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TITLE:
Immunologic Profiling of Human Metapneumovirus for the Development of Targeted Immunotherapy

ABSTRACT:
Human metapneumovirus (hMPV) is a respiratory virus detected in ≥9% of allogeneic hematopoietic stem cell transplant (HSCT) recipients, in whom it can cause significant morbidity and mortality. Given the lack of effective antivirals, we investigated the potential for immunotherapeutic intervention, using adoptively transferred T cells. Thus, we characterized the cellular immune response to the virus and identified F, N, M2-1, M, and P as immunodominant target antigens. Reactive T cells were polyclonal (ie, they expressed CD4 and CD8), T-helper type 1 polarized, and polyfunctional (ie, they produced interferon γ, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, and granzyme B), and they were able to kill autologous antigen-loaded targets. The detection of hMPV-specific T cells in HSCT recipients who endogenously controlled active infections support the clinical importance of T-cell immunity in mediating protective antiviral effects. Our results demonstrate the feasibility of developing an immunotherapy for immunocompromised patients with uncontrolled infections.

Donors and Cell Lines ::: MATERIALS AND METHODS:
Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors and allogeneic HSCT recipients with informed consent under Baylor College of Medicine Institutional Review Board–approved protocols (H-7634, H-36346, H-35436, and H-25064) and were used to generate hMPV-directed virus-specific T cells (VSTs) and phytohemagglutinin (PHA) blasts. PHA blasts were generated from PBMCs (5 × 105 cells/mL) in 24-well plates by using PHA (5 μg/mL; Sigma-Aldrich, St Louis, MO) and were maintained in VST medium (Roswell Park Memorial Institute 1640 [HyClone Laboratories, Logan, UT], 45% Click's [Irvine Scientific, Santa Ana, CA], 2 mM GlutaMAX TM-I [Life Technologies, Grand Island, NY], and 5% human AB serum [Valley Biomedical, Winchester, VA]) supplemented with interleukin 2 (100 U/mL; National Institutes of Health, Bethesda, VA), which was replenished every 3 days.

Pepmixes ::: VST Generation ::: MATERIALS AND METHODS:
For PBMC stimulation, we used pepmixes (peptide libraries of 15mers overlapping by 11 amino acids) spanning the antigens N, P, M, F, M2-1, M2-2, SH, G, and L derived from the hMPV strain CAN97-83 (hMPV substrain A2). All pepmixes were synthesized by JPT Peptide Technologies (Berlin, Germany). Lyophilized pepmixes were reconstituted at 400 ng/μL in dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) and stored at −80°C.

VST Activation ::: VST Generation ::: MATERIALS AND METHODS:
Fifteen million fresh/frozen PBMCs were pelleted in a 15-mL tube, pulsed for 30 minutes at 37°C with pepmixes at a concentration of 200 ng/peptide/15 × 106 PBMCs, and then resuspended in VST medium supplemented with 400 U/mL interleukin 4 and 10 ng/mL interleukin 7 (R&D Systems, Minneapolis, MN) and plated in either 24-well plates (2 × 106 cells/well) or transferred to a G-Rex10 device (15 × 106 cells/G-Rex10 devise; Wilson Wolf, Minneapolis, MN). Medium and cytokines were replenished on day 7, and cultures were split when they reached a density of >3 × 106 cells/well (for 24-well plate) or >50 × 106 cells (for the G-Rex10 device). On days 9–11, VSTs were harvested, counted, and used for phenotypic and functional studies.

VST Expansion ::: VST Generation ::: MATERIALS AND METHODS:
For the second stimulation, 1–2 × 107 hMPV-specific T cells were plated with 1 × 107 irradiated (30 Gy), pepmix-pulsed autologous PHA blasts. The cells were resuspended in 30 mL of VST medium supplemented with interleukin 4 and interleukin 7, and transferred to a G-Rex10 device. On days 3 and 7 (±1 day), cultures were replenished with fresh medium supplemented with 5 ng/mL interleukin 15 (CellGenix, Freiburg, Germany). On days 19–21, VSTs were harvested and used for further studies.

