Immunogenicity risk and mitigation in AAV-mediated gene transfer

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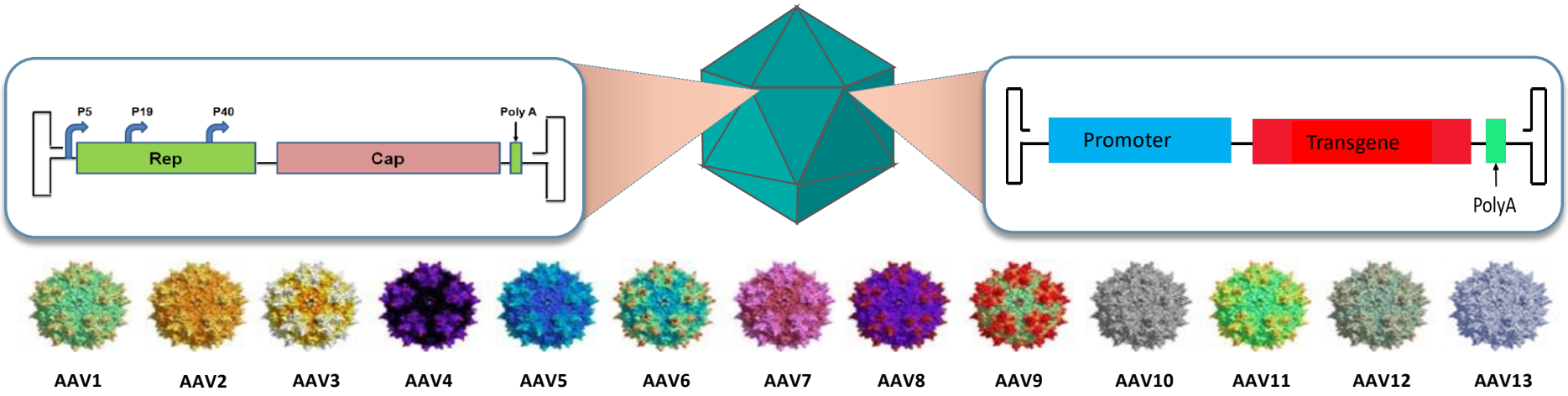
Recombinant Adeno-Associated Virus as a gene therapy vector

Wild-type AAV

- Parvovirus, Dependoparvovirus
- Non enveloped icosahedral capsid
- 4.7kb single-stranded DNA genome
- Several serotypes
- Naturally infects humans and non-human primates
- Non-pathogenic

Recombinant AAV (rAAV)

- Derived from WT AAV
- Broad tropism
- Quiescent cell transduction
- Mostly non-integrative (episomal)
- Long-term expression of the transgene
- Vector most commonly used for in vivo gene transfer



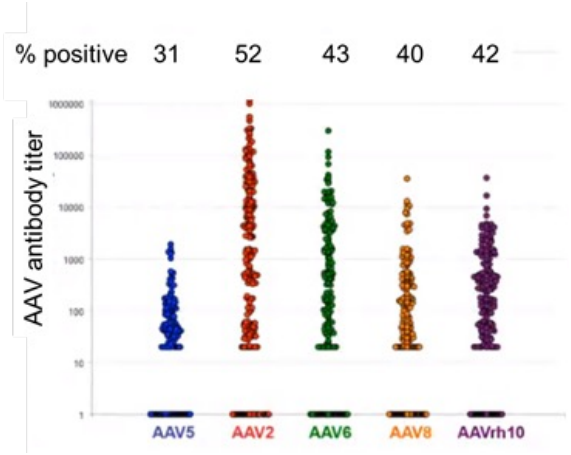
Adapted from Vance et al., 2015

Immunological challenges in AAV-mediated gene transfer

Pre-existing immunity to wild-type AAV

Pre-existing anti-AAV neutralizing antibodies prevent or lower the efficacy of gene transfer.

- Need to exclude patients with NAbs, or define a cut-off antibody titer
- Develop technologies to remove pre-existing Abs (IdeS, plasmapheresis, immunoadsorption,..)



Immune responses to AAV gene transfer

Anti-capsid antibodies

- Prevent re-dosing
- Activate complement and mediate TMA (Thrombocytic Microangiopathy)

Cytotoxic CD8+ T cell responses to AAV capsid

- Decrease the persistence of AAV-transduced cells and transgene expression, resulting in non-therapeutic PK/PD
- Mediate liver toxicities

Immune responses to the transgene product - Patient's immune system may recognize the transgene product as "foreign"

Anti-transgene antibodies

- Lead to non-therapeutic PK/PD (i.e. Factor IX inhibitors)

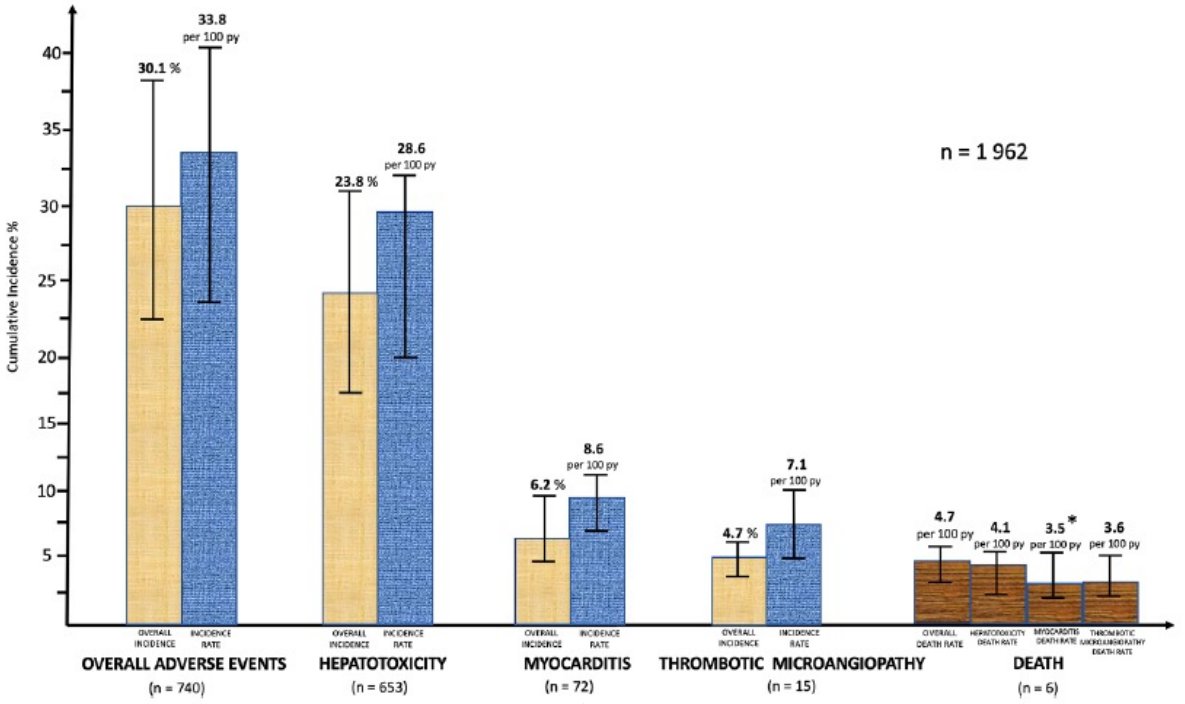
Cytotoxic CD8+ T cell responses to transgene product

- Decrease transgene expression
- Mediate tissue toxicities (i.e. T cell response to micro-dystrophin leading to Immune-Mediated Myositis)

Immune-mediated toxicities in systemic AAV gene transfer

- High-doses: order 1E12 - >1E14 vg/kg (plus empty capsids)
- At the highest doses, close to 50 mg of AAV capsid proteins for a 60 kg individual!

