APPLICATION NOTE

Proinflammatory cytokine evaluation from human PBMCs in response to AAV8 peptides

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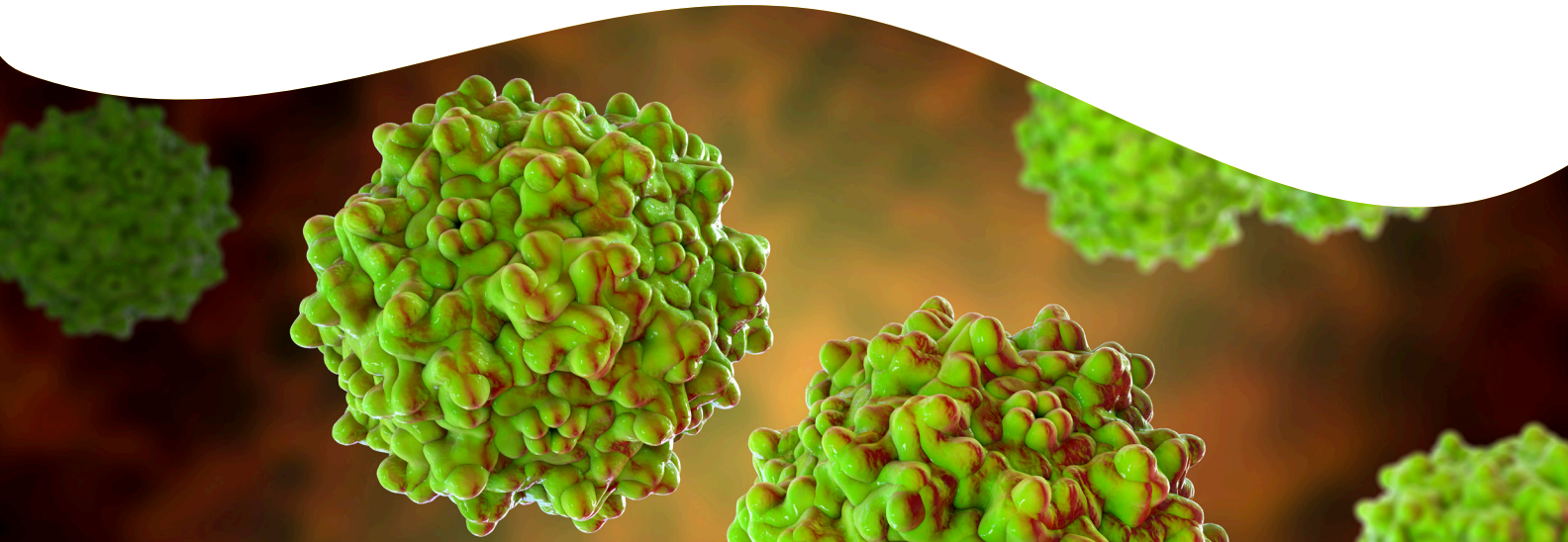
HTRF and ELISpot Technologies Introduction

The human adeno-associated virus (AAV) is a small (~25 nm), simple, non-enveloped parvovirus that was initially identified as a contaminant in adenovirus preparations.[1] Isolating and studying AAV revealed that it cannot replicate in the absence of a helper virus (e.g., adenovirus), and is not known to cause disease in animals or humans.[2] Due to these interesting biological properties, AAVs have been successfully engineered and utilized as a gene therapy vector in humans to treat a variety of genetic diseases, including retinal dystrophy, spinal muscular atrophy, and hemophilia.[3, 4, 5] As of April 2024, five AAV gene therapies have been approved for clinical use in the United States and UK with three approved in 2022 alone, with more currently in clinical trials. Generally, AAVs are considered to have high sustained efficacy with low toxicity and immunogenicity, however a rare cute immunogenic and toxic effects, even death have been reported in patients receiving AAV-related gene therapies.[6] Furthermore, host immune responses, such as the secretion of cytokines from B and T cells, may represent an immunological barrier that could limit the effectiveness of a particular gene therapy. Understanding how a candidate's immune system will respond to exposure to a viral vector, like AAV, is critical for assessing whether prophylactic measures, such as immunosuppression, need to be co-administered to ensure maximum therapeutic efficacy.[7] This is typically achieved by isolating the peripheral blood mononuclear cells (PBMCs) from the whole blood of patients or donors, exposing the PBMCs to AAV or AAV capsid-derived peptides, and measuring the production of pro-inflammatory cytokines in the cell culture supernatant using an ELISpot/ Fluorospot assay, or similar.[8]



