Lung CD8⁺ T Cell Impairment Occurs during Human Metapneumovirus Infection despite Virus-Like Particle Induction of Functional CD8⁺ T Cells

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ABSTRACT

Human metapneumovirus (HMPV) is a major cause of respiratory disease in infants, the elderly, and immunocompromised individuals worldwide. There is currently no licensed HMPV vaccine. Virus-like particles (VLPs) are an attractive vaccine candidate because they are noninfectious and elicit a neutralizing antibody response. However, studies show that serum neutralizing antibodies are insufficient for complete protection against reinfection and that adaptive T cell immunity is important for viral clearance. HMPV and other respiratory viruses induce lung CD8⁺ T cell (TCD8) impairment, mediated by programmed death 1 (PD-1). In this study, we generated HMPV VLPs by expressing the fusion and matrix proteins in mammalian cells and tested whether VLP immunization induces functional HMPV-specific TCD8 responses in mice. C57BL/6 mice vaccinated twice with VLPs and subsequently challenged with HMPV were protected from lung viral replication for at least 20 weeks postimmunization. A single VLP dose elicited F- and M-specific lung TCD8s with higher function and lower expression of PD-1 and other inhibitory receptors than TCD8s from HMPV-infected mice. However, after HMPV challenge, lung TCD8s from VLP-vaccinated mice exhibited inhibitory receptor expression and functional impairment similar to those of mice experiencing secondary infection. HMPV challenge of VLP-immunized μMT mice also elicited a large percentage of impaired lung TCD8s similar to mice experiencing secondary infection. Together, these results indicate that VLPs are a promising vaccine candidate but do not prevent lung TCD8 impairment upon HMPV challenge.

IMPORTANCE

Human metapneumovirus (HMPV) is a leading cause of acute respiratory disease for which there is no licensed vaccine. Virus-like particles (VLPs) are an attractive vaccine candidate and induce antibodies, but T cell responses are less defined. Moreover, HMPV and other respiratory viruses induce lung CD8⁺ T cell (TCD8) impairment mediated by programmed death 1 (PD-1). In this study, HMPV VLPs containing viral fusion and matrix proteins elicited epitope-specific TCD8s that were functional with low PD-1 expression. Two VLP doses conferred sterilizing immunity in C57BL/6 mice and facilitated HMPV clearance in antibody-deficient μMT mice without enhancing lung pathology. However, regardless of whether responding lung TCD8s had previously encountered HMPV antigens in the context of VLPs or virus, similar proportions were impaired and expressed comparable levels of PD-1 upon viral challenge. These results suggest that VLPs are a promising vaccine candidate but do not prevent lung TCD8 impairment upon HMPV challenge.

Human metapneumovirus (HMPV) is a paramyxovirus that was discovered by scientists in the Netherlands in 2001 (1, 2). The virus is a major cause of acute respiratory morbidity and mortality in infants, older adults, and immunocompromised individuals, although serological studies indicate that almost all humans have been infected by 5 years of age (2, 3). There are four subtypes of HMPV classified by genotype: A1, A2, B1, and B2 (4). The fusion (F) protein, which mediates viral fusion and entry, has high sequence identity (95 to 97%) between subgroups (2, 4, 5). F protein elicits neutralizing antibodies, whereas antibodies against the other proteins on the virion surface are nonneutralizing (6–10). Although HMPV subtypes are relatively conserved, reinfections occur throughout life, despite the presence of neutralizing antibodies (12, 48).

No licensed vaccine for HMPV is currently available. Several vaccine strategies against HMPV have been explored in animal models, including live attenuated, subunit protein, formalin-inactivated, and CDB⁺ T cell (TCD8) epitope vaccines (9, 13–17). However, live attenuated viruses are contraindicated in immunocompromised patients. Subunit vaccines tend to be less immunogenic than live attenuated and inactivated vaccines (18), and TCD8 epitope vaccines do not fully protect against challenge with live virus (19). Formalin-inactivated paramyxovirus vaccines, on the other hand, raise concerns for enhanced pulmonary disease, as illustrated by the results of the formalin-inactivated respiratory syncytial virus (RSV) vaccine trial in the...
1960s (20). Indeed, formalin-inactivated HMPV vaccines tested in animal models also resulted in enhanced disease after challenge with live virus (21, 22).

Virus-like particles (VLPs) formed from the assembly of viral structural proteins are an attractive alternative vaccine strategy (23). VLPs mimic virus structure and present antigens in a repetitive, ordered fashion, a characteristic that strongly triggers B cell responses (24). Studies in humans and animals show that they are capable of eliciting both humoral and cellular immunity (25–27). VLPs can be designed to incorporate specific viral proteins to direct host immune responses toward protective antigens. VLP vaccines currently licensed for use in humans include the human papillomavirus (HPV) and hepatitis B vaccines (28). In addition, VLP vaccines for several other viruses (such as influenza and chikungunya viruses) have been tested in clinical trials (29, 30).

