

1 **Immuno-peptidomic analysis of influenza A virus infected**  
2 **human tissues identifies internal proteins as a rich source of**  
3 **HLA ligands**

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30 **Running title:**

31 Immuno-peptidomics of influenza A virus infected human tissues

32 **Keywords:**

33 HLA, peptidome, influenza, antigen presentation

## 34 **Abstract**

35 CD8+ and CD4+ T cells provide cell-mediated cross-protection against multiple influenza strains  
36 by recognising epitopes bound as peptides to human leukocyte antigen (HLA) class I and -II  
37 molecules respectively. Two challenges in identifying the immunodominant epitopes needed to  
38 generate a universal T cell influenza vaccine are: A lack of cell models susceptible to influenza  
39 infection which present population-prevalent HLA allotypes, and an absence of a reliable in-vitro  
40 method of identifying class II HLA peptides. Here we present a mass spectrometry-based  
41 proteomics strategy for identifying viral peptides derived from the A/H3N2/X31 and  
42 A/H3N2/Wisconsin/67/2005 strains of influenza. We compared the HLA-I and -II  
43 immunopeptidomes presented by ex-vivo influenza challenged human lung tissues. We then  
44 compared these with directly infected immortalised macrophage-like cell line (THP1) and  
45 primary dendritic cells fed apoptotic influenza-infected respiratory epithelial cells. In each of the  
46 three experimental conditions we identified novel influenza class I and II HLA peptides with  
47 motifs specific for the host allotype. Ex-vivo infected lung tissues yielded few class-II HLA  
48 peptides despite significant numbers of alveolar macrophages, including directly infected ones,  
49 present within the tissues. THP1 cells presented HLA-I viral peptides derived predominantly  
50 from internal proteins. Primary dendritic cells presented predominantly viral envelope-derived  
51 HLA class II peptides following phagocytosis of apoptotic infected cells. The most frequent viral  
52 source protein for HLA-I and -II was matrix 1 protein (M1). This work confirms that internal  
53 influenza proteins, particularly M1, are a rich source of CD4+ and CD8+ T cell epitopes.  
54 Moreover, we demonstrate the utility of two ex-vivo fully human infection models which enable  
55 direct HLA-I and -II immunopeptide identification without significant viral tropism limitations.  
56 Application of this epitope discovery strategy in a clinical setting will provide more certainty in  
57 rational vaccine design against influenza and other emergent viruses.

## 58 **Author Summary**

59 Influenza infections present a significant global health challenge. High rates of mutation require  
60 reformulation of vaccines annually. Vaccines are designed to induce antibody responses to the  
61 surface proteins of the influenza virus, but the contribution of T cells to overall immunity is  
62 unclear. Here, we used several totally human laboratory models to show how the viral proteins  
63 are presented to the T cells to induce immunity. We found that CD8 T cells, which kill infected  
64 cells, and CD4 T cells which support the CD8 T cells as well as the antibody-producing B cells,  
65 mainly see proteins from inside the viral particle, not the surface ones which are targeted by  
66 antibodies. These internal viral proteins are more similar between different viral strains than the  
67 surface proteins, and therefore suggest that vaccines designed to induce T cell responses could  
68 be better protective if they target internal viral proteins.

## 69 Introduction

70 Influenza virus is a major cause of morbidity, with every individual predicted to have 1-2 illness  
71 episodes per decade. There are approximately 1 billion annual cases of influenza globally, of  
72 which 3-5 million are severe, resulting in up to 650,000 deaths [1]. Additionally, the risk of a  
73 pandemic is ever-present, with likely further global costs of billions of dollars. There is  
74 widespread viral resistance to antiviral medications such as amantadine [2] and developing  
75 resistance against oseltamivir [3]. Both reduce symptom severity and duration but, critically, do  
76 not protect against primary infection and are least effective in at-risk individuals [4]. The most  
77 effective anti-influenza prophylaxis is vaccination which has, on average, 40-60% efficacy  
78 across all current strains [5].

79 Seasonal immunization efficiently generates neutralizing antibodies against viral haemagglutinin  
80 (HA) specific to the immunizing strain, however high mutation rates, particularly in the viral coat  
81 proteins haemagglutinin and neuraminidase (NA), rapidly cause antigenic drift leading to  
82 immune escape, requiring vaccines to be reformulated annually, based upon epidemiological  
83 predictions of the predominant global strains. Production and manufacturing of a clinically  
84 proven influenza vaccine is lengthy (>5 months) and costly.

85 Influenza vaccines designed to induce strong neutralising antibody responses to haemagglutinin  
86 offer narrower and more short-lived immunity than naturally acquired infections, , which also  
87 induce antibody responses predominantly to HA, but also stronger responses to NA and some  
88 internal viral proteins [6,7]. Although neutralising antibodies provide key protection against initial  
89 infection, T cells play an equally important role in limiting the consequent illness [8].

90 T cells recognise viral peptides bound to class I and II major histocompatibility (MHC) molecules  
91 which are presented at the cell surface. CD8+ T cells recognise endogenously processed viral  
92 peptides presented by class I MHC molecules on the surface of infected cells, whereas CD4+ T

93 cells recognise exogenously processed peptides presented by class II MHC molecules mainly  
94 presented on the surface of professional antigen presenting cells such as dendritic cells and  
95 macrophages [9].

96 Targeting conserved viral protein sequences, which are more commonly derived from internal  
97 viral proteins, should confer greater vaccine induced cross-protection against multiple influenza  
98 strains, and early evidence in mice supports this [10]. Previous evidence has shown that the  
99 influenza virus nucleoprotein (NP) is a major target of immunodominant CTLs in direct infections  
100 [11], and acid polymerase T cell epitopes are more abundant in mouse cross-presentation  
101 models, but matrix protein (M1) and the RNA-directed RNA polymerase catalytic subunit (PB1)  
102 also contain conserved immunogenic sequences [12]. Viral NP and M1 are also major targets  
103 for immunodominant CD4 T cell responses [13]. Human infection trials suggest that pre-existing  
104 influenza-specific T cells, particularly those recognising conserved epitopes of internal viral  
105 proteins, are central to limiting disease severity following experimental challenge with different  
106 influenza strains [14].

107 Infections stimulate both CD4+ and CD8+ T cell subsets, and optimal humoral and cellular  
108 immunity is dependent upon the activation of CD4+ T helper cells, which support CD8+ T cell  
109 function but can also themselves have effector functions [15,16]. Virus-specific CD4+ and CD8+  
110 T cells specific for immunodominant influenza epitopes negatively correlate with disease  
111 severity and fever symptoms, respectively [17].

112 In animal models, long peptide vaccines designed to stimulate antibody and T cell responses  
113 have provided only minimal protection against infection, with limited evidence of symptom  
114 reduction [18]. Rationally designed T cell epitope targeted vaccines containing long peptide  
115 sequences from the extracellular domain of M2 (M2e) and NP have been tested in mice, but  
116 offered only limited to moderate protection with variable responses to each peptide [19]. This  
117 may arise because candidate T cell epitopes are commonly identified using machine learning

118 based algorithms to predict binding of 9-mer or 15-mer peptides to specific HLA-I and HLA-II  
119 HLA allotypes, respectively. Whilst peptide affinity predictions are reasonably accurate, at least  
120 for HLA-I, there are multiple other factors that influence the true efficacy of T cell epitopes,  
121 including, but not restricted to, the abundance of the source protein available for presentation by  
122 infected cells, the biochemical nature and structural stability of the epitope, the suitability of  
123 surrounding residues to endosomal processing, and the secondary structure of the source  
124 protein. Due to differences in mouse and human MHC, humanised mouse models must be  
125 utilised to examine influenza T cell epitopes in humans, but are then restricted to the transgenic  
126 allotypes, usually HLA-A\*02:01, the most prevalent global allotype.

127 Recent improvements in the sensitivity of mass spectrometry combined with  
128 immunoprecipitation of peptides bound to HLA-I and HLA-II have enabled the field of  
129 immunopeptidomics to be utilised in the search for optimal T cell epitopes [20]. Typically,  
130 influenza epitopes are identified from the elution of surface HLA-I associated peptides of an  
131 immortalised cell line infected with virus. [21]. Advantages of this approach are that the HLA-  
132 restricted peptide sequence of a known cell HLA allotype can be directly measured, showing  
133 that the peptide can be processed and presented, at least in-vitro. Such approaches have  
134 indicated protective immunopeptides across influenza A B and C strains [22].

135 A limitation of this immunopeptidomic strategy is the requirement for significant quantities of  
136 infected cell material (generally a minimum of  $10^8$  cells). For this reason, previous studies have  
137 utilised cell lines which grow readily in the laboratory e.g. HELA cells, but which do not fully  
138 reflect in-vivo cell targets. This may not be of concern if cells are infectable, however where a  
139 virus shows strong infection tropism for a particular cell type, often the case in freshly isolated  
140 strains which have not been adapted to laboratory conditions, this could become a significant  
141 challenge. We have previously demonstrated that ex-vivo infection models of lung tissues are

142 reflective of localised patterns of infection and subsequent inflammatory response, and can  
143 therefore be more accurately used to test respiratory viral inhibitors than cell models [23].

