Requirement of autophagy proteins Beclin-1 and Map1-LC3b in the induction of innate and adaptive immune responses to respiratory syncytial virus infection

by

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Abstract

Respiratory syncytial virus (RSV) is a ubiquitous human pathogen that produces severe lower respiratory disease in vulnerable individuals, characterized by bronchiolitis, excessive mucus production, and immune-mediated lung damage. Severe RSV bronchiolitis in infancy is strongly correlated with development of recurrent wheezing later in childhood, suggesting persistent alteration of the lung immune environment long after successful viral clearance. Within the lung environment, dendritic cells (DCs) direct innate and adaptive immune responses to viral pathogens through secretion of pro-inflammatory cytokines and type I interferon (IFN), as well as through activation of antiviral T cell responses. Signaling through the RNA-sensing intracellular toll-like receptors (TLR) -3 and -7 is required for robust production of type I IFN and antigen presentation function by DCs during viral infection. The intracellular process of macroautophagy (autophagy), or the sequestration of intracellular contents in a double-walled membrane for degradation by lysosomes, promotes TLR-dependent innate cytokine production and antigen-presenting cell function by RSV-infected DCs. The autophagic pathway further limits inflammation by degrading assembled inflammasome platforms and their substrate pro-IL-1β, suggesting that autophagy may also block excessive pro-inflammatory signaling during infection. However, the requirement for autophagy in promoting antiviral responses and limiting lung pathology during *in vivo* RSV infection has yet to be investigated.

Using a mouse model of lower respiratory tract infection, we found that deficiency in the autophagy proteins Beclin-1 (Becn1) or Map1-LC3b (LC3b) led to increased lung immunopathology upon infection, characterized by delayed viral clearance, increased mucus secretion, and altered T cell cytokine production. $Becn1^{+/-}$ and $LC3b^{-/-}$ DCs were impaired in antiviral cytokine production, and elicited Th2- or Th17- skewed responses from co-cultured CD4+ T cells, respectively. Furthermore, studies using bone-marrow chimeric mice revealed a critical role for LC3b in restricting

ER stress-dependent, inflammasome-mediated production of IL-1β by infected airway epithelial cells. Finally, neutralization of IL-17 or IL-1 receptor signaling *in vivo* ameliorated the development of lung pathology in LC3b$^{-/-}$ mice. Overall, these studies shed light on the importance of autophagy to the induction of antiviral immune responses and control of inflammation during RSV infection, and establish the autophagy pathway as a potential target for future therapeutic interventions.

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Chapter 1 – Introduction

Human respiratory syncytial virus (RSV) is an enveloped, single-stranded, negative-sense RNA virus in the family *Paramyxoviridae*. It is a ubiquitous pathogen which infects the majority of children during their first year of life (1). Since being first isolated from a sick chimpanzee in the 1950s (2) and later from infants with pneumonia (3), RSV has come to be recognized globally as the most important cause of lower respiratory tract infection among infants and young children. Worldwide, approximately 30 million cases of acute lower respiratory tract infection (LRTI) with RSV occur each year in children under the age of 5, with as many as 4 million requiring hospitalization (4). RSV is also a significant cause of morbidity among the elderly, immune deficient, and immunocompromised (5, 6), as well as for individuals with chronic pulmonary disease (7). Despite several decades of research, no safe and effective vaccine is yet available, and treatment for patients hospitalized with severe RSV infection largely consists of supportive care (8). It is therefore paramount to understand the immunological mechanisms underlying the development of severe RSV infections in order to develop novel pharmacological treatments for established infections, as well as to ultimately inform vaccine development.

1a. Respiratory Syncytial Virus

1a.i. Virology and immune evasion by RSV

The RNA genome of RSV consists of 10 genes, which encode 11 proteins: Two non-structural proteins (NS1 and NS2), nucleoprotein (N), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH), G glycoprotein (G), fusion glycoprotein (F), two matrix protein variants from alternate reading frames (M2-1 and M2-2), and finally the large RNA polymerase protein (L) (see Figure 1-1). The viral envelope is derived from the host cell plasma membrane, and contains the transmembrane G, F, and

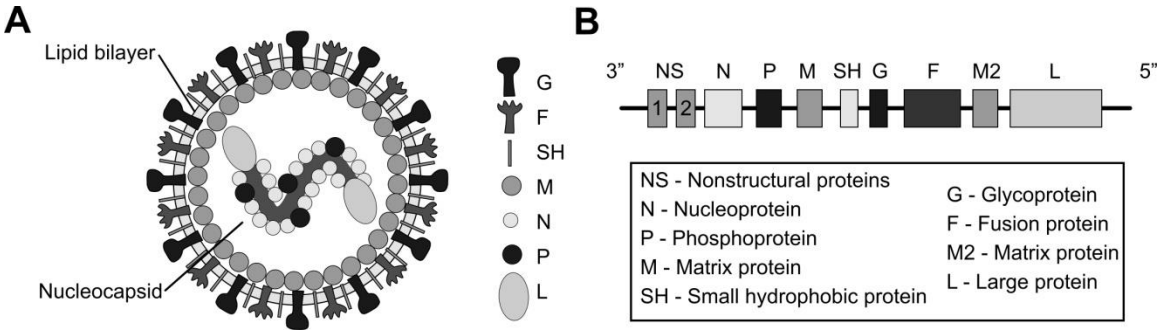

Figure 1-1 - Human respiratory syncytial virus. Virion structure (A) and ssRNA genome (B).

SH proteins. The M and M2 proteins assemble underneath the envelope, while the L, P, and N proteins associate with the single-stranded RNA (see Figure 1-1).

The G protein harbors heparin-binding domains that facilitate attachment to glycosoaminoglycans present on the surface of target cells (9, 10), while the F protein binds nucleolin and mediates fusion of the viral envelope and cell plasma membrane, releasing the nucleocapsid into the cytoplasm (11, 12). Upon entry, the L protein initiates 3’ to 5’ transcription of the negative-sense RNA, beginning at a single promoter near the 3’ end (13–16). As a result, a series of subgenomic mRNAs are produced, and relative protein abundance is dictated by proximity to the 3’ end of the viral genomic RNA (14). Virion assembly then takes place at the plasma membrane, where the nucleocapsid localizes with viral envelope proteins expressed on the host cell membrane. Virions bud off of the apical surface of polarized alveolar epithelial cells, where they form clusters of long filaments that extend from the cell surface (17).

RSV infection is typically restricted to the respiratory epithelium and intraepithelial immune cells lining the nasal and respiratory tracts (18, 19). Unlike influenza virus, RSV induces little cytopathology in *in vitro* models of polarized respiratory epithelium (20–22), at least partially due to its ability to delay programmed cell death or apoptosis of host cells. RSV infection induces the upregulation of numerous genes encoding inhibitors of apoptosis, including the Bcl-2 family members Bcl-XL and myeloid cell leukemia-1 (23–25). Other potential anti-apoptotic mechanisms employed by RSV include inhibition of tumor suppressor protein p53 and AKT activation (26) and phosphatidylinositol 3-kinase-dependent increases in ceramidase and sphingosine kinases (27, 28). Further evidence suggests that the NS-1, NS-2, and SH RSV proteins facilitate

viral replication through suppression of premature apoptosis, resulting in higher viral titers (29, 30).

In addition to inhibiting apoptosis in host epithelial cells, RSV modulates pattern-recognition receptor (PRR) signaling and resultant cytokine production by the host. Epithelial cell surface expression of TLR4 is upregulated within 24 hours of RSV infection *in vitro*, which primes cells for enhanced IL-6 and IL-8 production upon exposure to lipopolysaccharide (31, 32). In addition, the RSV F protein interacts with TLR4 and CD14 on human monocytes, promoting the production of TNF-α, IL-12, and IL-6 (33). Conversely, the NS-1 and NS-2 proteins inhibit production of type I interferons by infected host cells through promoting degradation of STAT-2 and through binding the cytoplasmic PRR retinoic acid-inducible gene-1 (RIG-I), thereby inhibiting activation of the downstream transcription factor interferon response factor 3 (IRF3) (34, 35). RSV further inhibits type I interferon production and pro-inflammatory cytokine production through antagonism of the TLR adaptor myeloid differentiation factor 88 (MyD88) (34), as well as through upregulation of suppressor of cytokine synthesis (SOCS) gene expression (36).

The RSV-G protein antagonizes host immune responses through several different mechanisms. The protein is produced in both a full-length, membrane-bound version (RSV-G_m), as well as in a truncated secreted form (RSV-G_s). As the G protein is an important immunological epitope, secreted G_s may serve as a decoy for neutralizing antibodies (37). RSV-G_s protein present in the cytoplasm of an infected cell blocks TLR3/4-dependent type I interferon production through binding the TLR adaptor protein TNF receptor-associated factor (TRAF) (38, 39). The RSV-G protein also bears a conserved CX3C motif, suggesting that RSV-G_s is capable of binding the fractalkine receptor CX3CR1 and altering the chemotactic activity of leukocytes. Whether this serves to recruit leukocytes to the lungs, or to block the effects of endogenous fractalkine, is not well understood (40). Finally, the conserved cysteine-rich region of RSV-G_s acts as a potent TLR antagonist *in vitro*, thereby down-regulating TLR-2, TLR-4, and TLR-9-mediated inflammatory cytokine production (37, 40).

Taken together, these varied mechanisms of viral evasion likely contribute to the unique epidemiological and immunological characteristics in human patient populations. Although RSV has a single serotype with two major antigenic subgroups, the induction of immunologic memory to RSV is short-lived, and reinfection throughout life is common (1). As no protective vaccine is currently available, it is of vital importance to understand the contribution of epidemiologic, immunologic, and environmental risk factors that predispose individuals to severe RSV infection.

1a.ii. Epidemiology of RSV infection

Respiratory syncytial virus (RSV) is a highly infectious and omnipresent pathogen, with up to 70% of infants being exposed within their first year of life (41–46). The typical clinical manifestation of disease is a mild upper respiratory infection, with 20-30% of children experiencing LRTI during the course of infection (47). Approximately 1-3% of the annual birth cohort develops LRT bronchiolitis necessitating hospitalization, with up to 10% of those hospitalized with RSV-associated bronchiolitis requiring admittance to the intensive care unit (41, 42, 45, 48, 49). While numbers vary by year, 132-172,000 infants and young children are hospitalized in the United States each year with RSV bronchiolitis (50). As many as 200,000 children under the age of 5 are estimated to die every year from severe RSV infections worldwide, predominantly in developing countries (4).

RSV is unusual in that reinfection is common among young children, with most individuals experiencing a decrease in severity of symptoms upon recurrent exposure (41, 45). While older children and adults have a low incidence of lower respiratory tract involvement upon reinfection, individuals who are immunocompromised or elderly are at higher risk of developing severe LRTI (5, 6, 51–54). This is especially true among individuals with chronic lung conditions such as asthma and chronic obstructive pulmonary disease, and is responsible for considerable morbidity among these patient populations (7, 55–57).

The global health burden presented by acute RSV infection is further compounded by a strong correlation between infant RSV infection and recurrent

wheezing later in childhood. The development of severe RSV bronchiolitis necessitating hospitalization is independently predictive of subsequent childhood asthma in multiple birth cohort studies (58–68), while others have identified a correlation between clinical severity of bronchiolitis and increased asthma risk (69). Further evidence of a causal relationship between RSV bronchiolitis and childhood asthma comes from studies of pre-term infants receiving prophylactic treatment with Palivizumab, a monoclonal antibody to RSV that significantly reduces the severity of RSV bronchiolitis (70). Among multiple cohorts of high-risk children, Palivizumab treatment significantly reduced the incidence of physician-diagnosed recurrent wheezing (70–74), with other studies finding reduced severity of asthma symptoms among treated children (75). Moreover, experimental evidence derived from mouse models of virally-induced chronic lung disease point to lasting alterations of the lung immune environment consistent with those observed in human asthma patients (76, 77). These sequelae of severe RSV infection point to the host immune response, and immune-mediated alteration of the lung environment, in precipitating subsequent development of chronic lung disease.

1a.iii. Immune-mediated lung pathology in patients during RSV infection

One of the most vivid illustrations of the contribution of host immune response to the development of severe lung pathology during RSV infection came from early attempts at vaccine development. During the 1960's, infants and children were injected with a vaccine containing formalin-inactivated RSV (FI-RSV) in an attempt to elicit protective antibody production. In addition to being a poor inducer of neutralizing antibodies, the vaccine primed for enhanced disease upon encounter with live RSV. As a result, up to 80% of vaccinees were hospitalized upon natural infection, and two children died (40). It was initially hypothesized that, given the link between RSV and Th2-polarized wheezing, the FI-RSV vaccine had induced a Th2-polarized immune response upon natural encounter with RSV, leading to infiltration of eosinophils and the over-production of interleukin (IL)-4, IL-5, IL-13, and possibly IL-10 (78, 79). While some experimental evidence supports this concept (80, 81), re-examination of post-mortem histological specimens found few eosinophils, and instead reported immune complex deposition and neutrophilia in the lungs (79, 82).

While the relevance of FI-RSV-induced disease to naturally-occurring RSV bronchiolitis is debated, other lines of evidence support over-exuberant immune responses as a causal factor of pathogenesis during RSV infection. Clinically, severe RSV infection is associated with rapid infiltration of immune cells such as neutrophils and monocytes (18, 83), as well as with excessive production of pro-inflammatory cytokines (discussed further in the next section). The virus does not appear to be highly cytopathic, and infection is generally restricted to the respiratory epithelium and intraepithelial lymphocytes (18). In agreement, infants infected with human immunodeficiency virus-1 do experience prolonged viral shedding upon RSV infection, but do not develop severe bronchiolitis necessitating hospitalization (84). Administration of neutralizing antibodies to RSV to a group of mechanically-ventilated, RSV-infected infants successfully reduced viral titers in tracheal aspirates, yet no significant differences in disease severity were observed (85). Conversely, the absence of a robust CD8+ cytotoxic T lymphocyte response among otherwise healthy infants contributes to worsened outcomes and prolonged illness, as the virus is not effectively cleared (19, 86). These observations point to a nuanced role for the T cell-mediated antiviral response to RSV by facilitating rapid viral clearance, but also exacerbating the development of lung pathology in the process.

1a.iv.Environmental and genetic risk factors associated with severe RSV infection

The risk of development of severe LRTI in infants is positively correlated with a number of factors, including premature birth (<35 weeks gestation), low birth weight, congenital heart or lung disease, congenital or acquired immunodeficiency, and inborn errors of metabolism (87). Despite the elevated risk conferred by these medical conditions, at least half of all infants hospitalized with severe LRTI were previously healthy and not considered at increased risk for severe infection (88, 89). Among otherwise healthy infants, increased risk of hospitalization is associated with young age (<6 months), male sex, older school-aged siblings, and exposure to tobacco smoke (49). Conversely, only two protective factors are well-established: Breast-feeding (88, 90, 91), and higher circulating levels of maternally-derived neutralizing antibodies to RSV (92, 93).

While no large genome-wide association studies comparing mild and severe cases of RSV have yet been conducted, concordance studies between Danish monozygotic and dizygotic twin pairs revealed a modest genetic contribution to the risk of hospitalization with RSV (94). This is supported by studies examining ethnic background and overall risk in infants in the United States, where children of Native Alaskan or American Indian heritage are far more likely to require hospitalization in comparison to the general infant population (95, 96), while infants of African ancestry experienced less severe bronchiolitis than their European-descended counterparts (97). Furthermore, studies of the same circulating strain of RSV during the same time period routinely find substantial variation in the severity of disease among infected individuals, ranging from mild rhinitis to severe LRTI (98).

Candidate gene studies utilizing 347 known single-nucleotide polymorphisms (SNPs) found that risk of hospitalization with RSV is associated with several innate immune gene variants, including VDR (vitamin D receptor), IFNA5 (interferon α5), and NOS2 (inducible nitric oxide synthase) (99, 100). Further studies identified an association between severe disease risk and SNPs located in other innate immune genes such as IFNG (interferon gamma), TGFBR1 (transforming growth factor beta receptor 1), and NFKBIA (nuclear factor kappa-B 1A), although the association was only significant among preterm children (100). Interestingly, polymorphisms in IL1RN (encoding IL-1 receptor antagonist) were protective among preterm infants, while increasing risk among full-term infants (100). Subsequent examinations of nonsynonymous SNPs present within single genes such as lung surfactant proteins and TLR4 (101–105) found overrepresentation of one of two SNP variants among infants with severe LRTI, although these findings are not always consistent (99, 106).

In addition to immunologically important receptor and signaling molecule genes, SNPs within the promoter- and coding-regions of critical cytokines are potentially associated with risk of severe RSV LRTI. Some studies of cytokine secretion in hospitalized infant cohorts found an association between excessive Th2 cytokine secretion and disease severity (107, 108), although others have reported little or no detection of Th2 cytokines (109–111). Several studies found overrepresentation of

common SNPs within the IL-4 gene and promoter regions (112–114), as well as in the IL-4 receptor alpha chain (115), among infants hospitalized with severe RSV infections in comparison to healthy controls. Polymorphisms within the gene encoding the Th2 cytokine IL-13 have also been associated with more severe disease, particularly in combination with certain IL-4 polymorphisms (113, 116). Numerous studies have also examined polymorphisms within the promoter of the Th2-promoting cytokine IL-10. Despite some indication that IL-10 promoter polymorphisms alter transcriptional regulation of the gene, evidence of higher risk of hospitalization among carriers of these SNPs is limited (117–119). One well-known disease-modifying locus on the human chromosome 5, labeled *5q31*, has been investigated as a potential modifier of RSV LRTI severity (113). This locus contains a cluster of cytokine genes, including the Th2 cytokine genes IL-4, -5, and -13, as well as IFN regulatory factor 1 (IRF1) and granulocyte-macrophage colony-stimulating factor (CSF2). The authors identified the highest hospitalization odds-risk ratio for infants carrying several SNPs across the IL-4 and IL-13 genes, especially among infants with no known risk factors (113). However, any correlation between haplotype-associated gene polymorphisms and cytokine production in response to RSV has yet to be examined.

As severe RSV LRTI is also associated with excessive influx of immune cells into the lungs, SNPs within genes encoding chemotactic cytokines have also been investigated for association with severe disease risk. One polymorphism within the promoter region of the IL-8 gene, whose product attracts neutrophils, was found to be overrepresented in infants hospitalized with severe RSV LRTI (120–122), although subsequent studies did not find significant associations with known SNPs within the IL-8 and IL-8 receptor genes (123, 124). Additional evidence exists to support increased risk of severe RSV among carriers of SNPs within the chemokine CCL5/RANTES (125, 126) and its receptor CCR5 (127), which along with CCL3/MIP-1 alpha recruit monocytes, basophils, eosinophils, and T cells.

These studies of genetic and environmental factors contributing to disease susceptibility provide interesting correlations with respect to the immune response to RSV, yet many findings await rigorous experimental validation. Further studies with

large patient populations will be needed to untangle the relationship between genetic, epigenetic, and environmental risk factors, and the ultimate development of pathological immune responses to RSV.

1b.Human and murine immune responses to RSV

In order to protect the host from pathogens, rapid detection of infection by the innate immune system is critical. This is accomplished by cells bearing germline-encoded pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) present on invading pathogens but absent on host cells. Three different classes of PRRs are involved in the detection of RSV: the toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (128). TLRs are type I transmembrane proteins expressed on the surface and inside endosomal compartments of epithelial cells, dendritic cells, macrophages, eosinophils, and neutrophils. A number of TLRs have been implicated in host detection of RSV, including TLR2, TLR3, TLR4, and TLR7 (128). The RNA helicases RIG-I and MDA5 are also critical to the detection of cytosolic viral RNA (129), while recent studies identify activation of the NLRs NOD2 and NLRP3 during RSV infection (130, 131). Upon detection of RSV, signaling pathways downstream of these PRRs converge at nuclear translocation of the transcription factors NFκB and IRF3/IRF7, initiating the production of pro-inflammatory cytokines and type I interferons (132).

The adaptive immune response to RSV is initiated by lung-resident dendritic cells (DCs), which are highly specialized in the detection and transport of viral antigens to the lung-draining lymph nodes. The instructional cytokines produced by DCs during antigen presentation to T cells are critical to the induction of robust, antiviral Th1-polarized CD4+ T cells and activated cytotoxic CD8+ T cells. The intracellular processes that dictate DC cytokine responses to viral pathogens are therefore capable of eliciting protective T cell responses to RSV, or inducing excessive or ineffective T cell responses that promote the development of immunopathology.

1b.i. Detection of RSV by pattern recognition receptors

The respiratory epithelium represents a primary interface between the host and environmentally-derived antigens and pathogenic organisms, and is the primary target of infection for RSV. As RSV infects target cells through fusion with the cell membrane, PRRs located in the cytosol of the infected cell are vital to rapid detection of viral RNA. The cytosolic RLRs RIG-I and MDA5 bind 5' triphosphate structures present on single- and double-stranded viral RNA using a conserved DExD/H box RNA-binding helicase domain, followed by interaction of N-terminal caspase-recruitment domains (CARDs) with the CARD domain of the mitochondrial membrane protein IPS-1. This interaction induces TBK-1-dependent phosphorylation of IRF3, which then homodimerizes and translocates to the nucleus to activate transcription of type I interferons (129). Mice deficient in IPS-1 expression (who are therefore unable to signal through RLRs) produced very little IFNβ in response to RSV infection, and exhibited worse airway neutrophilia and reduced viral clearance (133). RIG-I-dependent signaling is particularly critical to NFκB and IRF3 activation during the first hours of RSV infection, enabling the rapid production of IFNβ, CXCL10, and CCL5 by infected airway epithelial cells (134). RSV infection also induces the upregulation of RLR and intracellular TLR expression *in vitro*, in a manner dependent on IPS-1 expression (135). In agreement, RIG-I and MDA5 expression were found to be higher in RSV-infected infants in comparison to healthy controls (136). Finally, the importance of RLR signaling to enabling the host antiviral response is the finding that the RSV NS-1 and NS-2 proteins inhibit RLR signaling by blocking interaction of RIG-I with IPS-1 (35). These studies illustrate the importance of the RLR/IPS-1 pathway to the early detection of RSV, as well as to the development of robust innate immune responses to infection.

Some evidence exists to support an important role for members of the cytosolic NLR family in the detection of RSV and subsequent innate cytokine production. NLRs recognize a variety of different PAMPs such as cytosolic bacterial cell products and bacterial or viral nucleic acids (137). Recent studies indicate that NOD2 is capable of recognizing the single-stranded RNA of several different viruses, including RSV (130). Recognition of viral RNA led to a rapid translocation of NOD2 to the surface of

mitochondria, and to interaction with IPS-1. This activated both IRF3 and NFκB, leading to rapid production of IFNβ (130). Autocrine recognition of IFNβ subsequently leads to the upregulation of NOD2 expression, which upon encountering the bacterial antigen muramyl dipeptide may amplify pro-inflammatory cytokine production by infected cells. The importance of the rapid detection of RSV by NOD2 was further evidenced by impaired viral clearance, increased weight loss, and greater lung immunopathology in RSV-infected NOD2-deficient mice (130).

The cytosolic NLR cryoporin/NLRP3 has also recently been shown to recognize RSV, and to be required for the production of IL-1β and IL-18 in response to infection (131, 138). Direct interaction of RSV-derived PAMPs and NLRP3 has yet to be demonstrated, leaving the exact mechanism somewhat unclear. Upon activation, NLRP3 recruits the adaptor protein ASC to form large multi-protein complexes called inflammasomes, which then activates the cysteine protease caspase-1. IL-1β and IL-18 are translated in an inactive pro-form, which upon cleavage by caspase-1 become biologically active (139). In one recent study, mouse bone marrow-derived macrophages increased their expression of pro-IL-1β and NLRP3 in response to RSV infection, in a process requiring TLR2 and MyD88 (131). NLRP3/ASC inflammasome complexes formed in response to reactive oxygen species and potassium efflux, leading to the activation of caspase-1, pro-IL-1β cleavage, and secretion of mature IL-1β (131). These findings were recently verified in human bronchial epithelial cells, although the production of IL-1β by these cells was dependent on TLR4 expression rather than TLR2 (138).

In addition to cytosolic PRRs, airway epithelial cells express membrane-bound TLRs implicated in the detection of RSV, including TLR2, TLR3, TLR4, and TLR7 (128, 140). TLR2 and TLR4 are expressed on the cell surface, where TLR2 forms heterodimeric complexes with TLR1, TLR6, CD36, CD14, or dectin-1 to detect PAMPs (141). While comparatively little is known about the role of TLR2 in detection of RSV, it has been proposed as a potential cellular receptor for RSV, while TLR2/TLR6 signaling was shown to activate innate immune responses during RSV infection through promoting the production of tumor necrosis factor alpha (TNFα), IL-6, CCL2, and CCL5 (142).

In contrast, considerable evidence exists to support the importance of TLR4 expression to the outcome of the immune response to RSV. The involvement of TLR4 and CD14 was first identified through demonstration of interaction with the RSV-F protein (33), which stimulates NFκB-mediated innate cytokine production (143). Ligation of the RSV-F protein by TLR4/CD14 increases TLR4 expression on epithelial cells and stimulates production of IL-6 and IL-8 (31, 33, 144). Mice harboring a null mutation in the gene encoding TLR4 showed reduced NK cell trafficking and function in response to RSV, with decreased IL-12 production and delayed viral clearance in comparison to controls (33, 142, 145). Additionally, the expression of two non-synonymous SNPs in the ectodomain of TLR4, encoding Asp299Gly and Thr399Ile, has been epidemiologically correlated with increased risk of severe RSV bronchiolitis, especially among high-risk infants (102–104). Experiments using human bronchial epithelial cells expressing one of the two SNPs found reduced translocation efficiency of the receptor to the surface of the cell, resulting in decreased NFκB activation and lower production of IL-8, IL-10, IL-12p35, IL-18, TNFα, CCL8, and type I IFNs in response to stimulation (146). Conversely, the expression of TLR4 on blood monocytes was positively correlated with disease severity in one clinical study, suggesting potential involvement in the development of subsequent lung pathology (147).

TLR3 and TLR7 are expressed within intracellular compartments such as endosomes, where they detect double-stranded or single-stranded viral RNA, respectively (141). Upon ligation of the double-stranded RNA intermediate created during the RSV replication cycle, TLR3 recruits the adaptor protein TRIF, which activates both NFκB and IRF-3 leading to the production of IFNβ and the chemokines CCL5, CCL12, and CXCL10 (134, 148, 149). The expression of TLR3 does not seem to be required for clearance of RSV in mouse models of RSV infection (150), yet it plays an important role in modulating the immune environment and the ensuing development of immunopathology. Ligation of TLR3 promotes the expression of CXCL10 and CCL5 in RSV-infected cells, while RSV infection upregulates TLR3 expression in human lung fibroblasts and epithelial cells (148, 149). TLR3 expression is also critical to the development of a Th1-type antiviral response *in vivo*, as mice deficient in TLR3 produce

greater amounts of the Th2 cytokines IL-5 and IL-13, with more mucus production and infiltration of eosinophils into the airways (150).

TLR7 and its adaptor protein MyD88 are also required for host responses to RSV that minimize pulmonary immunopathology. In a manner similar to TLR3-deficient mice, TLR7-deficient mice develop altered T-cell cytokine responses characterized by increased production of IL-4, IL-13, and IL-17 in response to RSV infection (151). As both IL-13 and IL-17 promote airway mucus production (151, 152), RSV-infected TLR7-deficient mice produced significantly higher amounts of mucus in comparison to wild-type controls (151). Interestingly, DCs derived from TLR7-deficient mice preferentially produce the Th17-promoting cytokine IL-23 at the expense of the Th1-promoting cytokine IL-12, potentially contributing to an elevated Th17 response upon RSV infection (151).

1b.ii.Innate cytokine production and immune cell recruitment in response to RSV

A wide variety of cytokines and chemokines are produced by airway epithelial cells in response to RSV infection *in vitro*, including KC/CXCL1, MIG/CXCL9, IP-10/CXCL10, fractalkine/CX3CL1, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, IL-6, TNFα, IL-1α/β, and IFN-α/β (143, 153–155). In addition, airway-resident alveolar macrophages are an important source of CCL3, CCL5, TNFα, IL-6 and IFNα in mouse models of RSV infection (156, 157). The secretion of innate cytokines and chemokines in murine lungs increases early during infection, with bronchoalveolar lavage (BAL) concentrations peaking at 24-48 hours post-infection (155). Expression of IL-1, IL-6, and TNFα is also elevated in the early stages of infection, which facilitates retention of recruited immune cells and also contributes to their activation. The evidence regarding the role of TNFα expression in the clearance of RSV is somewhat contradictory, as it is required for viral clearance during the early stages of infection but may contribute to the development of lung immunopathology in later phases of infection (158–160). The concentration of IL-6 in BAL fluid is also correlated with viral load and disease severity, suggesting that it may promote lung pathology in later stages of infection (161).

Acute RSV-induced lung pathology in human and murine histological samples is characterized by bronchiolitis, edema of mucosal and submucosal tissues, and luminal occlusion (18). The plugs of material found occluding the airways are reported to be composed of apoptotic/necrotic epithelial cells, macrophages, fibrins, and mucins (162, 163). At the peak of inflammation 7 days post-infection, infiltrating monocytes/macrophages and lymphocytes congregate in high density in periarteriolar spaces, occasionally extending to adjacent peribronchiolar and alveolar spaces (18, 162, 163).

In mouse models of RSV infection, the rapid production of chemokines and innate cytokines by airway epithelial cells and alveolar macrophages results in an influx of immune cells into lung tissue and peribronchial airspaces. Neutrophils are recruited into the lungs as early as 24 hours post-infection in mice (161, 164–168), although the kinetics and magnitude of this response is dependent on the isolate of RSV and strain of mouse used (169). In contrast, eosinophils generally represent a minor but significant component of recruited immune cells in response to RSV (170–173), although some studies report no differences in lung eosinophil numbers between RSV-infected and control animals (174–176). The primary cell types present in the BAL of infected mice are usually monocytes and macrophages, although once again the kinetics of recruitment varies between studies (164). Natural killer (NK) cells play a critical role in viral clearance during the early stages of RSV infection, and appear in the lungs approximately 48 hours post-infection (165–168). Influx of NK cells peaks around day 3 or 4 post-infection, and are generally undetectable by day 8 post-infection (166).

