Envelope-Specific Adaptive Immunity following Transplantation of Hematopoietic Stem Cells Modified with VSV-G Lentivirus

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INTRODUCTION

Current approaches for hematopoietic stem cell gene therapy typically involve lentiviral gene transfer in tandem with a conditioning regimen to aid stem cell engraftment. Although many pseudotyped envelopes have the capacity to be immunogenic due to their viral origins, thus far immune responses against the most common envelope, vesicular stomatitis virus glycoprotein G (VSV-G), have not been reported in hematopoietic stem cell gene therapy trials. Herein, we report on two Fanconi anemia patients who underwent autologous transplantation of a lineage-depleted, gene-modified hematopoietic stem cell product without conditioning. We observed the induction of robust VSV-G-specific immunity, consistent with low/undetectable gene marking in both patients. Upon further interrogation, adaptive immune mechanisms directed against VSV-G were detected following transplantation in both patients, including increased VSV-G-specific T cell responses, anti-VSV-G immunoglobulin G (IgG), and cytotoxic responses that can specifically kill VSV-G-expressing target cell lines. A proportion of healthy controls also displayed preexisting VSV-G-specific CD4⁺ and CD8⁺ T cell responses, as well as VSV-G-specific IgG. Taken together, these data show that VSV-G-pseudotyped lentiviral vectors have the ability to elicit interfering adaptive immune responses in the context of certain hematopoietic stem cell transplantation settings.

Activated by human serum. More recent studies have attempted to engineer VSV-G to be more resistant to serum inactivation; however, these studies do not address the potential mechanism of natural serum inactivation. It has been shown that serum inactivation of vesiculoviruses is mediated by naïve immunoglobulin M (IgM) and complement even in nonimmune serum, hinting at a potential mechanism for immunogenicity in the context of innate immune responses. In vivo delivery of VSV-G-enveloped lentiviruses in mice has been shown to induce strongly neutralizing adaptive immune responses that can be subverted using heterologous boosts, indicating again that the immunogenicity of the viral glycoprotein envelope causes susceptibility to recognition and neutralization by the adaptive immune system.

Typically, bone marrow (BM) or hematopoietic stem cell (HSC) gene therapy involves a cytotoxic conditioning regimen with alkylating chemotherapy to aid engraftment and long-term persistence of transplanted modified stem and progenitor cells. However, for patients with genetic defects involving DNA repair, such conditioning regimens would result in increased toxicity and are thus avoided. For example, Fanconi anemia (FA) is characterized by deleterious mutations in a group of proteins responsible for DNA repair. While FA mutations have been linked to two different genes to date, approximately two-thirds of patients exhibit mutations in FANCA, making this the most common target for gene correction by way of FANCA-modified HSC transplantation.

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Current approaches for hematopoietic stem cell gene therapy typically involve lentiviral gene transfer in tandem with a conditioning regimen to aid stem cell engraftment. Although many pseudotyped envelopes have the capacity to be immunogenic due to their viral origins, thus far immune responses against the most common envelope, vesicular stomatitis virus glycoprotein G (VSV-G), have not been reported in hematopoietic stem cell gene therapy trials. Herein, we report on two Fanconi anemia patients who underwent autologous transplantation of a lineage-depleted, gene-modified hematopoietic stem cell product without conditioning. We observed the induction of robust VSV-G-specific immunity, consistent with low/undetectable gene marking in both patients. Upon further interrogation, adaptive immune mechanisms directed against VSV-G were detected following transplantation in both patients, including increased VSV-G-specific T cell responses, anti-VSV-G immunoglobulin G (IgG), and cytotoxic responses that can specifically kill VSV-G-expressing target cell lines. A proportion of healthy controls also displayed preexisting VSV-G-specific CD4⁺ and CD8⁺ T cell responses, as well as VSV-G-specific IgG. Taken together, these data show that VSV-G-pseudotyped lentiviral vectors have the ability to elicit interfering adaptive immune responses in the context of certain hematopoietic stem cell transplantation settings.
In this study, we describe a cohort of two FA patients who were enrolled in a clinical trial to receive corrective FANCA gene therapy via lentiviral gene transfer to HSCs. Due to known limitations in the positive selection of CD34-expressing (CD34+) cells in FA, an alternative lineage reduction strategy was devised to preserve CD34+ HSCs from both patients. These patients received lineage-reduced, gene-modified HSCs, transduced by a VSV-G-pseudotyped LV carrying FANCA under the control of a human phosphoglycerate kinase (PGK) promoter, in the absence of conditioning. Although modification of the cell product with the corrected transgene was achieved, long-term engraftment of corrected cells was not observed in either patient. We hypothesized that the mechanism of engraftment failure was related to immune responses derived against the transplanted cell product. To test this hypothesis, we quantified the antigen specificity and effector function of adaptive T cell and B cell immune responses against LV components used for gene modification of the cell product.