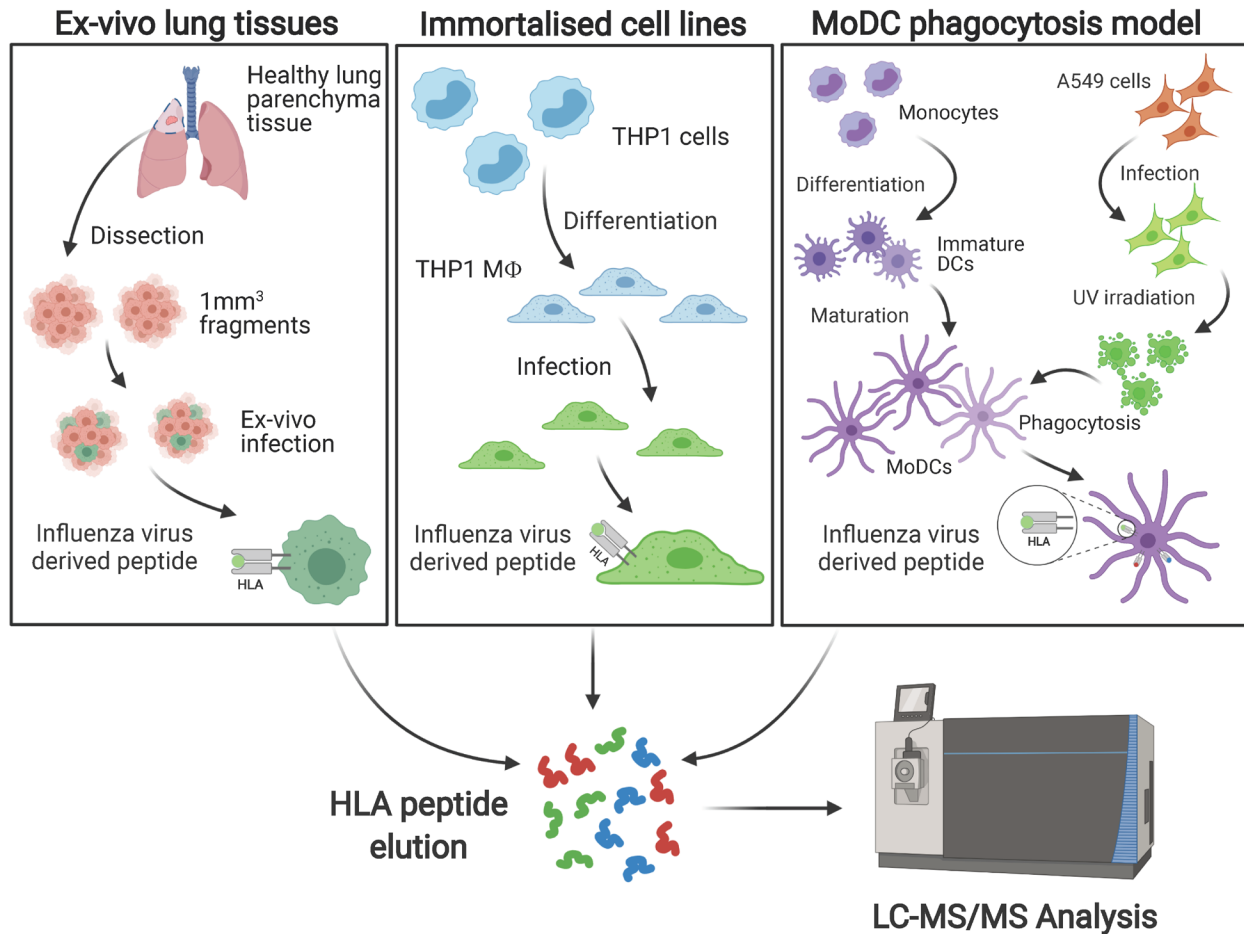
144 In the current study, we use this model to establish the antigenic landscape of human lung  
145 tissue 22 hours following influenza A virus (IAV) infection. By comparing immunopeptidomes  
146 recovered from infected explants with that of monocyte-derived dendritic cells (MoDC) following  
147 cross-presentation; and a directly infected monocyte/macrophages cell line, we show that the  
148 local lung repertoire of IAV peptides available for immune recognition is dominated by HLA-I  
149 bound ligands sourced from internal viral proteins.

150

151 **Results**

152 We used three different models as sources for immunopeptidome isolation, to identify HLA-I and

153 HLA-II restricted influenza immunopeptides (Fig 1) [24].



154

155 **Fig 1: Workflow of the approach to identify HLA-I and -II influenza immunopeptides**

156 **isolated from cell lines, dendritic cells and lung tissues.**

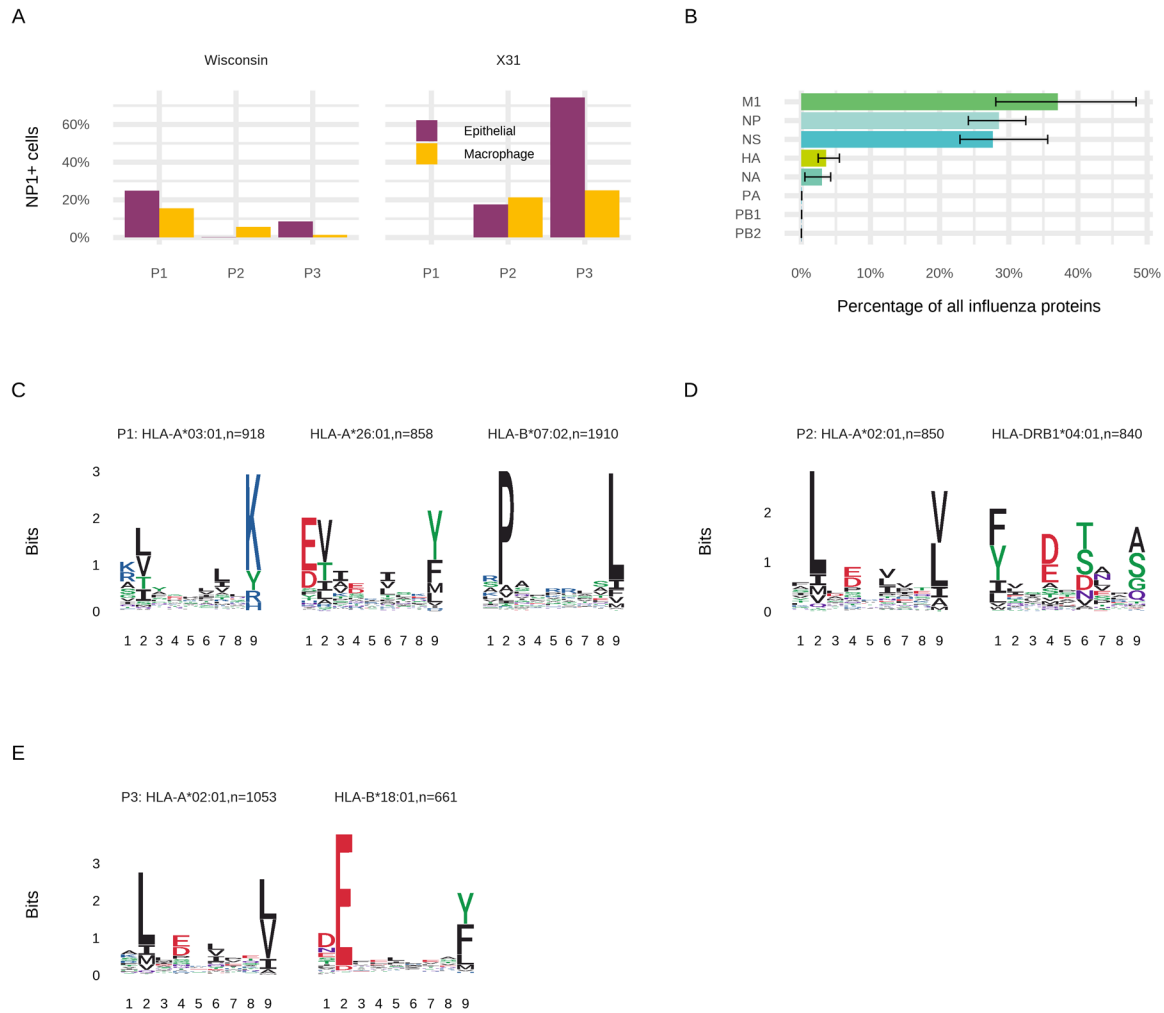
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158 **Infection of resected lung tissues reveals novel influenza HLA-I and –II restricted**  
159 **epitopes**

160 We examined infection rates and HLA-presented peptides using ex-vivo lung tissue samples  
161 from three different human donors with diverse HLA types (Table S1). Exposure of the ex-vivo  
162 lung parenchymal tissues: P1, P2 and P3 to the two viral strains studied herein led to variable  
163 infection rates in the two main cell types which have previously shown influenza susceptibility,  
164 epithelial and macrophage cells (Fig 2 A) [23]. We have previously shown that epithelial  
165 infection rates in resected lung tissues can be variable [23], and this study indicates that the  
166 viral strain also affects infection rate in the two target cell types. Infection rates varied from 2%-  
167 70% in epithelial and 1%- 15% in macrophage cells.

168 Following ex-vivo infection, we were able to identify a number of influenza-derived HLA-I  
169 restricted peptides across all samples derived principally from M1, NP and NS proteins (Table  
170 1). Similar to previous findings, despite consistent, if limited, expression of viral haemagglutinin  
171 and neuraminidase in the proteome, HLA-I peptides were generally of internal viral protein  
172 origins: M1, NP and Non-structural protein 1 (NS1) (Fig 2 B, Table 1). Following Wisconsin  
173 H3N2 influenza infection, we observed 7944 distinct class I HLA peptides deriving from 2603  
174 proteins for P1, 5304 distinct class I HLA peptides deriving from 1696 proteins for P2, and 6338  
175 distinct class I HLA peptides deriving from 1996 proteins for P3 (Table S2). We also observed  
176 similar numbers for the same tissues infected with X31.



177

178 **Fig 2: Characteristics of the ex-vivo lung tissues used to identify viral HLA ligands. (A)**

179 *Infection susceptibility of epithelial and macrophage cells of ex-vivo lung parenchymal tissues*

180 *for three samples to two influenza strains, A/H3N2/Wisconsin/67/2005 and A/H3N2/X31 (B)*

181 *Relative proportions of influenza proteins present in the three lung tissue samples (C-E) Class I*

182 *HLA allotype 9-mer binding motifs derived from host immunopeptides using unsupervised*

183 *clustering using MixMHCp matching influenza-specific peptides from the three lung tissue*

184 *samples*

185 Motifs following unbiased cluster analysis [25,26] of all the distinct observed peptides for each  
 186 sample (Fig 2 C-E) indicated the respective HLA allotypes for each observed influenza peptide,  
 187 which matched to the known allotypes of the individuals (Table S1). Due to the diverse nature of  
 188 the HLA allotypes in these randomly selected lung tissue samples, with the exception of the  
 189 well-known HLA-A\*02:01 peptide AIMEKNIML/AIMDKNIML, and the HLA-B\*15:01 peptide  
 190 SARPEDVSF, there was no overlap in the detected influenza peptides between the lung tissues  
 191 samples and THP1MΦ.

192 Excitingly from P3 we also identified one class II HLA peptide, derived from the Haemagglutinin  
 193 protein (Table 1). Although this potential CD4+T cell epitope is not yet proven as functional, it is  
 194 novel, and as the first such identification in ex-vivo infected tissues, it paves the way for the  
 195 identification of further CD4-stimulatory peptides. The presentation of the only observed CD4+ T  
 196 cell epitope derived from a membrane-resident protein is consistent with the predominantly  
 197 extracellular/membrane origin of the HLA-II sourced proteins.

