



# Activation of the NF- $\kappa$ B pathway by adeno-associated virus (AAV) vectors and its implications in immune response and gene therapy

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Because our *in silico* analysis with a human transcription factor database demonstrated the presence of several binding sites for NF- $\kappa$ B, a central regulator of cellular immune and inflammatory responses, in the adeno-associated virus (AAV) genome, we investigated whether AAV uses NF- $\kappa$ B during its life cycle. We used small molecule modulators of NF- $\kappa$ B in HeLa cells transduced with recombinant AAV vectors. VP16, an NF- $\kappa$ B activator, augmented AAV vector-mediated transgene expression up to 25-fold. Of the two NF- $\kappa$ B inhibitors, Bay11, which blocks both the canonical and the alternative NF- $\kappa$ B pathways, totally ablated transgene expression, whereas pyrrolidone dithiocarbamate, which interferes with the classical NF- $\kappa$ B pathway, had no effect. Western blot analyses confirmed the abundance of the nuclear p52 protein component of the alternative NF- $\kappa$ B pathway in the presence of VP16, which was ablated by Bay11, suggesting that AAV transduction activates the alternative NF- $\kappa$ B pathway. *In vivo*, hepatic AAV gene transfer activated the canonical NF- $\kappa$ B pathway within 2 h, resulting in expression of proinflammatory cytokines and chemokines (likely reflecting the sensing of viral particles by antigen-presenting cells), whereas the alternative pathway was activated by 9 h. Bay11 effectively blocked activation of both pathways without interfering with long-term transgene expression while eliminating proinflammatory cytokine expression. These studies suggest that transient immunosuppression with NF- $\kappa$ B inhibitors before transduction with AAV vectors should lead to a dampened immune response, which has significant implications in the optimal use of AAV vectors in human gene therapy.

Recombinant adeno-associated virus (AAV) vectors have been used successfully for *in vivo* gene transfer in numerous preclinical animal models of human disease and have been used successfully for long-term expression of a wide variety of therapeutic genes (1–6). AAV vectors have also generated long-term clinical benefit in humans when targeted to immune-privileged sites, e.g., in ocular delivery for Leber's congenital amaurosis (7–9). A major advantage of this vector is its comparatively low immune profile, eliciting only limited inflammatory responses and, in some cases, even directing immune tolerance to transgene products (10). Nonetheless, the therapeutic efficiency, when targeted to nonimmune privileged organs, has been limited in humans due to antibody and CD8<sup>+</sup> T-cell responses against the viral capsid, whereas in animal models, adaptive responses to the transgene product have also been reported (11–15). These results suggest that immune responses remain a concern for AAV vector-mediated gene transfer.

Recent studies have begun to define the initial activation signals that result from AAV gene transfer. One study found AAV-induced signaling through the Toll-like receptor 9 (TLR9)–myeloid differentiation factor 88 (MyD88) pathway to induce a type I IFN response in plasmacytoid dendritic cells (pDCs), thereby driving subsequent adaptive immune responses to the vector and transgene product upon gene transfer to murine skeletal muscle (16). These data indicate sensing of the DNA

genome by the endosomal TLR9 receptor in pDCs. No evidence for induction of proinflammatory cytokines following *in vitro* pulsing of dendritic cell (DCs) or macrophages with AAV was found. However, earlier reports demonstrated a rapid, albeit highly transient, Kupffer cell-dependent innate response to AAV vectors in the liver, which included expression of several inflammatory cytokines (17, 18).

Interestingly, the role of NF- $\kappa$ B, a key cellular responder to many stress- and pathogen-derived signals and a regulator of proinflammatory cytokine expression (19–21), has not been studied in the AAV life cycle. Here, we demonstrate that infection of human cells with AAV can lead to activation of the alternative NF- $\kappa$ B pathway, which substantially increases transgene expression, whereas inhibition of NF- $\kappa$ B blunts expression. *In vivo*, the canonical and alternative pathways are activated in consecutive order, likely reflecting interactions between the vector and different cell types. Prevention of inflammatory cytokine induction by transient inhibition of NF- $\kappa$ B reveals a role for NF- $\kappa$ B in the innate response to AAV *in vivo* and, importantly, does not interfere with long-term transgene expression.

