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Influenza A virus infection-induced macroautophagy facilitates MHC class II-restricted endogenous presentation of an immunodominant viral epitope

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Running title: IAV induced-autophagy facilitates endogenous MHC-II presentation

Key words: Influenza A virus, macroautophagy, MHC-II, antigen presentation, $CD4^+$ T cell

Abbreviations: 3-MA, 3-methyladenine; APCs, antigen presenting cells; *ATGs,* autophagy-related genes; BAL, bronchoalveolar lavage; BFA, brefeldin A; BMDCs, Bone-Marrow Derived Dendritic cells; CMA, chaperone-mediated autophagy; CLIP, class II-associated invariant chain peptide; CRISPR, clustered regularly interspaced short palindromic repeats; EBSS, Earle's Balanced Salt Solution; EBV, Epstein-Barr virus; ER, endoplasmic reticulum; IAV, influenza A virus; ICS, intracellular cytokine staining; IIV, inactivated influenza vaccines; LAIV, live-attenuated influenza vaccines; LC3, light-chain kinase 3; M2SR, M2-deficient single replication vaccine virus; MHC, major histocompatibility complex; MHC-I, MHC class I molecule; MHC-II, MHC class II molecule; MIIC, MHC class II compartment

Abstract

$CD4^+$ T cells recognize peptides presented by major histocompatibility complex class II molecules (MHC-II). These peptides are generally derived from exogenous antigens. Macroautophagy has been reported to promote endogenous antigen presentation in viral infections. However, whether influenza A virus (IAV) infection-induced macroautophagy also leads to endogenous antigen presentation through MHC-II is still debated. In this study, we show that IAV infection leads to endogenous presentation of an immunodominant viral epitope $NP_{311-325}$ by MHC-II to $CD4^+$ T cells. Mechanistically, such MHC-II-restricted endogenous IAV antigen presentation requires *de novo* protein synthesis as it is inhibited by the protein synthesis inhibitor cycloheximide, and a functional ER-Golgi network as it is totally blocked by Brefeldin

A. These results indicate that MHC-II-restricted endogenous IAV antigen presentation is dependent on *de novo* antigen and/or MHC-II synthesis, and transportation through the ER-Golgi network. Furthermore, such endogenous IAV antigen presentation by MHC-II is enhanced by TAP deficiency, indicating some antigenic peptides are of cytosolic origin. Most importantly, the bulk of such MHC-II-restricted endogenous IAV antigen presentation is blocked by autophagy inhibitors (3-MA and E64d) and deletion of autophagy related genes, such as *Beclin1* and *Atg7*. We have further demonstrated that in dendritic cells, IAV infection prevents autophagosome-lysosome fusion and promotes autophagosome fusion with MHC Class II compartment (MIIC), which likely promotes endogenous IAV antigen presentation by MHC-II. Our results provide strong evidence that IAV infection-induced autophagosome formation facilitates endogenous IAV antigen presentation by MHC-II to $CD4^+$ T cells. The implication for influenza vaccine design is discussed.

Introduction

$CD4^+$ T cells not only provide "help" to B cells to improve the humoral immune response, but also play a significant role in helping the $CD8^+$ T cell response, especially during T cell memory formation and recall [1]. In general, $CD4^+$ T cells recognize antigenic peptides presented by major histocompatibility complex class II molecules (MHC-II) on professional antigen presenting cells (APCs), including B cells, macrophages and dendritic cells (DCs). MHC-II generally present peptides derived from “exogenous” antigen engulfed by APCs, as they cannot normally pick up endogenous peptides in the endoplasmic reticulum (ER) because their peptide-binding clefts are occupied by the chaperone invariant chain [2]. Whereas $CD8^+$ T cell activation depends on the recognition of pMHC-I expressed on most cell types. MHC-I pick up "endogenous" peptides in the ER as they are largely derived from antigens synthesized inside the APC, degraded by the proteasomes and transported into the ER by transporters associated with antigen processing (TAP) [3]. However, there are

exceptions. For example, exogenous antigens can be presented by MHC-I through a well-established process known as "cross-presentation" [4, 5]. Similarly, endogenous antigens such as some self-antigens [6], tumor antigens [7-9], and viral antigens [10-12] can be presented by MHC-II to $CD4^+$ T cells. The endogenous IAV antigen presentation to $CD4^+$ T cells was first reported by Gueguen and colleagues [13], and later by Schmid *et al.* [14], and more recently, our lab showed that human $CD4^+$ T cells could recognize IAV-infected, EBV-transformed B cell lines [15]. However, the mechanism associated with endogenous IAV antigen presentation by MHC-II has been poorly established. Recently, some endogenous antigen presentation was reported to be related to macroautophagy activity [16-18]. EBNA1, a nuclear antigen from Epstein-Barr virus (EBV), was the first reported pathogenic antigen recognized by $CD4^+$ T cells through macroautophagy-involved endogenous antigen presentation [19]. Furthermore, virus infection-induced macroautophagy has been shown to activate HIV-specific $CD4^+$ T cells [20]. These findings suggest that macroautophagy activity could enhance endogenous antigen processing and presentation through the MHC-II-restricted pathway. As both EBV and HIV cause chronic infection, it is not clear whether acute virus infection, such as IAV infection, could also cause such endogenous antigen presentation by MHC-II.

Influenza virus infection causes half a million deaths annually worldwide [21]. Inactivated influenza vaccines (IIVs) are commonly used for protecting against influenza virus infection. However, their efficacy needs to be improved [22-24]. There are three potential reasons for the lower than expected efficacy of IIVs. First, as IIVs aim at inducing a humoral immune response towards the influenza virus surface proteins Hemagglutinin (HA) and Neuraminidase (NA) [25], the frequent mutations in HA and NA often render such vaccinated antibodies ineffective [26]. Second, IIVs induce limited $CD8^+$ T cell-responses [27, 28], as they do not carry sufficient internal antigens on one hand, whilst on the other hand the minimal internal antigens they do carry have to follow the less efficient cross-presentation pathway [29, 30]. Furthermore,

it is possible that $CD4^+$ T cell priming during IAV infection might rely on endogenous antigen presentation by MHC-II and the presented epitopes might be different from those derived from exogenous antigens. Therefore, understanding the nature of IAV antigen presentation to $CD4^+$ T cells during IAV infection could potentially provide insights into anti-IAV cellular and humoral immune responses for future novel influenza vaccine design.

Macroautophagy is a bulk degradation pathway by which intracellular constituents are cleared and recycled in the cytosol for the maintenance of cellular homeostasis [31, 32]. During this process, more than 36 autophagy-related genes (*ATG*s) are involved in regulating autophagosome formation and autophagic flux [31, 33]. Previous studies have shown that IAV infection induces autophagosome formation [34, 35]. Moreover, it was reported that the M2 protein played a critical role in blocking the fusion of autophagosomes with lysosomes [36, 37]. Furthermore, an IAV antigen M1 covalently linked to a key autophagy molecule LC3 led to M1 endogenous antigen presentation by MHC-II to $CD4^+$ T cells [14]. In contrast, more recently published data demonstrated endogenous IAV antigen presentation by MHC-II without the involvement of macroautophagy in this process [11, 38]. Thus, the role of macroautophagy during endogenous IAV antigen presentation through MHC-II-mediated pathway is still debated. Further characterizing IAV-infection-induced macroautophagy in APCs and its potential role (or the lack of it) in endogenous antigen presentation is of vital importance for better understanding the anti-IAV T helper response.

In this study, we have shown that IAV infection in DCs induces autophagosome formation, prevents autophagosome-lysosome fusion and promotes autophagosome fusion with MHC class II compartment (MIIC). As a result, we demonstrate for the first time that IAV infection-induced autophagosome accumulation contributes to endogenous IAV antigen presentation by MHC-II.

