Negative regulation and immune effects of

apoptotic cell clearance in the lung: SIRPα and miR34a

by

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To my wife Erin, who makes me laugh and reminds me that, while I

will be a doctor of philosophy, she has been a master of science for years

And to all the K-12 science teachers who inspire their students

and turn a spark of curiosity into a lifelong flame

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List of Abbreviations

AC   Apoptotic Cell

AE2   Alveolar epithelial type II cell

AMø   Alveolar macrophage

AMPK   AMP-activated protein kinase

BMDMø   Bone marrow-derived macrophage

CF   Cystic fibrosis

COPD   Chronic obstructive pulmonary disease

DAMPs   Damage-associated molecular pattern molecules

DC   Dendritic cell

GC   Glucocorticoid

GCAE   Glucocorticoid-augmented efferocytosis

GR   Glucocorticoid receptor

GRAF1   GTP-ase regulator associated with focal adhesion kinase-1

Glia   Microglia

ICS   Inhaled corticosteroids

iNOS   Inducible nitric oxide synthase

ITIM   Immunoreceptor tyrosine-based inhibitory motif

LPS   Lipopolysaccharide

Mø   Macrophage

MARCO   Macrophage receptor with collagenous structure

miRNA   MicroRNA

NO   Nitric oxide

PAF   Platelet-activating factor

PGE2   Prostaglandin E2

PMø   Peritoneal macrophage

PS   Phosphatidylserine

PTEN   Phosphatase and tensin homolog

SIRPα   Signal regulatory protein alpha

SIRT1   Sirtuin1

SOCS1   Suppressor of cytokine signaling 1

SOCS3   Suppressor of cytokine signaling 3

SP-A   Surfactant protein A

SP-D   Surfactant protein D

SR-A   Scavenger receptor A

TAM   Family of tyrosine kinase receptors: Tyro3, Axl and Mertk

TGF-β   Transforming growth factor beta

TLR   Toll-like receptor

TNFα   Tumor necrosis factor alpha

Abstract

Apoptotic cell clearance (efferocytosis) is the essential biological process by which dead cells, called apoptotic cells (AC), are engulfed and recycled by living cells called phagocytes. This process is regulated by many receptors that differentiate between apoptotic and viable cells by recognizing molecules expressed on apoptotic cells, particularly a phospholipid called phosphatidylserine (PS). Binding of recognition receptors to PS leads to intracellular signaling, culminating in engulfment of the bound AC. Little is known about how this process is negatively regulated; most variation in engulfment capacity has been explained by the differing repertoires of AC recognition receptors on different phagocytes.

In these chapters we describe two negative regulators of AC clearance: SIRPα and miR-34a. Both are highly expressed on the resident phagocyte of the alveolar space, the alveolar macrophage (AMø). Both contribute to a low AMø engulfment capacity. First, we show that expression of signal regulatory protein alpha (SIRPα), a previously described inhibitory receptor, is downregulated by treatment of AMø with glucocorticoids, leading to increased efferocytosis. Second, we show that this glucocorticoid-augmented efferocytosis (GCAE) increases the susceptibility of mice to bacterial pneumonia. Third, we show miR-34a is a master-regulator of AC clearance in Mø. We identify three target genes through which miR-34a can enhance AC uptake: Axl,

Sirtuin1 (SIRT1) and GTP-ase regulator associated with focal adhesion kinase-1 (GRAF1). Finally, we show that expression of miR-34a alone increases Mø bacterial killing. Collectively, these data suggest that limited AC uptake - rather than something to be "corrected" - is an important component of the AMø identity and is integral to maintaining appropriate responses to potential lung pathogens.

Chapter 1

Introduction

Biological Context of Apoptotic Cell Clearance

As multicellular organisms develop and grow, a single cell divides and differentiates to the myriad distinct cell types that make up the organs and tissues of the adult body. This process of growth also requires death. Significant programmed death occurs during embryogenesis (1, 2), but also continues throughout adult life, during which billions of cells die daily during normal cellular turnover in a healthy human. This huge burden of dead cells is a critical issue with which all multicellular organisms have evolved to contend through the highly conserved process of apoptotic cell (AC) clearance, also called AC engulfment or efferocytosis (3-6).

There are multiple types of cell death (7) including necrosis and pyroptosis, but the most common is termed apoptosis and refers to a 'programmed' cell death, such as occurs during breast tissue involution (8), CD8+ T cell clonal contraction following the resolution of infection (9), and the constant turnover of epidermal and epithelial tissues to maintain barrier integrity (10). Although apoptosis has been described as a 'quiet' death, uptake of AC has a dramatic impact on immunity. AC clearance is essential in maintaining self-tolerance, as well as impacting antimicrobial capacity and tissue repair (11-14).

Since the discovery of the first apoptotic cell recognition receptors in the early 1990s (15, 16), numerous components of this pathway have been identified, many of which are highly conserved through the worm, fly, mouse and human (17-19). Disrupting AC clearance can cause sterility (20, 21) and autoimmunity (22-25) in mice, demonstrating the great biological import of this pathway throughout higher eukaryotes. Both professional (i.e. macrophages) and non-professional phagocytes (i.e. epithelial cells) engulf AC (6), although the avidity of efferocytosis varies widely between cell types. Contrasting phagocytes with high and low rates of AC engulfment has been an important tool for identifying the myriad molecules involved in AC clearance, an approach also used throughout this thesis.

Mechanisms of AC clearance

The mechanism of AC clearance can be divided into four major steps: chemotaxis, recognition, engulfment, and degradation. Individual cell types complete these four steps using varied repertoires of molecules; the molecules expressed and used by each phagocyte contribute to differences in engulfment capacity. Although a plethora of molecules have been identified, the complex interactions that regulate this essential pathway or the selective pressures that restrict efficient AC uptake to specific cell populations are not fully understood. Additionally, many of the AC clearance components described in the following sections have only been identified as important in a single cell type; their expression in and relevance for uptake by other phagocytes is not fully characterized.

Chemotaxis. Phagocytes can express a number of cell surface receptors that recognize various molecules secreted by AC, allowing for directed movement of the phagocyte towards AC (**Fig. 1.1**) (6, 26). Three receptors are of particular interest: G2A, P2Y7 and CX3CR1, which respectively recognize oxidized phospholipids (27), ATP/UTP(28-30) and CX3CL1 (31) released by AC. AC also release general myeloid cell chemoattractants including the chemokines CCL2 (MCP-1) and CXCL8 (IL-8) (32) that assist in recruiting phagocytes. As the phagocyte must encounter the AC in order to engulf, enhancing this encounter through directed chemotaxis is an important mechanism by which AC clearance can be regulated.

Recognition. Phagocytes use a variety of receptors to differentiate live cells, which send negative signals that inhibit engulfment, from AC, which send positive signals triggering engulfment (**Fig. 1.2**) (5, 6, 33). A plethora of AC recognition receptors have been described. Some, such as integrins and TIM-4, are important for both binding and engulfing AC (15, 34, 35). Others, like Tyro3, Axl and Mertk (collectively called the TAM receptors) are only involved in engulfment after the AC has been "tethered"(11, 22, 35). Deletion of TAM receptors does not alter phagocyte binding to AC, although it significantly inhibits AC engulfment.

AC are primarily recognized via externalized phosphatidylserine (PS) (36, 37), although other molecules including pentraxin3 (38), calreticulin (39), and oxidized membrane lipids (40) can signal for engulfment. In viable cells, PS remains within the inner leaflet of the plasma membrane; during apoptosis, caspase-activated scramblases cause PS exposure on the outer leaflet (41-44). PS is recognized directly by receptors

including BAI-1 (45, 46), stabilin-1 (47), and TIM-4 (25, 48, 49). PS is recognized indirectly through bridge molecules including MFG-E8, Gas6, and ProteinS (23, 50-55). These serum proteins act as opsonins, binding to PS on AC and allowing recognition by $\alpha_v\beta3/5$ integrins and TAM receptors, triggering engulfment. The complement components C1q and iC3b, thrombospondin, surfactant proteins SP-A and SP-D, and mannose-binding lectin also bind AC and enhance subsequent engulfment (56-62).

In contrast to this vast and diverse collection of recognition receptors, only a small number of inhibitory receptors have been described (63). SIRPα is an inhibitory receptor. SIRPα can bind to CD47 expressed on viable cells or to secreted surfactant proteins (SP-), the lung collectins SP-A and SP-D. In the latter case, this binding causes SHP-1 signaling that inhibits Rac1 activation and AC engulfment (64, 65). CD300a similarly transmits inhibitory signals via its immunoreceptor tyrosine-based inhibitory motif (ITIM) domain upon binding to certain membrane lipids (66). Homophilic binding of CD31 expressed on AC to CD31 expressed on viable cells promotes subsequent detachment, preventing engulfment (67). Sufficient positive signals in the absence of inhibitory signals elicit the intracellular signaling that initiates engulfment.

Engulfment. There is some disagreement within the literature as to whether AC uptake occurs via phagocytosis or macropinocytosis, due to conflicting results of experiments examining the simultaneous uptake of AC and lucifer yellow, an accepted indicator of pinocytosis (39, 68). Regardless, engulfment requires actin mobilization by Rac1 (69-73) and is inhibited by RhoA (74). Other Rho family kinases and proteins that regulate Rho kinase activity may also be involved (75-77). PI3K signaling occurs downstream of AC

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recognition, producing phosphatidylinositol (PtdIns) (3,4,5)P3 that contributes to Rac1

activation (78). The molecule phosphatase and tensin homolog (PTEN) can

dephosphorylate PtdIns(3,4,5)P3 and thus inhibit Rac1 activation (78). Work in *C.*

elegans identified two recognition pathways that culminated in Rac activation via distinct

intracellular mediators: one via homologs of mammalian CrkII/ELMO/DOCK180 and

another through the homolog of mammalian GULP (79). Both of these pathways are

activated in mammalian cells following AC recognition (20, 72, 80-83). AC recognition

has been shown to activate Src and Syk family kinase signaling, but not Abl (84),

downstream of Draper (homolog of mammalian MEGF-10 and *C. elegans* CED-1) (85,

86). MEGF-10/CED-1 has been shown to interact with the heavy-chain of Clathrin and

AP2 during engulfment by phagocytes of human and worms (87, 88). AC engulfment

machinery involves contributions from a variety of cytoskeletal regulators and shares

many components with other types of uptake including endocytosis and Fc-mediated

phagocytosis.

Degradation. Following engulfment, the AC must be dealt with as a physical and

metabolic burden. At the completion of engulfment, the AC is contained within a

phagosome which acidifies and matures (89), degrading the engulfed cargo. Phagosome

maturation has been shown to require many of the same proteins as autophagy including

LC3 and beclin-1 (90-92). The degrading AC is broken down into lipids and proteins

that activate still more signaling pathways including AMP-activated protein kinase

(AMPK) (93), PPARδ (94), PPARγ (24, 95), RXRα (24), and LXRα (96). Signaling

through these pathways alters cellular metabolism to process the additional metabolic

burden of the AC (93). This signaling also upregulates components of AC engulfment machinery including Mertk (96) and MFG-E8 (94), enhancing the efficiency of further AC uptake. The entire process of AC clearance, from chemotaxis to degradation, is complex and interconnected.

Effects of AC clearance of the Phagocyte

In addition to the major metabolic changes wrought during the process of AC clearance, AC clearance significantly impacts immunity (12, 13). Most literature describes the tolerogenic capacity of efferocytosis. AC clearance has been shown to induce expression of suppressor of cytokine signaling 1 and 3 (SOCS1 and SOCS3) (97-99), Twist (100), and to prompt secretion of TGF-β (101-104), IL-10 (105, 106), platelet-activating factor (PAF) (107), and prostaglandin-E2 (PGE2) (108). Simply binding to AC can induce an anti-inflammatory response; antibody or Gas6 binding to TAM receptors is sufficient to induce SOCS1 and SOCS3 expression (109). Collectively, upregulation of these genes following AC clearance affects the engulfing phagocyte and the surrounding environment, facilitating suppression of the toll-like receptor (TLR) response, inhibition of cytokine signaling, induction of suppressive cells such as Tregs, and the initiation of tissue repair (11, 110). Murine models have shown that defective AC clearance leads to protracted inflammation (111, 112).

Uncleared AC can become necrotic, lose membrane integrity, and release their intracellular contents including inflammatory damage-associated molecular pattern molecules (DAMPs) such as DNA, histones, and HMGB1 (113-116). In addition to augmenting acute inflammation, the debris from necrotic cells can be cross-presented,

leading to activation of auto-reactive T cells and production of auto-antibodies (117).

Over time, defective AC clearance leads to lupus-like autoimmunity in multiple murine

models (23-25, 94, 96, 118, 119). Mutations in the C1q gene, a component of the

complement pathway that acts as an opsonin for AC, are associated with development of

lupus in humans (120). Importantly, engulfment of AC rather than necrotic cells is able

to produce the opposite response and induce "cross-tolerance" of self-antigens (12).

Thus, the tolerogenic, anti-inflammatory potential of AC clearance is important in wound

healing, resolution of inflammation, and maintaining self-tolerance.

However, there are exceptions to this rule and numerous contexts in which

clearance of AC is immunogenic. These contexts can be beneficial, such as in the cross-

presentation of tumor antigens from apoptotic tumor cells, inducing an anti-tumor

response (121). How phagocytes choose to treat an AC as immunogenic rather than

tolerogenic is not fully understood (13). The identity and activation state of the AC at the

time of apoptosis can bias towards immunogenic responses (122). The additional

presence of bacteria can result in immunogenic responses and contribute to the

development of Th17 cells (123). Importantly, the identity of the phagocyte that is

engulfing can also lead to an immunogenic response; AC clearance by inflammatory

monocytes rather than resident peritoneal macrophages (PMø) in mice leads to cross-

presentation of self-antigens and the development of autoimmunity (124). Directed AC

clearance to certain phagocyte subsets may be important in preventing cross-presentation.

This complex, context-dependent response to AC means that it is difficult to predict the

impact of death on the immune system. Whether the particular response is immunogenic

or tolerogenic, AC clearance is a vital event with notable impact on immunity.

The importance of AC clearance for lung health

The Curtis lab has a particular interest in AC clearance within the lung (125). Curiously, although AC clearance is an essential biological process, alveolar macrophages (AMø), the predominant leukocyte found within the alveolar space (126), have a low capacity for efferocytosis (127). Several factors contribute to the diminished capacity for AC uptake by AMø including reduced AC binding (128), low expression of PKCβII (129), and inhibition of Rac1 activity by lung surfactant proteins SP-A and SP-D signaling via SIRPα (64, 65). Other phagocytes contribute to AC clearance within the lung during inflammation including recruited monocytes and neutrophils (130-132).

Additionally, although AMø are the predominant leukocyte of the alveolar space, epithelial cells significantly outnumber AMø (133). Recent work has shown that epithelial cells are the important effectors of AC clearance during a model of allergic inflammation (134). Interestingly, alveolar epithelial type II cells are the source of the SP-A and SP-D that can both suppress efferocytosis by AMø through SIRPα signaling and opsonize AC, enhancing engulfment (135). Further work is needed to clarify the proportion of AC uptake performed by these diverse phagocyte subsets within the lung.

AMø from individuals with several chronic lung diseases, including chronic obstructive pulmonary disease (COPD) (136-138), asthma (139, 140), and cystic fibrosis (CF) (141), have decreased capacity for AC uptake relative to AMø from healthy subjects. These diseases are also characterized by an associated increase in uningested AC within the lung (142-144). It is likely that failed AC clearance exacerbates inflammation in chronic lung diseases; uncleared AC release numerous DAMPs that

drive local and systemic inflammation (113-115). Enhancing clearance has been shown to successfully decrease inflammation in multiple murine models of lung damage through reducing DAMP release and increasing phagocyte secretion of anti-inflammatory mediators such as TGF-β (103, 145-149). Conversely, inhibiting efferocytosis worsens inflammation (111, 112, 150, 151).

The disease-specific factors that may contribute to diminished efferocytosis in chronic lung disease are complex (**Fig. 1.3**). Surface expression of AC recognition receptors on AMø is decreased in CF and COPD (137, 151, 152). TNFα signaling, which is enhanced during inflammatory lung diseases (153-155), has been shown to suppress AC uptake (111, 156). Oxidant stress, also enhanced in inflammatory lung disease (157-161), has been shown to inhibit AC uptake through activation of RhoA (162). Further work is required to understand how these factors contribute to the increased burden of uncleared AC in lung disease and how that burden contributes to protracted inflammation.

In addition to effects on chronic lung disease, AC clearance within the lung modulates pathogen response and can both enhance and inhibit bacterial killing. Engulfment of AC leads to the secretion of PGE_2 and inhibition of subsequent bacterial killing (108). Instilling AC in the lung prior to infection worsens bacterial clearance and lung inflammation (108). However, clearance of AC that have undergone phagocytosis-induced cell death after engulfing bacteria contributes to effective clearance of numerous lung pathogens and to lung repair following infection (163-165). Instilling AC in the lung following infection improves bacterial clearance and decreases lung inflammation

(147), further evidence that the context of AC clearance determines the subsequent immune effects.

The possibility of therapeutic enhancement of AC clearance

The observations that uncleared AC can drive inflammation and that defective AC clearance occurs in human disease have led to speculation that enhancing AC clearance could have therapeutic use (133, 166, 167). Methods proposed include the use of pharmacologic agents to increase the engulfment potential of phagocytes, the addition of exogenous AC opsonins and, for the resolution of acute inflammation, increasing the availability of AC.

AC clearance can be augmented through phagocyte exposure to glucocorticoids (GC) (65, 168, 169), azithromycin (136, 137), statins (170), and antioxidants such as poractant alfa (171). GC, statins, and antioxidants culminate in increased Rac1 activity. GC increase Mertk expression and Rac activation (168, 172). In Chapter 2 we describe an additional mechanism by which GC enhance AC uptake by AMø: downregulation of SIRPα. Statins inhibit prenylation of Rho family kinases with a disproportionate effect on RhoA, thus preventing RhoA inhibition of Rac1 through altering subcellular localization (173-175). Antioxidants also act on RhoA, inhibiting its function and thus promoting Rac1 activity (162, 176-178). The mechanism by which azithromycin enhances AC clearance is less clear but may involve upregulation of mannose receptor (137). Interestingly, GC and azithromycin are commonly prescribed to patients with the chronic inflammatory disease COPD (133). It is unclear how their effect on enhancing AC engulfment may impact their efficacy in relieving the symptoms of COPD.

The availability of AC can be increased through inducing apoptosis in vivo or administering exogenous AC; both mechanisms have shown positive effects in murine models of acute inflammation. Enhancing apoptosis of neutrophils during respiratory infection can accelerate resolution as apoptotic neutrophils are engulfed (147). Direct instillation of AC into the peritoneum or lung during inflammation also promotes resolution via induction of TGF-β and hepatocyte growth factor following AC uptake (103, 148). Direct administration of exogenous AC opsonins has also been shown to beneficially accelerate resolution of inflammation in mice (145).

Although these murine results are promising, the current understanding of how AC impact immunity is a significant limitation in implementing the therapeutic enhancement of AC clearance. The response to AC is complex and the assumption that increased AC clearance contributes to the resolution of inflammation may be an oversimplification (**Fig. 1.4**) (12). Based on findings in the spleen, broadly enhancing AC engulfment could potentially cause a breakdown of directed AC clearance and lead to cross presentation of self antigens (124). In addition to the target effects on inflammation and tissue repair, AC clearance also leads to suppressed TLR response and bacterial killing. In a murine model this has been shown to increase susceptibility to infection (108). In Chapter 3 we ask how enhancing AC clearance using GC will impact susceptibility to bacterial pneumonia.

