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Tetramer-Based Enrichment of Preexisting Anti-AAV8 CD8⁺ T Cells in Human Donors Allows the Detection of a T_{EMRA} Subpopulation

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Pre-existing immunity to AAV capsid may compromise the safety and efficiency of rAAV-mediated gene transfer in patients. Anti-capsid cytotoxic immune responses have proven to be a challenge to characterize because of the scarcity of circulating AAV-specific CD8⁺ T lymphocytes which can seldom be detected with conventional flow cytometry or ELISpot assays. Here, we used fluorescent MHC class I tetramers combined with magnetic enrichment to detect and phenotype AAV8-specific CD8⁺ T cells in human PBMCs without prior amplification. We showed that all healthy individuals tested carried a pool of AAV8-specific CD8⁺ T cells with a CD45RA⁺ CCR7[−] terminally-differentiated effector memory cell (T_{EMRA}) fraction. *Ex vivo* frequencies of total AAV-specific CD8⁺ T cells were not predictive of IFN γ ELISpot responses but interestingly we evidenced a correlation between the proportion of T_{EMRA} cells and IFN γ ELISpot positive responses. T_{EMRA} cells may then play a role in recombinant AAV-mediated cytotoxicity in patients with preexisting immunity. Overall, our results encourage the development of new methods combining increased detection sensitivity of AAV-specific T cells and their poly-functional assessment to better characterize and monitor AAV capsid-specific cellular immune responses in the perspective of rAAV-mediated clinical trials.

Keywords: AAV, gene therapy, immune response, CD8⁺ T lymphocytes, tetramer-associated magnetic enrichment

INTRODUCTION

Over the past decade, recombinant adeno-associated virus-derived vectors (rAAV) have emerged as a powerful vector platform for *in vivo* gene delivery. With over a 100 gene therapy clinical trials worldwide, sustained therapeutic effect has been achieved in the frame of a variety of inherited diseases such as Leber's congenital amaurosis type 2 (1, 2), hemophilia B (3), M-type α -1 antitrypsin deficiency (4), or lipoprotein lipase deficiency (5). Already three different AAV-based gene therapy products have received market approval [Glybera (6), Luxturna (7), Zolgensma (8)]. Nevertheless, all these successes have been tempered by rising concerns over the immunogenicity of the AAV capsid in patients, especially when the vector was delivered *via* a systemic route.

Adeno-Associated Viruses (AAV) are small, non-enveloped, DNA dependo-viruses belonging to the *Parvoviridae* family. Though widely disseminated among the human population (6), wild-type (WT) AAV human infection has not been clearly associated to clinical outcome. Seroprevalence studies have indicated that initial exposure to WT AAV often occurs early during childhood (7, 8), when humoral and cellular immune responses directed against the AAV capsid might be mounted (9, 10). As such, memory AAV-specific T and B cells might be retained throughout lifetime and recalled upon rAAV-mediated gene transfer. While the prevalence of anti-AAV antibodies among the human population is widely studied today (11), and their impact on rAAV-mediated gene transfer is fairly well-documented (12), the detection and characterization of AAV-specific T cell responses remain somewhat more of a challenge even if this issue was first addressed more than 15 years ago (13).

Deleterious effects of anti-AAV cellular immune responses were first evidenced in a liver-directed gene transfer clinical trial for severe hemophilia B patients, where an AAV serotype 2 (AAV2) vector carrying the coagulation factor IX transgene was administered *via* the intrahepatic route (9). In this study, gradual loss of factor IX transgene expression correlated with transient rise in liver transaminase levels and increase in the frequency of AAV-specific CD8⁺ T lymphocytes (10). Those observations were further confirmed in the same clinical indication when the AAV8 serotype was administered intravenously (11). Tremendous amount of work has been done to understand the underlying mechanisms of AAV capsid-specific CD8⁺ T cell cytotoxicity. The current working model states that upon rAAV administration, transduced hepatocyte cells are able to process, and present capsid-derived epitopes onto major histocompatibility class I (MHC I) molecules. Those peptide-MHC (p-MHC) complexes serve as docking sites for recognition by memory capsid-specific CD8⁺ T cells which then activate and expand, leading to the destruction of the transduced cells (12). Notwithstanding, it is still currently impossible to predict the onset of AAV-specific CD8⁺ T cell responses in patients and their clinical impact as positive ELISpot responses don't always correlate with loss of transgene expression (3). One can put forward three main reasons for these limitations: (1) The absence of a relevant animal model recapitulating what is observed in patients; (2) An outcome shown to be variable between individuals and potentially dependent on the target tissue (i.e., liver vs. skeletal muscle) and route of rAAV delivery; and more importantly; (3) The difficulty to monitor AAV-specific CD8⁺ T cells *ex vivo* without prior amplification of PBMCs or splenocytes because of their scarcity leading to a lack of data on their phenotype and functionality.

As recent technological breakthroughs now allow direct *ex vivo* assessment of even scarce antigen-specific CD8⁺ T cell populations, we first addressed the issue of detecting low capsid-specific CD8⁺ T cell frequencies. We applied a p-MHC tetramer-based enrichment approach (later referred to as TAME, for Tetramer-Associated Magnetic Enrichment) (13, 14), with a flow cytometry-based read out, to analyze the presence and frequency of AAV-specific CD8⁺ T cells within the peripheral blood mononuclear cells (PBMCs) of healthy donors. We were able to

detect AAV-specific CD8⁺ T cells in a large cohort of donors and we further identified among them a subset of CD45RA⁺ CCR7[−] terminally-differentiated effector memory cells (T_{EMRA}). After flow cytometric cell sorting we generated primary AAV8-specific CD8⁺ T cell lines that were able to secrete IFN γ , TNF α as well as mediate cytotoxicity toward capsid-loaded targets *in vitro*. Importantly, there was no correlation between anti-AAV IFN γ ELISpot responses and *ex vivo* frequency of total AAV8-specific CD8⁺ T cells, suggesting that frequency of capsid-reactive T cells is not predictive of their functionality. However, we found a correlation between T_{EMRA} frequencies and IFN γ ELISPOT positive responses indicating that T_{EMRA} cells may play a role in recombinant AAV-mediated cytotoxicity in patients with preexisting immunity. In conclusion, our data emphasize the development of new methods combining increased detection sensitivity of AAV-specific T cells and their poly-functional assessment, to better understand capsid-specific cellular immune responses and ultimately predict their impact in rAAV-mediated clinical trials.

MATERIALS AND METHODS

All this work was performed under the control of our quality management system that is approved by Lloyd's Register Quality Assurance LRQA to meet requirements of international Management System Standards ISO 9001:2015. It has been implemented to cover all activities in the laboratory, including research experiments and production of research-grade viral vectors.

Vector Production

For ELISA and neutralization assays, single-stranded AAV serotypes 2 and 8 vectors were produced by the vector core facility in Nantes (<https://umr1089.univ-nantes.fr/plateaux-technologiques/cpv/>). Vectors were produced through co-transfection of Human Embryonic Kidney 293 cells, purified by cesium-chloride gradient, and titrated by dot plot and quantitative PCR assays. For rAAV vectors used in neutralization assays, the vector plasmid contained the LacZ reporter transgene.

Donor Samples

Cytapheresis samples were provided by the local Etablissement Français du Sang (EFS Nantes, Pays de la Loire, agreement N° PLER NTS 2016-25) and originated from consenting healthy donors living in the surrounding area (4 women, 61 men). Median age was 46.5 years old (min: 20; max: 72). Plasma were obtained by centrifuging undiluted cytappheresis samples, and were stored at -80°C . PBMCs were obtained by Ficoll density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare). A fraction of PBMCs was frozen in liquid nitrogen for ELISpot assays, while remaining fresh PBMCs were used for tetramer-based enrichment. HLA-A2 or HLA-B7 phenotyping of PBMCs was performed by flow cytometry using FITC-conjugated anti-HLA-A2 (clone BB7.2, BD Biosciences) or FITC-conjugated anti-HLA-B7 (clone BB7.1, Bio-Rad) antibodies.

Peptides and Peptide/MHC (pMHC) Tetramer Complexes

HLA-A2- and HLA-B7-restricted peptides were purchased from GL Biochem (Singapore) (Table S1). Immunodominant peptides (LIDQYLYYL and IPQYGYLTL) were provided by Proteogenix (France). Biotinylated A2- or B7-soluble MHC class I monomers loaded with conditional ligands (15) were produced by the Plateforme de Protéines Recombinantes (P2R, Nantes, France). Biotinylated A2- or B7- soluble monomers loaded with AAV2 or AAV8 capsid-derived peptides were obtained through UV-mediated peptide exchange as previously described (15). Oligomerization of pMHC monomers was performed with PE- or APC-labeled tetramer-grade streptavidin (PJRS27, Prozyme, and BD Biosciences *resp.*) during 1 h at 4°C, at a molar ratio of 4:1, to form low-order oligomers referred to as “tetramers.”