**Table 1:** Summary of the HLA-I and II immunopeptides isolated from lung tissues infected ex-vivo with two influenza strains

Protein	Protein Position	Length	Patient	Allotype	nM	Wisconsin	X-31
Matrix protein 1	243-252	10	P1	HLA-A*03:01	23.32	RMGVQMQRFK	-
Non-structural protein 1	122-131	10	P1	HLA-A*03:01	13.38	AIMEKNIMLK	-
Non-structural protein 1	142-150	9	P1	HLA-A*26:01	18.52	ETIVLLRAF	-
Non-structural protein 1	163-171	9	P1	HLA-B*07:02	21.2	LPSFPGHTI	-
Polymerase acidic protein	516-525	10	P1	HLA-A*26:01	14.82	DVVNFVSMEF	-
Hemagglutinin		15	P2	HLA-DRB1*04:01	-	AADLKSTQAAINQIN	-
Matrix protein 2	7-15	9	P2	HLA-B*44:27	-	-	VETPIRNEW
Non-structural protein 1	122-130	9	P2	HLA-A*02:01	23.35	AIMEKNIML	-
Nucleoprotein	305-313	9	P2	HLA-B*15:01	37.33	-	RLQNSQVY

**Table 1:** Summary of the HLA-I and II immunopeptides isolated from lung tissues infected ex-vivo with two influenza strains

Protein	Protein Position	Length	Patient	Allotype	nM	Wisconsin	X-31
Nucleoprotein	404-412	9	P2	HLA-B*15:01	26.29	-	GQISIQPTF
Nucleoprotein	450-458	9	P2	HLA-B*15:01	149.35	-	SARPEDVSF
RNA polymerase catalytic subunit	94-103	10	P2	HLA-B*44:27	-	-	FLEESHPGIF
RNA polymerase catalytic subunit	177-185	9	P2	HLA-B*44:27	-	-	EEMGITTHF
Matrix protein 1	111-119	9	P3	HLA-B*15:01	244.02	GAKEIALSY	-
Matrix protein 1	5-12	8	P3	HLA-B*18:01	107.2	TEVETYVL	TEVETYVL
Matrix protein 1	111-119	9	P3	HLA-B*15:01	107.01	-	GAKEISLSY
Non-structural protein 1	122-130	9	P3	HLA-A*02:01	23.35	AIMEKNIML	-
Nuclear export protein	109-116	8	P3	HLA-B*18:01	16.64	VEQEIRTF	VEQEIRTF
Nuclear export protein	111-118	8	P3	HLA-B*18:01	167.48	QEIRTFSF	QEIRTFSF
Nuclear export protein	33-40	8	P3	HLA-B*15:01	-	-	TQFESLKL
Nuclear export protein	90-99	10	P3	HLA-B*18:01	-	-	TENSFEQITF
Nucleoprotein	404-412	9	P3	HLA-B*15:01	26.29	-	GQISIQPTF
Nucleoprotein	450-458	9	P3	HLA-B*15:01	149.35	-	SARPEDVSF
Nucleoprotein	45-52	8	P3	HLA-B*18:01	126.91	-	TELKLSDY
Polymerase basic protein 2	52-61	10	P3	HLA-A*03:01	15.08	-	AMKYPITADK
RNA polymerase catalytic subunit	177-185	9	P3	HLA-B*18:01	35.56	-	EEMGITTHF

198 **Infection of THP1macs leads to selective presentation of HLA-restricted influenza**

199 **peptides**

200 Flow cytometry of THP1 cells differentiated into a macrophage-like phenotype (THP1MΦ)

201 indicated that all the cells expressed HLA-I but only a minority expressed HLA-II (Fig 3 A-B).

202 Following exposure to the Wisconsin H3N2 and X31 influenza strains at a MOI of 1.0,

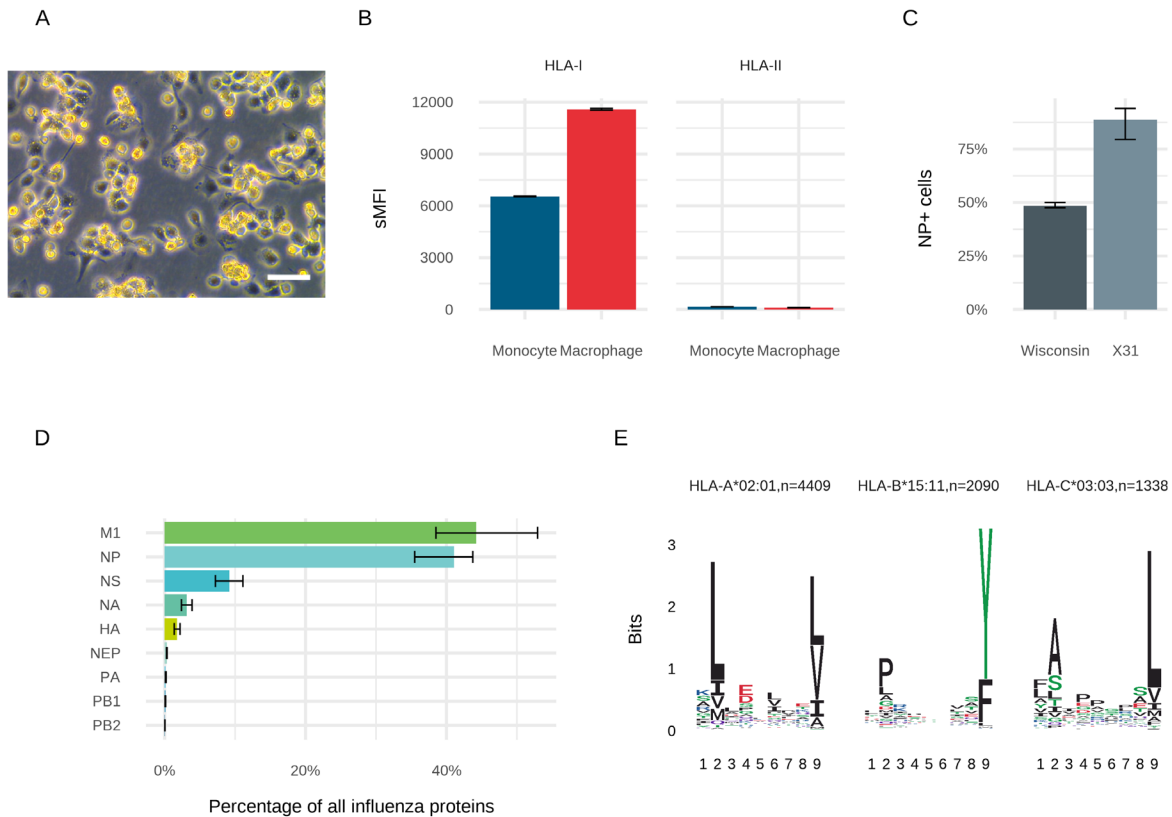
203 approximately 50% and 90% of the THP1 cells were infected respectively (Fig 3 C, Fig S2).

204 Proteomic analysis of the cell lysates using relative quantitation revealed that the intracellular

205 expression of the different influenza proteins showed a hierarchy of expression where the most

206 abundant proteins were matrix protein 1, nucleoprotein and non-structural protein-1, whereas  
207 others, such as polymerase basic proteins 1 and 2 were the least abundant (Fig 3 D). Matrix  
208 protein 2 and RRB2 were not detected in the analysis. This pattern was similar between the  
209 two influenza strains studied, with slight differences in the proportions of the most abundant  
210 proteins. This approximate pattern of expression has been previously observed in purified  
211 influenza virions [27,28], however our observation of the relatively high abundance of NP and  
212 NS1, similar to that observed in ex-vivo infected lung tissues, may be due to our examination of  
213 infected cells rather than virions [29], as these were over-represented in the infection models  
214 compared to our initial purified influenza stock (Fig S1). Notably, the five most abundant  
215 proteins are the same as those found to be principal targets for cell mediated immune  
216 responses in animal infection models [30].

217 Immunopeptidomic analysis of eluted HLA-1-bound peptides extracted from THP1MΦ infected  
218 with Wisconsin virus resulted in the detection of 10,709 unique peptide sequences matching  
219 3,064 host proteins in the Uniprot human database (Table S2). Cluster analysis of these  
220 peptides indicated the presence of three strong HLA-1 binding motifs (Fig 3 E), which were  
221 consistent with the HLA types of this cell line (Table S1). Of the observed peptides, 6,499 could  
222 be assigned to the homozygous HLA-A\*02:01 and -B\*15:11 allotypes on the surface of THP-1  
223 cells on the basis of motif presence. Similarly, infection with X31 resulted in 11,643 unique host  
224 peptide sequences derived from 3,308 host proteins in the Uniprot human database (Table S2).



225

226 **Fig 3: Characteristics of the THP1MF used to identify HLA-binding viral peptides**

227 **following direct infection with influenza virus. (A) Differentiation of THP1 cells alters cells to**  
 228 **a macrophage-like morphology viewed at 20X magnification using phase contrast**

229 **microscopy. White scale bar, 20  $\mu$ m (B) Differentiation into THP1MF increases cell surface HLA-**

230 **I expression but HLA-II expression remains low. (C) Infection rates following exposure to**

231 **Wisconsin and X31 influenza A strains at an MOI of 1.0. (D) Relative proportions of intracellular**

232 **influenza proteins present in three biological replicates of THP1MF (E) Class I MHC molecule 9-**

233 **mer binding motifs for distinct peptides from Wisconsin H3N2 infected THP1MF using**

234 **unsupervised clustering MixMHCp and assigned to HLA types on the basis of known motifs and**

235 **the known HLA types of these cells.**

236 From the three biological replicates used in the study of THP1MΦ, we detected 9 unique  
 237 influenza peptides associated with Wisconsin infection and 20 associated with X31 (Table 1).  
 238 HLA-restricted influenza peptides contained the correct binding motifs for the HLA types of  
 239 THP1 cells (Fig 3 E) [31]. There was only one unique Wisconsin strain peptide, which was  
 240 derived from PA-X, thus all but one of the peptides found in the Wisconsin strain were from  
 241 identical regions to those in X31 (with small like-for-like differences in amino acid sequence),  
 242 and the one unique Wisconsin strain sequence has an identical amino acid sequence in the X31  
 243 strain. The additional X31 peptides were potentially due to the higher infection rate of X31 in  
 244 these cells, leading to more intracellular viral protein.

**Table 2:** Summary of the HLA-I restricted immunopeptides isolated from THP1 macrophages infected with two influenza A viral strains, their predicted binding allotype and source protein.

\*Peptides in blue have mis-matched amino-acid sequences between the strains.

Protein	Protein Position	Length	Allotype	nM	Wisconsin	X-31
Hemagglutinin	28-36	9	HLA-A*02:01	322.25	TLCLGHHAV	TLCLGHHAV
Hemagglutinin	308-318	11	HLA-B*15:11	-	KPFQNVNRITY	KPFQNVNKITY
Hemagglutinin	310-318	9	HLA-B*15:11	-	FQNVNRITY	FQNVNKITY
Hemagglutinin	114-121	8	HLA-B*15:11	-	YPYDVPDY	YPYDVPDY
Hemagglutinin	251-259	9	HLA-C*03:03	94.67	-	TIVKPGDVL
Matrix protein 1	232-240	9	HLA-B*15:11	-	DLLLENLQTY	DLLLENLQAY
Matrix protein 1	134-142	9	HLA-A*02:01	23.5	-	RMGAVTTEV
Matrix protein 1	107-115	9	HLA-B*15:11	-	-	ITFHGAKEI
Non-structural protein 1	122-130	9	HLA-A*02:01	23.35	AIMEKNIML	-
Non-structural protein 1	68-77	10	HLA-B*15:11	-	ILKEESDEAL	ILKEESDEAL
Non-structural protein 1	122-130	9	HLA-A*02:01	53.78	-	AIMDKNIIL
Nucleoprotein	450-458	9	HLA-B*15:11	-	-	SARPEDVSF
Nucleoprotein	439-447	9	HLA-B*15:11	-	-	DMRTEIIRM
Nucleoprotein	256-264	9	HLA-C*03:03	419.04	-	LTFLARSAL
Protein PA-X	102-110	9	HLA-B*15:11	-	KPKFLPDLY	-
Protein PA-X	27-35	9	HLA-B*15:11	-	DLKIETNKF	DLKIETNKF
Protein PA-X	46-54	9	HLA-A*02:01	2.66	-	FMYSDFHFI

**Table 2:** Summary of the HLA-I restricted immunopeptides isolated from THP1 macrophages infected with two influenza A viral strains, their predicted binding allotype and source protein.

