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# Functional gene analysis of individual response to challenge of SIVmac239 in *M. mulatta* PBMC culture

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**Abstract**

It has previously been shown in macaques that individual animals exhibit varying responses to challenge with the same strain of SIV. We attempted to elucidate these differences using functional genomics and correlate them to biological response. Unfractionated PBMC from three rhesus macaques were isolated, activated, and infected with SIVmac239. Interestingly, one of the three animals used for these experiments exhibited a completely unique response to infection relative to the other two. After repeated attempts to infect the PBMC from this animal, little or no infectivity was seen across the time points considered, and corresponding to this apparent lack of infection, few genes were seen to be differentially expressed when compared to mock-infected cells. For the remaining two animals, gene expression analysis showed that while they exhibited responses for the same groups of pathways, these responses included differences specific to the individual animal at the gene level. In instances where the patterns of differential gene expression differed between these animals, the genes being differentially expressed were associated with the same categories of biological process, mainly immune response and cell signaling. At the pathway level, these animals again exhibited similar responses that could be predicted based on the experimental conditions. Even in these expected results, the degree of response and the specific genes being regulated differed greatly from animal to animal. The differences in gene expression on an individual level have the potential to be used as markers in identification of animals suitable for lentiviral infection experiments. Our results highlight the importance of individual variation in response to viral challenge.


*Keywords:* Gene expression; Microarray; Peripheral blood mononuclear cells; Simian immunodeficiency virus

## Introduction

The simian immunodeficiency virus (SIV) macaque model is an important system for studying HIV and AIDS progression. Macaques infected with SIV will develop AIDS in a manner dependent on the pathogenicity of the strain (Hirsch and Johnson, 1994). It has been demonstrated that in vivo, individual animals exhibit different responses to the viral challenge. In a group of macaques, certain individuals will progress rapidly with high levels of viremia within a normal time period for the onset of AIDS, while others will progress more slowly or not at all, with the latter termed long-term nonprogressors (Fultz, 1993). There are also reports of individuals progressing much faster than normal to AIDS disease. This phenomenon is very similar to that reported in HIV-infected humans (Sheppard et al., 1993).

In an attempt to identify the characteristics of individual macaques that define the differences seen in infectivity and disease progression, repeated SIVmac239 infections were performed with the peripheral blood mononuclear cells (PBMC) separated from the whole blood of three *Macaca mulatta*. In this study, we attempt to elucidate the effects of SIV infection utilizing global gene expression profiling on infected macaque primary cells.

Global gene expression profiling is increasingly used as a tool to study the complexities of virus–host interactions. Recently, microarray-based studies have been published on HSV (Kent and Fraser, 2005), rabies (Wang et al., 2005), and

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HCV (Helbig et al., 2005). In most cases, experiments are focused on in vitro infections that are performed using immortalized cell lines under conditions in which the majority of cells become infected. Although a high rate of infection is advantageous for measuring gene expression changes, it does not represent the typical state of in vivo infections, and cell lines by definition have properties that are quite distinct from primary cells. In addition, a cell line that supports the highest level of infection may not be representative of the natural target cell (in terms of tissue or species origin) (Kim et al., 2001), or for certain viruses, a cell line susceptible to infection may not be available. Another significant consideration is that, in vivo, changes in cellular gene expression may occur in direct response to virus infection or in reaction to intercellular signals from neighboring cells, which may be of a different type and which may or may not be infected. The use of cell lines excludes the ability to study this important aspect of the host response to infection.

Many of these factors come into play when studying cellular gene expression changes in response to infection with simian immunodeficiency virus. For example, although SIV readily infects a number of T-cell lines (Agy et al., 1991), these cells are of human origin and similar nonhuman primate lines are not available. We therefore sought to determine whether DNA microarray analysis could be applied to an experimental system that is more representative of SIV infection in vivo.

Gene expression profiling of SIV-infected PBMC presents a number of challenges. In particular, only specific subsets of cells within a population of PBMC are susceptible to SIV infection (Takahashi et al., 2005), and even among the potentially susceptible $CD4^+$ cells, the infection rate is low. This makes it difficult to detect gene expression changes that are specific to virus-infected cells. However, the in vitro infection of PBMC provides an easily manipulated experimental system in which to detect gene expression changes that result from intracellular as well as intercellular signaling mechanisms (Yu et al., 2005). This system therefore provides an opportunity to gain new insights into the genes and molecular pathways that are active in PBMC during an in vivo infection, such as the experimental infection of rhesus macaques.

This animal model is widely used for evaluating therapeutic and vaccine strategies and provides valuable insights into immunodeficiency virus pathogenesis (Reimann et al., 2005), the host response to infection (Staprans et al., 2004), and AIDS (Gardner, 1989). The studies described in this report contribute to an expanding knowledge base of the molecular events that occur in response to SIV infection and serve as a foundation for gene expression analyses performed on cells and tissues from SIV-infected macaques.