Currently, the functionality of T<sub>CD8</sub> elicted by VLP vaccination, compared to infection, is unclear. T<sub>CD8</sub> are important for viral clearance, and several studies have shown that they contribute to protection from HMPV (31, 32). It is known that several inhibitory receptors, including programmed death 1 (PD-1), mediate T<sub>CD8</sub> impairment during acute and chronic infections (33). We previously reported that HMPV lower respiratory tract infection led to impairment of lung T<sub>CD8</sub> function through PD-1 signaling, while splenic T<sub>CD8</sub> remained functional and PD-1<sup><sub>low</sub></sup> (34). The memory T<sub>CD8</sub> response to HMPV was also impaired via PD-1 signaling (35). Blockade or genetic ablation of PD-1 enhanced lung T<sub>CD8</sub> function in vitro and viral clearance in vivo during both primary and secondary infections, suggesting that a pulmonary T<sub>CD8</sub> memory response with low PD-1 expression might be correlated with enhanced protection.

In this study, we tested the hypothesis that immunization with HMPV VLPs containing the F and matrix (M) proteins could induce functional HMPV-specific T<sub>CD8</sub> and protection from viral challenge in mice. We found that VLP immunization conferred complete protection in the lungs of mice for at least 20 weeks postimmunization. VLPs elicited F- and M-specific T<sub>CD8</sub> responses in the lungs of mice, both after vaccination alone and after vaccination followed by HMPV challenge. VLP vaccination alone elicited lung T<sub>CD8</sub> that were more functional in vitro and expressed lower levels of PD-1 and other inhibitory receptors than those elicited by HMPV infection. However, when VLP-immunized mice were challenged with live virus, the improved in vitro function was seen after vaccination alone but not after challenge. Nonetheless, depletion of T<sub>CD8</sub> in immunized mice resulted in higher viral titers than in immunized nondepleted mice, indicating that VLP-induced T<sub>CD8</sub> still contributed to viral clearance despite higher PD-1 levels after challenge. These results suggest that VLPs containing the F and M proteins are a promising HMPV vaccine candidate and that T<sub>CD8</sub> contribute to the protection conferred by VLP vaccination.

**MATERIALS AND METHODS**

**Virus preparation and cell culture.** HMPV (clinical strain TN/94-49, subtype A2, as well as TN/89-515, subtype B2) was propagated in LLC-MK2 cells, as previously described (36). The B2 subtype was sucrose purified (37). 293-F cells were maintained in Freestyle 293 medium (Life Technologies) as recommended by the manufacturer.

**Generation of VLPs.** HMPV F and M amino acid sequences were derived from a pathogenic clinical strain of HMPV (subtype A2), sequenced for mammalian expression, and cloned into pcDNA3.1 (Life Technologies) as described previously (32). 293-F cells were transfected with plasmids encoding HMPV F and M proteins using pcDNA3.1-F (20 μg), pcDNA3.1-M (40 μg), and 293fectin transfection reagent (60 μl) as recommended by the manufacturer (Life Technologies). Mock VLPs were generated by transfecting 293-F cells with empty pcDNA3.1 vector. Eighteen hours posttransfection, the cell medium was changed, and 5 μg/ml trypsin was added to the flask. Three days posttransfection, the cells were pelleted, and the supernatant was centrifuged at 27,000 rpm for 90 min at 4°C using a SW32Ti rotor (Beckman Coulter) and pelleted through 20% sucrose. The VLP pellets were resuspended and snap-frozen in a dry-ice–alcohol bath for storage at −80°C.

**VLP protein content.** The protein content of VLPs was determined using the Bio-Rad protein assay as described previously (32). Western blot analysis was conducted to confirm incorporation of the F and M proteins. VLPs, mock VLPs, and HMPV samples were lysed with 1% SDS, heated in NuPAGE LDS sample buffer (Life Technologies) containing 5% β-mercaptoethanol (Sigma), run on 10% NuPAGE gels (Life Technologies), and transferred to polyvinylidene fluoride membranes (Life Technologies). The membranes were blocked with 5% milk in phosphate-buffered saline (PBS)-0.1% Tween for 30 min and then incubated with guinea pig anti-A2 F or rabbit anti-M serum diluted in 5% milk-PBS-0.1% Tween overnight at 4°C. The membranes were rinsed three times in PBS-0.05% Tween (PBS-T) and then incubated with secondary anti-guinea pig or anti-rabbit antibody for 1 h at room temperature. The membranes were rinsed three times in PBS-T and imaged using an Odyssey imaging system (LI-COR). The VLP F protein content was also determined by Western blotting using recombinant F protein to generate a standard curve. Band intensities were quantified using the Odyssey infrared imaging software. HMPV contained 120 ng/μl and VLPs contained 215 ng/μl of F protein. Although the amount of M protein was not formally quantified due to lack of a standard, its presence was confirmed by Western blotting; HMPV particles had approximately three times the amount of M protein as VLPs (not shown).