Maurizi et al., Incidence, timing, and clinical significance of adverse immune events after gene replacement therapy: A systematic review and meta-analysis, Molecular Therapy (2026) https://pubmed.ncbi.nlm.nih.gov/41520173/



T cell response to AAV capsid

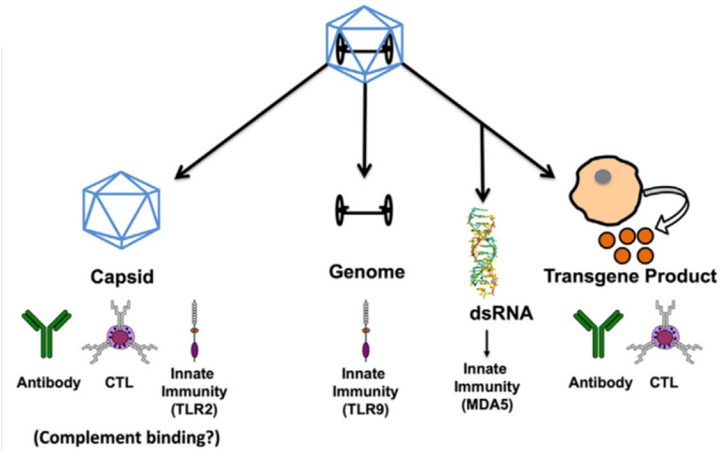
Innate IR
T cell response
Complement activation
Transgene overexpression

Ab response to AAV capsid
Complement activation

Immunogenicity risks of AAV-mediated gene transfer

Each component of a rAAV vector may affect its immunogenicity profile. Immunogenicity risks are evaluated separately for each component of the gene therapy

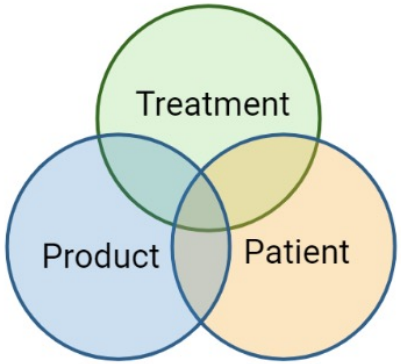
- ✓ Capsid serotype
- ✓ Genome composition
- ✓ Transgene product



JL Shirley et al. *Immune Responses to Viral Gene Therapy Vectors*. Molecular Therapy (2020)

Other factors may influence the immunogenicity risks:

- ✓ Vector dose (vg/kg, vp/kg) is the main factor
- ✓ Vector manufacturing (% empty capsids, impurities)
- ✓ Route of administration (systemic / local)
- ✓ Promoter (tissue-specific / ubiquitous)
- ✓ Surgical procedure
- ✓ Patients immune status, HLA genotype
- ✓ Disease-related factors



All parameters are considered for the Immunogenicity Risk Evaluation and Mitigation Strategy

Mitigating the rAAV product-associated immunogenicity risk

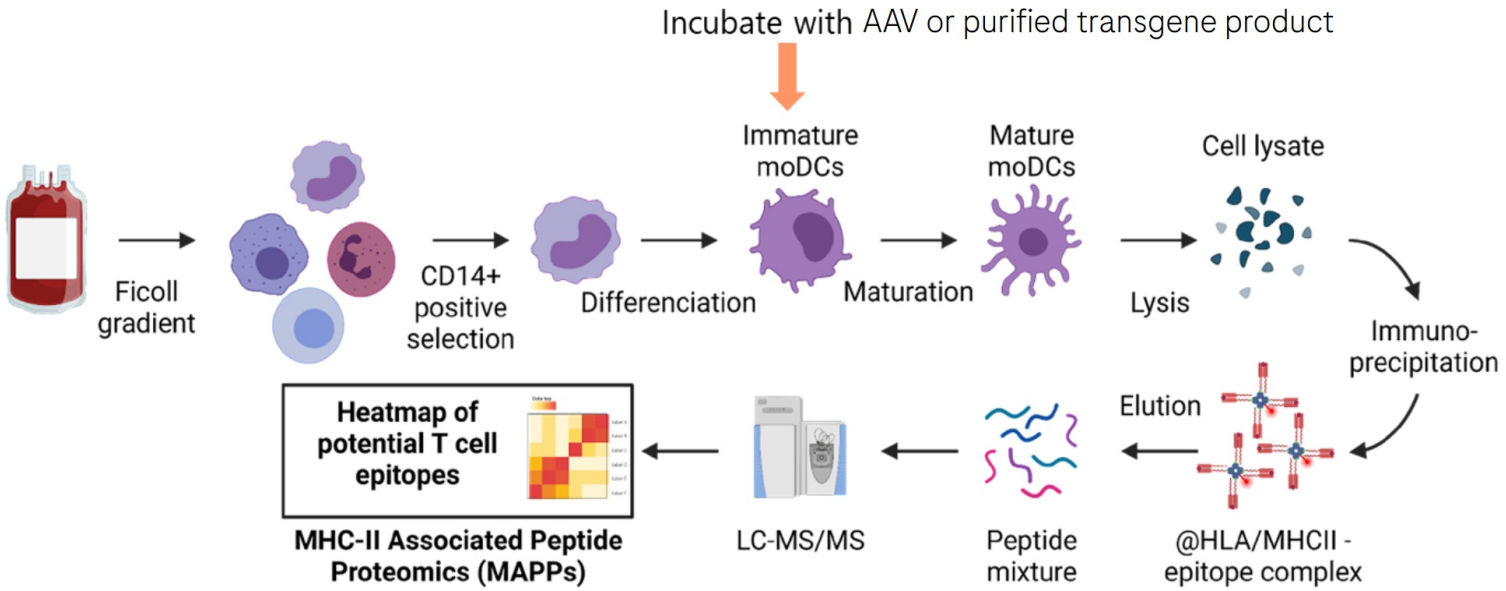
- **Need to consider HLA-I associated epitopes** (CD8 T cell responses) **in addition to HLA-II** (ADA responses)

- **Immunogenicity assessment tools** contributing to **CAPSID** and **TRANSGENE PRODUCT** candidate ranking and selection :
  - ✓ **In silico epitope prediction** using NetMHC: identifies peptide binding to 12 HLA-I and 13 HLA-II most common alleles
  - ✓ **MAPPs** (MHC-Associated Peptide Proteomics) for epitope identification; HLA-I and HLA-II
  - ✓ **Peptide/T cell** assays; CD4 and CD8 T cells
  - ✓ **Dendritic Cell/T cell** assays

- **Immunogenicity mitigation strategies** (transient prophylactic immunosuppression) in clinical development