## Results

**AAV-Inverted Terminal Repeats Contain Putative Binding Sites for the NF- $\kappa$ B-Responsive Transcription Factors.** We previously reported the existence of a cellular protein that interacts specifically with the single-stranded D[–] sequence in the left inverted terminal repeat (ITR) of the AAV2 genome (22). Because the ssD[+] sequence in the right ITR is complementary to the ssD[–] sequence in the left ITR, we reasoned that a putative cellular protein that interacts with the ssD[+] sequence in the right ITR might also exist. In electrophoretic mobility-shift assays using the ssD[+]–sequence probe, a distinct cellular protein was indeed detected, which we designated as the ssD[+]–sequence–binding protein (ssD[+]–BP) (22). Following purification and mass spectrometry, ssD[+]–BP was found to have partial amino acid homology to a cellular NF- $\kappa$ B–repressing factor, a negative regulator of transcription. Further *in silico* analysis with a human transcription factor database (TRANSFAC; <http://algggen.lsi.upc.es/>) demonstrated the presence of several binding sites for NF- $\kappa$ B–binding cofactors, such as p300, TFIIB, and SpII (Fig. S1). One of these is the p300/CREB transcription factor that has been recently shown to be associated with the AAV genome (23). Although it is not known whether the NF- $\kappa$ B sig-

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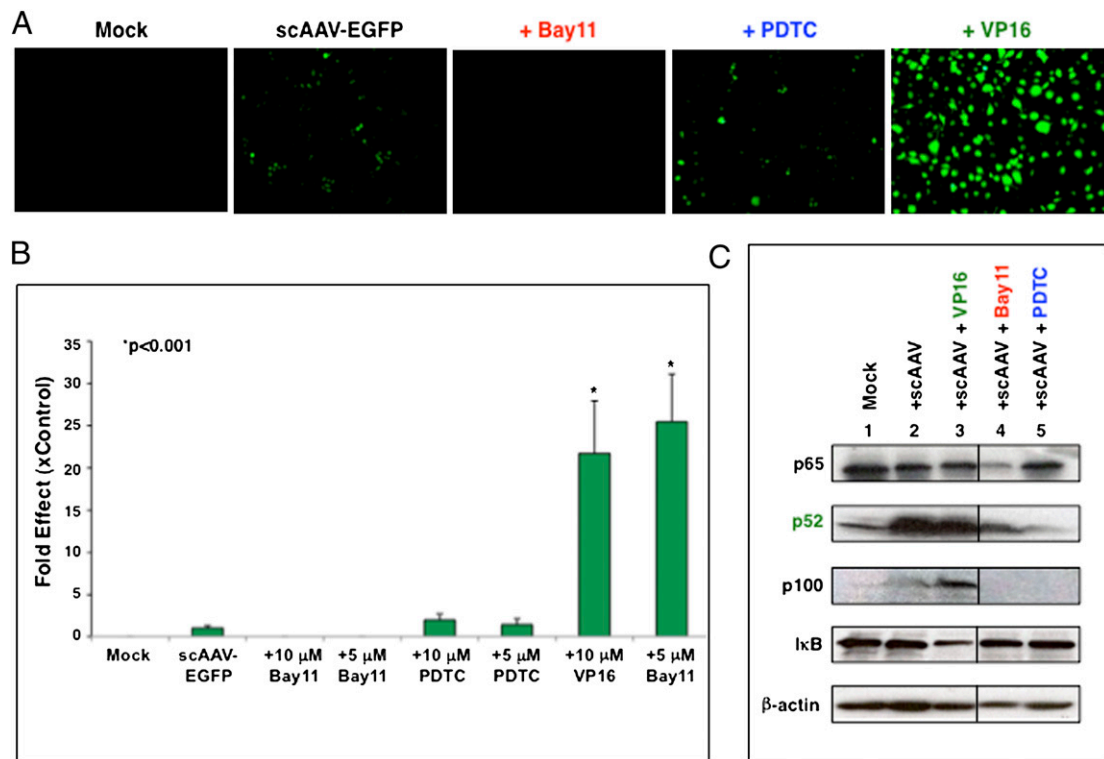
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naling is activated by AAV binding to the cell surface receptors/coreceptors, recent studies have demonstrated that the innate immune response could be triggered through the TLR9–MyD88 pathway or through the activation of the CD40 ligand on the cell surface in mouse models *in vivo* (16, 24). Both these ligands are known to interact downstream with NF- $\kappa$ B transcription factors during their biological activation (25, 26). Together, these data strongly suggested that the NF- $\kappa$ B is involved in the AAV life cycle. This possibility was tested experimentally as follows.

