# The Host Response to Adenovirus, Helper-dependent Adenovirus, and Adeno-associated Virus in Mouse Liver

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Understanding host responses to viral gene therapy vectors is necessary for the development of safe and efficacious *in vivo* gene transfer agents. We describe the use of high-density spotted complementary DNA microarrays in monitoring the *in vivo* host transcriptional responses in mouse liver upon administration of either a "first-generation" adenoviral (Ad) vector, a helper-dependent "gutless" adenoviral (HD) vector, or an adeno-associated viral (AAV) vector containing human factor IX (hFIX) expression cassettes. Since HD and AAV do not contain any viral genes, they allow us to assess the host response to the viral capsid and packaged nonviral DNA in whole animals. Comparison of the host response to Ad and HD helps assess the importance of leaky adenoviral gene expression. While all three vectors induced characteristic temporally sequenced programs of gene expression, the gene expression programs induced by the Ad and HD adenovirus vectors were remarkably similar, including the induction of a prominent type I interferon (IFN)-dependent cluster within 6 hours of administration. In contrast, the AAV-based vector caused far fewer alterations of host-gene expression. Our results indicate that recognition of the Ad capsid or double-stranded DNA (of nonviral origin) in the vector elicits a robust type I IFN response that is, however, not elicited by AAV-derived vector transduction.

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## INTRODUCTION

Viral infection in animals invokes both innate and adaptive immune responses resulting in a significant alteration of gene expression in a diversity of cell types. DNA microarrays are a powerful tool for examining virus–host interactions at the transcriptional level. Expression profiling of cultured cells is informative, but cannot capture the complex cross talk between cells *in vivo*. We therefore conducted transcriptional profiling of the host response to "first generation" adenovirus (Ad), helper-dependent Ad, and adeno-associated virus (AAV) after vascular delivery of these vectors into mouse liver.

Ad is a pathogenic nonenveloped DNA virus, and "first generation" serotype 5 Ads containing deletions in the E1 and E3 genes are common gene delivery vehicles. E1 and E3 deletion reduces expression of late Ad genes. Because Ad efficiently infects many cell types *in vivo*, there have been numerous Ad clinical trials (http://clinicaltrials.gov). However, Ad delivery to the liver induces innate and adaptive immune responses, resulting in transient transgene expression. Toxicity is in part due to leaky expression of the remaining viral genes, leading to cytotoxic T lymphocyte–mediated killing of transduced cells. Helper-dependent "gutless" adenoviral (HD) vectors replace all viral genes with mammalian DNA. However, HD still induces innate immune responses, although these may be attenuated. In 1999, a patient in a clinical trial died of acute toxicity shortly after receiving an Ad vector, emphasizing the urgent need to understand innate immune responses to viral vectors *in vivo*.

Single-gene studies have shown that Ad induces the expression of various chemokines[1] as well as proinflammatory cytokines such as tumor necrosis factor-$\alpha$, interleukins (ILs)[1] such as IL-6 and IL-1$\beta$[2,3] as well as interferon-$\gamma$ (IFN-$\gamma$).[4] Microarrays have been used to study the transcriptional response of cultured cells to wild-type Ads (ref. 5 and references therein). The transcriptional response to Ad has also been studied in cultured cells[6,7] and in mice.[8] Previous studies indicated that the capsid might be a major determinant of immune recognition.[9] Indeed, in cultured cells, empty Ad capsids elicited a similar response to that of Ad.[6] Recent studies have implicated Toll-like receptors (TLRs) in the recognition of Ad.[7,8,10] Another report suggests that viral double-stranded DNA might be important for Ad detection.[11]

AAV is a nonpathogenic, nonenveloped single-stranded DNA virus from the family, Parvoviridae. AAV serotype 2 vectors

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contain no protein coding viral DNA sequences, but do contain short inverted terminal repeats at both ends. Based on many preclinical studies, AAV is considered a promising gene therapy vector because of its low immunogenicity and long-term persistence. Compared to Ad, there are few studies of the host transcriptional response to AAV. A single-gene study in HeLa cells revealed no induction of IP-10, RANTES, MIP-1$\alpha$, or MIP-2, although *in vivo* administration revealed a demonstrable increase in these cytokines.[12] A microarray study in a human embryonic lung fibroblast cell line with AAV as well as empty capsids indicated a limited response to AAV.[13] Nevertheless, AAV vector administration in humans revealed apparently capsid-dependent cellular responses, and an inflammatory response leading to a transient transaminitis, and production capsid-specific $CD8^+$ T cells.

We are unaware of any reports of microarray analysis of the *in vivo* host response to HD or AAV. A comparison of the host response to Ad and HD vectors may provide insights into the role of the capsid and/or encapsidated DNA versus viral gene products in the host recognition of Ad. Likewise, since AAV also does not express viral genes, it also allows us to focus on the role of the capsid and/or encapsidated DNA and to determine if host responses to different viruses share common features. The response to AAV vectors may also provide insight into the transient inflammatory response and cellular immunity observed. Therefore, we used high-density spotted DNA microarrays to measure the host transcriptional response in mouse liver to infection with Ad, HD, and AAV.

## RESULTS

To assess host transcriptional responses in mouse liver, mice were injected via the tail vein with phosphate-buffered saline, Ad, HD, or AAV (see Materials and Methods) encoding human factor IX (hFIX). Because very different numbers of infectious particles are required to achieve the same levels of transgene expression for Ad versus AAV, viral doses were chosen to obtain similar peak serum hFIX levels (**Figure 1**). This would allow for comparison of the host response elicited (in response to the vector doses required to achieve the same level of transgene expression) and provided us with an objective criterion for deciding upon the doses of Ad, HD, and AAV in order to make this comparison. At the time points of 1 hour, 6 hours, 72 hours, or 4 weeks postinjection, RNA was prepared, and microarrays were hybridized (see Materials and Methods). Overall, 1,106 genes (including some represented more than once) were differentially expressed by an average of at least threefold under at least one time point or condition. **Figure 2** shows a clustergram of these genes. For brevity, fold changes for discussed genes are listed in **Table 1**, and **Table 2** shows the quantitative reverse transcription-PCR (RT-PCR) for a subset of genes. In **Table 1**, 14 genes that, to our knowledge, have not been previously shown to be differentially expressed in response to Ad, HD, or AAV are shown in bold. Ad and HD modulated the most genes with far fewer altered by AAV. A coherent cluster of genes was induced by Ad and HD at the time point of 1 hour and induction of these genes generally continued at later time points (**Figure 2II**). A large and coherent cluster, mostly of IFN-responsive genes, was

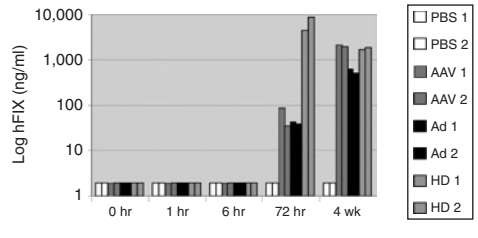


**Figure 1 Plasma human factor IX (hFIX) levels in mice after treatment with adenovirus (Ad), helper-dependent "gutless" adenovirus (HD), or adeno-associated virus (AAV) vectors.** Vector doses were chosen such that peak hFIX levels would be similar between groups. Two mice per group were injected with $5 \times 10^8$ transducing units of Ad or HD, or $3 \times 10^{11}$ vector genomes of AAV. PBS, phosphate-buffered saline.