\*Peptides in blue have mis-matched amino-acid sequences between the strains.

Protein	Protein Position	Length	Allotype	nM	Wisconsin	X-31
Protein PA-X	46-54	9	HLA-C*03:03	94.61	-	FMYSDFHFI
RNA polymerase catalytic subunit	162-170	9	HLA-A*02:01	23.34	-	RLIDFLKDV
RNA polymerase catalytic subunit	22-32	11	HLA-B*15:11	-	-	FPYTGDPYPYSH
RNA polymerase catalytic subunit	22-30	9	HLA-B*15:11	-	-	FPYTGDPY
RNA polymerase catalytic subunit	745-753	9	HLA-B*15:11	-	-	KICSTIEEL
RNA polymerase catalytic subunit	28-38	11	HLA-B*15:11	-	-	PPYSHGTGTGY

245 Two of the HLA-A\*02:01 immunopeptides, NS1 protein-derived peptide AIMDKNIIL and the M1  
246 peptide RMGAVTTEV have been previously observed following X31 infection in respiratory  
247 epithelial cells [32].

248 The majority of observed viral peptides were predicted to be strong binders to the HLA-B\*15:01  
249 whereas the majority of host immune-peptides were predicted to bind to HLA-A\*02:01 (Fig 3 E  
250 and Table 2). It is unclear whether this is due to preferential tracking of viral proteins to the B  
251 allotype, or simply the presence of favourable B allotype binding motifs in the viral proteins.

252 Previous MS studies have shown approximately similar numbers of HLA-A and -B immuno-  
253 peptides in THP1MΦ [33], the reasons for our observation of a greater abundance of HLA-A  
254 peptides is unclear, but may reflect technical differences in immunopeptide analysis. There was  
255 a notable bias towards presentation of highly conserved internal viral proteins in the HLA-I  
256 peptidome, with only four nested haemagglutinin peptides and no neuraminidase peptides  
257 detected in the infected cell line.



258 The majority of our observed peptides in THP1M $\Phi$  have been previously characterised by in-  
259 vitro binding/cytotoxicity assays and were present in the Immune Epitope Database (IEDB),  
260 although not derived from the two strains studied herein. Most reported positive ELISpot  
261 outputs, confirming that they led to functional responses. The majority of these immunogenic  
262 peptides were previously identified because many influenza strains have been intensively  
263 studied.

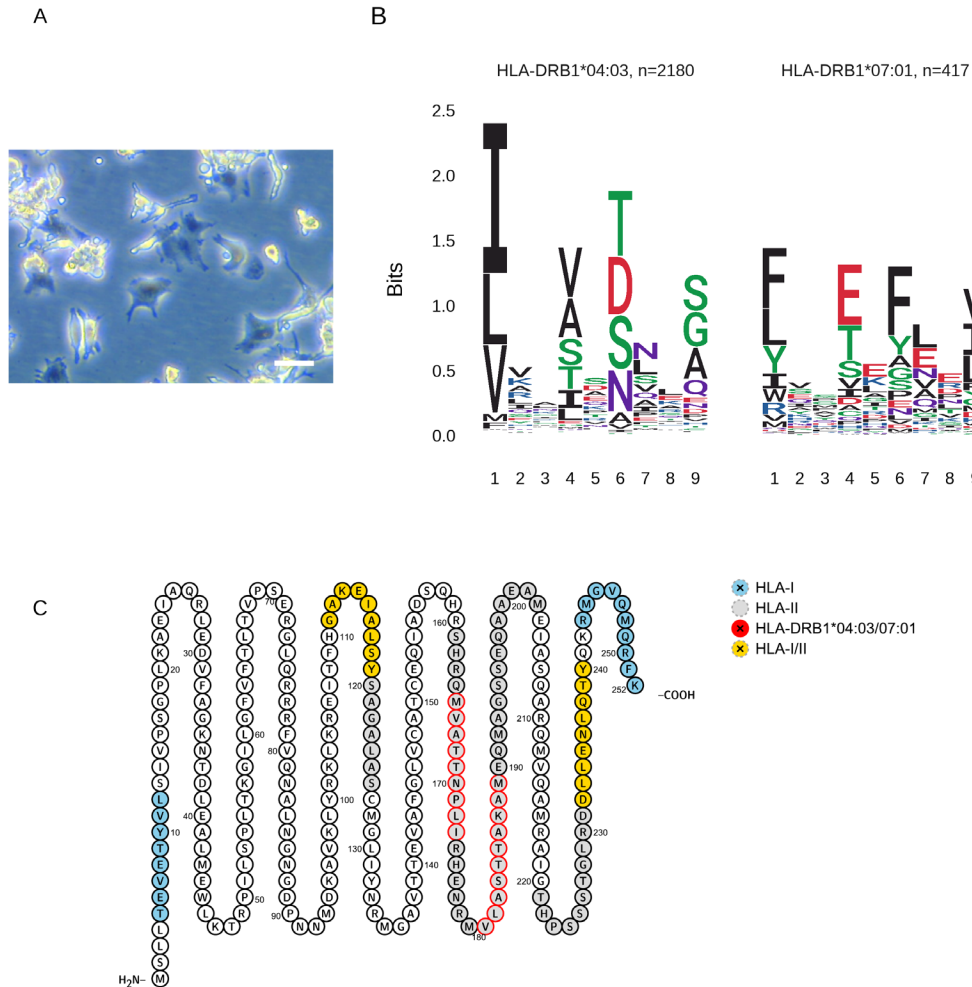
264 All of the novel peptides observed in our THP1M $\Phi$  study were predicted to bind the HLA-  
265 C\*03:03 allotype. The C allotype binding motifs are less clear than A and B allotypes, rendering  
266 allotype assignment by predictive algorithm less efficient. Further examination by functional  
267 assay would be required to confirm their functionality.

268 Very few host cell HLA-II peptides could be detected on these cells, consistent with our flow  
269 cytometry data, suggesting that expression of HLA-II on the cell surface was low (Fig 3 B). No  
270 influenza peptides were detected bound to HLA-II.

271 We found some influenza immunopeptides from proteins which were undetectable in the  
272 proteome of infected cells. Such a finding is consistent with previous reports that immuno-  
273 peptide selection is poorly correlated with source protein concentration [34], but may also reflect  
274 the challenges of detecting lower abundance proteins in a complex proteome such as that  
275 derived from infected cell lines.

## 276 **Phagocytosis of apoptotic influenza-infected MoDCs reveals multiple nested MHC-II** 277 **influenza epitopes**

278 Using Wisconsin H3N2-infected A549 cells (80% infected, see Fig S3) as the source of viral  
279 proteins, we UV irradiated these cells to drive them into apoptosis prior to feeding them to  
280 MoDCs from a heterotypic HLA type individual (Fig 4 A; Patient ID P4).



281

282 **Fig 4: MoDCs can be used to identify HLA-II binding immunopeptides following**

283 **phagocytosis of apoptotic influenza-infected respiratory epithelial (A549) cells. (A)**

284 *Dendritic cell morphology prior to exposure to apoptotic A549 cells, 20X magnification using*

285 *phase contrast microscopy. White scale bar, 50  $\mu$ m. (B) Motif deconvolution using MoDec yields*

286 *class II HLA-DRB1 molecule core binding motif logos for distinct peptides presented by MoDCs*

287 *following phagocytosis of A549 cells infected with Wisconsin H3N2. (C) The sequence of Matrix*

288 *protein 1 and all corresponding observed influenza peptides in our study: regions generating*

289 *class I and II epitopes are in yellow, class I only observed epitopes in blue, and class II only*

290 *observed epitopes in grey. The predicted HLA-DRB1 motifs are highlighted with red circles.*

291 Phagocytosis of these infected cells led to preferential presentation of HLA-II bound influenza  
 292 peptides (Table 3), with those peptides matching the HLA-II motifs from the DCs (Table S1, P4),  
 293 with no observable viral HLA-I peptides, despite robust host-derived HLA-I peptide presentation  
 294 in these cells (Table S3). This lack of evidence of cross-presentation of influenza HLA-I peptides  
 295 by human DCs has been previously observed when using an HLA-A\*02 cell line (BEAS-2B)  
 296 [32].

297 We observed 4,639 distinct class II HLA peptides deriving from 891 source proteins (Table S2).  
 298 Motif deconvolution [35] was able to assign 2,597 peptides to the respective HLA-DRB1  
 299 allotypes of P4 (Fig 4 B). Within these immunopeptidomes, there were 29 influenza A derived  
 300 HLA-II restricted peptide sequences. Contrary to viral presentation following direct infection of  
 301 cells or tissues, there was a very strong bias to the processing and display of the membrane-  
 302 bound proteins neuraminidase, haemagglutinin, and Matrix protein 1 in the detected HLA-II  
 303 peptides (Table 3).

**Table 3:** Summary of HLA-II restricted immunopeptides isolated from DCs following engulfment of influenza-infected A549 cells.

Peptide	Protein	Protein Position	Length	Motif	Allotype	nM	motif
ALNNRFQIKGVELK	Hemagglutinin	511-524	14		Unassigned	-	-
ENRMVLASTTAKAME	Matrix protein 1	176-190	15	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	161.63	VLASTTAKA LAST TAKAM
ENRMVLASTTAKAME	Matrix protein 1	176-190	15	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	78.62	VLASTTAKA LAST TAKAM
ENRMVLASTTAKAMEQ	Matrix protein 1	176-191	16	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
ENRMVLASTTAKAMEQM	Matrix protein 1	176-192	17	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
GAKEIALSYSAGAL	Matrix protein 1	111-124	14		Unassigned	-	-
GAKEIALSYSAGALA	Matrix protein 1	111-125	15		Unassigned	2455.17	-

**Table 3:** Summary of HLA-II restricted immunopeptides isolated from DCs following engulfment of influenza-infected A549 cells.

Peptide	Protein	Protein Position	Length	Motif	Allotype	nM	motif
GAKEIALSYSAGALAS	Matrix protein 1	111-126	16		Unassigned	-	-
HENRMVLASTTAKAME	Matrix protein 1	175-190	16	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQ	Matrix protein 1	175-191	17	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQM	Matrix protein 1	175-192	18	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQMA	Matrix protein 1	175-193	19	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQMAG	Matrix protein 1	175-194	20	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQMAGSSEQ	Matrix protein 1	175-198	24	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQMAGSSEQAA	Matrix protein 1	175-200	26	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQMAGSSEQAAE	Matrix protein 1	175-201	27	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQMAGSSEQAAEA	Matrix protein 1	175-202	28	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HENRMVLASTTAKAMEQMAGSSEQAAEAM	Matrix protein 1	175-203	29	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	-	VLASTTAKA LAST TAKAM
HGAKEIALSYSAGALAS	Matrix protein 1	110-126	17		Unassigned	-	-
IEEGKIVHTSTLSGSAQ	Neuraminidase	257-273	17	IVHTSTLSG	HLA-DRB1*04:03	-	IVHTSTLSG
IEEGKIVHTSTLSGSAQH	Neuraminidase	257-274	18	IVHTSTLSG	HLA-DRB1*04:03	-	IVHTSTLSG
LNNRFQIKGVELK	Hemagglutinin	512-524	13		Unassigned	-	-
NRMVLASTTAKAMEQ	Matrix protein 1	177-191	15	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	166.75	VLASTTAKA LAST TAKAM
NRMVLASTTAKAMEQ	Matrix protein 1	177-191	15	VLASTTAKA LAST TAKAM	HLA-DRB1*04:03/07:01	77.86	VLASTTAKA LAST TAKAM

**Table 3:** Summary of HLA-II restricted immunopeptides isolated from DCs following engulfment of influenza-infected A549 cells.

