Unique Roles of TLR9- and MyD88-Dependent and -Independent Pathways in Adaptive Immune Responses to AAV-Mediated Gene Transfer

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Key Words

Adeno-associated viral vectors · Gene therapy · Immune responses · Innate immunity · TLR9 · MyD88 · Antibody · CD8+ T cell

Abstract

The immune system represents a significant barrier to successful gene therapy with adeno-associated viral (AAV) vectors. In particular, adaptive immune responses to the viral capsid or the transgene product are of concern. The sensing of AAV by toll-like receptors (TLRs) TLR2 and TLR9 has been suggested to play a role in innate immunity to the virus and may also shape subsequent adaptive immune responses. Here, we investigated the functions of TLR2, TLR9 and the downstream signaling adaptor MyD88 in antibody and CD8+ T-cell responses. Antibody formation against the transgene product occurred largely independently of TLR signaling following gene transfer with AAV1 or AAV2 vectors, whereas loss of signaling through the TLR9-MyD88 pathway substantially reduced CD8+ T-cell responses. In contrast, MyD88 (but neither of the TLRs) regulated antibody responses to capsid. B cell-intrinsic MyD88 was required for the formation of anti-capsid IgG2c independently of vector serotype or route of administration. However, MyD88^{-/-}

mice instead produced anti-capsid IgG1 that emerged with delayed kinetics but nonetheless completely prevented in vivo readministration. We conclude that there are distinct roles for TLR9 and MyD88 in promoting adaptive immune responses to AAV-mediated gene transfer and that there are redundant MyD88-dependent and MyD88-independent mechanisms that stimulate neutralizing antibody formation against AAV.

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Introduction

Adeno-associated viral (AAV) vectors have been used in gene therapy applications for a wide variety of monogenetic disorders [1]. AAV is a replication-deficient, non-pathogenic parvovirus that normally contains a single-stranded DNA genome of 4.7 kb. Modification for use as a gene transfer vector involves the removal of all DNA encoding for viral proteins, leaving only the inverted terminal repeats which are required for packaging the recombinant genome. AAV is valued for gene transfer applications partly due to its ability to transduce quiescent cells and induce long-term transgene production in the absence of integration, mitigating the risks of insertional

mutagenesis. Additionally, the diversity of AAV serotypes allows for the transduction of numerous different target tissues. Although AAV generally provokes much weaker immune responses *in vivo* than other viral vectors such as adenovirus and lentivirus, both preclinical and clinical studies have revealed that immune responses to the transgene product as well as the input viral capsid can hinder the effectiveness of AAV-mediated gene transfer [2, 3].

AAV-mediated gene delivery for hemophilia B, a monogenic coagulation disorder caused by a loss in functional factor IX protein (F.IX), can provoke both antibody and CD8⁺ T cell-mediated immune responses to the human F.IX (hF.IX) protein, depending primarily on the route of administration and the underlying *F9* mutation [4]. We have previously demonstrated that hepatic gene transfer is tolerogenic, inducing antigen-specific regulatory T cells which can prevent or reverse ongoing immune responses against hF.IX [5, 6]. Muscle-directed gene transfer, on the other hand, typically provokes immune responses to hF.IX, although the endogenous expression of truncated, nonfunctional hF.IX can reduce the risk for transgene-specific immunity [4]. Other supplementary factors affecting transgene-specific immunity in mice include the vector dose, the AAV serotype and additional genetic factors which are not fully understood [7–9].

Clinical trials of AAV-mediated gene therapy for hemophilia B have also revealed unexpected roles for anti-capsid humoral and cellular immune responses in limiting therapeutic hF.IX expression. Extremely low-titer neutralizing antibody (NAB) to AAV (as low as 1:5) has been shown to prevent transduction *in vivo* following intravenous delivery [10]. In clinical trials of hepatic gene transfer for hemophilia B, memory CD8⁺ T-cell responses to the AAV capsid that can eliminate therapeutic expression in the absence of immunosuppression have also been observed [11–13]. Thus, understanding the mechanisms underlying transgene- and capsid-specific immunity is vital to developing successful AAV-mediated gene therapies.

One potential mediator of AAV vector immunogenicity is pattern recognition by toll-like receptors (TLRs), which can trigger an innate immune response and promote the development of adaptive immunity [14]. Although the innate immune response to AAV is severely limited in magnitude and duration, it has been suggested that the detection of the AAV DNA genome by TLR9, which senses unmethylated CpG DNA, plays a significant role in shaping adaptive immune responses

to both the transgene and the AAV capsid [15, 16]. Depletion of CpG motifs from the transgene reduced CD8⁺ T-cell responses to the AAV capsid and the transgene [17]. Likewise, the modification of AAV to encapsidate double-stranded DNA – termed self-complementary AAV (scAAV) – typically enhances transgene expression but also results in enhanced innate immune signaling through TLR9 and elevated capsid-specific immunity following hepatic gene transfer [18]. Intramuscular immunization with an scAAV vector expressing an HIV-derived protein provoked stronger antibody and CD8⁺ T-cell responses relative to single-stranded AAV (ssAAV) [19]. In the context of hemophilia B, scAAV vectors induced stronger CD8⁺ T-cell responses but comparable antibody responses to hF.IX following intramuscular gene transfer in hemophilic mice [20]. Human cells have been shown to sense AAV capsid through TLR2, a receptor recognizing various microbial protein and glycolipid structures, though no correlation has yet been made to adaptive immunity [21]. Finally, B cell-intrinsic MyD88, a downstream mediator of TLR2 and TLR9 signaling, has been suggested to be critical in the formation of Th1-dependent antibodies to AAV [22].

