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Novel HLA-A2-restricted human metapneumovirus epitopes reduce viral titers in mice and are recognized by human T cells

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ABSTRACT

Human metapneumovirus (HMPV) is a major cause of morbidity and mortality from acute lower respiratory tract illness, with most individuals seropositive by age five. Despite the presence of neutralizing antibodies, secondary infections are common and can be severe in young, elderly, and immunocompromised persons. Preclinical vaccine studies for HMPV have suggested a need for a balanced antibody and T cell immune response to enhance protection and avoid lung immunopathology. We infected transgenic mice expressing human HLA-A*0201 with HMPV and used ELISPOT to screen overlapping and predicted epitope peptides. We identified six novel HLA-A2 restricted $CD8^+$ T cell (T_{CD8}) epitopes, with $M_{39–47}$ (M39) immunodominant. Tetramer staining detected M39-specific T_{CD8} in lungs and spleen of HMPV-immune mice. Immunization with adjuvant-formulated M39 peptide reduced lung virus titers upon challenge. Finally, we show that T_{CD8} from HLA-A*0201 positive humans recognize M39 by IFNγ ELISPOT and tetramer staining. These results will facilitate HMPV vaccine development and human studies.

1. Introduction

Acute respiratory infections are a leading cause of childhood death worldwide, especially in low and middle-income countries [1]. Human metapneumovirus (HMPV) is a paramyxovirus discovered in 2001 and a major cause of acute respiratory infection [2]. The very young, elderly, immunocompromised persons, and those with underlying cardiopulmonary conditions are at the highest risk for severe HMPV disease [3–11]. Currently, no licensed vaccines or therapeutics exist for the virus, despite serological evidence that all individuals have been exposed to HMPV by the age of five years [2,12]. Humoral immunity alone appears to be insufficient for protection from infection with HMPV, since the level of neutralizing antibody response in previously infected individuals do not protect from reinfection with this virus [13–15]. The $CD8^+$ T cell (T_{CD8}) response contributes to control of HMPV, as is the case for the related respiratory syncytial virus (RSV); in the absence of T_{CD8}, mice exhibit higher viral titers and delayed clearance [16,17] and adoptively transferred CTL clones can reduce viral titer [18]. Moreover, humans with impaired T cell immunity experience more severe and fatal HMPV infection [6,7,9,19]. Recently, it has been shown that mechanisms exist which drive impairment of the lung T_{CD8} following HMPV infection in mice and humans [20,21] during primary and secondary infection, further bolstering evidence of the importance of this response in viral control.

Substantial progress has been made toward an effective vaccine, but HMPV vaccines have not yet entered clinical trials. Similar to the formalin-inactivated RSV vaccine tested in the 1960s [22], formalin-inactivated HMPV leads to enhanced lung pathology upon challenge in animal models [23,24]. Subunit vaccines based on HMPV F protein produce neutralizing antibodies [25,26], but T cell responses are thought to be important for protection. Live attenuated HMPV has a risk of reversion, which is particularly dangerous in immunocompromised populations, and often viral attenuation leads to significantly reduced immunogenicity [27–30]. CTL epitope vaccines can significantly reduce viral titers in mice [31], and recently a method utilizing virus-like particles (VLP), which incorporates benefits from both subunit and epitope-based vaccines, has shown promise for protection from HMPV [32,33]. In order to study T_{CD8} responses to vaccines and to natural HMPV infection in humans, it is necessary to map MHC I-restricted viral epitopes.

In this study, we used transgenic mice expressing human HLA-A*0201, and lacking major mouse MHC molecules ($Kb^{-/-}/Db^{-/-}$), to identify novel immune T_{CD8} epitopes recognized during HMPV infection. Immunization of mice with the immunodominant

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epitope reduced lung viral titers following challenge. We found that human T_{CD8} of HMPV-exposed HLA-A2 positive subjects were capable of recognizing the epitope by IFNγ ELISPOT and tetramer staining. Our results show that the transgenic mouse is a useful model for identification of HLA-A2-restricted HMPV epitopes, and suggest novel target/s for vaccination against HMPV.

2. Materials and methods

2.1. Mice, infections, and cells

HLA-A*0201 transgenic mice on a C57BL/6 (B6) background were obtained from Jackson Laboratories and MHC I K^b/D^b double knockout mice were obtained from Taconic. All animals were bred and maintained in specific pathogen-free conditions under guidelines approved by the AAALAC and the Vanderbilt Institutional Animal Care and Use Committee. Six to twelve-week-old age- and gender-matched animals were used in all experiments. HMPV (pathogenic clinical strain TN/94-49, genotype A2) was grown and titered in LLC-MK2 cells as previously described [34]. For all experiments, mice were anesthetized with intraperitoneal ketamine-xylazine and infected intranasally (i.n.) with 1×10^6 PFU of HMPV. For peptide vaccination (TriVax), mice were injected i.v. with a mixture of 200 μg M39 peptide, 50 μg anti-CD40 Ab (clone FGK4.5; BioXCell), and 50 μg polyinosinic:polycytidylic acid (InvivoGen) and allowed to rest for 4 weeks before infection. The dose of 200 μg peptide was used based on prior reports of TriVax in mice [35,36]. Viral titers in infected mouse lungs were measured by plaque titration as described previously [34]. HLA-characterized PBMCs from 5 unique human donors were obtained from Cellular Technology Limited (C.T.L.).