Peptide	Protein	Protein Position	Length	Motif	Allotype	nM	motif
PSGPLKAEIAQRLE	Matrix protein 1	16-29	14		Unassigned	-	-
QMVQAMRAIGTHPSSSTGLR	Matrix protein 1	211-230	20		Unassigned	-	-
QMVQAMRAIGTHPSSSTGLRDD LLENLQTY	Matrix protein 1	211-240	30		Unassigned	-	-
SHRQMVATTNPLIRHEN	Matrix protein 1	161-177	17	MVATTNPLI	HLA-DRB1*07:01	-	MVATTNPLI
SPRGKLSTRGVQIASN	Nucleoprotein	353-368	16		Unassigned	-	-

304 Multiple nested peptides were detected for the majority of these viral HLA-II epitopes, similar to  
305 those for the majority of host proteins, suggestive of permissive regions of these viral proteins  
306 for processing and presentation.

307 For example, three motifs corresponding to the HLA-DRB1 allotype could be identified in the M1  
308 protein. When overlaid onto the M1 protein sequence [36], these HLA-II motifs were located  
309 predominantly towards the C terminus of the amino acid sequence, whereas the HLA-I motifs  
310 were equally distributed over the protein, including regions also presented by HLA-II (Fig 4 C),  
311 possibly reflective of the different processing pathways involved in HLA-I and -II presentation.

312 Previous work has shown that, when pulsed with recombinant influenza haemagglutinin, MoDCs  
313 will present HLA-II immuno-peptides from select regions of the protein corresponding to the  
314 immune-dominant memory T cell population with higher avidity than naïve T cells [37]. Although  
315 we did not observe many haemagglutinin HLA-II peptides, we observed a similar pattern of  
316 multiple nested peptides from selected regions within the M1 protein. Cryo-EM has recently  
317 revealed the assembled structure of the M1 protein as multiple helical arrays that polymerise to  
318 form the viral endoskeleton, an assembly which unravels in low pH triggering disassembly of the  
319 M1 assembly in the endosome [38].

320 The studies on Haemagglutinin examined endocytosis of recombinant protein, identifying that  
321 the head portion of the protein results in the major T cell dominant clones, but our method using  
322 phagocytosis of infected respiratory cells renders the majority of viral proteins subject to antigen  
323 processing by APCs, potentially resulting in a wider range of immunodominant HLA-II peptides.  
324 The reason for the predominance of M1 protein in these HLA-II peptides is not clear but could  
325 reflect the high intracellular abundance of this protein.

326 Analysis of A549 cells infected with the Wisconsin strain identified only three HLA-I restricted  
327 peptides, and no HLA-II restricted peptides (Table S3). All were consistent with the HLA-I  
328 allotypes of these cells. We found no evidence of either HLA-I or –II peptides sourced from the  
329 A549 cells (i.e. matching their allotypes) following engulfment by DCs. This was perhaps not  
330 surprising considering the how few influenza peptides appear to be presented by these cells,  
331 and the fact that only  $10^6$  cells were used in the MoDC assay (only 10% of the amount normally  
332 required to achieve a peptidome of >1,000 unique peptides).

## 333 Discussion

334 Current subunit vaccine strategies to optimize T cell responses to influenza challenge are  
335 mostly directed towards the most mutable proteins such as Haemagglutinin and Neuraminidase.  
336 There is evidence to suggest that HLA-I restricted T cell responses are more directed towards  
337 the more highly conserved internal viral proteins, whereas humoral responses are dominated by  
338 envelope proteins [39]. Despite the proteome of influenza comprising of only a dozen proteins,  
339 this yields many thousands of potential T cell epitopes. Therefore identifying the epitopes most  
340 important for anti-influenza responses by predictive means is challenging. Most viral proteins  
341 will contain HLA binding motifs for multiple allotypes, but current evidence suggests that only a  
342 small minority of these will actually be presented [40].

343 Here we show how influenza epitope presentation is influenced by presence of HLA binding  
344 motifs, source protein abundance, and the HLA pathway. We confirm that only a few internal  
345 viral proteins provide the main source of HLA-I immunopeptides in lung tissues, and we find that  
346 select immunopeptides are favoured in different influenza strains. Viral protein abundance  
347 influences, but is not the only factor in HLA presentation. Using a MoDC model we show that  
348 viral membrane bound proteins such as NA, HA and M1 are preferentially presented by HLA-II,  
349 and that certain regions of these proteins may be more conducive to processing via the HLA-II  
350 pathway. These results demonstrate how peptidomics can reduce the potential pool of anti-  
351 influenza T cell epitopes from thousands to a few dozen. Furthermore, these candidates can be  
352 refined according to their relevant HLA pathway and helps guide predictive algorithm epitope  
353 selection more effectively.

354 To address the issue of viral tropism, we have taken an approach using ex-vivo human lung  
355 tissues as the targets for infection with different strains of influenza, one model strain  
356 (A/H3N2/X31) and one circulating strain from 2005 (A/H3N2/Wisconsin/2005/67).

357 Our work has previously demonstrated that these strains are capable of infecting both epithelial  
358 cells and macrophages in ex-vivo lung tissues and inducing disease-relevant inflammatory  
359 responses which can be modulated using either anti-viral or anti-inflammatory medications [23].  
360 We predicted that due to the presence of significant numbers of alveolar macrophages with high  
361 intrinsic HLA-DR expression, we would simultaneously generate HLA-I and -II peptides using  
362 this lung tissue model. However, despite demonstrable infection of both epithelial cells and  
363 macrophages in our experiments and identifying a number of novel HLA-I epitopes in these  
364 tissues with relative ease, we were only able to identify one HLA-II peptide. This observation  
365 may help to substantiate previous evidence that the primary function of alveolar macrophages is  
366 to kill and phagocytose infected cells [41], and that DCs are more efficient at presenting HLA-II

367 peptides than macrophages, whether this is by direct infection or following phagocytosis of  
368 infected cells.

369 CD8+ T cell responses are recognised as key components of the adaptive immune response to  
370 viral infections. Since they are mostly directed against conserved internal proteins of the  
371 influenza virus, they are thought to provide significant cross-protection between different strains.  
372 For this reason, vaccines designed to promote T cell protection against conserved T cell  
373 epitopes of multiple IFV strains are highly desirable. The diversity of HLA types in the human  
374 population has created challenges in the generation of universal T cell vaccines, as the selected  
375 optimal T cell epitopes must reflect the HLA restriction of the target population as much as  
376 possible.

377 To better understand the HLA-I viral immunopeptidome, we initially used a cell model (THP1)  
378 with the aim of identifying T cell epitopes for influenza. These cells were more susceptible to  
379 infection with the laboratory-adapted X31 strain than the more clinically relevant Wisconsin  
380 strain. We were able to identify a number of well-characterised HLA-A and B epitopes that had  
381 been previously observed in similar studies. The utility of this approach is limited by the  
382 molecular phenotype of THP1 cells which are homozygous for the three indicated haplotypes.  
383 Bioinformatic comparison of our observed epitopes with a predicted list found that most of the  
384 HLA-A and B epitopes detected using this cell model were predicted binders for the known  
385 THP1 allotypes, but only represented a small proportion of the predicted binders. The reasons  
386 for this are complex, and do not necessarily imply that others are not present, but rather that  
387 they may be unrecognised, since MS is biased to the detection of peptides with certain  
388 biophysical characteristics. For example, the well-characterised immunodominant M158-66  
389 peptide GILGFVFTL, is a high-ranking predicted HLA-A\*02:01 immunopeptide which is  
390 refractory to identification by mass spectrometry.



391 A number of the T cell epitopes we identified were derived from the identical region of the same  
392 protein in the two influenza strains despite small differences in amino acid sequence. This may  
393 arise because the HLA anchor positions were not altered, but is also suggestive of intrinsic  
394 properties of these protein regions being conducive to antigen processing and presentation.

395 Some of our observed influenza immunopeptides did not match with any allotype or were  
396 assigned to HLA-C, but with low predicted affinity. This may reflect the poor performance for  
397 predictive algorithms using the C allotype which is less well characterised. Often where the  
398 motifs for HLA binding are not clearly defined, prediction tools are less useful, meaning direct  
399 observation could play a more significant role, not only in identifying novel peptides, but also in  
400 improving the algorithms for future searches. HLA-II prediction algorithms are thought to be  
401 even less reliable [42].

402 Despite differentiation of THP1 cells into a macrophage-like phenotype, we did not generate  
403 significant numbers of host-cell HLA-II ligands, and saw no evidence of influenza HLA-II ligands,  
404 although the identified HLA-I binding viral peptides were consistent between biological  
405 experimental replicates confirming the robustness of our immunopeptidome isolation  
406 methodology. Recent work on tuberculosis using the THP1 cell line treated with a cytokine  
407 mixture designed to increase HLA-II expression identified some HLA-II epitopes [43], and a  
408 similar approach could be used for influenza in the future.

409 There are currently few reliable in-vitro cell culture methods of identifying HLA-II epitopes for  
410 any virus. These methods have the further limitation of being useful only for laboratory adapted  
411 strains of influenza which are capable of productive infection in cell lines, and can only be used  
412 to identify T cell epitopes for the HLA type of the cell line used.

413 To improve and increase the efficiency of identifying naturally processed and presented HLA-II  
414 epitopes, we employed a human infection model, where we infected a heterotypic monolayer

415 cell line (A549) with influenza virus to generate intracellular virus particles, and then drove those  
416 cells into apoptosis using UV irradiation to facilitate phagocytosis by in-vitro derived dendritic  
417 cells. This methodology resulted in robust generation of HLA-II epitopes of the outer coat  
418 proteins of influenza virus, in addition to matrix 1 proteins, but not of the internal core proteins of  
419 the virus. No evidence of influenza HLA-II peptides matching the HLA-types of the A549 cells  
420 were observed. Neither were viral HLA-I peptides generated using this method, suggestive of a  
421 lack of HLA-I cross-presentation, at least when using this influenza strain. This is the first time  
422 that candidate influenza HLA-II epitopes have been directly observed in a fully human infection  
423 model capable of generating personalised CD4+ T cell epitopes. Whilst we used a cell line  
424 infected with virus as the source material which could be susceptible to strain tropism issues, it  
425 would be entirely feasible to use any cells as the source material since their HLA type would not  
426 be relevant to the epitopes discovered, and very few cells express significant amounts of HLA-II  
427 apart from professional APCs. HLA-II help is essential for an effective vaccine, since although  
428 non-specific CD4+ help can promote CD8+ T cell effector functions, specific CD4+ T cells are  
429 required for the proper generation of memory CD4+ cells [44]. Recent evidence also suggests  
430 that CD4 cells reacting to peptides from core viral proteins such as NP and M1 are first  
431 responders to influenza challenge and can have important effector functions of their own, as  
432 they contain perforin and granzyme and produce IFN- $\gamma$  [14]. Furthermore, the requirement for  
433 CD4 T cell epitopes to facilitate CD8 T cell killing in addition to humoral responses means that  
434 the direct observation of HLA-II peptides will be extremely valuable in improving vaccines for  
435 combating potential influenza outbreaks in the future.

