

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

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## Supplementary Methods

### Flow Cytometry

#### *Immunophenotyping*

Multi-R-VSTs were surface-stained with monoclonal antibodies to: CD3, CD25, CD28, CD45RO, CD279 (PD-1) [Becton Dickinson (BD), Franklin Lakes, NJ], CD4, CD8, CD16, CD62L, CD69 (Beckman Coulter, Brea, CA) and CD366 (TIM-3) (BioLegend, San Diego, CA). Cells were pelleted in phosphate-buffered saline (PBS) (Sigma-Aldrich), then antibodies added in saturating amounts (5 $\mu$ l) followed by incubation for 15mins at 4°C. Subsequently, cells were washed, resuspended in 300 $\mu$ l of PBS and at least 20,000 live cells acquired on a Gallios™ Flow Cytometer and analyzed with Kaluza® Flow Analysis Software (Beckman Coulter).

#### *Intracellular Cytokine Staining (ICS)*

Multi-R-VSTs were harvested, resuspended in VST medium (2x10<sup>6</sup>/ml) and 200 $\mu$ l added per well of a 96-well plate. Cells were incubated overnight with 200ng of individual test or control pepmixes along with Brefeldin A (1 $\mu$ g/ml), monensin (1 $\mu$ g/ml), CD28 and CD49d (1 $\mu$ g/ml) (BD). Next, VSTs were washed with PBS, pelleted, surface-stained with CD8 and CD3 (5 $\mu$ l/antibody/tube) for 15mins at 4°C, then washed, pelleted, fixed and permeabilized with Cytofix/ Cytoperm solution (BD) for 20mins at 4°C in the dark. After washing with Perm/Wash Buffer (BD), cells were incubated with 10 $\mu$ l of IFN $\gamma$  and TNF $\alpha$  antibodies (BD) for 30min at 4°C in the dark. Cells were then washed twice with Perm/Wash Buffer and at least 50,000 live cells were acquired on a Gallios™ Flow Cytometer and analyzed with Kaluza® Flow Analysis Software.

### *FoxP3 Staining*

FoxP3 staining was performed using the eBioscience FoxP3 kit (Thermo Fisher Scientific, Waltham, MA), per manufacturers' instructions. Briefly,  $1 \times 10^6$  cells were surface-stained with CD3, CD4 and CD25 antibodies, then washed, resuspended in 1ml fixation/permeabilization buffer and incubated for 1 hour at 4°C in the dark. After washing with PBS, cells were resuspended in permeabilization buffer, incubated with 5µl isotype or FoxP3 antibody (Clone PCH101) for 30 minutes at 4°C, then washed and acquired on a Gallios™ Flow Cytometer followed by analysis with Kaluza® Flow Analysis Software.

### **Functional Studies**

#### *Enzyme-Linked Immunospot (ELISpot)*

ELISpot analysis was used to quantitate the frequency of IFN $\gamma$  and Granzyme B-secreting cells. Briefly, PBMCs, magnetically selected T cell sub-populations and multi-R-VSTs were resuspended at  $5 \times 10^6$  or  $2 \times 10^6$  cells/ml in VST medium and 100µl of cells was added to each ELISpot well. Cell selection was performed using magnetic beads and LS separation columns (Miltenyi Biotec, GmbH), according to manufacturer's instructions. Antigen-specific activity was measured after direct stimulation (500ng/peptide/ml) with the individual stimulating [NP1, MP1 (Influenza); N, F (RSV); F, N, M2-1, M (hMPV); M, HN, N, F (PIV-3)], or control pepmixes (Survivin, WT1). Staphylococcal Enterotoxin B (SEB) (1µg/ml) and PHA (1µg/ml) were used as positive controls for PBMCs and VSTs, respectively. After 20 hours of incubation, plates were developed as previously described, dried overnight at room temperature and then sent to Zellnet Consulting (New York) for quantification. Spot-forming cells (SFC) and input cell numbers were plotted and the specificity threshold for VSTs was defined as  $\geq 30$  SFC/ $2 \times 10^5$  input cells.