2.2. Epitope prediction and peptides

We used both predictopes and overlapping peptide approaches to identify MHC I-restricted HMPV epitopes. A previous report generated predictopes for the nucleoprotein (N), phosphoprotein (P), matrix (M), M2-1, M2-2, fusion (F), short hydrophobic (SH), and glycoprotein (G) using three separate online epitope prediction databases: SYFPEITHI (http://syfpeithi.de) [37]; BIMAS (http://www.bimas.cit.nih.gov) [38]; and PREDEP (http://margalit.huji.ac.il/Teppred/mhc-bind/index.html) [52]. To perform initial screening, 15-mer peptides overlapping by 9aa covering the entire HMPV M and N open reading frames were synthesized (GenScript). For predictopes, 9-mers were synthesized (GenScript).

2.3. IFNγ ELISPOT

ELISPOT assays were performed as previously described [20]. The mitogen concanavalin A (ConA, Sigma) was used as a positive control, while stimulation with an irrelevant peptide served as a negative control. The average number of spots counted from the negative control wells was subtracted from each of the HMPV epitope wells, and the data were expressed as spot-forming cells (SFC) per 10^6 lymphocytes. The antibodies used for the murine ELISPOT were anti-interferon-γ (IFN-γ) monoclonal antibody (mAb) clone AN-18 (5 μg/ml; eBioscience) and biotinylated anti-IFN-γ mAb R4-6A2 (2 μg/ml; eBioscience). For the human ELISPOT, the antibodies were anti-IFN-γ mAb 1-D1K (5 μg/ml; Mabtech) and biotinylated anti-IFN-γ mAb 7-B6-1 (2 μg/ml; Mabtech).

2.4. Flow cytometry

Cells were isolated from lungs and spleens of infected animals as previously described [20]. Briefly, lungs were rinsed in R10 medium (RPMI-1640 [Mediatech] plus 10% FBS, 2 mM glutamine, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and 50 μM β-mercaptoethanol [Life Technologies]), minced with a scalpel, and incubated with 2 mg/ml collagenase A (Roche) and 20 μg/ml DNase (Roche) for 1 h at 37 °C. Single-cell suspensions of spleens and digested lungs were obtained by pressing through a steel screen (80 mesh) and then passing over a nylon cell strainer (70 μm pore size). Erythrocytes were lysed using Red Blood Cell Lysis Buffer (Sigma-Aldrich). For labeling of HMPV-specific T_{CD8} single cell suspensions of mouse lung or spleen were stained with violet LIVE/DEAD dye (Life Technologies), Fc blocked with 1 μg per 10^6 cells anti-CD16/32 (BD Biosciences), and incubated with PE-labeled HLA-A*0201 tetramers (0.1–1 μg/ml), anti-CD8α (clone 53–6.7, BD Biosciences), and anti-CD19 (clone 1D3, eBioscience) [20]. Surface/tetramer staining was performed for 1.5 h at room temperature in PBS containing 2% FBS [39]. Staining for HMPV-specific T_{CD8} was normalized to the binding of an irrelevant PE-labeled tetramer loaded with a vaccinia virus (VACV) peptide to T_{CD8} (typically 0.05–0.1% of T_{CD8}). For all cell populations, FSC and SSC gating were used to define cells of appropriate size and shape. All flow cytometric data were collected using an LSRII or Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star).

2.5. Tetramer enrichment

Biotinylated HLA-A*0201 human monomers loaded with HMPV epitope were prepared by the exchange of conditional peptide ligand and then tetramerized with streptavidin-PE [40]. Briefly, recombinant HLA-A*0201 heavy and β2m light chain production [40], class I refolding with conditional peptide ligand KILGFVF**J**V [41], biotinylation and purification [42], UV-mediated exchange of conditional peptide with HMPV-derived peptides and quantification of peptide exchange were performed as described previously. Tetramerization with phycoerythrin (PE)-, or allophycocyanin (APC)-streptavidin conjugated fluorochromes (Life Technologies) were performed as described [43].

PBMC were briefly thawed, washed once with RPMI, re-suspended in 200 μl FACS (PBS containing 2% FBS and 50 nM dasatinib), and stained 30 min at RT with 10 μl of tetramer (25 μg/ml stock concentration) followed by addition of 50 μl FACS containing anti-CD8-Alexa Fluor700 (Life Technologies, 1:200 final dilution), anti-CD4-FITC (BD, 1:80 final dilution), anti-CD14-FITC (BD, 1:32 final dilution), and 1 h incubation at 4 °C. Cells were washed, re-suspended in 100 μl MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 8.0), and incubated with 10 μl of anti-PE beads (Miltenyi). Enrichment for tetramer+ (PE) T_{CD8} was performed with gravity MS magnetic column (Miltenyi). Both enriched and depleted fractions were collected for flow cytometric analysis; the entire enriched fraction and 10% of depleted fraction were analyzed. Accucheck counting beads (Life Technologies) were used to account for sample losses during flow cytometry and for more precise population counts. Dead cells were discriminated using propidium iodide. Up to 250,000 events were acquired using a 3-laser LSR-II (BD Biosciences).

2.6. Statistical analyses

Data analysis was performed using Prism v4.0 (GraphPad Software). Groups were compared using unpaired *t*-test or one-way ANOVA with post hoc Tukey test for multiple comparisons. $P < 0.05$ was considered significant by convention.