Monitoring cell-mediated immune responses in AAV gene therapy clinical trials using a validated IFN-γ ELISpot method

Kathryn S. Patton,[1] M. Travis Harrison,[2] Brian R. Long,[1] Kelly Lau,[1] Jennifer Holcomb,[1] Rachel Owen,[2] Theresa Kasprzyk,[1] Sylvia Janetzki,[3] Stephen J. Zoog,[1] and Christian Vettermann[1]

[1]Bioanalytical Sciences, BioMarin Pharmaceutical, 791 Lincoln Avenue, San Rafael, CA 94901, USA; [2]Immunology, Precision for Medicine, 2686 Middlefield Road, Redwood City, CA 94063, USA; [3]ZellNet Consulting, 555 North Avenue, Suite 25-S, Fort Lee, NJ 07024, USA

Adeno-associated virus (AAV)-based gene therapies have recently shown promise as a novel treatment for hereditary diseases. Due to the viral origin of the vector capsid, however, cellular immune response may be elicited that could eliminate transduced target cells. To monitor cellular immune responses in clinical trials, we optimized and bioanalytically validated a sensitive, robust, and reliable interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assay. For method performance validation, human peripheral blood mononuclear cells (PBMCs) were stimulated with peptides derived from AAV5 capsid proteins and the encoded transgene product, human blood clotting factor VIII (FVIII), in addition to positive controls, such as peptides from the 65-kDa phosphoprotein of cytomegalovirus. We statistically assessed the limit of detection and confirmatory cutpoint, evaluated precision and linearity, and confirmed specificity using HIV peptides. Robustness parameter ranges and sample stability periods were established. The validated IFN-γ ELISpot assay was then implemented in an AAV5-FVIII gene therapy clinical trial. Cellular immune responses against the AAV5 capsid were observed in most participants as soon as 2 weeks following dose administration; only limited responses against the transgene product were detected. These data underscore the value of using validated methods for monitoring cellular immunity in AAV gene therapy trials.

INTRODUCTION

Adeno-associated virus (AAV)-based gene therapy is a promising new modality to treat various genetic disorders.[1,2] The advantages of AAV vectors are their high transduction efficiency, tropism for various target tissues, low prevalence of preexisting immunity, and overall acceptable safety profile.[3–6] Wild-type AAVs cause no known human pathologies and are less immunogenic than other viruses (e.g., adenovirus).[5,7–9] AAV vectors are designed by replacing all viral genes with an expression cassette containing a promoter, coding region, and polyadenylation signal.[10,11] Upon administration, these vectors effectively deliver a functional transgene copy to patients with a genetic deficiency.[10,11] As a result, the patients express an essential protein they would otherwise lack, which can lead to substantial clinical improvements of their underlying disorder.[1,10]

AAV gene therapy vectors contain viral capsid proteins, and thus cellular immune responses against them can be triggered.[12,13] In addition, preexisting immunity may be an obstacle to the efficacy or safety of AAV gene therapies.[6,9,14–17] Seroprevalence studies show that 30%–60% of adults carry AAV capsid-specific antibodies (depending on the AAV serotype and geographic region), with AAV5 antibodies having among the lowest prevalence.[18,19] Adults can also have circulating capsid-specific $CD8^+$ and $CD4^+$ T cells resulting from natural exposure to AAV.[8] Immunity acquired to the wild-type virus, together with innate immunity, can later decrease the therapeutic efficacy of AAV gene therapy vectors.[5,8,9,13] In the context of liver-tropic AAV vectors, there is preliminary evidence that capsid-specific cellular immune responses, detected by interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assays, can be associated with elevated plasma levels of liver enzymes.[1,2,11,20] In some but not all cases, elevated liver enzymes (notably alanine transaminase [ALT]) were also associated with a decline in transgene expression levels.[2,20] The lack of consistency in correlating cellular immune responses with elevated ALT levels or declines in expression suggests that methodological standardization may be necessary. ALT increases can be mitigated through the use of corticosteroids, which often also stabilize transgene expression.[2,8] This led to the hypothesis that ALT is released into plasma through the targeting of vector-transduced cells by cytolytic immune cells.[2,13,20,21]

To monitor cell-mediated immunity against an AAV5-based vector that encodes for human B domain-deleted factor VIII (FVIII-SQ), we developed and validated an IFN-γ ELISpot for human peripheral blood mononuclear cells (PBMCs). This method format was chosen for 3 reasons: it is (1) semiquantitative, (2) sensitive to single-cell level, and offers (3) a broad dynamic range.[22] We selected IFN-γ, a cytokine primarily secreted by activated T cells and natural killer (NK) cells, as an established marker for cell-mediated
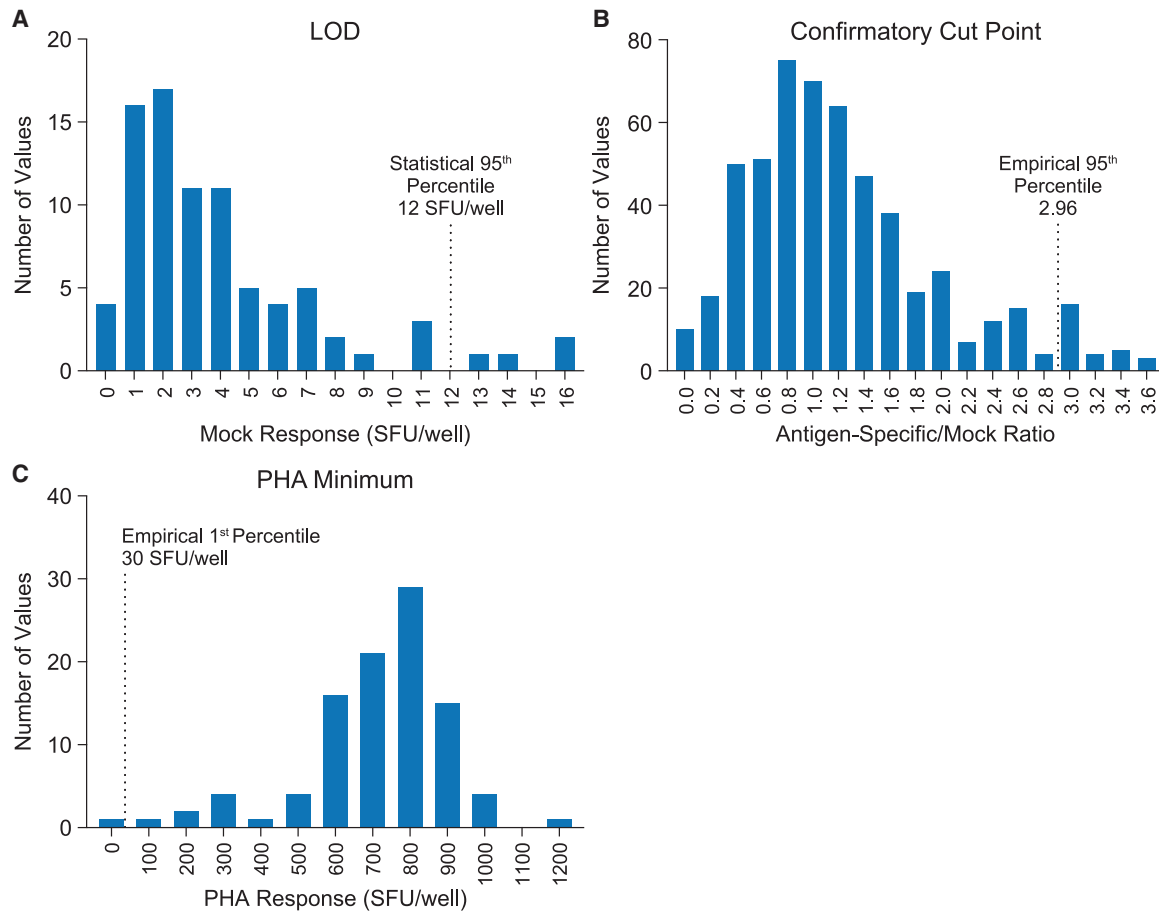
Figure 1. Distribution of screening responses for LOD, confirmatory cutpoint, and PHA minimum evaluation

PBMCs from 99 normal human donors were mock stimulated or stimulated with peptide pools from AAV5, FVIII, or CMVpp65 or PHA and tested by IFN-γ ELISpot. (A) The distribution of mock responses (outliers excluded) was used to establish the LOD as the statistical 95th percentile. (B) The pooled distribution of antigen-specific over mock response ratios (outliers excluded) was used to establish the confirmatory cutpoint as the empirical 95th percentile. (C) The distribution of PHA responses was used to establish the PHA minimum as the empirical 1st percentile.

immunity.[6,11,20,23] Reagent and protocol standardization are key for establishing reproducible ELISpot results across multiple test sites and analysts.[24–26] Regulatory guidance on ELISpot method validation is limited, yet standardized assay performance evaluations and acceptance criteria are essential for reliable data interpretation.[27] Thus, we identified an extensive list of key method performance parameters and used statistical methods to objectively assess and validate them: limit of detection (LOD), confirmatory cutpoint (CCP, based on mock/antigen response ratio), PHA (phytohemagglutinin-L) minimum response, precision (intra-triplicate, intra-assay, and interassay), linearity, upper limit of quantitation (ULOQ), lower limit of quantitation (LLOQ), specificity, robustness, and stability.[25,28,29] Due to the scarcity of AAV5- and FVIII-specific human PBMCs, cytomegalovirus phosphoprotein 65 (CMVpp65) was used as a surrogate antigen for some validation parameters. Using CMVpp65 also enables this method to serve as a platform technology for future gene therapy products. We used the validated method to monitor gene therapy participants for preexisting and acquired cell-mediated immunity targeting the AAV5 vector capsid or the expressed transgene product FVIII-SQ.