RESULTS

Neutralization of Clinical FA Vector by Post-transplantation Patient Serum

Four FA patients were screened for trial entry, but one subject (003) displayed evidence of spontaneous reversion of the FANCA mutation in blood cells, indicated by near-normal to normal hematologic parameters and a negative diepoxybutane (DEB) chromosome breakage test, the classic diagnostic test for FA. The three remaining FA patients were enrolled in a clinical trial to correct various mutations in FANCA via lentiviral delivery of wild-type FANCA cDNA under the control of a PGK promoter. An HIV-1-derived LV was pseudotyped with VSV-G envelope derived from the Indiana strain. Due to variation in available CD34+ cells, cell product formulation was varied between subjects (Table S1). In this retrospective analysis we focused on the two patients who received lineage-reduced cell products due to similarity of treatment, increased transduction and viability of gene-modified cell products administered, and lack of sample availability from the first patient enrolled (subject 001). In vitro transduction success and cell dosing in subjects 002 and 004 were previously reported. However, within the first 100 days following transplantation, engraftment of gene-modified cells was not observed in either subject (Figure 1).

To rule out potential immune response against either the vector or transgene, heat-inactivated and native serum from before and after HSC transplantation was incubated with the same viral vector used in the trial, except encoding a green fluorescent protein (GFP) reporter transgene. Following incubation with serum, the vector was used to transduce a highly permissive human fibrosarcoma (HT1080) cell line. Interestingly, only post-transplantation serum from subject 002 was found to interfere with vector transduction (Figure 2A), while serum from subject 004 neutralized vector transduction both before and after transplant (Figure 2B). Heat inactivation of serum at 56°C nominally restored the ability of the vector to transduce, although still at lower levels than control and pre-transplant sera. This observation indicated potential heat-labile serum effector molecules capable of binding the virus and preventing its ability to transduce cells. Notably, such molecules would have been absent during ex vivo manipulations of the gene-modified, infused cell products.

Increased T Cell Responses to VSV-G in Transplanted FA Patients

Previous studies have indicated the ability of the VSV-G envelope to remain on the surface of transduced cells and transduce secondary populations, both in vitro and in vivo. Due to the immunogenicity of the virally-derived VSV-G envelope and its potential retention on the surface of transduced cells, we next assessed adaptive immune responses to the envelope in the treated patients. Because inactivation of the VSV-G-enveloped lentivirus was observed in serum collected just 7 days after transplantation in subject 002, we postulated that a robust adaptive immune response was rapidly elicited. To determine whether an antigen-specific T cell response to VSV-G could be observed, a cohort of healthy patients was first assessed for T cell responses to VSV-G. Using an ex vivo stimulation strategy with recombinant VSV-G, we measured antigen-specific T cell responses in both the CD4+ and CD8+ memory compartments, using the costimulatory molecules CD154 and CD137 as respective readouts. Strikingly, we observed antigen-specific CD4+ (Figure 3A) and CD8+ (Figure 3B) responses following stimulation with VSV-G. These responses were observed across all memory T cell subsets evaluated in both the CD4+ and CD8+ lineages, including effector memory (TEM, CCR7+CD45RA−), central memory (TCM, CCR7+CD45RA−), and terminally-differentiated effector memory (TEMRA, CCR7−CD45RA+). Following the quantification of VSV-G-specific T cell responses in healthy controls, we next analyzed the FA patients 002 and 004 for responses before and after transplant.