436 Live vaccines confer more effective CD8+ T cell responses than attenuated ones which  
437 stimulate mostly CD4+ T cell responses [45]. The DC uptake model indicates strong  
438 compartmentalisation of CD4 and CD8 processing and peptide display and indicates that a  
439 combination of uptake and direct infection is likely to be most effective in vaccine generation.

440 It has been proposed that alternative pathways of antigen processing in infected APCs, rather  
441 than virion or infected cell uptake, is the primary driver of CD4+ T cell response to influenza  
442 infection [46]. Further work might reveal differential immunopeptidomes in MoDCs infected  
443 directly with influenza virus, rather than following uptake of infected cells as in our study. Here  
444 we have demonstrated the potential for fully human ex-vivo models as tools to identify viral  
445 immunopeptides which could be used to design strain-specific T cell vaccines against influenza.  
446 We have shown evidence that these epitopes will be conserved between different donors if they  
447 share the same HLA allotype.

448 Much of the vaccine design process employs machine learning algorithms to predict the  
449 relevant HLA-I CD8 T cell epitopes by searching for motifs and predicting their affinity in-silico.  
450 There is increasing evidence, including in our data, that epitopes that are actually presented are  
451 influenced in-vivo by a complex series of additional factors however, inclusive of source protein  
452 abundance, protease type in the proteasome, protein turnover, transporter protein expression,  
453 PTMs and many others, making the prediction algorithms liable to prioritise non-immunogenic  
454 peptides.

455 There are many potential combinations of HLA allotypes in humans, and the best T cell epitopes  
456 need to be selected. The current approach is limited by the quality of HLA-I and –II epitopes that  
457 can be predicted using the current algorithms. These algorithms are trained using mass  
458 spectrometry (MS) data from peptides eluted from the HLA complex, and thus, rarer HLA types  
459 often have less well-defined motifs, and the predictions are therefore less accurate. In the case  
460 of HLA-II this is even more apparent, as the paucity of eluted peptide datasets for each  
461 haplotype means the predictions are probably highly inaccurate. Furthermore, model cell lines  
462 may not be susceptible to pathogen infections.

463 Using our ex-vivo lung tissue and DC infection models, a panel of HLA-I and –II T cell epitopes  
464 for a known pathogen could be used to generate a vaccine based on real-world observations of

465 influenza A virus immunopeptides for a range of HLA types, potentially enhancing vaccine  
466 efficacy.

467

## 468 **Materials and Methods**

### 469 **Virus propagation**

470 *A/H3N2/X31* and *A/H3N2/Wisconsin/67/2005* seed stocks were obtained from the National  
471 Institute for Biological Standards and Control (NIBSC), UK, propagated in embryonated SPF-  
472 free chicken eggs and, subsequently, purified from egg allantoic fluid by sucrose density  
473 gradient ultracentrifugation (Virapur LLC, San Diego, USA). The X31 virus contains the six  
474 internal genes of PR8, an H1N1 laboratory-adapted influenza virus strain, but expresses H3N2  
475 surface proteins. Stock viral titre was determined by MDCK plaque assay using standard  
476 protocols. To generate inactivated UV virus, aliquots were irradiated for 30 min on ice using an  
477 ultraviolet microbicidal crosslinker (Steristrom 2537a) as previously described [47].

### 478 **Cell culture**

479 The acute myeloid leukaemia cell line THP1 was cultured in RPMI supplemented with 10% FCS  
480 and 1% penicillin-streptomycin. Cells ( $1.5 \times 10^8$  per treatment arm) were differentiated into  
481 macrophage-like cells (THP1M $\Phi$ ) by incubation with 100 ng/mL Phorbol-12-myristate-13-  
482 acetate (PMA) for 48h in complete medium followed by a 24 h rest period in complete medium  
483 without PMA, by which time the majority of cells were adherent to the culture surface.

484 A549 cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin  
485 and passaged at approximately 80% confluence.

### 486 **Cell infections**

487 Cell monolayers were rinsed three times with basal medium to remove serum proteins before 2  
488 h infection with either *A/H3N2/Wisconsin/67/2005* or *A/H3N2/X31* influenza virus in basal  
489 medium at the indicated multiplicity of infection (MOI), followed by a further incubation as  
490 specified. Mock infections with UV-inactivated virus were performed at the same dose.

491 Following infection, cell monolayers were rinsed twice with PBS and the cells were harvested by  
492 trypsin treatment, washed twice with 50 volumes of PBS prior to storage as a cell pellet at -  
493 80°C. A small aliquot of cells was preserved for flow cytometry.

#### 494 **Human lung tissue explants**

495 Resected human lung parenchymal tissues from three donors undergoing surgery for clinical  
496 reasons were obtained. Parenchymal tissue without evidence of visible abnormalities, distant  
497 from the resection margin (250 mg of fresh tissue per treatment), was dissected and placed into  
498 culture within 2 h. The study was performed in accordance with Research Ethics Committee  
499 (REC) approvals, (Southampton and South West Hampshire Research Ethics Committee,  
500 LREC no: 09/H0504/109). Parenchymal explants were first rested overnight in RPMI in 24-well  
501 culture in a humidified incubator at 37°C with 5% CO<sub>2</sub>, then cultured in glutamine-supplemented  
502 RPMI medium with log 7.4 pfu/well virus, either A/H3N2/X31 or A/H3N2/Wisconsin/67/2005  
503 (National Institute for Biological Standards and Control, UK or virus diluent as mock control.  
504 After 2 h incubation, the explants were washed in basal RPMI medium to remove excess virus  
505 and incubated further for 22 hours in glutamine-supplemented RPMI. At the end of the  
506 incubation, tissues were washed with 50 volumes of PBS and snap frozen as pellets prior to  
507 storage at -80°C. Approximately 20 mg of fresh tissue were removed prior to freezing for flow  
508 cytometric analysis.

#### 509 **Flow cytometry**

##### 510 *Assessment of infection in A549 cells and basal and differentiated THP-1 cells*

511 For analysis of influenza infection, monolayer cells were harvested and re-suspended in 200 µL  
512 of Fixation/permeabilisation solution (BD biosciences, San Jose, CA), and incubated on ice for  
513 30 min. The cells were then harvested by centrifugation (400g, 4°C, 5 min) and re-suspended in  
514 100 µL permeabilisation buffer (FACS buffer, 1% BSA, 1 mM EDTA in PBS containing 1 X BD

515 permeabilisation reagent). FITC-conjugated anti-influenza NP monoclonal antibody (Abcam  
516 clone 20921) was added (1  $\mu$ L per reaction) and incubated for 30 min on ice. Excess antibody  
517 was then removed by the addition of 2 mL of permeabilisation buffer followed by centrifugation  
518 as before. Cells were finally re-suspended in FACS buffer without permeabilisation reagents  
519 prior to flow cytometric analysis using either a BD FACSAria or a Guava EasyCyt flow  
520 cytometer, equipped with appropriate lasers and filters.

#### 521 *Analysis of MHC expression on differentiated THP1 cells*

522 THP1M $\Phi$  were harvested by centrifugation and re-suspended in 100  $\mu$ L FACS buffer containing  
523 10% FCS. 1  $\mu$ g/mL anti-HLA-I (W6/32) or anti-HLA-2 (HB-145) monoclonal antibodies were  
524 added and incubated for 30 min on ice. Excess antibodies were removed by centrifugation as  
525 previously described and cells were re-suspended in 100  $\mu$ L FACS buffer containing 1  $\mu$ L per  
526 reaction of FITC conjugated rabbit anti-mouse monoclonal antibodies and incubated for a  
527 further 30 min on ice. Cells were centrifuged as before and re-suspended in 200  $\mu$ L of 2% (w/v)  
528 formalin and incubated on ice for 30 min to fix the cells. Finally, cells were re-suspended in  
529 FACS buffer prior to analysis using a GUAVA EasyCyte benchtop flow cytometer (Merck  
530 Millipore) equipped with the relevant laser and filters to detect FITC fluorescence. Data were  
531 analysed using GUAVA software. HLA-I and -II FMO were calculated by gating against cells  
532 incubated with secondary antibody alone.

#### 533 *Assessment of influenza infection in resected human lung tissue samples*

534 To analyse influenza infection in resected tissue samples, post-infection, tissues were weighed  
535 and enzymatically dispersed with 1 mg/mL type I collagenase in RPMI as previously described  
536 24. Dispersed cells were re-suspended in 100  $\mu$ L FACS buffer containing human IgG (as  
537 Fcyblock) prior to the addition of antibodies directed against surface proteins: CD45-PECF594  
538 (to differentiate leukocytes from structural cells), CD3-PECy7, HLA-DR/APCH7 and CD326-

539 PerCPCy5.5 or relevant fluorophore-conjugated isotype controls, and incubated for 30 min on  
540 ice. Cells were then fixed and permeabilised as previously described, prior to intracellular  
541 staining to quantify viral infection using FITC-conjugated anti-viral nucleoprotein (NP) antibody.  
542 All flow cytometry was performed using a BD FACSAria equipped with relevant the relevant  
543 lasers and filters, and data were analysed using BD FACS DIVA software. Epithelial cells were  
544 identified using the following gating strategy: size/scatter, CD45-, CD326+. Macrophages were  
545 identified using the following gating strategy: size/scatter, CD45+, CD3-, HLA-DR++ (Fig S2).  
546 Infected epithelial and macrophage cell populations were identified by NP-FITC staining, gated  
547 against mock-infected controls using a 1% overlap (Fig S2 E-F).

#### 548 **Phagocytosis assay of MoDCs to identify HLA-II viral ligands**

##### 549 *Isolation and preparation of MoDCs*

550 PBMCs were isolated from buffy coats and allowed to adhere to tissue culture treated flasks for  
551 2 h in Promocell monocyte attachment buffer (Sigma, Dorset, UK). Monolayers were then rinsed  
552 thoroughly with Promocell DC generation medium to remove non-adherent cells and  
553 subsequently were cultured for 6 days in dendritic cell generation medium supplemented with  
554 cytokines (Sigma) to generate immature MoDCs.