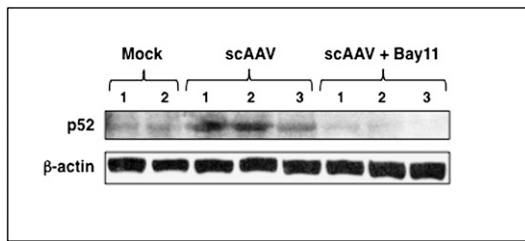
**AAV Infection Leads to Activation of the Alternative NF- $\kappa$ B Pathway *In Vitro* and in the Liver *In Vivo*.** We used small molecule activators and inhibitors of NF- $\kappa$ B signaling in HeLa cells transduced with a self-complementary AAV serotype 2 vector expressing EGFP (scAAV-EGFP). VP16, an NF- $\kappa$ B activator (27), augmented EGFP expression by ~25-fold (Fig. 1*A* and *B*). Bay11, which blocks the activity of both IKK $\alpha$  and IKK $\beta$ , totally ablated EGFP expression, whereas pyrrolidine dithiocarbamate (PDTC), which inhibits I $\kappa$ B degradation by blocking I $\kappa$ B ubiquitin ligase in the classical pathway (28), had no notable effect (Fig. 1*A* and *B*). Furthermore, VP16-mediated augmented transgene expression was completely ablated by Bay11, but not by PDTC (Fig. S24). Similar results were obtained with both single-stranded AAV (ssAAV) vectors (Fig. S2*B*) and the tyrosine triple-mutant scAAV vector (Y730+500+444F; TM-AAV), which we have described recently (29) (Fig. S2*C*). Thus, we concluded that AAV-mediated transgene expression is regulated by the alternative pathway of NF- $\kappa$ B. This conclusion was confirmed by Western blot analysis, which revealed an increase in the cytosolic p100 and the nuclear p52 protein components of the alternative NF- $\kappa$ B pathway of up to sixfold in the presence of VP16. Moreover, transduction with AAV vector by itself (i.e., in the absence of activator) increased p100 and

p52 (Fig. 1*C* and Fig. S3), whereas p65, the marker for the classical NF- $\kappa$ B pathway, was unaffected, indicating that AAV infection activates the alternative NF- $\kappa$ B pathway. This increase was ablated by Bay11 (Fig. 1*C* and Fig. S3). Using hepatic gene transfer as a model for *in vivo* responses to AAV, activation of the alternative pathway was observed at 9 h (but not earlier or at a 24-h time point) following scAAV vector delivery, which was effectively blocked by Bay11, as determined by Western blot analyses (Fig. 2).

**Activation of NF- $\kappa$ B Regulates Transgene Expression in Primary Antigen-Presenting Cells Following AAV Infection.** In primary human dendritic cells (DCs), on the other hand, although transgene expression was again substantially increased with the NF- $\kappa$ B activator (Fig. 3*A*), AAV infection by itself did not activate NF- $\kappa$ B (Fig. 3*B*). In the presence of VP16, an ~20-fold increase in EGFP expression was observed compared with scAAV vector-transduced DCs. Treatment with cytokines (TNF- $\alpha$ , IL-6, IL-1 $\beta$ , PGE2), known to activate the NF- $\kappa$ B pathway, led to a further increase in transgene expression to ~26%, which was reduced to ~12% following treatment with Bay11 (Fig. 3*A*). Western blot analyses of nuclear fractions further corroborated that the alternative pathway of NF- $\kappa$ B activation (accumulation of p52 proteins) was operational (Fig. 3*B*). We also tested the capability of NF- $\kappa$ B modulators and AAV to induce phenotypic changes in DCs. Flow cytometric analyses of two DC maturation markers, CD83 and CD86, indicated that VP16 and AAV alone were not able to induce maturation or to enhance the expression of costimulatory molecules when used together with the cytokine mixture. However, treatment with Bay11 led to inhibition of cytokine-mediated maturation of antigen-presenting cells (APCs), further implicating the involvement of NF- $\kappa$ B (Table 1). Thus, we hypothesized that suppression of NF-



**Fig. 1.** Effect of NF- $\kappa$ B pathway inhibitors and activator on AAV vector-mediated EGFP expression in HeLa cells *in vitro*. Cells were pretreated with various concentrations of inhibitors and activators for 12 h and transduced with  $2 \times 10^3$  AAV-EGFP vg per cell. (A) Transgene expression was detected by fluorescence microscopy 48 h postinfection. (B) Quantitative analyses of the data from A. Images from five visual fields were analyzed as described in *Materials and Methods*. \* $P < 0.001$ . (C) Western blot analysis of HeLa cell extracts transduced with scAAV vectors in the presence of NF- $\kappa$ B modulators. The samples were analyzed by using anti-p65 and anti-I $\kappa$ B antibodies (classical pathway) and anti-p100/p52 antibody (alternative pathway) for detection of NF- $\kappa$ B signaling. These results are representative of two independent experiments.



