

Therapeutics of Ebola Hemorrhagic Fever: Whole-Genome Transcriptional Analysis of Successful Disease Mitigation

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The mechanisms of Ebola (EBOV) pathogenesis are only partially understood, but the dysregulation of normal host immune responses (including destruction of lymphocytes, increases in circulating cytokine levels, and development of coagulation abnormalities) is thought to play a major role. Accumulating evidence suggests that much of the observed pathology is not the direct result of virus-induced structural damage but rather is due to the release of soluble immune mediators from EBOV-infected cells. It is therefore essential to understand how the candidate therapeutic may be interrupting the disease process and/or targeting the infectious agent. To identify genetic signatures that are correlates of protection, we used a DNA microarray-based approach to compare the host genome-wide responses of EBOV-infected nonhuman primates (NHPs) responding to candidate therapeutics. We observed that, although the overall circulating immune response was similar in the presence and absence of coagulation inhibitors, surviving NHPs clustered together. Noticeable differences in coagulation-associated genes appeared to correlate with survival, which revealed a subset of distinctly differentially expressed genes, including chemokine ligand 8 (CCL8/MCP-2), that may provide possible targets for early-stage diagnostics or future therapeutics. These analyses will assist us in understanding the pathogenic mechanisms of EBOV infection and in identifying improved therapeutic strategies.

Ebola virus (EBOV), a member of the *Filoviridae* family, causes severe and often lethal hemorrhagic fever in humans and nonhuman primates (NHPs) [1]. Although these agents are often associated with limited outbreaks characterized by impressive case fatality (25%–90%) in remote regions of Africa, they are also of significant

concern from a biodefense perspective. These agents are a potential biological threat agent of deliberate use because the viruses have low infectious doses and a clear potential for dissemination by the aerosol route. The recent development of several candidate therapeutics and vaccines for EBOV has been promising; however, there are no approved preventive vaccines or post-exposure treatments to date [2–12].

EBOV pathogenesis is characterized by the dysregulation of the normal host immune responses. Particularly notable events are the destruction of lymphocytes [13], the increase of circulating proinflammatory cytokines [14, 15], and coagulation disorders [16]. Previous studies have strongly suggested that much of the observed pathology resulting from virus infection is attributable to soluble immune mediators and is not the direct result of virus-induced structural damage [17, 18]. As the infection progresses, the accumulation

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of these mediators induces abnormalities with hypotension, coagulopathy, and hemorrhage leading up to fulminant shock and death [19]. As previously noted by others, these clinical presentations are remarkably similar to those associated with severe sepsis [20] and are accompanied by rapid and significantly reduced levels of protein C [16].

Based on these observations, the hypothesis was developed that blocking the development of coagulopathies during virus infection might limit pathogenesis in an animal model and that decreasing the hypercoagulation phenotype would increase survival following virus challenge. In 2 separate studies, EBOV-infected macaques were treated with recombinant nematode anticoagulant protein c2 (rNAPc2) [21] or with the anti-sepsis drug recombinant human protein C (rhAPC) [22]. Both rNAPc2 and rhAPC are unique anticoagulants that are reported to also have anti-inflammatory activities. rNAPc2 blocks the activation of Factor X by the tissue factor (TF):Factor VIIa complex. rhAPC is a serine protease that proteolytically inactivates Factor Va and Factor VIIIa. In addition to anti-inflammatory and anti-thrombotic activities, rhAPC has also been reported to be cytoprotective. Given the reports of early activation of coagulation and the development of cytokine storms and vascular leakage, it was proposed that these therapies may work by interrupting or targeting multiple critical EBOV-induced disease manifestations. Two of 11 animals in the rhAPC study survived *Zaire ebolavirus* (ZEBOV) challenge, whereas 3 of 9 animals in the rNAPc2 study survived ZEBOV challenge. In both studies, treated animals showed an increased mean time to death, compared with that for untreated controls animals. Thus, these studies were successful in demonstrating that post-exposure treatment to ameliorate coagulopathy decreased disease severity.

An unanswered question from these studies was the impact of rNAPc2 or rhAPC on the circulating immune response to virus infection. It was noted that animals that responded to anticoagulant therapeutics had reduced viremia, but it was unclear whether this was attributable to previously undetermined or undescribed mechanisms, an antiviral activity of the drug not detected in vitro, or simply the result of the animal better maintaining homeostasis and mounting a more effective immune response. To better understand the underlying mechanisms of successful EBOV intervention and to identify possible correlates of protection, we used a DNA microarray-based approach to compare the host transcriptional responses in sequential blood samples of NHPs from the aforementioned studies [21, 22]. Our results suggest that anticoagulant treatment did not have a generalized dampening effect on the overall immune response in treated animals, but that changes occurred in specific aspects of gene expression in circulating leukocytes. Furthermore, survivors showed significant differences in the expression of a unique subset of genes that may not only allow prediction of survival following interventions but also provide

critical insights into the pathogenicity and mechanisms of protection or anti-viral responses.