Herein, we provide a more defined role for the innate sensing of AAV, using genetically modified mice to determine the functions of TLR2, TLR9 and MyD88 in influencing adaptive immune responses. While antibody responses to the transgene were mostly independent of the innate immune sensors investigated, CD8⁺ T-cell responses substantially relied on the TLR9-MyD88 pathway. Anti-capsid IgG2c antibodies, on the other hand, depended on B cell-intrinsic MyD88 but not on either of the TLRs alone. Nevertheless, an alternative MyD88-independent pathway generated surprisingly robust IgG1 antibodies that completely prevented *in vivo* transduction with AAV.

Materials and Methods

Animal Strains and Experiments

Wild-type (WT) C57BL/6, TLR2^{−/−}, MyD88^{−/−}, μ MT, and STING^{gt/gt} mice were purchased from The Jackson Laboratory. TLR9^{−/−} mice had been kindly provided by Dr. Daniel Muruve (University of Calgary) and were bred in-house at the University of Florida [18]. IFN α R^{−/−} mice were housed at Baylor College of Medicine, as previously described [23]. All knockout mice were on a C57BL/6 background. The animals were housed under specific pathogen-free conditions at the University of Florida or Baylor College of Medicine and treated under approved protocols of the Institutional Animal Care and Use Committee. All animals were

male and 6–8 weeks old at the onset of the experiments; all cohorts contained at least 4 mice per group.

AAV vectors were administered intramuscularly or intravenously, as previously described [18, 20]. Plasma samples were collected by retro-orbital bleed into heparinized capillary tubes. TLR9i (ODN 2088; Invivogen) was delivered at 100 µg/mouse mixed with the vector formulation, as previously described [18]. MyD88 inhibitor (MyD88i) and control peptide (MyD88c; IMG-2005; Imgenex) were delivered intraperitoneally at 25 µg/mouse in 100 µl PBS, as previously described [24].

AAV Vectors

scAAV-CMV-hF.IX, ssAAV-CMV-hF.IX, scAAV-CMV-OVA, scAAV-CB-GFP, ssAAV-CB-GFP, ssAAV-hAAT-hF.IX, and scAAV-TTR-hF.IX vectors were constructed, as previously described [8, 20]. Vector genomes were packaged into AAV serotype 1 or 2 capsids by triple transfection of HEK-293 cells. Vector particles were purified by iodixanol gradient centrifugation, and vector titers were determined by dot blot hybridization and confirmed by Western blot using a reference standard of known titer for comparison.

Analysis of Plasma Samples

Plasma was analyzed for hF.IX expression, anti-hF.IX IgG1 or IgG2c, anti-OVA IgG1 or IgG2c, and anti-AAV1 or anti-AAV2 IgG1 or IgG2c by enzyme-linked immunosorbent assay (ELISA), as previously described [18]. For the anti-capsid antibody ELISAs, sample wells were coated with 2.5×10^9 viral genomes (vg)/well intact AAV particles.

Anti-AAV NABs were assayed, as previously described [25]. Briefly, serial dilutions of plasma were incubated with a fixed number of AAV vectors encoding GFP (MOI: 10^5 vg/cell) before infecting HEK-293 cells (AAV1) or HeLa cells (AAV2). Assays were performed with adenovirus coinfection. The final NAB titer was defined as the reciprocal of the dilution that showed >50% inhibition of transduction relative to virus alone.

Flow Cytometry

Following pretreatment with FcR Block (BD Biosciences), peripheral blood cells were stained with antibodies to CD8 (53-6.7), B220 (RA3-6B2), CD44 (IM7), CD62L (MEL14), and iTag MHC Tetramer (H2-K^b-SIINFEKL; Beckman Coulter). Red blood cell lysis was performed with VersaLyse (Beckman Coulter).

For intracellular cytokine staining, single-cell suspensions of splenocytes were prepared, as previously described [5]. Cells were resuspended in 96-well plates at 10^6 cells/well in 100 µl RPMI (supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin) and stimulated with media alone, SIINFEKL peptide (10 µg/ml) or PMA (5 ng/ml) and ionomycin (500 ng/ml) for 6 h in the presence of brefeldin A (3 µg/ml). After pretreatment with FcR Block (BD Biosciences), the cells were stained with antibodies to CD8 (53-6.7), CD107a (1D4B) and Fixable Viability Dye (eBioscience). Following permeabilization with a Cytotfix/Cytoperm kit (BD Biosciences), the cells were also stained with antibodies to IFN-γ (XMG1.2), IL-2 (JES6-5H4) and TNF-α (MP6-XT22). Data were analyzed in FCS Express (De Novo Software), using single-positive gates for each of the cytokines to create a matrix of all possible combinations. Data are reported upon subtraction of baseline data (SIINFEKL-stimulated minus unstimulated).

Adoptive Transfer

Naïve B cells were negatively selected and magnetically purified from a single-cell suspension of splenocytes using a B Cell Isolation Kit (Miltenyi Biotec.). MyD88^{-/-} mice received 5×10^7 B cells via intravenous injection in 200 µl PBS 1 day prior to vector delivery. µMT mice received 2×10^7 B cells 2 weeks prior to vector delivery, as previously described [26].