**Fig. 2.** Western blot analysis of liver homogenates from mice 9 h following mock injections ( $n = 2$ ) or injections with scAAV vectors, with and without prior administration of Bay11 ( $n = 3$  each). The samples were analyzed by using anti-p52 antibody for detection of NF- $\kappa$ B signaling in response to AAV exposure. Anti- $\beta$ -actin antibody was used as a loading control.

$\kappa$ B activation before vector administration might lead to a dampened innate immune response against AAV.

**AAV Vector-Mediated Hepatic Gene Transfer Leads to Rapid Activation of the Canonical NF- $\kappa$ B Pathway and Expression of Proinflammatory Cytokines, Which Is Effectively Blocked with Bay11.** Prior to activation of the alternate pathway of NF- $\kappa$ B by AAV vectors in the liver described above (Fig. 2), expression of genes encoding cellular proteins within the canonical NF- $\kappa$ B pathway and of proinflammatory cytokines was up-regulated within 2 h as shown by quantitative RT-PCR array (Fig. 4 *A* and *B*). However, Bay11 administration before vector injection prevented up-regulation of the NF- $\kappa$ B gene expression profile (Fig. 4*B*) and also abrogated induction of proinflammatory cytokines and chemokines, including IL-1 $\alpha$ , IL-6, TNF $\alpha$ , IL-12 $\alpha$ , KC, and RANTES, as well as of the TLR9-MyD88-signaling pathway (Fig. 4 *A* and *B*). A similar down-regulation trend of these innate immune response

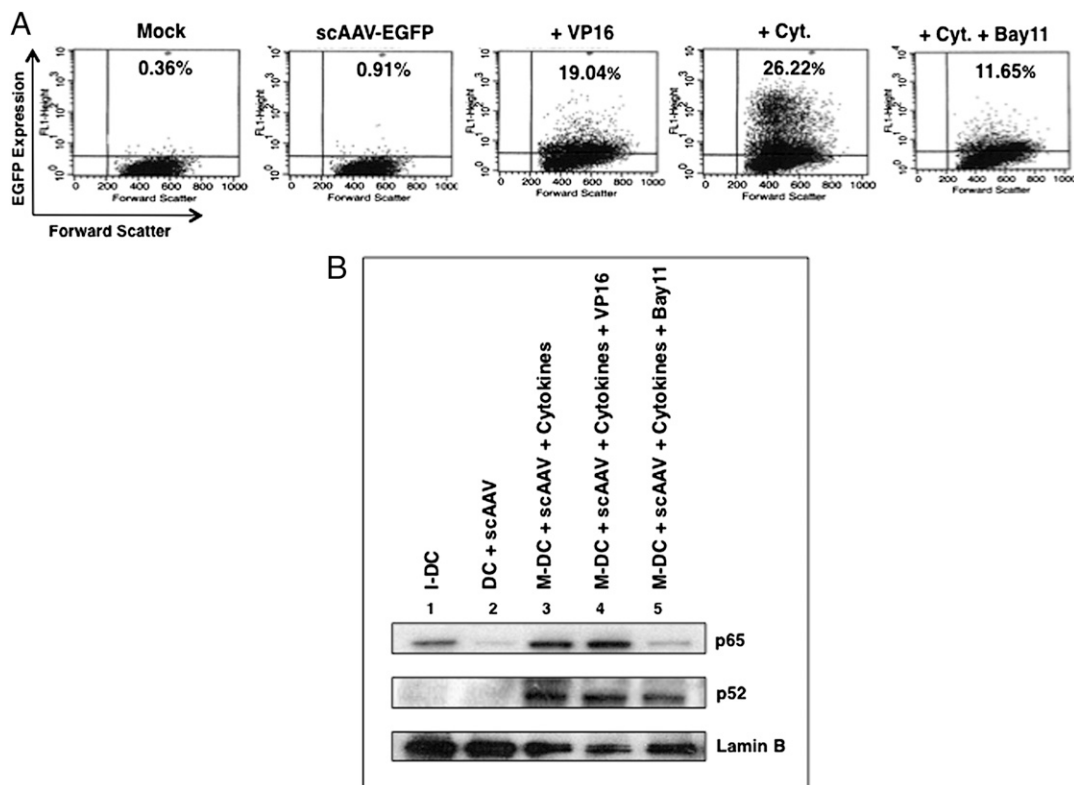
markers was seen in mice injected with the more efficacious tyrosine triple-mutant AAV vector (Y730+500+444F; TM-AAV) (29). Both wild type (WT)-AAV and TM-AAV vectors also induced increases in transcript levels from other innate immune markers that are not regulated by NF- $\kappa$ B (such as type I IFN). As expected, these transcripts were not affected by Bay11 (Fig. S4). Administration of Bay11 also significantly reduced the antibody response against AAV2 in these mice (Fig. 5). The sum of these results implies that the transient inflammatory cytokine response, typically seen during *in vivo* hepatic AAV gene transfer (17), is mediated by NF- $\kappa$ B activation.

