Different Innate Signatures Induced in Human Monocyte-derived Dendritic Cells by Wild-Type Dengue 3 Virus, Attenuated but Reactogenic Dengue 3 Vaccine Virus, or Attenuated Nonreactogenic Dengue 1–4 Vaccine Virus Strains

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DNA microarrays were used to assess the innate gene signature in human myeloid dendritic cells infected with chimeric dengue 1–4 vaccines, a wild-type dengue 3 virus, or a classically attenuated serotype 3 vaccine shown to be reactogenic in humans. We observed a very reproducible signature for each of the 4 chimeric dengue vaccines, involving stimulation of type I interferon and associated genes, together with genes encoding chemokines and other mediators involved in the initiation of adaptive responses. In contrast, wild-typeDEN3 virus induced a predominantly inflammatory profile, while the reactogenic attenuated serotype 3 vaccine appeared to induce a blunted response.

The 4 serotypes of dengue flaviviruses cause human disease with significant morbidity and mortality. Several candidate vaccines are being developed, including chimeric vaccines based on the yellow fever (YF) 17D virus vaccine (CYDs). The genes encoding the pre-membrane and envelope proteins of YF-17D have been replaced by those of a representative wild-type virus of each of the 4 dengue serotypes, generating CYD1–4 monovalent viruses. These vaccine viruses are safe and immunogenic in humans [1] and are presently under evaluation in large-scale efficacy trials (for a review, see [2]).

It is postulated that inappropriate immune responses, implicating both humoral and cellular components, could partially explain disease severity. Regarding early cellular responses, studies have demonstrated the permissiveness of human immature myeloid dendritic cells (mDCs) to wild-type (wt) dengue viruses, resulting in enhanced expression of some proinflammatory cytokines and cell-surface activation markers [3–5]. To further investigate the gene signature induced by dengue infection, DNA arrays have also been used with different cell types [6–8].

The CYD1–4 vaccine viruses replicate in human mDCsin vitro[9]. Using supernatant cytokine measurement, flowcytometry, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR), we previously explored the consequences of mDCs infection by CYD1–4 [10]. We observed an upregulation of HLA-DR, CD80, CD86, and CD83, as well as secretion of MCP1/CCL22IL6, high levels of type Iinterferons, low levels of TNFα, and no IL10, IL12, or IL1β. This pattern was similar to that observed with the parental wt dengue viruses but with less TNFα, more IL6, and more MCP1/CCL2. The chimeric viruses thus induced dendritic cells (DCs) maturation and a controlled response, accompanied by limited inflammatory cytokine production and significant expression of antiviral interferons, consistent with clinical observations of safety and immunogenicity.

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In another study, we similarly used human dendritic cells and the same endpoints and assays to compare the innate response induced by a classically attenuated live dengue virus vaccine (VDV3) and its parental serotype 3 wt virus (DEN3). We observed for VDV3 a pattern similar to the one induced by chimeric vaccines, mostly characterized by an antiviral signature. The VDV3 vaccine candidate turned out, however, to be reactogenic in humans [11], and although the cause may have been multifactorial (dose, nature, and kinetics of the adaptive response), we concluded that the limited set of responses measured in DCs was not fully predictive of the clinical outcome, particularly as regards safety.

Thus, in the present study, we used DNA arrays (22K and 44K Agilent chips) with the objective of identifying (1) a broader gene signature for CYD1–4 and (2) potential differences among these nonreactogenic vaccine viruses, a virulent wtDEN3 virus, and the reactogenic, former vaccine candidate virus, VDV3.