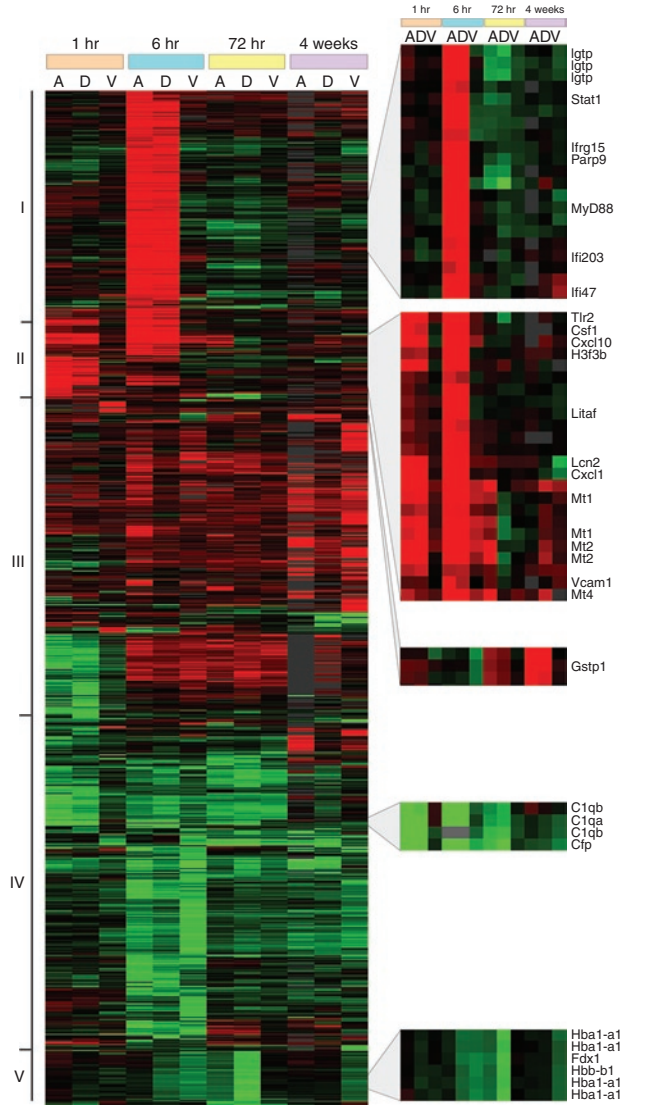


**Figure 2 Clustergram of genes differentially regulated upon infection with adenovirus (A), helper-dependent adenovirus (D) or adeno-associated virus (V).** Red and green indicate increased and decreased expression, respectively, relative to mock-transduced animals. Clusters 2I, 2II, and 2IV are discussed in the text, while cluster 2.V represents a deceptively large cluster of genes exhibiting uniformly weak repression by AAV at the time point 6 hours and Ad at 72 hours, and more powerful repression by helper-dependent "gutless" adenovirus (HD) at 72 hours. However, this cluster largely represents genes Hemoglobin-$\alpha$ adult chain (*Hba-a1*) and Hemoglobin-b pseudogene bh3 (*Hbbbh3*), which are repeatedly present on our arrays, as well as Ferritin heavy chain (*Fdx*), and H3 Histone family 3A (*H3f3a*). The significance of this cluster is unknown. Cluster 2III is discussed in **Supplementary Data S1**.