Epitope identification by MAPPs

MHC-Associated Peptide Proteomics

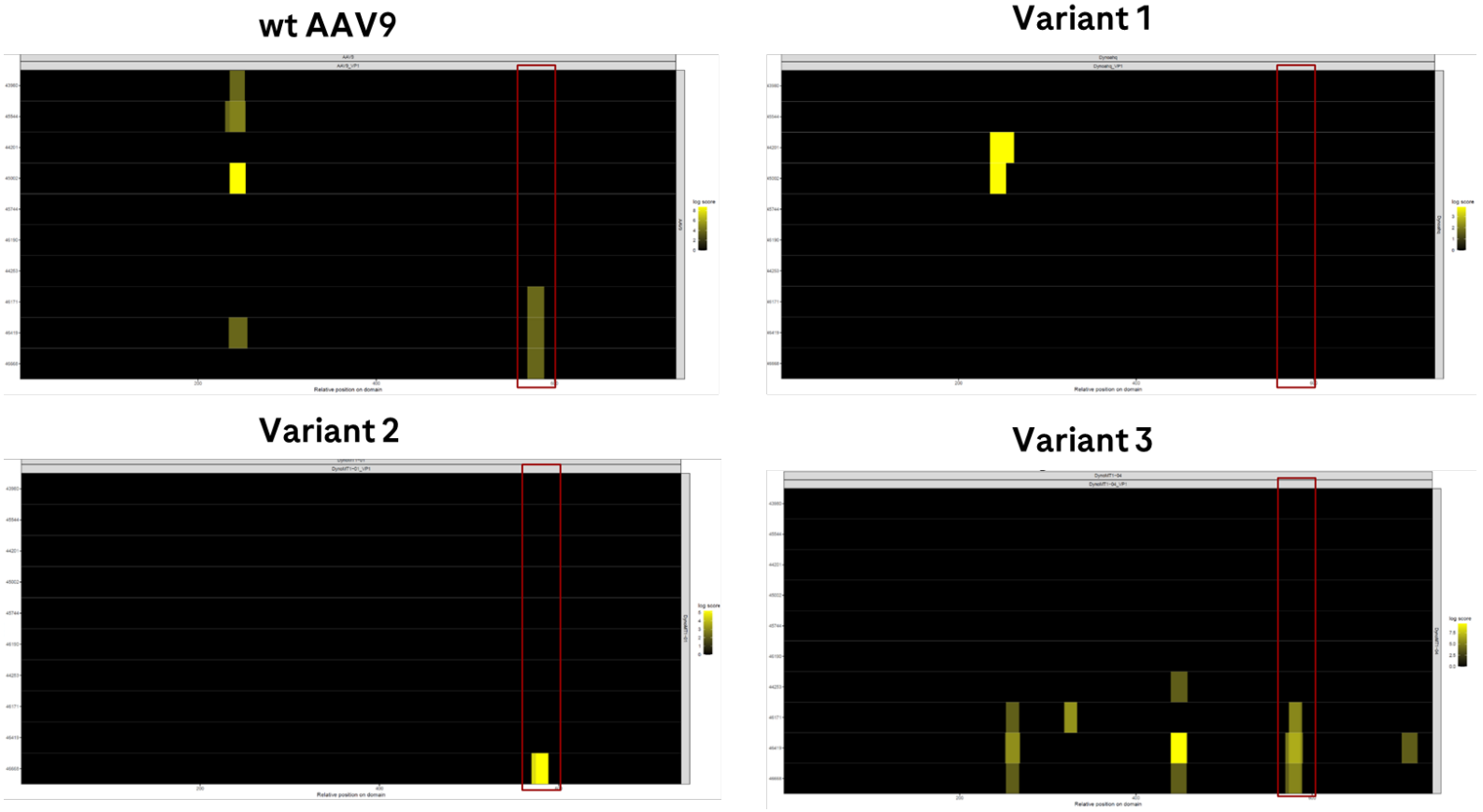


- Identifies peptides presented by dendritic cells in association with
  - ✓ HLA Class-II (DR) molecules → prerequisite for antibody formation
  - ✓ HLA Class-I (A,B,C) molecules → prerequisite for CD8 T cell response
- Generally performed on ≥ 10 blood donors

Identified peptides do not necessarily induce a T-cell response, T-cell assay needed.

MAPPs Class II

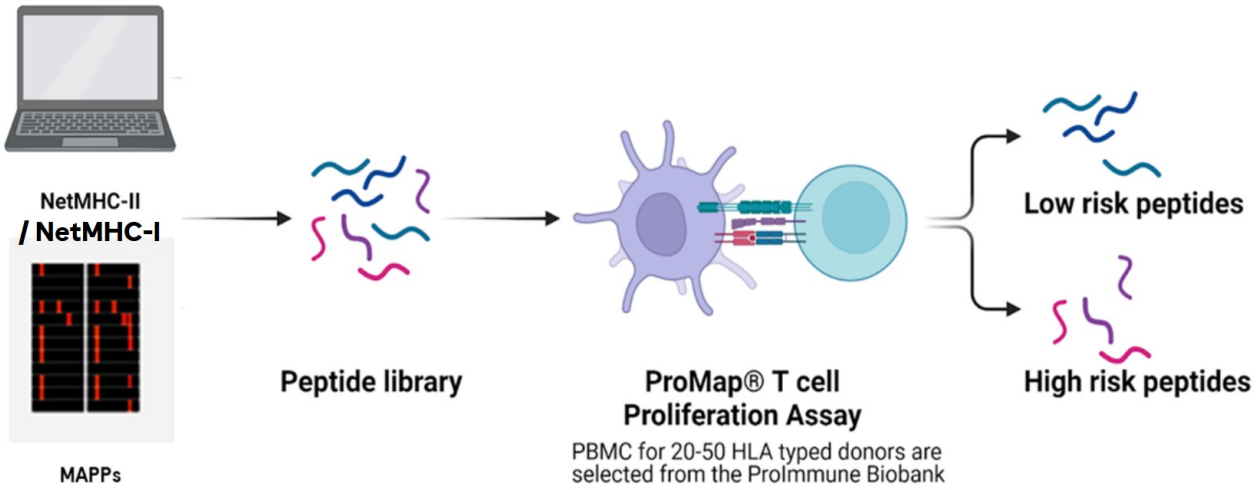
Sequence difference between the 4 capsids



✓ Less peptide clusters identified in AAV9 capsid variants 1 and 2, compared with variant 3

Peptide-T cell assays

- Measure T cell responses to peptides identified in silico or by MAPPs
- Readout: % donors showing T cell proliferation (above threshold), discriminates between low risk and high risk peptides
- For rAAV and transgene product (if feasible): performed with CD4+ and CD8+ T cells



PBMC for 20-50 HLA typed donors are selected from the ProImmune Biobank

A peptide library is synthesized according to project requirements

Donor PBMC are labelled with CFSE dye

Each peptide is co-cultured with the PBMC from each donor

Proliferation of CD4+ T cells in response to peptide causes dilution of the CFSE label

Flow cytometry analysis of CFSE in conjunction with staining for CD4, is used to quantitate cell division, and provide a measure of antigenicity.

