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CASE STUDY

Validation of an IFN-γ ELISpot Assay for Measuring T-Cell Responses Against Viral Antigens, in AAV-based Gene Therapy

Background

Adenovirus-associated virus (AAV)–based vectors are commonly used to deliver genes to various target tissues for long-term treatment of—or even to cure—genetic diseases. Although these vectors are generally well tolerated, preexisting and treatment-induced immune responses against both AAV capsids and encoded transgene products may occur, potentially adversely impacting the safety and efficacy of gene therapies.[1] An estimated 30%-60% of adults carry AAV capsid–specific antibodies, and a subset of those may also have circulating capsid-specific CD4+ and CD8+T cells due to natural exposure to AAV.[2]

Antigen-specific cellular immune responses can be measured by detecting T-cell activation, such as cytokine secretion, cell proliferation, or cytotoxicity. Detection of cytokine secretion is often the preferred approach due to ease of use.[1,3] IFN-γ is a common cytokine endpoint in ELISpot assays as it is produced and secreted primarily by activated CD4+ and CD8+ T cells, which make up 45%-70% of peripheral blood mononuclear cells (PBMCs).[4] Both enzyme-linked immunoassays and enzyme-linked immunospot (ELISpot) or FluoroSpot assays can detect secreted cytokines. However, unlike immunoassays, ELISpot/FluoroSpot can enumerate individual cytokine-producing cells and even measure the frequency of cytokine secretion at the single-cell level.[1]

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Challenges

To monitor treatment-specific cellular immunity in response to an AAV5 gene therapy, the sponsor sought to validate an interferon-gamma (IFN-γ)–based ELISpot assay for measuring T-cell responses against viral antigens in nonhuman primates. Although ELISpot testing has been used extensively in exploratory research settings, there are no universally accepted procedures for validating these assays for use in gene therapy studies.[1]

[1] Yang F, Patton K, Kasprzyk T, et al. Validation of an IFN-gamma ELISpot assay to measure cellular immune responses against viral antigens in non-human primates. *Gene Ther.* 2022;29:41-54.

[2] Stanford S, Pink R, Creagh D, et al. 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*. 2019;3:261-267.

[3] Mobs C, Schmidt T. Research techniques made simple: monitoring of T-cell subsets using the ELISPOT assay. *J Investig Dermatol*. 2016;136:e55-e59.

[4] Barabas S, et al. An optimized IFN-gamma ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. *BMC Immunol*. 2017;18:14.

Implementation

Precision for Medicine conducted a fit-for-purpose validation of this IFN-γ ELISpot assay using PBMCs from cynomolgus monkeys that were responsive to peptides derived from cytomegaloviruses (CMV). Since these viruses are endemic in cynomolgus monkey populations, CMV-derived peptides are ideal antigens for positive control stimulation.[1]

Initial characterization

A preliminary limit of detection (LOD) of 10 spot-forming units (SFUs)/well was verified by assessing the magnitude of the observed nonspecific background signal using PBMCs from 5 monkeys under unstimulated conditions in multiple independent runs. Because animals with suitable AAV5 capsid–specific responses were not identified, initial characterization of assay precision was performed using CMV-responsive PBMCs from 3 different monkeys in 3 independent runs over 4 days by 1 analyst, using stimulation with 2 CMV peptide pools. Overall, the results demonstrated that stimulation with CMV-derived peptide pools yielded spot counts that could be precisely quantified across a wide range of low to high antigen-specific responses.[1]

Assay validation

Following initial characterization, this IFN-γ ELISpot assay was validated using prespecified acceptance criteria (Table 1). In the absence of formal regulatory guidelines, these criteria were extrapolated, where feasible, from FDA guidance documents and industry white papers for immunogenicity and bioanalytical assays.[1] Further, the lack of positive control cells with a robust capsid-specific response limited the scope to a fit-for-purpose validation.

