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Cytotoxic T-Lymphocyte Epitope Vaccination Protects against Human Metapneumovirus Infection and Disease in Mice†

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Human metapneumovirus (hMPV) has emerged as an important human respiratory pathogen causing upper and lower respiratory tract infections in young children and older adults. In addition, hMPV infection is associated with asthma exacerbation in young children. Recent epidemiological evidence indicates that hMPV may cocirculate with human respiratory syncytial virus (hRSV) and mediate clinical disease similar to that seen with hRSV. Therefore, a vaccine for hMPV is highly desirable. In the present study, we used predictive bioinformatics, peptide immunization, and functional T-cell assays to define hMPV cytotoxic T-lymphocyte (CTL) epitopes recognized by mouse T cells restricted through several major histocompatibility complex class I alleles, including HLA-A*0201. We demonstrate that peptide immunization with hMPV CTL epitopes reduces viral load and immunopathology in the lungs of hMPV-challenged mice and enhances the expression of Th1-type cytokines (gamma interferon and interleukin-12 [IL-12]) in lungs and regional lymph nodes. In addition, we show that levels of Th2-type cytokines (IL-10 and IL-4) are significantly lower in hMPV CTL epitope-vaccinated mice challenged with hMPV. These results demonstrate for the first time the efficacy of an hMPV CTL epitope vaccine in the control of hMPV infection in a murine model.

Human metapneumovirus (hMPV) is a recently discovered pathogen first identified in respiratory specimens from young children suffering from clinical respiratory syndromes, ranging from mild to severe lower respiratory tract illness (55), and may exacerbate asthma and wheezing in young children (35). Seroprevalence studies have shown that hMPV has worldwide distribution and is acquired early in life, and by the age of 5 years, approximately 70% of all children develop antibodies to hMPV (33). The elderly and immunocompromised are also susceptible to hMPV respiratory tract infections (8, 17, 55).

hMPV is provisionally classified in the *Metapneumovirus* genus in the family *Paramyxoviridae*. This family contains several major human respiratory pathogens, including human respiratory syncytial virus (hRSV). Based on phenotypic and genotypic characteristics, hMPV is most closely related to avian metapneumovirus, inviting speculation of a species jump, though archival sera suggest that hMPV has been present in the human population for at least 50 years (55). The single-strand negative sense RNA genome of approximately 13 kb has been fully sequenced (5), and eight genes encode nine viral proteins: transmembrane surface glycoproteins (F, G, and SH), matrix protein (M), regulatory factors for transcription (M2-1) or RNA synthesis (M2-2), and nucleocapsid-associated proteins (N, P, and L). hMPV differs from hRSV in gene order and in the absence of the nonstructural NS1 and NS2 genes (6). Sequence analysis indicates two genotypes, A and B, of hMPV worldwide, with 80 to 88% similarity between the two types and 93 to 100% similarity within each type (55). The biochemical nature of hMPV proteins has not yet been fully characterized. hRSV has two major surface proteins, namely, the attachment (G) and fusion (F) proteins, which are important for infection, i.e., hRSV infects epithelial cells of the respiratory tract by interaction of heparin-binding domains on these surface proteins with glycosaminoglycans on the surface of the cell (50). However, the majority of virus binding is attributed to the G protein (10). In addition, the central conserved region of the G protein, which is nonglycosylated, contains a CX3C chemokine motif positioned at amino acids 182 to 186 and is capable of interacting with the CX3C chemokine receptor to aid infectivity (52). Based on the distribution of hydrophobic and hydrophilic regions along the predicted amino acid sequence, the putative hMPV G protein does not appear to have a CX3C chemokine motif; however, like that of hRSV, it consists of a type II mucin-like glycoprotein (37). The putative hMPV F protein, similar to that of hRSV, has two heptad repeats that may be required for viral fusion (4). Therefore, it is highly probable that the hRSV and hMPV G and F proteins have similar features necessary for infection.

The disease burden associated with hMPV infection is unclear, largely due to its cocirculation with hRSV (34), with up to 70% of infants with hRSV bronchiolitis carrying hMPV in some series (20). However, hMPV respiratory tract infection in the absence of other known respiratory pathogens is common (28, 33). The timing of the seasonal annual incidence of hMPV-versus-hRSV–associated disease differs (34) (M. Nissen, unpublished observations). There is evidence that disease associated with concomitant hRSV and hMPV infections has

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TABLE 1. hMPV CTL epitopes recognized by murine CTLs

Epitope[a]	hMPV protein	MHC restriction
^{164}VGALI**F**TK**L**^{172}	N	*H-2K*^{b}
^{56}**C**Y**L**ENIE**I**^{64}	M2-2	*H-2K*^{d}
^{32}**S**L**I**LIGITT**L**^{41}	G	HLA-A*0201
^{35}**K**L**I**LALLTF**L**^{44}	SH	HLA-A*0201

[a] Putative MHC "anchor" residues are in bold.

more severe long-term sequelae than that without hMPV involvement (9, 43).

No medications are known to inhibit the growth of hMPV either in vitro or in vivo. As for hRSV, a vaccine is highly desirable for this significant respiratory pathogen, and a vaccine eliciting neutralizing antibody alone is unlikely to be sterilizing or fully protective against disease. Indeed, it has recently been shown in a murine model of hMPV infection that hMPV persists despite the presence of neutralizing antibodies (1). T-cell involvement in the control of hMPV infection is indicated by the observation that T-cell deletion resulted in increased titers of hMPV in the lungs of infected mice (1). Similar T-cell involvement is reported in the control of hRSV infections (45). In the present study, we identify major histocompatibility complex class I (MHC-I)-restricted cytotoxic T-lymphocyte (CTL) epitopes in hMPV which, when administered to mice as a peptide vaccine, generate effector and memory CTL responses and Th1-type cytokine responses which are associated with protection against hMPV infection. In addition, lung histopathology shows reduced mononuclear cell inflammatory infiltration. These studies provide the foundation for the development of vaccines or treatment modalities for hMPV infection.

MATERIALS AND METHODS

Epitope prediction. The genome sequence of type A hMPV/NDL00-1 (NCBI accession no. NC_004148.1, derived from AF371337), with annotated coding regions and conceptual translations, was used to predict MHC-I-restricted T-cell epitopes in hMPV nucleoprotein (N), matrix protein (M), fusion protein (F), matrix proteins 2-1 and 2-2 (M2-1, M2-2), small hydrophobic protein (SH), and attachment glycoprotein (G) (55). Sequences from the type A hMPV/AUSQ01 strains were also used for the G protein (29) (NCBI protein identification no. AAQ01368-76) and F protein (unpublished data) (NCBI protein identification no. AAL35367, 35369, 35370).

Two independent online algorithms, SYFPEITHI with PAProc (27) (http://www.syfpeithi.de; http://www.paproc.de) and ProPred1 (46) (http://www.imtech.res.in/raghava/propred1), were used to predict proteasomal cleavage and MHC-I binding for five MHC alleles (mouse *H-2K*^{b}, *D*^{b}, *K*^{d}, and *L*^{d} and human HLA-A*0201). The following selection criteria were used. First, sequences with high MHC binding scores were preselected from within each protein. Next, sequences with the best MHC binding scores and a proteasomal cleavage site (C terminus) were selected from within the entire proteome. These sequences were ranked according to the MHC binding score for each online algorithm. Ultimately, the results from both algorithms were combined in a panel (consensus prediction) of limited size (n = 5/mouse allele, n = 16/human allele, total = 36). To assure prediction quality, "virtual polypeptides" were created with published ligands and T-cell epitopes listed in the SYFPEITHI database and were used to define confidence levels. Moderate confidence levels were set to include the 10 highest MHC binding scores for a particular allele. For SYFPEITHI, they were >21%, >23%, >24%, >21%, and >26% for the *K*^{b}, *D*^{b}, *K*^{d}, *L*^{d}, and A*0201 alleles, respectively. For ProPred1, they were >23%, >52%, >92%, >49%, and >42%. Any hMPV sequences with MHC binding scores in the moderate confidence level and/or high algorithm rank (1 to 4) were considered to be predicted epitopes or "predictopes" (Table 1).