RESULTS

LOD

To determine the LOD, which is used to distinguish positive from negative responses, the background signal in mock-stimulated PBMCs from 100 randomly selected, normal individual donors was evaluated. One donor did not meet the ≥70% viability criteria and was excluded. PBMCs from the remaining 99 donors were tested once over 8 runs by 2 analysts. The mock responses in spot-forming units per well (SFU/well) were determined and used to statistically establish the LOD (Figure 1A). A total of 16 donors were identified as statistical outliers (interquartile range method) and excluded from the analysis. Data from the remaining 83 donors were not normal by the Shapiro-Wilk test; therefore, values were $\log_{10}$ transformed, resulting in a normal distribution. The LOD was derived as the 95th percentile, anti-log transformed, and rounded to the nearest

Table 1. Intra-triplicate, intra-assay and interassay precision

Response level/range	Donor ID/stimulus	Average response level (SFU/well), N = 3 runs	Intra-triplicate precision		Intra-assay precision		Average response level (SFU/well) N = 10 runs	Interassay precision	
			%CV[a]	Average %CV	%CV	Average %CV		%CV	Average %CV
Low to medium[c] 50–300 SFU/well	Donor A/FVIII[b] pool 2	115.0	12.7		7.9		112.4	32.0	
	Donor A/FVIII pool 3	117.1	9.5		8.3		106.3	31.7	
	Donor B/CMVpp65[d]	260.0	6.0	10.8	4.7	7.6	277.4	14.1	35.9
	Donor C/FVIII pool 2	118.4	8.4		8.4		199.4	49.6	
	Donor C/FVIII pool 3	86.3	17.3		9.0		155.4	52.2	
High >300 SFU/well	Donor A/CMVpp65	517.5	4.4	4.2	2.8	3.2	548.8	15.7	27.4
	Donor C/CMVpp65	383.3	4.0		3.7		508.4	39.2	

[a]CV, coefficient of variation.
[b]FVIII, transgene product.
[c]No low (<50 SFU/well, but ≥30 SFU/well) responses were identified in the precision dataset even though the donors chosen for use in the validation had elicited such low responses upon screening.
[d]CMVpp65, 65kDa phosphoprotein of cytomegalovirus.

whole number, resulting in 12 SFU/well or 60 SFU/million PBMCs. Using mock responses in clinical trial participants at baseline (Figure S1A), we confirmed the suitability of this LOD in the disease population (hemophilia A).

CCP determination

For 20 of 99 donors above, the mean mock response (SFU/well) fell at or above the LOD. To ensure specificity if background signals are elevated, we statistically established a CCP. The CCP was based on the distribution of response ratios between antigen-specific responses (2 AAV5 peptide pools and 4 FVIII peptide pools) and corresponding mock response. Since the means and variances were equal across all 6 peptide pools by 1-way ANOVA, the datasets were pooled, 56 statistical outliers were excluded (interquartile range method), and the remaining 532 values were analyzed for normality using the Shapiro-Wilk test. The data were not normally distributed; therefore, the CCP was determined using the empirical 95th percentile, equaling 2.96 (Figure 1B). The CCP will be used to adjudicate samples, in which both peptide-specific responses and mock response are ≥LOD. These peptide responses are considered positive only if their response ratio is ≥2.96. Using mock responses from clinical trial participants at baseline (Figure S1B), we confirmed the suitability of this CCP in the disease population.

PHA minimum response

PHA induces polyclonal IFN-γ secretion in T cells, and thus a minimum PHA response serves as a quality control for the functionality of PBMC samples. To determine the PHA minimum response, the distribution of PHA responses from 99 individual donor PBMCs (viability ≥70%) were evaluated. PHA responses ranged from 30 to 1,201 SFU/well (Figure 1C; Table S1) but were not normally distributed by the Shapiro-Wilk test. Thus, the PHA minimum response was set empirically to the lower 1st percentile (30 SFU/well or 150 SFU/million PBMCs). The limit was chosen to achieve an ∼1% failure rate for the assay, as per US Food and Drug Administration (FDA) immunogenicity assay guidance.[30] However, using a low limit (30

SFU/well) for a positive-control stimulation that usually results in a 20-times-higher response (median: 736 SFU/well) may not be completely adequate to identify all of the samples with partially compromised functionality. Therefore, alternative PHA minimum response levels may be considered at the 5th percentile (260 SFU/well), or even more conservatively at the 10th percentile (457 SFU/well). The chosen PHA minimum criterion should be applied to all of the clinical samples tested to avoid reporting potential false-negative responses. No PHA maximum was set, since a high PHA response remains indicative of functional PBMCs.

Intra- and interassay precision

To evaluate precision, PBMCs from 3 CMVpp65 and/or FVIII-responsive donors were selected to cover a target range of low-to-medium (50–300 SFU/well) and high (>300 SFU/well) responses. Intra-triplicate and intra-assay precision were assessed by performing 3 assay runs, each with 3 donors and corresponding peptide pools. Each donor was plated in 3 sets of triplicate wells; each triplicate set was independently prepared with respective peptides. Intra-assay precision was assessed as the coefficient of variation (CV) across 3 independent peptide/PBMC preparations within each run for each donor/peptide pool that yielded a response ≥30 SFU/well. The average intra-triplicate CV was 10.8% and 4.2% (Table 1) for the low-to-medium and high responses, respectively, which met acceptance criteria (CV ≤ 30% for peptide responses with ≥30 SFU/well). The average intra-assay CV was 7.6% and 3.2%, for the low-to-medium and high responses, respectively, which met acceptance criteria (CV ≤ 50% for peptide responses with ≥30 SFU/well). Interassay precision was assessed across 9 different runs as the interassay CV for responses ≥30 SFU/well (9 days with 2 analysts, n ≥4 runs per analyst). The average interassay CV was 35.9% and 27.4% for the low-to-medium and high responses, respectively, which met acceptance criteria (CV ≤ 50% for peptide responses with ≥30 SFU/well). Hence, the method showed acceptable precision, but direct comparison of numerical results from different runs should take into account the expected range of potential interassay variability.
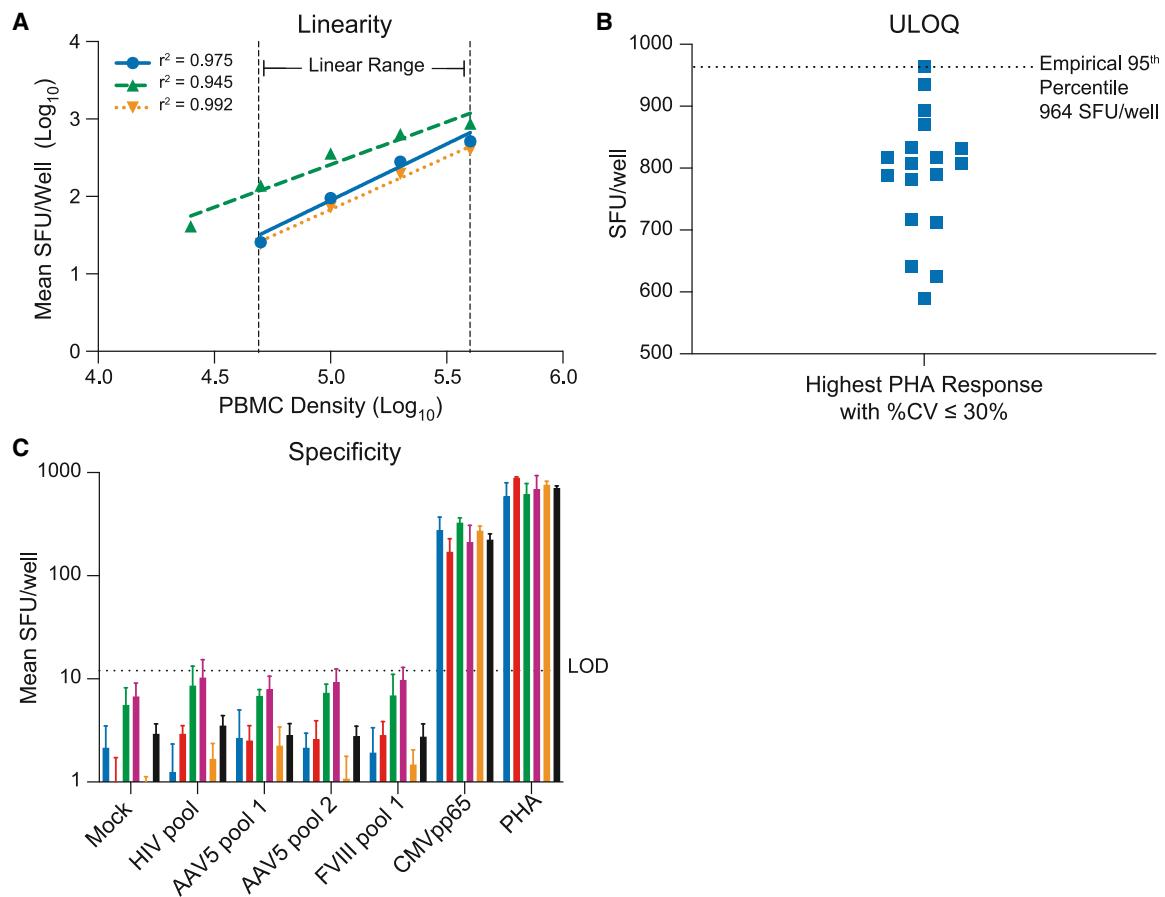
Figure 2. Linearity, ULOQ, and specificity evaluation

