NxGEN VECTOR SOLUTIONS

CpG Motifs within AAV Vectors Trigger Immune Activation upon Hepatic Gene Transfer

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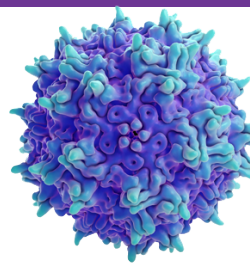
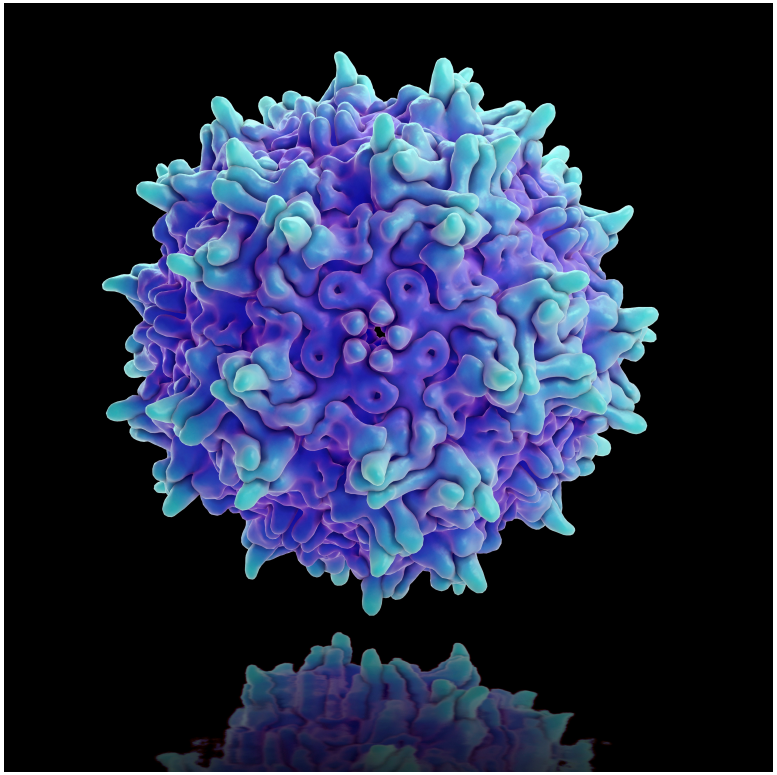


Background: Host Immune Response in Liver-Directed AAV Gene Therapy



- Immunological responses to the AAV vector can significantly impact the safety and longevity of any gene therapy trial.
- Unfortunately, the human body's immune defenses can respond to the engineered viruses just as they would to natural ones: as if they are a potentially deadly threat.
- The response can be innate and/or adaptive in nature and can occur *de novo*.
- Pre-existing immunity to AAV vectors can be due to memory B cells, which produce anti-AAV neutralizing antibodies or due to memory T cells, which can be reactivated to induce a memory T cell response.

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Background: Toll-like Receptors (TLRs)

