Rapid generation of multivirus-specific T lymphocytes for the prevention and treatment of respiratory viral infections

Ferrata Storti Foundation

Spyridoula Vasileiou, Anne M. Turney, Manik Kuvalekar, Shivani S. Mukhi, Ayumi Watanabe, Premal Lulla, Carlos A. Ramos, Swati Naik, Juan F. Vera, Ifigeneia Tzannou and Ann M. Leen

Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital and Houston Methodist Hospital, Houston, TX, USA

ABSTRACT

Respiratory tract infections due to community-acquired respiratory viruses including respiratory syncytial virus, Influenza, parainfluenza virus 3 and human metapneumovirus are detected in up to 40% of allogeneic hematopoietic stem cell transplant recipients in whom they cause severe symptoms including pneumonia and bronchiolitis and can be fatal. Given the lack of effective antivirals and the data from our group demonstrating that adoptively transferred *ex vivo*-expanded virus-specific T cells can be clinically beneficial for the treatment of both latent (Epstein-Barr virus, cytomegalovirus, BK virus, human herpesvirus 6) and lytic (adenovirus) viruses, we investigated the potential for extending this immunotherapeutic approach to respiratory viruses. We now describe a system that rapidly generates a single preparation of polyclonal (CD4+ and CD8+) virus-specific T cells reactive against 12 antigens derived from four viruses (respiratory syncytial virus, Influenza, parainfluenza virus 3 and human metapneumovirus) that commonly cause post-transplant morbidity and mortality. With a single *in vitro* stimulation we consistently generated Th1-polarized T-cell lines that produced multiple effector cytokines and were selectively reactive against viral-expressing targets, with no evidence of "off target" auto- or allo-reactivity. Finally, we demonstrated the clinical relevance of these virus-specific T cells by measuring responses in transplant recipients who successfully controlled active infections. These results support the clinical importance of T-cell immunity in mediating protective antiviral effects against community-acquired respiratory viruses and demonstrate the feasibility of utilizing a broad-spectrum immunotherapeutic in immunocompromised patients with uncontrolled infections.

Introduction

Acute upper and lower respiratory tract infections (RTI) due to community-acquired respiratory viruses (CARV) including respiratory syncytial virus (RSV), influenza, parainfluenza virus (PIV) and human metapneumovirus (hMPV) are a major public health problem.[1] For example, RSV-induced bronchiolitis is the most common reason for hospital admission in children under one year of age,[2-4] while the Center for Disease Control (CDC) estimates that, annually, Influenza accounts for up to 35.6 million illnesses worldwide, between 140,000 and 710,000 hospitalizations, annual costs of approximately $87.1 billion in disease management in the US alone, and between 12,000 and 56,000 deaths.

Thus, CARV are a leading cause of morbidity and mortality worldwide, with individuals whose immune systems are naïve (e.g. young children) or compromised being the most vulnerable. For example, in allogeneic hematopoietic stem cell transplant (HSCT) recipients, the incidence of CARV-related respiratory viral diseases is as high as 40%.[5] While most patients initially present with rhinorrhea, cough and fever, in approximately 50% of cases, infections progress to the lower respiratory tract and are characterized by severe symptoms including pneumonia and bronchiolitis and mortality rates of 23-50%.[6-9] There are neither approved preventative vaccines nor antiviral drugs for hMPV[10] and PIV[11] and for Influenza the preventative vaccine is not indi-

cated unless patients are at least six months post-HSCT.[12] Aerosolized ribavirin (RBV) has been approved by the US Food and Drug Administration (FDA) for the treatment of RSV, but it is extremely costly (5-day course = $149,756) and logistically difficult to administer, requiring a specialized nebulization device that connects to an aerosol tent surrounding the patient.[13-16] Thus, the lack of approved antiviral agents for many clinically problematic CARV, and the high cost and complexity of administering aerosolized RBV, underscores the need for alternative treatment strategies.

Our group has previously demonstrated that the adoptive transfer of *in vitro*-expanded virus-specific T cells (VST) can safely and effectively prevent and treat infections associated with both latent [Epstein-Barr virus (EBV), cytomegalovirus (CMV), BK virus (BKV), human herpesvirus 6 (HHV6)] and lytic [adenovirus (AdV)] viruses in allogeneic HSCT recipients.[17,18] Given that susceptibility to CARV is associated with underlying cellular immune deficiency,[1,5,6] in the current study, we explored the feasibility of extending the therapeutic range of VST therapy to include Influenza, RSV, hMPV and PIV-3.

We here describe a mechanism by which we can rapidly generate a single preparation of polyclonal (CD4+ and CD8+) VST with specificity for 12 immunodominant antigens derived from our four target viruses using Good Manufacturing Practices (GMP)-compliant manufacturing methodologies. The viral proteins used for stimulation were chosen on the basis of both their immunogenicity to T cells and their sequence conservation.[19-21] The expanded cells are Th1-polarized, polyfunctional and selectively able to react to and kill, viral antigen-expressing target cells with no activity against non-infected autologous or allogeneic targets, attesting to both their selectivity for viral targets and their safety for clinical use. We anticipate such multi-respiratory virus-targeted cells (multi-R-VST) will provide broad spectrum benefit to immunocompromised individuals with uncontrolled CARV infections.