In contrast to mice, neutrophil recruitment is a prominent feature of severe RSV infection in humans, as increased numbers are found in respiratory secretions of patients hospitalized with RSV (83, 177, 178). In studies of RSV-infected infants requiring intubation, 75-85% of cells in BAL fluid are neutrophils, with numbers being highest during the first few days of treatment and declining thereafter (83). Histological examination of lung tissue taken from patients who succumbed to RSV infection also revealed considerable numbers of neutrophils and monocytes (18, 179). However, some studies have found lower numbers of recruited neutrophils in the BAL fluid of

hospitalized, RSV-infected pre-term infants in comparison to full-term infants (83). Eosinophils are usually detectable in low numbers (<1%) in a minority of RSV-infected infants, although one study reported a subset of infants with an average of 3% eosinophils in BAL fluid (180).

In mice infected with RSV, CD4+ T cell recruitment begins early and levels out around day 4 post-infection, while CD8+ T cell numbers rise sharply around this time (157, 164–166). Numbers of both T cell subsets begin to decline around day 9 post-infection, but remain elevated in lung tissue through 20 days post-infection. This influx of T cells coincides with a second peak in the production of many chemokines in the lungs, including eotaxin, CCL3, and CCL5 (155, 161, 181). While T cells constitute a small fraction of cells in BAL samples from severe RSV patients (>2%), a significant increase in CD8+ T cells was observed in some studies in comparison to uninfected controls (177, 182). Histological data on the prevalence of CD8+ T cells in the lungs of patients with RSV suggest a protective function, as significant numbers were reported in the alveolar interstitium of one infant with non-fatal RSV (18), yet a second study of 9 fatal cases of RSV revealed a near absence of CD4+ and CD8+ T cells (19).

In both mice and humans, considerable numbers of dendritic cells (DCs) are recruited to mucosal sites during RSV infection. An increase of plasmacytoid DCs (pDCs) and monocyte-derived, 'conventional' DCs (cDCs) occurs in the lungs and lung-draining lymph nodes of mice infected with RSV (183–186). Similarly, an influx of cDCs and pDCs was noted in nasal washes from infants who were infected with RSV, along with decreases in numbers of these cells in peripheral blood (187, 188). The indispensable role of DCs in presenting antigen to T cells, and directing appropriate polarization of the ensuing adaptive immune response, has long been appreciated. However, recent studies suggest that DCs may also be responsible for the induction of aberrant CD4+ T cell responses to RSV under certain circumstances, implicating DCs in the development of lung immunopathology during RSV infection.

1b.iii. Induction of innate and adaptive immune responses to RSV by lung dendritic cells

Dendritic cells (DCs) are innate immune cells that are found throughout mucosal tissues, where they promote immune tolerance to innocuous environmental antigens and act as sentinels for invading pathogens. While many lung-resident cells such as epithelial cells and macrophages express PRRs and are capable of directing innate responses through the production of pro-inflammatory cytokines, DCs are uniquely specialized to transport pathogen-derived antigens to lung draining lymph nodes in order to activate the adaptive arm of the immune system (189). Thus, through the induction of antiviral CD4+ and CD8+ T cell responses and through the production of innate cytokines, DCs initiate and orchestrate both innate and adaptive immune responses to viral infection.

Three distinct DC subsets are known to reside in the lungs in the absence of inflammation, all of which perform specialized tasks upon detection of viral infection. Intraepithelial DCs, which express the E-Cadherin-binding integrin CD103 in mice (CD103+ DCs), intercalate with the epithelial cells lining the respiratory tract and project cellular extensions into the airway lumen. A second subset expressing the integrin CD11b (CD11b+ DCs) resides in the lamina propria of the major airways, immediately underneath the basement membrane (190). Both CD103+ DCs and CD11b+ DCs are referred to as myeloid or 'conventional' DCs (cDCs), and correspond to mDC1 and mDC2 subsets in human lungs (190). Plasmacytoid DCs (pDCs) can also be found in the conducting airways in steady-state conditions. Finally, during the course of an inflammatory response, monocyte-derived cDCs may be present in the lungs as well, characterized by surface expression of CD11b. the monocytic marker Ly6C, and the Fc epsilon receptor (FcεRI) (190).

DCs reside in the lungs in a functionally immature state, where they are poised to detect PAMPs such as viral nucleic acids and proteins. All subsets of cDC and pDC present within the lungs are capable of being infected by RSV *in vitro* (191, 192), resulting in surface upregulation of MHC class I and II and the costimulatory molecules CD80, CD83, and CD86 (183, 191, 193–196). Direct infection of at least some DCs is required for DC maturation in response to RSV, as UV-inactivated virus failed to stimulate DC maturation in several studies conducted *in vitro* (197, 198). Upon infection,

human monocyte-derived CD11b+ DCs produce the pro-inflammatory cytokines IL-1β, IL-6, IL-12, TNFα, and IFNγ (192, 194, 195), as well as chemokines such as CCL2, CCL3, CCL5, CXCL8, and CXCL10 (194, 195). In addition, murine bone marrow-derived DCs (BMDCs) produce the type I interferons IFNα and IFNβ in response to infection, which contributes to the surface upregulation of costimulatory molecules in these cells (199).

Upon detection of RSV, murine tissue-resident cDCs rapidly mature and upregulate expression of CCR7, enabling the transport of viral RNA and protein to lung-draining lymph nodes for presentation to T cells (200). The kinetics of migration differ depending on the subset of cDC, as numbers of CD103+ DCs in the lungs decrease rapidly in the first 24 hours post-infection, while an influx of monocyte-derived CD11b+ DCs contributes to increased numbers in the lungs by 7 days post-infection (201). In addition, some functional specialization has been noted among intraepithelial CD103+ DCs and lamina propria/monocytic CD11b+ DCs with respect to PRR expression, antigen acquisition, and T cell stimulation in response to viral infection. As CD103+ DCs reside in immediate contact with epithelial cells, these DCs are particularly adept at the uptake of apoptotic epithelial cells (202–204), aided by selective expression of TLR3, the scavenger receptor CD36, and the C-type lectin Clec9A (205–207). CD103+ DCs specialize in stimulating CD8+ T cells through cross-presentation of antigens in influenza infection models (208, 209), although this has not been exhaustively investigated in RSV. In contrast, CD11b+ DCs selectively express TLR2 and TLR7 (203), and are major producers of chemokines which attract effector cells to the lungs during pulmonary infection (210, 211). CD11b+ DCs are also reported to preferentially activate CD4+ T cells in the context of severe influenza infection (212) and studies of directly-infected CD11b+ DCs indicate proficiency in priming a CD8+ T cell response (213, 214). Less is known about the specialization of these cells in the context of RSV, although *ex vivo* co-culture experiments suggest that RSV-infected CD103+ DCs and CD11b+ DCs are capable of stimulating both CD8+ and CD4+ T cells (201).

Despite the ability of DCs to rapidly mature and produce innate cytokines in response to RSV infection, RSV appears to reduce the capacity of DCs to stimulate and

induce proliferation in naïve T cells (184, 192, 197, 198). Infected DCs release an as-yet unidentified soluble factor that suppresses T cell activation in a dose-dependent manner (197, 215), which is independent of IL-10, TGFβ, or the activity of regulatory T cells (184). This effect may be due to synergistic activity of IFNα and IFNλ (215), or to ligation of a combination of receptors to IFNα, IL-10 and IL-28 (215). In addition to stimulating the release of a soluble suppressor of proliferation, RSV may interfere with immune synapse formation that takes place during presentation of antigen to naïve T cells. One study found that naïve T cells fail to polarize their Golgi to the site of synapse formation during interactions with RSV-infected DCs, suggesting that inadequate DC-T-cell interaction may be responsible for observed impairment in T cell stimulation and proliferation (198). T cells stimulated with RSV-infected DCs were subsequently nonresponsive to proliferation signals such as IL-2 and anti-CD3 antibody treatment (197, 198), and were impaired in the production of IL-2, IL-4, IFNγ, and TNFα, all of which is suggestive of an anergic state (196, 197, 216). This phenomenon is only observed in DC-T cell co-cultures where the DCs are infected with replication-competent virus, as naïve T cells cultured with DCs exposed to UV-irradiated RSV showed no proliferation defects (197, 198). Similarly, RSV-infected DCs were able to efficiently stimulate proliferation and cytokine production from memory T cells, suggesting that RSV only interferes with naïve T cell stimulation (196). This subversion of DC antigen-presentation to naïve T cells likely contributes to weakened primary responses to RSV in human infants, potentially increasing the likelihood of severe LRTI.

1b.iv. Involvement of lung dendritic cells in protective and pathogenic responses to RSV

Lung DCs are uniquely positioned to promote protective or pathological immune responses to RSV infection through the induction of adaptive T cell responses and through production of pro-inflammatory cytokines that influence the immune environment of the lungs during infection. In order to develop an effective antiviral response to RSV, different DC subsets must work synergistically to facilitate swift viral clearance with minimal damage to uninfected tissues. On the other hand, evidence from clinical studies and mouse models of RSV suggest an immunopathological role for

certain DC subsets, highlighting the complexity of DC-mediated immune responses during infection.

While pDCs are poor presenters of RSV antigens to T cells, evidence suggest that they are critical sources of IFNα, pro-inflammatory cytokines, and chemokines upon exposure to RSV (183, 185, 217). Human pDCs isolated from peripheral blood also secrete innate cytokines and large amounts of IFNα in response to RSV infection, implicating these cells as important mediators of antiviral immunity (192, 218). In studies of RSV infection in mice, increased numbers of pDCs in the lungs appears to be protective, while depletion of pDCs results in increased viral replication, greater mucus production, and prolonged airway hyperreactivity in RSV-infected mice (183, 185). As numbers of pDCs in the lungs increase by day 3 post-infection and decrease below homeostatic levels prior to the onset of peak inflammation, IFNα production by pDCs is likely to be the most critical to early antiviral immune responses during RSV infection (183–185).

As cDCs within the lungs are tasked with the induction of CD4+ and CD8+ T cell-mediated responses to RSV, the ability of these cells to stimulate strong antiviral Th1-type responses is critical to successful viral clearance (189). By presenting antigen to naïve and RSV-reactive memory T cells, migratory lung cDCs induce the proliferation and differentiation of effector 'helper' CD4+ T cells and cytotoxic CD8+ T cells, which migrate to the lungs and clear virally-infected cells (190). While the actions of CD4+ and CD8+ T cells are critical to viral reduction and the eventual eradication of RSV, these cells are also involved in the development of lung immunopathology (219–223). By depleting CD4+ or CD8+ T cells during RSV infection in mice, it was found that both viral clearance and lung pathology were reduced (219). Similarly, the transfer of RSV-specific CD4+ and CD8+ T cells to infected mice increased viral clearance, but led to the development of increased lung pathology (220, 221). Sustained activity of T cells is also linked to increased airway hyperresponsiveness in RSV infected mice (220), further supporting the dual role of T cells in viral clearance and pulmonary damage during RSV infection.

Some evidence suggests that certain DC subsets also contribute to the production of Th2-type responses to RSV and lung immunopathology in mice. Studies in both mice and human infants note an influx of monocyte-derived DCs into the respiratory tract and draining lymph nodes during RSV infection (184, 186–188, 199), which may contribute to overall inflammation and hyper-responsiveness to both RSV and subsequent allergen challenges (186, 211, 224, 225). Blockade of migration of monocyte-derived CD11b+ DCs to the lungs or lung-draining LNs is protective against RSV-induced immunopathology in mice, resulting in more robust Th1 responses and enhanced viral clearance (226, 227). The relative abundance of these DCs within the lung environment is also implicated in the development of pathological responses to RSV *in vivo*, as the addition of monocyte-derived CD11b+ DCs increases Th2 responses and associated lung pathology during RSV infection of mice (217, 227, 228).

Because of the distinctive capacity of DCs to direct both innate and adaptive immune responses to pulmonary viral infections, the intracellular mechanisms which control DC responses to RSV are of particular interest. Through the transport of viral peptides to lung-draining LNs for antigen presentation, and through production of innate cytokines, DCs provide critical instructional signals that elicit antiviral Th1 responses by RSV-reactive T cells. As DC maturation and production of innate antiviral cytokines are dependent on recognition of RSV through TLRs (34, 229, 230), the processes which facilitate the transport of viral material to TLR-containing endosomes are of paramount interest. Recent studies into the process of macroautophagy, whereby cytosolic contents are enveloped in a double-walled membrane and delivered to endosomes, have revealed a crucial role for autophagosomes in the antiviral response of DCs. Through expanding the ancient homeostatic functions of autophagosome formation and maturation, DCs expedite the delivery of intracellular pathogen-derived nucleic acids and peptides to endosomal compartments, thereby facilitating the production of appropriate innate cytokines and the loading of viral peptides onto MHC-II molecules for presentation to CD4+ T cells.

1c. Autophagy in immune defense against viruses

The process of macroautophagy, hereafter referred to as autophagy, is thought to have initially evolved as a mechanism to enable survival of unicellular organisms during

periods of nutrient scarcity. To this end, autophagosomes envelop cytosolic organelles or proteins in a double-walled membrane, enabling the degradation of the contents upon fusion of the autophagosome with lysosomes. Macromolecules recovered from degraded autophagosomal contents may then be used to prolong cell survival until favorable conditions return (231). In addition to facilitating cell survival and enabling the removal of unneeded or damaged protein aggregates and organelles, the molecular machinery of autophagosome formation has been recruited for cellular defense against cytosolic pathogens in metazoan organisms (232). Recent studies have demonstrated additional immunological requirements for autophagy in DCs as a mechanism enabling the capture and delivery of cytosolic macromolecules to intracellular compartments containing TLRs and MHC-I/II peptide loading machinery (232). Conversely, autophagy antagonizes production of the pro-inflammatory cytokines IL-1β and IL-18 through selective degradation of assembled inflammasome platforms and their substrates pro-IL-1β and pro-IL-18 (233). The removal of depolarized mitochondria through mitochondrial autophagy (mitophagy) is also critical to the regulation of inflammasome activity within innate immune cells, as disrupted mitochondria are an important source of inflammasome-activating reactive oxygen species (ROS) (234, 235). Finally, signaling by sensors of ER stress, which activate the unfolded protein response (UPR) during pathogenic insult, also activate autophagosomal machinery in an attempt to restore homeostasis through removal of ER (ER-phagy), damaged mitochondria, and accumulated proteins (236). These processes collectively suggest an important immunomodulatory role for autophagy during viral infection, in both promoting critical antiviral cytokine production and APC function in DCs, as well as in minimizing pathological inflammation through the limitation of excessive pro-inflammatory cytokine production during infection.

1c.i. Autophagosome formation and maturation

The process of autophagosome formation is highly conserved among eukaryotic organisms, and the pathway was first elucidated with studies in yeast ((231), see Figure 1-2). Autophagy is initiated during starvation by the inhibition of the mammalian target of rapamycin (mTOR) and activation of AMP-activated protein kinase (AMPK), which phosphorylates ULK1 and cause the translocation of the ULK1 complex to the endoplasmic reticulum (ER) (237, 238). While the primary source of membrane in the formation of autophagosomes is thought to be the ER, some studies suggest that membrane material can also be contributed by mitochondria, the Golgi apparatus, the nuclear membrane, or even the plasma membrane (231). The phosphorylated ULK1 complex activates the VPS34-containing class III phosphatidylinositol-3-OH kinase (PI3K) complex located on the ER surface (VPS34-PI3K), which includes the class III PI3K VPS34, VPS15, autophagy protein (ATG) 14, and ATG6/Beclin-1 (Beclin-1) (237, 239). The VPS34-PI3K complex then produces phosphatidylinositol-3-phosphate, which recruits DFCP1 and WIFI family proteins that initiate the extension of source membrane into pre-autophagosomal structures termed omegasomes (240, 241).

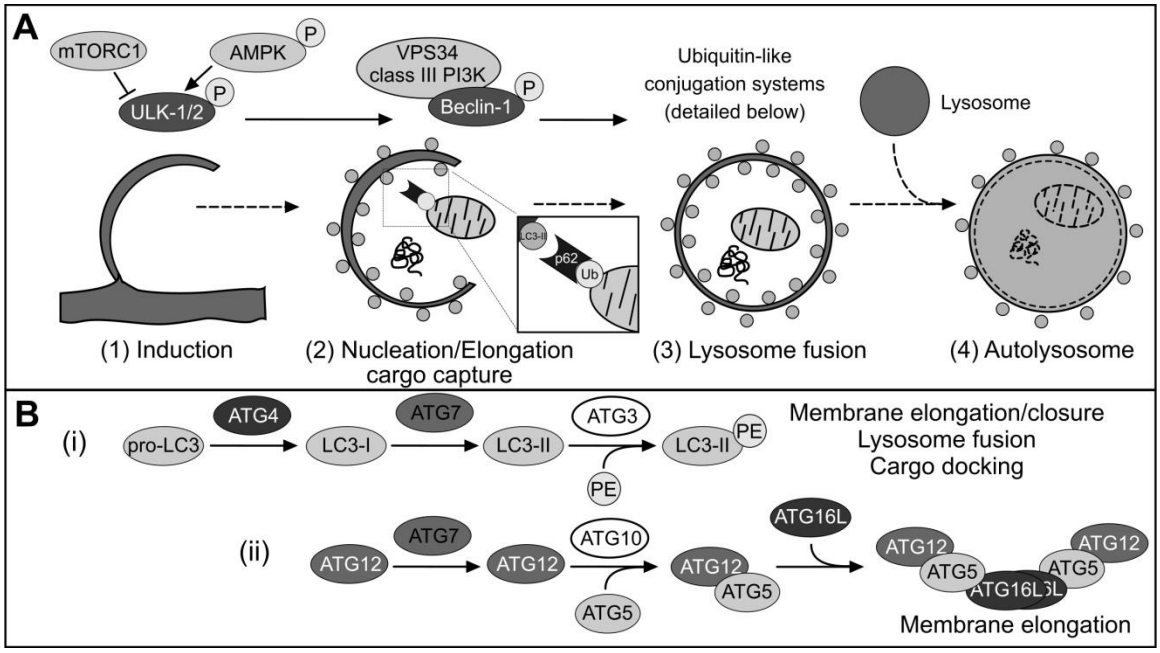

Figure 1-2 – **Autophagosome formation and maturation.** A) Autophagy induction is regulated by mTORC1 and AMPK in mammals. Phosphorylated ULK-1/2 (homologs of yeast ATG1) activates the VPS34-PI3K complex, which contains Beclin-1. Autophagic cargos are indisicriminantly obtained from the cytoplasm, or are targeted to nascent autophagosomes through sequesteosome proteins such as p62. Complete autophagosomes dock and fuse with lysosomes, leading to the degradation of the contents, as well as the inner autophagosomal membrane. B) Two ubiquitin-like conjugation systems, producing (i) PE-conjugated LC3-II, which is incorporated into outer and inner autophagosomal membranes. (ii) ATG5/12/16L complexes facilitate membrane elongation and curvature, and dissociate from the surface of fully-formed autophagosomes (not pictured).

The elongation and closure of the double-walled autophagosomal membrane is dependent on two ubiquitin-like conjugation cascades. The first involves creation of the ATG5-ATG12 conjugate through actions of the E1-like ATG7 and the E2-like ATG10. This conjugate then forms a dimeric complex with ATG16L1 on the outer membrane of nascent autophagosomes (242). The second conjugation system, which requires ATG7 and the E2-like ATG3, cleaves and conjugates ATG8 homologs with phosphatidylethanolamine (PE). The PE-conjugated ATG8 homologs, which in mammals are LC3, GATE16 and GABARAP, are incorporated into the inner and outer autophagosomal membranes (243, 244) (Figure 1-2). Finally, the maturation of the completed autophagosome is accomplished by fusion with acidic lysosomal compartments containing hydrolases that degrade the autophagosome contents, as well as the inner autophagosomal membrane. This occurs through the recruitment of the SNARE protein Syntaxin 17 to the outer membrane, which enables docking and fusion with lysosomes to form an autolysosome, whose contents are degraded (245).

The sensing of sudden nutrient scarcity caused by microbial invasion may have been the evolutionary basis for the involvement of autophagy in removal of invading intracellular pathogens. This is evidenced in modern innate immune cells through the integration of immunological and metabolic signaling which triggers autophagy, centered on the regulation of Beclin-1 (232). During starvation, the ULK1-mediated activation of VPS34-PI3K is accomplished through phosphorylation and release of Becn1 from its inhibitory interactions with the TAK-1-binding proteins (TABs) 2 and 3 (246, 247). The activating molecule in BECN1-regulated autophagy (AMBRA) protein is also phosphorylated by ULK1, leading to the recruitment of the E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) by Becn1 and AMBRA (248, 249). TRAF6 then ubiquitinates and stabilizes Becn1 and ULK1, enabling the progression of autophagosome formation (248, 249). This process is intersected by signaling events downstream of PRRs and cytokine receptors, enabling immunological control of autophagosomal machinery. Ligation of TLR4 triggers the interaction of the adaptor proteins MyD88 and Trif with Becn1, which disrupts the inhibitory interaction of Becn1 with BCL2 (250). TLR4 signaling also initiates autophagy through activation of TRAF6, leading to the ubiquitination of Becn1 and ULK1 (251). Some NLRs, such as NOD2, also

initiate autophagy through RIPK2-mediated ubiquitination of ULK1 (252). Signaling through the IL-1 receptor (253) and the IFNγ receptor (254–257) by IL-1 and IFNγ also trigger autophagy in macrophages through interaction with TRAF6 or Becn1, respectively. In contrast, the Th2 cytokines IL-4 and IL-13, along with IL-10 and IL-6, inhibit autophagy under certain circumstances (255, 258, 259). These examples illustrate the interconnected nature of PRR and cytokine receptor signaling and autophagosomal regulation.

1c.ii. Host autophagy and defense against intracellular pathogens

A requirement for host autophagy and autophagosomal machinery has been well-elucidated in the context of intracellular bacterial infection (260). Autophagosomal capture and degradation provides a critical mechanism for the removal of intracellular microorganisms, and is termed xenophagy (260). The degradation of bacteria such as *Listeria* and *Shigella*, which routinely escape phagosomal incarceration, is dependent on autophagosomal capture (261–263). Cytosolic bacteria are targeted to forming autophagosomes through the actions of sequesteosome-like receptors (SLRs), which bind ubiquitinated bacteria and interact with membrane-bound LC3 on the inner autophagosomal membrane (261, 262, 264–266). In addition, a non-canonical autophagic pathway termed LC3-associated phagocytosis (LAP) is employed to expedite the degradation of phagocytosed bacteria (267, 268), as well as extracellular objects such as apoptotic cells (269), TLR-ligand-coated particles (267), and immune complexes taken up by FcγR-dependent endocytosis (268, 270). The involvement of LC3 conjugation and Becn1-VPS34 complexes in the formation of a single-membrane phagosome targets the contents for degradation in the same manner as traditional double-membrane autophagosomes, which mature through fusion with lysosomes (271).

The importance of autophagy in defense against bacterial invasion is further evidenced by the variety of ways in which pathogens subvert or commandeer host autophagic machinery for their own ends. Many bacteria block the induction of autophagy or evade autophagosomal capture through the expression of proteins that interfere with autophagosomal targeting, formation, or maturation. For example, *Salmonella* avoid recognition by SLRs through expression of the deubiquitinase SseL,

which removes host ubiquitin tags utilized by SLRs to target bacteria to forming autophagosomes (272). Similarly, the *Listeria* proteins AktA and InIK interfere with ubiquitination through recruitment of cytosolic proteins to mask bacterial epitopes (262, 263), while *Shigella* evades capture through expression of the epitope-masking protein IcsB (273). Other bacterial effector proteins, such as the *Legionella* virulence factor RavZ, directly target host autophagosome formation through the cleavage of ATG8 homologs (274). Finally, the maturation of autophagosomes may be blocked by captured bacteria, as *Listeria* prevents autophagosomal acidification through the expression of the pore-forming protein listeriolysin O (275).

In contrast to bacterial infection, cell survival through direct autophagic control of viral replication is largely restricted to neurotropic viruses such as Sindbis virus (276, 277) and neurovirulent herpes simplex virus-1 (278), and is likely due to the irreplaceable nature of neurons. However, the importance of host autophagy to the limitation of viral replication is evidenced by the number of viral proteins which interfere with host autophagy, particularly by targeting Becn1. The herpes simplex virus 1 (HSV-1) virulence factor ICP34.5 inhibits host autophagy by binding Becn1 (278, 279), while the murine γ-herpesvirus 68 expresses M11, a viral homologue of BCL2, which inhibits Becn1-dependent autophagy (280). The influenza A M2 protein (281) and HIV protein Nef (282, 283) also bind Becn1 in order to prevent autophagosomal fusion with lysosomes. The virulence factors HIV-1 Nef, Hepatitis C virus NS3 and measles virus Mev3 were recently shown to interact with the autophagy factor immunity-related GTPase-family M (IRGM), although the functional consequences of these interactions have yet to be investigated (284). Interestingly, some evidence suggests that viruses such as poliovirus and HIV-1 may utilize autophagosomes as lipid-membrane scaffolds, thereby enhancing viral replication. Similarly, the induction of autophagy may function as a non-lytic mechanism for viral release, as the pharmacological stimulation of autophagy in cells infected with poliovirus (285) and HIV-1 (283) resulted in increased extracellular viral yields.

A potential requirement for autophagy in cell-autonomous control of RSV replication has not been investigated. However, the process may be indirectly involved

through modulation of DC antigen presentation and pro-inflammatory cytokine production in response to infection. Indeed, recent studies conducted by our laboratory have demonstrated that DCs rapidly upregulate autophagosome formation upon detection of RSV (Figure 1-3). Moreover, autophagy facilitates DC maturation, cytokine production, and antigen presentation function in the context of RSV infection, and is required for the stimulation of IFNγ production from RSV-reactive T cells (230).

1c.iii. Involvement of autophagy in DC function

Autophagy has been recruited to facilitate several aspects of DC maturation in response to pathogen detection. The process of autophagy provides a mechanism for delivery of cytosolic peptides to MHC-I and MHC-II loading compartments, which are subsequently presented to CD4+ or CD8+ T cells, respectively. In addition, the sequestration and transport of cytoplasmic contents enables surveillance of the intracellular environment by endosomal TLRs, thereby facilitating rapid upregulation of innate cytokine production and surface expression of MHC-I/II and costimulatory molecules that are vital to the activation of T cells during antigen presentation. The process of autophagy may be especially critical to the immediate detection of RSV, as the virus infects through fusion with the plasma membrane rather than entering and escaping the endosomal pathway as many viruses do (1).

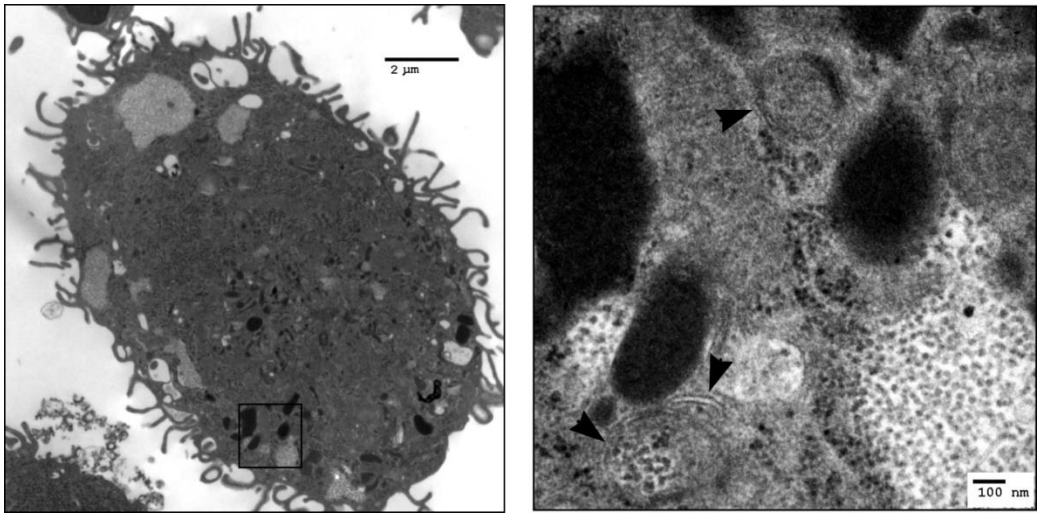

Figure 1-3- Autophagosome formation in RSV-infected murine bone marrow-derived DC. Right inset shows double-membrane autophagosomes containing cytoplasmic material and rough ER (black arrows).

The presentation of foreign antigens by DCs in the context of MHC-I/II, along with costimulatory molecules and instructional innate cytokines, is required for the activation of CD4+ T helper cell-mediated immune responses to pathogens. Peptides are loaded onto MHC-II molecules within endosomes or MHC class II-containing loading compartments (MIICs), thereby stabilizing MHC-II for export to the cell surface (189). Within DCs in the steady state, MIICs receive continuous input from both endocytosis and autophagy, enabling the loading of intracellular self-peptides or extracellular antigens for presentation to CD4+ T cells (286–288). The contribution of autophagy to the cross-presentation of cytosolic antigens via MHC-I is not as thoroughly investigated, but autophagy may contribute to the delivery of proteasome-degraded peptides to MHC-I-containing compartments (289–291). DCs frequently obtain peptides destined for cross-presentation from phagocytosed apoptotic cells, and autophagy in the peptide-donor cell prior to apoptosis contributes to this process (292, 293).

