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Use of Replication Restricted Recombinant Vesicular Stomatitis Virus Vectors for Detection of Antigen-Specific T Cells

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Abstract

Detection of antigen-specific T cells at the single-cell level by ELISpot or flow cytometry techniques employing intracellular cytokine staining (ICS) is now an indispensable tool in many areas of immunology. When precisely mapped, optimal MHC-binding peptide epitopes are unknown, these assays use antigen in a variety of forms, including recombinant proteins, overlapping peptide sets representing one or more target protein sequences, microbial lysates, lysates of microbially-infected cells, or gene delivery vectors such as DNA expression plasmids or recombinant vaccinia or adenoviruses expressing a target protein of interest. Here we introduce replication-restricted, recombinant vesicular stomatitis virus (VSV) vectors as a safe, easy to produce, simple to use, and highly effective vector for genetic antigen delivery for the detection of human antigen-specific helper and cytotoxic T cells. To demonstrate the broad applicability of this approach, we have used these vectors to detect human T cell responses to the immunodominant pp65 antigen of human cytomegalovirus, individual segments of the yellow fever virus polyprotein, and to various influenza proteins.

Keywords

VSV (vesicular stomatitis virus); ELISpot; intracellular cytokine staining (ICS); T cell assays; cytotoxic T cells; helper T cells

1. Introduction

The development and application of techniques such as MHC tetramer staining, ELISpot, and intracellular cytokine staining has transformed the study of T cell immune responses to microbes, tumors, auto-antigens, and vaccines. These assays—which permit detection of antigen-specific cells at the single cell level—do not require difficult to reproduce *in vitro* expansion protocols, and are widely regarded as the new “gold standards” for the characterization of T cell responses. When optimal peptide epitopes and their MHC restriction elements are mapped, MHC tetramers provide the most rapid method for detection of antigen-specific T cells and give direct access to physical phenotypes, but they

do not detect function. In contrast, the ELISpot and ICS assays detect a specific function—the ability to produce one or more cytokines upon short-term stimulation with antigen—and they are considerably more flexible with respect to the form and range of antigens that can be used in the assays. Neither the ELISpot nor the ICS assay requires mapping of MHC restriction elements or optimal MHC-binding peptide epitopes, and both assays are often performed with “complex” antigens that might contain multiple distinct epitopes that are recognized by the T cell population of interest.

Although a wide range of antigens may be used for ELISpot or ICS assays, each has one or more significant limitations. Antigens delivered in the form of recombinant proteins, microbial lysates, and lysates of infected cells are largely restricted to exogenous antigen-processing pathways, and are therefore effective for stimulation of CD4 T cells but ill-suited to efficiently stimulate CD8 T cells. Plasmid DNA has been used to deliver antigens for T cell assays (Tscharke et al. 2005), but since primary cells have low transfection efficiencies, its use requires highly transfectable cultured cell lines with an additional requirement for co-transfection with expression plasmids for one or more MHC alleles. Recombinant vaccinia viruses have been extensively used to deliver antigens for detection of antigen specific CD4 and CD8 T cells (Larsson et al. 1999; Xu et al. 2003), but their production requires lengthy protocols involving homologous recombination and multiple rounds of plaque purification. Furthermore, pre-existing immunity prevents their use in vaccinia-exposed individuals, including those who have received experimental vaccinia-based vaccines. Finally, pools of overlapping peptides representing entire proteins or even small viruses are now commonly used to stimulate both CD4 and CD8 T cells, but it is not economically feasible to routinely purchase all of the peptides that are required to contain all possible epitopes for large viruses, such as those in the pox and herpesviridae families, or for broad coverage for highly variable viruses, such as HIV or HCV. Under defined and common circumstances, each of these forms of antigen has significant theoretical and/or practical limitations, and new options for antigen delivery are needed.

Our search for a suitable antigen-delivery system began with the following criteria. (1) The antigen-delivery system had to be capable of stimulating both CD4 and CD8 T cells. (2) It had to do so using only fresh, *ex vivo* cell populations such as PBMC, without recourse to cultured stimulator cells, such as B lymphoblastoid cell lines (B-LCL). In practice, this narrowed the search to viral vectors. (3) Once the search was narrowed to recombinant viral vectors, they had to be easy to produce from plasmid DNA, without recourse to systems that require homologous recombination (effectively ruling out poxviruses). (4) The viral vector had to have reasonably broad tropism, both at the cellular and species level (it would be desirable if we could use the same constructs in mice, non-human primates, and humans). (5) There had to be little-to-no pre-existing immunity to vector epitopes; this made us leery of adenoviral vectors, because although many of them express negligible amounts of vector antigens upon infection, it is possible that the input adeno structural proteins could stimulate cells, especially CD4 T cells. (6) The vector should have the capacity to accommodate reasonably large insert sizes (up to 4 kb). (7) The vector had to give high levels of antigen expression early after infection. (8) The vector should have relatively low cytopathicity. (9) The vector should be inherently safe, at least at the BSL2 level. One virus that seems to meet all of these criteria is vesicular stomatitis virus (VSV).

Vesicular stomatitis virus is a member of the Rhabdoviridae family. It has an extremely compact, nonsegmented negative strand RNA genome with five non-overlapping genes coding for viral proteins. Reverse genetics systems for efficient production of recombinant VSV from plasmid DNA were developed in the mid-1990s by the laboratories of Rose and Wertz (Whelan et al. 1995; Lawson et al. 1995). These techniques were originally designed to genetically manipulate the VSV genome for RNA virus assembly and replication studies.

Further development of these methods soon made it possible to construct recombinant VSV encoding a foreign protein of interest in place of the viral glycoprotein (VSV-G) in the VSV genome (VSV-ΔG). VSV-ΔG vectors are safe for routine laboratory use, because even though the recombinant virions are coated with VSV-G, they lack VSV-G in the recombinant genome and are thus capable of only one round of replication. Additionally, VSV-ΔG has a relatively large insert capacity (≥4 kb). For these reasons, we have used VSV-ΔG as a vector to introduce antigen to T cells. We generated a panel of VSV-ΔG constructs encoding various viral antigens and have developed a method to employ these constructs for antigen delivery in T cell ICS and ELISpot assays. Our results demonstrate that VSV-ΔG vectors represent an alternative and efficient antigen source for detecting antigen-specific T cells.

2. Materials and Methods

2.1. ELISpot

The IFN-γ ELISPOT assay was performed as previously described (Larsson et al. 1999; Murali-Krishna et al. 1998). 2×10^5 to 5×10^5 human PBMC from a CMV-seropositive donor were infected with a recombinant VSV expressing the pp65 gene of human cytomegalovirus (VSV-ΔG.CMVpp65). PBMC from the same donor were also infected with a recombinant VSV encoding the nucleoprotein gene from lymphocytic choriomeningitis virus (VSV-ΔG.LCMV-NP). Since LCMV infection is uncommon in human populations, VSV-ΔG.LCMV-NP infection was not expected to stimulate cytokine production, and this vector was used as a negative control virus for our studies in human subjects.

2.2. Plasmid Construction

Plasmid pVSV-ΔG is a Bluescript-based plasmid that encodes the anti-genome RNA of VSV. In this plasmid, the coding region for VSV-G has been removed and replaced with a polylinker (Takada et al. 1997). Genes of interest were inserted into the polylinker region of pVSV-ΔG using *Kpn I*, *Sph I*, and *Asc I* restriction enzyme sites. For some constructs, a gene of interest was inserted into pVSV-ΔG using a ligation-independent cloning (LIC) method (Aslanidis and de Jong 1990). Briefly, the polylinker region of pVSV-ΔG was replaced with a LIC sequence that upon linearization with *Sma I* and subsequent treatment with T4 DNA polymerase and dATPs yields specific overhangs. In the absence of ligase, these overhangs anneal with complimentary sequences flanking genes of interest that have been amplified with LIC primers and treated with T4 DNA polymerase and dTTPs. The DNA primers used to amplify genes of interest from plasmid DNA or cDNA are listed in Table 1.

2.3. Recovery of Recombinant VSV-ΔG

Recombinant VSV-ΔG was produced as previously described (Takada et al. 1997; Robison and Whitt 2000; Whitt 2010). Briefly, baby hamster kidney cells (BHK-21;American Type Culture collection) were maintained in Dulbecco's modified Eagle's medium (DMEM)/5% fetal bovine serum (FBS) at 37°C. BHK-21 cells on 6-well plates (~95% confluent) were infected with recombinant modified vaccinia virus Ankara (MVA) expressing T7 polymerase (MVA-T7) at a multiplicity of infection (MOI) of approximately 1. Following a 90 min. incubation, plasmids encoding VSV-N, P, L, and G proteins and pVSV-ΔG containing a gene of interest (VSV-ΔG) were transfected at a ratio of 3:5:1:8:5, respectively, into the cells by using a liposome suspension of dimethyldioctadecyl ammonium bromide and L-α-dioleoylphosphatidylethanolamine for 4 hours. 48 hours after transfection, supernatants were 0.22μm syringe-filtered (to remove MVA-T7) on to BHK-21 cells previously transfected with pCAGGS-VSV-G (~85% confluent) using Lipofectamine reagent (Invitrogen). VSV-G must be introduced in trans, because recombinant VSV-ΔG

does not produce the viral glycoprotein. Following 24 to 48 hour incubation, successful recoveries were indicated by visualization of extensive cell rounding/cytopathic effect. Following recovery, virus was amplified on VSV-G-transfected BHK-21 cells, and viral titers were determined via plaque assay.

2.4 Cryopreservation and Thawing of PBMC

Following PBMC isolation, cells were washed with RPMI-1640/10% FBS (R10) and resuspended at a final concentration of 5–10^7/ml in R10 plus 10% DMSO (Sigma) in freezer vials. The cells were placed in a freezing container (Nalgene) and transferred to a −80°C freezer overnight before transfer to liquid nitrogen. Cells were thawed in a 37°C water bath and washed with R10. After washing, cells were resuspended in R10 and incubated overnight in a 37°C incubator. On the following day, cells were again washed with R10 prior to assay.