Methods

Donors and cell lines

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers and HSCT recipients with informed consent using Baylor College of Medicine institutional review board-approved protocols (H-7634, H-7666) and were used to generate phytohemagglutinin (PHA) blasts and multi-R-VST. PHA blasts were generated as previously reported[20] and cultured in VST medium [45% RPMI 1640 (HyClone Laboratories, Logan, UT, USA), 45% Click's medium (Irvine Scientific, Santa Ana, CA, USA), 2 mM GlutaMAX TM-I (Life Technologies, Grand Island, New York, NY, USA), and 10% human AB serum (Valley Biomedical, Winchester, VA, USA)] supplemented with interleukin 2 (IL2) (100 U/mL; NIH, Bethesda, MD, USA), which was replenished every two days.

Multi-respiratory virus-targeted cell generation
Pepmixes
Peripheral blood mononuclear cells were stimulated with peptide libraries (15mers overlapping by 11aa) spanning Influenza A (NP1, MP1), RSV (N, F), hMPV (F, N, M2-1, M) (JPT Peptide Technologies, Berlin, Germany) and PIV-3 antigens (M, HN, N, F) (Genemed Synthesis, San Antonio, TX, USA). Lyophilized pepmixes were reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -80°C.

Generation of virus-specific T cells
To generate multi-R-VST, PBMC (2.5×10^7) were transferred to a G-Rex10 (Wilson Wolf Manufacturing Corporation, St. Paul, MN, USA) with 100 mL of VST medium supplemented with IL7 (20 ng/mL), IL4 (800 U/mL) (R&D Systems, Minneapolis, MN, USA) and pepmixes (2 ng/peptide/mL) and cultured for 10-13 days at 37°C, 5% CO_2.

Flow cytometry
Immunophenotyping

Multi-R-VST were surface-stained with monoclonal antibodies to: CD3, CD25, CD28, CD45RO, CD279 (PD-1) [Becton Dickinson (BD), Franklin Lakes, NJ, USA], CD4, CD8, CD16, CD62L, CD69 (Beckman Coulter, Brea, CA, USA) and CD366 (TIM-3) (BioLegend, San Diego, CA, USA). Cells were acquired on a Gallios™ Flow Cytometer and analyzed with Kaluza® Flow Analysis Software (Beckman Coulter). See *Online Supplementary Appendix* for details.

Intracellular cytokine staining

Multi-R-VST were harvested, resuspended in VST medium (2×10^6/mL) and 200 μL added per well of a 96-well plate. Cells were incubated overnight with 200 ng of individual test or control (irrelevant non-viral, e.g. SURVIVIN, WT1) pepmixes, along with Brefeldin A (1 μg/mL), monensin (1 μg/mL), CD28 and CD49d (1 μg/mL) (BD). Intracellular cytokine staining (ICS) for IFNγ and TNFα was performed as described in the *Online Supplementary Appendix*.

FoxP3 staining

FoxP3 staining was performed using the eBioscience FoxP3 kit (Thermo Fisher Scientific, Waltham, MA, USA), per manufacturers' instructions and as detailed in the *Online Supplementary Appendix*.

Functional studies

Enzyme-linked immunospot

Enzyme-linked immunospot (ELIspot) analysis was used to quantitate the frequency of IFNγ and Granzyme B-secreting cells. PBMC and multi-R-VST were resuspended at 5×10^6 and 2×10^6 cells/mL, respectively, in VST medium and 100 μL of cells added to each ELIspot well. Antigen-specific activity was measured as previously described after direct stimulation (500 ng/peptide/mL) with the individual test or control pepmixes. See the *Online Supplementary Appendix* for more details.

Multiplex

The multi-R-VST cytokine profile was evaluated using the MIL-LIPLEX High Sensitivity Human Cytokine Panel (Millipore, Billerica, MA, USA), per manufacturer's instructions (see the *Online Supplementary Appendix*).

Chromium release assay

A standard 4-hour chromium (Cr^{51}) release assay was used to measure the specific cytolytic activity of multi-R-VST with autologous antigen-loaded PHA blasts as targets (20 ng/pepmix/1×10^6 target cells). Effector:Target (E:T) ratios of 40:1, 20:1, 10:1, and 5:1 were used to analyze specific lysis. The percentage of specific lysis was calculated [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. In order to measure the autoreactive and alloreactive potential of multi-R-VST lines, autologous and allogeneic PHA blasts alone were used as targets.
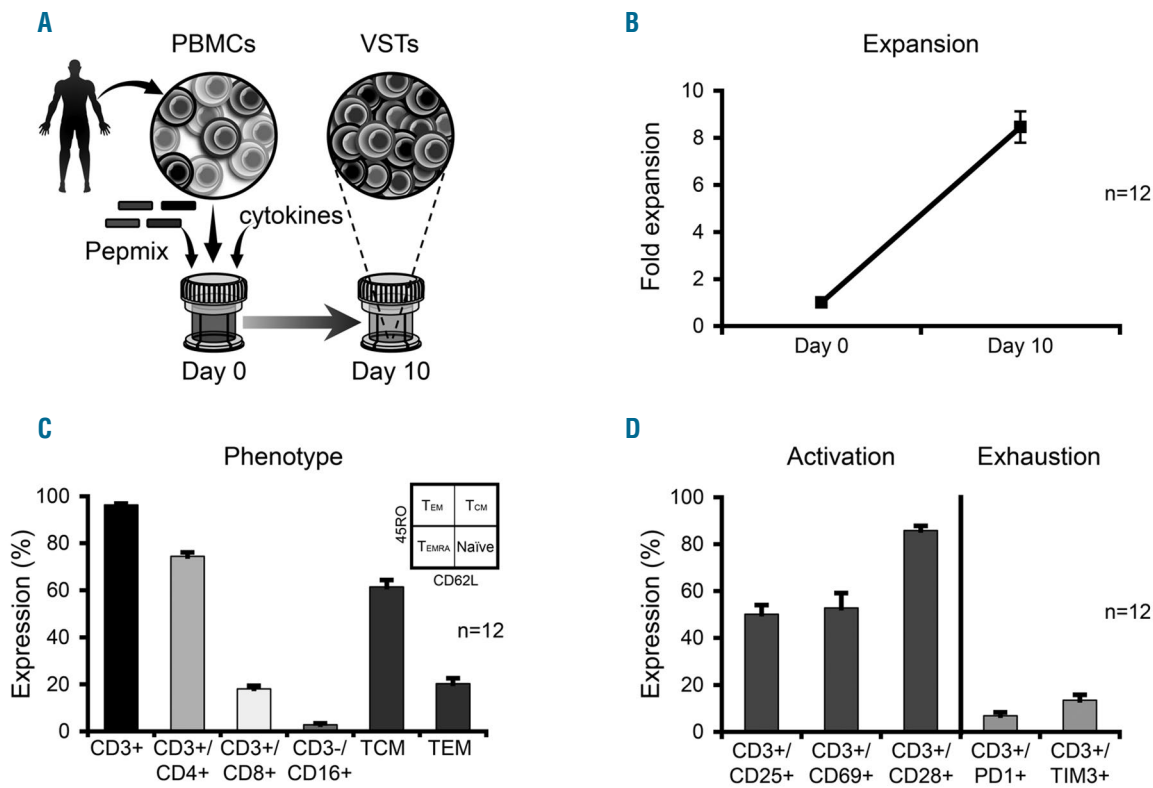
Figure 1. Generation of polyclonal multi-respiratory virus-targeted cells (multi-R-VST) from healthy donors. (A) A schematic representation of the multi-R-VST generation protocol. (B) Fold expansion achieved over a 10-13 day period based on cell counting using trypan blue exclusion (n=12). (C and D) Phenotype of the expanded cells (mean±Standard Error of Mean, n=12) (SEM).