MAPPs and peptide T cell assay results:
Focus on sequence differences between capsids VP1

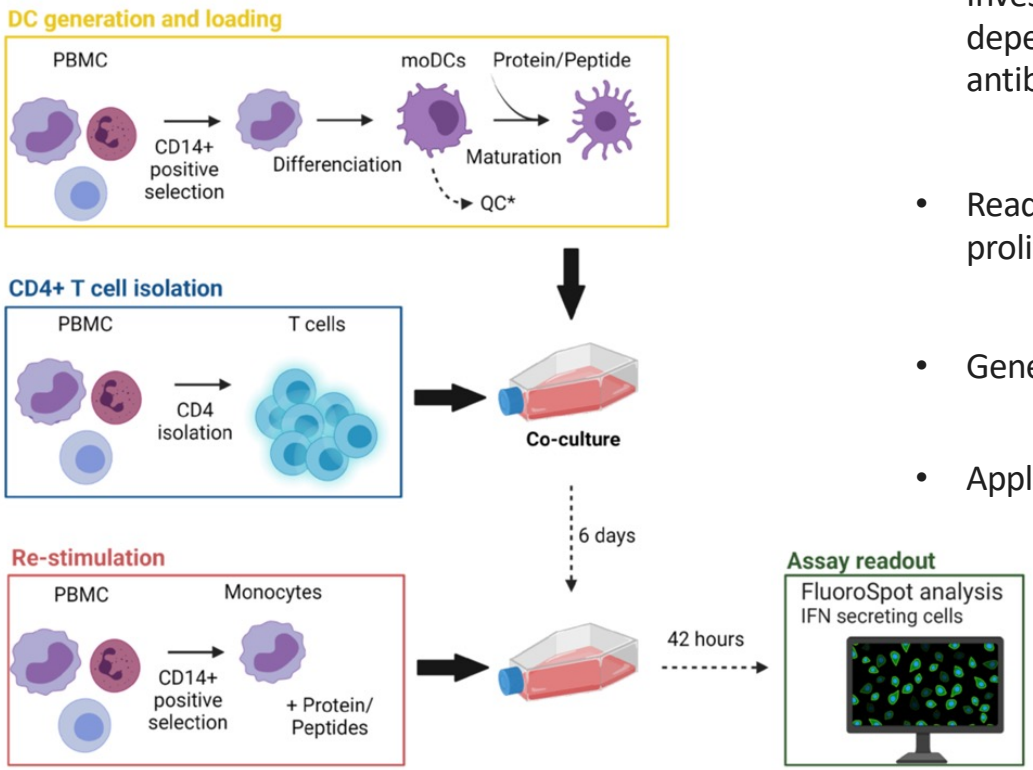
MAPPs Class-II peptide clusters

Peptide/CD4 T cell assay
- low risk
- moderate risk
- high risk

Peptide/CD8 T cell assay
- low risk
- moderate risk
- high risk

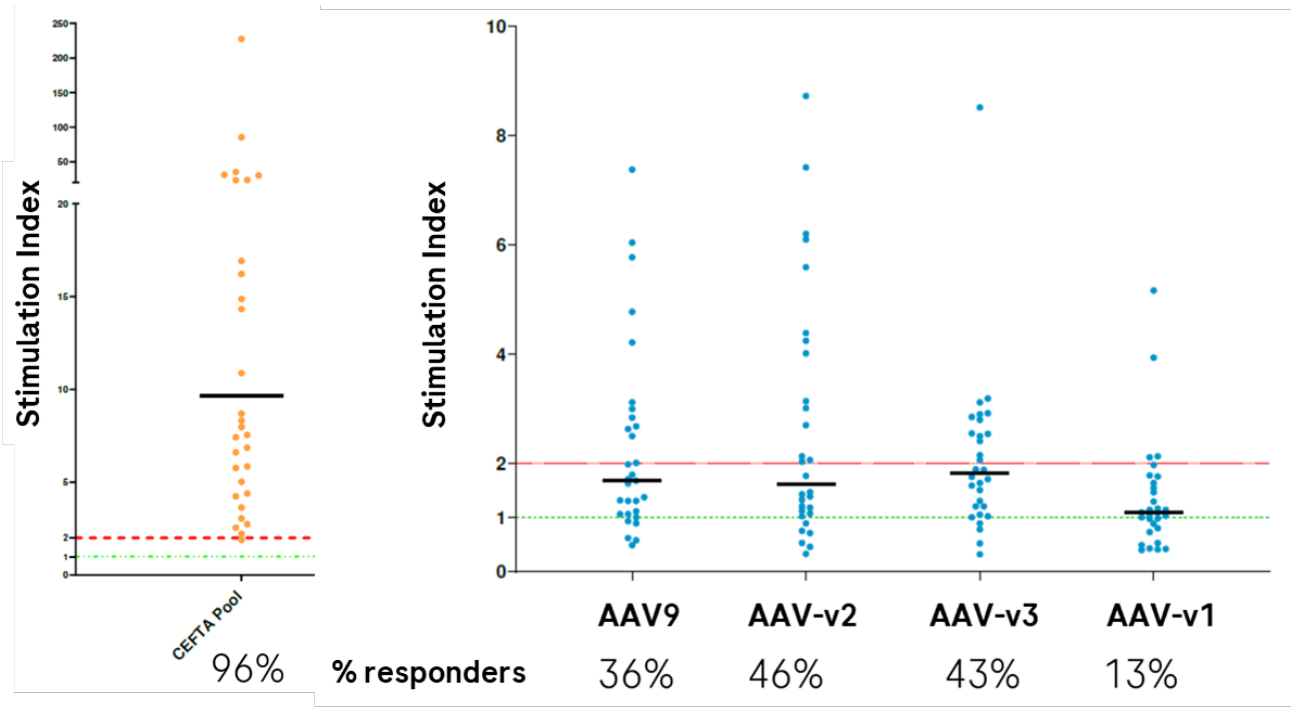
✓ Highest risk class-II peptide (45% CD4 T cell response) present in wt AAV9 and variants 2 and 3, while absent in capsid variant 1

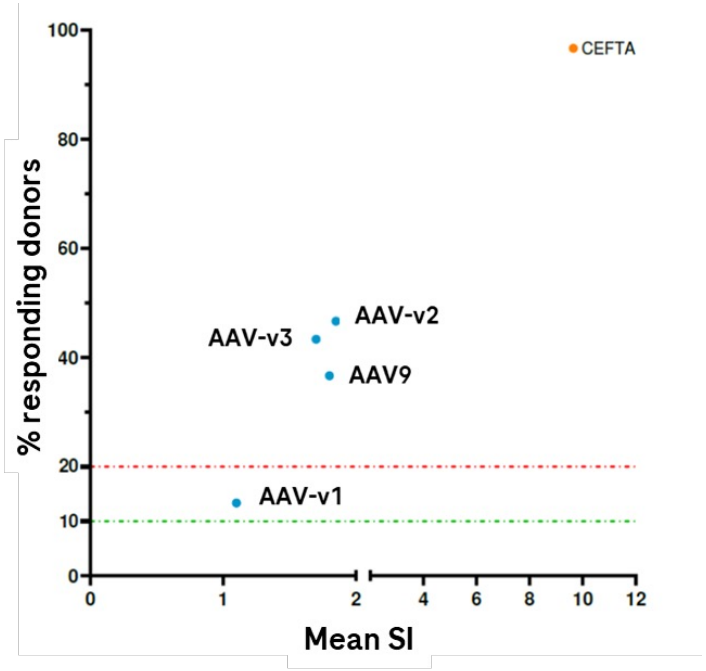
DC/T cell assay



- Investigates antigen-specific CD4 T cell stimulation (MHC-II dependent) by antigen-loaded dendritic cells, as a prerequisite to antibody formation
- Readout: (1) % donors showing T cell IFN-g production, or T cell proliferation; (2) Stimulation index
- Generally performed on 30 blood donors
- Applicable for purified transgene product or AAV vector