Results

IAV infection leads to endogenous IAV antigen presentation by MHC-II *in vitro*

We previously reported that IAV infection leads to endogenous IAV antigen presentation by MHC-II to $CD4^+$ T cells in EBV transformed human B cells [15]. To explore the associated molecular mechanisms, we utilized the H-2^b-restricted IAV-specific $CD4^+$ T cell epitopes identified previously [39] (Table 1). We first intranasally infected B6 mice with 100 pfu of PR8. 10 days post infection (dpi), T cells recovered in the bronchoalveolar lavage (BAL) and the spleen were assessed by an *ex vivo* intracellular cytokine staining (ICS) assay with the 17 reported MHC-II-restricted peptides, including NP epitopes ($NP_{260-273}$, $NP_{276-290}$, and $NP_{311-325}$), PB2 epitopes ($PB2_{91-105}$ and $PB2_{106-120}$) and PA epitopes ($PA_{316-330}$ and $PA_{456-470}$). We confirmed the $CD4^+$ T cell immunodominance hierarchy specific to the IAV peptides, among which $NP_{311-325}$-specific T cells were the most immunodominant both in the BAL and spleen cell populations (Figure 1A). Subsequently, we generated immunodominant $NP_{311-325}$-specific $CD4^+$ and $NP_{366-374}$-specific $CD8^+$ T cell lines [40], both with high purity and are highly sensitive to their cognate antigenic peptides as shown in Figure 1B. Using these T cell lines, we were able to demonstrate that PR8-infected BMDCs *in vitro* were able to present endogenous IAV antigens onto both MHC-I- and MHC-II-restricted pathways (Figure 1C). Meanwhile, $CD4^+$ T cell activation was abolished when BMDCs were infected with heat-inactivated PR8, although preheated NP protein was still presented to $NP_{311-325}$-specific $CD4^+$ T cells *via* the exogenous antigen processing and presentation pathway (Figure 1D). Additionally, we found that endogenous IAV antigen presentation by MHC-II was almost completely blocked by a protein synthesis inhibitor cycloheximide (CHX) [41] (Figure 1E) and totally blocked by an ER to Golgi transition inhibitor Brefeldin A (BFA) (Figure 1F). These results further confirm that $NP_{311-325}$ presentation after IAV infection relies on IAV infection and replication. The BFA blockade result likely indicates that the endogenous antigen presentation to $CD4^+$ T cells requires *de novo* synthesized MHC-II. In contrast, the subdominant, $PB2_{106-120}$-specific $CD4^+$ T cells could not respond endogenous IAV antigen presentation *in vitro*

(Figure 2), indicating that the efficiency of endogenous IAV antigen presentation to $CD4^+$ T cells *in vitro* is epitope dependent.

Endogenous IAV antigen processing and presentation by MHC-II is reduced by TAP *in vitro*

MHC-I binding peptides are usually generated in the cytosol by proteasomes and delivered into the ER through the TAP1 and TAP2 heterodimer [42]. In contrast, MHC-II presents exogenous antigens *via* a TAP-independent route. However, whether the MHC-II-mediated endogenous IAV antigen presentation required functional TAP was not known. To address this, *Tap1*-deficient BMDCs and mice were infected with IAV and the $CD8^+$ and $CD4^+$ T cell activation was then determined. We found that MHC-I-restricted endogenous IAV antigen presentation was totally blocked in IAV-infected $Tap^{-/-}$ BMDCs as expected (Figure 3A, left), and similar results were obtained from *in vivo* experiments (Figure 3B, left). Interestingly, the endogenous IAV antigen presentation of $NP_{311-325}$ to its specific $CD4^+$ T cells was enhanced in $Tap^{-/-}$ BMDCs *in vitro* (Figure 3A, right), although such an enhanced $CD4^+$ T cell response was not observed *ex vivo* (Figure 3B, right). Similarly, we verified that B6.$Tap1^{-/-}$ mice lacked the $CD8^+$ T cell population in their spleens, indicating their *Tap1* knockout status (Figure 3C) [43]. These results suggest that MHC-II-restricted endogenous IAV antigen processing and presentation is impaired by the presence of functional TAP.

IAV infection induce autophagosome accumulation in dendritic cells

IAV infection induces macroautophagy which enhances IAV replication [35]. To further elucidate the mechanisms of endogenous IAV antigen presentation, we investigated the potential involvement of autophagy pathway. The conversion of the soluble microtubule-associated protein 1 light chain kinase 3 (LC3-I) to lipidated LC3-II, which is subsequently associated with the autophagic membrane, is considered as the most important marker for autophagosome formation [44]. Besides, *Atg6/Beclin1*

activates phosphatidylinositol 3-phosphate (PI3P) deposition at membrane sites to facilitate autophagosome formation and *Atg7* contributes to autophagosome formation *via* its involvement in LC3 conversion [45]. Furthermore, the degradation of SQSTM1/p62 is commonly used as a marker for autophagic flux - reflecting autophagosome:lysosome fusion and the subsequent clearance of intracellular constituents [46]. To determine whether IAV infection regulates macroautophagy in APCs, these autophagy-related protein expression levels were assessed by Western blotting and confocal microscopy in IAV-infected BMDCs and DC2.4 cells, a DC-like cell line [47]. Our results showed that LC3 conversion increased in both PR8- and X31-infected BMDCs relative to that in mock-infected BMDCs (Figure 4A). In addition, the PR8- and X31-infected BMDCs showed increased expression levels of Beclin1 and Atg7 compared to non-infected group. More importantly, the autophagosome marker protein LC3-II increased in a time-dependent fashion in the infected BMDCs (Figure 4A). Similar results were obtained from PR8- and X31-infected DC2.4 cells (Figure 4B). However, here we could not detect p62 degradation within 24 h of virus infection, although IAV matrix protein M1 accumulated during this time (Figures 4A and 4B). Moreover, the increased Atg7 and Beclin1 expression, and the lipidated LC3-II in PR8- or X31-infected DC2.4 cells were also confirmed by confocal microscopy (Figure 4C). Taken together, we demonstrate that IAV infection increases Atg7 and Beclin1 expression and LC3 conversion in DCs, although there is no evidence of autophagosome fusion with lysosome as indicated by lack of p62 degradation.

To further confirm whether the IAV infection-induced macroautophagy leads to increased autophagic flux, we transduced DC2.4 cells with a tandem mCherry-EGFP-LC3B reporter utilizing a retroviral vector [48]. This reporter system detects autophagic flux based on the fact that GFP is unstable in acidic pH, while mCherry is not. Therefore, the green fluorescence will be decreased after autophagosomes fuse with lysosomes. Earle's Balanced Salt Solution (EBSS) was used as a positive control as it induces functional macroautophagy in cells through starvation [49, 50]. Western blotting

showed increased p62 degradation and LC3 conversion in EBSS-treated DC2.4 cells, compared to untreated control (Figure 4D).

Having established the system, we next examined PR8- or X31-infected, mock-infected or EBSS treated DC2.4-mCherry-EGFP-LC3B cells by flow cytometry. Importantly, reduced GFP fluorescence was only observed in the EBSS treated group (Figure 4E), indicating the autophagosomes did not fuse with lysosomes during IAV infection. Collectively, IAV infection induces autophagosome formation in the absence of autophagic flux in DCs.