## Results and discussion

### *PBMC from different rhesus macaques exhibit varying infection kinetics*

SIVmac239 infections in cell lines and primary cells rarely achieve a very high infection rate due to the inability to create high titer viral stocks. It is common for infection rates to be lower than 5% in primary cells, such as activated PBMC. However, this is more indicative of SIV/HIV infection in vivo (Zhu et al., 2004). To characterize our PBMC infections, we monitored cell viability, SIV gag protein (p27) level, and the percentage of infected cells. Cell viability was similar for each culture at each time point and remained in excess of 90% throughout the experiments (data not shown). SIV gag levels reflect normal progression of viral infection for animals 83 and 84 but indicate a lack of infection in animal 95 (Fig. 1). The PBMC for all three animals (animals 83, 84, and 95) were infected at the same $TCID_{50}$, and the infection rate was estimated using immunofluorescence (IFA). For animals 83 and 84, the infection rate was estimated to be 3–5% (Fig. 2). Exposure of PBMC from animal 95 to SIV yielded a very different result. After three attempts at infection, the best infection rate achieved was <1% (estimated by IFA at culture day 6). The viability of the cells was not affected by the virus, and the PBMC for all three animals had the same trend in viability, decreasing over time. This is due to the effects of primary cells in culture rather than the virus, since the infection rate is so low. Syncitia were not observed at any time point. The most notable difference between PBMC samples from animals 83 and 84 and animal 95 is seen in the FACS data (Table 1). PBMC from animal 95 were seen to contain overall lower percentages of CD2+ and CD4+/CD8+ cells and higher levels of CD20+ and CD16+ cells relative to the concentrations seen in the samples from animals 83 and 84. Also, while the CD16+(NK) cells were at a greater concentration in animal 95 samples, this population consisted of a high population of CD16+/CD8+/CD3+ cells (NKT cells). Animals 83 and 84 had much lower levels of NK cells overall, but in these animals that population consisted of almost equal parts CD16+/CD8+/CD3− and CD16+/CD8+/CD3+ cells. This more mixed population of cells is more typical of what is generally seen in PBMC. We speculate that at least some of the disparity in response between these animals is due to the disproportionately higher levels of B and NK cells and lower levels of T cells seen in animal 95.

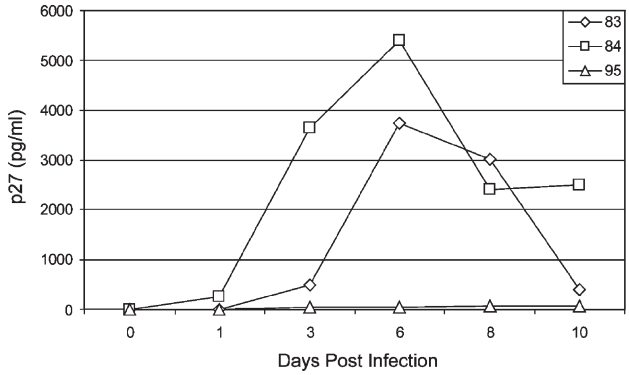


Fig. 1. Levels of SIV gag protein (p27) reflect the progression of viral infection. The chart tracks p27 levels for all three infected animals through 10 days post-infection. Animals 83 and 84 show "normal" progression with p27 levels peaking around 6 days post-infection. Animal 95 shows no progression of infection despite repeated attempts to expose these cells to virus.

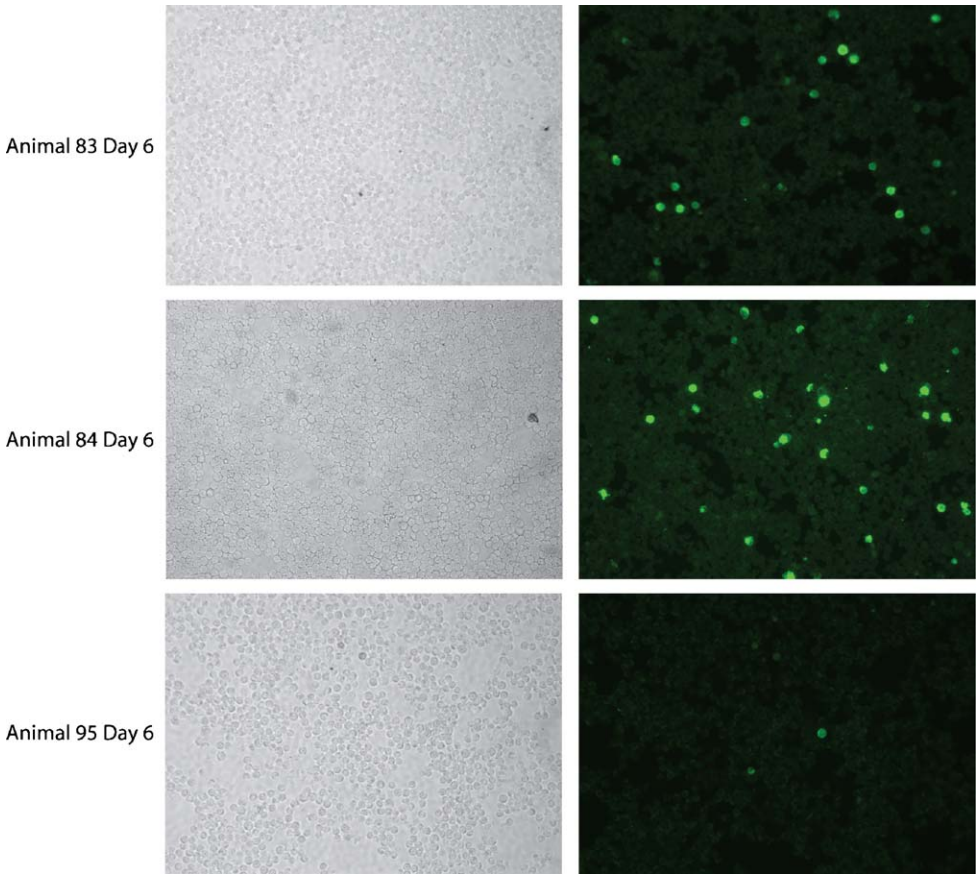


Fig. 2. Indirect Immunofluorescent Assay (IFA) in PBMC infected for 6 days with SIVmac239. On the left are phase contrast micrographs of virus-infected cells from all three animals used in the experiments. On the right are the immunofluorescence analyses of the identical microscopic field. Animals 83 and 84 exhibit an estimated infection rate of 3–5%, while the infection rate for animal 95 is <1%. There is approximately the same number of cells per field.

*Differential gene expression demonstrates that animal 95 has a unique response to viral challenge relative to animals 83 and 84*

To determine if these individual differences in response to infection can be categorized using functional genomics, we used differential gene expression analysis using human cDNA arrays. At the time these experiments were performed, the only array platform for macaque studies was the human array. Although the use of human cDNAs to measure gene expression changes in macaque cells is suboptimal, the high nucleotide sequence homology between humans and macaques makes this cross-species hybridization feasible (Baskin et al., 2004; George et al., 2003; Vahey et al., 2003). While macaques have been shown to have greater than 97% homology to humans at the DNA level, it is expected that some genes may be missed entirely or that cross-hybridization may confound the data. Without the availability of better macaque-specific resources, these issues until very recently had been unavoidable.