Immunohistochemistry

Immunohistochemistry was performed using fluorescent antibodies on frozen and cryosectioned tissue, as previously described [20]. Briefly, muscle tissue was harvested and frozen in liquid N₂-cooled 2-methylbutane. Cryosections (10 µm) of tissue were fixed in acetone at room temperature, blocked with 5% donkey serum (Sigma) and stained with rat anti-CD8α (eBioscience) and goat anti-hF.IX (Affinity Biologicals). Secondary antibody donkey anti-rat Alexa Fluor 488 and donkey anti-goat Alexa Fluor 568 (Life Technologies) were used for detection. Fluorescence microscopy was performed with a Nikon E800 microscope.

Statistics

Results are reported as means ± SEM. Significant differences between groups were determined with unpaired Student's *t* test, the Mann-Whitney U test, or two-way ANOVA with Bonferroni posttests, as appropriate. *p* values <0.05 were considered significant. Analyses were performed using GraphPad Prism (San Diego, Calif., USA).

Results

Antibody Responses to the Transgene

To assess the role of TLR signaling on antibody responses to the transgene product following AAV-mediated gene transfer, WT C57BL/6, TLR2^{-/-}, TLR9^{-/-}, or MyD88^{-/-} mice were injected intramuscularly with 10^{11} vg of scAAV1-CMV-hF.IX. Antibodies against hF.IX (which are typically of the IgG1 isotype) and circulating levels of hF.IX were measured subsequently for 4 weeks. Interestingly, the IgG1 antibody response to hF.IX was not dependent on signaling through any of the molecules tested, as all mice had comparable modest titers to hF.IX (fig. 1a). In this combination of vector and route, C57BL/6 mice do not produce IgG2c (the C57BL/6 equivalent of IgG2a) against hF.IX. Systemic expression of hF.IX was comparable in all backgrounds but reduced relative to RAG^{-/-} mice that are incapable of forming an adaptive immune response due to a lack of T and B cells, suggesting that these antibody levels impacted the amount of circulating transgene product (fig. 1b).

To evaluate an antigen that may elicit a less Th2-biased antibody response, we also investigated the role of these innate signaling molecules in antibody formation to OVA (ovalbumin). As before, WT, TLR2^{-/-}, TLR9^{-/-}, or MyD88^{-/-} mice were injected intramuscularly with 10^{11} vg of scAAV1-CMV-OVA, and antibody levels were tested over a 4-week period. Here, anti-OVA IgG1 levels trended towards a slight reduction in MyD88^{-/-} but not TLR2^{-/-} or TLR9^{-/-} mice (fig. 1c). The anti-OVA responses included the formation of IgG2c, which was more significantly reduced in MyD88^{-/-} mice (fig. 1d). Single TLR knockout mice developed IgG2c titers that were comparable to those in WT mice. Overall, the antibody response to OVA was relatively weak, probably be-

Fig. 1. Antibody responses to transgene. WT, TLR2^{-/-}, TLR9^{-/-}, or MyD88^{-/-} mice were injected intramuscularly with 10¹¹ vg of AAV vectors (n = 4/group) and plasma was collected weekly for 1 month. Circulating levels of anti-hF.IX IgG1 (**a**) and hF.IX (**b**) following intramuscular injection of scAAV1-CMV-hF.IX. Levels of hF.IX were also assessed in RAG^{-/-} mice 1 and 4 weeks after injection.

Following injection of scAAV1-CMV-OVA, anti-OVA IgG1 (**c**) and IgG2c (**d**) were assessed. WT and MyD88^{-/-} mice were injected with AAV2-CMV-hF.IX, and anti-hF.IX IgG1 (**e**) and IgG2c (**f**) were assessed. Data points are averages ± SEM. Statistical comparisons are made relative to WT mice. * p < 0.05, ** p < 0.01, *** p < 0.001. n.s. = Not significant.

cause the immune response to AAV-OVA is typically dominated by CD8⁺ T cells (see published data and below) [8].

Finally, we also investigated the antibody response following intramuscular administration of AAV2-CMV-hF.IX, which generally provokes stronger humoral immunity than AAV1 vectors. The antibody responses mirrored those observed with AAV1-OVA, with a trend towards slight reductions in IgG1 levels and a more significant reduction in IgG2c in MyD88^{-/-} mice relative to WT mice (fig. 1e, f). However, antibody formation against

hF.IX was dominated by IgG1, with IgG2c levels being >20-fold lower, so that the overall impact of the loss of MyD88 signaling on anti-hF.IX antibody formation was relatively minor. Although hF.IX expression in skeletal muscle persisted (online suppl. fig. S1A; for all online suppl. material, see www.karger.com/doi/10.1159/000369273), no hF.IX was detected in circulation (data not shown). Therefore, it is likely that the elimination of systemic expression was due to the high antibody levels and comparatively low expression expected from AAV2.

Fig. 2. Circulating CD8⁺ T-cell response to transgene. ns = Not significant. WT, TLR2^{-/-}, TLR9^{-/-}, or MyD88^{-/-} mice were injected intramuscularly with 10¹¹ vg of scAAV1-CMV-OVA (n = 4/group). **a** Frequencies of OVA-specific CD8⁺ T cells in circulation were assessed with an H2-K^b-SIINFEKL tetramer reagent. Results are shown as tetramer-positive percentage of CD8⁺B220⁻ cells. **b–e** 4 weeks after injection, expression of CD44 and CD62L on

OVA-specific cells was assessed. Median fluorescence intensities of CD44 (**b**) and CD62L (**c**) as well as representative plots of CD44 (**d**) or CD62L (**e**) expression among tetramer-positive and tetramer-negative CD8⁺ T cells. Data points are averages ± SEM. Statistical comparisons are made relative to WT mice. * p < 0.05, ** p < 0.01, *** p < 0.001.

