Human CD8$^+$ T cell cross-reactivity across influenza A, B and C viruses

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ABSTRACT

Influenza A, B and C viruses (IAV, IBV, ICV) circulate globally and infect humans, with IAV/IBV causing most severe disease. While $CD8^+$ T-cells confer cross-protection against different IAV strains, $CD8^+$ T-cell responses to IBV/ICV are understudied. We dissected the $CD8^+$T-cell cross-reactome against influenza viruses and provided the first evidence of $CD8^+$ T-cell cross-reactivity across IAV, IBV and ICV. Using immunopeptidomics, we identified immunodominant $CD8^+$ T-cell epitopes from IBV, protective in mice, and found prominent memory $CD8^+$ T-cells towards both universal and influenza type-specific epitopes in blood and lungs of healthy humans, with lung-derived $CD8^+$ T-cells displaying a tissue-resident phenotype. Importantly, effector $CD38^+Ki67^+CD8^+$ T-cells against novel epitopes were readily detected in IAV- and IBV-infected pediatric and adult patients. Our study introduces a new paradigm, whereby $CD8^+$ T-cells confer unprecedented cross-reactivity across all influenza viruses, a key finding for designing universal vaccines.

INTRODUCTION

Although 2018 marks the 100th anniversary of the catastrophic Spanish influenza pandemic, influenza viruses remain a constant, global health threat. Three types (genera) of influenza viruses infect humans: type A (or influenza A virus - IAV), type B (IBV) and type C (ICV). Two subtypes of IAV (A/H3N2 and A/H1N1pdm09) and two lineages of IBV (B/Yamagata/16/88-like and B/Victoria/2/87-like) co-circulate annually causing seasonal epidemics of mild, severe or fatal respiratory disease, while ICV causes severe disease in children[1, 2, 3, 4, 5]. Antigenically novel IAVs, generated by reassortment of the segmented genome and derived from animal reservoirs (aquatic birds and water fowl) or intermediate animal hosts (domesticated birds and swine) can also infect humans with high rates of morbidity and mortality[1]. When novel IAVs acquire the ability of sustained human-to-human transmissions, devastating influenza pandemics can occur.

The search for a long-lasting universal, broadly protective vaccine against influenza viruses is ongoing. Immune protection against influenza viruses is mainly mediated by adaptive humoral and cellular responses, although innate T cells also contribute to immune responses[1, 2]. Antibodies and B cells, induced by seasonal inactivated influenza vaccine (IIV), typically elicit strain-specific immunity by targeting the highly variable head domain of the surface glycoprotein hemagglutinin (HA). While these antibodies can provide neutralizing immunity, the constant antigenic drift of the HA protein makes them poor targets for broad cross-protection. Broadly cross-reactive antibodies predominantly targeted against the conserved stem of the HA molecule or at neuraminidase (NA)[6], can provide heterosubtypic cross-reactivity across either multiple IAV subtypes[7] or across IBVs, but not heterotypic cross-reactivity across IAVs and IBVs, with the reported exception of one rare antibody clone (CR9114)[8]. Conversely, cytotoxic $CD8^+$ T cells provide cross-protection across either seasonal IAVs[9, 10] or IBVs[11] as well as pandemic[12, 13, 14, 15] and avian[16, 17, 18] IAVs by recognizing highly conserved virus-derived peptides presented by Major Histocompatibility Complex class 1 (MHC-I) glycoproteins (Human Leukocyte Antigens (HLAs) in humans) on the surface of infected cells. To date, 195 $CD8^+$ T cell epitopes restricted by 24 different HLA alleles have been identified for IAVs, 7 epitopes (restricted by 2 HLAs; HLA-A*0201 or HLA-B*0801) for IBV and no T cell epitopes are currently known for ICV (Immune Epitope Database accessed on the 2nd Jan 2018). Following recognition of the peptide/MHC-I complex (epitope), $CD8^+$ T cells kill virally-infected cells and release anti-viral cytokines (IFNγ and TNF). The breadth of $CD8^+$ T cell cross-reactivity across antigenically-novel

viruses renders them promising targets for a universal vaccine. However, the current IIV formulation does not boost memory $CD8^+$ T cells[19]. Thus, novel vaccine formulations are needed to harness the potential of such cross-protective $CD8^+$ T cells.

The establishment of universal immune memory against influenza viruses requires prior knowledge of conserved antigenic regions to facilitate immunogen design and assessment of the immune response. While antibodies can be firstly isolated from serum and then mapped to epitopes, identification of antigen-specific $CD8^+$ T cells requires prior knowledge of the antigenic epitope, including both the peptide and the restricting HLA. While antibodies can be firstly isolated from serum and then used to map the epitopes, identification of antigen-specific $CD8^+$ T cells requires prior knowledge of the antigenic epitope, including both the peptide and the restricting HLA. Such knowledge can then be used to inform the antigenic composition of T cell-based vaccines, so they can be formulated as individual peptides, long epitope-rich peptides, mosaic peptides or even whole protein antigens to focus the immune response towards conserved and protective epitopes. Here, we defined the $CD8^+$ T cell cross-reactome against influenza A, B and C viruses and identified the antigenic specificity of IBV $CD8^+$ T cells using immunopeptidomics[20, 21]. We demonstrated that $CD8^+$ T cells can confer a previously unrecognized, broadly heterotypic cross-reactivity and characterized these responses in depth. Our data provide novel insights into universal $CD8^+$ T cell targets across IAV, IBV and ICV types and show that combining universal $CD8^+$ T cell peptide targets with B cell-based vaccines might lead to a broadly-protective influenza vaccine that does not require annual reformulation.

RESULTS

Universally cross-reactive $CD8^+$ T cell epitopes across IAV, IBV and ICV subtypes

To investigate the breadth of $CD8^+$ T cell cross-reactivity across IAV, IBV and ICV viruses, we first assessed the conservation of previously identified IAV-specific $CD8^+$ T cell epitopes across IAV, IBV and ICV types (**Fig. 1a, Supplementary Fig. 1**), as IAV-specific $CD8^+$ T cells have been the main research focus to date. Our conservation analysis of >67,000 influenza segment sequences identified 31 conserved epitopes (with >70% amino acid identity) across IAV and IBV as well as 8 epitopes across all IAV, IBV and ICV influenza types (**Supplementary Table S1**). Based on the prevalence of HLA-restricting molecules in the population and the nature of mutations within the peptide variants, we selected 9 epitopes across both HLA-A (HLA-A*01:01, HLA-A*02:01 and HLA-

A*03:01/A*11:01/*31:01/A*68:02) and HLA-B (HLA-B*07:02, HLA-B*44:02 and HLA-B*37:01) alleles (**Fig. 1b**) for further investigation.

To determine $CD8^+$ T cell immunogenicity towards these epitopes, we probed memory $CD8^+$ T cells within PBMCs obtained from healthy adults using *in vitro* peptide expansion and measured IFN-γ production after peptide re-stimulation. Our data indicate that three (A1/$PB1_{591}$ n=3, A2/$PB1_{413}$ n=5, B37/NP_{338} n=3) out of the nine conserved $CD8^+$ T cell epitopes recalled robust memory $CD8^+$ T cell responses across multiple donors (**Fig. 1c**). These conserved $CD8^+$ T cell peptides ($PB1_{591\text{-}599}$, $PB1_{413\text{-}421}$ and $NP_{338\text{-}345}$) are restricted by three prominent HLA molecules (HLA-A*01:01, HLA-A*02:01 and HLA-B*37:01, respectively), providing broad global coverage as ~54% of the population carry at least one of these three alleles, although some geographic regions would be underrepresented.

