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Transcriptional Activation of Interferon-Stimulated Genes but Not of Cytokine Genes after Primary Infection of Rhesus Macaques with Dengue Virus Type 1[▽]

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Macaques are the only animal model used to test dengue virus (DENV) vaccine candidates. Nevertheless, the pathogenesis of DENV in macaques is not well understood. In this work, by using Affymetrix oligonucleotide microarrays, we studied the broad transcriptional modifications and cytokine expression profile after infecting rhesus macaques with DENV serotype 1. Five days after infection, these animals produced a potent, innate antiviral immune response by inducing the transcription of signature genes from the interferon (IFN) pathway with demonstrated antiviral activity, such as myxoprotein, 2′,5′-oligoadenylate synthetase, phospholipid scramblase 1, and viperin. Also, IFN regulatory element 7, IFN-stimulated gene 15, and protein ligases linked to the ISGylation process were up-regulated. Unexpectedly, no up-regulation of IFN-α, -β, or -γ genes was detected. Transcription of the genes of interleukin-10 (IL-10), IL-8, IL-6, and tumor necrosis factor alpha was neither up-regulated nor down-regulated. Results were confirmed by real-time PCR and by multiplex cytokine detection in serum samples.

Dengue virus (DENV) is the second most important arthropod-borne tropical disease after malaria, with 50 to 100 million and 500,000 cases of dengue fever (DF) and dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), respectively, each year (29). These diseases are induced by DENV, a member of the *Flaviviridae* family that exists as four different serotypes (DENV-1, -2, -3, and -4). The mechanisms of DENV-induced disease and immune response are not fully understood, but two theories are the most discussed. The theory of antibody-dependent enhancement postulates that higher viremia occurs in secondary infections due to antibody-facilitated viral entry, causing more severe disease (32–35, 71). The cytokine-mediated immunopathology theory proposes a model of plasma leakage in DHF mediated through an interaction between DENV-infected monocytes/macrophages and memory $CD4^+$ and $CD8^+$ DENV-reactive T cells (58–61, 90, 91). This interaction leads to production of type 1 proinflammatory cytokines, mainly gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), which directly affects the vascular endothelium. This cytokine-mediated proinflammatory response is postulated to be more extended in a secondary infection than in primary infections due to the preferential expansion of memory T cells that recognize cross-reactive epitopes. However, it is well documented that DHF/DSS can occur after a single DENV infection (6, 7, 13, 50, 64, 77, 83, 108). In addition to IFN-γ and TNF-α, other cytokines, such as interleukin-18 (IL-18), IL-6, IL-2, and IL-10, have been detected in the sera of patients with DHF (6, 12, 22, 77, 82, 83). A pathogenic role has been assigned to monocytes/macrophages but also to B cells in humans (67). There is no appropriate animal model for DENV that reproduces DF/DHF/DSS symptoms. Recently, some interesting approaches have been taken using mice as a model (8, 99). However, in spite of its worth and the advance it represents in the study of DENV, macaques continue to be the standard model to test vaccine candidates or treatments against DENV. At present, there is no specific anti-DENV treatment, but several DENV vaccine formulations are being studied. All of them have been or will be tested using macaques before studies are conducted with humans (11, 21, 38, 84, 85, 87, 88, 102, 105). Previous work has been performed to characterize DENV replication in macaques (36, 37). However, little is known about the molecular mechanism of the macaque's immune response to DENV. In particular, the type or level of cytokines produced during DENV infection or the gene expression profile of infected cells

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is not known in these animals. The efficacy of DENV vaccine trials relies on the production of neutralizing antibodies after vaccination that can counteract viremia after challenge. Macaques do not develop symptoms that mimic the clinical outcomes of DF or DHF/DSS in humans, and only a transient viremia occurs without the development of relevant symptoms. This suggests a natural mechanism that might allow rhesus macaques to antagonize DENV infection better than humans. We have taken preliminary steps to better understand the innate immune response in rhesus macaques. Here, we have tested the profile of gene activation and cytokine production after infecting four rhesus macaques with a low-passage strain of DENV-1. Our studies provide the first evidence that rhesus macaques respond to DENV infection with a potent innate antiviral immune response although without measurable type I or II IFN or proinflammatory cytokine production in peripheral blood mononuclear cells (PBMC). These profiles are quite different from those reported for humans and could explain the absence of symptoms of DF or DHF/DSS in these animals. These findings have implications for vaccine efficacy studies in progress.

MATERIALS AND METHODS

Infection of animals and blood collection. Six male rhesus macaques that were negative for immunoglobulin G (IgG) and IgM antibodies to DENV were used for the study. Four animals were infected subcutaneously with a 1-ml suspension containing 1×10^4 PFU of a low-passage Western Pacific 74 DV1 strain (L. Markoff, Walter Reed Army Hospital). Two animals were mock infected with supernatant from uninfected LLCMK-2 cells. Sera were collected at days 1, 3, and 5 after infection, quick-frozen, and kept at −80°C until analysis. On day 5, PBMC were also collected using 8-ml Vacutainer CPT tubes (BD, Franklin Lakes, NJ). Tubes were centrifuged at $1,500 \times g$ for 30 min at 20°C. Collected PBMC were washed twice with phosphate-buffered saline, and platelets were removed by centrifugation at $200 \times g$ for 15 min at 20°C. PBMC were used directly for magnetic cell sorting. A fraction was frozen at −80°C for further RNA extraction. All work with animals was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to the use of animals in research. In addition, all procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Medical Sciences Campus, University of Puerto Rico, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Serologic tests. All animals were tested for the presence of anti-DENV IgM by the IgM antibody capture enzyme-linked immunosorbent assay in order to preclude recent natural infections in the monkeys. To preclude past infections, IgG antibody tests were performed using a quantitative enzyme-linked immunosorbent assay. Neutralizing antibodies to DENV-1 were quantified using a flow cytometry-based neutralization (FNT) assay on Vero cells. This neutralization assay is based on a fluorescence-activated cell sorter (FACS)-based dengue virus titration assay as described previously (63). In the flow cytometry-based neutralization assay, the ability of immune serum to neutralize the infectivity of DENV on Vero cells is measured at 24 h postinfection by enumerating cells that are positive for intracellular staining of DENV E protein by flow cytometry.