Tetramer-Based Enrichment Protocol

2×10^8 freshly isolated PBMCs were incubated with a mix containing 100 μ L of AAV capsid-specific PE-conjugated tetramers (20 μ g/mL) and 100 μ L of irrelevant APC-conjugated tetramers (20 μ g/mL). After washes, tetramer-stained cells were enriched using anti-PE antibody-coated immunomagnetic beads on LS columns (Miltenyi Biotec) according to manufactured instructions. After enrichment, cells were stained with an antibody panel that is summarized in Table S2. Counting beads (123count eBeads, eBioscience) were used to normalize results. Cells were acquired on a FACS Fortessa X20 cytometer, a FACS LSR II cytometer, or a FACS Aria III sorter (BD Biosciences) and analyzed with FlowJo software (version 10, Tree Star Inc.). For PE-labeled Tetramer⁺ CD8⁺ T cell detection and phenotyping, CD3⁺ CD8⁺ CD4[−] T cells were gated on single live dump[−] (CD14[−] CD16[−] CD19[−]) cells. CD45RA, CD45RO, and CCR7 expression was then assessed on PE⁺ APC[−] CD8⁺ T cells. Statistical analyses were performed using GraphPad Prism 8.0.1. Results were considered non-significant when $p > 0.05$.

Cell Sorting and T-Cell Lines

A2- or B7-restricted AAV8-specific CD8⁺ T cells were obtained after sorting PE-conjugated Tetramer⁺ CD8⁺ T cells with a FACS Aria III sorter (BD Biosciences). Cells were then expanded *in vitro* under non-specific conditions using rIL-2 (300 UI/mL, Novartis), phytohemagglutinin (PHA, 1 μ g/mL, Sigma-Aldrich), irradiated PBMCs, and B lymphoblastoid cells as previously described (14). T cell lines were maintained for 3 weeks without restimulation in RPMI 1640 medium containing 1 mM L-glutamine (Lonza), and supplemented with 10% FCS (Pierce-Hyclone) and 300 UI/mL rIL-2, before analysis. Additional rounds of amplification were performed with the same conditions if needed. Purity and specificity of CD8⁺ T cell lines were checked through staining with AAV8 capsid-specific PE-conjugated tetramers and irrelevant APC-conjugated tetramers.

Intracellular Cytokine Staining

To assess degranulation activity and cytokine expression of human primary A2- or B7-restricted AAV8-specific CD8⁺ T cell lines, A2⁺ TAP-deficient T2 cells or B7⁺ 7221.221 cells

were loaded overnight at 37°C with 10 μ g/mL of irrelevant peptides, or with A2- or B7- restricted AAV8 capsid-derived peptides (the same peptides used for TAME). A2- or B7-restricted AAV8-specific CD8⁺ T lymphocytes were then added at an effector / target *ratio* of 1/1. Effector and target cells were then incubated 4 h at 37°C in presence of anti-CD107a and anti-CD107b antibodies (clones H4A3 and H4B4 *resp.*, BD Biosciences) and 5 μ M of monensin sodium (Sigma-Aldrich). As a positive control, effector cells were stimulated with 10 ng/mL PMA (Phorbol 12-myristate 13-acetate, Sigma) and 250 ng/mL Ionomycin (Sigma). After incubation, cells were stained with eFluor 506-conjugated FVD and eFluor 605-conjugated anti-CD8. Cells were then fixed with PFA 1% (EMS) overnight at 4°C, and permeabilised with permeabilisation buffer (eBioscience). Finally cells were stained with PE-conjugated anti-IFN γ (clone B27, BD Biosciences) and APC-conjugated anti-TNF α antibodies (clone Mab11, BD Biosciences). Analysis was performed using a FACS Fortessa X20 or a FACS LSR II cytometer. For quantification of degranulation activity, CD107 expression was assessed on single live CD8⁺ T lymphocytes. For quantification of cytokine secretion, IFN γ and TNF α secretion was assessed on single live CD8⁺ T lymphocytes. Each cell line was assessed through three independent activation assays.

Cytotoxic Assay

To assess cytotoxic activity of human primary A2-restricted AAV8-specific CD8⁺ T cell lines, lactate dehydrogenase (LDH) release following target cell lysis was measured with CytoTox 96[®] non-radioactive cytotoxicity assay (Promega). 5×10^5 A2⁺ TAP-deficient T2 cells were loaded as described for functional assays. AAV8-specific CD8⁺ T lymphocytes were then added at an effector: target *ratio* of 5:1, 10:1, and 20:1. Effector and target cells were then incubated 4 h at 37°C and LDH release was measured after 4 h of incubation at 37°C. Target cell maximum LDH release was measured by adding medium with 1% Triton X-100. Each condition was made in triplicate. Medium background was subtracted from measurement and lysis percentages were calculated as follow:

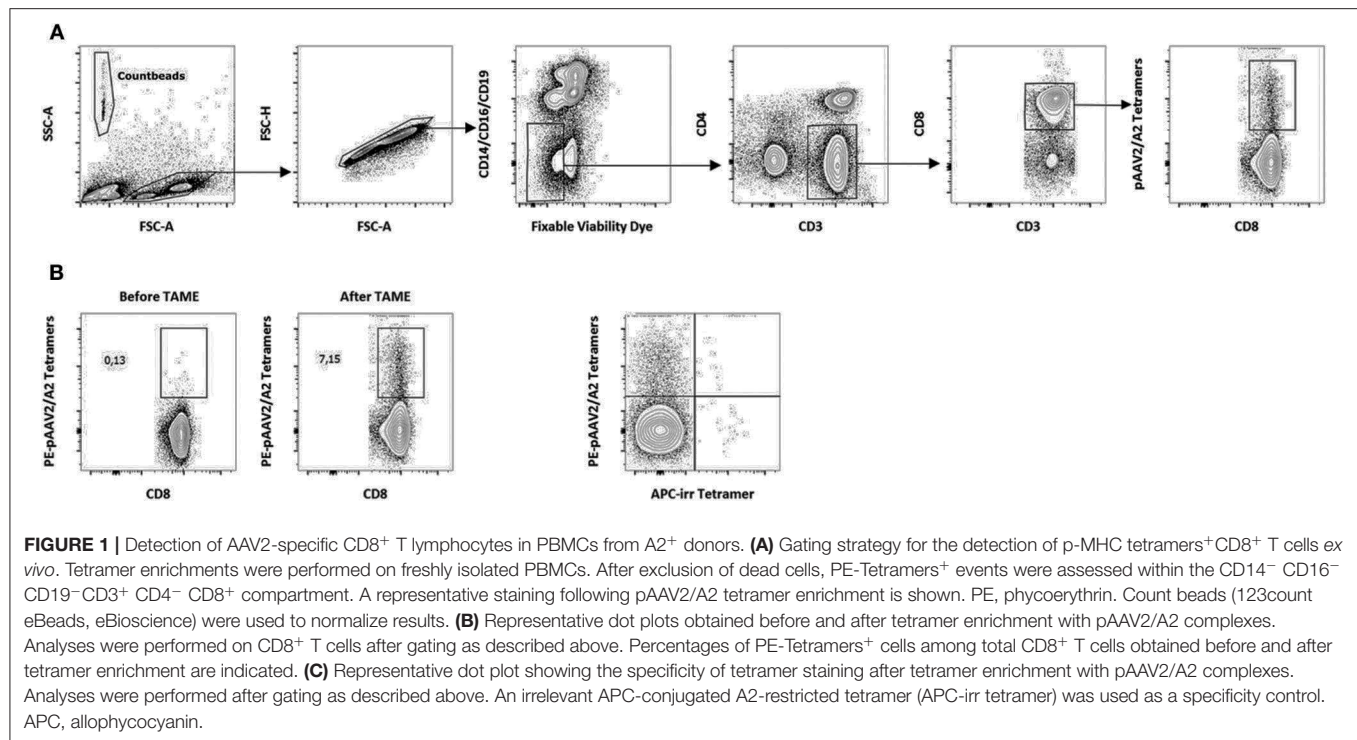
$$\% \text{ cytotoxicity} = (\text{Experimental} - \text{Effector spontaneous} - \text{Target spontaneous}) / (\text{Target Max} - \text{Target spontaneous}) \times 100.$$

CD4⁺ or CD8⁺ Depletion

CD4⁺ or CD8⁺ T cells have been depleted from total PBMCs using Stem Cell magnetic-based system (Easy Sep St Easy Sep Stem Cell kits #17852 and #17853) according to manufacturer instructions. Then, efficiency of depletion have been controlled by BD LSR II flow cytometer using anti-CD4 APC-H7 (BD Biosciences, #560251) and CD8 eFluor 605 (eBiosciences, #93-0088-42).

IFN γ ELISpot Assay

Anti-AAV2 and anti-AAV8 cellular immune responses were evaluated with an IFN γ ELISpot assay using overlapping peptide libraries covering the whole AAV2 or AAV8 capsid sequences (15 per 10 mers, PEPscreen, Sigma) split into three peptide pools each. MultiScreenHTS filter plates, with polyvinylidene difluoride membrane (PVDF, Millipore) were coated overnight at



4°C with human anti-IFN γ antibody (clone MT126L, Mabtech). After coating, 2×10^5 cells per well were plated and restimulated 48h with AAV2- or AAV8- derived peptide pools at a final concentration of 10 μ g/mL. Medium alone served as negative control, while cells stimulated with concanavalin A (Con A, 10 μ g/mL, SIGMA) served as a positive control. After incubation with a biotinylated anti-IFN γ antibody (clone 7-B6-1, MabTech) and Streptavidin-ALP (MabTech), enzymatic reaction was revealed using NBT/BCIP (MabTech). Spot number was determined using an ELISpot *iSpot* Spectrum reader (AID, Strassberg, Germany) and analyzed with AID ELISpot reader Software V7.0 (AID, Germany). Responses were considered positive when the number of spot-forming colonies per million cells was >50 and at least threefold higher than the medium alone negative control. For positive responses, statistical analyses were performed using a DFR(2 \times) test (Distribution Free Resampling).

