TLR9-Activating CpG-B ODN but Not TLR7 Agonists Triggers Antibody Formation to Factor IX in Muscle Gene Transfer

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Innate immune signals that promote B cell responses in gene transfer are generally ill-defined. In this study, we evaluate the effect of activating endosomal Toll-like receptors 7, 8, and 9 (TLR7, TLR7/8, and TLR9) on antibody formation during muscle-directed gene therapy with adeno-associated virus (AAV) vectors. We examined whether activation of endosomal TLRs, by adenine analog CL264 (TLR7 agonist), imidazolquinolone compound R848 (TLR7/8 agonist), or class B CpG oligodeoxynucleotides ODN1826 (TLR9 agonist), could augment antibody formation upon intramuscular administration of AAV1 expressing human clotting factor IX (AAV1-hFIX) in mice. The TLR9 agonist robustly enhanced antibody formation by the 1st week, thus initially eliminating systemic hFIX expression. By contrast, the TLR7 and TLR7/8 agonists did not markedly promote antibody formation, or significantly reduce circulating hFIX. We concurrently investigated the effects of these TLR agonists during muscle gene transfer on mature B cells and dendritic cells (DCs) in the draining lymph nodes including conventional DCs (CD11b⁺ or CD8 α ⁺ cDCs), monocyte-derived dendritic cells (moDCs), and plasmacytoid dendritic cells (pDCs). Only TLR9 stimulation caused a striking increase in the frequency of moDCs within 24 h. The TLR7/8 and TLR9 agonists activated pDCs, both subsets of cDCs, and mature B cells, whereas the TLR7 agonist had only mild effects on these cells. Thus, these TLR ligands have distinct effects on DCs and mature B cells, yet only the TLR9 agonist enhanced the humoral immune response against AAV-expressed hFIX. These new findings indicate a unique ability of certain TLR9 agonists to stimulate B cell responses in muscle gene transfer through enrichment of moDCs.

Keywords: adeno-associated virus, muscle, factor IX, antibody, Toll-like receptor

INTRODUCTION

GENE THERAPY FOR GENETIC DISEASES using adeno-associated virus (AAV) vectors has had multiple clinical successes in recent years, including recent Food and Drug Administration (FDA) approval for treatment of retinal dystrophy caused by biallelic RPE65 gene mutations and promising clinical trial results for the treatment of the X-linked bleeding disorder hemophilia.^{1,2} Although current gene therapy protocols for hemophilia are based on hepatic gene transfer, the first clinical trial was based on intramuscular gene transfer, an approach that continues to evolve in animal models of hemophilia B.³

The first gene therapy drug approved in the Western world also utilized AAV gene transfer to muscle.⁴ Besides for correction of muscle disorders, muscle-directed gene therapy has strong potential for delivery of therapeutic proteins into systemic circulation, including coagulation factors, α_1 -antitrypsin, lipoprotein lipase, monoclonal antibodies (for protection against infectious diseases, such as HIV infection), among others. A threat to the success of muscle-derived systemic delivery of proteins is the potential for neutralization by antibodies. This problem has become obvious with experiments in animal models of genetic disease

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and the formation of antibodies to AAV-expressed anti-HIV antibodies ("anti-antibodies").⁵ Therefore, exploring immune signals that trigger antibody formation in muscle gene transfer is critical.

AAV1 is one of several serotypes that efficiently transduce muscle fibers.⁶ During infection, the AAV vector binds to a primary host cell receptor and is internalized by receptor-mediated endocytosis into the endosomal compartment.^{7–11} AAV then undergoes conformational changes that expose capsid regions that allow endosomal escape and nuclear localization.^{10,12,13} Inside the nucleus, AAV is released by capsid uncoating, and conversion from single- to double-stranded DNA occurs either by annealing of plus and minus strands delivered to the same cell or by second-strand synthesis using host polymerase.^{14,15} However, while in the endosomal compartment, unmethylated CpG sequences of the viral vector DNA genome may activate Toll-like receptor 9 (TLR9).

TLR9 is an endosomal pattern recognition receptor for DNA and is, for example, expressed by murine dendritic cells (DCs) and B cells.^{16–18} When activated, TLR9 couples with adaptor signaling molecule myeloid differentiation factor 88 (MyD88), initiating a signaling cascade that increases expression of transcription factors nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1), and interferon regulatory factor-7 (IRF7).¹⁹ NF- κ B and AP-1 increase production of proinflammatory cytokines (interleukin [IL]-1, IL-6, IL-12, IL-18, interferon [IFN]- γ , and tumor necrosis factor [TNF]- α) and expression of surface costimulatory/presentation molecules (CD86, CD40, and MHCII).^{19–24} Primarily in plasmacytoid dendritic cells (pDCs), IRF7 increases production of type I interferons, which cause other cells to become resistant to viral replication and increase MHCII expression, as well as promote killing of virus-infected cells through increased cross-priming of cytotoxic T lymphocytes (CTLs), particularly by CD8 α^+ cDCs.^{19,25} TLR9 engagement of its ligand also promotes the humoral response both indirectly through CD4 $^+$ helper T cell activation by TLR-stimulated DCs (T cell-dependent route) and directly by way of TLR stimulation in B cells (T cell-independent route).^{19,26}

Several structurally distinct classes of synthetic CpG motif-containing oligodeoxynucleotides (ODNs) that are able to activate TLR9 and produce different immune responses include CpG-A, CpG-B, and CpG-C ODNs.^{27,28} CpG-A ODNs have a greater effect on increasing type I interferon production in pDCs, while CpG-B ODNs drive B cell proliferation and differentiation more strongly; CpG-C ODNs produce a combination of the two effects.^{27,28}