In an attempt to ameliorate some of these confounding factors, we have used stringent statistical analysis (2-fold change, $P \leq 0.01$).

It may be anticipated that use of PBMC would detect a larger number of differentially expressed genes than use of a cell line. A preliminary comparison of our results with gene expression changes associated with SIV infection of a human T-cell line (in which nearly 100% of the cells become infected) suggests that this is indeed the case (Li and Katze, unpublished observations). Similarly, gene expression measurements of human T-cell lines infected with human immunodeficiency virus (Corbeil et al., 2001; Geiss et al., 2000) also resulted in the detection of somewhat fewer differentially expressed genes but often with larger gene expression changes than we detected using PBMC. Thus, PBMC are better than less complex systems at revealing more subtle differential gene regulation during SIV infection, provided such changes are of a sufficient magnitude to detect when expressed by only a small percentage of cells.

Cellular gene expression patterns were profiled for each culture at days 3, 6, and 8 post-infection. The first step in analyzing the resulting gene expression data was to identify all genes that were differentially expressed by 2-fold or greater ($P \leq 0.01$) in response to SIV infection at any time point. In animals 83 and 84, this resulted in the identification of 1585 and 654 genes, respectively. Far fewer differentially expressed genes (310) were identified in the cultures from animal 95. Similarly, the greatest number of differentially expressed genes was detected in animals 83 and 84 at days 3 and 6, the time points that corresponded to the highest p27 levels for each culture. By day 8 post-infection, the cells were dying from culture conditions regardless of infection status. These results

Table 1
FACS analysis of whole blood and peripheral blood mononuclear cells (PBMCs) reveals distinct changes in lymphocyte subsets between infectible and uninfectible animals

| Animal Source | 83 Whole blood | 84 Whole blood | 95 Whole blood | 83 PBMC culture | 84 PBMC culture | 95 PBMC culture |
|---|---|---|---|---|---|---|
| CD2+ | 73.9 | 70.3 | 81.7 | 99.3 | 99.7 | 85.1 |
| CD4+/CD8− | 38.6 | 45.6 | 40.0 | 23.4 | 30.7 | 14.7 |
| CD8+/CD4− | 39.7 | 26.9 | 43.6 | 54.2 | 49.6 | 67.2 |
| CD4+/CD8+ | 0.9 | 1.6 | 1.2 | 12.4 | 19.0 | 2.6 |
| CD20+ | 24.5 | 28.0 | 16.7 | 0.3 | 0.0 | 5.8 |
| CD3+ | 67.7 | 68.9 | 79.1 | 99.3 | 98.5 | 94.0 |
| CD4+/CD3+ | 39.5 | 47.2 | 41.1 | 35.8 | 49.6 | 17.2 |
| CD8+/CD3+ | 23.9 | 20.4 | 36.7 | 65.6 | 66.8 | 68.1 |
| CD8+/CD3− | 16.7 | 8.0 | 8.1 | 1.0 | 1.8 | 1.7 |
| CD16+ | 13.3 | 13.2 | 16.3 | 2.0 | 2.6 | 11.2 |
| CD16+/CD8+/CD3+ | 11.4 | 13.6 | 51.6 | 51.0 | 38.3 | 91.7 |
| CD16+/CD8+/CD3− | 67.1 | 53.6 | 29.4 | 34.2 | 48.4 | 0.0 |

Whole blood and PBMCs were collected from the indicated macaques at time *X* post-infection with SIV. Macaque 95 was not readily infected and FACS analysis revealed significant changes in lymphocyte distribution in PBMC cultures from this animal. While there was not an overall change in T cell (CD3+) numbers in PBMC cultures from this animal, there was a skewed distribution of T cell subsets, with an increase in CD4−CD8+ cells and a decrease in CD4+CD8+ cells compared to macaques 83 and 84. Most strikingly, macaque 95 displayed a significant increase in CD16+ cells in PBMC cultures compared to animals 83 and 84. This was due to an increase in NKT cells (CD16+CD8+CD3+). The overall increase in CD16+ cells in PBMCs in macaque 95 masked a decrease in NK cells (CD16+CD8+CD3−) in this animal. While there was not an overall difference in total CD16+ cells in whole blood from macaque 95, the same trend that was observed in PBMC cultures from this animal was also observed in whole blood, with a relative increase in NKT cells and a decrease in NK cells compared to animals 83 and 84.

are skewed due to the high levels of cell death in the cultures and will not be considered in our comparisons (data not shown).

Hierarchical clustering was then used to compare and visualize gene expression changes over time for each culture. The resulting cluster diagram again revealed that animals 83 and 84 exhibited similarities in their patterns of gene expression on day 3 post-infection, and animal 95 exhibited a completely different pattern in response to the virus (Fig. 3). While there were similarities in the response patterns between animals 83 and 84, only 12% (184) of the overall genes with altered expression patterns on day 3 post-infection were in common between the two. Relatively few gene expression changes were common across multiple time points, highlighting the apparently ever-changing state of the cellular response to infection with many new gene expression changes occurring at each time point. Most likely, this is in response to the changing dynamics associated with viral replication.

### *SIV-induced changes in gene expression are associated with transcriptional regulation and immune response pathways*

To characterize further the cellular response to SIV infection, we classified differentially expressed genes into functional categories. This analysis was performed using FatiGO (Al-Shahrour et al., 2004), a World Wide Web-based tool for the extraction of Gene Ontology (GO) information (Harris et al., 2004). Not surprisingly, animals 83 and 84 showed perturbations in relatively large numbers of genes involved in transcriptional regulation (112 and 35 genes), immune response (61 and 27 genes), and protein metabolism (165 and 80 genes, respectively). Heat map patterns between animals 83 and 84 were visibly similar in these functional categories while the heat map of animal 95 clearly differed (Fig. 4). Expression changes in these genes most likely reflected the active transcriptional and translational processes associated with viral infection as the virus usurps cellular processes to support the generation of progeny virus and the host cells attempt to mount an immunological response to invasion. All of these perturbations should be predictable from the experimental conditions being tested. Animal 95, having considerably fewer altered genes overall (only 163 genes across all time points combined), exhibited perturbations in protein metabolism and, to a much lesser degree, regulation of transcription.