![Figure 1. Lentiviral Vector-Modified, Lineage-Depleted Cells Fail to Engraft in Fanconi Anemia Patients](image-url)
Applying the VSV-G-specific T cell assay to patient samples revealed increased antigen-specific T cell responses in both the CD4+ and CD8+ memory T cell compartments in subjects 002 and 004 following transplant (Figure 4A). These data demonstrate that VSV-G-specific T cells are induced in a proportion of healthy subjects and are strongly induced following autologous HSC transplantation that lacks a myeloablative conditioning regimen.

Efficient Killing of VSV-G+ Target Cells by Patient-Derived, VSV-G-Specific T Cells

The observation of increased VSV-G-specific T cell responses following transplantation with a VSV-G-pseudotyped LV led us to explore the functionality of these effectors on VSV-G targets, i.e., via direct killing (CD8+, Figure 4B) or aiding in the formation of Ig against VSV-G (CD4+, Figure 5). An adherent cell line expressing VSV-G was generated and validated for stable expression (Figure S1). This cell line and a control lacking VSV-G expression were then added to a real-time cell analysis (RTCA) system, which measures cellular adherence to substrate via electrical impedance. After cellular impedance of the VSV-G-expressing target cell line was established, a cytotoxicity assay was conducted using enriched CD137+ antigen-specific CD8+ T cells from transplanted FA patients 002 and 004 as effectors. Post-transplant CD8+ antigen-specific T cells were chosen for this assay because of their enriched frequency compared to control and pre-transplant samples, and because of their known cytotoxic potential. Compared to a parental target cell line lacking VSV-G expression, primary T cells from both patients 002 and 004 readily eliminated VSV-G-expressing targets while allowing the parental targets to grow out unperturbed (Figure 4B). These results indicate that the post-transplantation VSV-G-specific T cells efficiently target VSV-G in vitro.

Increased VSV-G-Specific Ig in Post-transplantation Patient Serum

The presence of VSV-G-specific T cells could have another confounding impact on immunity leading to engraftment failure, i.e., induction of neutralizing antibodies by VSV-G-specific CD4+ T cells, selecting for VSV-G-specific B cells. Pre-transplantation and post-transplantation serum samples from FA patients 002 and 004 were assessed for IgG, a class-switched soluble antibody molecule indicative of a T-dependent adaptive immune response directed toward VSV-G. Anti-(α-)VSV-G IgG increased dramatically following transplantation in both patients 002 and 004 (Figure 5A). This increase was significant compared to a control cohort and was maintained during 2 years following transplantation in subject 004 (Figure 5B). This finding strikingly indicates that a robust memory immune response to VSV-G was elicited and persisted following transplantation with VSV-G LV-modified HSCs.

Anti-VSV-G IgG Is Not Induced after Transplantation with Myeloablative Conditioning

