

The host response to smallpox: Analysis of the gene expression program in peripheral blood cells in a nonhuman primate model

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Smallpox has played an unparalleled role in human history and remains a significant potential threat to public health. Despite the historical significance of this disease, we know little about the underlying pathophysiology or the virulence mechanisms of the causative agent, variola virus. To improve our understanding of variola pathogenesis and variola–host interactions, we examined the molecular and cellular features of hemorrhagic smallpox in cynomolgus macaques. We used cDNA microarrays to analyze host gene expression patterns in sequential blood samples from each of 22 infected animals. Variola infection elicited striking and temporally coordinated patterns of gene expression in peripheral blood. Of particular interest were features that appear to represent an IFN response, cell proliferation, immunoglobulin gene expression, viral dose-dependent gene expression patterns, and viral modulation of the host immune response. The virtual absence of a tumor necrosis factor α /NF- κ B-activated transcriptional program in the face of an overwhelming systemic infection suggests that variola gene products may ablate this response. These results provide a detailed picture of the host transcriptional response during smallpox infection, and may help guide the development of diagnostic, therapeutic, and prophylactic strategies.

Smallpox, a calamitous, lethal disease, was endemic in many parts of the world until it was eradicated in 1977 by a systematic vaccination program by the World Health Organization (WHO) (1). Smallpox in nature appears to have been uniquely restricted to humans. The case fatality rate was estimated to be 30% (1, 2), and those who survived often had permanent, disfiguring scars (1, 3). After the eradication of smallpox in 1977, the worldwide vaccination program ceased. Despite the WHO mandate that all variola stocks be confined to two international repositories at the Centers for Disease Control and Prevention (Atlanta, GA) and the State Research Center of Virology and Biotechnology (Novosibirsk, Russia), there are concerns about reemergence of smallpox in modern society through use of smallpox as a bioweapon (2, 4). As a result, WHO and the U.S. National Academy of Sciences have recommended the development of a primate model of smallpox for testing vaccines and therapeutics.

Poxviruses have a large, complex double-stranded DNA genome and replicate in the cytoplasm of cells (5). Members of the poxvirus group display striking degrees of host species coadaptation and can provide insight into host immune defense (6). Poxviruses also display unique abilities to interfere with host innate immune defense mechanisms. At least 16 viral genes are thought to play a role in combating the host immune response (for review, see ref. 7). Although immune modulating genes of other members in the poxvirus family are well studied, little is known about the activity and function of these genes in variola. Other potential virulence mechanisms in variola also remain elusive; only four of the 197 predicted variola-encoded proteins have been purified and characterized (8–11). Although the smallpox genome has been fully

sequenced (11, 12), virtually no modern molecular biology has been applied to the study of live variola virus, because the eradication of smallpox predated modern recombinant DNA methods. Furthermore, until recently, there were no available animal models of variola virus-associated disease. Severe physical and procedural restrictions remain in place regarding any work with this virus within the Biosafety Level 4 laboratory environment. All of the work described below was performed at the Centers for Disease Control and Prevention.

Using serial blood samples from variola-infected cynomolgus macaques, we systematically analyzed the global host transcriptional responses during the course of a disease that closely mimicked human smallpox (13). The dynamic evolution of the gene expression program in response to physiological and pathological stimuli provides a richly detailed and revealing picture of the mechanisms underlying complex biological processes, such as host–pathogen interactions during infectious diseases (14). Peripheral blood is a uniquely accessible tissue for minimally invasive monitoring of physiological responses in living animals. In addition, it provides a window on the interactions of different components of the innate and adaptive immune system. Identification of distinctive gene expression patterns in blood samples after smallpox infection might provide a basis for early detection and diagnosis of poxviral infections. Our results provide a genome-wide investigation of an animal model of poxviral infection, and a genomic view of systemic interactions during smallpox infection.

Materials and Methods

Nonhuman Primate Model of Smallpox. In the first study, eight adult cynomolgus macaques (*Macaca fascicularis*) received either Harper or India 7124 variola strains through a combination of aerosol exposure [5×10^8 plaque-forming units (pfu)] and high-titer i.v. inoculation (10^9 pfu). The purpose of this inoculation scheme was to ensure subsequent productive infection. One of the animals died before sampling and was excluded from the microarray analysis. In the second study, four animals were exposed to the India 7124 strain via i.v. route alone (10^9 pfu). For the third study, viral dose was varied; two animals received 10^9 pfu, and three animals each received 10^8 , 10^7 , and 10^6 pfu of India 7124 through i.v. inoculation. Details of this animal model are described in ref. 13.