The neutralization titer for each serum sample was expressed as the reciprocal of the highest dilution of serum that neutralized the challenge virus by 50% (FNT_{50}) (63). Alanine aminotransferase and aspartate aminotransferase levels were determined using a commercial system (Dimension Expand; Dade Behring, Deerfield, IL).

Isolation of B cells and macrophages from PBMC. PBMC were obtained as described above and separated by magnetic sorting using superparamagnetic microbeads according to the manufacturer's instructions (all reagents and equipment for magnetic cell separation were purchased from Miltenyi Biotec, Auburn, CA). Macrophages were purified with anti-CD14 microbeads using LS columns. PBMC were CD3 depleted with the CD3 MicroBead kit using LD columns. B cells were subsequently purified with anti-CD20 microbeads using LS columns. The remaining fraction of PBMC and the eluted fraction from the CD3 depletion (which includes CD4, CD8, NK, and other cells) were labeled as other cells (OC) and kept frozen at −80°C for further analysis. The cells' purity was determined by FACS analysis using conjugated mouse anti-human monoclonal antibodies known to cross-react with rhesus monkey antigens. CD20 fluorescein isothiocyanate (2H7) and CD14 fluorescein isothiocyanate (M5E2) were used to label purified B cells or macrophages, respectively. In both cases, specific antibodies were combined with CD3 allophycocyanin (SP34-2), and $CD20^+/CD3^-$ or $CD14^+/CD3^+$ cells were quantified. Antibodies for FACS analysis were obtained from BD Biosciences (San Diego, CA).

Total cellular RNA preparation. RNA was extracted from 3×10^5 PBMC or from 3×10^6 isolated B cells, macrophages, or OC by using an RNeasy Mini kit (QIAGEN, Valencia, CA) and eluted in 30 μl of RNase-free double-distilled H_2O. For real-time PCR (RT-PCR), RNA was also extracted from frozen PBMC. The RNA was resuspended in diethyl pyrocarbonate-treated 0.01% distilled H_2O (Ambion, Austin, TX). The quality and quantity of RNA were estimated by the Agilent Bioanalyzer RNA Nanochip methodology. RNA samples from macrophages, B cells, and OC were used for microarray, and RNA from macrophages, B cells, and PBMC was used for RT-PCR.

Affymetrix GeneChip analysis. The Affymetrix protocol used was essentially described previously (109). Briefly, 100 picomoles of a T7-$(dT)_{24}$ primer was added to 10 μg total RNA. The primer and RNA were denatured at 70°C for 10 min and placed on ice, and cDNA was then synthesized using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Reactions were terminated by the addition of EDTA to a final concentration of 30 mM, and samples were placed on ice. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each cDNA sample and centrifuged at $16,000 \times g$ for 2 min. The aqueous upper layer was precipitated with 2.5 M ammonium acetate and a 2.5× final volume of 100% ethanol. Samples were centrifuged, and the resulting pellets were extensively washed with 80% ethanol, air dried, and resuspended in 12 μl diethyl pyrocarbonate-treated water. The in vitro transcription labeling reactions were done using ENZO BioArray (Affymetrix) to synthesize biotin-labeled cRNA targets. The labeled cRNA was cleaned using RNeasy Mini kits, and 15 μg was hybridized to rhesus macaque GeneChip arrays (Affymetrix, Santa Clara, CA) containing 56,867 probe sets, 47,400 transcripts, and 38,500 genes. The microarray procedure was performed at the Expression Analysis Laboratory (Durham, NC) using a GeneChip Scanner 3000 (Affymetrix). Probe intensity values were extracted from the array image using GCOS software (Affymetrix).

Data analysis. Genes were normalized to the mean of the controls, and data from corresponding negative controls were selected as a reference to allow the calculation of infected/uninfected gene expression ratios. Arrays were median normalized. Genes with low signals (defined as a signal of <100 above the background) were not considered. The remaining signals were $\log_2$ transformed prior to analysis so that relative increases and decreases in gene expression were represented on a linear scale. Gene expression profiles (txt version of the CHP files) were loaded into the GeneSifter microarray data analysis system (VizX Labs, Seattle, WA). The data were filtered by using the criteria absent (A), marginal (M), and present (P). To identify highly expressed genes, only genes with at least a fourfold increase in expression with a P value of <0.05 after t test and Benjamini and Hochberg correction were selected and displayed as scatter plots. For hierarchical clustering, only genes differentially expressed with a P value of <0.005 and called P were included. Analysis was performed individually for B cells, macrophages, or OC. When data from all three kinds of samples were grouped in a single project, the sample was considered to be PBMC.

Relative quantification of cytokine mRNA expression levels. Rhesus macaque cytokines and other gene mRNA levels were determined by RT-PCR as described previously (1). For this experiment, RNA isolated from PBMC, B cells, and macrophages was used. Briefly, samples were tested in duplicate, and the PCR for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene and the target gene from each sample were run in parallel on the same plate. The reaction was carried out on a 96-well optical plate (Applied Biosystems, Foster City, CA) in a 25-μl reaction volume containing 5 μl cDNA plus 20 μl Mastermix (Applied Biosystems). All sequences were amplified using the 7900 default amplification program. PCR conditions and cytokine and other protein mRNA expression levels were calculated from normalized ΔC_T values according to methods reported previously (1, 2). In the present study, a minimum of two normal samples for each kind of studied cell (B cells, macrophages, and PBMC) were analyzed to determine baseline mRNA levels for each cytokine and protein. The mean value for each cytokine mRNA level of all animals in each tissue (mean ΔC_T) and the statistical analysis were performed as previously described (2).

