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 vaccinomics11–15 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, MEG1 and TREML4).
Table 1. Effect of vaccinia stimulation on cellular gene expression

| Gene name | Gene description | Fold change | P-value<sup>a</sup> | FDR |
|-----------|------------------|-------------|----------------------|-----|
| **Cellular genes downregulated upon vaccinia stimulation** | | | | |
| 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 |
| PDG | 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 |
| TREM4 | Triggering receptor expressed on myeloid cells-like 4 | 0.74 | <1.00E−15 | <1.00E−15 |
| S100A10 | 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 |
| GPRB4 | G protein-coupled receptor 84 | 0.72 | <1.00E−15 | 8.86E−14 |
| CSF2R | TRAF-interacting protein with forkhead-associated domain | 0.73 | 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 | Heparin antimicrobial peptide | 0.67 | 3.73E−11 | 1.88E−09 |
| C1orf38 | Chromosome 1 open reading frame 38 | 0.75 | 7.43E−09 | 2.68E−07 |
| MYEOV | Myeloma overexpressed (in a subset of t11;14) positive multiple myelomas | 0.72 | 1.76E−06 | 4.16E−05 |
| C2orf103 | Chromosome 2 open reading frame 103 | 0.74 | 3.15E−06 | 6.96E−05 |
| SYT15 | Synaptotagmin XV | 0.68 | 3.79E−06 | 8.22E−05 |
| TRIM50 | Serine protease 50, involved in cellular proliferation | 0.55 | 7.23E−06 | 3.0E−05 |
| XCR1 | Chemokine (C motif) receptor 1 | 0.75 | 1.50E−05 | 0.003 |
| 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 |
| GBP1 | 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-acetylgalactosamine 6-O) sulfotransferase 6 | 0.52 | 0.000621449 | 0.007433113 |

| **Cellular genes upregulated upon vaccinia stimulation** | | | | |
| MFAP5 | Microfilibrar associated protein 5 | 0.74 | 0.000878039 | 0.00996752 |
| TNFRSF10D | Tumor necrosis factor receptor superfamily 10d, death domain | 1.90 | <2.00E−16 | <2.00E−14 |
| HIST4H4 | Histone cluster 1, H4i | 2.31 | <2.00E−16 | <2.00E−14 |
| HIST4H4H | Histone cluster 1, H4i | 3.86 | <2.00E−16 | <2.00E−14 |
| HIST4H4C | Histone cluster 1, H4i | 5.68 | <2.00E−16 | <2.00E−14 |
| HSPA4L | Heat shock 70kDa protein 4-like | 5.74 | <2.00E−16 | <2.00E−14 |
| HIST1H4E | Histone cluster 1, H4i | 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 | 3.35 | 2.22E−16 | 2.36E−14 |
| HIST1H2AM | Interleukin 3 cluster, H2ag | 3.77 | 1.34E−09 | 5.40E−08 |
| IFNβ | Interferon, beta 1, fibroblast | 4.49 | 1.56E−08 | 5.29E−07 |
| EPB3 | EPB 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 70kDa protein 6 (HSP70B); heat shock 70kDa protein 6 (HSP70B′) | 1.60 | 2.34E−06 | 5.34E−05 |
| HIST1H2BG | Histone cluster 1, H2bi | 2.46 | 2.85E−06 | 6.34E−05 |
| RNFL52 | 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 |
| WIPKIN | WIPKIN, a functional partner of WIPR | 1.52 | 0.000187 | 0.002686 |
| LVRN | Lateral, 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. *Corrected 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 $5 \times 10^{-5}$ and a false discovery rate cutoff value of 0.05. After applying these thresholds, three genes remained (see Table 2): KIR2DL3 (a killer cell
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 read counts. Table 2 shows that analyses 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. 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 10 740 for the A38L semaphorin gene. 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 10 740 for the A38L semaphorin gene. 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 10 740 for the A38L semaphorin gene.

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 |
|-----------|------------------|--------------------|------------------|---------|-----|
| KIR2DL3   | Killer cell immunoglobulin-like receptor, long cytoplasmic tail | 0.88 | 1.2 | 1.55E - 12 | 3.76E - 09 |
| TPSD1     | Trypsate, delta 1 | 1.15 | 0.81 | 1.68E - 11 | 3.49E - 08 |
| UNC13A    | 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