## Multiplex

The multi-R-VST cytokine profile was evaluated using the MILLIPIX High Sensitivity Human Cytokine Panel (Millipore, Billerica, MA).  $2 \times 10^5$  VSTs were stimulated with pepmixes (NP1, MP1, N, F, F, N, M2-1, M, M, HN, N, and F) (1 $\mu$ g/ml) overnight. Subsequently, supernatant was collected, plated in duplicate wells, incubated overnight at 4°C with antibody-immobilized beads, then washed and plated for 1 hour at room temperature with biotinylated detection antibodies. Finally, streptavidin-phycoerythrin was added for 30 minutes at room temperature. Samples were washed and analyzed on a Luminex 200 (XMAP Technology) using the xPONENT software.

## Supplementary Figure Legends

**Supplementary Figure 1:** Minimal detection of Tregs (CD4+CD25+FoxP3+) within the expanded CD4+ T cell populations (mean±SEM, n=8).

**Supplementary Figure 2:** Number of donor-derived VST lines responding to individual stimulating antigens.

**Supplementary Figure 3:** Specificity of virus-reactive T cells within expanded T cell lines following exposure to titrated concentrations of pooled stimulating antigens from each of the target viruses. Data is presented as mean±SEM SFC/2×10<sup>5</sup> (n=7).

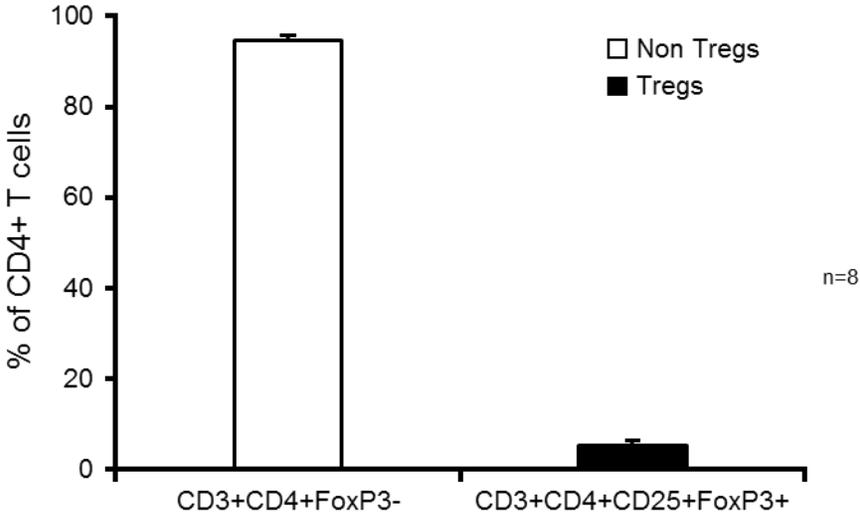
**Supplementary Figure 4:** Frequency of CARV-specific T cells in the peripheral blood of healthy donors following exposure to individual stimulating antigens from each of the target viruses. Data is presented as mean±SEM SFC/5×10<sup>5</sup> (n=12)

**Supplementary Figure 5:** Peripheral blood CARV-specific precursors are primarily detected within the CD4+ compartment. Shown here is the frequency of CARV-specific T cells in magnetically sorted CD4+ and CD8+ T cell populations isolated from the peripheral blood of healthy donors following exposure to individual stimulating antigens from each of the target viruses. Data is presented as mean±SEM SFC/5×10<sup>5</sup> (n=4).

