ORIGINAL ARTICLE

Transcriptomic profiles of high and low antibody responders to smallpox vaccine

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Despite its eradication over 30 years ago, smallpox (as well as other orthopox viruses) remains a pathogen of interest both in terms of biodefense and for its use as a vector for vaccines and immunotherapies. Here we describe the application of mRNA-Seq transcriptome profiling to understanding immune responses in smallpox vaccine recipients. Contrary to other studies examining gene expression in virally infected cell lines, we utilized a mixed population of peripheral blood mononuclear cells in order to capture the essential intercellular interactions that occur *in vivo*, and would otherwise be lost, using single cell lines or isolated primary cell subsets. In this mixed cell population we were able to detect expression of all annotated vaccinia genes. On the host side, a number of genes encoding cytokines, chemokines, complement factors and intracellular signaling molecules were downregulated upon viral infection, whereas genes encoding histone proteins and the interferon response were upregulated. We also identified a small number of genes that exhibited significantly different expression profiles in subjects with robust humoral immunity compared with those with weaker humoral responses. Our results provide evidence that differential gene regulation patterns may be at work in individuals with robust humoral immunity compared with those with weaker humoral immune responses.

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INTRODUCTION

Vaccinia virus (VACV) is the immunologically cross-protective orthopox virus found in the smallpox vaccine used in the eradication of smallpox.¹ Although smallpox has been eradicated, there continues to be significant public health interest in smallpox and other orthopox viruses for multiple reasons: biodefense against weaponized poxviruses; continuing outbreaks of zoonotic orthopox virus infections; the use of VACV as a vector for cancer immunotherapy and vaccines against other infectious agents.^{2–9} Thus, there continues to be a need for an increased understanding of poxvirus biology, host response to infection and the immunologic mechanisms behind immunity to poxviruses.

Next-generation sequencing is a powerful technology that holds tremendous promise in the areas of systems biology¹⁰ and vaccinomics^{11–15} for developing a deeper understanding of the host response to both vaccines and viral infections. Here we describe the use of next-generation sequencing mRNA-Seq to analyze transcriptomic changes occurring in peripheral blood mononuclear cells (PBMCs) from smallpox vaccine recipients after VACV stimulation, with a focus on early, innate responses to viral stimulation.

RESULTS

The high antibody (Ab) titer group ($n = 21$) had a median ID₅₀ titer of 433.4 (interquartile range: 400.7–481.9), whereas the low Ab titer group ($n = 23$) had a median ID₅₀ titer of 35.5 (interquartile range: 29.5–40.2). Of note, 19 of the low Ab group had Ab titers below the presumed protective threshold titer of 1:32.¹⁶ Each of

these 44 subjects had two samples (uninfected and vaccinia infected). We detected a similar number of reads between samples from high and low Ab responders. The read counts between the stimulated and unstimulated samples were also comparable. We detected a mean of 11.3 million reads in the high-titer stimulated samples (10.9 million reads mapped to the human genome and 350 000 reads mapped to the vaccinia genome). In the high-titer unstimulated samples we detected a mean of 11.7 million reads (of which a mean of only 530 reads mapped to the viral genome).

Viral stimulation and host gene expression

Viral stimulation had a dramatic effect on host gene expression, with over 1200 genes exhibiting significant upregulation or downregulation between unstimulated and stimulated samples from all subjects ($P < 0.001$ and false discovery rate < 0.01). A small subset of these genes with P -values < 0.001 , a false discovery rate < 0.01 and fold changes in expression > 1.5 or < 0.75 are listed in Table 1 (the Supplementary Table contains the list of 1000 genes, all with $P < 0.001$, which were used in the pathway analysis). These genes encode for a number of histone proteins, cytokines and growth factors (interleukin (IL)3, IL18, interferon- γ and bone morphogenic protein), chemokines and receptors (CXCL6 and XCR1), G protein-coupled receptors (GPBAR1, GPR84, ADORA3), proteins involved in lipid metabolism (APO2 and OLR1), heat shock proteins (HSPA4L and HSPA6), cellular receptors with immune function (CD14, PDCD1LG2 and TNFRSF10D), as well as a number of proteins specifically expressed in antigen-presenting cells (C5orf20, MPEG1 and TREML4).

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Table 1. Effect of vaccinia stimulation on cellular gene expression