This study evaluated cytokine profiles from PBMCs post-treatment with AAV8 peptides. Cytokines were detected using HTRF Human Cytokine Detection Kits (Revvity, Inc.) which accurately measured the level of five proinflammatory cytokines (IFNγ, TNFα, IL1β, Il2, and IL6) secreted from PBMCs isolated from the whole blood of four healthy donors when stimulated with AAV8 VP1-peptides. The measurement of IFNγ using the HTRF IFNγ kit were consistent with cell-based ELISpot assay using the IFNγ-specific kit (TSPOT.TB). Importantly, the quantification of the remaining cytokines using the HTRF kits revealed different immune profiles for each donor. Interestingly, treating the PBMCs isolated from donor 4 with AAV peptides resulted in a robust T-cell response with much higher TNFα, IL1β, and IL6 levels compared to the other 3 three. This suggests that the immune system of donor 4 is primed to react to the AAV8 antigen, likely through a variety of intrinsic factors, including the possibility of acquired immunity to wild-type AAV8 from previous exposure. These results illustrate how different individuals can have vastly different immune responses to the same antigen. Evaluating cytokine levels released by the isolated PBMCs of individuals using the HTRF kits provides a fast, reliable, convenient, and sensitive method to assess the risk of an adverse immune response to gene therapy prior to its administration. This type of assessment impacts the decision on whether pretreatment with immunosuppressors is warranted.

Methods

Isolation of PBMCs from donor whole blood

Whole blood from four anonymous healthy donors was ordered from BioIVT and was received in lithium heparin tubes containing 9 mL each of blood per donor. The blood was transferred to a 15 mL conical tube and mixed with 225 µL of T-cell Xtend by gently inverting the tube. T-cell Xtend is an antibody complex that is added to blood samples immediately before running TSPOT.TB (ELISpot) assays allows sample processing up to 32 hours after venepuncture without affecting the accuracy of the assay. The tube was incubated at room temperature for 20 minutes. A 5.4 mL volume of AIM V cell culture medium was added to the tube and mixed by inversion. The blood mixture was transferred to a leucosep tube and mixed with 1-part Ficoll to 3-parts diluted blood and centrifuged at 1000 x g for 10 minutes with a reduced brake, but not zero. After centrifugation, the plasma was removed with a Pasteur pipette and discarded. The buffy coat layer just above the frit was removed and transferred to a new tube. The cells were then washed 3 times in DPBS, centrifuged at 600 x g for 7 minutes and the supernatant was discarded after each wash. After washing, the cell pellet was resuspended in 500 µL of AIM V medium and the viable cells were enumerated using a Cellaca cell counter (Nexcelom).

Stimulation of PBMCs and preparation of the TSPOT.TB and CulturPlate

The 25 µg stock of lyophilized AAV8 VP1 peptide mixture (182 peptides at 15 nmol/peptide with 15 amino acid overlap - JPT) was initially diluted in 125 µL of 100% DMSO to generate a 0.2 mg/mL solution. A 30 µL volume of the reconstituted peptide was further diluted to 3 µg/mL in AIM V medium. DMSO was diluted to 1.5% in AIM V medium and used as a negative control. PHA was provided with the TSPOT.TB kit as a ready-to-use positive control. A 3x stock of LPS was prepared by diluting 12 µL of the 500x stock (00-4976-93, ThermoFisher) in 1988 µL of AIM V medium. The TSPOT.TB (ELISpot) and CulturPlate (Revvity) were loaded and treated identically, except that stimulants and PBMCs were added in triplicate to the CulturPlate while only single wells were used in the TSPOT.TB plate. The isolated PBMCs were diluted with AIM V medium so that 100 µL contained ~250,000 viable cells. A total volume of 50 µL of the 3x stocks of AAV8 peptide, PHA, LPS, and DMSO, as well as 50 µL of 1x AIM V medium was added to triplicate wells in both TSPOT.TB and CulturPlates for each donor. Each donor-specific well was loaded with 100 µL (2.5×10^5 cells) of the relevant PBMCs. Both plates were incubated at 37 °C under 5% CO_2 overnight (~16 hours). The A loading diagram for the TSPOT.TB and CulturPlate are provided in Figure 1. After the overnight incubation, the CulturPlate was centrifuged at 1,000 x g for 10 minutes with a reduced brake. The supernatant was recovered from triplicate wells, pooled in a microtube to mix like samples, and redistributed into 5 separate StorPlates (Revvity) loading 20 µL into each well using the well assignments shown for the CulturPlate in Figure 1. The StorPlates were stored at -80 °C until needed.