MATERIALS AND METHODS

Animals

Animal studies were performed as previously described [21, 22]. Peripheral blood mononuclear cells (PBMCs) were obtained from rhesus monkeys experimentally infected with ZEBOV and treated shortly after exposure with either rNAPc2 [21] or rhAPC [22]. Animal research was conducted at the United States Army Medical Research Institute for Infectious Diseases in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996.

RNA Processing and DNA Microarrays

PBMCs isolated from blood were placed in TRIzol (Invitrogen) and processed for microarray analysis. Total RNA was extracted from the TRIzol samples, linearly amplified [23], and hybridized to a whole human genome long-oligonucleotide microarray in a 2-color comparative format [24, 25] with a reference pool of messenger RNA (mRNA). Images were analyzed with GenePix Pro 6.0 [26] and stored in the Stanford Microarray Database [27]. The microarray dataset was submitted to the Gene Expression Omnibus (GEO) database [28], under series record GSE24943.

Data Analysis

Data were first background corrected and normalized using the Limma package in R [29–31], after which log intensity ratios (fold change) were generated and control probes were removed from the dataset. To eliminate animal-intrinsic expression profiles and to characterize the expression patterns in response to infection, data for each sample were normalized to the pre-infection samples for that animal. If >1 pre-infection array was available, the day 0 post-infection arrays were used. The resulting dataset was then filtered for differential expression. The data were hierarchically clustered using the Cluster program [32] and visualized using JavaTreeView [33]. Functional annotations of gene clusters were assigned using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [34].

RESULTS

Overview of the Dataset

Three experimental groups were analyzed, and the temporal host gene expression profiles were characterized: macaques that were infected with ZEBOV and subsequently treated with

Table 1. Overview of Peripheral Blood Mononuclear Cell Samples in the Dataset

Infection group	No. of days post-infection	Untreated				rNAPc2 treated								rhAPC treated										
		C1	C2	C3	C4	R1	R2*	R3	R4	R5	R6	R7*	R8	A1	A2	A3	A4	A5	A6*	A7*	A8	A9	A10	A11
Preinfection	-8			•	•		•	•		•			•	•	•	•				•	•	•	•	•
	0	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•					
Early infection	3	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Late infection	6	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	7	•				•		•		•	•													
	8	•								•														
	9	•											•											
Extended infection	10						•					•		•		•		•	•	•			•	
	13										•													
	14														•		•	•	•	•				
	16																				•			
	17						•					•												
	22															•								
Day of death		9	8	8	8	8	—	10	11	10	14	—	14	10	9	22	7	21	—	—	16	7	8	14

NOTE. Samples from untreated Zaire ebolavirus (ZEBOV) infected rhesus monkeys (untreated), ZEBOV-infected rNAPc2-treated rhesus monkeys (rNAPc2 treated), and ZEBOV-infected rhAPC-treated rhesus monkeys (rhAPC treated) were sorted temporally by the day on which the sample was obtained after infection, then divided into pre, early, late, and extended infection groups. Samples indicated with an asterisk (*) were obtained from ZEBOV-infected animals that survived following drug treatment.

rNAPc2; macaques that were infected with ZEBOV and subsequently treated with rhAPC; and macaques that were infected with ZEBOV and left untreated (experimental controls). A total of 4 untreated ZEBOV-infected control animals, 8 rNAPc2-treated, ZEBOV-infected animals, and 11 rhAPC-treated, ZEBOV-infected animals had sufficient samples available for complimentary DNA microarray analysis. Each of the drug-treatment groups included samples from animals that did not respond to treatment (ie, experienced no increase in the mean time to death), animals that responded but did not survive (ie, experienced an increase in the mean time to death), and those that survived (Table 1). The dataset consists of transcript abundance data from PBMC samples for 23 animals on a total of 91 DNA microarrays and consists of ~3 million total data points. Samples were separated into treatment categories and infection groups as follows: pre-infection (samples taken either 8 days prior to infection and/or on the day of infection), early infection (day 3 after infection), late infection (days 6–9 after infection), and extended infection (≥ 10 days after infection).

The Circulating Immune Response to ZEBOV Is Altered in the Presence of rNAPc2 Versus rhAPC Treatment

We observed significant differential expression (≥ 3 -log fold change in at least 3 animals) in 3043 probes corresponding to 2714 annotated genes (Figure 1). These genes fell into 3 major clusters corresponding to a general defense response (top cluster), innate immune response (middle), and vesicle trafficking (bottom). The complete gene list can be found in the GEO database (GSE24943). Much of the functional Gene Ontology (GO) annotations for these clusters were not unexpected when compared with earlier array analyses of ZEBOV infection, including similar increases in expression of genes from

inflammatory and cytokine responses, such as STAT1, IRF2, and IL6. Looking at the expression levels of interleukin (IL) 6, IL18, and tumor necrosis factor α , cytokines previously identified in ZEBOV-infected cynomolgus monkeys [35] as showing marked increases in both transcript and soluble cytokine levels, we found a similar expression upregulation in infected and untreated animals, indicating a good correlation between our results and previously published observations. The same transcriptional response appears to resolve to a certain extent during the extended infection of the treated animals.

