The role of Sirtuin 1 in the regulation of autophagy and Respiratory Syncytial Virus-induced immune responses

by

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"Of science and the human heart, there is no limit…"
:: ***Miracle Drug*, U2 ::**

"When you want something,
all the universe conspires in helping you to achieve it."
:: ***The Alchemist*, Paulo Coelho ::**

"Because the people who are crazy enough to think they can
change the world, are the ones who do."
:: Apple – Think Different ::

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DEDICATION

To the woman who taught me about perseverance and patience; to my fellow world-traveler and photographer; to my very first best friend; to my reality check—thank you for putting up with my drama, both big and small. I am eternally grateful for your support and belief in me, for our adventures into Nature, and for listening to my experimental and medical school woes. To become half the woman that you are would be a blessing and an honor.

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(A)EC ..... (Alveolar) epithelial cell
AHR ..... Airway hyperreactivity
APC ..... Antigen presenting cell
ATG ..... Autophagy
BAL ..... Bronchial-alveolar lavage
CTL ..... Cytotoxic T lymphocytes
DPI ..... Days post-infection
EAE ..... Experimental autoimmune encephalomyelitis
HPI ..... Hours post-infection
IFN ..... Interferon
IL ..... Interleukin
KO ..... Knockout
LDLN ..... Lung draining lymph node
LRTI ..... Lower respiratory tract infection
MHC ..... Major histocompatibility complex
NLR ..... Nod-like receptor
RLR ..... RIG-I-like receptor
SIRT ..... Sirtuin
SLE ..... Systemic lupus erythematous
SNP ..... Small nuclear polymorphism
TCR ..... T cell receptor
Th cells ..... T helper cells
TLR ..... Toll-like receptor
Tregs ..... T regulatory cells
UPR ..... Unfolded protein response
WT ..... Wild-type

ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract disease in children, and a significant source of morbidity and mortality among those susceptible, including infants, the elderly, and those with chronic lung diseases. Severe RSV infection during infancy is highly correlated with asthmatic symptoms later in life, suggesting a chronic alteration of the pulmonary immune environment even after viral clearance. Within the airways, dendritic cells (DCs) drive innate and adaptive immune responses to pathogens through the production of proinflammatory cytokines and the activation of T cell responses. Autophagy, a pathway that sequesters intracellular material within double-walled vesicles for degradation by lysosomes, enhances Toll-like receptor-dependent activation, cytokine production, and antigen presenting cell function within RSV-infected DCs. Of interest, key proteins that orchestrate autophagosome formation are targeted by SIRT1, a NAD^+-dependent histone and protein deacetylase. SIRT1 impacts many areas of biology and pathophysiology, including immune function. However, the role of SIRT1 in DC biology and its subsequent impact on adaptive immunity has not been elucidated.

We have demonstrated that SIRT1 regulates DC activation and autophagy-mediated processes during RSV infection, and that the absence of SIRT1 activity alters the antiviral immune response through the regulation of innate cytokine production. Upon infection, SIRT1 inhibitor (EX-527)-treated DCs, *Sirt1* siRNA-treated DCs, or DCs from conditional knockout (*Sirt1*$^{f/f}$*-CD11c-Cre*$^+$) mice failed to upregulate autophagy and cytokine production, but retained the capacity to present antigen to T cells. Additionally, RSV infection of *Sirt1*$^{f/f}$*-CD11c-Cre*$^+$ mice resulted in altered lung and lymph node cytokine responses, leading to exacerbated pathology. Overall, these studies highlight the essential role of SIRT1-mediated DC cytokine production in fine-tuning the antiviral adaptive immune response, and establish SIRT1 as a promising therapeutic target for the prevention of severe RSV-induced lung disease.

CHAPTER 1:
Introduction

Human respiratory syncytial virus (RSV), a non-segmented negative-sense single-stranded enveloped RNA virus of the Paramyxoviridae family, is a ubiquitous human pathogen. It was first isolated from chimpanzees in the 1950s and subsequently recovered from severely ill infants with lower respiratory tract (LRT) disease.[1, 2] As the leading global cause of lower respiratory tract infection (LRTI) among infants and young children, RSV is also responsible for significant morbidity and mortality among the young, the elderly, the immunocompromised, and those with chronic respiratory diseases.[3-5] Treatment of RSV infection relies heavily on supportive care, and despite decades of research efforts, no effective pharmacologic therapies exist.[6] Even with the knowledge from extensive studies on epidemiology, clinical course, diagnostic techniques, and animal models, the immunobiology of severe RSV infection is not fully understood. Deeper questioning of this virus's effect on the immune system is highly warranted to bring about therapeutic strategies aimed at severe pathology prevention and viral vaccine development.

Respiratory Syncytial Virus (RSV)

Virology & mechanisms of immune evasion

RSV's RNA genome of 10 genes encodes 11 proteins, including structural and envelope proteins. The viral envelope contains three transmembrane proteins: fusion (F) glycoprotein, G glycoprotein (G), and a small hydrophobic protein (SH). The G protein mediates host cell attachment while the F protein is responsible for fusion, cell entry, and the formation of the characteristic multinucleated epithelial cells, or syncytia.[7] Only the F and G proteins induce neutralizing antibodies, thereby making these proteins the most important determinants of viral pathogenicity.[8, 9] Five structural proteins compromise the remainder of the viral genome: a large RNA polymerase protein (L), nucleoprotein (N), phosphoprotein

(P), matrix protein (M), two matrix protein variants from alternate reading frames called transcription processivity factors (M2-1 and M2-2,) and two non-structural proteins (NS2, NS2). The M proteins assemble underneath the envelope, while the L, M2, N, and P proteins associate with the single-stranded RNA (Figure 1.1).

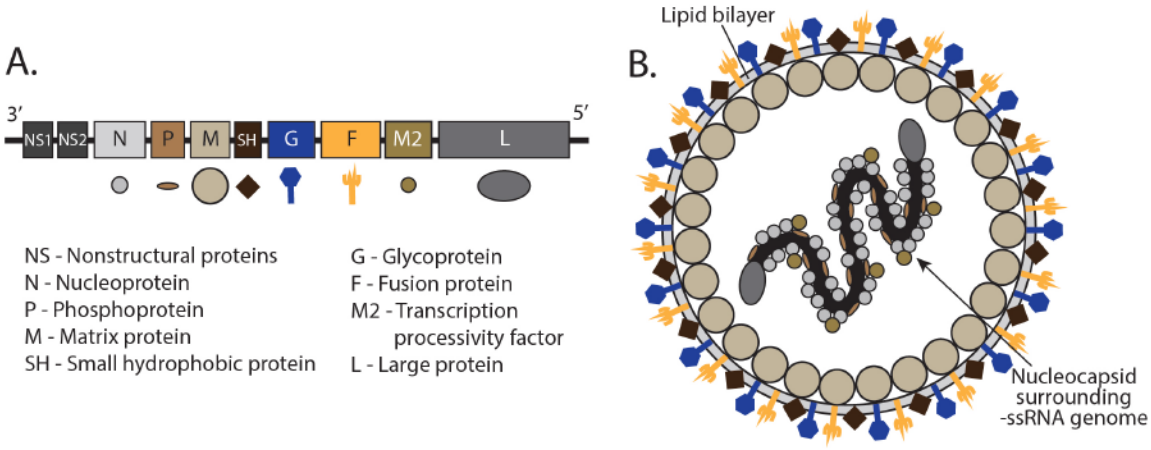


Figure 1.1: Human respiratory syncytial virus. ssRNA genome (A) and virion structure (B).[10, 11]

RSV-G binds glycosoaminoglycans present on the apical surface of target cells,[12, 13] while the F protein binds nucleolin, mediating the fusion to the host membrane and the release of nucleocapsid into the host cytoplasm.[14, 15] Upon entry, the L protein initiates transcription (3' to 5') of the negative-sense RNA.[16-18] Consequently, subgenomic mRNAs are produced, with relative protein abundance determined by proximity to the 3' end of the viral genomic RNA.[16] During virion assembly, the nucleocapsid localizes at the host's plasma membrane, which now expresses viral envelope proteins. New virions bud off of the apical surface of polarized alveolar epithelial cells, forming clusters of long, fragile filaments that extend from the cell surface.[19]

RSV primarily targets respiratory epithelial cells, particularly those that are ciliated, as well as the intraepithelial immune cells lining the airways.[20] In contrast to influenza virus, RSV induces scant cytopathology,[20-22] perhaps 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, such as the Bcl-2 family member myeloid cell leukemia-1.[23] Other probably anti-apoptotic mechanisms employed by RSV include p53 inhibition via Akt/Mdm2-mediated p53 degradation[24] and increased levels of prosurvival sphingolipid SP1 via enhanced ceramidase/sphingosine kinase activity.[23, 25] Moreover, it has been shown that

NS1, NS2, and SH RSV proteins facilitate viral replication, and thus higher viral titers, by suppressing premature apoptosis.[26]

RSV reinfections are common throughout life, suggesting that protective immunity is incomplete and short-lived.[7] This is likely in part due to the virus's numerous mechanisms to evade or sabotage the host immune response.[27] RSV-F protein binds Toll-like receptor 4 (TLR4), upregulating its surface expression and sensitizing respiratory epithelial cells to endotoxin.[28] Reports have shown an increase in the number of $TLR4^+$ peripheral blood monocytes in some infants with RSV bronchiolitis,[29] but the role of TLR4 *in vivo* is not clear.[30] RSV's NS1 and NS2 proteins have been shown to interfere with type I interferon (IFN) production, contributing to poor viral clearance and a skewed Th2 immune response.[9]

The G protein is heavily glycosylated, a modification that interferes with antibody recognition.[31, 32] RSV-G exists as both a full-length membrane-bound form and a truncated secreted form (RSV-Gs), the latter of which may act as a decoy for neutralizing antibodies.[27] In addition, the G protein contains a CX3C motif, which endows it with the ability to signal through the fractalkine CX3CR1 receptor and alter the chemotactic activity of leukocytes.[33] Whether this augments cellular recruitment to the lungs during RSV infection or inhibits the function of endogenous fractalkine is unclear.[9] Lastly, the conserved cysteine-rich region of RSV-Gs acts as a potent TLR antagonist *in vitro*, antagonizing TLR2, TLR4, and TLR9-mediated inflammatory cytokine production.[34]

Interestingly, studies suggest that RSV-G protein has greater potential than RSV-F protein to downregulate cellular responses. For example, sensitization of mice with recombinant vaccinia virus expressing the RSV G protein followed by subsequent RSV challenge primarily activated a Th2 response (allergy-like) with eosinophilia, while priming with RSV-F protein activated a Th1 response (antiviral) with lung inflammation.[35] Similarly, human RSV-G-specific T-cell lines produced interleukin (IL)-4 and IL-10 in response to RSV stimulation, whereas F-protein-specific T-cell lines produced Th1-dominated cytokines.[36]

The virology of RSV, in combination with the host's immune response and underlying health variables, contributes to the unique nature of RSV infection in the global human population. With no RSV vaccine available, it is vital to deeply ponder the

epidemiologic, environmental, genetic, and immunologic risk factors that predispose individuals to severe RSV infection.

Epidemiology & health burden

The single RSV serotype is classified according to the G protein subgroups A or B (RSV-A, RSV-B).[37, 38] As with other respiratory viral infections, the prevalence of each subgroup varies by region and season, with rates of viral infection and hospitalization peaking between mid-December and early February.[39, 40] No animal reservoirs exist, as humans are the only host for RSV, yet the reasons for the near absence of RSV between epidemics remain unclear.

RSV is a highly infectious, universal pathogen, such that every child experiences at least one RSV infection by the age of 2 years.[41] While infections usually pass in less than a week, they tend to be more severe in children aged 8 to 30 weeks.[42] It has been estimated that on an annual basis the virus causes about 34 million episodes of acute LRTI and nearly 4 million cases of severe acute LRTI requiring hospitalization.[4] Extrapolating from surveillance studies of laboratory-confirmed RSV infections to the entire US population, about two million children under 5 years of age require annual medical attention for RSV infection, with ~3% of them being hospitalized, another 25% of them being treated in emergency departments, and the remaining 73% being seen in pediatric practices.[7] Every year at least 66,000 deaths worldwide in children under 5 years of age can be attributed to severe RSV infection, most commonly in developing nations.[4]

Unfortunately, the total health burden of RSV disease is complicated by a strong relationship between infant hospitalization with RSV infection and the development of recurrent wheezing and allergic asthma later in life.[43-48] Studies involving Palivizumab, a monoclonal antibody to RSV-F protein that significantly reduces the severity of RSV bronchiolitis, further suggest a causal interaction between RSV infection and childhood asthma. Among cohorts of "high-sick" children, Palivizumab prevented hospitalization for RSV LRTI and decreased the incidence of less severe LRTI, thereby reducing the rate of physician-documented recurrent wheezing at the age of 3-4 years.[49] Similar findings have been reported where infants received prophylaxis with pooled immunoglobulins containing

high titers of RSV neutralizing antibodies.[50] Furthermore, data derived from mouse models of viral-induced chronic lung disease indicate lasting influences to the pulmonary immune milieu consistent with those observed in human asthmatics.[51] These complications of severe RSV infection suggest that the host antiviral immune response, as well as the immune-mediated alteration of the pulmonary environment, facilitate the development of chronic airway disease.

Unusually, RSV reinfection is common among young children, with most experiencing a decrease in symptom severity upon recurrent exposure.[52] While there is a low incidence of LRT involvement upon reinfection in older children and adults, the immunocompromised (e.g. transplant patients) and the elderly have a higher risk of developing severe LRTI.[3, 53] Likewise, RSV greatly exacerbates baseline pulmonary dysfunction in patients with conditions such as asthma and chronic obstructive pulmonary disease (COPD).[5] Overall, RSV infection contributes to extensive morbidity and mortality in these adult populations.

Clinical manifestation & diagnosis

RSV symptoms range from mild upper respiratory tract illness or otitis media to severe, life-threatening LRTI. The most common form of LRTI in RSV-infected infants is bronchiolitis, although pneumonia and croup are also observed. LRT signs, which lead to a clinical diagnosis of bronchiolitis, include tachypnea, hyperinflation, retractions of the intercostal muscles, and inspiratory crackles and expiratory wheezing on auscultation. High fever is uncommon, while apnea may be observed in very young and premature infants.[54]

Diagnosis of acute bronchiolitis is clinical, based on the patient's presenting respiratory signs and symptoms, possibly accompanied by lethargy, irritability, and poor feeding. While chest X-rays may show hyperinflation and patchy atelectasis, helping to distinguish bronchiolitis from pneumonia, this tool is reserved for cases of diagnostic uncertainty. Pulse oximetry is recommended for all patients reporting to the emergency department. Rapid antigen testing is routinely used for guiding cohort assignments, but its results rarely alter management decisions.[54] Antigen detection by immunofluorescence and viral culture are common for confirming RSV infection, but similar methods are not

available for all respiratory viruses. Hence, to investigate viral etiology for epidemiological studies, scientists use reverse transcription-polymerase chain reaction (RT-PCR) for RNA viruses and PCR for DNA viruses.[7]

Etiology of RSV hospitalization & severe infection

The risk factors for RSV-related hospitalizations and disease severity can be broken down into host-related and virus-related. From the host's perspective, well-established risk factors for hospitalization include pre-term birth, congenital heart or lung disease, acquired immunodeficiencies, interstitial lung disease, neuromuscular disease, liver disease, and inborn errors of metabolism.[7] In spite of the aforementioned risk factors, at least half of all infants that report to the hospital with severe RSV infection are otherwise healthy.[55, 56] Within this group, the most frequent and consistent risks include young age (< 6 weeks to < 6 months), male sex, presence of other children in the household, daycare attendance, tobacco exposure, lower family income, and lack of breast feeding.[7, 57] Conversely, only two protective factors have been identified: Breast-feeding[56, 58-61] and higher circulating levels of maternally-derived neutralizing antibodies to RSV.[62, 63]

Aside from these environmental and health risk factors that contribute to hospitalization rates, many studies have identified a multitude of genetic susceptibility factors for severe RSV disease. Concordance studies between Danish monozygotic and dizygotic twin pairs revealed a modest (~20%) genetic contribution to the risk of hospitalization with RSV.[64] Furthermore, studies on the ethnic background of RSV-infected US infants have shown that children of Native Alaskan or American Indian heritage are far more frequently hospitalized in comparison to the general US infant population,[65, 66] while African ancestry was found to be protective against severe bronchiolitis compared to European ancestry.[55, 67] Genetic risk factors are further supported by the finding that during the same season a single RSV strain can cause variable severity of disease among the infected, ranging from mild rhinitis to severe LRTI.[64]

Analysis of 347 single nucleotide polymorphisms (SNPs) from hospitalized RSV-infected children identified the risk of severe RSV disease was predominantly associated with innate immune genes, including *VDR* (vitamin D receptor), *IFNA5* (interferon α5), *NOS2A*

(inducible nitric oxide synthase), and *FCER1A* (high-affinity IgE receptor α-subunit).[68] Further studies identified additional immune gene SNPs associated with RSV disease severity in preterm infants, such as *IFNG* (interferon gamma; risk enhancing), *TGFBR1* (transforming growth factor beta receptor 1; protective), and *NFKBIA* (nuclear factor kappa-B 1A; protective).[69] Surprisingly, polymorphisms in *IL1RN* (IL-1 receptor antagonist) were protective among preterm infants, but disease enhancing among full-term infants.[69] Examination of the *TLR4* gene has identified the overrepresentation of two SNP variants among high-risk infants hospitalized with RSV compared to control infants,[70] though these findings are not always consistent.[68, 71] These data demonstrate that immunologically relevant receptor and signaling molecule genes contribute to host susceptibility to severe RSV LRTI.

Additionally, SNPs within the promoter- and coding-regions of critical cytokines may be associated with an increased risk of severe RSV disease. The role of Th2 cytokines, including IL-4, IL-5 and IL-13, in the immunopathology of human RSV infection is controversial since some studies have found an association between excessive Th2 cytokine secretion and disease severity,[72, 73] while others have reported little or no detection of Th2 cytokines.[74-76] Several studies reported overrepresentation of common SNPs within the *IL4* gene, IL-4 promoter, and the IL-4 receptor alpha chain, among RSV-infected hospitalized infants in comparison to healthy controls.[77-80] One well-studied locus on human chromosome 5 (5q31) has been identified as a potential modifier of RSV disease severity.[80] This locus contains a cluster of cytokine genes, including the Th2 cytokine genes *IL4*, *IL5*, and *IL13*, as well as IFN regulatory factor 1 (*IRF1*), granulocyte-macrophage colony-stimulating factor (*CSF2*), and T-cell transcription factor-7 (*TCF7*). The highest infant hospitalization odds-risk ratio involved several SNPs across the IL-4 and IL-13 genes, among patients with no known risk factors for RSV disease.[80] Future studies should investigate potential correlations between these haplotype-associated gene polymorphisms and RSV-induced cytokine production.

Lastly, SNPs within the genes of chemotactic cytokines have been studied in terms of risk for severe disease, since immune cells infiltrate the airways during RSV infection. For example, IL-8 attracts neutrophils, and the chemokines RANTES/CCL5 and MIP-1α/CCL3 recruit basophils, eosinophils, monocytes and T cells. While a polymorphism within

the *IL8* promoter region was found to be overrepresented in infants hospitalized with severe RSV LRTI,[81-84] subsequent studies did not find significant associations with known SNPs within the *IL8* and IL-8 receptor genes.[85, 86] However, evidence does support an increased risk among carriers of SNPs within *CCL5*[87-89] and its receptor, *CCR5*.[72]

Viral factors contributing to the risk of severe RSV disease include viral load, viral subgroup, and the presence of co-infection. There is some controversy over the extent viral load correlates with disease severity, but studies suggest it may be age stratified and dependent on other risk factors, such as co-infection with another respiratory virus (e.g. parainfluenza, influenza, adenovirus, or rhinovirus).[90, 91] Several human studies have shown RSV-A associated with higher disease severity compared to RSV-B, with the risk persisting after adjusting for age and other risk factors.[37, 38, 67] *In vitro* studies in primary airway epithelial cells (AECs) and epithelial cell lines demonstrate that "prototypic" RSV-A strains are better at inducing nuclear factor-κB (NF-κB) activation and thus pro-inflammatory cytokines (IL-6, IL-8) than RSV-B strains, supporting the clinical observations that RSV-A infections are more severe.[92] Other studies have yielded clear evidence that individual RSV-A isolates differ substantially in their infectivity, virulence, and immunopathogenicity.[93-97] Overall, these findings underline how viral characteristics interact with host susceptibility factors to dictate disease phenotype.

Taken together, the studies of risk factors contributing to RSV disease susceptibility, severity, and hospitalization highlight fascinating correlations with the RSV-induced immune response. However, most risk factor-disease relationships require investigation in much larger patient populations, so that significance can be attributed to the appropriate environmental, genetic, or viral risk factor involved in RSV pathology.

Treatment, prophylaxis, and vaccination

Management of acute bronchiolitis upon hospital admission largely consists of supportive care, such as nasal suction, nasogastric or intravenous fluids, supplemental oxygen, and nasogastric feeding. While it is common practice to administer bronchodilators (α and β adrenergics, anticholinergics, and nebulized epinephrine), there is no conclusive evidence that these positively impact disease outcome.[54] Likewise, there is a lack of evidence

for the use of inhaled or systemic corticosteroids in treating severe bronchiolitis.[98] One study showed that endogenous cortisol production during severe RSV bronchiolitis actually suppressed cytokines necessary for mediating antiviral responses,[99] suggesting that systemic corticosteroid treatment may not be advisable in severe RSV disease. Additionally, inhaled corticosteroids during the acute phase of RSV infection did not show a preventative effect on post-infection wheezing.[100]

The use of ribavirin, a FDA-approved antiviral agent used in nebulizer form to treat infants and children with severe bronchiolitis, is disputed. Meta-analysis indicates it may be effective in reducing duration of ventilation and length of hospitalization, but the studies have been too small in size and too variable to be conclusive.[101] Current AAP guidelines do not recommend ribavirin's routine use due to these uncertain studies, the potential health risk for caregivers (i.e. aerosolization of drug during administration), and the high cost, but reserve its use in high-risk infants with severe disease.[54]

Palivizumab is a monoclonal antibody (RSV-F protein) prophylaxis for serious RSV-induced LRTI in high-risk infants and young children. The FDA-approved dose is five monthly doses of 15 mg/kg body weight at the beginning of the regional RSV season. There is an international agreement that a full course of the antibody should be given to premature infants with a gestation age <32 weeks, but there is considerable variation in US recommendations for use in infants at the gestational age of 32-35 weeks. While Palivizumab is effective in infants <35 weeks of age,[102] providing such a prophylaxis to a population that comprises 3-5% of all annual births would be cost prohibitive. Thus, it is generally reserved for <32 week-old premature infants and those born with cardiopulmonary complications.[103]

Despite these imperfect treatments and prophylaxis for severe RSV infection, they are still achievements given the absence of a RSV vaccine. Early attempts at vaccine development vividly demonstrated just how much the host immune response contributes to RSV pathogenesis. The first formalin-inactivated (FI) RSV vaccine (1960s) not only failed to provide antibody protection, but also primed the infant participants for enhanced disease upon natural infection, such that 80% of the vaccinees required hospitalization and two even died.[62] Initially, it was hypothesized, given the link between RSV and Th2-polarized wheezing, that the FI-RSV vaccine had induced a Th2 immune response, leading to

eosinophilia and the upregulation of IL-4, IL-5, IL-13, and possibly IL-10.[104, 105] Re-examination of post-mortem histological specimens found few eosinophils, and instead reported immune complex deposition and neutrophilia in the lungs.[105, 106]

The amplification of RSV disease post-FI-RSV vaccine administration has been reproduced in mouse, cotton rat, primate, and bovine models.[107-110] Thus, numerous alternative approaches to vaccine development are being tested, including live attenuated cold-passaged and temperature-sensitive mutants,[111-113] recombinant RSV virus with deletions of one or more virus proteins,[114] recombinant RSV expressing host cytokines in order to boost vaccine responses,[115, 116] vectored vaccines, and single virus protein subunit or peptide vaccines.[117-119] Likewise, a variety of adjuvants are being tested for their abilities to magnify the immunogenicity of vaccine preparations and to prevent vaccine-enhanced Th2-skewing, some of which include CpG oligonucleotides and other TLR ligands.[120] To date, many of these vaccines and adjuvants have been shown to be highly protective in mice, but few have progressed to human testing. At the time of writing, 18 RSV vaccines, adjuvants, or combination therapies are currently in clinical trial (https://clinicaltrials.gov, search term: "RSV vaccine"). Therefore, a better understanding of how RSV interacts with the host, especially in terms of the aforementioned risk factors, must be achieved so that the road to RSV vaccine development is less replete with struggle.

Primary immune response to RSV (in mice & men)

Events during the first minutes and hours after viral infection are of paramount importance, not only in dictating the balance between viral replication and elimination but also in setting into motion the subsequent adaptive (acquired) immune response. In particular, cellular infiltrates and synthesized protein mediators must orchestrate a defense to clear the infection, while limiting injury to the surrounding healthy tissue. Knowledge of the early and late phases of RSV infection has been gained from animal studies and analysis of human respiratory secretions and autopsy specimens.

The most widely used animal models of RSV infection are inbred laboratory mouse strains, because of the ease of handling and housing, a wide variety of transgenic and knock-out models, and the availability of reagents. However, they are semi-permissive hosts,

requiring higher inoculum than humans to elicit symptomatic infection (10^5-10^7 vs. 1000 PFU (plaque forming units)).[121] Their lung anatomy is much simpler and the clinical signs of illness in mice are rather nonspecific, such as weight loss, lethargy, and ruffled fur. While acute RSV infection can cause airway obstruction and airway hyperreactivity (AHR)[122-125] as in human infants, certain strains, such as C57BL/6J mice, do not induce AHR.[93, 126] Peak viral load in the lungs of mice is seen 4-5 days post-infection (dpi) but becomes undetectable by plaque assay 8 dpi.[125, 127]

Histopathology

RSV-induced lung pathology is characterized by bronchiolitis, mucosal and submucosal edema, epithelial cell (EC) desquamation, mucus hypersecretion, as well as monocyte and granulocyte infiltration.[128] The ECs, mucin, and immune cells along with the edema contribute to the dangerous obstruction of small airways, especially in infants. While the sloughing of ECs is generally considered to reflect necrosis, markers of apoptosis are abundant in RSV-infected epithelium.[129, 130] The cellular infiltrate, consisting mostly of alveolar macrophages and recruited monocytes, is generally peribronchiolar and often extending into the alveoli, while neutrophils are localized to the submucosa.[128]

Detection of RSV by pattern recognition receptors (PRRs)

Host-viral interactions are initiated via host recognition of pathogen-associated molecular patterns (PAMPs). This recognition occurs through pattern recognition receptors (PRRs) that are expressed on a wide array of innate immune cells including alveolar epithelial cells (AECs), dendritic cells (DCs), macrophages, and neutrophils. Several PRR families, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs), are involved in viral detection. TLRs are present on the cell membrane and in endosomes, while RIG-I helicases and NLRs are intracellular microbial sensors. The engagement of PRRs by PAMPs results in the activation of multiple signaling pathways and transcription factors such as NFκB and members of the interferon regulatory factor (IRF) family, which regulate the expression of inflammatory, immune, and antiviral genes that facilitate viral eradication.[131]

Membrane-bound TLRs implicated in the detection of RSV include TLR2, TLR3, TLR4, and TLR7.[131] TLR2 is expressed in heterodimeric complexes with TLR1 and TLR6 on the cell surface of immune cells and AECs, and recognizes a complex array of proteins (bacterial and viral).[132-135] While relatively little is known about TLR2 in the context of RSV infection, knock mouse studies have shown that RSV-induced TLR2/6 signaling promotes the production of tumor necrosis factor alpha (TNFα), IL-6, CCL2, and CCL5.[136, 137] On the other hand, TLR4, which forms as a homodimer on cell surfaces, was the first PRR identified to play a role in RSV detection.[138] RSV-F protein ligation by TLR4/CD14 stimulates NFκB-mediated innate cytokine production.[138] In AECs, RSV-induced TLR4-signaling increases TLR4 surface expression and stimulates production of IL-6 and IL-8.[28, 139] Mice harboring a null mutation in the *Tlr4* gene failed to induce IL-6 production in response to RSV, and exhibited reduced NK cell trafficking and function, such as decreased IL-12 production and impaired viral clearance compared to controls.[138, 140] Two human gene polymorphisms within the extracellular domain of TLR4 have been correlated with increased risk of severe RSV bronchiolitis, especially among high-risk infants.[70, 141] Experiments using human bronchial ECs expressing one of the SNPs found reduced translocation efficiency of TLR4 to the cell surface, resulting in decreased NFκB-driven cytokine production and type I IFNs in response to stimulation.[142] Conversely, in another study, TLR4 expression on blood monocytes was positively correlated with disease severity.[29]

TLR3 and TLR7 are expressed within intracellular compartments such as endosomes, where they detect double-stranded (ds) or single-stranded (ss) viral RNA, respectively.[131] Upon ligation of the dsRNA RSV replication intermediate, TLR3 recruits the adaptor protein TRIF, which activates IRF-3/NFκB driven production of IFNβ and the chemokines CCL5, IL-8, and CXCL10.[143] RSV infection has been shown to upregulate TLR3 expression in human lung fibroblasts and epithelial cells.[143, 144] While TLR3 did not affect viral clearance in mouse models of RSV infection, it is important in modulating an antiviral Th1 immune response.[145] *Tlr3* knockout (KO) mice developed a Th2-biased immune response upon RSV challenge, resulting in increased IL-5 and IL-13 production, mucus secretion, and eosinophil infiltration into the airways.[145] Similarly, TLR7 detects ssRNA, thus activating NFκB and IRF-7 signaling in a MyD88-dependent manner to

mount host responses that minimize immunopathology. RSV-infected *Tlr7*-deficient mice experienced increased IL-4 production within the airways, in addition to IL-13 and IL-17, which promote AEC mucus secretion.[146, 147] Interestingly, DCs derived from *Tlr7*-deficient mice preferentially produced the Th17-promoting cytokine IL-23 at the expense of the Th1-promoting cytokine IL-12, likely causing the elevated Th17 response upon RSV infection.[146]

RLRs, expressed by cells such ECs and DCs, are intracellular PRRs that detect RSV-derived RNA upon direct cell fusion. Retinoic acid-inducible gene I (RIG-I) binds 5' triphosphate moieties on RNA, while melanoma differentiation-associated gene 5 (MDA-5) preferentially recognizes long, stable dsRNA.[131] These RLRs bind viral RNA with a conserved DEAD box RNA-binding helicase domain, followed by interaction of two N-terminal caspase-recruitment domains (CARDs) with the CARD domain of the mitochondrial membrane protein "IFNβ promoter stimulator 1" (IPS-1).[148] This interaction leads to dimer formation and subsequent activation of IKKα/β-dependent NFκB signaling and TBK-1-dependent phosphorylation of IRF-3 and IRF-7, which trigger transcription of type I IFNs.[149] RSV-infected *Ips1*-deficient mice, which are unable to signal via RLRs, produced very little IFNβ, and exhibited a Th1 phenotype with heightened airway neutrophilia and reduced viral clearance.[150] RSV bronchiolitis has been shown to upregulate gene expression of several PRRs in infants, especially *RIGI*, compared to those with non-RSV-induced bronchiolitis. Additionally, these RSV-infected infants demonstrated a positive correlation between *RIGI* mRNA levels and viral load.[151] Finally, RSV NS1/2 proteins antagonize RLR signaling by blocking the interaction of RIG-I with IPS-1.[152, 153]

NLRs are specialized intracellular cytoplasmic sensors that recognize a wide array of different PAMPS, including intracellular bacterial cell products and bacterial or viral nucleic acids.[131] One NLR implicated in RSV detection is NLRP3, yet the exact mechanism of interaction is unknown. Upon activation, NLRP3 recruits the adaptor protein ASC, forming large multi-protein complexes called inflammasomes, and leading to the processing and activation of pro-IL-1β and pro-IL-18 through the cysteine protease caspase-1.[154-156] In one study, RSV infection in mouse bone marrow-derived macrophages induced TLR2/MyD88-dependent upregulation of pro-*Il1b* and *Nlrp3* genes.[157] When another NLR, NOD2, recognizes RSV RNA, it rapidly translocates to the mitochondrial surface to interact with

IPS-1, modulating IRF-3/NFκB-dependent IFN production.[131] The importance of NOD2 in RSV detection is evidenced by impaired viral clearance, increased weight loss, increased proinflammatory cytokine and chemokine production, and greater lung immunopathology in RSV-infected *Nod2*-deficient mice.[158] All together, these studies on TLRs, RLRs, and NODs illustrate the importance of PRRs in early detection of RSV and the development of robust innate immune responses to infection.