(A) Using PBMCs from 3 medium to strong CMVpp65-responding donors, IFN-γ ELISpot was performed by stimulation to CMVpp65 in 3 different runs at 7 different cell densities (400,000–6,250 PBMCs/well). Each symbol indicates a different donor (●, ▲, and ▼). The linear range was defined as the range of cell densities with responses ≥LOD, through which a linear regression yields a coefficient of correlation $r^2 \geq 0.90$. (B) Human PBMCs from 6 donors were stimulated at varying concentrations with PHA in 3 runs. The ULOQ was defined as the 95th percentile of the highest SFU/well responses elicited that consistently showed an intra-triplicate CV ≤30% (total of 18 data points). (C) PBMCs from 6 donors were stimulated with 4 negative control peptide pools (HIV, AAV5 pool 1, AAV5 pool 2, and FVIII pool 1) along with positive (CMVpp65 and PHA) and mock controls across 4 runs. Each bar color indicates a different donor. Error bars represent the standard deviation from the mean for each donor across the 4 runs. Specificity met acceptance criteria if the SFU/well for the negative control peptide pools were <LOD or had a response ratio <2.96 (if both peptide and mock response were ≥LOD) in at least 5 of 6 donors in each of the 4 runs.

Assay linearity

Assay linearity was confirmed by showing that the number of responsive PBMCs plated was directly proportional to the number of measured SFU. Using PBMCs stimulated with CMVpp65 from 3 responsive donors per run, ELISpot was performed in 3 different runs at 7 different cell densities (ranging from 400,000 to 6,250 cells/well). Log10-transformed values for each donor were then plotted as the average number of SFU/well for CMVpp65 versus cell density, excluding cell densities that lead to responses <LOD. The linear range was defined between cell densities through which linear regression yielded a coefficient of correlation (r^2) ≥0.90. Values for r^2 ranged between 0.945 and 0.992 for a linear range between 50,000 and 400,000 cells/well for 2 donors, and 25,000 and 400,000 cells/well for the third donor

(Figure 2A), with an overall linear range across all 3 donors from 50,000 to 400,000 cells/well.

Range of quantitation

The range of quantitation between LLOQs and ULOQs defines where samples are quantifiable with acceptable precision. To assess the LLOQ, 6 donor PBMCs (2 negative, 2 positive near the LOD, and 2 low-positive responders) were tested in 6 runs over 6 days. These donors were stimulated with FVIII or CMVpp65 peptide pools in 2 sets of triplicate wells. For each donor/peptide combination, the mean SFU/well, standard deviation (SD), and %CV between triplicate wells were calculated for each run. The mean donor SFU/well was calculated across all sets of triplicates from all of the runs, separately for each donor/peptide

Table 2. LLOQ determination

Donor/peptide combination	Donor ID	Peptide stimulus	Mean SFU/well[a]	No. runs with CV ≤30%
1	Donor 4	CMVpp65[b]	221.5	6
2	Donor 1	FVIII[c] pool 2	82.2	6
3	Donor 1	FVIII pool 3	75.1	6
4	**Donor 4**	**FVIII pool 4**	**44.4**	**6**
5	Donor 5	FVIII pool 4	41.8	0
6	Donor 4	FVIII pool 2	39.3	3
7	Donor 5	FVIII pool 2	37.7	0
8	Donor 5	FVIII pool 1	33.2	1
9	Donor 2	FVIII pool 2	<LOD	N/A
10	Donor 2	CMVpp65	<OD	N/A
11	Donor 3	FVIII pool 2	<LOD	N/A
12	Donor 3	CMVpp65	<LOD	N/A
13	Donor 6	FVIII pool 2	<LOD	N/A
14	Donor 6	CMVpp65	<LOD	N/A

Bold indicates values selected as LLOQ.
[a]Cell-plating density was 200,000 PBMCs/well and viability was ≥70% for all donor samples across all runs.
[b]CMVpp65, 65 kDa phosphoprotein of cytomegalovirus.
[c]FVIII, transgene product.

combination. The LLOQ was defined as the lowest mean donor response ≥LOD for which both sets of triplicate wells showed an intra-triplicate %CV ≤30%, in at least 5 of 6 runs. The LLOQ was determined as 44.4 SFU/well or 222 SFU/million PBMCs (Table 2).

Since confluency can occur in wells with high spot counts, it was also critical to assess the ULOQ. PBMCs from 6 donors were stimulated in 3 runs with PHA or mock stimulation conditions. Each donor was tested at 7 different cell densities ranging from 400,000 to 6,250 PBMCs/well in triplicate wells. The ULOQ was defined as the 95th percentile of the highest SFU/well responses with intra-triplicate % CV ≤30% observed in each donor in each run (18 data points) and determined as 964.3 SFU/well or 4,822 SFU/million PBMCs (Figure 2B).

Assay specificity

To assess specificity, 4 runs were performed using PBMCs from 6 healthy donors responsive to CMVpp65 and non-responsive to human immunodeficiency virus (HIV), AAV5, and FVIII peptide pools (based on prior health status or previous results). To meet acceptance criteria, PBMC responses to HIV, AAV5, and FVIII peptide pools should remain negative in at least 5 of the 6 donors. A negative antigen-specific response was defined either as a peptide and mock response <LOD (12 SFU/well) or, if mock response was ≥LOD, then the peptide:mock response ratio must remain <2.96. Stimulation with HIV, AAV5, and FVIII peptides remained negative across all 6 donors (Figure 2C). Meanwhile, positive responses (≥LOD) were obtained after stimulation with CMVpp65, demonstrating that IFN-γ responses were antigen specific.

Robustness

To assess robustness, PBMCs from 3 antigen-responsive donors were tested with FVIII and CMVpp65 peptide stimulation, using small but deliberate changes in experimental conditions. To meet acceptance criteria, the mean SFU/well under robustness conditions must show an absolute relative difference ≤30% from the mean SFU/well under standard conditions for all donor/peptide combinations with responses ≥30 SFU/well. The concentration of stimulating peptides was successfully validated at 2 μg/mL, separately for each peptide pool; assays performed using 0.5 or 1 μg/mL were not robust (Figure 3A). The cell-stimulation time was successfully validated between 20 and 24 h; assays performed for 16 h were not robust (Figure 3B). The substrate development time was successfully validated between 3 and 4 min; assays performed for 2 min were not robust (Figure 3C). Plate drying time was successfully validated between 20 h and 80 days (Figure 3D); for testing purposes, a routine plate drying time between 20 and 100 h is recommended.

PBMC sample stability

To assess stability, multiple vials of donor PBMCs from the same collection were stored in liquid nitrogen (LN$_2$) over 18 months. On each testing occasion, the PBMCs from a single vial were incubated under mock conditions or stimulated with FVIII peptides, CMVpp65 peptides, or PHA. Responses were monitored continuously from baseline to 18 months. The sample remained negative (<LOD) under mock stimulation conditions on 49 of 50 test occasions; on 1 occasion, a low positive mock response was observed (Figure 4A). The sample consistently yielded positive responses (≥LOD of 60 SFU/million PBMCs) when stimulated with FVIII pool 2, FVIII pool 3, CMVpp65, and PHA, which fell within 3 SDs from the mean (Figures 4B–4E). In accordance, viability remained >79% at all times (Figure 4F). Hence, PBMCs stored in LN$_2$ remained stable for at least 18 months.

Clinical trial IFN-γ ELISpot results

The validated IFN-γ assay (validation summary in Table 3) was implemented in a clinical AAV5-FVIII gene therapy study (BMN270-301). For control purposes, a non-study-related donor was also tested to ensure consistent assay performance (Figures 4A–4E). Positive responses across both AAV5 peptide pools were combined to generate a heatmap of AAV5 reactivity for individual participants (Figure 5A). Fifteen (94%) of 16 tested participants were positive for cellular immune responses against the AAV5 capsid, starting as early as 2 weeks following dose administration. Responses to AAV5 ranged from 60 to 1,482 SFU/million PBMCs (median: 97); all of the baseline samples tested (12 of 16) were negative, thus confirming the specificity of post-baseline responses. Most AAV5-specific responses diminished over several weeks, reverting to negative. At week 52, 13 (92.9%) of 14 participants with available samples were negative for AAV5-specific responses.