In addition to direct effects on VSV-G-pseudotyped LVs, anti-VSV-G IgG could also interfere with engraftment of vector-transduced cells, for example by mediating opsonization or antibody-dependent cell cytotoxicity (ADCC) of HSCs displaying VSV-G protein at the cell surface. Unlike transplant patients who undergo myeloablative conditioning, following which immune responses would be limited due to the immunosuppressive nature of the conditioning regimen, FA patients are not conditioned, and these immune responses should be retained. To directly test the impact of the conditioning regimen on anti-vector immune responses, we examined the induction of VSV-G-specific IgG following autologous transplantation with myeloablative total body irradiation (TBI) in nonhuman primates.
CD34+ HSCs may not induce immunity in the context of immune-ure 6B). Interestingly, the only animal that showed a notable increase in anti-VSV-G IgG after transplantation also developed a strong anti-VSV-G-specific humoral and cellular immunity to VSV-G in transplanted patients. The rapid loss of engraftment of the autologous cell product induced antigen-specific immunity and functionality. IgG directed against VSV-G also showed a marked increase in both patients following transplantation, which was long-lived in the one patient who was followed. In stark contrast to the increases in VSV-G-directed IgG in patients without a conditioning regimen, a nonhuman primate cohort receiving a myeloablative conditioning regimen did not exhibit IgG increases against VSV-G following transplant. These data reinforce the commonly held notion of conditioning-dependent tolerization: development of immune memory responses against VSV-G and other vector-specific antigens is minimized when patients receive immunosuppressive conditioning regimens such as TBI.22–24

We conclude that the lack of conditioning in FA patients 002 and 004 was a contributing factor to the lack of long-term engraftment of these cells in vivo.

In addition to the role of the conditioning regimen, it is also possible that VSV-G-specific immune cells were generated ex vivo during manufacturing of the autologous cell product. CD34+ HSCs from FA patients are known to express the CD34 marker at lower levels, which may contribute to the failure due to low numbers of modified HSCs or immune-mediated clearance.

DISCUSSION

Although transplantation of gene-corrected, autologous HSCs is a highly promising curative strategy for FA patients, our study reveals limitations that should be considered to increase the success of this approach. Two patients transplanted with a lineage-depleted, VSV-G-pseudotyped LV gene-modified HSC product lacked measurable long-term engraftment of gene-modified cells. The rapid loss of gene-modified cells in vivo was consistent with either engraftment failure due to low numbers of modified HSCs or immune-mediated clearance. We found that serum collected from these patients following transplantation neutralized our LV in vitro. This led us to think that the lack of engraftment was possibly confounded by, or partially attributable to, an immune response. Furthermore, antigen-specific humoral and cellular immunity to VSV-G in transplanted patients drove adaptive immune responses that prevented engraftment of transduced HSCs; similar correlates of vector-specific immunity were also observed in a cohort of healthy controls.

Herein, we detail the mechanisms of anti-VSV-G immunity following transplant with cells modified by a VSV-G-enveloped vector. Our finding that anti-envelope adaptive immune responses can develop in the absence of standard conditioning regimens is extremely pertinent for the gene therapy field, especially for pathologies in which a preparative conditioning regimen is contraindicated. Transplantation with a lineage-depleted, gene-modified cell product induced antigen-specific CD4+ and CD8+ T cells against the VSV-G envelope. These T cells had the ability to suppress a VSV-G-expressing cell line following transplant as compared to a parental cell line lacking expression, indicating specificity and functionality. IgG directed against VSV-G also showed a marked increase in both patients following transplantation, which was long-lived in the one patient who was followed. In stark contrast to the increases in VSV-G-directed IgG in patients without a conditioning regimen, a nonhuman primate cohort receiving a myeloablative conditioning regimen did not exhibit IgG increases against VSV-G following transplant. These data reinforce the commonly held notion of conditioning-dependent tolerization: development of immune memory responses against VSV-G and other vector-specific antigens is minimized when patients receive immunosuppressive conditioning regimens such as TBI.22–24