**Long-Term AAV Vector-Mediated Transgene Expression in Murine Hepatocytes *In Vivo* Is Not Affected by Inhibition of NF- $\kappa$ B Activation.**

In view of our observation that Bay11 strongly inhibits AAV-mediated transgene expression in HeLa cells *in vitro* 48 h post-transduction (Fig. 1 *A* and *B*), which would be counterproductive to achieving long-term transgene expression *in vivo*, it was important to examine the effect of Bay11 in mice. As can be seen in Fig. 6*A*, animals injected with or without Bay11 had similar levels of EGFP expression from either vector when analyzed 2 wk after gene transfer. Transduction efficiency of the TM-AAV vector was ~12-fold higher than that of the WT-AAV vector (Fig. 6*B*), consistent with our recently published studies (29). These data suggest that Bay11 administration can safely and effectively down-regulate mediators of innate immune response without compromising long-term transgene expression.

**Discussion**

On the basis of preclinical data from murine models (30), AAV has been considered as minimally immunogenic. Moreover, hepatic gene transfer activates immune regulatory pathways, thereby promoting tolerance to the transgene product (31, 32). For example, multiyear stable expression of coagulation factor



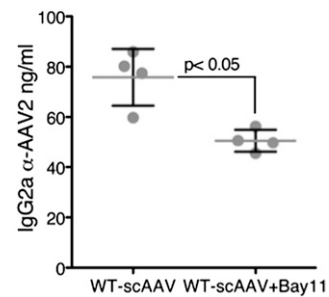
**Fig. 3.** AAV-EGFP vector-mediated transduction of primary human monocyte-derived dendritic cells in the presence of NF- $\kappa$ B modulators. (*A*) Transgene expression was detected by flow cytometry 48 h posttransduction. (*B*) Western blot analysis for NF- $\kappa$ B activation in nuclear extracts from DCs, mock-transduced or transduced with 2,000 vg/cell of scAAV vectors  $\pm$  NF- $\kappa$ B modulators. I-DC, immature dendritic cells; DC, dendritic cells; M-DC, mature dendritic cells.

**Table 1. FACS analyses of markers of maturation of primary human dendritic cells**

Group	Geometric means of levels of expression in cells expressing:	
	CD83	CD86
Immature DCs	10.38	7.04
DCs: no maturation supplement	18.08	13.63
Mature DCs + cytokines	20.60	26.80
DCs + AAV	18.29	12.65
DCs + VP16	16.48	13.70
Mature DCs + AAV + cytokines	24.25	23.75
Mature DCs + AAV + cytokines + VP16	19.92	21.92
Mature DCs + AAV + cytokines + Bay11	16.88	10.11

Data from a representative experiment are shown. Similar results were obtained in independent experiments using three different donor samples.

IX (F.IX) has been shown in animal models of hemophilia B (2, 30). In a clinical trial using AAV to deliver the human *F9* gene to the liver in subjects with hemophilia B, therapeutic levels (>10%) of F.IX expression were observed at a high dose of vector ( $2 \times 10^{12}$  vector genomes (vg)/kg body weight) (11). However, 4–6 wk after gene transfer, an AAV capsid-specific T-cell response that coincided with a rise in liver transaminases and a drop in F.IX transgene expression to baseline levels was observed. This CD8<sup>+</sup> T-cell-mediated immune response was unexpected, as it had not been observed in any preclinical animal models (12). Subsequently, a great deal of effort has been devoted to circumvent the host immune response to AAV vectors. These efforts include the use of alternate, naturally occurring AAV serotypes such as AAV1 (33) or AAV8 (34), the use of shuffled capsids (35) or surface-exposed tyrosine-mutant AAV2 (29) vec-