Autophagosomal capture and delivery of intracellular pathogen-derived peptides is critical to CD4+ T cell activation during certain viral infections. Presentation of peptides from the Epstein Barr virus (EBV)-encoded nuclear antigen 1 (EBNA1), a CD4+ T cell epitope consistently found in healthy carriers of EBV, requires autophagy in DCs (288, 294). The importance of DC-associated autophagy was demonstrated *in vivo* using mice selectively deficient in ATG5 in DCs, which showed decreased CD4+ T cell responses upon infection with HSV-1 (295). In addition, infection of DCs with HSV that lacked the Becn1-binding virulence factor ICP34.5 resulted in elevated CD4+ T cell responses in comparison to wild-type HSV (278). Further studies examining the ability of ATG5-deficient DCs to present extracellular peptides such as ovalbumin to ovalbumin-responsive CD4+ T cells also found decreased T cell responses (295), suggesting that autophagy machinery also promotes the presentation of extracellular peptides to T cells. This requirement is not universal, however, as CD4+ T cell responses to influenza A-infected DCs were unaltered by autophagy blockade (296).

In addition to sequestering viral peptides for presentation to T cells, autophagosomal transport of viral nucleic acids to endosomal TLRs enables the rapid induction of pro-inflammatory cytokine production in response to infection. This was

first established in pDCs, which express TLR7 and require the autophagy protein ATG5 to facilitate TLR7-dependent IFNα production in response to vesicular stomatitis virus (VSV) (297). Subsequent studies of human pDCs found a requirement for autophagy in the production of IFNα in response to HIV-1 (298) and the paramyxovirus Simian virus 5 (299). Interestingly, treatment of macrophages or DCs with certain TLR ligands was reported to upregulate autophagosome formation, suggesting that TLRs and autophagosomal proteins cooperate in the response to detected pathogens. The upregulation of autophagy in macrophages was first noted upon treatment with the TLR4 ligand lipopolysaccharide (LPS) (250), and subsequent studies found that this response is specific to agonists of TLR1/2, TLR3, TLR4, or TLR7 (300). Treatment of *M. tuberculosis*-infected macrophages with TLR4 or TLR7 ligands increased bacterial killing, suggesting that TLR-induced autophagy functions in clearance of intracellular pathogens (300). The upregulation of autophagy prior to microbial invasion facilitates the delivery of antimicrobial peptides to lysosomal compartments (301, 302), and may also mobilize autophagosomal proteins to forming phagophores, thereby expediting the acidification of pathogen-containing phagosomes (267). This TLR-induced mobilization of autophagy machinery ultimately contributes to enhanced antigen presentation and innate cytokine production by DCs in response to bacterial and viral infection (295, 303).

1c.iv. Autophagic regulation of pro-inflammatory signaling

In addition to promoting inflammatory responses to pathogens, the process of autophagy or autophagy proteins are implicated in the regulation of excessive inflammation. The first indication of this in chronic inflammatory human disease comes from genome-wide association studies, which revealed a correlation between SNPs in autophagy genes and risk of developing Crohns disease (304, 305), systemic lupus erythematosus (306–309), rheumatoid arthritis (310), and allergic asthma (311). These findings support a clinically relevant requirement for autophagy in the control of inflammation.

The proteins required for autophagosome formation, occasionally acting independently of the canonical autophagy pathway, are responsible for both direct and indirect suppression of certain pro-inflammatory complexes. The ATG5-ATG12 protein

complex negatively regulates the production of IFNβ by directly binding the CARD domains of the mitochondrial RLR adaptor protein IPS-1 and cytosolic RIG-I (312), while ATG9a suppresses IFNβ production in response to dsDNA (313). Similarly, the regulatory protein Rubicon binds CARD9, which is required for signaling downstream of RIG-I, thereby further inhibiting RIG-I-dependent IFNβ production (314). The mitochondrial protein NLRX1 simultaneously activates autophagy and inhibits RLR-dependent type I interferon production (315, 316), which occurs through interaction of ATG5-ATG12 and ATG16L with the NLRX1-interacting protein TUFM (317). Moreover, in the absence of autophagy, a buildup of depolarized mitochondria increases the abundance of IPS-1 and ROS, both of which contribute to elevated RLR signaling and IFNβ production (318). The regulation of RLR signaling by autophagy may represent a feedback mechanism by which IFN signaling is limited after successful induction, thereby preventing excessive or prolonged IFNβ release (253, 266, 319).

The negative regulation of IL-1β and IL-18 production, and therefore the development of IL-1β- and IL-18-dependent immunopathology, is also dependent on autophagy. IL-1β and IL-18 are produced in an inactive pro-form in response to NFκB activation, and require enzymatic cleavage by assembled cytoplasmic protein complexes called inflammasomes. A functional subunit of a multimeric inflammasome complex contains the enzyme pro-caspase-1, the adaptor protein ASC, and a cytosolic PRR such as AIM2 or one of the NLR family members (233). Autophagosomal regulation of inflammasome activity occurs on several levels: first, through the 'housekeeping' function of clearing the cytoplasm of protein aggregates and senescent mitochondria (234, 235); second, by sequestering and degrading pro-IL-1β and pro-IL-18 (320); and finally, through selective degradation of ubiquitinated inflammasome platforms (249).

The requirement for basal autophagy in the prevention of sterile inflammation was first demonstrated *in vivo* through the observation of increased IL-1β and IL-18 production by ATG16L-deficient mice in an experimental model of Crohns disease (321). Depolarized mitochondria accumulate in the absence of autophagy (234, 235), as autophagy is the only intracellular mechanism capable of sequestering and degrading mitochondria. Damaged mitochondria are a source of mitochondrial DNA (mtDNA) and

ROS, both of which function as endogenous inflammasome agonists (234, 235). The removal of compromised mitochondria is also critical to the limitation of inflammation during viral infection. A recent study utilizing a mouse model of influenza A infection found that mice deficient in NOD2 or its downstream target receptor-interacting protein kinase-2 (RIPK2) were hypersusceptible to infection, suffering increased lung pathology and mortality in comparison to WT mice (322). This was traced to a defect in mitochondrial clearance in the absence of RIPK2-mediated phosphorylation of ULK1. The failure to clear damaged mitochondria contributed to elevated release of ROS and increased inflammasome activation in RIPK2-deficient mice, ultimately resulting in greater IL-1β and IL-18 production *in vivo* (322). The importance of mitophagy likely extends to bacterial infection as well, as autophagy-deficient mice produce more IL-1β and suffer greater mortality in LPS-induced and cecal ligation-induced sepsis (235).

The induction of autophagy further serves to negatively regulate inflammasome activity through direct removal of ubiquitinated inflammasome proteins and their substrates pro-IL-1α, pro-IL-1β, and pro-IL-18. In response to LPS treatment, macrophages sequester pro-IL-1β inside autophagosomes, with increased secretion of IL-1β occurring upon treatment with the autophagy inhibitors 3-methyladenine (3-MA) or wortmannin (320, 321). Conversely, treatment with the autophagy inducer rapamycin promoted autophagosomal degradation of sequestered pro-IL-1β (320). A similar phenomenon was recently noted with respect to IL-1α secretion in response to LPS, although the production of IL-1α in this case was inflammasome-independent (323). In addition to promoting the degradation of inflammasome substrates, the process of selective autophagy facilitates the degradation of ubiquitinated inflammasome platforms (235, 249). The induction of AIM2 or NLRP3 inflammasomes in macrophages triggers autophagy, and is dependent on the expression of the inflammasome PRR (i.e. AIM2 or NLRP3) (235). Assembled inflammasomes undergo ubiquitination, and are targeted to nascent autophagosomes by the sequesteosome adaptor p62, which contains an ubiquitin-binding domain and an LC3-interacting domain (321, 324). A similar regulatory mechanism has been proposed for the NLR NOD2, which forms inflammasomes in response to bacterial ligands (325) but also activates the autophagy-promoting RIPK2 (322) and contributes to the spatial localization of autophagy machinery through

recruitment of ATG16L to sites of bacterial entry (326, 327). The simultaneous induction of autophagy and inflammasome assembly by the same cytosolic PRR provides a potential regulatory mechanism to limit pro-inflammatory IL-1β production.

1c.v. ER stress-induced autophagy and inflammasome activation

The endoplasmic reticulum is continually monitored in the steady state for signs of perturbation by several different ER-membrane-bound sensors, including inositol-requiring protein 1α (IRE-1α), activating transcription factor 6 (ATF6), and protein kinase-like ER kinase (PERK) (236). Upon detection of the accumulation of misfolded proteins or depletion of ER calcium gradient, these sensors of ER stress activate the unfolded protein response (UPR). The UPR reduces the production of new proteins while upregulating gene expression of ER chaperone proteins, which assist in the folding of peptides. Proteins that are improperly folded are removed through one of two ER-associated degradation (ERAD) pathways, either by ubiquitin-proteasome ERAD I or autophagosome-lysosome ERAD II (328, 329). The latter pathway utilizes K63-specific mono/polyubiquitin tags on misfolded proteins, which are bound by cytosolic SLRs and shuttled to forming autophagosomes (330).

Emerging research suggests that ER stress signaling may trigger autophagy-mediated ER clearance as a cellular survival strategy, thereby expediting the removal of misfolded proteins and a return to homeostasis (331–334). In the presence of strong ER stressors, the NLRP3 inflammasome is activated via a mitochondrial-dependent mechanism, ultimately leading to IL-1β production (335, 336). NLRP3 and ASC are spatially recruited to mitochondria-associated ER membranes (MAMs) upon activation, possibly in response to the release of pro-inflammasome ROS from the mitochondria (234). Moreover, recent studies identified MAMs as the source of autophagosomal membranes, suggesting that autophagy occurs in immediate proximity to both the ER and mitochondria (337). This spatial arrangement may facilitate rapid clearance of damaged mitochondria and impacted ER (331), while also enabling autophagic control of IPS-1-dependent signaling and IFNβ production (338).

Disruption of ER equilibrium may occur during viral infection through several different mechanisms. Rapid accumulation of viral proteins in the ER, especially those requiring glycosylation, may trigger ER stress (339–341). In addition, virally-encoded viroporin proteins such as the rotavirus NSP4 protein (342) and picornavirus 2B proteins (343) depolarize calcium differentials in the ER, which can trigger ER stress (344). The ER may be further perturbed by viral utilization of ER replication complexes, and ER membrane as source-membrane for newly-synthesized virions (236). Interestingly, RSV infection was recently shown to trigger IRE-1α and ATF6 signaling, with blockade of IRE-1α signaling leading to enhanced viral replication (345). The RSV SH protein was recently identified as a viroporin, expression of which was required for RSV-induced IL-1β production by human bronchial epithelial cells (138). However, any potential connection between ER stress, inflammasome activation, and autophagic control of IL-1β in the context of RSV infection remains to be explored.

1d. Summary of rationale and specific aims

Respiratory syncytial virus (RSV) is a ubiquitous human pathogen that produces severe lower respiratory disease in vulnerable individuals, characterized by lung infiltration of innate immune cells and a Th2-skewed adaptive immune response leading to excessive mucus production. RSV-associated bronchiolitis remains the leading cause of hospitalization among infants in the United States, and is responsible for considerable morbidity among patients who are elderly, immune compromised, and those with underlying chronic pulmonary illness. Hospitalization due to RSV in infancy is also strongly correlated with development of recurrent wheezing later in childhood, suggesting that infection causes lasting alteration of the lung immune environment and predisposition to chronic disease development.

Pulmonary dendritic cells (DCs) direct innate and adaptive immune responses to viral pathogens through secretion of pro-inflammatory cytokines and type I interferon (IFN), as well as through activation of antiviral T cell responses in lung-draining lymph nodes. Signaling through the RNA-sensing endosomal toll-like receptors (TLR) -3 and -7 is required for robust production of type I IFN and antigen presentation function by DCs during viral infection. As RSV infects target cells through membrane fusion rather than

through endocytosis, an alternative method of delivery of viral antigens to endosomal compartments is necessary for rapid detection of viral RNA. In agreement with other studies of virally-infected cells, work conducted by our laboratory has shown that macroautophagy (autophagy) modulates TLR-dependent maturation and antigen-presenting cell (APC) function by RSV-infected DCs.

Autophagosome formation is a conserved intracellular process by which cytosolic constituents are enveloped in a double-walled membrane and degraded by fusion with lysosomes. Moreover, autophagy within APCs facilitates MHC class II peptide loading and intracellular TLR signaling through acquisition and delivery of cytoplasmic viral proteins and nucleic acids to acidified endosomes. The autophagic pathway and its constituent proteins are conversely known to antagonize the assembled NLRP3 inflammasome and its substrate pro-IL-1β in both epithelial and myeloid immune cells, suggesting a potential dual role of autophagy as a negative regulator of inflammatory signaling during RSV infection. As the clinical manifestations of severe RSV infection are linked to aberrant innate and T cell cytokine production, we hypothesize that **autophagy proteins promote the effective induction of an antiviral immune response and clearance of RSV by facilitating DC maturation, while simultaneously minimizing excessive pulmonary inflammation through antagonism of inflammasome-mediated IL-1β secretion.** These studies will utilize human RSV Line-19, originally isolated from a hospitalized infant, and will be conducted *in vitro* and in a clinically relevant mouse model. This work will shed light on the basic mechanisms by which DCs orchestrate innate and acquired immune responses to RSV, which may have significant therapeutic implications for human disease.

We will first **examine the requirement ofATG6/Becn1 in DC-mediated induction of antiviral adaptive immune responses to RSV *in vivo*.** We hypothesize that impaired autophagy-dependent DC maturation and innate cytokine production in Becn1 haploinsufficient ($Becn1^{+/-}$) mice will result in an attenuated antiviral T cell response, decreased viral clearance, and increased lung pathology upon RSV infection. Results show that RSV-infected $Becn1^{+/-}$ mice develop Th2 cytokine-associated mucus production and eosinophil infiltration within the lungs, while RSV-infected $Becn1^{+/-}$ DCs

elicited Th2 cytokine production from co-cultured CD4+ T cells. Sensitization of WT mice with RSV-pulsed Becn1$^{+/-}$ DCs recapitulated the Th2 pathology observed in

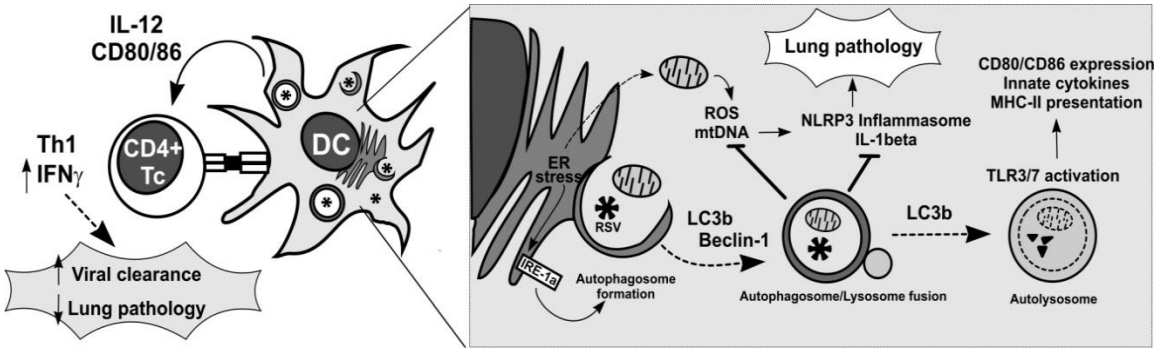

Figure 1-4 – **Schematic representation of autophagy-mediated promotion of DC-mediated elicitation of Th1-polarized CD4+ T cell responses to RSV, and autophagy-mediated regulation of lung pathology-inducing cytokine IL-1β**. A) Cytosolic viral peptides and RNA are captured by autophagosomes and fuse with lysosomes, thereby facilitating endosomal TLR activation, innate cytokine production, and upregulation of costimulatory molecules (B). This process promotes the production of IL-12, which polarizes CD4+ T cells towards a protective Th1 phenotype (C). D) In addition, autophagy suppresses NLRP3 inflammasome activation and production of its substrate IL-1β through sequestration of damaged mitochondria, and/or through direct removal of inflammasomes and pro-IL-1β. In the absence of autophagy, IRE-1α-mediated mitochondrial ROS/mtDNA release promotes NLRP3 inflammasome activation and IL-1β secretion, ultimately contributing to the development of lung pathology in response to RSV.

Becn1$^{+/-}$ mice upon RSV challenge, supporting the importance of Becn1-dependent autophagy in RSV-infected DC *in vivo.*

We will next **assess the requirement of ATG8/LC3b in innate and adaptive immune responses to RSV.** We hypothesize that impaired autophagy induction in LC3b-deficient (LC3b$^{-/-}$) mice will result in an altered antiviral T cell response and increased lung pathology upon RSV infection. Results show that LC3b$^{-/-}$ mice develop IL-17-associated lung mucus production and neutrophil infiltration upon RSV infection, while RSV-infected LC3b$^{-/-}$ DCs secrete IL-6 and IL-23, and elicit increased IL-17a production from co-cultured CD4+ T cells. Additionally, LC3b$^{-/-}$ lung epithelial cells produce increased amounts of IL-1β and IL-6 in response to RSV, with epithelial cell cytokine production contributing to Th17-dependent lung neutrophilia and mucus production. IL-1 receptor antagonist treatment reduced Th17 lung pathology, identifying IL-1R signaling as a driver of Th17 responses to RSV.

Finally, we will **investigate a requirement for LC3b expression in the control of ER stress signaling-dependent IL-1β production by RSV-infected cells.** We hypothesize that autophagy deficiency in LC3b$^{-/-}$ epithelial cells will promote ER stress signaling-dependent inflammasome activation and concomitant IL-1β secretion upon

RSV infection. Results demonstrate that LC3b$^{-/-}$ epithelial cells increase signaling through the ER stress sensor IRE-1α upon RSV infection, while treatment with an inhibitor of IRE-1α attenuated caspase-1 cleavage and IL-1β secretion. These results implicate IRE-1α signaling and its control through an LC3b-dependent mechanism in driving excessive inflammasome activation and IL-1β secretion during RSV infection.

Chapter 2 – Autophagy-inducing protein Beclin-1 in dendritic cells regulates CD4 T cell responses and disease severity during respiratory syncytial virus infection

2a. Abstract

Recent work has demonstrated the importance of macroautophagy in dendritic cell (DC) maturation and innate cytokine production upon viral infection through delivery of cytoplasmic viral components to intracellular toll-like receptors. To study the functional consequences of impaired autophagosome formation during a Respiratory Syncytial Virus (RSV) infection, mice harboring significant autophagy defects due to Beclin-1 haploinsufficiency ($Becn1^{+/-}$) were used. Upon RSV infection in vivo, lungs of $Becn1^{+/-}$ mice showed increased Th2 cytokine production, mucus secretion, and lung infiltration of eosinophils and inflammatory DCs. While isolated airway epithelial cells from $Becn1^{+/-}$ mice demonstrated little change compared to Wild-type, $Becn1^{+/-}$ pulmonary and bone marrow-derived DCs (BMDCs) showed decreased expression of MHC-II and innate cytokine production upon RSV infection. Further examination indicated that $Becn1^{+/-}$ DC stimulated less IFNγ and IL-17 production by co-cultured CD4+ T cells and increased Th2 cytokine production in comparison to wild-type controls. Finally, adoptive transfer of RSV-infected $Becn1^{+/-}$ DCs into the airways of wild-type mice produced severe lung pathology and increased Th2 cytokine production upon subsequent RSV challenge compared to wild-type DC transfer controls. These results indicate a critical role of autophagy in dendritic cells during pulmonary viral infection, facilitating appropriate antiviral adaptive immune responses.

2b. Introduction

Respiratory viral infections are associated with significant morbidity and mortality in susceptible patient populations, outcomes primarily linked to inappropriate inflammatory and immune responses that compromise lung function (55, 56). Respiratory

syncytial virus (RSV) is a single-stranded RNA virus of the paramyxoviridae family, with a double-stranded RNA intermediate (40). RSV is a ubiquitous human pathogen that predominantly causes mild respiratory tract infection, yet it remains one of the leading causes of respiratory infection-related hospitalization worldwide (4, 5, 346). Vulnerable individuals such as infants, the elderly, or the immunosuppressed often develop severe symptoms such as bronchiolitis and pneumonia, characterized by mucus secretion and pulmonary infiltration of monocytes and granulocytes (40). In addition, hospitalization due to RSV in infancy is associated with an increased risk of developing allergic asthma and recurrent wheezing later in life (61, 62). The epidemiological evidence of subsequent immune alteration after RSV infection, combined with the complex nature of host and viral factors contributing to disease pathogenesis, underscore the need to understand the host response to RSV and its contribution to viral clearance, as well as to immune-mediated lung pathology.

Within the lung environment, dendritic cells (DCs) direct innate and adaptive immune responses to viral pathogens through secretion of pro-inflammatory cytokines and type I interferon (IFN), as well as through migration and antigen presentation to T cells in lung-draining lymph nodes. DC activation is accomplished through detection of viral antigens by pattern-recognition receptors (PRRs) such as PKR, RIG-I, and both MyD88-dependent and TRIF-dependent toll-like receptors (TLRs) (139). Activation of RNA-sensing intracellular TLRs such as TLR3 and TLR7 is required for robust production of type I IFN and APC function in virally infected plasmacytoid and myeloid DC (347, 348). Acquisition of viral antigens may be accomplished through phagocytosis of virally-infected cells, yet recent work conducted by ourselves and others suggests that the intracellular process of macroautophagy (autophagy) within virally-infected DCs functions as an important driver of DC maturation and pro-inflammatory cytokine production (230, 297). This process may be especially important during RSV infection, as RSV directly enters the cytoplasm via membrane fusion (349), thus requiring delivery of cytosolic viral nucleic acid to endosomal TLRs (134, 349).

Autophagy is a highly conserved process through which cytoplasmic contents are enveloped in a double-walled membrane and degraded upon fusion with lysosomes.

Autophagosome formation is initiated in mammalian cells by release of ATG6/Beclin-1 (Becn1) from Bcl-2, enabling formation of the Becn1-containing VPS34-PI3K complex that is required for generation of pre-autophagosome structures (350). Becn1 is a frequent target of viral subversion, attesting to the importance of autophagy in clearance of intracellular pathogens from infected host cells (351). In addition, autophagy modulates several important functions within professional antigen-presenting cells (APCs) by enabling cytoplasmic antigen capture and MHC-mediated presentation to T cells (287, 352), by regulating inflammasome activity and IL-1β secretion (235, 249), and by promoting TLR-dependent DC maturation and type I IFN production through delivery of TLR ligands to endosomes (353). Furthermore, TLR ligation upregulates autophagosome formation through TRAF6-dependent ubiquitination and release of Becn1 from BCL-2, potentially serving as a positive regulation mechanism of TLR signaling (354). While the functions of autophagy in APCs infected *in vitro* have been examined, studies of autophagy *in vivo* have largely focused on host protection during bacterial infection, or host protection from encephalitis upon infection with neuropathogenic viruses (278, 355, 356). While viral subversion of autophagy and decreased CD4+ T cell responses to neurovirulent HSV-1 were recently elucidated (278, 352, 356), the role of autophagy in promoting DC maturation during pulmonary viral infection and the impact on CD4+ T cell responses is poorly understood.

Our laboratory recently reported that TLR-dependent innate cytokine production and maturation of RSV-infected DCs was attenuated upon blockade of autophagy, resulting in decreased production of IFNγ and IL-17a in co-cultured CD4+ T cells (230). In order to examine the importance of autophagosome formation during RSV infection *in vivo*, we used Becn1$^{+/-}$ mice that harbor defects in the upregulation of autophagosome formation upon stimulation (353). Altogether, these studies demonstrated the role of autophagy within DCs to facilitate priming of a robust antiviral adaptive immune response to RSV, as well as in the limitation of excessive pathology during pulmonary infection. The impact of these findings may have significant therapeutic implications for severe clinical disease and may contribute strategies for viral vaccine development.

2c. Materials and methods

Mice

Female C57Bl/6J, BALB/cJ, and B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) transgenic mice were purchased at 6-7 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Becn1$^{+/-}$ mice were originally obtained from Z. Yue (353), and a breeding colony was subsequently established at the University of Michigan (Ann Arbor, MI). D011.10 mice were bred in-house at the University of Michigan. All work involving animals was conducted in compliance with University of Michigan Committee on Use and Care of Animals policy.

Respiratory Syncytial Virus

Our laboratory uses antigenic subgroup A, Line 19 RSV, originally obtained from a sick infant at the University of Michigan Hospital System. This isolate has been shown in animal models to mimic human infection by eliciting airway mucus production upon inoculation with 1 x 10^5 pfu RSV (357). Mice were infected intratracheally with 1 x 10^5 pfu RSV.

Quantitative PCR

RNA was isolated from cell cultures and lung tissue using TRIzol, according to manufacturer's instructions (Invitrogen). 5μg of RNA was then reverse-transcribed to determine cytokine gene expression using pre-developed TaqMan Gene Expression Assay primer/probe sets and analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Transcription levels of *muc5ac, gob5, ifnb,* and *RSV-G, -F,* and *–N* proteins were assessed using custom primers as previously described (155). Gene expression was normalized using GAPDH expression as an internal control, and fold change values were calculated relative to an uninfected or wild-type control group assigned an arbitrary value of 1.

Dendritic cell culture

BMDC were cultured from whole bone marrow, obtained from WT C57Bl/6 mice, Becn1$^{+/-}$ mice, or WT littermates as indicated. Bone marrow cells were seeded into tissue culture flasks containing RPMI 1640-based complete media supplemented with 20ng/ml GM-CSF (R&D systems, Minneapolis, MN). Cells were fed on day 3 and 5, and

harvested on day 7. On day 7, cells were ≥85% CD11b+ CD11c+ BMDC by flow cytometric analysis.

Pulmonary CD103+ and CD11b+ DC were obtained from lungs and bronchi of C57Bl/6 mice by enzymatic digestion, through modification of previously-published methods (358). Minced tissue was incubated in RPMI-1640 with 200μg/ml Liberase TM (Roche Applied Science, Indianapolis, IN) and 200 U/ml DNase I (Sigma-Aldrich) for 45 minutes at 37°C, drawn through an 18-gauge needle/10cc syringe, and filtered through 40μm nylon mesh. The cell suspension was enriched for CD11c+ cells using anti-mouse CD11c microbeads and magnetic column separation (Miltenyi Biotec, Auburn, CA), then stained with PE-conjugated anti-CD11b and APC-conjugated anti-CD103 antibodies (eBioscience, San Diego, CA). DC subsets were sorted using an iCyt Synergy 3200 fluorescence-activated cell sorter (iCyt, Champaign, IL).

Alveolar Epithelial Cell culture

Whole lungs of naïve mice were digested in Dispase (BD Biosciences), filtered through 25μm mesh, and depleted of immune cells through labeling with biotinylated antibodies to CD16/32 and CD45 (BD Pharmingen), followed by labeling with anti-biotin microbeads and passage through a MACS column (Miltenyi Biotec). Depleted cell suspensions were adherence-purified overnight in DMEM-based complete media, and non-adherent cells cultured for 4 days in complete media within fibronectin-coated wells, yielding ≥90% e-cadherin positive cells. Cultures were RSV-infected at 1:1 MOI.

CD4+ T cell isolation and DC-T cell co-culture

RSV-responsive CD4+ T cells were isolated from mediastinal and cervical lymph nodes of C57Bl/6 mice infected 8 days previously with 1×10^5 pfu RSV. Ovalbumin-responsive T lymphocytes were isolated from minced spleens from OT-II or D011.10 transgenic mice as indicated. Lymph nodes or minced spleens were forced through a 40μm nylon strainer, then CD4+ T cells were isolated using magnetic bead selection, using a negative selection protocol yielding >95% pure CD4+ T cells (Miltenyi Biotec, Auburn, CA). T cells were subsequently plated at 5×10^5 cells per well in 96-well cell culture plates, on top of 5×10^4 DC treated two hours previously with 1:1 MOI RSV. Experiments conducted with OT-II or D011.10 T cells were treated with 200μg/ml whole ovalbumin protein as indicated. Co-cultures were incubated for 24 hours for mRNA analysis, or 48

hours for cell supernatant cytokine analysis on the BioRad Bioplex 200 system, according to manufacturer's protocol. Custom kits containing Ab-coated beads for mouse IL-4, IL-5, IL-13, IL-17a, and IFNγ were used to assay cytokine concentration (BioRad).

Flow Cytometry

Right lungs of control and RSV-infected mice were digested enzymatically in RPMI-1640 complete media containing 1mg/ml Collagenase A (Roche Applied Science, Indianapolis, IN) and 30μg/ml DNase I (Sigma). LDLN were forced through a 40μm nylon strainer. Cells were stained with Live/Dead Fixable Yellow (Invitrogen) followed by appropriate antibodies as indicated. Analysis was performed using FlowJo software (Treestar Inc, Ashland, OR).

Confocal Microscopy

BMDCs were cultured as described, then plated in Labtek chamber slides (Thermo Fisher Scientific). Cells were treated as indicated, then fixed in 4% paraformaldehyde for 20 minutes. Cells were blocked for one hour at room temperature in PBS containing 5% normal goat serum and 0.1% Tween-20, and stained with DyLight 550-conjugated anti-ATG5 antibodies (Novus Biologicals, Littleton, CO). Pro-long Gold anti-fade reagent plus DAPI was added (Invitrogen), and cells were imaged on a Nikon A1 Confocal Laser Microscope system under 60x oil immersion, using NIS Elements acquisition software (Nikon Instruments Inc.). Maximum intensity projection images were created from Z-stack images using ImageJ software (NIH).

Statistics

Data was analyzed and graphs generated using GraphPad Prism software. Statistical significance was assessed by one-way ANOVA, followed by Bonferroni post-test to obtain p values. Significant differences were regarded as $p \leq 0.05$.