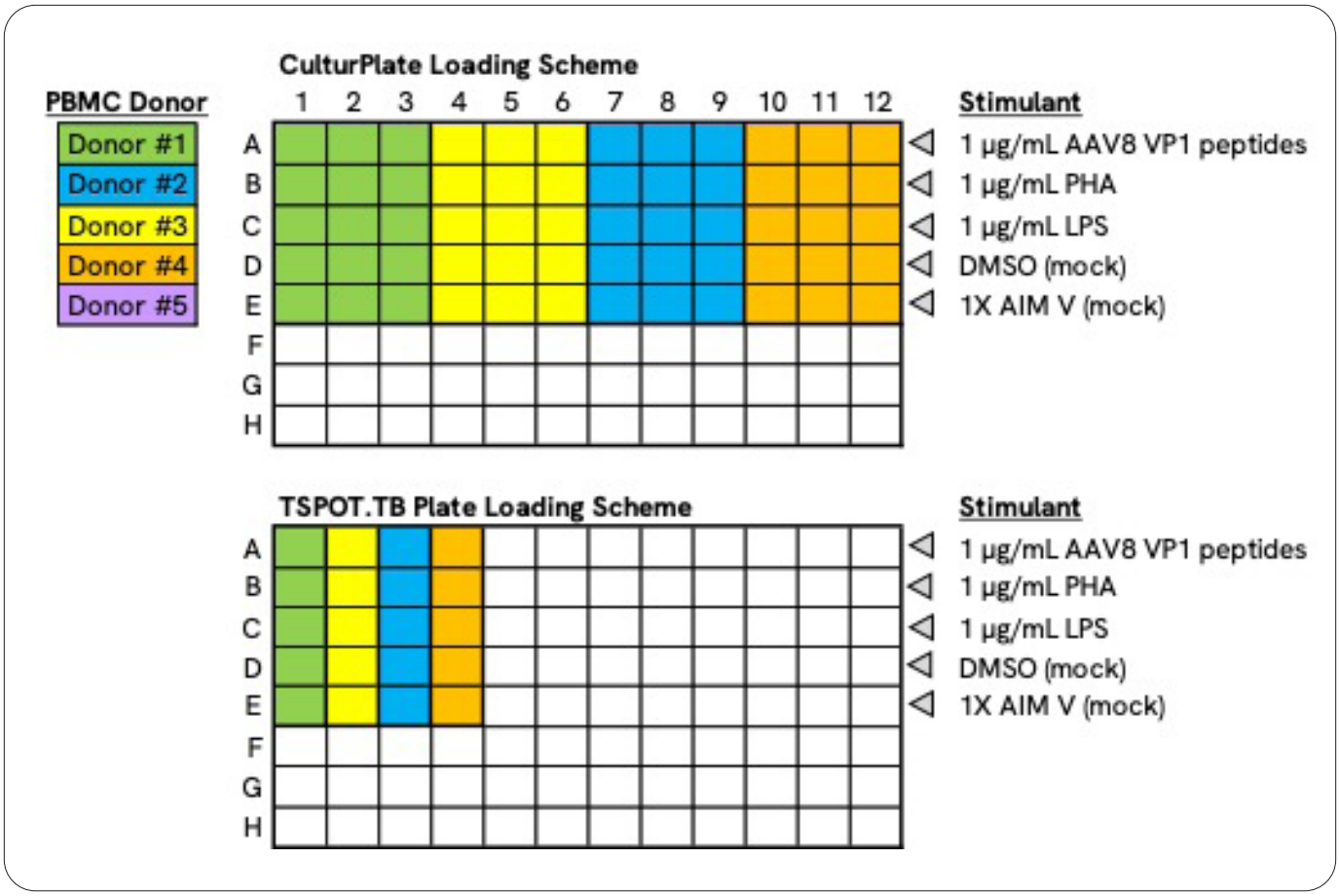


Figure 1: Assay plate loading diagram. The final concentrations are listed for the stimulants in each well. A total volume of 50 µL of the stimulants was added to the corresponding wells. 100 µl of PBMCs were loaded into each well. The final concentration of PBMCs from each donor was 2.5 x 10^5 cells/well.