Innate cytokine production & immune cell recruitment

Upon RSV infection, AECs signal their distress by secreting a wide variety of proteins, most of which have been detected in lung tissue or bronchoalveolar lavage (BAL) fluid of RSV-infected mice.[7] These cytokines and chemokines include: 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 IFNα/β.[121, 159, 160] In addition, resident alveolar macrophages are a key source of CCL3, CCL5, TNFα, IL-6 and IFNα in mouse models of RSV infection.[161-163] Proinflammatory cytokines, such as IL-1, IL-6, and TNFα upregulate the expression of adhesion molecules, thereby facilitating the retention of recruited immune cells and promoting their activation. Levels of these innate proteins increase early during mouse infection, with BAL concentrations peaking at 24-48 hours post-infection.[124, 125] Concentrations of IL-6 in the BAL positively correlate with viral load and disease severity, suggesting it may promote pathology at the later stages of infection.[125] Controversy also surrounds the role of TNFα in the clearance of RSV, as there are indications that it contributes to viral clearance during the early stages of infection but promotes lung immunopathology later on.[164-166]

During the initial phase of RSV infection, the rich, rapidly induced mixture of innate proteins attracts an army of immune cells into the airways. In mice, neutrophils are recruited as early as 24 hours post-infection (hpi),[95, 116, 122, 125, 167-169] although the kinetics and magnitude of the response are RSV- and mouse-dependent.[170] Natural killer (NK) cells play a critical role in viral clearance during the early phase of RSV infection, with levels rising ~2 dpi, peaking ~3-4 dpi, and becoming undetectable by 8 dpi in mice.[104, 116, 171, 172] Macrophages are always the major cell type in the BAL of mice, and may be further

upregulated by RSV infection, but the timing of recruitment varies by study.[169] In contrast, eosinophils generally represent a minor yet significant component of recruited immune cells in response to RSV,[168, 173-175] although some studies report no significant differences in their numbers between RSV-infected and control animals.[20, 176, 177]

While there is an early transient rise in neutrophils in RSV-infected mice, their recruitment is a predominant aspect of severe human RSV infection, making up 75-85% of the BAL.[178, 179] In a study on RSV-infected, mechanically ventilated infants, neutrophil numbers rose within the first few days of intubation and declined thereafter.[178] However, there were lower numbers of recruited neutrophils in the BAL fluid of pre-term infants in comparison to full-term infants. More variability has been seen in monocyte and lymphocyte proportions, perhaps due to differences in the time of sampling relative to the onset of symptoms.[178, 179] Eosinophils are detectable (<1% of BAL) in a minority of RSV-infected infants, although one study reported a subset of infants exhibiting asthma-attack-like characteristics, with ~3% eosinophils in BAL fluid.[180]

In RSV-infected mice, $CD4^+$ T "helper" lymphocyte recruitment begins early and plateaus ~4 dpi, while cytotoxic $CD8^+$ T cell numbers rise sharply around day 4.[104, 116, 161, 169] Numbers of both T cell subsets begin to decline ~8 dpi, but remain elevated in the lung through 20 dpi.[7] Lymphocyte recruitment coincides with a second peak in the production of certain chemokines, including eotaxin, CCL3, and CCL5.[124, 125, 160] At the peak of inflammation ~7 dpi, a dense peribronchiolar and perivascular infiltrate of macrophages, lymphocytes, and some neutrophils is present.[124, 181, 182] While T cells constitute a small fraction of BAL cells from severe RSV-infected infants (~2%), a significant increase in RSV-specific $CD8^+$ T cells does occur in comparison to uninfected controls.[179, 183] Interestingly, significant numbers of $CD8^+$ T cells were reported in the alveolar infiltrate of an infant with non-fatal RSV,[128] suggesting a protective function of these cells, yet a second study of 9 fatal cases of RSV revealed a near absence of $CD4^+$ and $CD8^+$ T cells on autopsy.[184]

Impressive numbers of dendritic cells (DCs) are recruited to respiratory mucosal sites of both RSV-infected mice and humans. An increase of monocyte-derived, "conventional" DCs (cDCs) and plasmacytoid DCs (pDCs) occurs in the lungs and lung-draining lymph nodes (LDLN) of RSV-infected mice.[185-187] Similarly, a large influx of DCs is noted in nasal

washes of RSV-infected infants, with decreases in both DC subsets in peripheral blood compared to healthy controls.[188, 189] These antigen-presenting cells (APCs) play an indispensable role in educating T cell maturation and differentiation, and in dictating the resultant immune environment within the lungs. However, under certain circumstances, DCs may be responsible for the inappropriate induction of aberrant Th2-skewed responses to RSV, implicating these APCs as unfortunate mediators of RSV immunopathology.

Pulmonary DCs: inducers of innate & adaptive immunity

The lungs are continuously exposed to foreign threats, such as microbes, organic and inorganic dusts, man-made pollutants, as well as invading pathogens. As a result, a network of DCs serves to recognize these foreigners, promote immune tolerance to innocuous particles, and activate the adaptive immune response in the case of pathogens. While AECs and macrophages express PRRs, and can direct innate immunity through the production of inflammatory cytokines, DCs are special in that they transport pathogen-derived antigens to LDLN.[190] Once here, these sentinel DCs stimulate naïve and RSV-reactive memory T cells through antigen-presentation, co-stimulation, and cytokine production. Effector $CD4^+$ and $CD8^+$ T cells then migrate to the lungs and clear virally-infected cells.[191] Thus, pulmonary DCs serve both the innate and adaptive arms of immunity.

At steady state, three distinct DC subsets reside in the lungs, all of which perform unique tasks upon viral infection. $CD11c^{high}$ $CD103^+$ $Langerin^+$ intraepithelial DCs, which express the E-cadherin-binding integrin CD103 in mice ($CD103^+$ DCs), intercalate with the epithelial lining of the respiratory tract and project their cellular processes into the airway lumen. $CD11c^{high}$ $B220^+$ $CD11b^+$ DCs, expressing the integrin CD11b ($CD11b^+$ DCs), reside immediately below the basement membrane in the lamina propria. $CD11b^+$ and $CD103^+$ DCs are characterized as monocyte-derived/"conventional" DCs (cDCs), and correspond to the mDC1 ($CD11c^+$ $CD1c^+$) and mDC2 ($CD11c^+$ $CD141^+$) human subsets, respectively. Thirdly, $CD11c^{dim}$ $CD11b^-$ $SiglecH^+$ plasmacytoid DCs (pDCs) are found at steady state conditions, though their precise anatomical location is unknown. When the lung is challenged with a foreign invader, such as RSV, additional $CD11b^+$ monocyte-derived cDCs (inflammatory DCs) are recruited into the conducting airways and lung parenchyma.

These DCs are characterized by the surface expression of CD11b, CD11c, Fc epsilon receptor (FcεRI), and the transiently expressed monocytic marker Ly6C.[191]

DCs reside in a functionally immature state in the periphery of the lung, but are poised to detect PAMPs such as viral nucleic acids and proteins. All lung DC subsets are capable of being infected by RSV *in vitro*,[192, 193] which results in their rapid maturation and the upregulation of both MHC class proteins and the co-stimulatory molecules CD40, CD80, CD83 and CD86.[185, 194] UV-inactivated virus failed to stimulate DC maturation in several *in vitro* studies,[195, 196] suggesting that a replicative virus is necessary for complete activation. Once infected, human monocyte-derived CD11b$^+$ DCs produce the pro-inflammatory cytokines IL-1β, IL-6, IL-12, TNFα, and IFNγ, and chemokines such as CCL2, CCL3, CCL5, CXCL8, and CXCL10.[197, 198] Additionally, RSV-infected mouse bone marrow-derived DCs (BMDCs) produce IFNα and IFNβ, which are required for the upregulation of co-stimulatory molecules.[199] Both human and mouse pDCs produce high levels of IFNα following *in vitro* RSV infection. [193, 196]

Upon RSV infection, the specialized immune functions of each DC subtype likely originate from maturation-induced differential PRR expression, surface protein expression, antigen processing, and T cell stimulation. The upregulation of surface expression of chemokine receptors CCR6 (ligands: CCL20, β-defensin) and CCR7 (ligands: CCL19, CCL21) on cDCs enables their transport of viral RNA and protein to LDLN for T cell presentation.[191, 200] Migration kinetics differ by cDC subset, as numbers of CD103$^+$ DCs within the lungs decrease rapidly in the first 24 hpi, while the influx of inflammatory DCs results in an increased number of CD11b$^+$ DCs in the lungs by 7 dpi.[201] On the other hand, pDC numbers peak by 3 dpi and decrease below baseline by 8 dpi.[185] CD103$^+$ DCs are particularly efficient at the uptake of apoptotic epithelial cells, given their intimate proximity to the epithelium, and aided by their selective expression of TLR3, CD36, and the C-type lectin Clec9A.[202] These migratory CD103$^+$ DCs are more potent in stimulating CD8$^+$ T cells through cross-presentation of antigens, natural (viruses)[202] and inorganic (labeled beads).[203] In contrast, CD11b$^+$ DCs selectively express TLR2 and TLR7,[202] and are major producers of chemokines that attract effector cells to the lungs during infection.[204, 205] CD11b$^+$ DCs preferentially activate CD4$^+$ T cells in the context of severe influenza infection[206] whereas

directly infected CD11b$^+$ DCs indicate proficiency in priming CD8$^+$ T cell responses.[207, 208] Less is known about DC specialization during RSV infection, although *ex vivo* co-culture experiments suggest that RSV-infected CD103$^+$ DCs and CD11b$^+$ DCs process RSV antigen equally as well, and are capable of stimulating both CD8$^+$ and CD4$^+$ T cells.[201]

Despite the induction of DC maturation and the production of inflammatory innate cytokines, RSV reduces the capacity of DCs to stimulate naïve T cells and induce their proliferation.[185, 193, 195, 209] In one model, suppression of T cell activation is mediated by an unidentified soluble factor produced by RSV-infected DCs, in a dose-dependent manner, independently of IL-10, TGFβ, or T regulatory cells (Tregs).[195, 210] This effect may be due to ligation of a combination of receptors to IFNα, IL-10 and IL-28.[210] In a second model, RSV infection of DCs disrupts proper immune synapse formation between DCs and naïve T cells, such that T cells fail to polarize their Golgi to the cell surface, resulting in impaired T cell stimulation and proliferation.[209] Studies have shown that these T cells subsequently entered an anergic state, becoming nonresponsive to proliferation signals such as IL-2 and anti-CD3 antibody treatment and producing decreased levels of IL-2, IL-4, IFNγ, and TNFα.[15, 195, 210] This phenomenon was only observed in co-cultures where the DCs were infected with replication-competent virus, as naïve T cells cultured with DCs incubated with UV-irradiated RSV showed no proliferation defects.[15] Similarly, memory T cells stimulated with RSV-infected DCs showed no defects in effector cytokine production or proliferation, demonstrating that RSV only interferes with naïve T cell stimulation.[211] Thus, perhaps a defect in the DC-T cell interaction contributes to less robust primary responses to RSV, increasing the likelihood of severe LRTI.

Immaturity of the infant immune system

While the immune system of neonates and young infants is capable of producing adult-like responses under certain conditions, most often there are quantitative or functional deficiencies in their innate and adaptive immune responses. Macrophage cytokine production is often attenuated in neonates and children as compared to adults, in part due to diminished expression or upregulation of PRRs. Neonatal DCs are reduced in frequency and differ in subset distribution, compared to adult DCs. These DCs show signs of poor antigen

presentation and T cell stimulation, because of decreased expression of MHC molecules, co-stimulatory molecules, and inflammatory cytokines, particularly IL-12p70.[212]

Interestingly, neonatal T cells display extraordinary plasticity, ranging from unresponsive to stimuli that normally elicit strong adult T cell activity to highly reactive under the appropriate stimuli. For the most part, however, $CD4^+$ T cells are diminished in their capacity to produce both Th1 and Th2 cytokines especially IFNγ, which does not reach adult levels until around adolescence.[213-215] However, Th2 skewing is not clearly shown in human infections despite this low IFNγ production,[212] although it is observed in neonatal mouse and human responses to environmental allergens.[216] Overall, neonates are capable of mature cytotoxic T lymphocytes (CTLs) responses to virus.[212]

Neonatal B cell antibody production is often delayed in onset, decreased in peak titers, and diminished in duration.[217, 218] Additionally, infant B cells undergo little somatic hypermutation after antigen encounter,[219] resulting in an antibody repertoire that displays lower affinity and decreased heterogeneity.[212] After about the age of 2 years, most severe RSV LRTI is restricted to the immunocompromised, highlighting the immaturity of the immune system as a key factor in the frequency of LRTI in infants during primary RSV infection.

RSV-induced protective vs. pathologic immunity

Induction of antiviral immunity is a double-edge sword. On one side, a virus, particularly if cytopathogenic, must be rapidly cleared. Yet on the flip side, exuberant inflammatory immune responses can damage tissue, leading to systemic illness or local organ damage. Lung DCs can set in motion protective or pathologic RSV-induced immune responses depending on the cytokines they produce and their antigen-presenting interactions with T cells. The aforementioned DC subsets must work in synergy to promote efficient, swift viral clearance with minimal injury to by-standing tissue. Alas, studies point out pathological roles for certain DC subsets and T cells, highlighting the complexity of the immune response to RSV infection.

Although pDCs are poor APCs,[196] studies suggest that they are critical sources of IFNα, pro-inflammatory cytokines, and chemokines upon exposure to RSV. RSV-infected human pDCs isolated from peripheral blood secrete innate cytokines and large amounts of

IFNα.[193] In RSV-infected mice, a rise in pDC numbers appears to be protective, while depletion of pDCs prior to RSV infection results in increased viral replication, prolonged AHR, enhanced lung inflammation, and mucus hypersecretion.[185, 196] Additionally, T cells restimulated with anti-CD3 from infected, pDC-depleted mice produced increased mRNA and protein levels of Th2-associated cytokines IL-4, IL-5, and IL-13, as well as the Th1 cytokine IFNγ.[185, 196] This suggests that pDCs may play a role in regulating Th2-associated responses. However, as poor stimulators of T-cell proliferation and their specialized production of IFNα, pDCs seem best oriented to an early antiviral response than toward the induction of an adaptive during RSV infection.

As pulmonary cDCs are better equipped at stimulating $CD4^+$ and $CD8^+$ T cells during RSV infection, their role in mounting effective antiviral Th1 responses is key to successful viral clearance.[190] Viral reduction and the eventual eradication of RSV infection depends on the actions of activated T cells, however it is evident that both T cell subsets contribute to immunopathology.[182 220, 221] Clinical illness was significantly reduced after the depletion of $CD4^+$ or $CD8^+$ T cells during RSV infection in mice, and was essentially absent after depletion of both T cell subsets.[182] In another study, transfer of RSV-specific cytotoxic T lymphocytes (CTLs) to RSV-infected animals resulted in rapid, yet dose-dependent viral clearance, but led to the development of increased lung pathology.[220, 221] Interestingly, while passive transfer of both $CD4^+$ and $CD8^+$ T cells enhanced neutrophil efflux from lungs of infected mice, transfer of only $CD4^+$ T cells was associated with pronounced eosinophilia.[220] These data support the dual roles of T cells in viral clearance and pulmonary damage during RSV infection.

Alas, evidence suggests that some cDC subsets may contribute to the production of Th2 responses and lung immunopathology during RSV infection in mice. Mouse and human infant studies note an influx of monocyte-derived $CD11b^+$ cDCs into the airways and LDLN during RSV infection,[185, 189, 199] which may contribute to overall inflammation and AHR in both RSV and subsequent allergen challenges.[187, 205, 222] Blocking migration of $CD11b^+$ cDCs to the lungs or LDLN protected mice against RSV-induced immunopathology, resulting in more robust Th1 responses and enhanced viral clearance[200, 223] Similarly, the addition of monocyte-derived DCs increased Th2 responses and lung

pathology RSV-infected mice.[223-225] Thus, the presence and relative number of cDCs within the lungs may direct pathological responses to RSV.

Given the dual capacity of DCs to promote and hinder antiviral innate and adaptive immune responses during RSV infection, questions emerge surrounding the intracellular mechanisms responsible for dictating DC function. From transporting viral antigen to LDLN, to the production of innate cytokines, DCs depend on RSV recognition via TLRs. One process that facilitates delivery of antigen to the endosomal TLRs (TLR3, TLR7) is macroautophagy, whereby cytosolic contents are enveloped in a double-walled membrane and delivered to endosomes. By utilizing a conserved pathway involved in cellular equilibrium to efficiently deliver intracellular pathogen-derived nucleic acids and proteins to endosomal compartments, DCs can promptly produce the necessary cytokines and stimulate reactive T cells via MHC-II presentation. Ultimately, DCs direct effective viral clearance, and reestablish immune system homeostasis.

Autophagy as an antiviral immune defense

Autophagy is an ancient intracellular membrane trafficking pathway whereby cytoplasmic material is sequestered within double-walled vesicles, which degrade upon fusion with lysosomes. Autophagy evolved as a mechanism by which unicellular organisms could survive periods of nutrient scarcity by reabsorbing macromolecules from autophagocytosed organelles and proteins.[226] Overall, autophagy maintains cellular metabolic equilibrium and promotes cell survival during physiological (aging, differentiation) and pathological (infection, degeneration, cancer) stress conditions.[227] Specifically, the molecular machinery of autophagosome formation plays critical roles in innate immunity, including the clearance of cytoplasmic pathogens, delivery of viral antigen to endosomal TLRs, and the loading of antigen onto MHC molecules for T-cell presentation.[228-230] Likewise, autophagy modulates immune cell function and inflammatory responses.[231] Lastly, signaling sensors of endoplasmic reticulum (ER) stress, which activate the unfolded protein response (UPR) during pathogenic insult, can also activate autophagic flux in an attempt to restore cellular homeostasis via removal of damaged organelles (ER-phagy, mitophagy) and unfolded/misfolded proteins.[232] Collectively, the data suggest important immunomodulatory

roles for autophagy during viral infection, whereby it promotes innate immune responses, such as APC function in DCs,[233] as well as adaptive immune responses, such as T cell development and activation.[234]

Autophagosomes: initiation to maturation

The process of autophagosome formation is highly conserved among eukaryotic organisms, and the pathway was first elucidated in yeast studies.[226] A family of autophagy-related (*Atg*) genes orchestrates the initiation, elongation/closure, and maturation of autophagosomes (Figure 1.2). Autophagy is initiated during starvation upon the inhibition of the mammalian target of rapamycin (mTOR) and the activation of AMP-activated protein kinase (AMPK), which phosphorylates Atg1/ULK1 (yeast/human) and causes the translocation of the Atg1/ULK1 complex to the ER.[235, 236] While the primary source of autophagosomal membrane is thought to be the ER, some studies suggest that the membrane precursor can be from the mitochondria, the Golgi apparatus, the nuclear membrane, or even the plasma membrane.[237] The phosphorylated Atg1/ULK1 complex, serving as an initiation scaffold, 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, Atg14/ATG14L, and Atg6/Beclin-1.[235, 238] The VPS34-PI3K complex then produces phosphatidylinositol-3-phosphate (PIP3), which recruits DFCP1 and Atg18/WIPI family proteins that initiate the extension of source membrane into pre-autophagosomal structures termed omegasomes.[239, 240]

The elongation and closure of this double-walled membrane is dependent on two ubiquitin-like conjugation systems. One involves creation of the ATG5-ATG12 conjugate through actions of the E1-like ATG7 and the E2-like ATG10. This conjugate then forms a 2:2:2 complex with ATG16L1 on the outer membrane of autophagic precursors.[241, 242] Since the ATG5-ATG5-ATG16L1 complex dissociates from the membrane upon closure, its protein members are frequently used as markers for early autophagosome formation.[243] The second conjugation system, which requires ATG7 and the E2-like ATG3, cleaves and conjugates Atg8/LC3 with phosphatidylethanolamine (PE). The PE-conjugated Atg8 homologs, which in mammals are LC3A/B/C, GABARAP, GABARAPL1/2/3 (hereafter

referred to as LC3), are incorporated into the inner and outer membranes of the developing autophagosome.[243] As LC3-II (conjugated LC3) stably associates with completed autophagosomes, it is commonly used in microscopic analysis of autophagy progression.[244] Finally, the closed autophagosome matures upon fusion with acidic lysosomal compartments via the recruitment of the SNARE protein Syntaxin 17.[245] The hydrolase-rich environment within the now-formed autolysosome degrades the autophagosome contents, as well as the inner autophagosomal membrane.[226, 245, 246]

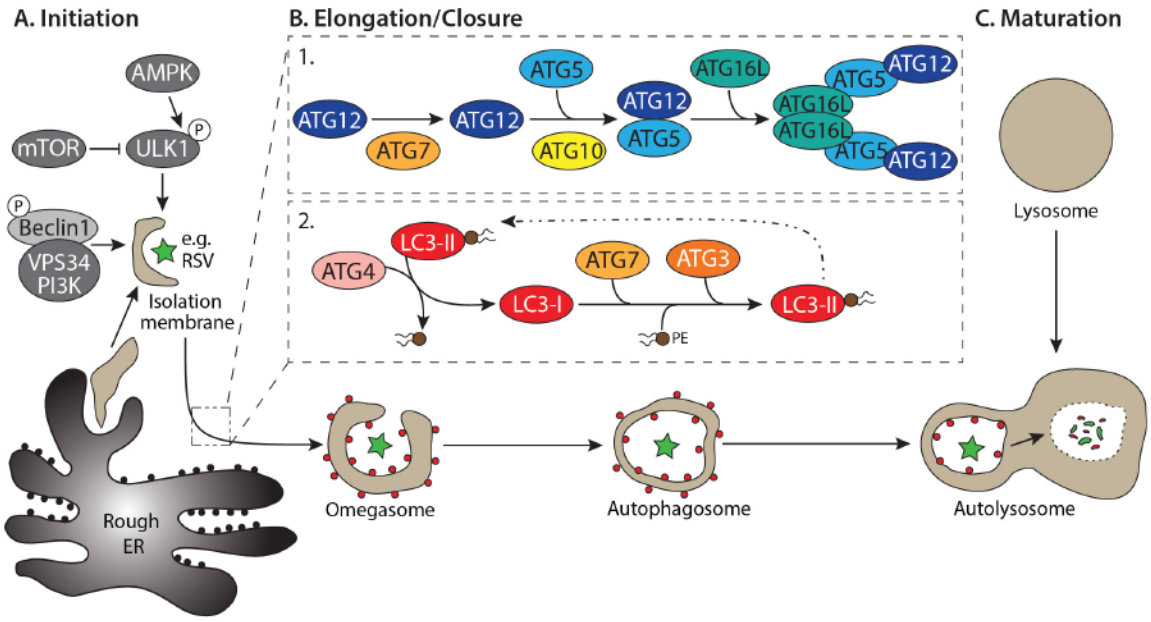


Figure 1.2: The life cycle of an autophagosome. (A) mTOR and AMPK regulate autophagy induction in mammals. In the presence of a stimulus, such as nutrient starvation or pathogen infection, ULK1 is phosphorylated and activates the VPS34-PI3K complex, which contains Beclin-1. These activated autophagy proteins associate with precursor membrane, often from the ER, to form an isolation membrane around general cytoplasmic content or cargo targeted to the phagophore by SLRs. (B) Elongation and shaping of the autophagosome are controlled by two ubiquitin-like conjugation systems producing: (1) ATG5/12/16L complexes and (2) PE-conjugated LC3-II, which is incorporated into both inner and outer autophagosomal membranes. Upon autophagosome closure, ATG5/12/16L and LC3 (delipidated by ATG4) are recycled. (C) Autophagic cargo, along with the inner membrane, is degraded upon maturation as the autophagosome fuses with an acidified lysosome.[228, 247]

Involvement of autophagy in cellular homeostasis

The physiologic functions of autophagy include: providing a cell-autonomous source of macromolecules and energy during times of cellular metabolic crisis or nutritional deprivation, prevention of cell death and senescence due to the accumulation of faulty

organelles and large protein aggregates,[248] and serving as a cell death modality.[249] All cells rely on constitutive autophagy to carry out basal housekeeping, including removal of organelles, such as depolarized mitochondria, damaged from regular wear and tear.[250] Autophagy is also necessary for normal development, as in the case of hematopoietic stem cell (HSC) maintenance and function.[251] However, when these day-to-day activities do not proceed as a result of dysregulated or dysfunctional autophagy, disease phenotypes emerge.[252] In addition, studies have found intersections between autophagy and immunity.[252]

Upon nutrient starvation, several events need to occur before autophagosome initiation can proceed. In the resting state, TAK1-binding proteins (TABs) 2 and 3 bind Beclin-1 and repress its activity.[253, 254] However, during starvation, ULK1-mediated activation of VPS34-PI3K is accomplished by the phosphorylation and release of Beclin-1 from its TAB inhibitors. BECN1-regulated autophagy protein 1 (AMBRA1), also activated by ULK1, along with Beclin-1, recruits E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6). TRAF6 then ubiquitinates and stabilizes Beclin-1 and ULK1,[255, 256] enabling the progression of autophagosome formation.

The requirement for autophagy in nutrient homeostasis is dramatically recognized in *Atg5*$^{-/-}$ or *Atg7*$^{-/-}$ neonatal mice. Although pups are born with few physical defects and in predicted Mendelian ratios, these autophagy-defective mice die within 24 hours after birth.[257, 258] Force-feeding can prolong survival, and analysis of metabolites confirms that these neonates suffer from systemic amino-acid deficiency and decreased glucose levels.[257, 258] Interestingly, in cultured wild-type (WT) hepatocytes, protein degradation increases by 3% total protein/hour upon starvation, with most of the increase attributable to autophagy.[257, 259] Therefore, under periods of acute starvation, autophagy is an indispensable stress response capable of temporarily restoring energy balance until nutrients are once again plentiful.

Sensing nutritional deficiency due to competition by microbial invasion is likely one ancestral danger signal that eukaryotes used to detect and eliminate pathogens through autophagy. In support of this concept, signaling downstream of amino acid starvation has been associated with antimicrobial autophagy in response to bacterial[260] and viral infection[261] in modern day immune cells. Other immune-sensing systems, such as PRR signaling upon recognition of PAMPs and damage-associated molecular pattern (DAMPs), also integrate

with autophagy. For example, TLR4 signaling leads to ubiquitylation of Atg1/Beclin-1 by the TRAF6,[262] releasing Beclin-1 from its inhibitor Bcl-2. TRAF6 also activates ULK1 through ubiquitylation[255] and thereby controls two key pathways that lead to autophagy. DAMPs, such as DNA complexes,[263] ATP,[264] and high-mobility group box 1 protein (HMGB1),[265] also activate autophagy. HMGB1 de-represses Beclin-1 by displacing Bcl-2, and can also activate autophagy extracellularly by interacting with its cell surface receptor RAGE (receptor for advanced glycation end products).[265] Signaling through the IL-1 receptor[266] and the IFNγ receptor[267-269] by IL-1 and IFNγ also trigger autophagy in macrophages through interaction with TRAF6 or Beclin-1, respectively. In contrast, the Th2 cytokines IL-4, IL-6, IL-10, and IL-13 can inhibit autophagy under certain circumstances.[268, 270, 271] These examples illustrate the complex integration of autophagy with PRR and cytokine receptor signaling.

Role of autophagy in human disease

Autophagy and autophagy genes have been implicated in a broad spectrum of human health issues including neurodegeneration,[272-274] cancer,[275, 276] and inflammation and immunity.[241] Two groups have identified novel mutations in *WDR45/WIP14*, a mammalian homologue of yeast Atg18, in patients with SENDA (static encephalopathy of childhood with neurodegeneration in adulthood).[277, 278] In lymphoblastoid cell lines derived from SENDA patients, researchers noted a severe reduction in WIP14 protein expression and the accumulation of aberrant autophagic $ATG9A^{+}$ $LC3^{+}$ (early stage) structures.[278] Amongst the many genes implicated in Parkinson's disease pathogenesis, a progressive neurodegenerative disorder, *PARK2/Parkin* and *PARK6/PINK1* lead to autosomal recessive or spontaneous juvenile-onset Parkinson's disease.[279, 280] PINK1 is a mitochondria-associated protein kinase that acts upstream of Parkin, an E3 ligase implicated in selective autophagy of mitochondria, or mitophagy.[281, 282] Consistent with this finding, excessive mitochondrial damage has been linked to Parkinson's disease,[283] suggesting that at least some forms of Parkinson's disease are caused by the lack of autophagy to clear these accumulated, defective organelles.

A connection between autophagy and cancer has long been proposed. Early on in cancer development, autophagy likely plays a preventive role, but once a tumor develops, the

cancer cells usurp autophagy for their own cytoprotection.[284, 285] A key role for autophagy in controlling the unregulated cell growth of tumor development has been shown through studies of *Atg1/BECN1*. The protein Atg1/Beclin-1 interacts with the anti-apoptotic protein Bcl-2, which prevents Bax-dependent release of mitochondrial cytochrome c.[286] Patients with a monoallelic deletion of *BECN1* have an increased predisposition for human breast, ovarian, prostate, and colorectal cancers, as well as a poorer prognosis.[252] Mutations in other autophagy proteins, such as ATG5 and UVRAG, are also correlated with the development of human cancers.[252]

Finally, given the evidence in support of cells applying autophagic machinery to promote antimicrobial responses, it is of no surprise that immune-mediated diseases have also been associated with defective autophagy function. Genome-wide association studies (GWAS) on non-synonymous SNPs have linked *ATG16L1* variants with susceptibility to Crohn's disease,[287, 288] a major type of inflammatory bowel disease that can affect any portion of the digestive tract. The ATG16L1 protein possesses a C-terminal WD repeat domain, within which or immediately upstream lies the Crohn's disease-associated mutation, T300A (Ala197Thr). However, studies have shown that this WD domain is not essential for ATG16L1's autophagic activity.[241, 289, 290] While the exact contribution of the *ATG16L1* T300A mutation to human Crohn's disease pathogenesis has yet to be clarified, *Atg16l1* mutant mouse studies suggest some possibilities. *Atg16l1*-deficient macrophages produced increased levels of the inflammatory cytokine IL-1β and IL-18 upon lipopolysaccharide (LPS) stimulation.[290] On the other hand, *Atg16l1* hypomorphic mice exhibited aberrant granule formation and ER stress in Paneth cells, which produce antimicrobial products and constitute a cardinal component of the intestinal stem cell niche.[291] Another report stated that ATG16L1 possesses an immunosuppressive role during intestinal bacterial infection.[292] Apart from ATG16L, other autophagy-related proteins, including IRGM (immunity-related GTPase M) protein,[293, 294] NOD2,[295, 296] autophagy-targeting factor SMURF1,[297] and ULK1[298] are reported to contribute to Crohn's disease. However, as some of these proteins hold other biological roles outside of promoting autophagy, it remains uncertain whether they relate to Crohn's disease pathogenesis via autophagy modulation.[252] Interestingly, population genetic analyses have also linked an *IRGM* gene variant (IRGM-261T) with

susceptibility to tuberculosis[299] and *IRGM* polymorphisms to systemic lupus erythematous (SLE).[300] Moreover, GWASs have linked *ATG5* variants with the development of asthma[301, 302] and a greater risk of SLE.[303, 304] Thus, autophagy shows clinical relevance as a result of various genetic connections to neurodegenerative, cancerous, immunological, and inflammatory disorders.