Strikingly, the NMLSTVLGV $PB1_{413\text{-}421}$ peptide in IAV (positioned as $PB1_{414\text{-}422}$ in IBV and ICV; referred to as $PB1_{413}$ hereafter) was universally (>98% of sequences) conserved (average identity >99.9%) across IAV, IBV and ICV, but not in influenza D viruses, where a L7F mutation was found, or other genera of the *Orthomyxoviridae* family like Infectious Salmon Anemia virus, Wellfleet Bay virus or Thogoto virus (**Fig. 1d**). The $PB1_{413\text{-}421}$ peptide has previously been reported as an IAV epitope[22, 23], that is share in sequence with IBV [24], however, $CD8^+$ T cell cross-reactivity has not been shown. To demonstrate the ability of A2/$PB1_{413}$-specific $CD8^+$ T cells to confer cross-reactivity across IAV, IBV and ICV subtypes, PBMCs obtained from HLA-A*0201-expressing donors were stimulated *in vitro* with autologous PBMCs infected with either IAV, IBV or ICV, followed by measuring A2/$PB1_{413}{}^+CD8^+$ T cell responses by IFN-γ on d10 (n=5) (**Fig. 1e**). In contrast to minimal IFN-γ production towards $PB1_{413}$ peptide directly *ex vivo* (**Fig. 1e**), 10-day culture with IAV-, IBV- or ICV-infected targets markedly increased the magnitude of A2/$PB1_{413}$-specific $CD8^+$ T cells (**Fig. 1e**), due to expansion of A2/$PB1_{413}{}^+CD8^+$ T cells towards all three IAV, IBV and ICV types. Our data thus provide the first evidence that memory A2/$PB1_{413}{}^+CD8^+$ T cells are activated following stimulation with either IAV-, IBV- and ICV-infected targets, introducing a new paradigm that $CD8^+$ T cells can exhibit universal cross-reactivity across IAV, IBV and ICV, and hence have a much broader cross-reactivity potential than previously thought.

Analysis of the remaining two conserved and immunogenic peptides ($PB1_{591\text{-}599}$ and $NP_{338\text{-}345}$ in IAV, $BPB1_{590\text{-}598}$ and $BNP_{394\text{-}401}$ in IBV) revealed variations at one or two amino acids (S2A and L8I for $PB1_{591}$ and F1Y within NP_{338}) between IAV and IBV viruses, and a

lack of conservation in ICV (**Fig. 1b**). *In vitro* expansion with either IAV- or IBV-derived peptides showed unidirectional cross-reactivity, with IAV-expanded $CD8^+$ T cells recognizing both IAV- and IBV-derived peptides, although the latter to a lesser extent. However, the IBV variants could not expand $CD8^+$ T cells directed at the cognate peptides, suggesting that the mutations may render these variants less immunogenic (**Fig. 1f-g**).

Collectively, our data demonstrate that human $CD8^+$ T cells can confer heterotypic cross-reactivity across IAV and IBV and ICV types. As the above findings are only based on the currently known IAV-derived epitopes and thus are mainly limited to IAV peptides presented by well-characterized HLA class-I molecules, such universal cross-reactivity might be broader than defined here. Furthermore, our data suggest a need for identification of novel $CD8^+$ T cell epitopes recognizing both IAV- and IBV-derived peptides restricted by a broad range of HLAs represented across different ethnicities.

Identification of novel HLA-A*02:01-restricted IBV epitopes by immunopeptidomics

As there is a general lack of $CD8^+$ T cell epitopes known for the clinically-relevant and understudied IBVs, we sought to address this knowledge gap. We embarked on the identification program of novel $CD8^+$ T cell epitopes derived from IBV viruses and presented by HLA-A*02:01, due to the high global prevalence of this allele. We utilized an immunopeptidomics approach to define peptides naturally processed and presented on the surface of IBV-infected cells. EBV-transformed B lymphoblastoid class I-reduced (C1R) cells lines stably expressing high levels of the HLA-A*02:01 molecule were used, together with the parental C1R cells, expressing background levels HLA-B*35:01 and HLA-C*04:01[25], to exclude peptides derived from these HLA molecules. Infection of C1R cells with the B/Malaysia resulted in high infection rates (~70% BNP^+ cells) and high cell viability (~93%) (**Supplementary Fig. 2a**). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of peptides isolated from HLA-A*02:01 molecules revealed predominantly 9-mer (n=1490), followed by 11-mer (n=695) and 10-mer (n=589) peptides (**Fig. 2a-b, Supplementary Fig. 2b**). These peptides mainly exhibited the canonical anchor residues of HLA-A*02:01 ligands (Leucine (L) at P2 and L or Valine (V) at the C-terminus[26] (**Fig. 2b**). Length distributions were similar for human peptides from uninfected cells and human and viral peptides from infected cells (**Supplementary Fig. 2c**). Analyses from two independent experiments yielded a total of 73 potential HLA-A*02:01-presented IBV-derived peptides, with ~64% overlap between the experiments (**Supplementary Table S2**). The IBV-derived peptides mainly originated from hemagglutinin (BHA) (22.3%), followed by BNP (16.4%)

and BM1 (11.9%), with all IBV proteins contributing to the HLA-A*02:01 immunopeptidome, except from BM2 and NB (**Fig. 2d**). In contrast, numerous peptides derived from BM2 were identified at high confidence in the analysis of peptides presented by the HLA class II of C1R cells (**Supplementary Fig. 2d, Supplementary Table S2**). Of these 73 HLA-A2 binding IBV peptides, 67 were synthesized for further investigation.

Prominence of novel IBV A2/BHA$_{543}$- and A2/BNS1$_{266}$-specific CD8$^+$ T cells IBV epitopes in healthy humans

To dissect IBV-specific CD8$^+$ T cell responses towards the 67 LC/MS-identified IBV-peptides, we firstly probed the memory CD8$^+$ T cell pools in HLA-A*02:01-expressing individuals. We randomly assigned the peptides in 6 pools of 10-12 peptides, avoiding overlapping peptides in the same pool (**Supplementary Table S2**). We established CD8$^+$ T cell lines specific for each of the 6 peptide pools, and then re-stimulated cells with the cognate pool in an IFNγ/TNF ICS assay (**Fig. 2fg**). CD8$^+$ T cell responses were predominantly targeted towards pool 2 (80% of donors responding, n=11), with smaller responses detected in some donors for pools 1, 3, 4 and 6 (**Fig. 2f, Supplementary Fig. 3a**). Dissection of pool 2 into individual peptides verified A2/BHA$_{543-551}$ as the prominent epitope amongst HLA-A*0201$^+$ donors (n=6) (**Fig. 2h**). Numerically smaller responses towards A2/BHA$_{538-551}$, A2/NS1$_{266-274}$, A2/BNS1$_{264-274}$ and BM1$_{132-140}$ were also detected in some donors **(Supplementary Fig. 3b**). To validate these responses independently of the peptide pools, we established CD8$^+$ T cell lines towards individual immunogenic peptides (**Fig. 2ij**). CD8$^+$ T cell responses to A2/BHA$_{543-551}$ were of the greatest magnitude (median 7.35% of CD8$^+$ T cells; n=6) and more frequent amongst donors (6/6) than the A2/NS1$_{266-274}$, A2/BNS1$_{264-274}$ and A2/BM1$_{132-140}$ (0.035% and 0.025% of CD8$^+$ T cells, respectively), each found in a single donor (**Fig. 2j, Supplementary Fig. 2b**). Thus, our thorough *in vivo* and *in vitro* analysis identified 5 novel peptides recognized by CD8$^+$ T cells in complex with the HLA-A*02:01 molecule, with BHA$_{543-551}$ being most prominent amongst the peptides tested.

Having identified novel IBV CD8$^+$ T cell epitopes, we determined the conservation of two most prominent peptides, BHA$_{543-551}$ and BNS1$_{266-274}$, across IBV strains. Both peptides were highly conserved (mean conservation of 99% and 98%, respectively) in >14,000 sequences per segment, spanning both lineages and 77 years of evolution (1940-2017) (**Supplementary Fig. 3c**). While some of the peptides identified by immunopeptidomics were highly conserved (>70%) in IAV (n=6 peptides) or in ICV (n=1) (**Supplementary Fig. 3c**), these were not immunogenic in the donors tested.

Overall, the immunopeptidomics approach identified 73 previously uncharacterized IBV-derived HLA-A*02:01 peptide-ligands, 67 of which were tested for immunogenicity, with $CD8^+$ T cell responses being targeted predominantly to $BHA_{543\text{-}551}$, highly conserved across IBV, but not IAV or ICV.