The published understanding of AC clearance has deepened in the past decades; researchers have identified AC recognition receptors, mapped the intracellular signaling during engulfment and identified and numerous effects of AC on immunity. However, much remains unknown and we continue to identify new components, new shared

pathways, and new complexity in AC clearance. Work presented in chapters 2 and 3 demonstrates a novel mechanism of GC-augmented efferocytosis and a negative consequence of pharmacologically enhanced AC clearance on immunity: enhanced susceptibility to bacterial infection. Chapter 4 identifies a novel master-regulator of AC clearance that is highly expressed in AMø and which acts on both known and novel components of the engulfment machinery. Together these studies increase understanding of the mechanisms and immune effects of AC clearance, unraveling more of the complex regulation that controls this essential biologic process.
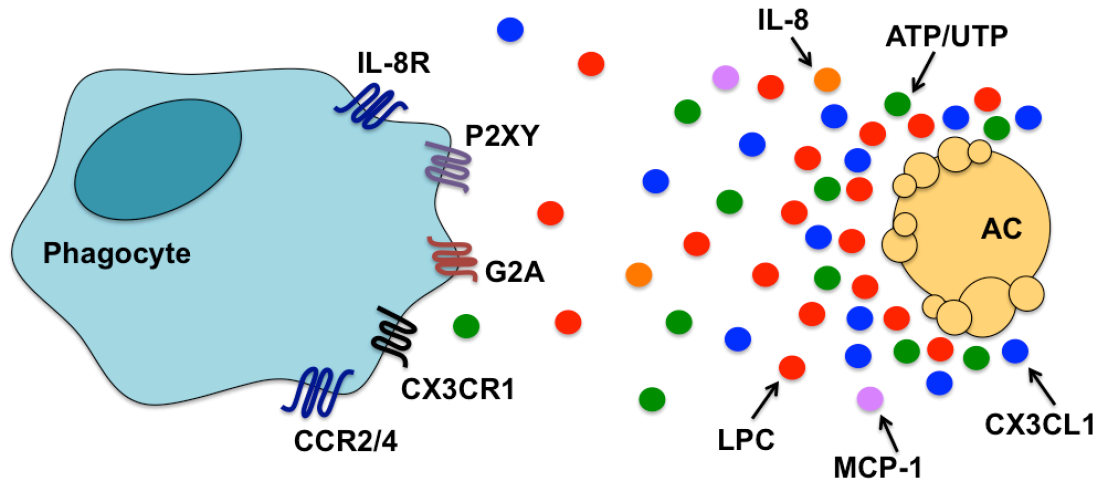
Figure 1.1 Chemotaxis during AC clearance. Release of CX3CL1, ATP, UTP, lysophosphatidylcholine (LPC), IL-8, and MCP-1 by apoptotic corpses causes directed movement of phagocytes by signaling through CX3CR1, P2XY, G2A, IL-8R, and CCR2/4.
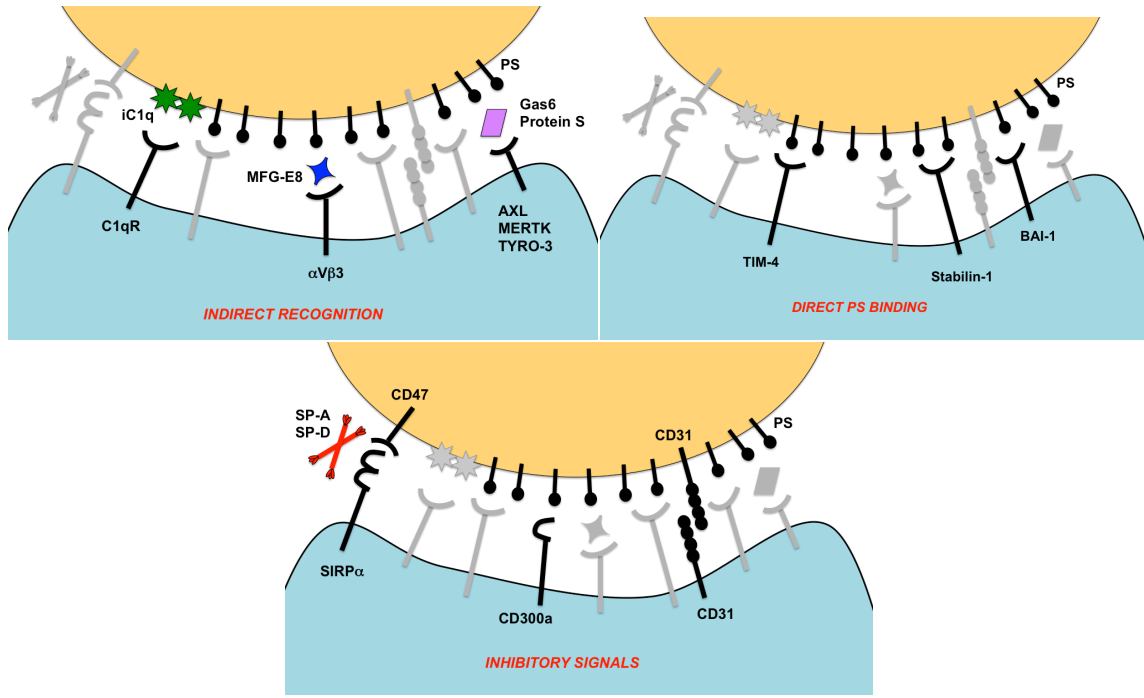
Figure 1.2 Receptors involved in AC recognition. AC are recognized indirectly through bridge molecules and opsonins MFG-E8, iC1q, Gas6 and Protein S binding to C1qR, $\alpha_v\beta3$ integrins, Axl, Tryo3 and Mertk. AC are recognized directly through TIM-4, Stabilin-1, and BAI-1 recognition of exposed phosphatidylserine (PS). AC engulfment can be inhibited by signaling through SIRPα, CD31 and CD300a.
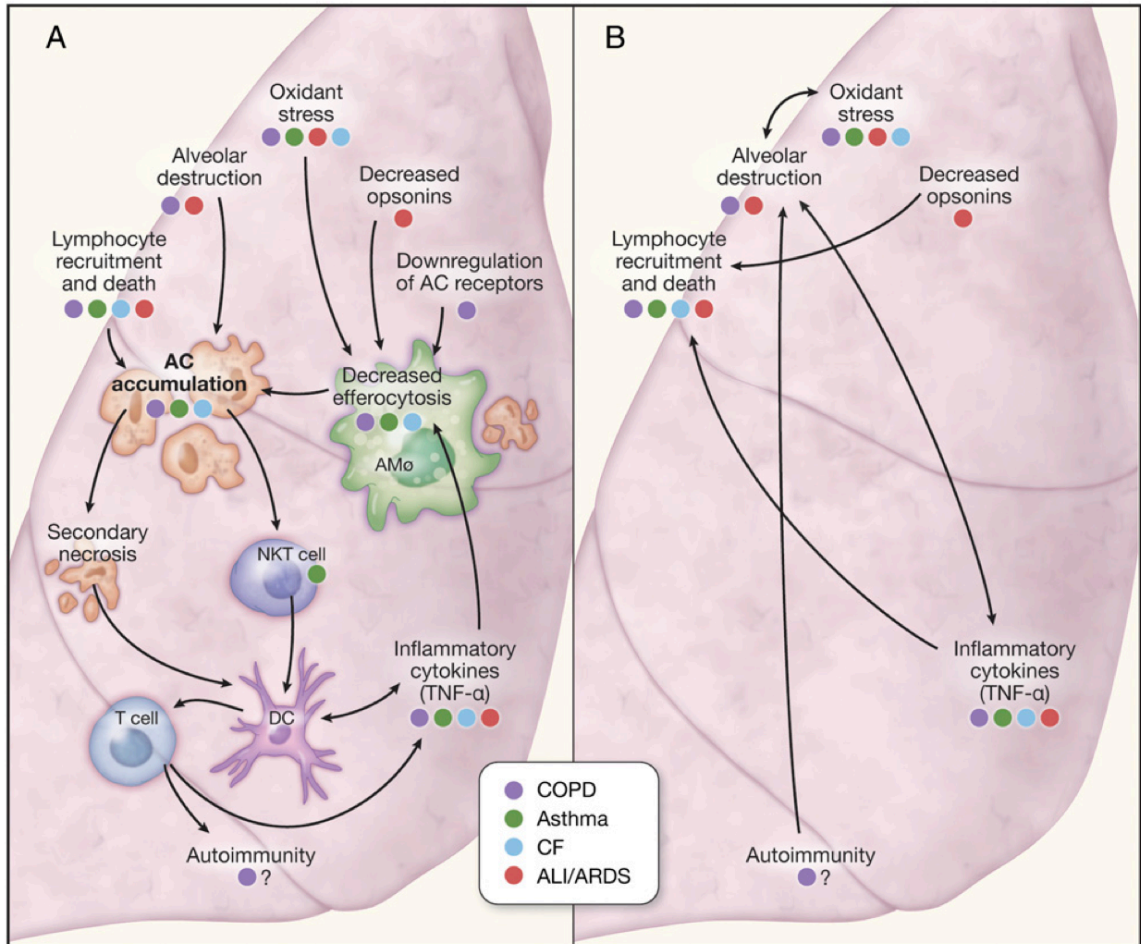
Figure 1.3 Interplay between lung disease and decreased AC clearance. A, Processes increasing AC accumulation in inflammatory lung diseases. Although the basal efferocytic capacity of resident AMø is low, oxidant stress and proteolytic events during inflammation can further reduce concentrations of efferocytic opsonins and cleave efferocytic receptors, leading to greater apoptotic cell accumulation. Uncleared apoptotic cells undergo secondary necrosis, which can expose autoantigens. Uningested apoptotic cells can also stimulate NKT cells to activate DCs, driving maturation of T cells, which can be pro-inflammatory or even autoreactive. The resulting release of inflammatory cytokines can both increase DC activation and further decrease efferocytosis. B, Feedback loops resulting from decreased efferocytosis. Oxidant stress, inflammatory cytokines, and autoimmunity can all amplify alveolar destruction, a potential source of ACs. Alveolar destruction itself amplifies inflammatory cytokine release and oxidant stress. Decreased efferocytic opsonins and increased inflammatory cytokines enhance leukocyte recruitment. Evidence linking a specific disease to any of these factors or consequences is noted with colored circles. AC - apoptotic cell; ALI - acute lung injury; AMø - alveolar macrophage; CF - cystic fibrosis; DC - dendritic cell; NKT - natural killer T; TNF - tumor necrosis factor. (Illustration by Haderer & Muller Biomedical Art, LLC.) Reproduced from McCubbrey and Curtis 2013.

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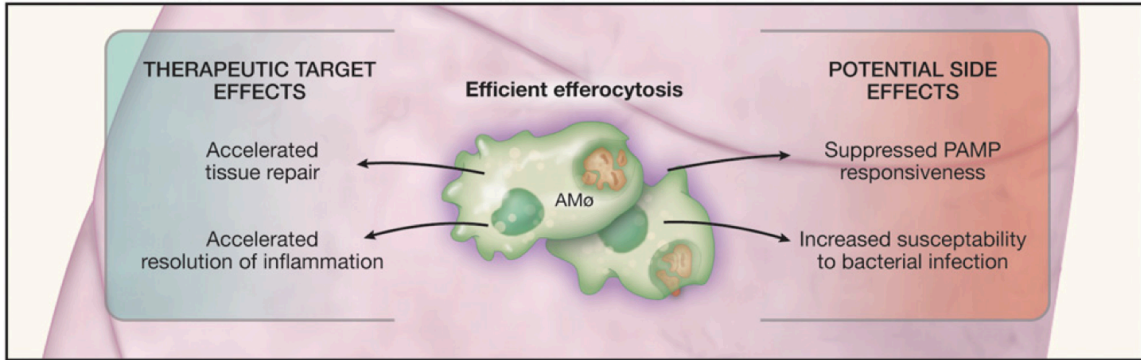
Figure 1.4 Theoretical positive and negative effects of therapeutic enhancement of AC clearance. (Illustration by Haderer & Muller Biomedical Art, LLC.) Reproduced from McCubbrey and Curtis 2013.

Chapter 2

Glucocorticoids relieve collectin-driven suppression of apoptotic cell uptake

in murine alveolar macrophages through downregulation of SIRPα[1]

Abstract

Apoptotic cell (AC) clearance or efferocytosis can be impacted by common

pharmacological therapies including glucocorticoids (GC), macrolides, and statins. Here

we provide evidence that AC engulfment by alveolar macrophages (AMø) but not their

peritoneal counterparts (PMø) is uniquely and rapidly increased by GC. Although

previous studies of GC treatment during human blood monocyte or murine bone marrow-

derived macrophage differentiation have identified a mechanism involving increased

translation of Mertk, we find that no new protein translation is required for the rapid

effect of the GC fluticasone on AMø efferocytosis. Fluticasone rapidly suppresses AMø

and PMø expression of signal regulatory protein alpha (SIRPα) mRNA and surface

protein. Lung collectins, which do not normally circulate in the peritoneum, have been

shown to inhibit AMø AC uptake by signaling through SIRPα. Following in vitro

treatment of PMø with the lung collectin surfactant protein D (SP-D), AC uptake by PMø

is inhibited. Although untreated PMø lack this capacity, PMø pre-treated with SP-D

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rapidly increase their capacity for AC clearance in response to fluticasone. In summary, the potent GC fluticasone increased AC uptake by murine AMø or PMø pre-treated with SP-D in a rapid, dose-dependent fashion through downregulation of SIRPα. These findings emphasize the importance of the unique lung environment and thus, more globally, of studying the effects of GC on primary AMø to fully understand the impact inhaled corticosteroid (ICS) treatment has on lung health.

Introduction

Apoptotic cell (AC) uptake by phagocytes, also termed efferocytosis (3), is an essential process that promotes the resolution of injury and inflammation, facilitating tissue repair in the lung and throughout the body (13, 14, 179). Impaired AC uptake has been found in phagocytes from human subjects with cystic fibrosis, asthma, and COPD (138, 139, 141, 151). Because defective AC clearance clearly contributes to autoimmunity in murine models (119, 180), and because there is growing evidence that human emphysema may have an autoimmune component (181, 182), potential therapies designed to bolster AC clearance in the lung have been proposed (133). This issue is of considerable importance, as COPD is now the third leading cause of death in the United States, and has been projected by the World Health Organization to become the leading worldwide cause of death by mid 21st century (183).

In seeming contradiction to the importance of AC clearance, the resident lung phagocyte, alveolar macrophages (AMø), bind and engulf AC less avidly than do other professional phagocytes (184-186). Reduced efferocytosis by AMø results in part from very restricted adhesion pathway usage (186, 187) and markedly decreased expression of

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PKCβII (186, 187). Importantly, however, the normal lung environment actively suppresses the ability of AMø to ingest AC, due to the inhibitory action of specific surfactant proteins, the lung collectins SP-A and SP-D, through their interaction with SIRPα (64). After AMø removal from the lung environment, this effect gradually decreases in vitro over days.

It is unclear whether increasing the ability of AMø to ingest AC would have beneficial heath affects. Inefficient AC clearance leads to autoimmunity in numerous murine models (24, 25, 96, 119). However, efficient AC clearance performed by improper phagocytes can also trigger the development of autoimmunity (124); clearance must be restricted to certain immune subsets to balance the tolerogenic and immunogenic response, although the identity of these subsets is poorly understood. A better understanding of the unique mechanisms and immune consequences of AMø interaction with AC is essential to guide the development of any future therapies involving altered AC clearance.

Problematically, several common pharmacological treatments used to relieve the symptoms of COPD have been shown to increase AC uptake with no understanding of the potential negative or positive consequences that altering AC uptake has on treatment effectiveness (136, 137, 169, 170, 175). Of particular import, GC have been shown to increase in vitro AC uptake by human blood-derived monocytes, macrophage cell lines, and, in a single report, human AMø (139, 169). The mechanism by which this occurs has only been described in human blood-derived monocytes, in which this increase is dependent on Mertk, increased Rac1 phosphorylation and altered surface sialylation (168, 172, 188). It is unclear whether GC act via these mechanisms in other cell types such as

AMø. Defining whether and how GC and other agents increase AC uptake by murine AMø is an essential step to develop murine models aimed to increase our understanding of how pharmacological manipulation of efferocytosis in the lung would affect lung health.

In this study, we report that the potent GC fluticasone increased AC uptake by murine AMø in a rapid, dose-dependent fashion through downregulation of SIRPα. Our data show a novel facet of GC action: a rapid decrease in the sensitivity of murine AMø to the collectin-rich, inhibitory environment of the lung, thus lifting tonic inhibition and increasing AC uptake.

Results

Potent GC rapidly increase murine AMø uptake and binding of AC. To study the effect of GC used clinically as inhaled corticosteroids (ICS) on AC uptake by murine AMø, we first performed in vitro phagocytosis assays following treatment with the potent GC fluticasone **(Fig. 2.1A-C)**. Pre-treatment with fluticasone significantly increased the ability of murine AMø to ingest AC after only 3 h, with peak effect by 6 h **(Figs. 2.1D, 2.1E)**. The magnitude of the effect was dose-responsive, increasing with higher doses of fluticasone; statistically significant increases were measured at and above 2 nM **(Fig. 2.1F, 2.1G)**. Fluticasone treatment also increased AMø uptake of UV-killed thymocytes **(Fig. 2.2A, 2.2B)**, implying that the effect did not depend on the method used to induce apoptosis. This pro-clearance effect was not restricted to fluticasone, as increased AMø AC uptake could also be seen following treatment with budesonide, another potent GC used clinically **(Fig. 2.2C, 2.2D)**. In contrast, AC uptake by resident murine PMø did not

increase within 6 h of fluticasone treatment **(Fig. 2.3A, 2.3B)**. Additionally, fluticasone

did not increase Fc-mediated clearance of IgG-opsonized Sheep red blood cells (SRBC)

(Fig. 2.3C, 2.3D) by murine AMø.

To study the effect of GC on murine AMø binding of AC, we next performed

adhesion assays **(Fig. 2.4A)**. Similar to the effect on AC engulfment, 4 h treatment with

fluticasone significantly increased the ability of murine AMø to bind AC, with the effect

peaking by 6 h **(Fig. 2.4B, 2.4C)**. The magnitude of the effect was also dose-responsive;

statistically significant increases were measured at doses above 200 pM **(Fig. 2.4D,**

2.4E). To determine if fluticasone initiated novel adhesion pathways, we pre-treated

AMø with mAbs to block CD11c and CD18, which we have previously shown mediate

the majority of adhesion of AC to murine AMø (186). Blocking either integrin subunit

reduced AMø binding to AC, regardless of treatment with fluticasone **(Fig. 2.4F)**. In

contrast, similar to the lack of effect on engulfment, fluticasone treatment did not

increase PMø binding to AC **(Fig. 2.4G)**.

Thus, GC pretreatment is associated with rapidly increased AC binding and

engulfment that is specific to AMø and not observed in a resting, fully-differentiated

tissue Mø from another mucosal surface. Further, the ability to increase AC uptake

appears to be a class effect of potent GC, which, however, does not alter phagocytosis by

murine AMø of other types of particles.

Fluticasone initiates reprogramming towards a pro-clearance phenotype and increases

AC uptake without a requirement for new protein synthesis. GC alter expression of large

numbers of target genes, for the most part via glucocorticoid receptor- α (GRα), a

member of the ligand-regulated family of nuclear receptors (189), but also by incompletely understood translation-independent mechanisms (190, 191). Blocking GRα using RU-486 completely inhibited the effect of fluticasone on AC uptake, indicating that fluticasone acts on efferocytosis through canonical GRα binding (**Fig. 2.5A, 2.5B**). To begin to define how fluticasone upregulates murine AMø uptake of AC, we assessed the expression of several genes known to be involved in AC clearance, including Mertk and Axl, members of the TAM family of receptor tyrosine kinases (192), CD91/LRP (193) and the negative regulator SIRPα (64). We also examined mRNA expression of the nuclear receptor PPARδ, a positive regulator of the expression of opsonins involved in bridging AC and of Mø surface receptors including Mertk (94). Within 3 h of fluticasone treatment, Mertk mRNA significantly increased, whereas SIRPα transcripts significantly decreased **(Fig. 2.5C)**. These changes are consistent with a GC-driven induction of a pro-clearance AMø phenotype, as previously described for human monocytes (194). Transcripts for Axl, LRP and PPARδ did not change during this period of fluticasone treatment.

The rapid kinetics of increased AC uptake in murine AMø led us to postulate that the rapid effect of fluticasone may be mediated through transrepression of SIRPα rather than induction of Mertk. To test that possibility, we blocked new protein synthesis using cycloheximide. Treatment of AMø with cycloheximide prior to an additional 5 h fluticasone treatment did not abrogate the increase in AC uptake **(Fig. 2.5D)**. Thus, although Mertk and likely other AC recognition molecules were significantly increased by fluticasone treatment, translation-dependent increases in Mertk or any other protein were not required for the rapid (< 5 h) effect of fluticasone. However, treatment of AMø

with cycloheximide prior to an additional 23 h fluticasone treatment partially blocked the

increase in AC uptake (**Fig. 2.5E**). This result indicates that both translation-dependent

and independent mechanisms are involved in the delayed effect of fluticasone on AC

engulfment. Following 24 h fluticasone treatment, surface expression of Mertk was

upregulated, while Axl expression was unchanged (**Fig. 2.5F**). It is likely that Mertk is

the cyclohexamide-inhibitable protein required for the optimal delayed effect of

fluticasone on AMø, in agreement with the Mertk requirement previously demonstrated

in studies of blood monocyte-derived macrophage differentiation (172), but Mertk

induction is not necessary for the rapid effect of fluticasone on AMø.

Fluticasone decreases protein expression of SIRPα. To test the significance of the

observed fluticasone-induced gene repression of SIRPα **(Fig. 2.5C)**, we examined protein

expression of SIRPα. Using flow cytometry, we found that surface expression of SIRPα

was decreased within 6 h of fluticasone treatment, with statistical significance reached by

24 h **(Fig. 2.6A, 2.6B)**.

We also tested the involvement of several pathways that have been implicated in

AC uptake by other types of tissue Mø, using pharmacological inhibitors or blocking

mAbs. Neither fluticasone-treated AMø, nor as we have previously described (185),

untreated murine AMø require CD36, α_v integrin or autocrine prostanoid signaling for

AC uptake **(Fig. 2.6C-H)**. These results complement those in which we blocked CD11c

and CD18 **(Fig. 2.4F)** in indicating that GC-augmented AC uptake does not require

engagement of new adhesion pathways but instead appears to result from increased

efficiency of the same pathways used in the resting state.

Azithromycin but not simvastatin has additive effects on GC-augmented efferocytosis. In addition to GC, AC uptake is known to be increased by other commonly prescribed pharmaceuticals including statins and macrolides (136, 137, 175). To study interactions between these medications, we treated murine AMø with combinations of fluticasone, simvastatin and azithromycin, and then assessed the effect on AC engulfment. Treatment with simvastatin or fluticasone alone each increased AC uptake, but the combination had no additive effect (**Fig. 2.7A, 2.7B**). By contrast, treatment of AMø with azithromycin and fluticasone was additive, resulting in near doubling of uptake capacity over either treatment alone (**Fig. 2.7C, 2.7D**).

Simvastatin affects AC uptake via the SIRPα pathway and mechanisms that require new protein translation. The lack of additive effect between simvastatin and fluticasone suggested that these agents likely affect AC uptake through the same molecular pathway. This possibility is supported by previous evidence that statin treatment decreases localization to the plasma membrane of RhoA, a downstream effector of SIRPα signaling; because RhoA antagonizes the essential action of Rac1 on AC uptake, the net effect is increased efferocytosis (24, 170). We used flow cytometry to test whether either simvastatin or azithromycin also affected SIRPα surface expression. Azithromycin did not change SIRPα expression compared to untreated AMø, but simvastatin significantly decreased SIRPα surface expression after 24 h (**Fig. 2.8A, 2.8B**). However, in contrast to fluticasone, simvastatin did not change SIRPα mRNA levels (data not shown).

To further differentiate possible mechanisms of action, we next blocked induction

of new protein synthesis by these two agents. Treatment of murine AMø with

cycloheximide prior to 24 h of treatment with simvastatin or azithromycin blocked the

ability of either agent to increase AC uptake over that of untreated AMø **(Fig. 2.8C)**.

These results indicate that, unlike fluticasone, both simvastatin and azithromycin do

require new protein synthesis to increase AC uptake in AMø. Thus, while simvastatin

and fluticasone both culminate in lowered SIRPα expression and inhibition of RhoA,

their upstream signaling differs.

SP-D treatment inhibits AC uptake by PMø, which is reversed with fluticasone treatment.