**Table 1 Microarray fold changes for genes discussed in the article**

| | 1 hour | | | 6 hours | | | 72 hours | | | 4 weeks | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ad | HD | AAV | Ad | HD | AAV | Ad | HD | AAV | Ad | HD | AAV |
| ***Bmpr1a*** | 1.7 | 2.0 | 1.3 | 2.5 | 1.5 | 1.9 | 1.7 | 1.7 | 1.3 | **4.6** | 2.4 | **4.4** |
| *C1qa* | | | −1.1 | | −11.2 | −3.3 | −3.3 | −4.1 | −1.4 | −1.3 | −2.0 | −1.6 |
| *C1qb* | −8.8 | −6.8 | −1.0 | −7.8 | −5.7 | −1.7 | −2.7 | −3.5 | −1.5 | −1.2 | −1.4 | −1.7 |
| *112* | **−14.9** | **−9.5** | −1.5 | | | −1.7 | **−3.0** | **−3.3** | −1.5 | −1.2 | −1.4 | −1.7 |
| *Ccl7* | 1.2 | −1.5 | | | 7.0 | | 1.8 | | | | | |
| ***Cd74*** | −1.5 | −1.6 | −1.2 | −2.3 | −2.8 | −1.7 | **−3.0** | **−3.5** | −1.4 | 2.1 | 1.9 | −1.2 |
| ***Cfp*** | | −3.7 | 1.2 | | −3.7 | −1.4 | −2.3 | −2.6 | −1.5 | | −1.6 | −1.4 |
| ***Cish*** | **3.4** | 2.9 | 1.5 | 1.7 | 1.7 | 2.2 | **−3.1** | **−4.0** | −1.6 | | −1.5 | 1.2 |
| *Csf1** | **3.2** | | 1.2 | **3.9** | **3.6** | 1.5 | −1.1 | −1.5 | 1.1 | | | |
| *Cxcl1* | **14.6** | **15.6** | −1.4 | **5.1** | **5.2** | 1.6 | 1.3 | 1.9 | 1.5 | | 1.4 | |
| *Cxcl10* | **19.6** | **17.4** | −1.3 | **69.8** | **60.3** | 2.3 | −1.2 | −1.2 | 1.2 | | 1.8 | 1.3 |
| ***Dusp6*** | 1.4 | −1.3 | −1.1 | −1.2 | 1.2 | −1.3 | 1.3 | −1.1 | −1.7 | −1.5 | −2.6 | **−5.7** |
| ***Fabp5*** | −1.8 | −1.6 | **−3.5** | −1.6 | −1.3 | −1.3 | **−3.2** | −1.8 | −1.1 | 2.4 | 1.1 | 1.3 |
| *Fdx* | 1.2 | 1.1 | 1.2 | 1.1 | −1.7 | −2.0 | −1.5 | **−3.2** | −1.1 | 1.4 | −1.4 | −1.5 |
| *Fos* | 1.1 | 1.1 | 1.1 | 1.4 | 1.1 | 1.3 | 1.4 | 1.3 | 1.2 | | −2.9 | **−3.4** |
| *Gstp2* | 1.4 | 1.4 | −1.4 | −1.2 | −1.2 | −2.0 | 2.3 | 1.9 | | **5.0** | **5.2** | |
| *Gzma* | **3.1** | 1.5 | 1.4 | **6.7** | | 1.9 | 1.6 | 1.4 | 1.1 | | | 1.8 |
| ***H2ab1*** | −1.3 | −1.2 | −1.2 | −2.5 | −2.4 | −2.7 | **−3.1** | −2.7 | −1.6 | 1.6 | 1.4 | −1.6 |
| *H3f3a* | 1.9 | −1.4 | −1.3 | −1.2 | −1.7 | −2.2 | −1.8 | **−7.6** | −1.3 | −1.2 | −1.2 | −1.7 |
| *Hbaa1* | 1.7 | −1.3 | −1.2 | −1.2 | −1.7 | −2.0 | −1.8 | **−3.4** | −1.1 | −1.6 | −1.1 | −1.7 |
| ***Hoxc4*** | **3.7** | **4.4** | 1.4 | **9.4** | **14.9** | **3.1** | 2.6 | −1.9 | | −1.2 | 1.7 | 1.3 |
| *Icam1* | **12.4** | **9.4** | 1.6 | 2.9 | 2.2 | 1.8 | 1.1 | 1.1 | 1.2 | 1.6 | 1.7 | 1.5 |
| *Ifi204* | 1.3 | −1.5 | −1.3 | **5.3** | **4.9** | 1.1 | | −1.5 | 1.5 | 1.3 | 1.1 | 1.3 |
| ***Ifnar2*** | 1.1 | 1.1 | −1.5 | **5.6** | **5.5** | −1.3 | −1.2 | −1.1 | −1.1 | −1.1 | −1.6 | −1.3 |
| *Igtp* | 1.5 | 1.4 | 1.4 | **19.7** | **24.3** | 1.3 | −1.6 | −1.5 | −1.3 | 1.2 | 1.1 | 1.3 |
| *Il1b* | **13.8** | **5.4** | 1.6 | **3.7** | **3.4** | −1.1 | −1.3 | −1.6 | −1.5 | −1.2 | 1.3 | 1.4 |
| *Irf1* | 2.1 | 2.2 | 0.4 | **47.8** | **45.6** | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 | 0.3 | 0.4 |
| ***Irf4*** | −0.1 | −0.2 | −0.6 | **6.0** | **6.7** | 0.9 | 0.7 | 0.6 | 1.0 | 1.1 | −0.8 | −0.7 |
| *Irf7** | −1.2 | −1.4 | −2.2 | **7.2** | **9.0** | −2.3 | −1.4 | −1.7 | −1.9 | 1.3 | −1.1 | −2.4 |
| *Isg20* | −1.1 | −1.7 | −1.3 | **11.2** | **8.8** | 1.4 | | 1.8 | 1.4 | | | |
| *Jun** | **3.0** | 2.8 | −1.2 | **5.6** | **3.8** | 1.8 | 1.3 | 1.4 | 1.3 | 1.4 | −1.9 | 1.5 |
| ***Junb*** | 2.3 | **3.1** | −1.7 | 2.3 | 1.9 | | −2.3 | −2.6 | −1.8 | **−3.0** | −2.9 | −2.7 |
| Lcn2 | **5.8** | **3.8** | 1.4 | **60.2** | **39.7** | 1.8 | 1.7 | 1.4 | 1.4 | 1.4 | −1.1 | −2.7 |
| *Mt1* | **3.2** | **4.8** | 1.6 | **9.0** | **7.8** | 2.8 | 2.0 | −1.9 | −1.5 | −1.2 | 1.6 | 1.4 |
| *Mt2* | **6.9** | **10.9** | 1.1 | **12.9** | **13.8** | 2.3 | **5.1** | −1.8 | 1.1 | 1.2 | 2.5 | 1.4 |
| *Mt4* | 2.8 | 1.9 | 1.6 | **10.8** | **9.8** | 2.4 | **7.0** | 1.3 | 1.8 | 1.4 | **3.0** | |
| *Myd88* | 1.8 | 1.3 | 1.4 | **5.0** | **5.4** | 1.1 | 1.1 | −1.1 | −1.2 | −1.6 | −1.4 | −1.5 |
| ***Psmb9**** | 1.1 | −1.4 | 1.2 | **6.4** | **5.7** | 1.4 | −1.1 | −1.8 | −1.1 | 2.6 | 1.8 | −1.4 |
| ***Psme2*** | **3.1** | 1.3 | 1.4 | **52.9** | **47.5** | 1.2 | −2.3 | **−3.0** | −1.6 | 1.7 | 1.6 | 1.3 |
| ***Socs1**** | | 1.7 | | **6.4** | **5.7** | 1.7 | 1.3 | −1.1 | −1.1 | | | |
| *Stat1* | 1.3 | −1.4 | 1.3 | **11.7** | **10.3** | −1.4 | −1.9 | −1.7 | −1.3 | −1.4 | −1.1 | −1.6 |
| *Stat2* | −1.5 | −1.6 | −1.1 | 2.5 | **3.1** | −1.3 | −1.6 | −1.8 | −1.7 | −1.3 | −1.3 | −1.8 |
| *Tlr2* | **7.3** | **5.7** | 1.7 | **17.7** | **13.5** | 1.6 | −1.1 | −2.2 | −1.1 | | 1.4 | −1.9 |
| *Usp18* | 1.6 | 1.1 | −1.9 | **26.2** | **26.5** | −1.6 | −2.9 | | −1.7 | | 1.2 | −1.9 |
| *Vcam1* | **3.8** | **3.4** | 2.1 | **36.7** | **7.3** | | **3.4** | −2.3 | 2.1 | | 2.1 | 3.1 |

The numbers in the columns represent fold changes relative to a mock-transduced animal. Fold changes in excess of threefold are shown in bold. If a gene was present on the array multiple times, the average value is shown. Genes marked with an asterisk were validated by quantitative reverse transcription-PCR (RT-PCR) and these values are listed in **Table 2**. Genes, that to our knowledge, have not been previously shown to be differentially expressed in response to adenovirus (Ad), helper-dependent "gutless" adenovirus (HD), or adeno-associated virus (AAV) are shown in bold. Blank spaces indicate that data for this condition did not pass our filtering criteria.

**Table 2 Quantitative RT-PCR validation of selected genes at selected times**

| | 1 hour | | | 6 hours | | | 72 hours | | | 4 weeks | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ad | HD | AAV | Ad | HD | AAV | Ad | HD | AAV | Ad | HD | AAV |
| *Irf7* | | | | **8.4** (1.6) | **8.3** (0.87) | | | | | | | |
| *Psmb9* | | | | **4.9** (1.0) | **7.9** (0.64) | | | | | | | |
| *Socs1* | | | | **97.5** (11) | **54.6** (7.1) | | | | | | | |
| *Il18bp* | | | | **2.5** (0.22) | **2.1** (0.14) | | | | | | | |
| *Tlr2* | **9.8** (0.64) | **9.3** (2.3) | | **13.5** (2.6) | **9.2** (0.33) | | | | | | | |
| *Csf1* | **11.6** (0.18) | **8.6** (0.11) | | **9.4** (0.12) | **6.1** (0.11) | | | | | | | |
| *Il1b* | **5.1** (0.34) | **3.5** (0.29) | | **1.4** (0.02) | **−2.0** (0.17) | | | | | | | |
| *Usp18* | **1.4** (0.36) | **1.1** (0.22) | **1.9** (0.41) | **48.7** (11) | **41.5** (9.2) | **2.1** (0.43) | **−2.0** (0.37) | **1.0** (0.12) | **1.0** (0.23) | **−2.0** (0.59) | **1.1** (0.14) | **−2.5** (0.68) |
| *Jun* | **3.4** (0.74) | **2.8** (0.43) | **−1.1** (0.28) | **6.0** (0.15) | **3.7** (0.64) | **1.1** (0.33) | **−5.0** (1.1) | **−1.1** (0.14) | **−2.5** (0.21) | **−2.5** (0.37) | **2.3** (0.41) | **−1.4** (0.31) |
| Mt1 | **3.5** (0.73) | **3.2** (0.61) | | | | | | | | | | |