Peptides. Predictopes were synthesized as a PepSet (>1 μmol scale; Mimotopes, Clayton, Australia). Peptides that were identified as epitopes were subsequently resynthesized (at >85% purity) and analyzed by high-performance liquid chromatography. Peptides were dissolved in dimethyl sulfoxide at 10 mg/ml (or dimethyl formamide if the sequence included Cys, Met, or Trp).

Mice. C57BL/6J (*H-2*^{b}), BALB/c (*H-2*^{d}), and A2Kb (*H-2*^{b}, HLA-A*0201) mice (female, 6 to 8 weeks) were supplied by Animal Resources Centre (Perth, Australia) and maintained under specific-pathogen-free conditions. (A2Kb × BALB/c)F_1 mice (female, 16 weeks) were also used and have been designated A2bdF_1 (HLA-A*0201, *H-2*^{b}, *H-2*^{d}). A2Kb mice express a chimeric HLA class I molecule, *A2.1K*^{b}, on a predominantly C57BL/6J (*H-2*^{b}) background (57). A2Kb mice are capable of making CTL responses restricted through both HLA-A*0201 and *H-2*^{b} class I molecules. BALB/c mice make CTL responses restricted through *H-2*^{d}. A2bdF_1 mice make CTL responses restricted through HLA-A*0201, *H-2*^{b}, and *H-2*^{d}.

Cells. EL4.A2 cells were derived by transfection of EL4 cells with *A2.1K*^{b} plasmid encoding the chimeric MHC-I heavy chain (described above) as described previously (14). EL4.A2 cells are susceptible to specific CTL lysis through both *H-2*^{b} and HLA-A*0201 restriction pathways. P815 (mastocytoma cell line) is susceptible to specific CTL lysis through the *H-2*^{d} restriction pathway. Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Mt. Waverley, Victoria, Australia) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). Vero E6 cells (used for virus propagation and plaque assay) were maintained in minimal essential medium (MEM; Invitrogen) supplemented with 10% FBS.

Immunizations. Mice were immunized subcutaneously at the tail base with either single-peptide epitopes (50 μg) or with an equimolar mix of peptides (60 μg total), together with tetanus toxoid (0.25 μg) as a source of T-helper epitopes and Quil A adjuvant (10 μg) as previously described (14). Mice were peptide immunized 8 days prior to evaluation of immune status by T-cell assays or 16 days prior to viral challenge.

Murine IFN-γ ELISPOT assay. Peptide-specific gamma interferon (IFN-γ)-secreting spleen cells were enumerated by an enzyme-linked immunospot (ELISPOT) assay essentially as described previously (21). Microwell plates (MultiScreen-HA; Millipore, North Ryde, New South Wales, Australia) were coated with capture antibody (rat anti-mouse IFN-γ, clone RA-6A2; BD PharMingen, San Diego, CA), blocked with Dulbecco's modified Eagle's medium and 10% FBS, and washed. Spleen cells, either ex vivo or after restimulation with peptide in vitro (1 μg/ml for 6 days), were added at various cell densities with recombinant human interleukin-2 (IL-2) (100 U/ml; Sigma, Castle Hill, New South Wales, Australia) and incubated with peptide (10 μg/ml) or without for 18 h at 37°C. The plates were washed, and detection antibody (biotinylated anti-mouse IFN-γ, clone XMG 1.2; BD PharMingen) was added for 2 h at 37°C. After being further washed, plates were developed using streptavidin-alkaline phosphatase (BD PharMingen) and Sigma Fast 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT) substrate. IFN-γ spots were counted using an AID EliSpot reader. Results were calculated as IFN-γ-positive cells/10^6 spleen cells (ex vivo) or 10^4 cells (postrestimulation) (means ± standard errors). Frequency (*f*) was defined as the number of positive cells with peptide minus the number of positive cells without peptide. The activation index (AI) was defined as the number of positive cells with peptide divided by the number of positive cells without peptide.

In vivo depletion of CD8$^+$ cells. C57BL/6J mice were injected intraperitoneally with ascitic fluid (0.1 ml) containing MAb 2.43 (anti-CD8.2) on days −6, −5, −4, and −1 before immunization. Depletion was maintained by injection at 3-day intervals (days 2 and 5). Flow cytometric analysis of peripheral blood cells using fluorescein isothiocyanate-labeled anti-CD8 monoclonal antibody (53-6.72; BD PharMingen) confirmed a >95% depletion of CD8$^+$ T cells at 1 day before and also 8 days after immunization.

^{51}Cr release cytotoxicity assay. Peptide-specific cytotoxicity of spleen cells was determined by a ^{51}Cr release assay as described previously (21). In summary, target cells were labeled with 100 μCi of ^{51}Cr (as sodium chromate) (MP Biomedicals, Seven Hills, Australia) for 90 min and then washed and incubated at 10^4 cells/microwell for 2 h with peptide (10 μg/ml) or without. Spleen cells, postrestimulation with peptide (1 μg/ml for 6 days), were added at the specified effector-to-target ratios; assay plates were incubated for 4 to 6 h at 37°C, and then radioactivity (counts per minute) in cell-free supernatant was determined by gamma counting. Results were calculated as percent cytotoxicities using the formula $[(E\text{-}S)/(T\text{-}S)] \times 100$, where E represents counts per minute released in the presence of effector cells, S represents spontaneous counts per minute with medium only, and T represents total counts per minute in the presence of 5% sodium dodecyl sulfate (S values were always less than 15% of T values). Peptide-

specific cytotoxicity was defined as the percent cytotoxicity for targets with peptide minus the percent cytotoxicity for targets without peptide.

Virus preparation. hMPV stocks were prepared from plaque-purified virus in Vero E6 cells as previously described (1). Briefly, subconfluent Vero E6 cells in serum-free medium (MEM) were infected with type A hMPV/CAN97-83. The virus was allowed to adsorb for 1 h at 37°C, followed by the addition of tissue culture medium. Infected cells were then incubated at 37°C for 3 days until more than 90% cytopathic effect was observed. Cells were harvested by removal of the medium and replacement with a small volume of serum-free MEM, followed by two freeze-thaw cycles at −70°C and 4°C. The lysates were collected and centrifuged at 4,000 × *g* for 20 min at 4°C to remove cell debris, and virus titer was determined by plaque assay as described below.

Infection of mice and tissue sampling. Immunized mice were anesthetized by intraperitoneal administration of 2,2,2-tribromoethanol (Avertin), followed by intranasal infection with 10^6 PFU of type A hMPV/CAN97-83. Four days later, lungs were collected on ice in phosphate-buffered saline (PBS; Invitrogen) for determination of virus titer by plaque assay or collected in RNAzol B (Biotech Laboratories, Houston, TX) for determination of chemokine/cytokine mRNA by reverse transcriptase (RT)-PCR. A portion of lung tissue was also collected in 10% formalin buffer for histology. Draining pulmonary lymph nodes were removed for cytokine protein determination by enzyme-linked immunosorbent assay (ELISA).

Determination of virus titer. hMPV titers from lungs were determined by plaque assay in Vero E6 cells using immunostaining to detect hMPV N protein. Lungs were collected and homogenized in 1 ml of PBS using a hand-held homogenizer (Fisher Scientific, Pittsburgh, PA). The lung homogenates were then placed on ice for 15 min to allow cell debris to settle. Clarified lung lysates were then diluted 10-fold in serum-free MEM (Invitrogen), added to subconfluent Vero E6 cells cultured in serum-free MEM in 24-well plates, and incubated for 1 h at 37°C, followed by 2 ml of medium overlay. At 72 h postinfection, the medium was removed, the wells were carefully washed with PBS, and the cells were fixed with acetone-methanol (60:40). After the cells were air dried, they were immunostained with purified hyperimmune serum reactive against a metapneumovirus N protein (kind gift of Ralph Tripp, University of Georgia) as previously described (1). The anti-N protein antibody was diluted in PBS containing blocking agents (Blotto; Bio-Rad, Hercules, CA) and detected using alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (Sigma, The Woodlands, TX), and the plaques were counted with 3′,3′-diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA).