DC/CD4 T cell assay with rAAV vectors





- ✓ Significant increases in mean SI and % responders with AAV9, AAV-v2 and -v3 -> suggests high risk of CD4 T cell response
- ✓ No significant increases with AAV-v1 -> suggests lower risk of CD4 T cell response
- ✓ Correlates with absence of high-risk peptide identified in the peptide/CD4 T cell assay

Persistence of transgene expression decreases with the vector genome CpG content

AAV Gene Therapy Clinical Trials for Hemophilia B

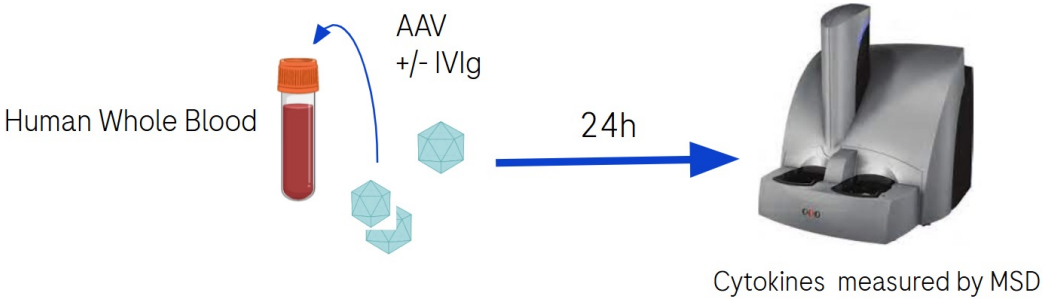
Sponsor	Serotype/Configuration[a]	No. of CpG in ORF	Production	Dose ($\times 10^{12}$)		Immunology		Outcomes	
				(vg/kg)	(~cp/kg)	IS[b]	CTL[c]	Peak FIX	Duration
CHOP, Stanford Avigen	AAV2-FIX/ss	19[d] (WT)	HEK	2	2	–	++	12% (n = 1)	<3 months
UCL, St Jude	AAV8-FIX/sc	0[d]	HEK	0.2–2	1–10	+	+	2%–11% (n = 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

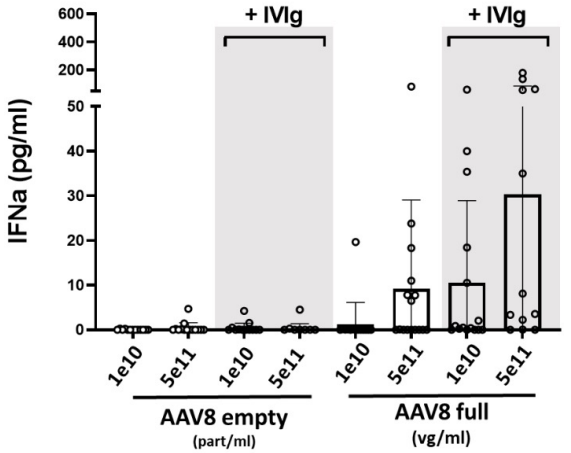
[a]Genome configuration: ss, single-stranded genome; sc, self-complementary genome.
[b]Immune suppression: –, not used; +, minority of subjects; ++, majority of subjects.
[c]Capsid-specific CTLs by IFN-γ ELISPOT: +, minority of subjects; ++, majority of subjects.
[d]Nathwani, 2019, American Society for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

Among the variables, including serotype, expression cassette configuration, production method, vector genome (vg) and estimated total capsid dose, and the use of immune-suppression, **low CpG content is the only parameter that fully correlates with long-term FIX expression.**

Wright JF. Codon Modification and PAMPs in Clinical AAV Vectors: The Tortoise or the Hare? Mol Ther. 2020 Mar 4;28(3):701-703.

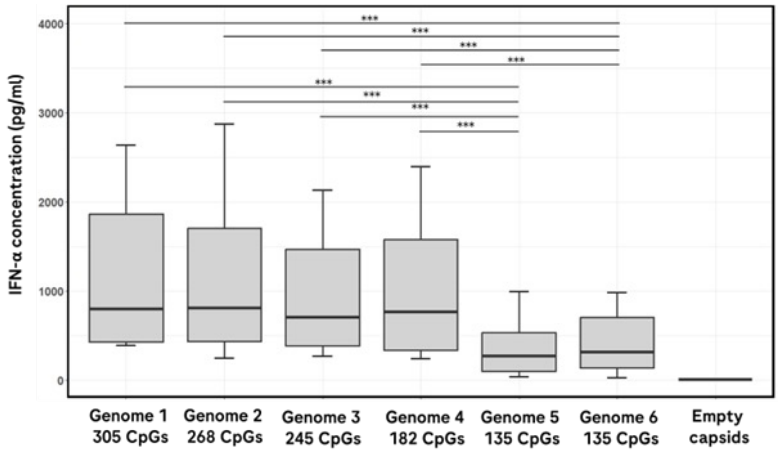
IFN-α release assay in whole blood assay to assess the effect of rAAV genome on innate immune response