2.5. Specimen Preparation and Assay Setup

Donor blood samples were collected in sodium citrate cell preparation tubes (Vacutainer, BD) and centrifuged at 1500g for 30 min. PBMC were then processed as directed by manufacturer and resuspended at 10^7/ml in R10. In a 5ml polypropylene tube, 10^6 human PBMC were infected with VSV-ΔG viral supernatant in a total volume of 200μl at an MOI of 10 unless otherwise noted. Costimulatory antibodies CD28/CD49d (FastImmune, BD) were added to each sample at a final concentration of 1μg/ml. A cytokine blocking reagent, GolgiPlug (Brefeldin A, BfA; BD), was added 4 hr post-infection, unless otherwise specified. Samples were then incubated overnight at 37°C/5%CO_2 and were processed for flow cytometry analysis on the following day. In some experiments, pools of overlapping CMVpp65 peptides (a kind gift from Holden Maecker) were used for PBMC stimulation at a final concentration of 1.75μg/ml. For these samples, BfA was added at the time of stimulation, unless otherwise stated.

2.6. Surface Staining and Intracellular Cytokine Staining

Monocyte and dendritic cell (DC) tropism: PBMC infected with a recombinant VSV expressing GFP (VSV-ΔG.GFP) for 6 hr were washed and assayed via flow cytometry. CD14-APC (Clone RM052, IO Test) was used to visualize monocytes. DCs were defined as staining positive for CD11c-APC (BD) and HLA-DR PerCP (BD) and negative for a lineage cocktail composed of (CD20, CD3, CD56, CD16, CD14)-PE (all BD). For monocyte viability studies, propidium iodide (BD) was included for live/dead discrimination.

T cell cytokine assays: Following VSV-ΔG infection and incubation, human PBMC were washed with PBS and permeabilized with FACS Permeabilizing Solution 2 (BD). Following permeabilization, the cells were again washed with PBS and stained with CD4-FITC (Coulter), CD3-PE (Coulter), CD8-PerCP (Coulter), and IFNγ-APC (BD). Samples were acquired on a FACSCalibur or LSRII flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.7 Sucrose Purification of Recombinant VSV-ΔG Viral Supernatant

A 30% sucrose in PBS solution was added to 38.5ml Ultra-Clear™ tubes (Beckman). Viral supernatant was slowly added to the tubes in order to prevent disruption of the sucrose layer. The tubes were then centrifuged for 90 min at 60,000 × *g* in an Optima™ L-70K ultracentrifuge (Beckman) using an SW-28 rotor (Beckman). After centrifugation, the supernatant was removed, and purified virus was resuspended in 500μl R10 before storage.

2.8. *In Vitro* T Cell Proliferation (CFSE)

A solution of 0.6μM CFSE in PBS was prepared. Human PBMC were washed and resuspended in PBS at a concentration of 0.5 to 1×10^7/ml. Equal volumes of PBMC and 0.6μM CFSE were mixed and incubated for 7 min at room temperature in the dark, vortexing every other minute. The CFSE-labeled PBMC were then washed twice with R10 to block CFSE and resuspended in R10 at a final concentration of 1×10^6/ml. 1ml CFSE-labeled PBMC was added to 5ml polypropylene tubes and infected with VSV-ΔG at an MOI of approximately 10 or stimulated with staphylococcal enterotoxin B (SEB, Sigma) as a positive control. Samples were then incubated at 37°C/5%CO_2 for 6 days. Following incubation, the cells were washed and stained with a live/dead discriminatory dye as well as CD8-PE (BD), CD4-APC (BD), and CD3 PacBlue (BD). Samples were acquired on a LSRII flow cytometer and analyzed using FlowJo software.

3. Results

3.1. VSV is Monocyte and Dendritic Cell Tropic

To determine the cell types in human PBMC that express an antigen of interest delivered by recombinant VSV-ΔG and are thus potentially capable of presenting antigen to T cells, we infected human PBMC with a recombinant VSV vector expressing GFP (VSV-ΔG.GFP; Fig. 1A). Following a 6 hr incubation, infected PBMC were stained for analysis by flow cytometry. GFP expression was found predominantly in $CD14^+$, $CD4^{low}$ cells, indicating a tropism for monocytes. GFP expression was also noted in an otherwise ungated $CD11c^+$ population, raising the possibility of VSV tropism in dendritic cells (DCs), which was demonstrated in Figures 1B and 1C. Little-to-no GFP expression was found in T, B, or NK cells (Fig. 1A). In short term culture (6 hr), GFP expression was not accompanied by significant cell death (data not shown). Kinetics studies were performed to determine the peak of GFP expression in monocytes and DCs (defined in this experiment as Lin^-, $HLA\text{-}DR^+$, $CD11c^+$ events) infected with VSV-ΔG.GFP. In monocytes, the peak of GFP expression occurred earlier (0–6 hr) than in DCs (6–20 hr; Fig. 1B, 1C). These results were observed in an additional donor and suggest that monocytes are the chief presenters of antigen in early *ex vivo* infection with VSV. Furthermore, DCs are a significantly more rare population in PBMC compared to monocytes.

We infected fresh PBMC with VSV-ΔG.GFP at various MOI (0.4–25) to maximize the percentage of monocytes expressing GFP and determine the effect on monocyte viability. As expected, GFP expression was proportional to MOI, but importantly, monocyte viability was similar at each MOI, indicating that VSV infection does not induce extensive cytopathology after 6 hr in *in vitro* cultures (data not shown). In an attempt to increase infectivity, experiments were conducted in the presence of polybrene, a cationic polymer that may serve to cluster virus and increase the number of particles that come into contact with infectable cells. The percentage of GFP positive monocytes increased only slightly when infected PBMC were incubated in the presence of polybrene (data not shown), and as a result, polybrene was not used in future experiments.

We tested the infectivity and viability of monocytes in fresh vs. cryopreserved PBMC for three donors, and we determined that monocyte viability following cryopreservation was similar to that of fresh PBMC (Supplementary Fig. 1). When compared to fresh PBMC, infection of cryopreserved PBMC with VSV-ΔG.GFP resulted in decreased but easily detectable percentages of monocytes expressing GFP (Fig. 1D). The average percentage decrease in GFP expression in the cryopreserved samples was 32%. These results indicate that while fresh PBMC are presumably optimal in VSV-ΔG assays, cryopreserved PBMC are also appropriate for use.

3.2. IFNγ ELISpot Assay

As an initial test of whether antigen delivered by VSV-ΔG could stimulate the production of cytokines by antigen-specific T cells, we employed an IFNγ ELISpot. Human PBMC from a CMV-seropositive donor were infected with several dilutions of VSV-ΔG.CMVpp65. The CMVpp65 gene codes for a tegument protein that has been previously characterized as highly immunodominant (Wills et al. 1996). PBMC from the same donor were also infected with a negative control construct, VSV-ΔG.LCMV-NP. In PBMC infected with VSV-ΔG.CMVpp65, an assayable number of spots representing individual IFNγ-producing T cells was observed. Samples infected with VSV-ΔG.LCMV-NP displayed only background levels of cytokine production equivalent to that of uninfected controls, demonstrating that the observed cytokine production was entirely dependent upon the CMVpp65 insert (data not shown).

3.3. Optimization of Time of Brefeldin A Addition

A limitation of the ELISpot assay is that it does not permit the direct discrimination between responding CD4 and CD8 antigen-specific T cells. In light of this, a flow cytometry-based intracellular cytokine staining (ICS) protocol was developed. 10^6 PBMC from a CMV-seropositive donor were infected with VSV-ΔG.CMVpp65 at an MOI of approximately 10. In parallel, PBMC from the same donor were stimulated with an overlapping 15-mer CMVpp65 peptide pool. Infection and CMVpp65 peptide pool stimulation were followed by Brefeldin A (BfA) addition at various time points. BfA is commonly used in ICS assays as a means to block cytokine secretion (Jung et al. 1993), but it has also been shown to inhibit cell surface presentation of class I MHC molecules (Yewdell and Bennink 1989). Thus, it was essential to determine the optimal time post-infection to add BfA in order to generate maximum IFNγ secretion by antigen-specific T cells. Following BfA addition and overnight incubation, IFNγ production was assayed via ICS and analyzed via the gating strategy depicted in Fig. 2A. CD4 T cells in the VSV-ΔG.CMVpp65-infected samples responded optimally through the 0–3 hr time points, with declines of ~30% and ~60% at 4 and 5 hours respectively. CD8 T cell responses were not detected until 3 hours and peaked at the 4–5 hour time points (Fig. 2B). Similar results were obtained after screening two additional donors (Supplementary Fig. 2). In all subsequent experiments, BFA was added at 4 hr post-infection, which represents a compromise for optimal detection of both CD4 and CD8 responders. In the CMVpp65 peptide pool-stimulated samples, optimal CD4 and CD8 T cell cytokine responses were attained when BfA was added at the time of stimulation (0 hr), consistent with previously published reports (Maecker et al. 2001). For the VSV-ΔG.CMVpp65-infected samples, the percentages of IFNγ-producing CD4 and CD8 T cells at the 4 hr time point was 0.5% (CD4) and 0.61% (CD8). These frequencies are somewhat smaller than those of the CMVpp65 peptide pool-stimulated samples at the optimal 0 hr time point (0.86%, CD4; 0.74%, CD8). Additionally, by comparing the positions of the IFNγ-producing CD4 and CD8 T cells overlaid on bulk T cell populations in CMVpp65 peptide pool-stimulated versus VSV-ΔG.CMVpp65-infected samples, we observed that the levels of CD4 and CD8 down-regulation were greater in the samples that were stimulated with the CMVpp65 peptide pool. This could be an indication that the CMVpp65 peptide pool is a stronger T cell stimulator than VSV-ΔG.CMVpp65, which may reflect previously published reports that potent inducers of T cell responses promote TCR down-regulation (Valitutti et al. 1995).

