



# Use of PEPscreen® Peptides to Investigate CTL Class I MHC and Th Class II Responses to AAV-hF.IX Gene Therapy<sup>1</sup>

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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.<sup>1,2</sup> To determine if AAV-hF.IX treatment could induce specific cytotoxic T lymphocyte (CTL) responses in mice, we used the prediction programs SYFPEITHI<sup>3</sup> and BIMAS<sup>4</sup> to identify potential CTL epitope peptides from hF.IX, AAV2 and AAV8 capsid proteins in the context of H2-K<sup>d</sup> and H2-D<sup>b</sup> (Table 1). The peptides were synthesized at Sigma Genosys and supplied in a PEPscreen format.

DBA/2 (D2, H2<sup>d</sup>), BALB/c (H2<sup>d</sup>) and C57BL/6 (B6; H2<sup>b</sup>) mice were IV injected with 2 x 10<sup>11</sup> vg of AAV2-hF.IX or AAV8-hF.IX, in which hF.IX expression is regulated by a liver-specific ApoE/hAAT promoter.<sup>5</sup> 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- $\gamma$  ELISPOT assay<sup>6</sup> using epitope peptides specific for hF.IX, AAV2 or AAV8 capsid as shown in Table 1. An irrelevant flu peptide AA<sub>240</sub>(YSTVASSL) restricted by H2-K<sup>d</sup> was used as a negative control.

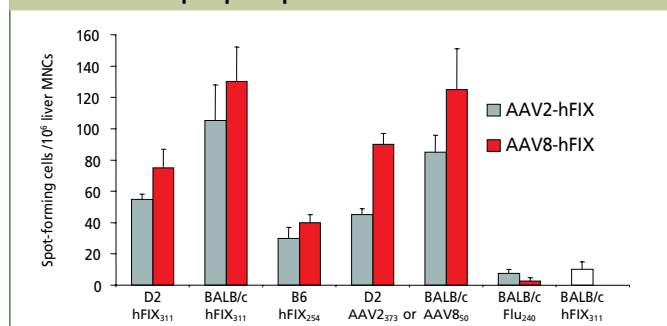
There was a significant increase in IFN- $\gamma$  spot-forming cells (SFC) specific for the hF.IX<sub>311</sub> peptide in H2<sup>d</sup> mice and hF.IX<sub>254</sub> in H2<sup>b</sup> mice, respectively (Figure 1). The highest CTL response to hF.IX was observed in BALB/c mice receiving AAV8-hF.IX. There was also a significant increase in SFCs specific for the AAV2<sub>373</sub> and AAV8<sub>51</sub> peptide in H2<sup>d</sup> mice, with the highest CTL response against AAV8 capsid in BALB/c mice receiving AAV8-hF.IX (Figure 1).

The *in vivo* CTL assay was performed to confirm the function of hF.IX specific CTLs (Figure 2). These results indicated that AAV2- or AAV8-hF.IX gene transfer can induce both vector and transgene specific CTLs in the liver of both H2<sup>d</sup> and H2<sup>b</sup> 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-hF.IX gene therapy delivery.

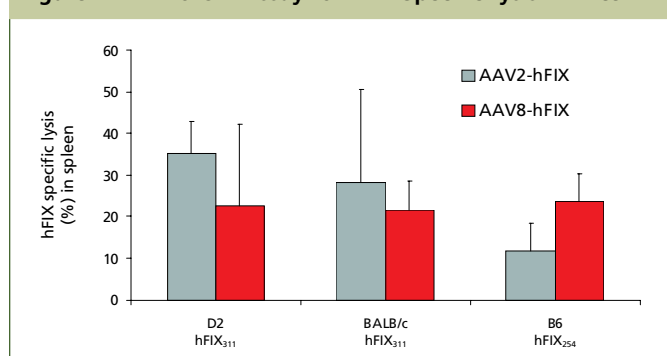
**Table 1.** MHC I binding peptides predicted by SYFPEITHI and BIMAS