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Abbreviations: PBMC, peripheral blood mononuclear cell; TNF- α , tumor necrosis factor α ; pfu, plaque-forming unit(s).

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Sample Acquisition and RNA Preparation. Peripheral blood samples (2.5 ml) were collected from each animal on multiple days before infection to define a robust baseline, and then on successive days after infection, until death or recovery. Peripheral blood mononuclear cells (PBMCs) were isolated from 1.5 ml of peripheral blood by using Histopaque (Sigma), and total RNA was extracted by using TRIzol (Invitrogen). RNA was linearly amplified as described (15) with some modifications (see *Supporting Protocols*, which is published as supporting information on the PNAS web site).

cDNA Microarrays and Hybridization. We used human cDNA microarrays containing 37,632 elements that represent $\approx 18,000$ unique genes. To determine whether these human arrays could efficiently capture monkey transcripts, we performed comparative genomic hybridization with DNA from two different human males and two different male monkeys vs. DNA from human male reference. A total of 99.8% of the array elements for which the fluorescent signal obtained with human genomic DNA was at least 1.4-fold over background also gave a signal of at least 1.4-fold over background for the macaque genomic DNA hybridization.

Arrays were produced as described (16). Fluorescently labeled cDNA prepared from amplified RNA was hybridized to the array in a two-color comparative format (16), with the experimental samples labeled with one fluorophore (Cy-5) and a reference pool of mRNA labeled with a second fluorophore (Cy-3) (17).

Data Filtering and Analysis. Array images were scanned by using an Axon Scanner 4000A (Axon Instruments, Union City, CA), and image analysis was performed by using GENEPIX PRO version 3.0.6.89 (Axon Instruments). Data were expressed as the \log_2 ratio of fluorescence intensities of the sample and the reference, for each element on the array (16). Data were filtered to exclude elements that did not have at least a 2.5-fold intensity/background ratio in at least 80% of the arrays. The time course data from each monkey were normalized to the averaged day-zero data from that animal, and the subset of elements that varied from the baseline by at least 3-fold in at least three samples was selected for further analysis. The data were hierarchically clustered by using the CLUSTER program (18), and displayed by using TREEVIEW (<http://rana.lbl.gov/EisenSoftware.htm>).

Nonhuman Primate Model of Ebola Infection, Sample Acquisition and Preparation, and Data Analysis. Twenty-one adult male cynomolgus macaques were inoculated intramuscularly in the thigh with 1,000 pfu of Ebola-Zaire (19). Animals were killed serially at days 1–6 after infection. PBMC samples were collected and processed exactly as described for the smallpox experiments. The genes representing the tumor necrosis factor α (TNF- α)/NF- κ B regulon, identified in a study of human PBMC responses to lipopolysaccharide (14), were extracted from both the smallpox and Ebola data (for list of genes see *Supporting Text*, which is published as supporting information on the PNAS web site). These data were filtered by selecting genes whose expression varied from the baseline measurements by at least 2.5-fold in at least three samples.

Hematology and Cytokine Production. White blood cell differentials were measured manually on Wright-stained blood smears. Levels of IFN- α , IFN- γ , and TNF- α in monkey sera/plasma were assayed by using commercially available ELISA kits (BioSource International, Camarillo, CA).

Immunohistochemistry. Tissues were immersed in 10% neutral buffered formalin for a minimum of 48 h and, to ensure viral inactivation, were subjected to 2×10^6 rad in a ^{60}Co irradiator. Immunohistochemistry was performed by using previously described methods and formalin-fixed tissues (19). Detection of apoptotic T cells was performed by using a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) as-

say (Apoptag, Intergen, Purchase, NY) in conjunction with a pan T cell marker, CD3 (DAKO).

Dose-Response Model. To model the effects of dose or time on gene expression, let variables T_i , D_i , and Z_i denote, respectively, time after infection ($T_i = 2, 4, 6, 8, 11, \text{ or } 14$ days), \log_{10} inoculum dose ($D_i = 6, 7, 8, \text{ or } 9$), and infection state ($Z_i = 0$ for preinfection and 1 for postinfection measurements). For a given gene let the relationship

$$Y_{ij} = \beta_{j0} + Z_i(\beta_{j1} + \beta_{j2}T_i + \beta_{j3}D_i) + \varepsilon_{ij} \quad [1]$$

represent the expected base 2 logarithm of expression of gene j in experiment i . This model specifies a linear time and dose response, where dose is measured on a logarithmic scale.