CD8⁺ T-Cell Responses to the Transgene Product

AAV vectors typically do not activate CD8⁺ T cells against hF.IX in C57BL/6 mice [2, 27]. In WT C57BL/6 mice, intramuscular injection of 10¹¹ vg of ss or scAAV1-CMV-hF.IX induces long-lasting hF.IX expression with little to no CD8⁺ T-cell infiltration (online suppl. fig. S1B). To investigate the role of TLR signaling in CD8⁺ T-cell responses to the transgene product, we injected mice intramuscularly with 10¹¹ vg of AAV1-CMV-OVA and measured the frequencies of OVA-specific CD8⁺ T cells with an H2-K^b-SIINFEKL tetramer. In contrast to the antibody responses, the CTL responses to the transgene product were much more dependent on the TLR9-MyD88 pathway. Frequencies of circulating OVA-specific CD8⁺ T cells were reduced substantially in TLR9^{-/-} and MyD88^{-/-} mice, whereas TLR2^{-/-} mice displayed frequencies

comparable to WT mice (fig. 2a; online suppl. fig. S2). At 4 weeks after injection, these cells displayed an effector phenotype, being primarily CD44^{hi}CD62L⁺ relative to the tetramer-negative CD8⁺ T cells (fig. 2b–e).

Concurrently, we measured the functionality of splenic OVA-specific CD8⁺ T cells by determining their ability to produce IFN-γ, IL-2, TNF-α, and CD107a (indicative of granular release). Following restimulation with SIINFEKL peptide, cells from mice of all genotypes expressed IFN-γ, CD107a and TNF-α (fig. 3a, b; online suppl. fig. S3A, B). The frequency and absolute number of IFN-γ⁺ cells mirrored that of tetramer-positive cells, being significantly higher in WT and TLR2^{-/-} mice relative to TLR9^{-/-} and MyD88^{-/-} mice. Although reduced, cytokine expression was still detectable in SIINFEKL-specific CD8⁺ T cells (above unstimulat-

Fig. 3. Polyfunctionality of transgene-specific CD8⁺ T cells. n.s. = Not significant. WT, TLR2^{-/-}, TLR9^{-/-} or MyD88^{-/-} mice were injected intramuscularly with 10¹¹ vg of scAAV1-CMV-OVA (n = 4/group). 4 weeks after injection, spleens were harvested and restimulated in vitro with SIINFEKL peptide in the presence of brefeldin A, then stained for surface CD107a and intracellular IFN-γ, TNF-α and IL-2. Results show frequencies of cytokine-expressing cells from stimulated samples minus unstimulated controls. **a** Frequen-

cy of CD8⁺ cells expressing each cytokine individually. **b** Number of IFN-γ⁺ cells per spleen. **c, d** A matrix of gates was created from the individual gates of each functionality marker. **c** Stacked bar graph showing the frequency of cells expressing 1, 2, 3, or 4 functionality markers. **d** Bar graph showing individual marker combinations. Combinations with frequencies less than 0.05% were excluded. Data points are averages ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

ed cells) in TLR9^{-/-} and MyD88^{-/-} mice. Consistent with the effector phenotype observed in circulation, very few of the cells produced IL-2.

In terms of polyfunctionality, most cells expressed either 1 or 2 cytokines, with some expressing 3 and very few expressing all 4 (fig. 3c; online suppl. fig. S3C). The most striking difference between WT/TLR2^{-/-} and TLR9^{-/-}/MyD88^{-/-} mice was the overall frequency of cytokine-producing cells, with the latter two being significantly reduced relative to the former. Nevertheless, there was no significant defect in the functionality of CD8⁺ T cells from TLR9^{-/-} mice, which had similar proportions of 1, 2 and 3 function cells as WT mice. While MyD88^{-/-} mice trended toward a somewhat higher proportion of monofunctional cells, they also generated bi- and trifunctional CD8⁺ T cells. Although major differences were not observed for most individual cytokine combinations within high-responding (WT, TLR2^{-/-}) or low-responding (TLR9^{-/-}, MyD88^{-/-}) cohorts, all knockout mice had elevated frequencies of CD107a single-positive cells compared to WT mice (fig. 3d). Thus, while the efficient activation of transgene-specific CD8⁺ T cells is strongly dependent on the TLR9-MyD88 pathway, the impact of this pathway on the functionality of cells that are still activated at low frequency in its absence may be more subtle, warranting further study.