Immunodominance of universal A2/$PB1_{413}^+$ over IBV-specific A2/$BHA_{543}^+CD8^+$ T cells in IBV infection

Our data so far identified three conserved HLA-A*02:01-restricted epitopes for IBV: the universal A2/$PB1_{413}$ and two IBV-specific (A2/$BHA_{543\text{-}551}$ and A2/$NS1_{266\text{-}274}$ hereafter A2/BHA_{543} and A2/$BNS1_{266}$) epitopes. To further understand the role of the universal A2/$PB1_{413}^+CD8^+$ T cells in the immunodominance hierarchy following either IAV or IBV infection, we established IAV- or IBV-specific $CD8^+$ T cell lines *in vitro* from PBMC of healthy adults (n=11) and assessed tetramer-specific $CD8^+$ T cell responses against IAV epitopes (A2/$M1_{58\text{-}66}$ (A2/$M1_{58}$), A2/$PA_{46\text{-}54}$ (A2/PA_{64}), A2/$PB1_{413}$) and IBV epitopes (A2/BHA_{543}, A2/$BNS1_{266}$, A2/$PB1_{413}$). Consistent with our IFNγ staining (**Fig. 1e**), A2/$PB1_{413}$-tetramer detected universal A2/$PB1_{413}^+CD8^+$ T cells within both IAV- or IBV-specific $CD8^+$ T cell lines, although they displayed differential immunodominance hierarchies following either IAV and IBV infection (**Fig. 2k-m**). Within the IAV-specific $CD8^+$ T cell lines, the A2/$M1_{58}$-$tetramer^+CD8^+$ T cell population was significantly dominant (median of 3.9% $tetramer^+$ of $CD8^+$ T cells; detected in all 11 donors) over the universal A2/$PB1_{413}^+$ (0.12%; detected in 10/11 donors) and the subdominant A2/$PA_{46}^+CD8^+$ T cells (0.05%) populations (**Fig. 2l**). Conversely, the universal A2/$PB1_{413}$ epitope within the IBV-specific T cell lines was immunodominant (0.3%; detected in 8/11 donors) over the IBV-specific A2/BHA_{543} (0.11%; detected in 10/11 donors) and A2/$BNS1_{266}$ epitopes (0.01%) (**Fig. 2m**). These data demonstrate that (i) the universal A2/$PB1_{413}$ as well as the newly-identified IBV-specific A2/BHA_{543} and A2/$BNS1_{266}$ $CD8^+$ T cells can be expanded following virus stimulation *in vitro*, and (ii) immunodominance of the universal A2/$PB1_{413}$ epitope depends on the type of influenza infection.

Recruitment of universal A2/$PB1_{413\text{-}421}^+CD8^+$ T cells following human IAV and IBV infection *in vivo*

To evaluate the recruitment and activation of universal A2/$PB1_{413\text{-}421}^+CD8^+$ T cells in humans during influenza virus infection, we analyzed PBMC samples from 3 different

clinical cohorts of PCR-confirmed IAV- or IBV-infected pediatric and adult individuals (**Fig. 3b**). Using a tetramer-associated magnetic enrichment (TAME) technique, we readily detected influenza-specific $CD8^+$ T cells directly *ex vivo* in IAV- and IBV-infected pediatric and adult patients (**Fig. 3a**)[27, 28, 29]. A healthy adult cohort and a cohort of HLA-A*02:01-positive patients who were hospitalized with a non-influenza respiratory illness (i.e. influenza-PCR negative but PCR-positive for other respiratory viruses such as RSV or picornavirus) were also analyzed for comparison (**Fig. 3b**). A2/$M1_{58}$- and A2/$PB1_{413}$-specific $CD8^+$ T cells were detected in 100% and 50% of IAV^+ individuals (n=16) respectively, while A2/BHA_{543}- and A2/$PB1_{413}$-specific $CD8^+$ T cells were detected in 75% and 87.5% of IBV^+ individuals (n=8). The frequency of A2/$M1_{58}$- and A2/$PB1_{413}$-specific $CD8^+$ T cells in the blood were significantly increased (4.3- and 6-fold increase, respectively) in IAV-infected patients, as compared to memory $CD8^+$ T cells in healthy donors (**Fig. 3c**). The numbers of A2/BHA_{543}- and A2/$PB1_{413}$-specific $CD8^+$ T cells in IBV-infected patients increased 2.2- and 2.6-fold, respectively, above the numbers in healthy donors, however these did not reach statistical significance, most likely due to the differential age distribution in IBV-infected ($p=0.0002$), but not IAV-infected ($p=0.27$) patients comparing to healthy controls. In the influenza-negative hispitalized cohort, $CD8^+$ T cells for all three specificities were in the same range as the healthy donors (**Fig. 3c**). Notably, tetramer-positive $CD8^+$ T cells for all 3 specificities could be detected across all age groups (**Fig. 3d**).

Tetramer-positive A2/$PB1_{413}{}^+CD8^+$, IBV-A2/$BHA_{543}{}^+$ and IAV-A2/$M1_{58}{}^+$ $CD8^+$ T cells detected in IAV- or IBV-infected patients displayed an increase in $CD38^+$/$Ki\text{-}67^+$ expression (**Fig. 3e, Supplementary Fig. 4**), which represents an activated/effector phenotype during human viral infections[30, 31, 32], suggesting their recruitment during human influenza virus infection. This was not seen in the influenza-negative cohort, suggesting that this activation is specific for acute influenza infection. The expression or upregulation of additional activation markers, like HLA-DR and PD-1, was also increased on some tetramer$^+CD8^+$ T cell populations (**Supplementary Fig. 5**). The variability in numbers and phenotype between tetramer$^+CD8^+$ T cells is likely due to (i) the age range and exposure history of the donors, and (ii) varying times of sampling following influenza virus infection, both within and between the cohorts (**Fig. 3b**). Indeed, $CD8^+$ T cell responses after human A/H1N1 infection peak within 7 days and then contract rapidly[33]. Additionally, the magnitude and activation status of $CD8^+$ T cells within the circulation can underrepresent virus-specific cells at the site of human respiratory virus infections[31].

These data show that A2/PB1$_{413}^{+}$CD8^{+} T cells are truly universal as they can be detected with an activated/effector phenotype in HLA-A*0201-expressing influenza-infected patients following either IAV or IBV infection. Additionally, activated/effector CD8^{+} T cells specific for A2/BHA$_{543\text{-}551}$, identified by immunopeptidomics, can be detected during human IBV infection, illustrating the power of mass-spectrometry in identifying novel peptide ligands.

Detection of tissue-resident memory universal A2/PB1$_{413}^{+}$ CD8^{+} T cells in human lungs

As human memory CD8^{+} T cells also reside outside the circulation[31], we used a rare set of human lung samples from deceased HLA-A*0201-expressing organ donors (n=8) to assess the presence of universal A2/PB1$_{413}^{+}$CD8^{+} T cells at the site of infection. We also used human spleens (n=11), tonsils (n=4) and lymph nodes (n=4) to assess the presence of influenza-specific CD8^{+} T cells in the secondary lymphoid organs (SLOs), where memory CD8^{+} T cells are enriched. CD8^{+} T cells specific for A2/M1$_{58}$ (4/5), A2/PB1$_{413}$ (2/5), and A2/BHA$_{543}$ (1/5) were detected within human lung CD8^{+} T cells (**Fig. 4a)**. Similarly, CD8^{+} T cells specific for A2/M1$_{58}$ (17/18), A2/PB1$_{413}$ (6/18), and A2/BHA$_{543}$ (4/18) were detected within human lung CD8^{+} T cells. Importantly, the majority of A2/PB1$_{413}^{+}$ and A2/BHA$_{543}$ CD8^{+} T cells exhibited a tissue-resident memory CD69^{+}CD103^{+}CD45RO^{+} phenotype in the human lung but not in SLOs (**Fig. 4b**), with central (CD27^{+}CD45RA^{-}) or effector (CD27^{-} CD45RA^{-}) memory-like phenotype dominating in SLOs (**Fig. 4c**). This analysis indicates the presence of universal A2/PB1$_{413}^{+}$ tissue-resident memory CD8^{+} T cell pools in the human lung as well as memory pools in human SLOs.