Immunophenotyping ::: Flow Cytometry ::: MATERIALS AND METHODS:
hMPV-specific T cells were surface stained with monoclonal antibodies to CD3, CD56, CD27, CD45RO, and CCR7 (Becton Dickinson [BD], Franklin Lakes, NJ) and to CD4, CD8, CD16, CD27,

and CD62L (Beckman Coulter, Pasadena, CA). For staining, cells were washed once with phosphate-buffered saline (PBS; Sigma Aldrich, St Louis, MO) and pelleted, and antibodies were added in saturating amounts (2–5 µL). After incubation for 15 minutes at 4°C in the dark, cells were washed twice and analyzed. Approximately 20000 live cells were acquired on a Gallios flow cytometer (Beckman Coulter, Brea, CA), and the data were analyzed using Kaluza flow cytometry analysis software (Beckman Coulter).

Intracellular Cytokine Staining ::: Flow Cytometry ::: MATERIALS AND METHODS:
VSTs were harvested, resuspended at a concentration of 2 × 106 cells/mL in VST medium, and plated at 200 µL/well in a 96-well plate. The cells were then stimulated with 200 ng of test or control pepmix in the presence of brefeldin A (1 µg/mL), monensin (1 µg/mL), CD28, and CD49d (1 µg/mL; BD) overnight. Subsequently, VSTs were washed with PBS, pelleted, and surface stained with CD8 and CD3 (5 µL/antibody/tube). After incubation for 15 minutes at 4°C in the dark, they were washed, pelleted, fixed, and permeabilized with Cytofix/Cytoperm solution (BD) for 20 minutes at 4°C in the dark. After washing with PBS containing fetal bovine serum and saponin (BD), cells were incubated with 20 µL of interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) antibodies (BD) for 30 minutes at 4°C in the dark. Cells were then washed twice with cold PBS containing fetal bovine serum and saponin, and at least 20 000 live cells from each population were analyzed with a FACSCalibur equipped with Gallios software. The data were analyzed using Kaluza flow cytometry analysis software (Beckman Coulter).

FoxP3 Staining ::: Flow Cytometry ::: MATERIALS AND METHODS:
FoxP3 staining was performed using the eBioscience FoxP3 kit per the manufacturer's instructions. Briefly, VSTs were rested in medium without cytokines for 48 hours, and 1 × 106 cells were washed with PBS and surface stained with CD3, CD4, and CD25 antibodies (BD) for 15 minutes. The cells were then washed, resuspended in 1 mL of fixation/permeabilization buffer, and incubated for 1 hour at 4°C in the dark. After washing with PBS, the cells were resuspended in permeabilization buffer and incubated with 5 µL of isotype or FoxP3 antibody (clone PCH101) for 30 minutes at 4°C. Following a final wash, cells were acquired and analyzed with a FACSCalibur. The data were analyzed using Kaluza flow cytometry analysis software (Beckman Coulter).

Enzyme-Linked Immunospot (ELISPOT) Assay ::: Functional Studies ::: MATERIALS AND METHODS:
ELISPOT analysis was used to quantitate the frequency of antigen-specific IFN-γ– and granzyme B–producing T cells. PBMCs and hMPV-specific T cells were resuspended at 5 × 106 and 2 × 106 cells/mL, respectively, in VST medium, and 100 µL of cells was added to each ELISPOT well. Antigen-specific activity was measured after direct stimulation (500 ng/peptide/mL) with each of the individual hMPV pepmixes (N, P, M, F, M2-1, M2-2, SH, G, and L) or control pepmixes (N and F [for respiratory syncytial virus], M and HN [for parainfluenza virus type 3], and IE1 and pp65 [for cytomegalovirus]). PHA (1 µg/mL) and staphylococcal enterotoxin B (1 µg/mL) served as positive controls for VSTs and PBMCs, respectively, while unstimulated cells served as a negative control. Each condition was run in duplicate, and results were considered positive if the frequency of reactive cells was ≥30 spot-forming cells (SFCs; defined as our potency threshold). After 20 hours of incubation, plates were developed as previously described, dried overnight at room temperature in the dark, and sent to Zellnet Consulting (New York, NY) for quantification. SFC and input cell numbers were plotted.

Multiplex ::: Functional Studies ::: MATERIALS AND METHODS:
The hMPV-specific T-cell cytokine profile was assessed using the Milliplex High Sensitivity Human Cytokine Panel (Millipore, Billerica, MA) per the manufacturer's instructions. A total of 2 × 105 VSTs were stimulated using N, P, M, F, or M2-1 pepmixes (200 ng/pepmix/well) overnight. Subsequently, supernatant was collected, plated in duplicate wells, incubated overnight at 4°C with antibody-immobilized beads, and 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 xPonent software.