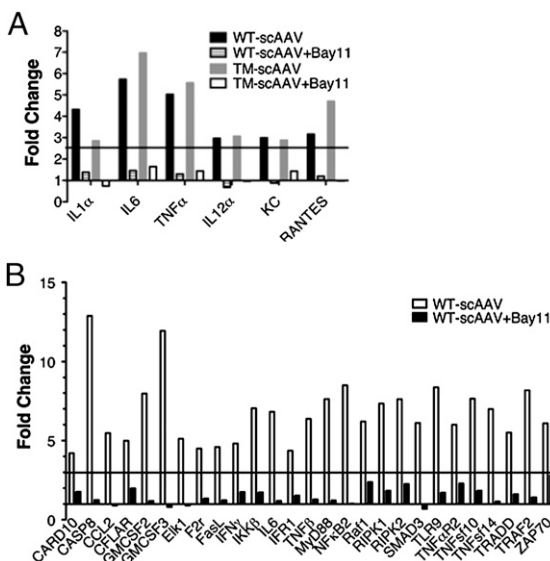
**Fig. 5.** Humoral response to AAV vectors in the absence or the presence of an NF- $\kappa$ B inhibitor. Anti-AAV2 IgG2a levels were determined in peripheral blood from mice at day 10 following injections with scAAV vectors, with and without prior administration of Bay11 ( $n = 4$  in each group).

tors, and the development of transient immune-suppression protocols (36, 37). Although such strategies have improved the safety of AAV gene transfer, their efficacy in humans remains to be seen. This underscores the need to better understand the specific interactions between the host cells and the vector to define activation of the innate immune response pathways and the underlying signaling pathways, which, in turn, may affect adaptive immune responses and transgene expression.

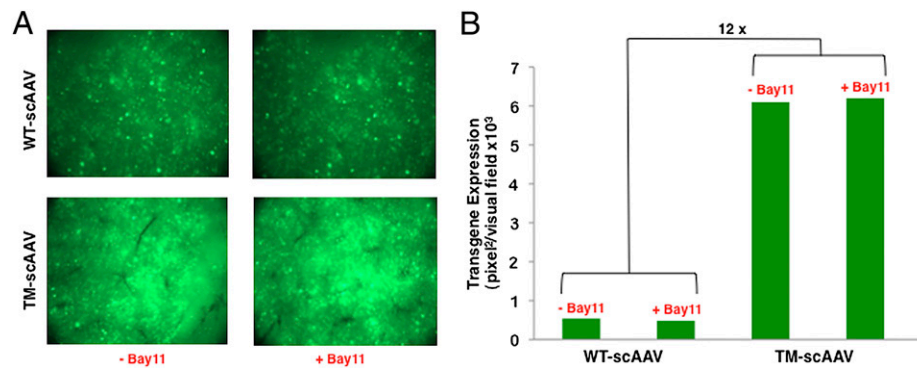
The basis for the present study was our observation that NF- $\kappa$ B can bind to the 20-bp D-sequence present in the AAV ITRs (22), which was identified by electrophoretic mobility-shift assays followed by mass spectrometry (Fig. S5). Our current studies provide evidence of the involvement of NF- $\kappa$ B in AAV infection by using a variety of pharmacological modulators, which have been extensively used by other investigators (27, 38). This is significant considering that activation of NF- $\kappa$ B is a fundamental immediate early step of inflammatory and immune activation (21) and that NF- $\kappa$ B signaling represents a prime candidate for viral susceptibility or interference (20, 39–43). On the other hand, many viruses disrupt the innate immune responses and NF- $\kappa$ B through the use of multifunctional viral decoy proteins that target specific aspects of the NF- $\kappa$ B pathway (20). It is possible that the alternative pathway of NF- $\kappa$ B is activated in part following AAV infection because AAV vectors lack complex structural gene elements necessary to develop any NF- $\kappa$ B-like decoy proteins.

The data presented here demonstrate consecutive activation of canonical and alternative NF- $\kappa$ B pathways, indicating that NF- $\kappa$ B plays a pivotal role in the early cellular responses to AAV gene transfer on multiple levels. Earlier studies have shown that AAV gene transfer to the liver results in a rapid but transient expression of proinflammatory cytokines, likely because of uptake of AAV vectors by resident antigen-presenting cells such as Kupffer cells (17). Our data demonstrate that this phenomenon is due to rapid activation of the canonical NF- $\kappa$ B pathway, which is effectively blocked by Bay11. More recently, it was shown that the innate immune response to AAV used the TLR9–MYD88 pathway, which generated a type I IFN response and was found to be essential for subsequent adaptive responses to vector and transgene product (16). However, TLRs are also known to activate the canonical NF- $\kappa$ B pathway. Future studies should reveal whether our observations of NF- $\kappa$ B and inflammatory responses are the result of endosomal DNA sensing by TLR9.