Although depletion of CpG motifs within the AAV genome significantly reduces CTL killing during AAV gene therapy, antibody formation against AAV capsid and transgene protein is relatively unaffected.^{29–32} In fact, we previously found that sensing of the AAV genome by TLR9 has a modifying effect on antibody formation, but is not required.^{31,33} This is in contrast to TLR9's critical role in activation of CTLs upon sensing of the AAV genome in pDCs. Nonetheless, TLR9 activation through an ODN ligand induces antibody formation against the transgene product.^{31,33} Specifically, we previously demonstrated that coadministration of AAV1 expressing human clotting factor IX (hFIX) under control of a CMV promoter (AAV1-hFIX) with ODN1826, a CpG-B ODN that activates TLR9, enhances antibody formation against hFIX.³¹ Antibody formation correlated with activation of monocyte-derived dendritic cells (moDCs) and enhancement of follicular helper T cell (T_{FH} cell) responses.³¹ In contrast, both intermediate-purity lipopolysaccharides (LPS) from *Porphyromonas gingivalis*, LPS-PG (TLR2/4 agonist), and high-purity LPS from *Escherichia coli*, LPS-EC (TLR4 agonist), failed to enhance anti-hFIX formation.³¹

Interestingly, in TLR9 $^{-/-}$ mice, the TLR9 agonist was still able to reduce hFIX levels and cause a less robust but still measurable increase in antibody formation.³¹ A separate study showed that TLR9 $^{-/-}$ mice retain some innate cytokine production in response to infection with murine cytomegalovirus, a double-stranded DNA virus; however, in TLR7 $^{-/-}$ TLR9 $^{-/-}$ double-knockout mice, innate cytokine production was completely ablated.³⁴ Others found that TLR7 agonists induce memory B cell activation against protein antigen VIII.^{35,36} Like TLR9, TLR7 is localized to endosomes and uses MyD88 as a cytosolic adapter molecule; however, the natural ligand recognized by TLR7 is viral single-stranded RNA rather than DNA.¹⁹ Nonetheless, TLR7 may also be stimulated by CpG ODNs to a lesser extent.

Patients receiving gene therapy might receive TLR7 ligand exposure during routine vaccination or incidentally with TLR7-stimulating RNA viruses (e.g., influenza virus), both of which could potentially hamper gene therapy if TLR7 stimulation enhances antibody formation.³⁷ In addition, the topical TLR7 agonist, imiquimod, is FDA approved for treatment of anogenital warts, actinic keratosis, and superficial basal cell carcinoma.^{38,39} TLR7 agonists are also being investigated for use in cancer vaccines,³⁹ hepatitis B and C virus treatment,^{40–42} reduction in allergen responsiveness,^{43,44} and respiratory disease treatment.⁴⁵ With continued development of both TLR7 agonist and AAV gene

therapies, there is increasing potential for overlap in patients, making investigation into the effects of activating TLR7 or other TLRs increasingly important. Therefore, we asked whether TLR7 activation may, as seen with TLR9, trigger antibody formation to AAV transgene products in muscle gene transfer.

We find that different TLR7 agonists have distinct effects on DC and B cell populations in the lymph nodes draining the injection site but, in contrast to TLR9-activating CpG-B ODNs, fail to trigger anti-hFIX formation in AAV gene transfer to skeletal muscle. Thus, TLR9 appears to have a unique capability among TLRs to induce antibody formation against transgene products in muscle gene transfer.

MATERIALS AND METHODS

Viral vectors

Single-stranded AAV serotype 1 vector expressing human F9 cDNA under control of the cytomegalovirus immediate-early enhancer/promoter (AAV1-hFIX) was as described previously.^{31,32,46} Vector was produced by triple transfection of HEK-293 cells with helper plasmid, pXYZ1, and vector plasmid, pAAV-CMV-hFIX.^{47,48} The virus was purified by iodixanol density gradient centrifugation, and titer was determined by Dot Blot using a gene-specific probe.⁴⁸

Animal experiments

Wild-type male C57BL/6J mice (4–6 weeks old) received 1×10^{11} vector genomes of AAV1-hFIX by intramuscular injection (50 μ L) by Hamilton syringe into the quadriceps muscle of a single hind leg for assessing transgene expression and antibody formation or into each hind leg for evaluating effects on DCs and B cells. TLR agonists (InvivoGen, San Diego, CA) were delivered mixed (50 μ g) with AAV1-hFIX and included class B CpG ODN1826 (TLR9 agonist), imidazoquinoline R848 (TLR7/8 agonist), or adenine analog CL264 (TLR7 agonist); $n = 8$ mice per experimental group for assessment of antibody titers and circulating hFIX and $n = 4$ mice per experimental group for evaluation of DCs and B cells. Plasma was obtained from the retroorbital plexus blood collected into heparinized capillary tubes every week for 6 weeks after vector administration. Cells from inguinal and popliteal lymph nodes of injected legs were isolated from euthanized mice 24 h after AAV administration using methods described previously and pooled for each mouse.^{31,49,50} Mice were purchased from Jackson Laboratory and housed at University

of Florida or Indiana University and treated under protocols approved by the Institutional Animal Care and Use Committee.

Enzyme-linked immunosorbent assay and flow cytometry

Cells isolated from draining lymph nodes (dLNs) were depleted of T cells using a CD3 ϵ MicroBead Kit (Miltenyi Biotec, Auburn, CA). B cells were subsequently isolated from DCs using a CD19 MicroBead Kit (Miltenyi Biotec). For flow cytometry, the CD3 $^{-}$ CD19 $^{-}$ cell fraction was used for the DC panel and the CD3 $^{-}$ CD19 $^{+}$ cell fraction was used for the B cell panel. Nonspecific binding of immunoglobulin to fragment crystallizable (Fc) receptors was blocked (CD16/32; S17011E; BioLegend, San Diego, CA) and dead cells were identified using amine-reactive fluorescent viability dye (BioLegend). The DC panel included a lineage gate with fluorophore-conjugated antibodies (BioLegend) that emit light at the same wavelength as the viability dye to exclude natural killer cells with CD49b (E50-2440), T cells with CD3 (17A2), B cells with CD19 (6D5), neutrophils with Ly-6G (1A8), and eosinophils with Siglec-F (HM α 2). The DC panel included fluorophore-conjugated antibodies (BioLegend) to CD11c (N418), CD11b (M1/70), CD8 α (53-6.7), CD64 (X54-5/7.1), MHCII (M5/114.15.2), CD86 (GL-1), CD40 (3/23), and Fc ϵ RI α (MAR-1). The B cell panel included fluorophore-conjugated antibodies (BioLegend) to CD93 (AA4.1), IgD (11-26c.2a), MHCII (M5/114.15.2), CD86 (GL-1), and CD40 (3/23). After staining, cells from both panels were fixed and permeabilized according to manufacturer's instructions (eBioscience, San Diego, CA). Flow cytometry was performed on an Attune NxT (Thermo Fisher Scientific, Waltham, MA). Levels of hFIX and hFIX-specific antibodies in plasma samples were determined by enzyme-linked immunosorbent assay as previously described.⁵¹