Host autophagy during infection by intracellular pathogens

The antimicrobial functions of autophagy provide a series of barriers against invading microorganisms. This includes the process of xenophagy, the selective degradation of intracellular pathogens in double-membrane autophagosomes.[305] Secondly, LC3-associated phagocytosis (LAP), which engages autophagic machinery, aids in expediting the degradation of bacterium and other extracellular objects such as TLR-ligand-coated particles.[306] LAP phagosomes are matured through the autophagic pathway, using Beclin-1-VPS34 complexes and LC3 conjugation, resulting in robust phagolysosomes.[306-308] Thirdly, a group of autophagic adaptors, known as sequestosome-1 receptors (SLRs), bind ubiquitinated bacteria and deliver them to autophagosomes via interactions with inner membrane-bound LC3.[309-312] Lastly, autophagy-associated factors can directly bind target microbial proteins. For example, ATG5 binds the *Shigella spp.* surface protein, VirG,[313] and along with ubiquitylation and SLR activity,[309, 314] herds the bacteria to autophagosomes for elimination.

Bacteria may also counteract or seize control over host autophagic machinery for their own benefit, highlighting the importance of autophagy in antibacterial defense. These inhibitory mechanisms include the expression of proteins that interfere with autophagosomal targeting, formation, or maturation. For example, *Listeria* proteins AktA and InIK interfere with host ubiquitin tags,[310, 315] while *Shigella* protein IcsB masks bacterial epitopes,[316] all of which assist the bacteria in evading recognition by autophagic machinery. *Salmonella* spp. deubiquitinase SseL cleaves off ubiquitin tags to thwart SLR-mediated targeting to nascent autophagosomes.[317] Some bacteria directly "attack" autophagy proteins, such as the *Legionella* virulence factor RavZ, which causes irreversible deconjugation of Atg8 homologs.[318] Lastly, captured *Listeria* blocks autophagosomal acidification, and thus maturation, via the pore-forming protein listeriolysin O.[319]

Likewise, host autophagy is important in curbing viral load, given the number of viral proteins that interfere with or utilize autophagic machinery for their own replication. The herpes simplex virus 1 (HSV-1) virulence factor ICP34.5,[320] influenza virus M2 protein,[321] HIV protein Nef,[322] murine γ-herpesvirus 68 M11 (BCL2 homologue),[323] and KSHV vBcl-2[324] all target Beclin-1 to either block autophagy or inhibit autophagosomal maturation via lysosomal fusion. HIV-1 Nef, hepatitis C virus (HCV) NS3, and measles virus Mev3 interact with IRGM, which has consequences yet to be investigated.[325] Interestingly, some viruses utilize the autophagosomal machinery to optimize their yield, including HCV, Dengue virus, poliovirus, Coxsackie B virus, and HIV.[326] For example, poliovirus tethers together autophagosomes, which serve as scaffolds for RNA replication and contribute to virion egress once viral particles within the cell reach a threshold level.[327]

Autophagic regulation of immune cell function

Autophagy functions as a bulk transporter of proteins from the cytoplasm into the lumen of antigen-processing compartments. As such, autophagy facilitates several aspects of DC maturation in response to pathogen detection. One, the process helps load cytosolic peptides onto MHC-I and MHC-II proteins, which are subsequently presented to $CD4^+$ or $CD8^+$ T cells, respectively. Second, the delivery of cytoplasmic contents to endosomal TLRs enables surveillance of the intracellular environment, thereby facilitating the upregulation of innate cytokine production, MHC-I/II expression, and co-stimulatory molecule expression that are vital to T cell activation during antigen presentation. The process of autophagy may be especially critical to the immediate detection of RSV, since the virus enters cells via non-receptor-mediated endosomes.[7]

A deficiency of autophagy in DCs can result in altered antigen-presentation in the context of MHC-I/II, leading to pathological T cell responses. In autophagy-deficient mice, both positive and negative selection of $CD4^+$ T cells but not $CD8^+$ T cells were affected.[328] Researchers transplanted *Atg5*-deficient thymi into WT mice, causing an infiltration of autoreactive $CD4^+$ T cells into multiple organs and the induction of autoimmune colitis.[328] This suggests that autophagy-enhanced MHC class II presentation has a role in thymic T cell selection. Other studies have shown that autophagy-dependent antigen presentation is

defective in DCs from patients with Crohn's disease carrying the *ATG16L1* or *NOD2* disease risk variants.[329] With regard to viral infection, DC presentation of the Epstein Barr virus (EBV)-encoded nuclear antigen 1 (EBNA1), a $CD4^+$ T cell epitope found in healthy EBV carriers, requires autophagy.[330, 331] Studies by our laboratory have demonstrated that DCs upregulate autophagosome formation upon RSV detection, and that autophagy facilitates DC maturation, cytokine production, and antigen presentation, and is thus required to stimulate IFNγ from RSV-reactive T cells.[233] The contribution of autophagy to MHC-I cross presentation is not fully understood, but it may contribute to the delivery of proteasome-degraded peptides to MHC-I-containing compartments.[332, 333] Of note, inhibition of *ATG16L* and *IRGM* expression in human DCs leads to hyperstable interactions with T cells and increases T cell activation.[334] Thus, while autophagy initially promotes antigen processing, at later stages it may help downregulate the response, such as through the disassembly of immunological synapses.[334]

In addition to sequestering viral peptides for presentation to T cells, the transport of viral nucleic acids to endosomal TLRs via autophagy induces pro-inflammatory cytokine production in response to infection. This was first established in pDCs, where ATG5 was necessary to enhance TLR7-dependent IFNα production in response to vesicular stomatitis virus (VSV).[335] Human pDC studies also demonstrated an autophagy-dependent IFNα production in response to HIV-1[336] and the paramyxovirus Simian virus 5.[337] Interestingly, macrophages or DCs treated with TLR ligands were reported to upregulate autophagosome formation, suggesting cooperation between TLRs and autophagic proteins in response to PAMPs. The upregulation of autophagy in macrophages was noted in response to agonists of TLR1/2, TLR3, TLR4, or TLR7.[338] Treatment of *M. tuberculosis*-infected macrophages with TLR4 or TLR7 ligands increased bacterial killing, suggesting that TLR-induced autophagy promotes intracellular pathogen clearance.[338] The upregulation of autophagy in advance of microbial invasion promotes the expression and the prompt delivery of antimicrobial peptides to lysosomes, and autophagosomal proteins to forming phagophores, thereby expediting the eventual acidification of pathogen-laden autophagosomes.[339-341] Therefore, enhanced recruitment of autophagy in response to TLR signaling positively modulates DC antigen presentation and cytokine production in response to antimicrobial infection.[342, 343]

Aside from its role in APC function, autophagy affects both the homeostasis and activity of effector cells involved in adaptive immunity, including T cells and B cells. After exiting the thymus, naïve T cells rely on autophagy and mitochondrial content reduction for their maturation.[344] Calcium ions sequestered in ER-like structures have been observed in *ATG7*-deficient T cells, suggesting the necessity of autophagy for ER maintenance.[345] Autophagy is also pro-survival in activated T cells by counteracting the pro-apoptotic function of CD95 (FAS) and CD95L (FASL), which are upregulated by TCR (T cell receptor) stimulation.[346] While autophagy may not be important for the survival of most mature B cells,[347] lymphoid precursor stages are affected by the absence of autophagy.[348] Interestingly, the lack of autophagy leads to excessive immunoglobulin secretion by plasma cells, indicating the importance of ER maintenance during conditions of high secretory demand.[349] Autophagy is also important for the preservation of the bone marrow plasma cell pool, which is relevant for long-lived humoral immunity.[349] Overall, autophagy plays crucial functions in both innate and adaptive immunity via influences on immune cell function.

Modulation of inflammation by autophagy

The recognition of autophagy's anti-inflammatory functions stemmed from the observed increase in IL-1β and IL-18 production in *Atg16l1*$^{-/-}$ mice with Crohn's disease. [298, 300] Several convergent reports show that autophagy has a negative role in inflammasome activation,[350, 351] likely explaining the observed cytokine profile in *Atg16l1*-deficient mice. Under sterile conditions, autophagy clears the cytoplasm of debris, protein aggregates, and defective organelles that could endogenously activate inflammasomes. Yet if autophagy is blocked, this leads to the accumulation of depolarized mitochondria, which leak inflammasome agonists such as mitDNA and reactive oxygen species (ROS). [350, 351] Studies with *Atg5*$^{-/-}$ macrophages have demonstrated how ROS in autophagy-defective cells activates both the inflammasome and the calpain pathways (protease which cleaves pro-IL-1α) that lead to the excess production of IL-1β and IL-1α, respectively. In the context of *M. tuberculosis* infection, this excess of IL-1α leads to magnified and prolonged Th17 cell responses, which contribute to lung tissue damage in mice.[352] However, during influenza A viral infection, the removal of damaged mitochondria is not passive. A NOS2-receptor-

interacting serine/threonine protein kinase 2 (RIPK2) pathway activates ULK1 to maintain or increase mitophagy, thereby reducing the set point of inflammasomal activation.[353] Importantly, autophagy may downregulate prolonged inflammasome activity by removing aggregated inflammasomal components.[256] Lastly, autophagy factors can degrade pro-inflammatory signaling factors, such as BCL-10 complexes[354] to reduce NFκB activation in antigen-activated T cells.[355] Therefore, basal autophagy protects cells from inadvertent inflammation via the elimination of microorganisms and endogenous irritants.

Interactions between ER-stress, autophagy, & viruses

Secreted and transmembrane proteins fold and mature in the endoplasmic reticulum (ER). However, ER homeostasis can be perturbed by physiological and pathological insults such as high protein demand, viral infections, environmental toxins, inflammatory cytokines, and mutant protein expression, all of which lead to the accumulation of mis- and unfolded proteins. Three ER-bound proteins monitor conditions in the ER lumen, sensing whether there is an insufficiency in protein folding capacity or a depletion of ER calcium gradient: inositol-requiring protein 1α (IRE1α), activating transcription factor 6 (ATF6), and protein kinase-like ER kinase (PERK). These trigger the unfolded protein response (UPR), an adaptive response aimed at restoring ER balance by three mechanisms: transcription of genes to increase folding capacity, transient reduction in protein translation, and ER-associated degradation (ERAD). In the case of unresolvable, chronic ER stress, the UPR initiates programmed cell death.[356, 357]

ERAD easily removes and degrades accumulated, soluble proteins via the proteasome, but its capacity is overwhelmed by protein aggregates and damaged organelles that often result during ER stress. [358] In such cases, the UPR has been shown to activate autophagy to mediate cellular survival.[358-360] For example neuroblastoma cells treated with ER stressors markedly induced the formation of autophagosomes, as assessed by microscopy, while inhibiting autophagy rendered cells vulnerable to upregulated ER stress.[359] Most recently, a microarray analysis of colon cancer cells subjected to ER stress showed the transcriptional upregulation of autophagy receptor genes SQSTMI/p62, NRI, and BNIP3L/NIX.[358] SQSTMI/p62 and NBR1 are ubiquitin-binding proteins with an LC3

interacting region (LIR), which targets ubiquitinated substrates to the autophagosome.[361, 362] BNIP3L/NIX is an adapter for the removal of damaged/depolarized mitochondria,[363] so the data indicate a role for mitophagy in protecting cells during ER stress by limiting apoptosis. IRE1α, the most conserved, sole branch in lower eukaryotes,[364] is consistently observed as a major regulator of this UPR-induced autophagic response.[358, 359] Of note, IRE1α's ribonuclease activity unconventionally splices an intron from mRNA encoding XBP1, which is translated into XBP1s, a transcription factor that mediates downstream IRE1α signaling.[356]

Viruses cause ER disequilibrium as well as hijack the UPR to their advantage, in congruence with their ability to commandeer the autophagy pathway. For example, HCV, poliovirus, human cytomegalovirus (HCMV), and HSV-I strictly depend on the ER for viral assembly and budding,[365] and thus are able to perturb host ER replication complexes, and ER membrane for source-membrane.[366] Viral proteins can induce ER stress via their competition for ER-mediated glycosylation, such as during influenza A virus, hepatitis virus, and Japanese encephalitis virus (JEV) infection.[367-369] In addition, the depolarization of ER-calcium differentials by viral viroporin proteins, such as the rotavirus NSP4 protein[370] and picornavirus 2B protein,[371] can trigger ER stress. Recently, RSV infection has been shown to induce IRE-1α and ATF6 signaling, while IRE-1α inhibition enhanced viral replication.[372]

As discussed, autophagy is critical in maintaining cellular equilibrium and mediating stressor-induced inflammatory and immune responses, such as during RSV infection. The fascinating relationships between autophagy, the UPR, and viral immunity are further intertwined when sirtuin 1 (SIRT1), a histone/protein deacetylase, is considered. Researchers have reported that three ATG proteins (ATG5, ATG7, ATG8/LC3) are deacetylated and activated by SIRT1.[373] Therefore, given the significance of DCs in dictating robust antiviral immune responses, and autophagy's crucial role in trafficking viral antigens to endosomal PAMPs, the exploration of SIRT1 activity within DCs is exceptionally warranted to better understand RSV-induced immunity.

Sirtuin 1: a regulator of immune responses

Sirtuins (SIRTs) are mammalian homologues to the yeast protein Sir2,[374] and were originally described as nicotinamide adenine dinucleotide (NAD^+)-dependent type III

histone deacetylases (HDACs). The deacetylation reaction converts NAD^+ into nicotinamide (NAM), a feedback inhibitor, and generates 2'-O-acetyl-ADP-ribose and a deacetylated protein substrate.[375] However, several sirtuins are now known to perform additional catalytic functions, including deacylation, demalonylation, and desuccinylation (Table 1.1). Phylogenetic analysis reveals that the 7 mammalian sirtuins can be divided into four classes: Class I, SIRT1-SIRT3; Class II, SIRT4; Class III, SIRT5; and finally Class IV, SIRT6-7.[374] While all sirtuins share a ~30 kDa core deacetylase domain, their N- and C-termini vary as well as their subcellular localization, protein targets, and regulatory functions (Table 1.1).

SIRT	Class	Localization	Activity	Examples of Targets	Example Regulatory Functions
SIRT1	I	Nucleus Cytosol	Deacetylation	PGC1α, FOXOs, p53, NFκB, HIF1α	Cancer, metabolism, inflammation, neurological
SIRT2	I	Nucleus Cytosol	Deacetylation	Tubulin, PEPCK, FOXO1, PAR3	Cell cycle, cancer, myelination
SIRT3	I	Mitochondria	Deacetylation Decrotonylation	LCAD, HMGCS2, GDH, SOD2, OXPHOS complexes	FA oxidation, antioxidant defense
SIRT4	II	Mitochondria	ADP-ribosylation Deacetylation	GDH, PPARα, NFκB, MCD, Caspase 3/7	FA oxidation suppression, insulin secretion
SIRT5	III	Nucleus Mitochondria Cytosol	Desuccinylation Demalonylation Deglutarylation	CPS1, SOD1, HMGCS2, SDH	Urea cycle, neurological
SIRT6	IV	Nucleus Cytosol	ADP-ribosylation Deacetylation Deacylation	H3K9, H3K56, CtIP, GCN5, PARP1	Metabolism, inflammation, genome stability
SIRT7	IV	Nucleolus	Deacetylation	p53, H3K18, PAF53, NPM1, GABP-β1	Ribosomal DNA transcription

Table 1.1: Properties and functions of mammalian sirtuins. CPS1, carbamoyl phosphate synthetase 1; CtIP, CtBP-interacting protein; FOXO, forkhead box O; GABP-β1, GA binding protein β1 subunit; GCN5, histone acetyltransferase GCN5; GDH, glutamate dehydrogenase; HIF1α, hypoxia-inducible factor 1α; HMGCS2, 3-hydroxy- 3-methylglutaryl CoA synthase 2; LCAD, long-chain acyl CoA dehydrogenase; MCD, malonyl CoA decarboxylase; NFκB, nuclear factor-κB; NPM1, nucleolar phosphoprotein B23; OXPHOS, oxidative phosphorylation; PAF53, polymerase-associated factor 53; PAR3, partitioning defective 3 homologue; PARP1, poly (ADP-ribose) polymerase 1; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, peroxisome proliferator-activated receptor-γ co-activator 1α; PPARα, peroxisome proliferator-activated receptor alpha; SDH, succinate dehydrogenase; SOD1,2, superoxide dismutase 1,2. [376-378]

The most studied sirtuin, SIRT1, was first noted to deacetylate histones, but thereafter shown to possess a wide array of targets, including p53 and NFκB.[379] It is a ubiquitous protein in the body, and expressed in neurons, heart, liver, kidney, blood and spleen, for example. SIRT1 impacts many areas of biology and pathophysiology (Figure 1.3). Like autophagy, it is induced in terms of expression and activity by nutrient deficiency,[380] critical during cell stress survival,[381-383] and implicated in various human health disorders.[384-386] In addition, recent studies have shown SIRT1 to regulate aspects of ER stress, including the UPR.[387-389] While SIRT1 influences immunity by regulating processes such as lymphocyte activation, T-cell proliferation and differentiation, and macrophage cytokine secretion,[390] its role in DCs is not well understood. This knowledge gap regarding the role of SIRT1 in DC function, and subsequent antiviral immune responses, prompted the studies discussed in this dissertation. Given the central role SIRT1 plays in numerous pathways, and therefore various disease processes, much effort has been directed at developing SIRT1 activators and inhibitors.

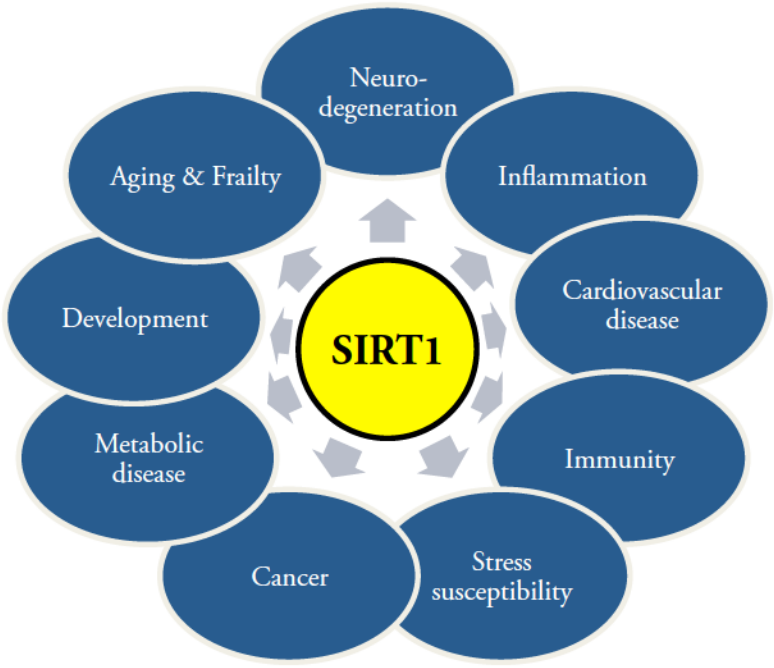


Figure 1.3: Sirtuin 1 – complex roles, many targets. SIRT1 interacts with a plethora of pathways and health-relevant processes including: genomic stability, stress responses, cellular development, metabolism, circadian rhythm, cardiovascular disease, cancer, neurodegeneration, anxiety control, inflammation, and immunity. [377, 390-393]

Pro-survival role of SIRT1 during stress responses

During cellular disturbances—including starvation, hypoxia, ER stress, genomic stress, oxidation, and inflammation—SIRT1 expression and activity are upregulated to facilitate the activation of necessary pro-survival pathways. The breadth of SIRT1 targets contributes to the complexity of the protein's role during different stress conditions, as in the case of autophagy induction. SIRT1 interaction with components of the autophagosomal machinery has been shown by co-immunoprecipitation of SIRT1 with ATG proteins.[373] Interestingly, global *Sirt1*$^{-/-}$ mice resemble *Atg5*$^{-/-}$ (autophagy defective) mice in phenotype, including the accumulation of damaged organelles in the cytoplasm, disruption of energy homeostasis, and early perinatal mortality.[373] Aside from direct deacetylation, SIRT1 can indirectly activate autophagy. For example, SIRT1 negatively regulates mTOR, which represses autophagy during nutrient-rich conditions, through the TSC1/2 complex.[394] Studies have also shown that SIRT1 mediates mTOR repression and autophagy formation during oxidative stress in embryonic stem cells.[382] In addition, nutrient surveillance by SIRT1 is dependent on various FOXO transcription factors, which sense insulin signaling and regulate longevity in lower organisms.[377,379] For example, SIRT1 deacetylation of FOXO mediates starvation-induced autophagy in cardiomyocytes.[390] Lastly, SIRT1 promotes autophagy in response to toxins, such as in the case of fluoride-induced ER stress in ameloblasts.[383]

Under hypoxic conditions, SIRT1 induces hypoxia-inducible factor 2α (HIF2α) activity by direct deacetylation to promote cell survival.[395] However, SIRT1 inhibits the related factor HIF1α by deacetylating at K674, blocking its association with p300, and thus limiting cell proliferation during hypoxia.[396] Furthermore, SIRT1 differentially regulates the transcriptional activities of HIF1α and HIF2α in a cancer cell-type dependent manner.[397] Together, these physiological responses illustrate how changing levels of NAD^+ and related metabolites during hypoxia can regulate cellular responses through SIRT1 to preserve energy homeostasis.

In an effort to preserve cell function, SIRT1 can also coordinate multiple stress response pathways simultaneously. Following a heat or protein aggregation challenge, SIRT1 promotes transcription of heat shock response (HSR) genes via direct deacetylation of heat

shock factor protein 1 (Hsf1).[398] Yeast studies have shown that the HSR and the UPR participate in a stress response network,[399, 400] and subsequent work has shown that activation of the HSR by the UPR is facilitated by Sir2 (yeast homolog of SIRT1).[401] Specifically, Sir2 was essential for HSR- and UPR-induced Hsf1 activation, and excess Sir2 compensated for UPR-dependent Hsf1 activation in UPR-defective yeast strains.[401] Likewise, while Sir2 was upregulated by the UPR, it also served as negative feedback to prevent the deadly consequences of chronic UPR.[401] Congruently, SIRT1 has been associated with the UPR in mammalian models, whereby it deacetylates XBP1s[387] to curb UPR-induced cell apoptosis and ER-stress mediated liver dysfunction.[388, 389]

While much research since SIRT1's discovery has been focused on its non-histone targets, the significance of SIRT1 in mediating genomic stability is well evidenced during hematopoietic stem and progenitor cell (HSPC) maintenance. Using bone marrow specific *Sirt1*-ablated mice, researchers observed aberrant HSPC expansion under 5-Fluorouracil-induced hematopoietic stress, which was associated with genomic instability, the accumulation of DNA damage, and the eventual loss of long-term progenitor populations. Likewise, SIRT1 was shown to bind the *Hox9* gene and deacetylate H4K16, thus promoting polycomb-specific repressive histone modifications. Together, these findings demonstrate two interconnected roles for SIRT1 in HSPC homeostasis, both via epigenetic regulation of a key developmental gene and by promoting genomic stability in adult stem cells.[402]

Additionally, SIRT1 possesses antioxidant and anti-inflammatory functions. For example, SIRT1-targeted DNA repair factor KU70 promotes survival by preventing BCL-2 associated X protein (BAX) from entering the mitochondria to activate oxidative stress-induced apoptosis.[403] SIRT1 deacetylation of FOXO1, FOXO3, and FOXO4 induces cell cycle arrest and resistance, while inhibiting apoptosis, during oxidative stress.[404-406] In starved mouse astrocytes, SIRT1 upregulation increased the expression of antioxidant enzymes (superoxide dismutase, catalase) in a FOXO4-dependent manner and suppressed the expression of pro-inflammatory cytokines.[407] Interestingly, while SIRT1 activity rises during acute inflammation, the protein plays dual regulatory roles. The deacetylation of the RelA/p65 subunit of NFκB[408] by SIRT1 leads to the transcriptional repression of NFκB-activated inflammatory cytokine expression,[409] while SIRT1 concurrently stimulates anti-

inflammatory gene expression by targeting RelB.[409] In contrast, SIRT1 activity diminishes during prolonged inflammation, which may explain the steady hyperactivation of NFκB in chronic inflammatory diseases such as obesity, atherosclerosis, diabetes, and COPD.[410]

Pleiotropic effects of SIRT1 on age-related diseases

As of now, there are few identified *SIRT1* polymorphisms associated with human disease, all of which are related to metabolism. Three *SIRT1* SNPs has been associated with differences in whole-body energy expenditure in Finnish subjects.[411] Recently, a study reported a link between *SIRT1* gene polymorphisms and obesity in children.[385] Finally, multiple family members affected by type 2 diabetes were found to carry a SIRT1-L107P point mutation, which contributed to insulin resistance and the overproduction of inflammatory mediators by pancreatic β-cells implicated in the pathogenesis of type 1 diabetes.[384] Nonetheless, given its central role in a plethora of cellular processes, SIRT1 has an intricate, two-sided association with cardiovascular health, cancer, and neurodegeneration, in addition to metabolic disease.

The maintenance of relatively constant blood glucose concentration is essential to provide fuel to tissues, which is regulated through various processes, including intestinal glucose uptake, hepatic glucose output and uptake, and utilization and storage in peripheral tissues.[376] Intriguingly, SIRT1 can suppress hepatic glucose production by deacetylating CREB-regulated transcription co-activator 2 (CRTC2), but can also stimulate gluconeogenic transcriptional programs via activation of FOXO1 and PGC1α.[412-414] Despite this duality, SIRT1 may serve as an insulin sensitizer, as SIRT1 activation protects mice from diet-induced and genetic insulin resistance.[415, 416] Moreover, SIRT1 can inhibit glycolysis via activation of PGC1α,[414] and increase mitochondrial oxidative metabolism via inhibition of HIF1α.[417] Lastly, SIRT1 promotes insulin secretion from pancreatic β-cells by transcriptional repression of mitochondrial uncoupling protein 2 (UCP2).[418]

Lipid homeostasis is likewise influenced by SIRT1 activity. The key transcription factor controlling genes involved in lipid synthesis, liver X receptor (LXR), is activated via SIRT1 deacetylation,[419] contributing to cholesterol metabolism. SIRT1 inhibits adipogenesis and enhances fat mobilization through lipolysis by suppressing the activity of PPARγ.[420]

Aside from inhibiting glycolysis, SIRT1-mediated PGC1α activation transcriptionally stimulates fatty acid uptake and/or β-oxidation.[421] In support of this, SIRT1 activation protects mice from diet-induced obesity by increasing fatty acid oxidation,[422] while *Sirt1*$^{-/-}$ mice are susceptible to hepatic steatosis under standard and high-fat diets.[421, 423]

Strong evidence exists in favor of SIRT1's cardioprotective properties. Overexpression of *Sirt1* is beneficial in both cardiac myocytes and in vascular cells,[424-428] and studies suggest that many of the positive effects of Resveratrol (details forthcoming in chemical modulator section), such as upregulation of antioxidant enzymes, are SIRT1-dependent.[429] Likewise, *SIRT1* overexpression in cultured endothelial cells decreased the expression and activity of tissue factor (initiator of coagulation), via NFκB-inhibition.[430] Another SIRT1 activator, SRT1720, significantly reduced the number of ischemic foci and attenuated inflammatory gene activation in the hearts of mice fed a high-fat diet.[431] However, while a study reported that 2.5- to 7.5-fold *Sirt1* overexpression reduced age-dependent cardiac dysfunction in mice, 12.5-fold overexpression increased cardiomyopathy and oxidative stress.[424]

SIRT1 has a fascinating yin-yang relationship with different types of cancers, serving as both an oncogene and tumor suppressor. On one hand, SIRT1 can be seen as a tumor promoter as it deacetylates p53, a tumor suppressor, leading to the inhibition of apoptosis in injured cells.[432] SIRT1 is significantly elevated in human prostate cancer, leukemia, primary colon cancer, and all non-melanoma skin cancers, among others.[433-435] Thus, higher SIRT1 levels may hamper p53-dependent cell cycle arrest and apoptosis in response to DNA damage and oxidative stress, two contributing factors to oncogenic mutations. SIRT1's oncogenic effect may also be attributed to loss of function in SIRT1's inhibitor, DBC1, as deletions in DBC1 in breast cell lines increase the risk for tumorigenesis.[436] Lastly, loss of global H4K16 acetylation combined with H4K20 tri-methylation is a hallmark of human cancers.[437] SIRT1 deacetylates H4K16 and H3K9 at promoters of several tumor suppressor genes, thereby recruiting methyltransferases and promoting gene silencing.[438, 439]

On the other hand, SIRT1 is often decreased in chronic inflammation, which is a risk factor for certain cancers. Downregulated *SIRT1* expression has been reported in human glioblastoma, bladder carcinoma, prostate carcinoma, ovarian cancer, and hepatic

carcinoma.[438] SIRT1 antitumor activity is supported by the fact that *SIRT1* deficiency hampers DNA repair, which can lead to severe genetic instability. Likewise, as mentioned, SIRT1 promotes mitochondrial fatty acid oxidation, which upsets tumor cells' reliance on glycolysis, known as the Warburg effect. Mutations in the tumor suppressor BRCA1 reduce *SIRT1* expression, thus increasing the risk for breast, ovarian, fallopian, and prostrate cancer in humans.[440] *Survivin*, an oncogene expressed in most cancer cell types, is repressed at the promoter by SIRT1-mediated deacetylation of H3K9. SIRT1 also blocks nuclear translocation of the oncogenic protein β-catenin, such that *Sirt1* overexpression in a mouse model of colon cancer significantly reduced tumor formation and morbidity.[441] Thus, the contribution of SIRT1 to cancer development is dependent on other cellular parameters, at both the genomic and epigenetic levels.

Finally, SIRT1 has shown beneficial roles in multiple models of neuropathology, primarily by deacetylating protein targets that protect against neurodegeneration.[442] Patients with Alzheimer's disease have lower *SIRT1* gene expression, which correlates with the accumulation of neurofibrillary tau tangles.[443] Evidence from *in vitro* and animal models indicates that increased SIRT1 activity, by genetic or chemical means, may be protective against Parkinson's disease by reducing α-synuclein-mediated toxicity.[444, 445] Three different mouse models have shown that SIRT1 can also protect against Huntington's disease by activating PGC1α to prevent huntingtin-induced mitochondrial dysfunction and to reduce protein aggregation.[446] Even in a mouse model of MS, experimental autoimmune encephalitis (EAE), SIRT1 activation may prevent neuronal damage and long-term dysfunction.[447] Despite the duality of SIRT1 activity in human pathologies, its beneficial role in preventing autoimmune demyelination and neurodegeneration is quite robust.

SIRT1 regulates immune cell differentiation and activation

The significance of SIRT1, especially with regards to immune function, is highlighted by studies in *Sirt1*-null mice. These *Sirt1* mutants are smaller and harbor notable developmental defects of the retina and heart, with most dying postnatally.[448] Younger *Sirt1*$^{-/-}$ mice are prone to eyelid inflammation, while mice 2 years of age present with increased anti-nuclear antigen antibodies in the sera, deposits of IgM and IgG immune complexes in the

liver and kidney, and exhibit a SLE-like phenotype.[449, 450] SIRT1-based therapies and various KO mouse models have helped delineate the role of SIRT1 in innate and adaptive immune responses, including those during infection and pulmonary disease. However, while studies have begun to elucidate the roles of SIRT1 in macrophages and T cells, much less is known about this protein's role in DCs.