The inhibitory effect of SIRPα on AC uptake by murine AMø is tonically maintained by

constant exposure in the alveolar space to high concentrations of the lung collectins SP-A

and SP-D (64). By contrast, although PMø express surface SIRPα (195), they receive

limited exposure to lung collectins. These considerations led us to hypothesize that the

absence of GC-augmented AC uptake by PMø **(Fig. 2.3A, 2.3B)** might reflect limited

activation of SIRPα in the peritoneal cavity. To test this possibility, we first used flow

cytometry to test whether SIRPα expression on PMø was altered by fluticasone treatment

in vitro. Similar to AMø, 24 h of fluticasone treatment significantly decreased PMø

expression of SIRPα surface protein, whether expressed as percentage positive relative to

isotype control or mean fluorescence index (MFI) **(Fig. 2.9A-C)**. Next, by pre-

incubating PMø with the SIRPα ligand SP-D, we investigated whether activation of

SIRPα could repress AC uptake by murine PMø. SP-D significantly inhibited AC uptake

within 4 h **(Fig. 2.9D)**. Finally, we tested whether fluticasone treatment could rescue

decreased PMø AC uptake following SP-D treatment. Although treatment with SP-D alone significantly inhibited AC uptake, subsequent incubation with fluticasone for 5 h completely reversed this inhibition **(Fig. 2.9D)**. These results provide a proof-of-concept that the rapid effect of GC on AC uptake by tissue Mø is mediated by release of collectin-induced repression acting via surface SIRPα expression (**Fig. 2.10**), and does not depend on GC-modification of other features of the AMø phenotype.

Discussion

The results of this study identify downregulation on AMø of the inhibitory receptor SIRPα, which releases them from tonic inhibition by lung collectins, as a novel mechanism by which clinically-relevant potent GC rapidly increase AMø uptake of AC. Using primary murine AMø, we found that treatment with fluticasone or budesonide increased both binding and uptake of AC within 3 h, without apparent induction of novel adhesive pathways. The effect did not require new protein synthesis, although its magnitude continued to increase through 6 h in association with significantly increased Mertk and decreased SIRPα expression. Reduced SIRPα surface expression would be fully anticipated to prevent SIRPα signaling via binding of SP-A and SP-D and thus prevent the subsequent downstream activation of RhoA and hence Rho Kinase to inhibit Rac (64), on which AC ingestion depends crucially (74, 196). Although fluticasone treatment of resting murine PMø did not show the same rapid effect on AC uptake, brief pre-treatment of PMø with SP-D induced a significant reduction in their AC uptake that was rapidly reversed by fluticasone treatment. These findings emphasize the importance

of the unique lung environment and thus, more globally, of studying primary phagocytes isolated from sites of interest in attempting to understand host defense of specific organs.

By defining a rapid, translation-independent effect on fully differentiated tissue Mø, these results extend previously described mechanisms of GC actions during Mø differentiation from precursors (168, 169, 172, 188, 197, 198). In contrast to the early SIRPα–dependent mechanism we show in mature AMø, results in those studies required new protein synthesis and more prolonged treatment, maximal when GC was added 3-5 days earlier. Thus, these studies were informative of the effects of systemic steroid treatments on Mø precursors, but not directly relevant to the question about how ICS might impact functions of resident AMø. Similarly, two groups have used microarray technology to define the effects of GC on gene regulation during in vitro differentiation of human monocytes (194, 199). They found alterations in a range of molecules plausibly involved in AC clearance, including integrins, scavenger receptors, receptor tyrosine kinases, bridging molecules, molecules associated with engulfment, nuclear receptors, and members of the interferon regulatory family genes. Our finding of upregulation of Mertk transcripts and surface protein expression is compatible with the initiation by GC of such a more prolonged multi-gene program in AMø, but the full range of such more delayed effects will require further study.

Our findings agree with and follow directly from recent publications that identified the importance of the alveolar environment to maintain a carefully regulated AMø phenotype (64, 126), particularly in terms of AC uptake. We believe that this line of investigation highlights the ability for elegant control of AMø function by altered expression of key receptors rather than by disruption of this fragile environment. SP-A

and SP-D serve at least three immunomodulatory functions in the alveolar space: modulating basal AMø signaling in the absence of AC; binding directly to AC to increase their uptake; and acting as opsonins of multiple lung pathogens (200). Maintaining the surface tension of alveoli for optimum gas exchange is not considered the primary role of SP-A or SP-D, as it is for SP-B and surfactant lipids (201). Breathing by SP-A-/- mice is completely normal (202), although SP-D-/- mice show an accumulation of surfactant lipid in the lung (203), which can lead to chronic low-grade pulmonary inflammation and fibrosis (204). Transgenic mice deficient in SP-A or SP-D have increased susceptibility to multiple viral, bacterial and fungal infections (reviewed in (205)). We speculate that regulating SP-A and SP-D signaling by altering SIRPα expression on AMø, rather than directly by modulation of lung collectin levels, permits the continuation of important SIRPα-independent signaling and particularly opsonic functions of the lung collectins.

Recent work has demonstrated that lung epithelial cells perform the essential majority of AC engulfment (134); the burden of clearance required to maintain tolerance is not upon the AMø. Interestingly, type II alveolar epithelial cells provide the main source of pulmonary surfactants (135) that signal through SIRPα on AMø and suppress AC clearance. Epithelial cells themselves do not appear to express significant SIRPα, implying that their rate of AC clearance would be unsusceptible to suppressive surfactant signaling (206).

A fascinating parallel in the peritoneal cavity demonstrates how such directed AC uptake may have substantial immune import. During sterile inflammation, PMø are significantly outnumbered by inflammatory monocytes, yet remain the primary phagocyte involved in AC clearance (124). This disparity depends on the production of

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oxidized lipid products by 12/15-lipoxegenase (12/15-LO) that act as negative regulators of AC uptake by inflammatory monocytes. In 12/15-LO deficient mice, both PMø and inflammatory monocytes readily engulf AC. Interestingly, in a model of induced lupus, 12/15-LO deficient mice fare significantly worse than their competent counterparts; AC clearance by their inflammatory monocytes leads to cross-presentation of AC-derived autoantigens and a break in self-tolerance (124). The division of AC clearance within the lung may be similarly restricted to maintain the essential balance between tolerance and immunogenicity, although there is no evidence to date that this division involves differential expression of 12/15 LO.

There is significant evidence that AC clearance is essential in maintaining self-tolerance. The development of lupus-like autoimmunity in mice lacking efferocytic genes including C1q (207), MFG-E8 (23), α_v integrins (208, 209) and the TAM receptors (22, 119, 210, 211) demonstrate that a failure to clear AC will result in autoimmunity as excess necrotic material leads to the prevalence of autoantigens and subsequent development of autoantibodies. Additionally, uptake of AC activates multiple anti-inflammatory pathways within phagocytes, notably through upregulation of SOCS1 and SOCS3 and subsequent inhibition of Jak-STAT signaling (97). AC clearance often leads to the release of anti-inflammatory mediators such as IL-10 and TGF-β that are important in the resolution of inflammation and tissue repair (107, 212).

In complement to this large literature on the tolerogenic effects of AC clearance, there is significant evidence that AC clearance can be immunogenic, for good or ill: clearance of normally anti-inflammatory AC by inflammatory monocytes leads to autoimmunity in 12/15-LO deficient mice (124), while clearance of apoptotic tumor cells

can lead to effective anti-tumor responses (213). It is important to recognize that directed

AC uptake is likely context dependent. In a model of sterile inflammation, similar to what

was observed in the peritoneum (124), clearance by inflammatory monocytes is

negligible within the lung and peritoneum (130). In contrast, in mice treated with

intratracheal lipopolysaccharide (LPS) to induce non-sterile inflammation, inflammatory

monocytes develop increased AC clearance (64). During an infection, this switch could

assist with cross-presentation of intracellular pathogens, leading to a more effective

adaptive immune response. It is unclear whether the increase in clearance is induced by

LPS-induced changes on the monocyte, the AC, the lung environment, or a combination

of these factors, but it is interesting to speculate how the dynamic regulation of negative

environmental inhibitors such as SIRPα in the lung or 12/15-LO in the peritoneum could

function as an important rheostat to control the appropriate immunogenic or tolerogenic

response to AC.

It is also interesting to consider how the low rate of efferocytosis in AMø may be

of evolutionary value. AMø antigen presenting capacity is suppressed by the alveolar

environment (126, 214). However, once removed from these external signals, as could

perhaps occur within a draining lymph node, AMø present antigen with high efficiency

(126); the sorted clearance that occurs within the alveolar space may be important in

preventing cross-presentation of autoantigens. Additionally, it is plausible that

preventing AC-induced SOCS1, SOCS3, and TGF-β expression may maintain AMø as

sentinel immune responders. The in vivo potential of such AC-induced suppression to

negatively impact host defense has been shown in a murine model, in which

intrapulmonary administration of large numbers of AC reduced phagocytosis and killing

of *Streptococcus pneumoniae* and impaired leukocyte recruitment through PGE_2-EP2-dependent signaling (108). If restricting AC clearance in AMø is important in maintaining the tolerogenic and immunogenic balance of the lung environment, GC enhancement of AC clearance may be disrupting an important regulatory mechanism that has evolved in AMø.

Increased AC uptake has been shown in various phagocytes in vitro using a number of pharmacological agents including GC, statins and macrolides. To our knowledge, this is the first report describing how simultaneous treatment with these drugs, commonly prescribed to individuals with respiratory disease, affects AC uptake in any cell type. The lack of additive effect between simvastatin and fluticasone is congruent with a shared mechanism of action: inhibition of RhoA leading to increased Rac activity. Of more interest is the additive effect of azithromycin and fluticasone on AC uptake, especially given the recent demonstration that azithromycin reduces the frequency of acute exacerbations of COPD (215). The mechanism for the positive effect of azithromycin on AC uptake remains undefined and will require considerable extra investigation; our results imply that azithromycin does not act on RhoA. Decreased AC uptake has been found in AMø from individuals with COPD (138) and asthma (139) when compared with healthy controls, which has prompted speculation that poor AC clearance may be contributing to various forms of inflammatory lung diseases. Our work does not address this hypothesis, but does identify a novel additive interaction between fluticasone and azithromycin that produces a robust increase in AC uptake and may be useful in future therapy.

The finding that SP-D can activate the pre-existing high levels of SIRPα on PMø merits discussion in relationship to acute lung injury, in which plasma concentrations of SP-A and SP-D increase significantly and correlate with clinical outcomes (216-218). Sepsis, the most common antecedent of acute lung injury, is associated both with massive apoptosis of circulating lymphocytes and with a delayed immunocompromised state. Results in murine models suggest that the first of these observations may explain the second, via the immunosuppressive effect of AC uptake on innate immunity (219, 220). Although our results strongly imply that SIRPα signaling is not active in resident PMø harvested from untreated mice, they do suggest that increased circulating levels of lung collectins could contribute to reduced efferocytosis through the body during acute lung injury. Moreover, signaling via SIRPα also suppresses Mø phagocytosis mediated by FcγR and complement receptors (221, 222). Thus, the possibility should be investigated that circulating SP-A and SP-D are not only biomarkers of severity during acute lung injury, but might also contribute to systemic immunosuppression that leads to the frequent superinfections that characterize this condition.

Defining how GC affect AMø is particularly important as a result of the widespread prescription of ICS for the treatment of lung disease. Multiple clinical trials have noted that receiving ICS is associated with increased hospitalization of COPD patients with pneumonia, compared to COPD patients receiving non-steroidal treatment, suggesting ICS treatment results in increased susceptibility to infection (223-233). In contrast, mice pre-treated with fluticasone had significantly reduced lung bacterial burdens 24 and 48 h after *Streptococcus pneumoniae* infection, suggesting that fluticasone is protective and increases bacterial clearance (234). Our findings in murine

AMø and previous findings in human AMø strongly suggest that GC treatment, by increasing AC uptake, will enhance the effects of AC on AMø including decreased TLR response and decreased bacterial killing. It would be interesting to test whether increased immunosuppression from AC within the lung may explain these opposing results between COPD patients and model systems regarding ICS use and pneumonia infection, particularly for emphysema patients where lung destruction generates large numbers of AC. Our finding that murine AMø efferocytosis is increased following GC, azithromycin or simvastatin treatment demonstrates that mice provide an appropriate model system with which to predict consequences of pharmacologically-augmented AC clearance on human lung disease.

In summary, to our knowledge, our study demonstrates for the first time that GC, azithromycin, and simvastatin increase AC uptake by murine AMø. We provide evidence that the rapid increase trigged by GC is caused by disruption of collectin-SIRPα signaling through downregulation of SIRPα transcript and surface protein, a novel GC mechanism. Finally, we demonstrate that regulation of AC uptake by SIRPα is not restricted to AMø and can be activated in PMø following exposure to SP-D.