Quantification of chemokine and cytokine mRNA expression in lungs. Total RNA was isolated from lungs by standard methods with RNA-Wiz (Ambion). An RT-PCR procedure was performed as previously described (31, 32) to determine relative quantities of mRNA for chemokines (RANTES, macrophage inflammatory protein 1α [MIP-1α], and Mig) and cytokines (IFN-γ, IL-12, IL-10, and IL-4). The primers and probes for all genes were purchased from GIBCO BRL (Invitrogen, Carlsbad, CA). Primer and probe sequences for Mig, IL-10, IL-12, IFN-γ, and hypoxanthine phosphoribosyltransferase (HPRT) have been published elsewhere (32). Other primer and probe sequences are RANTES (forward, CGCGGATCCCCACGTCAAGGAGTATTTCTACACC; reverse, CGG GATCCCTGGTTTCTTGGGTTTGCTGTG; and probe, GCTAGGACTAGAG CAAGCAATGACAGGGAA), Mip-1α (forward, CGCGGATCCCGGAAGATT CCACGCCAATTC; reverse, GGATCCGGTTGAGGAACGTGTCCTGAAG; and probe, GGAGATGGAGCTATGCAGGTGGCAGGAAT), and IL-4 (forward, GAATGTACCAGGAGCCATATC; reverse, CTCAGTACTACGAGT AATCCA; and probe, AGGGCTTCCAAGGTGCTTCGCA).

Forward and reverse primers are located in 5′ and 3′ positions of the gene, respectively, and were paired for PCR amplification. After the appropriate number of PCR cycles, the amplified DNA was analyzed by gel electrophoresis and Southern blotting and detected using probe sequence and enhanced chemiluminescence as recommended by the manufacturer (Amersham, Arlington Heights, IL.). PCR amplification with the HPRT reference gene was performed to assess variations in cDNA or total RNA loading between samples. Relative transcript levels were quantified in arbitrary units using ImageJ (Image Processing and Analysis in Java; http://rsb.info.nih.gov/ij/).

Quantification of cytokine protein expression in PBLNs. Cells from the peribronchial lymph nodes (PBLNs) were isolated and stimulated with UV-inactivated hMPV in mixed lymphocyte culture medium for 3 days at 37°C as described elsewhere (41, 42). The concentrations of IL-4, IL-10, and IFN-γ in the cell-free supernatants were determined by ELISA as described previously (23, 31). The sensitivities of detection were 0.1 ng/ml for IL-4 and IFN-γ and 15 pg/ml for IL-10.

Histopathology staining. Histopathological examination was performed in a blinded fashion by two independent observers on lung samples isolated from hMPV-infected mice as previously described (23). Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed in 10% phosphate-buffered formalin, sectioned, and stained with hematoxylin and eosin or alcian blue-periodic acid-Schiff for the enumeration of mucin-secreting cells.

Statistical analysis. Data are presented as means ± standard errors of the means (SEM). Mean values were compared by Student's *t* test, and a *P* value of <0.05 was considered significant.

Protein sequence alignments. Sequences for the N, M2-2, SH, and G proteins of hMPV strains ($n = 31$) were obtained from NCBI, and alignments were performed with CLUSTAL W (51). Representative hMPV strains were NDL00-1, CAN97-83, CAN97-82, and CAN98-75 for A1, A2, B1, and B2 subtypes, respectively. Protein sequences for other hMPV strains that have been detected in Australia, Canada, and Japan were also included in sequence alignments.

RESULTS

Prediction of MHC-I binding and proteasomal cleavage. MHC-I-restricted T-cell epitopes were predicted using two computer-based algorithms, SYFPEITHI (with PAProC) and ProPred1. SYFPEITHI is a database for MHC ligands and peptide motifs and also offers an epitope prediction algorithm (motif based) which results in a list of peptides (8-, 9-, and 10-mers) that have a high probability of being presented by MHC-I molecules (38). PAProC is a prediction tool for cleavages by human and yeast 20S proteasomes based on experimental cleavage data (27). ProPred1 is an online web tool for predicting peptide binding to MHC-I alleles and also allows prediction of the standard and immunoproteasome cleavage sites in an antigenic sequence (46).

We applied these algorithms to seven proteins (N, M, F, M2-1, M2-2, SH, and G) of type A hMPV for five MHC-I alleles (mouse *H-2K*^{b}, *D*^{b}, *K*^{d}, and *L*^{d} and human HLA-A*0201). The seven proteins were chosen by analogy to the related hRSV where the corresponding hRSV proteins elicit CTL responses during hRSV infection. Additionally, the large size of hMPV polymerase deters predictive algorithm or overlapping peptide epitope mapping. Twenty predicted epitopes ("predictopes") were selected for the mouse alleles, and 16 were selected for the human allele (not shown). For all MHC alleles, the frequency of predictopes was highest in the N and F proteins (11/36 and 6/36, respectively). For the mouse alleles, most predictopes were in the N and M proteins (8/20 and 4/20, respectively). For the human allele, most predictopes were in the F, N, and M2-1 proteins (5/16, 3/16, and 3/16, respectively).

Identification of hMPV CTL epitopes recognized by murine T cells. We asked whether any of the predictopes would function to elicit murine CTL responses restricted through the appropriate murine and human class I alleles. First, 10 groups of *H-2*^{b} mice (C57BL/6J; three mice per group) were immunized, each group with one of the predictope peptides restricted through *K*^{b} or *D*^{b}. An additional control group was immunized with a well-defined *H-2D*^{b}-restricted epitope of human papillomavirus E7 protein (18). Second, 10 groups of *H-2*^{d} mice (BALB/c; three mice per group) were immunized, each group with one of the predictope peptides restricted through *K*^{d} or *L*^{d}. An additional control group was immunized with a well-defined *H-2K*^{d}-restricted epitope of respiratory syncytial virus (25). Third, 16 groups of HLA-A*0201 transgenic mice (A2Kb; three mice per group) were immunized, each group with one of the predictope peptides restricted through HLA-A*0201. An additional control group was immunized with a well-defined HLA-A*0201-restricted epitope of influ-

