

Preclinical Studies in Gene Delivery Biodistribution & Host Response Examples from AAV

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Biodistribution Studies

- Preclinical animal studies to determine distribution of vector beyond intended therapeutic target or organ
- Identify potential target organs for toxicity
 - Distribution of vector to non-target sites
- Determine persistence and/or integration
- Determine potential for germline transmission
 - Distribution of vector to gonadal tissue
- Presence of vector determined by DNA PCR

Biodistribution Study Design

- Species: rodent/rabbits/NHP/Dogs
 - Depend on clinical application , if in conjunction with tox study
- Gender: M/F to reflect patient population
- Animal number: 3-5/sex/grp for small animals, maybe less with large animals (discuss with FDA)
- Dose: vehicle, low, med, high(max clin dose of MFD)
- Route of administration: intended clinical route
- Time points: 4-21, 90, (180) days
- Tissue panel
 - injection site, peripheral blood, gonads
 - highly perfused organs (brain, liver, lung, kidney, heart, spleen)
 - draining lymph nodes, bone marrow
 - others

DNA PCR: Recommendations

- Take 3 specimens from each tissue
- Test 3 samples per specimen
 - 2 unspiked , 1 spiked
- Each sample (or sum of 2 samples) should be 1 μg of genomic DNA or 10^5 cell equivalents
- Sensitivity of <100 copies/specimen
- 10 genomes per 100,000 cells ($1/10^4$)

Avoid CONTAMINATION: Segregate

- At necropsy: fresh instruments ,change gloves between tissues controls first, monitor collection, Pre-label collection tubes.
- DNA extraction: Dedicated space, reagents (single use, disposable), lab coat , gloves, sleeve covers, extractions first each day before other activities.
- PCR assembly: Prepare master mix & standards each in separate labs, Assemble reactions in following order:
 - Master mix (includes primers, probe and *Taq*)
 - DNA extracted from test specimens: close un-spiked tubes
 - DNA spike: close spiked tubes
 - Standards & controls
 - Remember “Go from low to high”

Biodistribution: Time and Cost

- Maybe 90 - 180 days in-life
- Sample analysis is labor intensive
- Number of animals maybe large (perhaps lower if NHP or for life threatening disease)
- Ideal n = 5/sex/group (10 per group)
- 3 doses (L, M, H) + vehicle (n=40)
- 3 time points (d5,90,180) (n=120)
- 15/16 tissues (1,860 tissues)
- 3 samples per tissue (5,580 samples)
- Cost (\$1000) is tens to hundreds