Our analysis revealed genes clusters whose expression level was altered due to treatment with either rNAPc2 or rhAPC during virus infection. Genes involved in immune response, including HLA-E, IRF1, and IFITM2, are strongly upregulated in untreated animals during early infection, as are genes found in the B cell receptor signaling pathway, NK cell mediated cytotoxicity, and lymphocyte activation. Expression of these genes then collapsed towards pre-infection levels during the late infection stage (Figure 2). In contrast, animals treated with rNAPc2 do not show the same upregulation of these gene pathways, whereas animals that underwent rhAPC treatment appear to have a sustained upregulation in this cluster.

A similar result was seen with genes involved with the activation and differentiation of lymphocytes, leukocytes, and T and B cells (Figure 2B). Untreated animals exhibited a distinct upregulation of these genes during early infection, but transcript levels decreased as the animal progressed to late infection. The downregulation of these genes appeared much earlier in animals treated with rNAPc2 and persisted throughout the infection course. However, although the upregulation of these genes in rhAPC-treated animals is more moderate than in the control animals, the expression also appears to be sustained to a lesser degree throughout the infection.

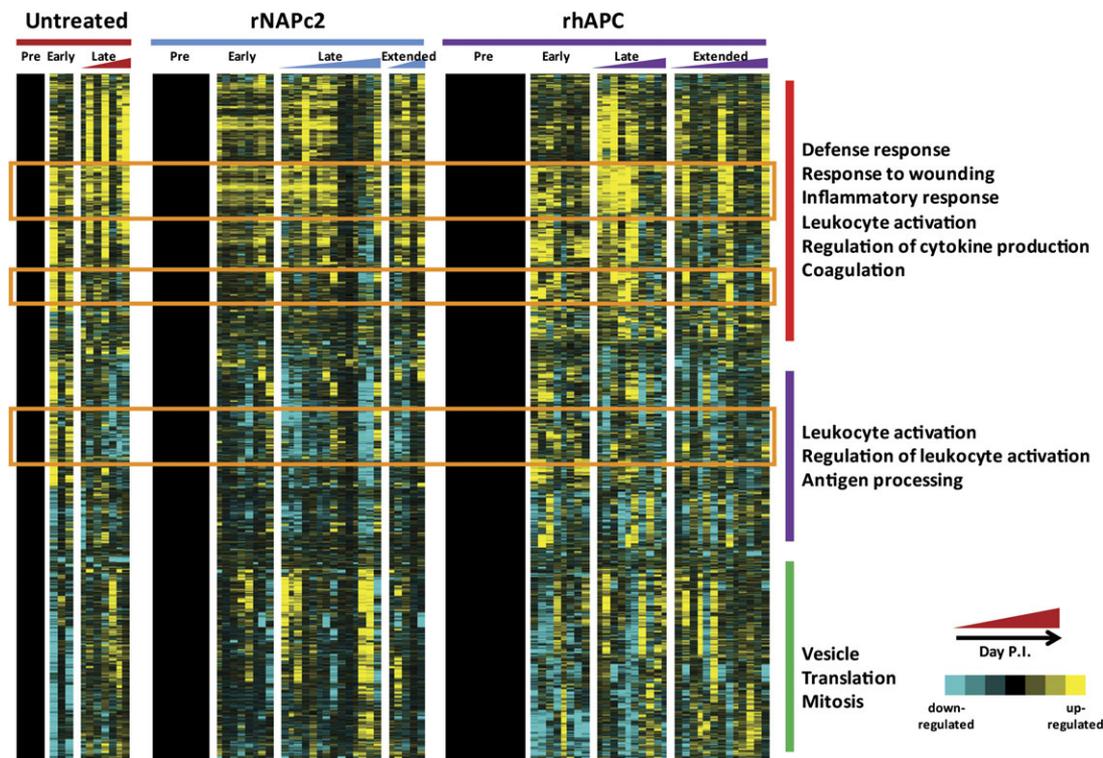


Figure 1. Overview of gene expression in peripheral blood mononuclear cells (PBMCs) from *Zaire ebolavirus* (ZEBOV)-infected rhesus monkeys. A total of 3043 probes (2714 annotated genes) exhibited a 3-log fold change or greater in at least 3 different animals. Arrays were grouped by treatment, then by infection stage, and data were hierarchically clustered. Data from individual genes are represented by row, and samples taken at different time points are represented in columns. Yellow and blue colors denote expression levels greater or less than baseline (black), respectively. Orange boxes identify the regions highlighted in Figure 2. The most significant gene ontology terms assigned to the dataset by the Database for Annotation, Visualization and Integrated Discovery ($P < .001$) are listed for the 3 major clusters.

One interesting consistency within the array data was that we also saw a similar general defense response among all 3 groups. Genes associated with the innate immune response, apoptotic regulation, and chemokine and Toll-like receptor signaling pathways (Figure 2C) are highly expressed throughout the infection course regardless of treatment. The responses seen in animals treated with either drug appear to be less acute.