Data analysis

Flow cytometry data were analyzed with FCS Express 6 software (De Novo Software, Glendale, CA) using standard methods. Statistical analysis was performed using GraphPad Prism version 8.1.1 (GraphPad Software, La Jolla, CA). Data were consolidated into the following groups for analysis: (1) circulating hFIX levels, (2) α -hFIX IgG1 antibody titers, (3) α -hFIX IgG2c antibody titers, and (4) cell frequency and cell activation. Unpaired *t*-tests were performed on each of the mentioned data groups comparing control mice receiving only AAV versus each other treatment group. Phosphate-buffered saline-treated mice

were also compared with naive mice. The unpaired *t*-tests were corrected for multiple comparisons using the Holm–Šidák method for calculating statistical significance. A one-way analysis of variance (ANOVA) (based on general linear model) was also performed on data groups 1–3, comparing control mice receiving only AAV with each other treatment group, which assumed Gaussian distribution of residuals and corrected for multiple comparisons using the Dunnett method for calculating statistical significance. All *p*-values given have been corrected for multiple comparisons and will refer to results of the unpaired *t*-tests unless otherwise specified.

RESULTS

Circulating hFIX is reduced and antibody formation against hFIX is enhanced by TLR9 activation, but not TLR7 or TLR8 activation during muscle AAV gene transfer

Previously, we found that class B CpG ODN1826 (TLR9 agonist), but not intermediate-purity LPS from *P. gingivalis*, LPS-PG (TLR2/4 agonist), or

high-purity LPS from *E. coli*, LPS-EC (TLR4 agonist), induce antibody formation to hFIX in muscle gene transfer with an AAV1 vector.³¹ The effect of the TLR9 agonist was mostly but not entirely dependent on TLR9, and ODNs may to some extent activate TLR7, which like TLR9 is located in the endosome.³¹ Therefore, in this study we tested whether one of two different potent and widely used TLR7 agonists that mimic the effects of single-stranded RNA, imidazoquinolone R848 (TLR7/8 agonist) or adenine analog CL264 (TLR7 agonist), coadministered with AAV1-hFIX, enhance antibody formation against hFIX. In parallel, we also tested the TLR9 agonist. Male C57BL/6J mice (*n* = 8 per experimental group) received intramuscular injections of the vector (1×10^{11} vg/mouse) by itself or mixed with one of the TLR agonists (50 μ g) into the quadriceps muscle of a single hind leg. In this strain of mice, intramuscular injection of AAV1 vector elicits only modest antibody responses to hFIX,³¹ which allows testing whether additional immune system signals provided at the time of gene transfer enhance antibody formation against the transgene product.

Figure 1. Effects of TLR agonists on circulating hFIX transgene expression and humoral immune responses to hFIX as a function of time after muscle-directed AAV gene transfer. Temporal comparison of (A) circulating hFIX levels, (B) anti-hFIX IgG1 antibody titers, and (C) anti-hFIX IgG2c antibody titers after administration of AAV1-hFIX vector alone (AAV) or in addition to the specified treatment: TLR7/8 agonist (50 μ g R848; AAV + TLR7/8), TLR7 agonist (50 μ g CL264; AAV + TLR7), or TLR9 agonist (50 μ g ODN1826; AAV + TLR9). Statistical significance was compared between the AAV group and each other group (*n* = 8 mice per group) by unpaired *t*-tests corrected for multiple comparisons using the Holm–Šidák method for calculating statistical significance. Statistically significant (*p* ≤ 0.05) *p*-values are shown. All *p*-values were adjusted for multiple comparisons. Bars represent means and error bars represent SEM. AAV, adeno-associated virus; SEM, standard error of the mean; TLR, Toll-like receptor.

Experimental mice showed similar levels of circulating hFIX (Fig. 1A) compared with vector-only controls 6 weeks after vector administration. Consistent with our previous findings,³¹ mice treated with AAV1-hFIX plus the TLR9 agonist (50 μ g/mouse) had significantly reduced transgene expression during the experiment than controls ($p = 0.0192$ by one-way ANOVA) and was not detected during the 2nd week. For the first 3 weeks, the TLR9 agonist-treated mice had much lower circulating hFIX levels ($p = 0.021$ – 0.0459) that subsequently rose to levels only moderately less than the control group by the 4th week ($p = 0.0822$) and afterward. Neither of the TLR7 agonists reduced hFIX levels significantly, although the TLR7-specific agonist did appear to transiently cause a mild reduction during the 3rd week ($p = 0.1254$).

Low-titer antibodies against hFIX formed in the TLR7 and TLR7/8 agonist-treated and control groups (Fig. 1B, C), with no significant differences in levels. The anti-hFIX antibodies that formed were mostly of the IgG1 subclass for all groups of mice. In contrast, TLR9 agonist-treated mice robustly increased IgG1 antibody titers to nearly 20-fold higher than controls ($p = 0.0122$) by the 2nd week. These IgG1 antibodies remained elevated by at least eightfold throughout the experiment ($p = 0.0203$ by one-way ANOVA). The TLR9 agonist also induced significant IgG2c antibodies during the 1st week ($p = 0.0056$) in most of the mice ($n = 7/8$), yet none of the mice in any other group ($n = 24$) had detectable levels of IgG2c antibodies during the 1st week, and their subsequent IgG2c responses against hFIX remained at very low titers.