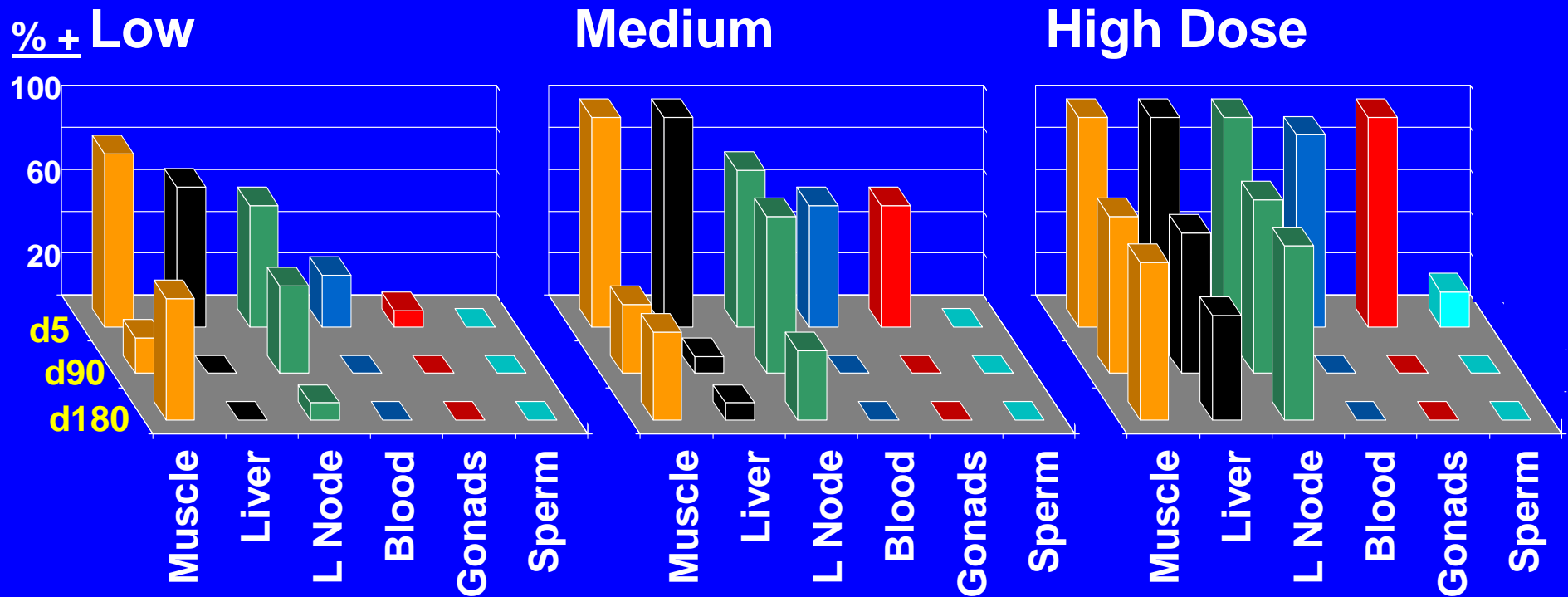
Persistence: rAAV in rabbit muscle

Schnepp, Clark, Johnson (CCRI), Munson (Targeted Genetics), ASGT Abs 2003

- Biodistribution
 - NZWR (n=144), single im dose rAAV2 or rAAV1
 - 3 doses ($3e^{10}$, $3e^{11}$, $3e^{12}$) , 5,90,180 d, n=10
 - 15 tissues collected, ~2,200 tissues analysed
- Episomal or integrated
 - Enzyme digestion or gel electrophoresis
- Integration frequency
 - PCR amplification from repeat sequences
 - C rpt in rabbits
 - B1 element in mice (Schnepp et al. J Virol 77:3495- 3504)

Biodistribution of rAAV2: IM in Rabbits

DNA PCR Specific tissues



Biodistribution of Vector: DNA PCR

- Sensitivity – 1 genome/ 10^4 cells
- 218 scored positive, all episomal
- Mostly at site of injection, highly perfused organs
- Dose dependent and decreased over time
- Gonads, epididymal sperm negative at day 90, 180
- Genome-wide analysis (C-rpt) PCR assay
 - rAAV vector integration is a rare event
 - 1 sample was \pm for amplification
 - At least 250-500 fold below the spontaneous mutation rate
- Spontaneous mutation rate 10^{-5} /gene
 - Cole & Skopek, 1994, Mutation Research 304:33-105

Integration Frequency

- AAV, Integration essentially undetectable
- Similar to Ad, plasmid in muscle
- Spontaneous mutation rate of 10^{-5} /gene
 - AAV 250 - 500 x lower
 - Plasmid 1000 x fold lower
- Retrovector
 - Integration frequency ~ 1.0 / cell (30,000 genes)
 - Similar to spontaneous mutation rate
- Forms basis of CBER/FDA guidance -tiered approach to Long term follow up

Predictability of Biodistribution

- Animal models
 - What is predictability of animal models?
 - Can localized delivery restrict biodistribution, transgene expression?
 - Is this reflected in clinical trials?
- Clinical trials
 - May be difficult to measure in clinical trial
 - Usually only blood samples and semen
 - (maybe tears, urine, sputum etc. for shedding)
 - Full biodistribution only from autopsy
- One example
 - rAAV2 intraarticular delivery of TNF α inhibitor
 - Animal study concordant with autopsy