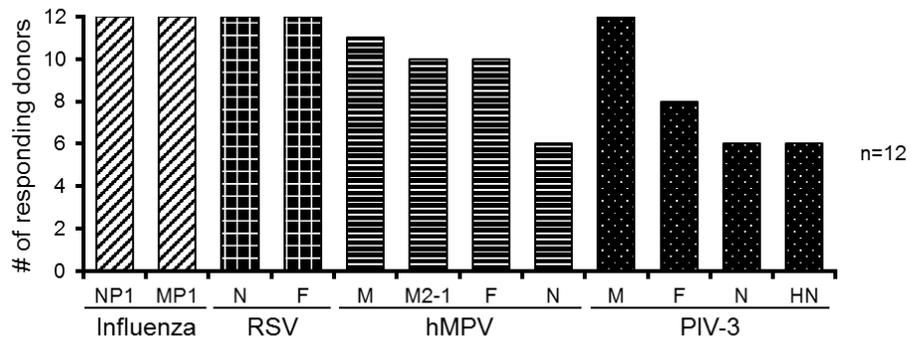
**Supplementary Figure 6:** Cytotoxic activity of multi-R-VSTs evaluated by standard 4-hour Cr<sup>51</sup> release assay using autologous pepmix-pulsed PHA blasts as targets (E:T 40:1, 20:1, 10:1, 5:1) with unloaded PHA blasts as a control. Results are presented as percentage of specific lysis (mean±SEM, n=8).

**Supplementary Figure 7:** Detection of RSV- and PIV3-specific T cells in the peripheral blood of HSCT recipients. PBMCs isolated from 3 HSCT recipients with 3 infections were tested for specificity against the infecting viruses, using IFN $\gamma$  ELISpot as a readout. Panels A and B show results from 2 patients with RSV- and PIV3-associated URTIs and LRTIs which were controlled, coincident with a detectable rise in endogenous virus-specific T cells. Panel C shows results from a patient with an ongoing PIV3-related severe URTI who failed to mount a T cell response against the virus. ALC: absolute lymphocyte count.