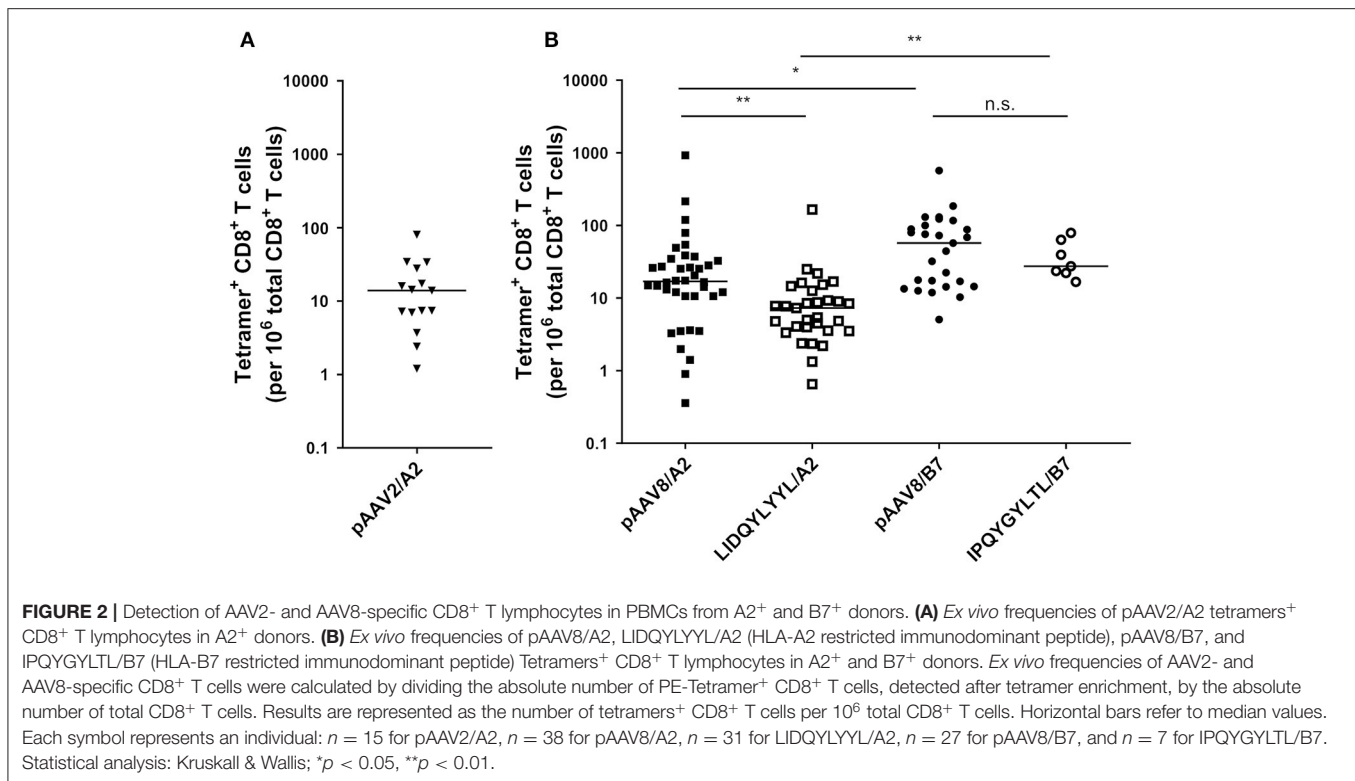
RESULTS

AAV2- and AAV8-Specific CD8⁺ T Cells Are Detectable in Peripheral Blood After *ex vivo* Tetramer Enrichment

Due to their low frequencies, detection of AAV-specific CD8⁺ T lymphocytes often requires prior amplification (or stimulation) of PBMCs or splenocytes (10, 16–18), a process which might induce some bias in the frequency or phenotype of those cells. To circumvent this limitation, we evaluated Tetramer-Associated Magnetic Enrichment (TAME), a flow cytometry-based method that can rapidly and efficiently detect scarce subpopulations of CD8⁺ T cells *ex vivo* (13, 14). We first proved the technical

feasibility of this strategy in our hands using HLA-A*0201 tetramers loaded with the Melan-A tumor-associated antigen ELAIGILTIV (pELA/A2). While Melan-A-specific CD8⁺ T cells displayed frequencies too low to be detected *ex vivo* through conventional tetramer flow cytometry assay, we were able to systematically detect them after TAME in a cohort of 31 HLA-A2⁺ donors, at frequencies ranging from 35 to 2,600/10⁶ total CD8⁺ T cells (median: 124/10⁶ total CD8⁺ T cells) (Figure S1).

As AAV2 was shown to be the most seroprevalent serotype in humans (19), and HLA-A2 the most prevalent allele among the Caucasian population (20), we first investigated the peripheral CD8⁺ T cell repertoire reactivity toward AAV2 capsid-derived peptides restricted to HLA-A*0201 allele (referred to as pAAV2/A2, p for pooled peptides) (Table S1). For that purpose, we used soluble phycoerythrin (PE)-labeled pAAV2/A2 tetramers obtained with a previously described UV-mediated peptide exchange technology (15). Thirteen A2-restricted AAV2 capsid peptides were selected using bioinformatical prediction databases tetramer. PBMCs isolated from A2⁺ donors were stained with pAAV2/A2 tetramers (13 tetramers pooled together), enriched with anti-PE para-magnetic microbeads, and analyzed through flow cytometry (Figure 1A for gating strategy; Figure 1B for an example of enrichment). Following TAME, we were able to readily detect CD8⁺ T cells stained with pAAV2/A2 tetramers *ex vivo* in all A2⁺ donors tested ($n = 15$, Figure 2A). Staining with an irrelevant allophycocyanin (APC)-labeled tetramer confirmed the specificity of pAAV2/A2 tetramer staining, as in all assays and for all donor samples, $>0.1\%$ of PE⁺ APC⁺ CD8⁺ T cells could be seen (Figure 1C for a representative staining). Absolute numbers of AAV2-specific CD8⁺ T cells were determined to calculate their *ex vivo* frequencies and ranged from 1.2 to 80/10⁶



total CD8⁺ T cells in donors (median: 14/10⁶ total CD8⁺ T cells) (Figure 2A), which is in the range of previously described T cell repertoires directed against some viral or tumoral antigens found in healthy donors (13, 14).

Following this proof of concept, we moved for the rest of the study to the AAV8 serotype as it is today a clinically relevant serotype in muscle- and liver-directed systemic gene transfer (21). Peripheral CD8⁺ T cell repertoire reactivity toward AAV8 capsid-derived antigens restricted by the HLA-A*0201 or HLA-B*0702 alleles (referred to as A2 or B7, respectively) was studied in healthy donors expressing at least one of those alleles. Using the same strategy described above, we constituted a pool of 9 pAAV8/A2 tetramers and a pool of 12 AAV8/B7 tetramers [notably containing the previously described HLA B7-restricted AAV8 epitope (10) (Table S1)] to identify AAV8-specific CD8⁺ T lymphocytes within the PBMCs of A2⁺ or B7⁺ healthy donors, respectively. Once again, following TAME, we were able to detect CD8⁺ T cells stained with pAAV8/A2 or pAAV8/B7 tetramers in all A2⁺ and B7⁺ donors tested without any amplification or stimulation of PBMCs beforehand. Specificity of the staining was systematically confirmed using irrelevant APC-labeled tetramers. *Ex vivo* frequencies of AAV8-specific CD8⁺ T cells ranged from 0.3 to 920/10⁶ total CD8⁺ T cells in A2⁺ donors (median: 17/10⁶ total CD8⁺ T cells, *n* = 38), and from 5 to 570/10⁶ total CD8⁺ T cells in B7⁺ donors (median: 57/10⁶ total CD8⁺ T cells, *n* = 27) (Figure 2B).

We further assessed the frequency of CD8⁺ T cells specific for the previously described immuno-dominant A2-restricted LIDQYLYL and B7-restricted IPQYGYLTL peptides (15, 22).

These peptides were used to construct PE-labeled tetramers for TAME on PBMCs from A2⁺ or B7⁺ healthy donors, respectively. Similar to our previous results using pooled tetramers with various peptide specificities, we were again able to detect AAV8-specific CD8⁺ T cells stained with single tetramers in all donors. In A2⁺ donors, these cells were detected at frequencies significantly lower than peptides pool (*p* = 0.0089) and comprised between 0.653 and 166/10⁶ total CD8⁺ T cells (median: 7.3/10⁶ total CD8⁺ T cells, *n* = 31). For B7⁺ donors, the frequencies were also slightly lower than peptides pool with no statistical significance: 16–78.8/10⁶ total CD8⁺ T cells in B7⁺ donors (median: 27.4/10⁶ total CD8⁺ T cells, *n* = 7) (Figure 2B). Interestingly, for both peptide pools and immunodominant peptides, AAV8-specific CD8⁺ T cells were found more frequent in B7⁺ donors than A2⁺ samples (Kruskal-Wallis statistical test: *p* = 0.0469 and *p* = 0.0037, respectively).