Cytokine production resultant from VSV-ΔG.CMVpp65 infection was compared in fresh vs. cryopreserved PBMC for two donors. Our results demonstrated that the frequencies of IFNγ-producing CD4 and CD8 T cells were only slightly diminished following infection of cryopreserved samples (Fig. 2C), a desirable result given the interest in assessing T cell responses in cryopreserved samples, such as from a vaccine clinical trial.

3.4. Effects of Carryover Antigen

For samples infected with VSV-ΔG.CMVpp65, IFNγ production by CD4 T cells was detected when BfA was added at the time of infection, suggesting that IFNγ production at this time point might be the result of carryover CMVpp65 antigen in our viral preparations. This was not unexpected as amplification of VSV vectors on BHK-21 cells presumably leads to some cell lysis as well as recombinant virus production, resulting in the subsequent release of antigen into the viral supernatant. To determine the contribution of carryover CMVpp65 antigen, we infected human PBMC from a CMV seropositive donor with VSV-ΔG.CMVpp65 viral supernatant at an approximate MOI of 5 under the following conditions: unpurified viral supernatant, unpurified/UV-inactivated virus, sucrose-purified virus, and sucrose-purified/UV-inactivated virus. BfA was added at 0, 2, and 4 hr post-infection, followed by overnight incubation. Whole protein antigen is known to be a very weak inducer of CD8 T cell responses, and as expected, UV inactivation of viral supernatant eliminated CD8 T cell responses (Fig. 3), indicating that these responses were driven by newly synthesized antigen resulting from VSV-ΔG.CMVpp65 infection. Carryover CMVpp65 antigen does play a significant role in generating CD4 T cell responses, as indicated by the production of IFNγ by CD4 T cells infected with UV-inactivated VSV-ΔG.CMVpp65 (Fig. 3). However, by comparing the frequencies of IFNγ-producing CD4 T cells in PBMC that were infected with sucrose-purified virus stock versus PBMC infected with sucrose-purified/UV-inactivated virus stock, it is demonstrated that VSV-ΔG.CMVpp65 infection is capable of generating detectable CD4 T cell responses.

3.5. MOI Optimization

Infection of human PBMC with recombinant VSV at an MOI of 25 results in optimal APC infection (data not shown). To determine if this MOI leads to maximum IFNγ production by responding T cells, we infected human PBMC from two CMV-seropositive donors with VSV-ΔG.CMVpp65 at an MOI range from 0.4–25. BfA was added 4 hr post-infection. For each donor tested, an MOI of 12.5–25 generated the most robust IFNγ responses by antigen-specific CD4 and CD8 T cells (Fig. 4A, B), although cytokine production was detectable at an MOI of less than 1. Based on these results, future assays were performed at a high MOI (≥10). To test for intra-assay variability, we performed several replicates of VSV-ΔG.CMVpp65 assays on two additional CMV-seropositive donors at an MOI of 10, and it was demonstrated that antigen delivery via the VSV-ΔG system yields highly reproducible results (Supplementary Fig. 3A, B). Similar high levels of reproducibility were seen in a donor with strong responses to the NP antigen from influenza A/Cal/04/09 (Supplementary Fig. 3C).

3.6. Range of Antigens

We have used recombinant VSV vectors to detect T cell responses resulting from vaccination with the yellow fever virus vaccine strain 17D (YFV-17D). YFV-NS3 and YFV-NS4 are nonstructural YFV proteins, one of which (NS3) has been shown to contain a dominant CD8 T cell epitope in YFV 17D-immunized mice (van der Most et al. 2002). PBMC from a YFV-17D-vaccinated individual (day 60 post-vaccination) were infected with VSV-ΔG constructs expressing YFV-NS3 (VSV-ΔG.YFV-NS3) and YFV-NS4A4B (VSV-ΔG.YFV-NS4A4B). We were able to detect IFNγ-producing CD4 and CD8 T cells specific for YFV-NS4A4B as well as CD8 T cell responses to YFV-NS3 (Figure 5A, B).

We also generated VSV-ΔG constructs encoding various influenza genes, including HA from A/New Caledonia/20/99 (A/NC/20/99) and B/Florida/04/06 (B/Fl/04/06) as well as internal influenza proteins, matrix protein 1 (M1) and nucleoprotein (NP) from swine-origin 2009 H1N1 influenza virus, A/California/04/09 (A/Cal/04/09). PBMC from a healthy adult donor were infected with VSV-ΔG.A/NC/20/99-HA, and PBMC from a donor vaccinated

with the 2008–2009 seasonal influenza vaccine were infected with VSV-ΔG.B/Fl/04/06-HA (day 14 post-vaccination; Fig. 5C,D). ICS analysis demonstrated that both vectors were capable of stimulating IFNγ production by influenza-specific CD4 T cells. We rarely detected CD8 responses to HA-expressing VSV-ΔG vectors (data not shown). This result was not unexpected, as recently published data indicates that CD8 T cell responses do not typically target influenza HA (Assarsson et al. 2008). We assayed PBMC from a healthy adult donor with VSV-ΔG constructs encoding M1 and NP from A/Cal/04/09 and were able to detect both CD4 and CD8 responses directed against these internal influenza proteins (Fig. 5E,F); these specific cells are likely to be cross-reactive against various influenza strains. In additional assays using an A/Cal/04/09 NP-expressing VSV-ΔG construct, variability within the assay was low (Supplementary Fig. 3c). Overall, these experiments demonstrate that the VSV-ΔG system is appropriate for investigating humans T cell responses across a broad range of antigens that are unrelated in sequence.

3.7. VSV-ΔG-induced Proliferation of Antigen-specific T cells

We determined that VSV-ΔG constructs are capable of effectively stimulating antigen-specific T cells in ICS assays. As an alternative to an ICS-based assay in elucidating antigen-specific T cells, we tested the ability of VSV-ΔG vectors to induce T cell proliferation *in vitro*. PBMC from a CMV-seropositive donor were labeled with CFSE and infected with VSV-ΔG.CMVpp65 or VSV-ΔG.LCMV-NP at an MOI of approximately 10. At 6 days post-infection, the percentage of $CFSE^{low}$ CD4 and CD8 T cells in samples infected with VSV-ΔG.LCMV-NP was equivalent to baseline levels observed in uninfected controls (Fig. 6A). In the samples infected with VSV-ΔG.CMVpp65, approximately 6% and 26% of CD4 and CD8 T cells, respectively, were $CFSE^{low}$ (Fig. 6A), demonstrating that VSV-ΔG vectors are capable of driving the proliferation of antigen-specific T cells. Additionally, we have used VSV-ΔG vectors to induce the proliferation of low frequency influenza-specific CD8 T cell populations (Fig. 6B). PBMC were prepared from a donor that was previously shown to possess CD4 and CD8 T cell populations specific for various A/California/04/09 gene products, including polymerase subunit B1 (PB1) and the viral nucleoprotein (NP; data not shown). These PBMC were infected with VSV-ΔG constructs encoding NP and PB1 from A/California/04/09 and cultured for 6 days. While both VSV-ΔG-influenza constructs were able to drive the proliferation of influenza-specific CD8 T cells, we were unable to detect the proliferation of CD4 T cells above background levels (Fig. 6B). It is possible that various culture manipulations, such as CD8 T cell depletion and the addition of cytokines, may promote a more favorable environment for the proliferation of CD4 T cells, but we have yet to explore these conditions.

4. Discussion

ELISpot and ICS assays are widely regarded as the most powerful tools for detecting antigen-specific T cells and studying their function. Traditional antigens for ELISpot and ICS assays include whole protein, viral lysates (Waldrop et al. 1997), plasmid DNA (Tscharke et al. 2005), recombinant vaccinia (Larsson et al. 1999; Xu et al. 2003), and overlapping peptide pools (Maecker et al. 2001), but these reagents each have significant limitations. Our aim was to develop an antigen delivery method that addressed these limitations while satisfying the previously discussed set of criteria established to ensure maximum assay utility. The most practical method for stimulating both CD4 and CD8 responses in PBMC would employ the use of antigen delivery by viral vectors, but recombinant vaccinia and adenovirus are less than ideal vectors due to the high frequency of the general population with pre-existing immunity. All things taken into consideration, the most promising candidate was recombinant VSV-ΔG.

VSV has been reported as capable of infecting a broad range of cell types (Coil and Miller 2004). We determined that following infection of PBMC with VSV-ΔG.GFP, the predominant GFP^+ cell type was monocytes while GFP expression in DCs was apparent but delayed by several hours. GFP expression was not seen in T cells, B cells, and NK cells, and though it is entirely possible that T cells were infected with VSV-ΔG, it was beyond the scope of our studies to differentiate between viral entry and neo-antigen expression. For our purposes, it was significant that T cells were not eliminated to a large extent in early infection and that antigen expression was limited to antigen-presenting cells. ICS assays were performed to measure IFNγ production followed overnight infection with recombinant VSV-ΔG, and in all of our studies, T cell viability remained high, consistently providing ample cells for the detection of antigen-specific populations (data not shown).

Optimization of recombinant VSV-ΔG vectors for use in ICS assays was dependent upon two key parameters: the time of BfA addition to infected PBMC and optimal MOI. Maximum IFNγ responses in CD4 and CD8 T cells were observed when BfA was added 3–4 hr following infection at an MOI of 10, a time point and MOI that is common in assays that employ recombinant vaccinia viruses for antigen delivery (Xu et al. 2003). Kinetic studies of monocyte and DC infection demonstrated that while a large percentage of monocytes express antigen delivered by VSV-ΔG 4 hr post-infection, few, if any, DCs expressed antigen at this time point. Given that addition of BfA 4 hr post-infection would be expected to largely inhibit surface presentation of MHC, this result is suggestive that monocytes are primarily responsible for activating antigen-specific T cell populations in overnight VSV-ΔG T cell assays.