Gene name	Gene description	Fold change	P-value ^a	FDR
<i>Cellular genes downregulated upon vaccinia stimulation</i>				
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	0.70	<1.00E-15	<1.00E-15
ARNT2	Aryl-hydrocarbon receptor nuclear translocator 2	0.71	<1.00E-15	<1.00E-15
PDPN	Podoplanin	0.72	<1.00E-15	<1.00E-15
CD14	CD14 molecule	0.73	<1.00E-15	<1.00E-15
S100A8	S100 calcium-binding protein A8	0.73	<1.00E-15	<1.00E-15
TREML4	Triggering receptor expressed on myeloid cells-like 4	0.74	<1.00E-15	<1.00E-15
S100A9	S100 calcium-binding protein A9	0.74	<1.00E-15	<1.00E-15
PDCD1LG2	Programmed cell death 1 ligand 2	0.74	<1.00E-15	<1.00E-15
THBS1	Thrombospondin 1	0.74	<1.00E-15	<1.00E-15
SIRPB2	Signal-regulatory protein beta 2	0.75	<1.00E-15	<1.00E-15
MPEG1	Macrophage expressed 1	0.75	<1.00E-15	<1.00E-15
GPR84	G protein-coupled receptor 84	0.72	<1.00E-15	8.86E-14
C5orf20	TRAF-interacting protein with forkhead-associated domain	0.75	3.42E-14	2.66E-12
IL18	Interleukin 18 (interferon-gamma-inducing factor)	0.71	3.57E-14	2.77E-12
OLR1	Oxidized low-density lipoprotein (lectin-like) receptor 1	0.72	1.42E-11	7.73E-10
HNMT	Histamine N-methyltransferase	0.75	2.89E-11	1.48E-09
HAMP	Hepcidin antimicrobial peptide	0.67	3.73E-11	1.88E-09
C15orf38	Chromosome 15 open reading frame 38	0.75	7.43E-09	2.68E-07
MYEOV	Myeloma overexpressed (in a subset of t(11;14) positive multiple myelomas)	0.72	1.76E-06	4.16E-05
C20orf103	Chromosome 20 open reading frame 103	0.74	3.15E-06	6.96E-05
SYT15	Synaptotagmin XV	0.68	3.79E-06	8.22E-05
TSP50	Serine protease 50, involved in cellular proliferation	0.55	7.23E-06	0.0001
XCR1	Chemokine (C motif) receptor 1	0.75	1.50E-05	0.0003
SEMA3A	Semaphorin 3A, secreted	0.73	4.04E-05	0.0007
SIRPD	Signal-regulatory protein delta	0.55	5.34E-05	0.0009
APOC2	Apolipoprotein C-II	0.73	6.50E-05	0.0011
BMP3	Bone morphogenetic protein 3	0.42	6.69E-05	0.0011
ADORA3	Adenosine A3 receptor	0.62	9.11E-05	0.0014
KCNJ10	Potassium inwardly rectifying channel, subfamily J, member 10	0.74	0.0001	0.0018
NDP	Norrie disease (pseudoglioma)	0.75	0.0002	0.0032
C4BPB	Complement component 4 binding protein, beta	0.66	0.0002	0.0033
GPBAR1	G protein-coupled bile acid receptor 1	0.74	0.0003	0.0043
PTGES	Prostaglandin E synthase	0.69	0.000364055	0.004745659
CHST6	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6	0.52	0.000621449	0.007433113
<i>Cellular genes upregulated upon vaccinia stimulation</i>				
MFAP5	Microfibrillar associated protein 5	0.74	0.000878039	0.009960752
TNFRSF10D	Tumor necrosis factor receptor superfamily 10d, decoy with truncated death domain	1.90	<2.00E-16	<2.00E-14
HIST4H4	Histone cluster 1, H4l	2.31	<2.00E-16	<2.00E-14
HIST1H4H	Histone cluster 1, H4l	3.86	<2.00E-16	<2.00E-14
HIST1H4C	Histone cluster 1, H4l	5.68	<2.00E-16	<2.00E-14
HSPA4L	Heat shock 70 kDa protein 4-like	5.74	<2.00E-16	<2.00E-14
HIST1H4E	Histone cluster 1, H4l	6.91	<2.00E-16	<2.00E-14
HIST1H1D	Histone cluster 1, H1d	9.37	<2.00E-16	<2.00E-14
HIST1H1E	Histone cluster 1, H1e	40.45	<2.00E-16	<2.00E-14
IL3	Interleukin 3 (colony-stimulating factor, multiple)	3.35	2.22E-16	2.36E-14
HIST1H2AM	Histone cluster 1, H2ag	3.77	1.34E-09	5.40E-08
IFNB1	Interferon, beta 1, fibroblast	4.49	1.56E-08	5.29E-07
EPHB3	EPH receptor B3	1.65	3.39E-08	1.09E-06
HIST1H2AE	Histone cluster 1, H2ae	1.85	3.09E-07	8.55E-06
HSPA6	Heat shock 70 kDa protein 7 (HSP70B); heat shock 70 kDa protein 6 (HSP70B')	1.60	2.34E-06	5.34E-05
HIST1H2BG	Histone cluster 1, H2bi	2.46	2.85E-06	6.34E-05
RNF152	Ring finger protein 152	1.82	4.33E-06	9.24E-05
CH25H	Cholesterol 25-hydroxylase	2.66	6.58E-06	0.000137
HIST2H2AC	Histone cluster 2, H2ac	1.90	3.11E-05	0.000545
WFIKN1	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1	1.52	0.000187	0.002686
LVRN	Laeverin, aminopeptidase Q	2.12	0.000247	0.003421
HIST1H2BN	Histone cluster 1, H2bn	1.81	0.000419	0.005343
IFNG	Interferon, gamma	2.57	0.00069	0.008117

Abbreviation: FDR, false discovery rate. ^aCorrected *P*-value.