CYD1–4 were produced in Vero cells and purified at Sanofipasteur (Marcy l'Étoile, France)[10], as were wtDEN3 Mahidol strain 16562 (batch 07/94) and the VDV3 strain [11]. Culture and infection (0.5 MOI) of human monocyte-derived DCs was also performed as described elsewhere[10–11]. DNA array analysis was done from RNA prepared 24 hours after infection, refined in a second step by qRT-PCR on a selected set of genes. For qRT-PCR, cytokine gene expression was normalized using the endogenous reference 18S rRNA gene, and mock-infected DCs were used as a reference sample [10]. For DNA arrays, the labeled cRNA were prepared using a Low RNA Input Fluorescence Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. The amount and the quality of labeled cRNA were assessed using Nanodrop ND-1000 spectrophotometer. Percentages of infection were monitored by flow cytometry 48 hours after infection.

The Agilent 22K and Whole Human Genome Oligo Microarray (44K) were used. The latter oligochip displays approximately 41,000 (60-mer) oligonucleotide probes representing the human genome and 3,000 controls, and the Human 1A microarray (V2) displays ~21,000 (60-mer) oligonucleotide probes representing the human genome and 1,000 controls. Information on the oligochip is available at http://www.chem.agilent.com/en-us/products/instruments/dnamicroarrays. Slides were hybridized according to the manufacturer's instructions and scanned using a GenePix Personal 4100A scanner (Axon Instruments). The signal intensities were measured using the GenePix PRO v6.1 software. Intensity files were then exported and analyzed using Rosetta Resolver software (Rosetta biosoftware V), with the appropriate error model according to the manufacturer's instructions. All oligochip experiments were performed in dye swap. For each experiment, the gene expression profile of infected cells was compared with the corresponding profile of uninfected cells. Intensity files (GenePix PRO software, versions 5.1 and 6) were exported and analyzed using Rosetta Resolver software (Rosetta biosoftware, version 4), with the appropriate error model according to the manufacturer's instructions. The thresholds for the selection of differentially expressed genes were set at a *P*-value of .001 and an expression ratio of at least 1.3. Gene expression was considered to be up- or downregulated if the foldchange was ⩾2. Data were submitted to the Gene Expression Omnibus database under the accession number GSE22329.

RESPONSES INDUCED BY CHIMERIC DENGUE VACCINE VIRUSES

A remarkably similar pattern in DNA array results from 3 donors, corresponding to biological replicates, was observed between serotypes at both qualitative and quantitative levels (Table 1). All 4 CYD viruses induced the cytosolic sensors RIG-I and MDA-5, critical for antiviral responses [12] and PTX3 leading to IRF3 activation and downregulation of deleterious inflammation involving neutrophils [13]. Transcription factors STAT1, also involved in innate and Th1 adaptive responses, and IRF1 were triggered, as was IFN-β and a large array of interferon-stimulated genes (ISGs). CYD4 was the most potent inducer of IFN-α. Several chemokine genes involved in both innate and adaptive responses were also induced, including CXCL9 and CXCL11, involved in APC, and NK and adaptive Th1 cell mobilization. Pro-inflammatory cytokine induction was almost absent, and an IL-6 response was detected only with CYD4. Some maturation markers involved in cellular activation were also induced, such as CD38, CD69, and CD83. In particular, type I IFN has been involved in CD38 upregulation and increased dendritic cell function [14]. Finally, some factors involved in apoptosis or dengue antiviral responses were triggered, such as TNFSF10 (TRAIL)[8]. No significant gene downregulation (more than 2-fold) was observed for any condition.

The pattern observed with the tetravalent (TV) vaccine mixture was similar to that observed with CYD4, which was the most potent of the 4 CYD viruses in these experiments. CYD4 was the most infectious, and CYD1 was the least infectious (mean infection level: 20% for CYD4, compared with 2.5% for CYD1, 11.5% for CYD2, 4.3% for CYD3, and 23% for TV), which may have contributed to the slight differences observed between the serotypes. Responses were nevertheless very comparable. Interestingly, CYD4 is the immunodominant serotype in humans in vivo[1–2] and the only one able to induce detectable (albeit low) viremia.

