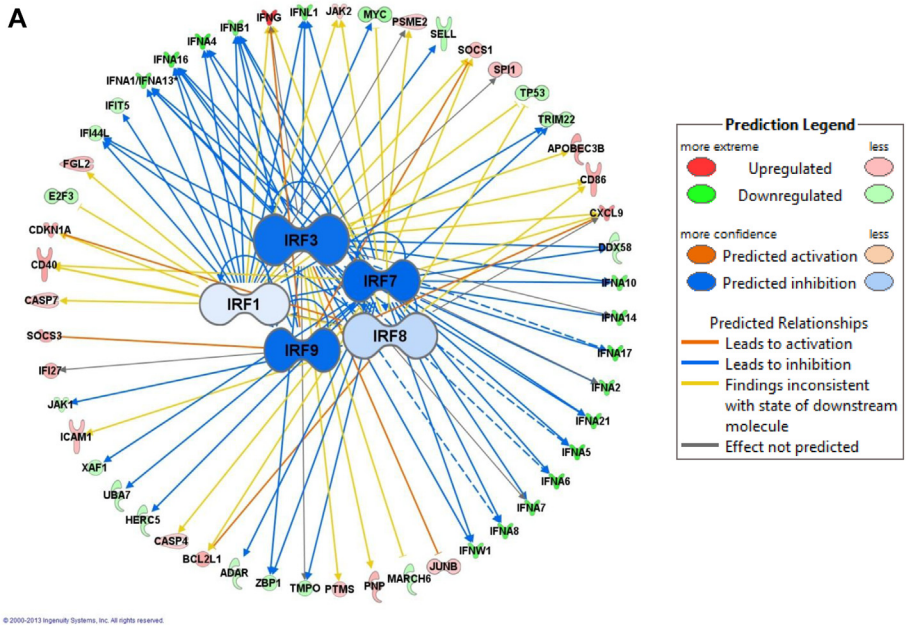
HSV-1 stimulation in all 3 diagnostic groups; however, the induction of *IRF7* in patients with ADEH+ was significantly reduced compared with that seen in healthy subjects and patients with ADEH− (Fig 5).

DISCUSSION

Diseases resulting from HSV infections are serious public health concerns. In the United States it is estimated that more than 100 million persons are infected with HSV-1 and at least 40 to 60 million subjects are infected with HSV-2.[28] Although most infected subjects are asymptomatic or have mild disease, others can have serious diseases, including herpes simplex encephalitis (HSE), keratoconjunctivitis, and the focus of our current study, ADEH+ disease. Despite acyclovir treatment, the fatality rate of HSE remains around 10%, and about 70% of patients with HSE recovered with neurological sequelae.[29] HSV ocular infections are the leading cause for corneal blindness.[30] ADEH+ can be severely disfiguring and life-threatening.[31] Therefore studies leading to a better understanding of human host immune defense mechanisms against HSV are needed for the development of more effective approaches to prevent and treat herpetic diseases.

The current study applied next-generation sequencing technology, RNA-seq, to explore changes in global transcriptome of PBMCs from patients with ADEH− and patients with ADEH+ with or without HSV-1 exposure *ex vivo*, with the goals

A



B

Upstream Regulator	# target genes	p-value of obtaining target genes	Activation z-score
IRF1	21	1.5×10^{-7}	-0.149
IRF3	**26**	$\mathbf{1.3 \times 10^{-12}}$	**-1.992**
IRF7	**31**	$\mathbf{1.2 \times 10^{-16}}$	**-3.591**
IRF8	14	1.8×10^{-5}	-0.320
IRF9	**7**	$\mathbf{9.8 \times 10^{-5}}$	**-2.369**

FIG 4. Upstream regulators of type I and type III interferons were inhibited in patients with ADEH+. **A,** IPA analysis demonstrated that 5 IRFs are predicted to be inhibited in patients with ADEH+. Each IRF is connected with its target genes; *dashed* and *solid connecting lines* indicate an indirect or direct relationship with the master regulator respectively. The colors of the lines connecting IRFs and their corresponding targets indicate the predicted activation status of the IRF gene based on expression patterns of its target genes. **B,** Statistical parameters of 5 IRFs.

of determining whether patients with ADEH+ have identifiable abnormalities in their immune systems and finding candidate genes that can serve as biomarkers of patients with AD at risk of disseminated HSV infection. The most striking finding from the RNA-seq data is that multiple type I and type III interferon genes are significantly decreased in PBMCs from patients with ADEH+ after HSV-1 exposure compared with patients with ADEH− (Table I). Using qPCR and ELISA, as well as additional subjects, we further confirmed, at both the transcription and protein levels, that IFN-α and IL-29 levels were significantly decreased in patients with ADEH+ compared with those seen in patients with ADEH− and healthy control subjects (Figs 2 and 3). Because type I and type III interferon signaling is the hallmark for antiviral innate immune responses,[32,33] these data demonstrate that innate immune responses are impaired in patients with ADEH+. All patients with ADEH+ had positive serological test results for anti–HSV-1 IgG (see Table E1), indicating that patients with ADEH+ are capable of establishing a specific adaptive immune response to HSV-1 infection. These data support the findings from other herpetic diseases and classic HSV vaccine studies that specific cell-mediated and humoral immune responses are not sufficient to protect human subjects from HSV infection–related diseases.[34,35]