DENV RT-PCR. DENV RNA was isolated from aliquots of serum using the QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) to a final volume of 50 μl. One hundred nanograms of RNA was used to amplify a 170-bp product in the capsid gene region of DENV by RT-PCR according to a methodology described

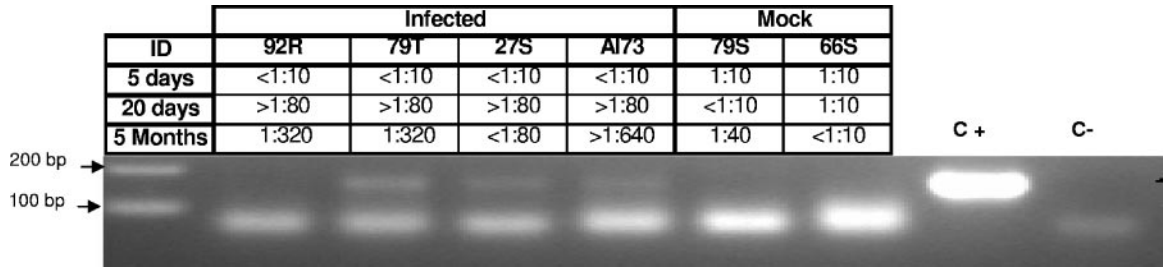

FIG. 1. Titers of neutralizing antibodies and serum DENV PCR. Specific antibodies to DENV-1 were measured at different points using a flow cytometry-based neutralization assay. Titers represent the FNT_{50} values. Twenty days after infection, all infected animals developed titers over 1:80. Five months later, all infected animals except one had neutralizing antibodies over 1:320. PCR for DENV was performed 5 days after the infection; the presence of the virus in serum was evidenced by this method in all infected animals except animal 92R. This animal showed a higher level of induction of antiviral genes (see the text for details). None of the mock-infected animals developed neutralizing antibodies or showed specific DENV PCR bands in serum.

previously (67). PCR products were separated by 1.5% agarose gel electrophoresis and visualized in a GelDoc station using Quantity One software (Bio-Rad, Hercules, CA).

Rhesus macaque cytokine detection. The detection of 20 nonhuman primate chemokines and cytokines was performed in a single sample using the Luminex^{100} system as previously described (25). Serum was collected at days 1, 3, and 5 after infection. The panel included granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IFN-γ, IFN-α, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p40), IL-17, IL-18, monocyte chemoattractant protein 1, macrophage inflammatory protein la, macrophage inflammatory protein 1β, RANTES, and TNF-α. The raw data (mean fluorescence intensity) from all the bead combinations tested were analyzed with Master-Plex QT quantification software (MiraiBio Inc., Alameda, CA) in order to obtain concentration values.

RESULTS

Animal infections and serological tests. To better understand immune responses in rhesus macaques, four animals were infected with a low-passage strain of DENV-1, and two animals were mock infected with supernatant from uninfected cells. All infected animals developed specific anti-DENV-1 neutralizing antibodies as measured by FNT. In Fig. 1, the titers of neutralizing antibodies at 5 and 20 days and then at 5 months after the infection are shown. Twenty days after infection, all infected animals had neutralizing antibody titers (FNT_{50}) above 1:80. At 5 months after infection, titers were higher in three out of four animals. One animal (animal 27S) showed a decrease in the antibody titer. Viral RNA was detected in serum by PCR 5 days after infection in all except one animal (Fig. 1). This animal, animal 92R, showed the highest levels of transcriptional induction of the genes involved in the innate immune response (data not shown). In particular, the aspartate aminotransferase level was increased in all animals, and in one of them (animal AI73), the alanine aminotransferase level was also increased (data not shown), possibly indicating different levels of hepatic injury. No other significant signs of illness were detected after DENV infection in these animals.

Cell separation. The magnetic microbead system was used to separate the cells. Cells were labeled with antibodies with known cross-reactivity to rhesus macaques according to the manufacturer's instructions. After separation, B cells ($CD3^-$/$CD20^+$) from infected animals were 89 to 95.4% pure (average, 92.75%), while the purity of the same cells isolated from control animals was 86% to 88.5% (average, 87.2%). Macrophage ($CD3^+$/$CD14^+$) purity was 90.6% to 95.6% (average, 93.1%) and 76% to 90.1 (average, 83.5%) when isolated from infected and uninfected animals, respectively.

Differential gene expression is cell specific. For microarray studies, we used RNA collected from different cells 5 days after infection. RNA from B cells, macrophages, and OC was run using rhesus macaque chips. Data obtained from all three kinds of samples were analyzed together and considered to be PBMC. Figure 2 shows the scattered plots obtained with samples from different kinds of cells. The overall pattern was similar for PBMC and macrophages, with more genes down-regulated than up-regulated (72.29% versus 27.7% and 74.49% versus 25.50%, respectively), while B cells showed three times more up-regulated than down-regulated genes (63.06% versus 36.93%). These results showed a cell type-specific effect in vivo, which can be coherent with the specific role for each type of cell. However, a common aspect in all cell types was the intensities of the differential modifications, with a stronger up-regulation and a limited down-regulation of the affected genes. Common ontologic pathways such as immune responses, inflammatory responses, antiviral responses, and defense were activated in all samples (data not shown) (a complete list of ontologic pathways can be found at http://ucm.rcm.upr.edu/geneexpression.html).

Analysis of gene expression in DENV-1-infected rhesus macaques. Gene expression profiles for rhesus macaques after experimental infection have not been studied previously. Following the procedure described above, a total of 812, 3,341, and 3,671 genes were differentially regulated in PBMC, B cells, and macrophages, respectively (Fig. 2). The complete gene lists for each type of cell can be reviewed at http://ucm.rcm.upr.edu/geneexpression.html. To identify specific genes differentially regulated with higher significance, conditions were set to P values of <0.005 and with signal calls detected as present (P). With these conditions, the number of up-regulated genes was set down to 20, 30, and 40 in PBMC, B cells, and macrophages, respectively. No down-regulated genes met these stringent conditions. A summary of all these genes is displayed in Table 1. The newly identified genes can be set apart into three categories: IFN-α/β-stimulated genes (IFN-induced proteins, IFN-stimulated gene 15 [ISG15], IFN-α-inducible protein 6-16 [G1P3 or ISG6-16], IFN-α-inducible transmembrane protein 27 [IFI27], IFN regulatory element 7 [IRF7], and phospholipid scramblase 1 [PLSCR1]), IFN-α/β-induced and virally induced