Analysis of selected genes by the more sensitive qRT-PCR assay in the same 3 donors together with a fourth one confirmed these results. Results were again very consistent among serotypes, with still some dominance for the TV formulation. IL6 was upregulated for all 4 serotypes and TV, with a mean-fold increase between 15 and 20. TNFα upregulation was also detected with CYD4 and TV (15-fold), compared with 5–10 for CYD1–3. Strong IFNβ responses (2,000- to 9,000-fold) were

Table 1. Comparison of CYD1 to 4 and tetravalent (TV) combination using a 44K DNA array (fold change)[a]

	CYD1		CYD2		CYD3		CYD4		TV	
Gene	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PRRs										
RIG-I	**23.7**	4.8	**26.2**	7.8	**23.0**	2.6	**24.3**	2.5	**26.6**	1.0
MDA-5	**9.7**	0.5	**10.5**	1.8	**8.7**	0.4	**10.0**	0.7	**9.6**	0.8
PTX3			**9.2**	4.5	**8.7**	5.1	**15.8**	7.4	**15.9**	12.4
Transcription Factors										
IRF1	**3.4**	1.1	**3.1**	1.1	**3.3**	1.3	**3.3**	1.2	**3.0**	1.1
STAT1	**7.3**	2.7	**8.5**	1.9	**7.4**	2.9	**7.2**	2.8	**7.3**	2.1
NFKBIA	**2.9**	0.8	**3.2**	1.1	**2.9**	0.5	**3.4**	0.7	**3.8**	0.7
NFKBIZ									**3.0**	1.4
IFNs/Interferon Stimulated Genes										
IFNB1	**20.0**	15.8	**19.6**	13.7	**14.7**	6.0	**30.0**	2.7	**31.9**	13.9
IFNA6			**6.6**	3.8	**5.9**	2.3	**10.3**	1.8	**12.0**	1.7
IFNA8					**4.7**	1.8	**8.0**	1.5	**11.0**	0.5
IFNA5			**4.0**	1.5	**3.8**	1.6	**7.8**	3.5	**8.5**	4.0
IFNA14							**6.0**	2.7	**5.6**	2.3
OASL	**15.3**	7.0	**15.7**	3.2	**12.1**	4.8	**17.6**	4.3	**15.4**	5.3
OAS3	**2.8**	0.9			**11.2**	2.3	**11.8**	3.4	**12.2**	2.8
OAS1	**5.5**	1.2	**5.5**	1.8	**5.4**	2.4	**6.1**	2.5	**5.3**	2.4
GBP1	**17.0**	6.7	**18.7**	3.5	**16.9**	6.7	**17.7**	7.5	**17.6**	5.4
GBP4	**20.7**	8.9	**25.1**	4.7	**22.2**	2.0	**21.7**	3.4	**26.8**	2.2
GBP5	**22.6**	7.4	**24.7**	8.2	**20.8**	4.6	**24.0**	6.1	**22.8**	3.4