*Abbreviations*: AAV, adeno-associated virus; Ad, adenovirus; HD, helper-dependent "gutless" adenovirus.
The numbers in the columns represent average fold changes relative to a mock-transduced animal. Values in parentheses represent the SD of the fold change above. Values were normalized to an internal $\beta$-actin control.

induced in 6 hours' time (Figure 2I). A pathway analysis of gene categories altered by Ad + HD, AAV, or all three viruses is shown in Table 3.

## HD and Ad stimulate a similar response which markedly differs from that to AAV

Despite the complete absence of viral genes in the helper-dependent vector, we observed very similar results for Ad and HD at all time points. The HD and Ad responses, especially at the critical 1-and 6-hour early time points, were dominated by genes involved in pathogen surveillance, host-defence, and an early innate immune response. This suggests that leaky viral gene expression does not play a significant role in the initial innate response. Other categories of genes induced by Ad and HD include nucleic acid and protein metabolism, signal transduction, and transport (Table 3). While limited by the amount of DNA that can be packaged, AAV vectors caused significantly fewer changes to host cell transcription, consistent with previous observations.[12,13]

## Host response to Ad and HD

***The program of gene expression is temporally sequenced: early responses.*** Ad and HD induced a temporally sequenced program of gene expression. At the time point of 1 hour postinjection, we observed a powerful response to Ad or HD that mostly persisted until at least the 6-hour time point (Figure 2II). These initial responses, which include proinflammatory cytokines, probably prime the subsequent and wider-spread responses (Figure 2I). Upregulation of *Il1b* was one of the first events induced in response to both Ad and HD (Tables 1 and 2). IL-1$\beta$ is a powerful proinflammatory cytokine that induces fever, expression of vascular adhesion molecules, and activates many genes involved in inflammation. IL-1$\beta$ signaling can lead to phosphorylation of mitogen-activated protein kinases such as p38, the activation of transcription factor nuclear factor $\kappa$B, and, ultimately, the secretion of IL-6 and tumor necrosis factor-$\alpha$, all of which have been described as responses to Ad vectors in the context of various systems. The importance of IL-1$\beta$ in mediating the early innate response is supported by the observation that Ad vectors elicited dramatically reduced acute inflammatory responses in IL-1$\beta$ knockout animals.[14]

Consistent with previous reports,[13] we observed induction (at the time point of 1 hour) of the immediate-early c-Jun oncogene (*Jun*). JUN is one component of the heterodimeric AP-1 transcription factor, which regulates processes such as proliferation, differentiation, and apoptosis. Ad induced *Jun* expression at the time point of 1 hour and Ad and HD induced *Jun* at the time point of 6 hours (Tables 1 and 2). Together with nuclear factor $\kappa$B, JUN induces transcription of a spectrum of proinflammatory cytokines, which in turn influence direct antimicrobial effects, the cell-mediated immune response and host cell apoptosis. JUN also induces proliferation and/or apoptosis in hepatocytes. The antimicrobial peptide, lipocalin 2, (*Lcn2*) is an AP-1 target gene. Interestingly, it is highly upregulated by Ad and HD, supporting the idea that they are activating the AP-1 pathway.

Two chemokines, *Cxcl1* and *Cxcl10*, were highly expressed early and coordinately with *Il1b*, possibly contributing to the staged response. CXCL1 is a neutrophil chemoattractant induced by IL-1$\beta$. Another early gene very highly activated by both Ad and HD infection was the chemokine CXCL10. It is important to note that the current study was conducted with whole liver, which is composed of multiple cell types as well as inflammatory infiltrates; it is therefore not known in which cell type these transcripts are induced and the significance of their induction in this organ must be interpreted with caution. These signals may serve to recruit immune effector cells from other organs. Studies of CXCL1 and CXCL10 expression levels in lymph nodes and the spleen would also be informative. Previous studies showed elevation of CXCL10 (also known as IP-10) in response to Ad (ref. 15), even in the absence of viral gene expression.[4,15] CXCL10 is a chemoattractant that stimulates monocytes, natural killer and T-cell migration, and is involved in T-cell maturation. It has been implicated in the host response to many viral pathogens including HIV, Hantavirus, herpes simplex virus type 1, and hepatitis C virus. *Cxcl10* is also induced in dendritic cells upon stimulation with

**Table 3 Biological process assignments for differentially expressed genes**

| | Ad + HD | AAV | Ad + HD | AAV | Ad + HD | AAV | Ad + HD | AAV |
|---|---|---|---|---|---|---|---|---|
| | 1 hour | 1 hour | 6 hours | 6 hours | 72 hours | 72 hours | 4 weeks | 4 weeks |
| Action potential propagation | | | | | 1 | | | |
| Amino acid biosynthesis | | | | | | 1 | | |
| Amino acid catabolism | | | | | | 1 | 1 | 1 |
| Amino acid metabolism | | | 1 | 5 | 1 | 2 | 1 | 2 |
| Amino acid transport | | | | 1 | | | | |
| Anterior/posterior patterning | | | | | 1 | | 1 | |
| Apoptosis | 3 | | 5 | 1 | 1 | 4 | 1 | 3 |
| Asymmetric protein localization | 1 | | | | 1 | 1 | | 1 |
| B-cell- and antibody-mediated immunity | 1 | | 1 | | | | | |
| Blood circulation and gas exchange | 1 | 1 | 1 | | 3 | 1 | | 1 |
| Calcium-mediated signaling | 2 | | 1 | | 1 | 1 | | 1 |
| Carbohydrate metabolism | | | 2 | | 1 | 1 | 3 | 1 |
| Carbohydrate transport | | | 1 | | | 1 | | 1 |
| Cation transport | | | | | 1 | | | |
| Cell adhesion | 1 | | 4 | 1 | 1 | | | |
| Cell adhesion–mediated signaling | 1 | | 3 | | | | | |
| Cell communication | 8 | 1 | 11 | 3 | 3 | 2 | 1 | 2 |
| Cell cycle | 3 | 1 | 2 | 2 | 2 | 2 | 3 | 2 |
| Cell cycle control | 3 | 1 | 2 | 2 | 2 | 2 | 3 | 1 |
| Cell motility | 2 | | 3 | | | | | |
| Cell proliferation and differentiation | 5 | 1 | 5 | 2 | 1 | 4 | 3 | 2 |
| Cell structure | | 1 | 3 | | 1 | | | |
| Cell structure and motility | 2 | 1 | 6 | | 2 | | | |
| Cell surface receptor–mediated signal transduction | 7 | | 13 | 1 | 3 | 4 | 2 | 4 |
| Cholesterol metabolism | | | | | | 1 | | 1 |
| Chromatin packaging and remodeling | 1 | | 2 | 1 | 2 | | | |
| Coenzyme and prosthetic group metabolism | 1 | 2 | | 1 | 5 | 1 | 1 | 1 |
| Complement-mediated immunity | 2 | | 3 | 1 | 3 | | | |
| Cytokine- and chemokine-mediated signaling pathway | 4 | | 8 | | | 1 | 2 | 1 |
| Cytokine-/chemokine-mediated immunity | 3 | | 3 | | 1 | | | |
| Detoxification | | | | | | | 2 | |
| Developmental processes | 5 | 1 | 7 | 3 | 7 | 3 | 3 | 3 |
| Ectoderm development | 2 | 1 | 3 | 1 | 2 | 1 | | 1 |
| Electron transport | | 1 | | | 2 | 1 | 1 | 1 |
| Endocytosis | | | 3 | | | | | |
| Exocytosis | | | | 2 | 1 | 1 | 1 | 1 |
| Extracellular matrix protein–mediated signaling | 1 | | | 1 | | 1 | | 1 |
| Extracellular transport and import | | | 1 | | | | | |
| Fatty acid metabolism | | 1 | | | 1 | | 1 | |
| Ferredoxin metabolism | | | | | 1 | | | |
| General mRNA transcription activities | | | | | | | 1 | |
| General vesicle transport | | | | | | 1 | 1 | 1 |
| Glycogen metabolism | | | | | 1 | | 1 | |
| Glycolysis | | | 1 | | | | 1 | |