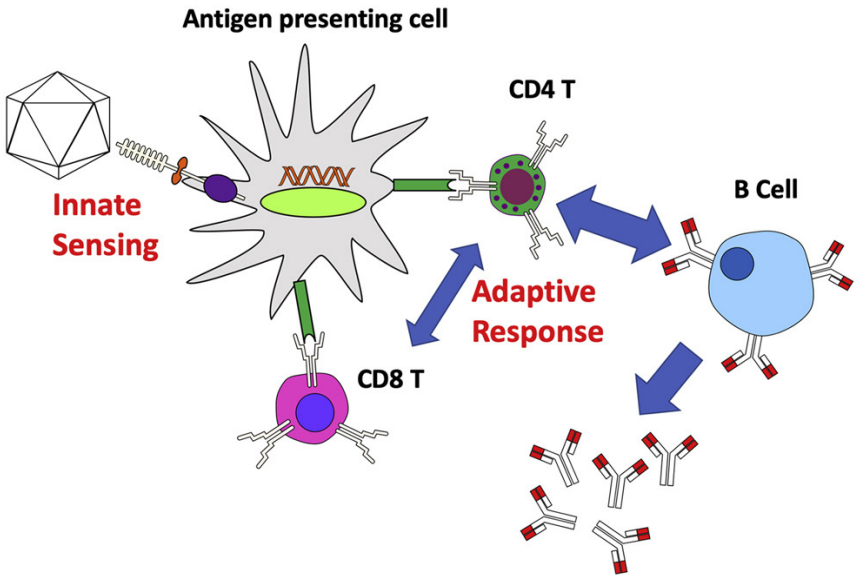

Image from Shirley et al., *Molecular Therapy*, 2020

- Toll-like receptors (TLRs) recognize molecules broadly shared by pathogens; distinguishable from host molecules.
- Expressed by innate immune cells: conventional and plasmacytoid DCs, and macrophages.
- TLR9 interacts with unmethylated CpG sequences in DNA molecules in the endosomal and lysosomal compartments, where TLR9 is expressed.
- Degradation (uncoating) of AAV vectors in the endosome results in the release of nucleic acids that are then able to signal through TLR9, resulting in the production of IL-1, TNF-α, and IL-6 and activation of the innate and adaptive immune response.

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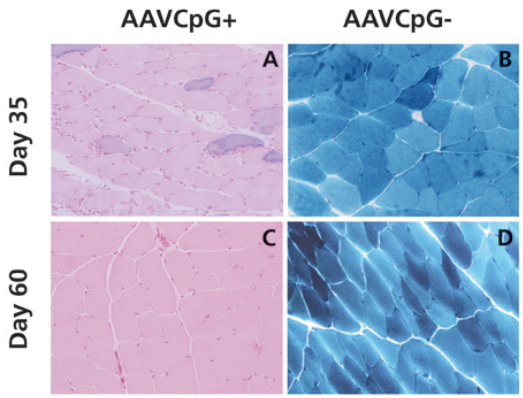
Background: CpG-Depleted AAV Vectors Inhibit Immune Responses and Result in Transgene Durability Following Intramuscular Gene Transfer

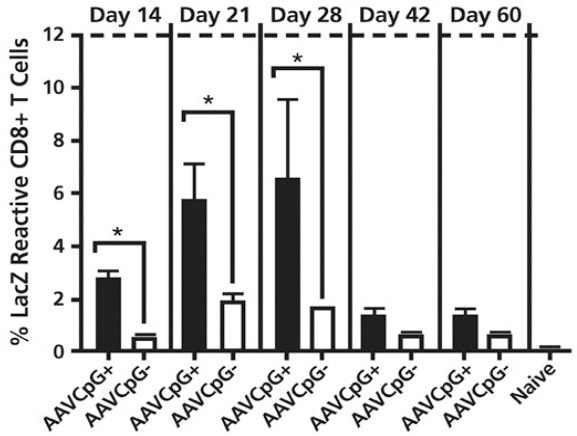
X-Gal Stain



CpG-depleted AAV-transduced murine muscle sections display robust and stable β-gal transgene expression.

Images from Faust et al., *JCI,* 2013

%LacZ Reactive CD8+ T cells



A significant reduction in the percentage of LacZ-responsive CD8+ T cells was observed in mice that received the AAVCpG- vector.

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Background: CpG Motifs in the AAV Vector are the Key Determinants To Long-Term Transgene Expression in Hemophilia B Human Clinical Trials

Table 1. AAV Gene Therapy Clinical Trials for Hemophilia B

Sponsor	Serotype/Configuration[a]	No. of CpG in ORF	Production	Dose ($\times 10^{12}$) (vg/kg)	Dose ($\times 10^{12}$) (~cp/kg)	Immunology IS[b]	Immunology CTL[c]	Outcomes Peak FIX	Outcomes Duration
CHOP, Stanford Avigen	AAV2-FIX/ss	19[d] (WT)	HEK	2	2	–	++	12% (n = 1)	<3 months
UCL, St Jude	AAV8-FIX/sc	0[d]	HEK	0.2–2	1–10	+	+	2%–11% (n = 10)	>1 year
Shire (BAX335)	AAV8-FIX Padua/sc	99[d]	HEK	0.2–3	ND	++	++	4%–45% (n = 8)	<3 months
CHOP	AAV8-FIX19/ss	94[e]	ND	1–2	ND	++	++[e]	ND	ND
Pfizer (SPK-9001)	AAVSPK-FIX Padua/ss	0[d]	HEK	0.5	1.5–2.5	+	+	34% (n = 10)	>1 year
Uniqure (AMT060)	AAV5-FIX/sc	0[d]	Bac	20	40	+	+	7% (n = 5)	>1 year
Dimension (DTX101)	AAVrh10-FIX/ss	96[d]	HEK	1.6–5	ND	++	++	3%–8% (n = 6)	<3 months
Uniqure (AMT061)	AAV5-FIX Padua/sc	0[d]	Bac	20	40	–	+	47% (n = 3)	>1 year