TLR agonists have distinct effects on DC and B cell populations during muscle AAV gene transfer

To understand the diverse outcomes that occurred in response to using different TLR agonists as adjuvant, we used flow cytometry to analyze DC (Fig. 2) and B cell populations in dLNs of male C57BL/6J mice ($n = 4$ per experimental group) 24 h after TLR agonist coadministration with AAV1-hFIX to quadriceps muscles of each hind leg. We assessed the effect of each TLR agonist on several DC subsets and mature B cells, including activation (Fig. 3) and frequency (Fig. 4). We analyzed pDCs (CD11c⁺ CD11b[−] PDCA⁺), two subsets of conventional DCs, namely CD11b⁺ cDCs (CD11c⁺ MHCII⁺ CD11b⁺ CD8 α [−]) and CD8 α ⁺ cDCs (CD11c⁺ MHCII⁺ CD8 α ⁺ CD11b[−]), and mature B cells (CD19⁺ CD93⁺ IgD⁺). pDCs express high levels of TLR7 and TLR9, and when activated, secrete large amounts of antiviral and proinflammatory cyto-

kines, including type I interferons, TNF- α , IL-6, and IL-12.^{19,52} CD11b⁺ cDCs also express high amounts of TLR7 and TLR9, but are associated with activating CD4⁺ T cells and inducing Th2 or Th17 responses.^{19,52} In contrast, CD8 α ⁺ cDCs express TLR9, but only lower levels of TLR7.⁵² CD8 α ⁺ cDCs are known to efficiently cross-present extracellular antigens on MHCI to CD8⁺ T cells, and are more closely associated with induction of Th1 and cytotoxic responses and cytokines such as IL-12 and IFN- γ .¹⁹ Activation status (Fig. 3) of these DC subsets was evaluated in the dLNs by measuring levels of surface molecules for costimulation (CD86 and CD40) and presentation to CD4⁺ T cells by I-A/I-E (MHCII).

Coadministration of TLR7/8 or TLR9 agonists with muscle gene transfer substantially increased levels of activation markers (CD86, CD40) for CD11b⁺ cDCs, CD8 α ⁺ cDCs, and pDCs and MHCII for CD8 α ⁺ cDCs compared with control mice that received vector only (Fig. 3). Although these increases were mostly similar in both the TLR7/8 and TLR9 agonist experimental groups for the cDCs (Fig. 3A, B), pDCs were more robustly activated by the TLR7/8 agonist and showed much higher levels of MHCII expression (Fig. 3C). Mature B cells in the lymph node also displayed significantly increased activation markers (MHCII, CD86, CD40) in response to the TLR7/8 and TLR9 agonists (Fig. 3E). However, the TLR7 agonist only marginally activated CD11b⁺ and CD8 α ⁺ cDCs and failed to induce activation of mature B cells or pDCs.

Stimulation of TLR9 by CpG-B ODNs, but not TLR7 agonists, substantially increased moDCs in dLNs during muscle gene transfer

Twenty-four hours after vector/agonist administration, there was an increased frequency of CD11b⁺ cDCs and CD8 α ⁺ cDCs ($p = 0.0138$ and 0.003 , respectively) for TLR7/8-treated mice (Fig. 4A, B). A reduction ($>75\%$, $p = 0.0154$) in pDCs occurred upon TLR9 agonist treatment, which also occurred for TLR7/8 agonist-treated mice but was even more exaggerated ($>90\%$ reduction, $p = 0.0035$; Fig. 4C). Yet this reduction appeared to be only temporary, as during 48 h after gene transfer, the pDCs were not significantly different from control mice for the TLR7/8 agonist-treated mice and somewhat recovered for the TLR9-treated mice ($p = 0.0494$, data not shown). Thus, these patterns in DC frequencies, like for activation markers, did not distinguish the antibody-inducing TLR9 agonist from TLR7 agonists. In addition, mature B cell frequency was unchanged for all groups.

TLR9 agonists have previously been shown to bias toward antibody responses by enhancing T_{FH}

Figure 2. Identification of DC subsets by flow cytometry. dLN cells were harvested from C57BL/6 mice ($n=4$ mice per group) 24 h after quadriceps muscle injection of either PBS, 1×10^{11} vector genomes AAV1-hFIX alone (AAV), or mixed with either 50 μg R848 (AAV + TLR7/8 agonist), 50 μg CL264 (AAV + TLR7 agonist), or 50 μg ODN1826 (AAV + TLR9 agonist). Cells were negatively sorted with CD3 and CD19 MicroBead Kits. Flow cytometry gating strategy is shown with examples of the final gate for moDCs for each different treatment. DC, dendritic cell; dLN, draining lymph node; moDCs, monocyte-derived dendritic cells; pDCs, plasmacytoid dendritic cells; PBS, phosphate-buffered saline.

cell differentiation, which is crucial to forming germinal centers necessary for generating high-affinity memory B cells and long-lived plasma cells.^{31,53} Although cDCs (CD11b⁺ cDCs and CD8 α ⁺ cDCs) can prime T_{FH} responses alone, it was previously shown that moDCs (sometimes also referred to as “inflammatory DCs”) are required for TLR9 agonist-induced enhancement of antibody formation.^{31,53} Although earlier studies have defined CD11c⁺ CD11b⁺ CD64⁺ cells as moDCs, we additionally used Fc ϵ RI α (MAR-1) to further distinguish moDCs (CD11c^{high} MHCII⁺ CD11b⁺

CD64⁺ Fc ϵ RI α ⁺) from other contaminating cells (Fig. 2).⁵⁴

Although there were no significant differences in activation marker expression in moDCs between groups (Fig. 3D), it is in the frequency of moDCs that we observed the most striking difference between TLR9 and the other agonists. Upon TLR9 agonist administration, moDCs significantly increased ($p=0.0079$) to a frequency >2% (of Live CD11c⁺ Lineage⁻ cells), which is more than seven-fold higher than any other experimental group (Fig. 4D). Thus, we found that TLR9 stimulation by