**Table 3 continued on next page**

**Table 3 (continued)**

| | Ad + HD | AAV | Ad + HD | AAV | Ad + HD | AAV | Ad + HD | AAV |
|---|---|---|---|---|---|---|---|---|
| | 1 hour | 1 hour | 6 hours | 6 hours | 72 hours | 72 hours | 4 weeks | 4 weeks |
| G protein–mediated signaling | 3 | | 4 | 1 | 3 | 2 | | 2 |
| Granulocyte-mediated immunity | 1 | | 3 | | | | | |
| Growth factor homeostasis | 1 | | | 1 | | 1 | | 1 |
| Hematopoiesis | | | 1 | | | | | |
| Homeostasis | 2 | | 2 | 1 | 1 | 3 | | 3 |
| Immunity and defense | 9 | | 22 | 1 | 7 | 4 | 6 | 4 |
| Induction of apoptosis | | | 1 | | | | 1 | |
| Inhibition of apoptosis | 1 | | 1 | | 1 | 1 | | |
| Interferon-mediated immunity | 1 | | 12 | | | | 2 | |
| Intracellular protein traffic | | | 4 | 4 | 3 | 2 | 2 | 2 |
| Intracellular signaling cascade | 7 | 1 | 6 | 1 | 4 | 3 | 1 | 3 |
| Ion transport | | | | | 1 | | | |
| JAK-STAT cascade | 1 | | 1 | | 1 | | | |
| JNK cascade | 3 | 1 | 2 | | 1 | 1 | 1 | 1 |
| Ligand-mediated signaling | 5 | | 7 | | 1 | 1 | | 1 |
| Lipid and fatty acid binding | | 1 | | | 1 | | | |
| Lipid and fatty acid transport | 1 | 1 | | | 2 | | | |
| Lipid, fatty acid, and steroid metabolism | 2 | 2 | | 2 | 4 | 2 | 2 | 2 |
| Lysosome transport | | | | | 1 | | | |
| Macrophage-mediated immunity | 4 | | 6 | | 1 | | 1 | |
| MAPKKK cascade | | | 1 | 1 | 1 | 1 | | 1 |
| Meiosis | | | | 1 | 1 | | | |
| Mesoderm development | | | 1 | | | 1 | 1 | 1 |
| MHCII-mediated immunity | | | | | 2 | | | |
| Monosaccharide metabolism | | | 1 | | | | 1 | |
| mRNA splicing | | | 1 | | 1 | 1 | | 1 |
| mRNA transcription | 5 | 1 | 11 | 5 | 6 | 6 | 7 | 6 |
| mRNA transcription regulation | 5 | 1 | 8 | 3 | 5 | 6 | 6 | 6 |
| Muscle contraction | 1 | | | | 1 | 1 | 1 | 1 |
| Natural killer cell-mediated immunity | | | 2 | | | | | |
| Neurogenesis | 1 | | 3 | 1 | 1 | 1 | | 1 |
| Neuronal activities | 3 | | 2 | 3 | 4 | 1 | 1 | 1 |
| Neurotransmitter release | | | | 1 | | 1 | 1 | 1 |
| NF-κB cascade | 1 | | 3 | | | | | |
| Nitrogen metabolism | | | | 1 | | | | |
| Nucleoside, nucleotide, and nucleic acid metabolism | 7 | 1 | 16 | 6 | 9 | 7 | 7 | 7 |
| Oncogene | 2 | 1 | 1 | | 1 | 2 | 2 | 2 |
| Oncogenesis | 3 | 1 | 7 | 1 | 1 | 3 | 2 | 3 |
| Peroxisome transport | | | | 1 | | | | |
| Phosphate metabolism | | | | | | 1 | 1 | |
| Pre-mRNA processing | | | 1 | | 1 | 1 | | 1 |
| Protein biosynthesis | 1 | | 1 | | 2 | | 1 | |
| Protein complex assembly | | | | | 1 | | | |
| Protein folding | | | | 1 | | 1 | 1 | 1 |

**Table 3 continued on next page**

**Table 3 (continued)**

| | Ad + HD | AAV | Ad + HD | AAV | Ad + HD | AAV | Ad + HD | AAV |
|---|---|---|---|---|---|---|---|---|
| | 1 hour | 1 hour | 6 hours | 6 hours | 72 hours | 72 hours | 4 weeks | 4 weeks |
| Protein glycosylation | | | | | | | 1 | |
| Protein metabolism and modification | 5 | | 11 | 2 | 11 | 6 | 10 | 6 |
| Protein modification | 1 | | 1 | | 3 | 3 | 4 | 3 |
| Protein phosphorylation | 1 | | 1 | | 1 | 3 | 3 | 3 |
| Protein targeting and localization | 1 | | | | 1 | 1 | | 1 |
| Proteolysis | 3 | | 9 | 1 | 6 | 2 | 4 | 2 |
| Purine metabolism | | | 2 | | | | | |
| Receptor-mediated endocytosis | | | 1 | | | | | |
| Receptor protein serine/threonine kinase signaling pathway | | | 1 | | | 1 | 1 | 1 |
| Regulation of lipid, fatty acid, and steroid metabolism | 1 | | | 1 | | 1 | | 1 |
| Regulation of vasoconstriction, dilation | 1 | | 1 | | | 1 | | 1 |
| RNA catabolism | 1 | 1 | 1 | | 1 | 1 | 1 | 1 |
| rRNA metabolism | 1 | | 1 | | | | | |
| Segment specification | 1 | | 1 | 1 | | | | |
| Sensory perception | | | | 2 | | | 1 | |
| Signal transduction | 17 | 2 | 19 | 5 | 10 | 11 | 4 | 11 |
| Skeletal development | | | | | | 1 | 1 | 1 |
| Small molecule transport | 1 | | 2 | | | 1 | | 1 |
| Steroid hormone–mediated signaling | | 1 | 1 | | 1 | | | |
| Steroid metabolism | | | | | 1 | 1 | | 1 |
| Synaptic transmission | | | | 1 | | 1 | 1 | 1 |
| T cell–mediated immunity | 1 | | 3 | | 2 | 1 | | |
| TGF-$\beta$ signaling pathway | 1 | | | | | | | |
| Translational regulation | | | | | 1 | | 1 | |
| Transport | 2 | 3 | 7 | 3 | 10 | 4 | 1 | 4 |
| Vision | | | | 2 | | | 1 | |
| Vitamin metabolism | 1 | | | | 2 | 1 | 1 | 1 |
| Vitamin/cofactor transport | | 2 | | | 3 | | | |