Cellular immune responses against the transgene product FVIII were much less frequent: positive responses were intermittently detected in only 5 (31%) of 16 subjects (Figure 5B). One participant was positive at baseline and at week 52, whereas the other 4 subjects
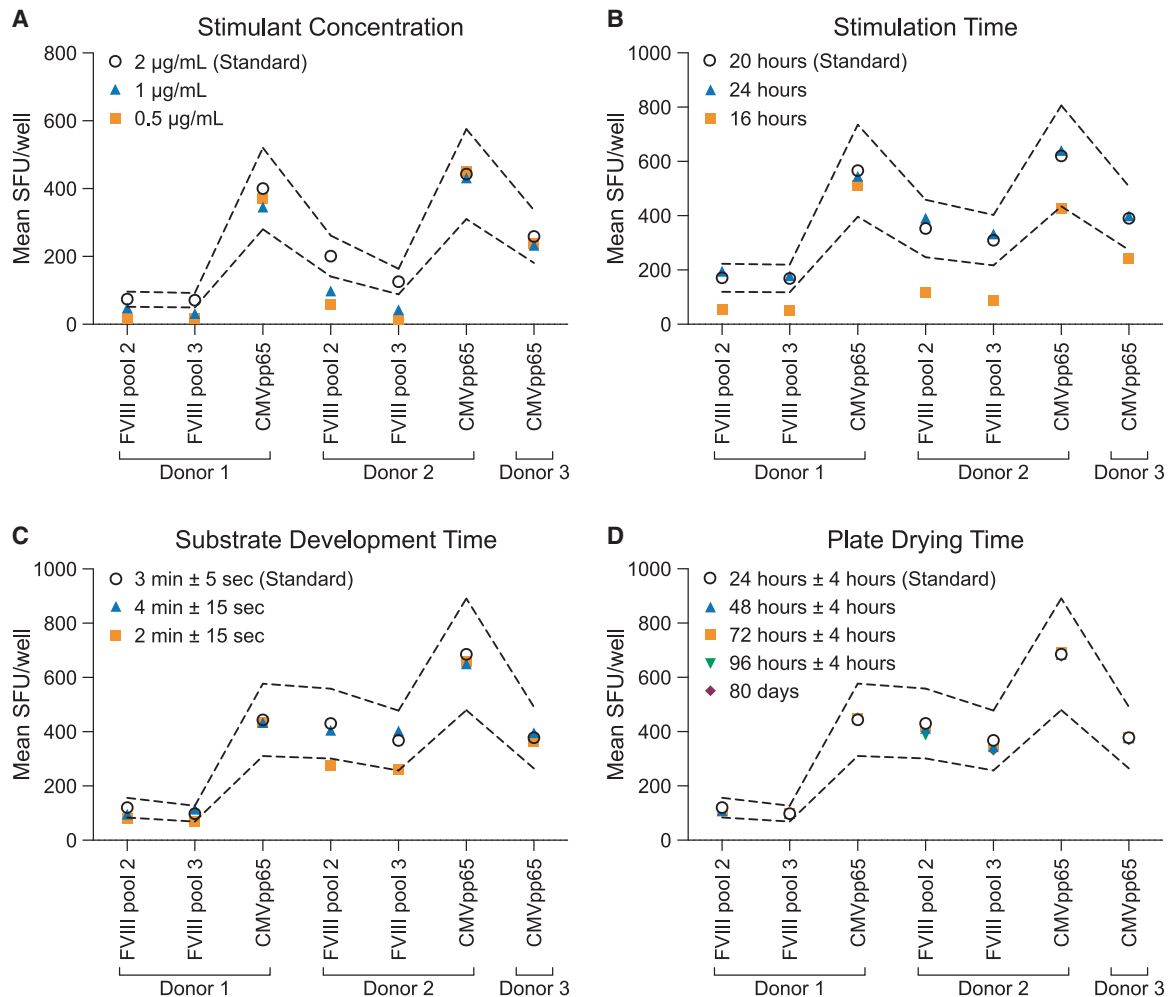
Figure 3. Robustness evaluation of critical assay parameters

Robustness was assessed using 3 donors across 1–3 stimulations for multiple critical assay parameters. (A–D) stimulant concentration (A), (B) stimulation time, (C) substrate development time, and (D) plate drying time. The SFU/well for the standard parameter condition is shown as open circles (○), the minimum parameter condition is shown as closed squares (■), and the maximum parameter conditions are shown as closed triangles (▲). Extended maximum parameter conditions were evaluated for plate drying time including 96 h indicated by closed inverted triangle (▼) and 80 days indicated by a closed diamond (◆). Substrate development time and plate drying times were performed within a window for each parameter condition as specified (e.g., ± 15 s or ± 4 h). Dotted range lines represent range for 30% relative difference of the SFU/well response observed for the standard parameter condition. Minimum, maximum, and extended parameter conditions must fall within this range to be considered acceptable.

were positive only at single time points (Weeks 10, 12, 20, and 44, respectively). Responses to FVIII ranged from 60 to 100 SFU/million PBMCs (median: 85). All of the subsequent time points were negative in 4 of these 5 subjects.

DISCUSSION

Immune monitoring using IFN-γ ELISpot is used in AAV gene therapy trials to detect potentially eliminatory T cell responses that may affect treatment outcomes.[22,28,29,31] Here, we devised a set of standardized bioanalytical validation parameters and assessments for IFN-γ ELISpots that ensure reliable clinical data generation. ELISpot method validation was performed in consideration of previous recommendations.[24,25,27–29,31,32] However, we went beyond previous

recommendations by including additional bioanalytical validation parameters and statistical assessments, borrowed from related regulatory guidance and white papers, as considered applicable to ELISpot methodology.[30,33,34] Method validation parameters included the following: LOD, confirmatory cutpoint, LLOQ, ULOQ, precision (intra-triplicate, intra-assay, and interassay), linearity, specificity, robustness, and long-term sample stability.

Since ELISpots rely upon the functionality of the stimulated cells, standardization of PBMC collection and cryopreservation are also critical to obtain reliable test results.[35] Suboptimal PBMC storage and shipment conditions, due to temperature fluctuations, can affect the responsiveness of PBMCs in IFN-γ ELISpot assays.[36–38] Thus, we
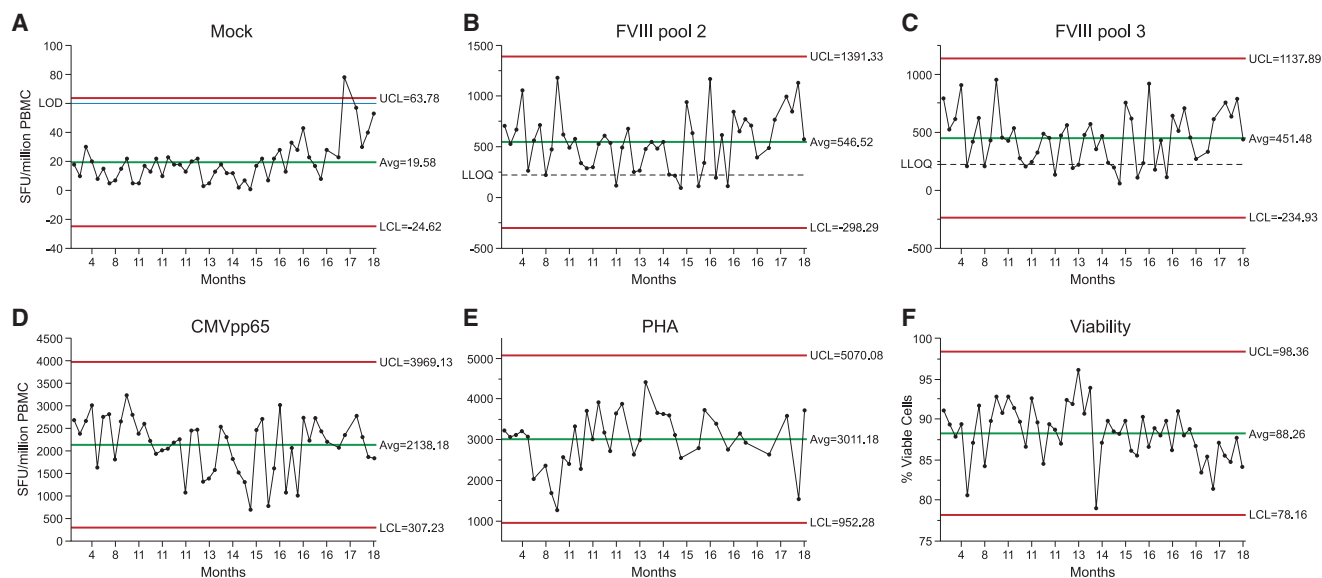
Figure 4. Trending and stability of control donor PBMCs

(A–E) PBMCs from a control donor were monitored during clinical sample testing for IFN-γ responses to (A) mock, (B) FVIII pool 2, (C) FVIII pool 3, (D) CMVpp65, and (E) PHA. Additional FVIII or AAV5 peptide pools were not tested since they did not generate a positive response in this control donor. Responses were analyzed for trends using Levey-Jennings plots. The blue line indicates the limit of detection (LOD, or 60 SFU/million PBMCs). The dashed black line indicates the lower limit of quantification (LLOQ, or 222 SFU/million PBMCs). The green line indicates the mean of each dataset. The red lines indicate the upper confidence limit (UCL) and lower confidence limit (LCL) of the dataset, representing 3 multiples of the standard deviation above and below the mean of all of the data points plotted, respectively. (F) PHA responses that were too numerous to count were not plotted since no numerical values could be assigned. PBMC viability remained >79% throughout.

restricted temperature fluctuations to ensure the highest PBMC sample quality. In addition, the absence of serum during PBMC processing reduces variability and increases reproducibility in IFN-γ responses.[39,40] Therefore, PBMC collection and cryopreservation during validation and clinical testing remained serum-free.