2d. Results

2d.i. Becn1$^{+/-}$ mice show increased lung pathology upon RSV infection

Homozygous deletion of many autophagy genes, including Becn1, results in early embryonic or neonatal lethality in mice (353, 359). In order to assess the importance of autophagosome formation during RSV infection in vivo, we chose to use Becn1$^{+/-}$ mice, which are viable but show defects in autophagosome formation (353). Becn1$^{+/-}$ and wild-type (WT) littermate mice were infected with RSV and sacrificed at day 8 post-infection

to assess lung pathology. Histological examination of paraffin-embedded lung sections revealed increased peribronchial inflammation, goblet cell metaplasia, and occlusion of airways by mucus and cellular debris in RSV-infected Becn1$^{+/-}$ mice (Figure 2-1**A**). Flow cytometric analysis of single-cell suspensions obtained from collagenase-digested lung tissue revealed increased numbers of eosinophils and DCs within the lungs of Becn1$^{+/-}$ mice (Figure 2-1**B**). Additionally, lung-draining lymph node (LDLN) cultures prepared from RSV-infected Becn1$^{+/-}$ mice secreted significantly greater amounts of Th2 cytokines, and significantly less IFNγ and IL-17a production upon restimulation with RSV *ex vivo* when compared to LDLN from WT mice (Figure 2-1**C**). As lung

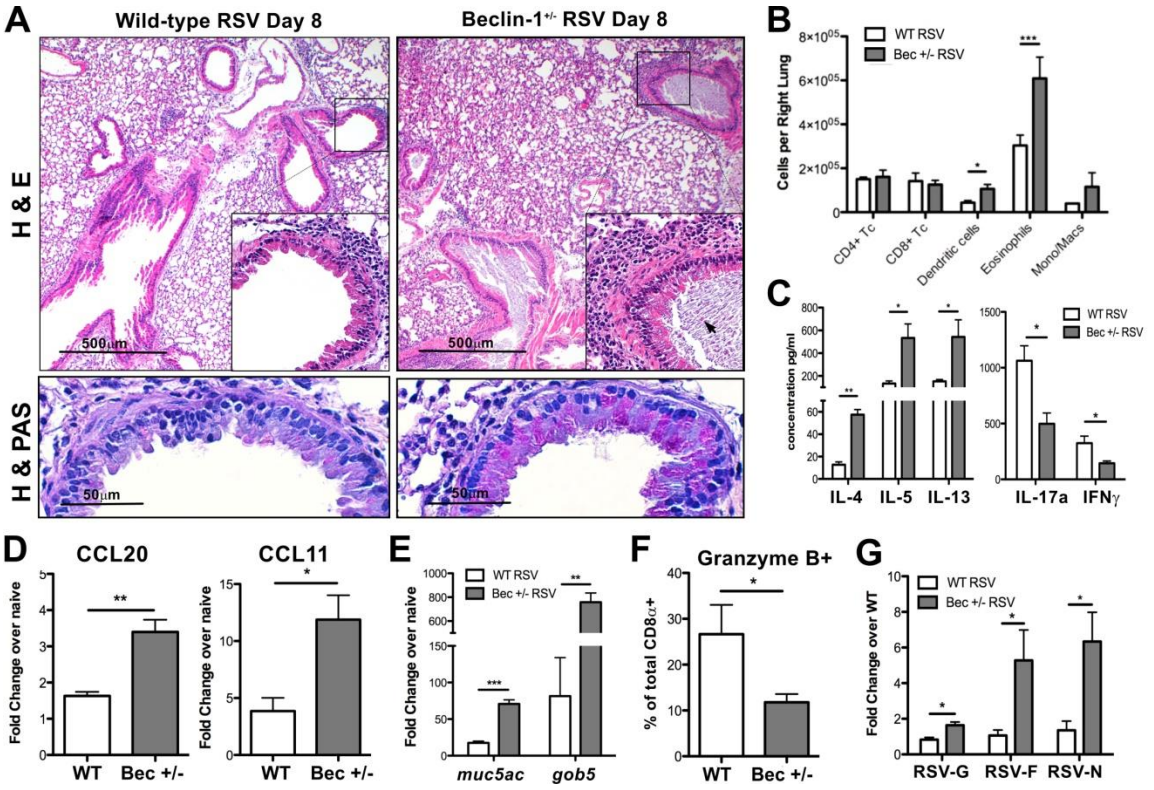

Figure 2-1 - **Beclin-1$^{+/-}$ mice show increased pulmonary immune infiltration and pathology upon RSV infection.** (A) Lung sections from RSV-infected Beclin-1$^{+/-}$ mice or WT littermate controls 8 days post-infection, stained with either haematoxylin and eosin (H&E, top panels) or haematoxylin and periodic acid schiff (H&PAS, bottom panels) stain. Arrow indicates apoptotic cell debris within airway mucus plug (B) Total numbers of lineage-positive immune cells obtained from collagenase-digested lungs of Beclin-1$^{+/-}$ or WT mice, 8 days post-RSV infection. (C) Lung-draining lymph nodes of Beclin$^{+/-}$ or WT mice, 8 days post-RSV infection, were dissociated into a single cell suspension and restimulated in culture with RSV. Cytokine concentrations in culture supernatants were assayed using a Bioplex system. Lung mRNA expression of CCL20 and CCL11/eotaxin (D) 6 days post-RSV infection, and (E) the mucus-associated genes *muc5ac* and *gob5* 8 days post-infection, was obtained using quantitative real-time PCR (qPCR) and compared to naïve controls. (F) Percentage of total lung CD8α+ T cells expressing Granzyme B by intracellular staining and flow cytometry, obtained from collagenase-digested lungs 8 days post-RSV infection. (G) Lung mRNA expression values of RSV-G, -F, and –N transcript was obtained by qPCR 8 days post-infection. Fold change was calculated relative to WT, RSV-infected lungs. Data are representative of three independent experiments, with four to six mice per group. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001.

recruitment of eosinophils and DCs during RSV infection was shown to be respectively dependent on secretion of CCL11/eotaxin (360) and CCL20 (226), these chemokines were assessed by qPCR and were found to be significantly increased in lungs of RSV-infected Becn1$^{+/-}$mice (Figure 2-1**D**). Increased mucus secretion within the lungs was observed by periodic acid schiff (PAS) staining of lung sections, while mRNA transcript levels of mucus-associated genes muc5ac and gob5 were significantly elevated in lungs of Becn1$^{+/-}$mice (Figure 2-1**A,** Figure 2-1**E**). Interestingly, overall numbers of CD8a+ T cells did not differ at day 8 post-infection (Figure 2-1**B**), yet fewer CD8a+ T cells from Becn1$^{+/-}$ lungs expressed the cytotoxic protease Granzyme B when compared to lungs from WT mice (Figure 2-1**F**). Finally, qPCR measurement of RSV-G, -F, and -N mRNA within lung tissue showed significantly increased viral mRNA expression in infected Becn1$^{+/-}$ mice in comparison to RSV-infected WT littermates, suggesting increased viral replication (Figure 2-1**G**). These data suggest that Becn1$^{+/-}$ mice are impaired in their ability to mount an effective antiviral adaptive immune response upon RSV infection, instead producing increased Th2 cytokine-associated lung pathology in vivo.

2d.i. Becn1$^{+/-}$ pulmonary DCs show impaired maturation upon RSV infection

Autophagosome formation and maturation is known to be critical in mediating innate viral recognition and presentation of cytoplasmic viral antigens by DCs to T cells (351). In agreement, previous studies conducted by our laboratory found that blockade of autophagosome formation within DCs resulted in impaired surface expression of MHC class II and costimulatory molecules CD80/CD86 upon RSV infection *in vitro* (230). We therefore examined the numbers and maturation status of DCs that were present in the lungs and LDLN of RSV infected Becn1$^{+/-}$ mice and compared them to control tissues from RSV-infected WT mice. Pulmonary DC subsets in enzyme-digested lungs and LDLN were identified and quantified by flow cytometry (361), and revealed sharply increased numbers of CD11c+ MHC-II^{high} CD11b^{high} DCs (CD11b+ DCs) in lungs of infected Becn1$^{+/-}$ mice, with no significant differences detected in numbers of CD11c+ MHC-II^{high} CD11b^{low} CD103+ intraepithelial DCs (CD103+ DCs) (Figure 2-2**A,** Figure 2-2**B**). Due to increased cellularity, greater numbers of both CD11b+ DCs and CD103+

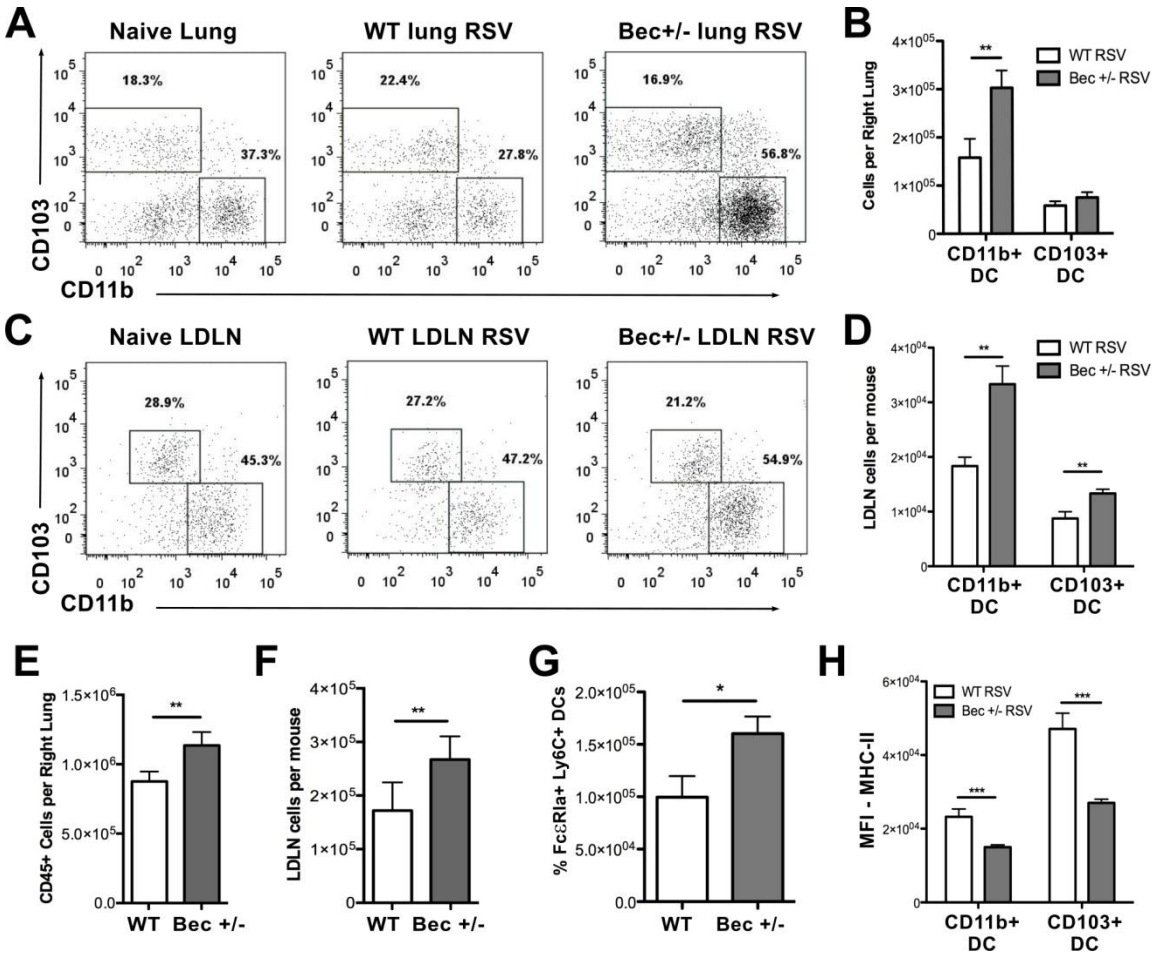

Figure 2-2 - **Beclin-1$^{+/-}$ mice recruit greater numbers of DCs displaying decreased MHC class II expression upon RSV infection**. Lungs and lung-draining lymph nodes (LDLNs) were harvested 8 days post-RSV infection, and tissues from Beclin-1$^{+/-}$ mice were compared to those from WT mice using flow cytometry. (A) Representative flow plots of CD11b+ and CD103+ DCs from collagenase-digested lungs of Beclin-1$^{+/-}$ or WT littermate mice. Total numbers of CD11b+ and CD103+ DCs (B) were calculated using percentages and total cell counts within lungs. (C) Representative flow plots of CD11b+ and CD103+ DCs in single-cell preparations of LDLNs of Beclin-1$^{+/-}$ or WT littermate mice. Total numbers of CD11b+ and CD103+ DCs (D) were calculated using percentages and total cell counts within LDLNs. (E) Total CD45+ cell counts from lungs, (F) LDLNs, and (G) lung inflammatory CD11b+ DCs co-staining with FcεRIα+ (clone MAR-1) and Ly6C+ were calculated by flow cytometry. (H) Median fluorescence intensity (MFI) of MHC-II surface staining on CD11b+ and CD103+ DCs from LDLNs. Data are representative of at least three independent experiments, with four to six mice per group. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001.

DCs were recovered from the LDLN of infected Becn1$^{+/-}$mice, although relative percentages did not reach significance (Figure 2-2**C,** Figure 2-2**D**). The lungs of RSV-infected Becn1$^{+/-}$ mice contained a significantly greater number of CD45+ cells (Figure 2-2**E**), while the mediastinal lymph nodes recovered from infected Becn1$^{+/-}$ mice appeared larger than those from WT littermates. This was reflected in significantly higher total numbers of lymph node cells recovered per mouse (Figure 2-2**F**). Further examination of surface molecule expression of CD11b+ DCs in the lungs revealed that a large percentage were FcεRIα+ Ly6C+ inflammatory DCs, and were present in greater

numbers in lungs of RSV-infected Becn1$^{+/-}$ mice (Figure 2-2**G**). Finally, assessment of DCs within the LDLNs revealed decreased MHC-II expression on both CD11b+ DCs and CD103+ DCs (Figure 2-2**H**), with no differences in expression of co-stimulatory molecules CD80, CD86, or CD40 in comparison to infected WT mice (data not shown).

In addition to modulating DC maturation, autophagy proteins have been shown to regulate cytokine production in virally-infected non-hematopoietic cells through delivery of viral antigens to intracellular TLRs, as well as through antagonism of signaling by cytosolic pattern recognition receptors such as RIG-I (362). As RSV predominantly infects the respiratory epithelium and intraepithelial DCs (363), we investigated innate cytokine responses of both RSV-infected epithelial cells and pulmonary DCs from Becn1$^{+/-}$ mice. Pro-inflammatory cytokine production by RSV-infected Becn1$^{+/-}$ primary airway epithelial cell cultures (AECs) revealed no significant differences in comparison to WT cultures when assessed by qPCR 24 hours post-infection (Figure 2-3**A**). Similarly, both WT and Becn1$^{+/-}$ RSV-infected AECs showed no significant upregulation of key autophagy genes 24 hours post-RSV infection (Figure 2-3**B)**. Further examination of autophagy induction through ATG5 punctate staining and confocal microscopy, as well as through accumulation of membrane-bound LC3-II by immunoblotting, revealed no significant changes in RSV-infected AECs compared to uninfected controls (data not shown).

In contrast, both CD11b+ and CD103+ pulmonary DCs from naïve Becn1$^{+/-}$ lungs showed reduced innate cytokine production upon *ex vivo* RSV infection (Figure 2-3**C)**. Pulmonary DCs from WT and Becn1$^{+/-}$ mice showed differential upregulation of autophagy genes upon infection, as RSV-infected Becn1$^{+/-}$ DCs failed to upregulate expression of ATG5, ATG6, and ATG7 in comparison to RSV-infected WT DCs (Figure 2-3**D**). Importantly, as a measure of antigen presentation function, RSV-infected Becn1$^{+/-}$ DCs co-cultured with CD4+ OT-II T cells elicited less IFNγ and IL-17a production in comparison to WT controls (Figure 2-3**E**). These results suggest that while Becn1$^{+/-}$ mice appear to have no innate defect in cytokine production by epithelial cells or CD4+ T cells, Becn1$^{+/-}$ pulmonary DCs are impaired in innate cytokine production and antigen presentation in response to RSV infection.

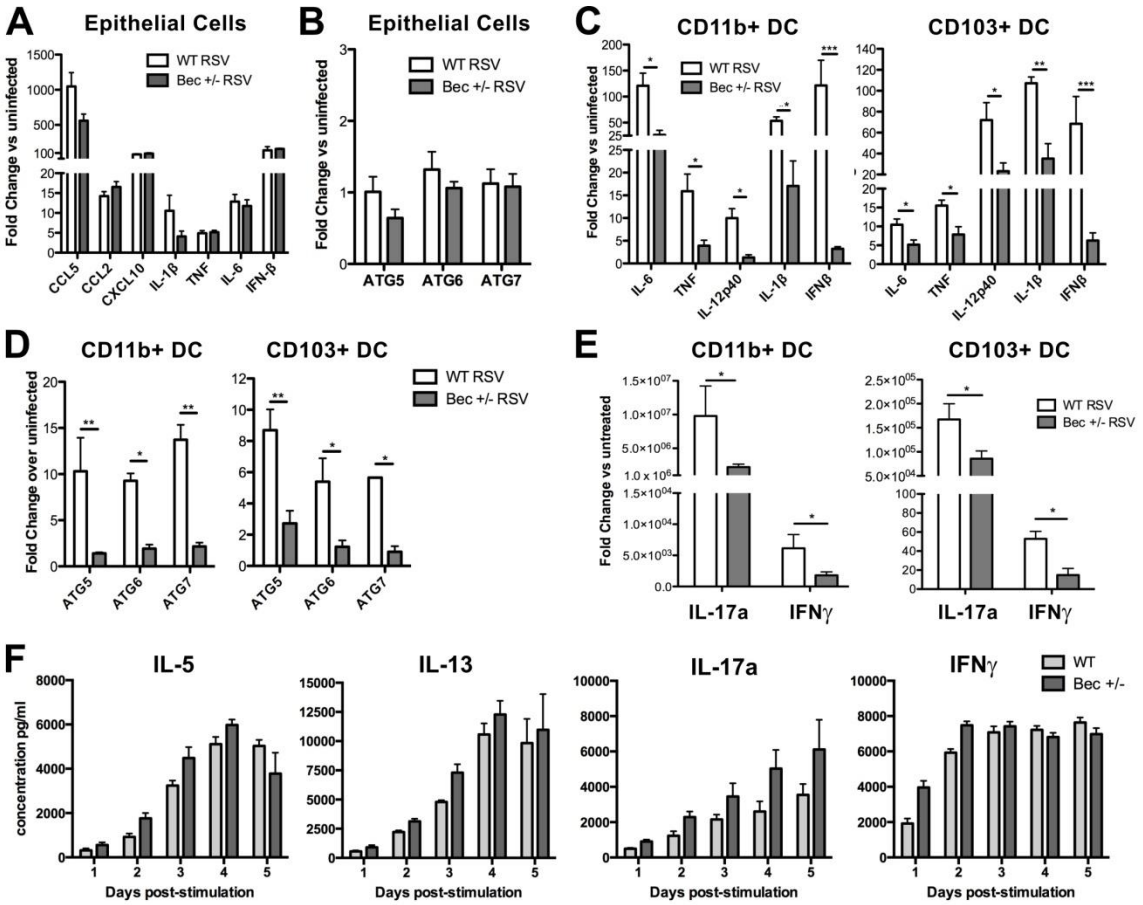

Figure 2-3 - **Beclin-1$^{+/-}$ lung epithelial cells are competent in innate cytokine responses to RSV infection, while pulmonary DCs are impaired in innate cytokine production, autophagy gene expression, and antigen presentation upon RSV infection.** (A) Cytokine production and (B) autophagy gene expression by Beclin-1$^{+/-}$ or wild-type mouse primary alveolar epithelial cells was assessed by qPCR 24 hours post-RSV infection. (C-E) Pulmonary DCs were fluorescently labeled and flow-sorted from collagenase-digested lungs of naïve Beclin-1$^{+/-}$ or wild-type mice, and infected with RSV at 1 DC: 1 pfu (1:1 MOI). Innate cytokine production and autophagy gene expression by CD11b+ lung DCs or CD103+ DCs (C) was assessed at 24 hours post-RSV infection by qPCR. Autophagy gene expression by CD11b+ DCs or CD103+ DCs (D) was measured by qPCR at 24 hours post-infection. Cytokine production by purified splenic CD4+ OT-II T cells, co-cultured with CD11b+ lung DCs or CD103+ DCs (E) treated concurrently with RSV and 200μg/ml whole ovalbumin protein, was assessed by qPCR at 24 hours. Data are representative of at least two independent experiments, with at least four replicates per group. Error bars represent SEM. (F) WT and Beclin-1$^{+/-}$ purified CD4+ splenocytes were cultured with antibodies to CD3 and CD28. Supernatants were assayed by Bioplex. *p<0.05, **p<0.01.

Controlled regulation of Becn1 expression has been documented during T cell development and activation, although the functional significance of this observation is not known (364). To ensure that Becn1$^{+/-}$ CD4+ T cells do not possess an intrinsic cytokine production deficiency or Th2 bias upon activation, we stimulated purified splenic CD4+ T cells from naïve Becn1$^{+/-}$ or WT littermate control mice with antibodies to CD3 and CD28. Measurement of cytokine secretion in culture supernatants across a five-day time course revealed no significant differences in production of IL-5, IL-13, IL-17a, or IFNγ by Becn1$^{+/-}$ T cells (Figure 2-3**F**).

2d.ii. Becn1$^{+/-}$ BMDCs are deficient in innate cytokine production and fail to mature upon RSV infection

Previous work conducted by our laboratory demonstrated that TLR-dependent DC maturation and innate cytokine production in response to RSV is dependent on autophagy (230). Having obtained similar results for lung-derived Becn1$^{+/-}$ DCs infected with RSV *ex vivo*, we utilized bone marrow-derived DCs (BMDCs) cultured from Becn1$^{+/-}$ mice to further study the impact of Becn1 insufficiency on the upregulation of autophagy and on DC maturation. Examination of punctate ATG5 staining by confocal microscopy, which identifies nascent autophagosomes (350), confirmed a defect in autophagosome formation within RSV-infected Becn1$^{+/-}$ BMDCs (Figure 2-4**A,** Figure 2-4**B**). Becn1$^{+/-}$ BMDCs similarly failed to upregulate autophagy gene mRNA expression in response to RSV (Figure 2-4**C**).

Becn1$^{+/-}$ DC maturation was next examined by assessing costimulatory molecule expression and innate cytokine production upon RSV infection. Analysis of surface staining of RSV-infected BMDCs by flow cytometry revealed that while WT BMDCs upregulated surface expression of MHC-II and CD80/86 co-stimulatory molecule expression in response to RSV infection, Becn1$^{+/-}$ BMDCs only weakly upregulated MHC-II expression and did not increase expression of CD80 or CD86 above the levels of uninfected controls (Figure 2-4**D**). In addition, qPCR of RSV-infected Becn1$^{+/-}$ BMDCs revealed significantly less type I interferon and pro-inflammatory cytokine production in comparison to WT controls (Figure 2-4**E**). These results confirm our previous findings that support the role of autophagy in promoting DC maturation, as Becn1$^{+/-}$ DCs fail to upregulate autophagosome formation, MHC-II expression, and produce innate cytokines upon RSV infection.

We next sought to verify the modulatory role of autophagy in altered cytokine production by Becn1$^{+/-}$ DCs. In agreement with our previous findings (230), induction of autophagy in WT BMDCs through amino acid starvation prior to RSV infection synergistically increased IFNβ and IL-6 production in comparison to RSV infection alone, as well as IL-12p40 and IL-1β to a lesser extent (Figure 2-4**F**). In contrast, Becn1$^{+/-}$ BMDCs produced significantly less of these cytokines in response to either RSV

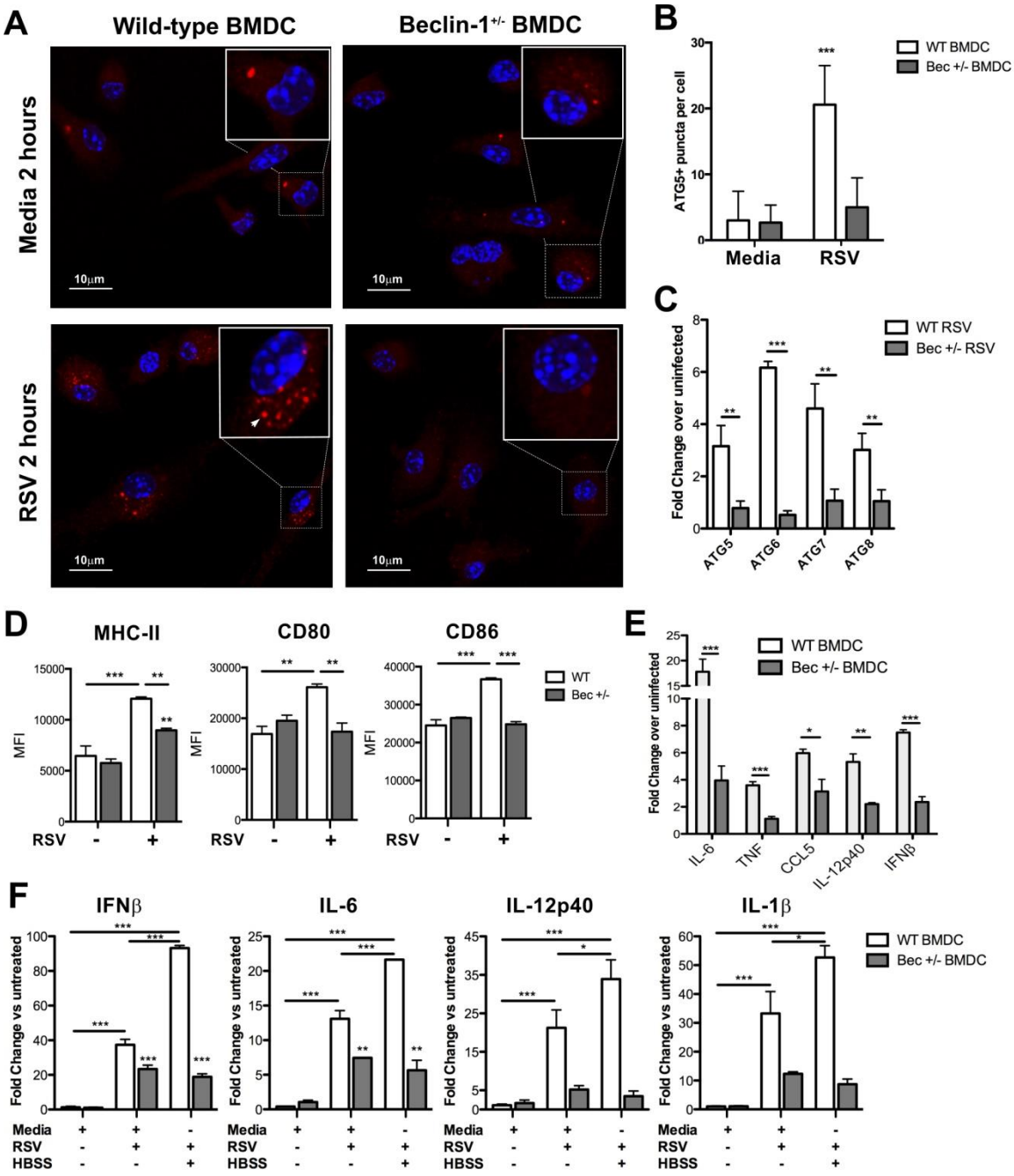

Figure 2-4 - **Altered autophagosome formation, autophagy-dependent innate cytokine production, and maturation in response to RSV infection by Beclin-1$^{+/-}$ DCs.** (A) Autophagosome formation by Beclin-1$^{+/-}$ or WT bone marrow-derived DCs two hours post-RSV infection (1:1 MOI) was measured by punctate ATG5 staining and confocal microscopy, quantified in (B). (C) Autophagy gene expression by Beclin-1$^{+/-}$ or WT BMDCs was assessed 24 hours post-RSV infection. (D) Surface costimulatory marker expression by Beclin-1$^{+/-}$ or WT BMDCs was measured by flow cytometry, 24 hours post-RSV infection. (E) Innate cytokine production by Beclin-1$^{+/-}$ or WT BMDCs, measured by qPCR 24 hours post-RSV infection. (F) Innate cytokine production by Beclin-1$^{+/-}$ or WT BMDCs, incubated in media or HBSS for two hours followed by 24 hour RSV infection, was assessed by qPCR. Data are representative of at least two independent experiments, with at least four replicates per group. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001.

infection or starvation-induced autophagy prior to RSV infection, with no synergistic increase in cytokine production observed with starvation (Figure 2-4**F**).

2d.iii.Becn1$^{+/-}$ DCs fail to stimulate antiviral cytokine production by CD4+ T cells in vitro

Innate cytokine production by DCs during antigen presentation is critical to the induction of Th1 adaptive immune responses, and data obtained thus far suggested that deficient autophagy-dependent maturation and pro-inflammatory cytokine production by Becn1$^{+/-}$ DCs may result in impaired antigen presentation capacity. We first verified our previously published finding that blockade of autophagy in RSV-infected BMDCs leads to attenuated IL-17a and IFNγ production by co-cultured CD4+ T cells (230). Treatment of BMDCs with the autophagy inhibitor 3-methyladenine (3-MA) prior to ovalbumin treatment and RSV infection blocked IFNγ and RSV-associated IL-17a production by ovalbumin peptide-recognizing CD4+ T cells (Figure 2-5**A**). Similar results were obtained using CD11b+ pulmonary DCs, flow-sorted from lungs of naïve C57Bl/6 mice and co-cultured with purified CD4+ OT-II T cells (Figure 2-5**B**).