Overall, we evidenced that all healthy donors tested possessed circulating AAV2 or AAV8-specific CD8⁺ T cells, though at very low frequencies among PBMCs, and that TAME is a suitable and feasible method to allow their *ex vivo* detection, even though it does not provide any functional insight.

Human Primary AAV8-Specific CD8⁺ T Cells Expanded *in vitro* Secrete Pro-inflammatory Cytokines and Can Specifically Mediate Cytotoxicity

In order to investigate the functional properties of AAV8-specific CD8⁺ T cells, tetramer positive CD8⁺ T cells were sorted by

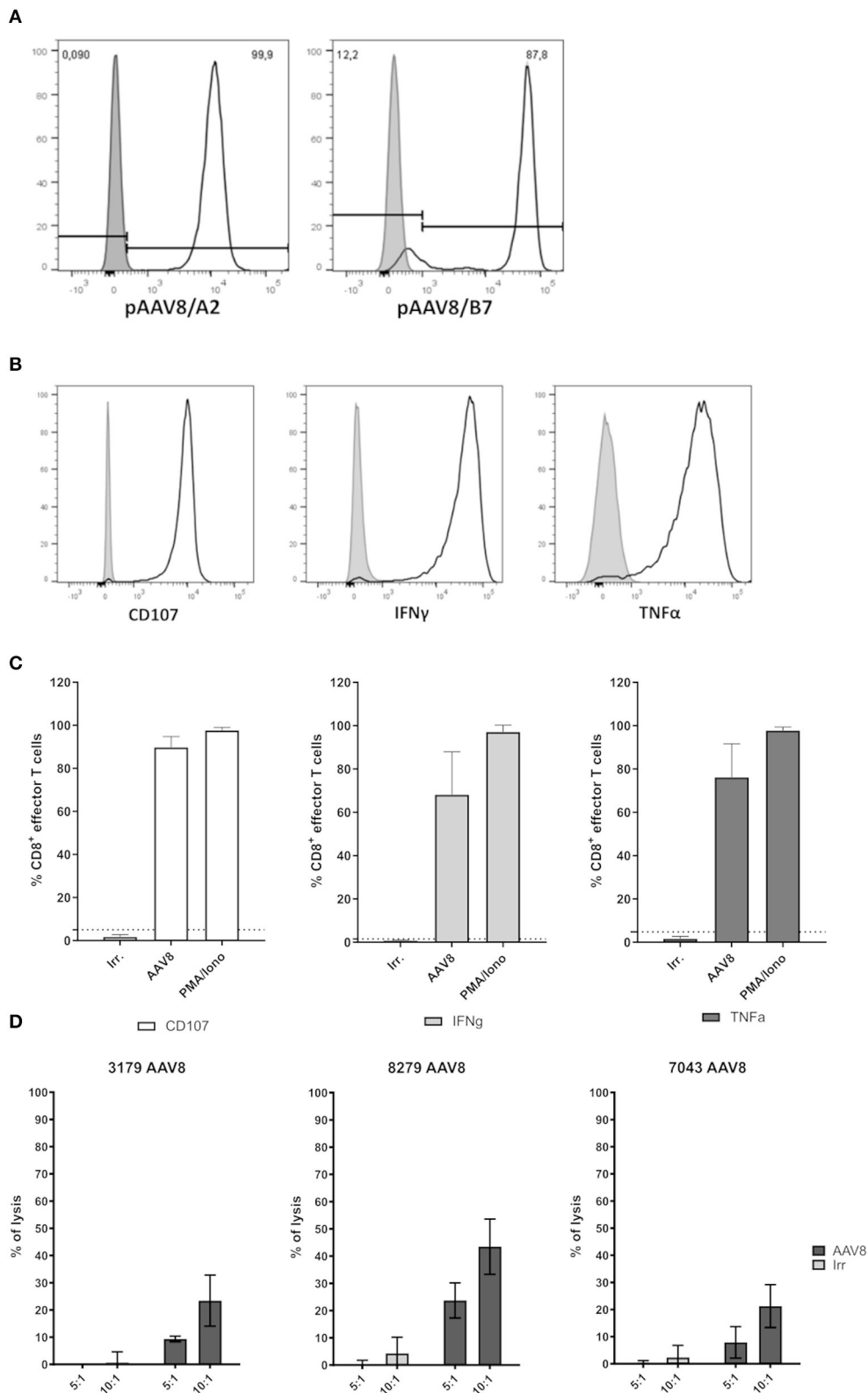


FIGURE 3 | Functional assessment of primary AAV8-specific CD8⁺ T cell lines derived from A2⁺ or B7⁺ donors. **(A)** Representative histograms of pAAV8/A2 or pAAV8/B7 tetramer staining of AAV8-specific CD8⁺ T cell lines generated from A2⁺ or B7⁺ donors. AAV8-specific CD8⁺ T cell lines were stained with relevant (white) (Continued)

FIGURE 3 | or irrelevant (gray) tetramers. The list of all AAV8-specific CD8⁺ T cell lines generated and their purity are indicated in **Table 1**. **(B)** Representative histograms of AAV8-specific CD8⁺ T cell lines activation. CD107 expression, IFN γ secretion, and TNF α secretion were assessed by flow cytometry on AAV8-specific CD8⁺ T cell lines after 4H of co-culture with target cells loaded with AAV8 peptides (light gray) or irrelevant peptides (dark gray). **(C)** Representative quantification of AAV8-specific CD8⁺ T cell lines activation. Each cell line was assessed through three independent activation assays. Results are represented as the mean percentage of AAV8-specific CD8⁺ T cells expressing CD107, IFN γ , and TNF α in response to target cells loaded with AAV8 peptides (AAV8) or irrelevant peptides (Irr.). Stimulation with PMA/ionomycin (PMA/Iono) served as positive control. AAV8-specific CD8⁺ T cells were considered positive when the expression of CD107, IFN γ , and TNF α in response to AAV8-loaded target cells was above the threshold of positivity (dotted line) calculated as the percentage mean of positive cells obtained in response to irrelevant peptides (mean + 3 SD). Overall activation profiles are shown in **Table 1**. **(D)** Representative quantification of AAV8-specific CD8⁺ T cell lines cytotoxicity. Each cell line was assessed for cytotoxicity through LDH measurement. Results are represented as the percentage of lysis of target cells loaded with AAV8 peptides (AAV8) or irrelevant peptides (Irr.) by AAV8-specific CD8⁺ T cells for different effector:target ratio (5:1 and 10:1).

flow cytometry after TAME and expanded *in vitro* from PBMCs of A2⁺ or B7⁺ donors. Indeed, low cell numbers were obtained following cell TAME-based enrichment and sorting and did not allow *in vitro* functional assays before amplification. The purity of all tetramer-sorted CD8⁺ T cell lines after expansion was assessed by pAAV8/A2 or pAAV8/B7 tetramer staining and was comprised between 80 and 99%, except for 2 T cell lines with a purity of 71 and 72%, respectively (**Figure 3A** for representative A2⁺ and B7⁺ tetramer staining on expanded T cell lines; **Table 1** for overall summary). AAV8-specific CD8⁺ T cell lines were tested for their functionality in the presence of A2⁺ or B7⁺ target cells loaded with AAV8 peptides vs. irrelevant peptides. The percentage of CD8⁺ effector cells expressing degranulation marker CD107, or secreting IFN γ and TNF α was analyzed by flow cytometry (**Figures 3B,C**) for representative responses; **Table 1** for overall results). Each T cell line was assessed through three independent activation assays. Responses were considered positive when the percentage of CD8⁺ effector cells expressing CD107, IFN γ , and TNF α in response to AAV8-loaded target cells was found above a threshold of positivity determined as the percentage mean of CD8⁺ effector cells positive for those markers in response to target cells loaded with an irrelevant peptide (mean + 3SD) (**Figure 3C**). Out of the 12 cell lines tested, 11 showed significant upregulation of at least two activation markers upon incubation with AAV8-loaded target cells. Of note, these markers correlated well with each other, as virtually almost all cells upregulating CD107 also expressed IFN γ and TNF α . For three T cell donors, we also investigated the cytotoxic activity of AAV8-specific CD8⁺ T cell lines using LDH measurement (**Figure 3D**). Each AAV8-specific CD8⁺ T cell line was tested with 2 different effector:target ratio: 5:1 and 10:1. AAV8-specific CD8⁺ T cell lines were able to mediate cytotoxicity specifically in response to AAV8 peptide-loaded target cells and not against irrelevant peptide-loaded target cells. Overall, these results indicate that *in vitro* expanded tetramer positive CD8⁺ T cells can secrete pro-inflammatory cytokines and mediate cytotoxicity against target cells loaded with AAV8 peptides.