M2 protein is not required for blocking the fusion of autophagosomes with lysosomes after IAV infection

Previous studies reported that the IAV M2 protein played a critical role in blocking autophagosome-lysosome fusion [36, 37]. To determine whether this is the mechanism in our study, an M2-deficient single-cycle replication virus (M2SR) on the PR8 backbone was utilized [51]. Unexpectedly, we did not observe p62 degradation in both PR8-infected DC2.4 cells and M2SR-infected DC2.4 cells assessed by Western blotting at different time points after virus infection. Virus M1 proteins were detected in both PR8 and M2SR infected cells confirming infected cell status, while virus M2 proteins only accumulated in PR8-infected cells, but not in M2SR-infected cells, confirming M2 knockout status (Figure 4F). Furthermore, the mCherry-GFP reporter system did not show any difference between the PR8 and M2SR infected cells, and the GFP fluorescence downregulation was again only detected in the EBSS-treated control group (Figure 4G). Taken together, in our system, M2 does not contribute to blocking fusion of the autophagosome with the lysosome during IAV-infection-induced macroautophagy.

Inhibition of autophagy impairs endogenous IAV antigen presentation by MHC-II

To verify the role of IAV infection-induced autophagosome accumulation during MHC-II restricted endogenous IAV antigen processing and presentation, autophagy inhibitors 3-methyladenine (3-MA) and E64d were used to downregulate autophagy activity. BMDCs or DC2.4 cells were IAV-infected in the presence of 3-MA or E64d for 24 h and autophagy-related proteins were assessed by Western blotting. LC3 conversion was decreased in IAV-infected BMDCs after 3-MA (at 5 mM) treatment, and no significant LC3-II turnover was observed in IAV-infected BMDCs with E64d (10 μg/ml) treatment, which again reflected IAV infection induced autophagy but in the absence of autophagic flux (Figure 5A). Importantly, drug treatment did not significantly affect cell viability as assessed by the AlamarBlue assay (Figure 5B). To determine whether antigen processing and presentation was altered, 3-MA or E64d treated IAV-infected BMDCs were used as APCs to stimulate $NP_{311-325}$-specific $CD4^+$ T cells. Both 3-MA or E64d treatment clearly diminished $CD4^+$ T cell activation (Figure 5C), although these inhibitors did not significantly inhibit IAV infection judged by NP staining and the mean fluorescence intensity (MFI) in NP^+ BMDCs (Figure 5D). Similar results were obtained from autophagy inhibitor-treated IAV-infected DC2.4 cells (Figures 5E and 5F). These data indicate endogenous IAV antigen presentation by MHC-II is at least partially autophagy dependent.

Knocking out autophagy-related genes *Beclin1* and *Atg7* decreases endogenous IAV antigen presentation by MHC-II

To further explore whether the IAV-infection-induced autophagosome accumulation contributed to endogenous IAV antigen presentation to $CD4^+$ T cells, we turned to a *Cre-loxP* based conditional *Beclin1* knockout mouse model (B6.BecConRosa) under the control of estrogen receptor [52-54]. Tamoxifen was given to B6.BecConRosa mice and control B6 mice for 5 days in order to specifically delete the *Beclin1* gene in these mice, resulting in nearly complete deletion of Beclin1 expression in various tissues examined, including BMDCs (Figure 6A). The lack of Beclin1 expression resulted in

slightly decreased IAV infection efficiency as slightly fewer Bec BMDCs expressed NP than those among B6 BMDCs. However, the MFI of NP^+ BMDCs were on a similar level between B6 BMDCs and Bec BMDCs (Figure 6B). Importantly, endogenous IAV antigen presentation was significantly decreased to less than 1/3 of that detected in B6 BMDCs (Figure 6C), indicating Beclin1 expression was essential for endogenous IAV antigen presentation by MHC-II.

As *Beclin1* is involved not only in autophagy but also other biological processes, such as apoptosis and endocytic trafficking [55-57], to more specifically address if autophagy is a key contributing factor, we turned to a lentiviral-based CRISPR/Cas9 system and created *Beclin1* and *Atg7* single knockout DC2.4 cell lines. Gene knockout was confirmed by Western blotting and Sanger sequencing (Figure 7A, 7B and 7C). Interestingly, DC2.4-*Beclin1*$^{-/-}$ cells, but not DC2.4-*Atg7*$^{-/-}$ cells, showed slightly reduced percentage of NP^+ cells after infection with IAV PR8 strain when compared to control DC2.4-*FgH1tUTG* cells (cells transduced with empty vector) and the MFI of NP^+ DC2.4 cells among these cells were at the same level (Figure 7D). Importantly, both DC2.4-*Beclin1*$^{-/-}$ and DC2.4-*Atg7*$^{-/-}$ cells showed significantly reduced, but almost identical, endogenous IAV antigen presentation to $NP_{311\text{-}325}$-specific $CD4^+$ T cells compared to again the IAV-infected DC2.4-*FgH1tUTG* control cells (Figure 7E). As Atg7's main role is in autophagy [58], these results further strengthen our previous observation made using the conditional *Beclin1* knockout BMDCs.

Comber *et al.* showed clearly that IAV infection induced macroautophagy activity in APCs. However, they did not observe decreased endogenous IAV antigen presentation using *Atg7*-siRNA or *Atg5*-siRNA knockdown BMDCs and *ex vivo* T cell readout in an ELISpot assay [38]. They therefore argued that macroautophagy did not play a significant role in promoting endogenous IAV antigen presentation by MHC-II. As our above experiments were all performed with a single epitope specific $CD4^+$ T cell line, we were concerned that the endogenous IAV antigen presentation might be significantly biased by such an *in vitro* T cell line in our study. We therefore performed

the assays described above, this time using *ex vivo* polyclonal T cells derived from PR8 intranasally infected B6 mice. Clearly, both DC2.4-*Beclin1*$^{-/-}$ and DC2.4-*Atg7*$^{-/-}$ cells showed significantly decreased endogenous IAV antigen presentation to primary CD4^{+} T cells derived from both BAL washes and spleens compared to the DC2.4-*FgH1tUTG* control cells (Figure 8A).

Miller *et al.* identified 17 CD4^{+} T cell epitopes in B6 mice through an overlapping peptide screen [11]. Thirteen of these peptides, ranging from 15AA to 17AA and including seven previously unreported ones (NP_{45-61}, NP_{47-63}, NP_{52-68}, HA_{16-32}, $NS1_{19-35}$, NA_{25-39}, NA_{41-55}, $NA_{110-124}$, PA_{15-31}, PA_{78-93}, $PB1_{119-135}$, $PB2_{18-34}$ and $PB2_{94-110}$), were used to assess endogenous IAV antigen presentation *ex vivo*, and they also concluded that macroautophagy activity did not contribute to endogenous IAV antigen presentation [11]. To see whether the different results obtained in theirs and our experiments were the consequence of using different peptides and assay methods, we used 18-mers peptides HA_{13-30} and HA_{19-36} to cover their HA_{16-32}; NA_{37-54} and NA_{43-60} to cover NA_{41-55}; NP_{43-60}, NP_{49-66} and NP_{55-72} to cover NP_{45-61}, NP_{47-63} and NP_{52-68}; and our NA_{25-42} contains their NA_{25-39}. These peptides stimulated the biggest T cell responses in their experiments. We then assessed CD4^{+} T cells specific to these 18-mer peptides along with our peptide penal and the immunodominant $NP_{311-325}$ peptide in an *ex vivo* ICS assay. Unexpectedly, the percentages of activated primary CD4^{+} T cells specific to these reported epitopes were very low both in the BAL wash and the spleen populations compared to those specific to $NP_{311-325}$, $PB2_{106-120}$, and $PA_{456-470}$ (Figure 8B). Taken together, our data acquired from conditional *Beclin1* knockout BMDCs and independently created *Beclin1* and *Atg7* knockout DC2.4 cell lines all demonstrate that the bulk of endogenous IAV antigen presentation by MHC-II is regulated by IAV-induced macroautophagy, which is true for the most immunodominant epitope $NP_{311-325}$ and is also true for the polyclonal CD4^{+} T cells.