IFIT1	**28.8**	3.9	**31.6**	13.0	**23.5**	4.5	**31.3**	8.6	**26.4**	7.9
IFIT2	**ND**	ND	**93.4**	11.4	**94.5**	8.7	**99.8**	0.4	**98.5**	2.5
IFITM1	**9.0**	1.9	**9.0**	0.5	**8.5**	1.7	**9.9**	1.9	**9.7**	0.9
IFI27	**11.5**	6.1	**12.4**	3.6	**12.8**	6.8	**13.5**	7.1	**13.8**	6.4
IFI44	**12.4**	0.5	**13.4**	2.4	**10.8**	0.9	**12.3**	0.8	**12.5**	1.6
MX1	**12.9**	0.4	**13.3**	2.3	**11.2**	0.8	**13.3**	0.1	**13.1**	2.6
MX2	**9.5**	2.6	**10.3**	0.2	**9.2**	3.2	**10.2**	3.7	**10.8**	4.3
ISG20	**18.4**	6.6	**18.5**	1.2	**16.4**	2.9	**20.4**	5.5	**18.0**	2.5
PNPT1	**11.2**	2.0	**12.0**	1.5	**10.1**	2.3	**11.5**	3.3	**10.8**	3.2
EIF2AK2 (PKR)	**6.7**	1.3	**6.6**	0.8	**6.2**	1.0	**6.0**	1.1	**6.4**	0.9
TRIM22	**5.7**	1.6	**6.1**	0.8	**5.4**	1.3	**5.9**	1.8	**5.6**	1.3
Pro-Inflammatory Cytokines										
IL6							**6.4**	4.6		
IL15	**4.5**	1.4	**4.1**	0.7	**4.1**	1.3	**4.3**	0.9	**4.4**	1.5
Chemokines										
CXCL9	**26.2**	19.7	**30.0**	15.5	**25.9**	18.8	**34.1**	22.0	**28.4**	18.8
CXCL11	**61.8**	15.0	**71.8**	24.8	**61.6**	6.3	**79.5**	11.2	**65.1**	11.6
Maturation Markers										
CD38	**13.7**	8.5	**12.6**	5.4	**13.8**	8	**16.0**	9.1	**17.5**	10.2
CD69	**7.8**	4.0	**7.3**	3.0	**7.5**	3.1	**7.0**	3.4	**5.5**	2.2
CD83	**3.6**	1.1	**4.0**	0.6	**3.8**	0.6	**4.1**	0.5	**4.6**	0.1
Other Mediators/Antigens										
APOBEC3A	**73.4**	28.5	**84.5**	5.8	**70.7**	15.2	**80.8**	17.4	**65.9**	14.3
INDO	**11.3**	6.7	**12.6**	1.9	**10.5**	4.2	**15.6**	3.8	**15.4**	6.1
USP18	**13.1**	6.2	**12.9**	6.4	**12.3**	7.6	**13.8**	8.6	**12.9**	8.3
TNFAIP6 (TSG-6)	**7.5**	4.2	**8.4**	1.6	**7.1**	2.2	**11.8**	2.2	**11.7**	1.8
TNFSF10 (TRAIL)	**10.9**	4.2	**12.1**	3.7	**10.3**	5	**10.8**	5.4	**9.9**	5.1
LAMP3	**5.0**	4.6	**3.4**	1.6	**5.1**	4.5	**5.8**	5.3	**7.2**	6.0
CASP1	**2.9**	0.8	**3.0**	0.8	**3.0**	1.0	**2.9**	0.9	**3.2**	1.5
CASP7	**4.8**	1.8	**5.3**	1.5	**5.2**	2.1	**4.8**	1.7	**5.2**	1.6