| Pathway | P-value |
|---------|---------|
| Ingenuity pathway | |
| TREM1 signaling | 2.43 x 10^-11 |
| Dendritic cell maturation | 6.17 x 10^-9 |
| NRF2-mediated oxidative stress response | 3.48 x 10^-8 |
| Role of PRR in recognition of bacteria and viruses | |
| FcR-mediated phagocytosis in macrophages and monocytes | 1.04 x 10^-7 |
| Metacore | |
| Immune response—alternative complement pathway | 1.03 x 10^-7 |
| Immune response—classical complement pathway | 2.08 x 10^-7 |
| Immune response—lectin-induced complement pathway | 1.79 x 10^-6 |
| Immune response—FcR-mediated phagocytosis in macrophages | 7.62 x 10^-5 |
| Apoptosis and survival—inhibition of ROS-induced apoptosis by 17b-estradiol | 2.01 x 10^-4 |
| Cell adhesion—chemokines and adhesion | 2.06 x 10^-4 |
| Immune response—TREM1 signaling pathway | 2.98 x 10^-4 |
| Inhibitory action of Lipoxins on superoxide production in neutrophils | 4.83 x 10^-4 |
| Immune response—inhibitory action of lipoxins on superoxide production induced by IL-8 and Leukotriene B4 in neutrophils | 4.83 x 10^-4 |
| Cell adhesion—ECM remodeling | 5.37 x 10^-4 |
| Development—EPO-induced MAPK pathway | 7.92 x 10^-4 |
| Immune response—CCR3 signaling in eosinophils | 9.21 x 10^-4 |
| Chemotaxis—Lipoxin inhibitory action on fMLP-induced neutrophil chemotaxis | 1.07 x 10^-3 |
| Immune response—HMGB1/RAGE signaling pathway | 1.16 x 10^-3 |

Abbreviations: CCR3, chemokine (C-C motif) receptor 3; ECM, extracellular matrix; EPO, erythropoietin; fMLP, formyl-Methionyl–Leucyl–Phenylalanine; HMBG1, 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.
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 transcriptional responses to VACV in a mixed cell population. As the subjects tested were taken from transcriptomic events that occur during innate responses to VACV and viral gene expression patterns after infection of 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.

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           | Homo sapiens ANKRD22, mRNA                        |
| CCNA1             | Homo sapiens cyclin A1 (CCNA1), mRNA             |
| CD163             | Homo sapiens CD163 molecule (CD163), transcript variant 1, mRNA. |
| CD177             | Homo sapiens CD177 molecule (CD177), mRNA.       |
| CLEC5A            | Homo sapiens C-type lectin domain family 5, member A (CLEC5A), mRNA. |
| CREBS             | Homo sapiens AMP responsive element binding protein 5 (CREBS), transcript variant 4, mRNA. |
| DAAM2             | Homo sapiens dishevelled associated activator of morphogenesis 2 (DAAM2), mRNA. |
| ECHDC3            | Homo sapiens enoyl Coenzyme A hydratase domain containing 3 (ECHDC3), mRNA. |
| GPR84             | Homo sapiens G protein-coupled receptor 84 (GPR84), mRNA. |
| IL1R1             | Homo sapiens interleukin 1 receptor, type I (IL1R1), mRNA. |
| KAAA1026          | Homo sapiens kazrin (KAAA1026), transcript variant 8, mRNA. |
| LOC400793         | PREDICTED: Homo sapiens hypothetical LOC400793, transcript variant 2 (LOC400793), mRNA. |
| LOC401233         | Homo sapiens similar to HIV TAT specific factor 1; cofactor required for Tat activation of HIV-1 transcription (LOC401233), mRNA. |
| LOC651612         | PREDICTED: Homo sapiens hypothetical protein LOC651612 (LOC651612), mRNA. |
| METTL7B           | Homo sapiens methyltransferase like 7B (METTL7B), mRNA. |
| MYO10             | Homo sapiens myosin X (MYO10), mRNA.             |
| OLAH              | Homo sapiens oleoyl-ACP hydrolase (OLAH), transcript variant 1, mRNA. |
| ORM1              | Homo sapiens oromucoid 1 (ORM1), mRNA.           |
| ORM2              | Homo sapiens orosomucoid 2 (ORM2), mRNA.         |
| PCOLCE2           | Homo sapiens procollagen C-endopeptidase enhancer 2 (PCOLCE2), mRNA. |
| PFKFB2            | Homo sapiens 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2), transcript variant 2, mRNA. |
| SLC1A3            | Homo sapiens solute carrier family 1 (glial high affinity glutamate transporter), member 3 (SLC1A3), mRNA. |
| SLC2A11           | Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 11 (SLC2A11), transcript variant 3, mRNA. |
| SYN2              | Homo sapiens synapsin II (SYN2), transcript variant Ia, mRNA. |
| TDRD9             | Homo sapiens tudor domain containing 9 (TDRD9), mRNA. |
| TLR2              | Homo sapiens Toll-like receptor 2 (TLR2), mRNA.  |
| ZDHHC19           | Homo sapiens zinc finger, DHHCC-type containing 19 (ZDHHC19), mRNA. |

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 colleagues9 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-κB 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 with those at 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).
in published reports using monkeypox and rabbitpox. The authors speculate that cellular histone proteins may have a function in the organization and compaction of the viral genome. 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. 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), which recognizes lipopolysaccharide, leading to activation of nuclear factor-κB, cytokine secretion and initiation of inflammatory responses. MPEG1 encodes for a macrophage-specific protein with limited homology to perforin. 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 upon 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, 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.