Autophagosomes fuse with MIIC leading to endogenous IAV antigen presentation by MHC-II

To further understand how IAV-infection-induced autophagosome accumulation promoted endogenous IAV antigen presentation by MHC-II, we investigated the relationship among IAV nuclear protein (NP), MHC-II, autophagosome and MIIC in IAV-infected DC2.4 cells through confocal microscopy. First, we detected significant fluorescent signals corresponding to LC3 puncta and NP in both PR8- and X31-infected DC2.4 cells at 24 hpi (Figure 9A and 9B). In contrast, the LC3 puncta accumulation was not observed in mock-infected cells (Figure 9C). A previous study demonstrated that the fusion of the autophagosome with MIIC in a human epithelial cell line and a dendritic cell line contributed to $CD4^+$ T cell activation in an artificial system in which IAV-M1 was fused with LC3 [14]. In order to determine whether fusion of autophagosomes with MIIC contributes to endogenous IAV antigen presentation by MHC-II after IAV infection, the PR8 or X31 infected DC2.4 cells were labelled by antibodies specific to LC3 and H2-M (a MIIC resident protein responsible for replacing the CLIP peptide with an antigenic peptide in murine APCs). Fluorescent puncta corresponding to LC3-II showed co-localization with H2-M (Figure 9A and 9B), which was not observed in mock-infected cells (Figure 9C). Next, the localization of NP and MHC-II was analyzed utilizing antibodies specific to NP or I-A^b, respectively. The fluorescence puncta of NP were co-localized with the fluorescence puncta of MHC-II I-A^b in IAV-infected DC2.4 cells (Figure 9A and 9B). These results indicate that MIIC obtains IAV antigens from autophagosomes, leading to endogenous IAV antigen presentation.

Discussion

We previously demonstrated endogenous IAV antigen presentation to human $CD4^+$ T cells by IAV-infected EBV-transformed B cell lines, although the mechanism was not further explored [15]. This phenomenon is now confirmed in murine DCs in this study

as $NP_{311-325}$-specific $CD4^+$ T cells recognized IAV-infected BMDCs *in vitro*. Such a conclusion was supported by several lines of evidence. First, $NP_{311-325}$-specific $CD4^+$ T cells only recognized DCs infected by live IAV, but not heat-inactivated IAV. Second, antigen presentation was completely blocked by BFA in IAV-infected DCs, indicating the lack of exogenous antigen presentation by surface MHC-II. Finally, $CD4^+$ T cell activation by IAV-infected BMDCs was almost completely blocked by protein synthesis inhibitor CHX.

In MHC-I-restricted endogenous and exogenous antigen cross-presentation, TAP plays a critical role [59]. In our study, the B6.*Tap*$^{-/-}$ mice were utilized to see whether endogenous IAV antigen presentation by MHC-II was also influenced by TAP function. Interestingly, the endogenous IAV antigen presentation by MHC-II was actually enhanced in *Tap*$^{-/-}$ BMDCs *in vitro*. However, the enhanced $CD4^+$ T cell response was not observed in IAV-infected B6.*Tap*$^{-/-}$ mice. It is possible that TAP also reduced endogenous IAV antigen presentation by MHC-II *in vivo*, however, as the endogenous IAV antigen presentation might be anyway above the level required for T cell priming, the $CD4^+$ T cell response in the *Tap*$^{-/-}$ mice remained unchanged. We do not believe that in our system the MHC-II presented peptides are transported by TAP into ER as MHC-II in the ER are blocked by the invariant chain [60]. Instead, we believe that some of the cytosolic antigenic peptides transported into the ER by the TAP molecules become unavailable for endogenous IAV antigen presentation by MHC-II. When TAP function is absent, such peptides become available to the MHC-II endogenous presentation pathway and thus the endogenous IAV antigen presentation is enhanced in *Tap*$^{-/-}$ APCs. Therefore, the functional TAP is not required for MHC-II-restricted endogenous IAV antigen processing.

Autophagy is essential for maintaining cellular homeostasis and plays an essential role in both innate and adaptive immune responses. Our results clearly demonstrated that IAV infection not only induced autophagosome accumulation in DCs as reflected by higher Atg7 and Beclin1 expression and increased LC3 conversion in infected cells, but

also decreased autophagosome-lysosome fusion reflected by lack of p62 degradation and GFP quenching in the GFP-mCherry reporter system in IAV-infected cells. Furthermore, since the M2SR infected cells also showed a lack of p62 degradation and GFP quenching, we excluded M2 as being the critical factor blocking autophagosome-lysosome fusion after IAV infection. These data contradict previous reports on M2 blocking autophagosome fusion with lysosome after IAV infection [36, 37]. However, in either report, no direct evidence was provided to demonstrate the role of M2 in autophagosome-lysosome fusion, for example, in the absence of M2 during IAV infection. Our highly sensitive GFP-mCherry tandem reporter system, coupled with an M2-deficient IAV infection provided direct evidence excluding a role of M2 in autophagosome-lysosome fusion.

Recent studies have shown that the autophagic pathways are involved in endogenous antigen presentation through MHC-II-restricted pathway [20, 61]. Our results from this study clearly showed that the MHC-II-restricted endogenous IAV antigen presentation was significantly inhibited by autophagy inhibitors, or by eliminating autophagy-related genes *Beclin1* and *Atg7 in vitro*. 3-MA and E64d treatment inhibit autophagosome formation [62-64] or inactivate lysosome enzymes respectively [65, 66]. As we were unable to detect autophagosome and lysosome fusion in IAV-infected DCs, yet $NP_{311-325}$ presentation was still significantly diminished by E64d-treated APCs indicating E64d might inhibit some other step of IAV-induced macroautophagy. This observation warrants further investigation. Importantly, the results from autophagy inhibitor-treated APCs were confirmed and strengthened by our results using IAV-infected *Beclin1*- or *Atg7*-deficient DCs.

Intriguingly, macroautophagy has been reported to not contribute to MHC-II- restricted endogenous antigen presentation during IAV infection [38]. This study showed that IAV infection induced functional autophagy in fibroblasts and BMDCs reflected by both autophagosome formation and autophagosome-lysosome fusion. However, no p62 degradation data were provided to support autophagic flux induced by IAV infection.

Furthermore, *Atg7* knockdown by siRNA in these cells did not affect endogenous IAV antigen presentation by MHC-II to *ex vivo* polyclonal $CD4^+$ T cells derived from PR8-infected mice. In contrast, our *Beclin1* and *Atg7* knockout APCs showed significant downregulation on endogenous IAV antigen presentation to similar *ex vivo* polyclonal $CD4^+$ T cells. We believe that this discrepancy might be a result of gene knockdown in their system, compared with total gene knockout used in our study. Hence, it is possible that the residual Atg7 expression might be sufficient to support endogenous IAV antigen presentation by MHC-II to polyclonal $CD4^+$ T cells. Additionally, Miller *et al*. extended such an observation using *ex vivo* $CD4^+$ T cells in an ELISpot assay [11]. As most $CD4^+$ T cell epitopes are about 12AA in length, we used 18-mer peptides covering or containing six of their peptides in an *ex vivo* ICS assay, which is similar to the ELISpot assay and was shown to yield comparable results [67]. None of the selected peptides stimulated major $CD4^+$ T cell response (Figure 8B). It is therefore possible that macroautophagy-induced changes to endogenous IAV antigen presentation might have not been properly assessed using such subdominant $CD4^+$ T cell populations as they are likely relatively insensitive due to limited overall signals. However, as we used a high purity T cell line specific to the most dominant IAV $CD4^+$ T cell epitope $NP_{311-325}$, our assessment is likely more sensitive to such change. Taken together, our results clearly demonstrate an essential role of autophagy in endogenous IAV antigen presentation by MHC-II *in vitro*. This conclusion is not only supported by single epitope-specific T cell line but also by *ex vivo* polyclonal T cells directly derived from IAV-infected mice.