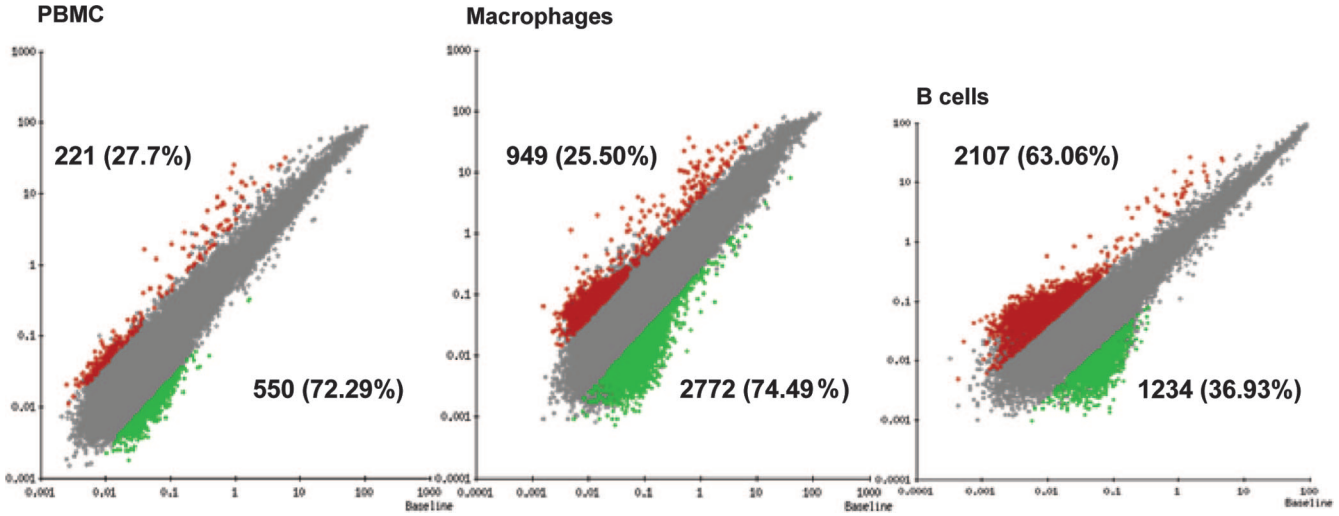

FIG. 2. Scatter plot of signal intensity comparisons of baselines and infected samples. The log of the mean intensity for the groups is plotted. Genes with fourfold up- or down-regulation and *P* values of <0.05 are represented. A cell type-specific response is noticeable by the number of modified genes (771, 3,721, and 3,341 for PBMC, macrophages, and B cells, respectively) and by its distribution according to the profile of modification (numbers and percentages are indicated). PBMC and macrophages showed a similar pattern of modification, with a higher percentage of genes being down-regulated. In contrast, in B cells, the higher number of modified genes was up-regulated. Only macrophages showed down-regulated genes with signals above 1 log. Genes with positive or negative changes are represented by red and green dots, respectively.

genes (2′,5′-oligoadenylate synthetase 1 [OAS1], OAS2, OAS3, Mx1, Mx2, and PRKR), and other genes with known or unknown antiviral function (cig5 or viperin, CKCL10, and epithelial-stromal interactor 1 [EPSTI1]). The increase (*n*-fold) of selected genes of each group is shown in Fig. 3A. Some of these genes (ISG15, ISG6, IFI27, EPSTI1, IFIPT-1, OAS, and viperin) were up-regulated in all three tested samples. ISG15 is an IFN-α/β-induced protein implicated in a process known as ISGylation (5, 17, 55). Its role in the antiviral response was first suggested because human influenza B virus was able to inhibit its conjugation to target proteins (112). Its direct antiviral activity against Sindbis virus, a member of the *Alphaviridae* family, has recently been demonstrated (66). Its role as an antiflavivirus has been documented in vitro for West Nile virus (23) and for hepatitis C virus (HCV) both in vitro and in vivo using chimpanzees as the animal model (10, 49). However, there was no previous evidence of ISG15 up-regulation in response to DENV in vitro or in vivo. The role of this protein in the anti-DENV immune response is reinforced in this work by the co-up-regulation of two members of the HERC protein ligase family (RLD5 and RLD6) linked to the ISGylation process (see references 24 and 43 and references therein) (Table 1). The up-regulation of ISG6-16 in response to DENV infection is documented for the first time in this work. This member of the ISG group has been shown to have an antiapoptotic function through inhibiting caspase-3 in cancer cell lines (106). Its role as anti-HCV (9, 113) and its induction after West Nile virus infection (23) have also been recently demonstrated. The induction of high levels of IFI27 (ISG12) and its antiviral role in response to HCV both in vivo and in vitro (9, 49) as well as its protective role in the development of lethal alphavirus encephalitis in mice have been well recognized (62). It has been quite interesting to identify the transcriptional activation of the EPSTI1 gene (Table 1), which has not been linked so far to the IFN signaling pathway. This gene was first identified as being highly upregulated in invasive breast carcinomas compared with normal breast cells (25, 78). More recently, it has been related to the pathogenesis of systemic lupus erythematosus (48). In concordance with this work, EPSTI1 and IFI27 were up-regulated in patients with systemic lupus erythematosus. To our knowledge, there were no previous reports of EPSTI1 activation after viral infection. Its induction after DENV infection in rhesus macaques will be further investigated.

In contrast to what has been reported for human cells (12, 109), we were unable to detect a significant modification in the level of cytokine gene transcription after a primary DENV infection. As shown in Fig. 3B, only a limited level of transcriptional activation was detected for IL-10 and IL-8. These data were obtained 5 days after infection. However, serum detection, using antibodies with known cross-reactivity to rhesus macaque cytokines, did not detect the presence of such cytokines at day 1, 3, or 5 after infection (data not shown).