NOTE. ND = not determined.

[a] Absent values: unchanged.

Table 2. Comparison of wtDEN3, VDV3, and CYD3 using a 22K DNA array (fold change)[a]

	wtDEN3		CYD3		VDV3	
Gene	Mean	SD	Mean	SD	Mean	SD
PRRs						
RIG-I	7.7	5.5	28.5	5.3	14.8	12.2
MDA-5	4.5	2.7	11.0	4.5	6.5	5.3
Transcription Factors						
IRF1	3.5	0.8	2.8	0.3		
IRF9			2.8	0.6		
STAT1	4.7	2.1	10.9	1.8	4.4	2.9
NFKB1	2.6	0.4				
NFKBIA	2.6	0.1				
NFKBIZ	2.8	0.4	2.5	0.3		
IFNs/Interferon Stimulated Genes						
IFNB1			6.0	3.8		
OASL	7.9	4.3	16.4	4.4	10.0	4.6
OAS3	7.3	1.2	12.2	0.3	6.0	3.5
OAS1	3.6	0.7	6.7	0.1	3.3	1.8
GBP4	6.8	6.7	11.1	6.8		
GBP5	8.4	7.2	20.4	10.7	7.5	7.5
GBP1	7.5	6.2	15.9	7.1	5.0	4.1
IFIT1	21.3	7.0	43.9	3.4	39.1	10.0
IFIT2	39.2	30.7	>100.0	NA	90.9	12.8
IFITM1	7.8	1.4	9.2	0.0	5.9	2.6
IFI27	21.1	10.0	15.0	9.4		
IFI44	11.1	3.2	18.7	0.3	9.6	4.6
MX1	10.4	0.9	15.3	2.7	11.7	0.9
MX2	8.2	3.7	17.8	4.7	9.5	6.6
ISG15	13.1	4.8	18.3	2.7	13.9	6.6
ISG20	13.2	6.1	18.0	1.7	10.2	7.2
PNPT1	3.3	1.7	10.5	0.7	5.5	3.3
EIF2AK2 (PKR)	4.9	3.3	9.0	1.1	6.0	3.4
TRIM22	3.8	2.6	10.8	1.0	5.4	3.9
Pro-Inflammatory Cytokines						
IL1B	32.2	3.4				
IL6	9.3	3.9				
IL12B	13.4	3.5				
IL15	2.5	1.2	3.45	1.25		
IL23A	7.5	3.7				
TNF-α	4.1	1.0				
Chemokines						
CCL2			6.9	6.8		
CCL4	5.5	1.9				
CCL5	3.9	0.4	2.7	0.8		
CCL8			20.1	24.3		
CCL13			2.8	0.4		
CXCL1	21.4	13.2				
CXCL2	5.0	2.4				
CXCL3	5.3	0.6				
CXCL8 (IL8)	5.8	0.8				
CXCL9	7.7	8.3	22.4	25.9		
CXCL10			19.0	2.8	11.97	11.42
CXCL11	16.2	19.1	60.5	48.8		

Table 2. (*Continued*)

Gene	wtDEN3		CYD3		VDV3	
	Mean	SD	Mean	SD	Mean	SD
Maturation Markers						
CD38			5.5	2.4		
CD69			6.5	2.3		
CD83	3.0	0.4				
Other Mediators/Antigens						
APOBEC3A	8.4	3.4	11.3	4.8		
APOBEC3B	18.0	9.2	25.4	8.2		
APOBEC3G			5.1	0.3		
INDO	6.6	5.9	7.3	7.0		
USP18	7.3	4.0	16.6	2.4		
TNFAIP6 (TSG-6)	4.5	2.3				
TNFSF10 (TRAIL)	6.3	5.3	24.0	11.4	9.9	8.7
LAMP3	3.6	1.5				
SIGLEC-1	3.6	1.0	5.0	0.7		
BCL2L14	3.4	2.0	2.8	0.9		
CASP1			5.5	0.7		

NOTE: [a]Absent values: unchanged.

detected. The major difference between qRT-PCR and DNA array data was the upregulation of chemokine genes encoding CCL2/MCP1 (15-fold), CCL3/MIP1a (10-fold), and CCL5/RANTES (50-fold), not detected with chips under any conditions. These chemokines are involved in the mobilization of APCs, NK cells, and T cells for CCL3 and CCL5.

The observed pattern induced by the 4 CYD vaccine viruses confirms and further extends our previous observations with a smaller set of genes [10]. DNA arrays identified a broader picture, including genes involved not only in antiviral defense but also in the interplay between adaptive and innate responses. We therefore decided to compare responses induced by the CYD vaccine candidates with those of a virulent wt virus (DEN3 Mahidol strain) and a reactogenic former vaccine candidate (VDV3[11]).

COMPARISON AMONG wtDEN3 (MAHIDOL), VDV3, AND CYD3

This second DNA array experiment in 2 additional donors confirmed the pattern observed with CYD3 in 44K chips, although some minor variations were noticed (Table 2). Differences were mostly quantitative; however, several additional chemokine genes were identified in the 22K chip.