Furthermore, we visualized autophagosomes and MIIC colocalization, indicating fusion of these two compartments, in IAV-infected DC2.4 cells by confocal microscopy. These results collectively demonstrate that IAV-specific $CD4^+$ T cell responses are regulated significantly by the autophagic pathway-mediated endogenous IAV antigen presentation. Our study for the first time demonstrates that IAV infection prevents the autophagosome from fusing with the lysosome, and as a result, the autophagosome

446 delivers cytosolic IAV antigens (including TAP-transportable peptides) into MIIC for
447 MHC-II presentation to $CD4^+$ T cells.
448 Our findings could potentially reshape our thinking on endogenous IAV antigen
449 presentation and, more importantly, future influenza vaccines. Most of the current
450 licensed influenza vaccines are IIVs. As they do not carry infectious IAV and are
451 composed largely soluble IAV proteins, they stimulate limited $CD4^+$ T cell response
452 through largely exogenous antigen presentation. Our data, and those from others [11,
453 38] demonstrate the importance of endogenous IAV antigen presentation by MHC-II.
454 It might be the case that the current IIVs could not provide sufficient $CD4^+$ T cell
455 stimulation as they largely do not access endogenous IAV antigen presentation [11, 39].
456 If some IAV-specific $CD4^+$ T cells, especially immunodominant $CD4^+$ T cells, are
457 primed more efficiently by endogenous IAV antigen presentation under physiological
458 conditions, and if such epitopes are not efficiently presented *via* the exogenous antigen
459 presentation provided by the IIVs, it is then possible that the vaccines might not provide
460 sufficient $CD4^+$ T cell stimulation. This will obviously affect the magnitude of both
461 $CD8^+$ T cell and B cell antibody responses, as $CD4^+$ T cell responses are required to
462 assist both $CD8^+$ T cell memory formation and B cell antibody affinity maturation and
463 class switching [68, 69]. We believe that a lack of these considerations might partly
464 explain the lower than expected protective efficacy of current IIVs against influenza
465 virus infection, especially against IAV infection, in recent years [23, 24]. Hence, the
466 more effective live-attenuated influenza vaccines (LAIV) should be taken into
467 consideration for controlling influenza virus infection. Based on our study, LAIVs
468 should not only prime specific $CD8^+$ T cell-mediated immune responses [70], but also
469 prime sufficient $CD4^+$ T cell response for supporting B cell-mediated high affinity,
470 class-switched antibody production. Since live vaccines may have some biosafety
471 concerns [71, 72], milder live-attenuated IAV viruses, such as the M2SR, would be a
472 better choice for designing future novel LAIVs. In our study, M2SR infection was
473 verified to induce autophagosome accumulation in infected DCs similar to that in the

PR8 infected DCs. in addition, novel M2SR live vaccines were reported to protect mice and ferrets against influenza virus [51, 73]. Therefore, M2SR or a similarly altered influenza virus might have potential as a novel LAIVs' design for the control of human influenza infections.

In summary, endogenous IAV antigens (synthesized within a cell) can be presented through both MHC-I- and MHC-II-restricted pathways. In the MHC-I-restricted pathway, endogenous IAV antigens followed the classical endogenous antigen processing and presentation pathway. In the MHC-II-restricted pathway, endogenous IAV antigens followed a pathway, which is autophagy-enhanced, influenced by TAP function and likely relies on newly synthesized viral proteins and MHC-II molecules.

Materials and methods

Antibodies and reagents

Antibodies for flow cytometry including rat-anti-mouse CD4 (APC and PE-Cy7, clone GK1.5), rat-anti-mouse CD8α (eV450, clone 53-6.7) and rat-anti-mouse IFN-γ (PE, clone XMG1.2) were purchased from eBioscience (Waltham, MA USA). Antibodies for western blotting and confocal microscopy were purchased from various companies: primary polyclonal rabbit-anti-Atg7, polyclonal rabbit-anti-p62 and polyclonal rabbit-anti-LC3B (Sigma-Aldrich); rabbit-anti-Beclin1 (clone D40C5) and rabbit-anti-GPAPDH (clone 14C10) (Cell signalling); anti-mouse MHC-II (I-A/I-E) (clone M5/114.15.2) and anti-mouse H2-M (clone 2E5A) (BD Biosciences). Anti-NP (clone HB-65) [74], anti-M1 (clone M2-1C6) [75], and anti-M2 (clone O19) [76] were kind gifts provided by Dr Jonathan Yewdell, NIAID, NIH (Bethesda, MD, USA). Secondary antibodies, goat-anti-mouse IgG, goat-anti-rabbit IgG were purchased from Jackson ImmunoResearch, goat-anti-mouse, Alexa Fluor 488, goat-anti-rabbit, Alexa Fluor 488, goat-anti-rabbit, Alexa Fluor 594, goat-anti-mouse, Alexa Fluor 555 and goat anti-rat, Alexa Fluor 555 from Invitrogen.

E64d and 3-MA were purchased from Sigma-Aldrich, EBSS from Gibco and AlamarBlue from Invitrogen. Cycloheximide (CHX) was a gift from Dr Jonathan Yewdell, NIAID, NIH (Bethesda, MD, USA).

Cell lines, plasmids and lentiviral or retroviral constructs

DC2.4 (a kind gift from Dr Kenneth L Rock, University of Massachusetts Medical School, MA) [47], 293T, M2CK (MDCK cells stably express M2) [51] and X63-GM-CSF [77] cell lines were cultured in RF-10 (RPMI 1640 containing 10% fetal calf serum (FCS), 2-mercaptoethanol (2-ME) ($5X10^{-5}$ M), and antibiotics). For upregulating MHC-II expression, DC2.4 cells were pretreated with 1 ng/ml of IFN-γ in RF-10 for 24 h.

DC2.4-*Beclin1*$^{-/-}$, DC2.4-*Atg7*$^{-/-}$ and DC2.4-*FgH1tUTG* cell lines were generated using CRISPR/Cas9 system [78]. Lentiviral particles were generated by transfection of FuCas9Cherry (Addgene#70182) along with packaging plasmids pMDL (Addgene#12551), pRSV-REV (Addgene#12553), and VSV-G (Addgene#12559) encoding lentiviral structural components into 293T cells for 48 h. DC2.4 cells were firstly transduced with FuCas9Cherry lentiviral supernatant containing 4 μg/ml polybrene through spinning infection at 2500 rpm, 32°C for 2 h. After constructing the DC2.4-Cas9 cell line, the lentiviral particles containing mBeclin sgRNAs, mAtg7 sgRNAs or FgH1tUTG empty vector (Addgene#70183, negative control) were generated from 293T cells with the same transfection method, then the DC2.4-Cas9 cells were transduced by these lentiviruses as described above. The transduced cells were cloned *via* single cell sorting on a FACS ARIA III and cultured in RF-10 containing 1 ng/ml doxycycline.

pBABE-puro-mCherry-EGFP-LC3B (Addgene#22418) is a retroviral based tandem report system for detecting autophagic flux [48]. Retroviral particles were generated by transfection LC3B plasmid along with packaging plasmids pCMV-Gag-Pol (Addgene#12263) and pCAG-Eco (Addgene#35617) encoding retroviral structural

components into 293T cells for 48 h. The DC2.4-mCherry-EGFP-LC3B cell line was generated with retroviral transduction.