RT-PCR confirmation of microarray results. From the microarray data, we attempted to confirm three central genes associated with IFN induction by RT-PCR: IRF7, OAS, and Mx. All of them were shown to be up-regulated by this method, confirming their increased induction found in the microarrays (Fig. 4). IRF7 was selected for confirmation because of its crucial role in the induction of the type I IFN response after viral infection (44–46). In addition to macrophages, this gene was found to be up-regulated in B cells. The transcriptional increase (*n*-fold) was higher by RT-PCR (compare Fig. 3A to Fig. 4). These variations could be attributable to differences in sample processing.

Both OAS and Mx proteins were amplified with generic primers that were unable to discriminate among different subtypes of these proteins. However, in both cases, an increase in gene transcription was also detected. As expected from the microarray data, both genes were activated in all three kinds of cells compared to baseline. OAS showed a peak of increase

TABLE 1. Genes up regulated after DENV-1 infection[a]

Gene	Description	Ratio (fold)			Function
		PBMC (20 genes)	MO (40 genes)	BC (30 genes)	
1	CBP/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain	NC	7.83	NC	Protein binding, transcription factor activity
2	10-kDa IFN-γ-inducible protein (CXCL10_1179)	NC	NC	5.69	Pleiotropic effects, stimulation of monocytes
3	5′ nucleotidase, cytosolic III	NC	4.12	NC	Nucleotide binding, hydrolase activity
4	CCL3 (chemokine ligand 3)	NC	11.93	NC	Immune response
5	Chromosome 17 open reading frame 27	NC	4.44	4	Protein binding
6	C-type lectin domain family 1, member B	NC	5.53	NC	Defense response, transmembrane receptor activity, response to virus
7	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	5.97	NC	NC	Innate immune response, protein binding
8	DNA polymerase-transactivated protein 6	NC	8.11	NC	DNA replication
9	DTFT5783 (hypothetical protein LOC388352)	NC	4.37	NC	?
10	Endothelial cell growth factor 1 (platelet derived)	NC	6.39	NC	Growth factor activity, thymidine phosphorylase activity
11	EPSTI1	6.13	10.29	4.64	?
12	FBJ murine osteosarcoma viral oncogene homolog B	NC	7.89	NC	Sequence-specific DNA binding, transcription factor activity
13	Full-length cDNA clone CS0DK002YF13 of HeLa cells, Cot25 normalized	NC	NC	5.53	?
14	G1P3, IFN-α-induced protein (ISG6-16)	5.01	7.17	5.01	Exonuclease activity
15	gb:AY044446.1 DB_XREF = gi:22074157 = Mmu.11363.1 (similar to glutathione)	NC	NC	4.22	?
16	GEN = PRIC285 peroxisome proliferator-activated receptor alpha-interacting complex protein 28S	4.29	NC	NC	?
17	gi:60301523 DEF = USP18_9254 rhesus (ubiquitin-specific protease)	NC	NC	5.08	Ubiquitin-specific protease
18	Hect domain and RLD5 (HERC family)	11.52	NC	9.63	Ligase activity, ubiquitin-protein ligase activity
19	Hect domain and RLD6 (HERC family)	5.7	NC	NC	Ubiquitin-protein ligase activity
20	HS-5-8-S rRNA ribosomal DNA complete repeating unit	NC	5.23	NC	?
21	Hypothetical protein FLJ20035 (no assigned function)	5.22	NC	5.08	Nucleic acid binding
22	Hypothetical protein LOC129607 (thymidylate kinase)	15.6	NC	22.17	Kinase activity
23	IBRDC3 (zinc ion binding metal ion binding protein binding)	NC	4.68	NC	?
24	Immunoglobulin heavy-chain variable region	NC	11.74	NC	Nucleotide binding, transferase activity, protein serine/threonine kinase
25	Immunoglobulin kappa constant	NC	10.1	NC	Antigen binding
26	IFI27	25.95	59.78	17.61	Immune response
27	IFN-induced protein 44	NC	NC	12.58	Response to virus
28	INF-induced transmembrane protein 1	13.96	11.73	17.15	Receptor signaling protein
29	IRF7	NC	4.37	NC	Transcription factor
30	IFN-induced protein with tetratricopeptide repeat 1 (IFIT1_6442)	NC	NC	17.15	Immune response
31	IFN-induced protein with tetratricopeptide repeat 2	10.52	NC	6.69	Immune response
32	IFN-induced protein with tetratricopeptide repeat 3	6.35	NC	10.27	Immune response
33	IFN-induced protein with tetratricopeptide repeat 4	6.17	NC	6.51	Immune response
34	IFN-induced protein with tetratricopeptide repeat 5 (IFIT5_6444)	NC	NC	5.6	Immune response
35	ISG15 ubiquitin-like modifier	22.85	34.5	18	Immune response
36	Kruppel-like factor 6	NC	4.51	NC	Nucleic acid binding
37	Lectin, galactoside-binding soluble 3 binding protein	NC	6.8	NC	Cell adhesion, activation of immune cells
38	Likely ortholog of mouse d11lgp2	4.64	NC	6.95	Nucleotide binding, hydrolase activity
39	Lymphocyte antigen 6 complex, locus E	NC	5.24	NC	Defense response, glycosylphosphatidylinositol anchor binding
40	Mx1	5.44	NC	5.7	Immune response, response to virus, GTP-binding protein
41	Mx2	NC	4.9	5.98	Immune response, response to virus, GTP-binding protein