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Service, Department of Veterans Affairs.
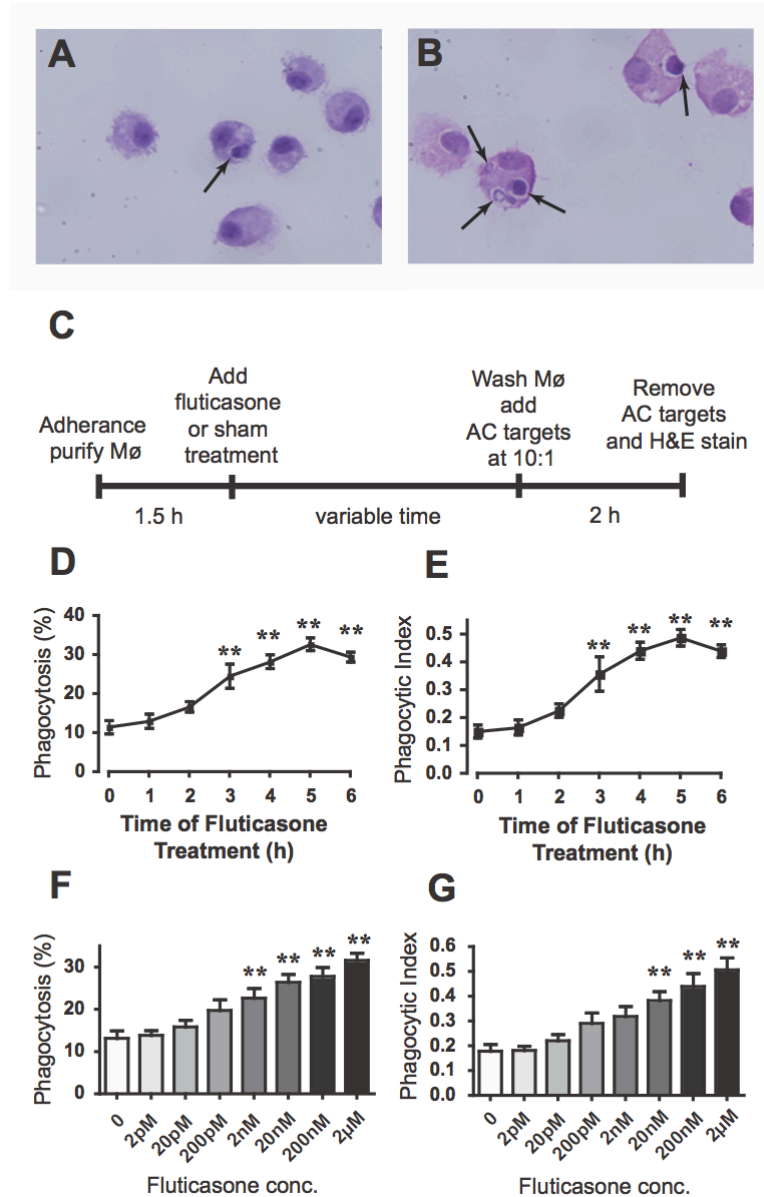
Figure 2.1. Fluticasone rapidly increases uptake of AC by murine AMø. Adherence-purified AMø from normal C57 BL/6 mice were treated in chamber slides with (A) control media or (B) 2μM fluticasone for 6 h, then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E and representative photographs were taken at 100X magnification under oil. Arrows denote ingested AC. C. Graphic timeline of a phagocytosis assay. D, E. Kinetics of GC-augmented AC uptake. F, G. Dose-response of GC-augmented AC uptake. Data are mean ± SE of 5-8 mice assayed individually in at least two independent experiments per condition. **, statistically significant, p<0.01 by One-Way ANOVA with Bonferroni post-hoc testing.
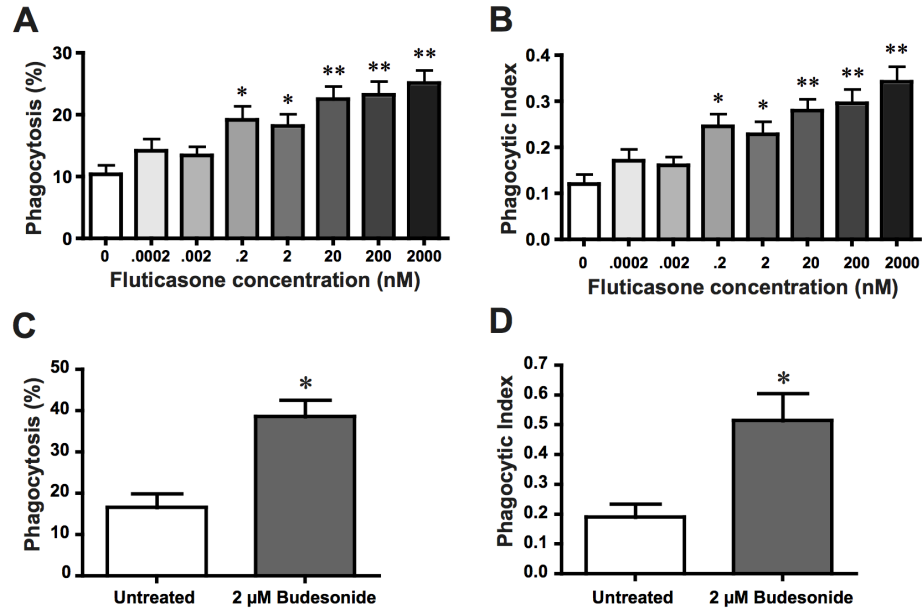
Figure 2.2. The effect of glucocorticoids on AC uptake by AMø is independent of the method by which target cell apoptosis is induced and independent of the type of glucocorticoid used. A, B. Adherence-purified AMø from normal C57 BL/6 mice were treated in chamber slides with 0.0002nM-2μM fluticasone for 6 h, then UV-killed AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. C, D. Adherence-purified AMø from normal C57 BL/6 mice were treated in chamber slides with control media or 2μM budesonide for 6 h, then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. SE of at least six mice assayed individually in at least two independent experiments per condition. *, statistically significant, $p < 0.05$ and **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
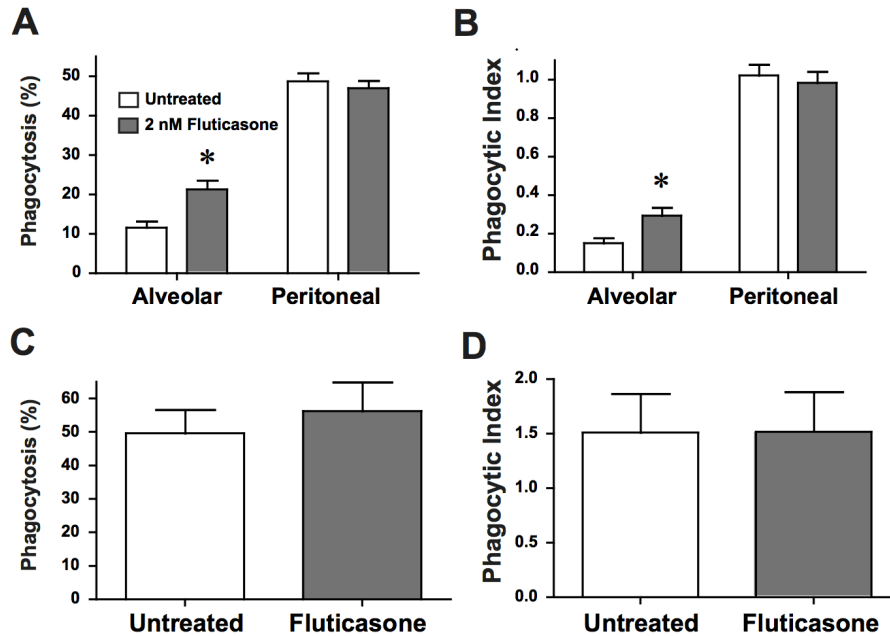
Figure 2.3. Fluticasone does not rapidly increase AC uptake by PMø or Fc-mediated uptake by AMø. A, B. Paired AC uptake by resident AMø and PMø. Adherence-purified paired AMø and PMø from normal C57 BL/6 mice were treated in chamber slides with control media or 2nM fluticasone for 6 h, then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. C, D. Opsonized SRBC uptake by AMø. Adherence-purified AMø from normal C57 BL/6 mice were treated in chamber slides with control media or 2μM fluticasone for 6 h, then opsonized SRBC targets were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested SRBC were counted at 100X magnification under oil. Data are mean ± SE of 4-8 mice assayed individually in two-three independent experiments per condition. *, statistically significant, $p < 0.05$ by One-Way ANOVA with Bonferroni post-hoc testing.
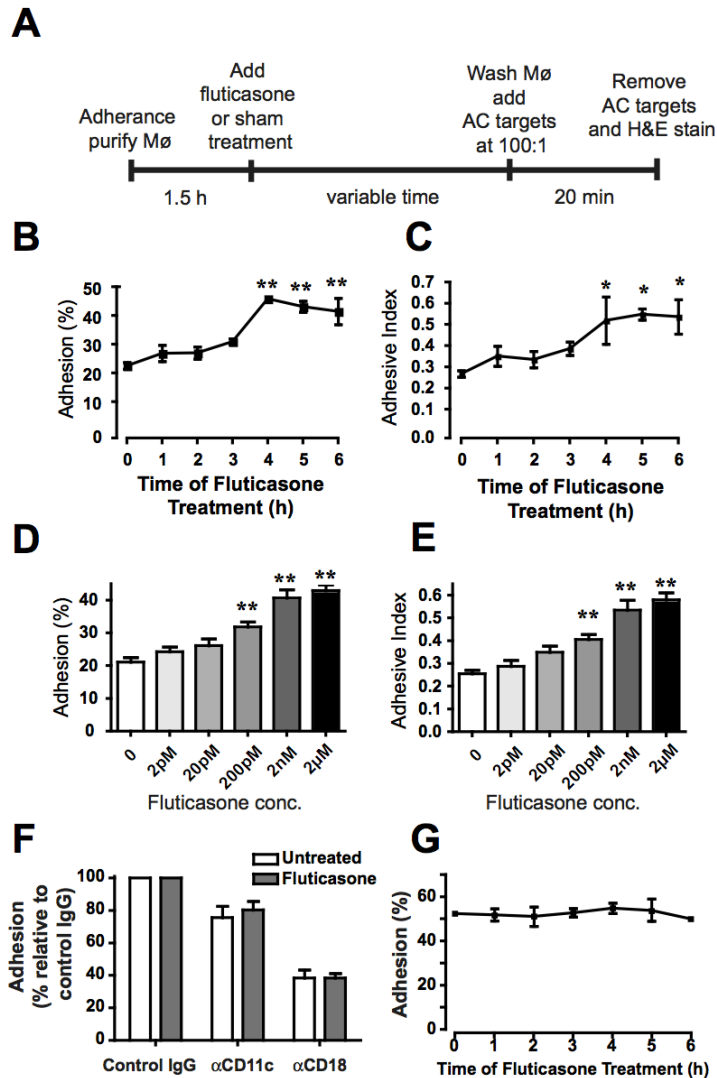
Figure 2.4. Fluticasone rapidly increases binding of AC by murine AMø. Adherence-purified AMø from normal C57 BL/6 mice were treated in chamber slides with fluticasone (2 nM unless indicated) for 0-6 h, then AC were added at a 100:1 ratio for 20 min. Slides were washed and stained using H&E, then surface bound AC were counted at 100X magnification under oil. A. Graphic timeline of a binding assay. B, C. Kinetics of GC-augmented AC binding. D, E. Dose-response of GC-augmented AC binding. F. AMø were pre-treated with blocking antibodies to CD11c, CD18, or isotype control at 5 μg/mL for 30 min followed by treatment with 2 nM fluticasone for 6 h prior to binding assay. G. Adherence-purified PMø from normal C57 BL/6 mice were treated in chamber slides with control media or 2nM fluticasone for 6 h, then AC were added at a 100:1 ratio for 20 min. Slides were washed and stained using H&E, then surface bound AC were counted at 100X magnification under oil. Data are mean ± SE of 5-8 mice assayed individually in at least two independent experiments per condition. **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
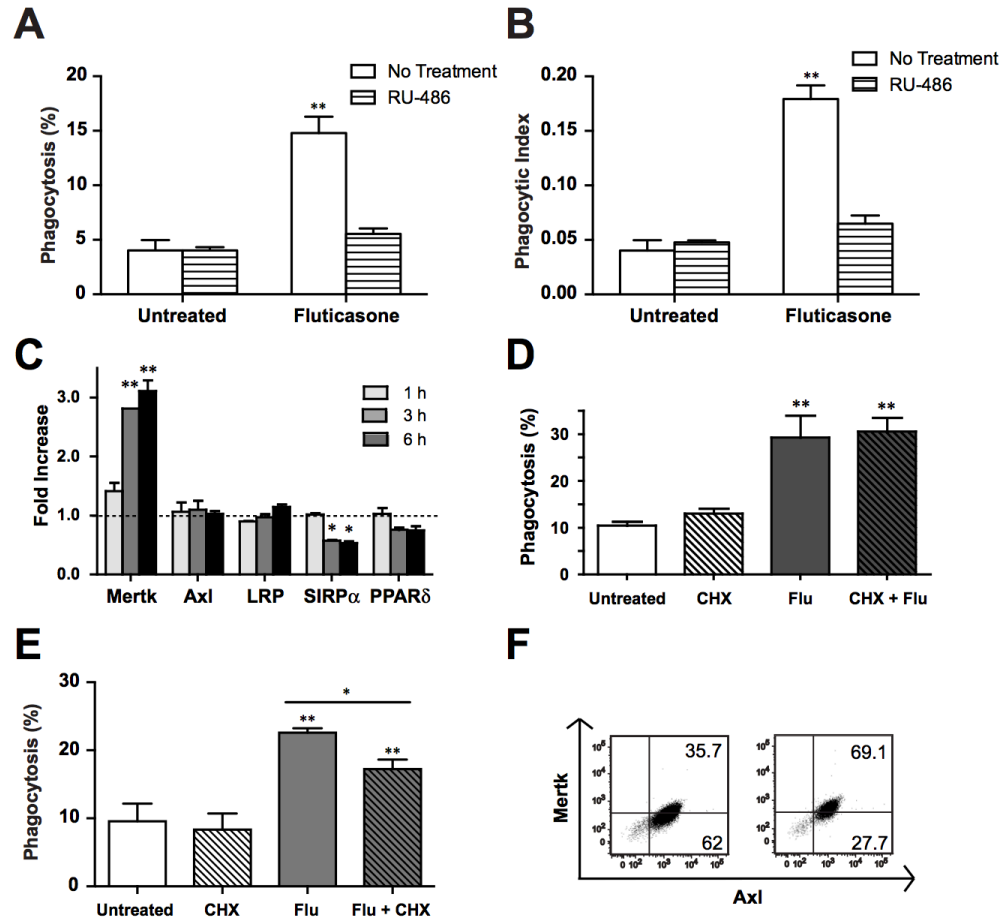
Figure 2.5. Fluticasone signals through the canonical GRα receptor to rapidly downregulate SIRPα and upregulate Mertk expression, increasing AMø efferocytosis without a requirement for new protein synthesis. A, B. Murine AMø were pre-treated with 5 μM RU-486 for 1 h followed by 2 μM fluticasone for 5 h, then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. C. Murine AMø were treated with 2 nM fluticasone for 0, 1, 3 or 6 h. RNA was collected at each time point and analyzed by real-time RT-PCR with GAPDH as the housekeeping gene; results are displayed as fold increase from untreated. D, E. Murine AMø were pre-treated with 5 μM cycloheximide (CHX) for 1 h followed by 2 μM fluticasone for 5 h (D) or 2 μM fluticasone for 23 h (E), then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. Data are presented as the mean ± SE of 5-7 individual mice from at least two independent experiments per condition. **, statistically significant, $p<0.01$ by One-Way ANOVA with Bonferroni post-hoc testing. F. Murine AMø treated with 2μM fluticasone for 24 h, then analyzed by flow cytometry for surface expression of Axl and Mertk. Representative dot plots. Cells shown are gated CD45^{+}CD11c+.
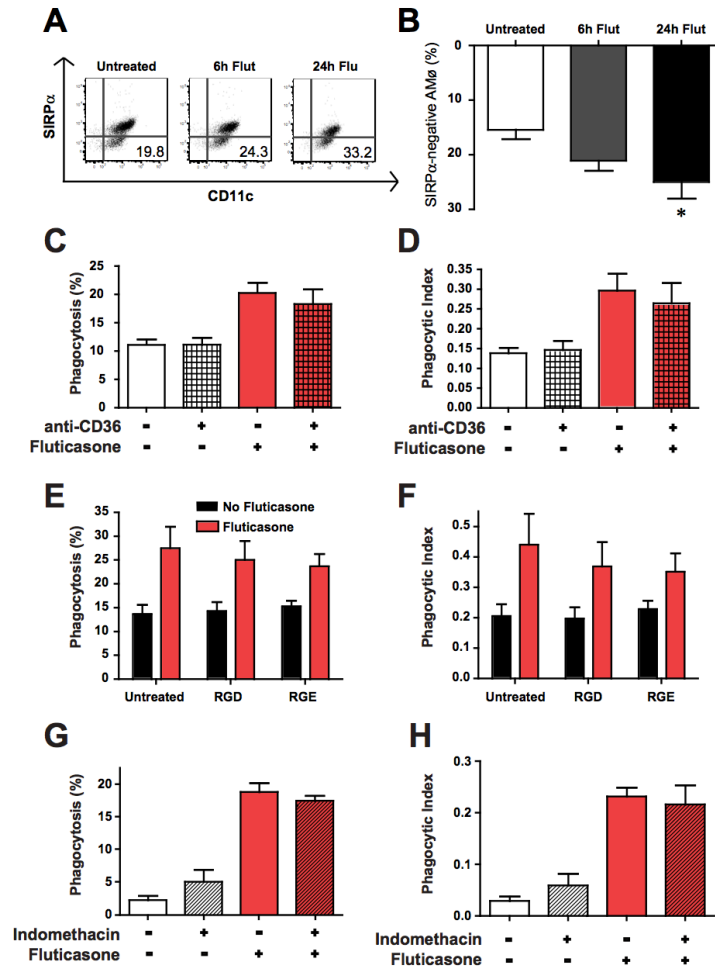
Figure 2.6. Fluticasone rapidly downregulates surface SIRPα and increases efferocytosis independent of CD36, α_V integrin or autocrine prostanoid signaling. A, B. Surface SIRPα protein. Murine AMø treated with 2μM fluticasone for 6 or 24 h, then analyzed by flow cytometry for surface expression of SIRPα. Cells shown are gated CD45^{+}CD19^{-}TCR^{-}. A. Representative dot plot. B. Average percent of CD11c^{+}SIRPα^{-} cells within gated CD11c^{+} population. C, D. CD36 Signaling. Adherence-purified AMø from normal C57 BL/6 mice were treated with 2 nM fluticasone for 5.5 h, followed by 2 μg/mL blocking antibody against CD36 or control Ig for 30 min, then AC were added at a 10:1 ratio for 2 h. E, F. α_V integrin signaling. Adherence-purified AMø from normal C57 BL/6 mice were treated with 2 nM fluticasone for 5.5 h, followed by 100 μM blocking peptide RGD or control peptide RGE for 30 min, then AC were added at a 10:1 ratio for 2 h. G, H. Autocrine prostanoid production. Adherence-purified AMø from normal C57 BL/6 mice were treated with 5 μM indomethacin for 30 min followed by 2 nM fluticasone for 5.5 h, then AC were added at a 10:1 ratio for 2 h. Following each of these types of treatments, slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. Data are mean ± SE of 5-7 individual mice assayed individually in at least two independent experiments per condition. *, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
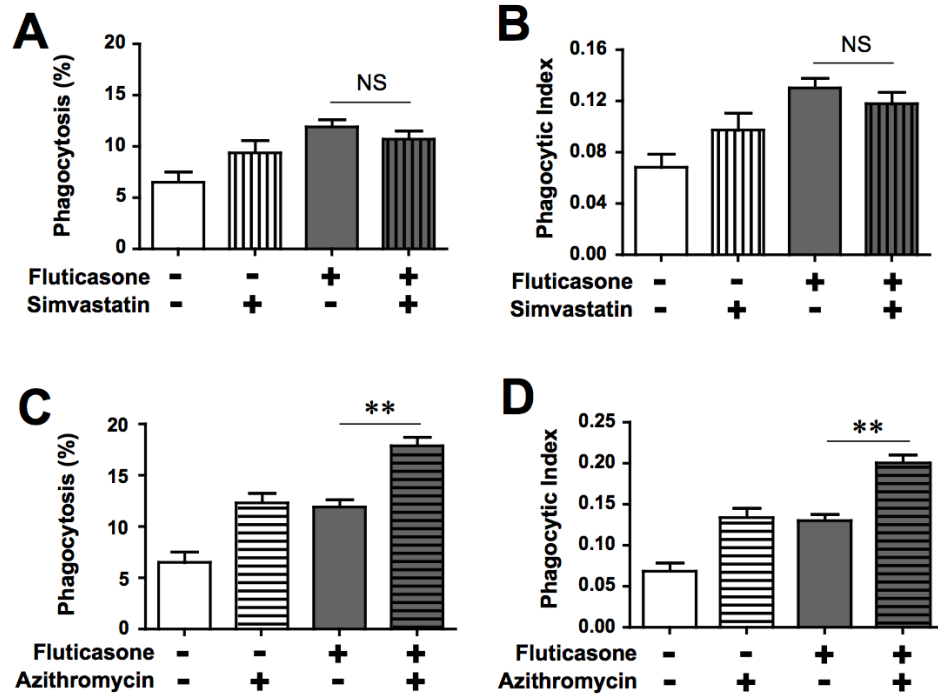
Figure 2.7. Azithromycin but not simvastatin has additive effects on efferocytosis by murine AMø. (A-D) Effect of multi-agent treatment on efferocytosis. Murine AMø were treated with Murine AMø were treated with 10 μM simvastatin, 500 ng/mL azithromycin, or media alone. After 18 h, 2 μM fluticasone was added for a further 6 h, then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. A, B. Simvastatin and Fluticasone. C, D. Azithromycin and Fluticasone. Data are presented as the mean ± SE of 7 individual mice from three independent experiments per condition. **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
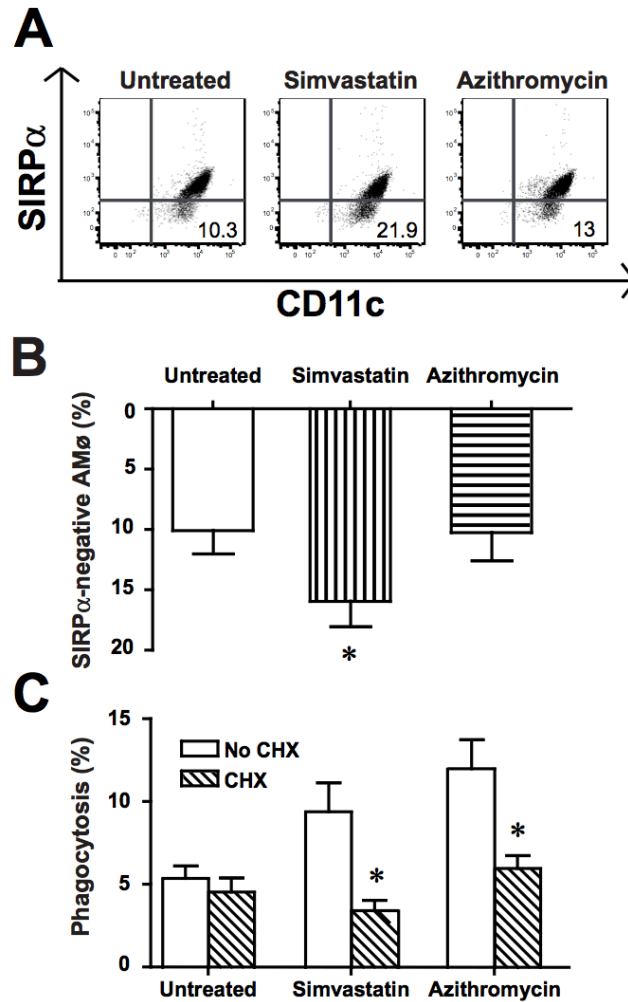
Figure 2.8. Simvastatin downregulates SIRPα expression while azithromycin does not. A, B. Surface SIRPα protein. Murine AMø treated with 10 μM Simvastatin or 500 ng/mL Azithromycin for 24 h, then analyzed by flow cytometry for surface expression of SIRPα. Cells shown are gated CD45^{+}CD19^{-}TCR^{-}. A. Representative dot plot. B. Average percent of CD11c^{+}SIRPα^{-} cells within gated CD11c^{+} population. C. Murine AMø were pre-treated with 5 μM cycloheximide for 1 h followed by 10 μM Simvastatin or 500 ng/mL Azithromycin for 24 h, then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. Data are mean ± SE of 5-7 individual mice assayed individually in at least two independent experiments per condition. *, statistically significant, $p<0.05$ by One-Way ANOVA with Bonferroni post-hoc testing.
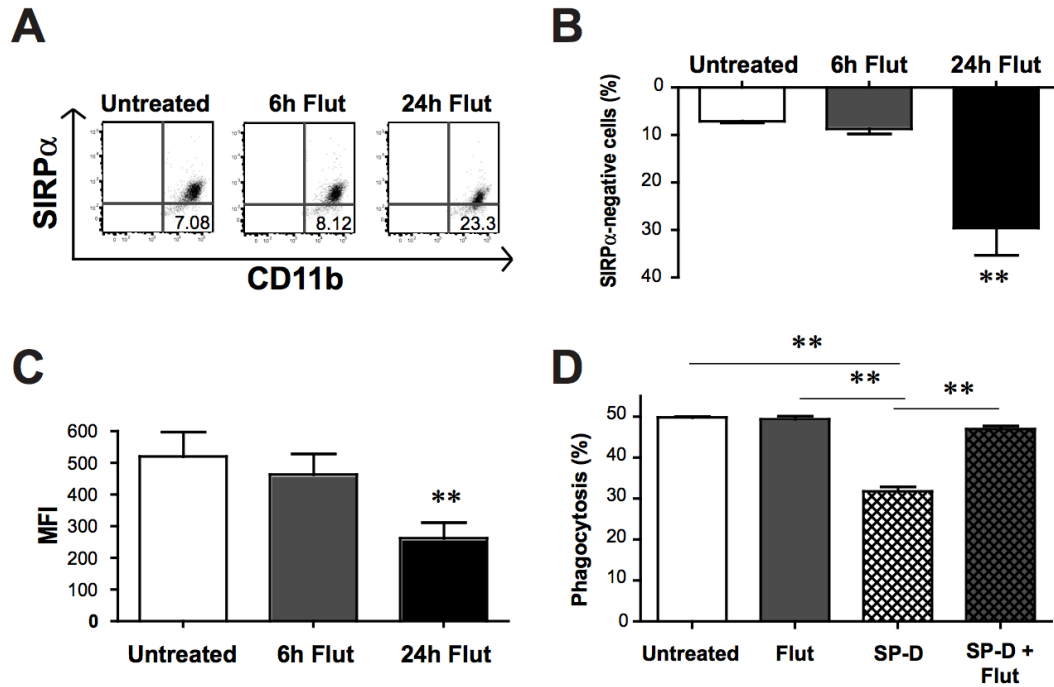
Figure 2.9. SP-D activates SIRPα pathway in PMø and makes PMø sensitive to fluticasone-driven increase in AC clearance. (A-C) Surface SIRPα protein. Murine PMø treated with 2μM fluticasone for 6 or 24 h, then analyzed by flow cytometry for surface expression of SIRPα. Cells shown are gated CD45$^+$CD19$^-$TCR$^-$. A. Representative dot plot. B. Average percent of CD11b$^+$SIRPα$^-$ cells within gated CD11b$^+$ population. C, Average MFI of SIRPα on gated CD11b$^+$ cells. D, Fluticasone rescues SP-D inhibition of AC uptake. Murine PMø were treated with 25μg/mL SP-D for 4 h, followed by control media or 2 μM fluticasone for 5 h, then AC were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. Data are mean ± SE of 5-8 mice assayed individually in at least two independent experiments per condition. **, statistically significant, p<0.01 by One-Way ANOVA with Bonferroni post-hoc testing.
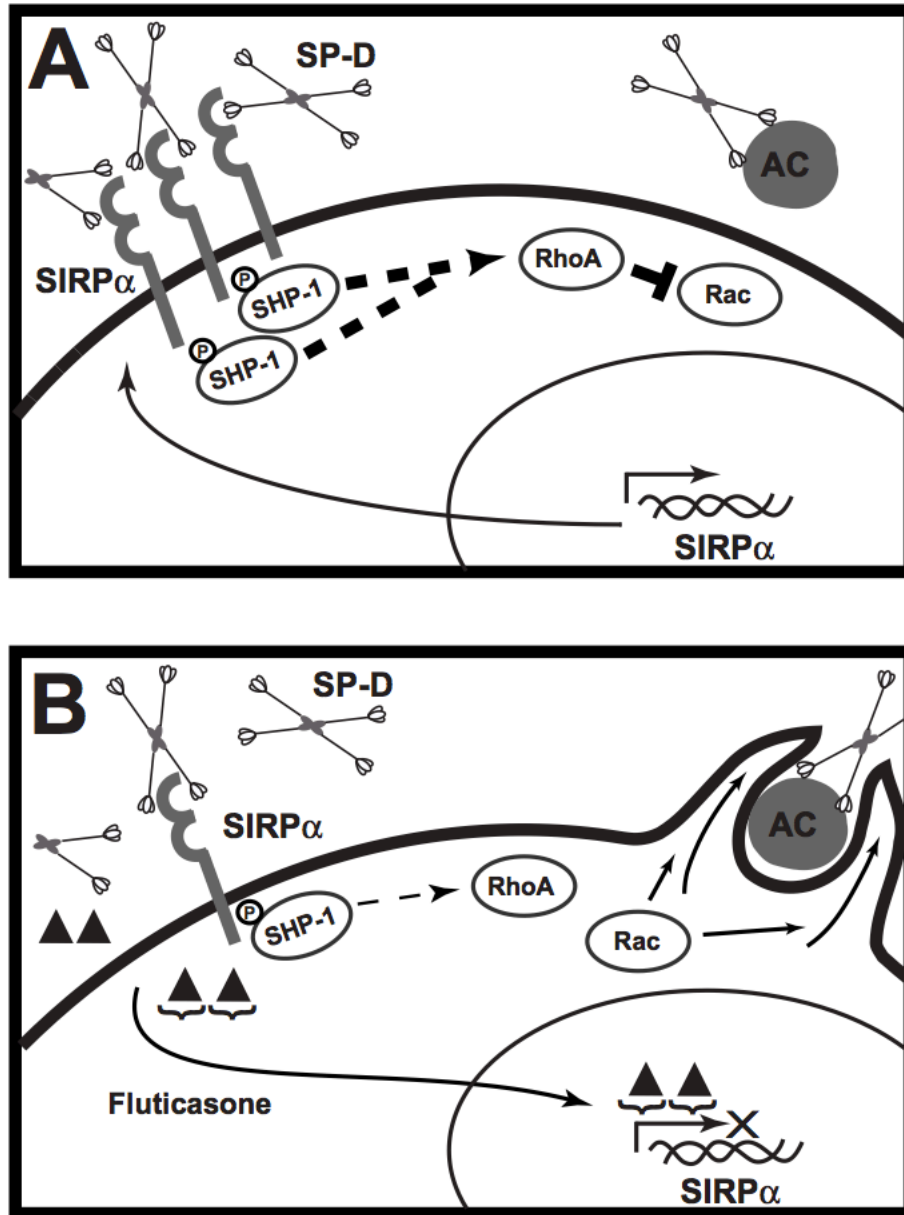
Figure 2.10. Model of GC regulation of SIRPα-mediated control of murine AMø efferocytosis. A. In untreated AMø, which express high amounts of SIRPα, lung collectins SP-D and SP-A (not shown) signal constitutively through SIRPα, activating SHP-1 and leading to downstream activation of RhoA. By inhibiting Rac-dependent mobilization of actin, the lung collectins tonically impede efficient uptake of AC by AMø, even though SP-A and SP-D can also bind AC. B. Treatment with fluticasone (triangles) reduces SIRPα surface expression, in part via transrepression of SIRPα by ligand-occupied GR homodimers (brackets). The consequent decreased activation of SHP-1 relieves inhibition of Rac, permitting efficient AC uptake. Based on data in the current study, plus previously published data (64, 74, 126, 196).