Biodistribution: rAAV2-TNFR:Fc intra-articular delivery

Species	Days	Dose (DRP/mL)	Non-joint Tissues Positive for Vector DNA
Rat Normal	4	3×10^{12}	heart, kidney, liver, lung, spleen
Arthritic		3×10^{12}	Kidney, liver, lung, ovary, spleen
Normal	30	3×10^{12}	Spleen
Arthritic		3×10^{12}	None detected
Normal	90	3×10^{12}	None detected

NHP	90	10^{11}	Injected joint 4 copies/cell Non-injected joint, lymph node and spleen
		10^{12}	Injected joint 425 copies/ cell Non-injected joint, lymph node and spleen
		10^{13}	Injected joint 6000 copies/cell Non-injected joint, lymph node and spleen

*Non-injected joint no dose dependent increase in copies/cell of vector DNA in synovial cells. Positive but LOQ (0.003)

Molecular Analysis of Autopsy Samples

DNA-PCR Results

Target Sequence	Tissue	Result (copies/ μ g)
tgAAC94	R. knee (injected)	450,000
	Tonsil	29
	Liver, brain, spleen	<22
	L. knee, adrenal, muscle, trachea, bowel, heart, lymph nodes, bladder, lung, kidney	< LLOQ
wtAAV2 <i>rep</i> gene	R. knee (injected)	435
	Trachea, heart	<4.3
	Liver, lung, spleen, L. knee, adrenal, costal muscle, bowel, lymph nodes, kidney, bladder, brain, tonsil	< LLOQ

tgAAC94: Lower Limit of Quantitation (LLOQ) = 22 copies/ μ g for most tissues;
44 copies/ μ g for kidney, adrenal, costal muscle, small bowel, bladder and heart.

rep: Lower Limit of Quantitation (LLOQ) = 4.3 copies/ μ g

Biodistribution Studies - Summary

- Science driven
- Relevant to clinical application
 - Safety, Toxicology
 - Persistence, Long term follow up
 - Comparison to autopsy when needed
- Combine with safety studies if possible
- Biodistribution data is only as good as:
 - specimen collection integrity
 - DNA PCR assay used for testing

Host immune responses to vector and transgene product

- Monitoring immune responses is an important endpoint in early clinical trial settings across many classes of therapeutics**
- Animal models can predict host immune responses to transgene product but have been less reliable at predicting human immune responses to AAV capsid**

Cellular and humoral immune responses to the transgene product

- Most reliable assessment is with a species-specific transgene
- Likelihood of antibody formation dependent on ROA.
 - Liver, CNS, subretinal space < skeletal muscle
 - Non-neutralizing antibodies may not be a problem, or may result in faster clearance of a secreted protein

(Arruda et al., Blood 2010; Haurigot et al., Mol Ther 2010)

Cellular immune responses to the transgene product

- **Formation of antibodies likely dependent on CD4+ T cell help**
- **CD8+ T cell responses to transgene product have been observed following IM administration of AAV vectors. Most informative analysis is based on extraction of lymphocytes from site of administration.**

T cell responses to transgene product

- **Many examples from skeletal muscle**
 - **Factor IX** (*Wang et al., Gene Ther 2005; Cao et al., Mol Ther 2010*)
 - **α -sarcoglycan** (*Fougerosse et al., Mol Ther 2007*)
- **From IFN- γ ELISPOT analysis of draining lymph nodes**

Correlation with clinical studies

- No evidence of T cell responses to F.IX in AAV-F.IX IM study, but they were not sought, and mechanisms in place to reduce likelihood of such responses
 - Only subjects with missense mutations were enrolled. Some level of tolerance to TG product
 - Vector dose/site was limited based on animal studies
- Dystrophin-oral report of T cell response to epitope of TG product at site of gene deletion in human subject, based on study of PBMCs

Immune responses to capsid

- Humoral responses-all animals and human subjects show rise in antibody titers following vector administration

Table. Neutralizing Antibody Titers Against AAV1

Time Postinjection	Subject 1	Subject 2	Subject 3
Pretreatment	1:100	<1:50	1:100
1 wk	1:25,600	1:400	1:200
2 wk	1:25,600	1:3,200	1:1,600
6 wk	1:12,800	1:3,200	1:3,200
12 wk	ND	1:3,200	1:3,200
26 wk	1:12,800	1:3,200	—

Endpoint titers determined using a cell-based reporter vector infection assay as previously described.²⁵ AAV1 = adeno-associated virus serotype 1; ND = not done because sample not available; — = to be collected.