*Abbreviations*: MAPKKK, mitogen-activated protein kinase kinase kinase; MHCII, class II major histocompatibility complex; mRNA, messenger RNA; NF-κB, nuclear factor κB; rRNA, ribosomal RNA; STAT, signal transducer and activator of transcription; TGF-$\beta$, tumor growth factor-$\beta$.
Pathway analysis was conducted using the Panther algorithm[48,49] to assign genes to functional categories. The number of differentially expressed genes in a given category for adenovirus (Ad) + helper-dependent "gutless" adenovirus (HD), adeno-associated virus (AAV) or Ad + HD and AAV are plotted. Note that each gene may be assigned to more than one biological process.

the TLR 9 ligand, CpG DNA. Significantly, *Cxcl10* is a "first wave" IFN-stimulated gene regulated directly by IRF3. Interestingly, *Cxcl1* and *Cxcl10* were specifically downregulated upon infection by wild-type Ad;[16,17] thus their regulation may be significant for the normal host response. This illustrates the point that E1 and E3 deletion may sacrifice the immunomodulatory effects of these regions. CXCL10 modulates expression of the cellular adhesion molecule intercellular adhesion molecule-1, which mediates lymphocyte extravasation into inflamed tissue. Ad and HD strongly upregulate *Icam1* by the time point of 1 hour. *Icam1* is induced by proinflammatory cytokines, including IL-1$\beta$, tumor necrosis factor-$\alpha$, IFN-$\gamma$, and IL-6.

In 6 hours Ad and HD induced chemoattractant (*Ccl7* by HD and *Csf1* by Ad and HD) and cell adhesion genes (*Vcam1*). Chemokine (C–C motif) ligand 7 attracts macrophages and colony stimulating factor 1 (CSF1) promotes the maturation and survival of mononuclear phagocytes. Vascular cell adhesion molecule-1, induced by IL-1$\beta$, promotes leukocyte adhesion and emigration at the site of vascular injury. Together, induction of these genes points to a coordinated host effort to recruit, promote, and retain subsets of effector cells. Again, however, because the current study was restricted to the liver, caution is warranted in interpreting these results. It would be interesting to test the levels of induction of these molecules in the spleen and lymph nodes.

Another prominent group with a similar early expression pattern was the metallothionein (MT) family (*Mt1*, *Mt2*, *Mt4*). MTs are stress-responsive metal-binding proteins induced by inflammation. They were highly elevated at early time points and more persistently by Ad, indicating the role played by leaky Ad gene expression in the maintenance of MT induction (**Figure 2II**). MT induction may protect cells from reactive oxygen species generated by immune effector cells. Expression of MTs is stimulated by many

factors, including IL-1$\beta$, NO, $H_2O_2$, and IL-6. Overexpression of MTs upregulates *Csf1* messenger RNA expression, suggesting another possible role for the powerful MT induction.

Other early-induced genes are involved in TLR or IL-1 pathways, such as *Tlr2*. *Tlr2* expression is stimulated by lipopolysaccharide, tumor necrosis factor-$\alpha$ or IL-1$\beta$, as well as by viral pathogens. TLR2 recognizes lipoproteins as well as the human cytomegalovirus, herpes simplex virus type 1 and measles virus. *Tlr2* was significantly upregulated by Ad and HD at the time point of 1 hour and highly upregulated by Ad and HD at 6 hours. TLR, IL-1, and IL-18 signaling occurs through the adapter protein MYD88. *Myd88* was upregulated fivefold by Ad and HD at the time point of 6 hours. The induction of *Myd88* could sensitize the cells to further stimulation by IL-1, IL-18, or detection of viral products via the TLRs. *Myd88*$^{-/-}$ mice fail to generate Th1 responses upon TLR stimulation; therefore, *Myd88* modulation may be important for creating a bias toward a Th1 response in some organs. Stimulation of TLRs by diverse TLR ligands is known to inhibit *in vivo* viral replication in a type I IFN-dependent manner. A number of genes linked to the TLR and IL-1$\beta$ signaling pathways were also upregulated by Ad and HD (see later). As described earlier, TLRs have recently been implicated in the recognition of Ad.[7,8,10] Another report suggests that viral double-stranded DNA might be important for viral detection,[11] consistent with the observation that empty Ad capsids stimulated far less gene expression than Ad in cultured cells.[13]

***A prominent IFN-dependent cluster.*** At the time point of 6 hours, a large cluster of genes, dominated by IFN-responsive genes, was activated by Ad and HD, but not AAV (**Figure 2I**). Induction of these genes likely results from both a primary IFN response and a secondary response following autocrine and paracrine detection of IFN. IFN responses occur in two waves, with infected cells initially secreting type I IFNs (IFN-$\beta$ and/or IFN-$\alpha$s). When bound by the type I IFN receptor, these lead to the activation of signal transducer and activator of transcription 1 (STAT1) and STAT2, and the formation of ISGF3, a transcription factor that stimulates a broader spectrum of IFN-responsive genes. *Stat1* is upregulated by Ad vectors, and is specifically inhibited by E1A in wild-type Ad.[18] However, the E1A region is deleted in most Ad vectors. STAT1 is an important link to adaptive immunity: engagement of the type II IFN receptor by IFN-$\gamma$ secreted from T cells and natural killer cells promotes STAT1 activation, inducing the expression of a large set of IFN-$\gamma$ responsive genes, which partially overlaps the set regulated by type I IFN. *Stat1*$^{-/-}$ mice are highly susceptible to viral infection. IRF7 is a regulator of type I IFNs via the TLR- and MYD88-dependent pathway and the virus-activated intracytosolic MYD88-independent pathway. Secreted type I IFNs also induce the expression and activation of IRF7 in lymphoid cells. IRF7 transactivation is important for the elaboration of a cellular antiviral state. Notably, both *Stat1* and *Irf7* are upregulated by Ad and HD at the time point of 6 hours. *Stat2* was 3-fold upregulated by HD (and 2.5-fold by Ad) at the time point of 6 hours. We also observe the induction of IRF1, which is required for induction of type I IFNs by double-stranded RNA in the mouse, is known to interact with MYD88,[19] and has been implicated in Ad clearance.[20] Further evidence of IFN feedback is the induction at the time point of 6 hours by Ad and HD of a gene encoding a component of the type I IFN receptor 2 (*Ifnar2*). Upregulation of *Ifnar2* likely contributes to a positive feedback loop making cells more responsive to type I IFNs, leading in turn to activation of ISGF3 via STAT1 and STAT2. Ad and HD induce *Myd88,* which could also sensitize the cells to further stimulation by IL-1, IL-18, or detection of viral products by the TLRs. Moreover, MYD88 (complexed with TRAF6) interacts with and activates IRF7. In addition to the genes mentioned earlier, we observe the induction of numerous IFN-responsive genes (see **Supplementary Data S1**).