[a]Genome configuration: ss, single-stranded genome; sc, self-complementary genome.
[b]Immune suppression: –, not used; +, minority of subjects; ++, majority of subjects.
[c]Capsid-specific CTLs by IFN-γ ELISPOT: +, minority of subjects; ++, majority of subjects.
[d]Nathwani, 2019, American Society for Hematology Annual Meeting, Ham Wasserman Lecture
[e]High and Anguela, 2016, USTPO 20160375110

WT FIX: 19 CpGs
Codon Optimized FIX: +94 CpGs

"CpG content is the single most important factor that determines the immune response towards an AAV vector [in human clinical trials for Hemophilia B]."

Wright, 2020

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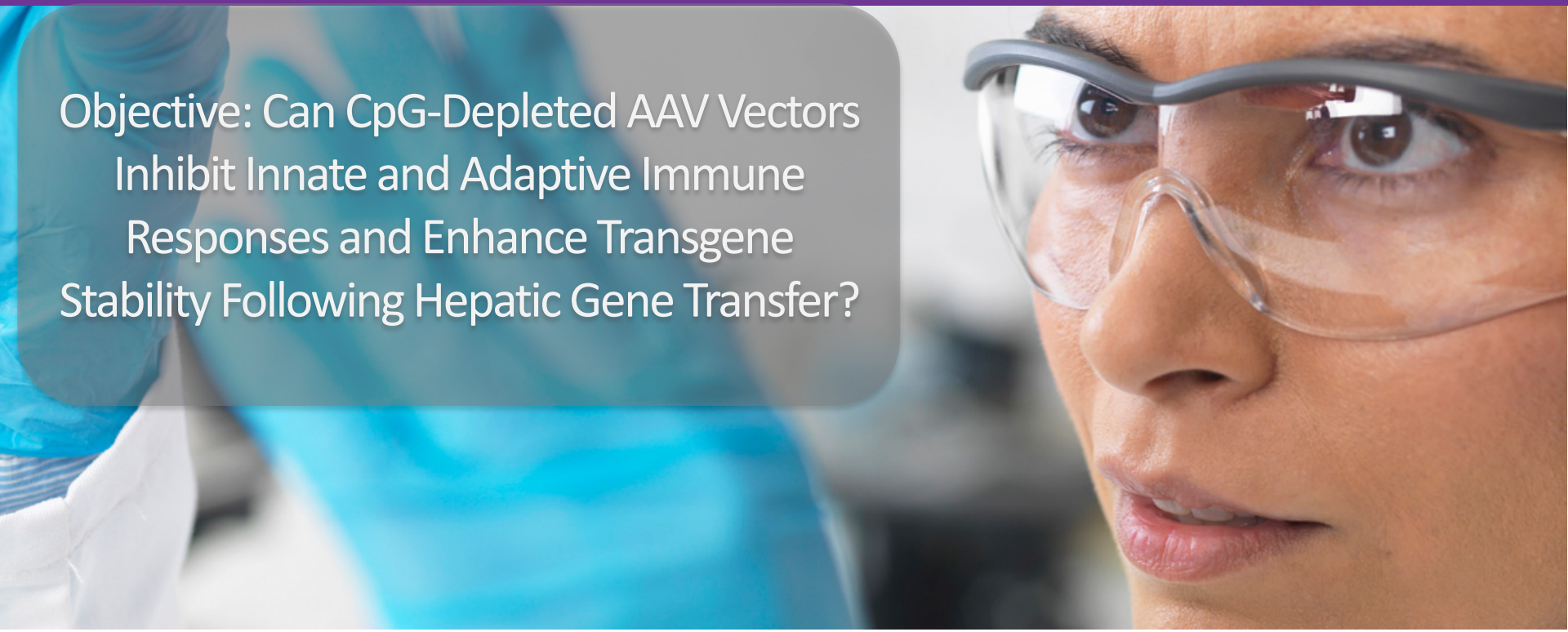

Objective: Can CpG-Depleted AAV Vectors Inhibit Innate and Adaptive Immune Responses and Enhance Transgene Stability Following Hepatic Gene Transfer?