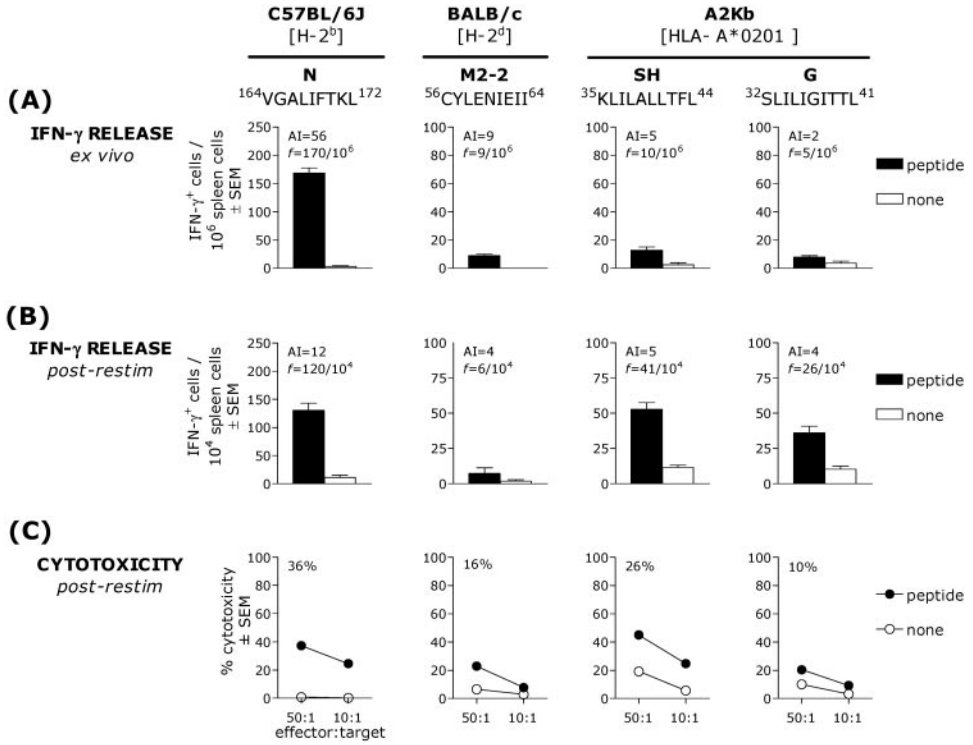

FIG. 1. Peptide immunization of mice with predictopes of hMPV elicits CTL responses. C57BL/6J (*H-2*^{b}), BALB/c (*H-2*^{d}), or A2Kb (HLA-A*0201) mice were peptide immunized with predictopes from the N, M2-2, SH, or G proteins of hMPV as indicated. Control mice were immunized with well-defined epitopes from other viruses: either human papillomavirus (HPV E7/*H-2*^{b}) (18), respiratory syncytial virus (hRSV M2/*H-2*^{d}) (25), or influenza virus (FLU M/HLA-A*0201) (19). Immune status was evaluated 8 days later by T-cell assays ($n = 3$ mice per group). IFN-γ-secreting T cells were quantified by ELISPOT assay using spleen cells (A) ex vivo or (B) postrestimulation (post-restim) with peptide for 6 days. Spleen cells were incubated for 18 h with or without peptide as shown. Results are expressed as IFN-γ-positive cells/10^6 spleen cells (ex vivo) or 10^4 spleen cells (postrestimulation) (means ± standard errors). For controls, *f* and AI values, respectively, were $120/10^6$ and 45 for HPV, $33/10^6$ and 21 for hRSV, and $37/10^6$ and 10 for FLU (ex vivo) or $280/10^4$ and 43 for HPV, $28/10^4$ and 4 for hRSV, and $65/10^4$ and 17 for FLU (postrestimulation). Cytotoxic T cells were measured by a ^{51}Cr release assay using spleen cells (C) postrestimulation with peptide for 6 days. Spleen cells were tested against target cells with or without peptide as shown. Results are expressed as percent cytotoxicities (means ± standard errors) (SEM was always <5%). Numbers represent peptide-specific cytotoxicities (psc) at an effector-to-target ratio of 50:1 (see Materials and Methods). For controls, psc values were 51% for HPV, 45% for hRSV, and 48% for FLU.

enza virus (19). Immune status was evaluated by T-cell assays 8 days later.

Effector response. To evaluate effector T-cell responses, ex vivo spleen cells were tested in IFN-γ ELISPOT assays. Spleen cells from *H-2*^{b} mice peptide immunized with the predictope ^{164}VGALIFTKL^{172} (from N protein) secreted IFN-γ when cultured ex vivo with this peptide (Fig. 1A). Spleen cells from *H-2*^{d} mice peptide immunized with the predictope ^{56}CYLENI EII^{64} (from M2-2 protein) secreted IFN-γ when cultured ex vivo with this peptide (Fig. 1A). Spleen cells from HLA-A*0201 transgenic mice peptide immunized with the predictope ^{35}KLILALLTFL^{44} (from SH protein) or with the predictope ^{32}SLILIGITTL^{41} (from G protein) secreted IFN-γ when cultured ex vivo with the relevant peptides (Fig. 1A). Effector responses by ex vivo spleen cells were not evident for any other predictopes.

Memory response. To evaluate memory T-cell responses, spleen cells postrestimulation with peptide (1 μg/ml for 6 days) were tested in IFN-γ ELISPOT and ^{51}Cr release cytotoxicity assays. Restimulated spleen cells from *H-2*^{b} mice immunized with the predictope ^{164}VGALIFTKL^{172} (from N protein) secreted IFN-γ when cultured with this peptide (Fig. 1B). Additionally, they specifically killed peptide-labeled *H-2*^{b} target cells (Fig. 1C). Similarly, restimulated spleen cells from *H-2*^{d} mice immunized with the predictope ^{56}CYLENIEII^{64} (from M2-2 protein) secreted IFN-γ when cultured with this peptide (Fig. 1B). Additionally, they specifically killed peptide-labeled *H-2*^{d} target cells (Fig. 1C). Furthermore, restimulated spleen cells from HLA-A*0201 transgenic mice immunized with the predictope ^{35}KLILALLTFL^{44} (from SH protein) or with the predictope ^{32}SLILIGITTL^{41} (from G protein) secreted IFN-γ when cultured with the relevant peptides (Fig. 1B). Additionally, they specifically killed peptide-labeled HLA-A*0201 target cells (Fig. 1C).

CTL responses are MHC-I-restricted and mediated by CD8$^+$ cells. The CTL responses to the above-described epitopes were restricted by the corresponding MHC-I allele. Thus, restimulated spleen cells from peptide-immunized mice were reacted in a ^{51}Cr release cytotoxicity assay with epitope-labeled target cells which expressed the corresponding MHC-I antigen and also with epitope-labeled target cells expressing a noncognate MHC-I antigen. Restimulated spleen cells from ^{56}CYLENIEII^{64}-immunized mice killed *H-2*^{d}-expressing target cells but not *H-2*^{b}-expressing target cells (Fig. 2A). Restimulated spleen cells