**Mice and immunizations.** C57BL/6 (B6) mice were purchased from the Jackson Laboratory. μMT mice were a kind gift from Mark Boothby (Vanderbilt University, Nashville, TN). HLA B6-K<sup>b</sup>B<sup>d</sup>-B7 transgenic (B<sup>7tg</sup>) mice were a gift from Alessandro Sette (La Jolla Institute for Allergy and Immunology, La Jolla, CA) and Francois Lemonnier (Institut Pasteur, Paris, France) (38). The animals were bred and maintained under specific-pathogen-free conditions. Six- to 10-week-old mice were used in all experiments. For intraperitoneal (i.p.) vaccinations, mice were injected with 100 μl of VLPs or mock VLPs mixed with 100 μl TiterMax Gold (TMG) adjuvant (Sigma). For intranasal (i.n.) vaccinations or infection with HMPV, mice were anesthetized intraperitoneally with ketamine-xylazine and inoculated intranasally with 6 × 10<sup>5</sup> PFU HMPV, mock VLPs, or VLPs in a 100-μl volume. For HMPV challenge, mice were anesthetized and inoculated i.n. with 6 × 10<sup>5</sup> PFU HMPV unless otherwise indicated. Serum was collected by submandibular bleed, and HMPV neutralizing titers were determined by plaque reduction assay as described previously (36).

**Viral titration.** Lung tissues were collected and pulverized in glass homogenizers before centrifugation at 1,200 rpm at 4°C for 10 min. Nasal turbinates (NT) were collected and ground with mortar and pestle prior to centrifugation. The supernatants were collected, aliquoted into cryovials, and snap-frozen in dry-ice–ethanol for storage at −80°C until further use. Viral titers were quantified by plaque titration as described previously (36).

**Tetramer analysis and staining for inhibitory receptors.** Lungs were minced and incubated for 1 h in 2 ml medium (RPMI 1640 with 10% fetal bovine serum [FBS], 2 mM glutamine, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and 50 μg/ml B-mercaptoethanol) at 37°C with 2 mg/ml collagenase A and 20 μg/ml DNase (both from Roche). Single-cell suspensions were obtained by passing digested lung tissue or whole spleens through metal screens and passing them over nylon cell strainers (BD...
Falc). Erythrocytes were lysed with red blood cell lysis buffer (Sigma-Aldrich). All antibodies were purchased from BD Biosciences unless otherwise indicated. After staining with LIVE/DEAD dye (Life Technologies) and blocking Fc receptors with anti-CD16/CD32, lymphocytes were incubated with allophycocyanin (APC)-labeled tetramers (0.1 to 1 μg/ml), anti-CD19 (clone 6D5; iCyt), anti-CD8α (clone 53-6.7), and anti-PD-1 (clone H43) or isotype control antibody (hamster IgG2e) in fluorescence-activated cell sorter (FACS) buffer (PBS-1% FBS) containing 50 nM dextran (LC Laboratories) for 1 h at room temperature. Background staining levels were determined by staining with a tetramer loaded with irrelevant (vaccine or influenza virus-derived) peptide. Tetramers were generated for the HMPV epitopes HLA-B*0702/M195–203 (APYAGLIMI) (M195), H2-Db/F528–536 (SGVTNNFGF) (F528), and H2-Kb/M94–112 (VALDEYSKL) (M94); the influenza virus epitope H2-Db/NP366–374 (ASNENMETM); and the vaccinia virus epitope HLA-B*1502/A34R82–90 (LPRPDTRHL) as previously described (39). For additional inhibitory receptors, cells were stained with TIM-3 (clone RMT3-23), LAG-3 (clone C9B7W), and 2B4 (clone m2B4 [B6] 458.1) or isotype control antibodies (Biolegend). The data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Intracellular cytokine staining. Lymphocytes were restimulated in vitro with 10 μM of the appropriate (i.e., M195, F528, or M94) peptide for 6 h at 37°C in the presence of anti-CD107a (clone 145-2C11), anti-CD19 (clone 6D5; iCyt), and anti-CD8α (clone 53-6.7). After extracellular markers were stained, the cells were fixed, permeabilized, and stained for intracellular interferon (IFN-γ) (clone XMG1.2) before flow cytometric analysis.

Lung histopathology. Mice were challenged 4 weeks postvaccination. Five days postchallenge, the left lung lobes were inflated with 10% formalin and fixed for 24 h, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on the Leica Bond Max automated stainer for B220 (BD Biosciences; 553086), CD3 (Santa Cruz; SC1127), neutrophils (Santa Cruz; SC71674), or major basic protein (Mayo Clinic; clone MR-14.7). The slides were scored by an experienced veterinary pathologist (K.L.B.) blinded to the compositions of the groups.

CD8⁺ T cell depletion. Mice were injected i.p. with 150 μg of CD8-depleting antibody (clone 2.43; BioXCell) 3 days prior to HMPV challenge and again with 100 μg of antibody on the day of challenge. Control animals received the same amounts of isotype control antibody. CD8⁺ T cell depletion was confirmed by flow cytometry.

Statistical analysis. Comparisons between two groups were performed using an unpaired two-tailed Student t test. Multiple-group comparisons were performed using one-way analysis of variance (ANOVA) with Tukey’s multiple-comparison test. A P value of <0.05 was considered statistically significant. The error bars in each graph represent standard errors of the mean (SEM) unless otherwise noted.