Figure 3. Effects of TLR agonists on activation of subsets of DCs and B cells in the spleen and dLNs during muscle AAV gene transfer. Lymph node cells were from mice ($n=4$ mice per experimental group or $n=3$ mice per group for naive) that were either untreated (naive) or received quadriceps muscle injects of both hind legs with PBS, 1×10^{11} vector genomes AAV1-hFIX alone (AAV) or mixed with either 50 μ g R848 (AAV + TLR7/8 agonist), 50 μ g CL264 (AAV + TLR7 agonist), or 50 μ g ODN1826 (AAV + TLR9 agonist). Cells were negatively sorted with CD3 MicroBead Kit (DCs and B cells) and positively (B cells) or negatively (DCs) sorted with CD19 MicroBead Kit. MFI of markers of activation (MHCII, CD86, and CD40) determined by flow cytometry for (A) CD11b⁺ cDCs (CD11c⁺ MHCII⁺ CD11b⁺ CD8 α ⁻) (B) CD8 α ⁺ cDCs (CD11c⁺ MHCII⁺ CD8 α ⁺ CD11b⁻), (C) pDCs (CD11c⁺ CD11b⁻ PDCA⁺), (D) moDCs (CD11c^{high} MHCII⁺ CD11b⁺ CD64⁺ Fc ϵ RI α ⁺), and (E) mature B cells (CD19⁺ CD93⁺ IgD⁺). Height of bars represents MFI. Statistical significance was compared between the AAV group and each other group by unpaired *t*-tests corrected for multiple comparisons using the Holm-Sidak method for calculating statistical significance. Statistically significant ($p \leq 0.05$) *p*-values are shown. All *p*-values were adjusted for multiple comparisons. Error bars represent SEM. MFI, median fluorescence intensity.

Figure 4. Effects of TLR agonists on frequencies of subsets of DCs and B cells in the spleen and dLNs during muscle AAV gene transfer. Lymph node cells were from mice ($n=4$ mice per experimental group or $n=3$ mice per group for naive) that were either untreated (naive) or received quadriceps muscle injects of both hind legs with PBS, 1×10^{11} vector genomes AAV1-hFIX alone (AAV) or mixed with either $50 \mu\text{g}$ R848 (AAV + TLR7/8 agonist), $50 \mu\text{g}$ CL264 (AAV + TLR7 agonist), or $50 \mu\text{g}$ ODN1826 (AAV + TLR9 agonist). Cells were negatively sorted with CD3 MicroBead Kit (DCs and B cells) and positively (B cells) or negatively (DCs) sorted with CD19 MicroBead Kit. Frequencies were determined by flow cytometry for (A) CD11b⁺ cDCs (CD11c⁺ MHCII⁺ CD11b⁺ CD8 α ⁻) (B) CD8 α ⁺ cDCs (CD11c⁺ MHCII⁺ CD8 α ⁺ CD11b⁻), (C) pDCs (CD11c⁺ CD11b⁻ PDCA⁺), (D) moDCs (CD11c^{high} MHCII⁺ CD11b⁺ CD64⁺ Fc ϵ R1 α ⁺), and (E) mature B cells (CD19⁺ CD93⁺ IgD⁺). Height of bars represents group mean percentage cells of live CD11c⁺ CD3⁻ CD19⁻ CD49b⁻ Ly-6G⁻ Siglec-F⁻ gated cells. Statistical significance was compared between the AAV group and each other group by unpaired t -tests corrected for multiple comparisons using the Holm-Šidák method for calculating statistical significance. Statistically significant ($p \leq 0.05$) p -values are shown. All p -values were adjusted for multiple comparisons. Error bars represent SEM.

CpG-B ODN induces moDCs in dLNs during muscle gene transfer, which matches previous findings that comprehensively showed that induction of moDCs is required for CpG-B ODN-mediated enhancement of antibody formation.^{31,53} Furthermore, the unchanged levels of antibodies to hFIX when TLR7 or TLR7/8 agonists were given during muscle gene transfer (despite widespread activation of DCs and B cells that occurred with the TLR7/8 agonist) correlated with the lack of the moDC induction for both TLR7 and TLR7/8 agonists.

DISCUSSION

Antibody formation against secreted transgene products is a major concern when using muscle gene transfer for systemic protein delivery, as inhibitory antibodies can neutralize the therapeutic effect. In a series of experiments, we sought to determine if activation signals derived from TLRs promote antibody formation against hFIX upon

AAV gene transfer to skeletal muscle. TLRs can be located on cell surfaces or in endosomes and function as innate receptors for the recognition of molecular structures associated with pathogens, such as bacterial or viral RNA and DNA, LPSs, and others. In prior studies, we found that agonists of either TLR2, TLR3, or TLR4 do not promote antibody formation against hFIX in AAV-mediated muscle gene transfer.^{31,33,55} Interestingly, *E. coli*-derived LPS (a TLR4 ligand) enhances antibody formation to intravenously injected proteins but not in AAV muscle gene transfer, which illustrates the importance of the context in which the signaling takes place. In this study, we find that both imidazoquinolone R848 (TLR7/8 agonist) and adenine analog CL264 (TLR7 agonist) also fail to induce B cell responses to muscle-derived hFIX. In sharp contrast, class B CpG ODNs (CpG-B ODNs) such as ODN1826 (TLR9 agonist) consistently induce high-titer antibody formation against hFIX. Thus, TLR9 appears to have a unique ability among

TLRs to promote antibody formation against a secreted transgene product in the muscle when stimulated in a certain way.

Unlike chromosomal DNA of the host cell, vector genomes contain CpG motifs that are not methylated during AAV production, and thus more strongly stimulate TLR9 signaling upon administration. Although ODN1826 enhances antibody formation in a largely TLR9-dependent manner,³¹ antibody formation is only modestly impacted through TLR9 pathways when AAV vectors are administered.³³ In addition, antibody formation to hFIX after AAV gene transfer can occur in the absence of TLR9 or MyD88 and, therefore, is independent of TLR9 sensing of the AAV genome.³³ Furthermore, ODN1826 coadministered with muscle AAV gene transfer in TLR9^{-/-} mice was shown to be able to induce a measurable increase in antibody formation against the transgene protein.³¹ Hence, both TLR9-dependent and TLR9-independent immune pathways exist that promote antibody formation. DExD/H-box helicase 9 (DHX9) is one possible TLR9-independent MyD88-dependent cytosolic sensor of CpG-B ODNs that may increase antibody formation, as it was previously shown to be important for cytokine responses by pDCs.^{56,57} Our new results here show that multiple bona fide TLR7 agonists failed to enhance antibody formation; therefore, off-target TLR7 or TLR8 activation is highly unlikely to account for the TLR9-independent effects of CpG-B ODN1826.