AAV8-Specific CD8⁺ T Cells Detected *ex vivo* Contain a T_{EMRA} Effector Memory Subset

Since CD8⁺ T cell capsid specificity was demonstrated using amplified cells, we further characterized AAV8-specific CD8⁺ T cell phenotype immediately after TAME and without the

TABLE 1 | Overview of *in vitro* functional responses obtained from primary AAV8-specific CD8⁺ T cell lines derived from PBMCs of A2⁺ and B7⁺ donors.

Cell lines	HLA restriction	Purity ^a (%)	<i>In vitro</i> functional responses			
			Upregulation of CD107 ^b	IFN γ secretion ^b	TNF α secretion ^b	IFN γ ^c
4374	HLA-A2	84	Positive	Positive	Positive	Negative
3179		87	Positive	Positive	Positive	Negative
4800		79.4	Positive	Positive	Positive	Negative
9946		98.9	Positive	Negative	Positive	Negative
6062		91.1	Positive	Positive	Positive	Negative
8279		99.3	Positive	Positive	Positive	Negative
3654	HLA-B7	97.4	Positive	Positive	Positive	Negative
2367		84.5	Positive	Positive	Positive	Positive
5576		99.2	Negative	Negative	Negative	Negative
8860		71.1	Positive	Positive	Positive	Negative
7043		82.1	Positive	Positive	Positive	Positive
6714		72.2	Positive	Positive	Positive	Positive
Total			11/12	10/12	11/12	3/12

^aPurity of pAAV8 tetramer-sorted CD8-specific CD8⁺ T cell lines was assessed through staining with relevant tetramers right before functional assessment.

^bActivation of AAV8-specific CD8⁺ T cell lines was assessed through flow cytometry-based degranulation assay monitoring the upregulation of CD107 expression, IFN γ and TNF α secretion in response to AAV8-loaded target cells. AAV8-specific CD8⁺ T cells were considered positive (gray) when the expression of CD107, IFN γ , and TNF α in response to AAV8-loaded target cells was above the threshold. Positive threshold (dotted line) was calculated using the percentage of positive cells obtained in response to irrelevant peptides (mean + 3 SD).

^cThe last column represents IFN γ ELISpot responses assessed on whole PBMCs from which the AAV8-specific CD8⁺ T cell lines were derived.

bias of prior amplification. For that purpose, we assessed the expression of CD45RA, CD45RO, and CCR7 T cell markers by flow cytometry. We first observed that virtually all AAV8-specific CD8⁺ T cells were CD45RA⁺ CD45RO⁻ (**Figure 4A**, lower panel and **Figure 4B**). In contrast, total polyclonal CD8⁺ T cells displayed a pool of CD45RO⁺ conventional memory cells, validating the anti-CD45RO antibody staining (**Figure 4A**, upper panel). To distinguish between the naïve cells and terminally differentiated effector memory (T_{EMRA}) phenotype among capsid specific CD45RA⁺ CD45RO⁻ T cells, we next looked at the expression of CCR7 (**Figures 4A,B**, right panels). CD45RA⁺/RO⁻ CCR7-positive naïve cells ranged from 53.8 to 93.67% (89.65% \pm 15.88 SD) of total AAV8-specific

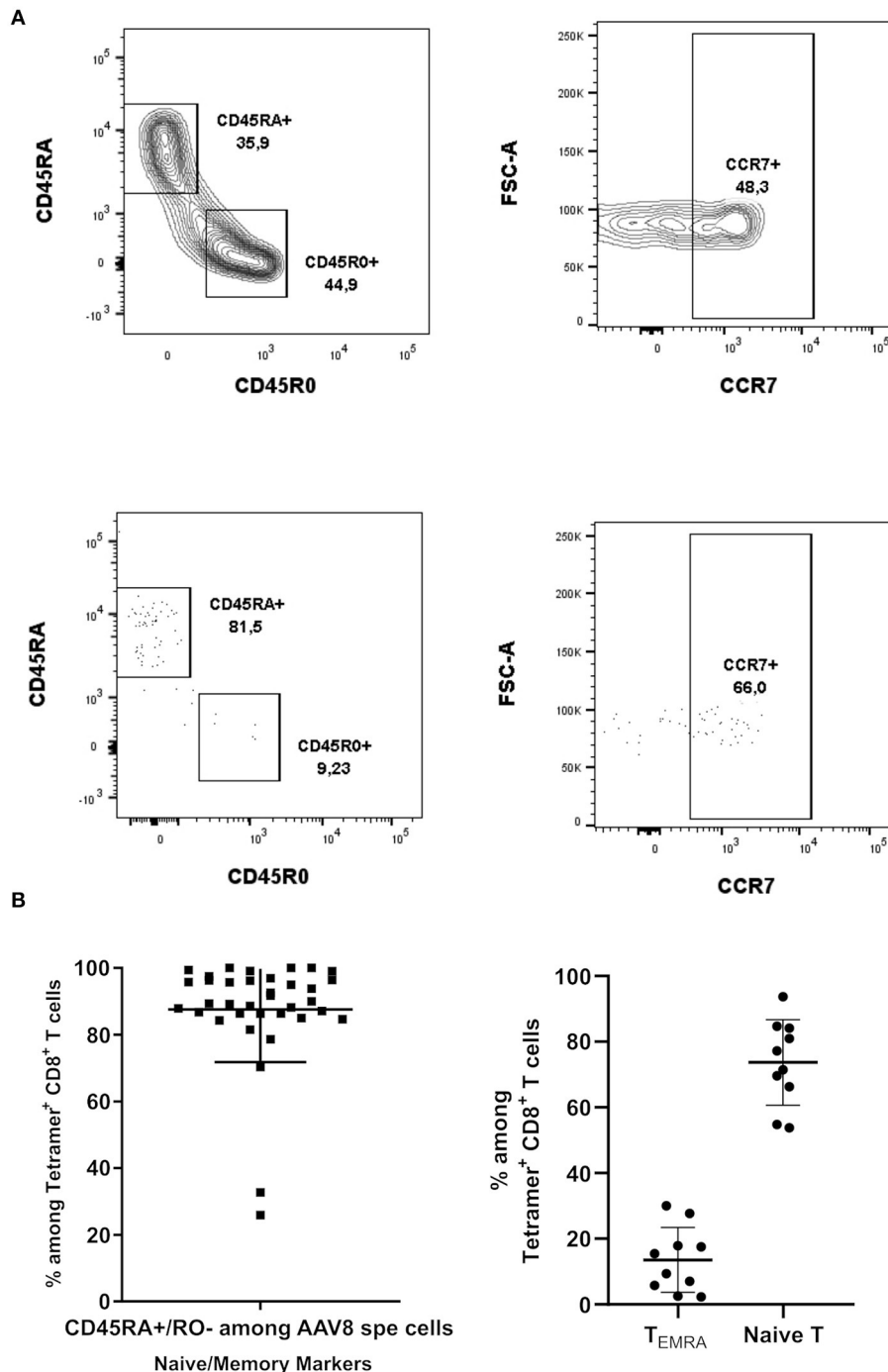


FIGURE 4 | Phenotypic characterization of AAV8-specific CD8⁺ T lymphocytes in PBMCs from A2⁺ and B7⁺ donors. **(A)** Representative dot plots of CD45RA, CD45RO, and CCR7 expression on Tetramer⁺ CD8⁺ T cells. Upper panel: cells were gated on total CD8⁺ T cells. Lower panel: cells were gated on Tetramer⁺ CD8⁺ T cells. **(B)** Overall phenotype of pAAV8 Tetramer⁺ CD8⁺ T cells. Percentages of AAV8-specific CD8⁺ T cells (isolated with AAV8 peptides pool tetramers) displaying a CD45RA⁺/RO⁻. Percentages of AAV8-specific CD8⁺ T cells (isolated with AAV8 immunodominant peptides tetramers) displaying a CCR7⁺ (Naive T cell) and CCR7⁻ (T_{EMRA}) phenotype among CD45RA⁺/RO⁻ T cells are represented. Each symbol represents an individual donor. Horizontal bars refer to median values.

CD8⁺ T cells, while CD45RA⁺ CD45RO⁻ CCR7-negative T_{EMRA} cells ranged from 2.27 to 30.07% (12.42 ± 9.8 SD) of total AAV8-specific CD8⁺ T cells. We therefore concluded that while most of the AAV8-specific CD8⁺ T

cells detected *ex vivo* had a naïve phenotype, all donors also displayed a subset of antigen-primed T_{EMRA} cells, a phenotype known to be associated with chronic viral infections in humans (23–25).