The human IFN-γ ELISpot assay was sensitive, precise, specific, and linear, with a broad range of quantitation. Average intra-triplicate precision (CV ≤ 10.8%), intra-assay precision (CV ≤ 7.6%), and interassay precision (CV ≤ 35.9%) were in accordance with the literature, with interassay variability being generally higher than intra-triplicate or intra-assay variability.[29,41] High interassay variability in 1 individual donor (52.2%; Table 1) represented an atypical result at the extreme end of the precision range. Potential factors contributing to increased variability in individual PBMC samples may be a certain degree of hemolysis during sample collection, lipid content, or other matrix effects.[42,43] The statistically derived LOD (60 SFU/million PBMCs) was similar to LODs previously reported for human IFN-γ ELISpot assays (21–55 SFU/million PBMCs).[22,28,41] Minor differences in LOD across methods could result from different instrument settings to enumerate spots. In summary, our method performance validation (Table 3) confirmed that the IFN-γ ELISpot was suitable for monitoring AAV5 capsid and FVIII-specific cellular immune responses in human PBMCs.

Validation data were also used to establish sample acceptance criteria and a result determination algorithm. A minimum PHA response was required to guarantee the functionality of PBMCs in clinical samples,

and a confirmatory cutpoint was established for samples with "high background" (i.e., mock response ≥ LOD), so that antigen-specific responses can still be distinguished. We implemented the following algorithm (LOD/CCP method) for determining a positive result: peptide response ≥LOD if mock is <LOD, or peptide response ratio ≥ 2.96-fold over mock, if mock is ≥LOD. Another common algorithm for ELISpots results is distribution-free resampling (DFR) analysis. DFR uses a non-parametric test that avoids distributional assumptions and separates permutations of each antigen-specific response with the corresponding mock control to determine positive responses.[44,45] We compared responders identified using our LOD/CCP method with responders identified using the DFR(2×) algorithm. Overall, DFR(2×) results were similar to those generated using our LOD/CCP method (Tables S1 and S2), even though LOD and CCP were established using a 5% false-positive rate, based on the overall distribution of mock responses.

In a clinical trial with AAV5-FVIII, capsid-specific cellular immune responses were broadly detectable following dose administration but generally diminished over several weeks. In contrast, only limited FVIII-specific responses were observed. Cellular immune responses to AAV gene therapies were observed in some but not all clinical trials to date.[46] As an explanation for the lack of AAV-specific T cell responses in some trials, different doses and routes of administration could affect the immunogenicity of AAV vectors.[6,47] Alternatively, the frequency of AAV-specific T cells in peripheral blood may sometimes be too low to be detected.[6] Consequently, enriching antigen-

Table 3. Human IFN-γ ELISpot assay validation summary

Assay parameter	Acceptance criteria	Validation results
LOD	the LOD is calculated using the 95th percentile of the mock-stimulation responses	LOD = 60 SFU/million PBMCs
CCP	the confirmatory cutpoint is calculated using the 95th percentile of the peptide/mock-stimulation response ratios	Confirmatory cutpoint = 2.96 response ratio
PHA minimum	the lower acceptance limit for a test sample under PHA stimulation is calculated empirically as the 1st percentile	PHA response $\geq$150 SFU/million PBMCs
Intra-triplicate precision	intra-triplicate CV $\leq$30% for responses $\geq$30 SFU/well, for each donor and peptide pool	Intra-triplicate CV average: Low to medium response level: 10.8% High response level: 4.2%
Intra-assay precision	intra-assay CV $\leq$30% for responses $\geq$30 SFU/well, for each donor and peptide pool	intra-assay CV average: low to medium response level: 7.6% high response level: 3.2%
Interassay precision	interassay CV $\leq$50% for responses $\geq$30 SFU/well, for each donor and peptide pool	interassay CV average: low to medium response level: 35.9% high response level: 27.4%
LLOQ	report the lowest peptide response that shows an intra-triplicate CV $\leq$30% in 2 independent sample preparations in at least 5 of 6 runs	222 SFU/million PBMC[d]
ULOQ	report the 95th percentile of the highest peptide responses at which single spots can be detected and intra-triplicate CV $\leq$30%	4,822 SFU/million PBMCs
Dilution linearity	report the range of cell densities through which a linear regression of responses shows an $R^2 \geq$0.90	50,000–400,000 PBMCs/well
Specificity	no positive response to irrelevant control peptides or previously non-stimulating peptides in $\geq$5 of 6 donors	no cross-reactivity to HIV peptides or previously non-stimulating peptides was observed in 6 of 6 donors
Stimulant concentration robustness	the response for each robustness condition should be within |RE| $\leq$ 30% of the response observed under the standard conditions	2 µg/mL; assays performed using less than the above-stated concentration of stimulating peptides were not robust
PBMC stimulation time robustness	the response for each robustness condition should be within |RE| $\leq$30% of the response observed under the standard conditions	20–24 h; assays performed at 16 h incubation time were not robust
Substrate development time robustness	the response for each robustness condition should be within |RE| $\leq$30% of the response observed under the standard conditions	3–4 min; assays performed at 2-min substrate development time were not robust
Plate drying time robustness	the response for each robustness condition should be within |RE| $\leq$30% of the response observed under the standard conditions	20 h–80 days
Long-term PBMC sample stability	passing performance of a control donor sample was monitored over time during sample testing	up to 18 months, if stored in the vapor phase of LN$_2$

CCP, confirmatory cutpoint; CV, coefficient of variation; LLOQ, lower limit of quantitation; LN$_2$, liquid nitrogen; LOD, limit of detection; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin-L; SFU, spot-forming units; ULOQ, upper limit of quantitation.

specific cells before ELISpot testing may offer enhanced detectability.[6,48] Analyzing T cells *in situ*, for example, by using liver biopsies, may also be considered. In addition, tumor necrosis factor-α (TNF-α) secreting capsid-specific memory CD8$^+$ T cells may be present in AAV-seropositive patients.[13] Thus, the evaluation of polyfunctional, multiple-cytokine-secreting T cells may provide further insights into AAV-specific cellular immune responses.

Overall, our clinical observations showed that in the context of the on-demand use of corticosteroid immune suppression following gene therapy administration, AAV5-FVIII gene therapy triggered manageable cellular immune responses, mainly targeting the AAV5 capsid rather than the FVIII transgene product. Humoral immune responses in a related clinical trial with AAV5-FVIII showed a similar antigenic preference.[49] Our data
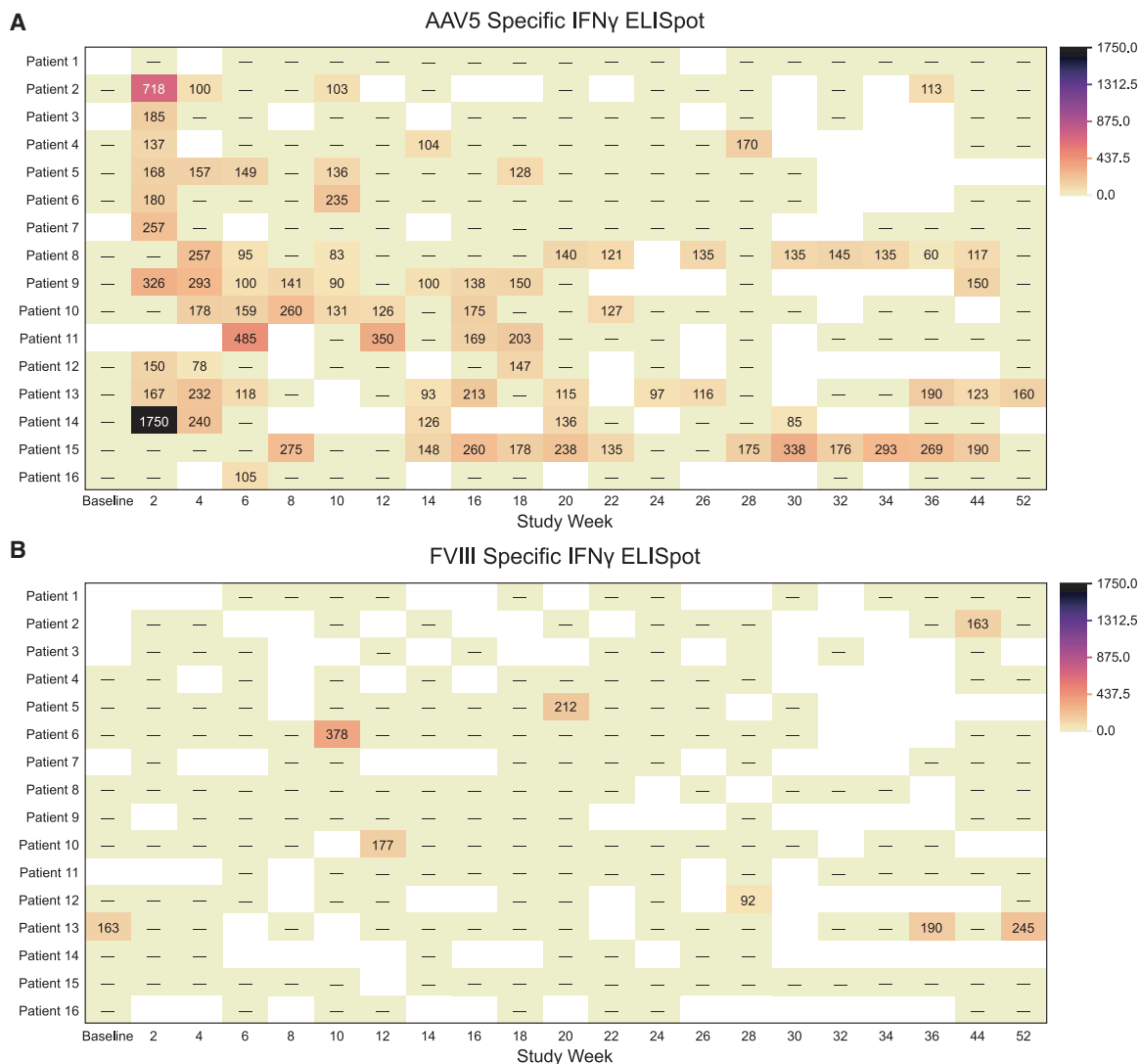
Figure 5. Clinical application of ELISpot assay: heatmap of AAV5 and FVIII positive responses