Chapter 3

Exposure to glucocorticoids and apoptotic cells prior to inflammatory stimulation inhibits AMø TLR signaling and bacterial killing with potential implications for ICS use in COPD patients

Abstract

Numerous studies have found that use of inhaled corticosteroids (ICS) by patients with COPD significantly increases their risk of developing pneumonia. The mechanism by which this increase occurs is not understood; murine models have not shown any consistent effect of glucocorticoid (GC) treatment on susceptibility to bacterial infection. Apoptosis is increased in the lungs of COPD patients and AC uptake has been shown to inhibit bacterial killing by murine AMø, increasing the severity of respiratory bacterial infection. We have previously shown that GC increase apoptotic cell (AC) uptake by murine alveolar macrophages (AMø). Here we provide evidence that GC-enhanced AC engulfment (GCAE) inhibits AMø TLR-stimulated response, decreases the expression of miRNA involved in regulating cytokine and TLR signaling, and decreases the capacity of AMø to kill bacteria in vitro and in vivo beyond any affect GC alone or AC alone have on these pathways. These findings suggest that individuals with high levels of AC within the lung, such as COPD patients, may respond uniquely to ICS: as ICS enhances uptake of AC by AMø, AMø lose important sentinel immune capabilities such as TLR response and bacterial killing, resulting in increased susceptibility to respiratory infection. This

may be one mechanism by which COPD patients on ICS become more susceptible to pneumonia.

Introduction

COPD is a respiratory disorder characterized by progressive airflow obstruction that is not fully reversible. Smoking, or exposure to other types of noxious particulate such as indoor biofuels, are significant risk-factors in the development of COPD (235, 236). When susceptible individuals receive sufficient lung insult, pathophysiologic changes occur, particularly of the distal airways (237, 238), which result in airway obstruction. COPD is the only leading cause of death with a rising incidence rate; WHO predicts COPD, currently the fourth leading cause of death, will be the third leading cause of death within twenty years (239).

COPD includes both emphysema and chronic bronchitis; individuals may simultaneously present with both conditions. In emphysema, peripheral bronchioles are damaged or destroyed leading to enlarged air spaces with reduced surface area and reduced efficiency of gas exchange (238, 240). There is speculation that this destruction may have an autoimmune component, as auto-antibodies against extracellular matrix proteins have been detected in COPD patients (181, 241). Importantly, studies have found evidence of increased apoptosis in the lungs of COPD patients, particularly those with emphysema (142, 242). The ability to engulf such apoptotic cells (AC) is decreased in alveolar macrophages (AMø) isolated from COPD patients (125, 138). Because defective AC clearance has been strongly linked to increased inflammation and the

development of autoimmunity (243), the use of therapies designed to bolster AC

clearance in the lung has been proposed (133, 166).

Although COPD is not fully reversible, numerous treatments exist that

temporarily relieve inflammation, decrease obstruction and diminish the rate of

exacerbations (244). Among the most commonly prescribed are inhaled corticosteroids

(ICS) such as fluticasone and budesonide. These GC reduce the rate of COPD

exacerbations in individuals with severe disease (245), likely through a combination of

inhibiting NF-kB signaling and inducing anti-inflammatory genes (246). However,

COPD patients are already at increased risk for developing pneumonia (247) and use of

ICS by these individuals is associated with a further 1.3 to 1.8-fold increase in the risk of

developing pneumonia severe enough to require hospitalization (248).

Over twenty years ago, observational evidence was published suggesting a link

between GC use in COPD patients and the development of non-resolving pneumonia

(249). Within the last decade, numerous clinical trials have confirmed that use of ICS,

particularly fluticasone, increases the risk of pneumonia in COPD patients (223-233).

The mechanisms by which this occurs remain unclear. Fluticasone treatment has been

shown to decrease platelet-activating factory receptor (PAFR) expression on human and

murine bronchial epithelial cells, decreasing invasion by *Haemophilus influenzae* and

Streptococcus pneumoniae and decreasing bacterial burden in a mouse model of *S.

pneumoniae* (234). Fluticasone treatment also reduced lung *Mycoplasma pneumoniae*

bacterial burden and lung inflammation (250). Thus, we have speculated that some

disease-specific factor alter the response of COPD patients to GC and this factor accounts

for the unexpected affect of ICS use on pneumonia in COPD patients.

AC uptake can induce a potent anti-inflammatory response including secretion of IL-10, TGF-β, PGE2, and inhibition of subsequent TLR and cytokine signaling in the phagocyte (107, 212). AMø capacity for bacterial killing in vitro is inhibited following AC uptake (108). Further, the presence of AC within the lung increases the lung colony-forming units (CFU) and dissemination of *S. pneumoniae* in a murine model (108). We and others have shown that GC such as fluticasone significantly increase AC uptake by AMø (125, 139); we speculated that the increased burden of AC found in COPD patients (142, 242) could contribute to an increased risk of bacterial infection, particularly when AC uptake was increased by ICS use.

In this study, we asked whether the additional presence of AC would alter the effect of fluticasone on *S. pneumoniae* infection in a murine model and using human AMø in vitro. Our preliminary findings show that the combination of GC and AC suppresses TLR responsiveness, lowers expression of miRNA involved in regulating cytokine and TLR signaling, and decreases the capacity of AMø to kill bacteria in vitro and in vivo beyond any affect GC or AC have on these pathways alone.

Throughout this chapter we will refer to the combined treatment of GC followed by AC as **G**luco**C**orticoid-**A**ugmented **E**fferocytosis (GCAE). Based on the data presented and previously published work regarding GC, we hypothesize that the ability of GC to augment efferocytosis is the salient mechanism by which the combination of GC and AC impacts bacterial clearance. Caveats of this interpretation and experiments that could further clarify the specific role of augmented efferocytosis will be discussed below. Importantly, our data that what we term GCAE suppresses bacterial killing supports our hypothesis that the increased burden of AC found in COPD patients (142, 242)

contributes to enhanced susceptibility of COPD patients to pneumonia when prescribed ICS (223-233), regardless of whether augmented efferocytosis is the mechanism by which this occurs. We hope that future work will study whether the presence of AC (i.e. emphysema) could be used as an exclusion criterion for prescribing ICS to COPD patients.

Results

Fluticasone increases uptake of apoptotic cells by, and expression of apoptotic cell recognition receptors on, human AMø. To understand the effects of inhaled corticosteroids on human AMø, we isolated AMø from consented research bronchoscopy subjects who were not taking ICS and performed in vitro phagocytosis assays following treatment with fluticasone, a potent GC we used at clinically-relevant doses. Pre-treatment with fluticasone significantly increased AC uptake by human AMø (**Fig. 3.1A**), as we had observed in murine AMø (65). Also similar to murine AMø (**Fig. 2.5F**), fluticasone treatment led to increased expression of Mertk on human AMø (**Fig 3.1B, 3.1C**). Unlike what had been observed in murine AMø (**Fig. 2.5F**), fluticasone treatment led to a concordant increase in Axl expression (**Fig 3.1D, 3.1E**). Although this last finding indicates that the mechanisms by which fluticasone acts to increase AC uptake differ slightly between murine and human AMø, AC uptake is significantly enhanced by GC treatment in both species.

Co-exposure to GC and AC suppresses LPS response in murine and human AMø. To understand the immune consequences of GC treatment followed by AC exposure

(GCAE), we pre-treated AMø with fluticasone, AC, or fluticasone followed by AC, then stimulated AMø with the TLR4 ligand LPS. Following co-exposure of murine AMø to GC and AC, secretion of TNFα, IL-6 and IL-12 was decreased in co-treated AMø compared to those pre-treated with GC alone, AC alone or AMø that did not receive any pre-treatment (**Fig. 3.2A-C**). This decrease reached statistical significance for TNFα (**Fig. 3.2A**). TNFα was also decreased following co-exposure of human AMø to GC and AC (**Fig. 3.2D**). The same decrease in TLR response by co-exposure to GC and AC was seen in human AMø stimulated with the TLR3 ligand PolyI:C and the TLR1/2 ligand Pam3Csk4 (data not shown).

Fluticasone pre-treatment alters SOCS1 but not SOCS3 expression in AMø following AC exposure. SOCS1 and SOCS3 are both induced by AC clearance and contribute to the immunosuppressive capacity of AC through inhibiting STAT signaling (97). We examined expression of SOCS1 and SOCS3 following AC exposure to test whether fluticasone affected this pathway. Using RT-PCR we found that, as previously published, exposure to AC induced SOCS1 and SOCS3 expression, however the kinetics of SOCS1 induction were altered by pre-treatment with fluticasone (**Fig. 3.3A, 3.3B**). SOCS1 expression increased more rapidly in response to AC in AMø pre-treated with fluticasone (**Fig. 3.3A**). However, by 3 h after AC induction SOCS1 expression was lower in AMø pre-treated with fluticasone than in AMø exposed to AC alone, similar to levels of SOCS1 in untreated AMø. The rate and amplitude of SOCS3 induction following AC exposure was not altered by pre-treatment by fluticasone.

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miR29c and let-7i are decreased following fluticasone and AC exposure while target genes SOCS1 and A20 are increased. To address whether GCAE is a unique immunosuppressive state or the additive suppression of GC and AC exposure we tested for the presence of regulatory factors whose expression was only altered in GCAE, not by GC or AC exposure alone. We performed a PCR miRNA-array on human samples, comparing RNA from untreated human AMø to RNA from AMø treated with fluticasone alone, AC alone, or fluticasone and AC. We identified miRNA uniquely altered in human AMø following GCAE (**Table I**). Of interest, two of these miRNA are known to target molecules that inhibit inflammatory signaling: both miR-29c and let-7i negatively regulate tumor necrosis factor alpha-induced protein 3 (TNFAIP3/A20) (251) while let-7i negatively regulates SOCS1 (252). Using quantitative real-time RT-PCR we confirmed that let-7i and miR-29c were decreased in human AMø following GCAE (**Fig. 3.4A, 3.4B**). mRNA expression of both miRNA target genes, A20 and SOCS1, was increased following GCAE (**Fig. 3.4C, 3.4D**). We performed a parallel experiment using murine AMø, comparing RNA from untreated AMø to RNA from AMø treated with fluticasone alone, AC alone, or fluticasone and AC. The let-7i, miR-29c and their target genes do not appear to be identically regulated by GCAE in murine and human AMø. miR-29c was decreased in murine AMø following GCAE, however, there was no significant decrease in let-7i (**Fig. 3.4E, 3.4F**). mRNA expression of the let-7i target SOCS1 was increased post-GCAE, but we found no increase in the miR-29c target A20 (**Fig. 3.4G, 3.4H**).

Fluticasone and AC exposure inhibits AMø killing of Streptococcus pneumoniae. To
establish a murine model by which to test the clinical import of GCAE, we first
administered a physiological dose of fluticasone via intranasal inoculation to observe
whether this altered murine AMø AC uptake and TLR response. AMø from mice treated
with in vivo fluticasone showed an increase in AC uptake in vitro (**Fig. 3.5A**), as had
been observed following in vitro fluticasone treatment. Importantly, an increase
following in vivo fluticasone treatment was also observed for in vivo AC uptake, where
apoptosis was induced in target cells in vitro and apoptotic target cells were then
administered intranasally following intranasal fluticasone inoculation (**Fig. 3.5B**).
Additionally, AMø from mice treated with in vivo fluticasone showed a decrease in their
in vitro LPS response (**Fig. 3.5C**). This finding indicated that the physiological dose of
in vivo fluticasone and in vivo inoculation of AC would lead to in vivo GCAE.

     We proceeded to induce GCAE in vivo and compared the response to *S.
pneumoniae* infection (253) between four treatment groups: 1) mice receiving PBS
control, 2) mice receiving fluticasone alone, 3) mice receiving AC alone, and 4) mice
receiving fluticasone followed by AC. However, when mice were pre-treated with
fluticasone and AC beginning 6 h prior to infection with *S. pneumoniae,* no subsequent
difference in bacterial CFU was observed in the isolated lung tissue from any group (**Fig.
3.6A**). There was also no change in CFU after 48 h and no change in bacterial
dissemination as measured by splenic CFU at 24 and 48 h following this acute induction
of GCAE (data not shown). However, a significant increase in bacterial burden was
observed in the lung tissue of infected mice given a longer pre-treatment of fluticasone
and AC. When mice received a chronic pre-treatment of fluticasone and AC beginning

24 h prior to infection, lung tissue isolated from mice co-treated with fluticasone and AC had the highest bacterial CFU 24 h after *S. pneumoniae* infection (**Fig. 3.6B**). To test whether bacterial killing by AMø was inhibited by GCAE and could contribute to this in vivo increase in bacterial burden, we performed an in vitro bacterial killing assay (**Fig. 3.6C**). Following pre-treatment with fluticasone and AC, murine AMø displayed a decreased ability to kill *S. pneumonia* (**Fig. 3.6D, 3.6E**). Collectively, this work provides preliminary data to support our hypothesis that GCAE inhibits important sentinel immune capabilities in AMø such as TLR response and bacterial killing, resulting in increased susceptibility to respiratory infection.

Discussion

The results of this study demonstrate how the combination of GC and AC alters AMø antimicrobial capacity through downregulating TLR response, decreasing expression of miRNA that indirectly regulate TLR-dependent cytokine production, and lowering the capacity of AMø to kill bacteria in vitro and in vivo. Using primary human AMø, we demonstrate that fluticasone enhances AC clearance, as we have previously shown in murine AMø (65). Using primary human and murine AMø we demonstrate that the combination of GC and AC inhibits cytokine production following LPS stimulation, beyond the inhibition caused by either GC or AC alone. We identify two miRNA uniquely decreased by the combination of GC and AC, let-7i and miR-29c, respectively involved in the regulation of SOCS1 (252) and A20 (251). Loss of these miRNA in human AMø correlates with increased expression of these negative regulators of TLR and cytokine signaling. Further, we establish a murine model to study the effects of GC and

AC on *S. pneumoniae* infection and provide preliminary data that this co-treatment decreases bacterial killing by AMø, increasing susceptibility to infection. These results suggest that increased AC may be a mechanism by which COPD patients on ICS develop increased susceptibility to pneumonia.

We continue to theorize that augmenting AC clearance by AMø prevents critical initial bactericidal capacity, resulting in lingering bacterial burden instead of resolution. Published work studying the effect of AC alone on in vivo *S. pneumoniae* infection demonstrates that the presence of AC leads to diminished clearance of bacteria (108). AMø perform essential bactericidal functions in initial *S. pneumoniae* infection (254-259). Bacterial killing by AMø is suppressed by the PGE2 produced following efferocytosis (108). In contrast to this inhibitory effect of AC, GC enhance bacterial killing (260), potentially through inhibiting PGE2 synthesis (261, 262). These data do not support the idea that diminished killing following treatment of GC and AC is explained by cumulative but separate effects of GC and AC on bacterial killing. We feel this data strongly suggest that the combination of GC and AC regulate bacterial killing by upregulating the inhibitory influence of AC through augmented efferocytosis: GCAE.

However, although we clearly document an effect of GC on AC uptake in vitro and in vivo, we lack data to prove that enhanced efferocytosis rather than unknown additive immunomodulatory effects of the GC and AC is responsible for this defective bacterial killing. There are experiments that can be performed to partially answer this concerns. We can treat AMø with AC before GC where there will be no enhancement of efferocytosis and compare this to our usual treatment of GC before AC where efferocytosis is enhanced. If comparable bacterial killing is measured in both groups this

would be strong evidence against our conclusion that the enhanced efferocytosis is key to the decreased bacterial killing. In contrast, better bacterial killing in the group treated with AC before GC would suggest that the enhanced efferocytosis (GCAE) is required for the decreased bacterial killing we observed in vitro and in vivo. Additionally, we could perform experiments where we attempt to normalize uptake, restricting the number of AC targets available following GC treatment to prevent enhanced uptake and comparing this to AMø pre-treated with AC alone. Diminished killing by AMø pre-treated with GC and restricted target AC would suggest that enhanced efferocytosis is not required. Such experiments will clarify the mechanism by which GC and AC inhibit bacterial killing, although our inability to conclusively demonstrate a role for augmented efferocytosis in our model does not diminish from the clinical implication that the presence of AC could increase susceptibility to bacterial pneumonia for COPD patients on ICS.

Our finding that in vitro GC treatment increases AC uptake by human AMø agrees with a previous report in asthmatics (139). We extend that study by demonstrating that GC exposure upregulates protein expression of the important AC recognition receptors, Axl and Mertk. This finding agrees with a study on human blood-derived monocytes that found an increase in mRNA expression of Axl and Mertk (199), but contrasts with our murine work showing that at the mRNA and protein level only Mertk is upregulated following GC treatment (**Fig. 2.5F**) (65). The import of this species difference is uncertain. Along with the less-studied Tyro3, Axl and Mertk belong to a family of structurally-related receptor tyrosine kinases (TAM family). Members of the TAM family of AC recognition receptors recognize phosphatidylserine (PS) expressed on

AC through bridge molecules including Gas6 and Protein S (263). Expression of TAM receptors varies on phagocytes (109, 264), although AMø express mRNA for all three receptors (unpublished data from Curtis lab). Mice lacking TAM receptors have a diminished capacity for AC clearance and a predisposition for autoimmunity (119, 265). Work in mice has shown that Axl is important for the resolution of inflammation: upregulation of Axl by inflammatory stimuli such as LPS is required for subsequent expression of SOCS1 and SOCS3 that complete a negative feedback loop to suppress cytokine signaling (97, 266). Mice lacking Axl or cells treated with blocking antibodies to Mertk show an exaggerated inflammatory response in addition to an increase in autoimmunity (99, 267). Knockout mice lacking multiple TAMs have stronger autoimmune phenotypes than single knockouts (11). The TAM receptors activate shared pathways but function in a non-redundant manner.

By upregulating both Axl and Mertk in human AMø, GC may more greatly enhance AC clearance by human AMø than murine AMø. This hypothesis is supported by our data showing that fluticasone alone led to a much stronger increase in AC uptake by human AMø (~6-fold above untreated) than murine AMø (~3-fold above untreated) (**Fig 2.1B, 3.1A**). Hence, GCAE and its consequences may be greater in human AMø, although further experiments will be needed to prove that conjecture.

In our study of the immune consequences of GCAE, we describe how AMø response to pathogens is altered in an environment where those AMø are pre-stimulated with GC and AC. We find that GCAE suppresses TLR response and bacterial killing by AMø. These changes coincide with increases in SOCS1 not seen in AMø treated with either GC or AC alone, consistent with a phenotype of immunosuppression. Importantly,

while this is true after only 1-2 h of AC exposure, by 3 h, AC exposure alone induces SOCS1 expression; GCAE only alters the kinetics of SOCS1 induction (**Fig. 3.3A, Fig. 3.4D, 3.4H**). SOCS1 and the related SOCS3 suppress STAT signaling downstream of cytokine receptors by binding to and inhibiting JAK activity (268). SOCS1/3 also suppress NFκB activity by decreasing p65 stability (269).

Expression of SOCS1 and SOCS3 is rapidly induced following Axl/Mertk recognition of AC. There is no change in the SOCS3 expression induced by AC alone following GCAE, suggesting that accelerated SOCS1 expression is not caused by alterations in this shared Axl/Mertk and STAT signaling. If changes caused by GCAE were simply a reflection of augmented AC clearance leading to an accelerated anti-inflammatory response to AC, we would expect both SOCS1 and SOCS3 to be increased. The lack of SOCS3 induction in GCAE supports our hypothesis that GCAE is a unique anti-inflammatory state beyond the independent effects of GC and AC.

The identification of several specific miRNAs decreased only by GCAE (**Table I**) further supports an interpretation of combinatorial rather than additive immunosuppression. Our finding that decreased let-7i expression coincided with increased SOCS1 agrees with the report that let-7i suppresses SOCS1 expression in DC (252). By contrast, let-7i is not known or predicted to target SOCS3, consistent with the lack of increased SOCS3 expression we found. Thus, let-7i is an attractive mechanism to explain the altered kinetics of SOCS1 but not SOCS3 by GCAE. This finding suggests that the observed differential kinetics of these two closely related immunoregulators are due to regulatory factors unique to GCAE.

GCAE also causes a unique upregulation of A20 not seen following either GC or AC alone. Increased A20 expression provides a direct mechanism by which GCAE could suppress TLR signaling; A20 is a ubiquitin editing protein that inhibits NFkB signaling by disassembling ubiquitination of TRAF6, preventing IKK complex activation and limiting signing through TLRs and other activators of NF-kB (270-272). GCAE-induced A20 in AMø could result in dampened TLR signaling, consistent with the diminished TLR response we observed following GCAE (**Fig. 3.2**). Coinciding with upregulation of A20, GCAE leads to decreased expression of miR-29c, which has been previously shown to inhibit A20 in B cells (251).

As with SOCS1/let-7i, the unique induction of A20 and repression of miR-29c following GCAE but not GC or AC exposure alone argues for a synergistic rather than additive relationship of GC and AC. However, further kinetics are necessary to confirm that longer exposure to AC alone does not induce A20. Interestingly, although there is no published record of AC exposure affecting A20 expression, A20 is necessary for AC-induced suppression of the TLR response via inhibition of IKK activity in murine BMDC (273), which suggests that either A20 expression or activity (274) is induced following AC exposure. Additionally, signaling through CD44, an AC recognition receptor (275, 276), induces A20 expression (277, 278). Kinetic studies are needed to clarify whether AC alone can induce A20 expression or alter its activity. Further experiments are also required to measure protein levels of both A20 and SOCS1, to confirm the changes we observed at a message level.