Mice

Female C57BL/6 (B6) were purchased from the Walter Eliza Hall Institute of Medical Research animal facility (Kew, Melbourne, Australia). B6.Beclin1 conditional floxed mice ($Becn1^{tm1a(KOMP)Wtsi}$) were purchased from genOway and crossed onto B6.ROSA26-CreERT2 mice [79] to generate the "BecConRosa" mouse strain. This enabled the ubiquitous deletion of the *Beclin1* gene following tamoxifen administration. B6.$Tap^{-/-}$ mice were a gift from Professor Luc van Kaer (School of Medicine, Vanderbilt University, Nashville, TN) [43]. Mice were bred under SPF conditions at La Trobe University. All animal experiments were approved by the La Trobe University Animal Ethics Committee and performed under the National Health and Medical Research Council of Australia guidelines.

Bone-Marrow Derived Dendritic cells (BMDCs)

For BMDC culture, 2 x 10^6 bone marrow cells collected from naive B6 mice, tamoxifen-induced B6.BecConRosa or control B6 mice were grown in a 10 cm petri dish (Corning) in 10 ml RF-10 containing 10% of X63-GM-CSF supernatant (containing-10 ng/ml granulocyte-macrophage-colony-stimulating factor, GM-CSF) [77]. Fresh culture medium (10 ml) was added on day 3 and day 6, respectively. On day 8, non-adherent BMDCs were harvested.

Antigen-specific T cell culture

The primary $CD8^+$ T cell lines were established according to our published method [80]. Briefly, splenic cells from IAV-infected memory mice were co-cultured with $NP_{366-374}$-pulsed and irradiated-BMDCs. For establishing IAV-specific $CD4^+$ T cell line, CD8-depleted splenocytes were co-cultured with $NP_{311-325}$- or $PB2_{106-120}$-pulsed and

557 irradiated-BMDCs. All the cell lines were cultured in RF-10 supplement with 1X MEM
558 NEAA (Gibco), 1X sodium pyruvate (Gibco) and hrIL-2 (10 unit/ml, PeproTech). The
559 IAV specific $CD4^+$ and $CD8^+$ T cell lines were then re-stimulated by peptide-pul sed
560 BMDCs regularly till high purity.
561
562 **Influenza A virus and infection**
563 Influenza A virus PR8 (A/Puerto Rico/8/34, H1N1) was obtained from Professor
564 Lorena Brown (University of Melbourne, Australia). M2SR is a PR8 based, M2
565 deletion mutant and prepared in M2CK cells (MDCK cells stably express PR8 M2) [51].
566 X31 (A/X-31, H3N2) was obtained from Dr Jonathan Yewdell, NIAID, NIH (Bethesda,
567 Maryland USA). PR8 and X-31 viruses were propagated in 10-day-old embryonated
568 chicken eggs, titered and stored as previously described [81]. Virus stock was heat
569 inactivated by incubation in a 65°C water bath for 1 h. For *in vitro* infection, cells were
570 infected at multiplicity of infection (MOI) of 1 of PR8, M2SR or X31 for 1 h in 200 µl
571 acidified RPMI-1640 medium, pH 6.8). RF-10 (2 ml) medium was then added, and the
572 cells were cultured for 24 h in a 37°C incubator with 5% CO_2. For *in vivo* infection,
573 mice were intranasally infected with 100 pfu PR8 or 1000 pfu X31 (in 40 µl PBS).
574
575 **Preparation of rVV-infected P815 cell lysates**
576 P815 cells were infected with rVV-CR19 (negative control), or rVV-NP or rVV-PB2
577 (kind gifts from Dr Jonathan Yewdell, NIAID, NIH, Bethesda, MD, USA) at a MOI of
578 10 for 1 h at 37°C in 0.1% (w/v) BSA/PBS, followed by addition of 10 volumes of RF-
579 10 medium followed by overnight incubation. Infected cells were pelleted and lysed
580 with 8 M urea [15]. The lysates were aliquoted and stored at -20°C until use.
581
582 **Peptides, intracellular cytokine staining (ICS) and flow cytometry**
583 All the peptides were synthesized by Mimotopes (Clayton, Melbourne, Australia).

For *in vitro* ICS assays, BMDCs and/or DC2.4 cells were infected with PR8 (MOI = 1) or X31 (MOI = 1), or pulsed with PR8 NP protein (10 μg/ml, Sino Biological, Beijing, China) or pulsed with rVV-infected P815 lysate (40 μmol, 5 μl in volume, equivalent to 10^5 infected cells). After 24 h, these BMDCs were co-cultured with IAV-specific $CD4^+$ or $CD8^+$ T cell line for 5 h in the presence of 10 μg/ml brefeldin A (BFA). Cells were then stained for surface makers CD8 and CD4, fixed by 1% (v/v) paraformaldehyde (PFA), and subsequently stained for intracellular IFN-γ in PBS containing 0.2% (v/v) saponin [82]. Cells staining was analyzed on a FACSCanto II flow cytometer (BD Biosciences) or Cytoflex (Beckman), and data were analyzed with FlowJo software (FlowJo VX, Ashland, OR, USA).

For *ex vivo* ICS assay, bronchoalveolar lavage wash and spleens were harvested from mice 10-day post IAV infection. Cells were stimulated in RF-10 medium with 10^{-5} M MHC-II-restricted peptides or 10^{-6} M MHC-I-restricted peptides for 5 h in the presence of 10 μg/ml BFA. Intracellular cytokine staining for IFN-γ and data analysis were performed as described above.

Western blotting

For adherent cell lines, cell monolayers were washed twice with cold PBS and treated with RIPA lysis buffer (Sigma-Aldrich) on ice. For suspension cells, cells were washed twice in cold PBS, pelleted and lysed with RIPA buffer. Lysate protein concentration was quantified by a Bio-Rad protein assay kit (Bio-Rad). Equal amounts of samples (10 μg) were separated on SDS-PAGE gels before being transferred onto nitrocellulose membranes (Thermo), incubated with primary and then corresponding secondary antibodies. The protein bands were visualized by an ECL Plus kit (GE Healthcare).

Confocal microscopy

DC2.4 cells infected with PR8 or X31 at MOI =1 for 24 h were washed with PBS and fixed with 4% (v/v) paraformaldehyde for 20 min at room temperature. Cell monolayers

were permeabilized with 0.2% (v/v) Triton X-100 for 10 min, blocked with PBS containing 5% bovine serum albumin (BSA) for 1 h at room temperature, and then incubated with primary antibodies (anti-LC3B, anti-Atg7, anti-Beclin1 anti-NP, anti-MHC-II or anti-H2-M) at 4 °C overnight, followed by 1 h incubation with secondary antibodies (goat-anti-rabbit 594, or goat-anti-mouse 488, or goat-anti-rat 555) at 37°C. The fluorescence signals were visualized with a Zeiss confocal fluorescence microscope 780 (Zeiss, German).

Author contributions

Perform experiments: JD, CLu, CLiu and SO; Experimental design: WC and JD; Manuscript writing: JD and WC; Resources: WDF, EFL, PB and HP; Funding acquisition: WC.

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Declaration of interest

PB is an employee of FluGen, the developer of the influenza vaccine candidate, M2SR.

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Figure legends

Figure 1. Endogenous IAV antigen presentation by MHC-II.