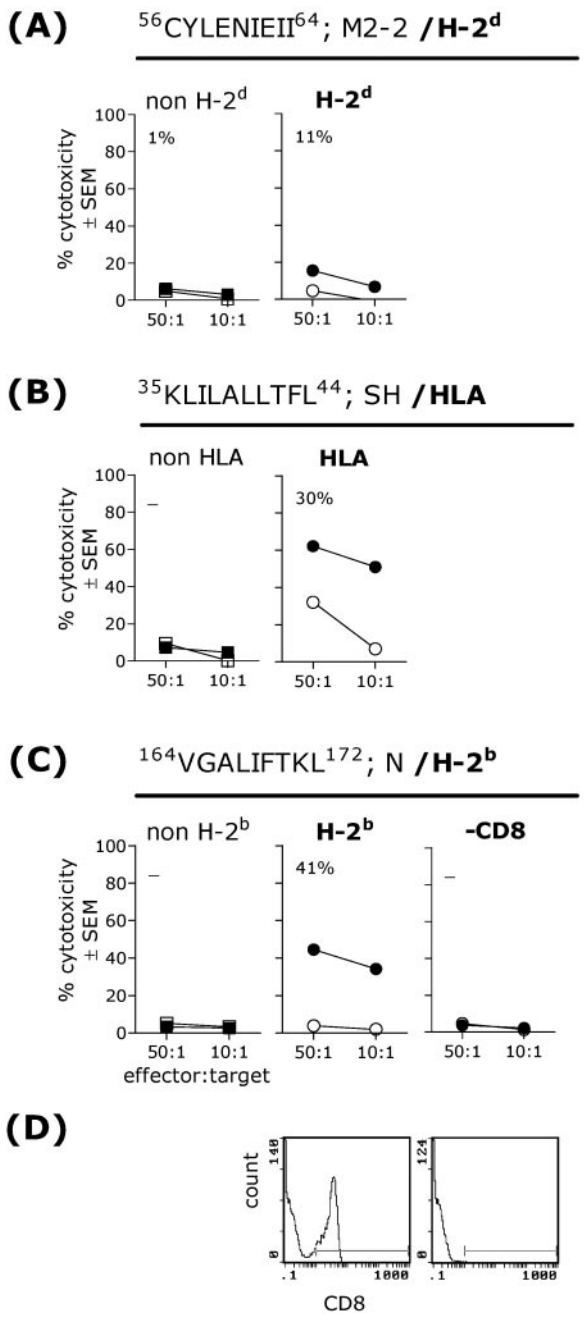

FIG. 2. hMPV-directed CTL response is MHC-I-restricted and mediated by $CD8^+$ cells. BALB/c (*H-2*^{d}), A2Kb (HLA-A*0201), or C57BL/6J (*H-2*^{b}) mice were immunized with peptide epitopes from the M2-2, SH, or N proteins of hMPV as indicated. Restimulated spleen cells were reacted (A) with *H-2*^{b} (EL4) and with *H-2*^{d} (P815) target cells, (B) with *H-2*^{d} and with HLA-A*0201 (EL4.A2) target cells, and (C) with *H-2*^{d} and with *H-2*^{b} target cells, with (closed symbols) or without (open symbols) peptide, as shown, in a ^{51}Cr release assay. (C, third panel) Restimulated spleen cells were derived from mice which had been depleted of $CD8^+$ cells. Results are expressed as percent cytotoxicities (means ± standard errors) (SEM was always <5%). Numbers represent peptide-specific cytotoxicities at an effector-to-target ratio of 50:1. (D) Flow cytometric analysis of $CD8^+$ cells in peripheral blood of representative ^{164}VGALIFTKL^{172}-immunized mice depleted of $CD8^+$ cells (right panel) or nondepleted (left panel).

from ^{35}KLILALLTFL^{44}-immunized mice killed HLA-A2-expressing target cells but not *H-2*^{d}-expressing target cells (Fig. 2B). Restimulated spleen cells from ^{164}VGALIFTKL^{172}-immunized mice killed target cells expressing *H-2*^{b} but not target cells expressing *H-2*^{d} (Fig. 2C).

We also inquired whether the response was mediated by $CD8^+$ cells. ^{164}VGALIFTKL^{172}-directed killing (Fig. 2C, second panel) was completely abolished if ^{164}VGALIFTKL^{172}-immunized mice were specifically depleted of $CD8^+$ T cells (Fig. 2C, third panel, and Fig. 2D).

The above data demonstrate that hMPV contains *H-2*^{b}-, *H-2*^{d}-, and HLA-A*0201-restricted CTL epitopes which, when administered to mice as peptide vaccines, elicit effector and memory CTL responses. The identified hMPV CTL epitopes are listed in Table 1.

Vaccination with hMPV CTL epitopes reduces viral load in the lungs of hMPV-challenged mice. We asked whether vaccination with the identified hMPV CTL epitopes would protect against viral replication in the lungs of hMPV-challenged mice. Groups of 10 $A2bdF_1$ mice were vaccinated with the identified CTL epitopes from hMPV (^{164}VGALIFTKL^{172}, N/*H-2*^{b}; ^{35}KLILALLTFL^{44}, SH/HLA-A*0201) or with "irrelevant" CTL epitopes from other viruses (human papillomavirus, E7/*H-2*^{b}; influenza virus, M/HLA-A*0201). Eight days after vaccination, immune status was evaluated in 3 of the 10 mice by T-cell assays, and 16 days after vaccination, the remaining 7 mice were hMPV challenged. Four days after challenge, lungs were removed for determination of virus titer.

Spleen cells from mice vaccinated with hMPV CTL epitopes secreted IFN-γ when cultured, ex vivo or postrestimulation, with the relevant peptides (Fig. 3A and B). Restimulated spleen cells also specifically killed relevant peptide-labeled target cells (Fig. 3C). These data confirm the induction of CTL responses directed to both of the hMPV CTL epitopes comprising the vaccine.

The remaining mice vaccinated with hMPV CTL epitopes and challenged with hMPV showed ~2 $\log_{10}$ reduction of viral load in the lungs compared to mice vaccinated with irrelevant CTL epitopes (Fig. 3D). Note that mice vaccinated with irrelevant CTL epitopes had good responses to these epitopes (Fig. 3), indicating that protection was associated with a vaccine response that was hMPV specific.

The above data demonstrate that vaccination with the identified CTL epitopes from the N and SH proteins of hMPV is protective in a mouse model of hMPV disease.

Vaccination with hMPV CTL epitopes upregulates expression of Th1-type cytokines in the lungs and peribronchial lymph nodes of hMPV-challenged mice. Cytokines and chemokines play crucial roles in mediating recovery from viral infections (39). It was important to establish whether the production of specific antiviral and/or regulatory cytokines and chemokines was altered in hMPV-challenged mice that had been vaccinated with either hMPV or irrelevant CTL epitopes.

RT-PCR analysis revealed that Th1-type cytokines (IFN-γ and IL-12) were expressed at significantly higher (150% or 50% higher, respectively) mRNA levels in lungs of mice vaccinated with hMPV CTL epitopes than in lungs of mice vaccinated with irrelevant CTL epitopes (Fig. 4B). Interestingly, mice vaccinated with hMPV CTL epitopes also had significantly lower (50% lower) mRNA levels of the Th2-type cytokine IL-10 and trend to reduction of the Th2-type cytokine