Interaction (high responders vs low responders) assessment of gene expression

Our study subjects were individuals from a large cohort, who had the highest and lowest neutralizing Ab titers following smallpox vaccination, allowing us to compare high responder gene

expression patterns following viral stimulation with the expression patterns in low responders. Given the large number of genes analyzed, we set a *P*-value cutoff of 5×10^{-5} and a false discovery rate cutoff value of 0.05. After applying these thresholds, three genes remained (see Table 2): *KIR2DL3* (a killer cell

Table 2. Differential effect of vaccinia stimulation on high and low responders

Gene name	Gene description	FC high responders	FC low responders	P-value	FDR
<i>KIR2DL3</i>	Killer cell immunoglobulin-like receptor, long cytoplasmic tail	0.88	1.2	1.55E-12	3.76E-09
<i>TPSD1</i>	Tryptase, delta 1	1.15	0.81	1.68E-11	3.49E-08
<i>UNC13A</i>	Unc-13 homolog A	0.83	0.62	2.96E-05	0.05

Abbreviation: FDR, false discovery rate.

Table 3. Pathways enriched in differentially expressed genes upon viral stimulation

	P-value
<i>Ingenuity pathway</i>	
TREM1 signaling	2.43×10^{-11}
Dendritic cell maturation	6.17×10^{-9}
NRF2-mediated oxidative stress response	3.48×10^{-8}
Role of PRR in recognition of bacteria and viruses	3.67×10^{-8}
FcγR-mediated phagocytosis in macrophages and monocytes	1.04×10^{-7}
<i>Metacore</i>	
Immune response—alternative complement pathway	1.03×10^{-7}
Immune response—classical complement pathway	2.08×10^{-7}
Immune response—lectin-induced complement pathway	1.79×10^{-6}
Immune response—FcγR-mediated phagocytosis in macrophages	7.82×10^{-5}
Apoptosis and survival—inhibition of ROS-induced apoptosis by 17β-estradiol	2.01×10^{-4}
Cell adhesion—chemokines and adhesion	2.06×10^{-4}
Immune response—TREM1 signaling pathway	2.98×10^{-4}
Inhibitory action of Lipoxins on superoxide production in neutrophils	4.83×10^{-4}
Immune response—inhibitory action of lipoxins on superoxide production induced by IL-8 and Leukotriene B4 in neutrophils	4.83×10^{-4}
Cell adhesion—ECM remodeling	5.37×10^{-4}
Development—EPO-induced MAPK pathway	7.92×10^{-4}
Immune response—CCR3 signaling in eosinophils	9.21×10^{-4}
Chemotaxis—Lipoxin inhibitory action on fMLP-induced neutrophil chemotaxis	1.07×10^{-3}
Immune response—HMGB1/RAGE signaling pathway	1.16×10^{-3}

Abbreviations: CCR3, chemokine (C-C motif) receptor 3; ECM, extracellular matrix; EPO, erythropoietin; fMLP, formyl-Methionyl-Leucyl-Phenylalanine; HMGB1, high mobility group box 1 protein; IL-8, interleukin 8; MAPK, mitogen-activated protein kinase; NRF2, nuclear factor-erythroid 2; PRR, pattern recognition receptor; TREM1, triggering receptor expressed on myeloid 1.

immunoglobulin-like receptor), *TPSD1* (a serine protease expressed in mast cells) and *UNC13A* (a phorbol ester receptor).

Pathway analysis

Recognizing that both infection and immune responses are the result of a highly complex, ordered series of events, and that contributions of individual genes may be quite small, we conducted both Metacore- and Ingenuity-based pathway analyses of host gene expression, using the top 1000 genes with lowest *P*-values (all genes were $P < 0.05$; see Supplementary Table S1). Our analyses indicated that 15 Metacore pathways and five Ingenuity pathways were significantly enriched for differentially expressed genes upon viral stimulation (Table 3). These pathways involved innate pattern recognition receptors, Fc receptor-mediated phagocytosis, dendritic cell maturation and oxidative stress responses. When comparing the high and low responders, we included the 593 genes with $P < 0.05$. The Ingenuity enrichment analysis indicated that five pathways (Table 4) were differentially activated by these two immune response groups.

Gene set analysis

Gene set analysis was also conducted using the transcriptional gene modules described by Banchereau *et al.*¹⁷ Almost two-thirds of the transcriptional modules examined (164/260) exhibited

significantly different ($P < 0.05$) expression patterns upon viral infection. In contrast, only a single transcriptional module, M7.35 (Table 5), varied between high and low Ab responders. Gene set analysis using a subset of the BROAD gene sets (those that included the keyword 'immune' in their title/functional description) indicated that 200 of the 234 immunology-related gene sets were differentially activated upon infection ($P < 0.05$), whereas gene set 132 (Innate_Immune_Response) was the only gene set with significant differences when comparing high and low Ab responders.