Data from the entire third study were filtered to exclude elements that did not have at least a 2.5-fold intensity/background ratio and regression correlation of at least 0.6 in at least 80% of the arrays. We used the method of least squares to fit the function in Eq. 1 to the nonzero-transformed data. The time course data from each monkey were then normalized to the averaged day-zero data from that animal. Genes with a significant ($P < 0.005$) dose effect were selected and filtered for the subset of elements that varied from the baseline by at least 2.5-fold in at least two samples, and hierarchically clustered.

Detailed experimental and data analysis procedures are available at <http://microarray-pubs.stanford.edu/smallpox>.

Results and Discussion

We characterized the host gene expression program in PBMCs of cynomolgus macaques during the response to smallpox. Responses were examined in 22 animals, infected with doses of virus varying over a 10^3 -fold range, and inoculated through various routes. The data set from these experiments comprises ≈ 6.51 million measurements of transcript abundance in individual specimens derived from 129 different blood samples by using 173 DNA microarrays. Fig. 1 provides an overview of the gene expression patterns in PBMC from the seven aerosol plus i.v. animals and the four i.v. animals (Table 1), focusing on the genes with the largest changes in transcript levels during the course of infection.

All animals had severe clinical responses to smallpox infection that closely paralleled the human disease, although the animals showed an accelerated disease course (13). The animals developed fevers of 1–2°F within 3 days, and enanthema and exanthema by day 3, with progression to vesicles and papules in the characteristic centrifugal distribution seen in humans. The total numbers of lesions exceeded 500 in animals surviving beyond day 7. In several of the animals that died by day 6, cutaneous erythema and hemorrhage appeared as early as day 2, with hemorrhagic pleural effusions and visceral and mucosal hemorrhage evident at the time of death (13). Nucleated red blood cell transcripts (Fig. 1 and Fig. 7, which is published as supporting information on the PNAS web site) were present in the circulating blood samples, also reflecting hematologic abnormalities. Immunohistochemical staining revealed viral infection of monocytes and macrophages within circulating blood and tissues, but no infection of lymphocytes (CD3⁺ or CD20⁺ cells). The presence of replicating virus in monocytes and macrophages (as opposed to phagocytosed antigen) was confirmed by electron microscopy and *in situ* hybridization (13).

Our data revealed stereotyped, highly choreographed patterns of gene expression (Fig. 1). The gene expression programs were remarkably similar in all animals, regardless of route of inoculation or viral strain used. Notable characteristics of these patterns included features that appeared to reflect the IFN response, a cell proliferation/cell cycle response, viral modulation of the TNF- α /NF- κ B pathways, Ig gene expression, lymphocyte-specific signatures (B cell/T cell), and features that varied with viral dose.

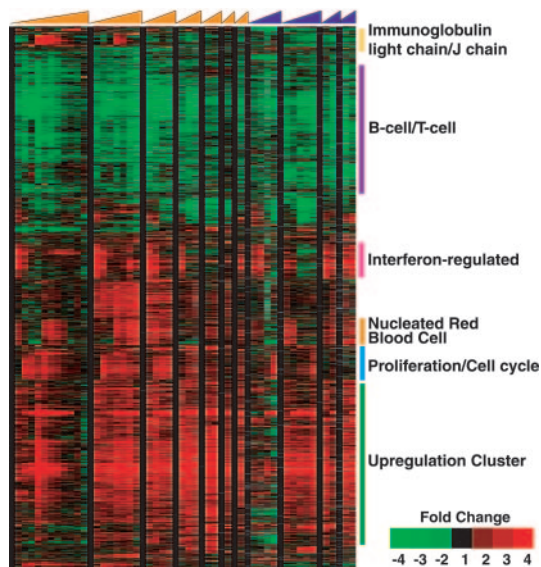


Fig. 1. Overview of gene expression in PBMCs from variola-infected macaques. A total of 2,387 elements displayed ≥ 3 -fold change in mRNA expression for greater than or equal to three different arrays. The data for these 2,387 clones were hierarchically clustered (21). Data from individual elements or genes are represented as a single row, and different time points in the time course (triangles) are shown as columns. Red and green denote expression levels greater or less, respectively, than baseline values (average of two to four samples taken at days -4 and 0 before inoculation). The intensity of the color reflects the magnitude of the change from baseline. Successive samples in the time course are displayed as consecutive columns following the black baseline column (for sample days, see Table 1). The orange triangles represent the time courses of seven animals from the first study (10^9 i.v. + aerosol; India 7124 and Harper strains). The blue triangles represent the time courses of four animals from the second study (10^9 i.v.; India 7124 strain). Animals are arranged from left to right based on their survival time.