As observed previously with OT-II T cells co-cultured with pulmonary CD11b+ DCs, RSV-infected WT BMDCs elicited greater production of IL-17a and IFNγ from co-cultured OT-II T cells in comparison to ovalbumin treatment alone (Figure 2-5**C**). In contrast, RSV-infected Becn1$^{+/-}$ BMDCs failed to stimulate greater production of IL-17a by co-cultured T cells, and elicited significantly less IFNγ production in comparison to both uninfected BMDCs and RSV-infected WT BMDCs (Figure 2-5**C**). Moreover, Becn1$^{+/-}$ BMDCs infected with RSV prior to treatment with ovalbumin stimulated increased production of the Th2 cytokines IL-5 and IL-13 from co-cultured OT-II T cells, in comparison to ovalbumin treatment alone (Figure 2-5**C**). These results were similar to those obtained through co-culture of WT or Becn1$^{+/-}$ BMDCs with CD4+ T cells purified from the LDLNs of RSV-infected C57Bl/6 mice, 8 days post-infection. In response to co-culture with RSV-infected Becn1$^{+/-}$ BMDCs, CD4+ lymph node T cells produced significantly greater quantities of Th2 cytokines and less IL-17a and IFNγ than T cells co-cultured with WT BMDCs (Figure 2-5**D**).

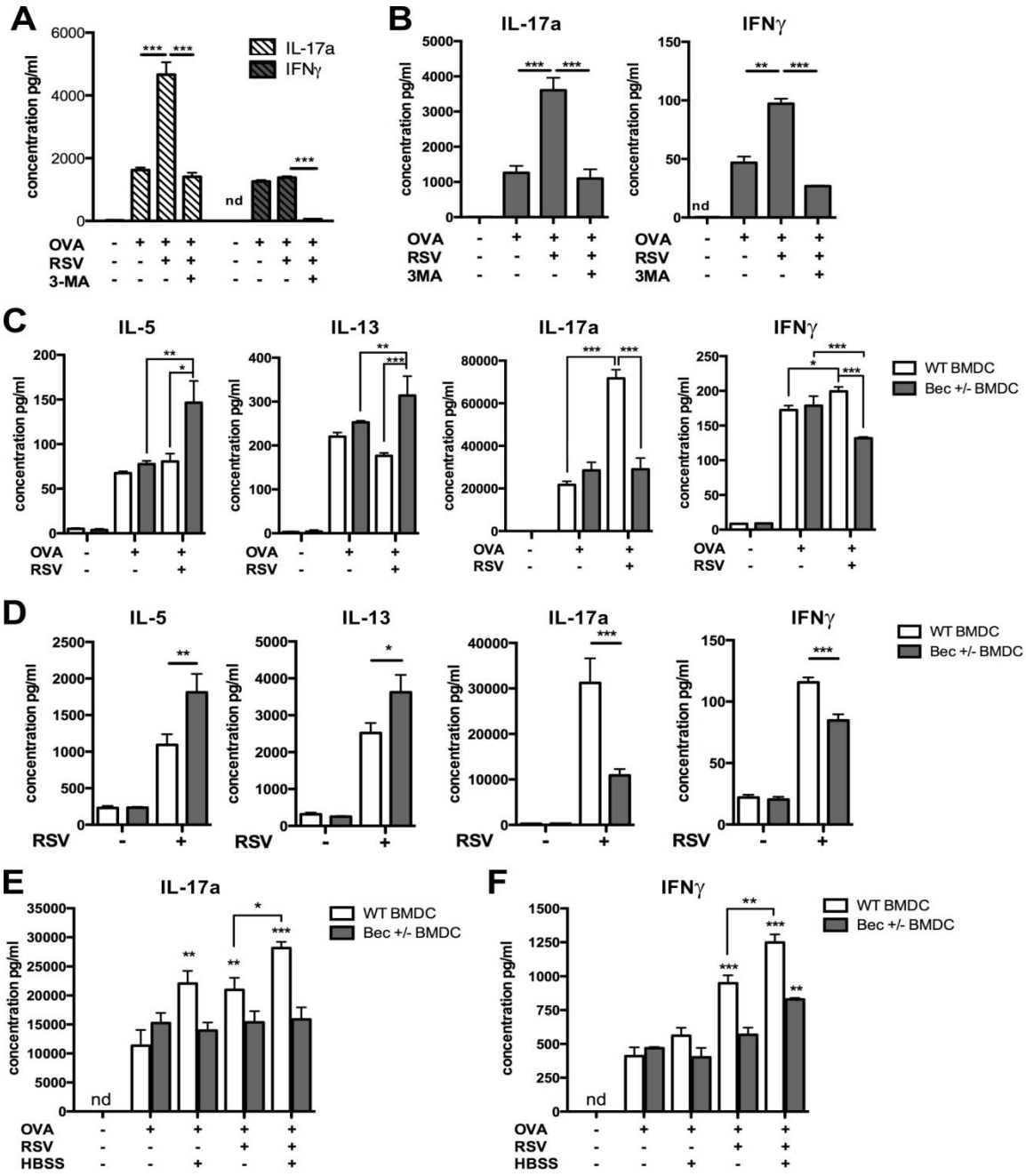

Figure 2-5 - **Elicitation of CD4+ T cell cytokine production by RSV-infected DCs is dependent on autophagy.** (A) WT BMDCs cultured from BALB/cJ mice were treated with saline or 5μM 3-methyladenine (3-MA), 30 minutes prior to the addition of 1:1 MOI RSV and 200μg/ml whole ovalbumin protein. Cells were co-cultured with purified splenic D011.10 CD4+ T cells, and culture supernatants tested at 48 hours by Bioplex assay. (B) CD11b+ DCs were flow-sorted from lungs of naïve C57Bl/6 mice and co-cultured with purified splenic OT-II CD4+ T cells (C) Cytokine production by CD4+ OT-II T cells, co-cultured with Beclin-$1^{+/-}$ or wild-type BMDCs infected with RSV and treated with 200μg/ml whole ovalbumin protein, was measured at 48 hours by Bioplex assay. (D)Cytokine production by CD4+ T cells purified from LDLNs of RSV-infected C57Bl/6 mice, 8 days post-infection. Cells were co-cultured with RSV-infected Beclin-$1^{+/-}$ or WT BMDCs for 48 hours, was measured in culture supernatants by Bioplex assay. (E,F) Beclin-$1^{+/-}$ or WT BMDCs were incubated in media or HBSS for 2 hours, placed in fresh media and treated with RSV and 200μg/ml whole ovalbumin protein as indicated. Production of IL-17a (E) and IFNγ (F) by co-cultured CD4+ OT-II T cells was measured in culture supernatants at 48 hours by Bioplex assay. Data are representative of at least two independent experiments, with at least four replicates per group. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001.

In order to confirm that DC elicitation of robust IL-17a and IFNγ production by

co-cultured CD4+ T cells is indeed autophagy-dependent, we used starvation by incubation in HBSS as a non-viral autophagy stimulus. In agreement with our earlier finding that autophagy induction prior to RSV infection synergistically increases innate cytokine production by WT BMDCs (Figure 2-4**F**), CD4+ OT-II T cells co-cultured with either starved or RSV-infected WT BMDCs produced greater quantities of IL-17a in comparison to co-cultures with BMDCs treated with ovalbumin alone (Figure 2-5**E**). Furthermore, autophagy induction prior to RSV infection in WT BMDCs augmented production of both IL-17a and IFNγ by co-cultured T cells in comparison to either treatment alone (Figure 2-5**E,** Figure 2-5**F**). In contrast, we previously found that innate cytokine production by starved or RSV-infected $Becn1^{+/-}$ BMDCs was muted or attenuated in comparison to WT BMDCs, with no observed augmentation of cytokine production in response to starvation prior to infection (Figure 2-4**F**). Accordingly, no increase in production of IL-17a or IFNγ by CD4+ OT-II T cells was observed upon co-culture with starved or RSV-infected $Becn1^{+/-}$ BMDCs, while only a slight increase in IFNγ production and no change in IL-17a production was observed in co-cultures with $Becn1^{+/-}$ BMDCs that were both starved and RSV-infected (Figure 2-5**E,** Figure 2-5**F**).

Surprisingly, we found no baseline defect in the ability of $Becn1^{+/-}$ BMDCs treated with ovalbumin alone to elicit cytokine production from co-cultured CD4+ OT-II T cells (Figure 2-5**C**). We also found no significant differences in the upregulation of MHC-II expression, costimulatory molecule expression, or cytokine production by $Becn1^{+/-}$ BMDCs treated with LPS, IFNβ, or ovalbumin protein, in comparison to WT controls (Figure 2-6**A-C**). Finally, confocal microscopy revealed no evidence of increased autophagy in WT BMDCs treated with ovalbumin alone, suggesting that our ovalbumin preparation does not induce autophagy (Figure 2-6**D**).

These results suggest that the autophagy-dependent production of innate cytokines by RSV-infected DCs is necessary for robust production of IL-17a and IFNγ by co-cultured CD4+ T cells, and that T cell cytokine elicitation is increased by autophagy induction prior to RSV infection of WT BMDCs. In contrast, while $Becn1^{+/-}$ BMDCs appear to be capable of normal presentation of ovalbumin protein in the absence of an autophagic stimulus, the lack of increased production of IL-17a and IFNγ by CD4+ T

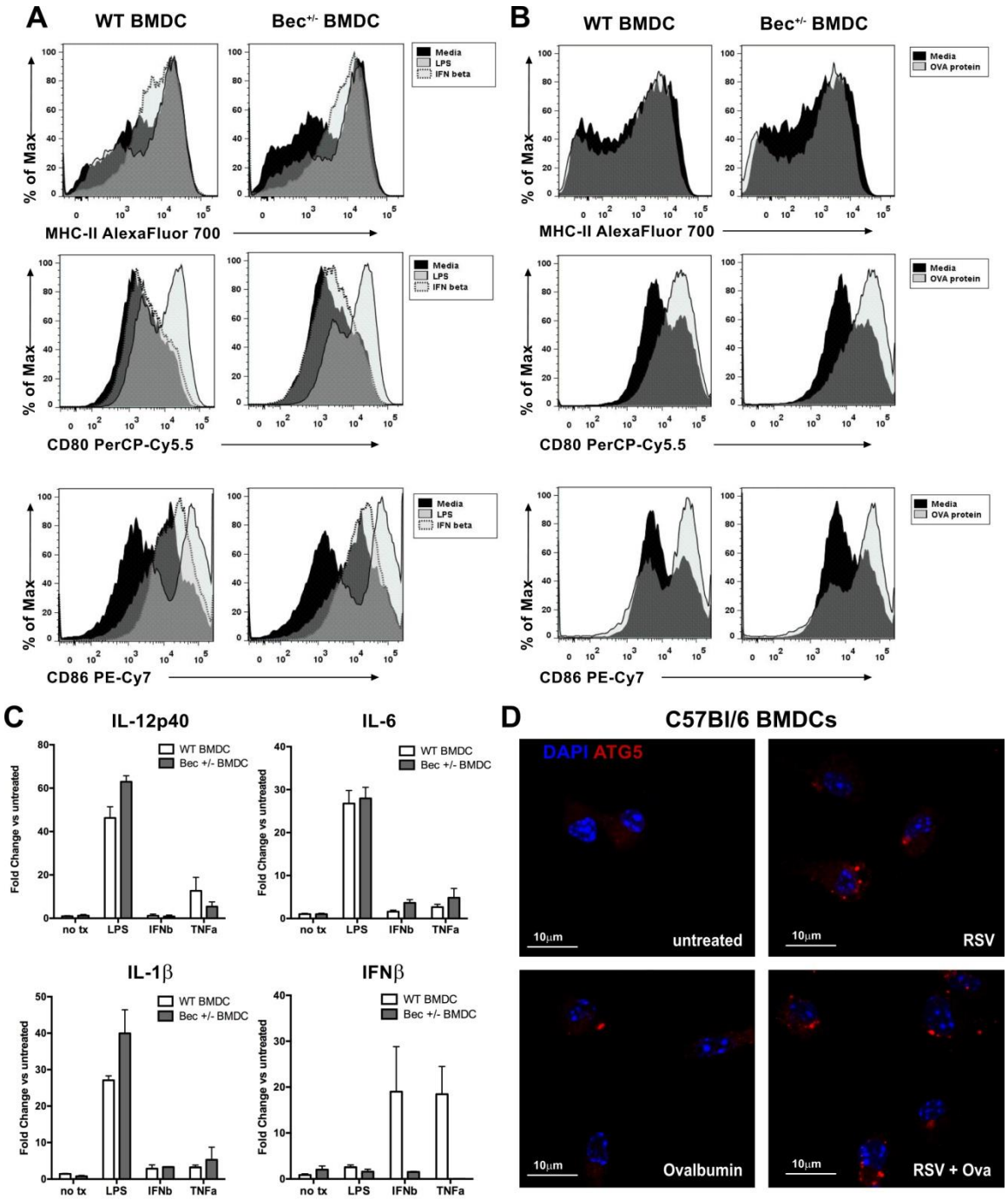

Figure 2-6 - **Beclin-1$^{+/-}$ BMDCs mature normally in response to LPS, Type I Interferon, or ovalbumin treatment.** (A) WT or Beclin-1$^{+/-}$ BMDCs were cultured 24 hours in media, or in media treated with 100ng/ml LPS or 100U/ml recombinant murine IFNβ. Histograms show representative cell surface expression of MHC-II, CD80, and CD86 as measured by flow cytometry. (B) WT or Beclin-1$^{+/-}$ BMDCs were cultured 24 hours in media, or in media treated with 200μg/ml whole ovalbumin protein. Histograms show representative cell surface expression of MHC-II, CD80, and CD86 as measured by flow cytometry. (C) WT or Beclin-1$^{+/-}$ BMDCs were cultured in media, or in media treated with 100ng/ml LPS, 100U/ml recombinant murine IFNβ, or 20ng/ml TNFα. Cytokine mRNA expression was measured by qPCR at 24 hours post-treatment. (D) Ovalbumin does not induce or inhibit autophagosome formation in vitro. BMDCs cultured from C57Bl/6 mice were treated for 2 hours with media, 1:1 MOI RSV and/or 200μg/ml ovalbumin protein as indicated. Cells were fixed and stained with antibodies to ATG5, followed by imaging by confocal microscopy.

cells co-cultured with either RSV-infected or starved Becn1$^{+/-}$ BMDCs suggests that the

results are due to the failure of Becn1$^{+/-}$ BMDCs to upregulate autophagy.

2d.iv. Adoptive transfer of RSV-infected Becn1$^{+/-}$ DCs into wild-type mice produces increased lung pathology upon subsequent RSV challenge

Data generated in the current study suggested that the primary defect leading to increased pathology during RSV infection in Becn1$^{+/-}$ mice was associated with altered DC activation, resulting in deficient induction of antiviral cytokine production and an altered CD4+ T cell response. In order to further address this mechanism, an adoptive transfer system was employed where WT C57Bl/6 mice were administered RSV-infected WT or Becn1$^{+/-}$ BMDCs into the trachea, followed by a live RSV challenge 28 days later (Figure 2-7**A**). Since BMDCs do not propagate RSV (227, 228), the use of this model allows the examination of airway sensitization with an autophagy defect in DCs alone, and assessment of the elicited anti-viral immune response through direct RSV challenge. Analysis of immune cell infiltrates in enzymatically digested lungs showed that C57Bl/6 mice administered RSV-infected Becn1$^{+/-}$ BMDCs had increased lung infiltration of DCs and macrophages 8 days post-RSV challenge (Figure 2-7**B**). Lung histology from mice sacrificed 8 days post-RSV challenge revealed peribronchial inflammation within the lungs of all mice airway-sensitized with RSV-infected BMDCs; however this inflammation was much more extensive in animals given RSV-infected Becn1$^{+/-}$ BMDCs (Figure 2-7**C**). In agreement with our findings on primary infection of Becn1$^{+/-}$ mice, only RSV-infected Becn1$^{+/-}$ BMDC recipients showed mucus plugging and granulocyte infiltration into the airways after challenge (Figure 2-7**C**). Additionally, mRNA levels of the mucus production-associated genes *muc5ac* and *gob5* were significantly elevated in lungs of RSV-infected Becn1$^{+/-}$ DC recipient mice in comparison to controls (Figure 2-7**D**).

In further concurrence with our findings in RSV-infected Becn1$^{+/-}$ mice, examination of T cell cytokine production from restimulated LDLNs of RSV-infected Becn1$^{+/-}$ BMDC recipient mice revealed increased production of IL-4, IL-5 and IL-13. Similarly, restimulated LDLNs produced less IL-17a in comparison to RSV-infected WT BMDC recipients, although IFNγ production was not significantly different (Figure 2-7**E**). Finally, intracellular staining and flow cytometry revealed decreased expression of

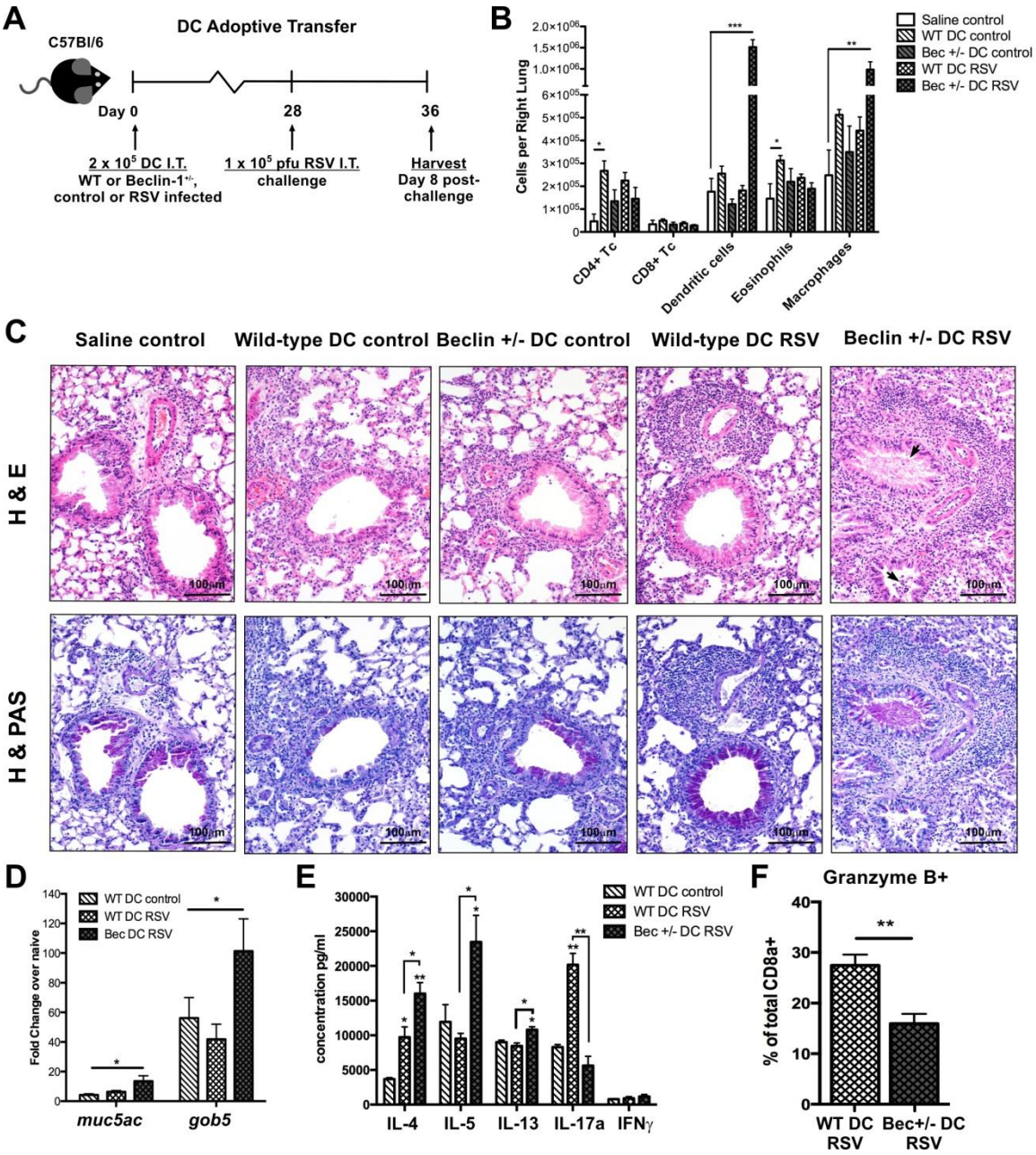

Figure 2-7 - **Adoptive transfer of RSV-infected Beclin-1$^{+/-}$ dendritic cells into C57Bl/6 mice produces severe lung pathology upon subsequent RSV challenge.** (A) DC adoptive transfer setup, with C57Bl/6 mice receiving media- or RSV-pulsed Beclin-1$^{+/-}$ or WT BMDCs i.t., followed by a live RSV challenge i.t. 28 days later. (B) Total numbers of lineage-positive immune cells obtained from collagenase-digested lungs of DC-sensitized mice, 8 days post-RSV challenge. (C) Lung sections from DC-sensitized mice 8 days post-RSV challenge, stained with either haematoxylin and eosin (H&E, top panel), or haematoxylin and periodic acid schiff (H&PAS, bottom panel) stain. (D) Lung expression of the mucus-associated genes muc5ac and gob5 8 days post-RSV challenge was obtained using quantitative real-time PCR (qPCR) in comparison to naïve controls. (E) Single-cell suspensions prepared from lung-draining lymph nodes of DC-sensitized mice, 8 days post-RSV challenge, were restimulated in culture with RSV. Cytokine concentrations in culture supernatants were assayed at 48 hours using a Bioplex system. (F) Intracellular expression of Granzyme B by CD8a+ T cells within lung digests was assessed using flow cytometry. Data are representative of three independent experiments, with four to six mice per group. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001.

Granzyme B in CD8+ T cells from lung digests of RSV-infected Becn1$^{+/-}$ BMDC

recipients (Figure 2-7**F**). These results provide additional evidence that the defect in the Becn1$^{+/-}$ response to RSV resides within the DC population, leading to the observed lung pathology and Th2 cytokine skewed responses in fully heterozygous mice.

2e. Discussion

Host inflammatory responses must balance pathogen clearance with minimal damage to healthy tissue, and this is particularly true in the respiratory tract. In the current study, we provide several pieces of evidence that autophagy is critical to DC-mediated induction of an effective antiviral adaptive immune response upon infection with RSV: 1) RSV-infected Becn1$^{+/-}$ mice showed increased lung pathology characterized by increased mucus production and infiltration of eosinophils and DCs into the lungs; 2) Pulmonary DCs from RSV-infected Becn1$^{+/-}$ mice show incomplete maturation in response to RSV, resulting in significantly less IFNγ and IL-17a production by co-cultured CD4+ T cells; 3) RSV-infected Becn1$^{+/-}$ BMDCs fail to upregulate innate cytokine production upon viral and non-viral autophagy stimulus; and 4) immunization of WT mice through adoptive transfer of RSV-infected Becn1$^{+/-}$ BMDC resulted in severe Th2-associated lung pathology upon subsequent challenge with RSV. Investigation of primary airway epithelial cells indicated that there was no induction of autophagy by RSV infection alone and no alteration in cytokine responses observed in the epithelial cells from Becn1$^{+/-}$ mice. Thus, the altered responses appeared to be focused upon DC activation during RSV infection. DCs are uniquely specialized for surveillance and rapid detection of invading pathogens, as well as for initiating and directing both innate and adaptive immune responses through antigen presentation and pro-inflammatory cytokine production. Histological staining of infected human lung tissue, as well as *in vitro* experiments conducted with human and mouse DCs, suggest that lung-residing intraepithelial DCs are capable of being directly infected with RSV (191, 201, 363). Infection of pulmonary DCs may be particularly important to the rapid acquisition of RSV antigens, as RSV infects through pH-independent cell membrane fusion and cytoplasmic entry (349), making autophagy mechanisms critical for rapid activation and antigen processing in DCs. In agreement with recent *in vivo* studies implicating immunological autophagy in the clearance of intracellular bacteria (356), and the

limitation of harmful inflammation while enhancing viral clearance within infected neurons (278), this study identifies a role for autophagy within pulmonary DCs in promoting virally-induced maturation, and the priming of an effective antiviral adaptive immune response during RSV infection.

Within antigen-presenting cells, autophagy modulates both pro- and anti-inflammatory events upon activation of pattern-recognition receptors (362). One mechanism whereby autophagy may promote DC maturation and innate cytokine production is through expedited delivery of cytoplasmic material to TLR-containing endosomes (297). Previous studies conducted by our laboratory support this, as autophagy induction within BMDCs prior to RSV infection synergistically increased innate cytokine production, while this effect was attenuated in BMDCs treated with autophagy inhibitors or cultured from TRIF- or MyD88-deficient mice (230). In agreement, the current findings of Th2-associated cytokine secretion, pulmonary eosinophilia, and mucus production in RSV-infected Becn1$^{+/-}$ mice are consistent with the RSV-induced phenotype observed in mice genetically deficient in TLR3 or MyD88 (150, 229). RSV-derived dsRNA and ssRNA, ligands specific to TLR3 and TLR7 respectively, are likely responsible for the induction of autophagy in infected DCs, as synthetic ligands to TLR3 and TLR7 induce autophagosome formation in murine macrophages and DCs (252, 300). Autophagy induction may therefore function as a positive feedback mechanism by increasing delivery of cytosolic viral components to endosomal TLRs, while simultaneously regulating other pro-inflammatory signals through inhibition of cytosolic pattern-recognition receptors (249, 318). Alternately, autophagy may indirectly promote TLR-dependent cytokine production by removing the non-structural RSV proteins NS1 and NS2 from the cytoplasm, as they disrupt TLR and IFN receptor signaling by decreasing intracellular expression of TRAF3 and STAT2 (34, 365). Further studies will be needed to address the mechanistic details of potential interaction of the autophagic pathway with other PRR pathways, as well as with virally-encoded proteins during *in vitro* and *in vivo* RSV infection.

In the current study, increased lung pathology in both RSV-infected Becn1$^{+/-}$ mice and RSV-infected Becn1$^{+/-}$ BMDC recipient mice was characterized by infiltration of

inflammatory DCs. Similarly, RSV-infected Becn1$^{+/-}$ BMDCs failed to mature upon RSV infection *in vitro*, and subsequently promoted increased Th2 cytokine synthesis from co-cultured CD4+ T cells. Inflammatory DCs, which are phenotypically similar to BMDCs (366) and are derived *in vivo* from blood monocytes (367, 368), are recruited in large numbers to the respiratory tract in both infected humans and mice (188, 201), and have been shown to drive Th2-associated lung pathology in mice during infection with the paramyxovirus Sendai virus (369) and in a mouse model of allergic asthma (366). Previous studies also noted that addition of BMDCs to the respiratory tract at the time of RSV infection increased lung pathology (229), while blockade of inflammatory DC emigration into lungs by genetic CCR6 deficiency or CCL20 neutralization increased viral clearance and reduced lung pathology upon RSV infection (226). In agreement, RSV-infected Becn1$^{+/-}$ mice showed elevated production of CCL20 and increased lung infiltration by inflammatory DCs. Furthermore, transfer of RSV-infected Becn1$^{+/-}$ BMDCs into WT host mice induced greater Th2 cytokine production and lung pathology upon subsequent challenge with RSV, ultimately suggesting that impaired maturation in inflammatory DCs may enhance Th2-associated pathology. In contrast, we found that while Becn1$^{+/-}$ CD103+ and CD11b+ lung DCs stimulated significantly less IFNγ and IL-17a production from co-cultured OT-II T cells upon infection with RSV, both WT and Becn1$^{+/-}$ RSV-infected lung-derived DCs elicited substantially less Th2 cytokine production in comparison to RSV-infected BMDCs of either genotype. Kinetic studies of DC migration upon RSV infection suggest that inflammatory DCs as well as lung-resident CD11b+ and CD103+ DCs transport viral RNA to the LDLNs (201), suggesting that the observed phenotype in RSV-infected Beclin$^{+/-}$ mice is due to impaired autophagy-dependent maturation in both lung-resident and inflammatory DCs. The impaired activation and maturation of lung-resident DCs may therefore result in insufficient induction of an RSV-specific Th1 response, enabling Th2 cytokine-driven pathology in its absence. The role of autophagy in various lung DC subsets and the associated contribution to CD4+ T cell activation during RSV infection remains to be explored.

While potential non-autophagic functions of Becn1 in DC activation and function cannot be ruled out at the present time, this study provides supportive evidence for the

critical role of autophagy in DCs in the induction of antiviral CD4+ T cell responses to RSV infection. This is supported not only by the current findings in Becn1$^{+/-}$ mice and in vitro studies of DC function, but more importantly through adoptive transfer of RSV-infected Becn1$^{+/-}$ DCs into fully wild-type mice. The recapitulation of increased Th2 cytokine production and occlusion of the airways with mucus and cellular debris in Becn1$^{+/-}$ BMDC recipients emphasizes that impaired autophagy within RSV-infected DCs alone is sufficient to induce much of the lung pathology observed in fully heterozygous mice. However, additional studies utilizing other models of autophagy deficiency will be necessary to further elucidate the exact mechanism by which autophagy in DCs contributes to host defense against RSV.

Genome-wide association studies recently identified autophagy gene polymorphisms associated with increased susceptibility to disease, including tuberculosis infection in human populations (370), as well as the development of Crohns disease (371). Interestingly, one recent study showed that viral infection interacting with underlying genetic susceptibility produces autoimmune illness in mice harboring the human ATG16L1 variant associated with increased Crohns disease risk (372). As severe RSV and other viral infections in infancy is associated with an increased risk of developing atopic asthma later in life (60, 62), it seems plausible that polymorphisms in autophagy genes may lead to pathological responses to respiratory viral infection early in life and the establishment of a Th2-skewed lung environment. Future studies addressing the importance of autophagy in different DC subsets may provide valuable information regarding respiratory viral infections and novel vaccination strategies.