Results

Generation of polyclonal multi-respiratory virus-targeted cells from healthy donors

To investigate the feasibility of generating VST-specific T-cell lines containing sub-populations of cells reactive against Influenza, RSV, hMPV, and PIV-3 we utilized a pool of overlapping peptide libraries spanning immunogenic antigens from each of the target viruses (Influenza – NP1 and MP1; RSV – N and F; hMPV – F, N, M2-1 and M; PIV-3 – M, HN, N and F) to stimulate PBMC before culture in a G-Rex10 in cytokine-supplemented VST medium (Figure 1A). Over 10-13 days we achieved an average 8.5-fold increase in cells (Figure 1B) [increase from 0.25×10^7 PBMC/cm² to mean $1.9 \pm 0.2 \times 10^7$ cells/cm² (median: 2.05×10^7, range: 0.6-2.82×10^7 cells/cm²; n=12)], which were comprised almost exclusively of CD3⁺ T cells (96.2±0.6%; mean±SEM), with a mixture of cytotoxic (CD8⁺; 18.1±1.3%) and helper (CD4⁺; 74.4±1.7%) T cells (Figure 1C), with no evidence of regulatory T-cell outgrowth, as assessed by CD4/CD25/FoxP3⁺ staining (*Online Supplementary Figure S1*). Furthermore, the expanded cells displayed a phenotype consistent with effector function and long-term memory as evidenced by upregulation of the activation markers CD25 (50.2±3.8%), CD69 (52.8±6.3%), CD28 (85.8±2%) as well as expression of central (CD45RO⁺/CD62L⁺: 61.4±3%) and effector memory markers (CD45RO⁺/CD62L⁻: 20.3±2.3%), with minimal PD1 (6.9±1.4%) or Tim3 (13.5±2.3%) surface expression (Figure 1C and D).

Anti-viral specificity of multi-respiratory virus-targeted cells

To next determine whether the expanded populations were antigen-specific, we performed an IFNγ ELIspot assay using each of the individual stimulating antigens as an immunogen. All 12 lines generated proved to be reactive against all of the target viruses (Table 1 and *Online Supplementary Figure S2*). Figure 2A summarizes the magnitude of activity against each of the stimulating antigens, while *Online Supplementary Figure S3* shows the response of our expanded VST to titrated concentrations of viral antigen. Of note, over the 10-13 days in culture we achieved an enrichment in VST of between 14.6±4.3-fold (PIV-3-HN) and 50.4±9.9-fold (RSV-N) (Figure 2B). The precursor frequencies of CARV-reactive T cells within donor PBMC are summarized in *Online Supplementary Figures S4* and *S5*. Taken together these data suggest that respiratory VST reside in the memory pool and can be readily amplified *ex vivo* using GMP-compliant manufacturing methodologies.

To next evaluate whether viral specificity was contained within the CD4⁺ or CD8⁺ or both T-cell subsets we performed ICS, gating on CD4⁺ and CD8⁺ IFNγ-producing cells. Figure 2C shows representative results from one donor with activity against all four viruses detected in both T-cell compartments [(CD4⁺: Influenza – 5.28%; RSV – 11%; hMPV – 6.57%; PIV-3 – 3.37%), (CD8⁺: Influenza – 2.26%; RSV – 4.36%; hMPV – 2.69%; PIV-3 – 2.16%)] while Figure 2D shows a summary of results for nine donors screened, confirming that our multi-R-VST are polyclonal and poly-specific.

Table 1. Reactivity of expanded virus-specific T cells lines against individual stimulating antigens.