Of particular interest, coagulation genes were also found to be highly significant in the dataset (Figure 1, top cluster). Further analysis showed clear treatment-specific differences in the expression of certain genes (Table 1; online only). Transcript levels for platelet factor 4 (PF4), which promotes blood coagulation, increased during late infection in control animals, while remaining visibly downregulated throughout infection in rNAPc2-treated animals and during the extended infection stage in rhAPC-treated animals. We also distinguished differences between the expression profiles of the two treatment groups. CD36, a thrombospondin receptor, and CD61/ITGB3, which is also located on platelets, both showed a decrease in transcript levels during the extended infection in rhAPC-treated animals when compared with both the control and rNAPc2-treated groups.

Given that the therapeutics in these studies target the coagulation cascade and that coagulation-associated genes seem to

be significantly driving the expression profiles, a closer look at all coagulation genes in the dataset was warranted. Using DAVID and GO terms, we identified 228 probes that tracked 146 annotated genes associated with coagulation in the dataset (Figure 1; online only). When data from the coagulation subset were separated into treatment groups, we found treatment-specific gene clusters that differed depending on disease outcome (Figure 3, A and B). In the expression profiles of survivors from both drug treatments, we see a slight sustained upregulation of the Factor VIII (F8) gene and CD49b beginning in the late infection stage, when compared with the untreated animals. A general downregulation of the vitronectin (VTN) and IL-10 alpha receptor (IL10RA) genes was also observed in surviving animals as early as day 3 post-infection in rNAPc2-treated animals and during the extended infection stage in rhAPC-treated animals, compared with the untreated animals.

From the expression profiles of the rNAPc2-treated animals (Figure 3A), we identified clusters of genes that showed either a sustained downregulation in the treated survivors and upregulation during early infection in the untreated controls or vice versa. Some of the genes identified were directly involved in coagulation, such as von Willebrand factor–cleaving protease (ADAMTS13/VWFPC), a metalloprotease that cleaves von

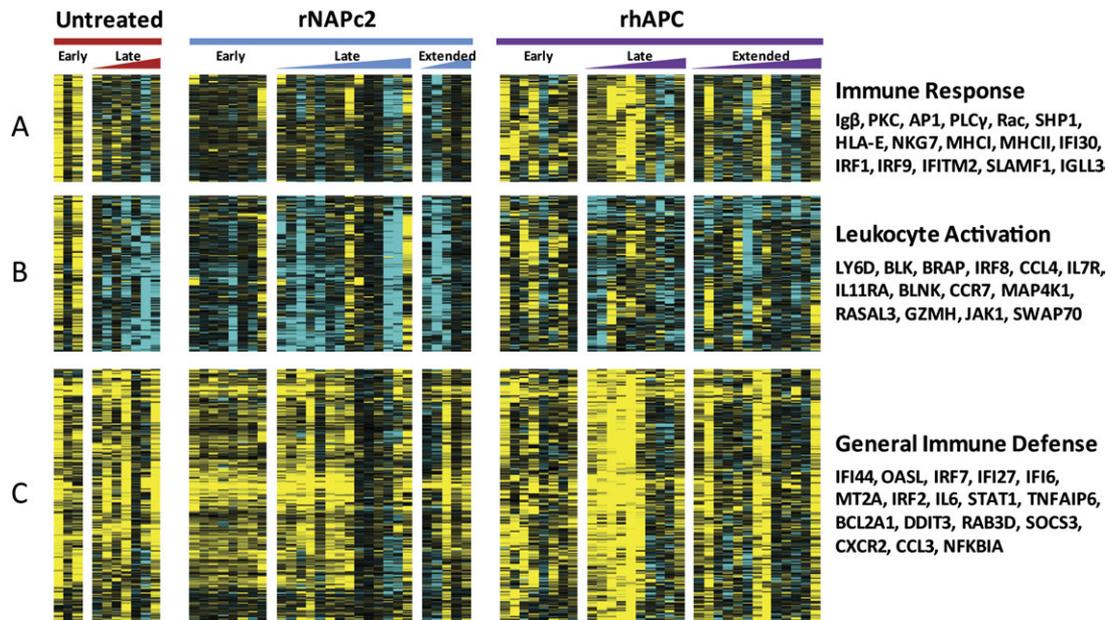


Figure 2. Comparison of differentially expressed gene clusters between the 3 treatment groups. An expanded view of significant expression clusters from Figure 1 that have altered profiles depending on treatment. Gene expression in immune response (A) and leukocyte activation clusters (B) appear downregulated for recombinant nematode anticoagulant protein c2 (rNAPc2)-treated rhesus monkeys, compared with untreated rhesus monkeys, whereas recombinant human protein C (rhAPC)-treated rhesus monkeys show a sustained upregulation. C, a general immune/defense response is seen to be strongly upregulated in all 3 treatment groups.

Willebrand factor, a large protein involved in blood clotting. Although not highly significant statistically, we did observe a slight downregulation of tissue factor pathway inhibitor (TFPI) and fibrinogen gamma in surviving animals during early infection that is not seen in either the untreated control animals or the treated nonsurvivors. An increase in transcript levels of the genes found in this cluster during late infection and a subsequent decrease in the extended infection were also observed.