Overall, pools of effector and memory IAV-A2/M1$_{58}^{+}$, IBV-A2/BHA$_{543}^{+}$ and universal A2/PB1$_{413}^{+}$ CD8^{+} T cells can be detected directly *ex vivo* in peripheral blood and SLOs of healthy individuals as well tissue-resident IAV-A2/M1$_{58}$-specific and universal A2/PB1$_{413}$-specific CD8^{+} T cells memory pools in the human lung.

Longitudinal single-cell RNA sequencing analysis of universal and IBV-specific CD8^{+} T cells

To further understand recruitment and activation phenotype of universal and novel IBV-specific CD8^{+} T cells at the molecular level during human influenza infection, we used single-cell RNA sequencing (scRNAseq) to assess the transcriptome of *ex-vivo* isolated tetramer^{+}CD8^{+} T cells from rare longitudinal PBMC samples obtained from an IBV-infected HLA-A*02:01-expressing individual. Infection with a B/Victoria strain was confirmed by

PCR[35] (**Supplementary Fig. 6a)** and serological analysis (**Supplementary Fig. 6b**). Blood samples were obtained at baseline (~3 months prior to infection), d14, 3 months and 1.5 years after IBV infection (**Fig. 5a**). Universal A2/PB1$_{413}^{+}$CD8^{+} T cells were readily detected at baseline at 19 tetramer^{+}/10^{6} CD8^{+} T cells, then increased 19-fold to 367 tetramer^{+}/10^{6} CD8^{+} T cells on d14 after infection and were maintained at a similar level (327 tetramer^{+}/10^{6} CD8^{+} T cells) up to 1.5 years after infection (**Fig. 5b**). Conversely, A2/BHA$_{543}^{+}$CD8^{+} T cells were undetectable at the baseline, suggesting this may have been the first IBV infection for this donor, despite a previous immunization against B/Yamagata strains, with an inactivated vaccine not eliciting CD8^{+} T cell responses[19]. A2/BHA$_{543}^{+}$CD8^{+} T cells increased to 73.4 tetramer^{+}/10^{6} CD8^{+} T cells on d14 after infection, 5-fold lower than universal A2/PB1$_{413}^{+}$CD8^{+} T cells, and close to the detection level at 1.5-year time-point. Thus, A2/PB1$_{413}^{+}$CD8^{+} T cells were assessed at all time-points, while IBV-specific A2/BHA$_{543}^{+}$CD8^{+} T cells were analyzed only on d14.

A total of 209 tetramer-positive CD8^{+} T cells were analyzed using scRNAseq, with an average of 1201 expressed genes identified per cell. Principal component analysis (PCA) revealed clear segregation of A2/PB1$_{413}^{+}$CD8^{+} T cells by time-point but no segregation between the two antigenic effector IBV-specificities (universal A2/PB1$_{413}^{+}$CD8^{+} T cells and IBV-specific A2/BHA$_{543}^{+}$CD8^{+} T cells) on d14 (**Fig. 5c**). Notably, differential expression analysis identified distinct gene expression signatures across time-points (**Fig. 5d**). Specifically, gene-set enrichment analysis revealed signatures of T cell activation and differentiation, cell division, immune cell migration and chemotaxis, which were enriched in d14 cells, as compared to those from baseline or 1.5 years (**Fig. 5e-f**).

We next analyzed specific expression of genes associated with T cell differentiation, activation, cytotoxicity and effector function (**Supplementary Fig. 6cd**). Importantly, effector CD8^{+} T cells across both IBV-specificities isolated from d14 upregulated genes associated with activation (CD74, CD52), cytotoxic molecules (PRF1, GZMB, GZMA, GZMK, GNLY, CTSW), cytotoxic receptors (NKG7, KLRK1) and effector cytokines (CCL5, CCL4). The expression profiles for some genes associated with differentiation and activation were confirmed by flow-cytometry (**Supplementary Fig. 6cd**).

Taken together, although these single-cell RNAseq data were obtained from one patient naturally-infected with IBV, this experiment provided a rare opportunity to examine baseline PBMC samples from a HLA-A*0201-expressing patient prior to the natural IBV infection as well as at the acute (d14), short-term memory (3 months) and long-term memory (1.5 years) time-points after the infection. Our results provide clear evidence of transcriptome

changes associated with differentiation and activation of A2/PB1$_{413}^{+}$CD8^{+} and A2/BHA$_{543}^{+}$CD8^{+} T cells during IBV infection. To the best of our knowledge, these are the first data on transcriptome changes within tetramer-specific CD8^{+} T cells at the single cell level from the baseline to long-term memory CD8^{+} T cells in humans. Thus, through the flow cytometric analysis of IBV-infected patients (n=8) and longitudinal scRNAseq analysis of an naturally infected individual, we demonstrate that A2/PB1$_{413}^{+}$CD8^{+} and A2/BHA$_{543}^{+}$CD8^{+} T cells are recruited to the immune response during IBV infection.

Immunodominance of A2/BHA$_{543}$- and A2/BNS1$_{266}$-specific CD8^{+} T cells during IBV infection of HHD-A2 mice *in vivo*

Having shown the recruitment of activated CD8^{+} T cells directed at the universal (A2/PB1$_{413}$) and novel IBV-specific (A2/BHA$_{543}$) epitopes during influenza disease in humans, we subsequently set to investigate their protective efficacy, especially as the role of CD8^{+} T cells in IBV infection remains unknown. To achieve this, we utilized our previously published HHD HLA-A2.1-expressing transgenic (HHD-A2) mouse model of influenza A infection[36] and established a HHD-A2 mouse model of influenza B and influenza C infection. HHD-A2 mice express a chimeric MHC-I monochain comprising of the human β2-microglobulin covalently linked to the HLA-A*02:01 α1 and α2 domains and the murine α3 and transmembrane domains[37] and thus can respond to many human HLA-A*0201-restricted epitopes, including IAV-derived A2/M$_{51}$[36] or cancer-derived A2/WT1A neoantigen[38]. These mice are not confounded by exposure infection history nor co-expression of other MHC-I molecules and thus provide an important model for both understanding influenza-specific CD8^{+} T cell responses *in vivo* as well as determining their protective role in influenza disease.

Firstly, to verify the immunogenicity of novel IBV-derived peptides, we infected HHD-A2 mice intranasally (i.n.) with 100pfu of B/Malaysia virus. On day (d) 10 after infection (**Fig. 6a**), we stimulated splenocytes with each peptide individually (out of 67 immunoproteomics-derived peptides) and measured production of IFNγ and TNF. As in humans (**Fig. 2j**), immunodominant CD8^{+} T cell responses were largely targeted towards A2/BHA$_{543\text{-}551}$ (mean of 5% of CD8^{+} T cells) and A2/BNS1$_{266\text{-}274}$ (mean of 1.8% of CD8^{+} T cells), with smaller subdominant responses observed for A2/HA$_{538\text{-}551}$ and A2/BNS1$_{264\text{-}274}$ (mean of <0.5% of CD8^{+} T cells), which overlap with A2/BHA$_{543\text{-}551}$ and A2/BNS1$_{266\text{-}274}$, respectively (**Fig. 6b-d**). We also assayed the peptides in 6 pools of 10-12 peptides, as for human studies (**Supplementary Table S2**) and assessed CD8^{+} T cell responses to each pool

at the site of infection represented by the bronchoalveolar lavage (BAL). $CD8^+$ T cell responses were targeted to pools 2 and 3 (**Fig. 6d**), containing the $BHA_{543-551}$, $BNS1_{266-274}$ and $BNS1_{264-274}$ peptides, as confirmed separately (**Fig. 6c**).