Overlapping peptide pools are among the most commonly used and effective reagents for stimulating both CD4 and CD8 T cells in *ex vivo* populations. Recombinant VSV-ΔG vectors performed comparably to overlapping peptide pools in ICS assays, a highly desirable result when considering the well-established effectiveness of overlapping peptide pools in detecting antigen-specific T cell populations. The primary limitation of peptide pools is that the cost of synthesis could potentially make their use economically prohibitive in some studies, such as vaccine trials that commonly generate massive numbers of samples to be processed or when mapping immunodominant epitopes in moderately large pathogens. VSV-ΔG replicates to a high titer in culture (10^8 to 10^9 infectious units/ml, typically), and expansion in a single 35mL tissue culture flask typically yields sufficient virus stock to perform hundreds of assays. This represents a significant advantage of recombinant VSV-ΔG in T cell assays – the potential to quickly and easily generate a large panel of reliable and effective reagents at little financial expense.

In addition to detecting T cell responses to immunodominant viral epitopes from HCMV in humans, VSV-ΔG vectors were effective in detecting T cell responses to YFV and influenza vaccination. Indeed, we believe that VSV-ΔG is an ideal tool for evaluating essentially any candidate vaccine designed to elicit T cell responses. Recombinant VSV-ΔG vectors are easy to produce, inexpensive, and potent stimulators of *in vitro* recall responses by antigen-specific T cells. Our results clearly demonstrate that these vectors represent a powerful alternative to traditional methods for stimulating antigen-specific T cells in ELISpot and ICS assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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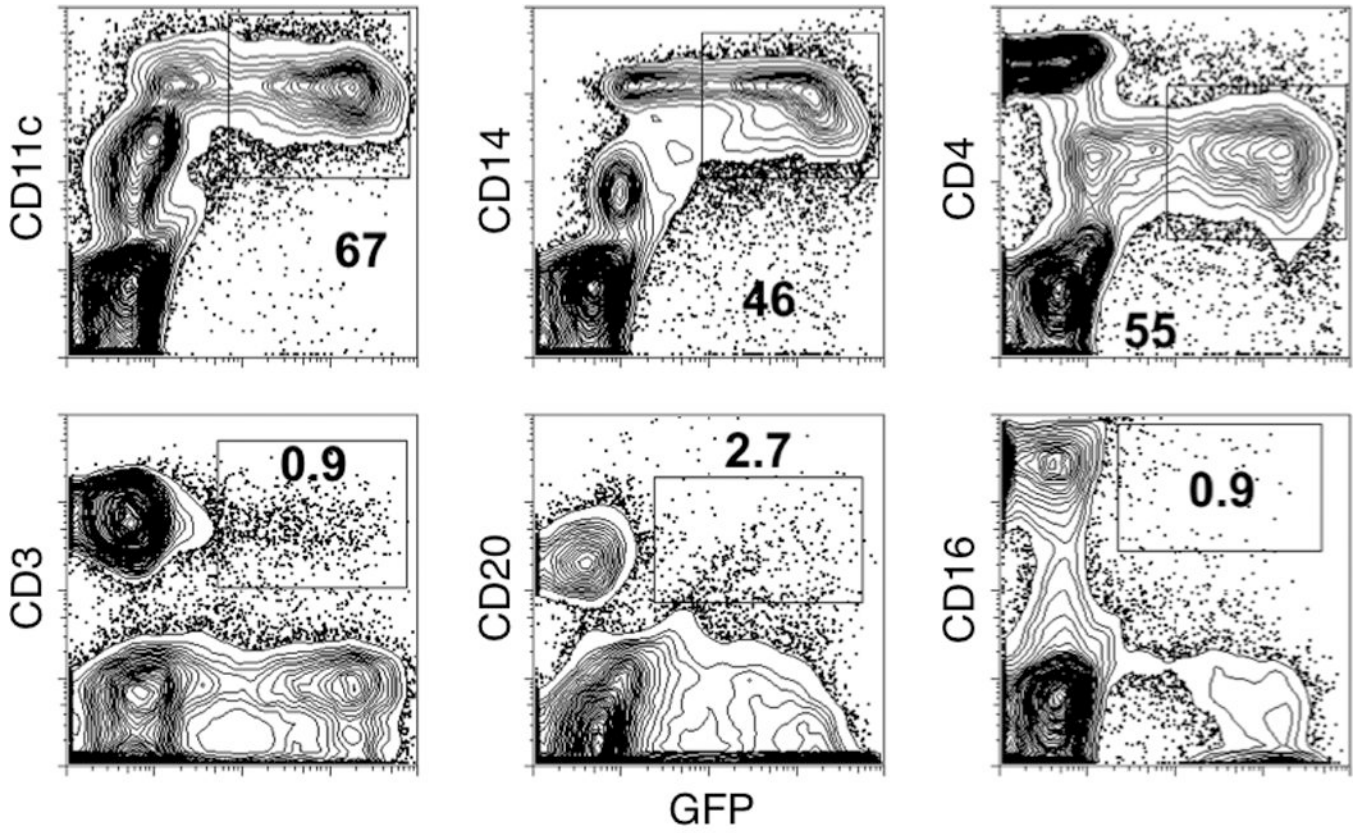

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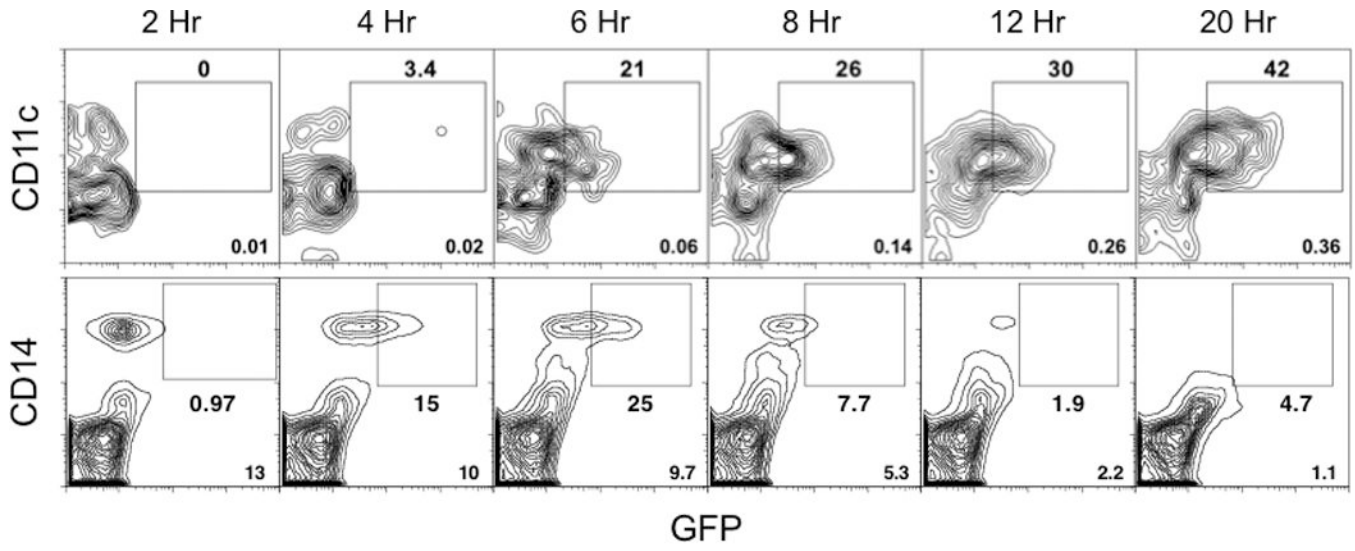

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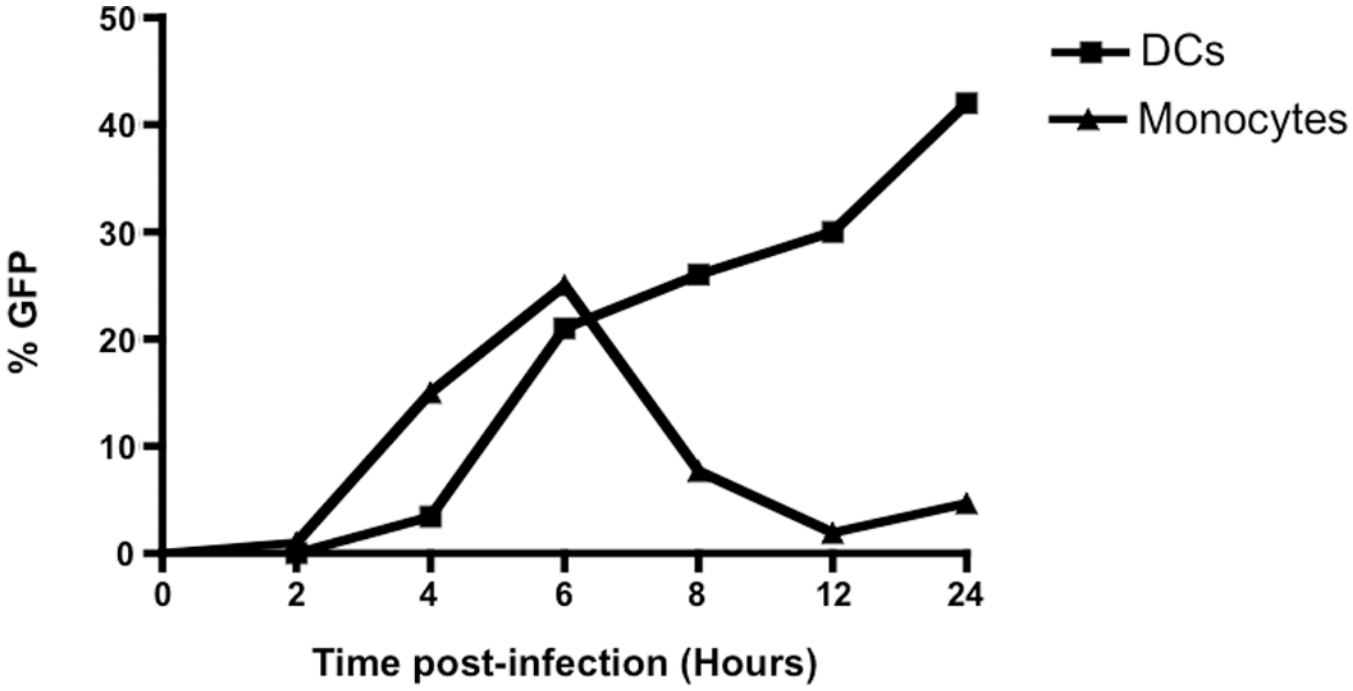