TSPOT.TB Assay

The immune response to infection with *Mycobacterium tuberculosis* is mediated predominantly through T-cell activation. As part of this response, T cells are sensitized to *M. tuberculosis* antigens, and the activated effector T cells, both CD4+ and CD8+, produce the cytokine interferon-gamma (IFN-γ) when stimulated by these antigens.[9, 10] The T-SPOT.TB test uses the enzyme-linked immunospot (ELISpot) methodology to enumerate *M. tuberculosis*-sensitized T cells by capturing interferon-gamma (IFN-γ) in the vicinity of T cells from which it was secreted.[11] Peripheral blood mononuclear cells (PBMCs) are separated from a whole blood sample, washed, and then counted before being added to the assay. Isolated PBMCs (white blood cells) are placed into microtiter wells where they are exposed to a phytohemagglutinin (PHA) control (a mitogenic stimulator indicating cell functionality), nil control, and two separate panels of *M. tuberculosis*-specific antigens. The PBMCs are incubated with the antigens to allow stimulation of any sensitized T cells present. Secreted cytokine is captured by specific antibodies on the surface of the membrane, which forms the base of the well, and the cells and other unwanted materials are removed by washing. A second antibody, conjugated to alkaline phosphatase and directed to a different epitope on the cytokine molecule, is added and binds to the cytokine captured on the membrane surface. Any unbound conjugate is removed by washing. A soluble substrate is added to each well; this is cleaved by a bound enzyme to form a (dark blue) spot of insoluble precipitate at the site of the reaction. Evaluating the number of spots obtained provides a measurement of the abundance of *M. tuberculosis*-sensitive effector T cells in the peripheral blood. Assay setup, plate loading information, spot development, and counting procedures were followed as described in the TSPOT.TB package insert.

HTRF Human Cytokine Detection Assay

HTRF is a homogeneous time-resolved fluorescence immunoassay technology that provides a simple, no-wash strategy to detect and quantify proteins. It combines standard fluorescence resonance energy transfer (FRET) technology with time-resolved measurement, eliminating short-lived background fluorescence. For a sandwich assay, two antibodies that recognize a protein of interest are used, with one antibody coupled to a donor, and the other to an acceptor. If the two antibodies recognize the analyte, the donor will emit fluorescence upon excitation and the energy will be transferred to the nearby acceptor resulting in specific long-lived acceptor fluorescence (Figure 2). In the HTRF human cytokine detection assays (Revvity: IFNγ – 62HIFNGPEG, TNFα – 62HTNFAPEG, IL1β – 62HIL1BPEG, IL2 – 62HIL02PEG, IL6 – 62HIL06PEG) an anti-cytokine-specific antibody is labeled with donor Europium and another anti-cytokine antibody is labeled with the d2 acceptor. The assay plates (HTRF 96-well low volume plate, Revvity, 66PL96025) were read on an EnVision® 2105 Multimode Plate Reader using laser excitation with APC 665 nm and Cy5 620 nm filters and the LANCE®/DELFIA® Dual bias mirror. Both the 665 nm and 620 nm signals were collected, and the HTRF ratio of the acceptor-to-donor signal shown in the results was calculated as (665 nm/620 nm) x 10^4. All kits come with analyte standards which are used to set up 7-point standard curves. The curves were constructed using the calculated HTRF delta Ratio following the guidelines provided in the technical data sheet (TDS) available for each cytokine kit. The concentration of cytokine in each sample was interpolated from the relevant analyte standard curve.