Donor	Influenza		RSV		hMPV				PIV-3			
	NP1	MP1	N	F	M	M2-1	F	N	M	F	N	HN
1	✓	✓	✓	✓	✓	✓	✓	X	✓	X	✓	✓
2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
4	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	X	X
5	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
6	✓	✓	✓	✓	✓	✓	✓	X	✓	X	X	✓
7	✓	✓	✓	✓	✓	X	✓	X	✓	✓	X	X
8	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	X
9	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	X	X
10	✓	✓	✓	✓	X	✓	X	X	✓	X	X	X
11	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓
12	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	X

Functional characterization of multi-respiratory virus-targeted cells

The production of multiple proinflammatory cytokines and expression of effector molecules has been shown to correlate with enhanced cytolytic function and improved *in vivo* T-cell activity. Hence, we next examined the cytokine profile of our multi-R-VST following antigen exposure. The majority of IFNγ-producing cells also produced TNFα (see Figure 3A for detailed ICS results from 1 donor, and Figure 3B, for summary results for 9 donors), in addition to GM-CSF, as measured by Luminex array (Figure 3C, left panel) with baseline levels of prototypic Th2/suppressive cytokines (Figure 3C, right panel). Furthermore, upon antigenic stimulation our cells produced the effector molecule Granzyme B, suggesting the cytolytic potential of these expanded cells (Figure 3D, n=9). Taken together, these data demonstrate the Th1-polarized and polyfunctional characteristics of our multi-R-VST.

Multi-respiratory virus-targeted cells are cytolytic and kill virus-loaded targets

To investigate the cytolytic potential of these expanded cells *in vitro*, we co-cultured multi-R-VST with autologous Cr[51]-labeled PHA blasts, which were loaded with viral pepmixes with unloaded PHA blasts serving as a control. Viral antigen-loaded targets were specifically recognized and lysed by our expanded multi-R-VST (40:1 E:T - Influenza: 13±5%, RSV: 36±8%, hMPV: 26±7%, PIV-3: 22±5%, n=8) (Figure 4A and *Online Supplementary Figure S6*). Finally, even though these VST had received only a single stimulation, there was no evidence of activity against non-infected autologous targets nor of alloreactivity (graft-*versus*-host potential) using HLA-mismatched PHA blasts as targets (Figure 4B), an important consideration if these cells are to be administered to allogeneic HSCT recipients.

Detection of CARV-specific T cells in hematopoietic stem cell transplant recipients

Finally, to assess the potential clinical relevance of multi-R-VST we investigated whether allogeneic HSCT recipients with active/recent CARV infections exhibited elevated levels of reactive T cells during/following an active viral episode. Figure 5A shows the results of Patient #1, a 64-year old male with acute myeloid leukemia (AML) who received a matched related donor (MRD) transplant with reduced intensity conditioning. The patient developed a severe upper respiratory tract infection (URTI) nine months post-HSCT that was confirmed to be RSV-related by polymerase chain reaction (PCR) analysis. He was not on any immunosuppression at the time of infection but was placed on prednisone the day of infection diagnosis to control pulmonary inflammation. Within four weeks his symptoms resolved without specific antiviral treatment. To assess whether T-cell immunity contributed to viral clearance, we analyzed the circulating frequency of RSV-specific T cells over the course of his infection. Immediately prior to infection this patient exhibited a very weak response to the RSV antigens N and F (6.5 SFC/5x10^5 PBMC). However, within a month of viral exposure, RSV-specific T cells had expanded *in vivo* (527 SFC/5x10^5 PBMC), representing an 81-fold increase in reactive cells (Figure 5A) which declined thereafter, coincident with viral clearance. Of note, the observed RSV-specific responses did not follow the overall increase in lymphocyte/CD4^{+} counts, thus indicating that T-cell expansion was virus-driven and not due to general immune reconstitution. Similarly, Patient #2, a 23-year old male with acute lymphoblastic leukemia (ALL) who received a matched unrelated donor (MUD) transplant with myeloablative conditioning, and developed a severe RSV-related URTI five months post HSCT while on tapering doses of tacrolimus. His infection symptomatically resolved within one week, coincident with the administration of ribavirin. To investigate whether endogenous immunity also played a role in viral clearance we monitored reactive T-cell numbers over time. Viral clearance was accompanied by an increase in the circulating frequency of RSV-specific T cells (peak 93 SFC/5x10^5 PBMC) with subsequent return to baseline levels (Figure 5B). The same patient was hospitalized seven months
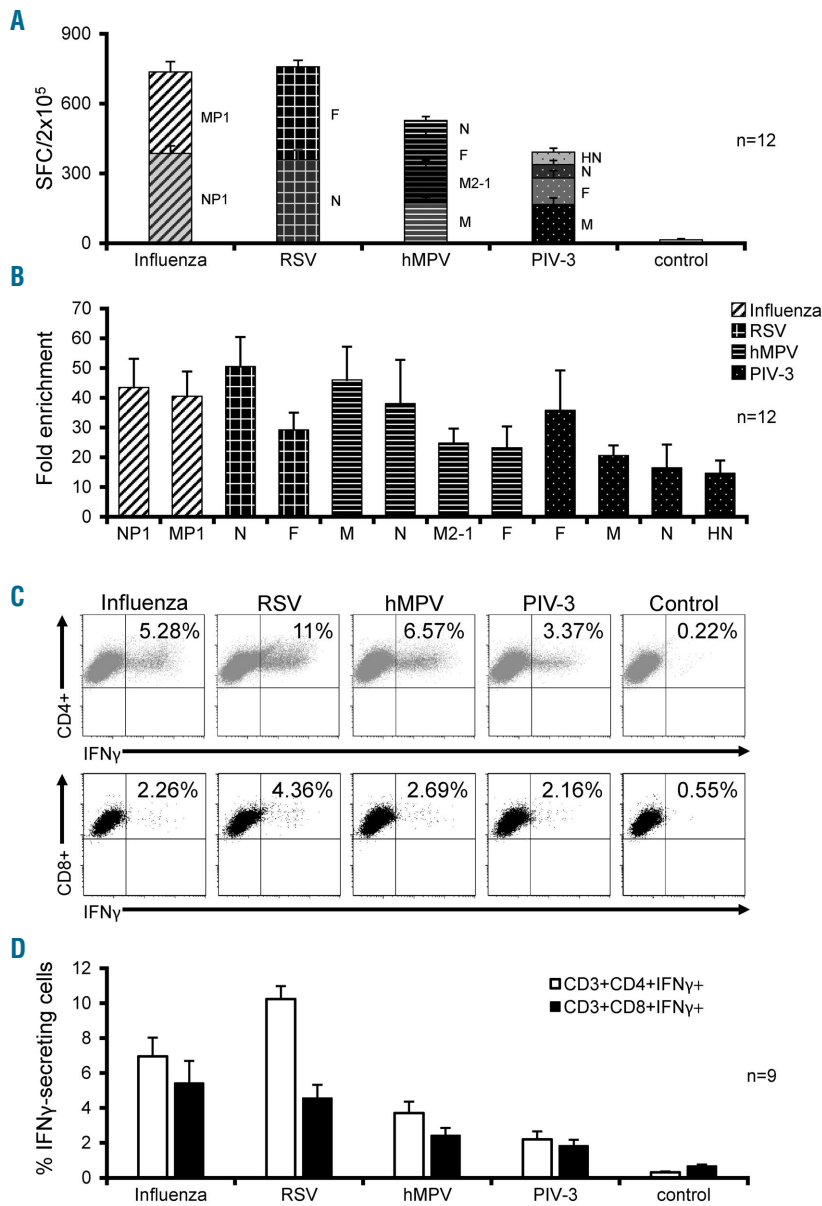
Figure 2. Specificity and enrichment of multi-respiratory virus-targeted cells (multi-R-VST). (A) The specificity of virus-reactive T cells within the expanded T-cell lines following exposure to individual stimulating antigens from each of the target viruses. Data are presented as mean± Standard Error of Mean (SEM) SFC/2x10^5 (n=12). (B) Fold enrichment of specificity [peripheral blood mononuclear cels (PBMC) *vs.* multi-R-VST; n=12]. (C) IFNγ production, as assessed by ICS from CD4 helper (top) and CD8 cytotoxic T cells (bottom) after viral stimulation in one representative donor (dot plots were gated on CD3$^+$ cells). (D) Summary results for nine donors screened (mean±SEM).