To compare these primary $CD8^+$ T cells directed at $BHA_{543-551}$ and $BNS1_{266-274}$ epitopes with secondary $CD8^+$ T cell responses, we firstly primed HHD-A2 mice i.n. with B/Malaysia, and then i.n. infected with the heterologous strain B/Phuket 6 weeks later (**Supplementary Fig. 7bc**). Assessment of $CD8^+$ T cell responses against the main A2/$BHA_{543-551}$ and A2/$BNS1_{266-274}$ epitopes in the spleen on d8 after challenge showed that the number of secondary $IFN\gamma^+TNF^+$ $CD8^+$ T cells in the spleen was ~27-fold higher than following a primary infection (**Supplementary Fig. 7bc**). Additionally, $CD8^+$ T cells for both specificities showed increased polyfunctionality ($IFN\gamma^+TNF^+IL\text{-}2^+$) following secondary infection (0.14% and 2.14% of $CD8^+$ T cells for BHA_{543}, n=4-5, p=0.013) (**Supplementary Fig. 7d**). Thus, using our model of IBV infection in HHD-A2 mice, we verified the novel (identified by immunopeptidomics) immunodominant IBV-specific A2/$BHA_{543-551}$ and A2/$BNS1_{266-274}$ epitopes in both primary and secondary IBV infections.

Lack of A2/$PB1_{413}{}^+CD8^+$ T cells in HHD-A2 mice

As universal A2/$PB1_{413}{}^+CD8^+$ T cells can be readily detected in both IAV- and IBV-infected patients, we next assessed A2/$PB1_{413}{}^+CD8^+$ T cell responses following IAV (A/X31), IBV (B/Malaysia) or ICV (C/Perth) infection of HHD-A2 mice. Unexpectedly, $CD8^+$ T cells specific for the A2/$PB1_{413}$ epitope could not be detected following either (i) primary IAV, IBV or ICV infection (**Supplementary Fig. 8a)**, (ii) secondary infection with either a heterologous virus (eg. A/X31→A/PR8) or a heterotypic virus (eg. A/X31→B/Mal) in all 4 possible combinations (A→A, A→B, B→B, B→A) (**Supplementary Fig. 8b)**, or (iii) tertiary (A→B→A, B→A→B) (**Supplementary Fig. 8c)** influenza infections (detailed description in Supplementary Results). Additionally, A2/$PB1_{413}{}^+CD8^+$ T cells in HHD-A2 mice could not be detected following well-established lipopeptide vaccination (**Supplementary Fig. 8d)** or peptide vaccination (**Supplementary Fig. 8f)** as well as using tetramer-enrichment in naïve mice. Thus, all of these above experiments (**Supplementary Fig. 8)** provide strong evidence for a lack of naïve A2/$PB1_{413}$-specific precursors in HHD-A2 mice, most likely due to a TCR repertoire hole in HHD-A2 mice towards the A2/$PB1_{413}$ epitope. Hence, the protective role of universal A2/$PB1_{413}{}^+CD8^+$ T cells towards IAV, IBV and ICV infection *in vivo* could not be assessed in HHD-A2 mice (Supplementary Results).

Protective capacity of A2/BHA$_{543}$- and A2/BNS1$_{266}$-specific CD8$^+$ T cells during *in vivo* infection of HHD-A2 mice

Previous studies using CD8$^+$ T cells depletion in mice lacking antibodies have demonstrated a role for CD8$^+$ T cells during IBV infection [39]. To determine the protective capacity of the novel IBV-derived CD8$^+$ T cell epitopes in HHD-A2 mice, we vaccinated mice with the BHA$_{543}$ and BNS1$_{266}$ peptides using a well-established prime/boost approach, then infected mice i.n. with 5x10^3 pfu B/Malaysia (**Fig. 7a**). On day 6 after boosting and prior to vaccination, no differences in the number of innate cells (neutrophils, macrophages or γδ Tcells) were observed between the mock (adjuvant alone) and peptide/adjuvant-vaccinated groups in blood (data not shown). Thus, any non-specific inflammatory or innate effects of vaccination are controlled for in the mock group.

Vaccination with peptides resulted in significantly higher numbers of total A2/BHA$_{543}$- and A2/BNS1$_{266}$-tetramer$^+$CD8$^+$ T cells in the spleen on d6 and d7 after IBV infection when compared to mock-vaccinated (adjuvant alone) mice (~5.6-fold $p<0.05$) (**Fig. 7bc)**. A2/BHA$_{543}{}^+$CD8$^+$ and A2/BNS1$_{266}{}^+$CD8$^+$ T cell numbers were comparable ($p>0.05$) in the BAL (with ~2-fold increase in immunized mice). Following immunization, however, there was an increase in recruitment of immunodominant A2/BHA$_{543}{}^+$CD8$^+$ T cells to the site of infection between d6 and d7.

Importantly, peptide-vaccinated mice exhibited significant protection against IBV, as shown by a significant ~65% reduction in viral titers in the lung and nose on d6 and 100% clearance in the lung on d7 after IBV infection when compared to the mock-immunized group ($p<0.05$) (**Fig. 7d**). Additionally, there was a significant decrease ($p<0.05$) in the levels of inflammatory cytokines (MIP-1β, IL-6, IL-1β, IFNγ) in d7 BAL of peptide-vaccinated mice in comparison to the mock-immunised animals (**Fig. 7ef**). Thus, CD8$^+$ T cells directed at our novel HLA-A2.1-restricted IBV-specific epitopes are protective, as they can markedly accelerate viral clearance and reduce the cytokine storm at the site of infection.

DISCUSSION

Cytotoxic CD8$^+$ T cells play a crucial role in protection from severe influenza disease in both human settings and animal models of influenza virus infection[1, 2]. CD8$^+$ T cells limit viral replication and promote clearance of infected cells, the recognition of which is dependent on presentation of viral peptides on the cell surface by MHC-I molecules. The high conservation of these peptides allows cross-recognition of cells infected by distinct IAV

strains, including pandemic and avian IAV viruses[1, 2]. Our study proposes and examines two levels of cross-reactivity by influenza-specific $CD8^+$ T cells: i) heterotypic cross-reactivity across IAV and IBV, and in some instances ICV, by $CD8^+$ T cells recognizing peptides derived from the most conserved regions of influenza viruses, and ii) IBV-wide cross-reactivity by $CD8^+$ T cells recognizing peptides derived from highly conserved regions of IBV (like BHA_{543} and $BNS1_{266}$).

Broadly-neutralizing antibodies (bNAbs) against the IAV and/or IBV HA stem have been the focus of the recent research. However, so far, such broadly cross-reactive antibodies have been rare and immuno-subdominant compared to strain-specific antibodies against the antigenically variable HA head[7]. Thus, combining such bNAbs with broadly cross-reactive and abundant (at the population level) $CD8^+$ T cells is important for optimal universal protection against distinct influenza strains and subtypes. Cross-reactivity across IAV and IBV is unprecedented for $CD8^+$ T cells and atypical for influenza-specific $CD4^+$ T cells and antibodies. Indeed, only one rare antibody (CR9114) that cross-recognizes a conserved region of the IAV and IBV HA stem regions has been identified[8] and its contribution in the immune response during human infection is unknown. Similarly, a highly conserved $CD4^+$ T cell epitope containing a peptide from the fusion peptide of the HA has been identified but remains poorly characterized[40]. Universal memory $A2/PB1_{413}$ $CD8^+$ T cells, however, are prominent in human peripheral blood and lung tissues and emerge as activated effector cells during both human IAV and IBV infections. Additionally, such $CD8^+$ T cells were found in the majority (80%) of donors tested, suggesting that such T cell responses are abundant across HLA-A*0201$^+$ donors. The heterotypic cross-reactivity demonstrated by our study is currently restricted to HLA-A*02:01, A*01:01 and B*37:01, which cover ~54% of the world's population. However, certain ethnic groups would not be sufficiently covered by these HLA alleles. Thus, while our study demonstrates the potential of heterotypic cross-reactivity by $CD8^+$ T cells, it also highlights the need for further identification of universal $CD8^+$ T cell epitopes across additional HLA alleles. The advent of immunopeptidomics, the unbiased identification of HLA-bound viral peptides using mass spectrometry, could facilitate such epitope-discovery endeavors.