Viral gene expression

mRNA-Seq analysis provides both host and viral gene expression data, and allowed us to investigate host-pathogen interaction at the gene expression level. Figure 1 illustrates the average read count across all samples (infected read count – uninfected read counts) for each of the ~250 open reading frames of VACV. Reads were mapped to the ACAM2000 sequence (Genbank: AY313848.1). We detected gene expression of each of the putative viral genes across a wide range of expression levels. Mean viral read counts ranged from 71 for the A38L semaphorin gene to 10740 for the H5R transcription factor. Closer examination of the data found that genes with the lowest expression values were predominantly expressed late in the viral life cycle, whereas genes classified as 'early' were expressed at significantly higher levels

(Figure 2). VACV possesses a number of immunomodulatory proteins, almost all of which were detected in our samples. Table 6 illustrates the expression levels of these viral genes. Interestingly, there were no significant differences between viral gene expression in samples from high and low Ab responders.

DISCUSSION

The transcriptional profiles of PBMCs from individuals vaccinated with the Dryvax smallpox vaccine were assessed after stimulation with VACV for 8 h. Our primary interest was to examine the early transcriptomic events that occur during innate responses to VACV in a mixed cell population. As the subjects tested were taken from

those with the two extremes of the humoral immune response after smallpox vaccination, we also compared gene expression patterns in individuals with high and low vaccinia-specific neutralizing Ab responses. Transcriptomic analysis of the effect of vaccinia infection on host cells has previously been reported primarily in immortalized cell lines or single cell subsets, and has typically utilized microarray technology. Here we report the use of mixed cell population PBMCs and next-generation sequencing technology to assess global gene expression changes (both cellular and viral).

Individual genes—stimulated/unstimulated

Previous reports of host gene expression after vaccinia infection indicate a generalized downregulation of gene expression with a few select genes being upregulated. Rubins *et al.*¹⁸ used microarrays to compare the effect of vaccinia and monkeypox infection on gene expression patterns in macrophages, fibroblasts and HeLa cells, and identified clusters of genes involved in innate immunity that were downregulated upon infection with VACV. Of the genes that they identified, in our experimental system only *CXCL3* and *STAT1* were downregulated, whereas the remaining genes exhibited insignificant changes in expression pattern. This is likely due to the different cell types examined. Rubins *et al.*¹⁸ reported that their three cell types responded to viral infection with distinct differences in the gene expression pattern. Moss and colleagues¹⁹ used RNA sequencing to simultaneously analyze host and viral gene expression patterns after infection of HeLa cells with VACV. Four hours post infection, they reported that 50–75% of host genes were decreased, while relatively few genes were overexpressed. They also indicated that expression of genes involved in nuclear factor- κ B signaling, apoptosis, signal transduction and other ligand-mediated signaling pathways was significantly altered. These results match our findings in a mixed cell population (Table 1), indicating that general features of vaccinia infection may be shared across cell type. Interestingly, a number of histone genes exhibited strong (2- to 40-fold) upregulation upon vaccinia infection. This is similar to the results obtained

Table 4. Pathways enriched in differentially expressed genes comparing high/low responders

Ingenuity pathway	P-value
Nitrogen metabolism	0.002
Mitochondrial dysfunction	0.002
Dopamine receptor signaling	0.003
eIF2 signaling	0.004
Corticotropin releasing hormone signaling	0.008
Biosynthesis of steroids	0.018
Aldosterone signaling in epithelial cells	0.020
Lysine biosynthesis	0.021
Endothelin-1 signaling	0.028
Starch and sucrose metabolism	0.030
Systemic lupus erythematosus signaling	0.030
T-helper cell differentiation	0.032
Signaling by Rho family GTPases	0.036
LXR/RXR activation	0.037
Sonic Hedgehog signaling	0.038
Melatonin signaling	0.038
Regulation of eIF4 and p70S6K signaling	0.040

Abbreviations: eIF2, eukaryotic initiation factor 2; eIF4, eukaryotic initiation factor 4; LXR, liver X receptor; RXR, retinoid X receptor.