- We compared the performance of two AAV vectors (AAVRh32.33) expressing a β-gal reporter gene that differed only by the abundance of CpG motifs. The AAV serotype that was used--AAVRh32.33—is unique in that it can can mimic in mice the inflammatory immune response that has been observed in human clinical trials.

- The AAVCpG+ vector contained 16 CpGs in the ITRs and 308 CpGs in the lacZ gene for a total of 324 CpGs. The AAVCpG- vector was void of CpGs in the lacZ gene, meaning its total CpG content was 16.

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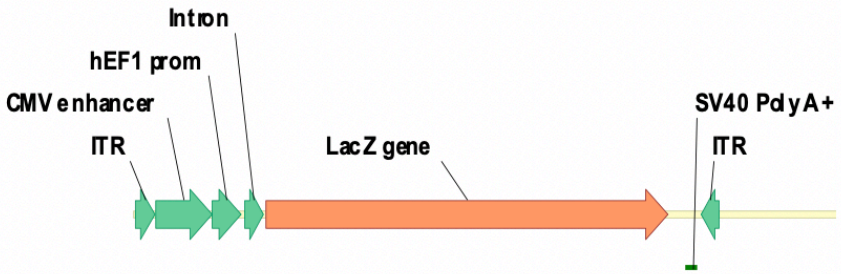
Research Design: Can AAV Vectors with Depleted CpG Motifs Evade Immunity and Result in Long-Term Transgene Expression?



- CpG+LacZ=324 CpGs
- CpG-LacZ=16 CpGs

Outside of the LacZ gene, the vectors were completely depleted of CpG motifs, including the human elongation factor 1α (E1Fα) promoter, CMV enhancer, intron, SV40, and 3' UTR. The ITRs contained 16 total CpGs motifs.

- We intravenously injected 1E11 viral particles of the unmodified (CpG+LacZ) or CpG-depleted AAVRh32.33LacZ vectors (CpG-LacZ) into C57BL/6 mice.
- Livers were recovered at day 28 post injection and X-gal stain was performed to assess βgal (transgene) expression.
- ELISPOT and Flow Cytometric analysis of transgene reactive T cells were performed at day 28 post-administration.
- Flow Cytometric analysis of plasmacytoid and conventional dendritic cell activation (CD80, CD86, and MHC II upregulation) was also assessed 18 hours post-administration.

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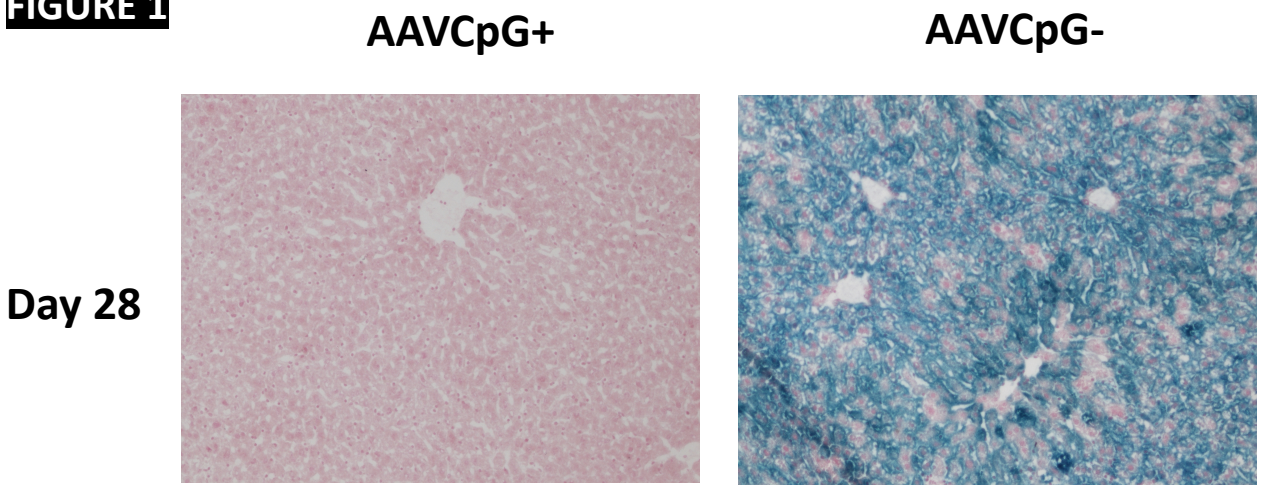
Results: CpG Depleted AAV Vectors Exhibit Stable Transgene Expression after Systemic Administration