### *Analysis of the pathways associated with differentially expressed genes highlights a number of hallmarks of viral infection*

In an attempt to attach more meaningful information to the lists of regulated genes in each animal, the data were next

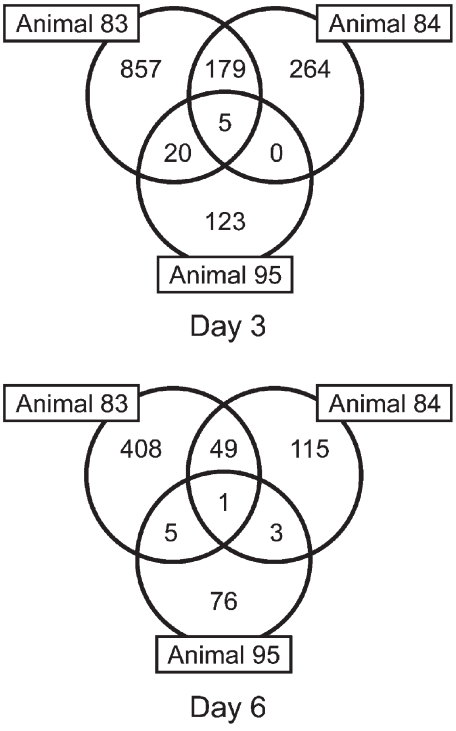


Fig. 3. Changes in gene expression in response to SIV infection vary between individuals. The Venn diagrams depict the number of differentially expressed genes (≥2-fold change, *P* value ≤0.01) that are unique to or common among the analyzed samples. Animal 95 exhibits little or no overlap with the other animals at any time point considered in this study. Animals 83 and 84 show common regulation of genes tightly associated with SIV infection (184 genes on day 3 and 50 on day 6) as well as a large number of genes uniquely regulated in each individual.

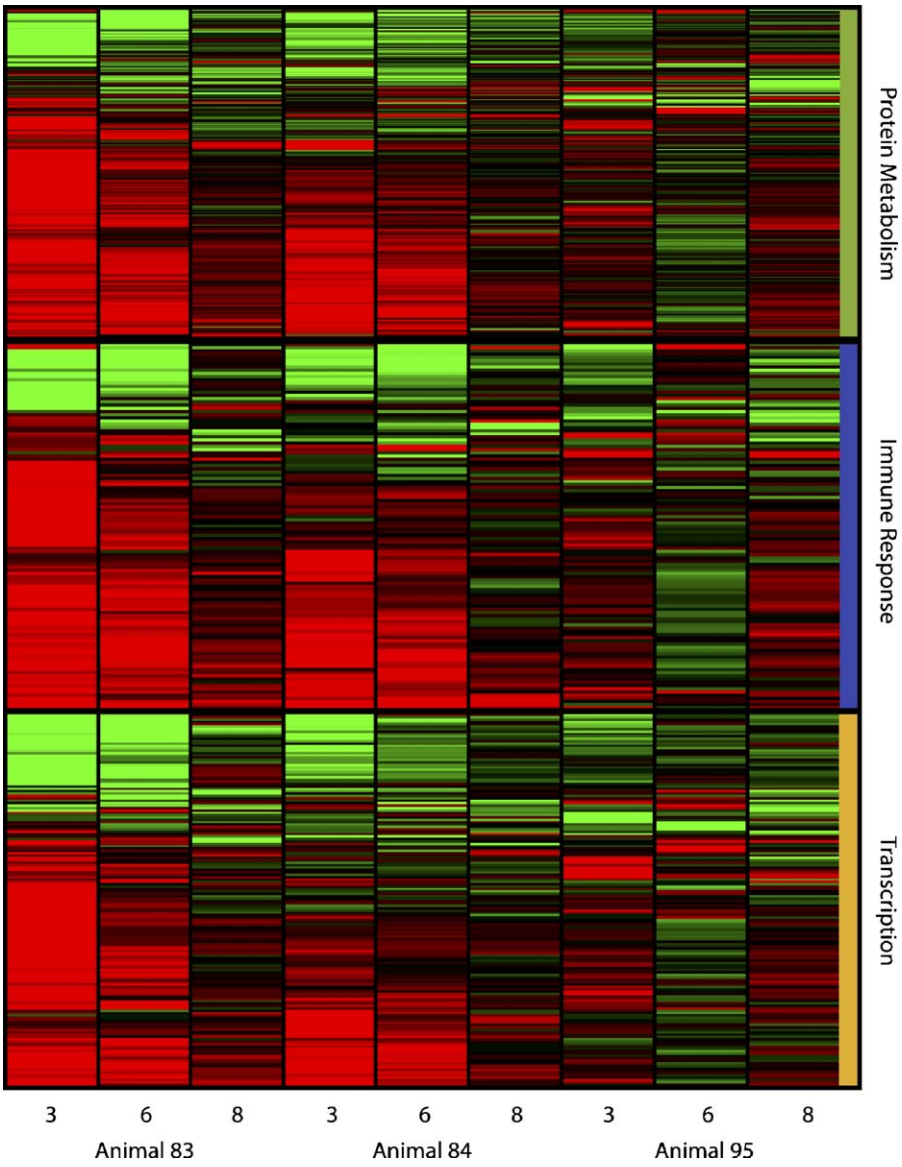


Fig. 4. Heatmap generated in Resolver™ provides a visual representation of similarities and differences seen in gene expression over three GO-derived functional categories. The images from animals 83 and 84 show clearly similar patterns in regulation of gene expression at days 3 and 6 post-infection in protein metabolism, transcription, and immune response—the top three categories exhibiting perturbation based on FatiGO analysis. Responses seen in samples from animal 95 vary greatly relative to those of the other animals as would be expected based on the IFA and p27 information for these samples.