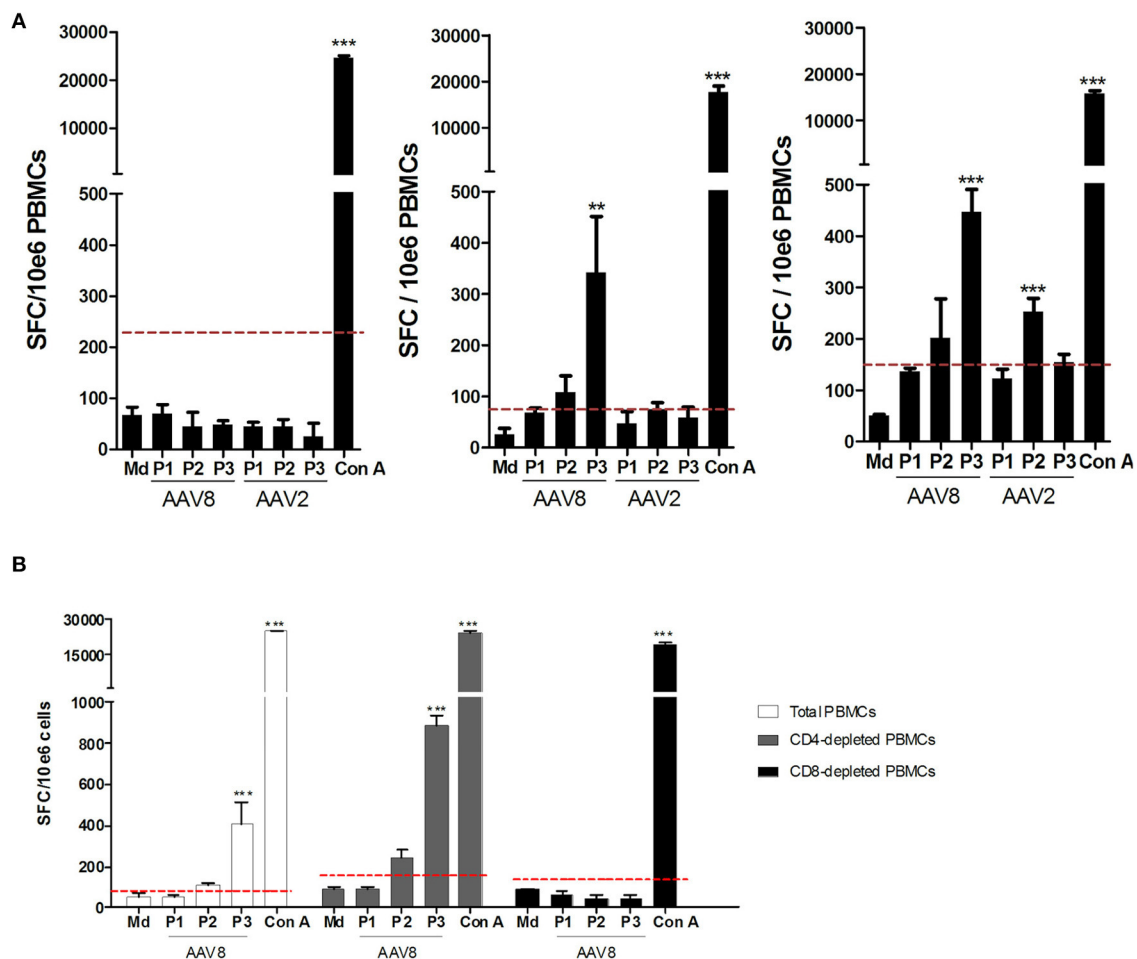


FIGURE 5 | Assessment of anti-AAV2 and anti-AAV8 IFN γ ELISpot responses on PBMCs from A2⁺ and B7⁺ donors. **(A)** Representative anti-AAV8 and anti-AAV2 IFN γ ELISpot responses. Anti-AAV2 and anti-AAV8 IFN γ ELISpot responses were assessed after 48H-stimulation of whole PBMCs from A2⁺ or B7⁺ donors with three pools of overlapping peptides (P1, P2, P3) covering the whole AAV2 or AAV8 capsid sequences. As negative control, PBMCs were cultured in medium alone (Md). Concanavalin A (Con A)-stimulated PBMCs served as positive control. Left: representative donor not responding to AAV2 nor AAV8. Middle: representative donor responding to AAV8 only. Right: representative donor responding to AAV2 and AAV8. Results are represented as the number of Spot Forming Cells (SFC) per million PBMCs. Positive threshold (dotted line) was calculated as three times the value obtained with medium alone or at least 50 SFC/10⁶ cells. Statistical significance was determined using the DFR(2x) statistical test. ** $p < 0.01$. *** $p < 0.001$. **(B)** Representative anti-AAV8 and anti-AAV2 IFN γ responses detected after CD4⁺ or CD8⁺ T cell depletion of PBMCs. CD4⁺ or CD8⁺ T cells were depleted from PBMCs of A2⁺ or B7⁺ donors through magnetic sorting, prior to IFN γ ELISpot assays which were performed as indicated above. Purity of CD4- or CD8-depleted fractions was assessed through flow cytometry (data not shown).

Ex vivo Frequencies of T_{EMRA} AAV8-Specific CD8⁺ T, but Not Total AAV8-Specific CD8⁺ T Cells, Show a Correlation With Anti-AAV8 IFN γ ELISpot Responses

We next wanted to parallel our results with the IFN γ ELISpot assay which is the conventional method used in clinical trials to monitor anti-AAV capsid cellular responses (26). Anti-AAV2 and anti-AAV8 IFN γ ELISpot assays were performed on all donors (A2⁺ and B7⁺) assessed through TAME using total PBMC as classically used for patient immunomonitoring. PBMCs from those donors were stimulated with 3 pools of overlapping peptides covering the whole AAV2 or AAV8 capsids (Figure 5A for a representative assay). Results from the whole cohort are

summarized in Table 2. Overall, 24.7% of the donors responded to AAV8 and 6.2% responded to AAV2. Unexpectedly, all donors responding against AAV2 responded also against AAV8. The majority of positive responses against AAV8 capsid were observed with peptide pools 2 and 3 (Table 2). Pool 2 contains the majority of peptides selected for AAV2-A2 tetramers (10/13) and for AAV8-A2 tetramers (5/9). Immunodominant peptides LIDQYLYYL and IPQYGYLTL are also in pool 2. Peptides selected for AAV8-B7 tetramers are contained in majority in pool 3 (5/12) and pool 2 (4/12) (Table S1). A more detailed analysis of our cohort revealed that B7⁺ donors seem to display a higher rate of positive anti-AAV8 IFN γ ELISpot responses compared to A2⁺ donors (40.0 vs. 28.6% responders, respectively), an observation that parallels the results obtained after TAME where higher *ex vivo* frequencies of AAV8-specific CD8⁺ T cells were observed in

TABLE 2 | Overview of anti-AAV8 and anti-AAV2 IFN γ responses assessed on PBMCs from A2⁺ and B7⁺ donors.

ANTI-AAV8 AND ANTI-AAV2 IFN γ RESPONSES						
HLA alleles	Donors tested	Positivity		Cross-reactivity		
		AAV2 positive	AAV8 positive	AAV2 only	AAV8 only	AAV2 and AAV8
A2 ⁺ /B7 ⁻	43	1 (2.3%)	5 (11.6%)	0	4 (9.3%)	1 (2.3%)
A2 ⁻ /B7 ⁺	15	2 (13.3 %)	8 (53.3%)	0	6 (40.0%)	2 (13.3%)
A2 ⁺ /B7 ⁺	7	1 (14.3 %)	3 (42.8%)	0	2 (28.6%)	1 (14.3%)
Total	65	4 (6.1%)	16 (24.6%)	0	12 (18.5%)	4 (6.2%)

ANTI-AAV8 IFN γ RESPONSES					
HLA alleles	Donors responding against AAV8	Pool 1	Pool 2	Pool 3	
A2 ⁺ /B7 ⁻	5	2 (40%)	4 (80%)	2 (40%)	
A2 ⁻ /B7 ⁺	8	0 (0%)	3 (37.5%)	8 (100%)	
A2 ⁺ /B7 ⁺	3	1 (33.3%)	1 (33.3%)	3 (100%)	
Total	16	3 (18.7%)	8 (50%)	12 (75%)	

ANTI-AAV2 IFN γ RESPONSES					
HLA alleles	Donors responding against AAV2	Pool 1	Pool 2	Pool 3	
A2 ⁺ /B7 ⁻	1	0 (0%)	1 (100%)	0 (0%)	
A2 ⁻ /B7 ⁺	2	0 (0%)	2 (100%)	1 (50%)	
A2 ⁺ /B7 ⁺	1	0 (0%)	1 (100%)	0 (0%)	
Total	4	0 (0%)	4 (100%)	1 (25%)	

B7⁺ donors (Table 2 and Figure 2B). It is nevertheless important to note that TAME and ELISpot methods have different read-outs (tetramer staining on one hand, IFN γ secretion on the other hand using total PBMC).

To further correlate the two assays, we first refined the analysis of anti-AAV cellular responses obtained through ELISpot assays by investigating which circulating lymphocyte sub-population (CD4⁺ or CD8⁺) was responsible for IFN γ secretion. Consequently, PBMCs from anti-AAV IFN γ ELISpot responders were tested a second time, but were subjected to CD4⁺ or CD8⁺ T cell depletion beforehand (Figure 5B for a representative donor). The efficiency of CD4 and CD8 depletions was confirmed through flow cytometry (purity from 94.27 to 99.71% for CD8-depleted fraction and 90.52 to 99.64% for CD4-depleted fraction). In all donors thus tested, CD4-depleted PBMCs responded in the same way as total PBMCs (with a higher sensibility even), while in CD8-depleted PBMCs the response was completely abrogated. These results suggest that AAV-directed IFN γ ELISpot responses were primarily mediated by CD8⁺ T cells.