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TABLE 1—*Continued*

Gene	Description	Ratio (fold)			Function
		PBMC (20 genes)	MO (40 genes)	BC (30 genes)	
42	Mx2 human-like protein	NC	4.92	NC	Immune response, response to virus, nucleotide binding, transferase activity
43	OAS1	NC	25.48	NC	Nucleotidyltransferase
44	OAS2	7.29	10.76	7.92	Immune response
45	OAS2 (69/71 kDa)	NC	NC	6.83	Immune response, nucleotidyltransferase, ATP/RNA binding
46	OAS3	4.2	16.62	NC	Immune response
47	Platelet factor 4 (CXCL4 variant 1)	NC	10.51	NC	Immune response, chemokine activity
48	Platelet factor 4 (CXCL4)	NC	5.57	NC	Leukocyte chemotaxis, immune response, chemokine activity
49	Platelet-derived endothelial cell growth factor/ thymidine phosphorylase	NC	4.61	NC	?
50	PRKR	NC	NC	4.16	Immune response, response to virus, negative regulation of cell proliferation
51	PTX3_4 pentaxin-related gene	NC	5.73	NC	Inflammatory response
52	Schlafen family member 5	NC	NC	8.74	DNA/RNA helicase
53	Scramblase 1	NC	4.11	NC	Response to virus, protein binding
54	Serum/glucocorticoid-regulated kinase	NC	5.95	4.5	Nucleotide binding, protein/serine/threonine kinase activity
55	STAT, 1.91 kDa (STAT1)	NC	NC	5.01	Transcription factor
56	Similar to MGC52679	NC	4.61	NC	Phosphorylase activity
57	Sterile alpha motif domain-containing 9	NC	6.14	NC	Binding
58	Syndecan 3 (*N*-syndecan)	NC	4.11	NC	Cytoskeletal protein binding
59	Thymidylate kinase activity	15.6	NC	NC	Kinase/transferase activity
60	Tribbles homolog 2 (*Drosophila melanogaster*)	NC	4.84	NC	Nucleotide binding, protein kinase activity, protein kinase inhibitor activity
61	Tripartite motif-containing 14 (TRIM14)	NC	NC	4.24	Protein binding, metal/zinc ion binding
62	Tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10)	NC	5	NC	Immune response, apoptosis, TNF receptor binding
63	Unc-93 homolog B1 (*Caenorhabditis elegans*)	NC	5.27	NC	?
64	Radical *S*-adenosyl methionine domain-containing 2 (cig5, viperin)	11.96	12.49	15.47	Catalytic activity, iron ion binding/antiviral

[a] Genes that were increased by 4.0-fold or greater were included. Conditions for this set of genes were a P value of <0.005 and call P. Twenty, 40, and 30 genes were detected under these conditions in PBMC, macrophages (MO), and B cells (BC), respectively. Gene names are given at the left and are organized alphabetically. The function attributable to each gene is given on the right. Information on how to access the full list of all genes, specific P values, and ontologic pathways is provided in the text. NC, no changes.

that duplicated the value detected by microarray. In this case, the detection of a whole pool of genes could account for these marked differences. As shown in Table 1, different members of this family were up-regulated in each type of cell (OAS1 in macrophages, OAS3 in macrophages and PBMC, and OAS2 in macrophages, PBMC, and B cells), suggesting a possible cell type-specific mechanism of activation of this pathway. The severalfold increases in Mx proteins detected by RT-PCR were comparable to those detected by microarray, possibly due to the existence of fewer variants of the proteins of this family. In summary, the three selected genes (Mx, OAS, and IRF) were found to be up-regulated upon DENV-1 infection, confirming their increased induction originally detected by the microarray method.

The induction of IRF7 was also confirmed by RT-PCR. In contrast to IRF3, the other key player in the type I IFN pathway, IRF7 is not constitutively expressed in most cell types, and it has a short half-life (69, 79, 93, 110). After activation by phosphorylation, IRF7 is translocated to the nucleus, where it induces the transcription of IFN-α/β genes (69, 93, 94). These aspects are very important in our work, because it was detected 5 days after infection with DENV, supporting the presence of a very recent stimulus. In addition, by the use of RT-PCR, we were able to confirm the lack of induction of cytokine genes like IFN-β, IFN-γ, and TNF-α 5 days after in vivo infection with DENV-1 (Fig. 4). This result was confirmed by cytokine detection in serum at days 1, 3, and 5 after infection (data not shown).

DISCUSSION

Presently, there is not an adequate animal model to study DENV pathogenesis or to test immune responses to DENV candidate vaccines. Interesting approaches using mice have been done in recent years (8, 99). It is well known that macaques respond to DENV infection with quite limited signs and symptoms compared to humans; however, they are the ultimate model of choice to test DENV vaccine candidates and their elicited immune responses. Nevertheless, the molecular basis of the immune response to DENV in macaques is remarkably unknown, and the continued use of this model requires a better understanding of underlying differences between human and macaque immune systems. We infected four rhesus macaques with a low-passage strain of DENV-1, and 5 days thereafter, by using microarrays and RT-PCR, we observed the gene transcriptional pattern. Also, the profile of