We identified several genes in rhAPC-treated animals that were downregulated in the untreated animals and saw a slight sustained upregulation of a subset of genes in the surviving animals (Figure 3B). These include plasminogen (PLG), which degrades fibrin blood clots, and serum amyloid A1, which regulates proinflammatory cytokines. We found genes that were upregulated to a lesser extent in surviving animals than in the untreated animals, including serpin peptidase inhibitor, clade E1, and CD61. We also distinguished genes that are downregulated in surviving animals during the extended infection stage when compared with the nonsurviving animals, such as PF4, VTN, and Factor XIIIa (F13A1).

A clear drug effect was noted on some of the blood coagulation genes (Figure 3C). Transcript levels for Wiskott-Aldrich syndrome and the interferon gamma receptor 2 were induced in all drug-treated samples, whereas genes like PLG and VTN seemed to be altered only following treatment with either rNAPc2 or rhAPC. Of particular interest was the identification of possible markers of disease outcome, such as

mRNAs that were altered in a manner specific to surviving animals. Three mRNAs showed correlation with survival: Factor IX (F9), TFPI, and podoplanin (PDPN). Expression levels of F9 appear slightly upregulated in surviving animals only. In contrast, TFPI, which inhibits Factor Xa and thrombin (Factor IIa), is moderately expressed in both untreated and nonsurviving animals, whereas its expression levels are clearly decreased in surviving animals. PDPN, which has been shown to induce platelet aggregation, also appears to be downregulated in surviving animals. These results suggest that tracking the expression of these 3 coagulation factors during infection may provide clues to disease outcome, although individually they might not be as strong a set of indicators as other mRNAs.

Responders versus Nonresponders

A critical question of our data analysis was whether computational analysis of the gene expression patterns found in the circulating immune response during drug treatment and virus infection would be able to yield genetic signatures that could discriminate between surviving and nonsurviving animals. Using a clustering algorithm that identifies patterns within arrays, the data were grouped by disease outcome and arranged temporally. This method of analysis showed distinct differences between treated and untreated animals as early as day 3 after infection (Figure 4). We observed differential expression of ≥ 3 -log fold change in 458 probes corresponding to 414 annotated genes (Figure 2; online only), with 4 major