The IBV-wide cross-reactivity resembles that of IAV-wide cross-reactivity provided by well-characterized $CD8^+$ T cell specificities, exemplified by $A2/M1_{58}$[36] but also other epitopes[12, 16]. While, the ability of $CD8^+$ T cells to cross-react across the two IBV lineages was previously reported[11], the antigenic specificity underpinning such cross-reactivity has been unknown. We demonstrate that $CD8^+$ T cells target peptides from the BHA and BNS1

proteins and that these responses are protective, as they accelerate viral clearance and reduce inflammatory cytokines in a murine model of human IBV infection. The observation that IBV-wide cross-reactivity can be conferred by peptides derived from the external HA protein is intriguing as it contests the belief that $CD8^+$ T cell cross-reactivity is conferred by peptides from the internal proteins of influenza viruses and contrasts the known immunodominance of responses to M1/NP-derived epitopes from IAV. Whether this is unique to the context of HLA-A2 or common across many HLA alleles during IBV responses is currently unknown, although an immunodominant epitope with a BHA peptide was also recently identified for murine H2-K^d [41]. Additionally, IBV-specific $CD4^+$ T cells predominantly recognize peptides from the BHA protein, as opposed to M1 and NP for IAV[42]. Given the high prevalence of HLA-A*02:01 and the clinical significance of IBV, our work implies that $CD8^+$ T cell-targeting vaccines need to be formulated with broader antigenic specificity not limited to NP and M1 antigens.

In our study, we provide direct evidence for the role of $CD8^+$ T cells in protection against influenza B viruses. This was demonstrated by immunization with the newly-identified IBV peptides (BHA_{543} and $BNS1_{266}$). Following IBV infection, peptide-immunized mice displayed milder influenza disease as shown by significant reductions in both viral titers and cytokine storm, when compared to the mock-immunized group. Unexpectedly, irrespective of the infection or immunization protocol used, A2/$PB1_{413}{}^+CD8^+$ T cells could not be detected in HHD-A2 mice. This strongly indicated that HHD-A2 mice lack naïve A2/$PB1_{413}{}^+CD8^+$ T cells TCR precursors, most likely as a result of a TCR repertoire hole in these mice. This lack of A2/$PB1_{413}{}^+CD8^+$ T cell responses in HHD-A2 mice is consistent with previous studies in HHD-A2/DRB1 mice[43]. While HHD-A2 mice express a chimeric MHC-I monochain comprising of the human β2-microglobulin covalently linked to the HLA-A*02:01 α1 and α2 domains and the murine α3 and transmembrane domains, T cell receptors remain murine. As a result, although HHD-A2.1-expressing mice respond to several human HLA-A*0201-restricted epitopes [36, 38, 44] TCRs for others may be lacking. Thus, while these mice are a useful tool for screening peptide libraries, results obtained from such screens warrant validation using human samples. This mouse model is however, an important tool in assessing the protective capacity of antiviral HLA-A2-restriced $CD8^+$ T cells *in vivo*. Indeed, by vaccinating HHD-A2 mice and challenging them with IBV, we provide the first evidence for a protective role of epitope-specific $CD8^+$ T cells in IBV infection following peptide vaccination, consistent with $CD8^+$ T cell depletion studies in mice lacking B cells [39]. In conjunction with the activation of these $CD8^+$ T cells in human patients and the presence of

T_{RM} cells in the human lung, these studies suggest that vaccinating against these epitopes may provide protection in humans.

The antigenic origin of such broadly cross-reactive epitopes is also of interest. PB1 is the most well-conserved protein across IAV and IBV, with ~60% amino acid identity, as opposed to 30% or less for the other proteins[4, 24, 45]. The $PB1_{413}$ peptide is derived from one of the most well conserved areas of the protein, namely motif B (residues 406-422 of IAV PB1 protein), one of the four core motifs present in viral RNA-dependent polymerases. Genome-wide mutational analysis, has shown that IAV cannot tolerate substitutions in these motifs[46]. More interesting, however, is the IBV-wide cross-reactivity conferred by the BHA_{543} peptide. This peptide is derived from the stalk region of the BHA molecule, which shows considerably higher conservation than the HA head domain[47]. Mutagenesis screens *in vitro* have also revealed limited tolerance to 15-nucleotide insertions of in the BHA molecule, particularly the stalk domain[47]. Thus, these universally cross-reactive $CD8^+$ T cells target epitopes with little sequence flexibility, making them ideal targets for a universal as well as IBV-wide influenza vaccine. Such extensive cross-reactivity across virus genera is uncommon and only resembles that of $CD8^+$ and $CD4^+$ T cells across the subfamily of *Alphaherpseviruses*[48] and to a lesser extend $CD8^+$ T cell cross-reactivity across the *Flavivirus* genus[49].

Overall, the ability of $CD8^+$ T cells to confer heterotypic cross-reactivity across IAV and IBV and the knowledge of cross-reactive epitopes across IAV/IBV types as well as within IBV strains, have substantial implications for the design of universal influenza vaccines that do not require annual reformulation. Pre-emptive influenza vaccines eliciting broadly-cross-reactive and long-lasting $CD8^+$ T cell immunity would reduce annual rates of IAV/IBV-induced morbidity and mortality globally. Additionally, an influenza vaccine eliciting immunity across influenza A, B and C viruses could also protect children from severe ICV disease[5]. Furthermore, T cell-targeted vaccines would also augment the numbers of universal IAV/IBV/ICV-, IAV- and IBV-specific $CD8^+$ T cell responses in individuals with previous influenza virus exposures (as currently these are mostly detectable *ex vivo* by tetramer enrichment in healthy donors) and thus would confer stronger protective immunity following infection[14]. Thus, it is critical to consider universal $CD8^+$ T cells, alongside with universal antibodies, for the design of universally cross-reactive influenza vaccines, especially as the current inactivated influenza vaccines do not elicit influenza-specific $CD8^+$ T cell responses[4].

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Conflicts of interest

SR is an employee of Seqirus Ltd and has no conflict of interest in the material presented. MK, KK and EBC are named as co-inventors in a patent application filed by the University of Melbourne (AU2017903652) covering the use of certain peptides described in the publication as part of vaccine formulation. The other authors declare no conflicts of interest.

Author Contributions

MK, KK, THON, PI, NAM, AWP, SG, DV, FL and PGT designed experiments. MK, PI, THON, NAM, AAE, EBC, SS, CYW, BYC, EKA, PD, LG, WZ and SG performed experiments. AH, IB, DCJ, TCK, ACC, MR, GPW, LMW, ST, SM, TL, BD, ME, PGT provided reagents and/or samples. MK, PI, THON, JCC, SS, SR, DT, DV, FL SG, JR, PGT, AWP and KK analyzed data. MK, THON and KK wrote the manuscript. All authors read and approved the manuscript.

References

1. Krammer, F. *et al.* Influenza. *Nat Rev Dis Primers* **4**, 3 (2018).

2. Nussing, S. *et al.* Innate and adaptive T cells in influenza disease. *Front Med* **12**, 34-47 (2018).

3. Paules, C. & Subbarao, K. Influenza. *Lancet* **390**, 697-708 (2017).

4. Koutsakos, M., Nguyen, T.H., Barclay, W.S. & Kedzierska, K. Knowns and unknowns of influenza B viruses. *Future Microbiol* **11**, 119-135 (2016).

5. Matsuzaki, Y. *et al.* Clinical features of influenza C virus infection in children. *J Infect Dis* **193**, 1229-1235 (2006).

6. Chen, Y.Q. *et al.* Influenza Infection in Humans Induces Broadly Cross-Reactive and Protective Neuraminidase-Reactive Antibodies. *Cell* **173**, 417-429 e410 (2018).

7. Corti, D. *et al.* Tackling influenza with broadly neutralizing antibodies. *Curr Opin Virol* **24**, 60-69 (2017).

8. Dreyfus, C. *et al.* Highly conserved protective epitopes on influenza B viruses. *Science* **337**, 1343-1348 (2012).

9. Hayward, A.C. *et al.* Natural T Cell-mediated Protection against Seasonal and Pandemic Influenza. Results of the Flu Watch Cohort Study. *Am J Respir Crit Care Med* **191**, 1422-1431 (2015).

10. McMichael, A.J., Gotch, F.M., Noble, G.R. & Beare, P.A. Cytotoxic T-cell immunity to influenza. . *New Eng J Med* **309**, 13-17 (1983).

11. van de Sandt, C.E. *et al.* Influenza B virus-specific CD8+ T-lymphocytes strongly cross-react with viruses of the opposing influenza B lineage. *J Gen Virol* **96**, 2061-2073 (2015).