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In addition to the role of the conditioning regimen, it is also possible that VSV-G-specific immune cells were generated ex vivo during manufacturing of the autologous cell product. CD34+ HSCs from FA patients are known to express the CD34 marker at lower levels,
making a CD34-based positive selection strategy inefficient. While our lineage depletion-based negative selection strategy was effective at reducing mature blood cell subsets, e.g., to enable transduction of a CD34-enriched cell population, there were residual populations of CD14+ monocytes, CD16+ macrophages, and CD19+ B cells remaining in the product prior to delivery of the corrected FANCA. It has been previously shown that VSVG is a potent inducer of innate immune responses, particularly by initiating antiviral interferon responses through Toll-like receptor 4 (TLR4) signaling in monocytes. VSVG has also been shown to trigger pattern recognition receptors on a host of antigen-presenting cells (APCs) through TLR13 signaling, particularly on monocytes and macrophages. Combined with evidence that HIV-based LVs are also sensed by TLR3 and TLR7 on professional APC-like dendritic cells and B cells, it is reasonable to assume that residual mature subsets in the lineage-depleted cell product were capable of initiating innate immune responses, potentiating antigen presentation, and synergizing with an endogenous immune system that was not suppressed by myeloablative conditioning. This model is consistent with the lack of vector-modified HSC engraftment that we observed in FA patients 002 and 004.

Notably, a recent clinical HSC gene therapy trial for FA by Río et al. applied the same lentiviral construct and gene transfer technique, but in contrast to the patients reported in the present study, these investigators used CD34+-enriched HSCs and were able to demonstrate long-term engraftment of gene-modified HSCs and progeny. This improved engraftment is likely due to several key distinguishing factors between the two studies. Río et al. collected and cryopreserved FA patient CD34+ cells prior to the patient’s bone marrow failure in order to enhance the CD34+ cell dose. The researchers then waited with the HSC gene therapy until the onset of hematologic decline, allowing for the enrichment and transplantation of a more robust cell product with increased CD34+ purity. In contrast, our patients received the gene-modified cell product immediately after cell collection and transduction. This is the most likely factor contributing to their success, as our initial protocol had to be altered due to low levels of CD34+ cells using positive selection after the discovery that CD34 expression was lower in HSCs in patients with declining bone marrow, making positive selection inefficient. This led to another major distinction in the preparation of the HSC product. In our study we transduced lineage-depleted or whole bone marrow while Río et al. used CD34+ modified cells. Thus, our product could have contained more APCs, contributing to the observed immune response and rejection of modified cells. While it is difficult to fully elucidate the mechanisms that prevented lack of engraftment in our study, it is mostly likely multifold, with product purity (lineage depletion versus CD34+ positive selection), robustness of the HSCs (collection after bone marrow failure versus early cryopreservation), and immune responses to vector components (contaminating mature blood cells in the lineage-depleted product) all contributing a role in long-term engraftment failure.

One approach to evade vector-specific immune responses is using alternative pseudotype envelopes that are less immunogenic. We have previously characterized an envelope derived from the VSV-related Cocal virus that is more resistant to serum neutralization than VSVG. Likewise, further work is required to determine whether anti-envelope immune responses can be modulated by distinct conditioning regimens. While VSVG-specific immune responses were not observed in our nonhuman primate cohort following transplantation with TBI, the impact of other conditioning regimens, e.g., busulfan and melphalan, remains unclear. Our data suggest that immunosuppressive conditioning should ablate immune responses to transplanted antigens, as observed in our nonhuman primate cohort. Because these traditional regimens are not feasible for

Figure 4. VSV-G-Specific T Cells Isolated from Two Patients following Transplant Can Suppress a VSV-G-Expressing Cell Line In Vitro

(A) VSV-G-specific T cells from FA patients 002 and 004 were assessed in a similar manner to healthy controls both before (C) and after (im) transplantation with a VSV-G pseudotyped, lentiviral vector-modified cell product. The “% VSV-G-specific of parent” is represented by CD154+ of CD4+ for CD4+ T cells and CD137+ of CD8+ for CD8+ T cells. (B) VSV-G-specific CD8+ T cells were isolated from patients 002 and 004 2 years following transplantation. These effectors were co-incubated with a VSV-G-expressing target cell line (red) in an in vitro real-time cell analysis assay to determine target-specific killing at a ratio of 10 effectors to 1 target cell. Gray line indicates mock target cell line not expressing VSV-G. Error bars represent duplicate data between both patients.
FA patients, next-generation conditioning regimens would be extremely useful toward addressing the limitations attributed to HSC transplantation for FA patients. As noted, an alternative to genotoxic TBI- or chemotherapy-based conditioning is antibody-based conditioning that is specifically targeted to the HSC niche.32 This approach has recently demonstrated success in supporting mismatched transplants in mice, and is being developed particularly in the context of FA, although impacts on immunosuppression as compared to traditional conditioning regimens have yet to be explored.33,34