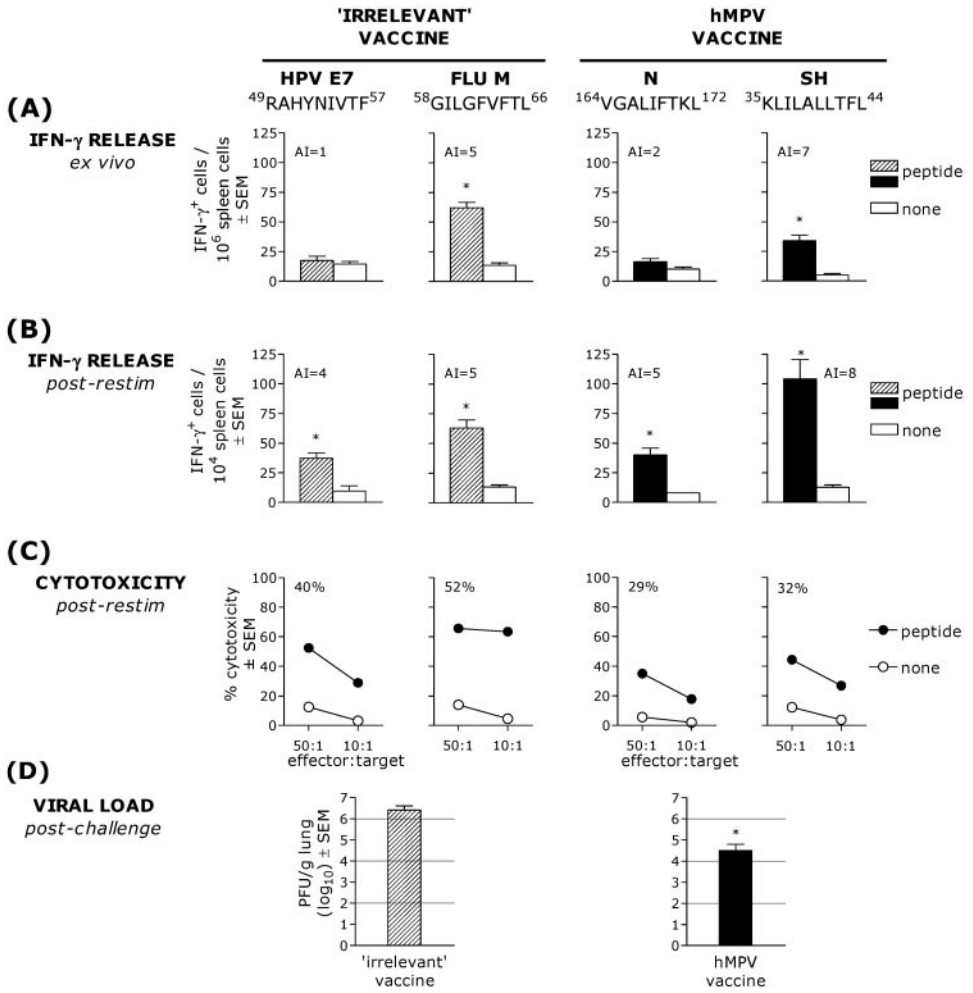

FIG. 3. Vaccination with hMPV CTL epitopes elicits CTL responses associated with a reduction of viral load in lungs of hMPV-challenged mice. A2bdF$_1$ (HLA-A*0201, *H-2*^{b}, *H-2*^{d}) mice were vaccinated with hMPV CTL epitopes (N/*H-2*^{b} and SH/HLA-A*0201). Control mice were vaccinated with irrelevant CTL epitopes from other viruses (human papillomavirus, HPV E7/*H-2*^{b}; influenza virus, FLU M/HLA-A*0201). Immune status was evaluated 8 days later by T-cell assays ($n = 3$ mice per group) or 16 days later by hMPV challenge. IFN-γ-secreting T cells were quantified by ELISPOT assay using spleen cells (A) ex vivo or (B) postrestimulation with peptide for 6 days. Spleen cells were incubated for 18 h with or without peptide as shown. Results are expressed as IFN-γ-positive cells/10^6 spleen cells (ex vivo) or 10^4 spleen cells (postrestimulation) (means ± standard errors). Significance was calculated using Student's *t* test (*, $P < 0.05$). (C) Cytotoxic T cells were measured by a ^{51}Cr release assay using spleen cells postrestimulation with peptide for 6 days. Spleen cells were tested against target cells with or without peptide as shown. Results are expressed as percent cytotoxicities (means ± standard errors) (SEM was always <5%). Numbers represent peptide-specific cytotoxicities at an effector-to-target ratio of 50:1 (see Materials and Methods). (D) Viral load ($n = 7$ mice per group) was quantified by plaque assay using lungs at 4 days postchallenge with hMPV. Results are expressed as $\log_{10}$ PFU/g lung (means ± standard errors). Significance was calculated using Student's *t* test (*, $P < 0.05$).

IL-4 (Fig. 4B). In order to determine whether this shift in cytokine profile at the mRNA level also occurred at the protein level, PBLN cells from vaccinated mice were stimulated in vitro with inactivated hMPV, and supernatants were analyzed for cytokine (IFN-γ, IL-4, and IL-10) proteins by ELISA. Th1-type cytokine (IFN-γ) was expressed at a significantly higher (at least four times higher) protein level in PBLN cells of mice vaccinated with hMPV CTL epitopes than in those of mice vaccinated with irrelevant CTL epitopes. Furthermore, mice vaccinated with hMPV CTL epitopes also had significantly lower (at least two to three times lower) protein levels of the Th2-type cytokines (IL-10 and IL-4) (Fig. 4D).

This cytokine profile (increased Th1 type and decreased Th2 type) in mice vaccinated with hMPV CTL epitopes was associated with reduced viral load in the lungs, suggesting that Th1-type cytokines are involved in vaccine-mediated immunity to hMPV.

The CXC chemokine Mig (monokine induced by IFN-γ) was expressed at a significantly higher (70% higher) mRNA level in lungs of mice vaccinated with hMPV CTL epitopes than in those of mice vaccinated with irrelevant CTL epitopes. In contrast, the CC chemokines (RANTES and MIP-1α) were not expressed at significantly different mRNA levels (Fig. 4A).

Vaccination with hMPV CTL epitopes reduces histopathology in the lungs of hMPV-challenged mice. Lung histopathology was examined in hMPV-challenged mice that had been vaccinated with either hMPV CTL epitopes or irrelevant CTL epitopes. Histopathology associated with hMPV infection was

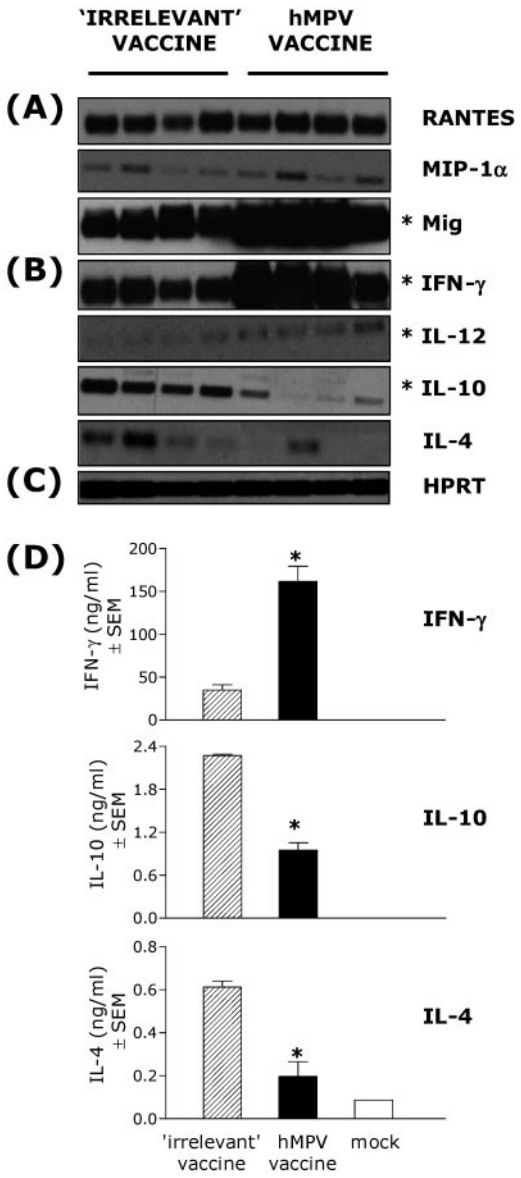

FIG. 4. Vaccination with hMPV CTL epitopes upregulates expression of Th1-type cytokines in lungs and PBLNs of hMPV-challenged mice. A2bdF$_1$ mice were vaccinated with either hMPV CTL epitopes or irrelevant CTL epitopes and then evaluated by T-cell assays (day 8) or challenged with hMPV (day 16) (see Fig. 2). Chemokine/cytokine expression was evaluated at 4 days postchallenge ($n = 4$ mice). Expression in lungs was quantified at the mRNA level for (A) chemokines and (B) cytokines with (C) HPRT reference gene. Expression in PBLNs was quantified at the protein level for (D) cytokines. Data are presented as means of triplicate cultures ± standard errors. Significance was calculated using Student's t test (*, $P < 0.05$; comparison between hMPV and irrelevant vaccine groups).

modest and characterized by mononuclear cell infiltration in the interstitium (Fig. 5A and B). Mice vaccinated with hMPV CTL epitopes showed reduced inflammatory cell infiltrates (Fig. 5C), which correlated with reduced viral load in the lungs (Fig. 3D), indicating that this vaccine was protective in a mouse model of hMPV disease. There was no significant difference in the number of mucin-secreting cells per representative high-power field between hMPV vaccine (11 ± 2.6 cells) and irrelevant vaccine groups (10 ± 1.1 cells, $P > 0.05$) Neutrophils were present around the airways of all hMPV-challenged mice but, interestingly, were significantly reduced in number per representative high-power field for mice vaccinated with hMPV CTL epitopes (8 ± 2.6 neutrophils) compared to those for mice immunized with irrelevant vaccine (23 ± 4.2 neutrophils, $P < 0.01$).

Identified CTL epitopes are present in type A (and some type B) hMPV strains (according to protein sequence alignments). In this study, type A hMPV protein sequences were used for epitope prediction. Since a human vaccine would ideally be protective against type A and type B hMPV, it is relevant to ask whether the identified CTL epitopes are present in both virus types. Sequence alignments for the N, M2-2, G, and SH proteins were performed for hMPV strains representing subtypes A1, A2, B1, and B2 (Fig. 6).

The N protein (394 amino acids) is highly conserved (>95% identity between types). The identified epitope (^{164}VGA LIFTKL^{172}, N/*H-2^b*) is located in a similarity region for the *Mononegavirales* order, probably a functional region for RNA binding/protein-to-protein interaction in the nucleocapsid (3). The identified epitope is present in all ($n = 23$) strains analyzed (Fig. 6A). The M2-2 protein (71 amino acids) is also conserved (~90% identity between types). The identified epitope (^{56}CYLENIEII^{64}, M2-2/*H-2^d*) is present in all ($n = 10$) strains analyzed (Fig. 6B). As the epitopes identified in the N and M2-2 proteins are completely conserved, protection afforded by a CTL epitope vaccine is expected to extend to both hMPV types.