post transplant for a subsequent pneumococcal pneumonia with concurrent detection (by PCR) of hMPV in sputum. His pneumonia was treated with antibiotics with subsequent resolution of disease and viral clearance, coincident with a marked expansion of hMPV-specific T cells (reactive against F, N, M2-1 and M), which increased from 4 SFC to a peak of 70 SFC and subsequent decline to baseline levels (Figure 5C). Again, the observed RSV-and hMPV-specific responses were independent of the overall increase in lymphocyte/CD4$^+$ counts.

Online Supplementary Figure S7 shows the results of three additional HSCT recipients who developed CARV infections. Patient #3 is a 15-year old female with AML who received a haplo-identical transplant with reduced intensity conditioning, and developed an RSV-induced URTI and LRTI while on tacrolimus five weeks post transplant. The patient was administered ribavirin and the infection resolved within four weeks. We monitored RSV-reactive T cells over time and viral clearance coincided with a striking increase in the frequency of RSV-specific T cells (from 0 to 506 SFC/5x10^5 PBMC) (*Online*

Supplementary Figure S7A). Similarly, Patient #4, a 10-year old male patient with ALL who received a MUD transplant with myeloablative conditioning, developed a PIV3-related URTI and LRTI one month after HSCT while on tacrolimus. His infection symptomatically resolved within five weeks, coincident with the administration of ribavirin. To investigate whether endogenous immunity also played a role in viral clearance, we monitored PIV3-reactive T-cell numbers over time. Viral clearance was accompanied by an increase in the circulating frequency of T cells specific for the PIV-3 antigens M, HN, N and F (peak 38 SFC/5x10^5 PBMC) with subsequent decline (*Online Supplementary Figure S7B*). Finally, we show Patient #5, a 3-year old male with chronic granulomatous disease who received a MRD transplant with myeloablative conditioning and developed a severe PIV-3-related URTI four months post HSCT while on cyclosporine. The patient received ribavirin but (at last timepoint assessed) continued to exhibit disease symptoms and failed to demonstrate PIV-3-specific T cells (*Online Supplementary Figure S7C*). Taken together, these data suggest the *in vivo* rele-

vance of CARV-specific T cells in the control of viral infections in immunocompromised patients.

Discussion

In the present study, we explored the feasibility of targeting multiple clinically problematic respiratory viruses using *ex vivo* expanded T cells. We have now shown that we can rapidly generate polyclonal, CD4+ and CD8+ T cells with specificities directed to a total of 12 antigens derived from four seasonal CARV [Influenza, RSV, hMPV and PIV-3] that are responsible for upper and lower respiratory tract infections in the immunocompromised host. These broad spec-

trum VST, generated using GMP-compliant methodologies, were Th1-polarized, produced multiple effector cytokines upon stimulation, and killed virus-infected targets without auto- or allo-reactivity. Finally, the detection of reactive T-cell populations in the peripheral blood of allogeneic HSCT recipients who successfully cleared active CARV infections suggests the potential for clinical benefit following the adoptive transfer of such multi-R-VST.