Study approval. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC).

RESULTS

VLP vaccination protects C57BL/6 mice from HMPV challenge. To determine whether VLP vaccination protects mice against HMPV challenge, we injected B6 mice i.p. with two doses of VLPs mixed with TMG, given 3 weeks apart. Control animals were injected with mock VLPs with TMG or were infected i.n. with HMPV (Fig. 1A). TMG was chosen as the adjuvant based on its immunogenicity in rodents (8, 9). Four weeks postvaccination, we challenged all mice i.n. with HMPV and collected tissues 5 days postchallenge (the day of peak viral titer during primary HMPV infection [34]). VLP-vaccinated mice had significantly reduced nose titers compared to mock-
vaccinated mice, and lung titers were undetectable (Fig. 1B). Previously infected mice had undetectable titers in both noses and lungs. Sera were collected from the mice after vaccination, and the presence of neutralizing antibodies was determined by a plaque neutralization assay. Both i.n. and i.p. VLP-immunized mice and HMPV-infected mice had neutralizing antibodies, whereas mock-immunized mice did not (data not shown), as previously reported (32).

To test the duration of immunity, we waited 20 weeks between the second VLP dose and HMPV challenge. VLP-immunized mice had significantly decreased titers in the nose and undetectable lung titers (Fig. 1C). Since i.p. vaccination did not fully protect the upper respiratory tract from HMPV challenge, we wondered whether the i.n. route of administration would elicit better protection in the nose. We repeated the experiment using the schedule in Fig. 1A with i.n. VLPs instead of i.p. VLPs and found that i.n. vaccination protected the lungs completely but did not fully protect the nose (Fig. 1D). Together, these results indicate that VLP vaccination protects B6 mice completely in the lower respiratory tract, the site of the most severe HMPV disease in humans, for at least 20 weeks postvaccination. Furthermore, both i.n. and i.p. vaccination induce partial protection in the upper respiratory tract.

VLP vaccination does not exacerbate lung histopathology. Previous reports of a formalin-inactivated HMPV vaccine found enhanced lung pathology when the animals were later challenged with virus (21). To determine whether VLP vaccination enhances lung pathology after HMPV challenge, we vaccinated mice i.n. or i.p. with two VLP doses with adjuvant 3 weeks apart or infected them with HMPV and challenged all the groups with HMPV 4 weeks postvaccination. The mice were euthanized 5 days postchallenge, and lung inflammation was scored by an expert pathologist blinded to the different groups. The inflammatory infiltrates post-HMPV challenge were not different between the groups. All the groups showed perivascular inflammation consisting of lymphocytes, macrophages, neutrophils, and plasma cells with no evidence of eosinophilia (Fig. 2A to D). VLP-immunized and previously infected groups showed similar levels of inflammation post-HMPV challenge, whereas the mock-immunized and challenged group had a lower level of inflammation (Fig. 2E). VLPs given alone without HMPV challenge elicited no inflammation (data not shown). Mice that were vaccinated with VLPs or infected with HMPV did not lose more weight postchallenge than mock-vaccinated mice (Fig. 2F), and none of the mice showed signs of illness. This is consistent with prior reports of HMPV infection (32), as mice are semipermissive hosts. These results indicate that VLP vaccination does not cause enhanced respiratory disease upon viral challenge.

VLP vaccination elicits functional HMPV F-specific T_{CD8$^+$.} To determine whether VLPs are able to elicit a T_{CD8$^+$} response after a single dose, we vaccinated mice i.p. with VLPs and TMG, i.p. with mock VLPs and TMG, or i.n. with VLPs alone, or infected them i.n. with HMPV (Fig. 3A). Seven and 10 days later, lungs and spleens were processed to quantify the HMPV-specific T_{CD8$^+$} response via flow cytometry (Fig. 3B). Previously, our group had identified several T_{CD8$^+$} epitopes in different HMPV proteins (34). In B6 mice, F528 is an immunodominant epitope and M94 is a subdominant epitope. VLP vaccination (i.p. and i.n.) and HMPV infection elicited F528-specific T_{CD8$^+$} in both lung and spleen detectable by tetramer assay, but mice that received mock VLPs i.p. did not show an F528-specific T_{CD8$^+$} response (Fig. 3C and data not shown). Mock VLPs given i.n. did not elicit F528-specific T_{CD8$^+$} (data not shown). Surface staining for CD107a, a marker of degranulation (42), and intracellular cytokine staining for IFN-γ showed that >50% of the F528-specific T_{CD8$^+$} were functional whether induced by VLPs or virus (Fig. 3D). Whereas there was no significant difference between the groups in terms of the percentages of functional cells, the IFN-γ mean fluorescence intensity (MFI) (a measure of the amount of cytokine produced) was higher in the VLP groups than in the HMPV group on day 10 (Fig. 3E). For the M94 epitope, only the HMPV-infected group had detectable levels of tetramer-positive T_{CD8$^+$} (data not shown). This may be because HMPV contains more M protein than the VLP preparations (as measured by Western blotting) or because HMPV M protein is more efficiently processed and presented on major histocompatibility complex class I (MHC-I) during viral replication.