Sensing of the AAV genome by TLR9 in pDCs is critical for activation of capsid-specific CD8⁺ T cells.⁵⁸ Similarly, TLR9-MyD88 signaling was shown to be crucial for CD8⁺ T cell responses to the transgene product in muscle gene transfer and can be blunted by editing out immune stimulatory CpG motifs in the viral vector genome.^{29,32,33} TLR9 agonist administration in the muscle of C57BL/6 mice has only a modest effect on CD8⁺ T cell induction, unless CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Tregs) are depleted.³¹ In fact, systemic hFIX levels recover over time if the TLR9 agonist is not repeatedly administered. This may be, in part, related to the activity of Tregs, which we have previously shown to also limit antibody formation in muscle gene transfer.³¹ However, in the absence of Tregs, the TLR9 agonist induces a potent inflammatory T cell response rather than antibody formation, resulting in a partial loss of transgene expression.³¹

Adaptive immune responses to hFIX and other transgene products are initiated locally in the dLNs of transduced muscle.^{59,60} Increased moDCs in dLNs of the TLR9 agonist-treated mice during muscle AAV gene transfer correlated with en-

hancement of antibody formation. This is consistent with other studies that found TLR9 agonists increase moDC secretion of IL-6, thereby inducing T_{FH} cell development and enhancing antigen-specific antibody formation.^{31,53} Although CpG-B ODNs also have direct effects on B cells, their ability to activate moDCs through TLR9 signaling is critical for augmenting antibody formation because of the resulting enhanced T_{FH} cell activation.⁵³ T_{FH} cells aid in germinal center formation and B cell activation, and we previously showed that ODN1826 generates a T_{FH} response when coadministered with AAV1-hFIX.³¹

In this study, we find that TLR9 and TLR7/8 agonists are both able to cause widespread and robust activation of cDCs (CD11b⁺ or CD8 α ⁺), pDCs, and B cells (to at least the same extent as the TLR9 agonist), yet no enhancement of antibody formation occurs. Thus, activation of B cells and DC subsets at the time of muscle gene transfer, in the absence of moDC induction, appears to not influence antibody formation against transgene proteins. Incorporation of the Fc ϵ RI α (MAR-1) marker allowed us to better distinguish moDCs (CD11c^{high} CD11b⁺ CD64⁺ Fc ϵ RI α ⁺) from monocytes and macrophages. Our findings support previous studies that showed the importance of moDCs for enhancing antibody responses to the transgene product when CpG-B ODNs are given during muscle gene transfer.^{31,53} A recent study by others correlated activation of moDCs with antibody formation against AAV capsid in humans.⁶¹ Future studies should investigate how this mode of TLR9 activation may influence antibody formation against AAV capsid.

A previous study showed that *in vivo* cytokine responses (IFN- α , TNF- α , and IL-12 p40) in either TLR7^{-/-} mice or MyD88^{-/-} mice given R848 (the same TLR7/8 agonist used here) are dependent only on TLR7.⁶² Additional studies also showed that TLR7^{-/-} mice do not respond to TLR8-specific agonists and natural single-stranded RNA does not activate murine TLR8, which led to the belief that TLR8 was nonfunctional in mice.⁶²⁻⁶⁴ However, it was later shown that administering polyT ODN (a TLR7/8 modulator that increases TLR8 signaling and abolishes TLR7 signaling) plus either a TLR8-selective agonist (3M-002) or TLR7/8 agonist (3M-003) initiates cytokine responses in both TLR7^{-/-} and TLR9^{-/-}, but not in MyD88^{-/-} mice.⁶⁵ This demonstrates that murine TLR8 is indeed functional and the TLR8-activating properties of R848, in the absence of other modulatory molecules, may have an effect only in humans.

Given that R848 is considered to only affect TLR7 in mice, it is interesting that we found it and

the TLR7-specific agonist (CL264) to have such dissimilar effects on DCs and B cells. One explanation is that R848 stimulates TLR7 more strongly than CL264. A recent study on *in vitro* stimulation of pDCs, by several TLR7 and TLR9 agonists, found that TLR7/8 agonists generally activated pDCs much more strongly than TLR7-specific agonists.⁶⁶ In response to different ligands, pDCs produced extremely different levels of cytokines, despite similar levels of activation markers.⁶⁶ For example, TLR7/8 agonists produced more than twice as much IFN- α compared with TLR7-specific or TLR9 agonists (CpG-A and CpG-C ODNs), but IFN- α production was absent for other TLR9 agonists (CpG-B ODNs).⁶⁶ Furthermore, in contrast to the enhancement of antibody formation seen here and in previous studies when CpG-B ODNs are coadministered with muscle gene transfer, CpG-A and CpG-C ODNs fail to induce antibodies.⁵³ Therefore, different TLR7 agonists and TLR9 agonists have diverse effects on DC populations and may, therefore, shape immune responses differently. Future studies will investigate the possibility of synergic or modulatory effects between TLR9 and TLR7 agonists, as the study discussed previously, for example, showed that TLR8 agonists are unable to activate murine TLR8 unless combined with polyT ODN.⁶⁵

In conclusion, a potent activating ligand of TLR9, such as CpG-B ODN, can substantially increase antibody formation against a secreted transgene product in AAV muscle-directed gene transfer. This is in contrast with activators of other

TLRs such as TLR7, indicating a unique property of the TLR9 signaling pathway. Induction of moDCs in the dLNs that we described here is likely a critical component of TLR9-induced antibody formation against the transgene product.