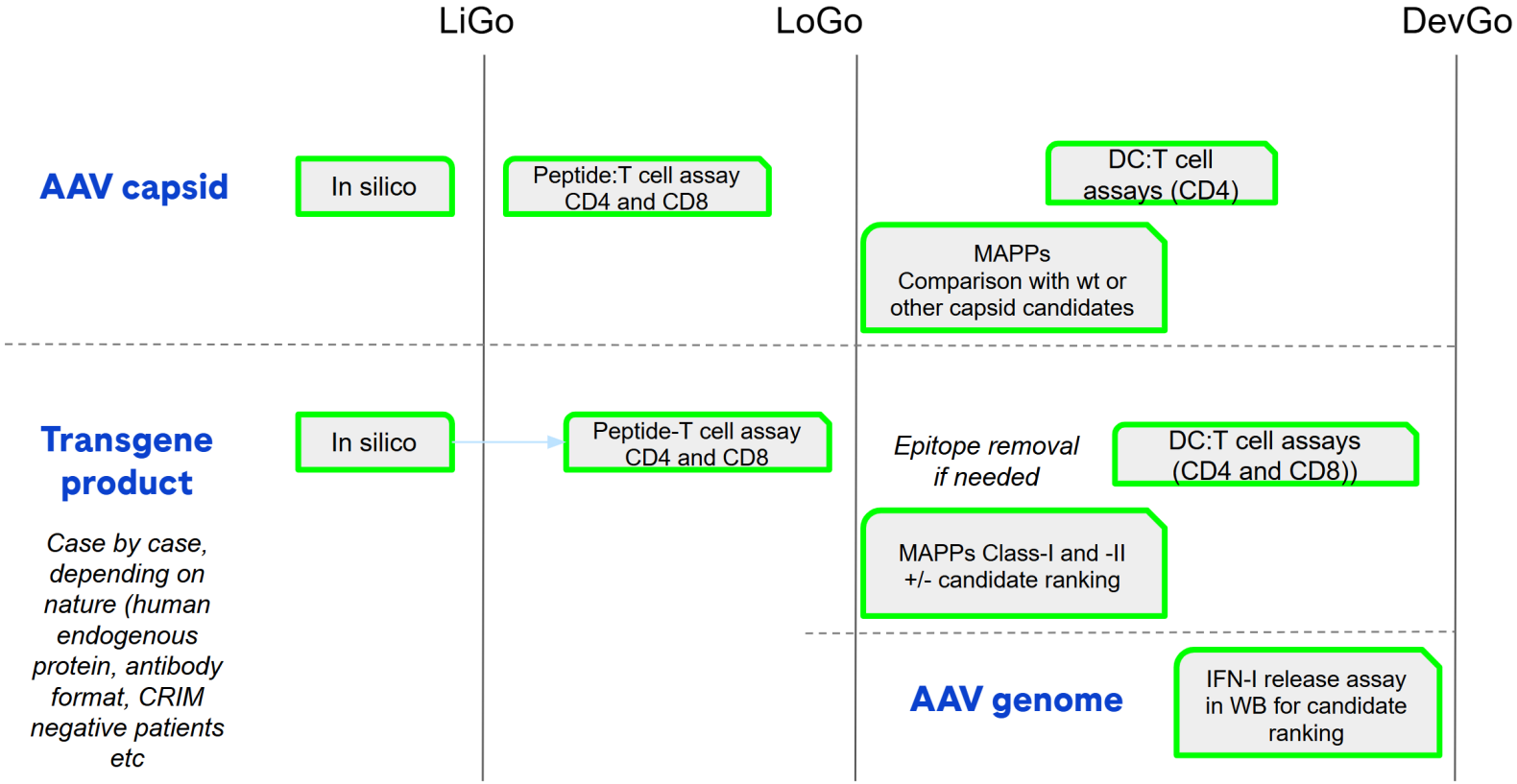
- ✓ Cytokines and chemokines are released in human whole blood incubated with rAAV
  IP-10, MCP-1, MIP-1α, MIP-1β, IFNγ, TNFα, IL-2, IL-1β, IL-2, IL-6, IL-10, IFNα
- ✓ Anti-AAV antibodies contribute to cytokine release
- ✓ Empty capsids do not elicit IFN-a release

IFN-α release in whole blood with IVIg reflects the effect of the vector genome on the innate immune response to rAAV



Xicluna R, et al. Innate immune response to AAV-based gene therapy vectors: Mechanisms of complement activation and cytokine release. Mol Ther Methods Clin Dev. 2025
https://pubmed.ncbi.nlm.nih.gov/40917687/

General assay strategy



Immunomonitoring in pre-clinical studies

General considerations

- Immunogenicity of transgene-derived protein in NHP may strongly affect the persistence of expression and potentially safety (cytotoxicity, inflammation). With a human transgene product, the risk of immune response in NHP is lower if the sequence homology is high.

- Antibody formation to AAV capsid carries the risk of activating the classical complement pathway. Alternative complement pathway activation is also observed in NHP studies. Both may affect safety.

- Immune response to AAV capsid differs between species (T cell response to capsid not always detectable in NHPs and in mice)

Impact of pre-existing anti-AAV antibodies on patient inclusion

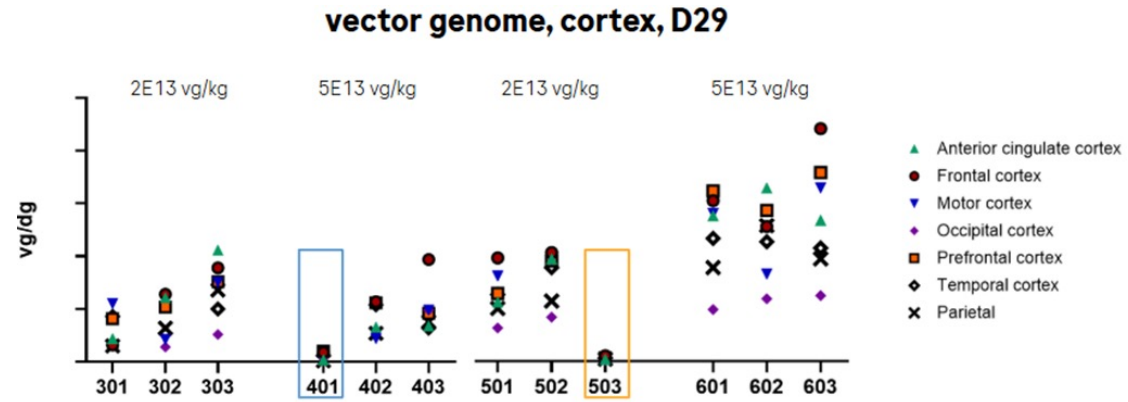
- Even low pre-existing anti-AAV Ab titers can prevent transduction by the systemic route
- Less drastic for local routes of administration (IVT, intra-CSF, intrathecal)
- Pre-existing anti-AAV Abs can affect safety in some cases
- Cut-off for patient inclusion should be determined preclinically, based on efficacy and safety

	Hemgenix	Roctavian	Zolgensma	Elevidys	Luxturna	Upstaza
	(Etranacogene Dezaparvovec)	(Valoctocogene Roxaparvovec)	(Onasemnogene Abeparvovec)	(Delandistrogene Moxeparvovec-rokl)	(Voretigene Neparvovec)	(Eladocagene Exuparvovec)
Serotype	AAV5	AAV5	AAV9	AAVrh74	AAV2	AAV2
Transgene	hFIXco-Padua	BDD hFVIII	SMN1	Micro-dystrophin	RPE65	AADC
ROA	intravenous infusion	intravenous infusion	intravenous infusion	intravenous infusion	subretinal injection	infused into putamen
Patient selection in pivotal trials	no patient selection	no AAV5 TAbs	TAb titer ≤50	AAVrh74 Ab titers <400	no patient selection	AAV2 NAb titers ≤20

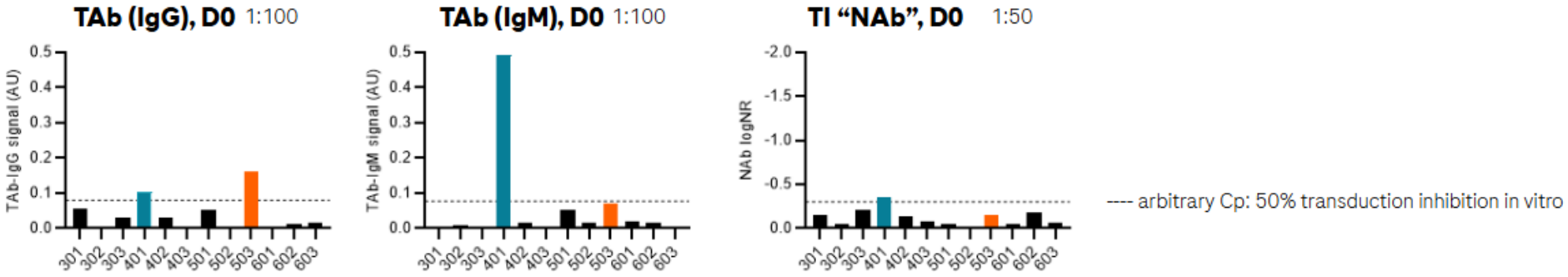
From: Braun M, Lange C, Schatz P, Long B, Stanta J, Gorovits B, Tarcsa E, Jawa V, Yang TY, Lembke W, Miller N, McBlane F, Christodoulou L, Yuill D, Milton M. Preexisting antibody assays for gene therapy: Considerations on patient selection cutoffs and companion diagnostic requirements. Mol Ther Methods Clin Dev. 2024. PMID: 38496304