***Negative regulators of IFN signaling.*** Because of potentially destructive consequences, the host inflammatory response is tightly regulated. Several genes that dampen inflammation were activated by Ad and HD infection. These include suppressor of cytokine signaling 1 (*Socs1*), cytokine-inducible SH2-containing protein (*Cish*) and ubiquitin specific protease 18 (*Usp18*) and IRF4. SOCS1, which is induced by IL-1$\beta$, serves to dampen cytokine signaling and directly inhibits TLR4 signaling.[21] SOCS1 also inhibits activation of intercellular adhesion molecule-1 by IFN-$\gamma$,[22] which might explain why expression of intercellular adhesion molecule-1 in response to Ad and HD does not persist. CISH is also a suppressor of cytokine signaling that is upregulated following stimulation of TLRs;[23] it is induced by Ad and HD at the time point of 1 hour and suppressed at 72 hours. As previously described for Ad,[7] Ad and HD also highly upregulate another negative regulator, USP18. *Usp18* is a "first wave" IFN-stimulated gene[7] encoding a negative regulator of type I IFNs which forms part of an auto-regulatory loop limiting IFN signaling. Finally, IRF4, which negatively regulates TLR-mediated proinflammatory gene expression,[19] was upregulated at the time point of 6 hours. We are not aware of any reports of Ad or HD induction of *Socs, Cish, or IRF4*. This highlights the utility of mouse models in which feedback between multiple cell types can be observed.

***Effectors of antigen presentation.*** Virus-infected cells elaborate a modified proteasome, called the immunoproteasome, to produce peptides for major histocompatibility complex class I presentation. At the time point of 6 hours, Ad and HD induce the immunoproteasome component, proteasome (prosome, macropain) subunit, $\beta$ type 9 (*Psmb9*; also called *Lmp2*), which can be induced by IFN-$\gamma$ via STAT1.[24] Psme2, which is required for immunoproteasome assembly,[25] was also highly induced at 6 hours. Wild-type Ad infection upregulates the expression of other immunoproteasome components, such as MECL1, PA28-$\alpha$, and PA28-$\beta$.[26] However, Ad 12 E1A downregulates *Psmb9* transcription by interfering with the binding of STAT1 to IRF1.[27] Likewise, the gp19K protein expressed from the E3 region blocks transport of class I antigens to the cell surface.[28] Again, deletion of E1A and E3 regions may have unintended consequences for immune surveillance. Intracellular viral antigens are classically thought to be presented through class I major histocompatibility complex, while peptides derived from extracellular sources are presented by class II major histocompatibility complex. Nevertheless, a number of viruses, including herpes simplex virus, HIV, and papillomavirus, all downregulate Class II pathway components. At the time point of 72 hours, Ad infection decreased the expression of histocompatibility 2, class II antigen A,

β 1 (*H2ab1*). At 72 hours, Ad and HD decreased the levels of the Ia-associated invariant chain (*Cd74*), which is required for Class II maturation.

***Complement.*** During the period from 1–72 hours, Ad and HD repress a cluster of genes encoding subunits of complement component C1Q (*C1qa*, *C1qb*, and *Cfp*, **Figure 2IV**). The significance of this is unclear, but the host transcriptional response to Ad is blunted in the absence of functioning complement.[29]

Additional information on IFN-regulated antiviral effectors, cell cycle control proteins, genes involved in regulation of apoptosis, Glutathione S-transferases and a hemoglobin repression cluster can be found in the **Supplementary Data S1**.

### The host response to AAV infection

***AP-1 transcription factor signaling.*** AP-1 is a heterodimer that can include JUN, JUN-B, and FOS. AAV infection alters the expression level of several AP-1 signaling pathway components, *Fos*, Junb, and *Dusp6*. In contrast to Ad and HD, AAV does not significantly upregulate *Jun*. At the time point of 4 weeks after AAV infection, we observed a significant (though slightly less than threefold) decrease in the *Junb* gene. Systemic tissue injury leads to release of acute phase reactants; *Junb* expression in rat liver leads to the production of hepatic acute phase proteins.[30] Thus *Junb* suppression by AAV may dampen the acute phase response. AAV infection caused decreased *Fos* expression at the time point of 4 weeks. It is difficult to ascribe a particular function to *Fos* downregulation, but it can control apoptosis in hepatocytes as well as inhibition of differentiation and activation of mononuclear phagocytes. Finally, AAV downregulated *Dusp6* at the time point of 4 weeks. DUSP6 is a negative regulator of AP-1 activation.[31]

***AAV modulates immunoregulatory genes.*** IL-12 is an immunoregulatory cytokine promoting Th1 differentiation and cell-mediated immunity. AAV downregulated two regulators of IL-12 signaling (*Fabp5* and *Fos*). Fatty acid–binding protein 5 (*Fabp5*) negatively regulates IL-12 expression in dendritic cells.[32] FOS[33] is a positive regulator of IL-12.

***AAV infection modulates members of the Wnt signaling pathway.*** The Wnt pathway is involved in pattern formation during development, tissue homeostasis, tissue regeneration as well as oncogenesis. At the time point of 4 weeks, AAV upregulates bone morphogenetic protein receptor, type 1A (*Bmpr1a*), a tumor growth factor family member encoding an upstream regulator of the Wnt pathway.[34] Intriguingly, the human homolog of *Bmpr1a* interacts with Bram1, which is known to interact with the Ad E1A protein.[35] The Wnt target gene, *Hoxc4* (ref. 36) is also upregulated at the time point of 6 hours.

## DISCUSSION

*Implications of response to different viruses*: Infection with Ad, HD, and AAV induced distinct and overlapping gene expression programs that evolved over time. There was some overlap with previous studies, but here we discuss 14 genes that (to our knowledge) have not been previously implicated in the response to Ad, HD, or AAV (bold in **Table 1**). This may be because our *in vivo* experiment captures cross talk between different cell types. Clearly AAV induced far fewer genes than Ad or HD. The fact that both HD and AAV do not express any viral genes, while Ad does, allows us to derive some useful conclusions. Ad and HD infection resulted in remarkably similar gene expression profiles. This suggests that the innate immune system primarily recognizes the viral capsid or some property of the encapsidated DNA (regardless of viral origin) rather than leaky viral gene expression. However, because the response to AAV was so blunted compared to HD, we can conclude that the nature of the capsid or encapsidated DNA can have a large impact on immune surveillance.