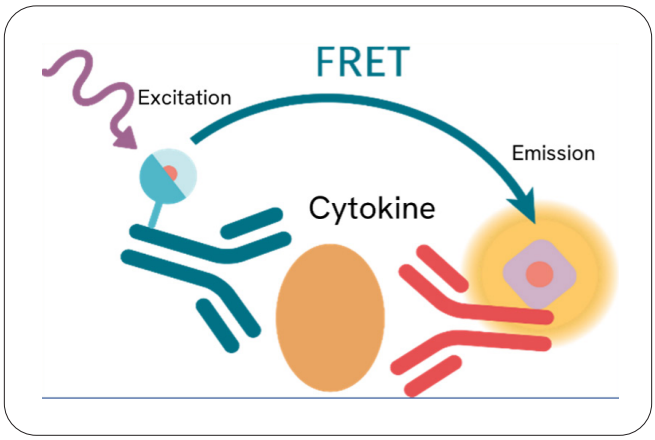
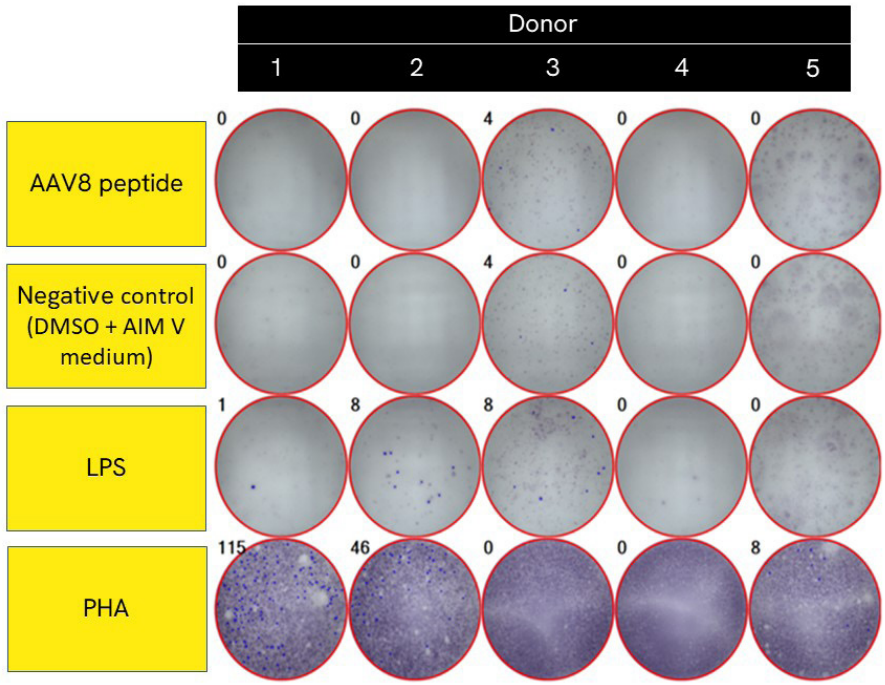


Figure 2: Assay principle for HTRF Human Cytokine Detection Kits.

Results

TSPOT.TB and HTRF IFNγ Detection Kit produced correlative results

The PBMCs from all four donors were stimulated with AAV8 VP1 peptides, PHA, LPS, and DMSO diluted in AIM V medium overnight. The TSPOT.TB plate results are shown in Figure 3. Stimulation with the AAV8 peptides did not produce detectable levels of IFNγ above the negative control (DMSO in AIM V medium) for all donors. This could be attributed to a low population or complete absence of AAV8 reactive cell types in the isolated PBMCs samples. Alternatively, IFNγ production may lag post-stimulation, and increasing the incubation time after treatment may result in an increase in IFNγ levels and an increased number of spots over the negative control. Stimulating with LPS results in a low number of measurable spots for donors 1 - 3. However, treating the PBMCs with PHA produced a significant number of spots in donors 1 and 2 with high background, and complete signal saturation in donors 3 and 4. PHA was used as a positive control as it is a well-known mitogen that stimulates T-cell proliferation and the secretion of IFNγ.

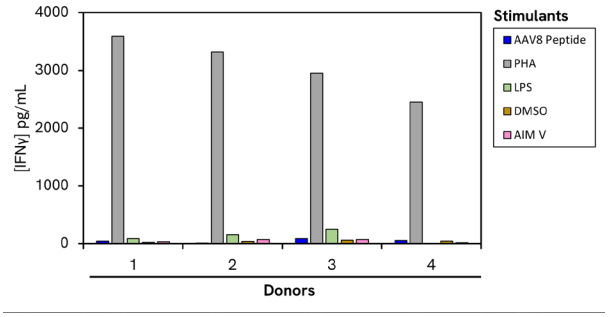


Quantification

Donor	Treatment		
	AAV8	LPS	PHA
	Spot Count		
1	0	1	115
2	0	8	700
3	4	8	700
4	0	0	700
5	0	0	700

Figure 3: TSPOT.TB IFNγ ELISpot assay. A visual representation of the wells is provided with the number of positive hits (spots) displayed at the top-left of each well. The spot quantification is also provided in the table to the right of the spots. A value of 700 is given for samples containing saturated signals in which the CTL spot reader artificially assigns a low value.

