

Use of PEPscreen™ Peptides to Investigate CTL Class I MHC and Th Class II Responses to AAV-hF.IX Gene Therapy¹

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Protein	MHC1	Epitope peptide
hF.IX	H2-Db	AA ₂₅₄₋₂₆₂ GSIVNEKWI
hF.IX	H2-Kd	AA ₃₁₁₋₃₁₉ KYNHDIALL
AAV2	H2-Kd	AA ₃₇₃₋₃₈₁ QYGSVSTNL

Table 1. MHC I binding peptides predicted by SYFPEITHI and BIMA

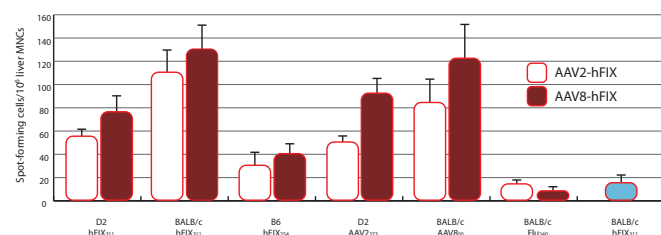


Figure 1. Density of IFN-γ Spot-forming Cells in Response to Different CTL Epitope Peptides

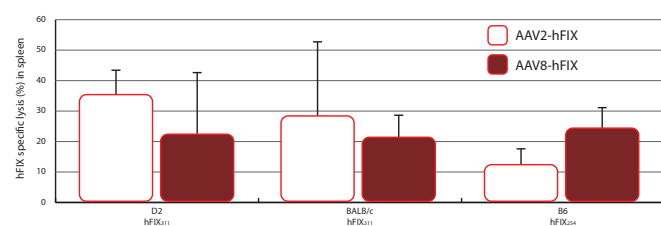


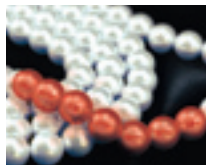
Figure 2. In Vivo CTL Assay for hFIX Specific Lysis in Mice

Recombinant adeno-associated virus (AAV)-mediated gene therapy expressing human coagulant factor IX (hF.IX) is a promising gene transfer strategy due to the long-term expression of hF.IX in the liver and weak immune response using a liver specific promoter. However, in humans and some animals, the F.IX expression declines over long time periods and immune responses to vector and transgene can occur.^{1,2} To determine if AAV-hF.IX treatment could induce specific cytotoxic T lymphocyte (CTL) responses in mice, we used the prediction programs SYFPEITHI³ and BIMAS⁴ to identify potential CTL epitope peptides from hF.IX, AAV2 and AAV8 capsid proteins in the context of H2-K^d and H2-D^b (**Table 1**). The peptides were synthesized at Sigma Genosys and supplied in a PEPscreen format.

DBA/2 (D2, H2^d), BALB/c (H2^d) and C57BL/6 (B6; H2^b) mice were IV injected with 2 x 10¹¹ vg of AAV2-hF.IX or AAV8-hF.IX, in which hF.IX expression is regulated by a liver-specific ApoE/haAT promoter.⁵ To obtain the optimal immunization, the mice were boosted 30 days later with the same route and dose of vector. Blood samples were collected for analysis of ALT and hF.IX.

The results showed that plasma ALT level was elevated by day 4 after administration and a second peak was observed 9 days after boost in all mice (p<0.05). There was no significant decrease in plasma hF.IX levels up to 9 days after boost. To determine the specificity of CTLs in the liver, mononuclear cells (MNC) were analyzed 9 days after boost by an IFN-γ ELISPOT assay⁶ using epitope peptides specific for hF.IX, AAV2 or AAV8 capsid as shown in **Table 1**. An irrelevant flu peptide AA₂₄₀(IYSTVASSL) restricted by H2-Kd was used as a negative control.

There was a significant increase in IFN-γ spot-forming cells (SFC) specific for the hF.IX₃₁₁ peptide in H2^d mice and hF.IX₂₅₄ in H2^b mice, respectively (**Figure 1**). The highest CTL response to hF.IX was observed in BALB/c mice receiving AAV8-hF.IX. There was also a significant increase in SFCs specific for the AAV₂₃₇₃ and AAV₈₅₁ peptide in H2^d mice, with the highest CTL response against AAV8 capsid in BALB/c mice receiving AAV8-hF.IX (**Figure 1**).