##### 555 *A549 cell infection and apoptosis*

556 Monolayer cultures of A549 cells were infected and sent into apoptosis essentially as previously  
557 described [48]. Briefly, 90% confluent monolayers of A549 cells were infected with  
558 A/Wisconsin/67/2005 influenza at an MOI of 1.0 in serum-free medium for 2 h, monolayers were  
559 rinsed twice with serum free medium to remove excess inoculum, and then cultured for a further  
560 12 h in serum-free DMEM supplemented with penicillin-streptomycin and l-glutamine for a  
561 further 12 h. The infection rate of >80% was confirmed by flow cytometry (Fig S3) using  
562 detection of intracellular viral NP protein as previously described. The infected cell monolayers



563 were rinsed twice with PBS and then irradiated with 150 mJ/cm<sup>2</sup> of UV light using a Stratalinker  
564 1800 (Agilent technologies, Santa Cruz, CA, USA) to induce apoptosis. The cells were then  
565 incubated for a further 2 h in serum-free medium, prior to enzymatic dispersal to collect the  
566 cells. These were then re-suspended at a concentration of 1x10<sup>7</sup>/mL in monocyte generation  
567 medium and added to the MoDCs for 3 h. DC activation supplement was then applied and the  
568 cells incubated for a further 4 h. Cells were then harvested by trypsinisation and washed twice  
569 by centrifugation in PBS before storage at -80°C.

## 570 **Immuno-peptide analysis**

### 571 *Purification HLA-I and -II immunopeptides*

572 Protein-A sepharose beads (Repligen, Waltham, Mass. USA) were covalently conjugated to 10  
573 mg/mL W6/32 (pan-anti-HLA-I) or 5 mg/mL HB145 (pan-anti-HLA-II) monoclonal antibodies  
574 (SAL Scientific, Hampshire, UK) using DMP as previously described [49]. Snap frozen tissue  
575 samples were briefly thawed and weighed prior to 30 S of mechanical homogenization using a  
576 150W handheld mechanical homogeniser with disposable probes (Thermo Fisher Scientific) in 4  
577 mL lysis buffer (0.02M Tris, 0.5% (w/v) IGEPAL, 0.25% (w/v) sodium deoxycholate, 0.15mM  
578 NaCl, 1mM EDTA, 0.2mM iodoacetamide supplemented with EDTA-free protease inhibitor mix).  
579 For cell cultures, frozen cell pellets were re-suspended in 5 mL of lysis buffer and rotated on ice  
580 for 30 min to solubilise.

581 Homogenates were clarified for 10 min @2,000g, 4°C and then for a further 60 min @13,500g,  
582 4°C. 2 mg of anti-HLA-I conjugated beads were added to the clarified supernatants and  
583 incubated with constant agitation for 2 h at 4°C. The captured HLA-  
584 I/β<sub>2</sub>microglobulin/immunopeptide complex on the beads was washed sequentially with 10  
585 column volumes of low (isotonic, 0.15M NaCl) and high (hypertonic, 0.4M NaCl) TBS washes  
586 prior to elution in 10% acetic acid and dried under vacuum. The MHC-I-depleted lysate was then

587 incubated with 1 mg of anti-HLA-II mouse monoclonal antibodies and MHC-II bound peptides  
588 were captured and eluted in the same conditions. Column eluates were diluted with 0.5 volumes  
589 of 0.1% TFA and then applied to HLB-prime reverse phase columns (Waters, 30 mg  
590 sorbent/column). The columns were rinsed with 10 column volumes of 0.1% TFA and then the  
591 peptides were eluted with 12 sequential step-wise increases in acetonitrile from 2.5-30%.  
592 Alternate eluates were pooled and dried using a centrifugal evaporator and re-suspended in  
593 0.1% formic acid.

#### 594 *LC-MS/MS analysis of HLA-I and -II peptides*

595 HLA peptides were separated by an Ultimate 3000 RSLC nano system (Thermo Scientific)  
596 using a PepMap C18 EASY-Spray LC column, 2 µm particle size, 75 µm x 50 cm column  
597 (Thermo Scientific) in buffer A (0.1% Formic acid) and coupled on-line to an Orbitrap Fusion  
598 Tribrid Mass Spectrometer (Thermo Fisher Scientific, UK) with a nano-electrospray ion source.  
599 Peptides were eluted with a linear gradient of 3%-30% buffer B (Acetonitrile and 0.1% Formic  
600 acid) at a flow rate of 300 nL/min over 110 minutes. Full scans were acquired in the Orbitrap  
601 analyser using the Top Speed data dependent mode, performing a MS scan every 3 second  
602 cycle, followed by higher energy collision-induced dissociation (HCD) MS/MS scans. MS  
603 spectra were acquired at resolution of 120,000 at 300 m/z, RF lens 60% and an automatic gain  
604 control (AGC) ion target value of 4.0e5 for a maximum of 100 ms. MS/MS resolution was 30,000  
605 at 100 m/z. Higher-energy collisional dissociation (HCD) fragmentation was induced at an  
606 energy setting of 28 for peptides with a charge state of 2-4, while singly charged peptides were  
607 fragmented at an energy setting of 32 at lower priority. Fragments were analysed in the Orbitrap  
608 at 30,000 resolution. Fragmented m/z values were dynamically excluded for 30 seconds.

## 609 *Data analysis for immunopeptidomics*

610 Raw spectrum files were analysed using Peaks Studio 10.0 build 20190129, with the data  
611 processed to generate reduced charge state and deisotoped precursor and associated product  
612 ion peak lists which were searched against a Uniprot database (20,350 entries, 2020-04)  
613 appended with the full sequences for both influenza strains: A/Wisconsin/67/2005(H3N2), 12  
614 entries or A/X-31(H3N2), 11 entries. A contaminants list (245 entries) in unspecific digest mode  
615 was applied. Parent mass error tolerance was set a 5ppm and fragment mass error tolerance at  
616 0.03 Da. Variable modifications were set for N-term Acetylation (42.01 Da), Methionine  
617 oxidation (15.99 Da) and carboxyamidomethylation (57.02 Da) of cysteine. A maximum of three  
618 variable modifications per peptide were set. The false discovery rate (FDR) was estimated with  
619 decoy-fusion database searches and were filtered to 1% FDR. The search results were further  
620 refined using the MS-Rescue package [50]. Downstream analysis and visualizations were  
621 performed in R using associated packages [26,51–53]. Peptide binding motifs were identified  
622 using unsupervised clustering methods MixMHCp2.1 [25] and MoDec [54], for class I and class  
623 II HLA peptides respectively. Peptide binding affinities predicted using NetMHC 4.0 [55,56] and  
624 NetMHCIIpan 4.0 [57] for class I and class II HLA peptides respectively.

## 625 **Proteomic profiling**

### 626 *Sample preparation*

627 100 µg of protein from HLA-I and –II depleted cell and tissue lysate were precipitated using  
628 methanol/chloroform extraction. Lysate containing 100 µg of protein were mixed with 600 µL of  
629 methanol and 150 µL of chloroform. 450 µL of water were added and the sample was  
630 centrifuged at 13,500 g for 10 min at room temperature. The upper aqueous later was removed  
631 and replaced with 450 µL of methanol and the sample centrifuged again to pellet the proteins.  
632 The protein pellet briefly air-dried prior to resuspension in 100 µL of 6M urea/50 mM Tris-HCl pH

633 7.4. The sample was sequentially reduced and alkylated by the addition of 5 mM dithiothreitol  
634 for 30 min @37°C and then 15 mM iodoacetamide for 30 min @ RT. 4µg trypsin/LysC mix  
635 (Promega) were then added and incubated for 4 h @37°C. 750 µL of Tris-HCl pH 8.0 were then  
636 added and incubated for a further 16 h at 37°C. The digestion was terminated by the addition of  
637 4 µL of TFA. The resultant peptide mixture was purified using HLB prime reverse phase µ-  
638 elution plates (Waters) by elution in 70% acetonitrile according to the manufacturers'  
639 instructions and dried under vacuum. Peptides were reconstituted in 0.1% formic acid and  
640 applied to a Fusion LTQ orbitrap instrument set up as previously described.

#### 641 *LC-MS/MS analysis of global proteome*

642 Tryptic peptides were reconstituted in 0.1% formic acid and applied to an Orbitrap Fusion Tribrid  
643 Mass Spectrometer with a nano-electrospray ion source as previously described. Peptides were  
644 eluted with a linear gradient of 3-8% buffer B (Acetonitrile and 0.1% Formic acid) at a flow rate  
645 of 300 nL/min over 5 minutes and then from 8-30% over a further 192 minutes. Full scans were  
646 acquired in the Orbitrap analyser using the Top Speed data dependent mode, performing a MS  
647 scan every 3 second cycle, followed by higher energy collision-induced dissociation (HCD)  
648 MS/MS scans. MS spectra were acquired at resolution of 120,000 at 300-1,500 m/z, RF lens  
649 60% and an automatic gain control (AGC) ion target value of 4.0e5 for a maximum of 100 ms  
650 and an exclusion duration of 40s. MS/MS data were collected in the Ion trap using a fixed  
651 collision energy of 32% with a first mass of 110 and AGC ion target of 5.0e3 for a maximum of  
652 100ms.

#### 653 *Data analysis for proteomics*

654 Raw data files were analysed using Peaks Studio 10.0 build 20190129. Parent ion tolerance  
655 was set to 10ppm and fragment ion tolerance set to 0.6 Da, and spectra were searched against  
656 the same database as used for immunopeptidomics. Fixed carbamidomethylation, variable N-

657 terminal acetylation and oxidation of methionine were specified. Variable modifications were set  
658 for N-term Acetylation (42.01 Da), Methionine oxidation (15.99 Da) and fixed  
659 carboxyamidomethylation modification (57.02 Da) of cysteine. A maximum of three variable  
660 modifications per peptide were set. The false discovery rate (FDR) was estimated with decoy-  
661 fusion database searches and were filtered to 1% FDR. Relative protein quantification was  
662 performed using Peaks software and normalized between samples using a histone ruler [58].  
663 Downstream analysis and visualizations were performed in R using associated packages  
664 [26,51–53].

665 The mass spectrometry proteomics data have been deposited to the ProteomeXchange  
666 Consortium via the PRIDE [59] partner repository with the dataset identifier PXD022884 and  
667 10.6019/PXD022884.

668

## 669 Acknowledgments

670 The authors would like to thank the staff at the Flow cytometry Core facility in the university of  
671 Southampton Faculty of Medicine for their assistance with this work. We would also like to thank  
672 Ben Johnson and Katie McCann in the Wessex Investigational Science Hub (WISH) for their  
673 assistance with patient samples.

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846

## 847 Supporting Information

**Table S1:** HLA allotypes of the cell lines, lung tissues and dendritic cells used in the study

ID	1	2	3	7	8	9	4	5	6	10	11	12
THP1	A*02:01	B*15:11	C*03:03	A*02:01	B*15:11	C*03:03	DRB1*01:01	DQB1*05:01	DPB1*02:01	DRB1*15:01	DQB1*06:02	DPB1*04:02
P1	A*03:01	B*07:02	C*07:02	A*26:01	B*07:02	C*07:02	DRB1*11:01	DQB1*03:01	DPB1*02:01	DRB1*15:01	DQB1*06:02	DPB1*16:01
P2	A*01:01	B*15:01	C*03:03	A*02:01	B*44:27	C*07:04	DRB1*04:01	DQB1*03:02	DPB1*03:01	DRB1*15:01	DQB1*06:03	DPB1*04:01
P3	A*02:01	B*15:01	C*03:04	A*03:01	B*18:01	C*05:01	DRB1*11:01	DQB1*03:01	DPB1*01:01	DRB1*15:01	DQB1*06:02	DPB1*04:01
P4	A*01:01	B*51:01	C*06:02	A*01:01	B*57:01	C*07:01	DRB1*04:03	DQB1*03:03	DPB1*04:01	DRB1*07:01	DQB1*03:05	DPB1*14:01
A549	A*25:01	B*18:01	C*12:03	A*30:01	B*44:03	C*16:01	DRB1*07:01	DQB1*02:02	DPB1*03:01	DRB1*11:04	DQB1*03:01	DPB1*06:01

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**Table S2:** Immunopeptides isolated from A549 cells following infection with A/Wisconsin/67/2005 influenza.