12. Gras, S. *et al.* Cross-reactive CD8+ T-cell immunity between the pandemic H1N1-2009 and H1N1-1918 influenza A viruses. *Proc Natl Acad Sci U S A* **107**, 12599-12604 (2010).

13. Greenbaum, J.A. *et al.* Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population. *Proc Natl Acad Sci U S A* **106**, 20365-20370 (2009).

14. Quinones-Parra, S.M. *et al.* A Role of Influenza Virus Exposure History in Determining Pandemic Susceptibility and CD8+ T Cell Responses. *J Virol* **90**, 6936-6947 (2016).

15. Sridhar, S. *et al.* Cellular immune correlates of protection against symptomatic pandemic influenza. *Nature medicine* **19**, 1305-1312 (2013).

16. Quinones-Parra, S. *et al.* Preexisting CD8+ T-cell immunity to the H7N9 influenza A virus varies across ethnicities. *Proc Natl Acad Sci U S A* **111**, 1049-1054 (2014).

17. Wang, Z. *et al.* Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8(+) T cells. *Nat Commun* **6**, 6833 (2015).

18. Wang, Z. *et al.* Clonally diverse CD38(+)HLA-DR(+)CD8(+) T cells persist during fatal H7N9 disease. *Nat Commun* **9**, 824 (2018).

19. Koutsakos, M. *et al.* Circulating TFH cells, serological memory, and tissue compartmentalization shape human influenza-specific B cell immunity. *Sci Transl Med* **10** (2018).

20. Ramarathinam, S.H. *et al.* Identification of Native and Posttranslationally Modified HLA-B*57:01-Restricted HIV Envelope Derived Epitopes Using Immunoproteomics. *Proteomics* **18**, e1700253 (2018).

21. Williamson, N.A. & Purcell, A.W. Use of proteomics to define targets of T-cell immunity. *Expert Rev Proteomics* **2**, 367-380 (2005).

22. Alexander, J. *et al.* Identification of broad binding class I HLA supertype epitopes to provide universal coverage of influenza A virus. *Hum Immunol* **71**, 468-474 (2010).

23. Rosendahl Huber, S.K. *et al.* Chemical Modification of Influenza CD8+ T-Cell Epitopes Enhances Their Immunogenicity Regardless of Immunodominance. *PLoS One* **11**, e0156462 (2016).

24. Terajima, M., Babon, J.A., Co, M.D. & Ennis, F.A. Cross-reactive human B cell and T cell epitopes between influenza A and B viruses. *Virol J* **10**, 244 (2013).

25. Zemmour, J., Little, A.M., Schendel, D.J. & Parham, P. The HLA-A,B "negative" mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. *J Immunol* **148**, 1941-1948 (1992).

26. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G. & Rammensee, H.G. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**, 290-296 (1991).

27. Alanio, C., Lemaitre, F., Law, H.K., Hasan, M. & Albert, M.L. Enumeration of human antigen-specific naive CD8+ T cells reveals conserved precursor frequencies. *Blood* **115**, 3718-3725 (2010).

28. Moon, J.J. *et al.* Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* **27**, 203-213 (2007).

29. Nguyen, T.H.O. *et al.* Perturbed CD8(+) T cell immunity across universal influenza epitopes in the elderly. *J Leukoc Biol* **103**, 321-339 (2018).

30. Chandele, A. *et al.* Characterization of Human CD8 T Cell Responses in Dengue Virus-Infected Patients from India. *J Virol* **90**, 11259-11278 (2016).

31. Jozwik, A. *et al.* RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. *Nat Commun* **6**, 10224 (2015).

32. Miller, J.D. *et al.* Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity* **28**, 710-722 (2008).

33. Hillaire, M.L. *et al.* Characterization of the human CD8(+) T cell response following infection with 2009 pandemic influenza H1N1 virus. *J Virol* **85**, 12057-12061 (2011).

34. Sathaliyawala, T. *et al.* Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* **38**, 187-197 (2013).

35. Allen, R.J., Koutsakos, M., Hurt, A.C. & Kedzierska, K. Uncomplicated Cystitis in an Adult Male Following Influenza B Virus Infection. *Am J Case Rep* **18**, 190-193 (2017).

36. Valkenburg, S.A. *et al.* Molecular basis for universal HLA-A*0201-restricted CD8+ T-cell immunity against influenza viruses. *Proc Natl Acad Sci U S A* **113**, 4440-4445 (2016).

37. Pascolo, S. *et al.* HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. *J Exp Med* **185**, 2043-2051 (1997).

38. Nguyen, T.H. *et al.* Understanding CD8(+) T-cell responses toward the native and alternate HLA-A*02:01-restricted WT1 epitope. *Clin Transl Immunology* **6**, e134 (2017).

39. Epstein, S.L., Lo, C.Y., Misplon, J.A. & Bennink, J.R. Mechanism of protective immunity against influenza virus infection in mice without antibodies. *J Immunol* **160**, 322-327 (1998).

40. Babon, J.A., Cruz, J., Ennis, F.A., Yin, L. & Terajima, M. A human CD4+ T cell epitope in the influenza hemagglutinin is cross-reactive to influenza A virus subtypes and to influenza B virus. *J Virol* **86**, 9233-9243 (2012).

41. Muralidharan, A. *et al.* Identification of immunodominant CD8 epitope in the stalk domain of influenza B viral hemagglutinin. *Biochem Biophys Res Commun* **502**, 226-231 (2018).

42. Richards, K.A., Treanor, J.J., Nayak, J.L. & Sant, A.J. Overarching Immunodominance Patterns and Substantial Diversity in Specificity and Functionality in the Circulating Human Influenza A and B CD4 T Cell Repertoire. *J Infect Dis* (2018).

43. Pedersen, S.R. *et al.* Immunogenicity of HLA Class I and II Double Restricted Influenza A-Derived Peptides. *PLoS One* **11**, e0145629 (2016).

44. Tan, A.C. *et al.* The design and proof of concept for a CD8(+) T cell-based vaccine inducing cross-subtype protection against influenza A virus. *Immunol Cell Biol* **91**, 96-104 (2013).

45. Jackson, D., Elderfield, R.A. & Barclay, W.S. Molecular studies of influenza B virus in the reverse genetics era. *J Gen Virol* **92**, 1-17 (2011).

46. Wang, L. *et al.* Functional Genomics Reveals Linkers Critical for Influenza Virus Polymerase. *J Virol* **90**, 2938-2947 (2015).

47. Fulton, B.O., Sun, W., Heaton, N.S. & Palese, P. The influenza B virus hemagglutinin head domain is less tolerant to transposon mutagenesis than that of the influenza A virus. *J Virol* (2018).

48. Jing, L. *et al.* Extensive CD4 and CD8 T Cell Cross-Reactivity between Alphaherpesviruses. *J Immunol* **196**, 2205-2218 (2016).

49. Lima, N.S., Rolland, M., Modjarrad, K. & Trautmann, L. T Cell Immunity and Zika Virus Vaccine Development. *Trends Immunol* **38**, 594-605 (2017).

FIGURE LEGENDS

Figure 1. $CD8^+$ T cell cross-reactivity across influenza A, B and C viruses. (a) Conservation of known IAV epitopes in IBV and ICV. The bars indicate the percent conservation (average amino acid identity) of each peptide across the three types of viruses in the indicated number of sequences. **(b)** Highly conserved peptides across IAV, IBV and ICV types were selected for dissection of cross-reactive $CD8^+$ T cell responses. **(c)**

Immunogenicity of memory $CD8^+$ T cells directed at conserved peptides in healthy adults. PBMCs were cultured with the peptides (as outlined in B) for ~10 days and responses were assessed in an IFN-γ ICS. Frequency of $IFN\gamma^+CD8^+$ T cells after subtracting 'no peptide' control and responding donors are shown. Dots indicate individual donors, median and interquartile range (IQR) shown. (n=3-5). **(d)** Conservation of $PB1_{413-421}$ in *Orthomyxoviruses*. Alignment of PB1 sequences derived from viruses representing each genus is shown. The box indicates the $PB1_{413-421}$ peptide. **(e)** $A2/PB1_{413-421}$-mediated cross-reactivity across IAV, IBV and ICV. PBMCs were stimulated with either virus for ~10 days and responses to the peptide were assessed in an ICS. $A2/PB1_{413-421}{}^+CD8^+$ T cell responses measured directly *ex-vivo* by ICS are shown for comparison. Representative concatenated FACS plots for IFNγ production are shown. Data points indicate individual donors, median and IQR. (n=6). Statistical significance was determined using the Mann-Whitney test, *$p<0.05$, **$p<0.005$. **(f)** $B37/NP_{338-345}$-mediated and **(g)** $A1/PB1_{591-599}$-mediated cross-reactivity across IAV and IBV. On ~day10 of peptide culture, $CD8^+$ T cell responses to either IAV or IBV variants were assessed by ICS. Dots indicate individual donors and bars indicate the mean (n=3-4). (e-f) 'No peptide' control was subtracted.