Table 1. **Assay Acceptance Criteria[1]**

Assay parameter	Acceptance criteria
LOD	The LOD is the 95th percentile distribution limit of mean SFU/well responses after DMSO mock stimulation
Confirmatory cut point	The confirmatory cut point is the 95th percentile distribution limit of peptide/mock response ratios in naïve animals
Precision	For responses ≥ 30 SFU/well: • Intra-triplicate CV ≤ 30% • Intra-assay CV ≤ 30% • Inter-assay CV < 50%
Positive control stimulation acceptance range	PHA responses > LOD
Linearity	Report cell densities through which a linear regression of responses shows an $R^2 \geq 0.95$
Specificity	No positive response to irrelevant HIV control peptides

The LOD was validated by applying a statistical approach using parametric calculations to the triplicate mean responses in SFU/well using PBMCs from 18 monkeys under unstimulated conditions. The validated LOD was calculated as 15 SFU/well.[1]

Since the triplicate mean SFU/well for 4 of the 18 monkeys fell at or above the validated LOD, it was necessary to establish and validate a confirmatory cut point. To do this, PBMCs from naïve monkeys were stimulated with 2 AAV5-derived peptide pools or 1 transgene product-derived peptide pool. Response was calculated as ratios in which SFU/well results for peptide-stimulated conditions were divided by the result for unstimulated conditions. Statistical analysis of these response ratios derived a confirmatory cut point of 1.69.[1]

The various dimensions of assay precision were validated using PBMCs from 3 CMV peptide–responsive monkeys. Six assay runs were performed. The coefficients of variation for intratriplicate, intraassay, interassay precision, and interanalyst all met acceptance criteria, demonstrating the consistency of measured PBMC responses to stimulating peptide antigens.[1]

Assay linearity, which refers to the ability to obtain results that are directly proportional to the concentration of the analyte being measured within a defined range, was validated using PBMCs from a monkey with medium to strong response to CMV peptides. The validated linear range of the assay was determined to be from 25,000 to 200,000 cells/well.[1]

Assay specificity was validated by stimulating CMV-responsive monkey PBMCs with an irrelevant HIV peptide pool at 2 concentrations, using CMV peptides as a peptide-responsive control. Responses to both concentrations of the HIV peptide pool remained below the LOD (Table 2), demonstrating that the IFN-γ ELISpot assay can specifically detect cellular immune responses after stimulation with antigenic peptide pools.[1]

Table 2. **Validation of Assay Specificity[1]**

Monkey ID	Stimulation	Run 1 Mean SFU/well	Run 2 Mean SFU/well	Run 3 Mean SFU/well
16	Mock (DMSO)	1	5	3
16	HIV (2 µg/mL)	2	3	2
16	HIV (1 µg/mL)	1	6	5
16	UL55 (1 µg/mL)	155	204	228
16	UL83 (1 µg/mL)	784	924	923
16	PHA (0.25 µg/mL)	470	551	545

PBMCs from a CMV-responsive monkey were stimulated with CMV peptide pools UL55 and UL83 or an irrelevant HIV-derived peptide pool. PHA stimulation and DMSO mock control were also included.

Results

The validated, fit-for-purpose assay was used in a cynomolgus monkey study to measure cellular immune responses to the vector capsid and encoded transgene product following AAV5-based gene therapy. A total of 72 PBMC samples were collected from 36 study animals allocated equally between 2 cohorts, 1 with a terminal time point on Day 28 (Cohort 1) and the other with a terminal time point on Day 56 (Cohort 2). Both cohorts were also sampled on Day 13 post gene therapy administration. All samples were stimulated with 2 AAV peptide pools and 1 transgene product peptide pool.[1]

All animals developed anti-AAV5 antibodies following administration of the gene therapy vector, confirming induction of an anticapsid humoral immune response. Conversely, AAV5-specific T-cell responses were detected in only 2 of 18 monkeys in Cohort 1 at Day 28 after vector administration, and no AAV5-specific T-cell responses were detected in either cohort at Day 13 or in Cohort 2 at Day 56. These T-cell responses did not correlate with liver enzyme elevations or transgene expression levels. Further, no transgene product–specific T-cell responses occurred in either cohort at any time point. Based on these results, the cellular immune responses evoked by the AAV5 capsid in these monkeys was less prevalent than the humoral response.

Conclusion

This case study describes an approach to validating a generic, commercial IFN-γ ELISpot assay using monkey PBMCs and stimulation with antigen-specific peptide pools. Using ELISpot assays to measure and monitor cellular responses targeting either the AAV capsid or the transgene product may provide insights into mechanisms that impact safety or efficacy in nonclinical gene therapy studies.

For more information on Precision for Medicine's expertise in developing and validating ELISpot and other immune monitoring assays, **please visit PrecisionforMedicine.com**.

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