\*nM is predicted affinity by NetMHC 4.0

Protein	Protein Position	Length	Allotype	nM	Peptide
Non-structural protein 1	142-150	9	HLA-A*25:01	24	ETIVLLRAF
Nucleoprotein	342-351	10	HLA-A*30:01	538	RLLSFIRGTK
Matrix Protein 1	47-56	10	HLA-A*30:01	22	KTRPILSPLT

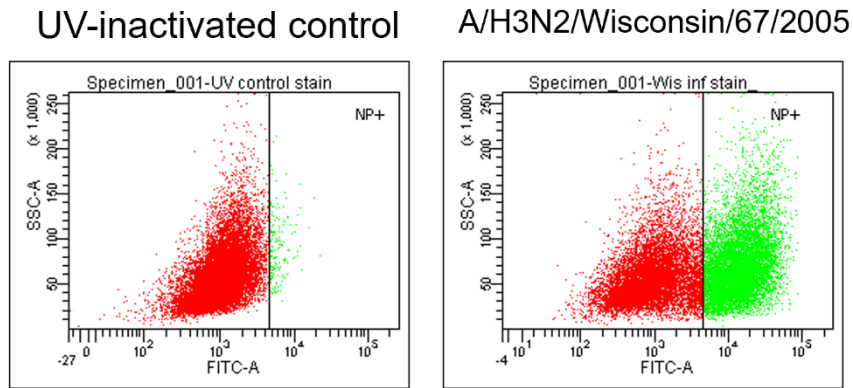
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**Table S3:** Summary of immunopeptidomes isolated from cell lines, lung tissues and dendritic cells

\*RsPa = resected lung parenchyma tissue, MoDC = monocyte-derived dendritic cells differentiated in vitro from PBMCs from donor P4.

ID	Sample type	No. peptides	No. proteins	Strain	HLA
THP1	Cell line	10,709	3,064	Wisconsin	I
THP1	Cell line	11,643	3,308	X-31	I
P1	RsPa	7,944	2,603	Wisconsin	I
P1	RsPa	1,038	324	Wisconsin	II
P2	RsPa	5,304	1,696	Wisconsin	I
P2	RsPa	1,826	438	Wisconsin	II
P2	RsPa	5,985	1,870	X-31	I
P2	RsPa	1,469	369	X-31	II
P3	RsPa	6,338	1,996	Wisconsin	I
P3	RsPa	1,091	327	Wisconsin	II
P3	RsPa	5,926	1,891	X-31	I
P3	RsPa	1,170	341	X-31	II
P4	MoDC+A549	5,432	2,614	Wisconsin	I
P4	MoDC+A549	4,639	891	Wisconsin	II

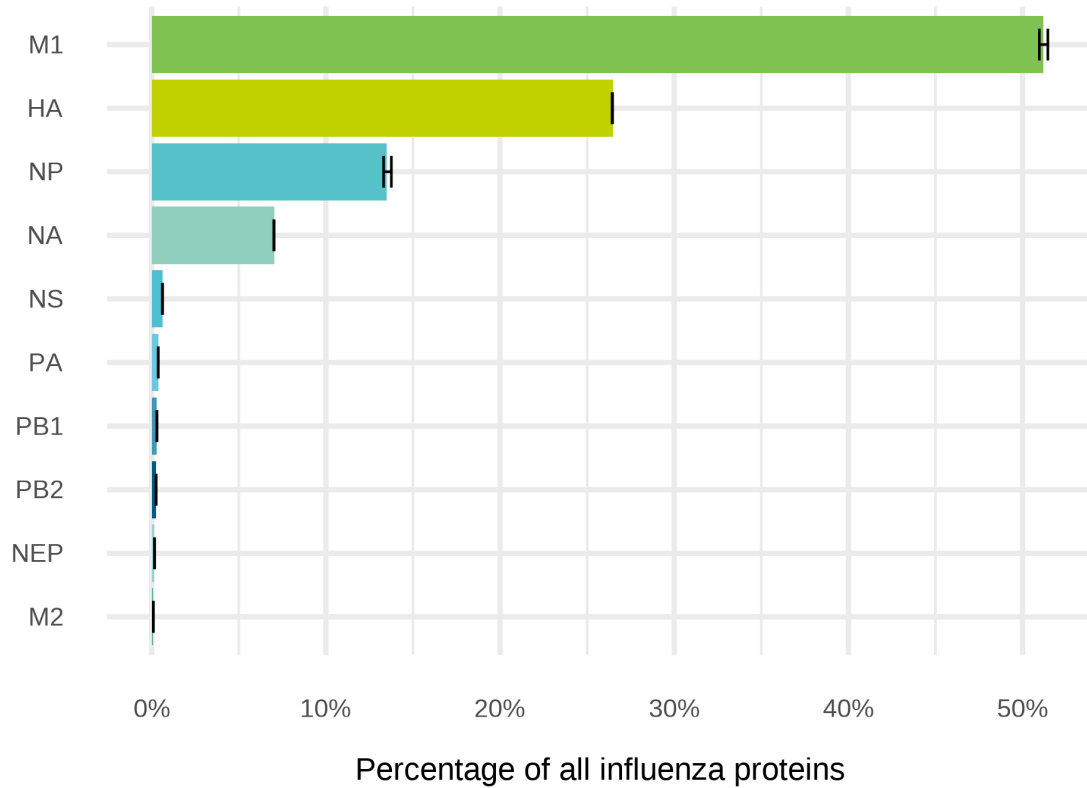
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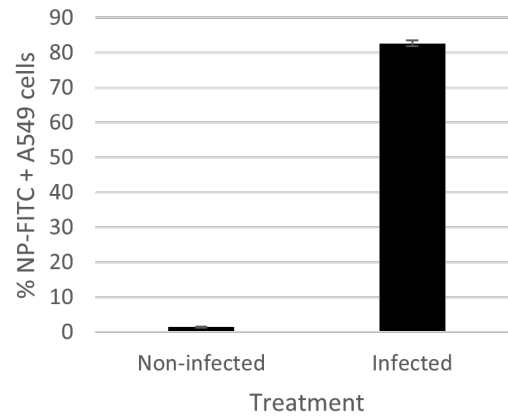
853 **Fig S1: Flow cytometric identification of infected THP1MΦ 24 h post-infection.** THP1MΦ  
854 were enzymatically dispersed from the culture surface prior to fixation and permeabilization of  
855 the cells. Cells were stained intracellularly with FITC-conjugated anti-nucleoprotein antibodies  
856 and analysed by flow cytometry. Infected cells were gated with respect to cells exposed to UV-  
857 inactivated virus as controls. Figure shown is representative of three independent experiments.





858

859 **Fig S2: Relative proportion of viral proteins in A/H3N2/Wisconsin stock.** Viral proteins  
860 were analysed by mass spectrometry proteomics, and the relative quantities of each protein  
861 was determined as described in the methods. Quantities are expressed as the percentage of  
862 the intensity of the top 3 peptides from each protein from 3 technical replicates.

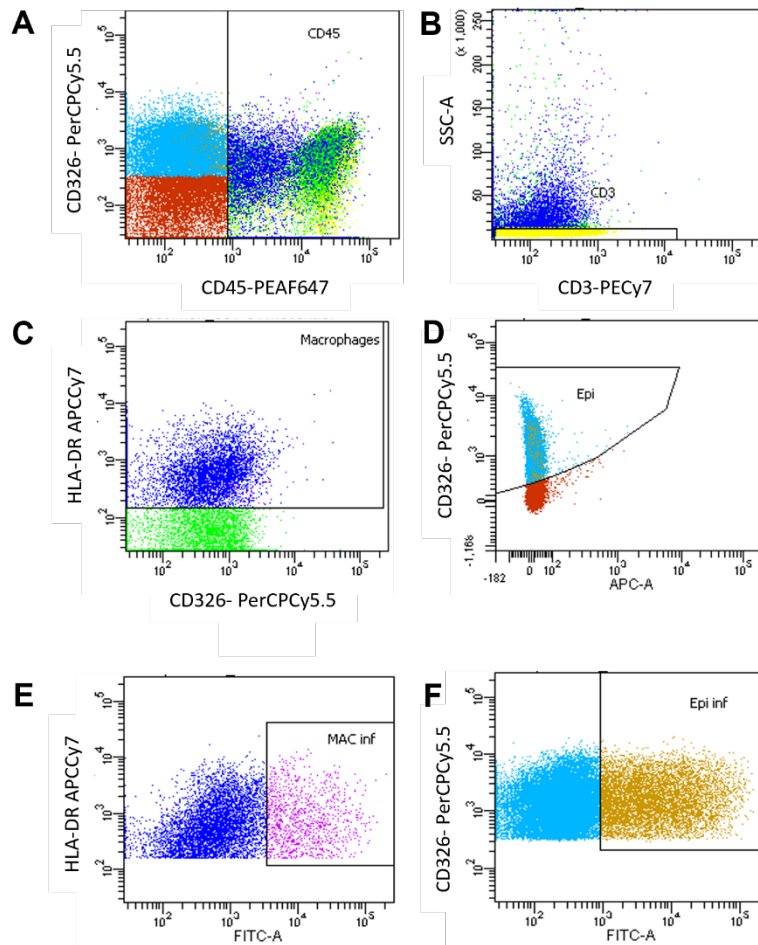


863

864 **Fig S3: Infection rates in A549 cells prior to DC engulfment.** A549 cells were infected at an  
865 MOI of 1.0 for 12 h, achieving >80% infection (Data are mean infection rates from replicate  
866 samples stained independently,  $n=3 \pm$  SD).

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870 **Fig S4: Flow cytometric gating strategy for identification of influenza infection in lung**  
871 **tissue sample cell subsets.** Lung tissue explants were infected with IFV-A ex-vivo and  
872 incubated post-infection for 24 h. Tissue samples were then enzymatically dispersed and the  
873 cells stained with monoclonal antibodies conjugated to cell-specific markers. Cell markers were  
874 used to identify (A) leukocytes (CD45-Horizon). (B) CD45+ cells were then gated to identify and  
875 exclude T cells (CD3-PECy7). (C) CD45+/CD3-/HLA-DR+ cells were macrophages. (D) CD45-  
876 CD326+ cells were identified as epithelial cells. (E) NP1/FITC staining was then used to identify  
877 infected macrophages (HLA-DR-APC/Cy7+/FITC+) and epithelial cells (F) (CD45-/CD326-  
878 PerCP/Cy5.5+/NP-FITC+).