The majority of SIRT1 functions observed in the immune system arise from the regulation of NFκB and AP-1 pathways. The NFκB pathway is a central signaling node in inflammatory cytokine stimulation and lymphocyte activation. As previously stated, SIRT1 deacetylation of the p65 subunit at K310 leads to reduced NFκB transcriptional activity.[408] Not surprisingly, p65 transcription positively feedbacks onto SIRT1, resulting in increased mRNA levels.[451] Activator protein 1 (AP-1, dimeric complex of JUN and FOS) transcriptional activity has an essential role in immune response activation, particularly in T cells, and is also targeted by SIRT1 deacetylation.[450]

Macrophages, the most abundant innate immune cell type, are a main source of pro-inflammatory cytokines TNFα, IL-6, and IL-1 in response to infection and inflammation. SIRT1's inhibitory role on NFκB signaling has an essential role in suppressing the pro-inflammatory phenotype of macrophages.[406, 452] For example, the ablation of *Sirt1* in macrophages, using a myeloid cell-specific *Sirt1* KO mouse, rendered NFκB hyperacetylated and resulted in increased transcriptional activation of proinflammatory target genes.[409] Interestingly, XBP1s is critical for IFNβ production in macrophages.[453] Given that SIRT1 inhibits XBP1s,[387] the suppression of the UPR in macrophages may serve to inhibit the activation of at least part of innate immune response.

Far less has been reported about the role of SIRT1 within DCs, which herald the presence of a foreign invader and initiate the adaptive immune response via cytokine production and antigen-presentation to T cells. However, the data suggests that SIRT1 programs DCs to promote inflammatory immune responses.[454, 455] One study examined DC-specific *Sirt1* KO mice and observed no changes in DC maturation, differentiation, or development compared to WT DCs; however, these *Sirt1*$^{-/-}$ DCs produced higher levels inflammatory cytokines upon TLR stimulation.[454] Moreover, these DC-specific *Sirt1* KO mice were resistant to myelin oligodendrocyte glycoprotein (MOG)-induced EAE, exhibiting

a significant reduction in central nervous system (CNS) pathology, much fewer Th17 and Th1 cellular infiltrates, and a significant increase in Treg cell populations.[454] A second study demonstrated that the lack of SIRT1 in DCs reduced macrophage and eosinophil lung infiltration in a murine model of OVA-induced airway disease.[455] The data suggest that SIRT1 repressed PPARγ activity within DCs, thereby promoting their maturation and pro-Th2 skewing activity. Accordingly, mice treated with pharmacological SIRT1 inhibitors had reduced Th2 responses and were protected against allergic airway inflammation.[455]

The role of SIRT1 in the adaptive immune response has been primarily focused on the activation and differentiation of T cells. SIRT1 protein levels are dramatically elevated in anergic T cells, compared to activated T cells or mature, naïve T cells.[450] *Sirt1*-null mice do not harbor aberrant T or B cell numbers as compared to WT littermates, indicating that SIRT1 may not be critical for these cells' development.[390] However, T cells from *Sirt1*-null mice are hyperproliferative and activated without CD28 co-stimulation; TCR stimulation alone leads to as much proliferation in *Sirt1*$^{-/-}$ T cells as TCR/CD28 co-stimulation.[450, 456] In addition, T cells isolated from OVA-sensitized *Sirt1*-null mice proliferate more upon OVA re-exposure and produce more IL-2, IFNγ, and IL-5.[450] This T cell hyperactivation results from the loss of suppression of NFκB and AP-1 transcriptional activity,[450] as well as a lack of SIRT1-dependent inhibition of BCLAF1, a BCL-2 binding protein required for T cell activation.[456] IL-2 provides a crucial signal for maintaining the viability and proliferation of activated CD25^{+} T cells by suppressing FOXO1, 3, and 4 to prevent apoptosis.[457] Additionally, data reveals that IL-2 can suppress *Sirt1* transcription by sequestering FOXO3a to the cytoplasm, thereby reversing T cell tolerance, which is characteristic of T cell anergy.[458] This may explain the aforementioned hyperproliferation and activation of *Sirt1*$^{-/-}$ T cells, and the greater mRNA and protein expression of SIRT1 in anergic T cells.[450, 456]

SIRT1 is implicated in the differentiation of activated T cells, although the specific mechanisms are not as clear. One group reported how ATF-like (BATF) directly suppresses the transcription of *Sirt1*, and demonstrated that *Batf*$^{-/-}$ CD8^{+} cells had lower acetylation at the TBET promoter and lower *Ifng*, *Ifngr1*, and *Il12rb2* expression.[459] Conversely, *Sirt1*$^{-/-}$ CD4^{+} T cells produced more IL-5 and IFNγ protein upon *in vitro* stimulation. SIRT1 may inhibit the differentiation of CD4^{+} T cells into inflammatory Th17 cells and the expansion

of Tregs by suppressing STAT3 activity.[460, 461] Moreover, SIRT1 is a negative regulator of Treg function via FOXP3 deacetylation, whereby hyperacetylation stabilizes the FOXP3 protein and enhances Treg suppressive activity.[462, 463] In agreement, *Sirt1$^{fl/fl}$-Foxp3-Cre^{+}* mice exhibited prolonged survival of MHC-mismatched cardiac allografts, while effector T cell responses were unaffected.[462] Likewise, administration of a SIRT1 activator in a mouse model of autoimmune uveoretinitis suppressed numbers of retinal infiltrating T helper cells, with a greater reduction in Tregs as compared to Th1, Th2 and Th17 cells.[464] Overall, SIRT1's regulation of NFκB, AP-1, and STAT3 points to SIRT1 being a suppressor of pro-inflammatory T cell responses.

SIRT1 and anti-microbial immune responses

Similar to autophagy, SIRT1 is called upon during cellular stress to reestablish cellular homeostasis. As all sirtuins require NAD^{+} for activity, their function is tightly linked to the metabolic status of the cell. Therefore, a stress such as bacterial or viral infection will upset the cell's energy balance, leading to the activation of sirtuin transcription and activity. Given that many of SIRT1's functions, including glycolysis inhibition and fatty acid oxidation, are contrary to what an actively replicating virus desires, SIRT1 may be an evolutionary antiviral defense. In fact, the *E. coli* sirtuin homologue, CobB, has been reported as a host defense mechanism against bacteriophages, whereby knockdown of *CobB* promoted phage plaque size and overexpression of *CobB* reduced phage titers.[465] Furthermore, all seven human sirtuins have recently been shown to have broad-range antiviral properties against both DNA and RNA viruses.[465]

In particular, the importance of SIRT1 during antiviral defense is underlined by the ways in which various viruses highjack the deacetylase. While SIRT1 has been shown to restrict viral replication of HCMV, HSV, adenovirus type 5 (Ad5), and H1N1,[465] HIV and hepatitis B virus (HBV) leverage SIRT1 activity to their advantage. The HIV Tat protein facilitates elongation of mRNA transcription, and once the transcripts are completed, Tat is recycled via SIRT1 deacetylation to allow for continued HIV genomic replication.[466] Conversely, Tat binds the catalytic domain of SIRT1, blocking its ability to deacetylate and inhibit NFκB. Interestingly, HIV has two NFκB binding sites, such that with Tat-induced

SIRT1 suppression, transcriptional activity of both NFκB-responsive genes and HIV-specific genes are enhanced.[467] During HBV infection of human cell lines, SIRT1 physically associates with the viral DNA template; as SIRT1 is upregulated in expression and activity, so is viral replication.[468] The HBV X protein blocks SIRT1 so it cannot repress the activity of β-catenin, known to promote the development of HBV-induced hepatocellular carcinoma.[469]

In contrast to SIRT1's involvement in viral infection, less is known about the role of SIRT1 in host defense against bacteria. One study, utilizing SIRT1 activators, SIRT1 inhibitors, and a myeloid-specific *Sirt1* KO mouse (*Sirt1^{ff}-LysM-Cre*), reported that SIRT1 has little influence on macrophage and neutrophil antimicrobial functions.[470] Likewise, myeloid *Sirt1* expression did not alter mortality in gram-negative toxin-induced shock or gram-positive bacteremia.[470] Thus, while SIRT1 serves to repress macrophage-induced inflammation,[409] it does not alter the intrinsic anti-bacterial characteristics of this innate immune cell.

Modulation of SIRT1 activity: natural and synthetic compounds

The activity and expression of SIRT1 are tightly regulated at many levels—from general mechanisms, such as substrate availability, tissue and subcellular localization, to gene-specific regulatory mechanisms, such as activation of transcription factors, regulation by microRNA, post-translational modification, and protein-protein interaction. SIRT1's activity is closely controlled by the availability of NAD^+, a required co-factor, and nicotinamide (NAM), a by-product of catalysis that serves as an endogenous inhibitor.[471] Another axis of regulation is the positive feedback between SIRT1 and AMP-activated kinase (AMPK), which upon sensing a rise in the intracellular AMP/ATP ratio facilitates gene transcription to increase ATP generation.[472, 473] AMPK activation also increases the intracellular NAD+/NADH ratio, leading to sirtuin activation.[474] Moreover, SIRT1 deacetylates LKB1, an activator of AMPK, thus further promoting its own activity.[475] In terms of SIRT1 expression, multiple transcription factors regulate SIRT1 mRNA levels, including p53, FOXO3a, E2F1, and c-Myc, in response to stimuli such as growth factors, cell cycle conditions, and apoptotic signals.[380, 476, 477] As previously noted, many of these transcription factors are also deacetylated by SIRT1, providing a feedback mechanism in the

regulation of SIRT1 expression. In addition, SIRT1 expression can be post-transcriptionally regulated by microRNA; miR-34a and miR-199 have been reported to suppress SIRT1 in prostate cancer lines and cardiomyocytes, respectively.[478, 479] Posttranslational modifications, such as phosphorylation by c-Jun N-terminal kinases 1 and 2 (JNK 1, 2), alter SIRT1 localization, activity, and stability.[480, 481] Finally, protein regulators influence SIRT1 activity via physical interaction, such as the active regulator of SIRT1 (AROS)[482] and the neuronal protein necidin,[483] which serve as enhancers, or DBC1, which inhibits SIRT1 by binding to the core catalytic domain.[436]

Given the prospective therapeutic benefits of promoting or blocking SIRT1 activity under different conditions, researchers have been enthusiastically studying SIRT1 modulators. Resveratrol (SIRT1 $EC_{1.5}$ = 46.2 μM),[416] a polyphenol naturally occurring in the grape *Vitis vinifera*,[484] was initially identified as a sirtuin activator and subsequently shown to expand lifespan in yeast.[485] Although Resveratrol can promote the activity of SIRT1, it is still inconclusive and hotly debated whether the effects are direct or indirect, such as via AMPK-dependent mechanisms.[486] Resveratrol also targets numerous other enzymes and acts as a powerful antioxidant on its own, making its effects difficult to interpret.[487] Structurally unrelated imidazothiazole-containing SIRT1 activators with increased potency have been synthesized by Sirtris Pharmaceuticals, including SRT1460, SRT1720, SRT2104, SRT2183, and SRT2530; however, like Resveratrol, there remains much dispute over their biological actions.[488, 489]

Regardless of these controversies over mechanism, SIRT1 activators have been confirmed to have numerous health benefits in various species.[377] For example, Resveratrol, SRT1460, SRT1720, and SRT2183 have been reported to improve insulin sensitivity, lower blood glucose, and increase mitochondrial capacity in diet-induced and genetically obese mice.[416] Likewise, SRT1720, the most potent and specific of the Sirtris compounds (SIRT1 $EC_{1.5}$ = 0.16 μM, SIRT2 $EC_{1.5}$ = 37 μM, SIRT3 $EC_{1.5}$ > 300 μM),[416] was shown to extend lifespan of adult mice on a high-fat diet, in addition to decreasing obesity health risks.[431] Recently, pulmonary studies utilizing SRT1720 have shown how SIRT1 activation protects against emphysema in a mouse model of COPD [490] and allergen-induced inflammation in a

mouse model of asthma.[491] Importantly, SRT2104 and SRT2379 are currently in human clinical trials to study their effects during sepsis and diabetes (https://clinicaltrials.gov).

Unlike the controversy-laden field of SIRT1 activators, there is more clarity with regards to SIRT1 inhibitors. Among them, Sirtinol was the first compound reported to inhibit the activity of sirtuins (SIRT1 IC_{50} =131 μM, SIRT2 IC_{50} =46 μM).[492, 493] Newer, Sirtinol-related inhibitors with far greater potency, such as Salermide (SIRT1 IC_{50} = 43 μM), have been developed.[494] NAM and NAM-related analogues serve as adequate inhibitors *in vitro* by occupying the NAM subpocket of the NAD+ binding site. Yet, with an IC_{50} of ~85 μM towards SIRT1, and a concentration of <1 μM in mammalian serum, NAM is less likely to impair SIRT1 *in vivo*.[495-497] Additional subclasses of SIRT1 inhibitors include Cambinol (SIRT1 IC_{50} = 56 μM, SIRT2 IC_{50} = 59 μM),[498] Tenovin-6 (SIRT1 IC_{50} = 20 μM),[499] and Suramin (SIRT1 IC_{50} = 0.3 μM, SIRT2 IC_{50} = 1.2 μM).[499] However, the indole-based EX-527 is one of the most potent and selective SIRT1 inhibitors today (IC_{50} = 38nM), with negligible potency against SIRT2 (IC_{50} =19.6 μM) or SIRT3 (IC_{50} = 48.7 μM), and no activity against class I/II HDACs.[500] EX-527 preferentially binds SIRT1 while it is in complex with the yet-to-be-released 2'-O-acetyl-ADP-ribose, locking the deacetylase in a "closed" state.[375]

Originally, the impetus for developing potent sirtuin inhibitors stemmed from a desire to modulate cell survival and proliferation. Several studies have reported the benefits of SIRT1 pharmacological inhibition in the setting of cancer.[501, 502] In fact, Cambinol was first developed as a potential antitumor drug[498] and later discovered to display anti-inflammatory properties *in vivo*.[503] Although contrasting to SIRT1's reported anti-inflammatory roles, SIRT1 inhibitors have been successfully used in animal models to treat certain inflammatory disorders.[503-506] For example, administration of Sirtinol or Cambinol has been shown to reduce allergic airway inflammation and Th2 cytokine responses.[455, 507]

Summary of rationale and thesis aims

The ubiquitous human pathogen RSV predominantly causes mild respiratory tract infection. Nonetheless, RSV infection remains the leading global cause of LRTI in children, and a source of significant morbidity and mortality among the vulnerable including infants,

the elderly, and those with chronic respiratory diseases. Infants hospitalized with a severe RSV infection are at a greater risk for developing allergic asthma and recurrent wheezing later in life, suggesting that a chronic alteration of the pulmonary immune environment ensues even after successful viral clearance.

Activated pulmonary DCs drive innate and adaptive immune responses to viral pathogens through the production of proinflammatory cytokines and the activation of T cell responses in LDLNs. Detection of viral antigens by DCs requires signaling through endosomal TLRs. As RSV enters the host cell cytosol directly through membrane fusion, an alternate antigen delivery is necessary to reach these endosomal compartments. Our lab and others have shown that DCs rely on autophagy to mediate TLR-dependent activation, cytokine production, and effective APC function.

Autophagy is a conserved intracellular membrane trafficking pathway whereby cytoplasmic material is sequestered within double-walled vesicles and degraded upon fusion with lysosomes. This process maintains cellular metabolic equilibrium and promotes cell survival during physiological and pathological stress conditions. Within APCs, autophagy facilitates TLR signaling and MHC molecule peptide loading through delivery of viral antigens to acidified endosomes.

Interestingly, key proteins that orchestrate autophagosome formation are targeted by SIRT1, a NAD^+-dependent histone and protein deacetylase. SIRT1 impacts many areas of biology and pathophysiology, and several *Sirt1* variants have been linked to human metabolic disorders. Importantly, SIRT1 diversely regulates immune function, such as lymphocyte activation, proliferation, and differentiation, and macrophage cytokine secretion. However, the role of SIRT1 in DC biology and its subsequent impact on adaptive immunity has not been elucidated.

The major aims of this dissertation were to determine (a) whether SIRT1 regulated DC activation and autophagy-mediated processes during RSV infection (Chapter 3) and (b) whether the absence of SIRT1 activity altered the antiviral immune response (Chapter 4). To address these questions, SIRT1 within DCs was chemically and genetically targeted and the effects were examined in a mouse model of RSV infection. Results demonstrate that RSV-infected DCs lacking functional SIRT1 failed to appropriately upregulate autophagy and

cytokine production, while retaining the capacity to stimulate T cells and present antigen. Likewise, DC-specific *Sirt1* knockout mice (*Sirt1*$^{f/f}$*-CD11c-Cre*$^{+}$) exhibited exacerbated, prolonged RSV-induced lung pathology, highlighting the essential role of SIRT1-mediated DC cytokine production in fine-tuning the antiviral adaptive immune response. Altogether, these findings expand our understanding of the innate immune response during RSV infection and identify SIRT1 as a potential therapeutic target for the treatment and prevention of severe viral-induced lung pathology.

CHAPTER 2:
Materials & Methods

Chemicals and reagents

Bafilomycin (Santa Cruz Biotechnology, Dallas, TX), EX-527 (SIRT1 Inhibitor III, Calbiochem, Darmstadt, Germany), SRT1720 (Calbiochem), and Tunicamycin (Sigma-Aldrich, St. Louis, MO) were reconstituted in DMSO and diluted in culture medium for *in vitro* work. Based on previous reports,[375, 500] we chose a 1 μM EX-527 dose *in vitro,* with no significant changes in DC cytokine production at greater concentrations. We observed comparable viability in control and EX-527-treated cells by flow cytometry (Live/Dead Fixable Yellow, Life Technologies, Carlsbad, CA) and exclusion dye stain (Trypan Blue). For *in vivo* experiments, mice received daily intraperitoneal (i.p.) injections of 100 μL (1 mg/kg) EX-527 reconstituted in DMSO and diluted in normal saline; controls received DMSO-saline. Dose response assays revealed that administrating 10 mg/kg EX-527 to RSV-infected mice caused a rebound in *Sirt1* and a reversal of the phenotype observed at the 1 mg/kg EX-527 dose. 3-methyladenine (3-MA, Sigma-Aldrich) and recombinant mouse TNFα (R&D Systems, Minneapolis, MN) were reconstituted in PBS + 0.1% BSA and used at 10 μM or 10 ng/mL, respectively. Imiquimod (R837, InvivoGen, San Diego, CA) was reconstituted in endotoxin-free water and used at 1 μg/mL. Ovalbumin (Sigma-Aldrich) was reconstituted in PBS to a stock concentration of 2 mg/mL. RPMI 1640 (Lonza, Walkersville, MD) and HAM-F12 (Life Technologies) media were used for cell culturing. To induce amino acid starvation, the cell culture medium was exchanged with HBSS (Life Technologies).

Cell lines

MLE-12 and LA4 cells were purchased from ATCC (Manassas, VA). MLE-12 cells were maintained in HITES medium, a supplemented RPMI 1640-based medium (1X

insulin transferrin selenium-X, 100 µg/ml streptomycin, 100 U/ml penicillin, 10 nM β-estradiol, 10 nM hydrocortisone, 2% FBS). LA4 cells were cultured in HAM-F12 medium supplemented with 1% Pen/Strep + 10% FCS.

Mice

C57BL/6J (BL6), B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II), B6;129-*Sirt1tm1Ygu/J* (*Sirt1*$^{f/f}$), and C57BL/6J-Tg(Itgax-cre,-EGFP)4097Ach/J (CD11c-Cre-GFP) mice were purchased at 6–7 weeks of age from The Jackson Laboratory (Bar Harbor, ME). *Sirt1*$^{f/f}$ mice, in which two loxP sites flank *Sirt1* exon 4, were crossed to CD11c-Cre-GFP transgene mice. As the *Sirt1*$^{f/f}$ mice were on a mixed C57BL/6J;129 background, we backcrossed the *Sirt1*$^{f/f}$-*CD11c-Cre* progeny to a C57BL/6J background for 6 generations. The resultant *Sirt1*$^{f/f}$-*CD11c-Cre*+ mice harbor CD11c^{high} cells producing a truncated, catalytically inactive SIRT1, mimicking a *Sirt1*-null genotype.[448] All breeding took place in-house at the University of Michigan (Ann Arbor, MI). Animal work protocols were reviewed and approved by the University of Michigan University Committee on Care and Use of Animals.

Deletion of exon 4 was verified by DNA analysis. Day 10 BMDCs were cultured from *Sirt1*$^{f/f}$-*CD11c-Cre* mice, and their DNA was extracted, purified, PCR amplified, and separated by 2% agarose gel. Excision was assessed by the presence of an excision band using primers flanking the *Sirt1* exon 4 excision site, according to the Jackson Labs genotyping protocol (Forward = 5'-AGGCGGATTTCTGAGTTCGA, Reverse = 5'-CGTCCCTTGTAATGTTTCCC). The absence of *Sirt1* exon 4 was verified using homemade SYBR primers flanking the interior of the exon (Forward: 5'-TGAGCTGGGTGTGGTGGCG, Reverse: 5'-ATTCAATAGCCACATGCAGTCACA).

1 cycle	94°C	3 min
25 cycles	94°C	30 sec
	56°C	30 sec
	72°C	30 sec
1 cycle	72°C	2 min
1 cycle	4°C	Indefinite

Table 2.1: PCR settings for homemade *Sirt1* exon 4 primers.

Respiratory syncytial virus

The RSV strain 2-20, kindly provided by Dr. Martin Moore (Emory University, Atlanta, GA), was originally isolated from a severely ill RSV-infected infant.[95] Line 19 RSV (antigenic subgroup A), originally obtained from a sick infant at the University of Michigan Hospital System, was shown in animal models to mimic human infection by eliciting airway mucus production upon inoculation with 1 x 10^5 PFU (plaque forming units) RSV.[93] RSV strains were propagated in our laboratory in HEp-2 cells (ATCC). Mice were infected intratracheally (i.t.) via tongue-pull with 1.5 x 10^5 RSV.

Lung histology

Serial 6 μm sections were obtained from paraffin-embedded, 10% formalin-fixed left lungs stained with hematoxylin and eosin (H&E). Intracytoplasmic and luminal mucin was assessed by periodic acid-Schiff (PAS) stain. 5 sections were analyzed per mouse lung, with 2 lung slices/section/mouse to select representative slides. PAS-stained slides were blindly scored for goblet cell hyperplasia by light microscopy. The following scoring system was used: 1, absent; 2, staining in multiple airways; 3, staining in multiple airways with mucus plugging; 4, severe mucus plugging in multiple airways.

BMDC culture

Bone marrow–derived DCs (BMDCs) were isolated from whole bone marrow of naïve C57BL/6 mice, *Sirt1*$^{f/f}$*-CD11c-Cre*$^{+}$ mice, or littermate controls. Bone marrow cells were seeded into tissue culture flasks containing RPMI 1640–based complete medium (10% FCS, 1% penicillin/streptomycin, 1% glutamate (Life Technologies), 1% Na Pyruvate (Life Technologies), 1% non-essential amino acids (Life Technologies), 50 μM β-mercaptoethanol) supplemented with 15 ng murine GM-CSF/mL (R&D Systems, Minneapolis, MN) at 10% CO_2, 37°C. C57BL/6 mouse-derived cells were fed on days 3 and 5 and harvested on day 7, a time point by which cells were ≥85% $CD11b^+$ $CD11c^+$ BMDCs by flow cytometric analysis. Cells derived from the *Sirt1*$^{f/f}$*-CD11c-Cre*± mice were cultured for 10 days (fed on days 3, 5, and 7) to achieve high Cre activity.

AEC culture

Alveolar epithelial cells (AECs) were isolated from whole lungs of naïve mice. Lungs were digested in Dispase (BD Biosciences), filtered through 25 µm mesh, and depleted of immune cells through labeling with biotinylated antibodies to CD16/32 and CD45 (BD Pharmingen, San Diego, CA), followed by labeling with anti-biotin microbeads and passage through a MACS column (Miltenyi Biotec, Auburn, CA). 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.

Lymph node re-stimulation and protein quantification

Lymph nodes were isolated by mechanical disruption, cultured, and then restimulated with RSV for 48 h before collecting supernatants for protein analysis on a Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA), according to the manufacturer's protocol. Custom kits containing antibody-coated beads for mouse IL-4, IL-5, IL-13, IL-17a, and IFNγ were used to assay cytokine concentration (Bio-Rad). Results are reported as fold difference over concentrations in control or unstimulated cells.

Stimulation of whole cell populations from mouse spleen

Total cell populations (APCs, T cell, etc.) were isolated from minced spleens from *Sirt1*^{ff}*-CD11c-Cre*± mice. Once minced, spleens were forced through a 100 µm nylon strainer. After red blood cell (RBC) lysis, single cell suspensions were plated at 2 x 10^6 in 24-well culture plates and stimulated with 2 µg/mL of anti-CD3 (eBioscience). After 72 h, cell supernatants were collected and analyzed by Bio-Plex, as described under "LN re-stimulation and protein quantification."

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

RSV-responsive CD4+ T cells were isolated from mediastinal LN of BL6 mice 8 dpi. Ovalbumin (OVA)-responsive T lymphocytes were isolated from minced spleens from OT-II transgenic mice. LN or minced spleens were forced through a 100 µm nylon strainer and

underwent RBC lysis. Then, $CD4^+$ T cells were isolated via magnetic bead selection: negative selection protocol for $CD4^+$ T cells; positive selection for $CD11c^+$ cells to eliminate residual APCs (Miltenyi Biotec). 5 x 10^5 T were plated per well in 96-well culture plates, on top of 5 x 10^4 DCs treated 2 h previously with 1:1 MOI RSV, and where indicated pre-treated with 10 µM EX-527 for 30 min prior to infection. In experiments conducted with OT-II T cells, DCs were treated with 200 µg/mL whole OVA for 4 h prior to co-culture with OT-II T cells. Co-cultures were collected after 24 h for mRNA analysis, or after 48 h for supernatant cytokine analysis by Bioplex, as described under "LN re-stimulation and protein quantification."

Quantitative PCR

RNA was extracted as per Trizol reagent protocol (Life Technologies) or per Qiagen RNeasy Mini Kit protocol (Hilden, Germany), and 5-10 µg were reverse transcribed into cDNA. mRNA was determined using pre-developed Life Technologies primer/probe sets, TaqMan Universal PCR Master Mix (Life Technologies) or SYBR Green PCR Master Mix (Life Technologies), and analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Transcription levels of *Muc5ac*, *Gob5*, *Ifnb*, and *RSV-G*, *RSV-F*, and *RSV-N* were assessed using custom SYBR primers, as previously described.[160] *Xbp1s* and *Xbp1us* were assessed with the following custom mouse-specific SYBR primers: Xbp1sF: 5'-CTGAGTCCGAATCAGGTGCAG; Xbp1s/usR: 5'-GTCCATGGGAAGATGTTCTGG; Xbp1usF: 5'-CAGCACTCAGACTATGTGCA. The cycling threshold value of the endogenous control gene, *Gapdh*, was subtracted from the cycling threshold (ΔC_T). Expression of target genes is presented as fold change (Δ) values relative to an uninfected, untreated, or wild-type (WT) control group ($2^{-\Delta\Delta CT}$), assigned an arbitrary value of 1.

Transfection and RNA interference

Small interfering RNAs (Dharmocon, Lafayette, CO: L-049440-00-0005, siSIRT1; D-001810-10-05, siControl) were introduced via electroporation at 20 µM/sample according to manufacturer's instructions with an Amaxa Nucleofector Kit and Nucleofector

II Device (LonzaCologene, Cologne, Germany). Transfection efficacy was verified by qPCR, demonstrating 75-96% decrease in *Sirt1* expression. Viability of cells after 48 h post-transfection, while maintained in DC growth medium, was ≥80% and not different from the transfection control cells. 48 h post-transfection, the DCs were infected with RSV for 2 h.

Immunoprecipitation and immunoblotting

Treated BMDCs were washed with PBS and treated with RIPA Lysis Buffer (#9806, Cell Signaling Technology) supplemented with PMSF (Sigma-Aldrich) for 30 min on ice, vortexed for 1 min at 15-min mark. After maximum centrifugation for 10 min, the supernatant was saved as whole cell lysate (WCL, input) and for immunoprecipitation (IP). For IP, cell lysates were mixed with acetyl-Lysine antibodies (#9441) at 4°C overnight followed by the addition of Protein A–Sepharose 4B beads (1:100 Life Technologies) for 2 h at 4°C. Immune complexes were washed 3x with lysis buffer. After boiling in NuPAGE LDS Buffer and Reducing Agent (Life Technologies), samples were run on NuPAGE precast gels (Life Technologies) and transferred to nitrocellulose by an iBlot Dry Transfer System (Life Technologies). Membranes were blocked with 5% milk or BSA (Sigma-Aldrich) in 1x TBST (10x TBS (Corning, Manassas, MA) + 0.1% Tween) at RT, incubated with the indicated 1° antibodies overnight at 4°C (Table 2.2), washed 3x in TBST, and finally incubated with the appropriate horseradish peroxidase-conjugated 2° antibodies for 1-2 h RT (Table 2.2). Bands were visualized by chemiluminesence (SuperSignal West Femto, Life Technologies) on a Bio-Rad Imager. As necessary, blots were stripped with Restore Stripping Buffer (Life Technologies). Captured gel images were inverted and saved as .tif files using Image Lab software (Bio-Rad). To calculate mean intensity of fluorescence (MIF), images were opened in Adobe Photoshop CS 6.0, transformed into gray scale, and rotated to level the bands. Using an identical marquee box, bands were sequentially selected to record the MIF off the histogram function. Mean background was calculated as the MIF of the area immediately below each band. Background was subtracted from each respective MIF value, and the corrected values are reported as ratios over β-actin or whole ATG protein.

Reagent	Company and Catalog #	Dilution
Acetylated-Lysine polyclonal Ab	Cell Signaling, #9441	1:100
Acetylated-Lysine rabbit mAb (HRP conjugate)	Cell Signaling, #6952	1:1000
β-actin anti-mouse mAb	Sigma-Aldrich A2228	1:5000
Sirt1 rabbit anti-mouse mAb	Cell Signaling, #3931	1:500
Atg-5 Rabbit mAb	Cell Signaling, #8540	1:1000
Atg-7 Rabbit mAb	Cell Signaling, #2631	1:1000
LC3A/B Rabbit polyclonal Ab	Cell Signaling, #4108	1:500
Goat anti-mouse IgG-HRP (2º)	Santa Cruz Biotechnology, sc-2031	1:50,000
Goat anti-rabbit IgG-HRP (2º)	Santa Cruz Biotechnology, sc-2004	1:2000
Anti-biotin, HRP-linked Ab	Cell Signaling, #7075	1:50,000
Biotinylated Protein Ladder	Cell Signaling, #7727	
EZ-Run pre-stained *Rec* Protein Ladder	Fischer Scientific, #BP3603500	

Table 2.2: Antibodies and ladders used for immunoblotting.

Confocal microscopy

LC3 staining: BMDCs were cultured as described, and then plated in Lab-Tek chamber slides (Thermo Fisher Scientific, Waltham, MA). Cells were treated as indicated, washed in PBS, fixed in 4% paraformaldehyde for 20 min, and then blocked for 1 h at room temperature (RT) in PBS + 5% normal goat serum + 0.1% Tween-20. Cells were subsequently incubated with 1º antibody rabbit polyclonal anti-LC3B (NB600-1384, Novus Biologicals, Littleton, CO) for 2 h at 37ºC, and then incubated with 2º antibody goat anti-rabbit Alexafluor 568 (A11011, Life Technologies). ProLong Gold antifade reagent plus DAPI (Life Technologies) was added before the cells were imaged on a Nikon A1 Confocal Laser Microscope system under oil immersion, using NIS Elements acquisition software (Nikon Instruments). The number of LC3 puncta/cell was counted in at least 15 cells/sample/condition/experiment. Images were converted to B&W in Adobe Photoshop to easily distinguish background staining from actual puncta.

Caspase-3 staining: After 2 h of HBSS-induced starvation or RSV-infection, 2 drops/mL of Caspase 3/7 Green ReadyProbe (Life Technologies) were added to each Lab-Tek. Cells were washed, fixed, and incubated with ProLong Gold, as described above.