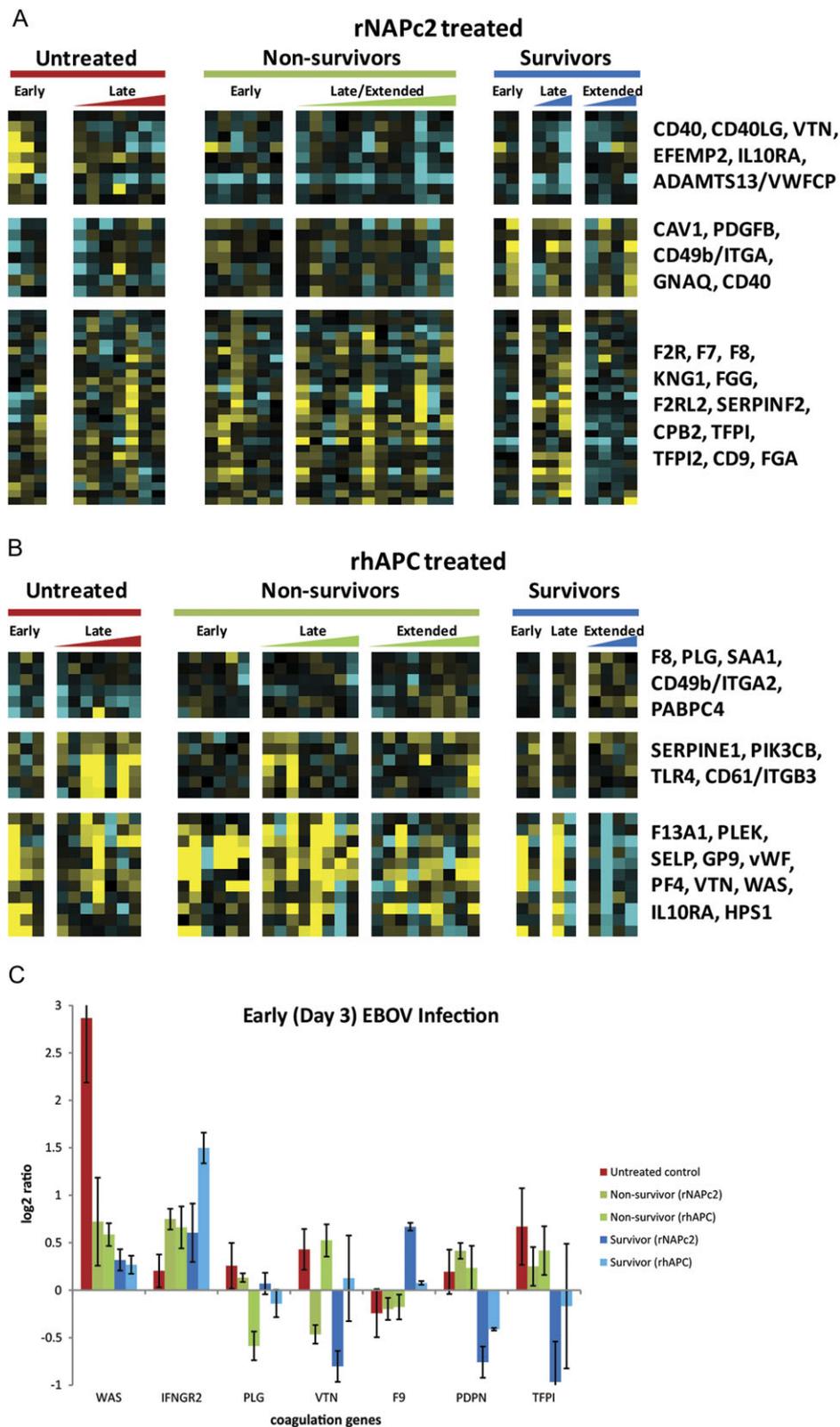


Figure 3. Identification of disease outcome–associated coagulation gene clusters of *Zaire ebolavirus* (ZEBV)–infected monkeys after treatment with recombinant nematode anticoagulant protein c2 (rNAPc2) or recombinant human protein C (rhAPC). The 228 probes from the dataset that were identified as “blood coagulation” associated were hierarchically clustered. We observed specific gene clusters that differed according to disease outcome in both rNAPc2-treated (*A*) and rhAPC-treated (*B*) animals. *C*, a graph of the mean log₂ ratios of a small subset of coagulation-associated genes at day 3 after infection clearly shows both drug-specific effects as well as possible correlate markers for disease outcome.

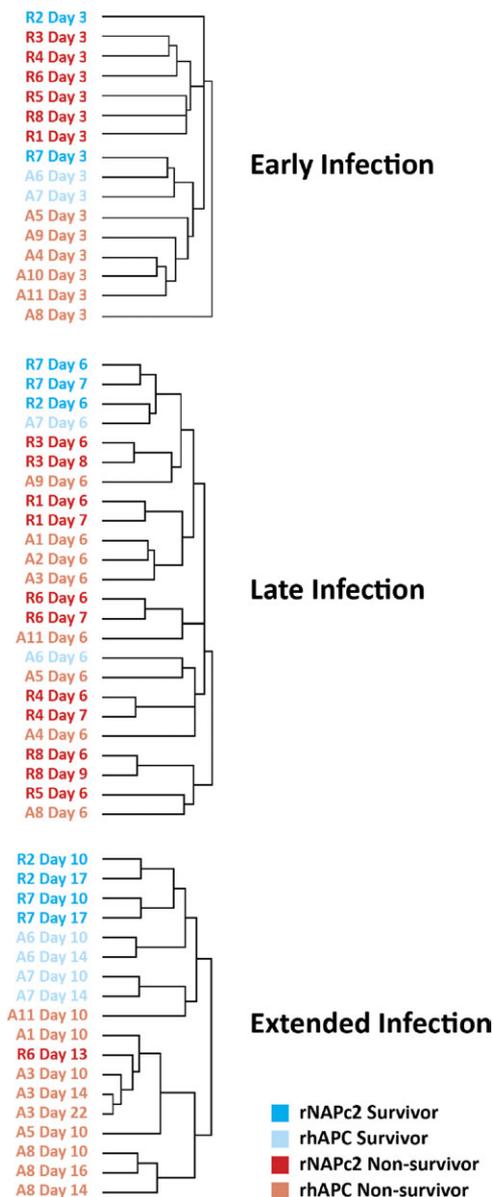


Figure 4. Microarrays cluster based on disease outcome. Arrays were arranged into an early infection group, consisting of all day 3 post-infection arrays; a late infection group, with arrays from days 6–9 after infection; and an extended infection group, with all arrays from ≥ 10 after infection. Using the Cluster program, arrays were clustered by average linkage, and the resulting array cluster tree is shown here. Arrays colored in shades of red were from nonsurviving *Zaire ebolavirus* (ZEBOV)-infected rhesus monkeys, and those in blue are from surviving animals.

clusters corresponding to functional annotations similar to those in Figure 1; however, there did not seem to be any overt systematic change in expression between survivors and non-survivors.

Further analysis of the data uncovered specific gene clusters that differed depending on disease outcome. Clusters specific for immune defense response (Figure 5A), inflammation and wound responses (Figure 5B), regulation of immune cell activation and apoptosis (Figure 5C), and viral response

(Figure 5D) showed similar patterns (Figure 5). Animals surviving ZEBOV infection after treatment with either drug exhibited a downregulation in genes found in these clusters past the late infection stage, whereas animals that died from infection did not show the same reduction in transcript levels.