IFN Response. The earliest consistent transcriptional response was an increase in transcript abundance by as much as 27-fold for a large cluster of IFN-associated genes, within 24 h after infection (Fig. 2A, average induction, 2.6-fold). This cluster of genes is markedly enriched for known IFN- α -, β -, and γ -regulated genes: 20 of 96 unique genes in the cluster have been shown to be IFN-regulated in two independent data sets (20, 21), as compared to 264 of 13,208 total unique genes on the array, $P < 0.0001$ by hypergeometric distribution. These 20 genes include the double-stranded DNA-activated protein kinase (PKR), signal transducer and activators of transcription (STAT1 and STAT2), myxovirus resistance (MX1 and MX2), chemokine (C-X-C motif) ligand 10 (IP-10), and 2'-5' oligoadenylate synthetases (OAS 1, 2, and 3).

The relative transcript abundance of these IFN-response genes increased markedly during days 1–4, and then tapered off through

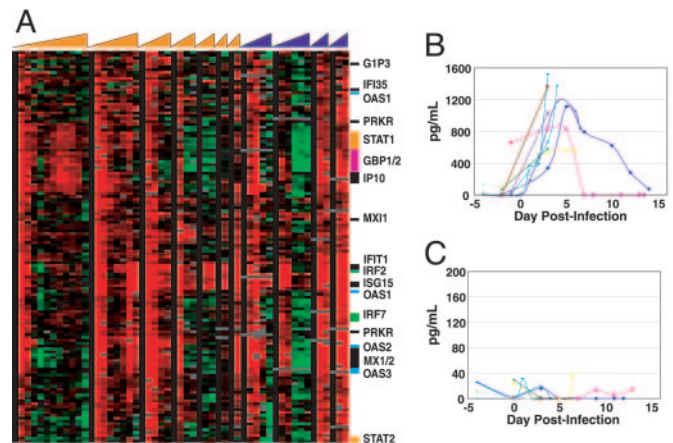


Fig. 2. IFN response. (A) Gene cluster associated with the IFN response. Colored bars indicate rows of data from two or more independent array elements representing the same gene. (B and C) IFN- γ (B) and IFN- α (C) production, as measured by ELISA in serum, for each animal over the time course.

day 6, in most animals. Interestingly, two of the four animals sampled at 24 h after infection had no overt clinical signs, yet at that time the transcript levels of IFN-regulated genes were already highly induced (first and fourth blue triangles, Fig. 2A). The one animal that developed a protracted disease course (second orange triangle, Fig. 2A) displayed a resurgence of IFN-related gene expression at days 11 and 13, immediately before death. The second peak of induction in this animal may have been a response to a surge of viral proliferation and spread; viral titers in circulating blood increased 2.5 logs from day 9 to day 11 (Fig. 8, which is published as supporting information on the PNAS web site).

The course of infection was rapidly fatal in the two animals that had minimal detectable IFN-associated gene induction (Fig. 2A, fifth and sixth orange triangles). These two animals also had the most advanced disease course at day 3 after infection, with macular lesions visible at many locations. It may be that with quickly progressing disease these animals did not have time to mount a successful IFN response. Alternatively, the failure to produce a sufficient early IFN response may have enabled the virus to quickly overwhelm the host.

IFN- γ protein levels rose rapidly, beginning as early as day 1 after infection (Fig. 2B), and peaked at days 3–5, closely paralleling the transcript levels of IFN-responsive genes. However, serum IFN- α levels were low throughout the entire time course (Fig. 2C). The vigorous IFN response is notable in light of variola genes that are predicted to encode IFN-modulating factors. (In variola India 1967 strain, the C3L ORF is predicted to encode an eIF2a homolog that prevents inhibition of translation; the E3L ORF is predicted to encode a double-stranded RNA binding protein that inhibits 2'5'-