Finally, while there is little known about the prevalence of anti-VSV-G immune responses in humans, VSV is endemic to various regions and can infect humans in close contact with livestock, typically in a subclinical fashion.35 The anti-VSV-G responses observed in our cohort of healthy controls could indicate cross-reactivity to similar infectious agents, such as other vesiculovirus G proteins,36 or perhaps subclinical exposure to VSV. Regardless, quantifiable immune responses to various virally derived lentiviral pseudotype envelopes in otherwise healthy subjects could be a key hurdle for LV gene therapy. Overcoming this barrier may represent an exciting step forward in the safety, efficiency, and persistence of numerous gene-modified cell therapies.

MATERIALS AND METHODS

Lentiviral Vectors

All SIN LVs were produced with a third-generation split packaging system and pseudotyped with VSV-G. LVs used to transduce healthy donor cells were encoded with either an enhanced GFP (EGFP) transgene (pRSC-PGK.EGFP-sW) or the full-length FANCA cDNA (pRSC-PGK.FANCA-sW), both regulated by an hPGK promoter. Research-grade vectors were produced by the Fred Hutch Vector Production Core (principal investigator, H.-P.K.). Clinical-grade LV (pRSC-PGK.FANCA-sW) was produced by the Indiana University Vector Production Facility (IUVPF, IN, USA) using a large-scale, validated process following good manufacturing practices (GMPs) standards under an approved Drug Master File held by IUVPF. The infectious titer was determined by serial transduction of HT1080 human fibrosarcoma-derived cells and evaluated either by flow cytometry for EGFP expression or by quantitative polymerase chain reaction (qPCR).

Manufacturing of Clinical Lineage-Depleted Cell Product

This procedure has been previously described.15 Patients underwent either bone marrow harvest or were mobilized with daily granulocyte colony-stimulating factor (G-CSF) (filgrastim; 16 μg/kg twice a day; days 1–6) and plerixafor (240 μg/kg/day; days 4–6) subcutaneously. Mobilized patients were subjected to large-volume leukapheresis when circulating CD34+ blood cell counts were ≥ 5 cells/μL. Immuno-magnetic beads were from Miltenyi Biotec (Auburn, CA, USA). Bone marrow red blood cells (RBCs) were debulked by hydroxyethyl starch (hetastarch) sedimentation prior to labeling on a CliniMACS Prodigy device (Miltenyi Biotec, Germany). Mobilized leukapheresis products were platelet-washed prior to labeling and custom lineage depletion on a CliniMACS Prodigy device (Miltenyi Biotec). CD34+ cells were cultured on recombinant fibronectin fragment (Takara Bio, Mountain View, CA, USA)-coated culture flasks at a density of 1 × 10⁶ cells/mL and 2.9 × 10⁵ cells/cm² in StemSpan animal component-free (ACF) media (STEMCELL Technologies, Vancouver, BC, Canada), supplemented with 4 μg/mL protamine sulfate (American Pharmaceutical Partners [APP], East Schaumburg, IL, USA), 100 ng/mL each of recombinant human stem cell factor (rhSCF), thrombopoietin (rhTPO), and Flt-3 ligand (rhFLT3L) (all from CellGenix, Freiburg, Germany), and 1 mM N-acetylcysteine (NAC) (Cumberland Pharmaceuticals, Nashville, TN, USA). Cells were immediately transduced at a multiplicity of infection (MOI) of 5–10 infectious units (IU)/cell for 12–24 h at 37°C, 5% CO₂ and 5% O₂ prior to harvest for infusion.