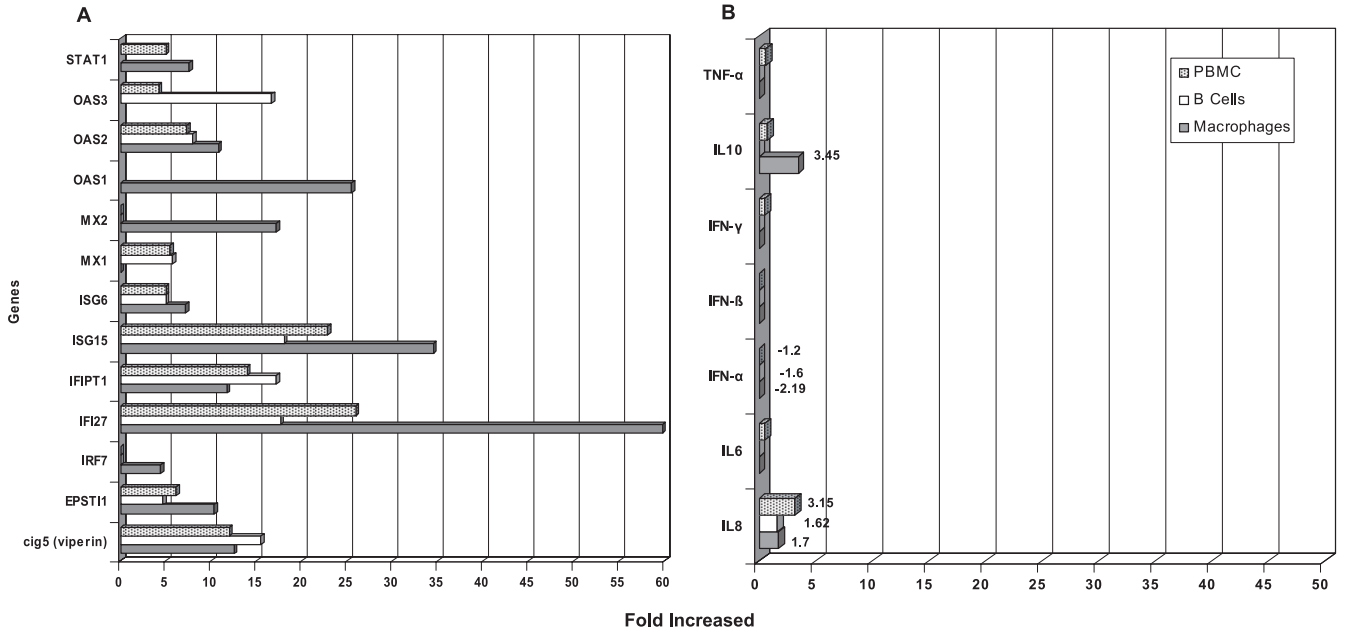

FIG. 3. Transcriptional increase (*n*-fold) detected by microarray. Selected genes were plotted for comparative purposes among samples and among genes. (A) Increment of the ISGs. The IFI27 and ISG15 genes were the most highly up-regulated, followed by the OAS genes Mx, viperin, and EPSTI1. (B) Transcriptional modifications of the cytokine genes. Only a limited increase in the transcription levels of IL-10 and IL-8 was detected. IFN-α was detected as being down-regulated in all kinds of samples. However, none of these changes were statistically significant, reflecting no changes in the transcriptional activity of these genes. The transcriptional increase (*n*-fold) was plotted on the abscissa. The names of the genes are shown on the ordinate.

serum cytokines was studied by multiplex cytokine detection after 1, 3, and 5 days of infection.

We found a robust innate immune response characterized by the induction of several genes implicated in the immune response to viral infections. Most of those genes were ISGs and virus-induced genes. A third group included some genes with no relation to the IFN signaling pathway. By RT-PCR, we confirmed that at least three of those genes (IRF7, OAS, and Mx) were indeed up-regulated.

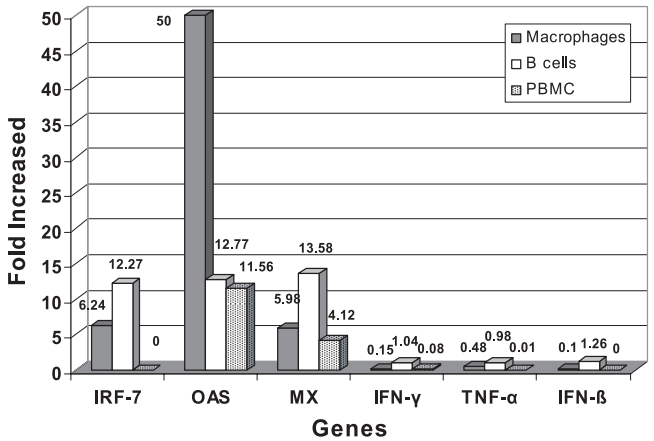

FIG. 4. Real-time confirmation of ISG gene but not of cytokine gene up-regulation. Three ISG genes (IRF7, OAS, and Mx) and three cytokine genes (IFN-γ, TNF-α, and IFN-β) were selected to be amplified by RT-PCR to confirm the microarray data. Figures represent increases (*n*-fold) in up-regulated genes compared to baseline samples. All three ISGs were up regulated except IRF7 in PBMCs. No increase in the transcription level of the studied cytokine genes was detected.

2′,5′-Oligoadenlyate is a system that is well documented as an endogenous player in the antiviral pathway (16, 89, 92). This protein is activated by dsRNA to produce 5′-phosphorylated, 2′,5′-linked oligoadenylates, whose function is to activate RNase L with subsequent RNA degradation. The implication of this system to control flavivirus infections has been demonstrated before in vitro and in vivo for West Nile virus (54, 70, 95) and HCV (56, 68, 75) and in vitro for DENV (109). Our finding in vivo of increased levels of OAS transcripts confirms the role of this system in controlling DENV replication in rhesus macaques.

The Mx protein family is well known for its role in antiviral responses in vivo and in vitro (31, 65, 80, 81), and the proteins are preferentially induced by IFN-α/β (20). Evidence from animal studies established that Mx alone is sufficient to block the replication of virus in the absence of any other IFN-α/β-inducible proteins (3, 30). The induction of Mx1, Mx2, and MxA genes was produced after in vitro infection of HUVEC cells with DENV-2 (110); however, in our study, only Mx1 and Mx2 were detected as being up-regulated by microarrays. These results together confirm the specific anti-DENV activity of Mx proteins.

In contrast with other key players in the type I IFN pathway such as IRF3, IRF7 is not constitutively expressed in most cell types, and it has a short half-life (69, 79, 93, 110). After activation by phosphorylation, IRF7 is translocated to the nucleus, where it induces the transcription of IFN-α/β genes (69, 93, 94). The detection of high levels of IRF7 5 days after the

infection with DENV-1 in rhesus monkeys could then be interpreted as evidence of the presence of a very recent stimulus. This is particularly remarkable in the lack of confirmed cytokine induction, including IFN-β, IFN-γ, and TNF-α, 5 days after DENV-1 infection (Fig. 4). These results were confirmed by cytokine detection in serum at days 1, 3, and 5 after infection (data not shown).

One of the key and unexpected outcomes of this study was the apparent lack of induction of three key cytokines in human innate immunity: IFN-β, IFN-γ, and TNF-α (Fig. 3 and 4). In addition, only limited but not significant up-regulation of IL-10 and IL-8 was detected by microarray, (Fig. 3). This is highly contrasting with what has been reported for humans. The high-level presence of IL-10, IL-8, IL-6, TNF-α, and IFN-γ is a hallmark of the development of DHF/DSS and has been associated with an increased risk of developing severe forms of disease in primary infections, which may be accentuated during secondary infections (7, 13, 18, 28, 40–42, 50, 52, 53, 64, 82, 86). Several groups documented an increased production of these proinflammatory cytokines after a primary infection (6, 7, 13, 50, 64, 77, 83, 108), even in cases without DHF/DSS manifestations (6, 64, 77, 83, 108).