Our findings are consistent with the notion that the canonical NF- $\kappa$ B pathway is primarily linked to inflammation, whereas the alternative pathway is important for adaptive immunity (44). For example, one study showed that activation of CD8<sup>+</sup> T cells by AAV vectors required the costimulatory molecule CD40L, a ligand known to activate the alternative NF- $\kappa$ B pathway upon binding to its cell surface receptor (24). The activation of the alternative pathway of NF- $\kappa$ B has been shown to result in maturation and T-cell priming by DCs over-expressing a mutated I $\kappa$ B $\alpha$  that blocks activation of the classical pathway (45). In



**Fig. 4.** AAV vector-induced innate immune and NF- $\kappa$ B response in mice in vivo. Gene expression profiling of innate immune mediators (A) or NF- $\kappa$ B activation (B) was performed as described in *Materials and Methods*. The data for fold changes in gene expression at the 2-h time point comparing AAV vectors with Bay11 (hatched or open bars) with AAV vectors without Bay11 (black or gray bars) are shown. The minimal threshold fold-increase (horizontal black line) was 2.5 (A) or 3.0 (B) by measuring the variability of duplicate  $\Delta$ CT (compared with GAPDH). Relative gene expression was determined for each group of treated and untreated animals and values >2.6 and <0.38 were considered significant.



**Fig. 6.** Transgene expression in murine hepatocytes 10 d postinjection of  $1 \times 10^{11}$  vg each of WT-scAAV-EGFP or TM-scAAV-EGFP vectors/animal via the tail vein. (A) Representative images are shown. Original magnification 400 $\times$ . (B) Quantitative analyses of the data from A. Images from five visual fields were analyzed quantitatively as described in the legend to Fig. 1.

alymphoplasia mice deficient in NF- $\kappa$ B-inducing kinase (NIK), the cross-priming of CD8<sup>+</sup> T cells to exogenous antigens in DCs is affected, suggesting the importance of this pathway in adaptive immunity (45). Mice deficient in alternative pathway components are also deficient in secondary lymphoid organ development and homeostasis (46). In addition, NIK is pivotal to the activation of DCs, whose activity is critical for priming of the antigen-specific T cells (47–49). NIK also enhances immune responses against a vector-encoded antigen and shifts them toward a T helper 1 immune response with increased IgG2a levels, T-cell proliferation, IFN- $\gamma$  production, and cytotoxic T-lymphocyte responses (47).

DCs and other antigen-presenting cells make up only a small proportion of liver cells compared with hepatocytes. Therefore, it is likely that the alternative NF- $\kappa$ B pathway that we observed in vivo at 9 h after vector administration using a less sensitive Western blot of soluble proteins from total tissue was the result of the target cell, i.e., of hepatocyte transduction. This interpretation is further supported by the in vitro HeLa cell data, demonstrating a link between transduction and alternative pathway activation. Because of the involvement of this pathway in regulating transgene expression, it was crucial that transient blockage of NF- $\kappa$ B did not affect long-term transgene expression (Fig. 6A and B). Further studies are warranted to determine the precise step during AAV infection of a target cell that results in activation of the alternative NF- $\kappa$ B pathway.

Inflammatory signals derived from the initial canonical pathway activation may have also contributed to activation of the alternative pathway, which would certainly be expected during the transition from innate to adaptive responses. The effects of Bay11 would therefore be twofold—preventing inflammatory signals and directly blocking the alternative pathway—thereby also down-regulating transgene expression in DCs. Both effects should dampen adaptive immune responses, as seen in a reduction in antibody formation against the vector. Although AAV vectors do not efficiently transduce DCs in vivo, a method to further suppress this process is desirable to prevent immune responses to the transgene product.

One possible strategy to negate undesired activation of transgene expression in antigen-presenting cells by NF- $\kappa$ B is to generate targeted mutations against the NF- $\kappa$ B-responsive transcription factor-binding sites in the AAV-ITRs. However, given the pleiotropic functions of NF- $\kappa$ B proteins in cellular physiology (19), it is possible that different NF- $\kappa$ B-responsive cytokine promoter-binding transcription factors might be operational in different cell types. Alternatively, a protocol for transient immunosuppression by targeting the NF- $\kappa$ B pathway might be universally applicable. Bay11 was able to down-regulate the activity of several key regulator cytokines (IL-1 $\alpha$ , IL-6, TNF $\alpha$ , IL-12 $\alpha$ , KC, and RANTES), suggesting the benefit of using this pharmacologic modulator to down-regulate the inflammatory response against AAV vectors.

However, Bay 11 did not prevent induction of type I IFN, which is not driven by NF- $\kappa$ B.