processed through Pathway Miner (Pandey et al., 2004). This web-based, analytical software overlays genes of interest onto functional pathway data from three open-source resources: KEGG (www.genome.ad.jp), BioCarta (www.biocarta.com), and GenMAPP at (www.genmapp.org). Using our list of genes with expression levels altered by greater than 2-fold ($P \leq 0.01$), we detected perturbations in a number of pathways already described in the literature as being affected by SIV (or HIV-1) infection. While few if any pathways were significantly perturbed in animal 95, the response seen in animals 83 and 84 showed a number of hallmarks of SIV infection, including perturbations in the mCalpain pathway (Fig. 5), which is responsible for cell motility in response to integrin signaling (Ghibelli et al., 2003) and which plays a role in modulating HIV-1 entry to CD4+ cells (Tardif and Tremblay, 2005), the MAPK signaling pathway (Popik and Pitha, 1998), the complement pathway (Horakova et al., 2004), and the T and B cell receptor signaling pathways (Luo and Peterlin, 1997). While these responses involved many of the same genes across animals and time points, some individual variation was seen as well. For example, in the case of the complement pathway, which is responsible for attracting immune cells and increasing phagocytotic clearance of infected cells during immune response, both animal 83 and 84 showed similar strong down-regulation of C8A expression, while animal 83 showed a much stronger regulation of the pathway overall, including changes in C5 and BF (data not shown).

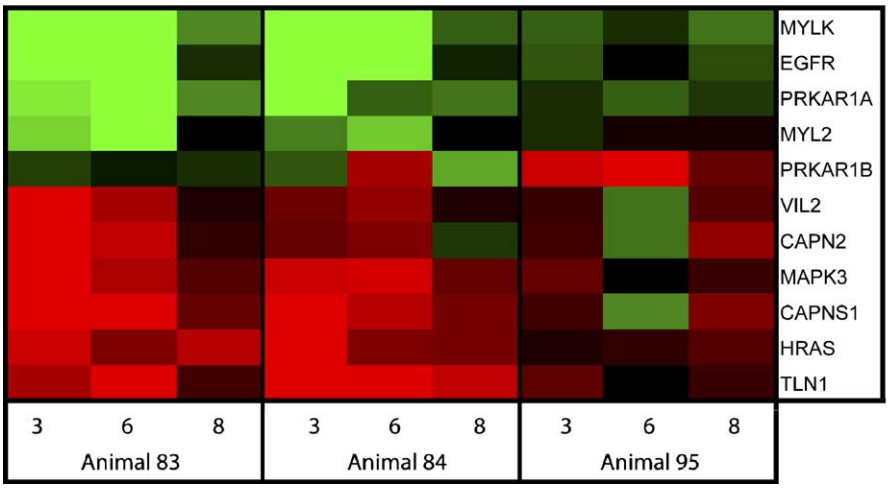


Fig. 5. The mCalpain pathway is instrumental in cell motility and cytoskeletal restructuring in response to integrin signaling. Recently, it has been shown that free lateral movement of LFA-1 and a dynamic cytoskeleton are important in viral entry into CD4+ T cells. The heatmap shows a total of 11 genes affiliated with the mCalpain pathway being regulated over the course of our infections for both animals 83 and 84.

*Trends in canonical pathway regulation differentiate the response of individual animals*

Finally, lists of regulated genes from each animal were run through the commercially available Ingenuity Pathway Analysis (IPA) Software. Similar to the Pathway Miner application, IPA imports genes of interest along fold change information and other statistical measurements and generates information regarding how these genes interact with one another and their potential effects in the samples of interest. IPA provides not only networks of interactions between genes and pathway information but also allows point and click access to information vetted from the literature by their scientific staff. Analysis of the canonical pathways in Ingenuity provides some results that are similar to those seen in Pathway Miner. For example, the ERK/MAPK and B- and T-cell receptor signaling pathways are, as expected, clearly represented in the highest level graphical view of the analysis.

The Antigen Presentation pathway – which one would expect to see regulated in most viral infections and is clearly reported as a regulated pathway in cases of immunodeficiency diseases (Steinman et al., 2003) – did not appear to be regulated in a significant fashion based on the Pathway Miner results (only 2 genes reported a significant fold change in animal 83 and only 1 in animal 84). Taking the literature information into account from the IPA software, this pathway shows regulation at 5 (animal 83) or 6 (animal 84) individual genes at day 3 and then exhibits a reduced response (regulation at 3 genes in each animal) for day 6. While the two animals show regulation of some genes in common (HLA-DQB1, HLA-DRB1, and HLA-DRB5), or at least genes from the same MHC class II subtypes (HLA-DPA1, HLA-DMA, and HLA-DQA2), they also show some genes that are only significantly regulated in each animal individually. Closer examination shows that while regulation of these "unique" responding genes does not meet our criteria for significance in both animals, the genes do exhibit some degree of regulation in both animals and are all considerably more perturbed in animals 83 and 84 than in animal 95 (Table 2).