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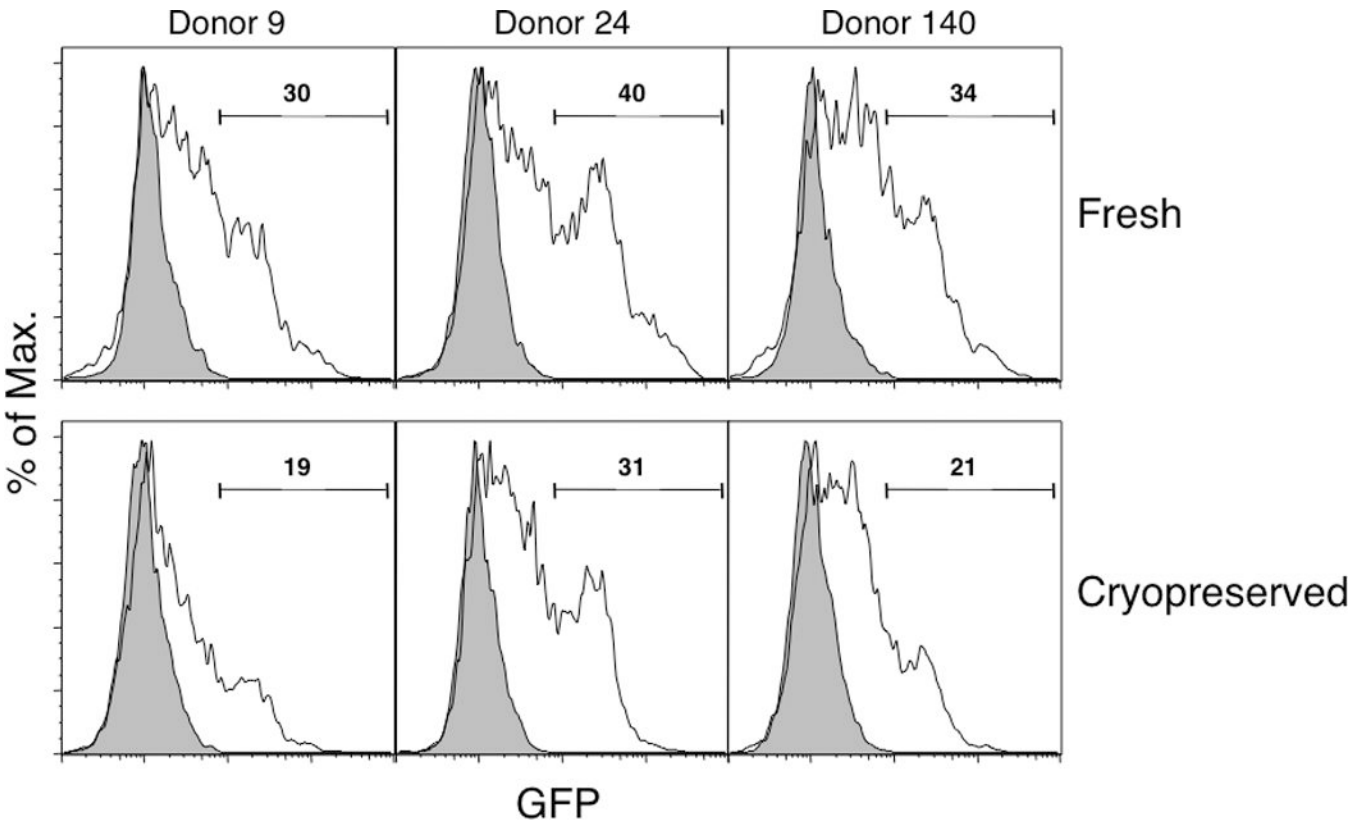
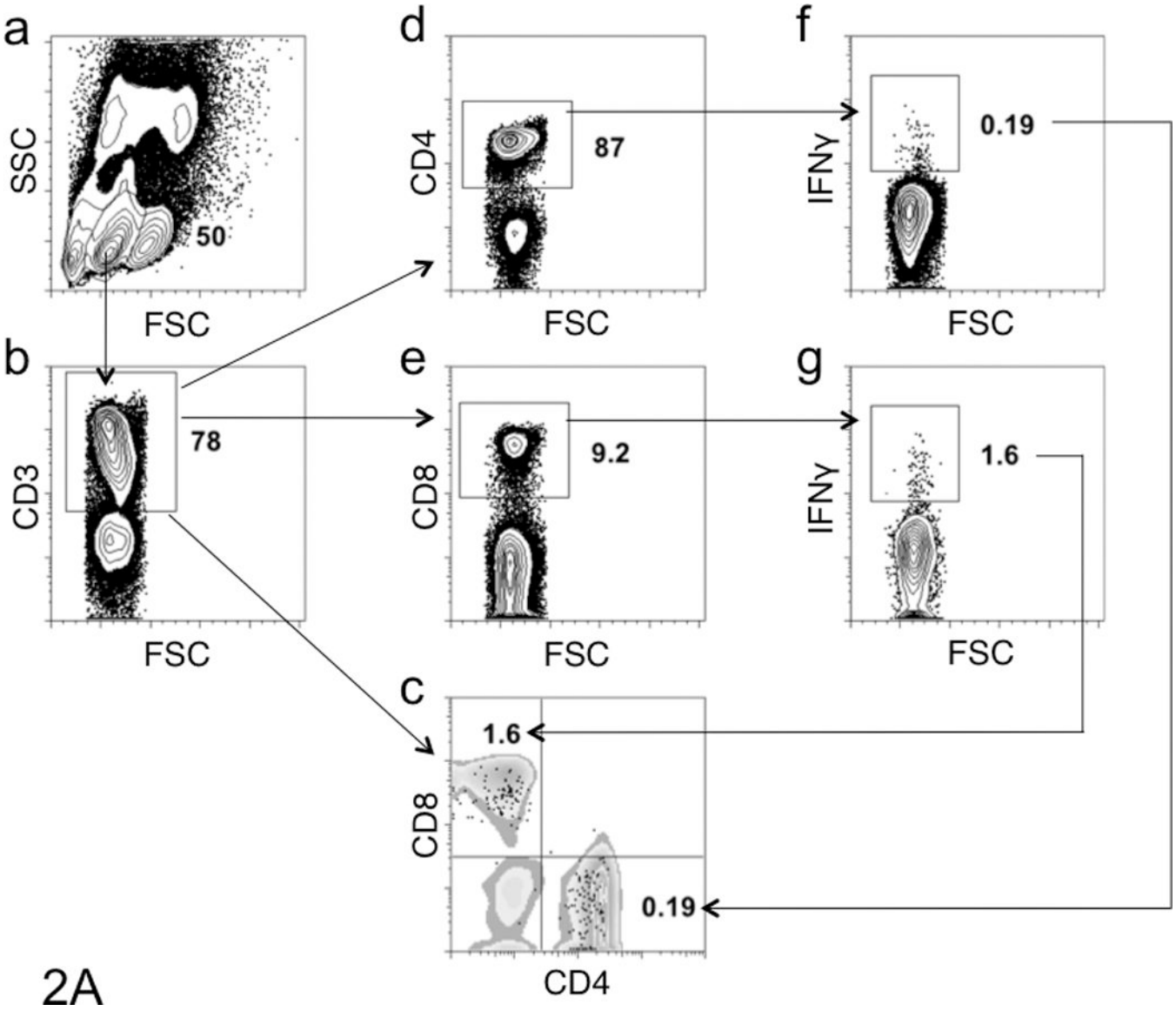