Table 5. Transcriptional module M7.35

Symbol	Description
ANKRD22	<i>Homo sapiens</i> ANKRD22, mRNA
CCNA1	<i>Homo sapiens</i> cyclin A1 (CCNA1), mRNA
CD163	<i>Homo sapiens</i> CD163 molecule (CD163), transcript variant 1, mRNA.
CD177	<i>Homo sapiens</i> CD177 molecule (CD177), mRNA.
CLEC5A	<i>Homo sapiens</i> C-type lectin domain family 5, member A (CLEC5A), mRNA.
CREB5	<i>Homo sapiens</i> cAMP responsive element binding protein 5 (CREB5), transcript variant 4, mRNA.
DAAM2	<i>Homo sapiens</i> dishevelled associated activator of morphogenesis 2 (DAAM2), mRNA.
ECHDC3	<i>Homo sapiens</i> enoyl Coenzyme A hydratase domain containing 3 (ECHDC3), mRNA.
GPR84	<i>Homo sapiens</i> G protein-coupled receptor 84 (GPR84), mRNA.
IL1R1	<i>Homo sapiens</i> interleukin 1 receptor, type I (IL1R1), mRNA.
KIAA1026	<i>Homo sapiens</i> kazrin (KIAA1026), transcript variant B, mRNA.
LOC400793	PREDICTED: <i>Homo sapiens</i> hypothetical LOC400793, transcript variant 2 (LOC400793), mRNA.
LOC401233	<i>Homo sapiens</i> similar to HIV TAT specific factor 1; cofactor required for Tat activation of HIV-1 transcription (LOC401233), mRNA.
LOC651612	PREDICTED: <i>Homo sapiens</i> hypothetical protein LOC651612 (LOC651612), mRNA.
METTL7B	<i>Homo sapiens</i> methyltransferase like 7B (METTL7B), mRNA.
MYO10	<i>Homo sapiens</i> myosin X (MYO10), mRNA.
OLAH	<i>Homo sapiens</i> oleoyl-ACP hydrolase (OLAH), transcript variant 1, mRNA.
ORM1	<i>Homo sapiens</i> orosomucoid 1 (ORM1), mRNA.
ORM2	<i>Homo sapiens</i> orosomucoid 2 (ORM2), mRNA.
PCOLCE2	<i>Homo sapiens</i> procollagen C-endopeptidase enhancer 2 (PCOLCE2), mRNA.
PFKFB2	<i>Homo sapiens</i> 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2), transcript variant 2, mRNA.
SLC1A3	<i>Homo sapiens</i> solute carrier family 1 (glial high affinity glutamate transporter), member 3 (SLC1A3), mRNA.
SLC2A11	<i>Homo sapiens</i> solute carrier family 2 (facilitated glucose transporter), member 11 (SLC2A11), transcript variant 3, mRNA.
SYN2	<i>Homo sapiens</i> synapsin II (SYN2), transcript variant IIa, mRNA.
TDRD9	<i>Homo sapiens</i> tudor domain containing 9 (TDRD9), mRNA.
TLR2	<i>Homo sapiens</i> Toll-like receptor 2 (TLR2), mRNA.
ZDHHC19	<i>Homo sapiens</i> zinc finger, DHHC-type containing 19 (ZDHHC19), mRNA.

Figure 1. Gene expression pattern across vaccinia genome.

Figure 2. Expression of vaccinia early and late genes.

in published reports using monkeypox and rabbitpox.^{20,21} The authors speculate that cellular histone proteins may have a function in the organization and compaction of the viral genome.²⁰ Our data support these earlier results; VACV elicits a similar effect as the other poxviruses. At this point, it is too early to determine whether this effect is the result of an antiviral host response or is necessary for the poxvirus life cycle.

Several groups have shown that monocytes are particularly susceptible to vaccinia infection.^{22,23} In our hands, flow cytometry analysis of PBMCs infected with VACV for 8 h indicate that the vast majority (>85%) of monocytes are infected (data not shown), and our mRNA-Seq results indicated that a large number of monocyte/macrophage-related genes (*CD14*, *MPEG1* and *PDCD1LG2*) have significantly altered expression. The *CD14* gene produces a surface antigen expressed on macrophages (and to a lesser extent by some granulocytes and dendritic cells),²⁴ which recognizes lipopolysaccharide,²⁵ leading to activation of nuclear factor- κ B, cytokine secretion and initiation of inflammatory responses. *MPEG1* encodes for a macrophage-specific protein with limited homology to perforin.²⁶ *PDCD1LG2* encoding for the programmed cell death 1 ligand 2 protein (a costimulatory molecule essential for T-cell proliferation and IFN γ production) was downregulated by vaccinia infection. Importantly, the *IL18* gene expression was also downregulated upon infection. This cytokine has been shown to have a critical function in cellular responses to poxvirus infection,²⁷ and we have previously reported that single-nucleotide polymorphisms in both *IL18* and *IL18R* genes are

associated with variations in immune response following smallpox vaccination.²⁸

Individual genes—interaction

In the interaction analysis (differential effect of vaccinia stimulation in high and low responders), the *KIR2DL3* gene was expressed at a significantly lower level in high responders and was downregulated upon vaccinia stimulation; in contrast, the low responders had higher baseline levels that increased upon viral stimulation. *KIR2DL3* is a killer cell Ig-like receptor with two immunoglobulin domains and a longer cytoplasmic tail containing the immune tyrosine-based inhibitory motif that inhibits natural killer cell lysis of target cells expressing human leukocyte antigen-C alleles. *KIR2DL3* has been linked to the resolution of hepatitis C virus infection, and this effect requires expression of both the natural killer receptor and its human leukocyte antigen-C1 ligand. It is possible that downregulation of this natural killer receptor in high responders allows for increased killing of vaccinia-infected cells; however, a cause and effect relationship, if one exists, between high responder status and *KIR2DL3* expression is unclear and will require additional study. The second gene of interest, *TPSD1*, encodes for a mast cell serine protease. *TPSD1* was expressed at similar levels in both high and low responders, but vaccinia stimulation resulted in increased expression in high responders and the opposite effect in low responders. *TPSD1* contains a premature stop codon that leads to the loss of the carboxy-terminal regions necessary for optimal catalytic activity, but has been implicated in autoimmune pathology.^{29,30} Although *TPSD1* is largely inactive, an increase in gene expression may serve as an indicator of mast cell activity. Elevated levels may be due to mast cell recognition of vaccinia through IgE, or may merely be an indirect effect of other immune recognition pathways. The third significant gene, *UNC13A*, is a phorbol ester receptor similar to protein kinase C that is integral to synaptic vesicle priming.³¹ *UNC13A* expression levels were lower to begin with in high responders and decreased 20% upon viral stimulation, whereas in low responders background levels were high to begin with and exhibited a dramatic decrease (~45%) upon stimulation ($P = 2.96 \times 10^{-5}$). *UNC13A* has also been shown to interact with Rab37 and control tumor necrosis factor- α secretion in activated macrophages;³² however, tumor necrosis factor- α mRNA expression did not differ between the high and low responder groups. The high Ab responders secreted greater amounts of tumor necrosis factor- α (224.7 pg ml⁻¹ vs 150.8 pg ml⁻¹ in low