Antibody Responses to the AAV Capsid

In addition to immune responses to the transgene product, we also investigated the role of TLR signaling in antibody responses to the AAV capsid. Since the antibody response to AAV is typically dominated by Th1-associated IgG2a/c, we started by measuring the levels of these antibodies. As with transgene-specific immunity, anti-capsid IgG2c levels following intramuscular injection of 10¹¹ vg of AAV1 were unaltered in TLR2^{-/-} or TLR9^{-/-} mice, whereas there was a significant drop in MyD88^{-/-} mice (fig. 4a). However, although the NAB levels were reduced in MyD88^{-/-} mice (but unchanged in TLR2^{-/-} or TLR9^{-/-} mice), a substantial neutralization of in vitro AAV transduction remained (fig. 4d). Some studies have suggested that Th1-associated IgG2c antibodies, but not Th2-associated IgG1, may be associated with MyD88 signaling [28, 29]. In the absence of MyD88 signaling, we found that anti-AAV1 IgG1 antibodies were significantly elevated (fig. 4b). Interestingly, the anti-capsid IgG1 response appears to be delayed relative to the formation of IgG2c. While IgG2c was detectable 1 week after injection, anti-capsid IgG1 did not emerge until between 2 and 3 weeks after vector administration. There was also a trend towards elevated IgG1 responses in TLR9^{-/-} mice, though these mice did not have reduced IgG2c; only in MyD88^{-/-} mice was the IgG2c/IgG1 ratio significantly altered (fig. 4b, c). This phenomenon was serotype independent, as the shift in the subclass of anti-

Fig. 4. Antibody responses to the AAV capsid. n.s. = Not significant. **a–d** WT, TLR2^{−/−}, TLR9^{−/−}, or MyD88^{−/−} mice were injected intramuscularly with 10¹¹ vg of scAAV1 (n = 4/group). Circulating levels of anti-AAV1 IgG2c (**a**) and IgG1 (**b**) were assessed 1, 2, 3, and 4 weeks later. 4 weeks after injection, the ratio of IgG2c/IgG1 (**c**) and NAB reciprocal titers (**d**) are shown. NABs were also measured for TLR2^{−/−} mice coinjected with scAAV1 and a TLR9 in-

hibitor. **e–h** WT or MyD88^{−/−} mice were injected intramuscularly with 10¹¹ of scAAV2 (n = 4/group). Circulating levels of anti-AAV2 IgG2c (**e**) and IgG1 (**f**) were assessed 1, 2, 3, and 4 weeks later. 4 weeks after injection, the ratio of IgG2c/IgG1 (**g**) and NAB reciprocal titers (**h**) are shown. Data points are averages ± SEM. Statistical comparisons are made relative to WT mice. * p < 0.05, ** p < 0.01, *** p < 0.001.

capsid antibodies in MyD88^{−/−} mice from IgG2c to IgG1 was also observed following intramuscular injection of AAV2 (fig. 4e–g). There was also a similar trend towards reduction in the NAB titer to AAV2 in these mice (fig. 4h).

As MyD88 is downstream of TLR2 and TLR9 signaling, we sought to determine whether the inhibition of both innate sensors would mimic the response seen in MyD88^{−/−} mice. TLR2^{−/−} mice coinjected with an oligonucleotide inhibitor of TLR9 developed comparable NABs to WT mice, suggesting that the effect in MyD88^{−/−} mice is not due to a combinatorial effect of TLR2 and TLR9 signaling (fig. 4d). Moreover, the daily administration of a peptide inhibitor of MyD88 (MyD88i), which blocks MyD88 activity by binding to TIR homology domains and preventing the homodimerization interactions that facilitate MyD88 signaling, failed to replicate the shift in anti-capsid antibody subclasses observed in MyD88^{−/−} mice (online suppl. fig. S4) [24]. It is, therefore, possible that the effect of MyD88 on antibody responses to the AAV capsid may be independent of its role in TLR signal transduction, albeit a solid conclusion on this point awaits further study, including an investigation on the effectiveness of treatment with the MyD88 inhibitor.

The fact that mice are capable of forming both MyD88-dependent and MyD88-independent antibodies to the AAV capsid suggests that an alternative sensing mechanism may be involved in the formation of MyD88-independent IgG1 antibodies. Given the DNA

genome packaged by AAV, we investigated whether cytoplasmic DNA sensing might have a role in anti-capsid antibody formation using mice deficient in the downstream signaling adaptor STING, which integrates signaling through multiple sensors of cytoplasmic DNA (including cGAS and IFI16) [30]. However, WT and STING^{gt/gt} mice produced comparably high levels of anti-AAV2 IgG2c and negligible IgG1, suggesting that this pathway is unlikely to be involved in antibody formation against capsid (online suppl. fig. S5).

Others have previously reported that B cell-intrinsic MyD88 signaling may be required for the induction of IFN-γ-producing CD4⁺ T cells and the subsequent formation of Th1-associated IgG2c antibody responses [22, 28, 29]. To verify this, we adoptively transferred WT B cells into MyD88^{−/−} mice 1 day prior to vector administration. In MyD88^{−/−} mice complemented with WT B cells, anti-capsid IgG2c levels were moderately increased and anti-AAV1 IgG1 was reduced relative to MyD88^{−/−} mice (fig. 5a, b). Together, these shifts partially restored the IgG2c/IgG1 ratio (fig. 5c), suggesting that MyD88 signaling in B cells is sufficient to produce anti-capsid IgG2c. Next, we investigated whether MyD88 signaling in other types of antigen-presenting cells could also restore the anti-capsid IgG balance. To ensure that other antigen-presenting cells would express MyD88, WT or MyD88^{−/−} B cells were adoptively transferred into μMT mice, which lack mature B cells due to a disruption of the IgM heavy chain. Unexpectedly, μMT mice that received either type of B cell formed anti-capsid

Fig. 5. Impact of B cell-intrinsic MyD88 in antibody responses to the AAV capsid. WT, MyD88^{-/-} or MyD88^{-/-} mice that received 5×10^7 naïve B cells 1 day prior were injected intramuscularly with 10^{11} vg of scAAV1 ($n = 4$ /group). Circulating levels of anti-AAV1

IgG2c (**a**) and IgG1 (**b**) were assessed 1, 2, 3, and 4 weeks later. **c** Ratio of IgG2c/IgG1 4 weeks after injection. Data points are averages $\pm$ SEM. Statistical comparisons are made relative to WT mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. = Not significant.