Interestingly, absence of A20 in vivo achieved by gene-targeting leads to a breakdown of directed AC uptake by splenic DC, cross-presentation of self-antigens, and

the development of spontaneous autoimmunity (273). Collectively, these data suggest that A20 promotes a tolerogenic response to AC-exposure. Verifying that increased expression of A20 in the engulfing phagocyte leads to tolerance, while beyond the scope of the current study, would have important implications for GCAE as a therapy in autoimmune disease. GC have modest benefit for treatment of COPD (248), but are an effective treatment for lupus (279). We speculate that the effectiveness of GC in controlling lupus is enhanced by GCAE; understanding this mechanism could lead to more targeted use of GC in these patients. We expect GCAE would partially reverse the disease-related defect in efferocytosis and, due to enhanced expression of A20 in the engulfing phagocytes, this increased AC clearance would be tolerogenic. Our data shows that GCAE can contribute to an increased susceptibility to bacterial infection, but it would be interesting to test whether the tolerogenic benefit outweighs the infectious cost in the context of autoimmune disease.

There is no evidence that changes in A20 or SOCS1 contribute to diminished bacterial killing following GCAE; diminished killing in GCAE likely occurs through amplification of the same mechanisms by which un-enhanced efferocytosis inhibits bacterial killing. Intracellular killing by AMø first requires bacterial phagocytosis. Uptake of *S. pneumoniae* is augmented by opsonization (280), which allows for Fc, scavenger, and complement receptor-mediated recognition and engulfment. In particular, scavenger receptor A (SR-A) (281, 282) and macrophage receptor with collagenous structure (MARCO) (283, 284) assist in recognition and engulfment of *S. pneumoniae* by AMø. Intracellular killing of *S. pneumoniae* occurs in the lysosome and involves indicible nitric oxide synthase (iNOS) generation of nitric oxide (NO) (280, 285-290). *S.*

pneumoniae killing by AMø occurs through another mechanism as well: phagocytosis-induced cell death. Phagocytosis of *S. pneumoniae* induce NO-dependent apoptosis in AMø; apoptotic AMø are engulfed and the bacteria are killed in a "double-walled" fashion (291-294). Altering prostaglandin signaling through EP2 or EP3 deletion increases generation of NO and improves bacterial phagocytosis and killing by AMø (288, 289). AC suppress bacterial killing through inducing the secretion of PGE2 which then signals through EP2 (108); we hypothesize that GCAE amplifies this pathway, leading to increased production of PGE2 and suppression of NO in the presence of AC, preventing both intracellular killing and killing via phagocytosis-induced cell death.

Multiple studies have attempted to understand why COPD patients on ICS have an increased risk of pneumonia with no conclusive results (233). Using murine models or in vitro treatments of human cells, some publications find that GC are protective (234, 250), while others find that GC increase the severity of infection (295, 296) or have no effect (297). Each study utilized a unique course of GC treatment and a different bacterial pathogen. Our preliminary results find no significant effect of GC on the severity of infection although a trend towards increased severity. Further, our results comparing a brief (**Fig. 3.6A**) GC pre-treatment to a more chronic (**Fig. 3.6B**) GC pre-treatment suggests that the length of pre-treatment has a significant impact on the study outcome, which may explain the aforementioned conflicting data. While a brief GC/AC/GCAE led to significant changes in vitro, brief GC/AC/GCAE pre-treatment showed no effect on *S. pneumoniae* CFU compared to mice pre-treated with a saline control (**Fig. 3.6B**). However, the more chronic pre-treatment showed a statistically significant increase in bacterial CFU in mice after GCAE and a trend towards increased

bacterial CFU in mice pre-treated with GC or AC alone (**Fig. 3.6C**).   The more chronic

GCAE treatment also decreased AMø *S. pneumoniae* killing in vitro. These data

demonstrate an inhibitory effect of GCAE on bacterial clearance and motivates further

study of the connection between GCAE and susceptibility to bacterial infection. It will

be important to study whether GCAE results in increased dissemination and mortality;

there is some suggestion that increased risk for community-acquired pneumonia with ICS

used is balanced by reduced mortality, however this early conclusion has since been

contested (223-226).

Our data also suggest that the use of a chronic GCAE model is the most likely to

provide significant results, as acute GC/AC/GCAE studies of bacterial susceptibility

following GCAE with chronic GC exposure (>1 week) are likely to be more relevant to

COPD patients receiving ICS. We speculate that chronic GCAE will further augment the

defect in bacterial clearance observed in our 24 h treatment, although it is unclear how

this could be impacted by induction of GC resistance with long-term ICS use. GC

resistance in COPD is believed to occur primarily because of decreased GR association

with NF-kB due to decreased histone deacetylase C2 (HDAC2) (231, 298); it is unknown

whether GC regulation of Mertk or SIRPα expression requires the GR-NFkB complex.

GC are just one of many pharmacologic agents that enhance uptake of AC and it

is unclear whether pre-treatment with other agents will result in similar

immunosuppression and susceptibility to infection. Statins, which also increase AC

uptake by AMø, have been observed to increase positive outcomes in pneumonia in mice

and humans (297, 299). Further work will need to investigate how statin-enhanced AC

uptake or azithromycin-enhanced AC uptake effect TLR response and bacterial killing in

the lung. Statins and azithromycin are common medications used by patients with COPD and neither has been connected to an increased risk of bacterial infection.

In summary, our data support the hypothesis that the increased exposure of AMø to intra-alveolar AC explains the association of therapeutic ICS use with increased incidence of community-acquired pneumonia in patients with COPD. This idea merits further investigation using our established murine model to better understand the mechanisms by which GCAE alter the anti-microbial response. Our study specifically suggests that COPD patients diagnosed with emphysema and prescribed ICS may be at the highest risk for bacterial pneumonia and subsequent hospitalization due to the increased presence of disease-associated AC. Avid engulfment of AC by AMø exposed to GC would lead to decreased TLR response, decreased bacterial killing, ineffective recruitment of monocytes and neutrophils, and hence increased hospitalizations for pneumonia in this population. No clinical trials of ICS have separated patients with emphysema from the larger COPD population. Our study motivates improved design of future trials to compare pneumonia risk between these two groups and potentially redefine appropriate interventions for individuals with emphysema to exclude the prescription of ICS. Further, it underscores that any use of therapeutic enhancement of AC uptake to inhibit inflammation and autoimmunity will first require a deeper understanding of the immune effects of enhanced AC clearance.

Jennifer Dolan for their shared expertise and time spent training me on their

Streptococcal pneumonia model.

Figure 3.1. Fluticasone increases uptake of AC by human AMø and their expression of Mertk and Axl. Adherence-purified AMø from human bronchoalveolar lavage were treated with control media or 2 µM fluticasone for 24 h. A. AC uptake by human AMø following fluticasone treatment. In chamber slides, AC were added at a 10:1 ratio for 2 h, then slides were washed and stained using H&E and ingested AC were counted at 100X magnification under oil. B. Western blot of Mertk in human AMø following fluticasone treatment. C. Western blot of Axl in human AMø following fluticasone treatment. D. Immunohistochemistry of Mertk in human AMø. E. Immuno-histochemistry of Axl in human AMø. Data are mean ± SE or representative data of AMø from two human subjects. Both humans were current smokers. All experiments shown were performed by Dr. Jill C. Todt.
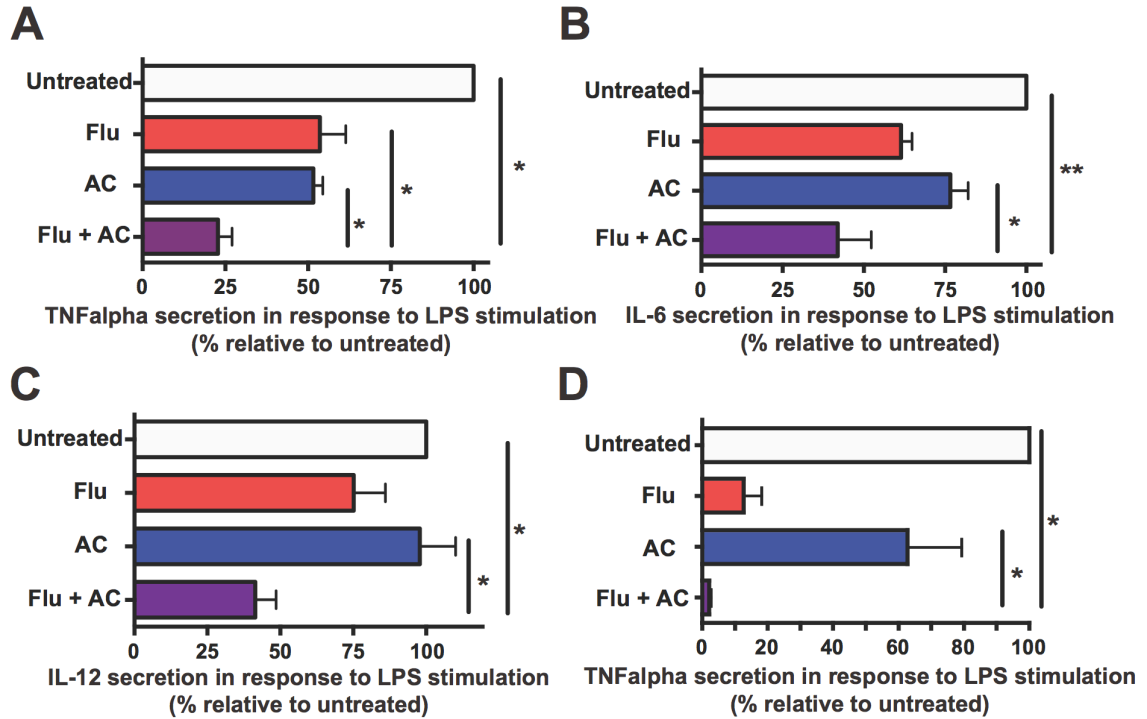
Figure 3.2. AMø LPS response is more greatly inhibited by GCAE than by AC or fluticasone alone. A-C. Adherence-purified murine AMø from normal C57BL/6 mice were treated with 2 μM fluticasone for 3 h (Flu), 10:1 AC for 2 h (AC) or 2 μM fluticasone for 3 h following by 10:1 AC for 2 h (Flu + AC), then stimulated with 1 ng/mL LPS. Supernatants were collected after 24 h and secreted TNFα (A), IL-6 (B) and IL-12 (C) were quantified by Luminex. D. Adherence-purified human AMø from human bronchoalveolar lavage were treated with 2 μM fluticasone for 3 h (Flu), 10:1 AC for 2 h (AC) or 2μM fluticasone for 3 h following by 10:1 AC for 2 h (Flu + AC), then stimulated with 1 ng/mL LPS. Supernatants were collected after 24 h and secreted TNFα was quantified by Luminex. Data are mean ± SE of four mice from two independent experiments or mean ± SE of AMø from two human subjects. Both humans were current smokers. *, statistically significant, $p < 0.05$ by One-Way ANOVA with Bonferroni post-hoc testing. Panel D is data from experiments performed by Dr. Jill C. Todt.
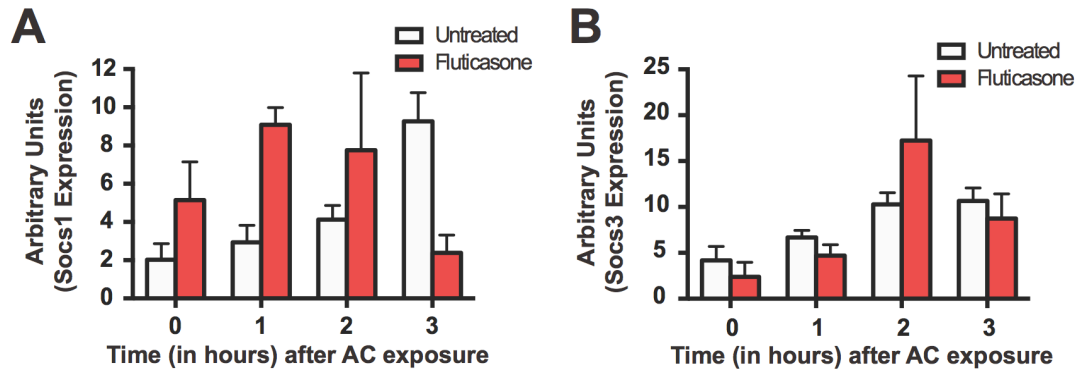
Figure 3.3. Kinetics of SOCS1, but not SOCS3, induction following AC exposure are altered by fluticasone pretreatment. Adherence-purified AMø from normal C57BL/6 mice were exposed to AC at ratio of 10 AC per AMø for 0-3 h following pretreatment with control media or 2 µM fluticasone for 6 h. Expression of SOCS1 (A) and SOCS3 (B) mRNA was measured by RT-PCR. Data are mean ± SE of three mice. No statistically significant differences by Two-Way ANOVA with Bonferroni post-hoc testing.

Table 3.1 Results of miRNA PCRArray screen of human AMø RNA after GCAE. Adherence-purified AMø from three healthy nonsmokers were treated with control media, fluticasone, AC, or fluticasone followed by AC. Changes in miRNA expression relative to control Mø were calculated for the three treatment groups. Six miRNA uniquely downregulated by GCAE were identified. PCRArray was performed by Sean Crudgington.

Fluticasone		Apoptotic Cells		Fluticasone + Apoptotic Cells	
hsa-miR-103a	(-2.5; 0.02)*			hsa-miR-103a	(-2.7; 0.02)
hsa-miR-22	(-5.6; 0.01)			hsa-miR-22	(-4.6; 0.01)
		hsa-miR-29a	(-5.0; 0.04)	hsa-miR-29a	(-4.9; 0.02
hsa-miR-188-3p	(-2.3; 0.05)	hsa-miR-188-3p	(-4.5; 0.04)	hsa-miR-188-3p	(-3.4; 0.03)
				hsa-let-7i	(-3.4; 0.03)
				hsa-miR-24	(-2.4; 0.006
				hsa-miR-27a	(-3.1; 0.01)
				hsa-miR-29c	(-4.3; 0.02
				hsa-miR-1248	(-3.8; 0.05)
				hsa-miR-3943	(-25.6; 0.0005)
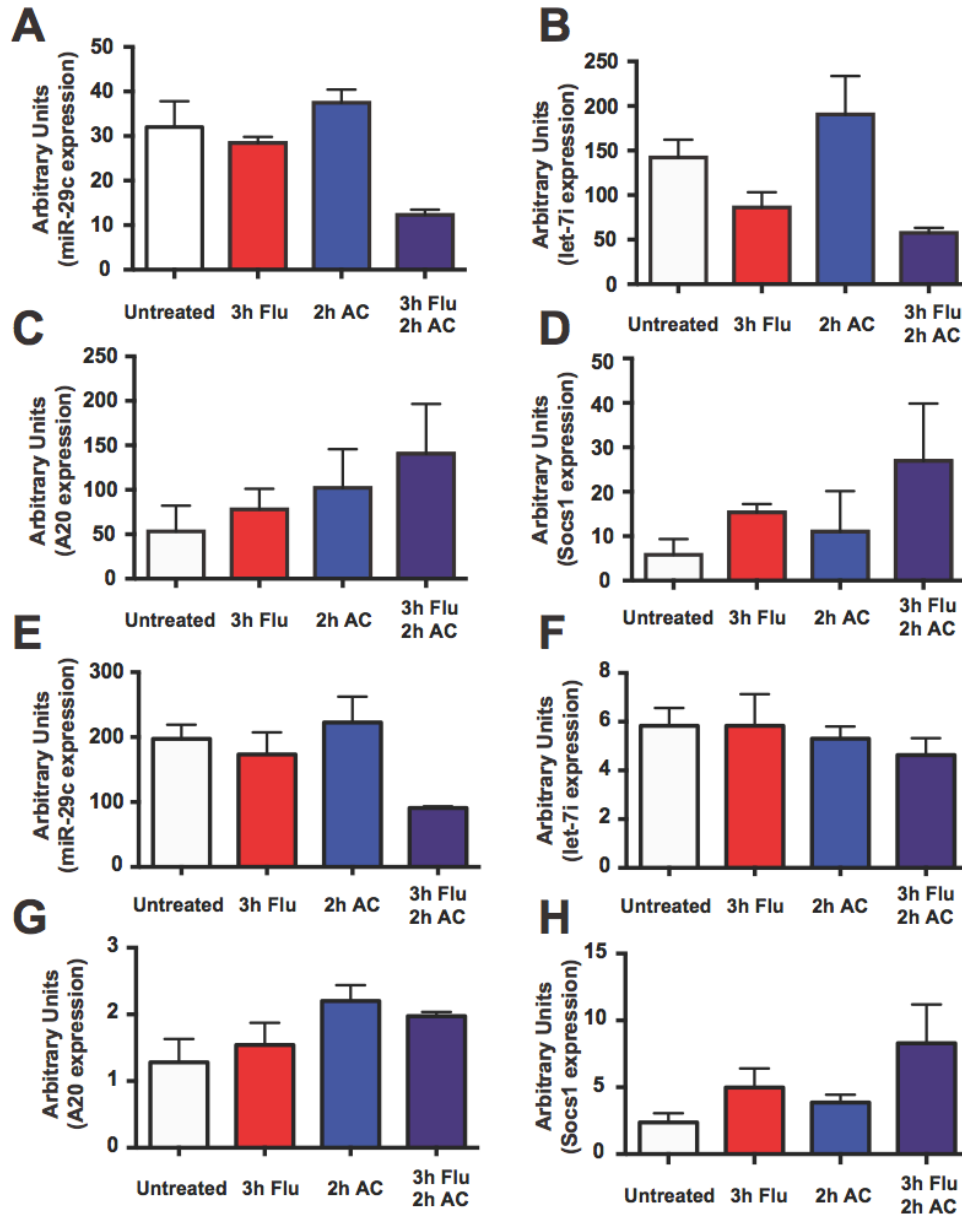
Figure 3.4. GCAE suppresses expression of let-7i and miR-29c and induces expression of respective target genes, SOCS1 and A20. A-D. Adherence-purified AMø from human bronchoalveolar lavage were treated with 2μM fluticasone for 3 h (Flu), 10:1 AC for 2 h (AC) or 2μM fluticasone for 3 h following by 10:1 AC for 2 h (Flu + AC). Expression of miR-29c (A), let-7i (B), A20 (C), and SOCS1 (D) was measured by RT-PCR. Data are mean ± SE of AMø from two human subjects. Both humans were current smokers. E-H. Adherence-purified AMø from normal C57BL/6 mice were treated with 2μM fluticasone for 3 h (Flu), 10:1 AC for 2 h (AC) or 2μM fluticasone for 3 h following by 10:1 AC for 2 h (Flu + AC). Expression of miR-29c (E), let-7i (F) A20 (G), and SOCS1 (H) was measured by RT-PCR. Data are mean ± SE of three mice from one experiment. No significant differences by One-Way ANOVA with Bonferroni post-hoc testing. PCR of human samples (A-D) was performed by Valerie Stolberg.
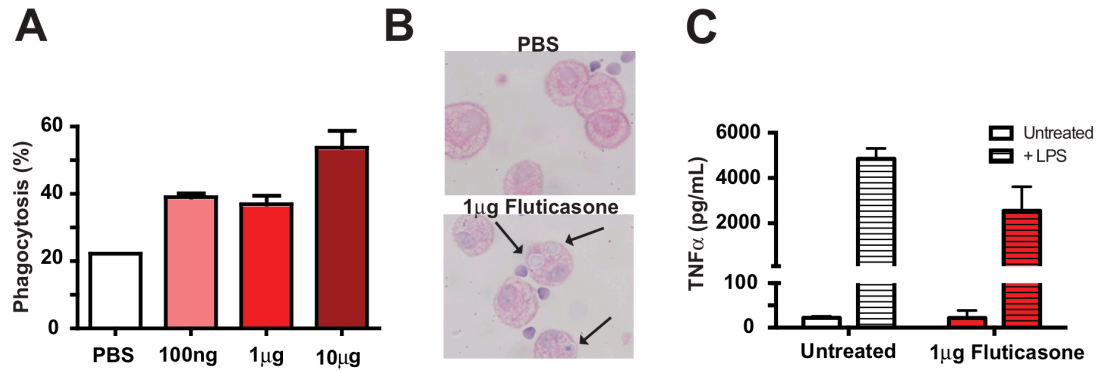
Figure 3.5. Intranasal administration of fluticasone affects in vitro and in vivo AMø responses. A. In vitro AC uptake following in vivo fluticasone treatment. AMø were adhesion-purified from bronchoalveolar lavage of normal C57BL/6 mice 6 h after intranasal treatment with 100ng-10µg fluticasone in 50µL PBS. AC were added at a 10:1 ratio for 2 h, then slides were washed and stained using H&E and ingested AC were counted at 100X magnification under oil. B. Representative cytospins showing in vivo AC uptake following in vivo fluticasone treatment. C57BL/6 mice were intranasally administered 1µg fluticasone in 50µL PBS, then after 6 h 10^7 AC were administered intranasally in 50µL PBS. Bronchoalveolar lavage was collected after 1 h, cytospins were stained using H&E and ingested AC were counted at 100X magnification under oil. C. Inhibition of in vitro LPS response following in vivo fluticasone treatment. AMø were adhesion-purified from bronchalveolar lavage of normal C57BL/6 mice 6 h after intranasal treatment with 1µg fluticasone in 50µL PBS, then stimulated with 1ng/mL LPS. Supernatants were collected after 24 h and secreted TNFα was quantified by Luminex. Data are mean ± SE of at least two mice.
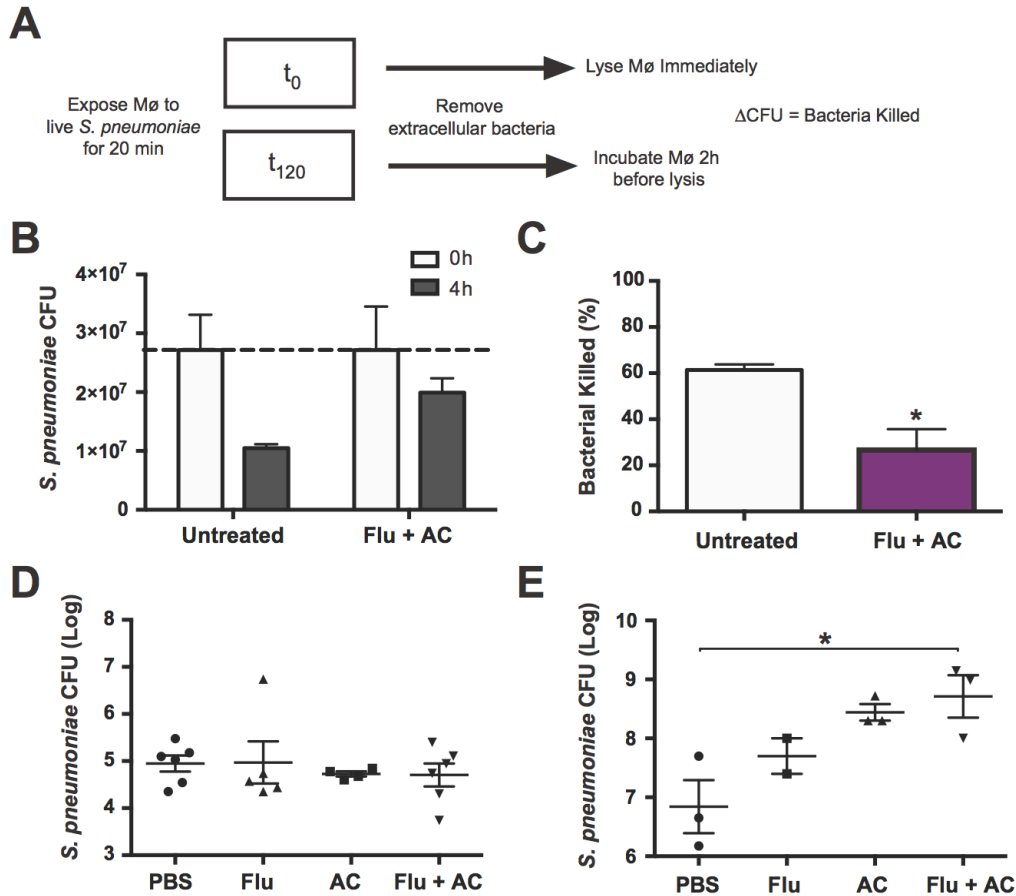
Figure 3.6. GCAE decreases bacterial killing of *Streptococcus pneumoniae* in vitro and in vivo. A. Graphic timeline of a bacterial killing assay. B,C. Bacterial killing of *S. pneumoniae* by murine AMø following GCAE. Adherence-purified AMø from normal C57BL/6 mice were treated with control media or 2μM fluticasone for 4 h followed by 10:1 AC for a further 18 h (Flu + AC) prior to exposure to 16x10^6 CFU *S. pneumoniae*. D. In vivo killing of *S. pneumoniae* following brief GCAE. C57BL/6 were pretreated with intranasal inoculations of saline alone, 1μg fluticasone at -6 h (Flu), 10^7 AC at -2 h (AC) or 1μg fluticasone at -6 h followed by 10^7 AC at -2 h (Flu + AC) in 50μL of PBS, then infected intratracheally with 50,000 CFU of *S. pneumoniae*. Lung homogenates were collected after 24 h and bacterial burden was quantified as CFU. E. In vivo killing of *S. pneumoniae* following chronic GCAE as measured by changes in whole lung CFU. C57BL/6 were pretreated with intranasal inoculations of saline alone, 1μg fluticasone at -24 and -6 h (Flu), 10^7 AC at -18 and -2 h (AC) or 1μg fluticasone at -24 and -6 h followed by 10^7 AC at -18 and -2 h (Flu + AC) in 50μL of PBS, then infected intratracheally with 50,000 CFU of *S. pneumoniae*. Lung homogenates were collected after 24 h and bacterial burden was quantified as CFU. Data are mean ± SE of at least three mice. *, statistically significant, $p < 0.05$ by One-Way ANOVA with Bonferroni post-hoc testing. Valerie Stolberg and Dr. Christine M. Freeman performed the in vitro bacterial killing assay (B, C) and collaborated on the in vivo experiment shown in panel E.