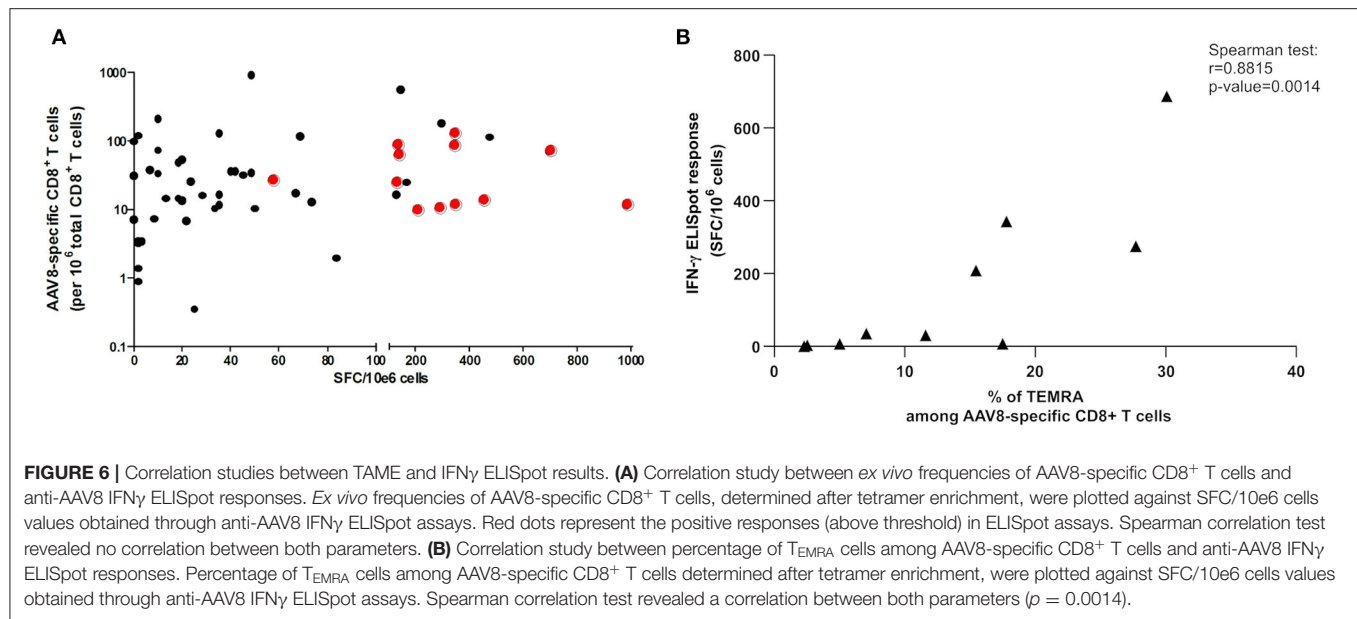
We then tested the possibility that anti-AAV responses detectable by IFN γ ELISpot assay stemmed from donors displaying the highest *ex vivo* frequencies determined after TAME. We therefore plotted tetramer⁺ AAV8-specific CD8⁺

T cell frequencies against anti-AAV8 IFN γ ELISpot responses (Figure 6A). We focused on AAV8 responses for which the total number of donors tested with tetramer pools reached 65 individuals. No obvious correlation could be found between the parameters assessed, and particularly, highest frequencies did not reflect in ELISpot responses above the threshold of positivity (represented by the red dots in Figure 6A).

Finally, we tested the correlation between the proportion of CD45RA⁺/RO⁻ CCR7⁻ T_{EMRA} memory cells after TAME selection and IFN γ ELISpot responses for AAV8 serotype. We observed a positive correlation between the two parameters validated through a Spearman correlation test (Figure 6B). This finding suggests that T_{EMRA} specific cells could be among the functional IFN γ -secreting T cell population after capsid AAV8 stimulation.

DISCUSSION

Up until now, phenotypical characterization of AAV-specific CD8⁺ T cells has almost exclusively relied on expansion or stimulation of PBMCs or splenocytes prior to phenotypical or functional assessment (10, 17, 18). Here, to circumvent biases inherent to *in vitro* cell amplification or cell stimulation (26), we used the highly sensitive pMHC-based tetramer enrichment method (TAME) to assess *ex vivo* frequencies and phenotypes of AAV2- and AAV8-specific CD8⁺ T cells using PBMCs of healthy A2⁺ and B7⁺ donors. We detected AAV2- and AAV8-specific CD8⁺ T cells in all donors tested (Figures 2A,B). Considering that anti-AAV seroprevalence studies have shown that at most 2/3 of human cohorts seem to have encountered WT AAV during their life (20), it is likely that we have detected naïve T cells that are not AAV-capsid antigen experienced as reported for other viral epitopes (13). Moreover, we cannot exclude that in some donors the tetramer-stained cells detected stem from potential HLA-cross reactive CD8 T cells as already shown for tumoral antigens using the same TAME method (27, 28). Indeed, a large number of cross reactive specific T cells recognizing epitopes within different proteins of the same pathogen, proteins from unrelated pathogens or even self-proteins have now been documented in both human or murine settings (22, 29–35). In our study, the detection of capsid tetramer-positive CD8 T cells in all donors might be explained by the fact that pools of tetramers relating to different epitope specificities were used for TAME, though when tetramer enrichment was performed using only the A2 or B7-restricted immunodominant epitope, both subsets were still detectable. The frequencies at which we detected AAV capsid-specific CD8⁺ T cells are coherent with other studies using TAME to assess pre-immune CD8⁺ T cell repertoires or virus-specific CD8⁺ T cells in the frame of chronic viral infections, i.e., situations where the frequencies of CD8⁺ T cells are too low to be evaluated using conventional flow cytometry (36–38). It is also likely that the frequencies of AAV-specific CD8⁺ T cells we calculated are somewhat underestimated, the main reasons being: (1) the potential loss of cells during the experimental procedure (14); (2) the AAV2- and AAV8-derived peptides selected to construct the tetramers



used for TAME (Table S1). Indeed, out of the many epitopes derived from AAV2 or AAV8 capsids, only the first 30 with the highest bioinformatics prediction scores were retained for UV-mediated peptide exchange screening. In the end, only 13 peptides served to construct pAAV2/A2 tetramers, nine peptides for pAAV8/A2's, and 12 peptides for pAAV8/B7's (Table S1). Of note, among the B7-restricted epitopes, our selection contained the immunodominant epitope identified by Mingozi et al. (10), a proof of the soundness of our bioinformatical epitope selection strategy. Therefore, we are far from covering the whole CD8⁺ T cell repertoire potentially associated to capsid antigenicity. Regarding serotype-specific response, the reactivity against the non-human primate serotype AAV8 in our cohort could also reflect epitope cross-reactivity as AAV8 capsid was shown highly cross-reactive with other AAV serotypes (10, 39). This is why we have not included A2-restricted AAV2 peptides in AAV8 peptide pools which could further underestimate specific CD8 T cell frequencies (Table S1).

During rAAV-based gene therapy clinical trials, destruction of transduced cells was shown to result from reactivation of capsid-specific cytotoxic CD8⁺ lymphocytes which had previously been established following natural infections with wild-type AAV (40). Consistent with this working hypothesis was the observation among different human populations that capsid-specific CD8⁺ T cells could exhibit a resting central memory phenotype (10), a central memory phenotype (17), or an effector memory phenotype (18). Nevertheless, up to now, phenotyping of capsid-specific CD8⁺ T cells has generally been performed after *in vitro* stimulation of PBMCs. Here, we addressed *ex vivo* phenotype of capsid-specific CD8⁺ T cells without prior stimulation and/or expansion, and we evidenced two subpopulations: naïve CD8⁺ T cells (CD45RA⁺/RO⁻ CCR7⁺) and T_{EMRA} effector memory CD8⁺ T cells (CD45RA⁺/RO⁻ CCR7⁻) (Figure 4). The discrepancy we observed with previous studies (where CD45RO⁺ cells were detected) could be explained

by the difference in human populations that were analyzed, but could also stem from the fact that we assessed phenotype *ex vivo*, while other studies did so after *in vitro* stimulation. Indeed, in our hands, when we expanded primary AAV8-specific CD8⁺ T cell lines from PBMCs, we observed a change in phenotype, with virtually all cell lines switching from CD45RA⁺/RO⁻ to CD45RA⁻/RO⁺ phenotype (data not shown). The detection of capsid-specific T_{EMRA} effector memory cells in the AAV context could help understanding the natural history of AAV infection in humans. T_{EMRA} effector memory CD8⁺ T cells represent the most differentiated memory T cell subset. They exhibit low proliferative capacities, but they express high levels of IFN γ and cytotoxic molecules, such as perforin and Fas ligand, upon TCR activation (26, 41). It is generally admitted that the presence of T_{EMRA} effector memory CD8⁺ T cells results from chronic exposure of CD8⁺ T cells to their antigens, as can be the case in particular clinical contexts such as graft-vs.-host disease (41), cardiac transplantation (42), chronic viral infections with CMV, HIV, or HCV (23–25) or in AAV infection (35). While the chronicity of WT AAV infection has never been clearly established, a putative evidence of its occurrence is the observation that a significant portion of individuals, aged >60 years old, who harbor anti-AAV1 IgG antibodies also exhibit anti-AAV1 IgM antibodies, an immunoglobulin subtype classically encountered during the course of a running infection (43, 44). Our own results seem to indicate that some AAV8-specific CD8⁺ T cells might be chronically exposed to their antigens, however whether this occurs through WT AAV reinfection cycles or through helper-mediated re-expression of capsid antigens from latent AAV genomes is still up to debate. The detection of whole infectious AAV particles in the bloodstream or in tissues would constitute the most accurate proof of ongoing AAV infections. However, this approach is hardly feasible considering the sensitivity of the detection techniques, and

instead it is the presence of infectious AAV DNA in cells that is assessed (45–51).