FIGURE 1



CpG depleted AAV-transduced liver sections display robust and stable β-gal transgene expression.

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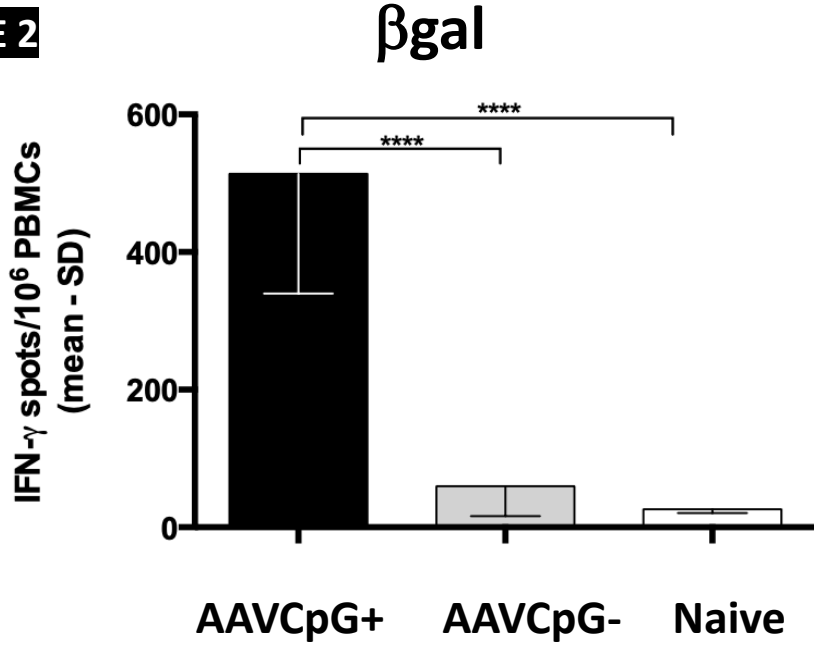
Results: CpG Depleted AAV Vectors Evade the Immune Response after Systemic Administration

FIGURE 2



A significant decrease of primed transgene antigen-reactive IFN-γ ELISPOT responses was observed in mice that received the AAVCpG-depleted vector.

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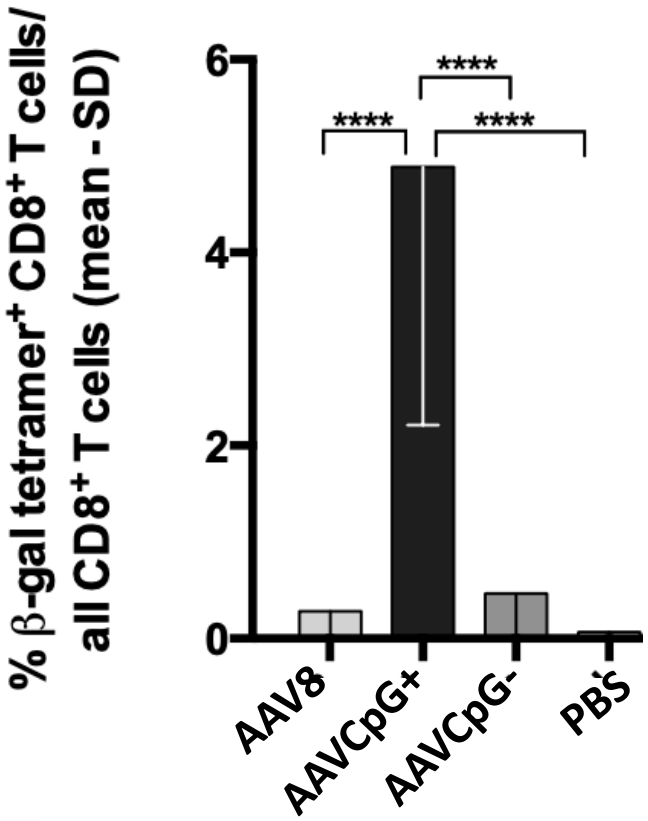
Results: CpG Depleted AAV Vectors Evade the Immune Response after Systemic Administration