Transmission electron microscopy (TEM)

Cells were spun down into a gelatin capsule with a clinical centrifuge. Cell pellets were fixed in 4% gluteraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1 h. The cell pellets were washed 2x in 0.1 M cacodylate buffer, postfixed with 2% Osmium tetroxide for 1 h on ice and then rinsed 2x with 0.1M cacodylate buffer. The fixed cell pellets were dehydrated through an ethanol (ETOH) dilution series up to 100% ETOH and then immersed in propylene oxide (PO) for 10 min 2x. Next, pellets were infiltrated in a 1:3 (1 h), 1:1 (2 h), and finally 3:1 (overnight) eponate resin: PO mixture while rotating at RT, and subsequently embedded in 100% Eponate resin in gelatin capsules and allowed to harden in a 65°C oven overnight. After hardening, tissue blocks were ultra-thin-sectioned at a 70 nm thickness and placed on 200 mesh copper grids. Grids were counterstained with saturated uranyl acetate and lead citrate and then viewed on a Philips CM-100 electron microscope. 5 grids, ~ 15-20 cells/grid, were studied per treatment.

Flow cytometry

Right lungs of control and RSV-infected mice were digested enzymatically in RPMI-1640 complete media containing 1 mg/mL Collagenase A (Roche Applied Science, Indianapolis, IN) and 30 µg/mL DNase I (Sigma). BMDCs were washed from culture treatment. Cells were incubated with Live/Dead Fixable Yellow (Life Technologies) for 30 min on ice, blocked with anti-CD16/CD32 for 20 min RT with gentle shaking, and stained for 20 min RT with gentle shaking with appropriate antibodies as indicated in Table 2.3. Flow-Count Fluorospheres (Beckman Coulter, Pasedena, CA) were added right before flow cytometry to quantify absolute cell counts. Data were collected on a BD Biosciences LSR II flow cytometer and on a BD Biosciences FACS Aria, and analyzed using FlowJo software (Treestar Inc, Ashland, OR).

Sorted pulmonary cell populations

Pulmonary CD11b$^+$ DCs, CD103$^+$ DCs, and alveolar macrophages were isolated from lungs/bronchi of *Sirt1$^{f/f}$-CD11c-Cre*± mice by enzymatic digestion as previously published.[225] Briefly, minced tissue was incubated in RPMI-1640 with 200 µg/mL Liberase

TM (Roche Applied Science, Indianapolis, IN) and 200 U/ml DNase I (Sigma-Aldrich) for 45 min at 37°C, drawn through an 18-gauge needle/10cc syringe, and filtered through 40 µm nylon mesh. The cell suspension was enriched for $CD11c^+$ cells using anti-mouse $CD11c^+$ microbeads and magnetic column separation (Miltenyi Biotec); stained with anti-CD11b, anti-CD103, and anti-SigF antibodies (Table 2.3, Lung Sort); and finally sorted with an iCyt Synergy 3200 cell sorter (iCyt, Champaign, IL).

Antibody	Conjugate	Company	Catalog #	Dilution	Purpose
Ly6C	PE	BioLegend	128008	1:400	Lung Sort
CD103	APC	BioLegend	121414	1:200	Lung Sort
MHCII	Alexa Fluor 700	BioLegend	107622	1:1000	Lung Sort
CD11c	APC-Cy7	BioLegend	117324	1:200	Lung Sort BMDC Sort
CD11b	eFluor 450	eBioscience	48-0112-82	1:400	Lung Sort BMDC Sort
CD3	PE	BDPharmigen	553063	1:200	Lung Sort
B220	PerCP-Cy5.5	BDPharmigen	552771	1:200	Lung Sort
CD19	APC	BioLegend	115512	1:200	Lung Sort
CD4	APC-Cy7	BioLegend	100414	1:200	Lung Sort
CD8α	Pac Blue	BioLegend	100725	1:200	Lung Sort
ILR5α	PE	BDPharmigen	555902	1:200	Lung Sort
Ly6G	PE-Cy7	BDPharmigen	560601	1:400	Lung Sort
Siglec-F (SigF)	PE	BDPharmigen	552126	1:200	Lung Sort
CD11b	PerCP-Cy5.5	BDPharmigen	550993	1:200	Splenic Sort
CD11c	APC	BDPharmigen	550261	1:200	Splenic Sort
CD4	PE-Cy7	BDPharmigen	552775	1:200	Splenic Sort
CD8	Alexa 700	eBioscience	56-0081-82	1:200	Splenic Sort
B220	APC-Cy7	BioLegend	103224	1:200	Splenic Sort
CD115	PE	eBioscience	12-1152-83	1:200	Splenic Sort
CD40	PE	BDPharmigen	553791	1:200	BMDC Sort
CD80	PerCP-Cy5.5	BioLegend	104722	1:200	BMDC Sort
CD86	PE-Cy7	BioLegend	105014	1:200	BMDC Sort
MHCII	APC	eBioscience	17-5321-82	1:1000	BMDC Sort
CD16/32	--	BioLegend	101302	1:200	All
Live/Dead	--	Life Technologies	L-34968	1:2000	All

Table 2.3: Antibodies for mouse flow cytometry and flow sorting.

Cell apoptosis assay

The extent of necrosis and apoptosis in RSV-infected *Sirt1*^{ff}*-CD11c-Cre* BMDCs was determined using Annexin-V-APC and propidium iodide (PI). BMDCs were infected 1:1 with RSV 2-20 for 2 h in a 96-well culture plate. After thorough washing, cells were incubated with Annexin-V-APC (1:200) for 15 min at 25 °C in the dark, washed, incubated under the same conditions with PI (1:200), and assessed immediately on a BD Biosciences LSR II flow cytometer. Raw data was analyzed as described above. Viable cells are negative for both PI and Annexin-V; early stage apoptotic cells are positive for Annexin-V and negative for PI; necrotic cells were positive for PI and negative for Annexin-V; late stage apoptotic cells are positive for both Annexin-V and PI. Readings were recorded as mean fluorescence intensity (MFI).

Statistics

All data are presented as mean ± SEM. Data were evaluated and graphs were generated using GraphPad Prism software. Statistical significance was assessed by one-way ANOVA, followed by Newman–Keuls post-test. Significant differences were regarded as $p \leq 0.05$.

CHAPTER 3:
SIRT1 regulates DC activation & autophagy-mediated processes

Sirt1 is significantly upregulated in DCs during RSV infection

In Chapter 1, we described the importance of SIRT1 immune function and several studies studying SIRT1 in the context of pulmonary disease. To determine whether *Sirt1* regulates RSV-induced disease, *Sirt1* mRNA levels were measured in RSV-infected C57BL/6J WT mice. Maximal *Sirt1* expression was observed 4 days post-infection (dpi) in lung tissue, coinciding with peak viral load (Figure 3.1).[7]

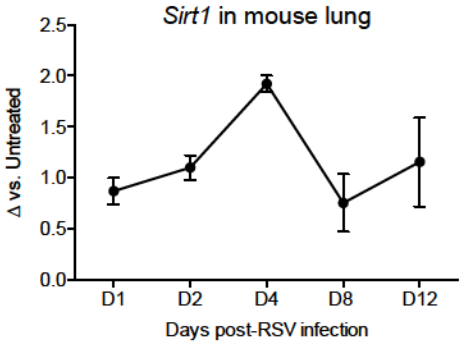


Figure 3.1: *Sirt1* is upregulated in a mouse model of RSV infection. Lung mRNA expression of *Sirt1* in RSV-infected WT BL6 mice was obtained using qPCR and compared with naive controls. Values represent mean ± SEM, 5 mice per group.

We next assessed whether there were cell-specific differences in *Sirt1* expression. *Sirt1* mRNA levels were examined in: mouse BMDCs; primary mouse pulmonary DC subsets; primary mouse alveolar macrophages; human monocyte-derived DCs; two immortalized pulmonary epithelial cell lines, MLE and LA4; and primary mouse AECs (Figure 3.2A-E). While all cell populations showed increased *Sirt1* expression over untreated controls, the DCs had the highest increase (BMDCs ~400-fold; pulmonary DCs and hDCs: ~4-fold). Additionally, *Foxo3* expression rose in sync with *Sirt1* in RSV-infected DCs, supporting SIRT1's roles in cellular metabolism and stress response (Figure 3.2A). Due to the central role of DCs in directing immune responses, these cells became the focus of our studies.

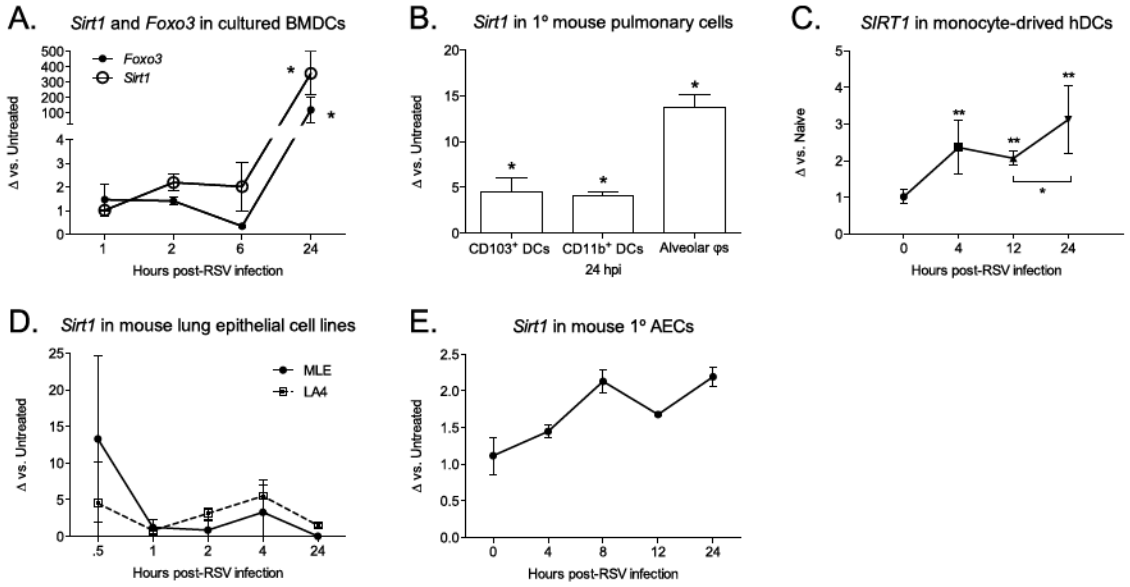


Figure 3.2: RSV-infected DCs greatly upregulate *Sirt1*. mRNA expression of *Sirt1* and *Foxo3* in WT BL6 BMDCs, primary WT BL6 pulmonary DCs and alveolar macrophages, human monocyte-derived DCs, MLE cells, LA4 cells, and primary WT BL6 mouse AECs, as analyzed by qPCR and compared to untreated controls. Data are representative of at least 2 independent experiments, with at least 3 replicates/group. Values represent mean ± SEM. $*p < 0.05$.

SIRT1 promotes efficient DC activation and autophagosome formation

Our laboratory has previously demonstrated that efficient TLR-dependent DC maturation and innate cytokine production in response to RSV depend on autophagy.[233] Given the reported relationship between SIRT1 and autophagy proteins,[373] DC activation upon RSV infection was assessed in the presence of SIRT1 inhibition compared to autophagy inhibition. Cultured BMDCs infected for 2 hours in the presence of the SIRT1 inhibitor EX-527 or 3-methyladenine (3-MA), an autophagy inhibitor, expressed lower levels of inflammatory cytokine genes compared to RSV-infected BMDCs (Figure 3.3A). These studies also demonstrated a significant reduction in cytokine production within EX-527 or 3-MA treated DCs stimulated with imiquimod, a TLR7 agonist (Figure 3.3B). Additionally, SRT1720, a SIRT1 activator, caused a modest increase in cytokine production in RSV-infected BMDCs (Figure 3.3C). Thus, SIRT1 is necessary for endosomal TLR stimulation and efficient DC cytokine production and autophagy in response to RSV infection.

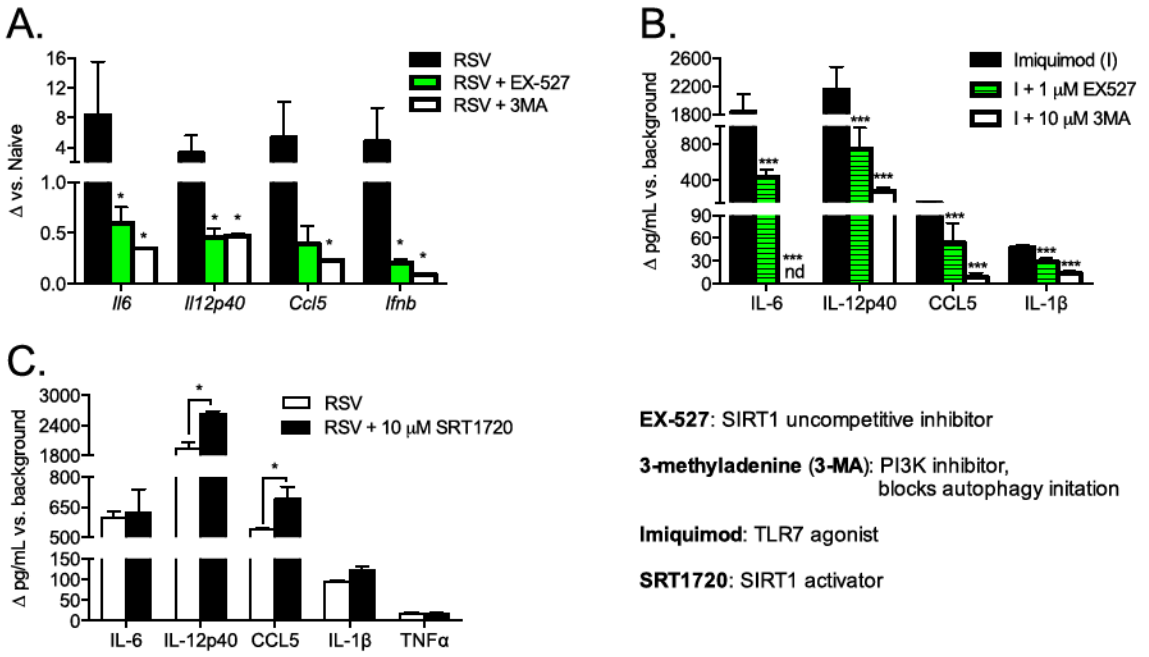


Figure 3.3: Modulation of SIRT1 activity affects DC cytokine production. (A) WT BMDCs cultured from BL6 mice were treated with DMSO, 1 μM EX-527, or 10 μM 3-MA for 30 min before being infected with Line 19 RSV (1:1 MOI) for 24 h. Cytokine gene expression was determined by qPCR. (B) WT BL6 BMDCs were pre-treated as in (A), and then stimulated for 4 h with 1 μg/mL imiquimod. (C) WT BL6 BMDCs were treated with 10 μM SRT1720 for 30 min before infection as in (A). Cytokine protein concentrations (B, C) were measured by Bio-Plex assay. Data are representative of 2 independent experiments, at least 3 replicates/group. Values represent mean ± SEM. For (A, B) stars indicate significance vs. black treatment bars. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

The aforementioned results obtained using EX-527 and 3-MA prompted experiments to determine whether SIRT1 was necessary for efficient autophagy in DCs. Cultured BMDCs were treated with EX-527, infected with RSV for 2 hours, and immunostained for LC3 (ATG8), a key marker for autophagosomes. Confocal staining for LC3 revealed an increase in autophagosomes during RSV infection, supporting previous reports from our lab (Figure 3.4A).[225, 233] However, EX-527 treatment of RSV-infected BMDCs dramatically reduced the number of autophagosomes compared to infected control cells (Figure 3.4A, B). In addition, the EX-527-treated, RSV-infected cells did not have an activated morphology, i.e. the formation of dendrite-like projections. On the other hand, SRT1720-treated, RSV-infected BMDCs had similar numbers of autophagosomes as RSV-infected controls, suggesting SIRT1 activation may reach a threshold in the presence of a viral stimulus (Figure 3.4A, B).

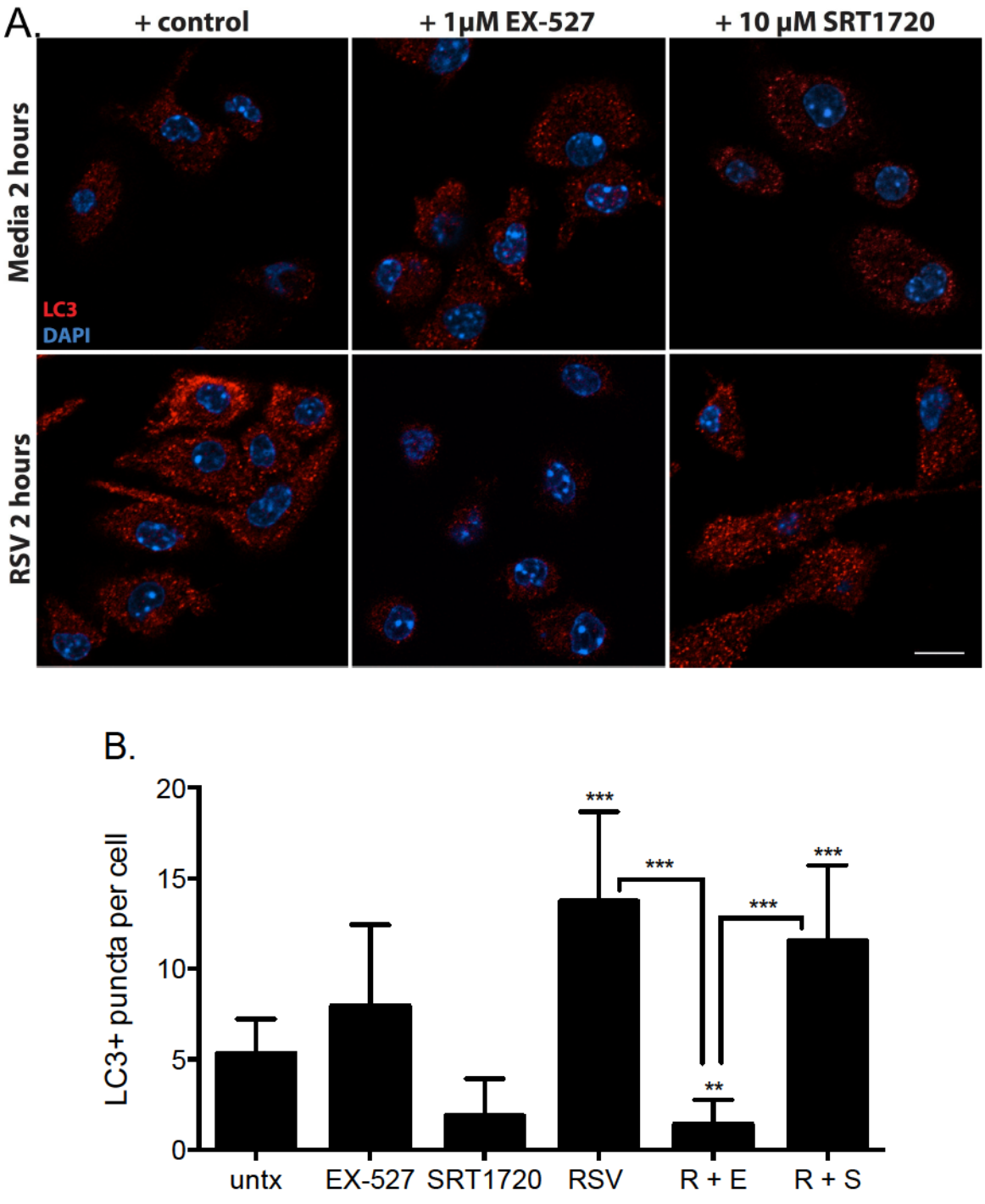


Figure 3.4: Inhibition of SIRT1 attenuates autophagy in RSV-infected DCs. (A) Number of autophagosomes in WT BL6 BMDCs by 2 h post-RSV 2-20 infection (R) ± 1 μM EX-527 (E) or ± 10 μM SRT1720 (S) was assessed by punctate LC3 staining and confocal microscopy (scale bar = 10 μm); data was quantified in (B) compared to respective controls. Data are representative of 2 independent experiments, 3 replicates/group. Values represent mean ± SEM. $^{**}p < 0.01$. $^{***}p < 0.001$.

To further explore SIRT1's promotion of autophagy, DCs were depleted of SIRT1 using *Sirt1*-specific siRNA. Examination by confocal microscopy demonstrated that, in presence of *Sirt1* siRNA, fewer autophagosomes were found in RSV-infected BMDCs (Figure 3.5A, B). Additionally, *Sirt1* knockdown significantly downregulated *Ifnb* expression during RSV infection and reduced the levels of other innate cytokines, *Il1b* and *Ccl5* (Figure 3.5C). Overall, these results illustrate that SIRT1 is crucial for effective autophagy-dependent DC-activation using both pharmacological and siRNA knockdown approaches.

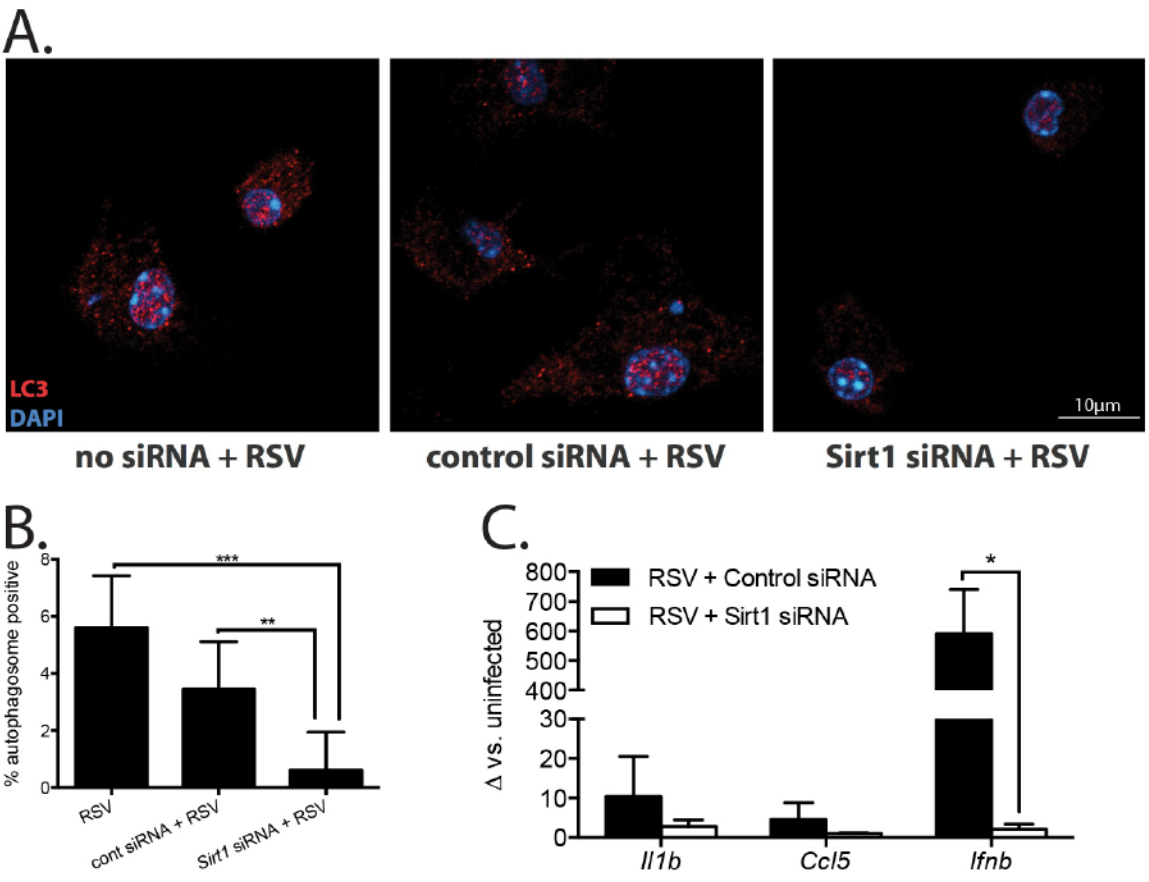


Figure 3.5: Repressed autophagy and autophagy-dependent innate cytokine production in *Sirt1* siRNA-treated BMDCs. (A) WT BL6 BMDCs were transfected by electroporation with appropriate siRNA 48 h prior to RSV 2-20 infection (MOI 1:1). 2 h later, autophagosomes were observed by confocal microscopy, and quantified in (B). Scale bar = 10 μm. (C) Innate cytokine gene expression assessed by qPCR in WT BL6 BMDCs 24 h post-RSV infection. Cells were transfected with control or *Sirt1*-specific siRNA 48 h prior to infection. Data are representative of 2 independent experiments, 3 replicates/group. Values represent mean ± SEM. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

DCs from *Sirt1$^{f/f}$-CD11c-Cre^{+}* mice have altered cytokine production and autophagy in response to RSV

To investigate the role of *Sirt1* within DCs during RSV-induced responses in a more physiologic setting, we generated conditional knockout mice (*Sirt1$^{f/f}$-CD11c-Cre)*, where the Cre^{+} progeny express catalytically inactive SIRT1 in CD11c^{+} myeloid cells. We verified *Sirt1* excision in Cre^{+} BMDCs (Figure 3.6A). We also observed no baseline differences in the number of pulmonary immune cell subtypes from naive *Sirt1$^{f/f}$-CD11c-Cre* mice or in splenic cell subsets from RSV-infected *Sirt1$^{f/f}$-CD11c-Cre* mice (Figure 3.6B-D).

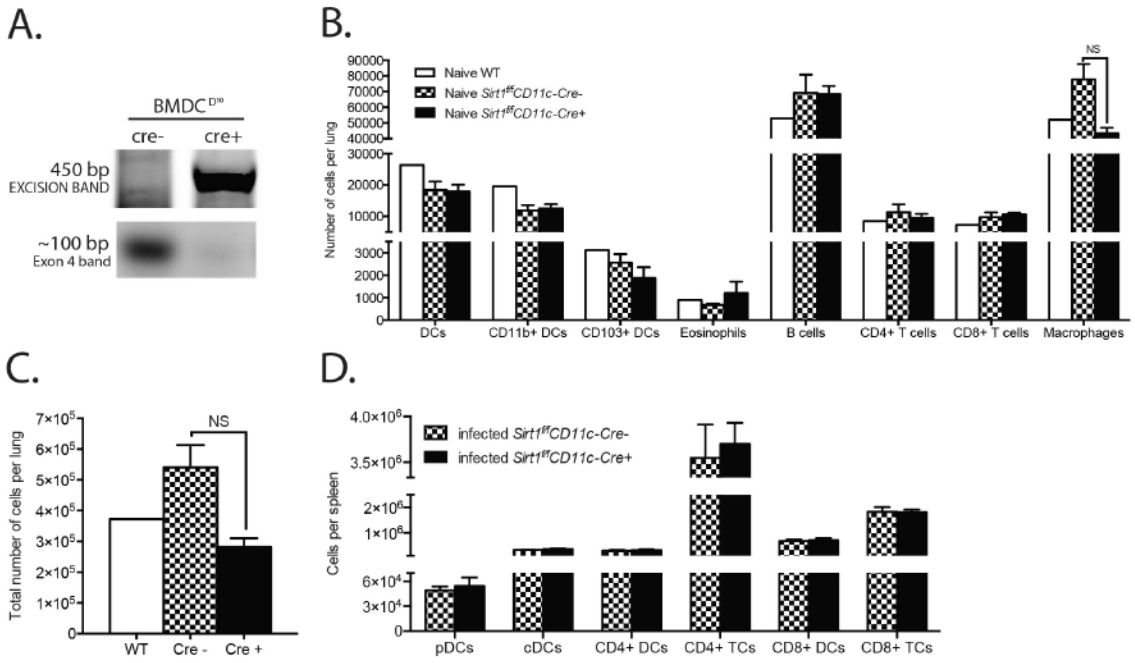


Figure 3.6: Characterization of *Sirt1$^{f/f}$-CD11c-Cre* mice. (A) DNA from Day 10 BMDCs cultured from DC-*Sirt1* KO mice was extracted, purified, PCR amplified, and finally separated by 2% agarose gel. The top row illustrates the expected excision band created using primers flanking the *Sirt1* exon 4 excision site in Cre^{+} mice and the absence of this excision band in Cre^{-} mice. Note the negligible amount of *Sirt1* exon 4 DNA in Cre^{+} BMDCs. Data representative of >5 independent samples. (B, C) Total numbers of pulmonary immune cell populations obtained from collagenase-digested lungs of 3 naive WT BL6, 3 *Sirt1$^{f/f}$-CD11c-Cre^{-}*, or 3 *Sirt1$^{f/f}$-CD11c-Cre^{+}* mice, as determined by flow cytometry. (D) Flow cytometric analysis of the total numbers of DC and T-cell populations isolated from the spleens of *Sirt1$^{f/f}$-CD11c-Cre* mice 8 dpi, 5 mice/genotype.

As shown in Figure 3.7A, RSV-infected CD11b$^+$ pulmonary DCs isolated from *Sirt1$^{f/f}$-CD11c-Cre$^+$* mice showed reduced cytokine gene expression compared to Cre$^-$ controls. Similarly, Cre$^+$ BMDCs did not upregulate inflammatory cytokine genes in response to RSV, in contrast to Cre$^-$ BMDCs (Figure 3.7B). Moreover, TLR7-stimulated Cre$^+$ BMDCs produced significantly less cytokine as compared to Cre$^-$ BMDCs (Figure 3.7C). This data further supports the hypothesis that SIRT1 is essential for robust TLR-mediated DC cytokine production during RSV infection.

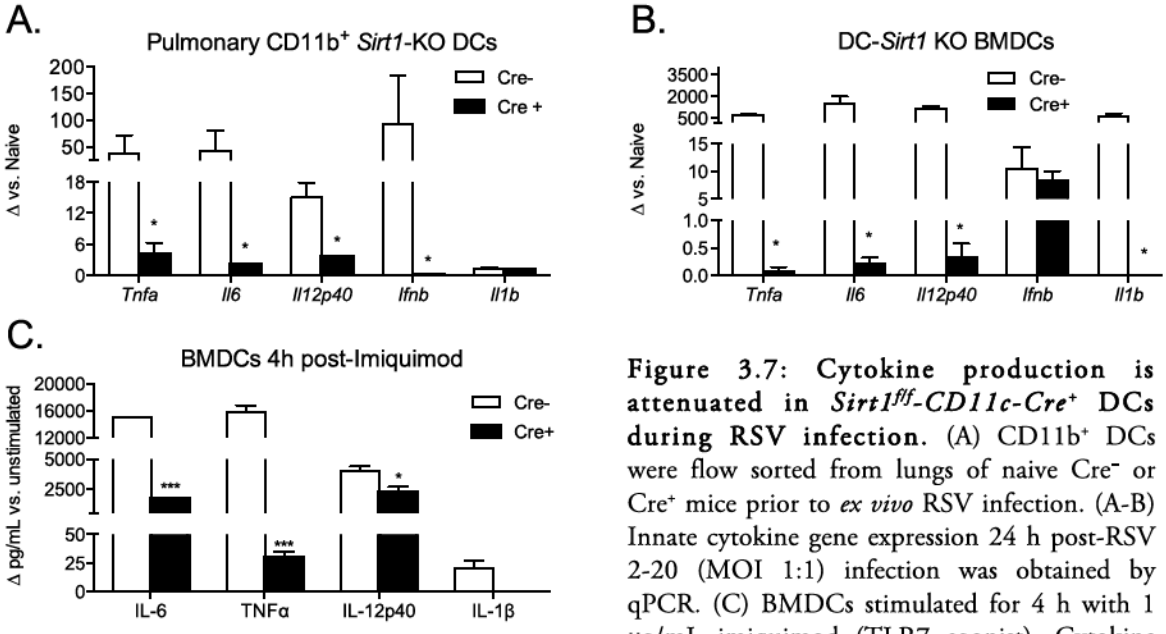


Figure 3.7: Cytokine production is attenuated in *Sirt1$^{f/f}$-CD11c-Cre$^+$* DCs during RSV infection. (A) CD11b$^+$ DCs were flow sorted from lungs of naive Cre$^-$ or Cre$^+$ mice prior to *ex vivo* RSV infection. (A-B) Innate cytokine gene expression 24 h post-RSV 2-20 (MOI 1:1) infection was obtained by qPCR. (C) BMDCs stimulated for 4 h with 1 μg/mL imiquimod (TLR7 agonist). Cytokine concentrations were measured by Bio-Plex assay. Data are representative of at least 2 independent experiments. Values represent mean ± SEM, 3 replicates/group, 5 mice/sort. Stars indicate significance vs. infected Cre$^-$ DCs. *$p < 0.05$. ***$p < 0.001$.