The presence of circulating naïve self-specific and virus-specific CD8⁺ T cells in contexts where these cells should have exhibited antigen experience has already been evidenced (27, 36). It is now generally known that the distribution of virus-specific CD8⁺ subsets (naïve, central memory, effector memory, T_{EMRA} effector memory) is closely related to the course of viral natural infection and virus wild-type's life cycle, particularly in cases where viruses are able to establish chronic infections or immune tolerance/exhaustion. A history of chronic and repeated infections could also shift CD8 T cell compartment toward the newly described virtual memory subpopulation (T_{VM}) in humans (52, 53). T_{VM} is an emerging T cell memory concept where T cells are phenotypically naïve-like (CD45RA⁺) whereas they harbor memory-like and antigen-specific features allowing an immune response. They were described as negative for CCR7 as T_{EMRA} cells and could be confounded with this T cell memory population. A potential contribution of T_{VM} cells cannot be excluded in our context but still remains to be explored. Additional markers should be used to differentiate true naïve and memory or memory-like T cells. Moreover, because very few things are known about the natural life cycle of WT AAV throughout lifetime (that might in addition differ among serotypes), it is still very difficult to infer immunological status to state of infection in the context of AAV-specific immunity.

To assess the specificity and functionality of AAV8 capsid-reactive CD8⁺ T cells, we sorted cells after TAME to expand primary AAV8-specific CD8⁺ T cell lines *in vitro*. Out of the 11 cell lines that we generated, 10 were responsive, i.e., were able to upregulate CD107, IFN γ and TNF α upon incubation with AAV8-loaded target cells (**Figures 3B,C** and **Table 1**). Three cell lines were also tested for cytotoxicity and exhibit a cytotoxic activity against AAV8 peptides loaded cells (**Figure 3B**). These results were in line with the results obtained by Kuranda et al. who observed secretion of granzyme B by T_{EMRA} and T_{EM} cells in response to stimulation with AAV2 peptides (35).

We noted higher, but not significant, *ex vivo* frequencies of AAV8-specific CD8⁺ T cells in B7⁺ donors compared to A2⁺ individuals, an observation paralleled for anti-AAV8 IFN γ ELISpot responses where responders were more frequent in B7⁺ than in A2⁺ subjects (**Figure 2A** and **Table 2**). Interestingly, the first report of AAV2 capsid-specific immunity in hemophilia B clinical trial happened to have been described in a B7⁺ patient (10). Whether B7⁺ individuals are significantly more likely to develop anti-AAV cellular responses, and as such should be more carefully monitored during clinical trials, is a question that remains to be investigated.

While TAME permitted the detection of AAV2- or AAV8-specific CD8⁺ T cells in A2⁺ or B7⁺ donors PBMCs, only 24.6% of donors on the whole were positive when evaluated by anti-AAV2 and anti-AAV8 IFN γ ELISpot assays (**Figure 2** and **Table 2**). Surprisingly, when assessed through IFN γ ELISpot assays, cellular anti-AAV8 responses appeared to be more frequent than anti-AAV2's in our cohort (**Table 2**). This is unexpected because: (1) the wild-type AAV8 serotype was first isolated from non-human primate samples, and is thought to

circulate at a lower rate among the human population (48); (2) according to the literature, AAV2 has always shown a higher seroprevalence than AAV8 in humans (20). Concerning AAV-directed cellular responses, all donors responding to AAV2 peptide pool stimulation also responded to AAV8, which again likely reflects a cross-reactive response, though not complete, between both serotypes, as discussed above, and has been already reported in other studies (39). In our study, anti-AAV8 IgG antibodies were detected in 51.7% of donors (median titer: 1:2,560) and neutralizing factors were detected in 50% of donors (median titer: 1:1,000) (**Table S3**). Overall, in our cohort, anti-AAV8 humoral responses surprisingly seemed to be more prevalent than in the literature where seroprevalence was generally reported between 20 and 40% (19, 20, 54, 55). A positive correlation was found between anti-AAV2 and anti-AAV8 IgG antibody titers (data not shown) confirming again the cross-reactivity between the two serotypes. When investigating the correlations between anti-AAV8 cellular and humoral responses, we found no correlation between antibody titers and *ex vivo* frequencies of AAV8-specific CD8⁺ T cells assessed by TAME (**Figure S2A**) or anti-AAV8 IFN γ ELISpot responses (**Figure S2B**). In addition, frequencies of AAV8-specific T cells assessed by TAME were found quite similar between AAV8 IgG positive and negative donors (**Figure S2C**). These observations parallel the results already reported for AAV1 (18) and AAV2 (10). Regarding the discrepancy between anti-capsid humoral vs. cellular immune responses, a recent study highlighted two new distinct profiles (56). Indeed, in seronegative individuals, they have shown transient activation of a subset of IFN γ -secreting CD16^{bright}CD56^{dim} NK cells known to rapidly respond to infection without prior sensitization. In contrast, seropositive “AAV-experienced” individuals presented capsid-specific memory CD8⁺ T cells that secrete TNF α upon capsid peptide stimulation.

Ex vivo frequencies of total AAV-specific CD8⁺ T cells were not predictive of the functionality of AAV-specific CD8⁺ T cells, at least in terms of IFN γ secretion, as we could not evidence any correlation between TAME results and anti-AAV IFN γ ELISpot responses. Appraisal of other parameters [TNF α as reported by Kuranda et al. (56), Granzyme B, Perforin, IL-10...] might have yielded to different conclusions. In addition, we noticed that depletion of CD4⁺ T cells prior to ELISpot assay noticeably increased the intensity of responses (which is expected as when CD4⁺ T cells are removed, CD8⁺ T cells are proportionally more frequent in samples), without increasing the background signal (**Figure 5B**). As such, CD4⁺ T cell depletion is a quick and an efficient method that might be used to enhance ELISpot detection sensitivity before or during rAAV-mediated clinical trials immunomonitoring.

Interestingly, we evidenced a correlation ($p = 0.0042$) between the proportion of T_{EMRA} effector memory cells within AAV-specific CD8⁺ T cells detected through TAME method and IFN γ positive ELISpot responses using total PBMCs. As we have shown that IFN γ secretion is mediated by CD8 T cells in all positive donors tested using CD4-depleted PBMC ($n = 16$, **Figure 5**), this correlation is likely evidenced between comparable T cell populations. Because TNF α was recently shown to play a role

in preexisting T cells immunity (56), it could be interesting to determine whether TNF α secretion by capsid specific CD8⁺ T cells is also related to a potential TEMRA phenotype. Whether anti-capsid effector responses reported in gene therapy trials is due to the reactivation of T_{EMRA} preexisting cells remains to be investigated. For instance, higher proportion of CD8⁺ T_{EMRA} cells in early phase of HIV infection were shown to be associated to superior antiviral activity and lower viral load (57). It remains important to better characterize AAV-specific T_{EMRA} CD8⁺ T cells to determine their functionality and how they are potentially recalled after administration of rAAV. Indeed, because AAV could result in a viral chronic infection setting as CMV or EBV with changes in immunological memory (24), T_{EMRA} CD8⁺ cells could have an exhausted/non-effector functional phenotype with PD1 regulation in some individuals or infection settings. This could explain why all patients displaying AAV positive IFN γ ELISpot assay did not show transaminitis and subsequent loss of transgene expression in hemophilia gene therapy trials (58).

In conclusion, overall, our study evidenced the difficulty to predict the impact of AAV capsid-specific cellular immunity on the safety and efficiency of rAAV-based gene transfer as it is not only a matter of detection sensitivity, but also a matter of functionality. Both qualitative and quantitative aspects of the CD8⁺ T-cell response need to be considered. Therefore, the development of assays combining both improved detection sensitivity and multiparametric polyfunctional assessment should prove to be the way to efficient clinical pre-screening and monitoring of patients. Emerging data from current and future rAAV-based clinical trials would help understanding T cell memory biology in the context of AAV immunity and its impact on the outcome of gene transfer following the delivery of recombinant AAV vectors. Ultimately, as others before us have underlined, setting up an animal model recapitulating observations done in humans is still an ongoing quest for the field.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Ethical approval was not required for this study on human participants because blood human samples from healthy donors

(cytapheresis) were obtained after written informed consent and under an ethical agreement with the local Etablissement Français du Sang (EFS Nantes, Pays de la Loire, agreement N° PLER NTS 2016-25). The samples are only used for a research purpose.

AUTHOR CONTRIBUTIONS

CV, XS, PM, and OA conceived the scientific strategy. CV, RX, LH, XS, MD, and OA conceived and designed the experiments. CV, RX, MD, NJ, MG, CC, and JL performed the experiments. CV, RX, MD, NJ, MG, CC, and OA analyzed the data. CV, RX, MG, OA, XS, and LH contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.03110/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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