Table 1. Inoculation and time course of various infections

Monkey	Tattoo no.	Time points, days after infection	Route of infection	Strain
H-1	C099	0, 3, 5, 7, 9, 10, 12, 14, 17, 20, 35, 42	i.v. + aerosol	Harper
I-4	C713	0, 3, 5, 7, 9, 11, 13	i.v. + aerosol	India 7124
H-4	C881	0, 3, 5, 6	i.v. + aerosol	Harper
I-2	C651	0, 3, 4	i.v. + aerosol	India 7124
I-1	C171	0, 3	i.v. + aerosol	India 7124
H-2	C625	0, 3	i.v. + aerosol	Harper
H-3	C681	0, 3	i.v. + aerosol	Harper
I-7	C437	0, 1, 2, 4, 6	i.v. only	India 7124
I-6	C088	0, 1, 2, 3	i.v. only	India 7124
I-5	C373	0, 1, 2	i.v. only	India 7124
I-8	C956	0, 1, 2	i.v. only	India 7124

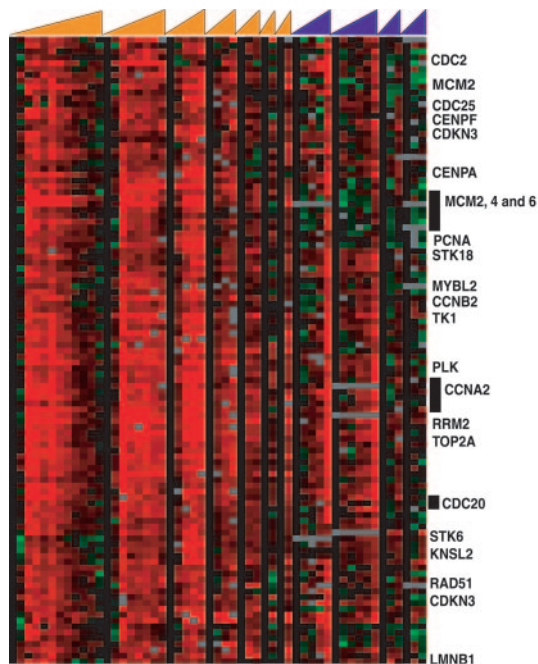


Fig. 3. Proliferation/cell-cycle response. Gene cluster associated with the cell-cycle/proliferation response. Colored bars represent sets of independent measurements of the same gene.

OAS, the RNaseL system, and PKR; and the B20R and B9R ORFs are predicted to encode soluble and cell surface receptors that could act as competitive antagonists for type I and type II interferons, respectively; for review, see ref. 7). B9R has been shown to bind human IFN- γ (8); however, the expression patterns, activities, and roles in pathogenesis of these IFN-modulating genes remain to be established.

Serum IFN- γ levels and transcript levels of IFN-responsive genes increased during the infection, despite the putative variola system for blocking the IFN response and widespread tissue distribution of replicating virus. Although the observed induction of IFN responsive genes was considerable, it is possible that an even greater or more substantial response would have been seen without these viral immunomodulatory proteins. Moreover, the products of the C3L and E3L ORFs are predicted to inhibit the intracellular action of key IFN responsive proteins, rather than preventing their induction. Localized or intracellular action of the variola IFN-modulating proteins might mediate a more favorable environment for the virus in target cells or tissues, notwithstanding the activation of the IFN response in circulating PBMCs.

Cell-Cycle/Proliferation Response. The induction of a large group of genes characteristically expressed during cell proliferation was a prominent feature of the observed gene expression program (Fig. 3). The majority of the genes in the cluster are periodically expressed during the cell cycle and encode proteins involved in progression through the cell cycle, DNA replication, and chromosome segregation. Of the 73 genes in the proliferation cluster, 39 were identified as cell cycle-regulated in a previous study of HeLa cells (22), compared to 646 of the 13,208 total unique genes on the array ($P < 0.0001$ by hypergeometric distribution). This proliferation/cell-cycle signature first appeared at day 3 after infection and was most pronounced at days 5 and 6. This response may reflect lymphocyte activation, because it is highly correlated with numbers of atypical lymphocytes (ATL) in each of the specimens (the average Spearman rank correlation for genes in the cluster is 0.54; 33 of the 73 genes are significantly correlated with ATL counts, $P < 0.001$). Increased abundance of a more rapidly dividing immune cell

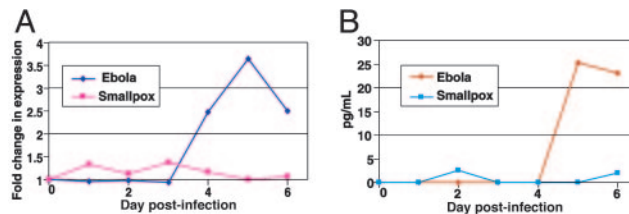


Fig. 4. TNF- α /NF- κ B responsive genes. (A) Average expression of genes associated with a TNF- α /NF- κ B response. The set of genes representing the TNF- α /NF- κ B regulon present in a previously published lipopolysaccharide stimulation data (14) was extracted from both the smallpox and Ebola data sets (K.H.R., unpublished data). (B) TNF- α production, as measured by ELISA in serum for monkeys infected with smallpox (blue line) or Ebola (pink line).

subpopulation could also account for the observed gene expression response.