Immune responses to capsid

- AAV-F.IX in liver
 - Studies in dogs and non-human primates negative by IFN- γ ELISPOT for evidence of T cell responses to capsid in PBMCs, but these were documented in human subjects

(Mingozzi et al., Blood 2007; Haurigot et al., Mol Ther 2010; Manno et al., Nat Med 2006; Mingozzi et al., Nat Med 2007)

ELISPOT

- **Advantages**
 - Attractive as a screening assay, can detect even low frequency cells (1 in 10^5)
 - Does not require *in vitro* expansion of cells
 - Uses small volume of primary cells
- **Disadvantages**
 - Cannot distinguish CD4+ from CD8+ T cells
 - Cannot distinguish memory from effector

Design of a Peptide Library Derived from the AAV-2 and F.IX Protein Sequence to Investigate Capsid and Transgene T cell Responses

MAADGYLPDWLEDNLSEGIREWDDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGP
 FNGLDKGEPVNAADAAALFHDKAYDQQLKAGDNPYLRYNHADAFAERLQEDTSF
 GGNLGRAVFAQKRVLEPLGNVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKGTGQQ
 PAKKRLNFGQTGDSESVDPDPQLCEPPATPAAVGPTTMSGGGAPMADNNEGADG
 VGNASGNWHCDSTWLGDRTVITSTRTWALPTYNHLYKQISSASTGASNDNHYFG
 YSTPWGYFDNRFHCHFSRWDQRLINNNWGFPRKRLNFKLFNIQVKEVTNDGV
 TTIANNLTSTVQVFSDEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGS
 QAVGRSSFYCLEYFPPSOMLRGTGNNFTFSYTFEEVPEFHSSYAHSQSLDRLMNPLID
 QYLYLNRQTQNSGSAQNKDLLFSRGSFAGMSVQPKNNLPGPCYRQQRVSKTKTD
 NNNSNFTWTGASKYNLNGRESIINPGTAMASHKDEDEKFFPMMSGVMIFGKESAGA
 SNTALDNVMI TDEEELKATNPVATERFGTVAVNFQSSSTDPATGCVHAMGALPGM
 VWQDRDVLQGP IWAKLPHTDGHFHPSPMLGGFGLKNPPPQILIKNTPVPANPPA
 EFSATKFASFITQYSTGQVSVIEIWEWELQKENSKRWNPEVQYTSNYAKSANVDFTV
 DNNGLYTEPRPIGTRYLTRL