Table 6. Immunomodulatory gene expression by vaccinia virus

ACAM name	COP name	Function	Median (IQR)	Median
			No. of reads stimulated	No. of reads unstimulated
001	C23L, B29R	Chemokine binding protein	1006 (485–1546)	1
002	C22L, B28R	TNF α R	166 (103–223)	0
003		TNF α R	231 (122–305)	0
013	C12L	Serpin 1,2,3	3640 (2026–5,187)	7
014	C11R	EGF	1254 (577–1973)	2
015	C10L	IL1R antagonist	1244 (672–1886)	2
018		Apoptosis, host defense mod	92 (45–145)	0
019		IL18 binding prot	2147 (1158–3,661)	4
031	C4L	IL1R antagonist	502 (241–706)	1
032	C4L	IL1R antagonist	666 (304–882)	1
033	C4L	IL1R antagonist	155 (66–210)	0
037	N1L	NF- κ B inh./virokine	3230 (1684–4,651)	4
042	M2L	NF- κ B Inh	4554 (3176–7,382)	8
045	K3L	IFN resist, PKR inh	335 (215–785)	1
049	K7R	Inh IFN β signal	572 (238–750)	1
069	E3L	IFN resist, PKR inh, dsRNA binding	9325 (5180–15,798)	16
183	A46R	IL-1 signal inhibitor TLR-like	4233 (2232–6,450)	8
189	A52R	TLR/IL-1 signal inhibitor	486 (243–666)	1
201	B8R	IFN γ receptor	10,664 (6994–14,488)	16
206	B13R	SPI-2, crmA, IL-1 convertase,	4110 (2351–5,452)	5
207	B14R	SPI-2, crmA, IL-1 convertase	7536 (4242–10,295)	9
212	B19R	Secreted IFN α/β receptor	7452 (4629–10,948)	13
224		IL18 binding	1235 (700–2270)	2
229	C12L	SPI-1	4506 (2566–6,306)	8
239		TNF α R like	210 (140–310)	0
240		TNF α R like	187 (110–223)	0
034	C3L	Complement binding	72 (46–134)	0
044	K2L	Serpin, SPI-3, host defense modulator	263 (167–513)	0
190	A53R	TNFR Crm C	538 (279–789)	1
209	B16R	IL-1 β receptor	73 (42–125)	0

Abbreviations: ACAM, ACAM2000 strain; COP, Copenhagen strain; Crm c, cytokine response modifiers; EGF, epidermal growth factor; IFN, interferon; IL, interleukin; IQR, interquartile range; NF- κ B, nuclear factor- κ B; PKR inh., protein kinase R inhibitor; SPI, *Salmonella* pathogenicity island 1; TLR, Toll-like receptor; TNF α R, tumor necrosis factor- α receptor; TNFR, TNF receptor.

responders), although the difference did not reach statistical significance ($P = 0.14$).

Pathway analysis

Our pathway analyses indicated several important immune recognition pathways exhibiting differential activation upon viral stimulation, including complement, pattern recognition receptor, dendritic cell maturation pathways, as well as oxidative stress response, TREM1 triggering receptor expressed on myeloid cells 1 signaling and Fc γ R-mediated phagocytosis. In spite of high-level transcription of immunomodulatory genes, some innate immune recognition pathways are still activated in response to vaccinia infection, while others are suppressed. These results further illustrate the dynamic tension that exists between a host and a pathogen.

TREM1 encodes for an immunoglobulin superfamily receptor expressed on monocytes and neutrophils, and is upregulated in response to pathogen-associated molecular patterns resulting in monocyte activation, secretion of IL-8, tumor necrosis factor- α and monocyte chemotactic protein-1, and upregulation of adhesion and costimulatory molecules (ITGB1 and CD40).³³ Flow analysis of PBMCs from these subjects infected with VACV revealed a strong propensity for VACV to infect CD14⁺ monocytes (data not shown). Our transcriptomic data indicate that *TREM1* and multiple downstream components of its signaling pathways are decreased upon viral infection, which might impair the immune function of infected monocytes and may lead to a decreased ability to prime adaptive immune responses.