Figure 5. Increase in Anti-(α-)VSV-G IgG Following Transplant with Long-Term Persistence

(A) Serum from transplanted FA patients 002 and 004 was assessed for α-VSV-G IgG before and after transplantation. (B) α-VSV-G IgG levels were compared in transplanted patients (red bar, ■) and healthy controls (gray bar, ○). Dotted line indicates limit of detection for VSV-G ELISA detection of IgG.
Quantitative Real-Time PCR-Based Measurement of Vector Copy Number

This procedure has been previously described. Briefly, the vector copy number (VCN) per genome equivalent was assessed by quantitative real-time PCR with an LV-specific primer/probe combination (forward, 5'-TGCGCGCTTCAG; reverse, 5'-CCGTGCGGCTTCAG; probe, 5'-AGCTCTCTCTGCCGAGACTCGGC [Integrated DNA Technologies (IDT), Coralville, IA, USA]). A separate reaction with a β-globin-specific primer/probe combination (forward, 5'-CTATCA-GAAAGTGGTGGCTGG; reverse, 5'-TTGGACAGCAA-GAAAGTGAGCTT; probe, 5'-TGCTAAATGCCCTGGCCCA-CAAGTA [IDT]) served as a positive control for intact genomic DNA (gDNA). The standard curve method was used to quantify the VCN from patient samples. The standard curve for the LV was established by serial dilution of gDNA isolated from a human cell line (HT1080) confirmed to contain a single integrant of the same LV backbone. The standard curve for β-globin was established from serial dilution of peripheral leukocytes collected from a healthy donor.

In Vitro Serum Neutralization of Clinical-Grade VSV-G-Pseudotyped Vector

This assay was adapted from previously published experiments. Serum from CD34+ hematopoietic stem and progenitor cell (HSPC)-transplanted nonhuman primates receiving VSV-G-pseudotyped, lentiviral vector-modified cells was assayed for the presence of α-VSV-G IgG following transplant. Serum was mixed with VSV-G-pseudotyped vector preparations at 37°C for 30 min, and then added to 1 × 10^5 HT1080 cells (ATCC, Manassas, VA, USA). A vector-only control was also incubated at 37°C for 30 min. Heat inactivation of serum was conducted for 30 min at 56°C, and then incubated in the same conditions. Gene transfer was evaluated by flow cytometry for EGFP expression 3 days after vector exposure, and the percentage of EGFP-expressing cells after incubation in the serum was determined relative to the percentage of EGFP-expressing cells in the vector-only control to determine the fold increase or decrease in titer after exposure to serum.

CD154/CD137 Antigen-Specific T Cell Assays

Antigen-specific T cells assays for CD4+ and CD8+ responses were modified from previously published methods. Isolated peripheral blood mononuclear cells (PBMCs) were incubated in the presence of 4 μg/mL recombinant VSV-G (Alpha Diagnostic International, San Antonio, TX, USA) for 16–18 h at 37°C in addition to anti-CD40 (Miltenyi Biotec). Following incubation, cells were stained with CD154-phycocerythrin (PE) and CD137-PE-Cy7. After washing, incubation with anti-PE microbeads (Miltenyi Biotec) preceded magnetic enrichment on an MS column (Miltenyi Biotec). Following enrichment, cells were stained with the remaining antibodies against lineage and memory antigens and analyzed by flow cytometry.

ELISA Detection of VSV-G-Specific Igs

Patient and nonhuman primate serum was diluted 1:50 in PBS prior to analysis. Detection of IgG against VSV-G was conducted using commercially available kits (Alpha Diagnostic International) using the manufacturer’s recommended protocol. Each sample was run in duplicate in accordance with the manufacturer’s recommendation.