(A) B6 mice were intranasally infected with 100 pfu of PR8, the $CD4^+$ T cell response in the BAL or spleen of IAV-infected mice were assessed using 17 MHC-II-restricted peptides on 10 dpi *via* standard ICS assay. NIL indicates no peptide addition. (B) $NP_{311-325}$-specific $CD4^+$ T cell line and $NP_{366-374}$-specific $CD8^+$ T cell line were stimulated by $NP_{311-325}$ and $NP_{366-374}$ peptides, respectively, at various peptide concentrations in the

892 presence of BFA for 5 h. Error bars indicate the ± SEM of 5 independent experiments
893 (n=5). (C) Endogenous IAV antigen presentation was assessed by PR8 infected
894 BMDCs (MOI=1, 24hpi). Infected-BMDCs were then co-cultured with $NP_{311-325}$-
895 specific $CD4^+$ T cells and $NP_{366-374}$-specific $CD8^+$ T cells in the presence of BFA for 5
896 h. (D) BMDCs infected with PR8 (MOI=1, 24h), inactivated-PR8 (65°C for 1 h, MOI=1,
897 24h), pulsed with exogenous NP (10 μg/ml, 24h) or pulsed with heated-NP (65°C for 1
898 h, 10 μg/ml, 24h). These APCs were then co-cultured with $NP_{311-325}$-specific $CD4^+$ or
899 $NP_{366-374}$-specific $CD8^+$ T cells in the presence of BFA for 5 h. (E) BMDCs were
900 pretreated with or without 25 μg/ml CHX for 20 min before being infected with PR8
901 (MOI=1) and for further 24 h before being co-cultured with $NP_{311-325}$-specific $CD4^+$ T
902 cells in the presence of BFA for 5 h. (F) BMDCs infected with PR8 at MOI=1 were
903 treated with or without 10 μg/ml BFA for further 24 h before being co-cultured with
904 $NP_{311-325}$-specific $CD4^+$ T cells in the presence of BFA for 5 h. The IFN-γ productions
905 in the above experiments were detected by standard ICS assays (APCs: T=2:1), and
906 IFN-γ^+ T cells were expressed as a percentage of total $CD4^+$ or total $CD8^+$ T cells,
907 respectively. Experiments were performed twice with similar results (A, D, E, F; n=2).
908
909 **Figure 2. Endogenous IAV antigen presentation by MHC-II *in vitro* is epitope-**
910 **dependent.**
911 (A) $NP_{311-325}$-specific $CD4^+$ T cells (B) $PB2_{106-120}$-specific $CD4^+$ T cells were co-
912 cultured with PR8-infected BMDCs, or relevant rVV lysate-pulsed BMDCs in the
913 presence of BFA for 5 h (APCs: T=2:1). (C) $NP_{311-325}$-specific $CD4^+$ T cells or (D)
914 $PB2_{106-120}$-specific $CD4^+$ T cells were stimulated by $NP_{311-325}$ peptide or $PB2_{106-120}$
915 peptide, respectively, at various peptide concentrations in the presence of BFA for 5 h.
916 IFN-γ production was detected by standard ICS assay, and IFN-γ^+ $CD4^+$ T cells were
917 expressed as a percentage of total $CD4^+$ T cells. Experiments were performed twice
918 with similar results (A, B, C, D; n=2).
919

Figure 3. Endogenous IAV antigen presentation by MHC-II is reduced by functional TAP.

(A) WT BMDCs or *Tap*$^{-/-}$ BMDCs were infected with PR8 (MOI=1) or X31 (MOI=1). 24 hpi, the IAV-infected APCs were co-cultured with $NP_{366-374}$-specific $CD8^+$ or $NP_{311-325}$-specific $CD4^+$ T cells in the presence of BFA for 5 h (APCs: T=2:1). IFN-γ production was detected by standard ICS assays, and IFN-γ^+ T cells were expressed as a percentage of total $CD4^+$ or total $CD8^+$ T cells. Error bars indicate the ± SEM of experimental replicates. Two independent experiments were performed with similar results, and each independent experiment included 3 biological replicates (n=6). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 and ns P > 0.05 (determined by unpaired two-tailed Student's *t*-test). (B) WT B6 mice and B6.*Tap1*$^{-/-}$ mice were infected with 1000 pfu of X31. Antigen-specific T cells in the BAL washes were assessed using 17 MHC-II-restricted peptides and 4 MHC-I-restricted peptides *via* standard ICS assays at 10 dpi. IFN-γ^+ T cells were expressed as a percentage of total $CD4^+$ or total $CD8^+$ T cells. NIL indicates no peptide addition. Error bars indicate ±SEM of 3 mice per group. Experiments were performed twice with similar results (n=6). (C) Spleen T cell phenotypes of WT B6 and B6.*Tap1*$^{-/-}$ mice (n=3).

Figure 4. IAV infection induces autophagosome accumulation in dendritic cells.

(A) BMDCs or (B) DC2.4 cells were mock-infected or infected with PR8 or X31 for 6, 12 and 24 h. At the end of the infection, the expression levels of Atg7, Beclin1, p62, M1, LC3 and GAPDH (loading control) were analyzed by western blotting. Experiments were performed 3 times (n=3) with similar results. (C) DC2.4 cells were mock-infected or infected with PR8 or X31. At 24 hpi, the cells were fixed and then assessed by indirect immunofluorescence using antibodies against Beclin1, Atg7 or LC3, respectively, followed by the corresponding secondary antibody conjugated to FITC. The cell nucleuses were stained by DAPI. The fluorescent signals were visualized by confocal microscopy, scale bar: 10 μm. The fluorescence intensity of

Beclin1, Atg7 and LC3 in each group was graphed on the right side. Error bars indicate the ± SEM of 60 cells per group from two independent experiments (n=60). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ and ns $P > 0.05$ (determined by one-way ANOVA followed by a Dunnett test). (D) DC2.4 cells were treated with or without EBSS for 24 h and the expression levels of p62, LC3 and GAPDH (loading control) were analyzed by western blotting. (E) DC2.4-mCherry-EGFP-LC3B cells were mock-infected, infected with PR8 or X31, or treated with EBSS for 24 h. Cells were analyzed by flow cytometry for GFP and mCherry fluorescence intensity. (F) DC2.4 cells were PR8-infected or M2SR-infected for 6, 12, or 24 h (MOI=1). The expression levels of p62, M1, LC3, M2 and GAPDH (loading control) were analyzed by western blotting. (G) DC2.4-mCherry-EGFP-LC3B cells were mock-infected, infected with PR8 or M2SR, or treated with EBSS for 24 h. Cells were analyzed by flow cytometry for GFP and mCherry fluorescence intensity. Experiments were performed 3 times with similar results (D, E, F, G; n=3)

Figure 5. Autophagy inhibitors downregulate endogenous IAV antigen presentation by MHC-II.

(A) BMDCs were infected with PR8 or X31 in the presence or absence of 3-MA (5 mM) or E64d (10 μg/ml). At 24 hpi, cell lysates were prepared and analyzed by western blotting using anti-LC3, anti-p62, anti-M1 and anti-GAPDH (loading control). (B) The cell viability of BMDCs or DC2.4 cells were detected by AlamarBlue kit at 24 h after cells were treated by control, 3-MA or E64d. Error bars indicate ± SD of 5 biological replicates (n=5) per group. Experiments were performed twice with similar results. (C) BMDCs were infected with PR8 or X31, or mock infected, in the presence or absence of 3-MA or E64d. At 24 hpi, endogenous IAV antigen presentation by these BMDCs were assessed with $NP_{311-325}$-specific $CD4^+$ T cells in the presence of BFA for 5 h (APCs: T=2:1) and revealed by standard ICS assays. IFN-γ^+ T cells were expressed as a percentage of total $CD4^+$ T cells. (D) Virus infection efficiency and the MFI of

infected BMDCs described in (C) were assessed by IAV NP expression *via* flow cytometry. (E) DC2.4 cells were infected and their endogenous IAV antigen presentation (E) and virus infection efficiency and the MFI of infected DC2.4 cells (F) as described in (C and E). Error bars indicate ± SEM of experimental replicates. Similar results were obtained from two independent experiments with 3 biological replicates (C, D, E, F; n=6). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ and ns $P > 0.05$ (determined by one-way ANOVA followed by a Dunnett test). NA means not available.