Some poxviruses encode homologs of the mammalian epidermal growth factor (EGF) (23) that bind ErbB family members (24) and are potent stimulators of cell proliferation (25). The purified variola EGF homolog (D4R) has been shown recently to bind ErbB1 on human epithelial cells (10). However, ErbB family members do not appear to be expressed by blood cells, with the exception of occasional expression of ErbB4 on plasma cells and macrophages (26, 27).

TNF- α /NF- κ B Regulated Genes. We previously found that a major feature of the global gene expression response in PBMCs *in vitro* to Gram-negative or Gram-positive bacteria is a pronounced induction of genes regulated by the transcription factor NF- κ B, including genes associated with NF- κ B autoregulatory mechanisms (14). Comparison of the transcriptional response of PBMCs during variola infection *in vivo* with these previous findings revealed a contrast in the behavior of the NF- κ B- and TNF- α -regulated genes (Fig. 9, which is published as supporting information on the PNAS web site), in that there were only small increases in levels of expression of a few of these genes in the face of an overwhelming and fatal variola infection. To investigate whether the absence of the TNF- α /NF- κ B response might be a specific feature of variola infection, we compared this response to the gene expression program in PBMCs collected during the course of a different fatal systemic viral infection in the same host species, i.e., Ebola virus infection in cynomolgus macaques. Our findings from Ebola-infected macaques are the only other available data on the genome-wide responses of primates in peripheral blood to viral infection (K.H.R., unpublished data). PBMC samples from the Ebola-infected animals were collected and processed in the exact same manner as the smallpox-infected monkeys. Our analysis showed a prominent induction of NF- κ B- and TNF- α -regulated genes during the course of Ebola infection, in contrast to the negligible modulation of expression of NF- κ B- and TNF- α -regulated genes during variola infection (Fig. 4A).

Soluble TNF- α was measured by ELISA in serum samples from the same macaques, and was only detected at low levels at two of the time points in variola-infected animals (Fig. 4B). In contrast, TNF- α protein levels rose to consistently higher levels in the Ebola-infected animals (Fig. 4B).

Modulation of TNF- α pathways is a common theme in studies of viral subversion of the host immune response (28), presumably because TNF- α signaling is so crucial to the antiviral response (29). Poxviruses are known to encode several homologs of the TNF- α receptor (30). CrmB is the only TNF-receptor homolog encoded by variola that is predicted to be functional (12). The absence of a TNF- α response (at the level of gene expression and protein secretion) during smallpox infection, in contrast to the TNF- α response during Ebola virus infection, may represent the first evidence for variola CrmB activity, and for specific disruption of a