Community-acquired respiratory virus-associated acute upper and lower RTI are a major public health problem with young children, the elderly and those with suppressed or compromised immune systems being the most vulnerable.[1-3] These infections are associated with symptoms including cough, dyspnea, and wheezing, and
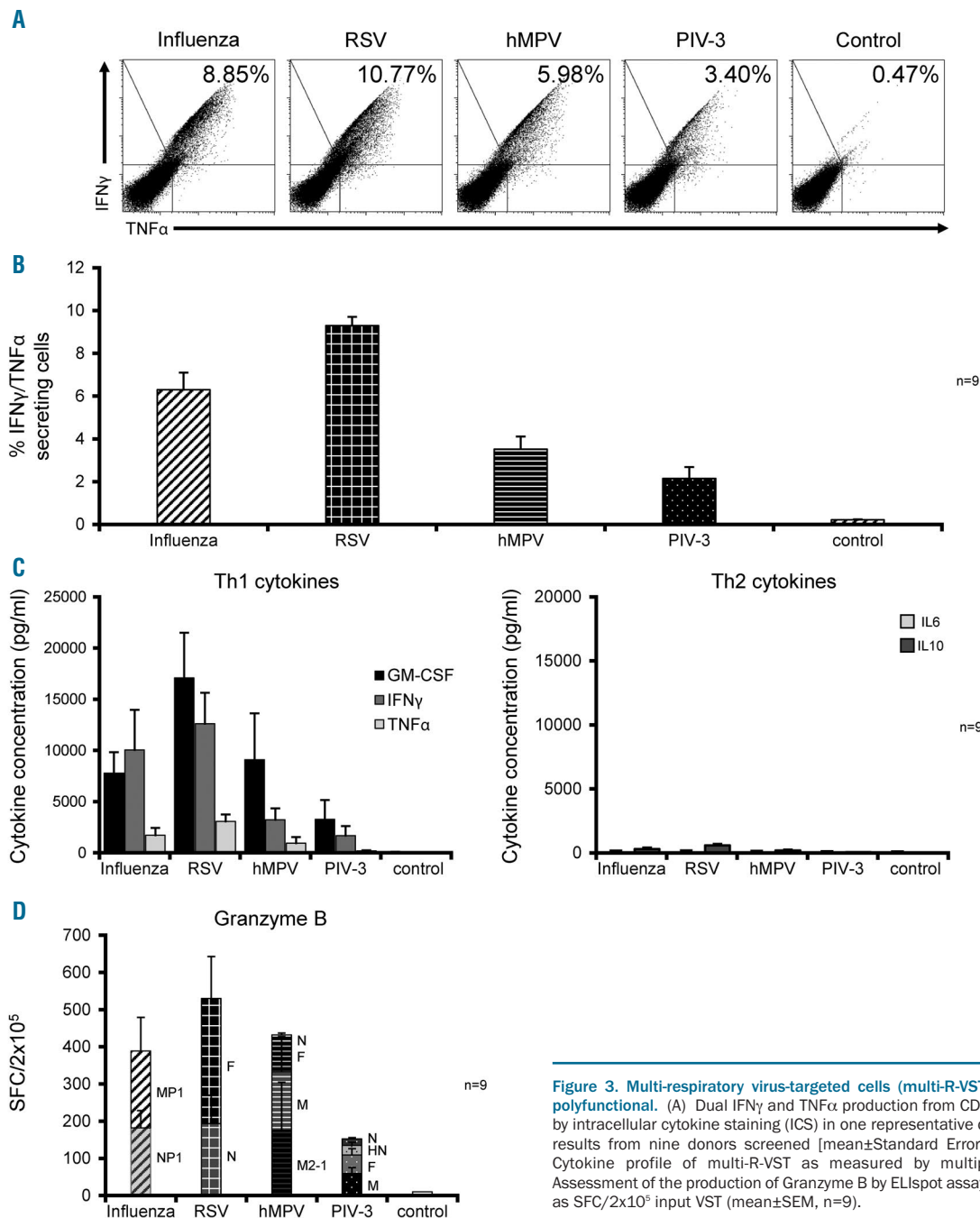

Figure 3. Multi-respiratory virus-targeted cells (multi-R-VST) are polyclonal and polyfunctional. (A) Dual IFNγ and TNFα production from CD3+ T cells as assessed by intracellular cytokine staining (ICS) in one representative donor. (B) Summary of results from nine donors screened [mean±Standard Error of Mean (SEM)]. (C) Cytokine profile of multi-R-VST as measured by multiplex bead array. (D) Assessment of the production of Granzyme B by ELIspot assay. Results are reported as SFC/2x10^5 input VST (mean±SEM, n=9).