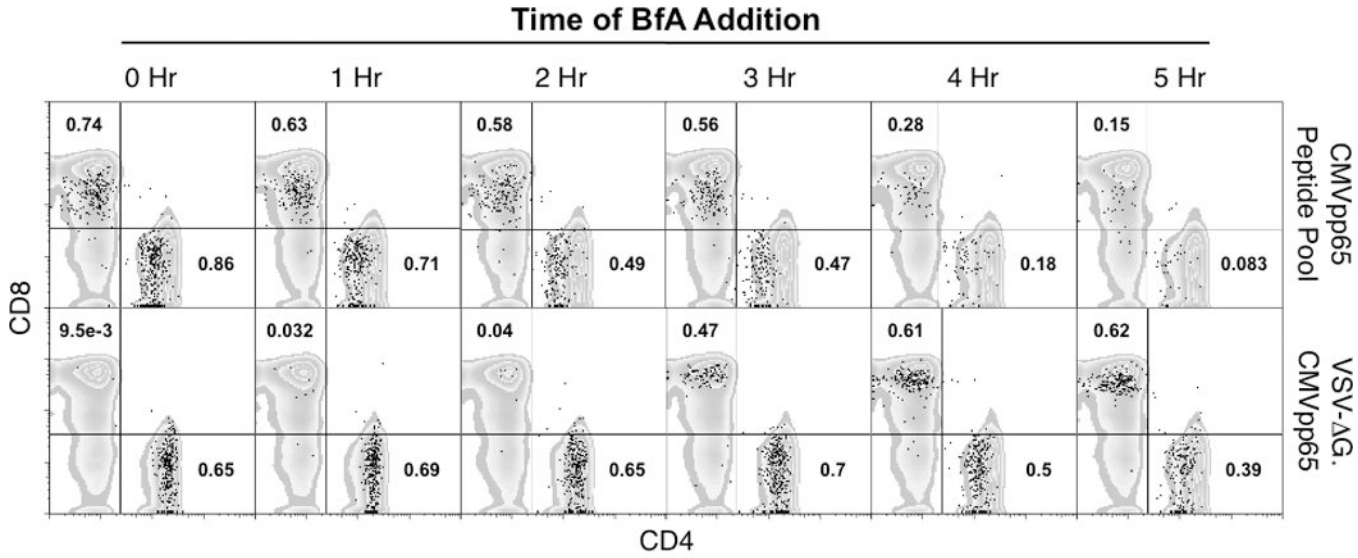
1D

Figure 1. VSV is monocyte tropic

(A) GFP expression in monocytes following 6 hr infection of human PBMC with VSV-ΔG.GFP. Gated populations represent the percentages of GFP-expressing $CD11c^+$, $CD14^+$, $CD4^+$, $CD3^+$, $CD20^+$, or $CD16^+$ cells. (B) Kinetics of GFP expression in DCs (top) and monocytes (bottom) following VSV-ΔG.GFP infection. The percentages of GFP-expressing DCs and monocytes, respectively, are shown as gated populations with the frequencies of DCs and monocytes in total PBMC populations shown in the lower right corner of each plot. DCs are defined as $CD11c^+$, lin^- (CD20, CD3, CD56, CD16, CD14), $HLA\text{-}DR^+$ events, and monocytes are visualized by CD14 staining. (C) Graphical representation of GFP expression in VSV-ΔG.GFP-infected DCs (squares) and monocytes (triangles). (D) GFP expression in monocytes from fresh vs. cryopreserved PBMC for three donors following VSV-ΔG.GFP infection. The histograms shown are gated on live, $CD14^+$ events, and the percentage of GFP-expressing monocytes is shown. The percentage decrease in GFP expression in the cryopreserved samples was 37%, 23%, and 38% for donors 9, 24, and 140, respectively.



2A



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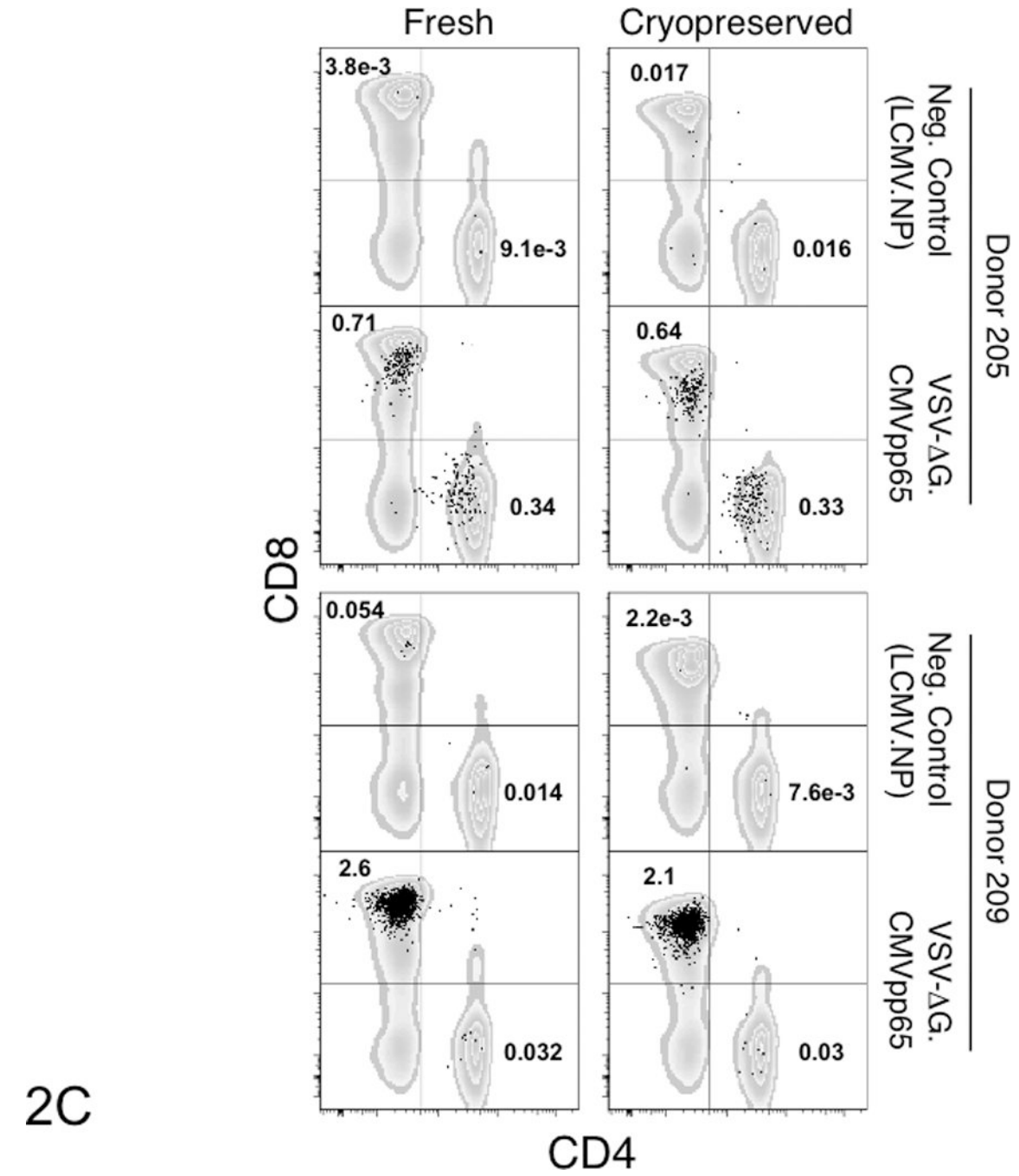


Figure 2. Optimization of assay parameters for flow cytometric detection of T cell responses by VSV-ΔG antigen delivery

(A) Gating strategy. Lymphocyte gating is shown in a. From this point $CD3^+$ T cells are gated (b), and bulk $CD3^+$ populations are shown in c. From $CD3^+$ T cell populations, CD4 T cells (d) and CD8 T cells (e) are gated, and the percentage of $IFN\gamma^+$ CD4 and CD8 T cells is depicted in f and g, respectively. $IFN\gamma^+$ events in f and g are then overlaid on bulk $CD3^+$ populations (c). (B) Optimization of time of BfA addition. PBMC from a CMV-seropositive donor were stimulated with a CMVpp65 peptide pool (top) or infected with VSV-ΔG.CMVpp65 (bottom). BfA was added at various time points post-stimulation/infection. $CD3^+$ lymphocytes are shown. IFNγ-producing cells (black) are overlaid on bulk CD4 and

CD8 T cell populations, and statistics represent the percentages of IFNΔ-producing CD4 and CD8 T cells. (C) Comparison of IFNγ production in fresh vs. cryopreserved PBMC for two CMV-seropositive donors following VSV-ΔG.CMVpp65 infection. Shown are $CD3^+$ lymphocytes. IFNγ-producing cells (black) are overlaid on bulk CD4 and CD8 T cell populations, and statistics represent the percentages of IFNγ-producing CD4 and CD8 T cells.