AAV-2

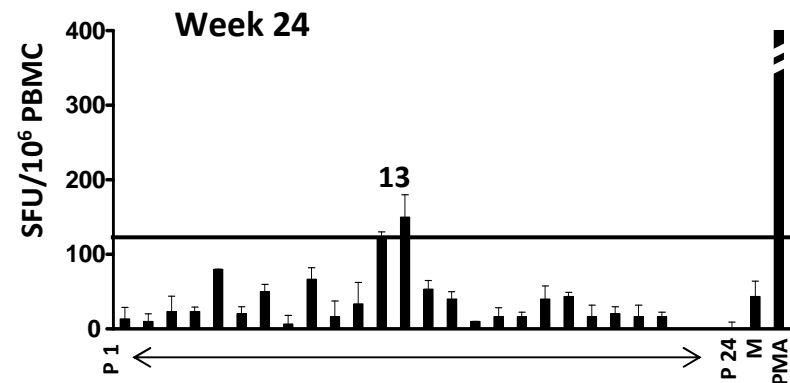
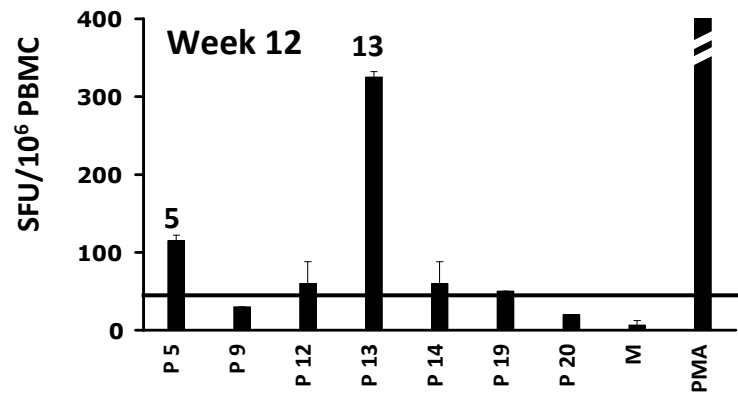
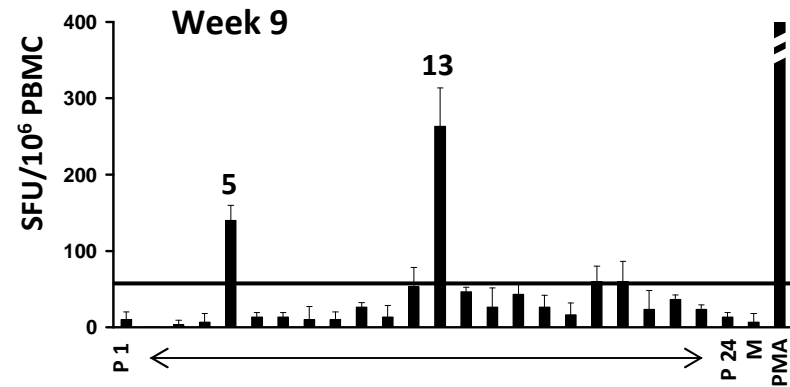
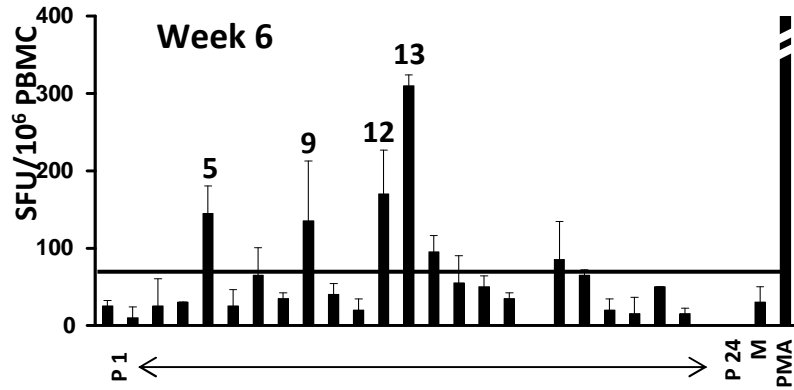
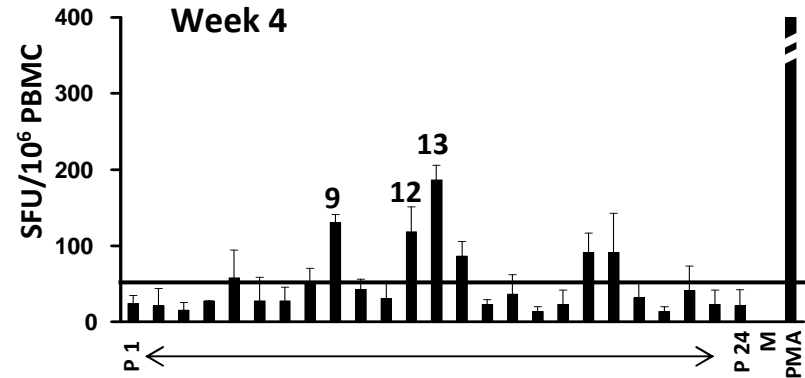
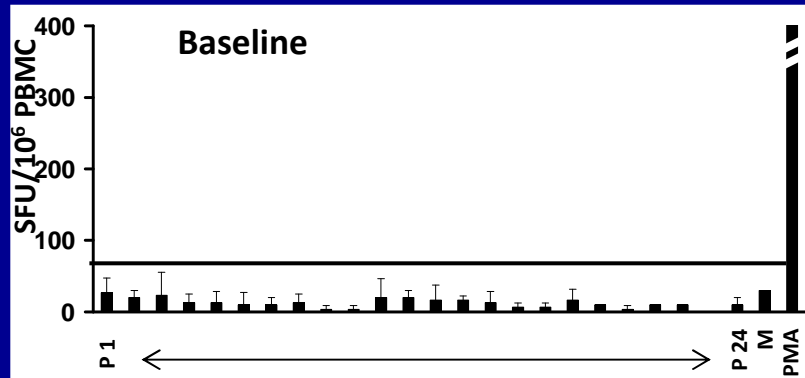
Peptide 1 MAADGYLPDWLEDNL
 Peptide 2 YLPDWLEDNLSEGIR
 Peptide 3 LEDNLSEGIREWDDL