Heatmaps show results from a validated IFN-γ ELISpot assay for individual patients at baseline and every 2 weeks through 52 weeks post-dose administration. Colors signify the magnitude of response by SFU/10^6 PBMCs as indicated. Positive responses were summarized across peptide pools and displayed numerically. (A) Results of the IFN-γ ELISpot assay following stimulation with AAV5-derived peptide pools and (B) transgene expressed protein-derived peptide pools. White squares indicate time points at which there were too few viable PBMCs to test, or where they have not yet been tested. Samples testing negative with a result <60 SFU/10^6 PBMCs are shown in yellow with a dash (-). SFU counts were summarized across separate stimulation pools when positive; 2 pools of AAV5 peptides and 4 pools of FVIII peptides, to represent the total number of SFU reactive across the entirety of the protein sequence.

further demonstrate the value of extensive pre-study and in-study bioanalytical validations of ELISpot methodology to reliably measure cellular immune responses against AAV gene therapy.

MATERIALS AND METHODS

This section was written in compliance with the Minimal Information about T Cell Assays (MIATA) guidelines[26] (http://miataproject.org/miata-guidelines/final-guidelines-2/).

Human PBMC preparation

Healthy Human PBMCs (AccuCell) cryopreserved in a proprietary serum-free freezing media were sourced from Precision for Medicine (Frederick, MD, USA). The cryopreserved PBMC were shipped overnight on dry ice and stored until use in the vapor phase of LN_2. Cryovials of normal healthy human PBMCs were removed from LN_2 storage and kept on dry ice until thawing. The PBMC vials were thawed quickly at 37°C, and the volume was transferred to a 50-mL conical tube when only an ice crystal remained. Drop-wise 9 mL 1× CTL-Wash media (Cellular Technology Limited,

Shaker Heights, OH, USA) diluted with RPMI (ThermoFisher, Waltham, MA, USA) containing 50 U/mL Pierce Universal Nuclease (ThermoFisher) was added, with gentle agitation. The PBMC were pelleted by centrifugation (350 × g for 8 min at room temperature [RT]), resuspended, and washed a second time with 10 mL 1× CTL-Wash media diluted with RPMI containing 50 U/mL Pierce Universal Nuclease. After another pelleting by centrifugation, the PBMCs were gently resuspended into 10 mL CTL-Test media (Cellular Technology Limited) containing 1× Glutamax (LifeTechnologies, Carlsbad, CA, USA). After 1 final centrifugation, the PBMC pellet was resuspended into CTL-Test media and counted. For counting, the PBMCs were stained using the Cellometer AOPI stain (Nexcelom, Lawrence, MA, USA). PBMC viability was determined as the percentage of cells that are live as compared to total cells (live and dead). The viable cell count is the quantity of live cells per milliliter of original volume multiplied by the AOPI dilution factor. Based on the resulting PBMC concentration, the original PBMC sample was diluted to a concentration of 2.0×10^6 live PBMCs/mL with CTL-Test media. The final cell density after adding 100 μL PBMC to each well was 2.0×10^5, unless otherwise specified. PBMC viability must be ≥70% to be plated in the ELISpot assay.

Antigen and control stimulant preparation

Overlapping peptide pools (15mers overlapping by 10 amino acids [aa]) of AAV5 capsid protein VP1 and transgene product (FVIII-SQ) were custom synthesized with capping (to avoid any deletion peptides) by JPT Peptide Technologies GmbH (Berlin, Germany). AAV5 capsid proteins VP2 and VP3 are N-terminal truncations of VP1 with otherwise identical sequence; therefore, peptide pools derived from VP1 can detect cellular immune responses to all 3 capsid proteins. Due to the size of the protein sequences, the AAV5 VP1 sequence (UniProt: Q9YIJ1) was split into 2 peptide pools (AAV5 pool 1 and AAV5 pool 2) and the FVIII-SQ sequence (UniProt: P00451.1) was split into 4 peptide pools (FVIII pool 1, FVIII pool 2, FVIII pool 3, and FVIII pool 4), each pool containing ~70 peptides each with ≥90% purity. Each vial of peptide pool was reconstituted in dimethyl sulfoxide (DMSO) and diluted in CTL-Test media and stored in single-use aliquots at −80°C until use. A single-use aliquot of each peptide pool was thawed on each assay day and further diluted in CTL-Test media to a final in-well concentration (1×) of 2 μg/mL for 79 donors and 1 μg/mL for the remaining 20 donors, and 0.25% DMSO or 0.125% DMSO, respectively (Sigma-Aldrich; St. Louis, MO, USA), unless otherwise specified.

Aliquots of lyophilized human CMVpp65 peptide pool (JPT Peptide Technologies GmbH) containing 138 peptides with >70% purity were purchased. Each vial of CMVpp65 peptide pool (15mers overlapping by 11 aa) was reconstituted in DMSO, diluted in CTL-Test media, and stored in single-use aliquots at −20°C until use. For each ELISpot assay, a single-use aliquot of CMVpp65 peptide pool (50 μg/mL) was thawed and further diluted in CTL-Test media to a final in-well concentration of 2 μg/mL of each peptide and 0.25% DMSO, unless otherwise specified.

Background IFN-γ responses were monitored using a negative control that mimicked the DMSO concentration present in the antigen peptide wells. The negative control (mock) stimulation contained a final in-well concentration of 0.25% DMSO diluted in CTL-Test media. PHA, a lectin from the red kidney bean (*Phaseolus vulgaris*), was used as a positive-control stimulant due to its mitogenic nature and ability to activate T cells in a universal manner. For use in each ELISpot assay, a single-use aliquot of PHA was thawed and further diluted in CTL-Test media to a final in-well concentration of 0.25 μg/mL, unless otherwise specified. DMSO and PHA were both sourced from Sigma-Aldrich.

IFN-γ ELISpot assay procedure

Pre-coated plates from human IFN-γ ELISpot ALP Kit (Mabtech, Cincinnati, OH, USA) were prepared and developed according to the manufacturer's instructions (version 2018-11-16), with some modifications. Using sterile conditions and aseptic technique, the 96-well pre-coated assay plate were blocked with CTL-Test media containing 1× Glutamax for 2–6 h at RT. Stimulants were prepared at a 2× concentration, so that their final in-well concentration after the addition of the PBMC volume was 1×. To each well of a blocked pre-coated ELISpot plate, 100 μL 2× stimulant was added in triplicate. To each well of stimulant, 100 μL cells at 2.0×10^6 PBMC/mL was added for a final cell density of 2.0×10^5 PBMC/well, unless otherwise specified, and a final stimulant concentration of 1×. PBMCs were stimulated for 20–24 h at 37°C, 5% CO_2, unless otherwise specified. Cells were removed from the plate and IFN-γ was detected using sequential incubations of a biotinylated anti-IFN-γ antibody (0.22 μm filtered) and a streptavidin alkaline phosphatase-coupled detection antibody. Spots were visualized following the addition of 100 μL 0.22 μm filtered 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT)-plus substrate for 3 min ± 15 s, unless otherwise specified. Spot development was stopped using a water wash and plates were air dried overnight at RT, avoiding exposure to light. SFU in each well were enumerated using an automated spot counter (ImmunoSpot CTL S6 Micro Analyzer, Cellular Technology Limited) within 24–96 h of development, unless otherwise specified. The settings used on the Immunospot to detect the secreted IFN-γ spots were sensitivity of 145, background balance of 10, spot separation of 1, counting mask size of 90% (not normalized), minimum spot size of 0.0015 mm^2, and a maximum spot size of 9.6466 mm^2. The spot counts in each well were visually quality controlled by an analyst to ensure that the spot detection was appropriate, before finalization.

Statistical evaluations

Statistical analysis was performed using GraphPad Prism version 7 software (GraphPad, San Diego, CA, USA). Briefly, statistical outliers were removed using the interquartile range method; where the upper limit of the interquartile range was determined as 1.5×, the difference between the 1st and 3rd quartiles added to the 3rd quartile, and the lower limit of the interquartile range was determined as 1.5× the difference between the 1st and 3rd quartiles subtracted from the 1st quartile. The Shapiro-Wilk test was used to evaluate the normality of the

data distributions.[50] The upper 95% distribution limit for the LOD was calculated as the anti-log of the [grand mean + (1.645 × SD) of $\log_{10}$ transformed values]. A 1-way ANOVA was performed in GraphPad Prism to determine whether the means and variances were equal across datasets.[51] The empirical 95th percentile is equal to the value where 95% of the values in the datasets are below and only 5% are above. CV was calculated as the SD divided by the mean for a given dataset, multiplied by 100.