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AUTHORS' CONTRIBUTIONS

J.S.S.B., M.B., J.L.S., S.R.P.K., and A.S. performed experiments; J.S.S.B., M.B., S.R.P.K., and R.W.H. designed experiments; J.S.S.B., M.B., S.R.P.K., J.L.S., C.T., and R.W.H. analyzed and interpreted data; J.S.S.B. and R.W.H. wrote the article; and C.T., C.L., and R.W.H. supervised the study.

AUTHOR DISCLOSURE

R.W.H. serves on the scientific advisory board of Applied Genetic Technologies Corporation (AGCT).

REFERENCES

- Russell S, Bennett J, Wellman JA, et al. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: A randomised, controlled, open-label, phase 3 trial. *Lancet* 2017; 390:849–860.
- George LA, Sullivan SK, Giermasz A, et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *N Engl J Med* 2017;377: 2215–2227.
- Arruda LCM, Malmegrim KCR, Lima-Júnior JR, et al. Immune rebound associates with a favorable clinical response to autologous HSCT in systemic sclerosis patients. *Blood Adv* 2018;2:126–141.
- Haddley K. Alipogene tiparvovec for the treatment of lipoprotein lipase deficiency. *Drugs Today (Barc)* 2013;49:161–170.
- Martínez-Navio JM, Fuchs SP, Pedreño-López S, et al. Host anti-antibody responses following adeno-associated virus-mediated delivery of antibodies against HIV and SIV in Rhesus Monkeys. *Mol Ther* 2016;24:76–86.
- Louboutin J-P, Wang L, Wilson JM. Gene transfer into skeletal muscle using novel AAV serotypes. *J Gene Med* 2005;7:442–451.
- Akache B, Grimm D, Pandey K, et al. The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *J Virol* 2006;80:9831–9836.
- Bartlett JS, Wilcher R, Samulski RJ. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* 2000;74: 2777–2785.
- Duan D, Li Q, Kao AW, et al. Dynamin is required for recombinant adeno-associated virus type 2 infection. *J Virol* 1999;73:10371–10376.
- Xiao P-J, Samulski RJ. Cytoplasmic trafficking, endosomal escape, and perinuclear accumulation of adeno-associated virus type 2 particles are facilitated by microtubule network. *J Virol* 2012;86: 10462–10473.
- Huang L-Y, Patel A, Ng R, et al. Characterization of the adeno-associated virus 1 and 6 sialic acid binding site. *J Virol* 2016;90: 5219–5230.
- Liu Y, Joo KI, Wang P. Endocytic processing of adeno-associated virus type 2 capsids with externalized VP1/VP2 trafficking domains are generated prior to passage through the cytoplasm and are maintained until uncoating occurs in the nucleus. *J Virol* 2006; 80:11040–11054.
- Nonnenmacher M, Weber T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther* 2012;19:649–658.