Patterns of response similar to that seen in the Antigen presentation pathway (high initial response on day 3 with

Table 2
Genes from the antigen presentation pathway show significant changes early in infection for both animals 84 and 83 with regulation decreasing over time

| Gene | Day post infection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Animal 83 | | | Animal 84 | | | Animal 95 | | |
| | 3 | 6 | 8 | 3 | 6 | 8 | 3 | 6 | 8 |
| CD74 | 2.20 | 1.85 | 1.36 | 1.78 | 1.93 | 1.24 | 1.00 | 1.30 | 1.64 |
| HLA-DMA | 1.25 | 1.26 | 1.31 | 2.72 | 1.72 | 1.21 | 1.07 | −1.14 | −1.35 |
| HLA-DPA1 | 2.10 | 1.73 | 1.08 | 1.40 | 1.48 | −1.09 | −1.10 | −1.16 | 1.53 |
| HLA-DPB1 | 1.76 | 1.90 | 1.15 | 2.32 | 1.71 | 1.12 | 1.03 | 1.09 | 1.98 |
| HLA-DQA1 | 1.45 | 1.44 | 1.00 | 2.03 | 1.72 | 1.25 | 1.31 | 1.19 | −1.03 |
| HLA-DQB1 | 2.34 | 2.61 | 1.25 | 2.44 | 2.42 | 1.36 | 1.06 | 1.19 | 1.14 |
| HLA-G | 1.64 | 1.80 | 1.33 | 2.24 | 1.16 | −1.25 | 1.17 | 1.31 | 1.63 |
| PSMB6 | 1.43 | 2.10 | 1.53 | 1.95 | 1.45 | 1.24 | 1.19 | 1.19 | 1.64 |

Again, animal 95 exhibits no significant changes. The gray boxes indicate fold-changes that meet our statistical cut-offs.

diminishing numbers of genes regulated across time) can also be seen in animals 83 and 84 in the endoplasmic reticulum (ER) stress, integrin signaling, IGF-1 signaling, apoptosis signaling, and ERK/MAPK pathways (Fig. 6). Response patterns between the two animals are similar for a number of other pathways and correspond to hallmarks of persistent viral infection reported in the literature. These include chemokine and Jak/STAT signaling (Mellado et al., 2001), TGFB signaling (Bettaccini et al., 2005), and PI3K/AKT signaling (Mizutani et al., 2004) (data not shown).

We were able to find a consistently regulated network of genes that were common to both animals 83 and 84 (Fig. 7A). Networks are sets of genes that are biologically related based on references in the literature. A heat map of the genes from this network clearly shows the consistency of regulation between the two infected animals as well as the distinct pattern seen in the apparently uninfected animal 95 (Fig. 7B). At day 3 post-infection, this entire group of genes was seen to be regulated in both animals, and two of these genes, Egfr and Angpt2, show consistent regulation in both animals at day 6 post-infection. This particular network of genes centers around Egfr, a known target for down-regulation by *gag* (Valiathan and Resh, 2004). The up-regulation of Cdkna1 seen in these samples may be important, as a deficiency in this particular protein reportedly ameliorates systemic autoimmunity (Lawson et al., 2004). Other genes contained in this network worth noting include Mcm7 and Mcm5 which are essential for initiation of eukaryotic genome replication (Tsao et al., 2004), and Fkbp4 which interacts with Irf4 to play a role in immunoregulatory gene expression in T and B lymphocytes (Mamane et al., 2002).

### *SIV Tat and/or Vpr may play a direct role in regulating the expression of select host genes*

As a means to further identify differentially expressed genes that may be relevant to immunodeficiency virus infection, we compared our list of differentially expressed genes with the cellular genes listed in the HIV-1, Human Protein Interaction Database (NIAID HIV Protein Interaction Project). Using this approach, we identified genes with ≥2-fold change in expression (in at least one experiment) that encode proteins that have a previously reported direct association with one or more HIV-1 proteins. Although the bulk of the reports in the HIV-1-Human Protein Interaction Database describe protein–protein interactions, a few deal specifically with the regulation of gene expression. In this regard, our microarray analyses detected the up-regulation of ARHGDIA and VIL2 (Matarrese et al., 2000), RELA (Leifert et al., 2003), CCR2 (McManus et al., 2000), and ITGA5 (Maroder et al., 1996) which have been reported previously in association with Vpr and/or Tat. In contrast, several genes reported to be up-regulated by Tat (HLA-E and HLA-G; Leifert et al., 2003) or Vpr (CDH1 (Matarrese et al., 2000) and IFNG (Rautonen et al., 1994)) were either down-regulated or unchanged in our analyses.

Although it can be difficult to make comparisons across divergent experimental platforms, we envision the use of such databases and the ability to integrate gene expression, proteomic, and protein interaction data will be at the foundation of future efforts aimed at a comprehensive understanding the response of cells and tissues to viral infection. For example, it is apparent that not all changes in gene expression result in a corresponding change in the level of protein production and

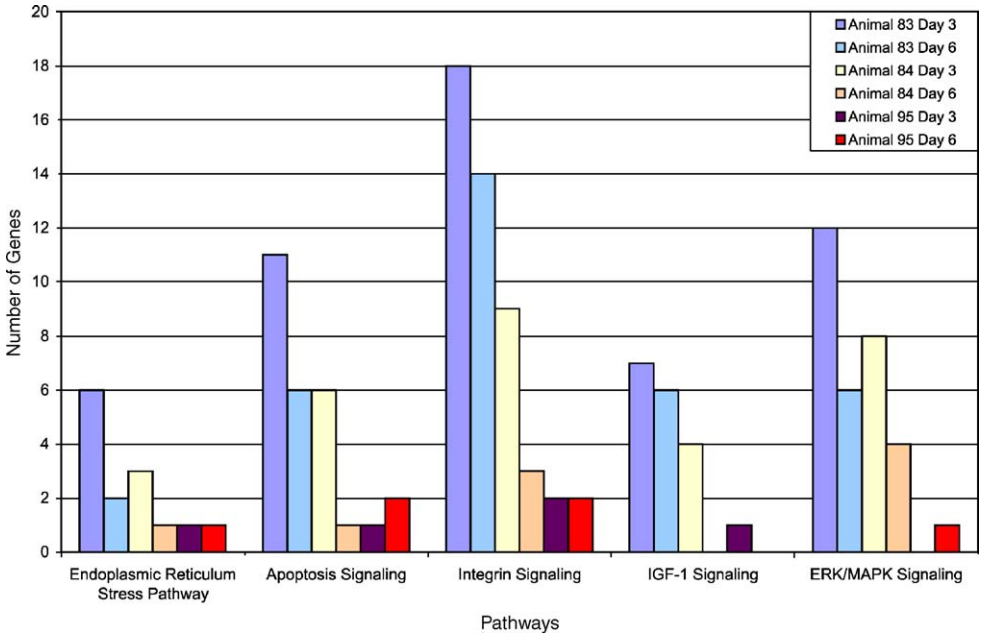


Fig. 6. Similarities in pattern of response to viral infection between animals 83, 84, and 95 at days 3 and 6 post-infection highlight common hallmarks of viral infection. IPA software was used to generate lists of pathways seen to be significantly impacted by our SIV infections in these samples. Consistent patterns of response across time points for pathways, including antigen presentation, endoplasmic reticulum stress, and ERK/MAPK, IGF-1, integrin, and apoptosis signaling, are readily recognizable using this software.