Chapter 3 - Deficiency of autophagy protein Map1-LC3b mediates IL-17-dependent lung pathology during respiratory viral infection via ER stress-associated IL-1

3a. Abstract

While recent studies suggest that IL-1β production is modulated by macroautophagy or sensors of ER stress upon pro-inflammatory insult, autophagy and IL-1β production during viral infection has not been fully investigated. This was addressed using respiratory syncytial virus (RSV), which is associated with lung immunopathology, IL-1, and IL-17a secretion in severely infected patients. Mice deficient in the autophagy-associated protein Map1-LC3b ($LC3b^{-/-}$) developed increased IL-17a-dependent lung pathology upon infection. RSV-infected $LC3b^{-/-}$ DCs fail to upregulate autophagosome formation, secrete IL-1β and IL-6, and elicit IL-17a production from CD4+ T cells. Bone marrow chimeras revealed both structural and hematopoietic LC3b deficiency contribute to the development of IL-17a-dependent lung pathology *in vivo*. Further investigation revealed airway epithelium as the primary source of IL-1β during infection, while inhibition of the ER-stress sensor IRE-1 in primary airway epithelial cells reduced IL-1β production identifying a primary ER stress pathway. Finally, blockade of IL-1 receptor signaling in RSV-infected $LC3b^{-/-}$ mice abolished IL-17a-dependent lung pathology. These findings provide novel mechanistic insight into the contribution of autophagy- and ER stress-dependent cytokine production that initiate and maintain aberrant Th17 responses, while identifying IL-1 as a potential therapeutic target in the treatment of severe respiratory viral infections.

3b. Introduction

Human respiratory syncytial virus (RSV) remains the leading cause of infant hospitalization in the United States (373, 346), and is responsible for considerable morbidity among infants, the elderly, and individuals with chronic respiratory illnesses

worldwide (5, 55, 56). Hospitalization with RSV as an infant is also highly correlated with the development of recurrent wheezing in childhood, suggesting lasting immune environment alteration in the lung post-infection (62). The severity of lower-respiratory tract RSV infection in otherwise healthy individuals is largely driven by an over-exuberant immune response to the virus, and is characterized by bronchiolitis, epithelial cell sloughing, mucus hyper-secretion, and infiltration of neutrophils into the airways (18, 37). Elevated levels of the pro-inflammatory cytokines IL-1, IL-6 and IL-17a have recently been noted in respiratory aspirate samples from patients hospitalized with severe RSV infections (51, 374, 375), while experimental evidence suggests that IL-17a production drives mucus hypersecretion, neutrophil infiltration, and the suppression of CD8+ T cell responses in the context of RSV infection (374, 376, 377). The mechanisms by which Th17 immune responses to RSV are initiated and maintained remains poorly understood.

Within the lung environment, resident dendritic cells (DCs) detect invading pathogens and mediate both innate and adaptive immune responses through cytokine secretion and antigen presentation to T cells. Classical induction of IL-17a production by murine CD4+ T cells requires expression of the innate cytokines IL-6 and TGFβ by antigen-presenting cells (APCs), while IL-23 is required for the expansion and survival of Th17 cells (378, 379). In contrast, the induction of IL-17a secretion by human CD4+ T cells requires IL-1 and IL-6 but not TGFβ (380), while IL-1 receptor signaling is required for the production of IL-17a by both human and mouse CD4+ T cells (381). The production of IL-23 by DCs in response to TLR ligands is further driven by IL-1β (382), suggesting a critical role for IL-1 in the maintenance of Th17 responses.

The IL-1 family proteins IL-1β and IL-18 are synthesized upon TLR activation in an inactive precursor state, and regulated via post-translational cleavage by assembled inflammasome complexes such as AIM2 and NLRP3 (233). The secretion of IL-1β and IL-18 by DCs and macrophages upon TLR activation is also negatively regulated by macroautophagy (autophagy), a homeostatic mechanism by which cytosolic constituents are enveloped in a double-walled membrane and delivered to lysosomes for degradation. Pro-IL-1β and pro-IL-18 are rapidly sequestered within autophagosomes upon the

induction of autophagy (320), while ubiquitinated inflammasomes are bound by sequestosome-1/p62 and selectively targeted to nascent autophagosomes through p62 binding to membrane-bound Map-1LC3b (LC3b)(249). It was recently reported that RSV infection activates the NLRP3 inflammasome and induces IL-1β secretion from human bronchial epithelial cells (383), as well as murine macrophages and epithelial cells (131). Our laboratory has found evidence supporting a critical role for autophagy in DCs by promoting activation, cytokine secretion, and antigen presentation upon RSV infection in vitro (230), while autophagy deficiency in DCs enhances RSV-induced pathology in vivo (228). However, the potential modulatory role of autophagy in controlling excessive inflammasome activation and IL-1β secretion during RSV infection has yet to be examined.

Since RSV infects via a membrane fusion event that allows direct entry of its genetic material into the cytoplasm, activation of critical innate TLR responses rely on autophagy to transport viral PAMPs and promote appropriate anti-viral responses (230). To investigate the potential role of autophagy in mitigating RSV-induced lung pathology *in vivo*, mice deficient in the critical autophagy protein LC3b ($LC3b^{-/-}$) were used. These mice have been previously reported to express greater NLRP3 inflammasome activation, IL-1β secretion, and increased susceptibility to experimental sepsis (235). Our results demonstrate that LC3b is critical to the regulation of Th17-induced lung pathology during RSV infection, as $LC3b^{-/-}$ mice show greater mucus hypersecretion, lung neutrophil infiltration, and IL-17a production, which was ameliorated by IL-17a neutralization. These disease-associated pathologies were associated with increased IL-1β and IL-6 production in the LC3b deficient mice during RSV infection that were related to ER stress responses based upon specific pathway blockade. Importantly, the blockade of IL-1 receptor signaling *in vivo* substantially reduced IL-17a secretion and immunopathology in the lungs of $LC3b^{-/-}$ mice. These findings suggest that neutralization of IL-1 may represent a potential therapeutic approach to alleviating severe RSV-induced responses.

3c. Materials and methods

Mice

Female C57Bl/6J and B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6;129P2-*Map1lc3b*^{tm1Mrab}/J were obtained from The Jackson Laboratory and repeatedly backcrossed to C57Bl/6J mice to produce WT or LC3b-deficient (LC3b$^{-/-}$) mice of ≥98% C57Bl/6 background. All *in vivo* experiments utilized age- and sex-matched WT or LC3b$^{-/-}$ littermates.

Bone marrow chimeras were created by irradiating WT or LC3b$^{-/-}$ littermates at 900rad, followed by tail-vein injection of 2 x 10^6 whole bone marrow cells of the genotype indicated. Chimeras were allowed to reconstitute for 8 weeks prior to RSV infection. All work involving animals was conducted in accordance with University of Michigan Committee on Use and Care of Animals policy.

Respiratory Syncytial Virus

All experiments utilized antigenic subgroup A, Line 19 strain RSV, originally isolated from a sick infant in the University of Michigan Health System. Viral stocks were maintained in Hep-2 cell cultures.

Dendritic Cell Isolation and Culture

Bone marrow-derived dendritic cells (BMDCs) were cultured in complete media as previously described (228), from whole bone marrow in the presence of 20ng/ml GM-CSF (R&D Systems, Minneapolis, MN). Lung-resident CD11b+ and CD103+ DCs were isolated by enzymatic digestion of lungs of naïve WT or LC3b$^{-/-}$ mice as previously described (228). Briefly, minced lung tissue was incubated with 200μg/ml Liberase TM (Roche Applied Science, Indianapolis, IN) and 200U/ml DNase I (Sigma-Aldrich), drawn through an 18-gauge needle, and filtered through 40μm mesh. Cells were positively selected for CD11c expression using anti-mouse CD11c microbeads and magnetic column separation (Miltenyi Biotec, Auburn CA), followed by staining with PE-labeled anti-CD11b and allophycocyanin-labeled anti-CD103 Abs (eBioscience, San Diego, CA). Cells were sorted on an iCyt Synergy 3200 fluorescence-activated cell sorter (Champaign, IL), followed by overnight culture in complete media.

For DC-T cell co-culture experiments, RSV-responsive CD4+ T cells were obtained from mediastinal lymph nodes of RSV-infected C57Bl/6J mice, 8 days post-infection. CD4+ OT-II T cells were isolated from minced spleens of OT-II transgenic mice. Cells were purified using a magnetic column negative-selection protocol, yielding untouched CD4+ T cells (Miltenyi Biotec). CD4+ T cells were added to culture at a 10:1 ratio to DCs. For RSV-reactive T cell co-cultures, DCs were infected with RSV at an MOI of 1, 2 hours prior to the addition of T cells. For OT-II T cell co-cultures, DCs were treated with 200μg/ml whole ovalbumin protein, or concurrently treated with ovalbumin and 1:1 MOI of RSV, for 2 hours prior to the addition of T cells. Culture supernatants were harvested at 48 hours and analyzed using a custom BioRad Bioplex PRO kit on a BioRad Bioplex 200 system, according to manufacturer instructions (BioRad).

Airway Epithelial Cell culture

Airway epithelial cell cultures were prepared from lungs of naïve mice by digestion in Dispase (BD Biosciences), followed by filtration through 25μm nylon mesh. Immune cells were depleted using biotinylated anti-CD45 antibodies and streptavidin-conjugated Dynabeads (Thermo Fisher Scientific). Cells were plated on 10cm tissue culture dishes and adherence-purified in DMEM-based complete media, followed by 4 day culture in fibronectin-coated wells.

Reagents

Recombinant mouse IL-1Ra was obtained from R&D systems. Neutralizing antibodies to mouse IL-17a were generated and purified in-house, as described previously (374). The ER stress inhibitors 3,5-dibromosalicylaldehyde and 4-phenylbutyrate were purchased from Santa Cruz Biotechnology.

Quantitative PCR

Total RNA was isolated from cell cultures and lung tissue using TRIzol reagent, according to manufacturer's instructions (Invitrogen). RNA was reverse-transcribed, and cytokine gene expression was assessed using TaqMan Gene Expression Assay primer/probe sets on an ABI Prism 7500 Sequence Detection System (Applied

Biosystems, Foster City, CA). Custom primers were used to assess transcription levels of *muc5ac, gob5, RSV-G* and *RSV-F*, as previously described (155). Fold change expression was calculated from gene expression values normalized to GAPDH, relative to the indicated control group.

Flow Cytometry

Cells were isolated from the right lungs and mediastinal LNs by digestion in 200μg/ml Liberase TM (Roche Applied Science, Indianapolis, IN) and 200U/ml DNase I (Sigma-Aldrich). Cells were stained with Live/Dead Fixable Yellow (Invitrogen), followed by fluorescent antibodies as indicated. Analysis was performed using FlowJo software (TreeStar, Ashland OR).

Immunofluorescence and Confocal Microscopy

BMDCs and AECs were cultured as described above, and plated in Labtek chamber slides (Thermo Fisher Scientific). After indicated treatment, cells were fixed in 4% paraformaldehyde, followed by blocking in DPBS containing 5% normal goat serum and 0.1% Tween-20. Cells were stained with DyLight 550-conjugated anti-ATG5 antibodies (Novus Biologicals, Littleton, CO), or rabbit anti-Caspase-1 p10 (Santa Cruz Biotechnologies) followed by Alexa Fluor 488-conjugated goat anti-rabbit antibodies (Jackson ImunoResearch, West Grove, PA). Slides were mounted in ProLong Gold antifade reagent plus DAPI, and imaged using 40x oil immersion objective on a Nikon A1 Confocal Microscope System using NIS Elements software (Nikon Instruments). Maximum intensity projection images were generated using ImageJ software (National Institutes of Health).

For fluorescent immunohistochemistry. formalin-fixed paraffin-embedded lung sections were de-parrafinized, rehydrated, and incubated overnight in antigen retrieval buffer (0.01M Citric acid, pH 6.0). Slices were blocked in DPBS + 2% normal goat serum, followed by incubation with rabbit anti-mouse IL-1β (Novus Biologicals, Littleton, CO) and biotinylated anti-E-cadherin (BD Biosciences). After washing in blocking buffer, slices were incubated with anti-rabbit Alexa Fluor 568 and streptavidin-conjugated Alexa

Fluor 488 (BD Biosciences). Images were taken using a 40x objective on an Olympus BX43 fluorescent microscope.

Statistics

Data were analyzed and graphed using GraphPad Prism software. Statistical significance was determined by one-way ANOVA and Bonferroni post-test to obtain p values.

3d. Results

3d.i. LC3b$^{-/-}$ mice develop increased IL-17a-dependent lung pathology upon RSV infection

Upon intratracheal infection with RSV, LC3b$^{-/-}$ mice developed increased lung pathology in comparison to RSV-infected WT littermates, with increased periarteriolar/peribronchiolar immune cell infiltration visible by haematoxylin and eosin (H & E) histological staining at 8 days post-infection (Figure 3-1**A**). Additionally, greater periodic acid-schiff (PAS)-positive mucus staining was visible along the apical epithelial surface of the major airways of RSV-infected LC3b$^{-/-}$ mice (Figure 3-1**A**), while mRNA transcripts for the mucus-associated genes *muc5ac* and *gob5* were elevated in the lungs of RSV-infected LC3b$^{-/-}$ mice (Figure 3-1**B**). In order to assess lung viral replication, qPCR for mRNA transcripts encoding the RSV-G and –F proteins was performed on whole lung homogenates. This revealed increased viral transcripts in the lungs of infected LC3b$^{-/-}$ mice, which suggests decreased viral clearance relative to WT littermates (Figure 3-1**C**). Analysis of immune cells in collagenase-digested lung tissue revealed significantly higher numbers of neutrophils and eosinophils in the lungs of RSV-infected LC3b$^{-/-}$ mice (Figure 3-1**D**), while fewer Granzyme-B positive CD8a+ T cells were present in comparison to WT littermates (Figure 3-1**E**).

Experimental evidence has shown a causative role for T cell-associated cytokines, most recently IL-17a, in the development of severe lung pathology during RSV infection (374). Therefore, lung expression of IL-17a and IFNγ was examined and results demonstrated that the expression of IL-17a mRNA was elevated in the lungs of RSV-infected LC3b$^{-/-}$ mice (Figure 3-1**F**). Conversely, mRNA expression of IFNγ was significantly lower in comparison to WT littermate controls (Figure 3-1**F**). In addition,

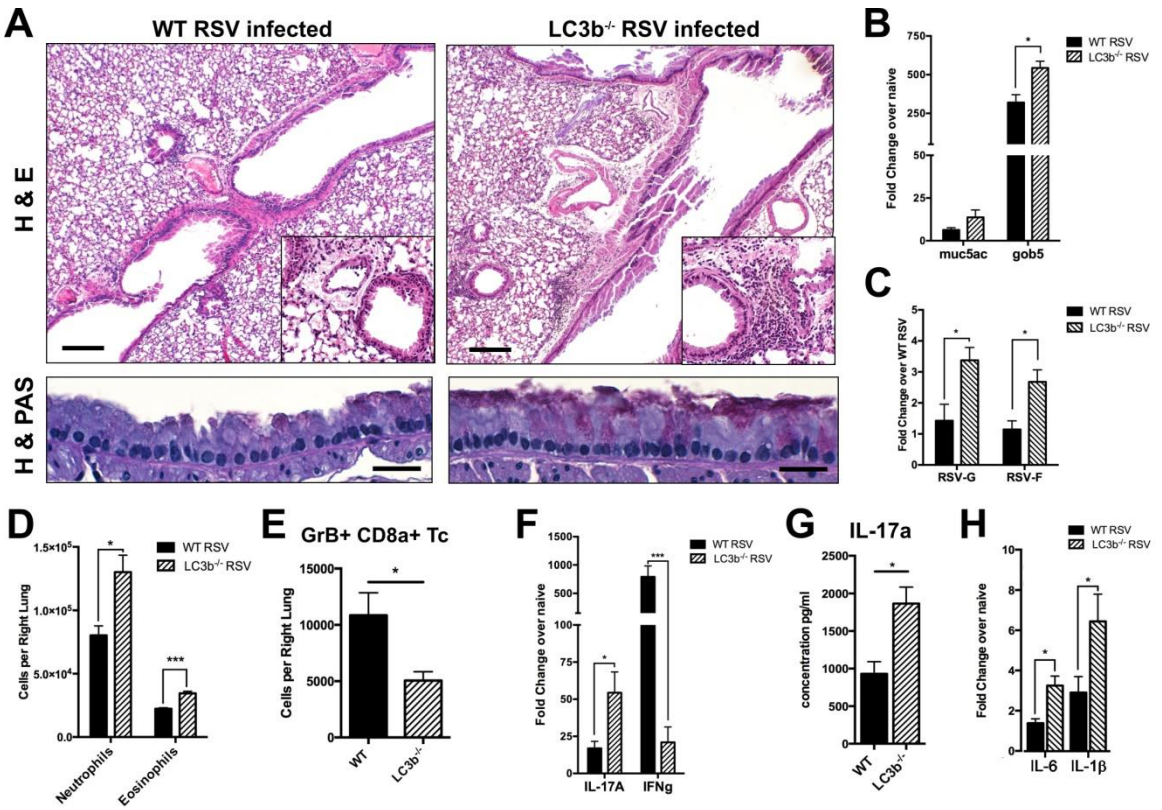

Figure 3-1 - **LC3b$^{-/-}$ mice develop increased lung pathology upon RSV infection.** A) Lung sections of WT or LC3b$^{-/-}$ mice, 8 days post-infection (DPI) with RSV, were stained with either haematoxylin and eosin (H & E, upper panels) or periodic acid schiff and haematoxylin (H & PAS, lower panels) to visualize mucus along airway epithelium. Scale bars = 500µm for upper panels, 20µm for lower panels. B) Lung mRNA expression of *muc5ac* and *gob5* at 8 DPI was obtained by qPCR, compared to naïve controls. C) mRNA transcripts from lungs, encoding RSV-G and –F proteins, were measured by qPCR and normalized to values of WT RSV-infected lung samples. D) Neutrophils and eosinophils in right lungs of WT and LC3b$^{-/-}$ mice at 8 DPI were quantified by flow cytometry, following enzymatic digestion of lungs. Total granulocytes were gated CD11b^{hi} CD11c^{lo} SSC^{hi} Auto^{lo}, with neutrophils gated Ly6G^{hi} Ly6C^{+} and eosinophils gated Ly6C^{+} Siglec F^{+}. E) Granzyme-B positive (GrB+) CD3^{+} CD8a^{+} T cells in enzymatically-digested lung tissues were quantified by flow cytometry. F) Lung IL-17a and IFNγ values were obtained by qPCR. Fold change values were calculated relative to naïve control mice. G) Single-cell suspensions were prepared from mediastinal lymph nodes of RSV-infected WT or LC3b$^{-/-}$ mice at 8 DPI, followed by restimulation in culture with RSV. Cell supernatants were analyzed at 48 hours by Bioplex assay. H) Lung expression of IL-1β and IL-6 at 6 DPI was assessed by qPCR. Results are representative of three independent experiments, n≥4 mice per group. *p≤0.05, ***p≤0.001.

mediastinal lymph node (MedLN) cells prepared from RSV-infected LC3b$^{-/-}$ mice restimulated *ex vivo* with RSV secreted greater quantities of IL-17a (Figure 3-1**G**). As IL-1, IL-6, and TGFβ are required for the induction of mucosal Th17 responses in mice (379, 381), lung mRNA expression was examined and IL-1β and IL-6 mRNA were found to be elevated in the lungs of LC3b$^{-/-}$ mice (Figure 3-1**H**), while TGFβ expression did not differ in comparison to RSV-infected WT littermates (data not shown).

Previous studies conducted by our laboratory found that IL-17a production contributes to lung inflammation, excessive mucus production, and the suppression of IFNγ production during RSV infection (151, 374). To assess whether the increased lung pathology in RSV-infected LC3b$^{-/-}$ mice was IL-17a-dependent, LC3b$^{-/-}$ mice were

treated with neutralizing antibodies to IL-17a. In comparison to control antibody treatment, administration of anti-IL-17a antibodies to RSV-infected LC3b$^{-/-}$ mice substantially decreased lung inflammation and mucus secretion at 8 days post-infection (Figure 3-2**A**). In agreement, anti-IL-17a antibody treatment significantly decreased *muc5ac* expression in LC3b$^{-/-}$ mice at 8 days post-infection (Figure 3-2**B**), while reducing lung neutrophil infiltration in comparison to control antibody administration (Figure 3-2**C**). Furthermore, depletion of IL-17a in LC3b$^{-/-}$ mice reduced lung viral mRNA to levels equivalent to WT controls (Figure 3-2**D**), and increased lung IFNγ expression (Figure 3-2**E**). These results collectively suggest that the development of increased lung pathology in LC3b$^{-/-}$ mice is IL-17a-dependent.

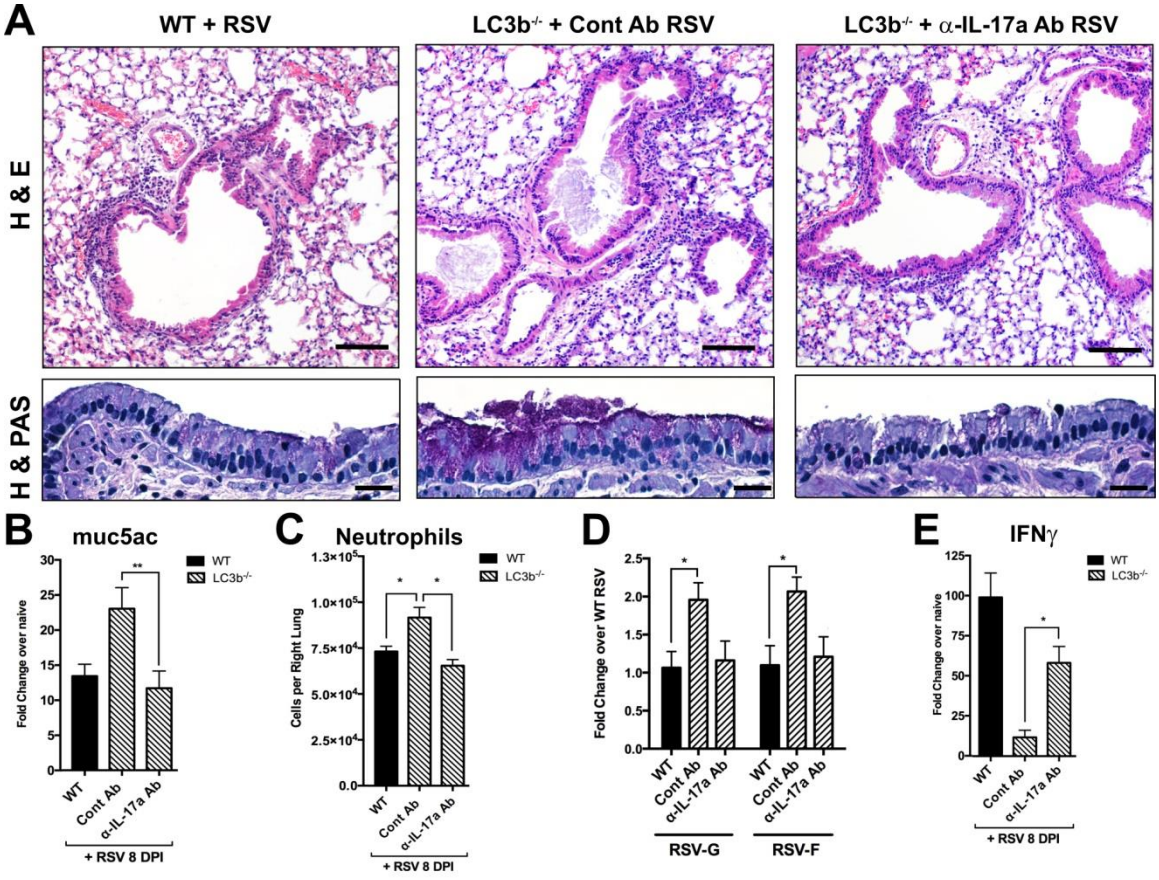

Figure 3-2 - **Increased RSV-induced lung pathology in LC3b$^{-/-}$ mice is IL-17a-dependent.** LC3b$^{-/-}$ mice were treated with 2.5mg non-specific control antibody, or polyclonal antibody to mouse IL-17a, immediately prior to RSV infection and at days 2, 4, and 6 post-infection. A) Lungs were harvested at 8 DPI, sectioned, and stained with haematoxylin and eosin (H & E) or haematoxylin and periodic acid schiff (H & PAS) to visualize mucus. Scale bars = 100μm on upper panels, 20μm on lower panels. B) Lung expression of the mucin gene muc5ac was measured at 8 DPI by qPCR, relative to naïve controls. C) Neutrophils were quantified by flow cytometric analysis of enzymatically-digested lungs, 8 DPI. Neutrophils were gated as indicated in Figure 3-1. D) Lung mRNA expression encoding RSV-G and RSV-F was assessed at 8 DPI, relative to RSV-infected WT. E) Lung IFNγ expression was measured at 8 DPI by qPCR. Results are representative of three independent experiments, n≥4 mice per group. *p≤0.05, **p≤0.01, ***p≤0.001.

3d.ii.Altered autophagy, innate cytokine production, and CD4+ T cell cytokine elicitation by LC3b$^{-/-}$ CD11b+ DCs in response to RSV infection

Recent work identified a requirement for CD11c^{high} CD11b+ tissue-resident DCs in the induction of mucosal Th17 immune responses (384, 385). As pharmacological blockade of autophagy increases IL-23 secretion by DCs and macrophages in response to TLR agonists (382), the ability of LC3b$^{-/-}$ DCs to upregulate autophagosome formation and secrete innate cytokines in response to RSV was investigated. Confocal microscopy revealed robust formation of ATG5+ autophagosomes by WT bone marrow-derived DCs (BMDCs) in response to RSV infection (Figure 3-3**A**). In contrast, LC3b$^{-/-}$ BMDCs failed to increase formation of ATG5+ puncta in response to RSV (Figure 3-3**A).** In agreement with these observations, intracellular levels of the sequesteosome protein p62 decreased in WT BMDCs by 8 hours post-infection, while LC3b$^{-/-}$ BMDCs continued to accumulate p62 over a 24 hr period (Figure 3-3**B**). Analysis of cytokine secretion by RSV-infected BMDCs revealed significantly higher production of IL-1β and IL-6 by LC3b$^{-/-}$ BMDCs (Figure 3-3**C**). LC3b$^{-/-}$ BMDCs expressed less of the IL-12-specific subunit IL-12p35 and the IL-12/IL-23 common subunit IL-12p40 by qPCR, in comparison to WT BMDCs (Figure 3-3**D**). Conversely, the RSV-infected LC3b$^{-/-}$ BMDCs significantly upregulated IL-23p19 expression in comparison to WT BMDCs (Figure 3-3**D**). These findings were confirmed using CD11c^{high} CD11b^{+} lamina propria DCs (CD11b^{+} LP DCs), flow-sorted from lungs of naïve WT and LC3b$^{-/-}$ mice and infected *ex vivo* with RSV. LC3b$^{-/-}$ CD11b^{+} LP DCs secreted greater amounts of IL-1β and IL-6 upon RSV infection, while IL-12p35 and IL-12p40 mRNA expression were significantly reduced in comparison to WT controls (Figure 3-3**E**, **F**).