IgG1 antibodies rather than IgG2c (online suppl. fig. S6). This was possibly due to an intrinsic defect in these mice (e.g. lymph node structure or CD4⁺ T cell functionality) [31]. Hence, B cell-intrinsic MyD88 is sufficient for anti-capsid IgG2c, whereas the role of other antigen-presenting cells remains unclear.

Next, we wanted to determine the impact of MyD88-independent anti-capsid antibody formation in vivo. WT or MyD88^{-/-} mice were injected intravenously with 10^{11} vg of scAAV2-GFP, and 4 weeks later received 10^{11} vg of AAV2-hf.IX to measure transduction capacity in the presence of preexisting MyD88-dependent or MyD88-independent antibodies. As previously observed, WT mice formed an IgG2c-dominated antibody response and MyD88^{-/-} mice primarily produced anti-capsid IgG1 (fig. 6a–c), suggesting that this phenomenon was not dependent on intramuscular immunization. In both groups of mice, 4 weeks after the second injection, no circulating levels of hf.IX were detected, whereas control mice (which did not receive an initial immunization and were thus naïve to AAV capsid) produced systemic hf.IX levels (fig. 6g). In order to rule out a role of enhanced TLR9 activation by scAAV, we repeated the study using ssAAV2-GFP. As with scAAV, the antibody subclass shift was observed in MyD88^{-/-} mice following an injection of ssAAV2 (fig. 6d–f). Even though the antibody levels in both cohorts were somewhat reduced compared to mice that received scAAV, they were still sufficient to completely prevent systemic hf.IX expression 4 weeks after the second injection (fig. 6g). Therefore, MyD88-independent antibodies are sufficient to completely prevent the readministration of either ss or scAAV vectors.

Role of Type I IFN in Antibody Responses to the Transgene Product and Capsid

One of the major signaling pathways downstream of TLRs results in the induction of type I IFN (interferon). To investigate the role of this pathway in antibody responses to AAV-mediated gene transfer, IFN- α receptor-deficient mice (IFN α R^{-/-}) were injected intramuscularly with 10^{11} vg of scAAV1-hf.IX. Through 10 weeks, circulating levels of hf.IX and anti-hf.IX IgG1 were comparable in WT and IFN α R^{-/-} mice, suggesting that type I IFN signaling is

not required for antibody responses to the transgene product (fig. 7a, b). Similarly, AAV1 NAB levels 4 weeks after injection were not affected by the loss of type I IFN signaling (fig. 7c). However, when mice were injected intravenously with an scAAV2 vector, a significant reduction in NAB levels was observed (fig. 7d). These results suggest that the role of type I IFN signaling may be contingent on the route of vector administration, with antibody formation following intravenous, but not intramuscular, injection being partially dependent on type I IFN signaling.

Discussion

Clinical studies of AAV-mediated gene therapy for hemophilia B have revealed that adaptive immune responses to the AAV capsid, including NAB and CD8⁺ T cells, pose a significant barrier to the efficacy of therapeutic gene transfer [11, 12]. Preclinical studies have highlighted the risk of transgene product-specific adaptive immunity during muscle gene transfer due to a local immune response [4, 8, 9]. Rare anti-transgene product CD8⁺ T-cell responses have also been observed in clinical trials [3]. However, these studies have mostly enrolled patients with missense mutations that have a reduced risk to develop anti-transgene immunity. The danger of these responses in a broader patient population is not yet known.

Bridging Innate and Adaptive Immunity to AAV-Mediated Gene Transfer

To better understand the mechanism of immune responses to AAV-mediated gene transfer, we were interested in the ability of innate immune responses to shape

Fig. 6. In vivo neutralizing capacity of MyD88-dependent and MyD88-independent antibodies. WT or MyD88^{-/-} mice were injected intravenously with 10¹¹ vg of scAAV2-GFP (**a–c**) or ssAAV2-GFP (**d–f**; n = 4/group). 4 weeks later, mice received 10¹¹ vg of ssAAV2-hAAT-hF.IX. Circulating levels of IgG2c (**a, d**) and IgG1 (**b, e**) were assessed 4 and 8 weeks after the initial administration. The ratio of IgG2c/IgG1 is also shown for mice initially immunized with scAAV (**c**) or ssAAV (**f**). **g** Circulating levels of hF.IX 4 weeks after the second injection compared to naïve mice (n = 4) that did not receive an initial immunization. Data points are averages ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001. n.s. = Not significant.