15. Zhou X, Zeng X, Fan Z, et al. Adeno-associated virus of a single-polarity DNA genome is capable of transduction in vivo. *Mol Ther* 2008;16:494–499.
16. Kaisho T, Akira S. Toll-like receptor function and signaling. *J Allergy Clin Immunol* 2006;117:979–987.
17. Bekeredjian-Ding I, Jegou G. Toll-like receptors—sentries in the B-cell response. *Immunology* 2009;128:311.
18. Gururajan M, Jacob J, Pulendran B. Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. *PLoS One* 2007;2:e863.
19. Murphy K, Weaver C. *Janeway's Immunobiology*. New York, NY: Garland Science/Taylor & Francis Group, LLC, 2017.
20. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004;4:499–511.
21. Klinman DM, Yi AK, Beaucage SL, et al. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci U S A* 1996;93:2879–2883.
22. Overstreet MG, Freyberger H, Cockburn IA, et al. CpG-enhanced CD8+ T-cell responses to peptide immunization are severely inhibited by B cells. *Eur J Immunol* 2010;40:124–133.
23. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000;408:740–745.
24. Ballingall KT, MacHugh ND, Taracha ELN, et al. CpG motifs of DNA vaccines induce the expression of chemokines and MHC class II molecules on myocytes. *Eur J Immunol* 2001;31:301–310.
25. Joffre OP, Segura E, Savina A, et al. Cross-presentation by dendritic cells. *Nat Rev Immunol* 2012;12:557–569.
26. Defranco AL, Rookhuizen DC, Hou B. Contribution of Toll-like receptor signaling to germinal center antibody responses. *Immunol Rev* 2012;247:64–72.
27. Huang X, Yang Y. Targeting the TLR9-MyD88 pathway in the regulation of adaptive immune responses. *Expert Opin Ther Targets* 2010;14:787–796.
28. Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 2004;4:249–259.
29. Faust SM, Bell P, Cutler BJ, et al. CpG-depleted adeno-associated virus vectors evade immune detection. *J Clin Invest* 2013;123:2994–3001.
30. Anderson RA, Reid RF, Soutar I. Intramolecular excimer formation in macromolecules-III. Energy migration and excimer formation in copolymers of vinyl naphthalene and methyl acrylate. *Eur Polym J* 1979;15:925–929.
31. Herzog RW, Cooper M, Perrin GQ, et al. Regulatory T cells and TLR9 activation shape antibody formation to a secreted transgene product in AAV muscle gene transfer. *Cell Immunol* 2017 [Epub ahead of print]; DOI: 10.1016/j.cellimm.2017.07.012.
32. Rogers GL, Martino AT, Zolotukhin I, et al. Role of the vector genome and underlying factor IX mutation in immune responses to AAV gene therapy for hemophilia B. *J Transl Med* 2014;12:25.
33. Rogers GL, Suzuki M, Zolotukhin I, et al. Unique roles of TLR9- and MyD88-dependent and -independent pathways in adaptive immune responses to AAV-mediated gene transfer. *J Innate Immun* 2015;7:302–314.
34. Zucchini N, Bessou G, Traub S, et al. Cutting edge: Overlapping functions of TLR7 and TLR9 for innate defense against a herpesvirus infection. *J Immunol* 2008;180:5799–5803.
35. Diebold SS, Kaisho T, Hemmi H, et al. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* (80-) 2004;303:1529–1531.
36. Simchoni N, Cunningham-Rundles C. TLR7- and TLR9-responsive human B cells share phenotypic and genetic characteristics. *J Immunol* 2015;194:3035–3044.
37. Jeisy-Scott V, Kim JH, Davis WG, et al. TLR7 recognition is dispensable for influenza virus A infection but important for the induction of hemagglutinin-specific antibodies in response to the 2009 pandemic split vaccine in mice. *J Virol* 2012;86:10988–10998.
38. Oldfield V, Keating GM, Perry CM. Imiquimod. *Am J Clin Dermatol* 2005;6:195–200.
39. Sabado RL, Pavlick A, Gnjatic S, et al. Resiquimod as an immunologic adjuvant for NY-ESO-1 protein vaccination in patients with high-risk melanoma. *Cancer Immunol Res* 2015;3:278–287.
40. Pockros PJ, Guyader D, Patton H, et al. Oral resiquimod in chronic HCV infection: Safety and efficacy in 2 placebo-controlled, double-blind phase IIa studies. *J Hepatol* 2007;47:174–182.
41. Menne S, Tumas DB, Liu KH, et al. Sustained efficacy and seroconversion with the Toll-like receptor 7 agonist GS-9620 in the Woodchuck model of chronic hepatitis B. *J Hepatol* 2015;62:1237–1245.
42. Lanford RE, Guerra B, Chavez D, et al. GS-9620, an oral agonist of Toll-like receptor-7, induces prolonged suppression of hepatitis B virus in chronically infected chimpanzees. *Gastroenterology* 2013;144:1508.e1–1517.e10.
43. Greiff L, Ahlström-Emanuelsson C, Alenäs M, et al. Biological effects and clinical efficacy of a topical Toll-like receptor 7 agonist in seasonal allergic rhinitis: A parallel group controlled phase IIa study. *Inflamm Res* 2015;64:903–915.
44. Delaney S, Biffen M, Maltby J, et al. Tolerability in man following inhalation dosing of the selective TLR7 agonist, AZD8848. *BMJ open Respir Res* 2016;3:e000113.
45. Lebold KM, Jacoby DB, Drake MG. Toll-like receptor 7-targeted therapy in respiratory disease. *Transfus Med Hemother* 2016;43:114–119.
46. Arruda VR, Schuettrumpf J, Herzog RW, et al. Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. *Blood* 2004;103:85–92.
47. Zolotukhin S, Potter M, Zolotukhin I, et al. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* 2002;28:158–167.
48. Crosson SM, Dib P, Smith JK, et al. Helper-free production of laboratory grade AAV and purification by iodixanol density gradient centrifugation. *Mol Ther Methods Clin Dev* 2018;10:1–7.
49. Perrin GQ, Zolotukhin I, Sherman A, et al. Dynamics of antigen presentation to transgene product-specific CD4+ T cells and of Treg induction upon hepatic AAV gene transfer. *Mol Ther Methods Clin Dev* 2016;3:16083.
50. Markusic DM, Hoffman BE, Perrin GQ, et al. Effective gene therapy for haemophilic mice with pathogenic factor IX antibodies. *EMBO Mol Med* 2013;5:1698–1709.
51. Mingozzi F, Liu Y-L, Dobrzynski E, et al. Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. *J Clin Invest* 2003;111:1347–1356.
52. Edwards AD, Diebold SS, Slack EMC, et al. Toll-like receptor expression in murine DC subsets: Lack of TLR7 expression by CD8 α + DC correlates with unresponsiveness to imidazoquinolines. *Eur J Immunol* 2003;33:827–833.
53. Chakarov S, Fazilleau N. Monocyte-derived dendritic cells promote T follicular helper cell differentiation. *EMBO Mol Med* 2014;6:590–603.
54. Plantinga M, Guillems M, Vanheerswynghels M, et al. Conventional and monocyte-derived CD11b+ dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 2013;38:322–335.
55. Cao O, Herzog RW. TLR3 signaling does not affect organ-specific immune responses to factor IX in AAV gene therapy. *Blood* 2008;112:910–911.
56. Kim T, Pazhoor S, Bao M, et al. Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells. *Proc Natl Acad Sci* 2010;107:15181–15186.
57. Zhang Z, Yuan B, Lu N, et al. DHX9 pairs with IPS-1 to sense double-stranded RNA in myeloid dendritic cells. *J Immunol* 2011;187:4501–4508.
58. Rogers GL, Shirley JL, Zolotukhin I, et al. Plasmacytoid and conventional dendritic cells cooperate in crosspriming AAV capsid-specific CD8+ T cells. *Blood* 2017;129:3184–3195.
59. Wang L, Dobrzynski E, Schlachterman A, et al. Systemic protein delivery by muscle-gene transfer is limited by a local immune response. *Blood* 2005;105:4226–4234.

60. Wang L, Herzog RW. AAV-mediated gene transfer for treatment of hemophilia. *Curr Gene Ther* 2005; 5:349–360.
61. Kuranda K, Jean-Alphonse P, Leborgne C, et al. Exposure to wild-type AAV drives distinct capsid immunity profiles in humans. *J Clin Invest* 2018; 128:5267–5279.
62. Hemmi H, Kaisho T, Takeuchi O, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* 2002;3:196–200.
63. Pulendran B, Palucka K, Banchereau J. Sensing pathogens and tuning immune responses. *Science* (80-) 2001;293:253–256.
64. Jurk M, Heil F, Vollmer J, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848 [1]. *Nat Immunol* 2002;3: 499.
65. Gordon KKB, Qiu XX, Christine CA, et al. Modifiers and PolyT oligodeoxynucleotides. *Blood* 2011;177: 6584–6587.
66. Wu J, Li S, Li T, et al. pDC Activation by TLR7/8 ligand CL097 compared to TLR7 ligand IMQ or TLR9 ligand CpG. *J Immunol Res* 2019;2019: 1–10.

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