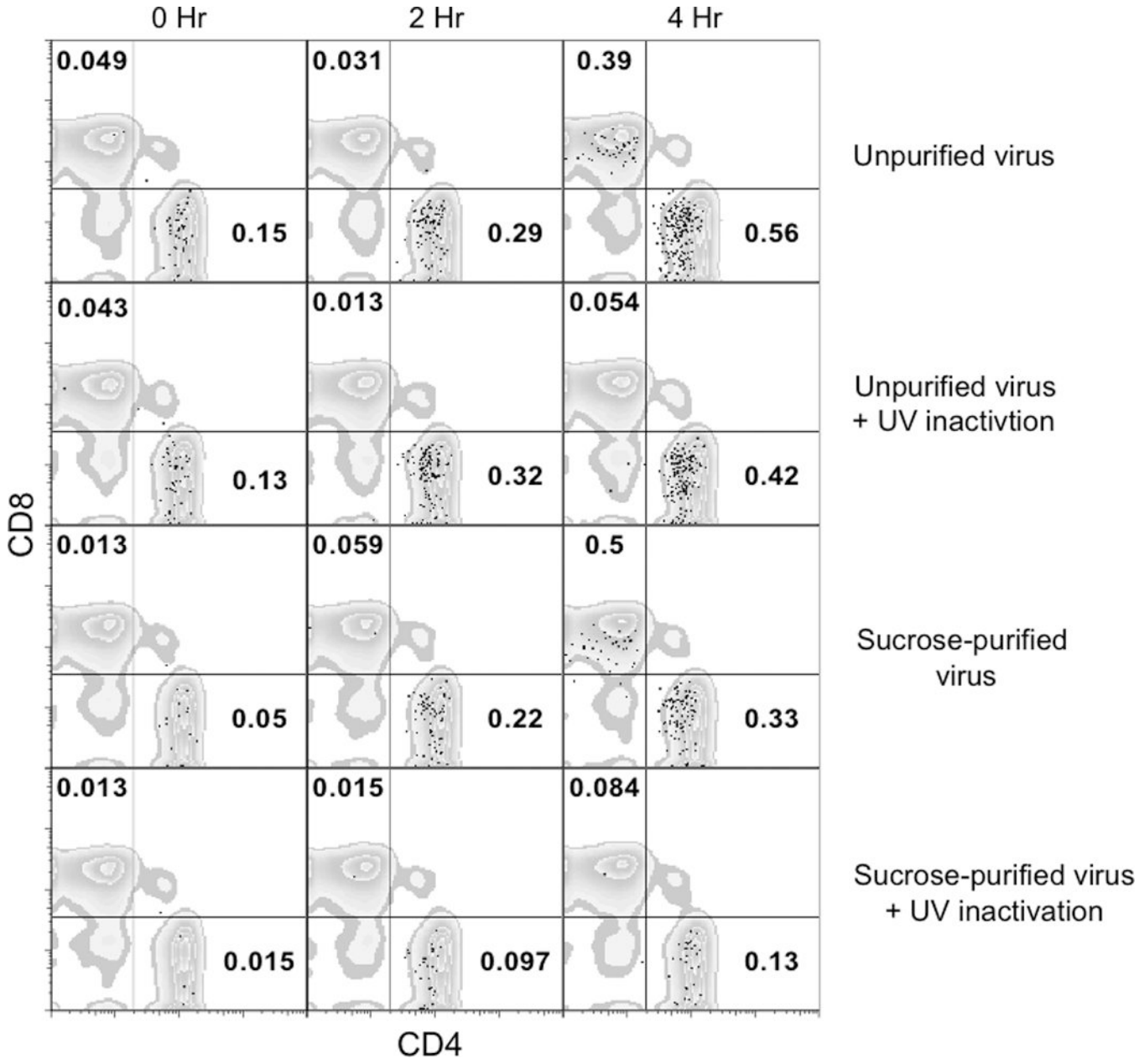


Figure 3. Effects of carryover antigen

PBMC from a CMV-seropositive donor were infected with unpurified VSV-ΔG.CMVpp65, unpurified/UV inactivated virus, sucrose-purified virus, and sucrose-purified/UV inactivated virus. BfA was added at 0, 2, and 4 hr post-infection. Shown are CD3-gated lymphocytes. IFNγ-producing cells (black) are overlaid on bulk CD4 and CD8 populations, and statistics given represent the percentages of IFNγ-producing CD4 and CD8 T cells.

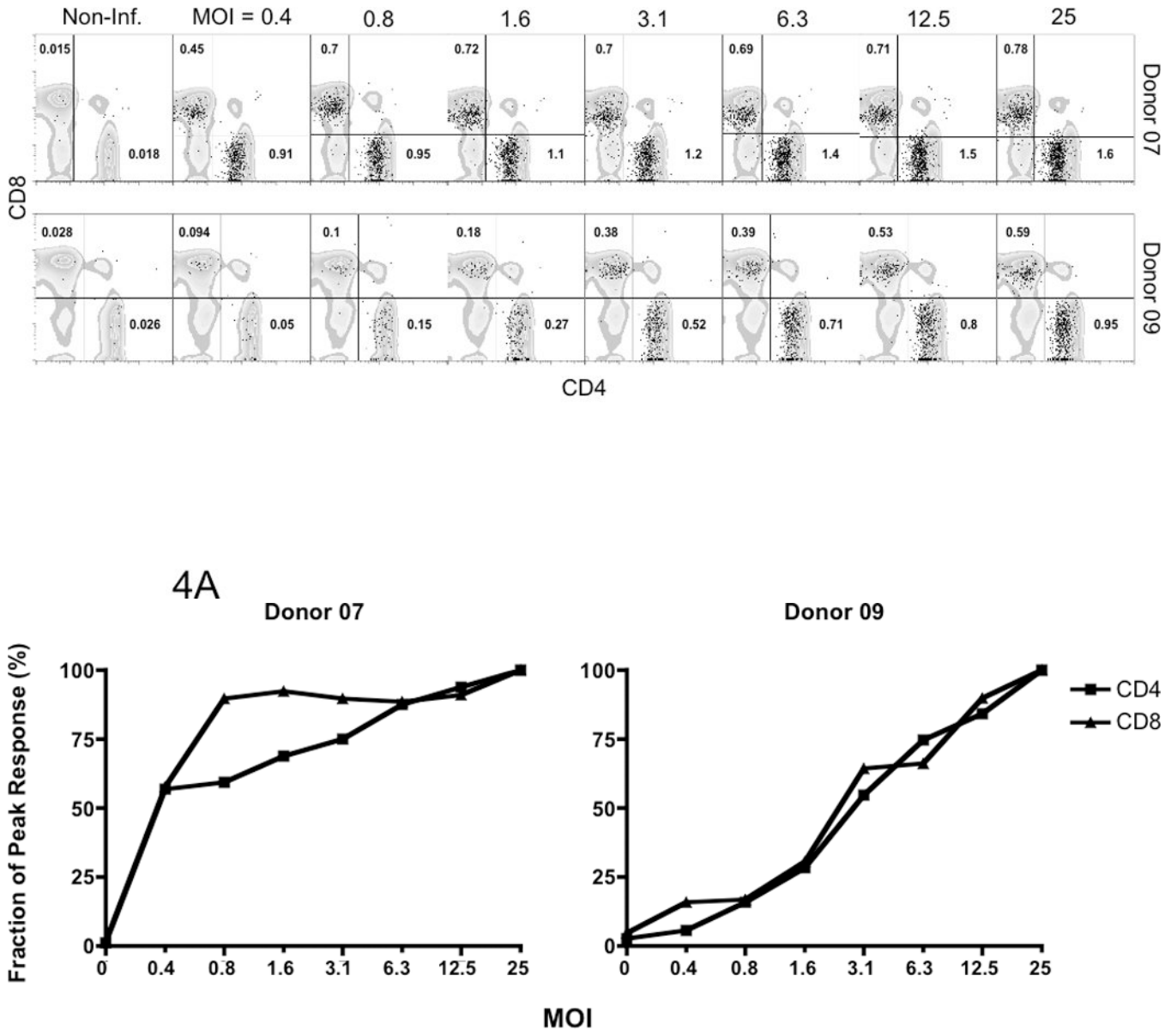


Figure 4. Optimization of MOI

(A) PBMC from two CMV-seropositive donors were infected with VSV-ΔG.CMVpp65 at an MOI range of 0.4–25. CD3-gated lymphocytes are shown. IFNγ-producing cells (black) are overlaid on bulk CD4 and CD8 populations, and statistics given represent the percentages of IFNγ-producing CD4 and CD8 T cells. (B) The graph represents the fraction of the peak IFNγ response for CD4 (squares) and CD8 (triangles) T cells as a function of MOI for the donors in (A).