VSV-G Target RTCA Cytotoxicity Assay

In vitro cytotoxicity of a VSV-G-expressing cell line was determined using RTCA on the xCelligence DP platform (ACEA Biosciences, San Diego, CA, USA). Targets were prepared by stably expressing VSV-G in an HT1080 cell line (ATCC) using a VSV-G helper plasmid under selective pressure as previously described. VSV-G expression was determined using western blot, and surface expression was determined using flow cytometry (Figure S1). Cells were plated overnight and allowed to adhere to the RTCA plate for 18–24 h. During this initial period, a “normalized cell index” was established based on the number of cells that adhered to substrate, which was quantified via changes in electrical impedance across the substrate. This value increased as cells adhered to the plate and decreased as they released from the monitored substrate. Next, CD137+CD8+ antigen-specific
Flow Cytometry
Antibodies used in this study included the following: CD45RA-fluorescein isothiocyanate (FITC) (5H9, Becton Dickinson [BD], Franklin Lakes, NJ), TIGIT-peridinin chlorophyll protein (PerCP)-eFluor 710 (MBSA43, Invitrogen, Waltham, MA, USA), CD69-Alexa Fluor 647 (FN50, BioLegend, San Diego, CA, USA), Ki67-Alexa Fluor 700 (B56, BD), PD-1-allophycocyanin (APC)-eFluor 780 (J105, eBioscience), CCR7-PE (3D12, Thermo Fisher Scientific), CD154-PE-CF594 (TRAP1, BD), CD137-PE-Cy7 (4B4-1, BioLegend), CD25-V450 (4E3, eBioscience), CD4-Brilliant Violet (BV)570 (OKT4, BioLegend), CD3-BV605 (SP34-2, BD), human leukocyte antigen (HLA)-DR-BV711 (G46-6, BD), CTLA-4-BV786 (BNI3, BD), PD-1-allophycocyanin (APC)-eFluor 780 (J105, eBioscience), CCR7-PE (3D12, Thermo Fisher Scientific), CD154-PE-CF594 (TRAP1, BD), CD137-PE-Cy7 (4B4-1, BioLegend), CD25-V450 (4E3, eBioscience), CD4-Brilliant Violet (BV)570 (OKT4, BioLegend), CD3-BV605 (SP34-2, BD), human leukocyte antigen (HLA)-DR-BV711 (G46-6, BD), CTLA-4-BV786 (BNI3, BD), PD-1-allophycocyanin (APC)-eFluor 780 (J105, eBioscience), CCR7-PE (3D12, Thermo Fisher Scientific), CD154-PE-CF594 (TRAP1, BD), CD137-PE-Cy7 (4B4-1, BioLegend), CD25-V450 (4E3, eBioscience), CD4-Brilliant Violet (BV)570 (OKT4, BioLegend), CD3-BV605 (SP34-2, BD), human leukocyte antigen (HLA)-DR-BV711 (G46-6, BD), CTLA-4-BV786 (BNI3, BD), CD45-UV395 (D058-1283, BD), and CD8-BUV737 (SK1, BD). Anti-VSV-G-PE (F-6, Santa Cruz Biotechnology, Dallas, TX, USA) was used to detect VSV-G expression. All flow cytometry was collected and analyzed on a FACSsymphony using FACSDiva software.

Statistical Analysis
For comparisons of statistical significance between stimulated and unstimulated antigen-specific T cells, multiple unpaired t tests were applied between groups (n = 10), using the Holm-Sidak method, with alpha = 0.05. Each row was analyzed individually, without assuming a consistent standard deviation (SD). For comparisons of anti-VSV-G IgG between controls (n = 10) and transplant patients (n = 2), an unpaired t test using Welch’s correction was used. For comparisons of anti-VSV-G IgG between nonhuman primate samples (n = 7) before and after transplant, a paired t test was used. All statistical analysis was done in Prism v7 (GraphPad, San Diego, CA, USA).

SUPPLEMENTAL INFORMATION
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