Peptide Matrix

Pools

	1	2	3	4	5	6	7	8	9	10	11	12
13	1	2	3	4	5	6	7	8	9	10	11	12
14	13	14	15	16	17	18	19	20	21	22	23	24
15	25	26	27	28	29	30	31	32	33	34	35	36
16	37	38	39	40	41	42	43	44	45	46	47	48
17	49	50	51	52	53	54	55	56	57	58	59	60
18	61	62	63	64	65	66	67	68	69	70	71	72
19	73	74	75	76	77	78	79	80	81	82	83	84
20	85	86	87	88	89	90	91	92	93	94	95	96
21	97	98	99	100	101	102	103	104	105	106	107	108
22	109	110	111	112	113	114	115	116	117	118	119	120
23	121	122	123	124	125	126	127	128	129	130	131	132
24	133,145	134	135	136	137	138	139	140	141	142	143	144

Follow up on capsid T cell responses with IFN- γ ELISpot assay



Immune response to capsid after IM injection of AAV vectors is **dose-dependent**

Serotype	Transgene	Dose (vg)	IFN- γ ELISPOT	Magnitude SFU/10 ⁶ cells	Time point first detected	Reference
AAV 1	Sarcoglycan	3.25 x 10 ¹¹	1/3	60-70	2 wks, 6 wks	Annals Neuro 2009
AAV 2.5	Mini-Dystrophin			0		
AAV 1	SERCA2a (cardiac muscle)	3 x 10 ¹²	1/3	90	4 wks, 6 wks	J. Cardiac Failure 2009
AAV 1	LPL	7.0 x 10 ¹²	2/4	~300	12 wks	Blood 2009
	LPL	2.1 x 10 ¹³	3/6	~300 Different kinetics	4-6 wks	
AAV 1	Alpha 1-Antitrypsin	2.2 x 10 ¹³	2/2 tested	~100	2 wks	PNAS 2009
	Alpha 1-Antitrypsin	6.0 x 10 ¹³	3/3 tested	<200 - 428	2-4 wks	

Low doses = low responses.