dual/multiple co-existing infections are common, with frequencies that may exceed 40% among children under 5-years of age and are associated with increased risk of morbidity and hospitalization.[22-26] Among immunocompromised allogeneic HSCT recipients up to 40% experience CARV infections that can range from mild (associated symptoms including rhinorrhea, cough and fever) to severe (bronchiolitis and pneumonia) with associated mortality rates as high as 50% in those with LRTI.[5-9] The therapeutic options are limited. For hMPV and PIV-3, there are currently no approved preventative vaccines nor therapeutic antiviral drugs, while the off-label use of the nucleoside analog RBV and the investigational use of DAS-181 (a recombinant sialidase fusion protein) have had limited clinical impact.[10,11,27,28] The preventative annual Influenza vaccine is not recommended for allogeneic HSCT recipients until at least six months post transplant (and excluded in recipients of intensive chemotherapy or anti-B-cell antibodies), while neuraminidase inhibitors are not always effective for the treatment of active infections.[12] For RSV, aerosolized RBV is FDA-approved for the treatment of severe bronchiolitis in infants and children, and it is also used off-label for the prevention of upper or lower RTI and treatment of RSV pneumonia in HSCT recipients.[13,15,16] However, its widespread use is limited by the cumbersome nebulization device and ventilation system required for drug delivery, as well as the considerable associated cost. For example, in 2015, aerosolized RBV cost $29,953 per day, with five days representing a typical treatment course.[14] Thus, the lack of approved treatments combined with the high cost of antiviral agents led us to explore the potential for using adoptively-transferred T cells to prevent and/or treat CARV infections in this patient population.

The pivotal role of functional T-cell immunity in mediating viral control of CARV has only recently attracted attention. For example, a retrospective study of 181 HSCT patients with RSV URTI, reported lymphopenia (defined as ALC ≤100/mm^3) as a key determinant in identifying patients whose infections would progress to LRTI, while RSV neutralizing antibody levels were not significantly associated with disease progression.[29] Furthermore, in a recent retrospective analysis of 154 adult patients with hematologic malignancies with or without HSCT treated for RSV LRTI, lymphopenia was significantly associated with higher mortality rates.[30] Both of these studies are suggestive of the importance of cellular immunity in mediating protective immunity *in vivo*.

Our group has previously demonstrated the feasibility and clinical utility of *ex vivo*-expanded VST to treat a range of clinically problematic viruses including the latent viruses CMV, EBV, BKV, HHV-6 and AdV.[17,31-33] Our initial studies (and those of others)[34-37] explored the safety and activity of donor-derived T-cell lines, but more recently we have developed an "off the shelf" universal T-cell platform whereby VST specific for all five viruses (CMV, EBV, BKV, HHV-6, AdV) were prospectively generated and banked, thus ensuring their immediate availability for administration to immunocompromised patients with uncontrolled infections. Indeed, in our recent phase II clinical trial, we administered these partially HLA-matched VST to 38 patients with a total of 45 infections that had proven refractory to conventional antiviral agents and achieved an overall response rate of 92%, with no significant toxicity.[18] This precedent of clinical success using adoptively transferred T cells, as well as the absence of effective therapies for a range of CARV, prompted us to explore the potential for extending the therapeutic scope of VST therapy to Influenza, RSV, hMPV and PIV-3 infections post HSCT. In this context, one could consider the option of prophylactic VST administration seasonally to high-risk patients [e.g. young (<5 years) and elderly adults, patients with impaired immune systems]. Alternatively, these cells could be used therapeutically in patients with URTI who have failed conventional antiviral medications in order to prevent LRT progression.

Thus, using our established, GMP-compliant VST manufacturing methodology, we demonstrated the feasibility of generating VST reactive against a spectrum of CARV-derived antigens chosen on the basis of both their immunogenicity to T cells and their sequence conservation [Influenza – NP1 and MP1;[20,38,39] RSV – N and F;[15,16,20] hMPV – F, N, M2-1 and M;[21] PIV3 – M, HN, N and F[19]] from 12 donors with diverse haplotypes. The expanded cells were polyclonal (CD4$^+$ and CD8$^+$), Th1-polarized and polyfunctional, and were able to lyse viral antigen-expressing targets while sparing non-infected autologous or allogeneic targets, attesting to both their virus specificity and their safety for clinical use. Finally, to assess the clinical significance of these findings we examined the
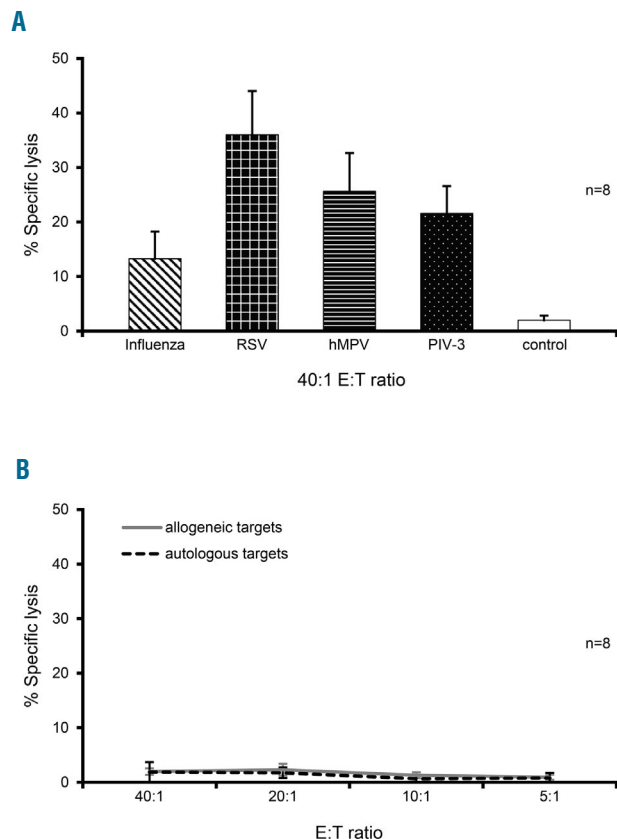