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The *in vivo* CTL assay was performed to confirm the function of hFIX specific CTLs (Figure 2). These results indicated that AAV2- or AAV8-hFIX gene transfer can induce both vector and transgene specific CTLs in the liver of both H2^d and H2^b mice. These results show that the use of specific peptides, ELISPOT and the *in vivo* killing assay provides sensitive methods to measure CTL response and will be useful in the design of effective and safe vectors and protocols for AAV-hFIX gene therapy delivery.

It was previously shown that C3H (H2^k) mice express the highest levels of anti-hFIX antibodies, which requires the I-A^kα, I-A^kβ and IL-10 region of Chromosome 1.⁷ We utilized MHC class II peptides in the context of I-A^k and I-E^k to specifically detect Th cells in AAVhFIX immunized C3H mice. First, we used a prediction program, SYFPEITHI, to predict MHC II binding peptides from hFIX, AAV2 and AAV8 capsid proteins (Table 2). The peptides were then synthesized at Sigma Genosys and supplied in a PEPscreen format. C3H mice were IV injected with 2 x 10¹¹ vg of AAV2- or AAV8-hFIX, in which hFIX expression is regulated by a liver-specific ApoE/haAT promoter. The mice were boosted 30 days later. Blood samples were collected for analysis of ALT and plasma hFIX. The plasma ALT level was significantly elevated (p<0.05) at day 9 after boost with AAV2- or AAV8-hFIX. There was no significant decrease in plasma hFIX levels up to 9 days after boost.

To determine the frequency of hFIX, AAV2 and AAV8 specific CD4 Th cells in the liver, MNC were prepared and analyzed by peptide specific ELISPOT assays for production of Th1 cytokine IFN-γ and Th2 cytokine IL-4, respectively. Liver MNCs were co-cultured with irradiated antigen presenting cells pulsed with the peptides specific for hFIX, AAV2 or AAV8. The number of IFN-γ spot-forming cells (SFC) was determined 48 hours later (Table 2). For hFIX, the strongest class II ELISPOT assay was observed for I-A^k peptide AA₁₀₈₋₁₂₂ and I-E^k peptide AA₄₄₄₋₄₅₈. For both peptides, the ELISPOT assay revealed the induction of both a Th2 response indicated by IL-4 ELISPOT

response as well as a Th1 response, indicated by an IFN-γ ELISPOT response (Table 2). For AAV8-hFIX infected mice, an I-E^k restricted AAV8 epitope AA₁₂₆₋₁₄₀ was found to induce more IFN-γ SFCs than IL-4 SFCs (p<0.05). Similar results were seen in spleen of immunized mice. Other predicted peptides failed to induce IFN-γ or IL-4 SFCs in immunized mice (data not shown).

These results indicated that AAV2- or AAV8-hFIX gene transfer induced both vector and transgene specific Th cells with the highest response in IFN-γ production to hFIX epitope peptide AA₁₀₈₋₁₂₂. Moreover, these epitope peptides could function differently on Th1 and Th2 cells to drive a Th response towards a CD8 T cell response or B cell activation. These findings will facilitate a better understanding in CD8 T cell response or antibody response to AAV-hFIX gene therapy.

For more information, please visit wherebiobegins.com/pepscreen

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Protein	MCHII	Epitope Peptide	IFN-γ SFC/10 ⁶ Liver MNCs	IL-4 SFC/10 ⁶ Liver MNCs	SFCs in naïve mice (IFN-γ/IL-4)
HFIX	I-A ^k	AA ₁₀₈₋₁₂₂ CKDDINSYECWCWCPFG	250 ± 23 (AAV2-hFIX)	55 ± 17 (AAV2-hFIX)	0/0
	I-E ^k	AA ₄₄₄₋₄₅₈ YTKVSRVYVNWIKKEKT	85 ± 21 (AAV8-hFIX)	65 ± 11 (AAV8-hFIX)	0/0
AAV2	I-E ^k	AA ₄₇₅₋₄₈₉ QVSVEIEWELQKENS	30 ± 12	90 ± 17	0/0
AAV8	I-E ^k	AA ₁₂₆₋₁₄₀ LEPLGLVEEGAKTAP	75 ± 26	25 ± 15	5 ± 5/0
Ovalbumin	I-E ^k	AA ₁₁₆₋₁₃₀ PEYLQCVKELYRGGL	10 ± 5	0	0

Table 2. Summary of CD4T cell responses against different peptide epitopes

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