Higher doses = greater magnitude, faster kinetics

Data from human subjects

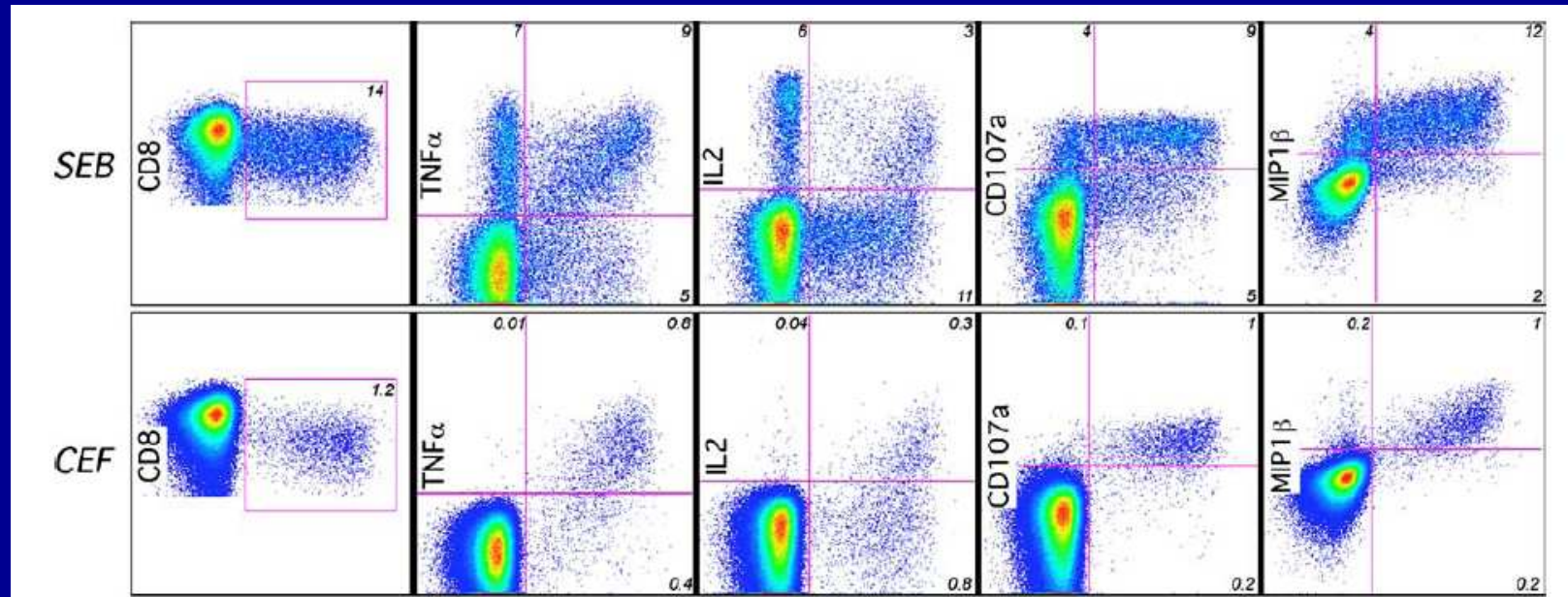
- T cell responses to capsid occur.
- The biological significance of the T cell response to capsid is unclear
- Whether CD8⁺ T cells will have a biological effect will depend on:
 - Levels of expression of MHC Class I in the target tissue-normally low on healthy muscle
 - Kinetics of capsid degradation and presentation
 - Whether transgene product itself is immunomodulatory

T cell responses to capsid

- Pre-clinical and clinical studies can use IFN- γ ELISpot, or polyfunctional T cell analysis using flow cytometry-based techniques
- Former is more straightforward, latter more informative
- Standardization of assays and data interpretation can be challenging and requires significant experience/efforts

(Janetzki et al., Cancer Immunol Immunother 2008; Hobeika et al., J Immunother 2005; Britten et al., Cancer Immunol Immunother 2008)

Polyfunctional analysis of T cell responses



(Makedonas and Betts, Springer Semin Immun 2006)

- Simultaneous detection of multiple cytokines and activation/function markers of T cells
- Allows assessment of both quality and magnitude
- Analyzed by flow cytometry
- Requires good knowledge of flow cytometry and proper controls/data analysis

T cell responses to capsid

- For studies in humans, ideally need ~20 ml blood per point analyzed, yielding $\sim 2 \times 10^7$ PBMCs
- PBMC isolation and cryopreservation is one of the most critical steps in immunomonitoring
- Costs are ~\$1/antibody/sample. Multiple controls needed.
- NIH Immune Tolerance Network. Assays done by Cellular Technology Ltd. www.elispot-analyzers.de

Immunomonitoring

- **Preclinical studies:**

Important to define the immunogenicity of transgene product, less informative for capsid T cell responses

- **Clinical studies:**

Important for both capsid and transgene. Need to use standardized methods, better if performed by or in collaboration with “expert hands”.