With regard to autophagosome numbers, *Sirt1*-deficient BMDCs had significantly attenuated punctate LC3 staining during HBSS starvation-induced autophagy, compared to their littermate controls (Figure 3.8A, B). While the Cre$^+$ BMDCs showed an increase in LC3 puncta after RSV infection, the number of autophagosomes was significantly reduced compared to infected Cre$^-$ BMDCs (Figure 3.8A, B). Interestingly, *Atg8/Map1lc3b* mRNA expression was upregulated in Cre$^-$ BMDCs, but downregulated in Cre$^+$ BMDCs during RSV infection, suggesting that SIRT activity may also promote autophagy at the transcriptional level (Figure 3.8C). Therefore, these data provide genetic evidence of our

previous observations and further indicate that *Sirt1*-deficient DCs harbor defects in their ability to mount productive anti-RSV responses.

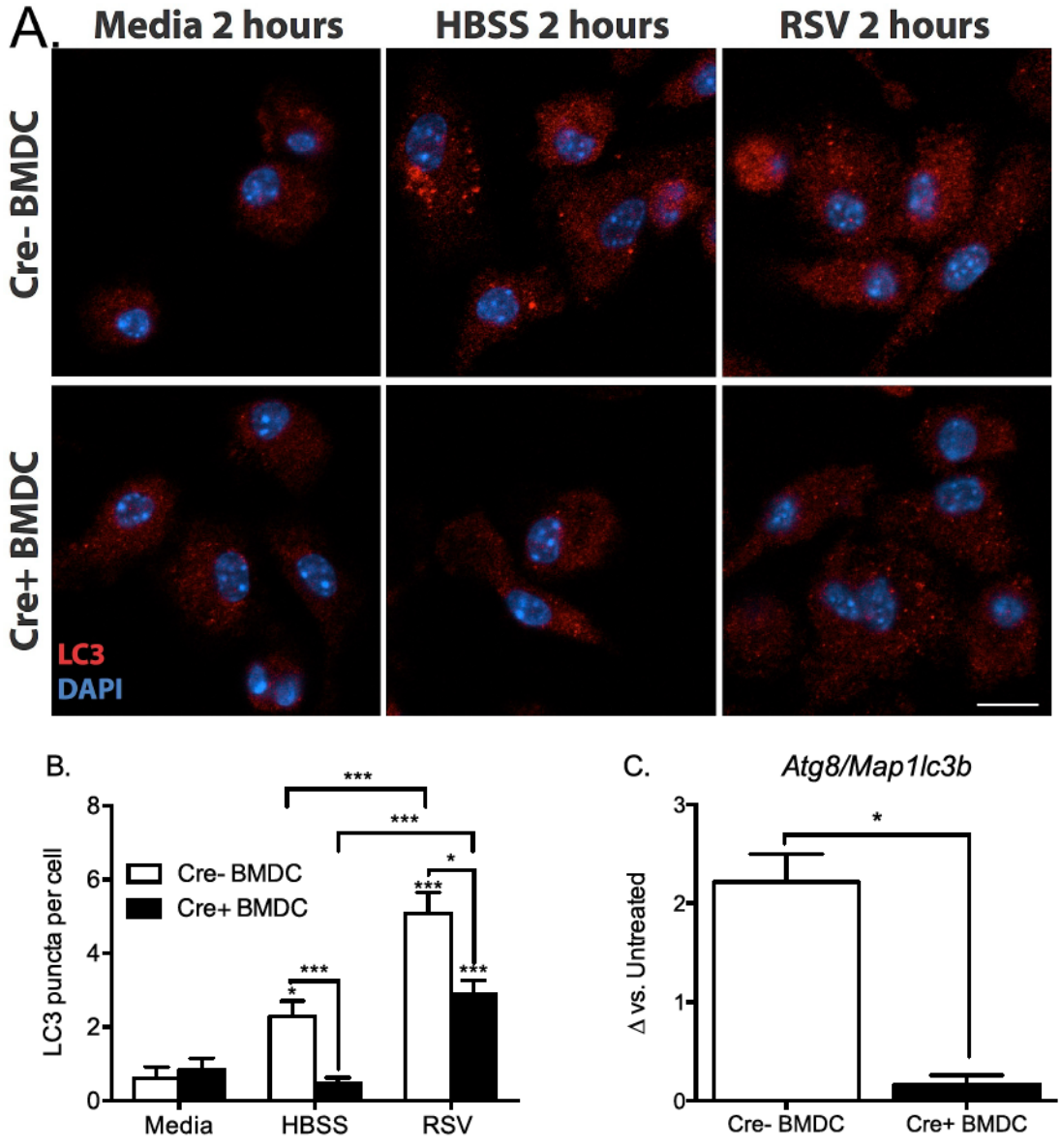


Figure 3.8: *Sirt1*$^{f/f}$*-CD11c-Cre*$^{+}$ DCs form fewer autophagosomes in response to starvation or RSV infection. (A) Number of autophagosomes in Cre^{-} and Cre^{+} BMDCs 2 h after no treatment, HBSS treatment (starvation medium), or RSV 2-20 infection (MOI 1:1), as assessed by LC3 immunostain and confocal microscopy. Data from (A) quantified in (B) as compared to media-only controls. Scale bar = 10 μm. (C) *Atg8* gene expression 24 h post-RSV 2-20 (MOI 1:1) infection obtained by qPCR. Data are representative of 3 independent experiments. Values represent mean ± SEM, 3 replicates/group. *$p < 0.05$. ***$p < 0.001$.

Recently it has been reported that LC3 can be recruited to other membranes, including single-membrane vesicles, in a process known as LC3-associated phagocytosis (LAP).[508] While LAP and autophagy both produce punctate LC3 immunostaining, a key distinguishing ultrastructural feature between these processes is the formation of a single versus double-walled vesicle.[244] To verify that the *Sirt1*-deficient DCs were not forming autophagosomes, starved or RSV-infected Cre⁻ and Cre⁺ BMDCs were examined by transmission electron microscopy (TEM). Double-walled and single-walled membranes were observed in Cre⁻ BMDCs at baseline, after starvation, and after RSV infection, whereas double-walled autophagosomes or autolysosomes were undetected in *Sirt1*-deficient BMDCs under the same conditions (Figure 3.9). These TEM data validate the confocal microscopy data that the formation of autophagosomes was severely impaired in Cre⁺ DCs.

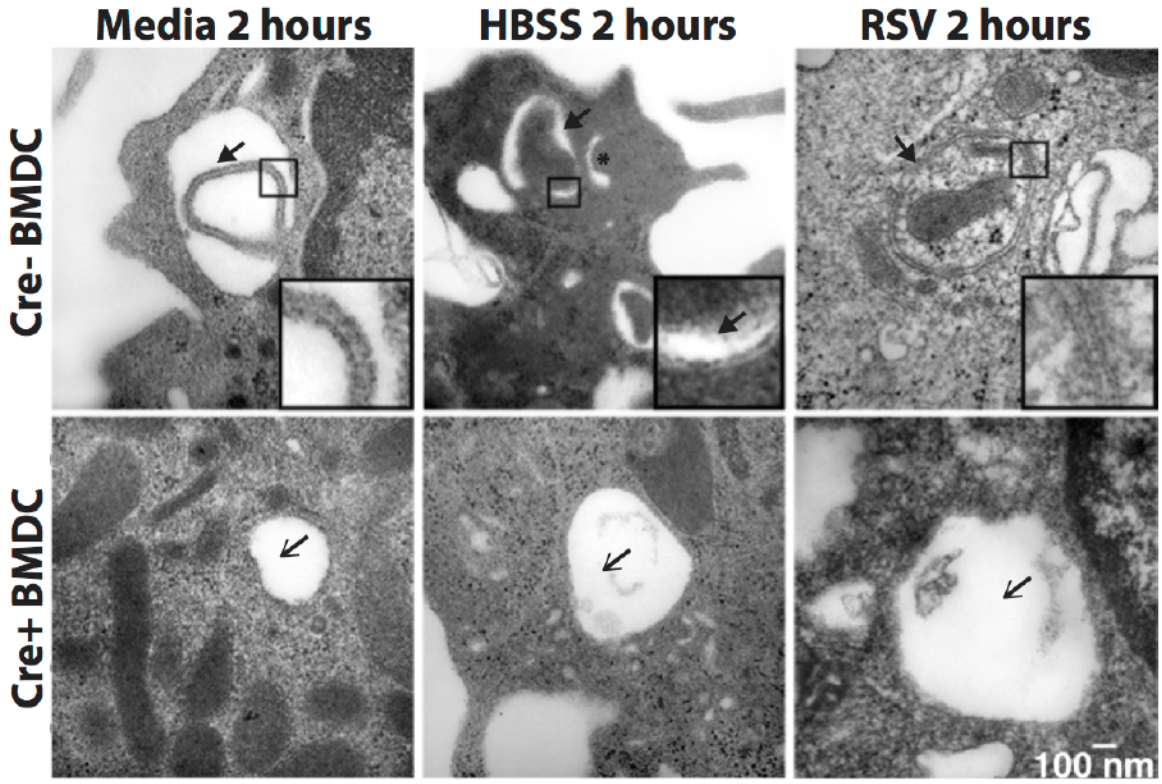


Figure 3.9: Ultrastructural analysis of BMDCs from *Sirt1$^{f/f}$-CD11c-Cre* mice during stress conditions. Representative TEM images of Cre⁻ and Cre⁺ BMDCs, which were untreated (media only), starved with HBSS medium, or infected with RSV 2-20 for 2 h. Scale bar = 100 nm. Note the double-membranes in the insets (Cre⁻ panel). Thick arrowheads indicate autophagosomes. The star indicates the initiation of an autophagosome, with an isolation membrane beginning to encompass cytosolic material. Thin arrowheads indicate single-membrane vacuoles, some containing cellular material. 5 grids, ~ 15-20 cells/grid, were studied per treatment.

Interestingly, several autophagosome-engulfed mitochondria were observed in RSV-infected Cre$^-$ BMDCs (Figure 3.9). The absence of mitophagy in the Cre$^+$ BMDCs in the presence of RSV was striking, given the necessity of eliminating damaged mitochondria.[253, 353] As cell death is a common response to unresolved cell stress, studies to visualize mitochondrial-caspase activation were performed. During nutrient starvation or RSV infection, Cre$^+$ BMDCs contained greater staining for active caspase 3/7, an early indication of apoptosis, suggesting that the cells were destined for cell death (Figure 3.10). However, cell analysis by flow cytometry revealed equal proportions of Cre$^-$ and Cre$^+$ BMDCs destined for death after RSV infection (Figure 3.11). Thus, while *Sirt1*-deficient DCs appear to respond negatively to nutritional or viral stress, their survival is not altered.

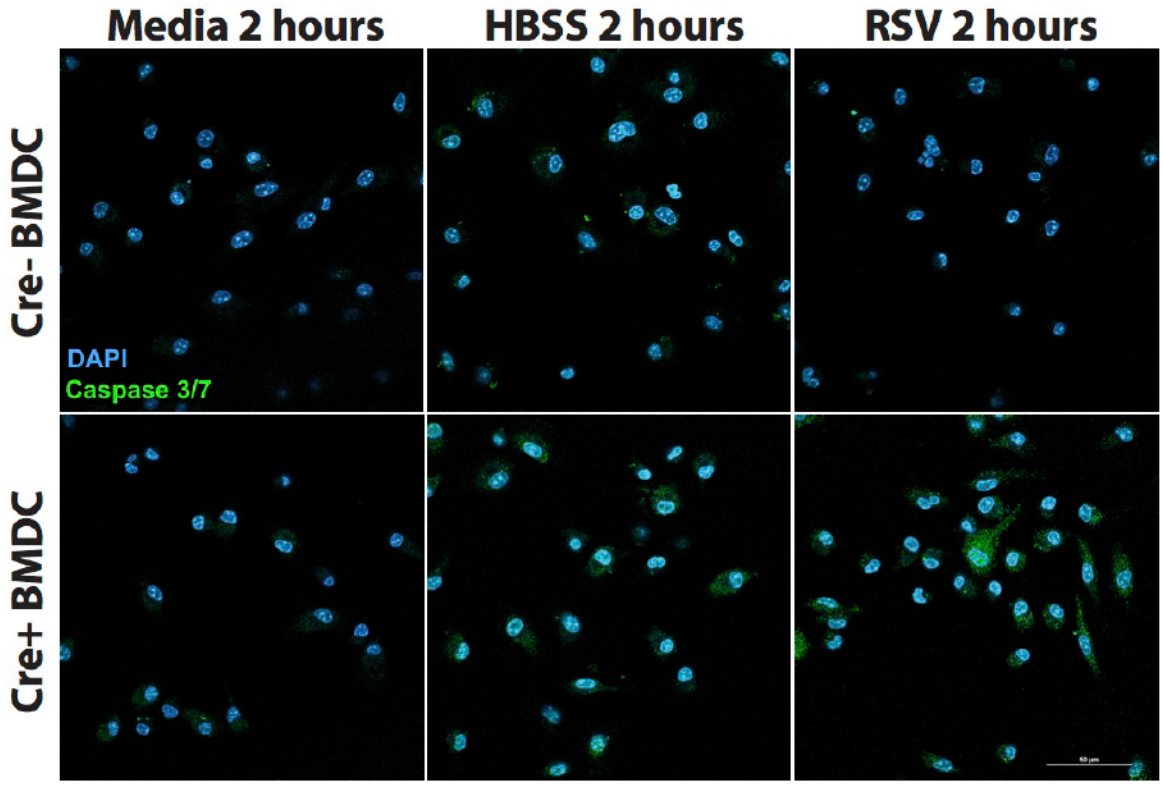


Figure 3.10: BMDCs from *Sirt1$^{f/f}$-CD11c-Cre$^+$* mice express more activated caspase 3 during stress conditions. Caspase 3/7 staining in Cre$^-$ and Cre$^+$ BMDCs 2 h after no treatment (media only), HBSS treatment (starvation medium), or RSV 2-20 infection (MOI 1:1), visualized by confocal microscopy. Scale bar = 50 µm. Data are representative of 2 independent experiments.

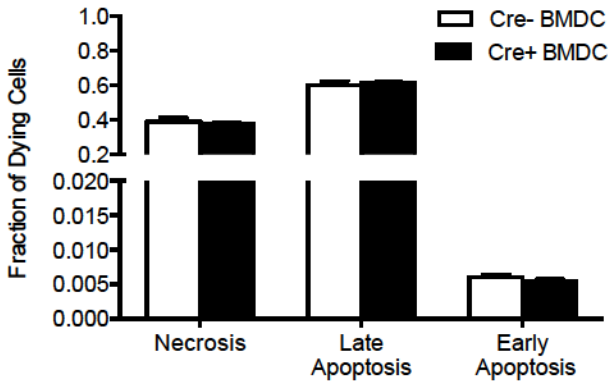


Figure 3.11: The deficiency of SIRT1 does not alter cell survival during RSV infection. Annexin-PI flow cytometric analysis of cell death in BMDCs isolated from *Sirt1^{fff}-CD11c-Cre* mice 24 h after RSV 2-20 infection (MOI 1:1). Data are representative of 2 independent experiments. Values represent MFI fractions ± SEM, 3 replicates/group. No significance between groups. See Chapter 2 for detailed characterization of these death stages.

APC function is not SIRT1-dependent

Attenuation of cytokine production and autophagy in the absence of functional SIRT1 prompted APC function studies. As described in Chapter 1, DCs promote adaptive immune responses by stimulating naïve and RSV-reactive memory T cells in the LN via cytokine production, co-stimulation, and antigen presentation. Flow cytometric studies were used to examine SIRT1's affect on the expression of DC maturation markers (CD40, CD80, CD86), which serve as co-stimulatory proteins for T cell activation. BMDCs from *Sirt1^{fff}-CD11c-Cre*± mice equally upregulated the expression of these surface proteins in response to RSV (Figure 3.12). Thus, while SIRT1 significantly affects DC cytokine production during viral infection, it is not necessary for APC co-stimulatory marker expression.

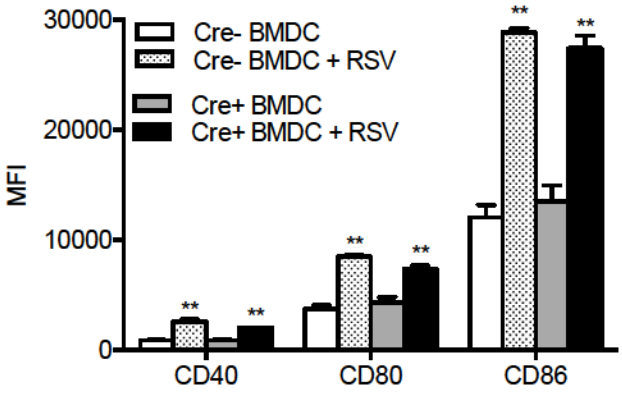


Figure 3.12: SIRT1 does not influence the expression of DC maturation markers. Surface maturation (co-stimulatory) marker expression by Cre$^-$ or Cre$^+$ BMDCs, isolated from *Sirt1^{fff}-CD11c-Cre* mice, as measured by flow cytometry 24 h after RSV 2-20 infection (MOI 1:1). Data representative of 4 independent experiments. Values = MFI ± SEM, 3 replicates/group. Stars indicate significance compared to respective naïve controls. $^{**}p < 0.01$.

This observed proficiency in DC co-stimulatory marker expression was tested *in vitro*. Whole splenic cell isolates from *Sirt1^{fff}-CD11c-Cre*± mice, containing a mixture of

APCs and T cells, were stimulated with an antibody against CD3, a T cell co-receptor necessary for transmitting activation signals. In this manner, we could observe the co-stimulatory function of the Cre^- and Cre^+ splenic DCs in the presence of a strong TCR stimulus. Figure 3.13 shows no differences in T cell cytokine production between Cre^- and Cre^+ splenic whole cell cultures. Therefore, SIRT1 is neither necessary for APC co-stimulatory marker expression nor for T cell co-stimulation.

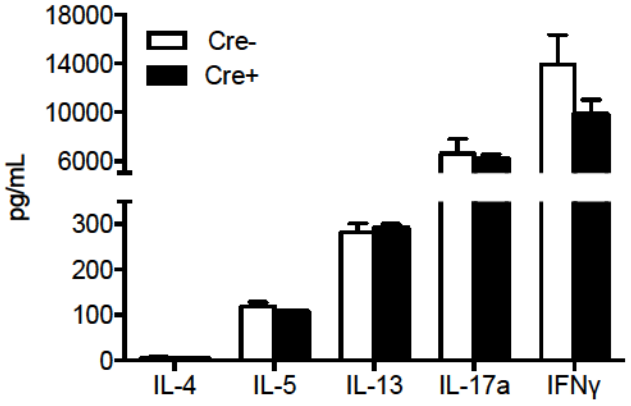


Figure 3.13: T cell co-stimulation is not dependent on SIRT1. Cell cultures from whole spleen, prepared from *Sirt1$^{f/f}$-CD11c-Cre*± mice, were stimulated with 2 µg/mL of anti-CD3 for 72 h. T cell cytokine concentrations were measured by Bio-Plex assay. Values represent mean ± SEM, 5 independent mice per group. No significance between groups.

Finally, to directly evaluate the antigen presentation capacity of DCs that harbored dysfunctional SIRT1, co-cultures of DCs and T cells were performed. In the presence of the SIRT1 chemical inhibitor, EX-527, BL6 RSV-infected BMDCs retained the capacity to present viral antigen to RSV-specific $CD4^+$ T cells (Figure 3.14A). To analyze the APC function in context of *Sirt1*-deficiency, *Sirt1$^{f/f}$-CD11c-Cre*± BMDCs were co-cultured with OVA-specific or RSV-specific $CD4^+$ T cells. Despite some inconsistency in IL-4 production, T cells were equally stimulated regardless of the status of SIRT1 in DCs (Figure 3.14B, C). These data reveal that even in the absence of functional SIRT1, DCs are proficient at antigen presentation.

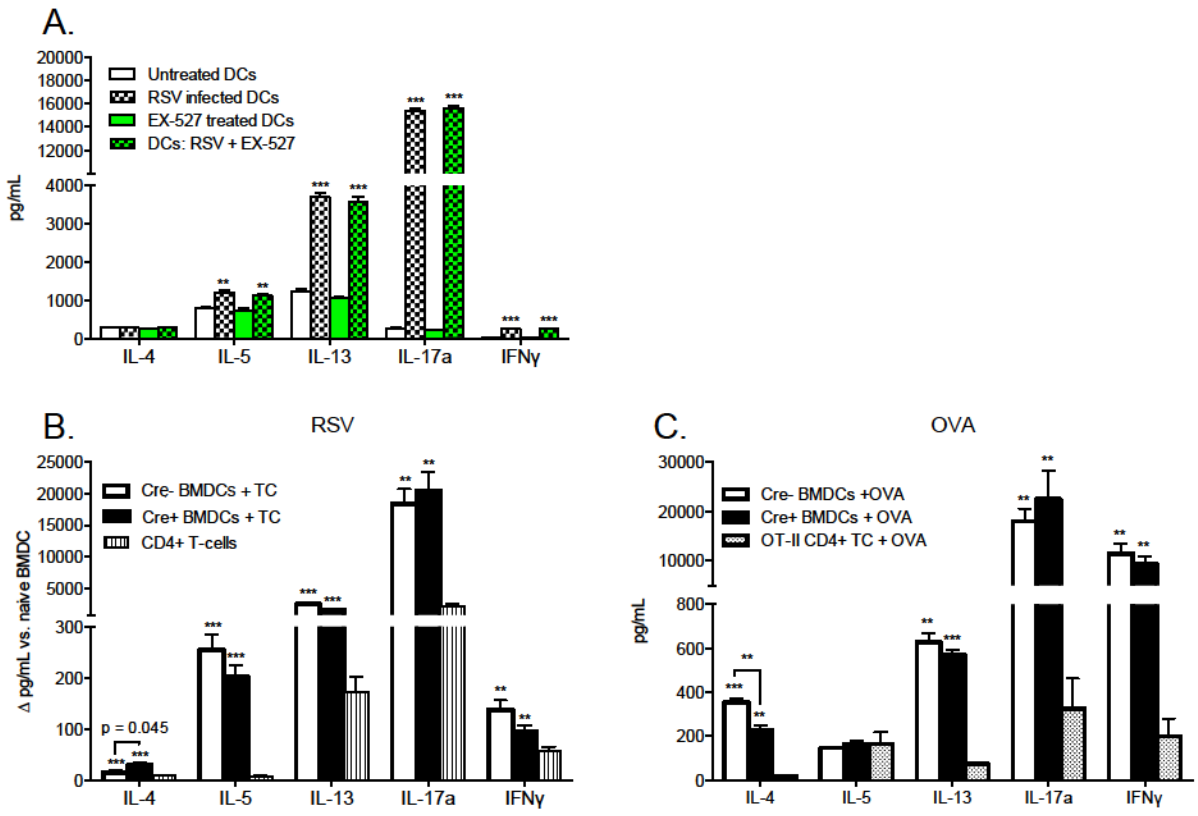


Figure 3.14: *Sirt1*-deficient DCs retain the capacity to present antigen to T cells. (A) $CD4^+$ T cells were purified from mediastinal LN of BL6 RSV-infected mice, 8 dpi. Cells were plated at a 10: 1 ratio over ± EX-527-treated BMDCs, infected 2 h previously with 1:1 MOI RSV. (B) An equivalent experiment as in (A) using *Sirt1*$^{f/f}$*-CD11c-Cre*± BMDCs instead of EX-527. (C) *Sirt1*$^{f/f}$*-CD11c-Cre*± BMDCs were pulsed with whole OVA protein followed by the addition of 10:1 purified splenic OT-II $CD4^+$ T cells. Cytokine concentrations in culture supernatants were assayed at 48 h by Bio-Plex. Results are representative of at least two independent experiments. Unless indicated, stars represent significance as compared to T cell + OVA controls. $^*p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

Summary

Chapter 3 focused primarily on SIRT1's influences on cellular processes that are critical for DC function during RSV infection. As antigen-presenting cells (APCs), DCs are responsible for T cell differentiation and maturation, and the T cell response in turn shapes the pulmonary immune environment. Activated DCs provide two signals required for successful T cell activation: antigen presentation via MHC molecules (to stimulate the TCR) and co-stimulatory marker presentation (to stimulate the T cell receptors CD28L and CD40). In the presence of TCR stimulation alone, T cells undergo apoptosis or enter an

inactivated state called anergy.[509] DC-specific cytokines provide a third instructive signal to activated T cells, fine-tuning the adaptive immune response to the invasive pathogen.

EX-527-inhibited and *Sirt1*$^{f/f}$*-CD11c-Cre*$^{+}$ DCs displayed a unique, altered phenotype, which included attenuated cytokine production and autophagy in the setting of undisrupted antigen presentation and T cell co-stimulation. These findings suggest that SIRT1 enhances autophagy-dependent cytokine production in DCs, but does not influence the essential APC signals necessary for effective T cell activation. Likewise, this suggests that SIRT1 is necessary but not sufficient for autophagy, as some autophagosomes were still detectable in SIRT1-inhibited or *Sirt1*-deficient DCs. In contrast, our laboratory has previously shown that inhibition or knockdown of autophagy in RSV-infected DCs prevents DC maturation, decreases cytokine production, and disrupts antigen presentation.[233]

Given that cytokines provide critical communication signals between effector cells and APCs, we hypothesized that this observed deficiency in DC cytokine production would alter the overall immune response *in vivo*. To more thoroughly understand the impact of DC-specific *Sirt1* dysfunction on both innate and adaptive immunity, we performed studies (described next in Chapter 4) using a mouse model of RSV infection.

CHAPTER 4:
SIRT1 promotes the development of efficient antiviral immune responses during RSV infection

Systemic inhibition of SIRT1 augments RSV-induced lung pathology

To test whether *Sirt1* modulated the outcome of RSV infection, C57BL/6J WT mice were infected with RSV on day 0, and received daily intraperitoneal injections of EX-527 (1 mg/kg), a SIRT1-selective chemical inhibitor. At 8 dpi, a time point that corresponds to maximum lung pathology,[42] the animals were sacrificed to assess pathological parameters. Histological examination of lung sections from RSV-infected mice revealed greater peri-bronchial inflammation (Figure 4.1A) and goblet cell hyperplasia (Figure 4.1B) in EX-527-treated, RSV-infected lungs than in infected controls. Gene expression analysis revealed similar upregulation of mucus-related genes, *Muc5ac* and *Gob5*, in response to RSV infection regardless of EX-527 treatment (Figure 4.1C). There were significant increases in the expression of *Ifng, Il5,* and *Il10* in the EX-527-treated, RSV-infected mice compared to control RSV-infected mice (Figure 4.1D). The increased mRNA levels of RSV proteins in the EX-527-treated, RSV-infected animals suggested that viral clearance was reduced in the absence of functional SIRT1 (Figure 4.1E). RSV-restimulated lymph nodes from the EX-527-treated, RSV-infected mice produced significantly higher levels of the Th2 cytokines IL-4, IL-5, and IL-13 than RSV-infected controls (Figure 4.1F), with no differences in IL-17a or IFNγ production. Therefore, this *in vivo* study demonstrates that systemic SIRT1 inhibition exacerbates RSV-induced lung pathology and alters the cytokine milieu within the lungs and lymph nodes, suggesting that SIRT1 promotes efficient antiviral immune responses.

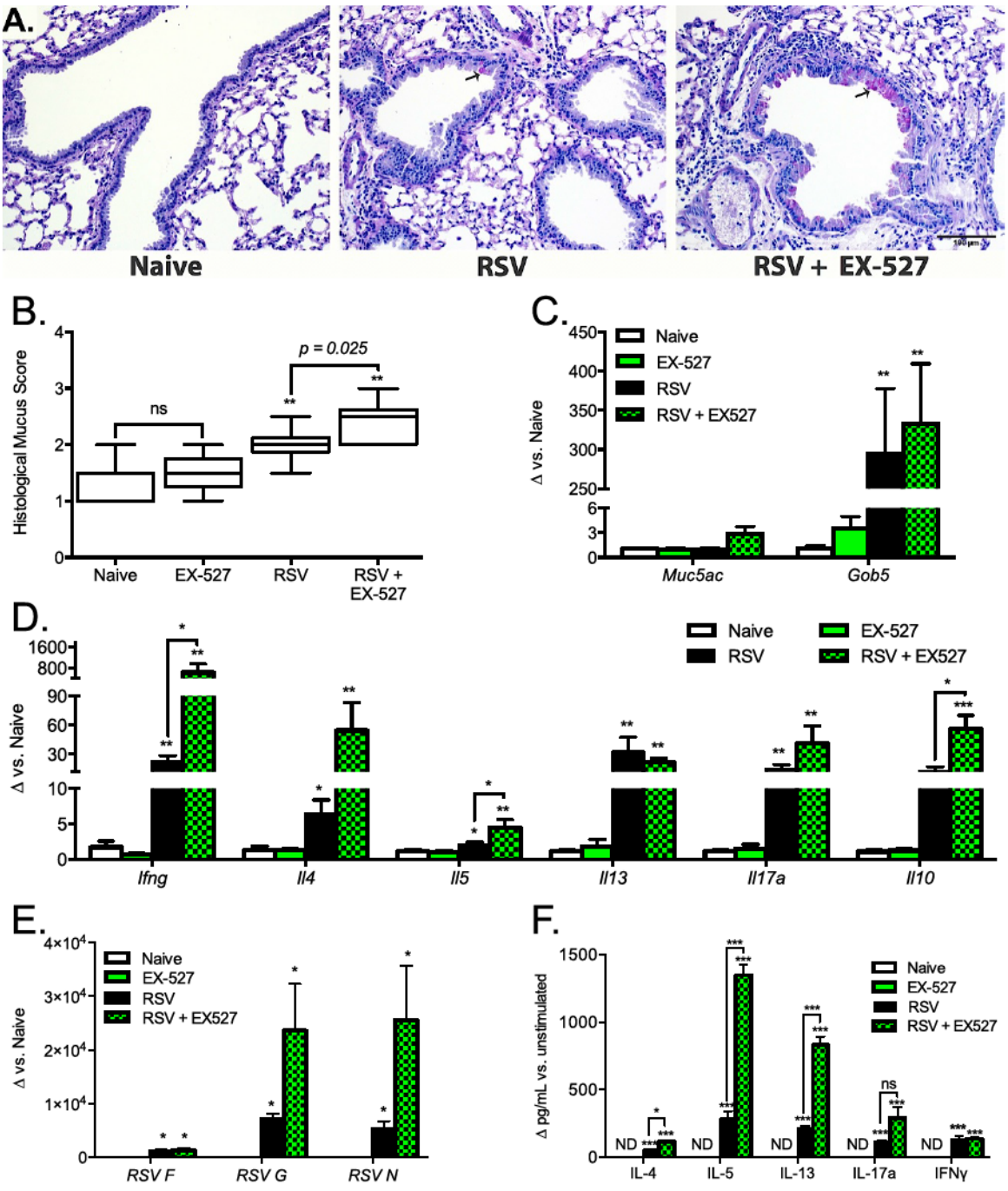


Figure 4.1: Systemic SIRT1 inhibition augments RSV-induced lung pathology. (A) Representative lung histology from naive and infected (Line 19 RSV) mice treated with DMSO-saline control or EX-527 (8 dpi) stained with hematoxylin and PAS. Arrows = goblet cells. Scale bar = 100 μm. (B) Histological mucus scores as assessed from lung sections of control and experimental groups 8 dpi. Stars indicate significance compared to naïve and EX-527 control mucus scores. Lung mRNA expression of (C) mucus-associated genes *Muc5ac* and *Gob5*, (D) cytokine genes, and (E) viral protein transcript 8 dpi was obtained by qPCR, compared with naive controls. Note naïve and EX-527 controls were not statistically different. (F) LDLNs from each treatment group were restimulated for 48 h in culture with RSV. Cytokine concentrations were assayed by Bio-Plex. Data are representative of 3 independent experiments. Values represent mean ± SEM, 5 mice/group. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

Sirt1$^{f/f}$*-CD11c-Cre*$^{+}$ mice experience exacerbated lung pathology and delayed resolution of inflammation following RSV infection

During the first three days of RSV infection the host response is dominated by innate immunity. This response includes the activation of resident DCs, the secretion of early inflammatory mediators, and the recruitment of Natural Killer cells and neutrophils.[42] Since DC influx begins as soon as 2 dpi,[186] we used this time point to analyze early immune responses to RSV in *Sirt1*$^{f/f}$*-CD11c-Cre* mice. There were significant increases in lung mRNA levels of potentially pathogenic cytokines *Il13, Il17a*, and *Il10* in Cre^{+} mice compared to Cre^{-} mice (Figure 4.2). Additionally, crucial innate cytokines, including *Il6*, *Il12p40*, and *Tnfa*, were downregulated in the lungs of Cre^{+} mice post-infection, recapitulating our *in vitro* observations in DC subsets. Overall, at 2 dpi, immune cells were infiltrating the lungs in similar proportions (Figure 4.3, top right).