Impact of preexisting anti-AAV Abs on brain biodistribution

Vector genome biodistribution in NHPs dosed IV with rAAV9 capsid variants



- Even low pre-existing antibody titers at the time of dosing prevented brain transduction
- For1st-in-NHPstudies using the systemic route of administration, need to select AAV-seronegative animals

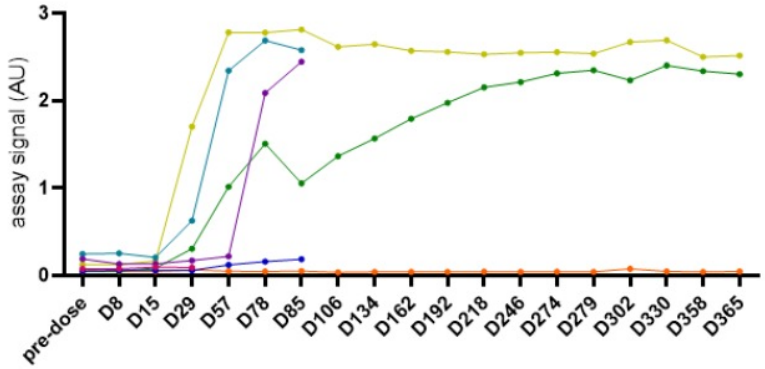


Florian Neff, Francesca Ros, Kerstin Hahn and team

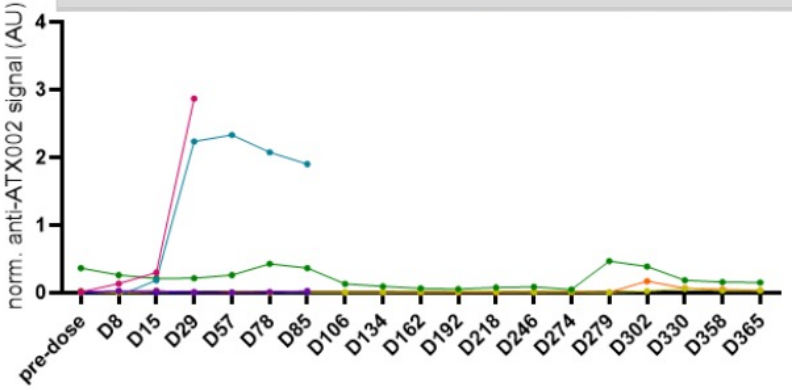
ADA incidence and pathology finding correlations

Antibody monitoring in NHPs dosed with a rAAV through the intra-vitreal route, 5E9 vg/eye

Anti-transgene Abs (serum)



Anti-AAV capsid Abs (serum)



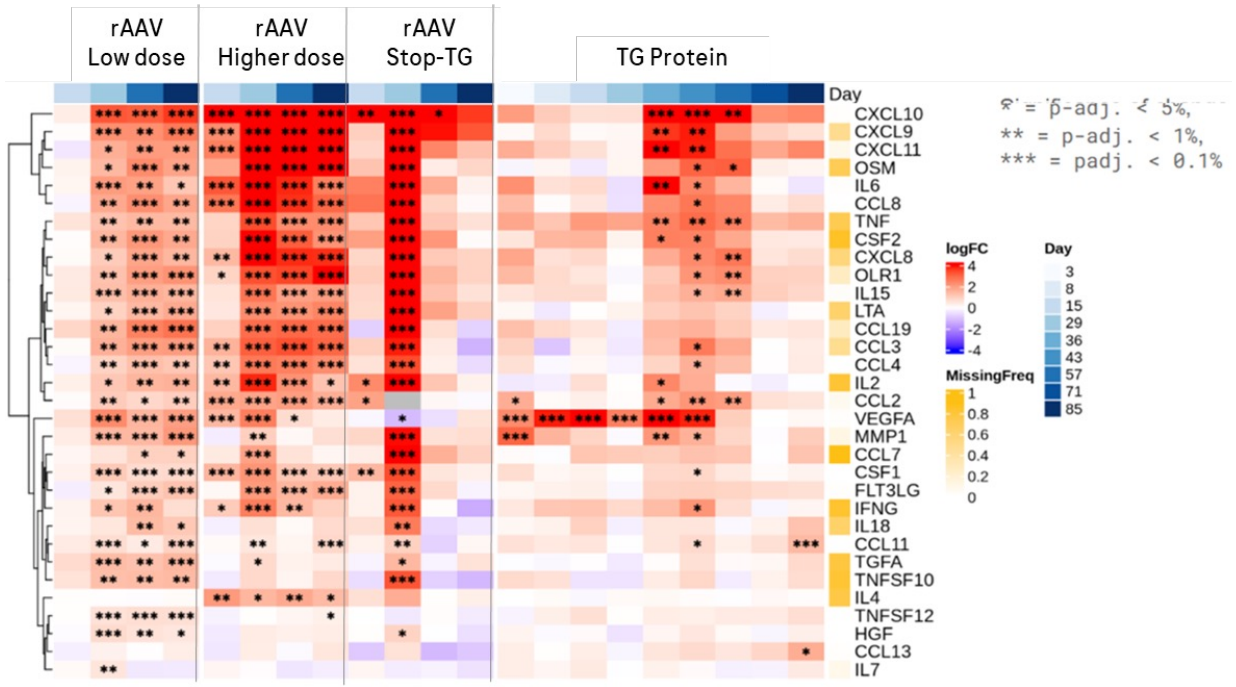
- **Positive correlation** between early-onset (day 29–57) and high-level (>2 AU) **anti-transgene Ab** formation, mononuclear cell infiltrates, and incidence of pathology findings

- **NO correlation** between **capsid ADAs** formation and incidence of pathology findings

Florian Neff, Barbara Lenz, Jacek Krol and team

Cytokine and chemokine release mediated by AAV capsid vs transgene product

Olink analysis of aqueous humor in NHPs injected IVT with rAAV or TG protein
No prophylactic immunosuppression, mild post-treatment immunosuppression