Previous studies from mice and human subjects have demonstrated that type I, type III, or both interferons are the first line of host defense combating HSV infection. Murine infection models demonstrated that production of type I interferons (IFN-α/β) during the first several hours of viral exposure is critical in control of HSV-1 infections.[36] Type I interferons can inhibit the onset of HSV immediate early gene expression,[37] limit viral spread into the nervous system,[38] and activate the natural killer cell defense system.[39] The type III interferons IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B), which were discovered in 2003, were shown to protect human cells from HSV infection as well.[40] The studies of human HSE further highlight the importance of type I and type III interferons in the control of severe human HSV-related diseases. Loss-of-function mutations in signal transducer and activator of transcription 1 and TYK2,

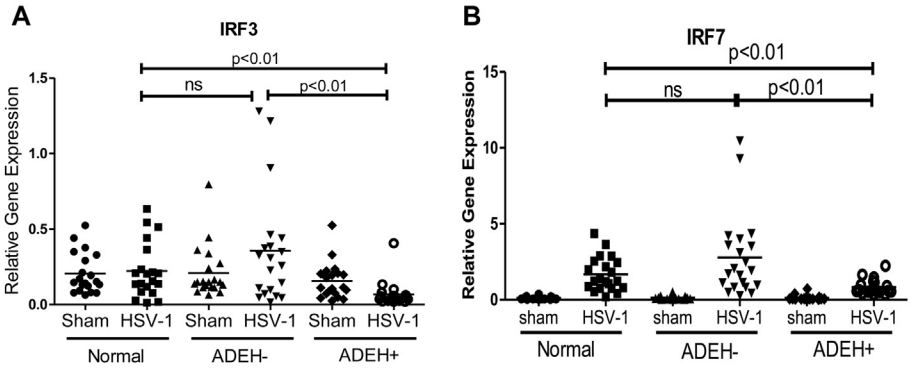


FIG 5. Expression of *IRF3* and *IRF7* is significantly decreased in HSV-1–stimulated PBMCs from patients with ADEH+. *IRF3* **(A)** and *IRF7* **(B)** mRNA levels were evaluated by using real-time qPCR.

the 2 important signal components in the downstream signaling pathway of type I interferon receptors, predisposes to the development of HSE.[41,42] Inborn gene mutations in the upstream signaling pathway of type I, type III, or both interferons have also been found in patients with HSE. The reported gene mutations include *TLR3*, unc-93 homolog B1 *(UNC93B)*, Toll-like receptor adaptor molecule 1 *(TRIF)*, and TNF receptor-associated factor 3 *(TRAF3)*.[43-46] TLR3 recognizes double-strained RNA from HSV-1 intermediates, binds UNC93B and TRIF (the downstream adaptors) and subsequently activates IRF3 and IRF7 to transcribe type I, type III, or both interferons. Cells isolated from autosomal recessive UNC-93B–deficient patients, autosomal dominant TLR3–deficient patients, and autosomal dominant TRIF– and autosomal dominant TRAF3–deficient patients have been reported to be more susceptible to HSV-1 infection. The loss-of-function mutations in these genes lead to either completely abolished or reduced type I and type III interferon production in innate immune cells after TLR3 agonist polyinosinic-polycytidylic acid stimulation. Based on the significance of type I/type III interferons in combating HSV-1 infections in both mice and human subjects, our findings of decreased type I and type III interferon production in patients with ADEH+ suggest that the major innate antiviral defense mechanism in patients with ADEH+ is compromised. This could be the mechanism underlining the increased propensity for disseminated HSV-1 in patients with ADEH+. Recently, several case reports demonstrated that application of synthesized type I interferons can effectively cure human severe HSV-1 infection manifestation.[47,48] The current study has found that expression of the 2 master regulators of the innate immune response, *IRF3* and *IRF7*, are suppressed in patients with ADEH+, suggesting that abnormalities of ADEH+ exist upstream of *IRF3* and *IRF7* signaling pathways. These data open new research avenues for future investigation into the cause of ADEH+.

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Key messages

- **PBMCs from patients with ADEH+ demonstrated distinct gene expression profiles after HSV-1 stimulation.**
- **Type I and type III interferon responses are impaired in patients with ADEH+.**
- ***IRF3* and *IRF7* gene expression are reduced in patients with ADEH+.**

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