Clinical PBMC sample procedures and testing

PBMC samples were collected from 16 hemophilia A participants enrolled in an AAV5-FVIII gene therapy clinical trial (BMN 270-301, NCT03370913, EudraCT 2017-003215-19), approved by the institutional review board (IRB), independent ethics committee (IEC), and research ethics board (REB). Participants screened negative for preexisting anti-AAV5 antibodies received a single vector dose of 6 E13 vg/kg AAV5-hFVIII-SQ and were treated with corticosteroids on demand. In the trial described here, as of the data cut of April 30, 2019, 10 of 16 participants received corticosteroids on demand, starting at 60 mg/day as early as 10.9 weeks after dosing for an average duration of 20.4 weeks (including tapering periods). Blood collected using sodium heparin vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) was shipped to a central lab for PBMC isolation within 24 h. Isolated PBMC were cryopreserved in the same serum-free freezing media as was used during the validation, frozen at $-80°C$ in a cell freezing container, and stored long term in the vapor phase of LN_2. PBMCs were shipped to the testing lab using LN_2 cryo-shippers and upon receipt were promptly stored in the vapor phase of LN_2 until testing. Upon thaw, PBMC samples with confirmed viability >70% were tested for antigen-specific responses to AAV5 and FVIII peptide pools. PHA was included as a positive control stimulation for all of the samples; mock stimulation (0.25% DMSO) was included as a negative control. All 8 conditions were plated in triplicate for each sample unless there were insufficient PBMC quantities; in this case, a sample plating priority plan was implemented. One or 2 PHA wells were omitted, with at least 1 PHA well remaining. If there were still not enough cells, then sets of triplicates for individual peptide pools were omitted in a pre-defined order until there were enough cells to plate at least 1 PHA well, triplicate for mock and triplicate for at least 1 peptide pool, or else the sample was not plated at all. Potential alternatives to consider when sample quantities are insufficient may be the use of combined peptide pools or plating lower cell numbers per well, even though these deviations could affect assay performance, including sensitivity. Results were only reported if the sample elicited a PHA response greater than the PHA minimum (30 SFU/well or 150 SFU/million PBMCs). Positive and negative responses were adjudicated using the following algorithm: (1) if an antigen-specific response is <LOD (12 SFU/well or 60 SFU/million PBMCs), then it is negative; (2) if a sample had a mock response <LOD and an antigen-specific response is ≥LOD, then the antigen-specific response is positive; and (3) if both mock and antigen-specific response is ≥LOD, then the antigen-specific response is only positive if the response ratio is equal to or greater than the CCP (i.e., ≥2.96-fold over the mock response). If the %

CV for a triplicate with a mean response ≥LLOQ (44.4 SFU/well or 222 SFU/million PBMCs) was >30%, an outlier could be removed using a Dixon's Q test, median absolute deviation, or other statistical method. The final results were reported as SFU/million PBMCs. A validated Excel spreadsheet was used to apply all of these acceptance criteria during clinical sample analysis. A control donor (a responder to FVIII pool 2, FVIII pool 3, and CMVpp65) was tested for FVIII pool 2, FVIII pool 3, CMVpp65, PHA, and mock responses, along with every batch of clinical samples. The results generated by this donor were used for trending and reagent bridging purposes and were not used for assay acceptance.

AUTHOR CONTRIBUTIONS

K.S.P., S.J., M.T.H., and C.V. designed and implemented the validation study experiments. M.T.H., B.R.L., and K.S.P. analyzed the data. K.S.P., B.R.L., and C.V wrote the manuscript. R.O., B.R.L., K.L., K.S.P., C.V., and J.H. implemented and/or monitored the clinical sample testing. M.T.H., R.O., S.J., J.H., T.K., and S.J.Z. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

K.S.P., J.H., T.K., B.R.L., K.L., S.J.Z., and C.V. are current employees and/or shareholders of BioMarin Pharmaceutical. M.T.H. and R.O. are both current employees of Precision for Medicine. S.J. provided consultancy on the project and is currently employed by Zellnet Consulting.

REFERENCES

1. Nathwani, A.C., Reiss, U.M., Tuddenham, E.G.D., Rosales, C., Chowdary, P., McIntosh, J., Della Peruta, M., Lheriteau, E., Patel, N., Raj, D., et al. (2014). Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N. Engl. J. Med. 371, 1994–2004.

2. Manno, C.S., Pierce, G.F., Arruda, V.R., Glader, B., Ragni, M., Rasko, J.J., Ozelo, M.C., Hoots, K., Blatt, P., Konkle, B., et al. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat. Med. 12, 342–347.

3. Balakrishnan, B., and Jayandharan, G.R. (2014). Basic biology of adeno-associated virus (AAV) vectors used in gene therapy. Curr. Gene Ther. 14, 86–100.

4. Ritter, T., Lehmann, M., and Volk, H.D. (2002). Improvements in gene therapy: averting the immune response to adenoviral vectors. BioDrugs 16, 3–10.

5. Colella, P., Ronzitti, G., and Mingozzi, F. (2017). Emerging Issues in AAV-Mediated In Vivo Gene Therapy. Mol. Ther. Methods Clin. Dev. 8, 87–104.

6. Verdera, H.C., Kuranda, K., and Mingozzi, F. (2020). AAV Vector Immunogenicity in Humans: A Long Journey to Successful Gene Transfer. Mol. Ther. 28, 723–746.

7. Naso, M.F., Tomkowicz, B., Perry, W.L., 3rd, and Strohl, W.R. (2017). Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs 31, 317–334.

8. Ertl, H.C.J., and High, K.A. (2017). Impact of AAV Capsid-Specific T-Cell Responses on Design and Outcome of Clinical Gene Transfer Trials with Recombinant Adeno-Associated Viral Vectors: An Evolving Controversy. Hum. Gene Ther. 28, 328–337.

9. Nidetz, N.F., McGee, M.C., Tse, L.V., Li, C., Cong, L., Li, Y., and Huang, W. (2020). Adeno-associated viral vector-mediated immune responses: Understanding barriers to gene delivery. Pharmacol. Ther. 207, 107453.

10. Wang, D., Tai, P.W.L., and Gao, G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. Nat. Rev. Drug Discov. 18, 358–378.

11. Mingozzi, F., and High, K.A. (2017). Overcoming the Host Immune Response to Adeno-Associated Virus Gene Delivery Vectors: The Race Between Clearance, Tolerance, Neutralization, and Escape. Annu. Rev. Virol. 4, 511–534.

12. Basner-Tschakarjan, E., and Mingozzi, F. (2014). Cell-mediated immunity to AAV vectors, evolving concepts and potential solutions. Front. Immunol. 5, 350.

13. Kuranda, K., Jean-Alphonse, P., Leborgne, C., Hardet, R., Collaud, F., Marmier, S., Costa Verdera, H., Ronzitti, G., Veron, P., and Mingozzi, F. (2018). Exposure to wild-type AAV drives distinct capsid immunity profiles in humans. J. Clin. Invest. 128, 5267–5279.

14. Falese, L., Sandza, K., Yates, B., Triffault, S., Gangar, S., Long, B., Tsuruda, L., Carter, B., Vettermann, C., Zoog, S.J., and Fong, S. (2017). Strategy to detect pre-existing immunity to AAV gene therapy. Gene Ther. 24, 768–778.

15. Rogers, G.L., Martino, A.T., Aslanidi, G.V., Jayandharan, G.R., Srivastava, A., and Herzog, R.W. (2011). Innate immune responses to AAV vectors. Front. Microbiol. 2, 194.

16. Long, B.R., Sandza, K., Holcomb, J., Crockett, L., Hayes, G.M., Arens, J., Fonck, C., Tsuruda, L.S., Schweighardt, B., O'Neill, C.A., et al. (2019). The Impact of Pre-existing Immunity on the Non-clinical Pharmacodynamics of AAV5-Based Gene Therapy. Mol. Ther. Methods Clin. Dev. 13, 440–452.

17. Mingozzi, F. (2018). AAV Immunogenicity: A Matter of Sensitivity. Mol. Ther. 26, 2335–2336.

18. Stanford, S., Pink, R., Creagh, D., Clark, A., Lowe, G., Curry, N., Pasi, J., Perry, D., Fong, S., Hayes, G., et al. (2019). Adenovirus-associated antibodies in UK cohort of hemophilia patients: a seroprevalence study of the presence of adenovirus-associated virus vector-serotypes AAV5 and AAV8 neutralizing activity and antibodies in patients with hemophilia A. Res. Pract. Thromb. Haemost. 3, 261–267.

19. Boutin, S., Monteilhet, V., Veron, P., Leborgne, C., Benveniste, O., Montus, M.F., and Masurier, C. (2010). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum. Gene Ther. 21, 704–712.

20. Mingozzi, F., Maus, M.V., Hui, D.J., Sabatino, D.E., Murphy, S.L., Rasko, J.E.J., Ragni, M.V., Manno, C.S., Sommer, J., Jiang, H., et al. (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. Nat. Med. 13, 419–422.