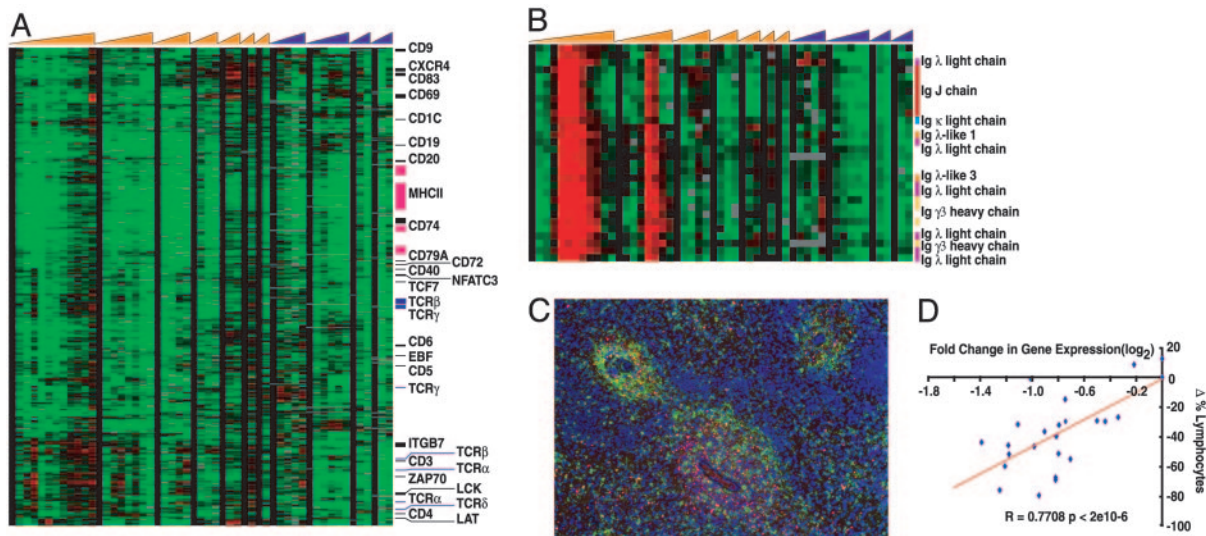


Fig. 5. Ig/Lymphocyte genes. (A) Gene cluster associated with lymphocyte gene expression. (B) Ig gene cluster. (C) T cell (green) depletion and apoptosis (red) in the periarteriolar lymphoid sheath (PALS) of the spleen; blue is 4',6-diamidino-2-phenylindole (DAPI) staining. (D) Correlation ($P < 2 \times 10^{-6}$) between the decrease in percent lymphocytes and fold change in gene expression in the lymphocyte cluster.

host response system by variola virus during *in vivo* smallpox infection. In addition, variola may inhibit other components of the NF- κ B signaling pathway (as was seen with the recent discovery that the vaccinia Western Reserve K1L gene inhibits I κ B α degradation (31).

Lymphocyte and Ig Responses. A set of 328 genes showed a decrease in their relative transcript abundance in PBMC samples following smallpox infection (Fig. 5A). This group of genes is highly enriched in canonical T and B lymphocyte markers, including CD19, CD20, MHCII, CD83, CD69, CD74, and CD79A (B cells); and T cell receptor, CD3, TCF7, CD4, and ZAP70 (T cells). Transcript abundance for genes in this cluster was strongly correlated with the relative abundance of lymphocytes in the corresponding blood sample (Fig. 5D, $P < 0.0001$). The decrease in levels of transcripts during infection therefore most likely reflects a decrease in the relative abundance of lymphocytes, rather than physiological regulation of their transcription.

The relative lymphopenia may be due to loss of T cells in the lymphatic tissues. Fig. 5C shows a considerable depletion of T cells, and a large number of apoptotic cells in the splenic periarterial lymphatic sheath (PALS) compared to the PALS of an uninfected animal (Fig. 10, which is published as supporting information on the PNAS web site). Geisbert *et al.* (19) demonstrated a pronounced loss of lymphocytes early during the course of Ebola virus infections and suggested that this lymphocyte depletion was caused by apoptosis and was responsible, at least in part, for the inability of the host to mount a productive immune response. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) stains performed on tissues from smallpox-infected animals suggest that apoptosis occurs predominantly in lymphocytes; however, occasional apoptotic monocytes/macrophages are also detected (13). It is possible that virus replicates in antigen-presenting cells in the lymphatic tissues, leading either directly or indirectly to lymphocyte apoptosis (32).

In contrast to the decrease in most lymphocyte-related transcripts during the course of infection, transcripts for the majority of the Ig genes represented on the array (heavy chains μ and γ , light chains λ and κ , and J-chain) showed a slight initial decrease in relative abundance on days 3–5 after infection (average decrease 0.6-fold) and then increased an average of 3-fold from baseline on days 9–12 (Fig. 5B). It is intriguing that the two animals that

survived beyond day 6 both demonstrated a strong induction of Ig genes. This pattern may reflect either an early antibody response or increase in plasma cell number, which may have contributed to prolonged survival of the host.

Dose Response. We examined the gene expression responses over time in the peripheral blood of macaques that were inoculated intravenously with 10^9 ($n = 2$ animals), 10^8 ($n = 3$), 10^7 ($n = 3$), or 10^6 ($n = 3$) pfu of variola India 1967 strain. In addition to the opportunity to examine inoculum dosage effects, these experiments provided an opportunity to examine the reproducibility of our previous results by using a separate set of animals, with samples and data processed and analyzed independently. The overall gene