In order to assess whether LC3b$^{-/-}$ DCs preferentially elicit increased IL-17a secretion from CD4+ T cells, RSV-infected BMDCs were co-cultured with purified CD4+ T cells derived from MedLNs of RSV-infected WT mice. CD4+ MedLN T cells secreted significantly greater quantities of IL-17a in co-culture with RSV-infected LC3b$^{-/-}$ BMDCs, in comparison to T cells co-cultured with WT BMDCs (Figure 3-3**G**). To further investigate the elicitation of CD4+ T cell IL-17a secretion as a consequence of autophagy deficiency, purified CD4+ splenic OT-II T cells were co-cultured with

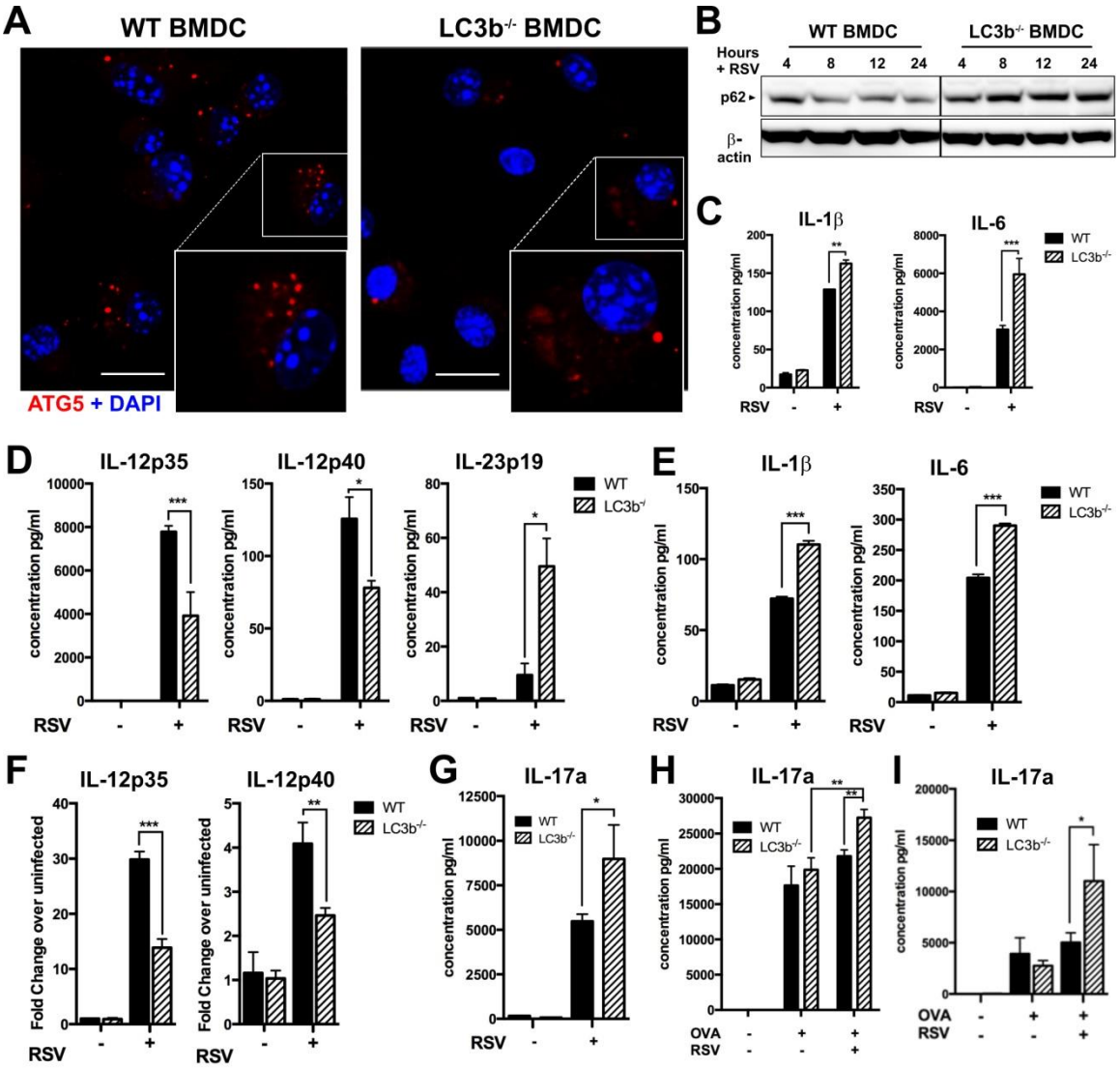

Figure 3-3 - **Altered autophagic flux, innate cytokine production, and elicitation of CD4+ T cell IL-17a production by LC3b$^{-/-}$ DCs in response to RSV.** Bone marrow-derived DCs (BMDCs) were cultured from naïve WT or LC3b$^{-/-}$ mice, then infected for 4 hours with RSV. Cells were fixed, stained with DAPI and fluorescent antibody to ATG5, and imaged by confocal microscopy. Scale bars = 10μm. B) BMDCs were cultured and infected with RSV, followed by harvest at 4, 8, 12, and 24 hours post-infection. Whole cell lysates were run on SDS-PAGE gels, followed by membrane transfer and immunoblot for p62 or β-actin as indicated. C) BMDCs were infected with 1:1 MOI RSV and cell supernatants analyzed at 48 hours post-infection by Bioplex assay. D) BMDCs were infected as above, harvested at 24 hours post-infection, and gene expression analyzed by qPCR. E) Lung-resident CD11c^{hi} Auto^{lo} CD11b^{+} lamina propria DCs (CD11b^{+} LP DCs) were flow-sorted from enzymatically-digested lungs of naïve WT and LC3b$^{-/-}$ mice. Cells were infected *ex vivo* with 1:1 MOI RSV, and cell supernatants harvested at 48 hours post-infection by Bioplex assay. F) Lung-resident CD11b^{+} LP DCs were isolated and infected with RSV as previously described, followed by gene expression analysis by qPCR at 24 hours post-infection. G) CD4+ T cells were purified from mediastinal lymph nodes of WT RSV-infected mice, 8 days post-infection. Cells were plated at a 10:1 ratio over WT or LC3b$^{-/-}$ BMDCs, infected 2 hours previously with 1:1 MOI RSV. Culture supernatant IL-17a concentrations were measured at 48 hours by Bioplex assay. WT or LC3b$^{-/-}$ BMDCs (H) or CD11b^{+} LP DCs (I) were pulsed with whole ovalbumin protein in the presence or absence of 1:1 MOI RSV, followed by the addition of 10:1 purified splenic OT-II CD4+ T cells. Culture supernatant IL-17a concentrations were measured at 48 hours by Bioplex assay. All results are representative of at least three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001.

BMDCs pulsed with ovalbumin protein in the presence or absence of RSV, as ovalbumin treatment alone does not induce autophagy. While co-cultures in which WT and LC3b$^{-/-}$

DCs were treated with ovalbumin alone produced similar quantities of IL-17a, cultures in which LC3b$^{-/-}$ DCs were concurrently infected with RSV elicited significantly greater IL-17a production from the CD4+ OT-II T cells (Figure 3-3**H**). These findings were confirmed using CD11b^{+} LP DCs isolated from naïve WT and LC3b$^{-/-}$ mice, with RSV-infected and ovalbumin-pulsed LC3b$^{-/-}$ CD11b^{+} LP DCs eliciting significantly higher quantities of IL-17a from co-cultured OT-II T cells (Figure 3-3**I**). These results support impaired autophagy and altered innate cytokine production by LC3b$^{-/-}$ DCs as primary mechanisms in the initiation of Th17-skewed CD4+ T cell responses to RSV infection.

3d.iii. Non-Immune cell deficiency in LC3b augments Th17-associated RSV pathology through increased IL-1 secretion by airway epithelial cells

Results thus far indicated that LC3b$^{-/-}$ CD11b+ DCs elicit increased IL-17a production from CD4+ T cells in response to RSV. As LC3b expression in epithelial cells is an important modulator of lung pathology upon exposure to cigarette smoke (386) or hyperoxia (387), bone marrow chimeric mice were created to assess relative contributions of structural and hematopoietic cells to observed RSV-induced lung pathology. Histological examination of lung tissue 8 days post-infection revealed that WT and LC3b$^{-/-}$ mice reconstituted with LC3b$^{-/-}$ bone marrow experienced comparable significant increases in lung inflammation, infiltration of neutrophils, and IL-17a expression in comparison to fully WT chimeric animals (Figure 3-4**A-C**). These findings support the *in vitro* findings described above, confirming that hematopoietic LC3b deficiency is sufficient to induce increased IL-17 production and neutrophil infiltration in response to RSV *in vivo.*

Unexpectedly, reconstitution of LC3b$^{-/-}$ mice with WT bone marrow produced an augmented inflammatory response, as significantly higher numbers of neutrophils were found in the lungs in comparison to all other treatment groups (Figure 3-4**B**). These PMN accumulation data correlated well with qPCR analysis that revealed augmented expression of IL-17a in lung tissues (Figure 3-4**C**) and reduced expression of IFNγ (Figure 3-4**D**), with the WT to LC3b$^{-/-}$ chimera having the most significant effect. Elevated IL-17a secretion was similarly observed in MedLN cultures stimulated *ex vivo* with RSV (Figure 3-4**E**). Finally, investigation of innate cytokine expression in the lungs

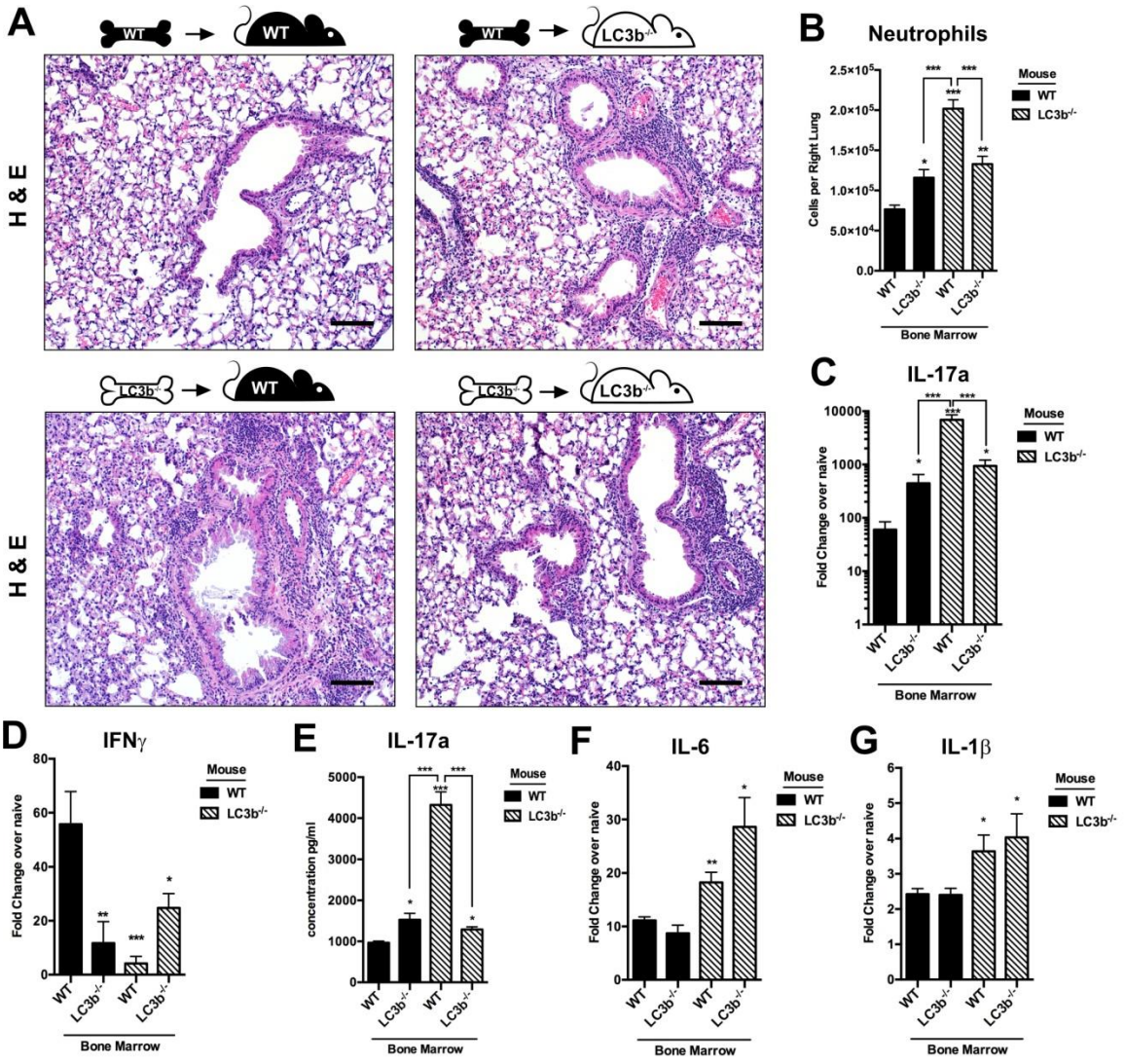

Figure 3-4 - **Both structural and hematopoietic LC3b deficiency contribute to increased lung pathology during RSV infection.** WT and LC3b$^{-/-}$ mice were lethally irradiated and reconstituted with whole bone marrow from WT or LC3b$^{-/-}$ donors, as indicated. Chimeric mice were infected with RSV and sacrificed 8 days post-infection. A) Lung sections were stained with haematoxylin and eosin (H & E) to visualize inflammation. Scale bars = 100μm. B) Neutrophils were quantified from enzymatically-digested lungs by flow cytometry. Neutrophils were gated as indicated in Figure 3-1. Lung mRNA expression of IL-17a (C) and IFNγ (D) was measured by qPCR, relative to naïve controls. E) Single-cell suspensions were prepared from mediastinal lymph nodes, followed by restimulation in culture with RSV. Culture supernatant IL-17a production was measured at 48 hours by Bioplex assay. F,G) Lung mRNA expression of IL-6 and IL-1β was measured by qPCR. Results are representative of three independent experiments, n≥4 mice per group. *p≤0.05, **p≤0.01, ***p≤0.001.

of chimeric mice revealed increased IL-6 and IL-1β mRNA transcript in RSV-infected LC3b$^{-/-}$ chimeric mice, independent of WT or LC3b$^{-/-}$ bone marrow reconstitution (Figure 3-4**F,G**), implicating structural cells as the primary source of these cytokines in the lungs. These findings collectively suggest that while LC3b$^{-/-}$ bone marrow is sufficient for the development of increased Th17-dependent lung pathology in response to RSV, LC3b$^{-/-}$

lung structural cells may augment production of IL-17 in the lungs through increased secretion of innate cytokines.

3d.iv.Airway Epithelial cells deficient in LC3b have enhanced inflammasome activation and increased cytokine production due to ER Stress

To further examine non-hematopoietic innate cytokine production in response to RSV, fluorescent immunohistochemistry was used to examine IL-1β protein expression in lung sections of infected mice. This revealed greater staining intensity in lungs of RSV-infected LC3b$^{-/-}$ mice, with the vast majority of IL-1β-positive cells co-staining for the epithelial marker E-Cadherin (Figure 5**A**). To verify these findings, primary airway epithelial cell (AEC) cultures were derived from lungs of naïve mice and infected with

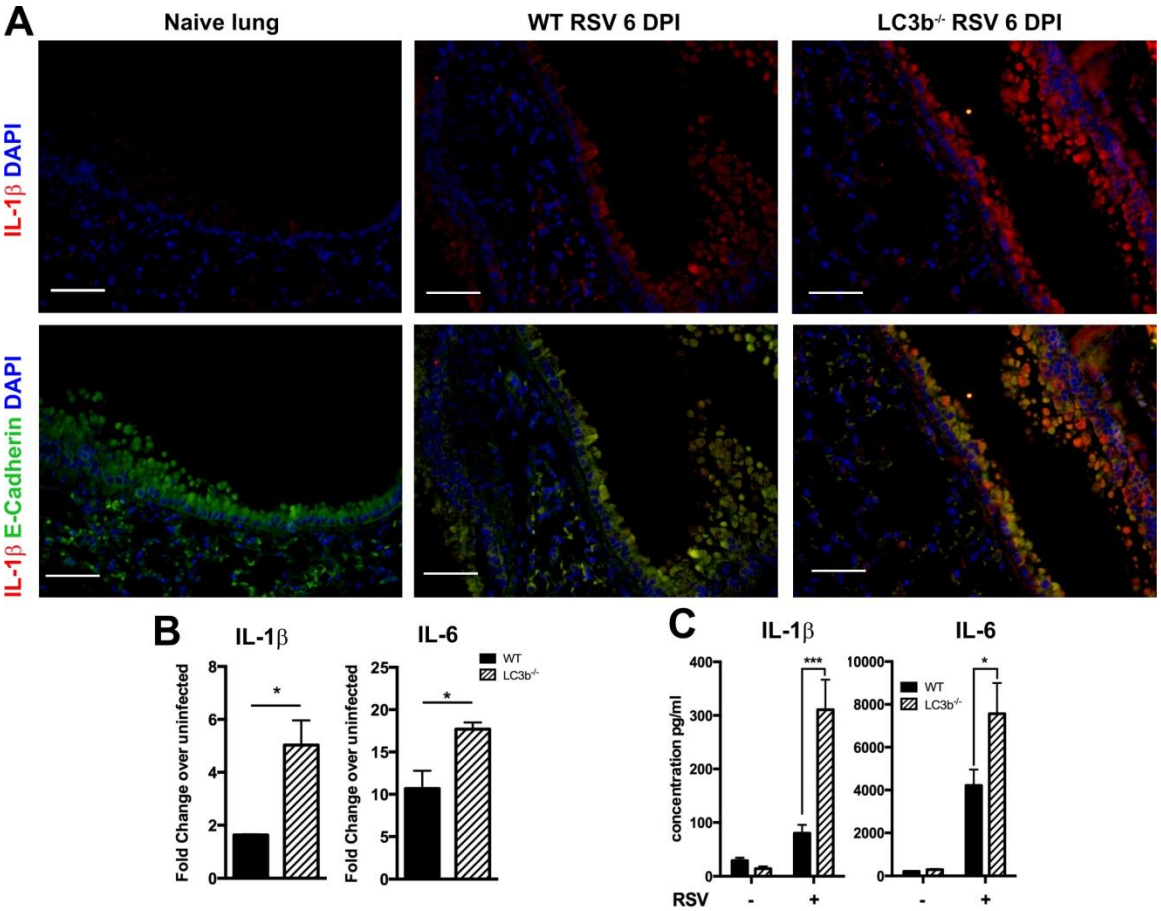

Figure 3-5 - **Increased IL-1β and pro-inflammatory cytokine production by LC3b$^{-/-}$ airway epithelial cells upon RSV infection.** A) Lung sections from WT and LC3b$^{-/-}$ mice, 6 days post-infection, were stained for IL-1β protein expression using antibodies to mouse IL-1β and E-cadherin. Sections were stained with DAPI and imaged on an Olympus BX43 fluorescent microscope. Scale bars = 40μm. Airway epithelial cell (AEC) cultures were prepared from lungs of naïve WT or LC3b$^{-/-}$ mice, and infected with 1:1 MOI RSV. B) IL-1β and IL-6 expression was measured at 12 hours post-infection by qPCR, while C) IL-1β and IL-6 secretion was measured in culture supernatants at 24 hours by Bioplex assay. *p≤0.05, **p≤0.01, ***p≤0.001.

RSV *in vitro*. In agreement with previous observations in lungs of LC3b$^{-/-}$ chimeric mice, LC3b$^{-/-}$ AECs upregulated IL-1β and IL-6 mRNA and secreted greater quantities of protein upon RSV infection, in comparison to WT cultures (Figure 5**B,C**).

RSV infection is a known inducer of both ER stress and NLRP3 inflammasome activation in bronchial epithelial cells (345, 383). As the accumulation of damaged mitochondria in the absence of autophagy is a known driver of NLRP3 inflammasome activation (234, 235), mitochondrial sequestration was examined in RSV-infected AEC cultures. While mitochondrial signal colocalized with distinct ATG5-positive structures in WT AECs at 24 hours post-infection, LC3b-/- AECs amassed large aggregates of mitochondria in response to RSV infection (Figure 3-6**A**). In addition, prolonged ER stress causes mitochondrial damage and triggers NLRP3 inflammasome activation (336). The expression levels of several genes activated during ER stress were therefore examined in RSV-infected AEC cultures. In agreement with the previous report of signaling through the ER stress-sensing receptor IRE-1α upon RSV infection (345), mRNA expression of Edem-1 and cleaved XBP-1 (XBP-1s) were significantly elevated in RSV-infected LC3b$^{-/-}$ AEC cultures at 12 hours post-infection (Figure 3-6**B**). Furthermore, pre-treatment of LC3b$^{-/-}$ AECs with the IRE-1α inhibitor 3,5-dibromosalicylaldehyde (DBSA) reduced the secretion of IL-1β and IL-6 to levels comparable to WT AECs (Figure 3-6**C,D**). Finally, in order to assess the contribution of ER stress to inflammasome activation during RSV infection, expression of the active p10 subunit of caspase-1 was measured in primary AEC cultures infected with RSV. Confocal imaging and fluorescence quantification of caspase-1 p10 antibody staining revealed increased signal in LC3b$^{-/-}$ AECs 12 hours post-treatment with RSV, in comparison to WT control cultures (Figure 3-6**E**). Pre-treatment of LC3b$^{-/-}$ AECs with DBSA abolished this difference (Figure 3-6**E**). Collectively, these results suggest that elevated RSV-induced production of IL-1β and IL-6 by the LC3b$^{-/-}$ airway epithelium is due to increased inflammasome activation and ER stress, and may have a critical role in shaping the ongoing immune response during RSV infection.

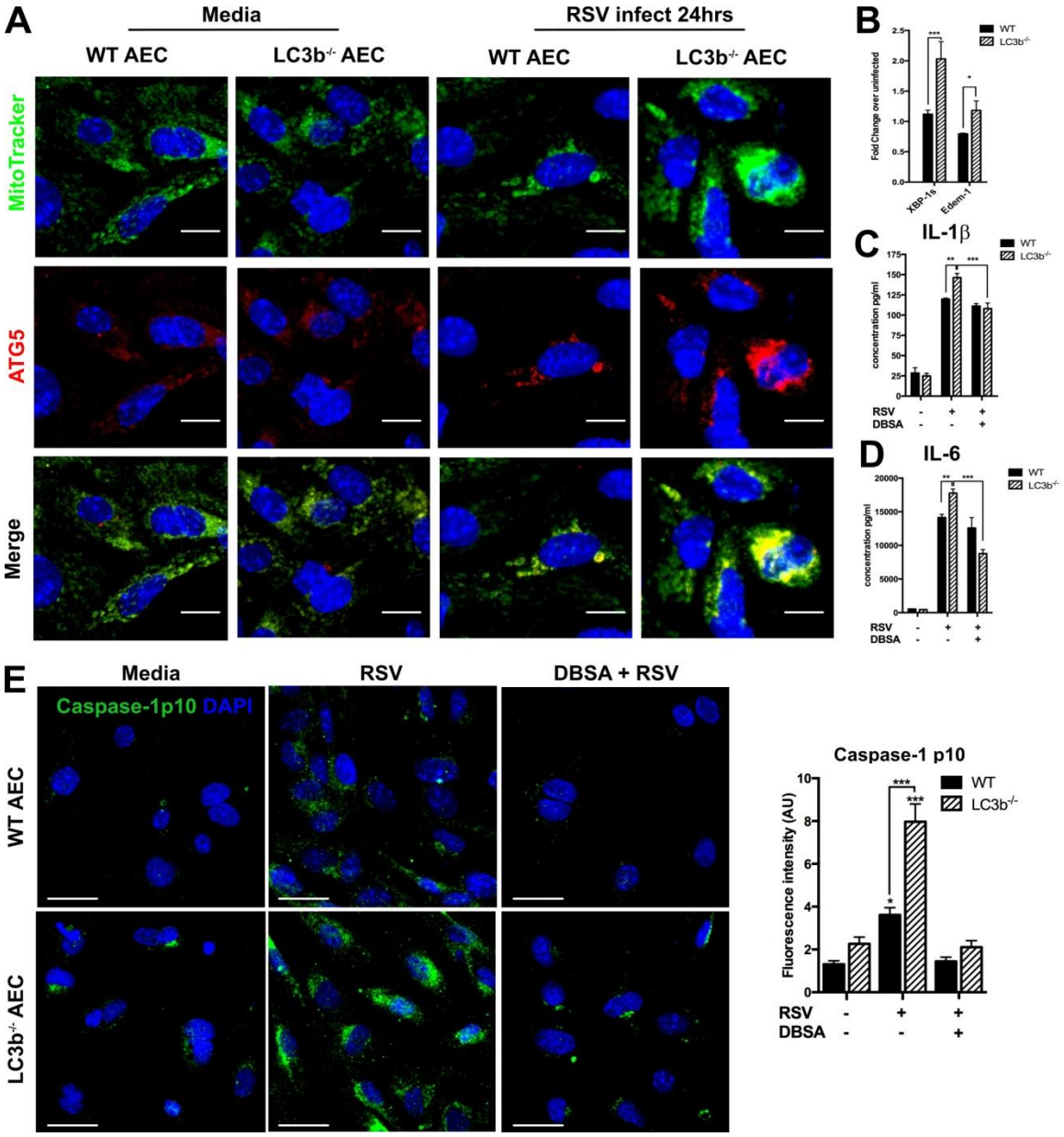

Figure 3-6 – Mitochondrial accumulation and IRE-1α-dependent increase of IL-1β production in RSV-infected LC3b$^{-/-}$ AECs. A) AECs were pulsed with Mitotracker 30 minutes prior to infection with 1:1 MOI RSV. Cells were stained at 24 hours post-infection with anti-ATG5 antibodies and DAPI, followed by imaging on a Nikon A1 confocal microscope. Scale bar = 10μm. B) mRNA transcripts of IRE-1α-associated genes Edem-1 and spliced XBP-1 were assessed in AECs at 12 hours post-infection by qPCR. (C,D) AECs were treated with the IRE-1α inhibitor 3,5-dibromosalicylaldehyde (DBSA) at 40μM 30 minutes prior to 1:1 MOI RSV. Concentrations of IL-1β (C) and IL-6 (D) were measured in cell supernatants at 24 hours post-infection by Bioplex assay. E) AECs were infected for 12 hours with 1:1 MOI RSV, then fixed and stained with antibodies to the Caspase-1 p10 subunit and DAPI. Cells were imaged on a Nikon A1 confocal microscope. Fluorescence intensity per cell was measured and quantified using maximum intensity projections, using ImageJ software. Scale bars = 30μm. Results are representative of at least two independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001.

3d.v. Blockade of IL-1 receptor signaling ameliorates IL-17a-associated pathology in LC3b$^{-/-}$ mice in vivo

In order to assess whether excessive IL-1 production was driving the development of the elevated Th17 response in LC3b$^{-/-}$ mice, murine IL-1 receptor antagonist (IL-1Ra) was administered immediately prior to and during the course of RSV infection. Strikingly, LC3b$^{-/-}$ mice treated with IL-1Ra during RSV infection showed substantially reduced peribronchial inflammation in comparison to LC3b$^{-/-}$ saline treatment controls (Figure 3-7**A**). Treatment with IL-1Ra decreased PAS-positive mucus in the airways of RSV-infected LC3b$^{-/-}$ mice (Figure 3-7**A**, lower panels), which was verified through significantly reduced mRNA expression of muc5ac in lung homogenates (Figure 3-7**B**). Quantification of lung neutrophils by flow cytometry revealed decreased neutrophil infiltration in IL-1Ra-treated LC3b$^{-/-}$ mice, in comparison to saline-treated controls (Figure 3-7**C**). Examination of cytokine production in lungs by qPCR revealed

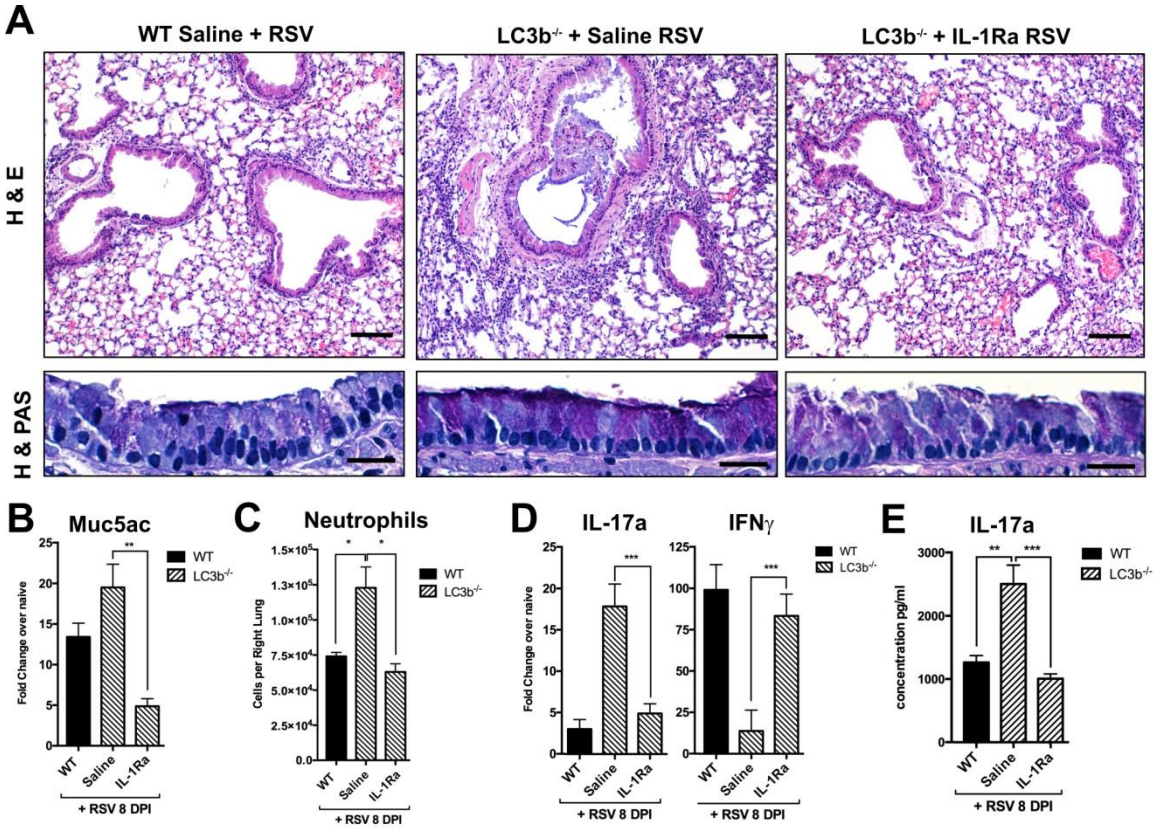

Figure 3-7 - **Increased IL-17a-dependent lung pathology in RSV-infected LC3b$^{-/-}$ mice requires IL-1 receptor signaling.** LC3b$^{-/-}$ mice were injected I.P. with 25mg/kg mouse IL-1 receptor antagonist (IL-1Ra) immediately prior to RSV infection, then treated once daily until sacrificed at 8 days post-infection. A) Lung sections were stained with haematoxylin and eosin (H & E) or haematoxylin and periodic acid schiff (H & PAS) to visualize mucus. Scale bars = 100µm on upper panels, 20µm on lower panels. B) Lung expression of *muc5ac* was measured by qPCR. C) Neutrophils were quantified in enzymatically-digested lung tissue by flow cytometry, using cell surface antigens detailed in Figure 3-1D) Expression of IL-17a and IFNγ were measured in lungs by qPCR. E) Single-cell suspensions prepared from mediastinal lymph nodes were restimulated in culture with RSV. IL-17a concentrations in supernatants were measured at 48 hours post-infection by Bioplex assay. Results are representative of two independent experiments, n≥5 mice per group. *p≤0.05, **p≤0.01, ***p≤0.001.

significantly reduced IL-17a expression in IL-1Ra-treated LC3b$^{-/-}$ mice, with a concomitant increase in IFNγ production in response to RSV infection (Figure 3-7**D**). Finally, IL-17a secretion by MedLN cells restimulated *ex vivo* was significantly reduced in cultures from LC3b$^{-/-}$ mice who received IL-1Ra treatment (Figure 3-7**E**). These results ultimately suggest that elevated IL-17a production and associated lung pathology in RSV-infected LC3b$^{-/-}$ mice are driven by IL-1 receptor signaling.