The results from the HTRF IFNγ Detection Kit are provided in Figure 4 and show that stimulating PBMCs with AAV8 peptide resulted in the detection of < 100 pg/mL of IFNγ from all donors. This concentration is consistent with the background IFNγ levels measured in the DMSO and AIM V negative controls. Treating the PBMCs with LPS resulted in a slight increase in IFNγ over the AAV8 peptide-stimulated samples and negative controls. The relative donor-specific trend in the LPS data mirrors the number of spots observed in the ELISpot results. Stimulation of the PBMCs with PHA triggered the secretion of comparatively high levels of IFNγ, which is consistent with the results obtained from the TSPOT.TB assay.



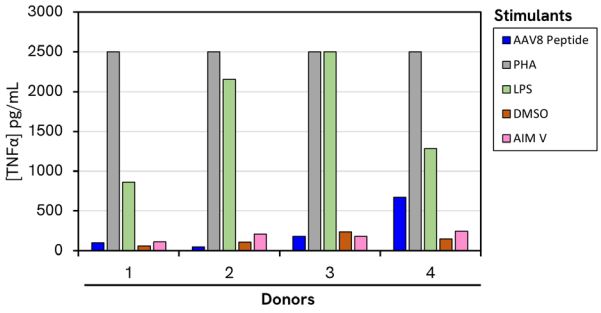
Stimulant	[IFNγ] pg/mL			
	Donor 1	Donor 2	Donor 3	Donor 4
AAV8 peptide	39	12	87	53
PHA	3594	3321	2955	2450
LPS	84	152	248	2
DMSO	18	39	59	44
AIM V	32	67	68	15

Figure 4: Results from HTRF IFNγ Detection Kit. The IFNγ standard curve was prepared in 1x AIM V medium and is provided on the left. The comparative concentrations of IFNγ from each sample are shown in the histogram (middle) and table (right).

Measuring TNFα, IL1β, IL2, and IL6 in donor PBMC supernatants reveals divergent immunogenicity to AAV8 peptides

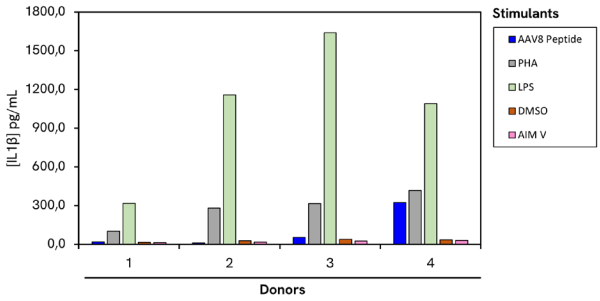
In addition to IFNγ, the levels of TNFα, IL1β, IL2, and IL6 proinflammatory cytokines were quantified in the PBMC supernatants of each donor using HTRF Human Cytokine Detection Kits and are provided in Figure 5. The TNFα level in the AAV8 peptide-treated PBMC supernatants was higher than IFNγ but was still lower than the negative controls (DMSO and AIM V medium) for donors 1 – 3 (Figure 5A). However, donor 4 had TNFα levels that were significantly higher than the other 3 donors and the negative controls. Stimulating with PHA and LPS positive controls resulted in TNFα levels that exceeded the top end of the standard curve (> 2500 pg/mL*) in some samples. Stimulating donor PBMCs with AAV8 peptides resulted in low IL1β levels (Figure 5B), on par with the negative controls for donors 1 - 3. Again, stimulation of the PBMCs from donor 4 resulted in increased IL1β levels compared to the other donors with a concentration ~10x higher than the negative controls. The IL2 levels in the PBMC supernatants shown in Figure 5C revealed that only PHA sufficiently stimulated IL2. All other stimulants failed to raise IL2 to detectable levels including the LPS positive control. When challenged with AAV8 peptides, the PBMCs from donors 1 – 3 secreted low levels of IL6 (Figure 5D), below the concentration detected for the negative controls. PHA and LPS stimulated differential IL6 secretion for each donor to levels higher than the negative controls in most cases, except LPS for donor 3. Stimulating the PBMCs from donor 4 with AAV8 peptides resulted in the secretion of a very high level of IL6, dwarfing the negative controls and equal to the IL6 levels quantified in PBMC samples stimulated with PHA and LPS positive controls.