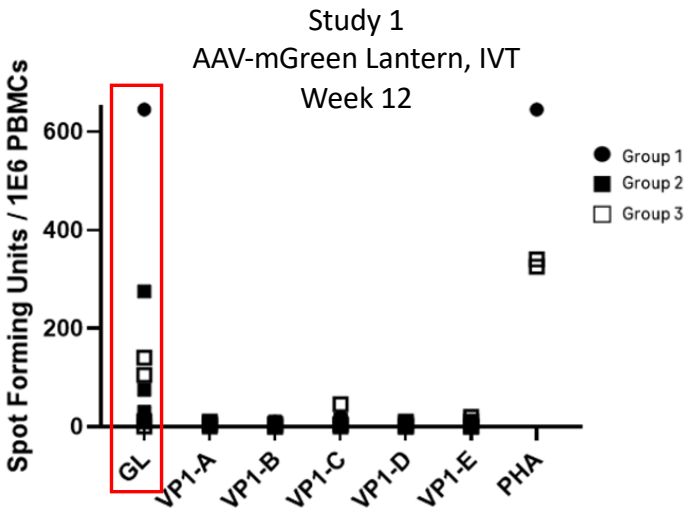


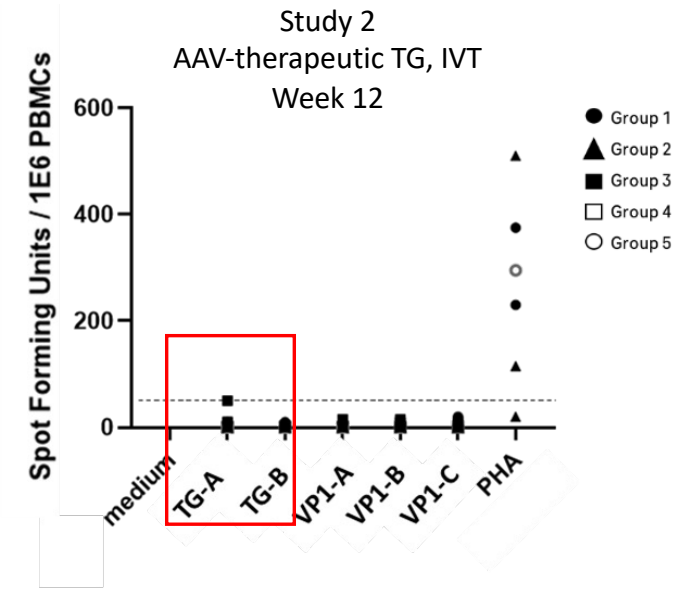
- Early inflammation induced by rAAV (capsid/DNA genome/mRNA)
- Delayed inflammation linked with transgene protein expression
  Note: ADA formation to the therapeutic transgene (human) was observed in all animals
- Mitigation strategy: prophylactic local immunosuppression

Juliane Siebourg-Polster, Florian Neff, Stefan Seeber, Nicole Hellbach, Jacek Krol and team

Impact of T cell response to the transgene product

IFN-ɣ response to the transgene product (Fluorospot) 12 weeks post-IVT dosing of AAV-GL or AAV-therapeutic transgene in NHPs



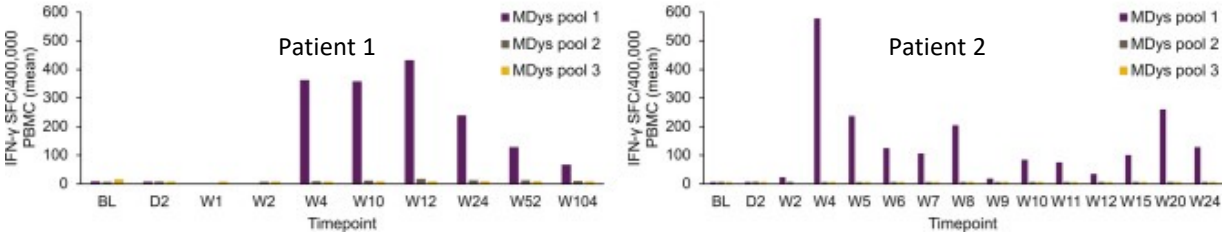


- Strong response to the mGreenLantern transgene product in Study 1, with pathological findings
- No IFN-ɣ cell response to the therapeutic transgene product (weakly positive in 1/18 animals) in Study 2
- Only discrete IFN-ɣ cell responses to the AAV capsid VP1 protein

Clinical case: Immune response to micro-dystrophin transgene leading to Immune Mediated Myositis in patients with DMD treated with Elevidys

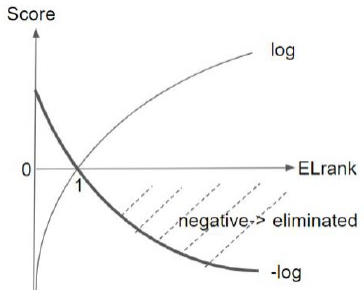
- Severe adverse events of Immune-Mediated Myositis (IMM) occurred in patients treated with several DMD gene therapies, attributed to T cell responses against micro-dystrophin.

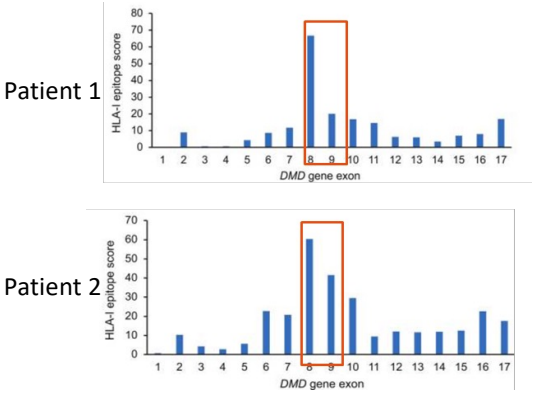
IFN-γ ELISpot response to micro-dystrophin in 2 patients with IMM



Sohrab Khan
Potter et al, 2025

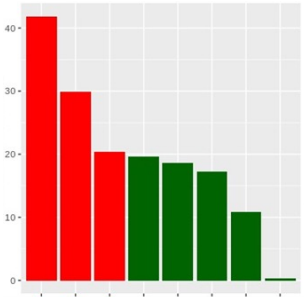
- All patients with IMM had deletions of the DMD gene encompassing exons 8 and 9; however, not all patients with mutations in exons 8-9 developed IMM.
- *In silico* epitope mapping and scoring based on patients' HLA-I* alleles showed that exons 8 and 9 carried the highest risks of CD8 T cell response. Patients who developed IMM displayed high epitope scores for these exons.





Epitope scoring for exon 9 of the DMD gene correlates with the risk of IMM in patients with mutations in exons 8 or 9

HLA-I score for exon 9



Guido Steiner, Andreas Hollenstein, Hélène Haegel

- The label now excludes patients with deletions in exons 8 and/or 9

Final considerations

- AAV-mediated gene transfer represents a "one-and-done" therapeutic approach but carries inherent immunogenicity risks.
- Antibody and T cell responses to the AAV vector and transgene product can significantly influence pharmacokinetics (PK), pharmacodynamics (PD), and safety profiles.
- By nature, AAV capsids are immunogenic. To address this, ongoing efforts aim to engineer capsids that can evade neutralizing antibodies or eliminate dominant T cell epitopes.
- Additionally, research is focused on developing methods to remove pre-existing anti-AAV antibodies, thereby allowing seropositive patients to access AAV gene therapies.
- There is a critical need to enhance prophylactic immunosuppression strategies to enable AAV redosing, which is currently not feasible.

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- Pathology and Applied Safety Sciences
- Gene Therapy Portfolio squad

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Kerstin Hahn

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Nicole Hellbach

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Christoph Wandel

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Tim Hickling

Doing now what patients need next