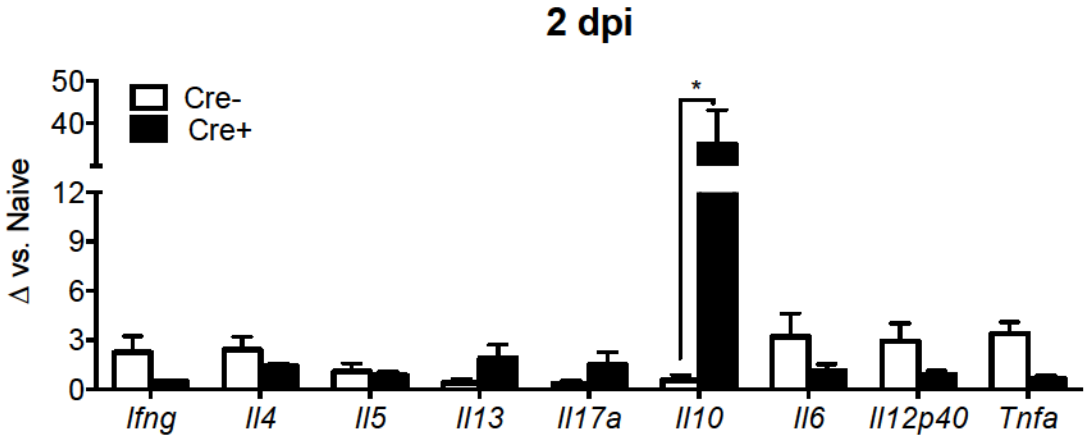


Figure 4.2: RSV-infected *Sirt1*$^{f/f}$*-CD11c-Cre*$^{+}$ mice develop a pro-inflammatory lung immune environment by 2 dpi. Gene expression of lung cytokines 2 dpi with RSV 2-20 was obtained using qPCR and compared with naive controls. Data representative of 2 independent experiments, 3-5 mice/group, values = mean ± SEM. *$p < 0.05$.

To specifically examine the impact of *Sirt1* deletion in CD11c^{+} APCs during the height of RSV-induced lung pathology, we analyzed *Sirt1*$^{f/f}$*-CD11c-Cre*$^{+}$ mice and littermate controls 8 dpi. Histological examination revealed that Cre^{+} mice had increased levels of airway inflammation and goblet cell hyperplasia compared to Cre^{-} controls (Figure 4.3, bottom left). RSV-infected Cre^{+} mice expressed higher levels of pathogenic cytokine genes,

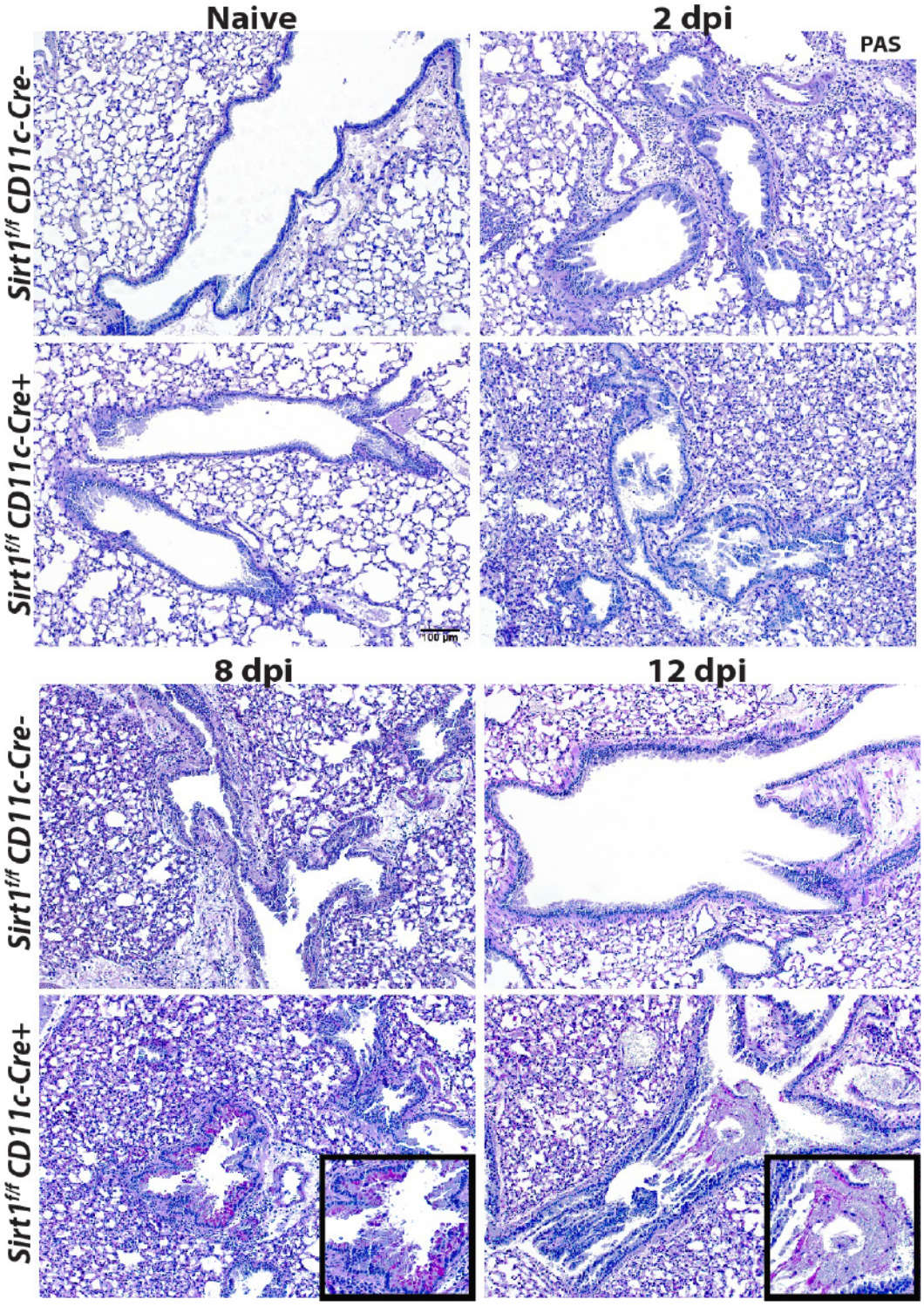


Figure 4.3: Increased inflammatory infiltrates and mucus production in airways of RSV-infected *Sirt1$^{f/f}$-CD11c-Cre^{+}* mice. Representative lung histology from naive and RSV 2-20-infected *Sirt1$^{f/f}$-CD11c-Cre* mice 2, 8, and 12 dpi stained with hematoxylin and PAS. Insets highlight goblet cell hyperplasia. Note the mucus plug occluding the Cre^{+} airway 12 dpi. Scale bar = 100 µm. Data representative of 2 independent experiments, 3-5 mice/group.

including *Il4, Il13,* and *Il17a* (Figure 4.4A), and mucus-related genes (Figure 4.4B). Histological mucus scoring correlated well with the mRNA expression data (Figure 4.4C). More RSV protein mRNA transcript in the lungs of Cre^+ mice as compared to Cre^- mice once again suggested a reduction in viral clearance at 8 dpi (Figure 4.4D). Of interest, LDLN cultures prepared from RSV-infected Cre^+ mice secreted significantly greater amounts of IL-17a and IFNγ upon restimulation with RSV than LDLN cultures from Cre^- mice (Figure 4.4E).

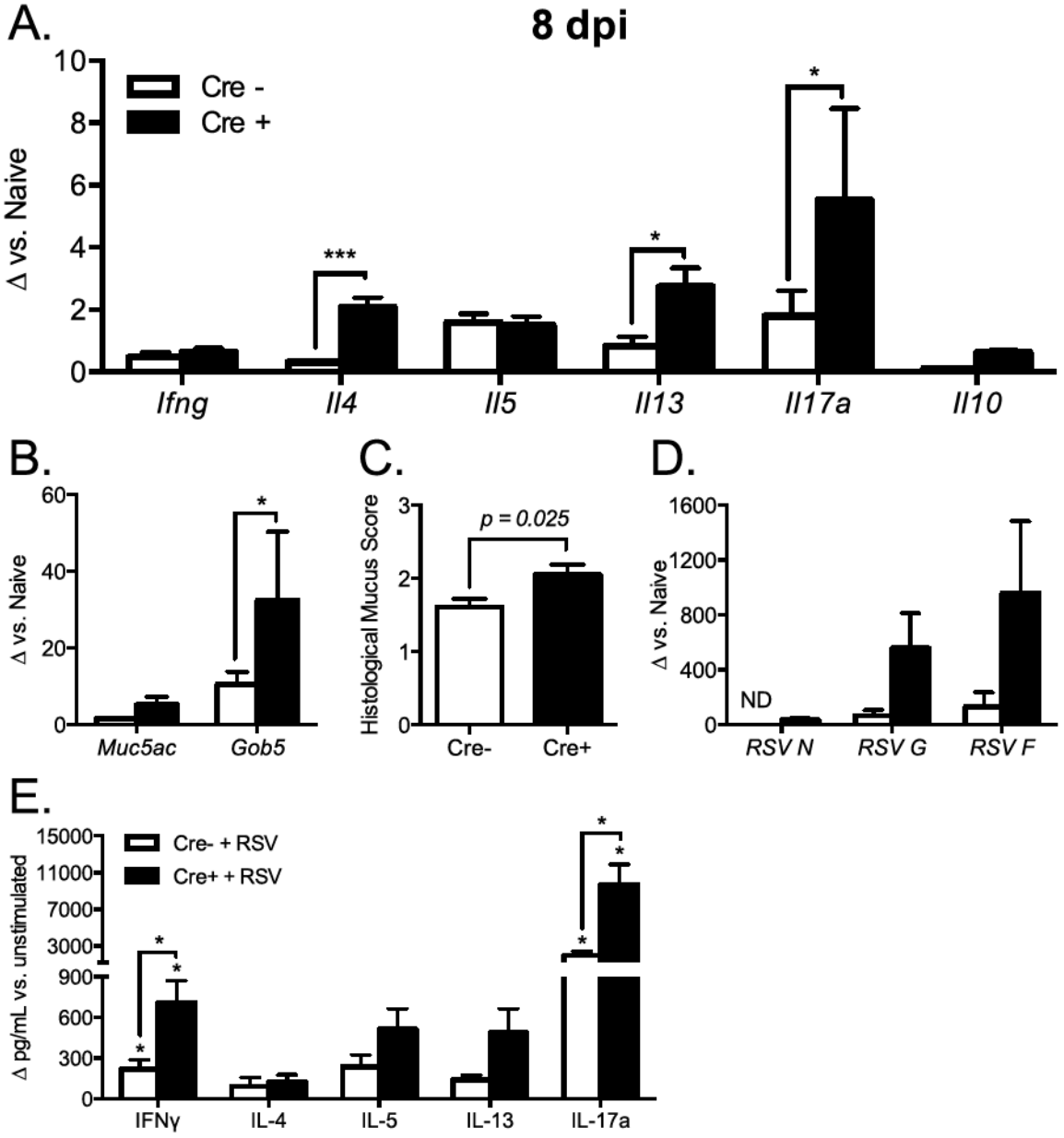


Figure 4.4: *Sirt1*^{fff}*-CD11c-Cre*$^{+}$ mice suffer from exacerbated RSV-induced lung pathology at 8 dpi. (A) mRNA expression of lung cytokines, (B) mucus-associated genes *Muc5ac* and *Gob5*, and (D) viral protein were obtained using qPCR and compared with naive controls. (C) Histological mucus scores as assessed from lung sections of Cre^{-} and Cre^{+} mice 8 dpi. (E) LDLNs from RSV-infected Cre^{+} and Cre^{-} mice 8 dpi were dissociated into a single-cell suspension and restimulated in culture with RSV 2-20. Cytokine concentrations were assayed by Bio-Plex. Data representative of 2 independent experiments, 3-5 mice/group, values = mean ± SEM. $^{*}p < 0.05$. $^{***}p < 0.001$.

Since the *Sirt1*^{fff}*-CD11c-Cre*$^{+}$ animals showed a proinflammatory lung environment by 2 dpi and a heightened pathology at 8 dpi, the 12 dpi time point was assessed to investigate the resolution of the response. Cre^{+} mice had significantly elevated mRNA levels of *Ifng, Il4*, and *Il5,* coupled with three-fold higher *Gob5* expression in their lungs (Figure 4.5A, B). However, there were no significant differences in cytokines produced by LDLN harvested at 12 dpi and restimulated with RSV (Figure 4.5C). Despite viral elimination as 12 dpi, as indicated by the absence of RSV protein mRNA (data not shown), histological examination of the lungs from Cre^{+} mice revealed prolonged, persistent pathology and inflammation (Figure 4.3, bottom right).

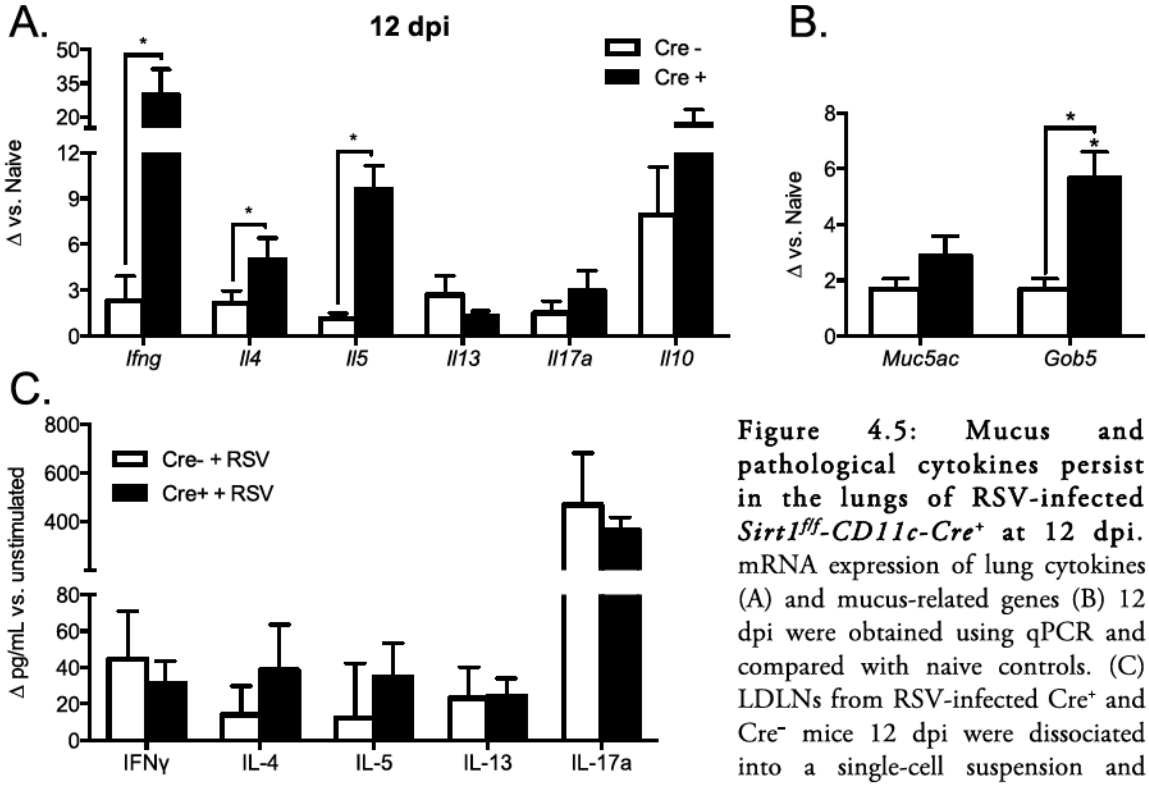


Figure 4.5: Mucus and pathological cytokines persist in the lungs of RSV-infected *Sirt1*^{fff}*-CD11c-Cre*$^{+}$ at 12 dpi. mRNA expression of lung cytokines (A) and mucus-related genes (B) 12 dpi were obtained using qPCR and compared with naive controls. (C) LDLNs from RSV-infected Cre^{+} and Cre^{-} mice 12 dpi were dissociated into a single-cell suspension and restimulated in culture with RSV 2-20. Supernatant cytokine concentrations were assayed by Bio-Plex. Values = mean ± SEM, 5 mice/group. $^{*}p < 0.05$.

Alveolar macrophages are unique amongst other macrophages located throughout the body in that they express high levels of CD11c. To verify that the observed RSV immune responses were in fact due to DC-specific dysfunction, we sorted primary macrophages from the lungs of *Sirt1*$^{f/f}$*-CD11c-Cre* mice. Unlike the significant decrease in cytokine production in CD11b^{+} pulmonary DCs upon RSV infection (Figure 3.7), alveolar macrophages were still able to efficiently upregulate inflammatory cytokines during *ex vivo* RSV stimulation (Figure 4.6). Therefore, *Sirt1*-deficiency in alveolar macrophages is likely not relevant in these *in vivo* experiments, especially given that DCs are the superior APCs responsible for mediating the resultant T cell response. Overall, the results of the conditional KO mouse studies parallel the results of our EX-527 studies, and support the concept that *Sirt1* in DCs promotes RSV-induced immunity while limiting lung pathology.

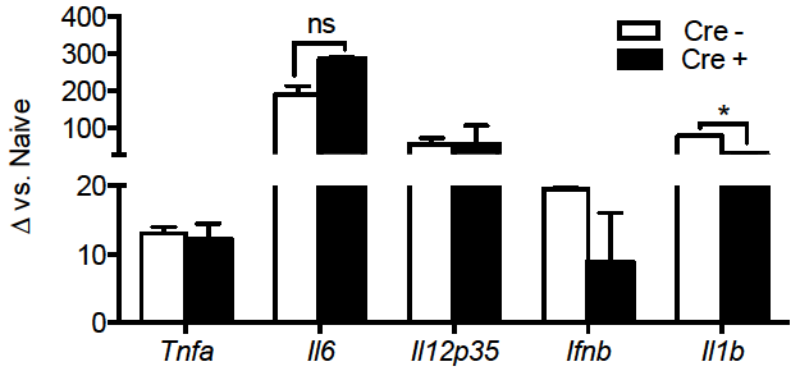


Figure 4.6: RSV-infected CD11c^{+} alveolar macrophages from *Sirt1*$^{f/f}$*-CD11c-Cre*$^{+}$ mice are proficient at inflammatory cytokine production. CD11c^{+} macrophages were flow sorted from lungs of naive Cre^{-} or Cre^{+} mice prior to *ex vivo* RSV infection. Innate cytokine gene expression 24 h post-RSV 2-20 (MOI 1:1) infection was obtained by qPCR. Data are representative of 2 independent experiments. Values represent mean ± SEM, 3 replicates/group, 5 mice/sort. $^{*}p < 0.05$.

SIRT1 does not alter RSV gene expression within DCs

In the aforementioned mouse experiments, there were greater increases in viral transcript within the airways of EX-527-treated or DC-specific *Sirt1*-deficient mice over infected controls (Figure 4.1E, Figure 4.4D). This we attributed to a poorer immune response, which resulted in reduced viral clearance. As discussed in Chapter 1, a recent study has described SIRT1 as a viral restriction factor, capable of inhibiting replication of several

viruses.[465] While it may be possible that SIRT1 directly regulates viral processes in AECs, the primary replicative targets of RSV infection, DCs do not propagate RSV. Viral mRNA detection within DCs is quickly attenuated, suggesting viral particles do not have time to successfully form intact virions.[225] To determine whether SIRT1 dysfunction could alter RSV gene expression within DCs, RSV protein transcript was analyzed. Figure 4.7 demonstrates that deficiency of *Sirt1* expression (KO model) or activity (EX-527) did not significantly change the levels of detectable RSV protein mRNA in either pulmonary DCs or BMDCs. Therefore, these results strengthen our hypothesis that SIRT1 regulates DC function during RSV infection without direct influence over viral gene expression.

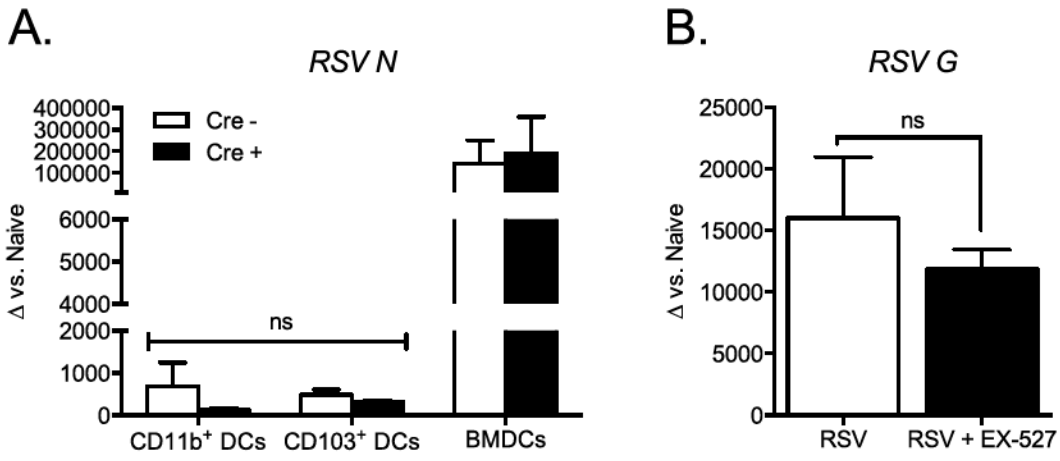


Figure 4.7: SIRT1 does not alter DC-specific RSV gene expression levels. (A) Pulmonary DCs and cultured BMDCs were isolated from *Sirt1$^{f/f}$-CD11c-Cre*± mice, and infected with RSV 2-20 (1:1 MOI) for 24 h. (B) WT BMDCs cultured from BL6 mice were treated ±1 μM EX-527 30 min before infection with Line 19 RSV (1:1 MOI) for 24 h. Cytokine gene expression was determined by qPCR. Data are representative of > 5 experiments. Values = mean ± SEM, 3 replicates/group, 5 mice/sort.

Summary

Chapter 4 described how both global SIRT1 inhibition (using EX-527) and DC-specific *Sirt1*-deficiency translates to heightened and prolonged RSV-induced lung pathology. This data demonstrates that (a) SIRT1 dysfunction augments respiratory illness in RSV-infected mice and (b) SIRT1 plays a crucial antiviral role during the immune response to RSV infection. Moreover, these *in vivo* studies suggest that SIRT1 enables DC-

directed immune responses important for efficient viral clearance, rapid resolution of pulmonary inflammation, and minimal damage to adjacent healthy tissue.

Sirt1$^{f/f}$*-CD11c-Cre*$^{+}$ mice displayed elevated viral gene expression levels in their lungs compared to Cre^{-} animals, indicating either excessive viral replication or retention of viral antigens within DCs, likely due to a decrease in autophagy. As *Sirt1*-deficient DCs harbored no apparent defects in T cell activation, as discussed in Chapter 3, the lack of efficient viral resolution suggests that the miscommunication between the APCs and effector T cells is primarily a result of poor DC cytokine production. Therefore, while not necessary for T cell differentiation and maturation, DC-made cytokines are significant in instructing the adaptive immune response, which in the absence of functional SIRT1 within DCs was skewed towards a pathological Th2/Th17 response.

Interestingly, our results are contrary to the previous studies, which suggest SIRT1 programs DCs to promote pathological inflammatory immune responses.[454, 455] In one experiment, DC-specific *Sirt1* KO mice were resistant to chemically-induced EAE, exhibiting a significant reduction in central nervous system pathology, much fewer Th17 and Th1 cellular infiltrates, and a significant increase in Tregs.[454] Another study demonstrated that the lack of SIRT1 in DCs reduced innate immune cell infiltrates in the airways of mice during OVA-induced asthma.[455] The data suggest that SIRT1 promotes DC maturation and pro-Th2 skewing activity. Likewise, upon treatment with pharmacological SIRT1 inhibitors, these DC-specific *Sirt1* KO mice experienced reduced Th2 responses and were protected against allergic airway inflammation.[455] Of note, these contradictory results to our EX-527 study may be due to the differential specificity of the SIRT1 inhibitors. Overall, our RSV studies suggest that SIRT1 plays a differential role during infectious vs. noninfectious diseases.

CHAPTER 5:
Discussion, Conclusions, & Future Directions

As discussed in Chapter 1, adults often mistake an RSV infection for the common cold, most experiencing only mild upper respiratory tract symptoms. However, this highly infectious virus is the principle cause of LRTI among neonates and children, predisposing these young patients hospitalized by an RSV infection to develop recurrent wheezing and asthma later in life.[3-5, 43-48] Additionally, RSV contributes significantly to the morbidity and mortality among the elderly, the immunocompromised, and those living with pulmonary dysfunction, such as in the case of allergic asthma or COPD.[3-5] Overall, the complications of severe RSV infection suggest that the host antiviral immune response, and the subsequently altered pulmonary environment, facilitate the genesis and exacerbation of chronic airway disease. Alas, an effective, safe vaccine or an affordable, widely accessible treatment has yet to be synthesized. Therefore, studies aimed at further elucidating the RSV-host immune system interaction are imperative to the development of successful pharmacologic therapies.

The deacetylase SIRT1 is a ubiquitous protein that impacts numerous areas of biology and pathophysiology, including stress responses, development, metabolism, cardiovascular disease, cancer, neurodegeneration, inflammation, and immunity.[377, 390-393] While SIRT1 regulates immune responses such as lymphocyte activation, proliferation, differentiation, and macrophage cytokine secretion,[390] its role in DC biology has yet to be elucidated. Given the significance of DCs in dictating innate immunity and priming adaptive immune responses, the unknown function of SIRT1 in DCs prompted the studies described in Chapter 3 and 4. Our results shine new light on SIRT1's complex relationship with various cellular pathways (i.e. autophagy) and disease-related processes (i.e. respiratory viral infections).

SIRT1 promotes DC-mediated antiviral immunity and limits RSV-induced pathology

Appropriately activated APCs are instrumental in achieving immune responses that effectively clear an infection while limiting injury to surrounding tissue. The studies addressed in this dissertation indicate that SIRT1 is necessary to promote DC activation and autophagy during RSV infection, and that in the absence of active SIRT1 within DCs, mice experience a pathological antiviral immune response. We have demonstrated the importance of SIRT1 in DC biology by three independent approaches: (1) chemical inhibition of SIRT1 with EX-527, (2) siRNA knockdown of *Sirt1*, and (3) genetic ablation of *Sirt1* in DCs. Blocking *Sirt1* by any of these methods resulted in attenuated cytokine production and inhibited autophagosome numbers within RSV-infected DCs. Additionally, global inhibition of SIRT1 (EX-527) and conditional ($CD11c^+$ cell-specific) *Sirt1* deficiency in an RSV infection model led to the exacerbation of pulmonary pathology. These latter results suggest the adaptive immune response is skewed towards an allergic (Th2) phenotype in the setting of DC-specific *Sirt1* deficiency. Furthermore, the decrease in viral clearance at 8 dpi may have contributed to the lack of resolution in pathology and inflammation at 12 dpi in *Sirt1$^{f/f}$-CD11c-Cre$^+$* mice (Figure 4.5). Thus, these dissertation experiments are the first to characterize *Sirt1* as having a beneficial impact on an antiviral response, and to link *Sirt1* to autophagy and innate cytokine activation within virally infected DCs. As summarized in Figure 5.1, these functions of SIRT1 appear to have a significant role in directing the development of an efficient antiviral, minimally pathologic immune environment.

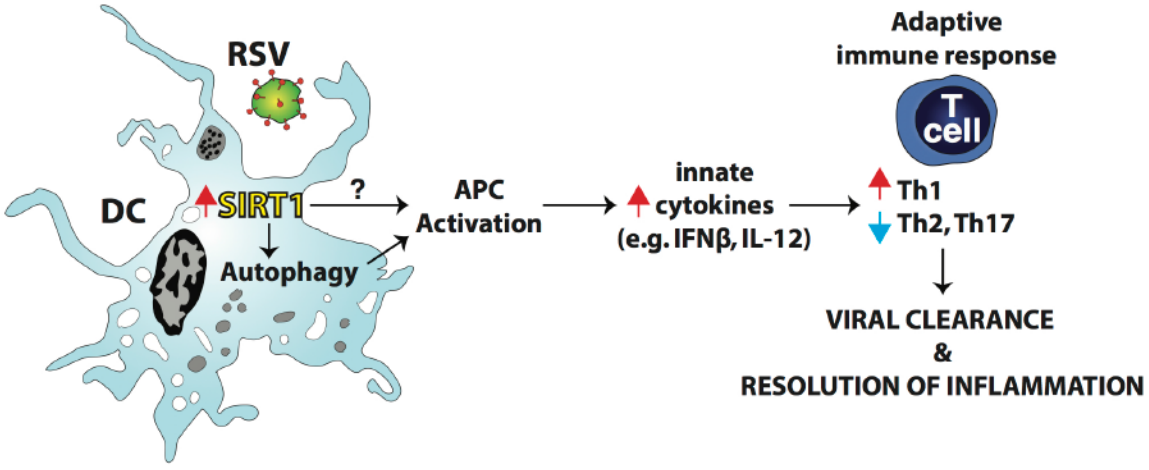


Figure 5.1: Sirtuin 1 promotes effective antiviral adaptive immune responses by driving DC activation and autophagy. Upon uptake of viral antigens, DCs upregulate *Sirt1* expression. SIRT1 contributes to the activation of autophagic processes within the DC, such as by deacetylating key ATG proteins, which indirectly promote APC function. Likewise, SIRT1 may directly promote DC function (not elucidated), given its broad involvement in many cellular pathways. Once activated, DCs produce innate cytokines, skewing T-cell differentiation toward an antiviral Th1 adaptive immune response, while suppressing pathologic Th2 and Th17 responses. As a result, SIRT1 within DCs dictates the development of an immune environment that efficiently clears the RSV and resolves the associated inflammation.