Protein	MHC I	Epitope peptide
hF.IX	H2-Db	AA <sub>254-262</sub> GSVINEKWI
hF.IX	H2-Kd	AA <sub>311-319</sub> KYNHDIALL
AAV2	H2-Kd	AA <sub>373-381</sub> QYGSVSTNL

**Figure 1.** Density of IFN- $\gamma$  Spot-forming Cells in Response to Different CTL Epitope Peptides



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





It was previously shown that C3H (H2<sup>k</sup>) mice express the highest levels of anti-hf.IX antibodies, which requires the I-A<sup>k</sup> $\alpha$ , I-A<sup>k</sup> $\beta$  and IL-10 region of Chromosome 1.<sup>7</sup> We utilized MHC class II peptides in the context of I-A<sup>k</sup> and I-E<sup>k</sup> to specifically detect Th cells in AAV-hf.IX immunized C3H mice. First, we used a prediction program, SYFPEITHI, to predict MHC II binding peptides from hf.IX, 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 10<sup>11</sup> vg of AAV2- or AAV8-hf.IX, in which hf.IX 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 hf.IX. The plasma ALT level was significantly elevated ( $p < 0.05$ ) at day 9 after boost with AAV2- or AAV8-hf.IX. There was no significant decrease in plasma hf.IX levels up to 9 days after boost.

To determine the frequency of hf.IX, 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- $\gamma$  and Th2 cytokine IL-4, respectively. Liver MNCs were co-cultured with irradiated antigen presenting cells pulsed with the peptides specific for hf.IX, AAV2 or AAV8. The number of IFN- $\gamma$  spot-forming cells (SFC) was determined 48 hours later (**Table 2**). For hf.IX, the strongest class II ELISPOT assay was observed for I-A<sup>k</sup> peptide AA<sub>108-122</sub> and I-E<sup>k</sup> peptide AA<sub>444-458</sub>. 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- $\gamma$  ELISPOT response (Table 2). For AAV8-hf.IX infected mice, an I-E<sup>k</sup> restricted AAV8 epitope AA<sub>126-140</sub> was found to induce more IFN- $\gamma$  SFCs than IL-4 SFCs ( $p < 0.05$ ). Similar results were seen in spleen of immunized mice. Other predicted peptides failed to induce IFN- $\gamma$  or IL-4 SFCs in immunized mice (data not shown).

These results indicated that AAV2- or AAV8-hf.IX gene transfer induced both vector and transgene specific Th cells with the highest response in IFN- $\gamma$  production to hf.IX epitope peptide AA<sub>108-122</sub>. 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-hf.IX gene therapy.

## References

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

Protein	MHCII	Epitope Peptide	IFN- $\gamma$ SFC/10 <sup>6</sup> Liver MNCs	IL-4 SFC/10 <sup>6</sup> Liver MNCs	SFCs in naïve mice (IFN- $\gamma$ /IL-4)
HF.IX	I-A <sup>k</sup>	AA <sub>108-122</sub> CKDDINSYECWCPCFG	250 $\pm$ 23 (AAV2-hf.IX)	55 $\pm$ 17 (AAV2-hf.IX)	5 $\pm$ 5/0
	I-E <sup>k</sup>	AA <sub>444-458</sub> YTKVSRYYNWIKEKT	85 $\pm$ 21 (AAV8-hf.IX)	65 $\pm$ 11 (AAV8-hf.IX)	0/0
AAV2	I-E <sup>k</sup>	AA <sub>475-489</sub> QVSVEIEWELQKENS	30 $\pm$ 12	90 $\pm$ 17	0/0
AAV8	I-E <sup>k</sup>	AA <sub>126-140</sub> LEPLGLVEEGAKTAP	75 $\pm$ 26	25 $\pm$ 15	5 $\pm$ 5/0
Ovalbumin	I-E <sup>k</sup>	AA <sub>116-130</sub> PEYLQCVKELYRGGL	10 $\pm$ 5	0	0