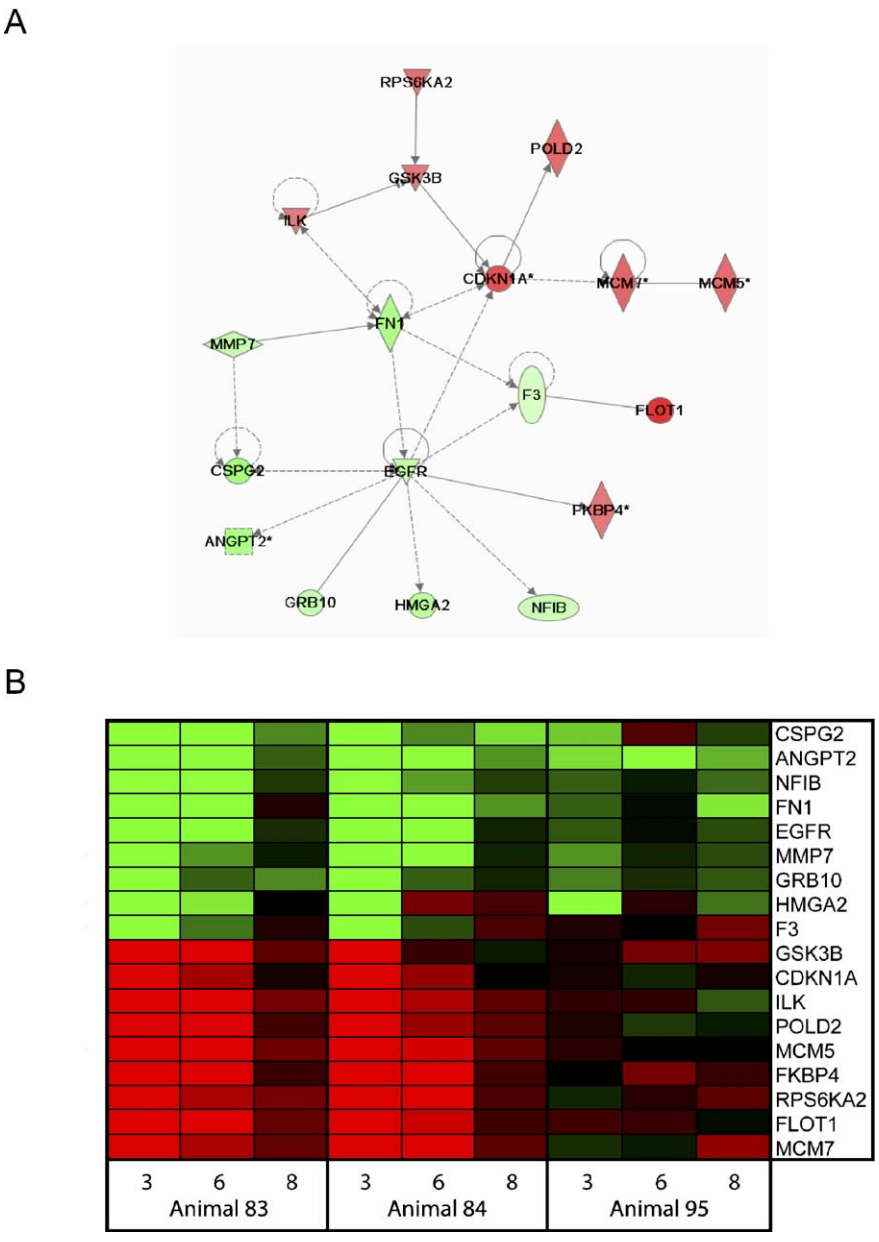


Fig. 7. Network of inter-related genes based on literature information that are seen to be similarly regulated in infections from animals 83 and 84. (A) An example of an Ingenuity Network describing a group of genes regulated in animal 83. This network of genes centers around Egfr, a known target for down-regulation by *gag*. (B) Heat map of all genes represented in the network. The heat map illustrates the consistency of regulation between the two infected animals as well as the distinct pattern seen in the apparently uninfected animal 95.

changes in protein level may not always correlate with protein function or activity (Ideker et al., 2001). Therefore, by integrating diverse types of data, we can obtain a clearer picture of the biological significance of the individual cellular responses detected. Protein interaction data may be particularly important in this regard, and software tools are now available for modeling complex protein-interaction networks (Shannon et al., 2003), and gene expression and proteomic data can be overlaid onto the network to enhance our understanding of the biological processes at work (Prinz et al., 2004; Rand Corporation and Elisa Eiseman, 2004).

Our observations of SIV-induced changes in the expression of genes associated with lymphocyte response and metabolic processes, such as cell growth and maintenance, nucleic acid or protein metabolism, or metabolic regulation, are in general similar to those reported (Vahey et al., 2002) in studies examining gene expression changes in human PBMC responding to HIV infection. HIV was also observed to elicit a transcriptional response in genes associated with cellular activation, transcription, translation, and the immune response. Similar to our findings, the majority of genes differentially expressed in response to HIV exhibited an increase in expression and decreases in gene expression occurred primarily at later time points. Coordinated patterns of cellular gene expression were also reported, but similar to our results, the number of genes that exhibited a coordinated

or sustained response over the duration of the experiment was limited.