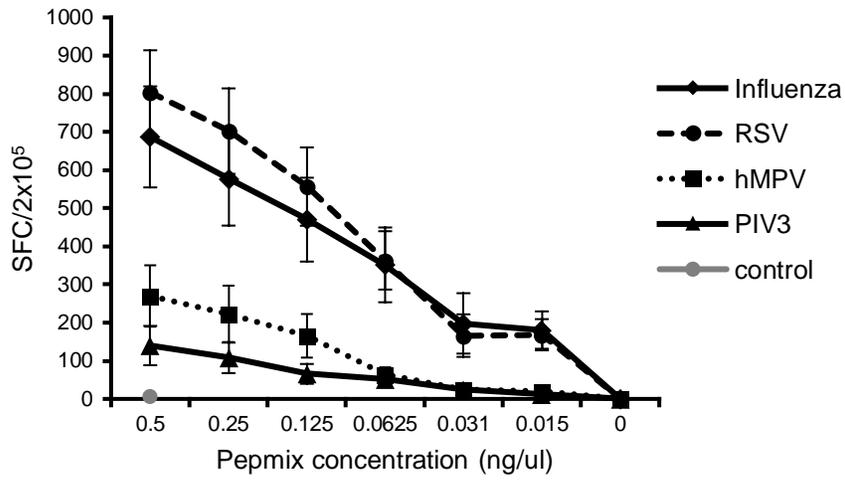
Supplementary Figure 1



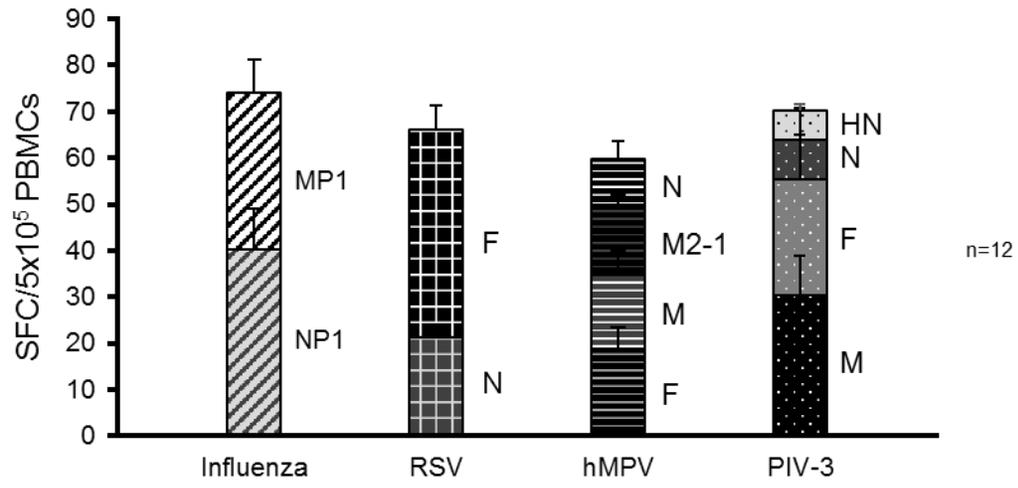
Supplementary Figure 2



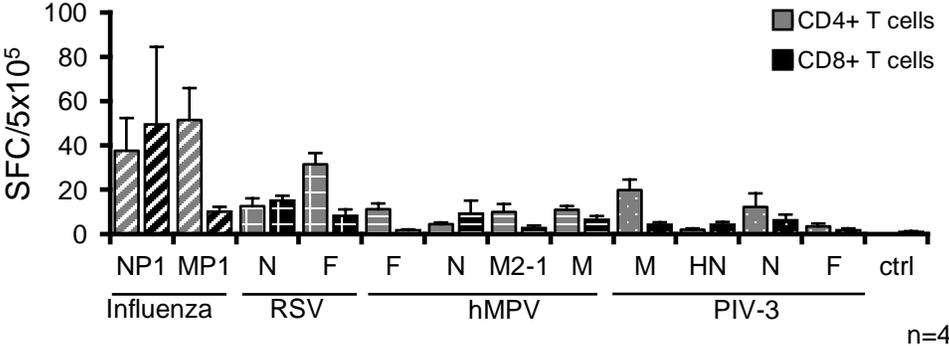
Supplementary Figure 3



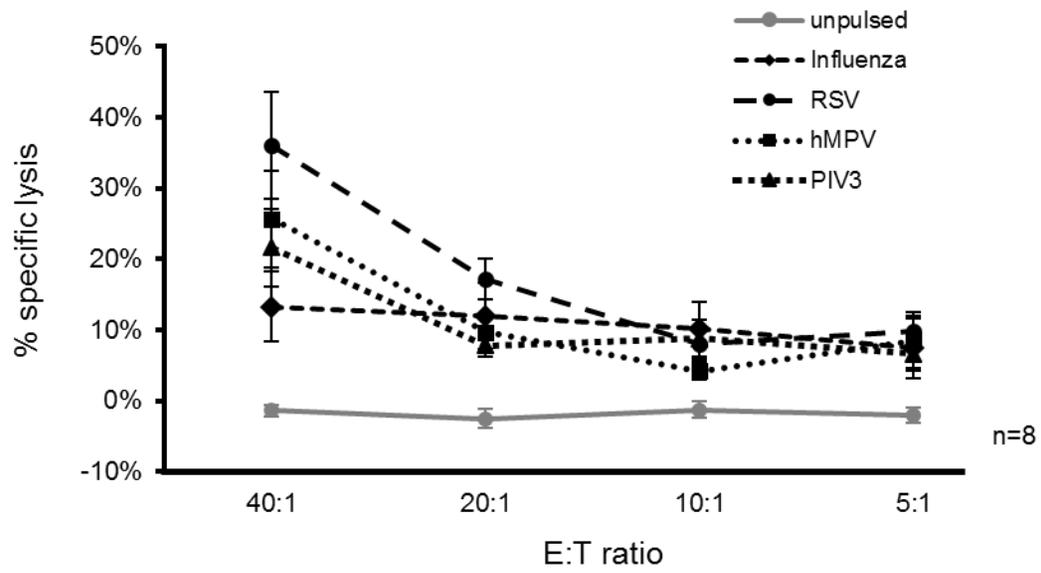
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7