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. 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. 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 × 10^{-5}). UNC13A has also been shown to interact with Rab37 and control tumor necrosis factor-α secretion in activated macrophages; 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 responders.
Our transcriptomic data indicate that propensity for VACV to infect CD14+ PBMCs from these subjects infected with VACV revealed a strong monocyte chemotactic protein-1, and upregulation of adhesion response to pathogen-associated molecular patterns resulting in expressed on monocytes and neutrophils, and is upregulated in pathogen.

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 FcγR-mediated phagocytosis. In spite of high-level transcription of immunomodulatory genes, some innate immune recognition pathways are still activated in response to vaccinia 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 (ITGβ1 and CD40). 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.

Table 6. Immunomodulatory gene expression by vaccinia virus

| ACAM name | COP name | Function | Median (IQR) | No. of reads stimulated |
|-----------|----------|----------|--------------|------------------------|
| 001       | C23L, B29R | Chemokine binding protein | 1006 (485–1546) | 1 |
| 002       | C22L, B28R | TNFαR | 166 (103–223) | 0 |
| 003       | TNFαR | 231 (122–305) | 0 |
| 013       | C1L | Serpin 1,2,3 | 3640 (2026–5,187) | 7 |
| 014       | C1R | EGF | 1254 (577–1973) | 2 |
| 015       | C10L | IL-1R antagonist | 1244 (672–1886) | 2 |
| 018       | Apoptosis, host defense mod | 92 (45–145) | 0 |
| 019       | IL-18 binding prot | 2147 (1158–3,661) | 4 |
| 031       | C4L | IL-1R antagonist | 502 (241–706) | 1 |
| 032       | C4L | IL-1R antagonist | 666 (304–882) | 1 |
| 033       | C4L | IL-1R antagonist | 155 (66–210) | 0 |
| 037       | NTL | NF-κB in/n/virokine | 3230 (1684–4,651) | 4 |
| 042       | M2L | NF-κB inh | 4554 (3176–7,382) | 8 |
| 045       | K3L | IFN resist, PKR inh | 335 (215–785) | 1 |
| 049       | K7R | Inh IFNβ 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 | IFNy receptor | 10,664 (6994–14,488) | 16 |
| 206       | B13R | SPI-2, crmA, IL-1 convertase, | 4110 (2351–5,450) | 5 |
| 207       | B14R | SPI-2, crmA, IL-1 convertase | 7536 (4242–10,295) | 9 |
| 212       | B19R | Secreted IFNα/β receptor | 7452 (4629–10,948) | 13 |
| 224       | IL-18 binding | 1235 (700–2270) | 2 |
| 229       | C12L | Alpha-1 | 4506 (2566–6,306) | 8 |
| 239       | TNFαR like | 210 (140–310) | 0 |
| 240       | TNFαR 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-1R 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-κB, nuclear factor-kB; PKR inh., protein kinase R inhibitor; SPI, Salmonella pathogenicity island 1; TLR, Toll-like receptor; TNFαR, tumor necrosis factor-α receptor; TNFR, TNF receptor.

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, IL1R2). 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, and (3) the ORM1 and ORM2 proteins involved in transport of lipophilic compounds in the blood. The ORM proteins are also thought to regulate immune function during acute phase responses. 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, CR2AM 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 incubation. 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. 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, is likely to alter the local microenvironment and the individual cell response to infection. The response of a mixed T-cell:macrophage population may not 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 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, IFNβ, IFNγ 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.

MATERIALS AND METHODS

Subject recruitment
Details regarding the cohort from which we selected subjects for use in this study have been previously published. 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, and 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 (NIH/AID, 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.

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 2010 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 at 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 SC5 v2.01 and v2.4. Image processing utilized the Illumina Pipeline Software v1.5 and FireCrest, bastard, ELAND and CASAVA. 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 modeling, assuming a negative binomial distribution. An offset of the 75th percentile 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.n estimates via edgeR in an empirical Bayes-like manner, sharing variance information across genes (moderated dispersion) with the prior.n estimates. Self-contained gene set tests were conducted using the self-contained gene set test package. Both R35 and SAS36 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 PAVVAX 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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