Because our study was performed 5 days after infection, we cannot rule out later cytokine gene induction and cytokine production. One of the advantages of the macaque model is that we have the opportunity to analyze the very early virus-host interaction, which is often not possible for humans. DENV is able to induce the activation of PBMC in mice as early as 3 days after infection (97) and in humans with less than 72 h of fever/viremia (27). Also, the production of cytokines has been detected in children presenting less than 72 h of fever (26–28, 60). Considering that disease outcome is likely to be decided by those early events, it is remarkable that we were unable to find the inflammatory response that is so typical for disease in humans. Nevertheless, in the future, we plan to conduct similar studies at later time points following infection.

Our new findings may explain why rhesus macaques do not develop DF or DHF/DSS after a primary DENV infection. Indirectly, these results confirm the proposed role of TNF-α and IFN-γ in the development of DHF/DSS. Thus, it might be argued that a therapy aimed at the suppression of inflammation/activation may reduce the incidence of DHF/DSS in humans. It can be affirmed that a different profile could be expected after a secondary infection of these animals. However, our data show that the cytokine profile after secondary infection with DENV-2 is very similar to the one described after primary infection (C. A. Sariol et al., unpublished results).

The up-regulation of OAS and Mx proteins shown by microarrays and RT-PCR was expected, as they constitute a standard response after infection with several viruses (3, 30, 47, 54, 92, 103, 109, 110) and are a confirmation of the role of the innate immune response generated after DENV infection in macaques. The increased detection by both methods reinforces the importance of this type of immune response in controlling DENV replication in rhesus macaques. Additionally, several ISGs such as ISG15 and ISG16-56, which are acting during the innate immune response, were found to be highly up-regulated by microarrays.

Induction of ISGs without increased levels of type I or type II IFN has been reported for HCV-infected chimpanzees (10). Similar to data from that report, we found an apparent contradiction in that the induction of ISGs may not necessarily be accompanied by a higher induction of IFN. Additionally, increases in serum levels of IFN-α or IFN-γ were not detected by the multiplex cytokine array even as early as 24 h after the infection (data not shown). However, the following explanations for the apparent discrepancy may account for this fact. An early transcriptional activation of these genes before the day of the sampling for microarray and RT-PCR study (5 days after infection) with a restricted level of expression below the limit of detection of our assay but still able to stimulate the ISG transcription detected 5 days later may have occurred. Second, only the first wave of the IFN response, characterized by the production of IFN-β (69, 93, 94), was allowed to occur (animal or virus restriction). Because of the lack of a reagent with reactivity for this rhesus macaque cytokine (IFN-β), we were unable to test its levels in serum, and we were missing a crucial and early-phase activation of the innate immune response. For the same reasons, we may be missing the possible expression of IFN-λ, which has been shown to have an active role in the activation of the immune response (57, 96). Third, anti-IFN activity of DENV protein NS4B through the inhibition of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway has been documented in vitro using monkey kidney cells (72, 73). This mechanism may lead to the partial impairment of the IFN pathway. Fourth, novel virus-host interactions may limit the transcriptional activation and expression of IFN-α and IFN-γ and other cytokines, such as TNF-α, IL-6, IL-8, and IL-10, linked to the immunopathogenesis of DENV.

The detection of IRF7 up-regulation by microarray and confirmation by RT-PCR 5 days after infection also support the existence of alternative mechanisms in this animal model to regulate type I IFNs and activate ISGs. The role of IRF7 as a master regulator in the induction of type I IFN is well known (44–46). It was long believed that the activation of IRF7 was dependent exclusively on basal levels of IFN-α/β or in response to an early wave of IFNs following virus infection, which in turn amplified IFN-α/β production through the increase in the intracellular level of IRF7 (69, 76, 93, 94, 107). However, it has recently been shown that the regulation of IRF7 transcription includes a virus-dependent but IFN-independent signaling pathway (79). In such alternative mechanisms, the viral infection induces virus-activated factor complex formation (including IRF7/IRF3), which directly binds to specific regions of the IRF7 promoter. One essential component of this pathway is the recruitment of cyclic AMP response element binding protein (CREB)-binding protein (CBP)/p300 for IRF3 activation (79). However, it has also been shown that this activator has an opposing activity by negatively modulating the IRF7 DNA binding (14). Supporting this mechanism in our model, as an alternative induction of IRF7 without detectable transcriptional activation or serum levels of type I IFN, is the transcriptional up-regulation of the CBP/300 gene detected by microarray (Table 1). It could act in synergy with the anti-IFN activity of the nonstructural proteins of DENV, particularly NS4B through the JAK/STAT pathway (39, 51, 72, 73). Although we have no data to support the occurrence of this mechanism in vivo in our model, it is an alternative to be addressed in further studies.

Other genes such as PLSCR1 or EPSTI1, here detected as being up-regulated by microarray, are also interesting targets for further studies. PLSCR1 is a membrane protein implicated in the synthesis and translocation of phospholipids to the cell surface in response to cell activation, injury, or apoptotic stimulus (111), and it is able to strengthen the antiviral activity of IFNs (19). This gene was previously found to be activated after DENV-2 infection in HUVEC cells (109). Our in vivo results support the anti-DENV activity of this protein. Confirmation of the up-regulation of EPSTI1 by other methods will be performed.

The immune response to DENV described in this work is quite different from the immune response characterized in mice (4, 97–100) and in humans in vivo or in vitro using human cells (12, 13, 15, 18, 41, 83, 99, 104, 109). This strongly suggests interspecies differences in the complex molecular mechanisms of the IFN response. This reinforces the need to search for a proper animal model to study the pathogenesis and immune response to vaccines against DENV.

This balance of innate immune response without cytokine induction can be an example of a long-term host-parasite evolutionary process. Similar situations have been demonstrated for sooty mangabeys infected with simian immunodeficiency virus. These animals are naturally infected without developing symptoms. The key feature of that process is a controlled activation of the immune response (74, 101).

Results documented in this work prompted us to consider other interventions in addition to vaccine approaches, like the modulation of the innate immunity, to control the development of severe forms of this disease.

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