FIGURE 3



A significant reduction in the percentage of LacZ-responsive CD8+ T cells was observed in mice that received the AAVCpG-depleted vector.

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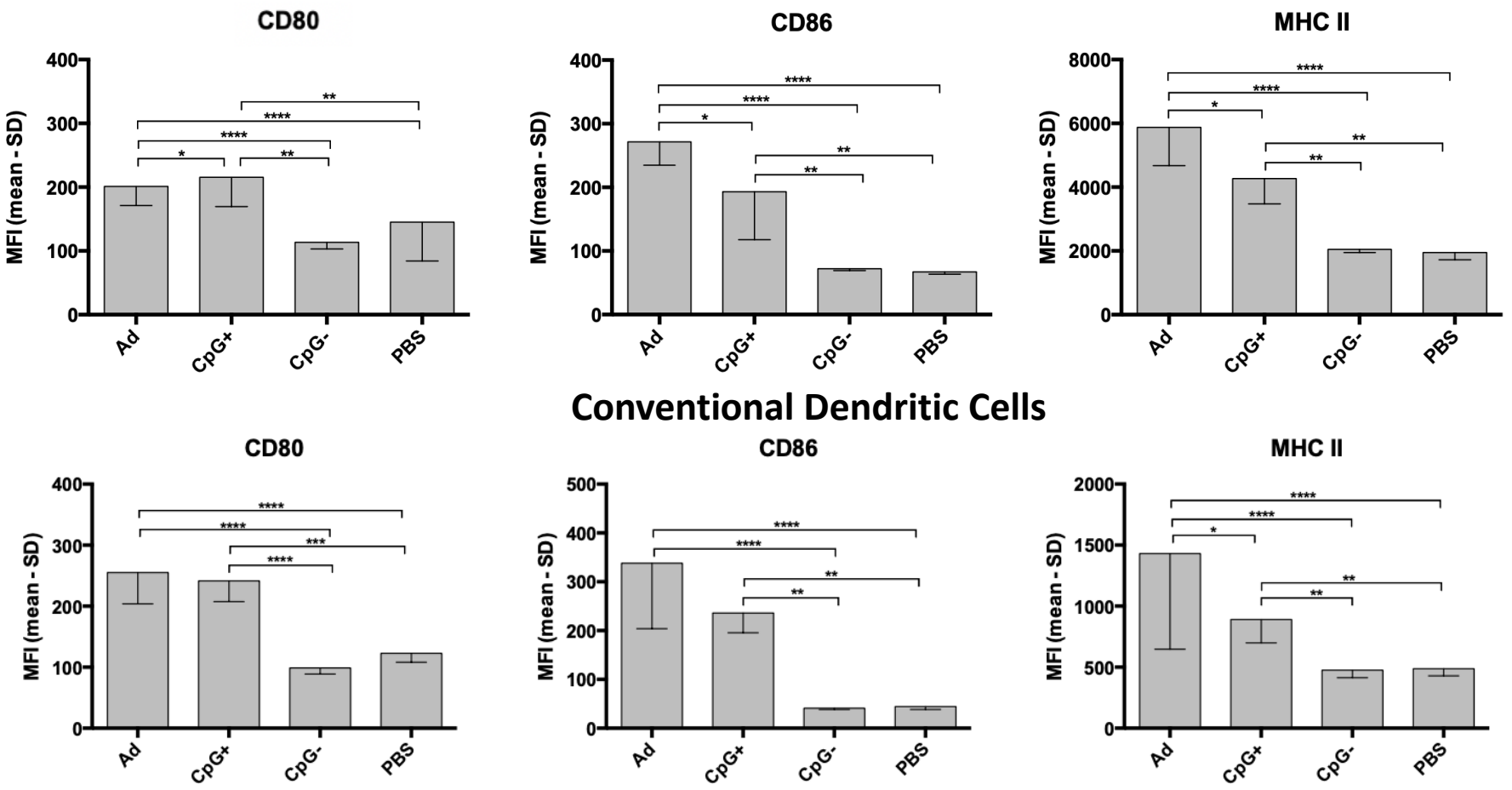
Results: CpG Depleted AAV Vectors Evade the Immune Response after Systemic Administration and it Begins with Innate Immune Cells