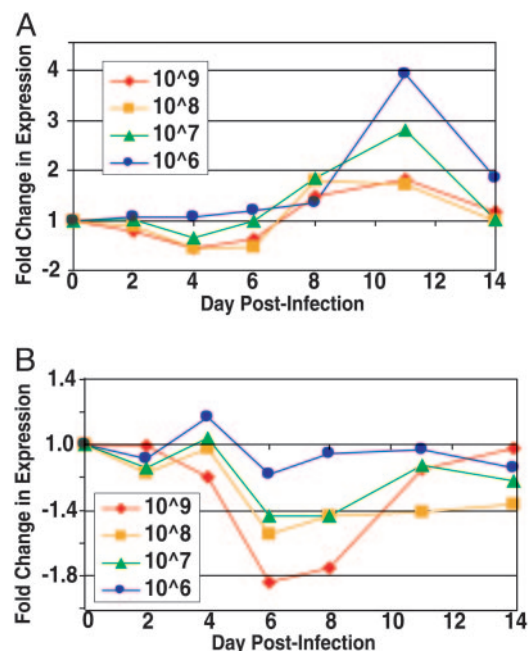


Fig. 6. Dose-responsive genes. Data for Ig (A) and MHC class II (B) gene clusters were averaged within each dose group.

expression patterns were very similar between the two data sets (Fig. 11, which is published as supporting information on the PNAS web site).

We analyzed the effects of inoculum dose on gene expression by using a linear mixed effects modeling approach (see *Supporting Text*). Using a cutoff of $P < 0.005$ for the dose effect, 465 genes demonstrated a direct relationship between level of gene expression and dose; of these, 179 genes had at least a 2-fold change from baseline in relative transcript levels in at least two of the samples (Fig. 12, which is published as supporting information on the PNAS web site). A total of 342 genes demonstrated an inverse relationship with dose; of these, 107 genes had at least a 2-fold change from baseline in relative transcript levels in at least two of the samples (Fig. 13, which is published as supporting information on the PNAS web site). Two of the most interesting clusters were inversely dose-responsive (Fig. 6). Levels of both the Ig and MHC class II gene transcripts decreased with higher doses. This pattern suggests that, at the higher doses, the viral assault may have inhibited antibody production by damaging immune system components required to develop an effective adaptive response (e.g., apoptosis and depletion of splenic T cells on day 3 after infection in an animal inoculated with 10^9 pfu, Fig. 5C). A large cluster of MHC class II genes shows almost no change at lower doses, but a decrease in the transcript levels relative to baseline at higher doses. Several other viruses have been shown to down-regulate transcript levels of MHC class II, including cytomegalovirus (33), varicella-zoster virus (34), human parainfluenza virus type 3 (35), and HIV (36). It is possible that smallpox could down-regulate MHC class II to escape CD4⁺ T cell immune surveillance, representing yet another poxviral mechanism of immune evasion.

Conclusions/Future Directions

Although smallpox was one of the deadliest diseases in human history, it is one of the least-studied on the molecular level, and much remains to be learned about the mechanisms underlying its virulence. We therefore carried out a detailed and systematic comparative analysis of the molecular features of smallpox infections *in vivo* by using a recently developed primate model (13). These results provide intriguing clues to pathogenesis mechanisms and raise many questions for future investigation. One of the most intriguing aspects of our results is the glimpse

they provide of the complex interaction between host immune system and virus. Although only 2% of smallpox ORFs have been characterized, there are several proteins predicted to have host immune-modulating functions. The systemic host response of these nonhuman primates suggests that one or more of these smallpox immune-modulating gene products may be functional. Further analysis of the host IFN, cell-cycle/proliferation, and TNF- α /NF κ B responses to smallpox will help to clarify this issue, as well as the potential role of these factors in disease pathogenesis.

A mixed cell population, such as PBMCs, presents a more complex and comprehensive picture of gene expression during the systemic responses of a host to infection than does any individual cell type. However, data from mixed cell populations can be more difficult to interpret. Although correlative analysis based on numbers of PBMC types may provide the basis for attributing variation in expression to specific cell subsets, future experiments examining variola infection in less heterogeneous cell populations will assist in identifying cell type-specific responses. Likewise, examination of different tissues during the course of infection will reveal the molecular anatomy of host responses to smallpox on an organism-wide basis.

Our results provide a useful foundation for future testing of therapeutic and diagnostic approaches. Gene expression analysis opens the door for more effective and efficient development and testing of new drugs and less reactogenic vaccines. The use of lower inoculum doses that produce a less fulminant disease may allow us to identify early molecular signatures associated with smallpox. Such diagnostic and predictive markers could be of great importance for guiding early intervention and allocation of critical medical resources in the event of a smallpox outbreak.

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