Chromium Release Assay ::: Functional Studies ::: MATERIALS AND METHODS:
We measured the cytotoxic specificity of the hMPV-specific T cells in a standard 4-hour Cr51 release assay, using effector to target ratios of 40:1, 20:1, 10:1, and 5:1. VSTs were used as

effectors, and the targets were autologous PHA blasts pulsed with pepmixes. Autologous PHA blasts alone were used as specificity controls. The percentage of specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Respiratory Virus Panel ::: Patient Screening ::: MATERIALS AND METHODS:
Nasopharyngeal wash samples (from patients with upper respiratory tract infection) or bronchoalveolar lavage samples (from patients with lower respiratory tract infection) were tested for hMPV and other pathogens, including adenovirus, coronavirus, rhinovirus/enterovirus, influenza virus, parainfluenza virus, respiratory syncytial virus, Bordetella pertussis, Chlamydophila pneumoniae, and Mycoplasma organisms, using a multiplex reverse transcription polymerase chain reaction assay. Samples were analyzed by Viracor Eurofins Diagnostics (Lee's Summit, MO) or Houston Methodist Hospital (Houston, TX).

Identification of Immunogenic hMPV Antigens ::: RESULTS:
Since little is known about the T-cell response to hMPV, we isolated PBMCs from healthy donors, stimulated them with pepmixes spanning all 9 viral antigens, and evaluated the frequency of antigen-specific T cells circulating in peripheral blood, using an IFN-γ ELISPOT assay. As shown in Figure 1C, the magnitude of activity in peripheral blood was low. For example, in 18 donors screened (age range, 24–59 years; mean age [±standard error of the mean {SEM}], 35 ± 2.1 years), we detected the following mean numbers (±SEM) of antigen-reactive SFCs/5 × 105 input PBMCs: 7.7 ± 1.3 for N, 5.7 ± 2.1 for P, 8.2 ± 1.9 for M, 12.0 ± 2.6 for F, 7.4 ± 1.7 for M2-1, 2.0 ± 0.7 for M2-2, and 2.0 ± 0.4, 3.4 ± 1.1, and 3.2 ± 0.7 for SH, G, and L, respectively. This magnitude of activity was similar to that for antigens of respiratory syncytial virus, a genetically and clinically closely related family member (mean [±SEM], 12 ± 1.4 and 15 ± 2.8 antigen-reactive SFCs/5 × 105 input PBMCs for N and F, respectively; n = 18) [32], and of parainfluenza virus type 3 (mean [±SEM], 18 ± 3.1 and 8 ± 1.5 antigen-reactive SFCs/5 × 105 input PBMCs for M and HN, respectively; n = 18)] [33], but it was substantially less than the response against the immunodominant cytomegalovirus antigens IE1 and pp65 (mean [±SEM], 164 ± 42 and 561 ± 59 SFCs/5 × 105 input PBMCs, respectively; Figure 1C).

Amplification of hMPV-Reactive T Cells In Vitro ::: RESULTS:
To determine whether the apparent paucity of hMPV-reactive T cells in peripheral blood simply reflected a frequency of cells that was below the limit of detection of the ELISPOT assay, we performed a single in vitro stimulation assay to selectively amplify specific cells. Thus, we exposed PBMCs from 29 healthy individuals (age range, 21–59 years; mean age [±SEM], 32 ± 1.6 years) to a mastermix of the hMPV peptide libraries and allowed reactive T cells to expand for 9–11 days. Overall, we achieved a mean fold-increase (±SEM) 4.3 ± 0.2 in total cell numbers (n = 29; Figure 2A), and the expanded populations comprised CD3+ T cells (91.7% ± 1%), with a mix of cytotoxic (CD8+) T cells (29.5% ± 2.3%) and helper (CD4+) T cells (61.8% ± 2.3%; Figure 2B), with no evidence of regulatory T-cell outgrowth, as assessed by CD4+CD25+FoxP3+ staining (Supplementary Figure 1). Furthermore, the expanded cells expressed both central (CD45RO+CD62L+; mean [±SEM], 38.6% ± 2.4%) and effector (CD45RO+CD62L−; mean [±SEM], 26.9% ± 2.2%) memory markers (n = 29; Figure 2B). To next determine whether we had enriched for hMPV-reactive cells, we assessed specificity by the IFN-γ ELISPOT assay, and, as shown in Figure 3A, we were readily able to expand populations that were specific for all antigens except M2-2. The fold enrichment achieved is summarized in Figure 3B. These data suggest that hMPV-specific T cells reside in the memory pool and can be readily amplified ex vivo.
We then sought to identify a hierarchy of immunodominance based on (1) the number of donors who positively responded to each antigen (≥30 SFCs/2 × 105 cells) and (2) the magnitude of the response (based on the absolute number of IFN-γ–producing SFCs per 2 × 105 input cells; Table 1). F was recognized by 28 of 29 donors screened (97%) and induced the highest frequency of specific cells (mean [±SEM], 300 ± 52 SFCs/2 × 105 input cells), followed, in descending order, by N (218 ± 28; n = 25), M2-1 and M (167 ± 21 and 154 ± 24, respectively; n = 23 for both), P (130 ± 24; n = 20), L (125 ± 21.7; n = 13), and G (62 ± 18; n = 9). Reactivity against SH was detected in only 1 donor screened (34 SFCs/2 × 105 input cells), whereas M2-2 was not immunogenic. Notably, the majority of donors screened recognized ≥5 of the expressed antigens (Figure 3C). Thus, for subsequent functional studies, we chose to focus on the top 5 immunogenic target antigens (F, N, M2-1, M, and P).