Nonetheless, a protocol for transient immunosuppression by targeting the NF- $\kappa$ B pathway might be universally applicable to limit immunotoxicities. Indeed, a recent report showed decreased AAV capsid antigen presentation by the use of a proteasomal inhibitor, Bortezomib (Velcade) (50). Bortezomib has a considerable antimyeloma efficacy (51), which is likely due in large part to repression of NF- $\kappa$ B signaling. It may therefore be possible to simultaneously block MHC I presentation of capsid and inflammatory signals or use more selective NF- $\kappa$ B-targeted therapies, such as Bay11 in our study, or the newer IKK inhibitors to further enhance the safety and therapeutic efficacy of AAV vectors.

## Materials and Methods

A brief summary of materials and techniques used is provided here. A more detailed account is presented in *SI Materials and Methods*.

**Recombinant AAV Vectors.** Highly purified stocks of ssAAV and scAAV2 vectors with the WT or the triple tyrosine-mutant (TM; Y730+500+444F) capsids described recently (29) and containing the EGFP gene driven by the chicken  $\beta$ -actin promoter, were generated (WT-scAAV2-EGFP, TM-scAAV2-EGFP) as described previously (52, 53).

**Recombinant AAV Vector Transduction Assays in Vitro.** Optimal concentration of NF- $\kappa$ B-modulating compounds was determined as described previously (27, 38). VP16 or Bay11 (5 or 10  $\mu$ M, final concentration), and PDTTC (25 or 50  $\mu$ M final concentration) were used either alone or in activator/inhibitor combinations. Approximately  $1 \times 10^5$  HeLa cells were pretreated with these compounds 24 h before transduction with 500 or 2,000 vg/cell of recombinant WT-AAV or TM-AAV vectors as described previously (29). Primary human dendritic cells were transduced with AAV vectors at 2,000 vg/cell and incubated for 48 h. Transgene expression was assessed as total area of green fluorescence (pixel<sup>2</sup>) per visual field (mean  $\pm$  SD) or by flow cytometry. Analysis of variance was used to compare test results and the control, which were determined to be statistically significant.

**Recombinant AAV Vector Transduction Studies in Vivo.** Groups of 6-wk-old normal C57BL/6J mice (Jackson Laboratories) were administered intraperitoneally with a single dose (20 mg/kg) of the NF- $\kappa$ B inhibitor Bay11 in a 200- $\mu$ l volume diluted in DMSO (day 0). Animals injected with only the DMSO carrier solvent were considered as the baseline (mock) group ( $n = 75$ ) and animals injected with Bay11 were our test group ( $n = 75$ ). At this point, the animals from mock and Bay11 groups were randomized to receive either PBS (pH 7.4) or WT-AAV or TM-AAV vectors ( $n = 25$  mice in each group). On day 1,  $\sim 1 \times 10^{11}$  vg particles of WT-AAV2-EGFP or TM-AAV2-EGFP vectors or PBS were administered i.v. via the tail vein. To measure the modulation of immune response to AAV, three to five animals each from PBS-, WT-AAV-, or TM-AAV vector-injected groups were killed at different time points post-vector administration (2, 6, 10, and 24 h and day 10). Hepatic lobes were cross-sectioned and evaluated for EGFP expression (from day 10 mice). All

animal experiments were conducted in accordance with the University of Florida Institutional Animal Care and Use Committee guidelines.

**Gene Expression Analysis of Innate Immune Response to AAV Vectors by Real-Time Quantitative PCR Assay.** Groups of 6-wk-old normal C57BL/6J mice were administered intraperitoneally with Bay11 and injected with PBS or with  $\sim 1 \times 10^{11}$  vg of the WT-AAV-EGFP vectors or the TM-AAV-EGFP vectors i.v. as described above ( $n = 5$  mice in each group). At 2 h post-vector administration, gene expression profiling of the innate immune response was performed, which included Toll-like receptors 1–9, MyD88, MIP-1, IL-1 $\alpha$ , IL-1 $\beta$ , IL-12 $\alpha$ , IL6, KC, TNF $\alpha$ , RANTES, MCP-1, IFN $\alpha$ , IFN $\beta$ , and IP-10. Data were captured and analyzed using ABI Prism 7500 Sequence Detection System Version 1.1 software (Applied Biosystems). The baseline was determined automatically for 18S rRNA and for other genes. Thresholds were determined manually for all genes. Gene expression was measured by the comparative threshold cycle

( $\delta$  Ct) method. The parameter threshold cycle (Ct) was defined as the cycle number at which the reporter fluorescence generated by the cleavage of the probe passed a fixed threshold above baseline.

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