A



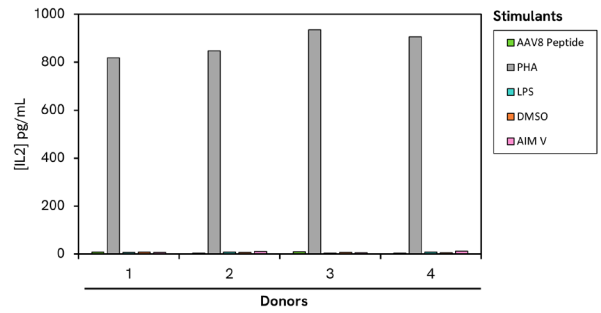
Stimulant	[TNFα] pg/mL			
	Donor 1	Donor 2	Donor 3	Donor 4
AAV8 peptide	103	50	179	673
PHA	2500	2500	2500	2500
LPS	862	2157	2500	1284
DMSO	60	109	238	148
AIM V	114	209	179	247

B



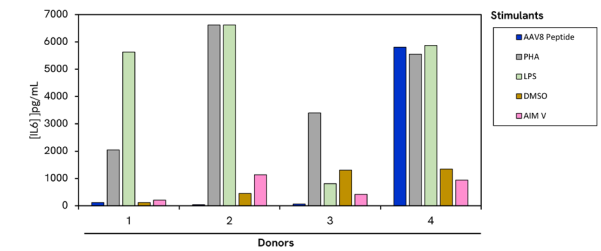
Stimulant	[IL1β] pg/mL			
	Donor 1	Donor 2	Donor 3	Donor 4
AAV8 peptide	18.3	10.3	54.6	323.3
PHA	100.5	279.9	315.6	417.3
LPS	316.8	1157.3	1638.2	1090.3
DMSO	15.2	27.0	39.5	33.8
AIM V	12.4	16.3	24.7	30.6

C



Stimulant	[IL2] pg/mL			
	Donor 1	Donor 2	Donor 3	Donor 4
AAV8 peptide	9	5	10	5
PHA	819	848	936	906
LPS	7	9	5	8
DMSO	8	8	7	6
AIM V	8	11	5	12

D



Stimulant	[IL6] pg/mL			
	Donor 1	Donor 2	Donor 3	Donor 4
AAV8 peptide	123	37	61	5808
PHA	2044	6614	3405	5557
LPS	5627	6621	813	5868
DMSO	119	455	1306	1343
AIM V	212	1139	422	937

Figure 5: Evaluation of proinflammatory cytokines levels in donor PBMC supernatants using HTRF Human Cytokine Detection Kits. Cytokine (analyte) standard curves were prepared in 1x AIM V cell culture medium. The standard curves used for data interpolation, comparative histograms, and data tables are provided for the following HTRF cytokine detection kits: A. - TNFα, B. - IL1β, C. - IL2, and D. - IL6.

Both ELISpot (T-SPOT.TB) and HTRF assays yielded correlative results despite the lack of IFNγ secretion from the PBMCs isolated from the four donors in this study. The T-cells in the PBMC samples from each donor were successfully activated by one or both positive controls (PHA and LPS) resulting in the secretion of each of the cytokines tested using HTRF technology. The secretion of cytokine in PBMCs treated with negative controls (DMSO and AIM V medium) was comparatively much lower than the positive controls and represented the basal or low-level secretion of each cytokine from the donor-derived PBMCs. Interestingly, the AAV8 VP1 peptides failed to stimulate an immune response in donors 1 - 3, with cytokine levels consistent with the negative controls. However, the PBMCs of donor 4 secreted TNFα, IL1β, and IL6 at concentrations higher than the negative controls and donors 1 - 3, providing critical insights into how the immune systems of each donor might respond if treated with a gene therapy utilizing the AAV8 capsid as a vector.

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