In contrast, gene patterns differed for wtDEN3, VDV3, and CYD3. All 3 viruses induced ISGs, STAT1, and the cytosolic sensors RIG-I and MDA-5, with higher levels typically observed for CYD3, which was the only virus to induce IFN-β in these conditions. However, although these antiviral genes were almost the only ones induced by VDV3, both wtDEN3 and CYD3 induced numerous additional genes, although with different profiles. Pro-inflammatory cytokines (dominated by IL1β) and NFKB1 were only induced by wtDEN3. Several chemokine genes involved in both innate and adaptive responses were induced by both wtDEN3 and CYD3, including CCL5, CXCL9, and CXCL11. Only wtDEN3 induced genes such as CXCL1, 2, and 3 (GROα, β, γ), involved in neutrophil migration and activation, while CYD3 induced chemokines such as CCL2 (MCP1), CCL8 (MCP2), CCL13, and CXCL10, which contribute to both innate and subsequent adaptive responses. Maturation markers CD38 and CD69 were only induced by CYD3. Other genes involved in antiviral activities or apoptosis were additionally triggered by both wtDEN3 and CYD3, similar to those identified with the 44K chip, including TRAIL, APOBEC genes (rather known for their modulation of retroviral infection through DNA editing), and Caspase 1. No significant downregulation was observed with any virus.These differences were not related to infectiousness, and the level of infection of VDV3 was higher in the 2 donors (8% and 3.5%) than those of wtDEN3 (4% and 1%) and CYD3 (5% and 1.5%).

With use of qRT-PCR, the expression of selected genes was further assessed in 4 donors, including the 2 analyzed by DNA arrays. As above, qRT-PCR was more sensitive. In agreement with the DNA array data, all 3 viruses induced high IFN-β levels, with the highest fold change observed for CYD3 (mean value 920), followed by VDV3 (350) and wtDEN3 (160). In contrast, wtDEN3 induced the highest fold changes for pro-inflammatory IL1β, IL6, and TNF-α (57, 63, and 8, respectively), compared with 1, 7, and 2.5 for CYD3 and 1.5, 3.5, and 1.5 for VDV3. IL12p40 was mostly induced by wtDEN3 (35), compared with

3- and 2-fold for VDV3 and CYD3, respectively. CCL5 and CCR7 were also increased by both wtDEN3 (7 and 4.5, respectively) and CYD3 (4 and 2.5) but not by VDV3.

DISCUSSION

Our findings document the innate profile induced in human mDCs by the CYD candidate vaccine viruses, revealing a limited inflammatory response in the presence of type I IFN genes and associated ISGs and of maturation markers and chemokines involved in both innate and adaptive immunity. These results confirm and expand upon those of our previous studies [10–11].

The observed gene profile of the CYD viruses was consistent with clinical trial observations of safety and immunogenicity. The gene profile of the virulent wtDEN3 strain concords with previous reports of other wt dengue viruses and includes a strong inflammatory profile with induction of chemokines involved in neutrophil attraction, although the innate immune pattern induced may also depend on the wt strain used in each study [15]. In contrast, while initial studies performed on a limited set of parameters showed a similar pattern for VDV3 and CYD viruses (and similar expectations regarding safety), we found markedly different profiles here. The reactogenic VDV3 virus induced an almost exclusively antiviral profile, with limited induction of genes involved in early innate or subsequent adaptive immune responses. In contrast to Sariolet al.[6], who proposed that such a blunted response contributes to protection in macaques, we hypothesize that VDV3 induces a weak early immune response that is insufficient to prevent or control a second and higher round of viral replication, which then contributes to the observed high reactogenicity of VDV3 in clinical trials, concomitant with peak viremia at day 8 [11]. In conclusion, our findings further support the safety and immunogenicity of YF-17D-based chimeric dengue virus vaccine candidates and highlight the interest of determining a broad innate signature of candidate vaccines in preclinical studies to better predict the outcome in humans.

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