A lower percentage of F528-specific T_{CD8$^+$} in lungs of i.p. VLP-immunized mice expressed surface PD-1 than for i.n. VLP-immunized and HMPV-infected animals on days 7 and 10, whereas i.n. VLP-immunized animals had a lower percentage of PD-1-positive T_{CD8$^+$} than infected animals only on day 10 (Fig. 3F). The difference between i.n. and i.p. vaccination was likely due to the higher lung antigen load after i.n. vaccination (since the VLPs were given directly into the respiratory tract), as previous studies showed that a higher antigen load correlated with greater T_{CD8$^+$} impairment (43, 44). In contrast, splenic T_{CD8$^+$} expressed lower PD-1 levels than lung T_{CD8$^+$} and were fully functional in all three groups (data not shown). This is consistent with our previously published findings that HMPV infection elicits fully functional T_{CD8$^+$} in the spleen but that a significant percentage of lung T_{CD8$^+$} did not degranulate or produce IFN-γ (34, 35).

With regard to other inhibitory receptors that had previously been shown to be important for T_{CD8$^+$} functional impairment (45–47), infected mice had the highest levels of lung T_{CD8$^+$} TIM-3 on both days 7 and 10 (Fig. 3G). The lower percentage of PD-1-positive T_{CD8$^+$} in VLP-vaccinated mice likely correlates with the higher IFN-γ MFI seen in Fig. 3E, but the differences between i.n. and i.p. VLP vaccination suggest that the route of vaccine administration plays a role in these different results. Together, the results indicate that VLP vaccination elicits functional T_{CD8$^+$} specific for the dominant F528 epitope. T_{CD8$^+$} in VLP-vaccinated mice had lower expression of the inhibitory receptors PD-1 and TIM-3 than those of infected mice, especially on day 10, which corresponds to a higher IFN-γ MFI.

VLP vaccination elicits functional HMPV M-specific T_{CD8$^+$}. We questioned whether the lack of a detectable T_{CD8$^+$} response to the subdominant M94 epitope in VLP-vaccinated B6 mice was because VLPs did not elicit M-specific T_{CD8$^+$} or if our assay was not sensitive enough to detect a subdominant response after one dose of VLPs. To distinguish between these two possibilities, we repeated the experiment shown in Fig. 3 using B7tg mice, which have T_{CD8$^+$} restricted by human HLA-B*0702 (38) and recognize M95 as an immunodominant HMPV epitope (34).

One dose of VLPs was sufficient to elicit M195-specific T_{CD8$^+$} in mice, with the i.n. VLP group producing a stronger response than the i.p. VLP group (Fig. 4A). The HMPV-infected group had the highest percentage of M195-specific T_{CD8$^+$}. Functionally, the i.n. VLP group had the highest proportion of degranulating and IFN-γ-producing T_{CD8$^+$} (Fig. 4B). Both VLP groups had a higher IFN-γ MFI in M195-specific T_{CD8$^+$} than the HMPV group.
(Fig. 4C). The percentage of PD-1-expressing M195-specific T<sub>CD8</sub> was lower in both VLP groups than in the HMPV-infected group on day 10, whereas only that of the i.p. VLP group was lower on day 7 (Fig. 4E). With regard to other inhibitory receptors, the HMPV-infected group had the highest levels of TIM-3 (Fig. 4F). This higher level of inhibitory receptor expression in HMPV-infected mice correlated with the lower IFN-γ/H9253 MFI shown in Fig. 4C. These data indicate that VLP vaccination elicits functional T<sub>CD8</sub> with lower inhibitory receptor expression than those of infected mice.

VLP vaccination elicits F- and M-specific T<sub>CD8</sub> that are similar in function and inhibitory receptor expression to those of previously infected mice after HMPV challenge. VLP vaccination elicited HMPV-specific T<sub>CD8</sub> with lower inhibitory receptor expression and improved in vitro function (IFN-γ production). We therefore hypothesized that the VLP response to challenge might be similar to our previous findings in the setting of genetic ablation or antibody blockade of PD-1, where increased T<sub>CD8</sub> function led to enhanced viral clearance during primary infection of wild-type (WT) B6 mice or secondary infection of MT mice (34, 35). B6 mice were given two doses of VLPs (i.n. or i.p.) or mock VLPs or were infected with HMPV and were challenged 4 weeks postvaccination (Fig. 5A). Seven days postchallenge, all groups had F528- and M94-specific lung T<sub>CD8</sub> (Fig. 5B and C). The i.n. VLP group had the highest percentage of F528-specific T<sub>CD8</sub>, whereas the HMPV group had the highest proportion of M94-specific T<sub>CD8</sub>. Both VLP groups had a higher absolute number of F528<sup>+</sup> T<sub>CD8</sub> than the other two groups, whereas the HMPV group had the highest number of M94<sup>+</sup> T<sub>CD8</sub> (Fig. 5D and G). Mock-immunized mice had the highest percentage of
There were no significant differences between the VLP groups and the HMPV group for TCD8 IFN-\(\gamma\)/H9253 MFI (data not shown). The percentage of PD-1 expressing F528-specific TCD8s was lowest in the mock-immunized group, whereas there were no significant differences between the two VLP groups and the HMPV group (Fig. 5F). There was a similar trend for M94-specific TCD8s (Fig. 5I). Taken together, these results indicate that while both VLP vaccination and prior HMPV infection enhanced the number of memory T CD8s responding to HMPV challenge compared to primary infection (the mock-immunized group), there was a greater proportion of TCD8s that did not degranulate or produce IFN-\(\gamma\)/H9253 in mice that had previously encountered HMPV antigens in the context of either VLPs or previous infection. Furthermore, while VLP vaccination alone (without viral challenge) elicits epitope-specific TCD8s that appear to be more functional and less likely to express PD-1 or TIM-3, HMPV challenge overcomes this increased functionality.