Functional Characterization of hMPV-Specific T Cells ::: RESULTS:

To evaluate whether hMPV specificity was mediated predominantly by the CD4+ and/or CD8+ T-cell subsets, we performed intracellular cytokine staining studies and gated on IFN-γ– and TNF-α–producing CD3+CD4+ and CD3+CD8+ T cells. For all 5 antigens, specificity, as determined by production of either cytokine, was detected predominantly in the CD4+ T-cell compartment, with a minor CD8-reactive component. Figure 4A shows fluorescence-activated cell-sorting data from a representative donor, while Figure 4C summarizes data from all 14 donors studied.
Production of multiple proinflammatory cytokines and effector molecules has been reported to correlate with enhanced cytolytic function and improved in vivo activity. Thus, we comprehensively explored the functional profile of our expanded VSTs and found that the majority of IFN-γ–producing cells (Figure 5A) also produced TNF-α (detailed results from 1 donor are shown in Figure 4B; data for 14 donors are summarized in Figure 4D). Furthermore, the lines produced granulocyte-macrophage colony-stimulating factor (GM-CSF; Figure 5B), as well as granzyme B (mean [±SEM], 99 ± 22 SFC/2 × 105 input cells for F, 85 ± 20 for N, 96 ± 14 for M2-1, 60 ± 17 for M, and 40 ± 10 for P; n = 14 donors screened; Figure 6A) with production of Th2 cytokines, including interleukin 6 and interleukin 10, detected at levels similar to control VSTs (Figure 5C–D). Thus, our expansion of hMPV-reactive cells induced polyclonal, Th1-polarized T-cell lines.

hMPV-Specific T Cells Are Cytolytic and Kill Virus-Infected Targets ::: RESULTS:
To determine whether the expanded hMPV-specific T cells were cytolytic, we cocultured hMPV-specific T cells with Cr51-labeled, peptide-loaded autologous PHA blasts with unloaded PHA blasts serving as a control. As shown in Figure 6B, hMPV-loaded targets were specifically recognized and lysed by our expanded hMPV-specific T cells at all effector to target ratios tested (mean percentage lysis [±SEM], 22% ± 1.9% for the ratio of 40:1, 18% ± 3% for 20:1, 18% ± 3.7% for 10:1, and 12% ± 2% for 5:1; n = 10). In contrast, control target cells were not killed (mean percentage lysis [±SEM], 6.5% ± 1.5% for the ratio of 20:1).

Detection of hMPV-Specific T Cells After In Vivo Viral Reactivation ::: RESULTS:
Finally, to assess the clinical relevance of hMPV-specific T cells, we investigated whether allogeneic HSCT recipients had evidence of amplified levels of T cells directed against our identified immunodominant target antigens in their peripheral blood during and/or after clearance of hMPV infection. Figure 6C shows the results for patient 1, a 20-month-old girl who developed an upper respiratory tract infection 18 days before her scheduled receipt of a matched related donor transplant for acute lymphoblastic leukemia. She presented with symptoms including congestion, rhinorrhea, and cough, and hMPV was detected by respiratory virus panel (RVP) screening performed on a nasal wash sample. Since her symptoms had resolved by day 5 before transplant receipt, her myeloablative transplantation proceeded on schedule, but hMPV remained detectable by RVP PCR until day 45 after transplantation. To assess whether reconstituting hMPV-specific T cells contributed to viral clearance, we collected blood specimens before and 1, 2, 3, 4, and 5 weeks after HSCT receipt and measured specific T-cell activity. As shown in Figure 6C, this patient exhibited strong T-cell activity as early as 3 weeks after transplantation, which subsequently declined coincident with viral clearance. We were able to detect similar patterns of T-cell activity in 3 additional transplant recipients with confirmed hMPV infections that were cleared. These results are summarized in Supplementary Table 1 and Supplementary Figure 2A–C.