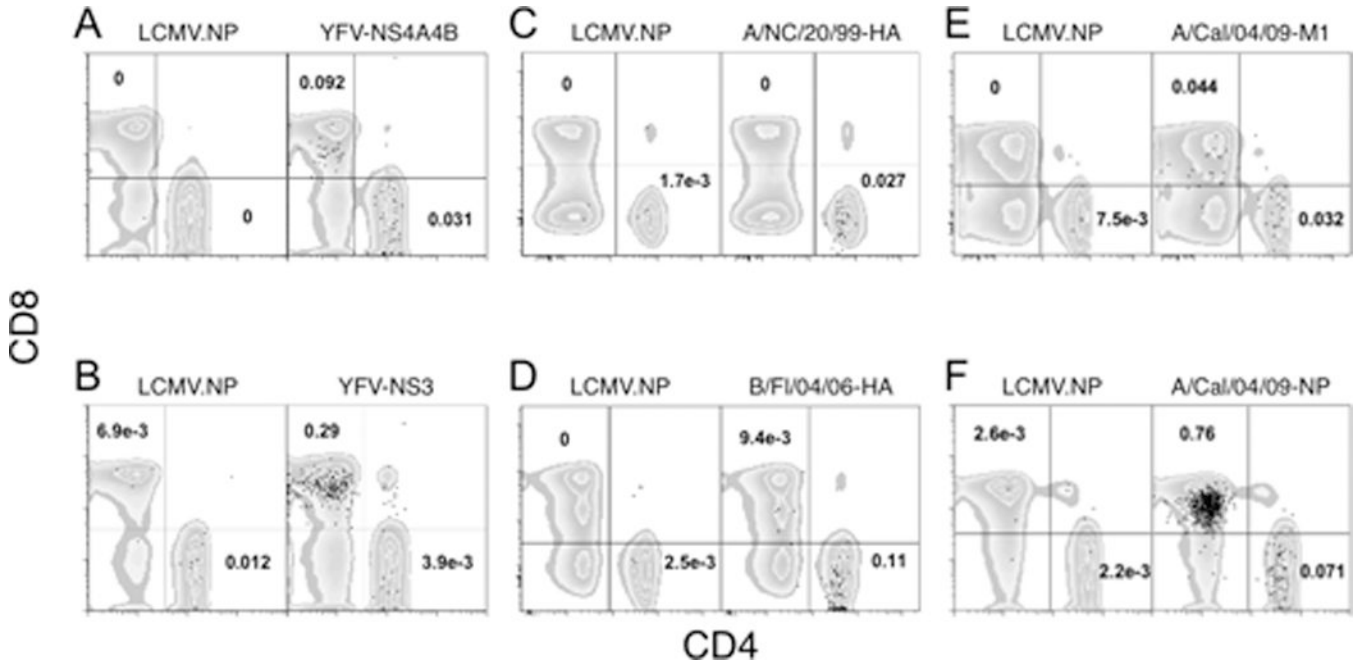
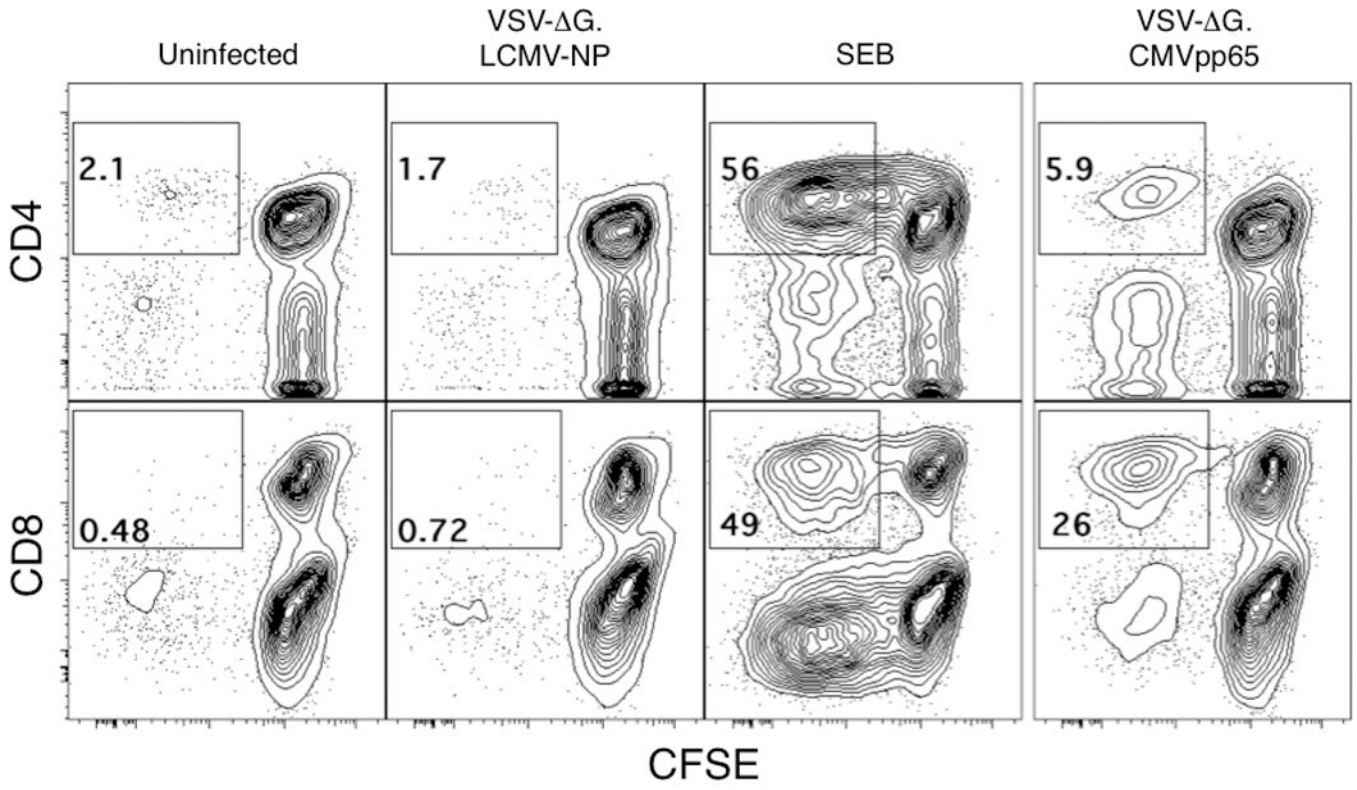
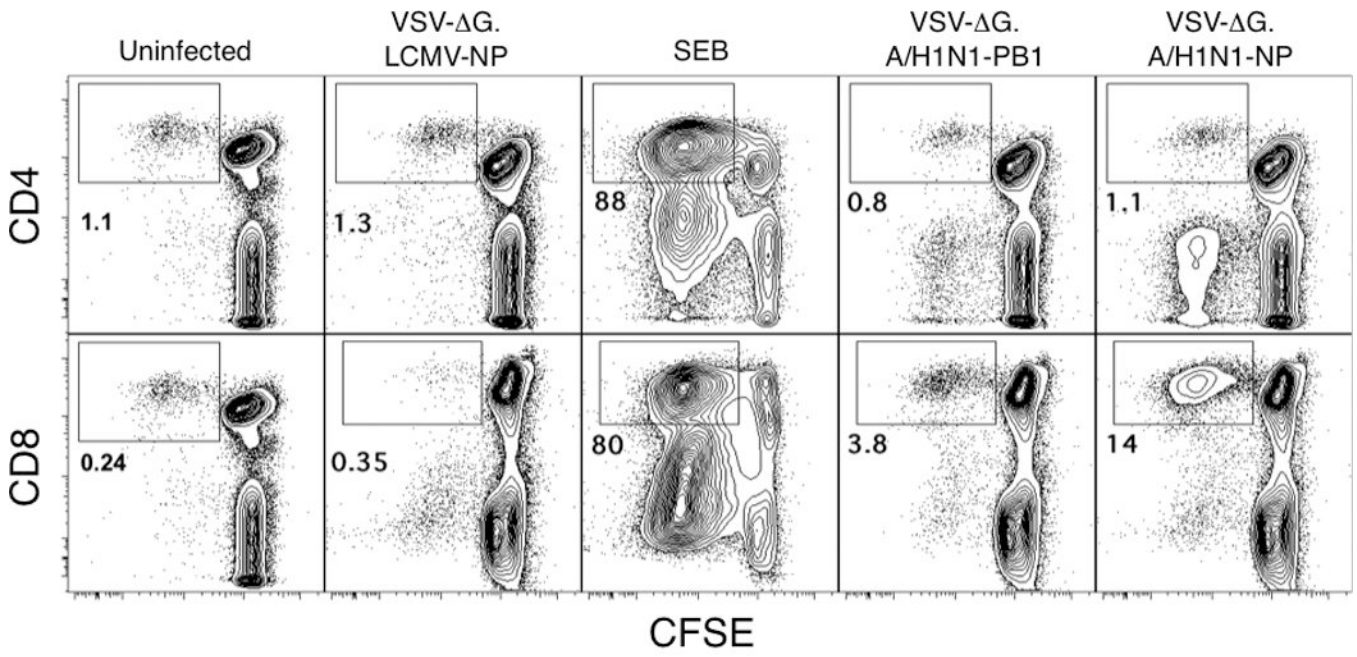


Figure 5. Evaluation of T cell responses to a range of antigens encoded by VSV-ΔG vectors

PBMC from a YFV-17D-vaccinated donor (day 60 post-vaccination) were infected with VSV-ΔG.YFV-NS4A4B (A) and VSV-ΔG.YFV-NS3 (B). PBMC from a healthy donor were infected with VSV-ΔG.A/NC/20/99-HA (C), and PBMC from a donor vaccinated with 2008–2009 seasonal influenza vaccine (14 days post-vaccination) were infected with VSV-ΔG.B/Florida/04/06-HA (D). PBMC from a healthy donor were assayed with VSV-ΔG constructs encoding M1 and NP from A/Cal/04/09 (E,F). For all samples, BfA was added 4 hr post-infection. CD3-gated lymphocytes are shown, and IFNγ-producing cells (black) are overlaid on bulk CD4 and CD8 populations. Statistics represent the percentages of IFNγ-producing CD4 and CD8 T cells.



6a



6B

Figure 6. VSV-ΔG vectors drive the proliferation of antigen-specific T cells *in vitro*

(A) PBMC from a CMV-seropositive donor were CFSE-labeled and infected with VSV-ΔG.CMVpp65; (B) PBMC from a donor that has previously been demonstrated to possess influenza-specific T cell populations were CFSE-labeled and infected with VSV-ΔG vectors encoding PB1 or NP from A/Cal/04/09. Samples from (A) and (B) were cultured for 6 days prior to flow cytometry analysis, and the plots are gated on live, $CD3^+$ events. As a positive control, PBMC were stimulated with staphylococcal enterotoxin B (SEB). The statistics shown represent the percentages of $CFSE^{low}$ CD4 or CD8 T cells.

Table 1

VSV-ΔG Construct	Primer DNA Sequence
CMVpp65.s	CGCGGCGCCTGCCCAGCCACCATGGAGTCGCGCGGTCGCCGTTG
CMVpp65.a	TGCTCGTGGCTCCCATCACTTGTCGTCATCGTCTTTG
YFV-NS4A4B.S	ACGGGGTACCGCCACCATGGCGTCTGAATTTATTAAGTTTGCTG
YFV-NS4A4B.a	AATTGGCGCGCCATCAGACTTCACCCAAAGTTTTTCC
YFV-NS3.s	ACGGGGTACCGCCACCATGGCTGGGTGGCTGTTTCATGTC
YFV-NS3.a	AACATGCATGCATCATTCACTCAGCACAACTAGCAC
A/NC/20/99 HA.s	ACGGGGTACCGCCACCATGAAAGCAAAACTACTGGTCC
A/NC/20/99 HA.a	AATTGGCGCGCCTCAGATGCATATTCTACACTGC
B/FI/04/06 HA.s	CGCTGCGTCTGCCCAGCCACCATGAAGGCAATAATTGTACTACTC
B/FI/04/06 HA.a	TGCTCGTGGCTCCCATCATAGACAGATGGAGCAAGAAACATT
A/Cal/04/09 M1.s	CGCTGCGTCTGCCCAGCCACCATGAGTCTTCTAACCGAGGTCGAA
A/Cal/04/09 M1.a	TGCTCGTGGCTCCCATCACTTGAACCGTTGCATCTGCACCCC
A/Cal/04/09 NP.s	CGCTGCGTCTGCCCAGCCACCATGGCGTCTCAAGGCACCAAACGA
A/Cal/04/09 NP.a	TGCTCGCTGGCTCCCATCAACTGTCATACTCCTCTGCATTGTC
A/Cal/04/09 PB1.s	CGCTGCGTCTGCCCAGCCACCATGGATGTCAATCCGACCTTACTT
A/Cal/04/09 PB1.a	TGCTCGTGGCTCCCATCATTTTTGCCGTCTGAGCTCTTCAATG

Note: VSV-ΔG.GFP was provided as a gift from Michael Whitt, and VSV-ΔG.LCMV-NP was constructed by blunt end cloning of LCMV-NP from plasmid DNA into VSV-ΔG.