Chapter 4

MiR-34a is a master-regulator of apoptotic cell clearance in macrophages[2]

Abstract

MicroRNA, small regulatory RNA that inhibit translation of target genes, have been implicated in the control of myriad biological pathways, however, the role of miRNA in regulating apoptotic cell (AC) engulfment is largely unknown. Here we provide evidence that the microRNA miR-34a is a negative regulator of AC engulfment, acting through repression of multiple target engulfment genes. In alveolar macrophages (AMø), microglia (glia), bone marrow-derived macrophages (BMDMø) and peritoneal macrophages (PMø), transiently induced reduction or overexpression of miR-34a expression increased or decreased efferocytosis, respectively. By crossing miR-34a-flox mice with LysM cre mice we generated mice that were miR-34a haploinsufficient in the myeloid lineage (miR-34a+/-). AC uptake by AMø from miR-34a+/- mice was increased compared to wild-type (WT) mice. We demonstrate that miR-34a targets at least three components of AC engulfment machinery: Axl, SIRT1, and GRAF1 (ArhGAP26). Both SIRT1 and GRAF1 are novel components of the AC uptake pathway. We further show that miR-34a does not solely depend on altered expression of Axl to modulate AC

engulfment.   These results imply that miR-34a alters AC clearance through the combined effects of multiple target engulfment genes including Axl, SIRT1 and GRAF1.   Finally, we show that miR-34a augments the capacity for bacterial killing. The dual role of miR-34a in inhibiting AC uptake and enhancing bacterial killing suggests that miR-34a may act as a master-regulator of the balance between AC clearance and Mø antimicrobial function.

Introduction

MicroRNA (miRNA) are short, regulatory RNA that bind to target mRNA and inhibit translation by triggering mRNA degradation or blocking proper ribosome association (300-302). MiRNA are processed from pri-miRNA down to a final 21-25 nucleotide length by Drosha/DGCR8 and Dicer, then interact with Ago2 in a RISC complex to bind target mRNA (303, 304). Generally, a seed sequence of 5-8 nucleotides within the 5' region of the miRNA binds a complementary region within the 3'-UTR of target mRNA, although binding within the 5'-UTR, promoter, or open reading frame has also been described (305, 306). Numerous miRNA have been implicated in the regulation of important Mø functions; miR-145, miR-146a, and miR-155 have all been shown to inhibit TLR signaling (307-312); miR-144 and miR-33a to regulate cholesterol efflux (313, 314); and let-7c and miR-125a to influence M1/M2 polarization (315-317). How miRNA may regulate AC clearance, another important macrophage function, remains largely unknown. A single miRNA, miR-21, has been described to regulate AC uptake positively in human blood-derived monocytes (318), likely through targeting Phosphatase and tensin homolog (PTEN).

While the known positive regulators of AC uptake are numerous, much less is known regarding the negative regulation of AC engulfment. SIRPα (63, 64) and CD300a (66) have been described as inhibitory engulfment receptors. PTEN (78), MTM-1 (319) RhoA/ROCK1 (74, 162) have been described to negatively regulate downstream engulfment signaling. No miRNA have previously been identified that negatively regulate AC uptake. Here we describe miR-34a as a negative regulator of AC uptake that targets multiple components of engulfment machinery in resident tissue macrophages.

miR-34a is a target of p53 that is well-described as a negative regulator of many types of cancer, mediating its anti-tumor effects through targeting pathways controlling cell cycle (320, 321), glycolysis (322, 323), invasion (324-328), and cell death (329, 330). The positive effect of miR-34a on cell death has been shown to have negative consequences as well; tissue death and scarring is enhanced following ischemia when cardiac myocytes express miR-34a (331, 332). Additionally, miR-34a can inhibit the differentiation of B cells (333, 334) and is required for differentiation of dendritic cells (335) and neurons (336, 337). Interestingly, increased levels of miR-34a have been observed in the liver during aging (338, 339) and obesity (340, 341), and the lung tissue of patients with COPD (342). These three diseases are associated with decreased AC clearance (138, 343-346).

In this study, we asked whether miR-34a could directly inhibit AC engulfment and the mechanisms by which this could occur. We demonstrate that miR-34a is a master-regulator of AC engulfment, inhibiting AC uptake through targeting multiple genes within the engulfment pathway. Interestingly, the AC recognition receptor Axl is also an oncogene and direct target of miR-34a (326, 347). We confirm that miR-34a

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inhibits Mø expression of Axl, a known AC recognition receptor. Further, we provide evidence that two miR-34a target genes, a protein deacetylase named SIRT1 and a Rho-family GTPase activating protein (RhoGAP) named GRAF1, are novel components of AC engulfment machinery. Of interest, miR-34a expression is particularly high in alveolar macrophages (AMø). Finally, we document a positive role for miR-34a in killing engulfed bacteria, which suggests that AMø function may be biased towards bacterial killing and away from AC clearance.

Results

Knockdown or overexpression of miR-34a respectively increases and decreases AC uptake in murine and human Mø. We observed a clear inverse relationship between AC uptake, expressed as phagocytic index, and miR-34a expression, as measured by quantitative real-time RT-PCR in murine AMø, glia, BMDMø and PMø (**Fig. 4.1A**). To understand whether this relationship was correlative or causative, we transiently transfected primary Mø to knockdown or over-express miR-34a using the lipofectamine-based RNAiMAX system. Transfection of a specific miR-34a antagomir led to significant knockdown of miR-34a expression as measured by quantitative real-time RT-PCR (**Fig. 4.1B**). We measured uptake of FITC-labeled miR-34a and control constructs using flow cytometry and confirmed transfection occurred in >90% of cells (**Fig. 4.1C**). Although miR-34a has been shown to regulate apoptosis, we observed no changes in cell viability following overexpression in PMø (**Fig. 4.1D**). AC uptake in vitro was increased by knockdown of miR-34a in murine AMø (**Fig. 4.2A, 4.2B**), murine microglia and murine BMDMø (**Fig. 4.2C, 4.2D**). Conversely, overexpression of miR-34a in PMø

inhibited AC uptake (**Fig. 4.2E**). Collectively, miR-34a negatively regulated AC uptake

in all four macrophage types tested. Knockdown of miR-34a in human AMø also

increased AC uptake, indicating this regulation is not species specific (**Fig. 4.2F**).

MiR-34a+/- AMø have increased AC uptake. To confirm the results of our transient

transfection experiments, we crossed miR-34a flox+/+ mice with LysM cre mice. This

breeding generates mice where miR-34a expression is deleted in LysM-expressing cells

(i.e. myeloid cells including Mø). We have not yet produced sufficient numbers of miR-

34a flox+/+ LysM cre mice to study miR-34a-/- Mø. However, we were able to study the

heterozygous miR-34a flox+/- LysM cre mice (miR-34a+/-), whose myeloid cells have

only one copy of miR-34a. miR-34a expression was halved in AMø from miR-34a+/-

mice; no further decrease in the already low miR-34a expression of PMø was observed

(**Fig. 4.3A**). Interestingly, miR-34a+/- mice showed a slight though non-significant

increase in weight and a more pronounced increase in visceral white adipose tissue

(WAT) size (**Fig. 4.3B, 4.3C**). Because LsyM cre-targeting should not impact adipose

cells of our miR-34a+/- mice, these results imply that miR-34a-directed gene expression

changes within myeloid cells must be sufficient to affect WAT growth. Aside from

increased WAT size in miR-34a+/- mice, we observed no differences between WT and

miR-34a+/- mice. WT and miR-34a+/- mice showed similar splenic composition of

immune cells as quantified by flow cytometry (**Fig. 4.3D**) and similar histology of

immune organs (**Fig. 4.3E-H**).

Exactly as predicted by our transfection experiments in primary AMø, in vitro AC

uptake by isolated miR-34a+/- AMø and differentiated BMDMø was significantly

increased compared to WT AMø and BMDMø (**Fig. 4.4A, 4.4B**). As expected, there was no change in AC uptake by PMø (**Fig. 4.4C**).

MiR-34a does not regulate Fc-mediated uptake. To test specificity, we measured Fc-mediated uptake of opsonized particles following miR-34a knockdown or overexpression. Following knockdown of miR-34a in AMø there was no change in uptake of Ig-opsonized sheep red blood cells (SRBC) (**Fig. 4.5A, 4.5B**). Similarly, following overexpression of miR-34a in PMø there was no change in uptake of opsonized SRBC (**Fig. 4.5C**) or opsonized heat-killed *S. aureus* (**Fig. 4.5D**). Global engulfment is not affected by miR-34a; AC engulfment is specifically altered.

MiR-34a targets Axl, but that effect of miR-34a is not required to limit AC uptake. Previous studies have identified the receptor tyrosine kinase Axl, a receptor for AC ingestion, as a direct target of miR-34a (323, 326, 347). To test whether miR-34a regulated Axl expression in macrophages, we used flow cytometry to measure surface expression of Axl following miR-34a knockdown. Following knockdown of miR-34a in AMø, MFI of Axl nearly doubled (**Fig. 4.6A**). We also observed slight but significant increase in MFI of Mertk (a kinase in the same family as Axl, and also an AC receptor) and CD80 but not of TIM-4 or CD206 (**Fig. 4.6A**). As expected, we also found an increase in Axl expression by flow cytometry of miR-34a+/- BMDMø compared to WT BMDMø (**Fig. 4.6B, 4.6C**).

To test whether Axl was required for the effect of miR-34a on AC uptake, we obtained bone marrow from Axl-/- mice (generously provided by the Lemke Laboratory,

Scripps Institute). Differentiation using GM-CSF induced Axl expression in WT

BMDMø but not in Axl-/- BMDMø (**Fig. 4.7A**).   AC uptake was increased by

knockdown of miR-34a in Axl-/- BMDMø (**Fig. 4.7B**), as had been observed in WT

BMDMø (Fig 4.2D). This result indicated that miR-34a reduction of efferocytosis does

not require inhibition of Axl expression, and thus must involve other pathways.

The miR-34a target SIRT1 positively regulates AC uptake but not Fc-mediated uptake.

Previous studies have identified SIRT1 as a direct target of miR-34a (348-350), although

SIRT1 has not been connected to AC clearance. SIRT1 expression was increased in

miR-34a+/- AMø (**Fig 4.8A**). To test whether SIRT1 could have a heretofore unknown

function in regulating AC clearance, we treated WT macrophages with SIRT1 agonists

and antagonists, then measured the effect on efferocytosis. Treatment of PMø with the

SIRT1 antagonists Sirtinol or EX-527 inhibited AC uptake (**Fig. 4.8B-D**). Treatment of

AMø with the agonist Resveratrol increased AC uptake (**Fig. 4.8E**). Treatment of PMø

with Sirtinol did not alter Fc-mediated uptake of opsonized SRBC (**Fig. 4.8F, 4.8G**).

SIRT1 acetylates and thus activates LXRα (351), which is upstream of the AC

engulfment receptor Mertk (96).   We asked whether inhibition of SIRT1 would reduce

Mertk expression. Surface expression of Mertk on PMø, as measured by flow cytometry,

showed a dose-dependent decrease following Sirtinol treatment (**Fig. 4.8H**). Positive

regulation of the LXRα/Mertk axis may be one mechanism by which SIRT1 positively

regulates AC engulfment.

GRAF1 is increased in miR-34a+/- Mø and positively regulates AC and not Fc-mediated uptake. We identified GRAF1 as a potential direct target of miR-34a using *in silico* screening (**Fig. 4.9A**). The predicted miR-34a binding site within the 3'-UTR is highly conserved among eukaryotes; TargetScan 6.2 calculated a PCT of 0.9, indicating a high probability that this site within the GRAF1 3'-UTR is conserved due to selective maintenance of miRNA targeting (352). Selective maintenance suggests that a miR-34a/GRAF1 interaction has functional import. To test whether GRAF1 could regulate AC uptake, we transiently knocked down GRAF1 expression in PMø using siRNA. Knockdown of GRAF1 in PMø inhibited AC uptake (**Fig. 4.9B, 4.9C**) but not Fc-mediated uptake of opsonized SRBC (**Fig. 4.9D**). Although we have not confirmed whether miR-34a directly binds to its predicted site within the GRAF1 3'-UTR, we showed that GRAF1 expression is increased in miR-34a+/- AMø, indicating it is at least an indirect target of miR-34a (**Fig. 4.9E**).

miR-34a enhances Mø killing of Streptococcus pneumoniae. We were interested in whether miR-34a might regulate other macrophage functions. Pervious work has shown that miR-34a can inhibit the macrophage TLR response (353). To test whether miR-34a could affect bacterial killing by Mø we measured the ability of PMø to kill *S. pneumoniae* following overexpression of miR-34a. Overexpression of miR-34a increased bacterial killing by PMø (**Fig. 4.10A**).

Discussion

The results of this study identify miR-34a as a negative regulator of Mø AC clearance through targeting multiple components of engulfment machinery: Axl, SIRT1 and GRAF1. Using primary murine and human AMø as well as culture-differentiated murine glia and BMDMø, we show that transient or genetic knockdown of miR-34a enhances AC uptake. Conversely, transient overexpression of miR-34a in primary murine PMø inhibits AC uptake. We demonstrate that SIRT1 and GRAF1 are novel components of engulfment machinery; pharmacologic inhibition of SIRT1 in PMø and transfecting PMø with siRNA against GRAF1 both decrease AC uptake. We demonstrate that SIRT1, GRAF1, and Axl are all inhibited by miR-34a in macrophages. Finally, we show that in addition to its role as a negative-regulator of AC clearance in Mø, miR-34a also enhances bacterial killing. We hypothesize that miR-34a may act as a master-regulator of Mø function.

Although small in size, miRNA have the ability to regulate the expression of hundreds to thousands of genes (300, 302, 306, 354). Each miRNA has numerous direct targets, many of which themselves influence transcription of other genes, meaning that each miRNA influences the expression of additional, indirect targets. MicroArray and pSILAC analysis of miR-34a targets in kidney cells identified at least 228 genes as direct or indirect targets of miR-34a (323). KEGG pathway analysis found that many miR-34a targets were regulators of DNA replication, cell cycle, apoptosis and metabolism (323). Our data identify a new group of known and novel targets of miR-34a that we demonstrate are grouped by their common involvement in AC engulfment and explain how miR-34a negatively regulates efferocytosis.

Our results complement a recent publication identifying miR-21 as a positive regulator of AC engulfment in human blood monocyte-derived macrophages that is upregulated in these cells following AC exposure (318). miR-21 directly targets PTEN (355, 356) which is known to regulate both NF-kB signaling (357) and AC engulfment (78). It is unclear whether miR-21 may target additional engulfment genes. Of interest, an inverse relationship between miR-34a and miR-21 expression has been previously described in the cancer literature. miR-34a functions as a tumor-suppressor while miR-21 is tumorigenic (358-361). Additionally, an inverse relationship between miR-34a and miR-21 expression has been observed following LPS (353, 362), TGF-β (363, 364), and hypoxia (365, 366). There is some evidence that miR-34a may negatively regulate miR-21 expression through CD24 and Src (367). Although further experiments are necessary, we speculate that miR-21/PTEN is yet another target through which miR-34a may negatively regulate AC uptake.

Our finding that reduced Axl expression is not required for the inhibitory effect of miR-34a of AC uptake supports our assertion that miR-34a acts as a master-regulator of AC clearance, altering the program of AC engulfment, cumulatively inhibiting efferocytosis by small changes to multiple components of engulfment machinery. Interestingly, our lab has observed that basal Axl expression is normally very high in AMø and less high in PMø (unpublished data). The lack of correlation between Axl expression and miR-34a levels or engulfment in AMø and PMø further supports our finding that Axl alone does not determine engulfment capacity and is not the only mechanism by which miR-34a regulates AC uptake.

Although our data show that the effect of miR-34a on AC clearance is conserved in both mice and humans, it is unclear whether miR-34a will affect AC uptake in simpler organisms. Evolutionarily, miR-34a arose from an ancestral miR family (368) and is found as a single miR-34 transcript in *C. elegans* and *D. melanogaster*. miR-34 has subsequently diversified in both mice and humans into miR-34a-c. While both worm and fly encode SIRT1 homologs (369, 370), and GRAF1 homologs (NCBI database search), neither encode Axl homologs (11). It would be interesting to test whether miR-34a retains its function as a negative regulator of AC clearance in *C. elegans* and *D. melanogaster*. Such a result would further support our finding that miR-34a-mediated repression of AC clearance does not require Axl. It would also be interesting to test whether miR-34b or miR-34c could also regulate AC uptake; related miRNA have similar seed sequences and thus overlapping target pathways (360, 371). MiR-34c, like miR-34a, has recently been shown to repress SIRT1 (372). MiR-34b/c are highly expressed in the lung (360), although we have not examined specific expression by AMø or how that may compare to PMø.

Our finding that the miR-34a target SIRT1 positively regulates AC uptake extends the previous observation that SIRT1-/- mice develop a lupus-like autoimmunity (373) and have increased numbers of apoptotic germ cells in the testes (374-376). As defective efferocytosis is known to manifest in vivo with autoimmunity and an increase in uncleared AC (20, 119, 377), we hypothesize that defective AC clearance in SIRT1-/- mice may contribute to both of these previously described phenotypes.

The mechanism through which SIRT1 acts on AC uptake remains unclear. SIRT1 functions as a protein deacetylase of nuclear and cytoplasmic proteins (378). Many

targets of SIRT1 have been identified, although how acetylation changes their function is often unknown. LXRα functional activity is augmented following acetylation by SIRT1 (351). LXRα is known to regulate both basal AC engulfment and the ability to enhance secondary engulfment following initial AC exposure (96). In particular, the enhanced secondary engulfment has been shown to involve LXRα-dependent upregulation of Mertk in response to AC (96). We observed a small but significant increase in Mertk expression following miR-34a knockdown in AMø (**Fig. 4.6A**), although there is no evidence that Mertk is a direct target of miR-34a. We also observed a decrease in Mertk expression following SIRT1 inhibition in PMø (**Fig. 4.8H**). We theorize that SIRT1, through acetylation of LXRα, enhances expression of Mertk, and thus miR-34a can decrease expression of Mertk through inhibiting SIRT1 translation. Preliminary experiments to test this hypothesis could involve examining the nuclear translocation of LXRα (379, 380), testing for increased basal nuclear localization or increased translocation in response to AC within cells with decreased miR-34a expression. This would indicate increased LXRα activity.