Epitope-specific TCD8s are impaired in lungs but remain fully functional in spleens postchallenge. Previously, our group found that after i.n. HMPV infection, TCD8s in lungs became impaired starting on day 7 postinfection, when only a fraction of lung TCD8s responded to antigen by degranulating or producing IFN-\(\gamma\)/H9253 (34, 35). In contrast, splenic TCD8s remained fully functional postinfection. To determine the TCD8 kinetics after vaccination and challenge, we vaccinated B6 mice as shown in Fig. 5A and then analyzed the functional responses on days 3, 5, and 7 postchallenge. F528-specific TCD8s increased in both spleens and lungs from day 3 to day 7 postchallenge (Fig. 6A and B).
In the spleens, we observed a high concordance between tetramer staining and CD107a mobilization or IFN-γ production in TCD8s on both days 5 and 7 postchallenge (Fig. 6C). However, in the lungs, TCD8s were beginning to show impairment on day 5 postchallenge, and the impairment was more pronounced on day 7, with only 40% or less of tetramer-positive TCD8s degranulating or producing IFN-γ in antigen-experienced mice (Fig. 5E and 6D). Due to the small percentage of F528-specific TCD8s on day 3, we were unable to perform functional assays for that time point. These results, in combination with those shown in Fig. 5, indicate that a large percentage of pulmonary TCD8s in antigen-experienced mice were impaired and failed to respond to antigen. On the other hand, splenic TCD8s remained fully functional.
VLP vaccination facilitates HMPV clearance in μMT mice in the absence of antibodies. Mice are semipermissive hosts for HMPV, and wild-type B6 mice cannot be reinfected with HMPV, whereas reinfections in humans are common (11, 12, 41). For this reason, we performed additional experiments in μMT mice, which lack mature B cells and can be reinfected with HMPV. μMT mice (on a B6 background) and wild-type B6 mice demonstrated similar HMPV replication kinetics in the lungs and nasal turbinates during primary infection (32). However, while wild-type mice were completely protected against viral replication during HMPV challenge, μMT mice had peak titers similar to those in primary infection.

To determine the effect of VLP vaccination in μMT mice, we immunized them with two i.n. or i.p. doses of VLPs or mock VLPs or infected them i.n. with HMPV. Four weeks after the second VLP dose, we challenged them all the animals i.n. with HMPV (Fig. 7A). While VLP vaccination did not reduce the viral titers in the nose or lungs on day 5 postchallenge, by day 7, the vaccinated animals had lower viral titers in both noses and lungs. By day 10 postchallenge, most vaccinated animals had cleared HMPV from the lungs, whereas mice in the mock-immunized group still had detectable viral titers (Fig. 7B and C). This suggests that in the absence of antibodies, VLP vaccination can still facilitate HMPV clearance in the host.

VLP vaccination elicits F- and M-specific TCD8s in μMT mice. Since TCD8s have a known role in clearing viral infections (49, 50), we next sought to determine the magnitude of the HMPV-specific TCD8 response. After analyzing lung lymphocytes from μMT mice on day 7 postchallenge using tetramer and intracellular cytokine staining assays, we found that the VLP groups had the highest percentages and numbers of lung F528-specific TCD8s (Fig. 8A), whereas the HMPV group had the highest percentages and numbers of lung M94-specific TCD8s (Fig. 5), the mock-immunized group had the highest percentage of functional TCD8s after challenge for both epitopes (Fig. 8B and D).

TCD8s mediate HMPV clearance in μMT mice. To determine whether the faster clearance of HMPV in vaccinated animals was due to TCD8s or another component of the immune system, we vaccinated μMT mice i.p. with VLPs plus TMG (or mock VLPs...
plus TMG) and injected a CD8-depleting antibody or isotype control antibody 5 days before and on the day of HMPV challenge (Fig. 9A). T_{CD8} depletion was confirmed by flow cytometry (data not shown). T_{CD8} -depleted mice had higher viral titers than mice treated with isotype control antibody in both noses and lungs (Fig. 9B and C). In the nasal turbinates, titers in depleted mice were the same as those in mock-immunized mice, suggesting that T_{CD8} were responsible for the decreased viral titer seen in VLP-immunized, non-T_{CD8} -depleted animals. Conversely, while lung titers were lowest in the VLP-vaccinated, isotype antibody-treated group, titers in depleted animals were still lower than those in mock-VLP-treated animals. This suggests that in the lungs, T_{CD8} do play an important role in VLP-mediated HMPV clearance, but another component of the immune response (likely CD4$^+$ T cells) also contributes.