Figure 2. Identification of novel protective IBV $CD8^+$ T cell epitopes by immunopeptidomics. (a) Immunopeptidomics outline. **(b)** Peptide binding motifs for host and IBV HLA-A*02:01 ligands generated from combined non-redundant lists of 9mer, 10mer and 11mer, using Icelogo by the static reference method against the swiss-prot human proteome. **(c)** Length distribution of filtered HLA-A*02:01 ligands (non-redundant by sequence) from uninfected (single experiment) and B/Malaysia infected (2 experiments) CIR.A*02:01 cells. Numbers of peptides of each length identified from the Human proteome (5% FDR cut-off) and B/Malaysia proteome (all confidences) are shown. **(d)** Distribution of IBV-derived HLA ligands (non-redundant by sequence) across the B/Malaysia proteome, identified as likely HLA-A*02:01 ligands. Pooled data from 2 independent experiments. **(e-h)** *In vitro* screening of novel peptides in human HLA-A*02:01-expressing PBMCs. **(e)** Experimental outline of screening. **(f)** Representative concatenated FACS plots for each peptide pool are shown, with a mock (unstimulated) control outlined for comparison. Frequency of $IFN\gamma^+TNF^+CD8^+$ T cells for each pool. Dots indicate individual donors, median and IQR are shown (n=11). **(g)** Frequncy of responding donors for each pool (n=11). **(h)** Frequency of $IFN\gamma^+TNF^+CD8^+$ T cells directed towards individual peptides from pool 2

(n=6), median and IQR are shown. **(i-j)** *In vitro* validation of immunogenic peptides. **(i)** Experimental outline of validation. **(j)** Representative FACS plots for a positive $CD8^+$ T cell response directed towards each peptide. Frequency of $IFN\gamma^+TNF^+CD8^+$ T cells. Donors are color-coded, medians and IQRs are shown. (n=6). **(k-m)** Immunodominance of universal $CD8^+$ T cells during *in vitro* IAV or IBV infection. **(k)** Experimental outline. **(l)** Responses during IAV infection against $A2/M1_{58}$, $A2/PA_{46}$ and $A2/PB1_{413}$ and **(m)** during IBV infection against $A2/BHA_{543}$, $A2/BNS1_{266}$ and $A2/PB1_{413}$. Bar charts show the contribution of each specificity to the total measured (sum of $tetramer^+$) response. ND: not detected.

Figure 3. Prominance of memory and effector pools of universal $CD8^+$ T cells in healthy adults, influenza-infected individuals and human tissues. (a-d) Tetramer-specific $CD8^+$ T cells in healthy and influenza-infected individuals. (**a**) *Ex-vivo* TAME on PBMCs from healthy and infected donors. Representative FACS plots are shown. **(b)** Characteristics of healthy and influenza-infected or influenza-negative ILI cohorts used in this study. ILI: influenza-like illness. (**c**) Precursor frequency of $tetramer^+$ cells in healthy, influenza-infected individuals and influenza-negative ILI patients (n=6-24). Statistical significance was determined using the Mann-Whitney test, *p<0.05, **p<0.005. Median and IQR are shown. (**d**) Precursor frequency of $tetramer^+$ $CD8^+$ T cells in healthy and influenza-infected individuals across age. (**e**) Expression profiles of $tetramer^+$ $CD8^+$ T cells for activation/effector markers CD38 and Ki-67. Representative FACS plots are shown. Frequency of $CD38^+/Ki\text{-}67^+$ $tetramer^+$ $CD8^+$ T cells from healthy controls (n=3-5) and influenza-infected donors (n=6-26). Statistical significance for changes in the frequency of $CD38^-Ki\text{-}67^-$ cells was determined using the Mann-Whitney test A, *p<0.05, **p<0.005.

Figure 4. Universal $CD8^+$ T cells with a tissue-resident phenotype in the human lung. (a) *Ex-vivo* detection of universal $CD8^+$ T cells in human lung and secondary lymphoid organ (spleen, tonsils and lymph nodes) samples. Frequency of $tetramer^+$ $CD8^+$ T cells (n=8 lungs, n=11 spleens, n=4 tonsils, n=4 lymph nodes). **(b)** Phenotype of $tetramer^+$ $CD8^+$ T cells based on CD103 and CD69 expression. **(c)** Phenotype of $tetramer^+$ $CD8^+$ T cells based on CD27 and CD645RA expression. Representative FACS plots are shown. SLO: secondary lymphoid organs.

Figure 5. Single-cell RNA sequencing of universal $CD8^+$ T cells in an IBV-infected individual. (a) Timeline of infection and number of $tetramer^+CD8^+$ T cells isolated from

each sample. (**b**) FACS plots and precursor frequency of tetramer$^+$ CD8$^+$ T cells prior to, during and after IBV infection. (**c**) Principal component analysis (PCA) of tetramer$^+$ CD8$^+$ T cells sequenced. Timepoints are distinguished by colour and specificity by shape. (**d**) Heatmap illustrating expression of differentially expressed genes identified across all the timepoints compared to the baseline as reference using MAST. Cells grouped by epitope and timepoint. **(e)** Heatmap representing the gene sets enrichment of up-regulated (pink) and down-regulated (green) genes of tetramer$^+$CD8$^+$ T cells sorted at d14 compared to baseline. **(f)** Heatmap representing the gene set enrichment of up-regulated (pink) and down-regulated (green) genes of tetramer$^+$CD8$^+$ T cells sorted at d14 compared to 1.5-year time-point.

Figure 6. *In vivo* CD8$^+$ T cell responses to novel IBV peptides in HHD (A2$^+$) mice. (a) Experimental outline of screening. **(b)** Representative FACS plots for immunogenic peptides. **(c)** Frequency of IFNγ$^+$TNF$^+$CD8$^+$ T cells in the spleen of IBV-infected mice towards each peptide. Mean and SEM are shown (n=4-12). **(d-e)** CD8$^+$ T cell responses in the BAL. **(d)** Cytokine responses to each peptide pool. Data from two independent experiments in which the BAL of multiple (n=3-5) mice were pooled. **(e)** Cytokine responses to individual immunogenic peptides in the BAL (n=4). Mean and SEM are shown.

Figure 7. CD8$^+$ T cells against novel epitopes mediated protection from IBV challenge. (a) Detailed experimental plan of vaccination. **(b-d)** Tetramer-specific CD8$^+$ T cells responses on day 6 and 7 after infection in BAL and spleen; **(b)** representative FACS plots; **(c)** number of total (A2/BHA$_{543}$ and A2/BNS1$_{266}$) tetramer$^+$ CD8 T cells in the spleen on day 7 after IBV infection. **(d)** number of individual A2/BHA$_{543}^+$ and A2/BNS1$_{266}^+$ tetramer$^+$ CD8 T cells in the spleen on day 7 after IBV infection. **(e)** Viral titers in the lungs and nose of peptide-vaccinated and mock-vaccinated mice, following IBV infection. Days 5 (n=5) and d6 (n=5) were assessed in an independent experiment to d7 (n=4-5). **(e-g)** Cytokine responses in the BAL on d7 after IBV challenge (n=4-5). Means and SEM are shown (n=5). Statistical significance was determined using an unpaired t-test. *p<0.05, **p<0.005. ****p<0.0001.