The G protein (236 amino acids) is highly variable (33 to 37% identity between types) (3, 26). The identified epitope (^{32}SLILIGITTL^{41}, G/HLA-A*0201) is located in the transmembrane region and is present in all ($n = 16$) type A strains analyzed. However, it differs in all ($n = 4$) type B strains (Fig. 6C). The SH protein (183 amino acids) is also highly variable (58 to 59% identity between types) (3, 26). The identified epitope (^{35}KLILALLTFL^{44}, SH/HLA-A*0201) is located in the transmembrane region and is present in both ($n = 2$) subtype A1 strains analyzed. However, it differs in all ($n = 8$) other subtypes (Fig. 6D).

DISCUSSION

While hMPV ranks between hRSV and parainfluenza type 3 (PIV3) in severity of the disease that it causes (30), it is nonetheless a global and significant respiratory pathogen. As for hRSV and PIV3, a vaccine for hMPV is highly desirable (15), particularly in view of recent reports that dual infection with hRSV and hMPV is strongly associated with exacerbated illness compared with hRSV infection alone (9, 43) and that hMPV infection is associated with asthma in children (35). hMPV establishes productive infection in mucus epithelial cells of the upper and lower respiratory tract. By analogy with other epitheliotropic human viral infections (e.g., HPV, hRSV), hMPV proteins expressed during infection are likely to be targets of CD8$^+$ CTL responses, which may be associated with reduced viral titers and disease. To develop a successful vaccination strategy, viral antigens that activate both protective CTL and humoral responses may be

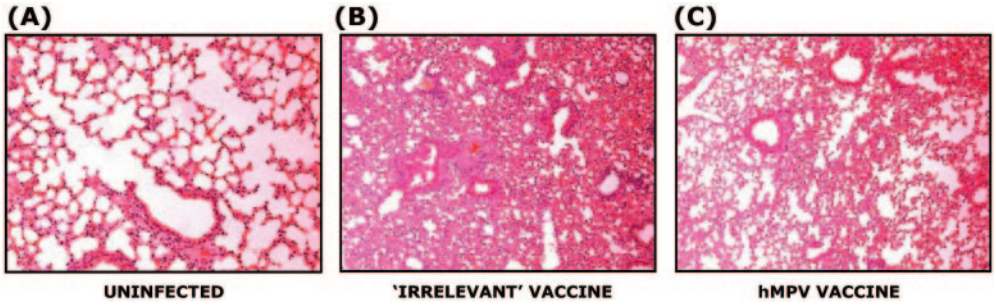

FIG. 5. Vaccination with hMPV CTL epitopes reduces histopathology in lungs of hMPV-challenged mice. A2bdF$_1$ mice were vaccinated with either hMPV CTL epitopes or irrelevant CTL epitopes and then evaluated by T-cell assays (day 8) or challenged with hMPV (day 16) (see Fig. 2). Histopathological changes in lungs at 4 days postchallenge were examined by hematoxylin and eosin staining. Representative sections are shown for mice that were (A) uninfected, (B) vaccinated with irrelevant CTL epitopes, or (C) vaccinated with hMPV CTL epitopes (magnification, ×10).

necessary. While progress has been made in defining the hMPV-directed humoral response both during natural infection (1) and following vaccination (48), nothing is hitherto known of hMPV epitopes targeted by the CTL response, nor whether a vaccine-induced hMPV-directed CTL response might contribute to the control of infection. In a recent study, Alvarez and Tripp examined aspects of the innate and adaptive immune response associated with hMPV infection in a BALB/c mouse model and showed that primary hMPV infection is associated with delayed CTL activity that coincides with decreased hMPV titer in the lung, suggesting that hMPV-directed CTL might contribute to the control of infection (2). There is compelling evidence that CTLs directed to specific epitopes are sufficient to eradicate or control disease in a number of viral infections, e.g., Epstein-Barr virus (40), bovine leukemia (24), and paramyxoviruses (25). For hRSV, CTL-mediated protection is recorded in a murine model (13, 45), and $CD8^+$ T cells are thought to play a pivotal role in recovery from hRSV infection in infants (12, 53).

In this study, we examined the hMPV proteome to define CTL epitopes recognized by murine CTLs relevant to immunological sequelae and vaccinology of hMPV infection. Epitopes were identified in four of the seven proteins examined (N, M2-2, SH, and G but not M, F, or M2-1) from the hMPV proteome (nine proteins). Epitopes were predicted for five MHC alleles and identified for three alleles. The success of each prediction algorithm (number of identified epitopes as a percentage of predictopes) varied by allele from 0 to 50%. Three of the four identified epitopes were predicted by both algorithms. However, some predictopes were not identified as epitopes. This likely reflects that events other than MHC binding and proteasomal cleavage, e.g., transmembrane transporter binding and T-cell receptor (TCR) binding, are also necessary for epitope functionality. Algorithms for the integrated prediction of MHC binding, transmembrane transport, and proteasomal cleavage will soon be available online, but prediction of TCR binding is not yet possible. Nevertheless, an immunoinformatics approach to epitope identification can be successful, as demonstrated here. Starting with the viral genome, it was possible to derive a viral immunome (a set of epitopes and antigens that interface with the immune system).

We show for the first time that vaccination of mice with hMPV CTL epitopes reduces both viral load and hMPV-associated histopathology in the lungs. We find that vaccination significantly enhances expression of Th1-type cytokines IL-12 (in lungs) and IFN-γ (in lungs and pulmonary lymph nodes), presumably predisposing to a local Th1 milieu. Interestingly, control mice (vaccinated with irrelevant CTL epitopes and challenged with hMPV) exhibited higher expression levels of Th2-type cytokines IL-4 and IL-10 (in lungs and pulmonary lymph nodes). Our findings are consistent with the observation of Alvarez and Tripp that hMPV infection is associated with low levels of IFN-γ expression and high induction levels of the Th2-type cytokine IL-10 (2).

As is true for most respiratory viruses, humoral immunity to hMPV may be adequate to at least partially control disease but not to provide full sterilizing immunity. Naturally acquired humoral immunity to hMPV is apparently unable to fully control infection since children more than 12 years of age have higher virus neutralizing antibody levels than those aged 5 years (55), which suggests that reinfections may frequently occur in spite of the presence of circulating hMPV antibody. In the mouse model of hMPV infection, in addition to humoral immunity, there is evidence of a T-cell response to hMPV. Lungs of infected mice exhibit mononuclear cell infiltration and an inflammatory environment conducive to CTL response induction. A CTL response can be demonstrated when viral load starts to diminish, following viral challenge (2). Furthermore, depletion of T cells or NK cells results in increased viral titers in the lungs of infected mice, indicating at least some T-cell-mediated, as well as antibody-mediated, immune control of viral persistence (1). Nonetheless, virus persists at a low level in spite of the T-cell response, reinforcing a view that immunity acquired by natural infection or viral challenge with hMPV is insufficient to eliminate infection. That hMPV infection in the mouse model does not engender a prominent T-cell response is supported by the observation of low IFN-γ expression levels by CD8 cells and increased IL-10 expression levels by CD4 and CD8 cells, coinciding with a peak of virus replication in the lung, and by the lack of up-regulation of activation and adhesion molecules in bronchoalveolar lavage leukocytes (2).