VLP vaccination protects DBA/2 mice against heterosubtypic HMPV challenge. We used the A2 subtype of HMPV to perform all the experiments to this point, as the subtype replicates most efficiently in a mouse model (references 34 and 37 and unpublished observations). Since clinical HMPV isolates fall into four subtypes, all of which infect humans (51, 52), we decided to determine whether VLP vaccination is protective against challenge with a different HMPV subtype. While the VLPs in our protocol were generated using F and M sequences derived from a clinical isolate of the A2 subtype, there is high amino acid sequence homology to the F and M proteins of different HMPV

FIG 6 Epitope-specific T_{CD8} are impaired in lungs but remain fully functional in spleens postchallenge. B6 mice were vaccinated with VLPs or infected with HMPV and later challenged with virus, as described in the legend to Fig. 5. Spleens and lungs were collected on days 3, 5, and 7 postchallenge for flow cytometric analysis. (A and B) F528 tetramer-positive CD8$^+$ T cells as a percentage of total CD8$^+$ T cells in spleens (A) and lungs (B). (C and D) F528 tetramer-positive, degranulating (CD107a$^+$), and IFN-γ$^+$ CD8$^+$ T cells as percentages of total CD8$^+$ T cells in spleens (C) and lungs (D). * P < 0.05 (this group is significantly different from all other groups; one-way ANOVA with Tukey’s multiple-comparison test); #, P < 0.05 (2-tailed paired t test). The data are representative of the results of one experiment with three mice per group. The error bars show standard deviations.

FIG 7 VLP vaccination facilitates HMPV clearance in μMTmice in the absence of antibodies. (A) μMT mice were vaccinated i.n. or i.p. with VLPs or VLPs and TiterMax Gold adjuvant or with mock VLPs or were infected i.n. with HMPV. Three weeks later, the vaccinated mice were given a second dose of VLPs. All the mice (including the mock-vaccinated group) were challenged i.n. with HMPV 4 weeks after the second VLP dose, and tissues were collected postchallenge. (B and C) Nose (B) and lung (C) titers were calculated. *, P < 0.05 (this group is significantly different from all other groups; one-way ANOVA with Tukey’s multiple-comparison test). The bars show the means of individual data points combined from the results of two independent experiments with two to four mice per group per experiment. The error bars show standard deviations.
subtypes. We previously showed that the DBA/2 mouse model is permissive for all four subtypes of HMPV (37). Since the B2 subtype diverges more from the A subtypes than the B1 subtype (5), we vaccinated DBA/2 mice i.p. with VLPs plus TMG and challenged them with either the A2 or B2 HMPV subtype using the same protocol shown in Fig. 1A. Similarly to B6 mice, DBA/2 mice were completely protected from A2 challenge in the lungs and partially protected in the nose (Fig. 10A and B). In addition, B2-challenged animals had undetectable lung titers and significantly reduced titers in the nose. Our results suggest that VLP vaccination protects mice against different HMPV subtypes.

**DISCUSSION**

HMPV is a major cause of disease in young infants and elderly and immunocompromised individuals. Reinfection also occurs in healthy adults, despite the presence of serum antibodies, indicating that antibody-mediated protection is insufficient to prevent HMPV infection in humans. T cell immunity is also thought to be important.
in the immune response against HMPV. In humans, immune compromise leads to more severe HMPV infections (53, 54), and fatal RSV disease in infants was characterized by a lack of T cells in the lung (55). Several studies in rodent models also suggest that T cells contribute to protective immunity (31, 34). Therefore, an ideal HMPV vaccine would elicit both antibody and T cell responses.

In this study, we found that VLP vaccination protected mice against HMPV challenge for at least 20 weeks postvaccination. The protection was complete in the lungs but partial in the nose. This is probably due to the nonreplicating nature of VLP vaccines, as live viruses tend to be more efficient at eliciting mucosal immunity (56). However, since HMPV causes the most severe disease in the lower respiratory tract of humans and much milder disease in the upper respiratory tract, VLPs are an attractive vaccine candidate for protecting against severe disease.

Previously, Cox et al. described a protocol for generating HMPV VLPs containing F and M proteins (32). While M protein did not elicit neutralizing antibodies and VLPs containing M protein alone (without F protein) did not protect mice from HMPV challenge, we chose to incorporate M into our VLP vaccine because it enhances the VLP yield (R. G. Cox, unpublished observations). Furthermore, both F and M proteins contain TCD8 epitopes (34, 35).

Since HMPV VLPs are fusion competent (57), this indicates that at least some of the F proteins on VLPs are in the prefusion form. This is an important consideration, since the prefusion state of the RSV fusion protein is the target of most neutralizing antibodies in humans (58). Nonneutralizing antibodies have been associated with enhanced respiratory disease by fixing complement and causing tissue damage (59). However, we observed no evidence of enhanced disease in animals vaccinated with HMPV VLPs. Antibodies in vaccinated animals were neutralizing, and lung histopathology revealed no eosinophilia or increased inflammation. In addition, our group previously reported that VLPs elicited a balanced T_{H1}/T_{H2} cytokine response (32).