Fig. 7. Role of type I IFN in antibody responses to AAV-mediated gene transfer. n.s. = Not significant. WT (n = 9) or IFNαR^{-/-} (n = 8) mice were injected intramuscularly with 10¹¹ vg of scAAV1-CMV-hF.IX. Circulating levels of hF.IX (**a**) and anti-hF.IX IgG1 (**b**) were assessed weekly for 10 weeks. **c** NAB reciprocal titers

4 weeks after injection. **d** WT or IFNαR^{-/-} mice were injected intravenously with 10¹¹ vg of scAAV2 (n = 8/group) and NAB reciprocal titers were measured 4 weeks after injection. Data points are averages ± SEM. * p < 0.05.

adaptive immunity. Due to its weak and transient nature, innate immunity to AAV was ignored for a number of years [15]. More recently, it was suggested that TLR9 signaling was critical for both humoral and cellular immune responses to the AAV capsid and the transgene product [16]. The role of TLR9 in CD8+ T-cell responses has been reaffirmed by others and is further supported by our data here [17, 18]. We found a significant but incomplete reduction in the number of transgene-specific CD8+ T cells in TLR9^{-/-} and MyD88^{-/-} mice. Surprisingly, these cells still possessed the capacity to produce multiple cytokines in response to peptide stimulation. These somewhat opposing results could be due to the divergent natures of the transgene (e.g. inherent immunogenicity or secreted vs. cytoplasmic) or the AAV serotype. Alternatively, differences in T-cell functions may have eluded detection by our panel, which was limited to only 4 of the many factors that are produced by T cells upon recognition of their antigen.

In contrast, the role of innate immunity in antibody responses is less clear. As opposed to the initial findings, contradictory data in the literature suggest an incomplete role for TLR9 signaling or a function for MyD88 but not TLR9 in shaping antibody responses [18, 20, 22]. As a whole, our data agree with the latter conclusions. Although TLR9 slightly modulated the anti-capsid antibody response by suppressing IgG1 formation, only the loss of MyD88 significantly reduced IgG2c and NAB formation. Transgene product-specific antibody responses were largely independent of TLR signaling. While a reduction in anti-transgene product IgG2c antibodies was observed in MyD88^{-/-} mice, there was minimal modulation of the dominant IgG1 response to hF.IX [4]. We have previously shown that TLR9 signaling in B cells induced by plasmid transfection can enhance anti-hF.IX antibody responses, so these marginal effects might be related to the low magnitude of innate immune signaling or temporal separation of inflammation and transgene expression during AAV-mediated gene transfer [14, 32]. Finally, we observed no effect of the loss of TLR2 signaling on humoral or cellular immune responses to transgene or capsid in mice.

Interestingly, circulating hF.IX was detected in mice injected with AAV1 but not AAV2 vectors. Expression was, however, detected in the tissue of these mice in the absence of CD8+ T-cell infiltration. MyD88^{-/-} mice would not be expected to mount a CTL response to hF.IX, as our data with OVA suggest that these mice are significantly deficient in anti-transgene CD8+ T-cell responses. Moreover, WT C57BL/6 mice are hyporesponsive to hF.IX and typically do not mount these responses

during AAV-mediated gene transfer [2, 27]. The use of an scAAV vector did not significantly alter this, as hF.IX expression persisted for at least 25 weeks with minimal CD8+ T-cell infiltration in mice treated with ss or scAAV1 vectors. Instead, it is likely that the transgene neutralization in AAV2-injected mice was due to the humoral immune response. AAV2 vectors are known to induce lower transgene levels than AAV1 during intramuscular gene transfer. Silencing of the CMV promoter due to inflammation, particularly with an scAAV vector, might play an additional role in further reducing hF.IX expression [20]. However, silencing of the CMV promoter is more typically seen in liver gene transfer. Thus, circulating hF.IX was probably neutralized due to a combination of the higher antibody levels and lower expression induced by AAV2 vectors relative to AAV1, as previously observed in hemophilic mice [4, 20].

MyD88-Dependent and MyD88-Independent Antibodies to the AAV Capsid

In accordance with previous reports using AAV vectors lacking a transgene ('null' vectors), we found that B cell-intrinsic MyD88 was sufficient to induce IgG2c formation to transgene-expressing AAV vectors [22]. However, despite the loss of Th1-associated IgG2c antibodies, MyD88^{-/-} mice produced an unexpectedly robust anti-capsid IgG1 response. MyD88-independent antibodies were able to completely prevent in vivo readministration of AAV vectors, regardless of whether ss or scAAV vectors were used. Of note, the kinetics of the anti-capsid IgG1 response were delayed relative to IgG2c. The overall antibody response was not retarded, however, as we detected anti-capsid IgM 1 week after injection in both the WT and MyD88^{-/-} mice (data not shown), suggesting a slower class switch to IgG1. These differential kinetics suggest that the underlying mechanisms for MyD88-dependent and MyD88-independent antibodies may differ.

Although the loss of IgG2c antibodies in MyD88^{-/-} mice has previously been linked to a reduced number of IFN- γ -secreting CD4+ T cells [22, 28, 29], it is unlikely that MyD88-independent IgG1 antibodies are formed independent of T-cell help, as robust humoral immune responses to AAV have previously been shown to be T dependent [33]. Instead, this may be due to a shift in the Th1/Th2 ratio among CD4+ T cells, as other studies have related MyD88 signaling and cytokine production in B cells to Th-polarization and IgG subclass induction [34, 35]. As some studies have suggested, it is possible that the response by default switches to Th2 in the absence of Th1-polarizing stimuli [36], which may, for example, be provided by CpG

motifs contained in viral particles, resulting in a direct MyD88-dependent adjuvant effect on B cells [29]. Alternatively, stimulation of the TLR9-MyD88 pathway, particularly in monocyte-derived DCs, may drive differentiation of follicular helper T cells and promote robust IgG2c production by B cells/plasma cells to soluble OVA [37].