Figure 4. Multi-respiratory virus-targeted cells (multi-R-VST) are exclusively reactive against virus-infected targets. (A) Cytolytic potential of multi-R-VST evaluated by standard 4-hour Cr^{51} release assay using autologous pepmix-pulsed PHA blasts as targets (E:T 40:1; n=8) with unloaded phytohemagglutinin (PHA) blasts as a control. Results are presented as percentage of specific lysis (mean±SEM). (B) Demonstration that multi-R-VST show no activity against either non-infected autologous or allogeneic PHA blasts, as assessed by Cr^{51} release assay.
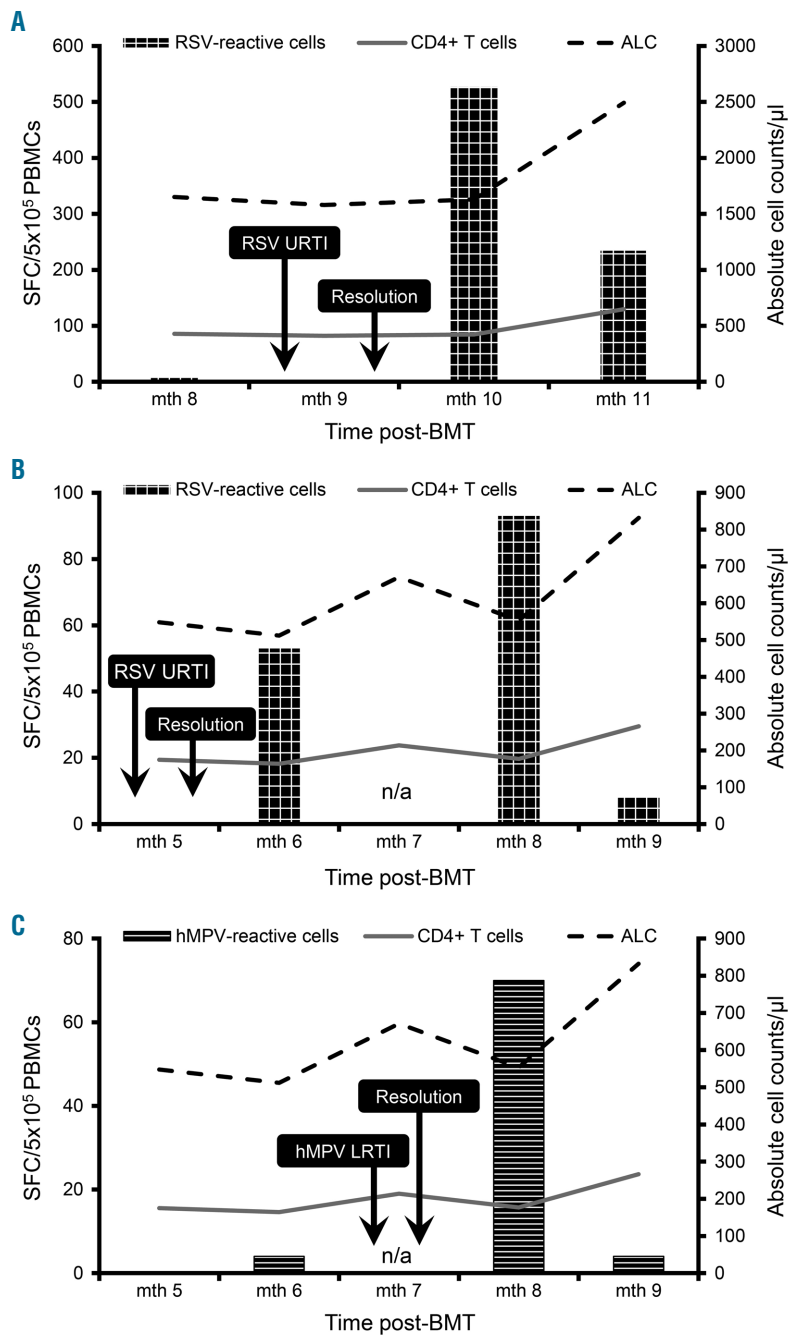
Figure 5. Detection of respiratory virus-targeted (RSV)- and human metapneumovirus (hMPV)-specific T cells in the peripheral blood of hematopoietic stem cell transplant (HSCT) recipients. Peripheral blood mononuclear cells (PBMC) isolated from two HSCT recipients with three infections were tested for specificity against the infecting viruses, using IFNγ ELIspot as a readout. (A and B) Results from two patients with RSV-associated upper respiratory tract infection (URTI) which was controlled, coincident with a detectable rise in endogenous RSV-specific T cells. (C) Clearance of an hMPV-lower respiratory tract infection (LRTI) with expansion of endogenous hMPV-specific T cells. ALC: absolute lymphocyte count.

peripheral blood of five allogeneic HSCT recipients with active RSV, hMPV and PIV-3 infections. Four of these patients successfully controlled the viruses within 1-5 weeks, coincident with an amplification of endogenous reactive T cells and subsequent return to baseline levels upon viral clearance, while one patient failed to mount an immune response against the infecting virus and has equally failed to clear the infection to date. These data suggests that the adoptive transfer of *ex vivo*-expanded cells should be clinically beneficial in patients whose own cellular immunity is lacking.

In conclusion, we have shown that it is feasible to rapidly generate a single preparation of polyclonal multi-respiratory (multi-R)-VST with specificities directed to Influenza, RSV, hMPV and PIV-3 in clinically relevant numbers using GMP-compliant manufacturing method-

ologies. These data provide the rationale for a future clinical trial of adoptively transferred multi-R-VST for the prevention or treatment of CARV infections in immunocompromised patients.

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