21. Nathwani, A.C., Tuddenham, E.G.D., Rangarajan, S., Rosales, C., McIntosh, J., Linch, D.C., Chowdary, P., Riddell, A., Pie, A.J., Harrington, C., et al. (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N. Engl. J. Med. 365, 2357–2365.

22. Barabas, S., Spindler, T., Kiener, R., Tonar, C., Lugner, T., Batzilla, J., Bendfeldt, H., Rascle, A., Asbach, B., Wagner, R., and Deml, L. (2017). An optimized IFN-γ ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. BMC Immunol. 18, 14.

23. Boehm, U., Klamp, T., Groot, M., and Howard, J.C. (1997). Cellular responses to interferon-γ. Annu. Rev. Immunol. 15, 749–795.

24. Janetzki, S., Panageas, K.S., Ben-Porat, L., Boyer, J., Britten, C.M., Clay, T.M., Kalos, M., Maecker, H.T., Romero, P., Yuan, J., et al.; Elispot Proficiency Panel of the CVC Immune Assay Working Group (2008). Results and harmonization guidelines from two large-scale international Elispot proficiency panels conducted by the Cancer Vaccine Consortium (CVC/SVI). Cancer Immunol. Immunother. 57, 303–315.

25. Butterfield, L.H., Palucka, A.K., Britten, C.M., Dhodapkar, M.V., Hâkansson, L., Janetzki, S., Kawakami, Y., Kleen, T.-O., Lee, P.P., Maccalli, C., et al. (2011). Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. Clin. Cancer Res. 17, 3064–3076.

26. Britten, C.M., Janetzki, S., Butterfield, L.H., Ferrari, G., Gouttefangeas, C., Huber, C., Kalos, M., Levitsky, H.I., Maecker, H.T., Melief, C.J.M., et al. (2012). T cell assays and MIATA: the essential minimum for maximum impact. Immunity 37, 1–2.

27. Maecker, H.T., Hassler, J., Payne, J.K., Summers, A., Comatas, K., Ghanayem, M., Morse, M.A., Clay, T.M., Lyerly, H.K., Bhatia, S., et al. (2008). Precision and linearity targets for validation of an IFNgamma ELISPOT, cytokine flow cytometry, and tetramer assay using CMV peptides. BMC Immunol. 9, 9.

28. Dubey, S., Clair, J., Fu, T.M., Guan, L., Long, R., Mogg, R., Anderson, K., Collins, K.B., Gaunt, C., Fernandez, V.R., et al. (2007). Detection of HIV vaccine-induced cell-mediated immunity in HIV-seronegative clinical trial participants using an optimized and validated enzyme-linked immunospot assay. J. Acquir. Immune Defic. Syndr. 45, 20–27.

29. Körber, N., Behrends, U., Hapfelmeier, A., Protzer, U., and Bauer, T. (2016). Validation of an IFNγ/IL2 FluoroSpot assay for clinical trial monitoring. J. Transl. Med. 14, 175.

30. Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, US Food and Drug Administration (2019). Immunogenicity testing of therapeutic protein products — developing and validating assays for anti-drug antibody detection, https://www.fda.gov/regulatory-information/search-fda-guidance-documents/immunogenicity-testing-therapeutic-protein-products-developing-and-validating-assays-anti-drug.

31. Banas, B., Steubl, D., Renders, L., Chittka, D., Banas, M.C., Wekerle, T., Koch, M., Witzke, O., Mühlfeld, A., Sommerer, C., et al. (2018). Clinical validation of a novel enzyme-linked immunosorbent spot assay-based in vitro diagnostic assay to monitor cytomegalovirus-specific cell-mediated immunity in kidney transplant recipients: a multicenter, longitudinal, prospective, observational study. Transpl. Int. 31, 436–450.

32. Piccoli, S., Mehta, D., Vitaliti, A., Allinson, J., Amur, S., Eck, S., Green, C., Hedrick, M., Hopper, S., Ji, A., et al. (2019). 2019 White Paper on Recent Issues in Bioanalysis: FDA Immunogenicity Guidance, Gene Therapy, Critical Reagents, Biomarkers and Flow Cytometry Validation (Part 3 – Recommendations on 2019 FDA Immunogenicity Guidance, Gene Therapy Bioanalytical Challenges, Strategies for Critical Reagent Management, Biomarker Assay Validation, Flow Cytometry Validation & CLSI H62). Bioanalysis 11, 2207–2244.

33. Center for Drug Evaluation and Research, Center for Veterinary Medicine, US Food and Drug Administration (2018). Bioanalytical Method Validation: Guidance for Industry, https://www.fda.gov/media/70858/download.

34. European Medicines Agency (2011). Guideline on Bioanalytical Method Validation, https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf.

35. Britten, C.M., Walter, S., Janetzki, S., Mingozzi, F., and Ferreira, V. (2013). Immunological monitoring to rationally guide AAV gene therapy. Front. Immunol. 4, 273.

36. Smith, J.G., Joseph, H.R., Green, T., Field, J.A., Wooters, M., Kaufhold, R.M., Antonello, J., and Caulfield, M.J. (2007). Establishing acceptance criteria for cell-mediated-immunity assays using frozen peripheral blood mononuclear cells stored under optimal and suboptimal conditions. Clin. Vaccine Immunol. 14, 527–537.

37. Bull, M., Lee, D., Stucky, J., Chiu, Y.L., Rubin, A., Horton, H., and McElrath, M.J. (2007). Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. J. Immunol. Methods 322, 57–69.

38. Olson, W.C., Smolkin, M.E., Farris, E.M., Fink, R.J., Czarkowski, A.R., Fink, J.H., Chianese-Bullock, K.A., and Slingluff, C.L., Jr. (2011). Shipping blood to a central laboratory in multicenter clinical trials: effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability and immunologic function. J. Transl. Med. 9, 26.

39. Filbert, H., Attig, S., Bidmon, N., Renard, B.Y., Janetzki, S., Sahin, U., Welters, M.J.P., Ottensmeier, C., van der Burg, S.H., Gouttefangeas, C., and Britten, C.M. (2013). Serum-free freezing media support high cell quality and excellent ELISPOT assay performance across a wide variety of different assay protocols. Cancer Immunol. Immunother. 62, 615–627.

40. Janetzki, S., Price, L., Britten, C.M., van der Burg, S.H., Caterini, J., Currier, J.R., Ferrari, G., Gouttefangeas, C., Hayes, P., Kaempgen, E., et al. (2010). Performance of serum-supplemented and serum-free media in IFNgamma Elispot Assays for human T cells. Cancer Immunol. Immunother. 59, 609–618.

41. Patton, K., Aslam, S., Lin, J., Yu, L., Lambert, S., Dawes, G., Esser, M.T., Woo, J., Janetzki, S., and Cherukuri, A. (2014). Enzyme-linked immunospot assay for detection of human respiratory syncytial virus f protein-specific gamma interferon-producing T cells. Clin. Vaccine Immunol. *21*, 628–635.

42. Vitale, J.J., and Broitman, S.A. (1981). Lipids and immune function. Cancer Res. *41*, 3706–3710.

43. Dutra, F.F., and Bozza, M.T. (2014). Heme on innate immunity and inflammation. Front. Pharmacol. *5*, 115.

44. Moodie, Z., Huang, Y., Gu, L., Hural, J., and Self, S.G. (2006). Statistical positivity criteria for the analysis of ELISpot assay data in HIV-1 vaccine trials. J. Immunol. Methods *315*, 121–132.

45. Moodie, Z., Price, L., Gouttefangeas, C., Mander, A., Janetzki, S., Löwer, M., Welters, M.J.P., Ottensmeier, C., van der Burg, S.H., and Britten, C.M. (2010). Response definition criteria for ELISPOT assays revisited. Cancer Immunol. Immunother. *59*, 1489–1501.

46. Ferreira, V., Petry, H., and Salmon, F. (2014). Immune responses to AAV-vectors. The Glybera example from bench to bedside. Front. Immunol. *5*, 82.

47. Gernoux, G., Wilson, J.M., and Mueller, C. (2017). Regulatory and Exhausted T Cell Responses to AAV Capsid. Hum. Gene Ther. *28*, 338–349.

48. Vandamme, C., Xicluna, R., Hesnard, L., Devaux, M., Jaulin, N., Guilbaud, M., Le Duff, J., Couzinié, C., Moullier, P., Saulquin, X., and Adjali, O. (2020). Tetramer-Based Enrichment of Preexisting Anti-AAV8 CD8$^+$ T Cells in Human Donors Allows the Detection of a T$_{EMRA}$ Subpopulation. Front. Immunol. *10*, 3110.

49. Long, B.R., Veron, P., Kuranda, K., Hardet, R., Mitchell, N., Hayes, G.M., Wong, W.Y., Lau, K., Li, M., Hock, M.B., et al. (2021). Early Phase Clinical Immunogenicity of Valoctocogene Roxaparvovec, an AAV5-Mediated Gene Therapy for Hemophilia A. Mol. Ther. *29*, 597–610.

50. Shapiro, S.S., and Wilk, M.B. (1965). An analysis of variance test for normality (complete samples). Biometrika *52*, 591–611.

51. Zar, J.H. (2010). Biostatistical Analysis, Fifth Edition (Prentice Hall).