Gene set analysis

Our gene set analysis comparing uninfected and infected samples indicated that a large number of transcriptional modules were differentially expressed. These modules were identified from subjects with a variety of immunologic conditions and, hence, it is not surprising that many of the same modules would be affected by a viral infection. With the exception of module M7.35, we did not see significant differences between subjects with robust or weak Ab responses to smallpox vaccine. A potential functional role for this module has not yet been determined; however, several genes within the module are integral to innate and inflammatory responses (*IL1R1*, *TLR2*). Other genes include (1) the monocyte marker *CD163*, (2) *CLEC5A*, a C-type lectin that serves as a macrophage recognition receptor for dengue virus stimulating pro-inflammatory responses,³⁴ and (3) the ORM1 and ORM2 proteins involved in transport of lipophilic compounds in the blood.³⁵ The ORM proteins are also thought to regulate immune function during acute phase responses.³⁶ Further examination of the contributions of these genetic elements in host responses to vaccinia infection is warranted and may provide additional insights into host–pathogen interactions. The Broad gene set M3064 (annotated by GO:0045087), containing 23 innate immune response genes, was the only gene set with significantly different expression when comparing high and low Ab responders. This gene set includes several defensins (*DEFB1*, *DEFB118*, *DEFB127*), *IL12A* and *IL12B*, as well as receptors involved in natural killer cell activity (*CD1D*, *CRTAM* and *NCR1*). It is possible that high Ab responders have more innate responses that more readily recognize and react to VACV, and that this increased innate

activity promotes stronger adaptive responses culminating in higher vaccinia-neutralizing Ab titer.

Viral gene expression analysis

Examination of the expression levels of viral genes indicated robust viral gene expression, predominantly in early genes (Figure 2). We saw a striking similarity of viral gene expression between individuals with high and low immune response to the smallpox vaccine. One possible explanation may be the experimental setup in which subjects' PBMCs were isolated, frozen, thawed and placed in tissue culture with growth medium for an 8-h *in vitro* infection. These conditions abrogate immediate binding by virus-specific serum Ab and may not allow sufficient time for the differential cellular immune reactivity between these two groups to alter the initial and early rounds of viral replication.

Assarsson *et al.*³⁷ used a genome-tiling array to measure expression kinetics of 223 vaccinia genes after infection of HeLa cells and found that a majority of the viral genes were detected at the 8-h time point. They found several genes were not expressed at any of their studied time points, however those genes were expressed in our experiments (mean read counts are as follows: WR092 = 41 reads; WR097 = 766 reads; WR145 = 378 reads; WR162 = 141 reads; WR206 = 5022; see Figure 1). Our data indicates that each of the 241 ACAM2000 open reading frames were expressed in the stimulated samples, and for some viral genes we identified relatively high expression levels (5000–10 000 read counts), indicating that strain and, more likely, cell-specific differences can dramatically affect viral gene expression and care must be taken when comparing expression data across studies.

A limitation of this study is the possible dilution of observable effects, given that each individual cell type may respond differently to vaccinia infection. On the other hand, this system more closely matches the *in vivo* environment during infection, and allows for the myriad cell–cell interactions that will occur during an infection or vaccination event. These interactions, involving either cell-to-cell contact, or mediated through soluble factors, are likely to alter the local microenvironment and the individual cell response to infection. The response of a mixed T cell:macrophage population may neither reflect the response of a pure T-cell population nor that of a pure macrophage population, but rather will include at least four components: (1) a T-cell response to infection; (2) a macrophage response to infection; (3) a T-cell response to infected macrophages and (4) a macrophage response to infected T cells. However, further studies on purified cell populations, such as monocytes (given our findings with multiple monocyte-specific genes), or alternatively B cells or Th cells (given the neutralizing Ab titer-based subject selection) will yield valuable additional information. Our subjects were selected based on differences in humoral immunity and are likely to have different numbers of vaccinia-specific memory T and B cells. It is possible that interindividual differences in the memory lymphocyte pool contribute to the differences seen in this study. We selected an 8-h time point in order to allow for the development of early innate responses, while minimizing the contribution of the memory T or B cells.

A strength of this study is the combined use of both individual analyses and pathway/gene set analyses along with highly sensitive NGS technology. Taken together, these separate analyses can identify individual components of the immune response and the interaction of multiple signaling components. The gene set-type analyses have the additional benefit of reducing the number of associations to help offset false discovery.