FIGURE 4



Ad: Adenovirus (Positive Control)
PBS: Saline (Negative Control)

Conclusion/Future Directions: Vector Design Strategies to Decrease the Number of CpG motifs in the Expression Cassette is Imperative for Gene Therapy Clinical Success

- The steady loss of LacZ transgene expression and robust cellular immunity in a mouse model of liver gene transfer with an AAV vector is dependent on vector genome CpG motifs, just as it is observed in human clinical studies for Hemophilia B gene therapy.

- CpG dinucleotide motifs in the AAV vector can trigger activation of the innate immune response (plasmacytoid and conventional dendritic cells) and a CTL response, which leads to therapeutic transgene loss following hepatic AAV gene transfer.

- CpG-depleted AAV vectors provide a ***novel and effective strategy*** for suppressing AAV associated immunity and for generating durable, long-term transgene expression.

- ***Future Direction: The most urgent improvement/innovation that can be made to an AAV vector to maximize the probability of clinical success is to reduce the CpG content of the AAV vector.***

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Note: This data was submitted in a manuscript to **Molecular Therapy in 2014 and rejected for not being novel enough to publish because the reviewers stated that since CpG-depleted AAV vectors minimized immunity and lead to long-term transgene expression following intramuscular injection (Faust et al. 2013), it naturally followed these vectors would function identically in liver, and that the data was confirmatory in nature.*

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RE: Manuscript MT-E-14-423, "CpG-Motifs within AAV Vectors Trigger Immune Activation upon Hepatic Gene Transfer"

Thank you for allowing us to review your manuscript "CpG-Motifs within AAV Vectors Trigger Immune Activation upon Hepatic Gene Transfer", which has now been through the peer review process. Reviewer comments are below.

Unfortunately, the comments below indicate that your manuscript could not successfully compete for space with other articles that have been recently submitted to us. We are currently receiving a very high number of outstanding papers, and the competition for space is severe.

Therefore, we regret to inform you that we cannot consider your article for publication in Molecular Therapy.
We hope that you appreciate that less than one in three submissions receives a priority high enough for publication, and that our decision was therefore based on editorial priorities, and not on the technical aspects of your work or the quality of your science.

Reviewer #1 (Remarks to the Author):

In this manuscript, Faust et al., describe that AAV vectors containing CpG motifs trigger immune activation following hepatic gene transfer in mice. Some of the same authors from this group have previously reported that CpG-depleted AAV vectors evade immune detection following gene transfer to skeletal muscle in mice (J. Clin. Invest., 123: 2994-3001, 2014). Thus, beyond providing evidence for the role of CpG motifs in immune activation, albeit in a different tissue target, these studies are largely confirmatory in nature, and in my opinion, do not reach the significance to warrant publication in Molecular Therapy.

Reviewer #3 (Remarks to the Author):

This manuscript describes attenuation of immune response to a recombinant AAVrh32.33 vector upon CpG depletion in the vector genome after systemic delivery, targeting liver.

This work is an extension of the group's prior work examining the same phenomenon in murine skeletal muscle (Faust et al. JCI. 2014) with a minor change in route of administration (iv vs im) and promoter (E1Fa vs CB) and it seems the results in this work is the same mechanism that has been described in the prior paper. The additional information that is provided by this manuscript include some direct evidence that pDCs and cDCs in spleen are activated and that a CpG-deleted AAVrh32.22 vector can result in increased transduction in mouse liver. While the work is sound and of some interest, as liver-directed gene transfer with AAV vectors is still being avidly pursued both pre-clinically and clinically, the novel information in this work is very incremental and may better be suited in a different journal as is.

Minor comment: Label in Fig 1A is incorrect - both panels are labeled as RhCpG+.

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- Or contact me at sfaust@nxgenvectorsolutions.com