We found that VLPs elicit TCD8 responses against both F and M proteins after a single dose given either i.n. or i.p. Most vaccines licensed for use in humans are given intramuscularly, including the VLP vaccines for human papillomavirus and hepatitis B virus, but the i.n. route of administration has been used in animal models (60). The addition of adjuvant increases neutralizing antibody titers when the VLPs are given i.p. (32). Although TiterMax Gold is not licensed for use in humans, other adjuvants have been approved for use in humans, and aluminum adjuvants are a component of both the HPV and hepatitis B virus vaccines (61, 62).

The mechanism of VLP-induced T_{CD8} responses is unclear. One possibility is that dendritic cells present extracellular antigens on MHC-I via cross-presentation (63, 64). Since VLPs are fusion competent, another possibility is that VLP-cell membrane fusion might deliver HMPV proteins into the cytosol, leading to the presentation of HMPV epitopes onto MHC-I molecules. Indeed, studies have shown that VLPs are capable of delivering proteins to the cytosol (65).

In the absence of antibodies, μMT mice that had been vaccinated or previously infected could be reinfected with HMPV. However, these mice were able to clear virus more rapidly than mice that had not previously encountered HMPV antigens. T_{CD8} depletion resulted in higher viral titers in both nasal turbinates and lungs of mice than in their nondepleted counterparts. This result is congruent with previous studies showing that T cell depletion resulted in higher HMPV titers (31, 66) and that passive transfer of HMPV-specific T_{CD8} protected mice against HMPV challenge (67). Since μMT mice, but not wild-type B6 mice, can be reinfected with HMPV, this suggests that while T_{CD8} help to clear virus once an infection has already taken place, antibodies are important in preventing infection, at least in a semipermissive mouse model.

While VLP-immunized μMT mice depleted of T_{CD8} had higher lung titers than nondepleted mice, their titers were still lower than those of mock-immunized mice. Therefore, it is likely that another component of the immune system plays a role in HMPV clearance, in addition to T_{CD8}. We speculate that this contribution is made by CD4^{+} T cells, as both CD4^{+} and CD8^{+} T cells are important in terminating infection by HMPV and RSV (31, 68). However, HMPV MHC class II epitopes have not yet been reported, limiting the ability to study HMPV-specific CD4^{+} T cells.

While we focused on three HMPV T_{CD8} epitopes, F528, M94, and M195, in the present study, our laboratory has identified additional HMPV epitopes in B6 mice (unpublished data), and other murine and human epitopes have been reported (19, 67, 69, 70). Both M epitopes analyzed in this study are well conserved between all HMPV subtypes. F528, which is found in the cytoplasmic tail of the fusion protein, is generally well conserved, but several clinical isolates of other HMPV subtypes show two amino acid sequence changes compared to the epitope in TN/94-49, the clinical isolate...
(A2 subtype) used in most of our experiments in this study. It remains to be seen whether VLPs containing other HMPV proteins can elicit HMPV-specific T\textsubscript{CD8} responses.

The F protein expressed on VLPs was derived from an A2 subtype of HMPV; however, VLP vaccination protected mice completely in the lungs from the B2 subtype of HMPV. This result is in concordance with the findings of Levy et al. (10), which showed that vaccinating mice with HMPV F/G VLPs elicited cross-protective antibodies, and with other reports showing that passive antibody transfer protects mice from HMPV infection (71, 72). Furthermore, given that the F and M proteins of one HMPV subtype share high amino acid sequence homology with those of different HMPV subtypes, there may also be shared T\textsubscript{CD8} epitopes between the different subtypes that contribute to heterosubtypic immunity. One study found that T\textsubscript{CD8} in humans previously infected with HMPV recognized epitopes that were conserved across HMPV subtypes (69).

Various studies have indicated that the lung environment contributes to impaired T\textsubscript{CD8} function in response to viral infection (73–75). Previously, our group reported that T\textsubscript{CD8} responding to HMPV vaccine candidate, even though they do not prevent lung T\textsubscript{CD8} impairment after viral challenge. Vaccination protects mice completely in the lungs and partially in the nasal turbinate against both homosubtypic and heterosubtypic HMPV strains and elicits neutralizing antibodies and functional HMPV-specific T\textsubscript{CD8}. Future directions include testing different types of adjuvants and dosing strategies, important considerations for any HMPV vaccine to be used in humans.

**ACKNOWLEDGMENTS**

We thank D. Flaherty, B. Matlock, and C. Warren at the Vanderbilt Flow Cytometry Shared Resource. The VMV Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA64845) and the Vanderbilt Digestive Disease Research Center (DK058404). This work was supported by R01 AI85062 (J.V.W.), A0412284 (S.I.), and T32 GM07347 from the National Institute of General Medical Studies for the Vanderbilt Medical Scientist Training Program.

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