Previous work has revealed that SIRT1 can block immune and inflammatory processes, including cytokine production in APCs,[390, 484] likely due to its transcriptional repression of NFκB (RelA/p65) via deacetylation.[408] Furthermore, it has been demonstrated that the ablation of *Sirt1* in macrophages, using a myeloid cell-specific *Sirt1* knockout (Mac-*Sirt1* KO) mouse, rendered NFκB hyperacetylated and resulted in increased transcriptional activation of proinflammatory target genes, including *Il6, Il12, Tnfa* and *Il1b*.[409] These researchers concluded that by targeting NFκB, SIRT1 acts as a brake on metabolically detrimental inflammatory cytokine production by macrophages in a model of diet-induced diabetes. However, during RSV infection, DCs depend on autophagy for the trafficking of viral components to mediate TLR-induced innate cytokine upregulation. As evidenced in Chapter 3, the lack of SIRT1 in DCs reduced critical cytokine production, including IFNβ and IL-12, which we suggest contributed to a pathological Th2/Th17 immune response within the airways. Importantly, when DCs were stimulated with TNFα in the presence of EX-527, increased IL-6 production was observed (Appendix 1). Thus, lack of SIRT1 does not intrinsically impair cytokine gene transcription and secretion. This preliminary data also suggests that under certain stimuli, SIRT1-inhibited DCs do not rely on autophagy-mediated processes, and that instead, blockade of SIRT1 leads to cytokine production driven by increased NFκB acetylation. It would be interesting to explore gene expression and activity levels of the NFκB signaling pathway within *Sirt1*-deficient DCs during RSV infection. Corollary studies could also include the overexpression of NFκB pathway components in the context of RSV infection, to observe whether this would overcome attenuated cytokine production in *Sirt1*-deficient DCs. Two independent studies examining DC-specific *Sirt1* KO mice have reported no changes in DC maturation, differentiation, or development as compared to WT DCs,[454, 510] in agreement with our observations examining

co-stimulatory marker expression and DC immune populations (Figure 3.6, Figure 3.12). However, in contrast to our TLR7 agonist results, one of these studies demonstrated that *Sirt1*$^{-/-}$ DCs produced higher levels of cytokines upon TLR1, TLR4, or TLR3 stimulation.[454] Thus, our data indicate that SIRT1 has a unique role in modulating DC cytokine production in the context of a viral infection, such as RSV, that relies on autophagy-mediated PAMP delivery to endosomal TLRs.[511]

Sirt1$^{-/-}$ mice share phenotypic similarities with autophagy-defective *Atg5*$^{-/-}$ mice,[373] highlighting the significance of these proteins' interactivity. Given that the lack of functional SIRT1 attenuated DC-specific autophagy (Figure 3.4, Figure 3.5, Figure 3.8), experiments were performed to verify the previously reported physical interaction between SIRT1 and autophagosomal proteins within DCs.[373] Overall, preliminary results suggest that levels of acetylated-ATG proteins were increased or at least stabilized by EX-527 treatment, compared to untreated controls, indicating the chemical inhibitor was blocking SIRT1 activity (data not shown). During autophagosomal formation, the protein LC3 is proteolytically modified into LC3II, and upon vesicular maturation LC3II is recycled. Bafilomycin can block this maturation event by inhibiting lysosomal acidification and thus autophagosome-lysosome fusion.[244] Protein analysis of RSV-infected DCs isolated from *Sirt1* conditional KO mice showed lower levels of LC3II/I, LC3II, and LC3I protein ratios, suggesting a decrease in autophagy flux (Appendix 2). These protein analyses recapitulate our observation that *Sirt1*-deficient BMDCs are still capable of low levels of autophagosome formation upon RSV exposure (Figure 3.8). All together, it is possible that SIRT1 enhances autophagy flux, but it is not absolutely required. Further biochemical studies should be conducted to thoroughly characterize SIRT1's influence on the autophagosomal machinery.

The significance of SIRT1 in regulating autophagy-related DC function can be expanded to emerging studies on the pathogenesis of neurodegenerative diseases. Among these neuronal diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, the main pathological phenotype is the accumulation of toxic intracellular aggregates of mutant proteins. Several neurodegenerative diseases have been associated with defective autophagy, most in part caused by specific mutations in autophagy genes.[252, 512] Additionally, the evidence for SIRT1's protective activity against protein-aggregate-related

neurodegeneration is quite expansive. Thus, it is intuitive to propose a crucial interaction between SIRT1 and autophagy in promoting neuronal survival. We can speculate that in neurons susceptible to degeneration, a dysfunction in autophagy is due to either low expression or activity of SIRT1, defective autophagy proteins (as reported), or a combination of SIRT1 and autophagy protein deficiencies. While there is active investigation into the independent contributions of SIRT1 and autophagy in the genesis and progression of these diseases, it would be highly enlightening to study these processes together. For example, a subset of Parkinson's disease is linked to mutation in *PARK2/Parkin*.[279] Do patients with this disease mutation display reduced SIRT1 activity? α-synuclein has been shown to interfere with Parkin solubility and distribution, contributing to the pathogenesis of Parkinson's disease.[513] If neuronal cells isolated from these individuals were treated with SIRT1 activators, would there be a reduction in α-synuclein-mediated toxicity due to an upregulation of autophagosome formation? Therefore, future studies focusing on the interaction of SIRT1 and autophagy within neurons may yield fruitful discoveries that could contribute to cell survival in patients plagued with such neurodegenerative conditions.

On the other hand, the interaction of SIRT1 and autophagy may also contribute to disease pathology, such as during the chronic inflammation of rheumatoid arthritis (RA). This autoimmune disease is characterized by persistent synovial hyperplasia and inflammation due to immune infiltrates, leading to the destruction of joint cartilage and bone.[514] Innate immunity has been shown to be important in the development of chronic arthritis, including TLR and NLR activation within synovial cells, which leads to IL-6 and TNFα overproduction.[515, 516] Of note, TNFα-induced overexpression of *Sirt1* has been reported to contribute to this chronic inflammation by promoting cytokine production and inhibiting apoptosis in RA synovial fibroblasts.[505] Interestingly, knockdown of *Sirt1* by siRNA or inhibition by EX-527 decreased TNFα production in human monocytes and fibroblasts,[505] much like blocking SIRT1 significantly reduced cytokine levels in our DCs. Likewise, independent studies have noted that TNFα promoted autophagy in osteoclasts, enhancing their bone resorptive capacity.[517] Human synovial fibroblasts from RA patients displayed increased autophagy and protection from apoptosis, thus contributing to their persistent inflammatory cytokine production.[518] This begs the question whether there is a

feedback loop between SIRT1 and autophagy, in which enhanced SIRT1 activity contributes to additional autophagy flux, leading to more TNFα production. Therefore, the described relationship between SIRT1 and autophagy in our study suggests the possibility of a parallel, yet detrimental relationship in the context of RA.

Tissue- and context-dependency of SIRT1's effects may underlie the variability in the protein's role in many diseases, including diabetes, cancer, and immunity. Data suggest that SIRT1 serves to dampen the pro-inflammatory nature of activated macrophages and T cells (Chapter 1), which parallel our *in vivo* results where mice experienced greater RSV-induced lung pathology and inflammatory cytokine production with EX-527 treatment (Figure 4.1). As EX-527 was administered systemically, it is possible that the aggravated pathology was due to a synergistic effect of global SIRT1 inhibition on multiple cell types, including AECs, alveolar macrophages, and T cells. However, this EX-527 *in vivo* data mirrored the results obtained when DC-specific ($CD11c^+$) *Sirt1* KO mice were infected with RSV (Figure 4.3, Figure 4.4), highlighting the significance of SIRT1 dysfunction specifically in DCs. Despite reports that SIRT1 mediates pro-inflammatory responses in DCs,[454, 455] we observed an attenuation of DC cytokine production, but a retention of APC function, when SIRT1 was absent or defective (Chapter 3). Therefore, our *in vivo* experiments stress the essential role of SIRT1-mediated DC cytokine production during RSV infection in fine-tuning the T cell-mediated adaptive immune response.

This modulating role and tissue-specificity of SIRT1 is further exemplified in the setting of metabolic disease. In the case of several family members diagnosed with Type 1 diabetes due to a *Sirt1* point mutation (SIRT1-L107P), their insulin-producing pancreatic β-cells overproduced nitric oxide, the cytokines TNFα and IL-1β, and the chemokine CXCL1, a neutrophil chemoattractant.[384] As a result, these patients suffered from hyperglycemia and insulin dependence, and they were positive for β-cell autoantibodies. The puzzling aspect of this mutation is its location outside of the conserved enzymatic core, which causes no changes in protein stability, subcellular localization, or protein-protein interactions with major binding partners. Therefore, the alteration in SIRT1's ability to modulate the activity of specific proteins over a transient period of time in pancreatic β-cells seems to cause the diabetic phenotype observed at the organismal level. Perhaps in the presence of insulin

resistance due to SIRT1 dysfunction, stress accelerates β-cell death resulting in the release of autoantigens and endogenous "danger signals" capable of promoting pathologic self-antigen presentation.[519] In this disease context, the ability of SIRT1 to suppress TNFα expression[452] was partially ablated by the L107P mutation, which is significant given that TNFα antagonism has been shown to improve diabetic symptoms.[520] Thus, SIRT1 fine-tunes insulin sensitivity by affecting cytokine production in β-cells, much as it modulates antiviral immunity by affecting cytokine production in DCs.

Data discussed in Chapter 4 allow for further speculation toward specific pulmonary disease states. Baseline respiratory dysfunction, as in the case of chronic obstructive pulmonary disease (COPD) and allergic airway disease (i.e. asthma), can be exacerbated during RSV infection.[5] Of note, cigarette smoke reduces the levels of SIRT1 in the lungs of patients with COPD and in rat models, as well as in monocyte-macrophage cell lines.[521] Perhaps mechanistically related, SIRT1 deacetylates target proteins such as FOXO3, p53, matrix metalloproteinase 9 (MMP 9), and NFκB, all of which are implicated in the pathogenesis of COPD.[521] Thus, reduction of SIRT1 may promote acetylation of these proteins, thereby enhancing disease factors including autophagy, cellular senescence, emphysema, fibrosis, and inflammation. Targeting SIRT1 in pre-clinical pulmonary disease models has yielded disparate results. In asthma mouse models, administration of pharmacological sirtuin inhibitors (Sirtinol and Cambinol) reduced allergic airway inflammation and Th2 cytokine responses.[455, 507] However, in a separate study using the SIRT1 activator SRT1720, inhibition of Th2 responses was observed during OVA-induced airway disease.[491] Thus, compared to our studies of RSV-induced responses, SIRT1 may have a differential effect in a non-infectious setting. These seemingly contradictory results may also be attributed to the specificity of the inhibitors. As noted in Chapter 1, Sirtinol antagonizes SIRT2 more potently than SIRT1, while Cambinol inhibits SIRT1 and SIRT2 with similar IC_{50} values. In contrast, EX-527 is a potent and selective SIRT1 inhibitor, with negligible potency against SIRT2 or SIRT3, and no activity against class I/II HDACs.[500] The present dissertation demonstrates the exacerbation of RSV infection in the context of a highly selective SIRT1 inhibitor and in $CD11c^{+}$-specific *Sirt1* KO mice, suggesting that during viral infection, SIRT1 promotes a protective immune environment linked to

autophagy within DCs. These observations may be especially important, as most severe exacerbations in asthma and COPD are associated with viral infections.[5]

Ongoing and Future Studies

Adjuncts can boost the immunogenicity of vaccines, but careful selection is required, as common alum-based adjuvants have shown to promote a Th2-associated response[522, 523] that is frequently associated with vaccine-enhanced disease symptoms.[108, 524] Based on the CD11c-specific *Sirt1* KO mouse experiments, *Sirt1*-deficient $CD11c^+$ DCs drive a Th2 response in the airways and in restimulated T cells isolated from infected animals. Perhaps SIRT1 activating compounds, such as Resveratrol (natural) or SRT1720 (synthetic), could serve as potential adjuvants in combination with a RSV vaccine to stimulate a more Th1-driven response that would prevent disease exacerbation. These SIRT1 activators could also be considered as part of a preventative regimen for immunocompromised or immunodeficient individuals. Of note, Resveratrol treatment of RSV-infected human cell lines has been shown to downregulate IL-6 production and to partially inhibit RSV replication.[525, 526] Currently, we are administering daily Resveratrol by oral gavage to BALB/c mice, which have been infected with RSV Line 19. Given the augmented pathology observed in RSV-infected mice receiving EX-527 (Figure 4.1), but the protection from emphysema or asthma in mice treated with SRT1720,[490, 491] we predict that the Resveratrol treatment group will clear RSV better by 8 dpi, displaying little to no mucus hyperplasia and insignificant levels of lung inflammatory cytokines, as compared to RSV-infected controls. While the exact influence of Resveratrol on SIRT1, and other possible biological targets, is not clearly understood,[486,487] the results of our study would be the first to describe the effects of the compound on the immunopathology of RSV infection.

SIRT1 has been shown to induce autophagy as a protective mechanism during stress conditions, including hypoxia, oxidation, and the accumulation of toxic/unfolded proteins, in a wide range of cell types.[382, 527, 528] We have demonstrated that in the absence of SIRT1, and therefore adequate levels of autophagy, DCs upregulate caspase 3/7 activity (i.e. apoptotic pathway) compared to control cells (Figure 3.10), yet these *Sirt1*-deficient cells do not perish in a greater proportion or rate (Figure 3.11). Interestingly, a separate study also

observed the upregulation of caspase-3 without cell death during $CD8^+$ T cell proliferation *in vivo*.[529] This prompts the question whether other SIRTs are compensating for the lack of SIRT1 to maintain organelle function, such as the mitochondria-specific SIRT3, 4, or 5. For example, we observed evidence of mitophagy in RSV-infected Cre^- BMDCs, yet could not identify autophagocytosed, damaged mitochondria in *Sirt1*-deficient Cre^+ BMDCs (Figure 3.9). Studies have already shown that mitochondrial SIRTs can serve as tumor suppressors and promoters, regulate fatty acid metabolism, promote cardiac and HSC stress resistance, and reduce ROS levels, all of which encompass functions of SIRT1, too.[378, 530] Specifically, SIRT3 has been reported to regulate mitochondrial biogenesis via interactions with PGC1α (also a SIRT1 partner), inducing mitochondrial DNA replication and synthesis of mitochondrial proteins.[531] Likewise, another study has described how SIRT3 orchestrates antioxidant machinery, as well as mitophagy, via the mitochondrial UPR during proteotoxic stress.[532] Perhaps, mitochondrial SIRTs are capable of regulating mitochondrial homeostasis during RSV infection, in such a way as to mitigate the necessity of mitophagy in context of SIRT1/autophagy dysfunction. In that regard, it would be wise to study the effects of *Sirt1*-deficiency on the expression and activity of the other six mammalian SIRTs, in an effort to identify any overlapping sirtuin functions in the realm of DC biology.

Since virus-induced endoplasmic reticulum (ER) stress is a known biological response,[232] RSV-infected *Sirt1$^{fl/fl}$-CD11c-Cre*$^+$ DCs may be experiencing altered ER stress due to *Sirt1* deficiency, contributing to the observed caspase-3 activity. Preliminary studies suggest that *Sirt1*-deficient BMDCs and pulmonary DCs experience exponentially greater ER stress than control cells in response to RSV infection, as indicated by the upregulation in gene expression of several UPR proteins including *Xpb1s*, which mediates IRE1α signaling (Appendix 3A-C).[356] UPR induction was verified with Tunicamycin, an N-acetylglycosamine transferase inhibitor that halts glycosylation of newly synthesized glycoproteins, leading to the accumulation of unprocessed proteins in the ER.[357] At two observed time points, *Sirt1*-deficient BMDCs expressed greater mRNA levels of UPR proteins in response to Tunicamycin (Appendix 3D). These data are similar to the ER stress changes we noted in autophagy-deficient AECs, in which mRNA expression of *Edem1* and *Xbp1s* were significantly elevated in RSV-infected *Lc3b*$^{-/-}$ AEC cultures 12 hpi.[533] Thus, *Sirt1* deficiency

and autophagy deficiency parallel each other both in terms of DC cytokine reduction (Figure 3.3A) as well as in the case of UPR activation, providing further evidence in support of a reciprocal relationship between SIRT1 and the autophagy pathway.

Studies have shown that co-treating macrophages with chemical ER stressors and TLR3/4 activators significantly enhanced IFNβ production in an XBP1s-dependent manner.[534] TLR ligation was also reported to suppress PERK signaling while simultaneously activating and re-routing XBP1s signaling away from canonical UPR targets toward proinflammatory genes.[535, 536] Thus, protein-folding homeostasis may be suppressed in favor of innate immune signaling. Additionally, SIRT1 and inflammatory cytokine protein levels were upregulated upon TLR4 signaling in a human monocyte cell model of endotoxin tolerance.[537] Once endotoxin tolerance developed, SIRT1 promoted epigenetic reprogramming to downregulate cytokine production, by binding to the *Tnfa* and *Il1b* promoters and recruiting the anti-inflammatory NFκB subunit, RelB.[537] As SIRT1 has been attributed with the attenuation of ER stress,[5, 388, 389] including the inhibition of XBP1s transcriptional activity,[387] it is plausible that SIRT1 initially promotes UPR-related signaling until the stressor (e.g. pathogen) is eliminated, at which point the deacetylase curbs proinflammatory signaling to restore homeostasis. Therefore, the observed upregulation of ER stress protein gene expression in *Sirt1*-deficient DCs may be downstream of incomplete TLR-activation, which leads to unsuccessful upregulation of inflammatory cytokines in the absence of SIRT1. If proven correct, this would be another example of SIRT1's coordination of multiple pathways (e.g. UPR and inflammatory) with an ultimate goal of cellular survival and maintenance.

In relationship to pulmonary disease, IRE1α-mediated XBP1s-activation may sustain the amplified ER calcium stores frequently observed in inflamed airway mucosa,[538] as well as the excessive IL-13-driven mucus production observed in allergic airway disease.[77] While further genetic studies are necessary to firmly determine whether and how observed UPR activation contributes to inflammatory airway disease, we can speculate that ER stress may at least partially explain why viral infection, such as RSV, often instigates or exacerbates baseline pulmonary pathology.[5] Future experiments should address the mechanistic details of the potential interaction(s) of the UPR and SIRT1 during RSV infection—such as UPR

transcription factors directly regulating SIRT1 expression—both in DCs and the primary targets of RSV infection, the AECs.

Chapter 1 addressed how both autophagy and SIRT1 participate in nutritional toggling, effectively promoting cellular homeostasis. At the organismal level, *Atg5*$^{-/-}$ or *Atg7*$^{-/-}$ mice suffer from systemic nutritional deficiency and extreme glucose deficits, while *Sirt1*$^{-/-}$ mice are susceptible to hepatic steatosis, mitochondria dysfunction, and metabolic inefficiency upon dietary challenge.[421, 539, 540] Awareness of energy imbalance likely evolved as a mechanism by which eukaryotes detected and eliminated pathogens through autophagy, while SIRT1's enzymatic requirement for NAD^+ intimately links it to a cell's metabolic state. Additionally, SIRT1 interacts with nutritional pathways to mediate cellular energy balance, including the repression of mTOR and the activation of FOXO transcription factors to induce autophagy.[380, 394] These interactions were verified in our studies by the upregulation of *Foxo3* gene expression alongside *Sirt1* expression in RSV-infected DCs (Figure 3.2A), which coincided with an increased autophagosome formation (Figure 3.4, Figure 3.8).

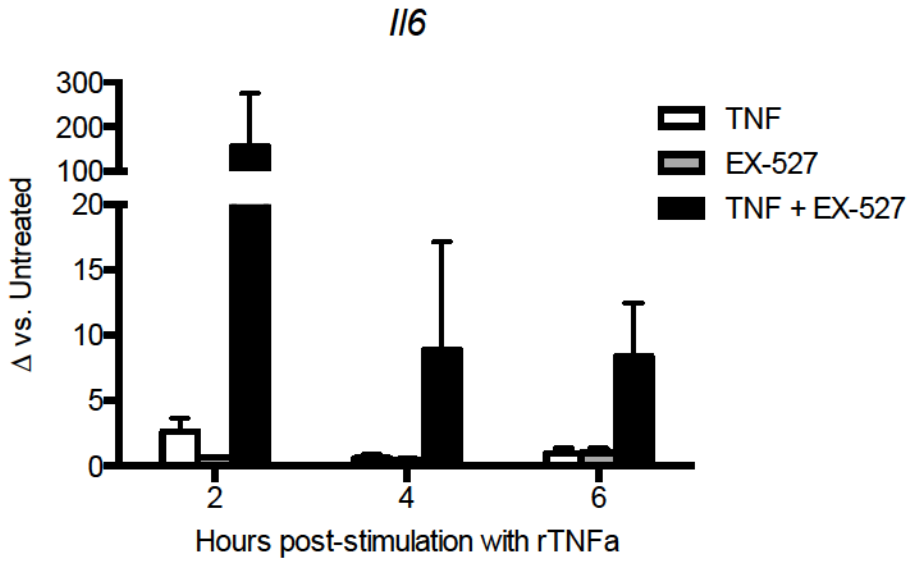
Given these relationships, a fascinating line of research would be investigating the metabolic alternations—and how they relate to SIRT1—within DCs specifically infected with RSV, and how these hinder or facilitate DC activation and APC function. Metabolic regulation and cell signaling are tightly and ubiquitously linked with immune responses, and well studied in the case of lymphocyte biology.[541-543] DCs must adjust their extracellular and intracellular environments as they migrate to nutrient or oxygen-deficient sites, and during the reprogramming that proceeds after inflammatory stimulation (i.e. resulting from a microbial invader). Recent studies have indicated that TLR signaling-mediated metabolic reprogramming facilitates DC maturation and antigen presentation.[544, 545] TLR stimulation and Type 1 IFN drive the switch from oxidative phosphorylation to aerobic glycolysis, a less efficient but more rapid energy-producing process, similar to the Warburg effect in cancer cells.[544, 545] This shift is likely required to meet the energy demands of activated DCs, thus preventing premature death in order to sustain an immune response. Additionally, dysregulated mTOR or AMPK activity (inhibitor and activator of autophagy, respectively, Figure 1.2) impairs DC development and maturation, suggesting the significance of a metabolic checkpoint in promoting DC function. As SIRT1 can directly suppress mTOR via

deacetylation, our results suggest that SIRT1 deficiency does not dramatically alter this metabolic checkpoint, as we did not observe attenuated DC maturation or development. However, SIRT1 dysfunction negatively affected downstream DC cytokine production, which greatly impacted the resultant adaptive immune response.

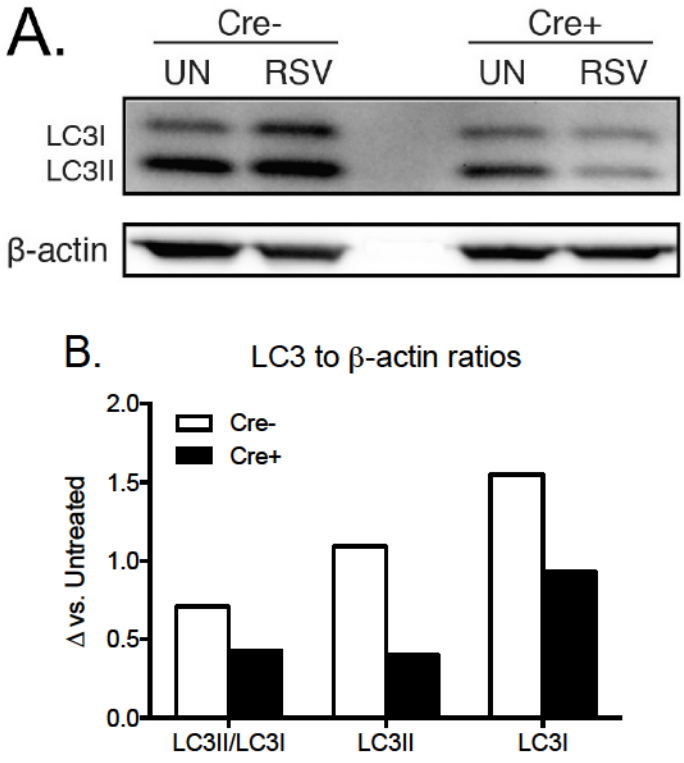
Furthermore, Chapter 1 explored how viruses exploit baseline cellular proteins and pathways to their own advantage, such as for progeny propagation by commandeering autophagosomal machinery or ER function. What aspects of DC biology does RSV counteract or seize control over in an attempt to prevent detection? While autophagy significantly heightens PRR activation, via delivery of viral antigen to endosomal TLRs, other cytoplasmic PRRs participate in RSV detection. Do RLRs or NRLs interact with stress pathways, like the previously described TLR association with the UPR, or metabolic pathways? The plausibility of SIRT1 influencing a majority of these changes is quite high as it targets such a tremendous array of proteins, and this could be preliminarily addressed with a microarray panel analysis of immune and metabolic pathways. Moreover, it would be highly warranted to investigate SIRT1's activity at the epigenetic level, as it was initially described as a histone deacetylase and obviously influences cytokine production and cell stress survival in this manner.[537, 402]

In conclusion, this thesis work suggests crucial roles for the protein deacetylase SIRT1 in innate immunity, specifically in the activation of DCs through autophagy during RSV infection. Our findings also suggest SIRT1 may be particularly beneficial during an antiviral immune response to limit pathological outcomes. Thus, SIRT1 pharmacological activators, such as Resveratrol, may serve a part in preventative therapies aimed at fortifying weak or insufficient immunity in RSV-susceptible patients. Likewise, the creation of adjuvants containing SIRT1-activating components may be beneficial, as this could facilitate the development of a successful RSV vaccine.

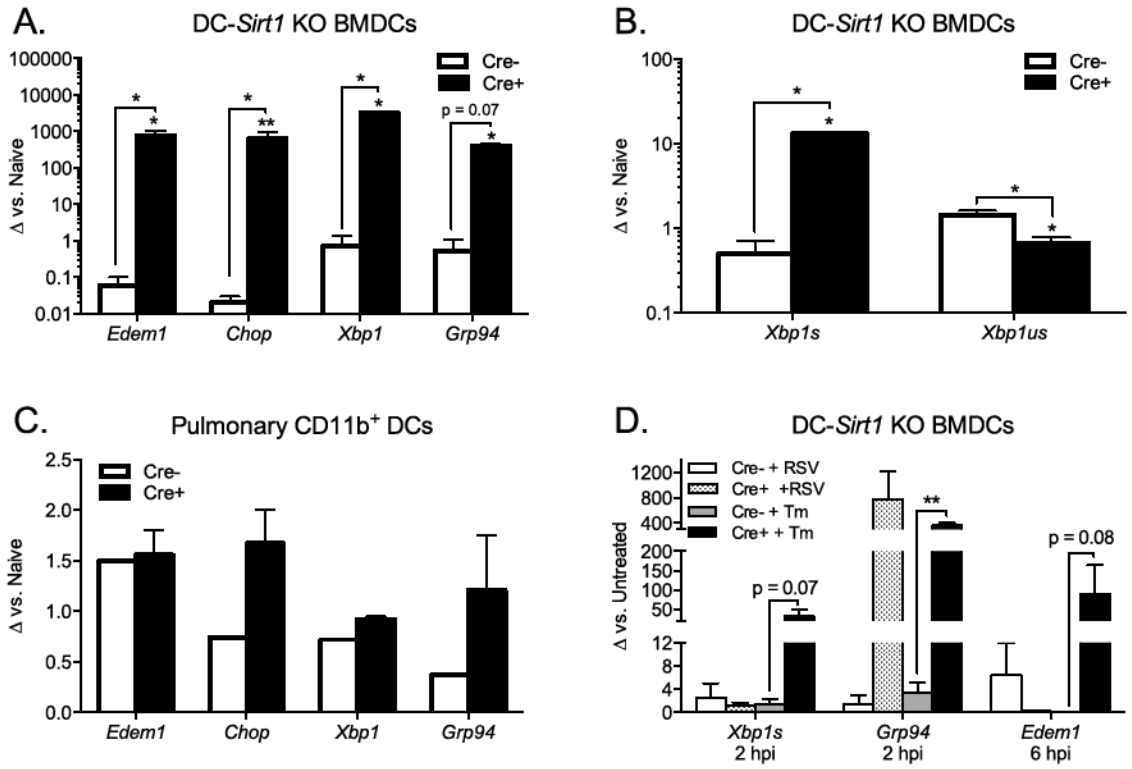
APPENDICES



Appendix 1: TNFα-stimulated NFκB-dependent IL-6 production is amplified in the presence of a SIRT1 inhibitor. Previous work has revealed that SIRT1 can block immune and inflammatory processes, including cytokine production in APCs,[390, 484] due to its transcriptional repression of NFκB (RelA/p65) via deacetylation.[408] TNF signals via the NFκB pathway to promote IL-6 production; of note, NFκB binds the promoter region of IL-6. This particular experiment indicates that in the presence of SIRT1 inhibition and TNF stimulation, DCs are still proficient at cytokine production. Therefore, lack of SIRT1 does not intrinsically impair cytokine gene transcription and secretion. Likewise, this data suggests that under certain stimuli, SIRT1-inhibited DCs do not rely on autophagy-mediated processes, and that instead, blockade of SIRT1 leads to cytokine production driven by increased NFκB acetylation. Experimental Design: WT BMDCs cultured from BL6 mouse legs were treated ±1 μM EX-527 30 min prior to stimulation with 10 ng/mL recombinant TNFα for 2, 4, or 6 hours. Cytokine gene expression was determined by qPCR. Data are representative of 2 experiments. Values = mean ± SEM, 3 replicates/group.



Appendix 2: *Sirt1*-deficient BMDCs have attenuated proteins levels of LC3 in response to RSV infection. During autophagosomal formation, the protein LC3 is proteolytically modified into LC3II, which is recycled upon vesicular maturation. The chemical Bafilomycin blocks this maturation event by inhibiting lysosomal acidification and thus autophagosome-lysosome fusion.[244] Protein analysis of RSV-infected DCs isolated from *Sirt1* conditional KO mice shows lower levels of LC3II/I, LC3II, and LC3I protein ratios, suggesting a decrease in autophagy flux. This data verifies our previous observation that *Sirt1*-deficient BMDCs undergo low levels of autophagy during RSV infection (Figure 3.8). Therefore, it is possible that SIRT1 enhances autophagy, but it is not absolutely required. Experimental Design: (A) BMDCs isolated from *Sirt1*^{ff}*-CD11c-Cre* mice were treated with Bafilomycin 30 min prior to infection with RSV Line 220 for 2 h. After cell harvest and protein lysis, samples were run on SDS-PAGE gels (20 µg per well), followed by membrane transfer and immunoblot for LC3I/II or β-actin. UN = untreated controls. (B) Protein levels were quantified as mean band fluorescence intensity ratios over β-actin protein band intensity, compared to uninfected cells. Data are representative of 2 independent experiments, 2 replicates/group.



Appendix 3: *Sirt1*-deficient DCs undergo greater levels of ER stress upon RSV infection or Tunicamycin treatment compared to *Sirt1*-proficient DCs. These experiments suggest that *Sirt1*-deficient BMDCs and pulmonary DCs experience exponentially greater ER stress than control cells in response to RSV infection, as indicated by the upregulated gene expression of UPR proteins including *Xpb1s*, which mediates IRE1α signaling.[356] UPR induction was verified with Tunicamycin, an N-acetylglycosamine transferase inhibitor that halts glycosylation of newly synthesized glycoproteins, leading to the accumulation of unprocessed proteins in the ER.[357] At two time points, *Sirt1*-deficient BMDCs expressed greater mRNA levels of UPR proteins in response to Tunicamycin. Experimental Design: (A-B) BMDCs isolated from *Sirt1^{fff}-CD11c-Cre* mice were infected with RSV 2-20 for 24 h. (C) Sorted pulmonary CD11b^{+} DCs from *Sirt1^{fff}-CD11c-Cre* mice were infected with RSV 2-20 for 24 h. (D) BMDCs cultured from *Sirt1^{fff}-CD11c-Cre* mice were infected with RSV 2-20 or treated with Tunicamycin (ER stress inducer) for 2 or 6 h. Cytokine gene expression of select UPR proteins was determined by qPCR, and compared to naïve/untreated controls. Data are representative of 2 experiments. EDEM1, ER degradation-enhancing alpha-mannosidase-like 1; CHOP, C/EBP homologous protein (aka GADD153 = DNA damage-inducible gene 153); GRP94, glucose-regulated protein 94 kDa (aka HSP90B1 = Heat shock protein 90 kDa beta member 1). Values = mean ± SEM, 3 replicates/group. $^{*}p < 0.05$. $^{**}p < 0.01$.

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