Figure 6. Autophagy-related gene *Beclin1* contributes to endogenous IAV antigen presentation by MHC-II.

(A) *In vitro* cultured BMDCs from tamoxifen-treated B6 or B6.BecConRosa mice were assessed for Beclin1 and GAPDH (loading control) expression by western blotting. Experiments were performed 3 times (n=3) with similar results. (B) BMDCs in (A) were mock infected or infected with PR8 or X31. At 24 hpi, virus infection efficiency and the MFI of infected BMDCs were assessed by IAV NP expression *via* flow cytometry. (C) Endogenous antigen presentation by BMDCs described in (B) was assessed by $NP_{311-325}$-specific $CD4^+$ T cells *via* standard ICS assays (APCs: T=2:1). Error bars indicate ±SEM of the experimental replicates. Two independent experiments were performed with similar results, and each independent experiment included 3 biological replicates (B, C; n=6). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ and ns $P > 0.05$ (determined by one-way ANOVA followed by a Dunnett test). NA means not available.

Figure 7. Not only *Beclin1* but also *Atg7* contributes to endogenous IAV antigen presentation by MHC-II.

(A) Knockout DC2.4 cell lines were confirmed by western blotting for lack of the targeted protein expression. Experiments were performed 3 times (n=3) with similar

1004 results. (B) DC2.4-*Beclin1*$^{-/-}$ knockout status was confirmed by Sanger sequencing. A
1005 part of cDNA sequence and its amino acid sequence translated from related cDNA
1006 between WT DC2.4 cells and DC2.4-*Beclin1*$^{-/-}$ cells were displayed. (C) DC2.4-*Atg7*$^{-/-}$
1007 knockout status was confirmed by Sanger sequencing. A part of cDNA sequence and
1008 its amino acid sequence translated from related cDNA between WT DC2.4 cells and
1009 DC2.4-*Atg7*$^{-/-}$ cells were displayed. (D) DC2.4-*FgH1tUTG* (control), DC2.4-*Beclin1*$^{-/-}$,
1010 and DC2.4-*Atg7*$^{-/-}$ cells were mock infected or infected with PR8 or X31. At 24 hpi,
1011 virus infection efficiency and the MFI of infected DC2.4 cells were assessed by IAV
1012 NP expression *via* flow cytometry. (E) Endogenous antigen presentation by DC2.4 cells
1013 treated as described in (E) was assessed by $NP_{311-325}$-specific $CD4^+$ T cells *via* standard
1014 ICS assays (APCs: T=2:1). Error bars indicate ±SEM of the experimental replicates.
1015 Two independent experiments were performed with similar results, and each
1016 independent experiment included 3 biological replicates (D, E; n=6). *$P < 0.05$; **$P <$
1017 0.01; ***$P < 0.001$; ****$P < 0.0001$ and ns $P > 0.05$ (determined by one-way ANOVA
1018 followed by a Dunnett test). NA means not available.
1019
1020 **Figure 8. Lack of *Beclin1* or *Atg7* decreases endogenous IAV antigen presentation**
1021 **to *ex vivo* polyclonal $CD4^+$ T cells.**
1022 B6 mice were intranasally infected with 100 pfu of PR8. Primary T cells were collected
1023 from BAL washes and spleens at 10 dpi. Endogenous IAV antigen presentation to (A)
1024 primary $CD4^+$ T cells in BAL washes (top) and spleens (bottom) were assessed by
1025 mock-infected or PR8-infected DC2.4-*FgH1tUTG* (control), DC2.4-*Beclin1*$^{-/-}$, or
1026 DC2.4-*Atg7*$^{-/-}$ cells *via* standard ICS assays (APCs:T=2:1). (B) Selected peptides
1027 (HA_{13-30}, HA_{19-36}, NA_{25-42}, NA_{37-54}, NA_{43-60}, NP_{43-60}, NP_{49-66}, and NP_{55-72}) covering the
1028 reported novel epitopes (NP_{45-61}, NP_{47-63}, NP_{52-68}, HA_{16-32}, NA_{25-39}, and NA_{41-55}) and 3
1029 other positive control peptides ($NP_{311-325}$, $PB2_{106-120}$, and $PA_{456-470}$) were used to assess
1030 $CD4^+$ T cell response to IAV infection in the BAL washes and the spleens by standard
1031 ICS assays. Cells in the BAL washes from 4 independent mice were pooled together

for the analysis, while the cells in each spleen were separately assessed. NIL indicates no peptide addition. Error bars in the spleen group indicate ± SEM of 4 mice (n=4) per group. Experiments were performed twice with similar results (n=8). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ and ns $P > 0.05$ (determined by one-way ANOVA followed by a Dunnett test).

Figure 9. Autophagosomes fuse with MIIC after IAV infection.

DC2.4 cells were infected with (A) PR8 or (B) X31 (MOI=1) or (C) mock-infected for 24 h before being stained by anti-LC3, anti-NP, anti-I-A^b and anti-H2-M antibodies. Scale bar: 10 μm. The intensity profile of the region of interest (ROI) lines show the consistent intensity of green and red channels respectively, which present the co-localization between the two channels. Experiments were performed 3 times (n=3) with similar results.

Table 1 Peptide information

Peptide name	**Sequence**
$HA_{211-225}$	YVQASGRVTVSTRRS
$HA_{261-275}$	INSNGNLIAPRGYFK
$HA_{276-290}$	MRTGKSSIMRSDAPI
$HA_{326-340}$	KQNTLKLATGMRNVP

NP_{91-105}	KTGGPIYRRVNGKWM
$NP_{136-150}$	MMIWHSNLNDATYQR
$NP_{146-160}$	ATYQRTRALVRTGMD
$NP_{201-215}$	INDRNFWRGENGRKT
$NP_{211-225}$	NGRKTRIAYERMCNI
$NP_{260-273}$	ARSALILRGSVAHK
$NP_{276-290}$	LPACVYGPAVASGYD
$NP_{311-325}$	QVYSLIRPNENPAHK
$NP_{316-330}$	IRPNENPAHKSQLVW
$PB2_{91-105}$	VSPLAVTWWNRNGPM
$PB2_{106-120}$	TNTVHYPKIYKTYFE
$PA_{316-330}$	GWKEPNVVKPHEKGI
$PA_{456-470}$	RATEYIMKGVYINTA
$PA_{224-233}$	SSLENFRAYV
$PB1F2_{62-70}$	LSLRNPILV
$PB1_{703-711}$	SSYRRPVGI
$NP_{366-374}$	ASNENMETM
NP_{43-60}	MCTELKLSDYEGRLIQNS
NP_{49-66}	LSDYEGRLIQNSLTIERM
NP_{55-72}	RLIQNSLTIERMVLSAFD
HA_{13-30}	AAADADTICIGYHANNST
HA_{19-36}	TICIGYHANNSTDTVDTV
NA_{25-42}	QIGNIISIWISHSIQTGS
NA_{37-54}	SIQTGSQNHTGICNQNII
NA_{43-60}	QNHTGICNQNIITYKNST

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