It appears that Ad infection leads to production of type I IFNs followed by upregulation of a spectrum of IFN-responsive genes. Indeed, IL-6 and IL-12 production in response to Ad is attenuated in IFN-α/β receptor knockout mice.[10] Our data, and that of others, suggest that the viral capsid and/or encapsidated DNA is being recognized. Consistent with our observed upregulation of TLR pathway members (*Tlr2*, *Myd88*) and 2nd wave IFN genes, several recent articles suggest that TLRs are involved in the recognition of Ad.[7,8,10,37,38] Plasmacytoid dendritic cells recognize Ad in a TLR9-dependent fashion.[10,37] TLR9 recognizes CpG DNA.[39] However, Kupffer cells recognize Ad DNA via a cytosolic sensor in a TLR-independent manner.[10] Consistent with the idea that Ad DNA is what is recognized, empty adenoviral capsids elicited far fewer responses than Ad.[13]

As noted above, AAV induces a much more modest host response than HD, indicating that the AAV capsid and/or encapsidated DNA is recognized differently by the host. Of note, viral double-stranded DNA is expected to be present in the cytoplasm of Ad-infected cells, while AAV second strand formation occurs in the nucleus, secluded from cytosolic sensors. Future studies will be required to determine the significance of changes in gene expression due to the innate host response to AAV.

## MATERIALS AND METHODS

***Virus production, infection, and serum hFIX measurements.*** Ad, HD, and AAV vectors, each containing an hFIX expression cassette were produced and titered. First generation Ad was produced and amplified as previously described.[40,41] Briefly, Ad was purified by one CsCl gradient ultracentrifugation followed by an equilibrium CsCl ultracentrifugation purification. Transducing units were determined by performing a plaque assay in HEK293 cells. Virus was serially diluted, HEK293 cells were infected and overlaid with agarose containing medium. Approximately 2 weeks later plaque forming units were counted.

Viral production, purification, and characterization of HD vectors was as previously described.[41,42] HD vectors were purified by one CsCl gradient ultracentrifugation followed by two equilibrium CsCl ultracentrifugation purifications. Helper virus contamination was >1.0% as determined by alkaline-phosphatase staining. The number of HD transducing particles was determined as previously described.[42] HeLa cells were infected with different volumes of the HD vector preparation. For comparison, HeLa cells were infected at different defined multiplicities of infection with Ad to generate a standard curve. Cells were incubated for 3 hours, and the genomic DNA was isolated; this was followed by a Southern blot probed with an hFIX complementary DNA probe. The intensity of the bands for the HD vector was compared with the Ad standard curve. Spectrophotometry was used for physical titration of adenoviral vectors. The viral particle titer was also determined by an optical density$_{260}$ assay.

Briefly, the AAV vector was purified by two cycles of CsCl gradient ultracentrifugation as previously described.[43] Therefore, contamination by empty particles should be minimal. AAV viral titer was determined using a quantitative dot blot assay.

Six-week-old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), with two mice per group were injected with $5 \times 10^8$ transducing units of Ad or HD, or $3 \times 10^{11}$ vector genomes of AAV. The optical particle units injected (of Ad and HD) were $1.2 \times 10^{10}$ and $2.3 \times 10^{10}$, respectively. The amount of injected virus was chosen such that similar peak levels of plasma hFIX would be reached for each virus. Plasma hFIX levels were measured by enzyme-linked immunosorbent assay as described.[44] Animal experiments were performed according to the guidelines for animal care at Stanford University.

***RNA isolation.*** Total RNA was isolated using an RNeasy Midi Kit (Qiagen, Valencia, CA). At indicated times postinjection, livers were removed from mice, the gall bladders were discarded and the entire liver was dounce homogenized in 10 ml of RLT buffer. Homogenate was passed through an 18-gauge needle five times. Four milliliter of lysate was purified using an RNeasy Midi Kit according to manufacturer's instructions. PolyA RNA was purified using FastTrack II kits (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

***DNA microarrays.*** For each microarray, fluorescently (Cy-5) labeled complementary DNAs corresponding to messenger RNA from a treated mouse were hybridized relative to a Cy-3 labeled complementary DNA derived from a matched time point mock-treated mouse. Data were normalized and archived using the Stanford Microarray Database[45] (http://smd.stanford.edu/cgi-bin/publication/viewPublication.pl?pub_no=684). Gene expression data are also available through the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Genes differentially expressed relative to the mock treatment were identified by removing spots with a signal less than twofold above background in both channels, and/or a regression correlation <0.6, followed by averaging of available technical replicates prior to a best-effort average calculation of aggregate biological replicates. Genes with an absolute average fold change exceeding threefold in at least one time point or condition was sufficient for inclusion in a master gene list, and best-effort averages of replicates were calculated. Genes with <75% good data were excluded and remaining data were hierarchically clustered[47] using the single-linkage method, followed by manual refinement of the resulting clustergram (**Figure 2**).

***Quantitative RT-PCR.*** Two microgram of polyA-purified RNA was treated with one unit of DNase I (Ambion, Austin, TX) for 30 minutes. Ten millimolar final EDTA was added and samples were incubated at 70 °C for 20 minutes. RNAs were ethanol precipitated and washed with 70% ethanol. Pellets were resuspended in 10 mmol/l tris-hydroxyamino methane (pH 7.5). SYBR green quantitative RT-PCR analysis was conducted with a RT$^2$ Real-Time SYBR Green kit (Bio-Rad, Hercules, CA). Quantitative PCR with primers specific for $\beta$-actin was also conducted in parallel to normalize signal intensities to account for differences in recovery and loading. Quantitative RT-PCRs were conducted in triplicate. Quantitative RT-PCR primer sequences are listed below: *Csf1*: GTCCTGCAGCAGTTGATCGA and GGCAATCTGGCATGAAGTCTC; *Il1b*: CCAAAAGATGAAGG GCTGCT and TCATCTGGACAGCCCAGGTC; *Il18bp*: CGCTTCCCC TACTTCAGCAT and AATGAAGGAACCATTGCCCA; *Irf7*: AAGACC AACTTCCGCTGTGC and GCGCAAGATAAAACGCCCT; *Mt1*; CGTG GGACTCCGTCTGTCTC and GAGGTTGGCTCTGGCTCTCC. *Psmb9*: CGTGAGGACTTGTTAGCGCA and CTCACATTGGTCCCAGCCA; *Socs1*: TCGAGTAGGATGGTAGCACGC and GGAGATCGCATTGTC GGCT; *Tlr2*: ACCCTTGGTCGCGCTTAAC and AAGAACAGCGAT AGGCGGC; *Usp18*: CCCCAAACCTTGACCATTCA and CCTCGAG TTCCTGGCAGAGA; *Actb*: AGGTGTGATGGTGGGAATGG and GCCTCGTCACCCACATAGGA.

***Pathway analysis.*** Pathway analysis was carried out using the Panther Classification System[48,49] (Applied Biosystems, Foster City, CA).

## SUPPLEMENTARY MATERIAL

**Data S1.** Supplementary Results and References.

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