Discrete Functions of TLR9 and MyD88

Clearly, TLR9 and MyD88 mediate distinct effects on adaptive immune responses to AAV-mediated gene transfer. The literature is inconsistent with regard to this phenomenon, with some studies reporting distinct roles for single TLR and MyD88 knockouts, whereas others indicate comparable phenotypes [38]. Though the effect of MyD88 could potentially be a combinatorial effect of TLR2 and TLR9 signaling, this seems unlikely, since the use of an oligonucleotide inhibitor of TLR9 in TLR2^{-/-} mice had no effect on NAB formation. Rather, three potential explanations for this distinction seem more likely. Firstly, there may be a yet unidentified TLR upstream of MyD88 facilitating the production of IgG2c. Secondly, the role of MyD88 in antibody responses may be unrelated to TLR signaling. The most obvious other potential role for MyD88 is in IL-1 β signaling. However, we have previously shown that AAV does not induce IL-1 β synthesis. Furthermore, it has been previously demonstrated that the antibody response to AAV is unaltered in IL-1R^{-/-} mice [18, 22]. Instead, the function of MyD88 may be ancillary to its TIR domain, which binds to both TLRs and the IL-1R via homodimerization interactions. For instance, MyD88 has been shown to bind to the signaling receptor TACI on B cells through a unique site, and mutation of this site in human cells resulted in impaired cooperation between TACI and TLR9 signaling and reduced IgG1 (equivalent to murine IgG2a/c) class switch recombination [39, 40]. This theory is supported by the fact that a peptide inhibitor of MyD88 that binds to the TIR domain had no effect on the antibody response to AAV. Though we cannot rule out the possibility that the inhibitor dose was too low, it is likely that at least a partial effect on the IgG2c/IgG1 ratio would be expected since mixed responses were observed in other cases. Finally, it is possible that MyD88-independent antibodies emerge in a manner inconsistent with the classical model of pattern recognition-induced inflammation.

Influence of the Route of Administration on Anti-Capsid Antibodies

The matter of the anti-capsid antibody response is further complicated by our results in IFN α R^{-/-} mice. Al-

Fig. 8. Model of innate immune pathways involved in AAV-mediated gene transfer. **a** The TLR9-MyD88 pathway, which senses the AAV genome, is involved in CD8⁺ T-cell responses. It may also be involved in suppressing anti-capsid IgG1 antibody responses when MyD88 signaling is intact. **b** Through an unknown pathway, B cell-intrinsic MyD88 regulates the production of capsid-specific Th1-associated IgG2c antibodies. **c** An unidentified mechanism leads to the production of MyD88-independent anti-capsid IgG1.

though anti-AAV antibody formation in MyD88^{-/-} mice was comparable regardless of the route of administration, in IFN α R^{-/-} mice, we found that NAB activity was significantly reduced following intravenous, but not intramuscular, injection. Historical data as well as our own findings suggest that the serotype probably has a minimal influence on the antibody response to capsid [16, 22]. It seems more likely that this is a result of the route of administration, since intramuscular inoculation is generally believed to be more immunogenic than intravenous injection. It is important to note that, in addition to type I IFN, AAV also provokes NF- κ B-mediated proinflammatory pathways that have been shown to affect anti-capsid antibody formation [41]. In addition, our results suggest an impact of the route of administration on anti-capsid immunity.

Implications for Gene Therapy

These results reinforce the idea that, despite their transient nature and limited magnitude, innate immune responses to AAV vectors can influence adaptive immune responses to the capsid and transgene product (fig. 8).

However, the effects we observed were not absolute. TLR9^{-/-} mice formed a low number of transgene-specific CD8⁺ T cells, and MyD88^{-/-} mice produced anti-capsid IgG1 that precluded vector readministration. These observations add to the growing evidence that the TLR9-MyD88 signaling pathway is much more critical for the activation of CD8⁺ T cells than for antibody formation. One aspect not explored here is the effect of vector dose on the immune response. All studies were performed using a dose of 10¹¹ vg/mouse (about 4 × 10¹² vg/kg), which is comparable to the high dose cohorts in several clinical trials [11, 12]. Since immune responses to AAV-mediated gene transfer are dose dependent, it is possible that a lower vector dose would pass below a threshold for these responses [3, 7]. While MyD88-independent antibodies showed reduced efficacy *in vitro*, they were completely neutralizing *in vivo* at 10¹¹ vg/mouse. It is possible that inhibition of MyD88 signaling may be more effective in preventing NAB formation at lower vector doses. Alternatively, combination with other immunosuppressive therapies may be useful. Other situations may also be more favorable in preventing transgene product-specific immune responses. For instance, we have previously shown that, while scAAV vectors induced elevated CD8⁺ T-cell responses to hF.IX in hemophilia B mice with a null

mutation, neither the antibody nor CTL responses were observed when the vector was delivered to transgenic mice expressing a nonfunctional form of hF.IX [20]. Moreover, anti-hF.IX antibodies were observed in transgenic mice receiving AAV2 but not AAV1 vectors [4, 20]. Thus, the role of innate immune signaling may be more marked in the context of therapeutic gene transfer. Overall, these findings suggest that blocking innate immune signaling may be of benefit in preventing immune responses to AAV-mediated gene transfer.

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Disclosure Statement

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