In conclusion, our results from these experiments show that individuals supposedly on the same path to disease (such as animals 83 and 84) still exhibit individual variation at the level of gene transcription. We also saw that our animal 95 could provide insight into how certain infected individuals become long-term nonprogressors.

These results highlight the importance of individual variation in response to viral infection. It will be important to discover with greater precision the factors involved in determining these different responses to viral challenge, as they could provide insights into potential cures or vaccines for HIV as well as allow for more efficient use of declining nonhuman primate resources in these types of experiments. These data also provide a novel resource for members of the HIV research community as the first whole animal, nonhuman primate, microarray-based experiment of its kind. Our continuing development of a rhesus-specific microarray will allow for greater elucidation of the mechanisms and pathways being altered in these kinds of infections.

## Materials and methods

### *PMBC preparation*

Peripheral blood was collected from three healthy rhesus macaques (*M. mulatta*), termed animals 83, 84, and 97, and PBMC were recovered by standard Ficol density-gradient centrifugation. The recovered cells were then washed, counted, and placed at a density of $2 \times 10^6$ cells/ml in complete RPMI 1640 growth medium containing 10% fetal bovine serum and 5% lectin-purified IL-2 (Roche Diagnostics). Phytohemagglutinin (Sigma Chemical) was then added at a concentration of 5μg/ml, and after a 24-h incubation period, the medium was replaced with fresh complete medium and the culture was incubated at 37 °C for 3 days. The resulting phytohemagglutinin-activated cells were then filtered to remove dead and aggregated cells, counted, and divided.

### *Virus infection*

Unfractionated PBMC ($5 \times 10^6$ cells/ml) from each of the three animals were infected with SIVmac239 (grown in CEMx174 cells) at a multiplicity of 0.01 TCID50/cell or mock-infected with a volume of culture supernatant from uninfected CEMx174 cells equal to the volume of virus stock. After a 30-min incubation period at 37 °C, unattached virus was removed by 3 washes with phosphate-buffered saline. Mock-infected cells were washed in the same manner. The cells were then divided into 12-well tissue culture plates at $2 \times 10^6$ cells/well in 3 ml of complete medium per well per animal. For each animal, a single well of cells was harvested at each time point, and cells in the remaining wells were maintained by replacing 1.5 ml of medium with fresh medium at 48-h intervals. Cells were counted at harvesting and the percentage of viable cells was determined by trypan blue dye exclusion. The cells were then collected by centrifugation and lysed in solution D as described previously (Chomczynski and Sacchi, 1987). Culture supernatants were monitored for p27 levels by antigen-capture ELISA (ZeptoMetrix Corp.). The percentage of infected cells was estimated by indirect immunofluorescence assay using pooled sera from SIV-infected macaques (Agy et al., 1991).

### *Microarray analysis*

For cDNA array analysis, total RNA was isolated by the acid guanidinium thiocyanate-phenol/chloroform extraction method (Chomczynski and Sacchi, 1987) and amplified twice using the RiboAmp RNA amplification system (Arcturus). The quantity and quality of total amplified RNA were evaluated by capillary electrophoresis using an Agilent Technologies 2100 Bioanalyzer. First-strand cDNA synthesis, probe labeling, and microarray hybridizations were performed as described previously (Geiss et al., 2001). Labeled probes derived from SIV-infected cells were co-hybridized with probes derived from time-matched mock-infected control samples on microarrays containing duplicate spots of cDNAs representing approximately 13,000 human genes (Agilent Technologies). Slides were scanned with an Agilent DNA microarray scanner and image analysis was performed using Agilent Feature Extractor Software. Each microarray experiment was done using a dye-swapping technique, thereby providing 4 technical replicates for each measurement (Kerr and Churchill, 2001). Initially, genes were selected as differentially expressed based on two criteria: a greater than 99% probability of being differentially expressed ($P \leq 0.01$) and an expression level change of 2-fold or greater. All data were entered into a custom-designed database, Expression Array Manager, and then uploaded into Resolver 4.0 (Rosetta Biosoftware) and DecisionSite for Functional Genomics (Spotfire Inc.) for analysis. Biological gene sets (referred to as Biosets) were compiled for various cellular processes by selecting genes of interest that were both represented on the microarray and which had Gene Ontology (GO) annotation (Harris et al., 2004). Once compiled, Biosets were used to characterize and compare gene expression patterns between experiments. These Biosets were also analyzed using Pathway Miner (Pandey et al., 2004) and the Ingenuity Pathway Analysis (IPA) Software (Ingenuity Systems, Mountain View, CA) to assign functional information and biological relevance to the groups of genes being regulated. In accordance with proposed standards (Brazma et al., 2001), all data described in this report, including sample information, intensity measurements, gene lists, error analysis, microarray content, and slide hybridization conditions, are available in the public domain through Expression Array Manager at http://www.expression.washington.edu/public.

### *qRT-PCR*

Quantitative real-time PCR (rtPCR) was used to validate the gene expression changes. Total RNA samples were treated with DNase using DNA-*free* DNase Treatment and Removal

Reagents (Ambion Inc.). Reverse Transcription was performed using TaqMan Reverse Transcription Reagents from Applied Biosystems (ABI). Primer and probe sets for each of the target sequences were designed using macaque EST sequences available from Macaque.org and mapping them to the *M. mulatta* genome available from Baylor. rtPCR was performed on the ABI 7500 Real-time PCR System, using TaqMan chemistry (ABI). Each target was run in quadruplicate, with 20 μL reaction volumes of TaqMan 2× PCR Universal Master Mix (ABI). GAPDH and 18S were chosen as endogenous controls to normalize quantification of the target. Quantification of each gene relative to the calibrator was calculated by the instrument using the standard equation in the ABI Sequence Detection Software version 1.3:$2^{-\Delta\Delta CT}$. The probe used for SIV gag was previously described (Leutenegger et al., 2001).

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