Previous reports of gene expression in the context of vaccinia infection have focused on established cell lines or on primary cultures of single cell types. Our results indicate that numerous innate genes and pathways are activated upon vaccinia infection of a complex mixture of PBMCs. A number of chemokines,

cytokines, interferons and macrophage-associated genes exhibited significant downregulation upon infection. Upregulated genes included histones, IFN β , IFN γ and heat-shock proteins. Our data also indicate that notable differences in gene expression between high and low responders to the smallpox vaccine exist. It is possible that these differences are the result of divergent immunoregulatory processes in high and low responders. Further investigation of the effect of these loci on immune responses to viral vaccines may lead to important findings regarding genetic control of immune responses and the ability to use such information in engineering new vaccine candidates.^{10,12,15}

MATERIALS AND METHODS

Subject recruitment

Details regarding the cohort from which we selected subjects for use in this study have been previously published.^{28,38–40} Briefly, we selected 44 subjects from a cohort of 1076 recipients of Dryvax (Wyeth Laboratories, Marietta, PA, USA). All subjects had been vaccinated 1–48 months before enrollment, were generally healthy, had received no more than one dose of the smallpox vaccine and were successfully immunized as evidenced by the characteristic vaccine 'take.' Subjects were enrolled at the Mayo Clinic (Rochester, MN, USA) and at the Naval Health Research Center (San Diego, CA, USA). Institutional Review Board approval from both centers (Mayo Clinic and Naval Health Research Center) was obtained before subject enrollment, and informed consent was obtained in writing from all subjects. We selected subjects from among those individuals with the highest ($n=21$) and those with the lowest ($n=23$) vaccinia-specific neutralizing Ab titers.

Viruses and cell lines

The NYCBOH strain of VACV was purchased from ATCC (Manassas, VA, USA), whereas the vSC56 strain of VACV was graciously provided by B Moss (NIAID, Bethesda, MD, USA). All virus strains were grown and titered according to established protocols. HeLa, HeLa S3 and Vero cells were also obtained from ATCC.

VACV neutralization assay

VACV-specific, neutralizing Ab titers from each subject's serum sample were obtained using a high-throughput neutralization assay developed at the Food and Drugs Administration and further optimized in our lab as previously described.^{38,41}

Cell cultures, RNA extraction, mRNA-Seq

PBMCs were stimulated for 8 h with or without live VACV NYCBOH at amultiplicity of infection of 5. Following the incubation, RNAprotect reagent (Qiagen, Valencia, CA, USA) was added to each culture and total RNA was extracted by RNeasy Plus mini Kit (Qiagen). The quantity and quality of each RNA sample were determined by Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA) and by an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cDNA libraries were created using the mRNA-Seq 8 Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's directions. Sample preparation was performed in the Advanced Genomics Technology Center, Gene Sequencing core facility at the Mayo Clinic. Poly-A RNA was isolated using two rounds of magnetic purification with oligo-dT-coated beads. The purified poly-A RNA was fragmented and reverse transcribed into double-stranded cDNA fragments, which were attached to Illumina adaptor sequences. Library validation and quantification was carried out using DNA 1000 Nano Chip kits on an Agilent 2100 Bioanalyzer (Agilent). cDNA libraries were loaded onto individual channels of each flow cell at a concentration of 5–7 pM. Single-end read sequencing was performed using the Genome Analyzer GAIIX (Illumina), with Illumina's Single Read Cluster Generation kit (v2) and 50 Cycle Illumina Sequencing Kit (v3). Flow cells were then analyzed with SCS v2.01 and v2.4. Image processing utilized the Illumina Pipeline Software v1.5 and FireCrest, bustard, ELAND and CASAVA.⁴² Viral gene expression was determined by mapping sequencing reads to the VACV ACAM2000 (GenBank: AY313847.1) reference genome using Bowtie.

Statistical modeling and analyses

Randomized block principles were utilized to allocate specimens to flow cell and lane. Specifically, samples were randomly allocated to library preparation batch, flow cell and lane, with the constraints that: (1) high and low responders were evenly balanced across flow cell and lane assignment and (2) all samples for a given subject were assayed on the same flow cell.

Per-gene tests of statistical significance used generalized linear models⁴³ assuming a negative binomial distribution.⁴² An offset of the 75th percentile⁴⁴ was included as a normalization factor. Predictor variables were response status, stimulation status and the interaction of these two variables. The dispersion was allowed to vary across genes and was estimated via edgeR in an empirical Bayes-like manner, sharing variance information across genes (moderated dispersion) with the prior parameter set to 3.^{45,46} Because of the natural correlation between unstimulated and stimulated specimens within a subject, generalized estimating equations⁴⁷ with an exchangeable correlation structure were utilized to properly estimate the variance. Genes with counts of at least 10 per response $\times$ stimulation combination or total counts of least 440 (that is, an average of five counts per single lane) were analyzed; those with fewer counts were filtered out after calculating the normalization factor. False discovery rates were computed by using standard methods.^{48,49} Self-contained gene set tests⁵⁰ were conducted using the Fisher's method^{51,52} with restandardization.⁵³ Gene sets were defined using immunologically based transcriptional modules.⁵⁴ Both R⁵⁵ and SAS⁵⁶ computing packages were utilized.

CONFLICT OF INTEREST

Dr Poland is the chair of a Safety Evaluation Committee for investigation vaccine trials being conducted by Merck Research Laboratories. Dr Poland offers consultative advice to Merck & Co. Inc, CSL Biotherapies, Avianax, Sanofi Pasteur, Dynavax, Novartis Vaccines and Therapeutics, and PAXVAX Inc. These activities have been reviewed by the Mayo Clinic Conflict of Interest Review Board and are conducted in compliance with Mayo Clinic Conflict of Interest policies. The content is the sole responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health. The remaining authors declare no conflict of interest.

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