3e. Discussion

In this manuscript, we provide evidence for the importance of the autophagy protein LC3b in both innate cytokine production and the induction of adaptive immune responses to RSV. Mice deficient in LC3b developed greater IL-17a-dependent lung pathology upon RSV infection, characterized by neutrophil infiltration, mucus hypersecretion, and decreased viral clearance in comparison to WT littermates. While cells such as γδ T cells and NK cells are capable of producing IL-17a, previous observations suggest that CD4+ T cells are the primary source of IL-17a at 8 days post-RSV infection (374). In agreement with previous work showing a requirement for autophagy in innate cytokine production by DCs (230, 382), RSV-infected LC3b$^{-/-}$ DCs produced IL-1β, IL-6, and IL-23 at the expense of IL-12 and preferentially elicited IL-17a production from CD4+ T cells (Figure 3-8**A**). In further support of DC-mediated induction of deleterious Th17 responses, hematopoietic LC3b deficiency was sufficient for the development of elevated lung pathology in response to RSV. Unexpectedly, structural deficiency in LC3b augmented the development of IL-17-dependent lung pathology, as infected LC3b$^{-/-}$ airway epithelial cells secreted greater amounts of IL-1β in a manner dependent on elevated IRE-1α signaling (Figure 3-8**B**). Finally, treatment of LC3b$^{-/-}$ mice with IL-1Ra attenuated IL-17a production and associated lung pathology in response to RSV, implicating enhanced IL-1 receptor signaling in the development of IL-17-driven lung pathology. The identification of these innate pathways may lead to new strategies to alleviate pathophysiologic changes during RSV infection that lessen severity and subsequent long term sequelae of pulmonary damage associated with RSV.

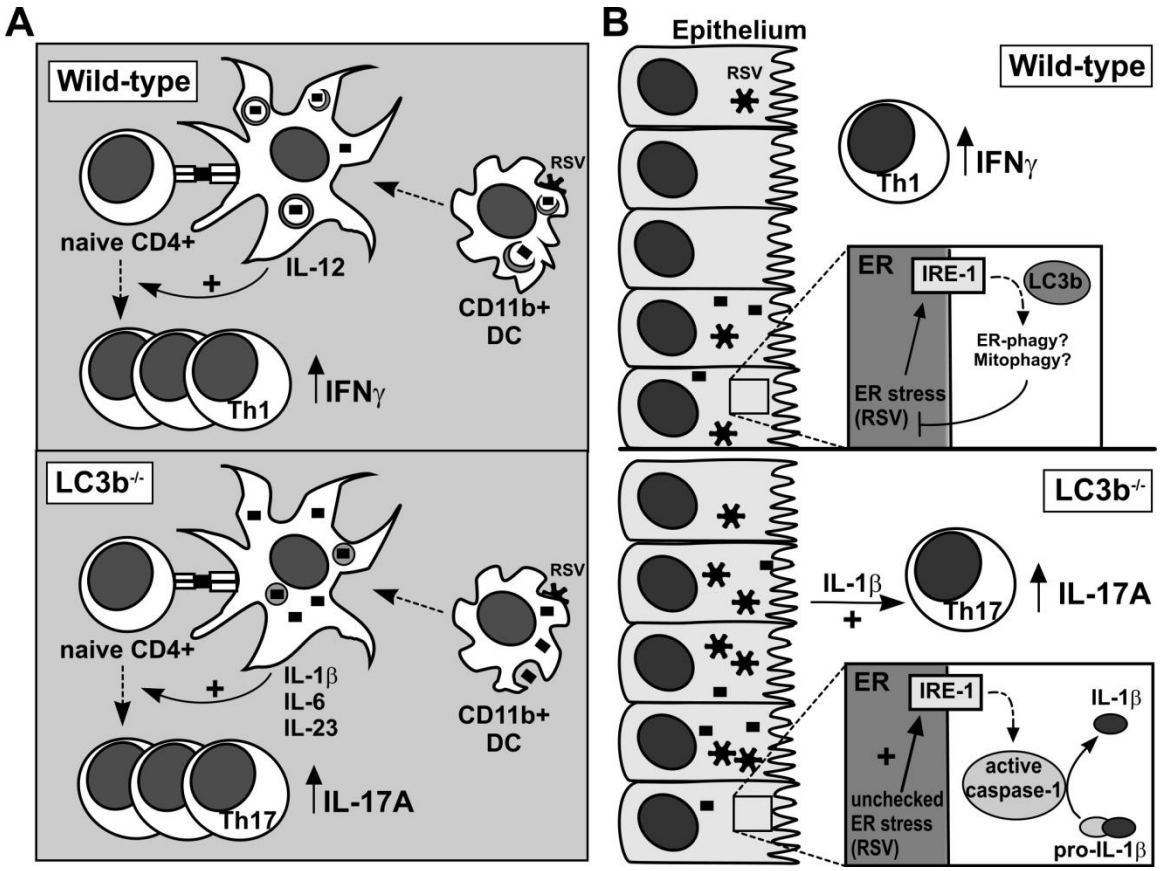

Figure 3-8 - **Proposed mechanisms of the induction and maintenance of CD4+ T cell responses to RSV in wild-type or LC3b$^{-/-}$ mice.** A) Upon detection of RSV by WT lung DCs, rapid induction of autophagy facilitates IL-12 production, ultimately promoting the development of a Th1 response by RSV-reactive CD4+ T cells. In contrast, LC3b$^{-/-}$ DCs fail to upregulate autophagy upon encountering RSV, resulting in the production of IL-1β, IL-6, and IL-23. This cytokine environment skews RSV-reactive CD4+ T cells toward a Th17 phenotype. B) RSV-infected lung epithelial cells upregulate signaling through the ER stress sensor IRE-1, potentially triggering LC3b-dependent autophagosomal degradation of ER and damaged mitochondria, thereby alleviating RSV-induced stress and minimizing IL-1β production. In the absence of LC3b expression, RSV-infected epithelial cells experience increased IRE-1 signaling owing to unchecked ER stress, resulting in caspase-1 cleavage and mature IL-1β production. This enhanced IL-1β secretion stimulates further IL-17a production from CD4+ effector T cells through IL-1 receptor signaling.

Since the discovery of Th17 cell lineage, aberrant or excessive Th17 immune responses have been found to enhance pathology in the context of immune-mediated disorders such as rheumatoid arthritis (388) and allergic asthma (389), as well as during viral infection (390). Studies of severe RSV infection suggest that a Th17-skewed response contributes to lung pathology in at least a subset of patients, as elevated IL-17a expression has been reported in tracheal aspirates of infants hospitalized with RSV (374, 391). In addition, lung expression of IL-6 and IL-1 are correlated with severity of disease in both infants and adults infected with RSV (51, 392). IL-17a may drive lung pathology by inducing mucus gene expression (393) and potentiating IL-8 production in RSV-infected bronchial epithelial cells, thereby augmenting lung neutrophil recruitment (391).

This is supported by previous findings in our laboratory in animal models of RSV infection, as administration of neutralizing antibodies to IL-17a attenuated lung mucus production and neutrophil infiltration (151, 374). In the present studies treatment of LC3b$^{-/-}$ mice with anti-IL-17a antibodies during RSV infection significantly decreased lung neutrophil infiltration and mucus hypersecretion, while also elevating lung IFNγ expression. Moreover, *in vivo* blockade of IL-1 receptor signaling through IL-1Ra treatment mitigated lung production of IL-6 and IL-17a in RSV-infected LC3b$^{-/-}$ mice, resulting in reduced lung pathology. These findings not only provide further evidence in support of the critical role of LC3-dependent autophagy secretion during infectious insult (235), but also provide novel evidence supporting the role of autophagy in counteracting deleterious Th17 responses to RSV through blocking inflammasome-mediated production of IL-1β.

The production of IL-23 by DCs and macrophages is also indirectly regulated by autophagy, through autophagy-mediated inhibition of IL-1 secretion (382). The alteration of autophagy reduces the ability to shuttle RSV RNA to endosomic TLR pathways, which have been shown to be associated with regulation of IL-23 and IL-17a (151). Accordingly, we found that RSV-infected LC3b$^{-/-}$ DCs that failed to upregulate autophagosome formation secreted more IL-1β, IL-6, and IL-23, and preferentially induced IL-17a expression from co-cultured CD4+ T cells in comparison to WT controls. Experiments with chimeric mice further support a causative role for autophagy deficiency in DC-mediated induction of Th17 responses to RSV, as WT mice reconstituted with LC3b$^{-/-}$ bone marrow developed similar lung pathology as mice fully deficient in LC3b. Conversely, the development of T cell-dependent lung pathology in fully LC3b$^{-/-}$ mice may be dampened not only by inefficient antigen presentation by autophagy-deficient DCs (352, 394), but may also be due to a requirement for autophagy proteins in the survival and proliferation of effector T cells (395, 396). In support of this, the reconstitution of LC3b$^{-/-}$ mice with WT bone marrow augmented Th17-dependent lung pathology above levels observed in fully LC3b$^{-/-}$ mice. Ultimately, these results suggest critical roles for LC3b-dependent autophagy in both structural and hematopoietic cells in mitigating inflammasome activation and the development of Th17-associated lung pathology during RSV infection.

Several non-exclusive mechanisms may contribute to increased IL-1β secretion by LC3b$^{-/-}$ DCs, macrophages, and airway epithelial cells in response to RSV infection. Our findings of increased IL-1β secretion and active caspase-1 by LC3b$^{-/-}$ AECs concurs with two recent reports of RSV infection inducing IL-1β secretion in macrophages (131) and primary human airway epithelial cells (383) through an NLRP3- and caspase-1-dependent mechanism. While LC3b-dependent autophagy directly regulates inflammasome activity in macrophages through selective degradation of ubiquitinated NLRP3 inflammasome platforms (249) and pro-IL-1β (320), further work will be required to establish this mechanism in epithelial cells during RSV infection. The inability of LC3b$^{-/-}$ cells to regulate inflammasome activation through autophagy may be further exacerbated by an inability to remove damaged mitochondria through mitophagy (235). Reactive oxygen species released by damaged mitochondria are required for NLRP3 inflammasome activation and caspase-1-mediated IL-1β production (234, 397), and this was recently verified in RSV-infected macrophages (131). Autophagy-deficient LC3b$^{-/-}$ macrophages accumulated damaged mitochondria and mitochondrial ROS in response to LPS and ATP treatment, resulting in greater NLRP3 inflammasome activation and IL-1β secretion (235). As RSV infection was previously reported to increase numbers of morphologically altered mitochondria in human airway epithelial cells (398), an inability to sequester damaged mitochondria via LC3b-associated mechanisms likely contributes to increased inflammasome activation upon infection.

Importantly, these data uncovered evidence of increased ER stress in LC3b$^{-/-}$ AECs upon RSV infection. Recent data indicated that RSV infection of A549 cells activates ER stress response signaling through IRE-1 and ATF6 (345). ER stress is known to upregulate pro-survival autophagy (332, 333), while numerous studies have shown a requirement for IRE-1 pathway signaling in the regulation of autophagy during the ER stress response (332, 399). It was also recently reported that the induction of ER stress activates the NLRP3 inflammasome and increases IL-1β secretion in a manner dependent on mitochondrial integrity (336). It is therefore plausible that ER stress in RSV-infected cells may serve the dual purpose of promoting cell survival through autophagy-mediated removal of viral nucleic acids and proteins, while simultaneously controlling NLRP3 inflammasome activation through degradation of damaged

mitochondria and assembled inflammasome platforms. Further studies will be required to elucidate the exact mechanisms underlying increased RSV-induced inflammasome activation and IL-1β secretion by LC3b$^{-/-}$ cells.

These findings provide additional evidence for the role of autophagy during RSV infection in promoting the induction of antiviral Th1 responses to RSV *in vivo*. Furthermore, these results suggest that LC3b associated responses and autophagy serves as a critical negative regulator of inflammation during RSV infection, through limitation of IL-1β production by lung epithelial cells. The present studies highlight the unique, cell-specific contribution of autophagy proteins to inflammatory processes, and provide valuable insight into the induction and development of Th17-skewed responses during RSV infection. Ultimately, this information may inform novel treatments for severe RSV infection, and ultimately aid in vaccine design strategies.

Chapter 4 – Discussion and Future Directions

4a. Autophagy in DC-mediated limitation of RSV-induced pathology *in vivo*

The studies described in this dissertation provide novel evidence of a requirement for autophagy in the successful induction of antiviral responses to RSV. These findings agree with recent studies of immunological autophagy in the direct clearance of intracellular bacteria and viruses from infected cells, yet to our knowledge this represents the first demonstration of an *in vivo* requirement for immunological autophagy in the induction of protective responses to a respiratory virus.

Our findings build on previous work that established the importance of autophagy in DCs in the production of pro-inflammatory cytokines and antigen presentation to T cells, and extend this knowledge to a translationally relevant animal model of RSV infection. Our studies documenting the development of pathological Th2 and Th17 responses to RSV in mice deficient in different autophagy proteins is reminiscent of Th2-biased and Th17-biased subsets of patients with severe RSV (107, 180, 375). The potential of dysregulated autophagic machinery to cause pulmonary disease has drawn considerable scientific interest in recent years, and autophagy gene variants are now associated with disease pathogenesis in COPD (400), cystic fibrosis (401), cigarette smoke-induced emphysema (386, 387, 402), and asthma (311, 403). The well-established correlation between severe RSV infection and subsequent development of chronic lung disease, combined with evidence of genetic predisposition precipitating these responses, may point to a common mechanism driving pathological responses to both RSV infection and environmental insults. While any relationship between impaired autophagy and neonatal illness susceptibility has yet to be investigated, there are several plausible ways in which subtle defects in the autophagic response could contribute to the development of severe RSV infections. Insufficient neonatal immune responses are partly due to the failure of infant DCs and macrophages to mature in response to antigen, as these cells

express lower levels of pattern recognition receptors and produce less cytokine in comparison to myeloid cells from adults (404). The reasons for this functional deficit are not fully understood, and it is plausible that an underlying failure to rapidly upregulate autophagy could underlie incomplete neonatal DC maturation. Our findings indicate that blockade of autophagy inhibits DC maturation and cytokine production in response to RSV, and attenuates production of the critical antiviral cytokine IL-12 (228, 230). Neonatal DCs are similarly deficient in IL-12 production (405) and in the induction of cytotoxic T cell responses to RSV (404). Any impaired autophagic upregulation could in turn be exacerbated by the Th2-biased adaptive immune system of infants, as the Th2 cytokines IL-4 and IL-13 are reported to suppress autophagy in macrophages (255). While further studies would be required to prove a causal link between dysregulated autophagy and RSV-induced pathology in humans, these findings suggest that genetic or epigenetic disruption of autophagy may plausibly contribute to the development of pathogenic responses to RSV in human patients.

We have also provided novel evidence of autophagy limiting inflammasome activity and IL-1β secretion by airway epithelial cells during RSV infection. It was recently demonstrated that RSV infection induces NLRP3 inflammasome activation in human airway epithelial cells and murine macrophages (131, 383), and our finding of increased IL-1β secretion by LC3b$^{-/-}$ DCs and epithelial cells correlates with the established role of autophagy in counteracting inflammasome activity in immune cells. IL-1β stimulates IL-17 production from CD4+ T cells (406), and elevated IL-1, IL-6 and IL-17 concentrations are observed both in lungs of RSV-infected LC3b$^{-/-}$ mice and in the airway aspirates of infants hospitalized with RSV (374, 391, 392). Finally, our experiments provide evidence of the efficacy of IL-1Ra treatment in the amelioration of IL-17-dependent lung pathology. Recombinant human IL-1Ra has already been approved for patient use (anakinra, trade name Kineret), and further studies into the effectiveness of treating established RSV infection with IL-1Ra may provide evidence for a novel treatment option for severe RSV infections. Overall, these studies shed light on the importance of autophagy to the induction of antiviral immune responses and control of the inflammatory environment of the lung during RSV infection, and could potentially

inform the development of novel RSV vaccine formulations or the development of DC- or epithelium-targeted therapeutics.

4b. Differential responses of Becn1$^{+/-}$ and LC3b$^{-/-}$ mice to RSV infection

Several interesting discrepancies were uncovered between the responses of Becn1$^{+/-}$ mice and LC3b$^{-/-}$ mice to RSV infection (see Table 4-1). Both Becn1$^{+/-}$ and LC3b$^{-/-}$ mice were impaired in viral clearance and developed increased lung pathology in response to RSV. Nevertheless, Becn1$^{+/-}$ responses were characterized by increased Th2 cytokine expression and eosinophil infiltration into the lungs, while LC3b$^{-/-}$ mice developed lung neutrophilia and an increased Th17-associated cytokine secretion profile. RSV-infected Becn1$^{+/-}$ and LC3b$^{-/-}$ DCs were both deficient in MHC-II surface expression, and both elicited aberrant CD4+ T cell responses, yet these responses were skewed toward Th2- and Th17-associated cytokines, respectively. These results reinforce the critical role of autophagy in DC function, and in DC initiation of antiviral CD4+ T cell response to RSV. However, the differential responses of Becn1$^{+/-}$ and LC3b$^{-/-}$ cells to RSV infection point to other

	Becn-1$^{+/-}$	**LC3b$^{-/-}$**
Lungs		
Viral Clearance	Delayed	Delayed
Mucus production	Increased	Increased
Lung T cell cytokines	IL-5, IL-13	IL-17a
Granulocytes	Eosinophils	Neutrophils
DCs		
MHC-II expression	Decreased	Decreased
IL-12 production	Decreased	Decreased
IL-1β , IL-6 production	Decreased	Elevated
Elicited CD4+ T cell cytokines (co-culture)	Th2 (IL-5, IL-13)	Th17 (IL-17a)
Epithelial cells		
IL-1β production	No difference	Elevated
IL-6 production	No difference	Elevated
Mitochondrial clearance	Not investigated	Impaired

Table 4-1- **Summary of responses of Becn1$^{+/-}$ and LC3b$^{-/-}$ mice to RSV infection**.

intracellular processes intersecting with the autophagy machinery, and demonstrate how targeting different aspects of a conserved intracellular pathway can produce very different immunological results at the organismal level.

One marked difference uncovered in these studies was the different cytokine responses of LC3b$^{-/-}$ and Becn1$^{+/-}$ epithelial cells upon RSV infection. LC3b$^{-/-}$ epithelial cells produced more of the IRE-1-signalling-dependent cytokine IL-6 in response to RSV, while expression of IL-6 by RSV-infected Becn1$^{+/-}$ epithelial cells was identical to wild-type controls ((228), see Figure 2-3 and Figure 3-5). The induction of ER stress signaling in RSV-infected Becn1$^{+/-}$ epithelial cells was not investigated, yet these cells are likely to function normally with respect to the unfolded protein response (UPR). While Becn1 expression is required for ER stress-induced autophagy, ER stress signaling and the induction of the UPR proceed normally in the absence of Becn1 (407). Of note, the authors used silencing RNA to eliminate Becn1 production, rather than utilizing Becn1$^{+/-}$ cells as in the current studies. As the induction of the UPR triggers an upregulation of Becn1 gene expression (334, 407), it is plausible that cells with one functioning copy of the Becn1 gene may express sufficient Becn1 for some autophagic mitigation of ER stress to occur. In contrast, LC3b$^{-/-}$ epithelial cells are unable to remove damaged ER through ER-phagy, as LC3b expression is required for this process (331). This inability to alleviate ER stress likely augments IL-1β production by RSV-infected LC3b$^{-/-}$ cells, as prolonged ER stress activates the NLRP3 inflammasome by triggering mitochondrial ROS production (335, 336, 397). Finally, ER stress-driven inflammasome activation in LC3b$^{-/-}$ cells is exacerbated by an inability to degrade mitochondria, as membrane-bound LC3b is a critical receptor for proteins targeting depolarized mitochondria to forming autophagosomes (331, 408). Thus, the ability to mitigate ER stress and sequester damaged mitochondria during RSV infection may ultimately explain the differential cytokine expression profiles of Becn1$^{+/-}$ and LC3b$^{-/-}$ epithelial cells.

In contrast to epithelial cells, TLR-dependent DC maturation and innate cytokine production requires the rapid upregulation of autophagosome formation in response to RSV, with baseline autophagosome numbers increasing 4-fold in as little as 30 minutes post-infection (timing observation unpublished; see Figure 2-4). Although Becn1$^{+/-}$ DCs

have one functional copy of the gene encoding Becn1, we found that this was not sufficient to allow the immediate upregulation in autophagosome formation observed in WT DCs in response to RSV. In keeping with our previous studies identifying a requirement for autophagy in TLR activation and MHC-II surface expression in RSV-infected DCs (230), both $Becn1^{+/-}$ and $LC3b^{-/-}$ DCs were deficient in production of the key innate cytokine IL-12 and in surface upregulation of MHC-II. Consequently, autophagy-deficient $Becn1^{+/-}$ and $LC3b^{-/-}$ DCs were impaired in the ability to elicit antiviral CD4+ T cell responses. These findings reinforce the importance of rapid autophagosomal delivery of viral RNA and peptides to endosomal compartments, thereby facilitating TLR-dependent DC maturation, IL-12 production, and antigen presentation function.

While both $Becn1^{+/-}$ and $LC3b^{-/-}$ DCs displayed altered innate cytokine production profiles in response to RSV, secretion of IL-1β and IL-6 by RSV-infected $LC3b^{-/-}$ DCs was significantly greater in comparison to WT controls. In contrast, $Becn1^{+/-}$ DCs produced less IL-1β and IL-6 in response to RSV. It is possible that this discrepancy in IL-1β and IL-6 production is due to the ability of RSV-infected $Becn1^{+/-}$ DCs to manage ER stress, while $LC3b^{-/-}$ DCs are unable to do so. The pharmacological induction of ER stress in macrophages augments IL-6 production in response to TLR agonists (409), which may explain elevated IL-6 secretion by $LC3b^{-/-}$ DCs. The differential secretion of IL-1β by RSV-infected $Becn1^{+/-}$ and $LC3b^{-/-}$ DCs could also be due to an inability of $LC3b^{-/-}$ DCs to clear depolarized mitochondria, as the targeting of damaged mitochondria to forming autophagosomes requires LC3b expression (408). Indeed, inhibition of autophagy in LPS- and ATP-treated $LC3b^{-/-}$ macrophages promoted rapid cytosolic accumulation of depolarized mitochondria, leading to increased NLRP3 inflammasome activation and IL-1β secretion (235). While the authors reported a slight accumulation of depolarized mitochondria in $Becn1^{+/-}$ macrophages, $LC3b^{-/-}$ macrophages showed a far more pronounced increase in mitochondrial accumulation, cytosolic ROS production, and IL-1β secretion in response to LPS and ATP treatment (235). This raises the possibility that $Becn1^{+/-}$ macrophages may be able to remove some damaged mitochondria when stimulated with a less-potent inflammasome activator, and therefore do not produce excessive IL-1β as $LC3b^{-/-}$ macrophages do when infected with RSV. Further studies

would be required to fully elucidate the potential relationship between ER stress signaling, mitochondrial clearance, and inflammasome activation in RSV-infected Becn1$^{+/-}$ and LC3b$^{-/-}$ DCs.

Despite common findings of impaired viral clearance, increased mucus production, and granulocyte infiltration into lungs of RSV-infected Becn1$^{+/-}$ and LC3b$^{-/-}$ mice, some key differences were found. Elevated Th2- and Th17-associated cytokine production observed in lungs of RSV-infected Becn1$^{+/-}$ and LC3b$^{-/-}$ mice correlated with the induction of Th2- and Th17-biased CD4+ T cell responses by Becn1$^{+/-}$ and LC3b$^{-/-}$ DCs, respectively. The Th2-associated lung pathology observed in Becn1$^{+/-}$ mice is primarily due to a deficiency in DC-mediated induction of antiviral CD4+ T cell responses, as RSV-pulsed Becn1$^{+/-}$ DCs administered into the lungs of WT mice elicited increased Th2 cytokine responses and lung pathology upon subsequent RSV challenge. This was further upheld by cell culture experiments, where no differences in Becn1$^{+/-}$ epithelial cell cytokine production were detected in response to RSV.

In contrast, examination of LC3b-deficient mice revealed increased IL-17-dependent lung pathology in response to RSV, as neutralization of IL-17 ameliorated lung inflammation and restored IFNγ production and viral clearance. This finding concurred with previous studies in our laboratory, which found that IL-17 directly inhibits viral clearance through suppressing IFNγ production and cytotoxic activity by CD8α+ T cells (374). In agreement with findings in Becn1$^{+/-}$ mice, the skewed CD4+ T cell cytokine environment in the lungs of infected LC3b$^{-/-}$ mice is due to altered DC-mediated induction of antiviral CD4+ T cell responses. This is evidenced by increased elicitation of IL-17 production from CD4+ T cells by RSV-infected LC3b$^{-/-}$ DC, and further supported by the development of elevated lung pathology in RSV-infected WT chimaeric mice reconstituted with LC3b$^{-/-}$ bone marrow. However, we found a substantial contribution of airway epithelial cell cytokine production to the development of Th17-associated pathology in RSV-infected LC3b$^{-/-}$ mice, as reconstitution of LC3b$^{-/-}$ mice with WT bone marrow produced augmented lung neutrophil recruitment and IL-17 production. This ultimately suggests that, in the absence of functional autophagy, an

excessively inflammatory lung environment amplifies the skewed CD4+ T cell response initiated by autophagy-deficient DCs, leading to augmented lung pathology.

4c. Future studies

Much of our knowledge of the mechanisms of epithelial cell and DC responses to pathogenic insults are derived from studies utilizing transfected cell lines, purified PRR ligands, and pharmacological agents. While these studies are of considerable worth to elucidating the intracellular mechanics of immune cell responses to specific stimuli, they are not always easily translated to the context of live viral infection. Viruses are dynamic entities that have been evolutionarily selected to commandeer host cell processes for their own ends, particularly in order to replicate and inhibit host antiviral signaling. Any study of host defense against viral infection in cultured cells, or at the organismal level, must therefore take viral subversion into account.

One interesting avenue to pursue would be untangling the interaction between RSV-encoded virulence proteins NS1/2 and SH (410), and host autophagy, ER stress, and inflammasome activation. Many viruses block or induce autophagy activation, yet it is currently unknown whether RSV modulates autophagy in this way. The RSV nonstructural proteins NS1 and NS2 colocalize with mitochondrial IPS-1 in infected cells (35, 365, 411), promote the degradation of IPS-1-binding RIG-I and its downstream signaling molecules IRF3 and Iκκε (411, 412), and delay host cell apoptosis (30). IPS-1 localizes to the junction between mitochondria and ER mitochondria-associated membranes (ER-MAMs) (413), which is also the site of autophagosome generation (337). As NS1/2-mediated degradation of RIG-I is only partially dependent on proteasome activity (412), the remainder could be due to autophagosomal sequestration and degradation of bound mitochondria. RSV is known to form viral ‘inclusion bodies’ in the cytoplasm of host epithelial cells, which colocalize with mitochondrial markers (414). A recent study of a novel phlebovirus found that NS-dependent inclusion bodies function to sequester TBK1, Iκκε, and IRF3, thereby enabling viral suppression of IFNβ production (415). As RSV NS1 is reported to interact with Iκκε and promote its degradation through a proteasome-independent mechanism (365, 411), it seems plausible that autophagy could be involved in this process. Furthermore, the induction of

mitophagy by NS1/2 could expedite viral blockade of RIG-I/IPS-1-mediated IFNβ production through removing mitochondrial IPS-1 and RIG-I (35). The induction of mitophagy could also potentially explain NS1/2-dependent delayed intrinsic apoptosis (30) through the removal of mitochondria from the cytosol. Our own findings indicate that RSV-induced, mitochondria-containing inclusion bodies in epithelial cells are ATG5-positive (see Figure 3-6), warranting further investigation to answer these questions.

The studies detailed in this dissertation demonstrate that inflammasome-mediated secretion of IL-1β is an important driver of Th17-dependent lung pathology *in vivo*, and that LC3b expression is critical to the control of IL-1β production. It is known that RSV induces NLRP3 inflammasome activation through a mechanism requiring potassium efflux and the generation of mitochondrial ROS (131, 383), but exactly how RSV induces this response remains to be elucidated. The RSV protein SH was recently identified as a viroporin targeting the Golgi, ER-MAMs, and mitochondria, and was required for RSV-induced IL-1β production (383). This raises the possibility that this protein could directly induce ion transfer to or from mitochondria, or perhaps indirectly promote inflammasome activation through the induction of ER stress. Viroporins encoded by other viruses have been shown to activate ER stress by altering ER calcium ion gradients (342, 343), and calcium release from the ER is a potent inducer of both autophagy (416) and NLRP3 inflammasome activation (417). The induction of NLRP3 inflammasome activation during ER stress is known to be dependent on ion channel expression (336), yet any contribution of the SH protein to ER stress remains to be addressed.

Future studies could also examine the relative contributions of different ER stress signaling pathways, and their interaction with TLR signaling, in the production of pro-inflammatory cytokines. The pioneering report of ER stress induction by RSV found evidence of IRE-1α-dependent control of viral replication in infected epithelial cells, but no evidence of PERK activation (345). Interestingly, pharmacological induction of signaling downstream of PERK attenuates IRE-1α-dependent IL-6 production by primary epithelial cells undergoing ER stress (418), suggesting that the induction of PERK may suppress signaling through other ER stress sensors. PERK signaling may also reduce

viral replication in RSV-infected epithelial cells (419). Additional experiments are needed to illuminate the signaling pathways downstream of IRE-1α and PERK that lead to reduced viral replication and/or IL-6 production in the context of RSV.

Finally, the relative importance of ER stress signaling and autophagy is likely dependent on the cell type, and future studies addressing cell-specific responses may lead to better-designed and properly-targeted therapeutic development. Airway epithelial cells are primary replicative targets for respiratory viruses, providing considerable evolutionary incentive for the emergence of viral proteins that suppress epithelial antiviral signaling. In the never-ending conflict between host and pathogen, the detection of rapid viral protein synthesis through sensors of ER stress provides another critical danger signal to the cell. Several viruses, including RSV, are now known to modulate IRE-1α signaling in human bronchial epithelial cells in order to enhance viral replication (345, 420, 421). In contrast, our previous data indicates that RSV replication is quickly attenuated within infected WT DCs (228), suggesting that these cells respond so rapidly that RSV may not have the opportunity to generate severe ER stress under normal circumstances. It may also be to the advantage of professional antigen-presenting cells to gather more nuanced, PRR-dependent information about encountered pathogens, rather than sending out general distress signals. The findings and additional questions raised by the current studies will ultimately contribute to the effort to design novel treatments for established RSV infections, and will hopefully aid in the eventual design of an effective vaccine.

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