The IFN-γ release and cytotoxicity results we obtained for the CTL epitopes from hMPV compare favorably with those obtained for previously well-documented protective CTL epitopes from other viruses (Fig. 1 and 3). The identified epitopes are recognized by murine CTLs and are found within four (N, M-2, G, and SH) of the nine hMPV putative proteins. All may be

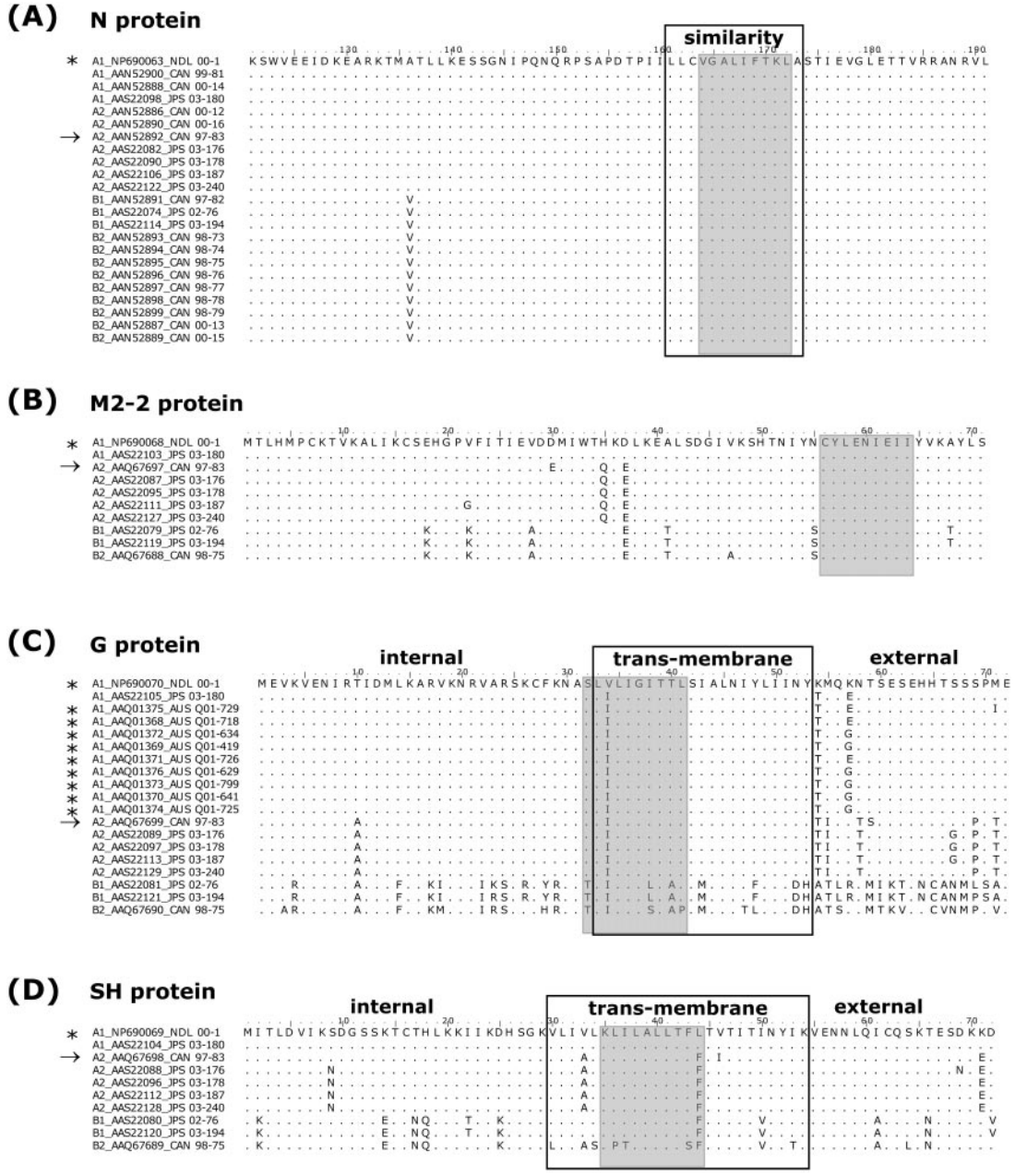

FIG. 6. Identified CTL epitopes are present in type A (and some type B) hMPV strains according to protein sequence alignments. (A) N protein (amino acids 120 to 191 of 394) with similarity region (54) boxed. (B) M2-2 protein (amino acids 1 to 71 of 71). (C) G protein (amino acids 1 to 72 of 236) with transmembrane domain (54) boxed. (D) SH protein (amino acids 1 to 72 of 183) with transmembrane domain (54) boxed. Identified CTL epitope sequences are shaded. * indicates virus types used for epitope prediction; → indicates virus type used for challenge.

biologically relevant since, as has been demonstrated with other viruses (e.g., EBV [7]), CTLs to all virally derived nascent proteins may be generated by cross-presentation.

The identified epitopes in the N and M2-2 proteins are completely conserved among hMPV types. A similar situation in humans would engender optimism that a vaccine comprising CTL epitopes restricted through the major HLA class I supertypes (44) would offer protection to most of the population against most hMPV types. Cross-reactive immunity is important not only from a community vaccine perspective but also because heterologous hMPV infections may occur in individuals (36), including infants (16).

Both the epitopes from the G and SH proteins are located in transmembrane regions, which exhibit minimal variability in these otherwise variable surface glycoproteins, and are restricted by the HLA-A*0201 allele, which has a binding motif of a 9- or 10-mer sequence with hydrophobic amino acids (A, I, L, M, V, and T) at anchor positions (position 2 and C terminus). The epitope from the G protein is present in type A hMPV. For type B, however, the amino acid sequence is iden-

tical at the anchor positions (MHC binding should still be possible) and differs at a few nonanchor positions, some of which may be involved in TCR binding (note that promiscuity in nonanchor residues can be tolerated for TCR recognition). The epitope from the SH protein is present in subtype A1 hMPV. For subtypes A2 and B1, however, the amino acid sequence differs at an anchor position (MHC binding may be compromised). For subtype B2, the sequence differs further (L→P, F→S) at the anchor positions (MHC binding is not expected). As the epitopes identified in the G and SH proteins are conserved mainly in type A hMPV, protection afforded by a CTL epitope vaccine could extend to some subtypes, with the probable exception of subtype B2. In general, CTL epitopes in variable proteins may be less suitable for consideration for inclusion in a vaccine designed to elicit cross-protective immunity. As with the G protein of hRSV (11, 56), immune pressure during natural infection may contribute to the accumulation of amino acid changes in the G protein of hMPV.

Vaccine approaches to hMPV infection have hitherto involved the induction of humoral responses. As with hRSV and PIV3 proteins, the surface glycoproteins F and G are major targets of a neutralizing antibody response. In a hamster model of hMPV infection (49), vaccine-induced neutralizing antibodies to hMPV F protein (induced by a chimeric bovine/human PIV3 vector harboring hRSV F and hRSV G proteins as well as hMPV F protein) protected against hMPV challenge. Since hMPV F protein (unlike hMPV G protein) is highly conserved (4, 55), antibody to F protein is likely to provide cross-reactive immunity for all hMPV subtypes (A1, A2, B1, and B2) (47), and this is indicated in a primate model of infection (48). Recently described reverse genetics systems provide the possibility of developing attenuated derivative hMPVs as candidates for antibody-inducing vaccines (6, 22).

In summary, we have identified epitopes in a number of hMPV proteins as targets for protective T-cell immunity. CTL epitopes are likely to be important in understanding the dynamics of hMPV immune control in natural infection in clinical settings and to be relevant in vaccine development. The use of CTL epitope vaccines for infections with paramyxoviruses is likely to obviate concerns of Th2-mediated enhancement of disease which occurs in infected individuals following specific vaccination, as has occurred with RSV (41). While our studies establish proof of principle of protection against hMPV infection by immunization with hMPV CTL epitopes, extrapolation to humans would require human-appropriate vaccine formulations validated in clinical trials. A vaccine approach which elicits both CTL and humoral responses against the major protagonists of respiratory disease (hRSV, hMPV, and PIV) would be particularly relevant (49).

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Karen A. Herd and Suresh Mahalingam contributed equally to this work and should be considered joint first authors.

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