DISCUSSION:
In this study, we characterized the T-cell immune response against all 9 virally encoded hMPV antigens and, based on both the frequency of responding donors and the magnitude of reactive cells, were able to define a hierarchy of immunodominance, with F being the most immunogenic, followed in descending order by N, M2-1, M, P, L, G, and SH; M2-2 was not immunogenic. Reactive T cells could be readily enriched in vitro and were polyclonal, Th1-polarized, polyfunctional (producing IFN-γ, TNF-α, GM-CSF, and granzyme B), and able to kill autologous antigen-loaded target cells. Finally, the potential for clinical benefit following the adoptive transfer of such cells was inferred by the detection of reactive T-cell populations in the peripheral blood of allogeneic HSCT recipients who successfully cleared active hMPV infections.
The lack of effective treatments for hMPV in immunocompromised patients led our group to investigate the potential for using adoptively transferred donor-derived T cells to prevent and/or treat hMPV infections, given the success of this approach against other clinically problematic viruses, including cytomegalovirus, Epstein-Barr virus, BK virus, human herpesvirus 6, and adenovirus [9]. Thus, we first sought to identify appropriate hMPV antigens against which an

effector T-cell response might be generated. Prior to the current study, there was limited information on immunity to hMPV, although there is some evidence to suggest the importance of cell-mediated over humoral antiviral activity. For example, in immunocompromised individuals (eg, human immunodeficiency virus–infected patients and steroid-treated HSCT recipients), hMPV infections can be severe and sometimes fatal [23, 34–36], and treatment with intravenous immunoglobulin has not proven beneficial [23, 30]. In murine studies, Kolli et al intranasally infected BALB/c mice with a laboratory strain of hMPV (CAN97-83) or exposed animals to UV-inactivated hMPV and demonstrated that the depletion of either CD4+ or CD8+ T cells compromised the animals' ability to control/clear the viral challenge [37]. Similarly, Hastings et al vaccinated HLA-A2 transgenic C57BL/6 mice with either an hMPV M–derived CD8+ T-cell epitope or irrelevant control, challenged them with a live hMPV strain (TN/94-49), and demonstrated that the viral load in the lungs of vaccinated animals was significantly lower than that for control mice [38]. Finally, Herd et al demonstrated that T cells with an effector profile, characterized by production of both prototypic Th1 cytokines (IFN-γ, GM-CSF, and interleukin 12) and proinflammatory chemokines (macrophage inflammatory protein 1α and IFN-γ–inducible protein 10), were able to accumulate at the lung following hMPV infection [39]. However, since immune studies of human viruses performed in animals do not always faithfully recapitulate studies performed in the natural host, we sought to analyze the T-cell response to hMPV in healthy human subjects and hMPV-infected HSCT recipients. We initiated our studies in healthy adults and discovered that the frequency of circulating hMPV-reactive T cells was at or below the threshold of our detection assay (IFN-γ ELISPOT). However, reactive cells could readily be expanded to numbers that were clinically relevant [7, 9, 40] with a single in vitro stimulation.

To identify which (if any) of the 9 encoded antigens induced protective immunity, we took a 2-pronged approach. First, we profiled the expanded T-cell product to determine which antigens were most frequently recognized by the majority of donors. After screening 29 healthy individuals, we identified a clear hierarchy of immunodominance, with F being the most frequently recognized, followed by N, M2-1, M, P, L, G, and SH; M2-2 was not immunogenic. Second, we examined peripheral blood specimens from 4 allogeneic HSCT recipients with active hMPV infections (confirmed by RVP PCR) who successfully cleared hMPV coincident with an amplification of reactive T cells, with subsidence upon viral clearance. Finally, to establish the feasibility of using adoptively transferred T cells as a prophylactic or therapeutic approach in immunocompromised patients, we demonstrate that hMPV-reactive T cells with a Th1-polarized effector profile can be successfully expanded to clinically relevant numbers in vitro by means of manufacturing approaches compliant with good manufacturing practice. Taken together, these studies support the development of a future clinical trial of adoptively transferred hMPV-specific T cells specific for either prophylactic or therapeutic use in immunocompromised patients.