To identify genes that could serve as correlates of protection, we compared the profiles of the untreated control animals with those of treated survivors and nonresponders (Figure 6A). We looked for differentially expressed genes that appeared in 100% of the surviving animals, but not the untreated control animals or nonresponders, and vice versa, and we were able to distinguish 7 probes that corresponded to 6 annotated genes. Although the significance of most of the genes in this group was unclear, 1 gene did appear to be highly noteworthy. Chemokine ligand 8 (CCL8/MCP-2) was upregulated in 4 of 4 surviving animals, whereas there was little-to-no upregulation in untreated animals (0 of 3) and in nonresponders (1 of 4). CCL8/MCP-2 has been previously shown to be highly expressed in animals during EBOV infection, with the upregulated gene expression apparent at day 6 after infection [35]. As expected, this expression pattern was seen in our untreated control animals. We were therefore surprised to find that, in all four surviving animals, CCL8/MCP-2 expression was extremely high as early as day 3 after infection (Figure 6B). When compared over multiple days, this upregulation appeared to be sustained in the surviving animals, leading to the speculation that earlier expression of CCL8/MCP-2 in the surviving animals may play an important role in protection against EBOV.

DISCUSSION

Here we report on the genome-wide transcriptional response of blood leukocytes to direct infection with EBOV in a lethal animal model and the identification of detectable changes in this response following treatment of infected animals with factors that block activation of the coagulation pathway. Prominent in our findings are discernable differences in the circulating immune response as early as 3 days after infection when transcriptomes from infected animals are compared with transcriptomes from infected animals treated with coagulation blockers. A discernibly different immune response in rNAPc2- or rhAPC-treated animals was not necessarily the expectation, because these treatments do not themselves have a direct effect on transcription. Indeed, we were surprised to discover coagulation genes that correlated with expression profiles of survival. However, this supports the hypothesis that controlling coagulopathy early in infection can have a notable impact not only on the pathogenesis of infection but also on the circulating leukocyte response to hemorrhagic fever.

Although changes in the overall transcriptome were observable when untreated animals were compared with treated animals, this “Ebola response” appeared to be limited in scope. The

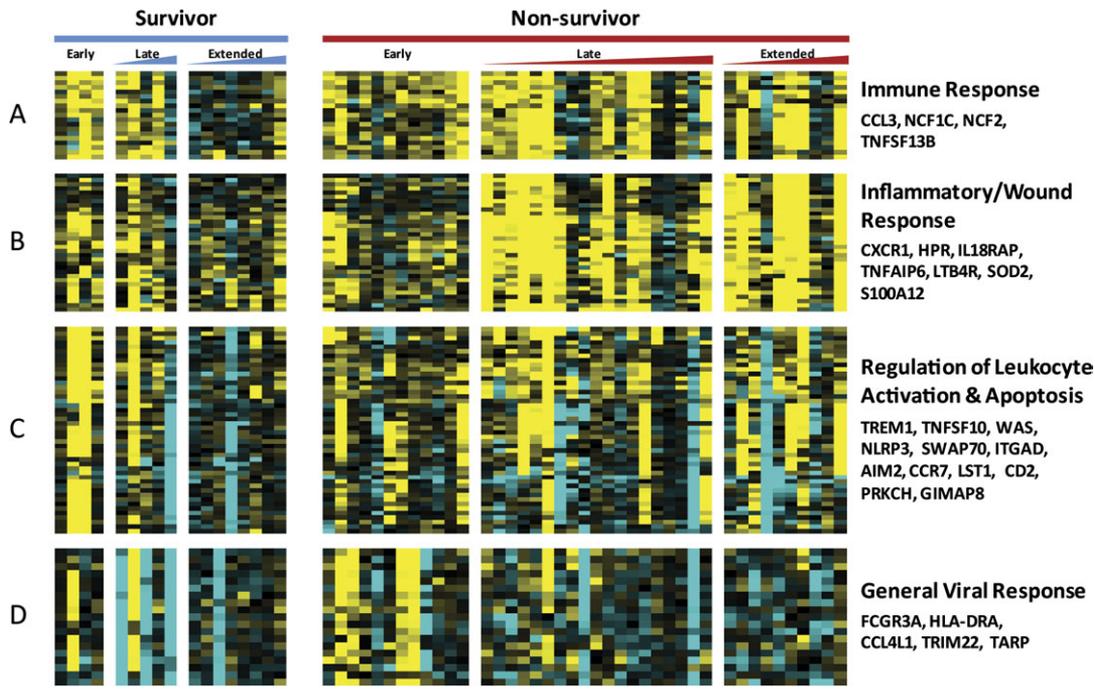


Figure 5. Differential expression of survivors and nonsurvivors can be distinguished in specific clusters. Specific gene clusters from Figure 4 reveal distinct differences in the expression patterns of surviving and nonsurviving *Zaire ebolavirus* (ZEBOV)-infected rhesus monkeys. There is a decrease in transcript levels for surviving animals during the extended infection stage in clusters relating to (A) immune defense responses (A), inflammatory and wound responses (B), and regulation of leukocyte activation and apoptosis (C). In surviving animals, there is also a strong decrease in the gene expression of a cluster relating to general viral response (D), when compared with nonsurviving animals.

overall immune response to infection with ZEBOV remained very similar whether animals underwent drug treatment or not. In both treated and untreated animals, markers of the interferon response, such as IFIT1, GBP1, and MX1, were clearly discernible, as was a robust transcriptional upregulation of cytokines, such as IL-6, IL-10, and IL-15, that are also involved in T and B cell activation. The changes in cytokine response are consistent with findings from other groups that analyzed the transcriptional response to other negative-sense RNA viruses and demonstrated upregulation of these types of cytokines. These results have been taken to suggest that disease severity can be modulated by changes in gene expression outside of core immune responses [36]. In the case of coagulation inhibitor treatment of EBOV infection, the results are surprising, given that both rNAPc2 and rhAPC are both purported to be anti-inflammatory and that direct measurement of circulating cytokines in the serum samples of animals showed decreased levels in those that responded to either therapy. This suggests that the disconnect between mRNA upregulation and protein translation and secretion is an important consideration in the overall conclusions of these types of studies.

The earlier upregulated expression of CCL8/MCP-2 in surviving animals, compared with expression in the untreated and nonresponding animals, suggests that, in surviving animals, certain important aspects of immune response are different than in nonresponding animals. A major player in immunoregulatory

and inflammatory processes, CCL8/MCP-2 has also been shown to specifically stimulate the directional migration of immune cells and may play an essential role in the recruitment of immune cells [37]. The early accumulation of CCL8/MCP-2 mRNA in the surviving animals may be inducing host immune responses at an earlier time point in the disease progression, which can be crucial in determining survival. This suggests that CCL8/MCP-2 and other related genes may aid in therapeutics, and the possibility of using these genes as early stage diagnostic markers should be further examined. Although the transcript levels of CCL8/MCP-2 are increased in surviving animals, more work is needed to verify a similar increase in protein levels.

A clear effect on the coagulation pathway was seen in animals treated with either drug. Anticoagulant treatment prevented the upregulation of the coagulation promoting PF4 and led to downregulation of thrombospondin and CD61. It is unclear at this point whether these findings are a result of the inhibition of coagulation leading to the preservation of cells that express these genes in the circulating population or whether this represents an effective reprogramming of the coagulation response by these protein drugs. Regardless, these findings are intriguing, because rNAPc2 predominantly targets the extrinsic cascade by blocking the TF-mediated activation of Factor X by the TF:Factor VIIa complex, whereas rhAPC proteolytically inactivates proteins Factor Va and Factor VIIIa in the common and intrinsic pathways, respectively.

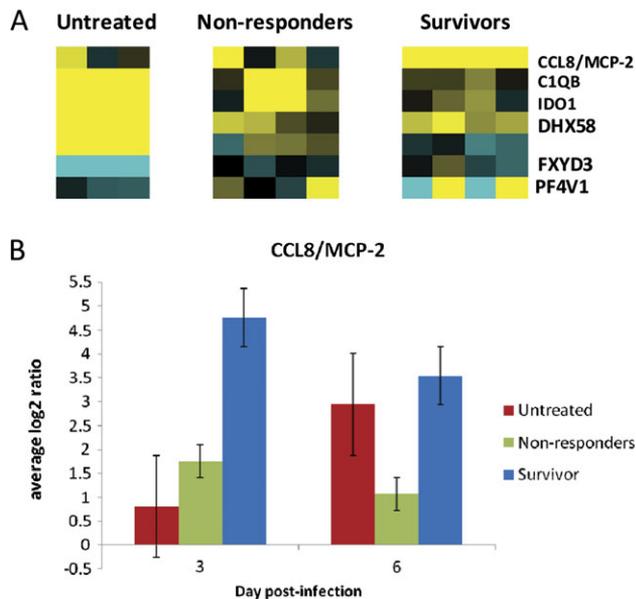


Figure 6. Chemokine ligand 8 (CCL8/MCP-2) appears to correspond with survival of Ebola virus (EBOV) infection. *A*, genes that exhibited a 3-log fold-change or greater were further filtered to identify genes that were differentially expressed in 100% of the surviving animals during early infection and none in either the untreated animals or nonresponding animals. A total of 7 (6 annotated genes) were found. *B*, the log₂ ratios of CCL8/MCP-2 in each group were averaged and plotted, showing that, whereas the expression levels for the untreated control animals increased at day 6 after infection, the gene was highly expressed in the surviving rhesus monkeys as early as day 3. Error bars represent the standard error.

As noted in Figure 4, clustering analysis of the gene arrays showed that, even as early as day 3, the response of treated animals that survived infection was different than the response of treated animals that later died from infection, and this becomes more easily discernable as infection progresses. The discovery that CCL8/MCP-2 and coagulation-associated genes TFPI and PDPN correlate with survival in this study introduces the possibility of developing survivor diagnostic tools using these various probes as potential markers of infection. Although the changes in expression levels of the coagulation genes are not dramatic and their overall significance may be mitigated, their correlation with survival warrants further investigation. Though the current study is underpowered to guarantee clear identification of biomarkers, our data clearly show that gene expression patterns that signal survival following infection with ZEBOV can be obtained using this approach, and expansion of these studies with additional samples from animals that survive other treatments and animals infected with other high consequence pathogens will advance the identification of unique sets of biomarkers for early pathogen identification as well as predictions of disease severity and survival.

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