Early studies in *C. elegans* described a role for SIRT1 in promoting autophagy, which shares some machinery with AC uptake, particularly during AC degradation (90, 92, 381, 382). Although our phagocytosis assays are biased towards measuring changes in engulfment and not necessarily degradation, accelerating degradation could impact subsequent engulfment and this possibility deserves further thought. A more intriguing possibility for SIRT1 is that it acts on AC clearance by altering other components of cell metabolism. Altering metabolism through Ucp2 and AMPK within the phagocyte has been shown to affect AC uptake (93, 383). Of particular interest is the connection

between AMPK and AC uptake; there is substantial evidence of crosstalk between AMPK and SIRT (384-389). Resveratrol, a SIRT1 agonist that we demonstrate can enhance AC uptake by AMø (**Fig. 4.8E**), has been shown to activate AMPK through a SIRT1-dependent mechanism (389, 390). AC engulfment triggers AMPK activation and blocking AMPK abrogates AC uptake: efferocytosis both regulates and is regulated by AMPK signaling (93). It is possible that SIRT1 is required for this activation of AMPK following AC uptake. We speculate that SIRT1 regulates AC uptake both through activation of AMPK and activation of LXRα, although further experiments are required to test this hypothesis.

The ability to decrease mitochondrial membrane potential has been connected to the ability of both AMPK and Ucp2 to increase AC uptake; in both cases, pharmacologic decrease of mitochondrial membrane potential alone was sufficient to increase AC uptake (93, 383). Unexpectedly, there is evidence of the inverse relationship with miR-34a and SIRT1: miR-34a inhibits mitochondria membrane potential in Y79 retinoblastoma cells (391) but we show that it inhibits AC uptake by Mø (**Fig. 4.2**). Both SIRT1 and Resveratrol are known to increase mitochondrial membrane potential in C2C12 myoblast cells (389), but we show they also increase AC uptake by Mø (**Fig. 4.8**). We have not directly examined mitochondrial membrane potential in our macrophages following manipulation of miR-34a and SIRT1. There may be cell-specific differences; previous studies of AMPK and Ucp2 have only measured mitochondrial membrane potential in elicited PMø and a variety of tumor cell lines which are likely metabolically distinct from many resident tissue macrophages. This will be an important issue to clarify.

MiR-34a alters metabolism through SIRT1-dependent and -independent mechanisms. LDHA, required for the production of lactate, is a direct target of miR-34a (323). MiR-34a has been shown to inhibit glycolysis through repression of additional glycolytic enzymes (322). Early work showed that AMø possess a highly oxidative metabolism when compared to the more glycolytic metabolism of PMø (392). Although the full import of macrophage metabolism is unknown, recent work has demonstrated that M1/M2 polarization involves a switch between glycolysis and oxidative phosphorylation (393-396). As miR-34a expression inhibits glycolysis, this suggests that miR-34a may inhibit M1 polarization and favor a more M2-like phenotype. Additionally, previous work has shown that miR-34a expression is induced by TGF-β (364) and decreased by LPS (353), classic M2 and M1 polarizers, respectively. However, it is unclear how applicable the phenotypes of M1 and M2 polarization are to resident tissue Mø. Most importantly, M2-like cells have been shown to have improved AC clearance (397-399), the inverse effect that miR-34a expression has on AC clearance. When we looked at M1/M2 markers (400, 401), we found no decrease in expression of the M2 marker CD206 following miR-34a knockdown in AMø, although we did see a slight increase in the M1 marker CD80 expression (**Fig. 6A**). Thus, although there are certain similarities, our data are not fully consistent with describing AMø as M1 or M2-like cells or describing miR-34a as driving an M1 or M2-like phenotype in resident tissue Mø.

It is also important to recognize that the alveolar environment may contribute to a unique metabolism for AMø. The alveolar space is actively maintained as very glucose-poor (402-404), potentially to provide an inhospitable environment for survival of inhaled pathogens. AMø do not express GLUT1 (405), the primary glucose transport protein;

even with increased availability of glucose AMø may not be able to effectively perform glycolysis. Thus, the bias of AMø away from a glycolytic metabolism, potentially through mechanisms involving miR-34a, is unsurprising. Studying how resident tissue Mø metabolism regulates their function, including AC uptake, will be a rich and important field for inquiry. Our current understanding of resident Mø is limited by the extensive use of monocyte-derived Mø populations to study Mø biology.

In addition to a novel role for SIRT1 in AC uptake, we describe a novel role for GRAF1. both in AC uptake and as a target of miR-34a. GRAF1 is a Rho-family GTPase-activating protein that assists in deactivation of Rho kinase family members (406). Most publications have found that GRAF1 preferentially regulates cdc42 and RhoA (407-409), although there is some suggestion that it is truly specific for RhoA (410). Through regulating the activity of Rho-family kinases, GRAF1 alters cell spreading, motility, adhesion, chemotaxis and clathrin-independent endocytosis (409, 411). Of interest, cells deficient in GRAF1 (409) or SIRT1 (412) and those over-expressing miR-34a (413) all show similar defects in chemotaxis following a confluent culture scratch model of wound healing. GRAF1 knockout cells (409, 410) and miR-34a overexpressing (328) cells show particularly similar cytoskeletal limitations, both lacking stable filopodia formation and exhibiting increased adhesion to tissue culture dishes. MiR-34a has been shown to alter cytoskeletal motility through indirect effects on the activity of RhoA and Rac1 (328, 413). It is interesting that GRAF1 has been shown to regulate endocytosis, as there is debate as to whether AC engulfment occurs through endocytosis, macropinocytosis, or true phagocytosis. Components of Clathrin and AP2 are required for efficient AC uptake (88), suggesting a connection with Clathrin-dependent endocytosis. However, there are

conflicting reports of finding lucifer yellow in phagosomes with AC (68, 414), which would indicate a mechanistic connection with macropinocytosis. Although we find no effect of miR-34a on Fc-mediated uptake of opsonized sheep red blood cells (SRBC) or Ig-opsonized *Staphylococcus aureus*, chemotaxis towards numerous factors such as AC-released ATP, UTP, and CX3CL1 (29, 31) may be inhibited by miR-34a through decreased expression of target protein GRAF1. Such a change would further contribute to the restriction of AC clearance caused by miR-34a expression. There may be alterations in endocytosis and macropinocytosis that would alter the uptake of various particles through non-Fc-mediated pathways. In future studies it will be important to understand how other types of engulfment and cytoskeletal movement may be regulated by miR-34a and GRAF1; we have only described the effect of miR-34a and GRAF1 on AC and Fc-mediated uptake.

Unexpectedly, miR-34a+/- mice showed an increase in white adipose tissue (WAT). As miR-34a was only deleted conditionally in LysM expressing cells and WAT does not express LysM (415), this observation is even more surprising. SIRT1 is known to regulate adiposity, but in the reverse direction: increasing SIRT1 expression through knockdown of miR-146a induces hypertrophy of WAT (416). Additionally, SIRT1 overexpression prevents infiltration of adipose tissue macrophages during high fat diet (417). These phenotypes, however, are due to SIRT1 expression in adipocytes. The phenotype we observe in our miR-34a+/- mice is due to an effect of decreased miR-34a on myeloid cells. This data is preliminary and, importantly, non-littermate C57BL/6 mice were used as WT controls in our experiments. Further study will be required to

confirm that the effect we saw on WAT adiposity was in fact due to miR-34a+/-

expression in myeloid cells and not confounding factors.

In addition to the novel role of miR-34a in negatively regulating AC uptake, we

find that miR-34a enhances bacterial killing of *S. pneumoniae*. The mechanism by which

this occurs is unclear. Chondrocytes lacking miR-34a are unable to induce iNOS

expression (418) suggesting miR-34a may positively regulate iNOS expression. iNOS

and NO have a known role in *S. pneumoniae* killing (280, 285-290). MiR-34a favors an

oxidative over a glycolytic metabolism (322, 323), which could enhance ROS production

for bacterial killing through iNOS-dependent or independent mechanisms. An alternate

possibility is that the observed increase in bacterial killing with increased miR-34a is due

to increased apoptosis of Mø. Successful killing of *S. pneumoniae* (291-294) and other

select bacteria (164, 259, 419-421) can involve phagocytosis-induced apoptosis of Mø.

Apoptotic Mø containing bacteria are engulfed and degraded by other phagocytes

through AC clearance (422). MiR-34a has been well-documented to increase

susceptibility to apoptosis in certain cell types (320, 330, 332, 348, 360, 391), however,

the ability of miR-34a to regulate apoptosis specifically in Mø has not been studied. We

did not observe increased apoptosis in PMø following over-expression of miR-34a alone

(**Fig. 4.1D**), however, we have not tested the possibility that miR-34a over-expressing

cells are more sensitive to phagocytosis-induced apoptosis, thus augmenting *S.*

pneumoniae killing. Further work is required to detail the mechanisms by which miR-

34a enhances Mø bacterial killing.

Adaptations to prevent bacterial infection are likely of extreme import within the

alveolar space as the average adult human breathes >5 liters of air every minute, inhaling

airborne pathogens and aspirating oral bacteria in the process. As AMø are the predominant leukocyte of the alveolar space (423) they provide the first line of defense against pathogens. We hypothesize that AMø ability to kill bacteria is of greater survival value than AC clearance and, since AC clearance itself inhibits bacterial killing, actively suppressing AC clearance through miR-34a would further protect against respiratory infection.

Collectively our data demonstrate a novel role for miR-34a as a master-regulator of AC clearance in Mø. MiR-34a targets Axl, SIRT1 and GRAF1 to inhibit AC clearance in all four Mø studied. Further, miR-34a augments the capacity of Mø for bacterial killing. Future work may identify additional miR-34a targets that contribute to a cumulative repression of AC engulfment and may deepen our understanding of how miR-34a might act, beyond a master-regulator of AC uptake, as a master-regulator of the balance between efferocytosis and Mø antimicrobial function.

Figure 4.1. miR-34a expression inversely correlates with AC uptake and can be manipulated by transient transfection in primary macrophages. A. Relationship between phagocytic index (open bars) and miR-34a expression (black bars) in select Mø cell types. Quantitative real-time RT-PCR was performed on RNA from AMø, glia, BMDMø and PMø, shown as arbitrary units miR-34a relative to the control nucleic acid sno-142, contrasted with apoptotic cell uptake of each cell type, quantified by in vitro phagocytosis assays. B, C. Successful transfection of miR-34a in primary murine AMø. Murine AMø were transfected using RNAiMAX lipofectamine with either no construct (-), control scramble construct (scramble), or miR-34a antagomir (miR-34a). At 24 h after transfection, (B) MiR-34a levels were measured by quantitative real-time RT-PCR shown as arbitrary units miR-34a/sno-142 and (C) efficiency of transfection was quantified by flow cytometry tracking FITC-positivity of either control (scrambled) construct or miR-34a antagomir, gated on CD45+ cells. Shown as representative histograms. D. MiR-34a overexpression does not induce apoptosis in PMø. Murine PMø were transfected using RNAiMAX lipofectamine containing no construct (untransfected), control scramble construct (control), or miR-34a mimic (miR-34a). At 48 h after transfection, we quantified apoptosis in these three groups by flow cytometry of Annexin-PI staining. Shown as representative dot plots. Data are mean ± SE of 4-7 mice assayed individually in at least two independent experiments per condition. *, statistically significant, $p<0.05$ and **, statistically significant, $p<0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
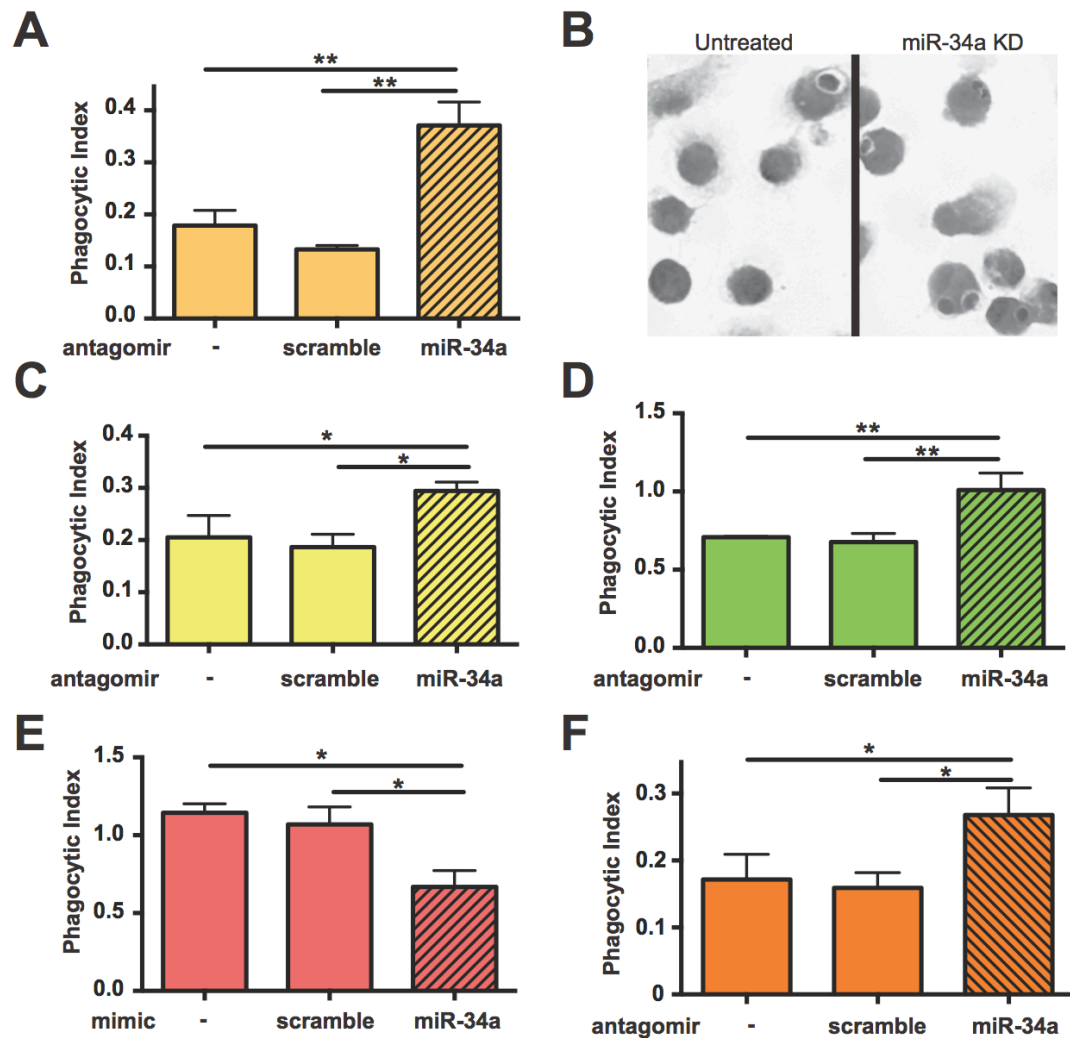
Figure 4.2. MiR-34a negatively regulates AC uptake. Mø were plated in chamber slides and transfected using RNAiMAX lipofectamine containing no construct (-), control scramble construct (scramble), or miR-34a antagomir/mimic (miR-34a). 24 h after transfection with antagomir or 48 h after transfection with mimic, 10:1 AC were added for 1.5 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. A,B. AC uptake following knockdown of miR-34a in murine AMø. A. Quantification. B. Representative photos of AC engulfment. C-F. AC uptake following manipulation of miR-34a in various Mø: C, knockdown in murine microglia. D. knockdown in murine BMDMø. E. overexpression in murine PMø. F. knockdown in human AMø. Data are mean ± SE of 3-7 mice assayed individually in at least two independent experiments per condition. *, statistically significant, $p<0.05$ and **, statistically significant, $p<0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
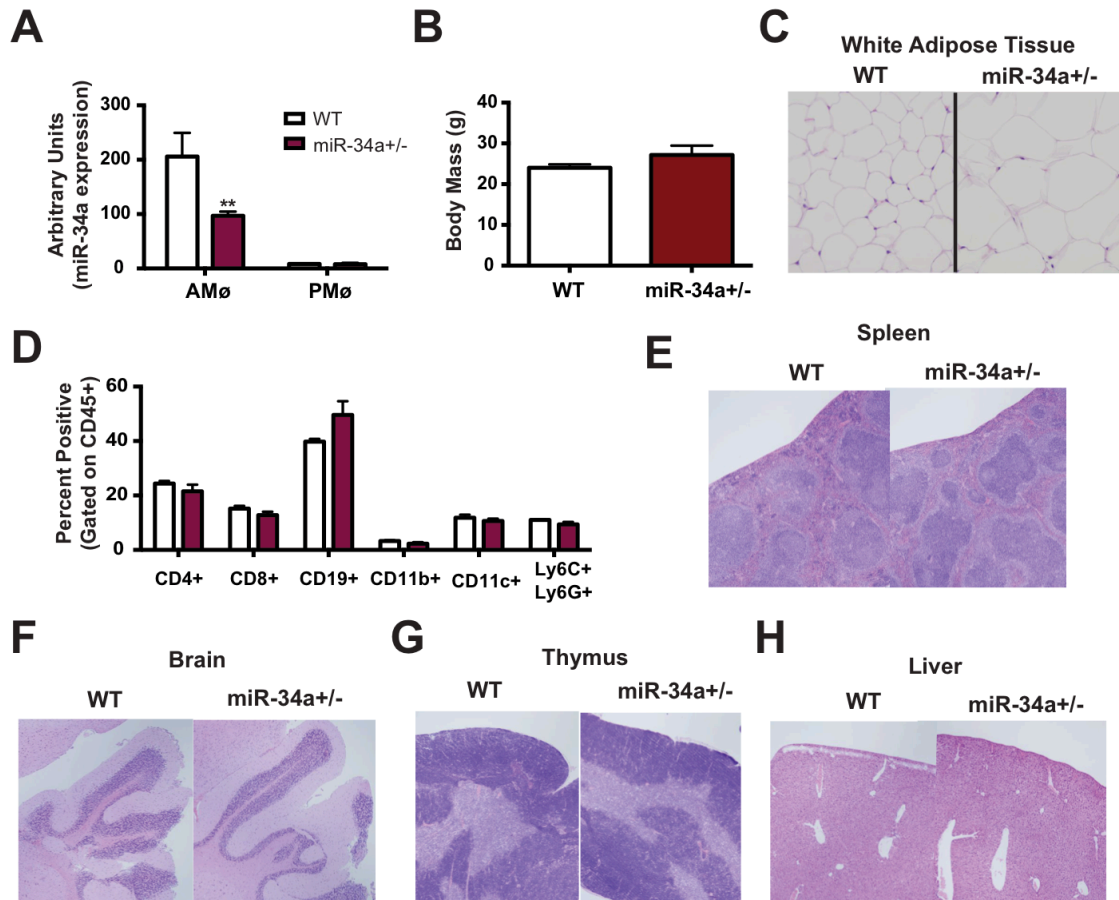
Figure 4.3. Phenotype of MiR-34a+/- mice. MiR-34aflox mice were crossed with LysMcre mice to develop miR-34aflox+/-LysMcre mice (miR-34a+/-). A. MiR-34a expression was decreased in AMø but not PMø from miR-34a+/- mice. MiR-34a levels were quantified by RT-PCR shown as arbitrary units miR-34a/sno-142. B. WT and MiR-34a+/- weights at 16w. C. Histology of white adipose tissue from WT and miR-34a+/- mice, stained with H&E, shown at 20X. D. Flow cytometry to quantify relative immune compartments within murine spleen. Populations shown were gated CD45+. Histology of organs from WT and miR-34a+/- mice, stained with H&E, shown at 4X. E-H. Histology of (E) spleen, (F) brain, (G) thymus, and (H) liver. Data are mean ± SE of 6 mice of each genotype assayed individually in two independent experiments. **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
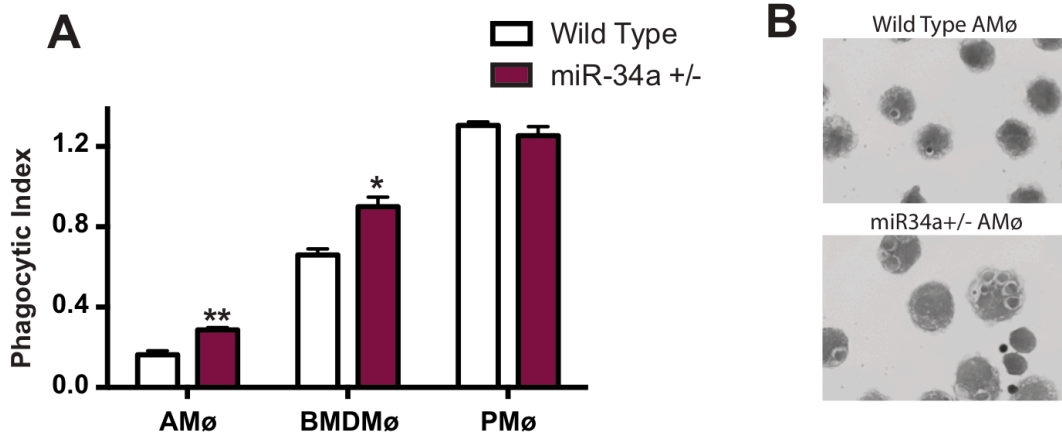
Figure 4.4. Alveolar macrophages and Bone Marrow-Derived Macrophages from miR-34a+/- mice have increased AC uptake. Mø were plated in chamber slides and 10:1 AC were added for 1.5 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. A. AC uptake by WT and miR-34a+/- AMø, BMDMø and PMø. B. Representative photos of AC engulfment by AMø. Data are mean ± SE of 3 mice of each genotype. *, statistically significant, $p < 0.05$ and **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
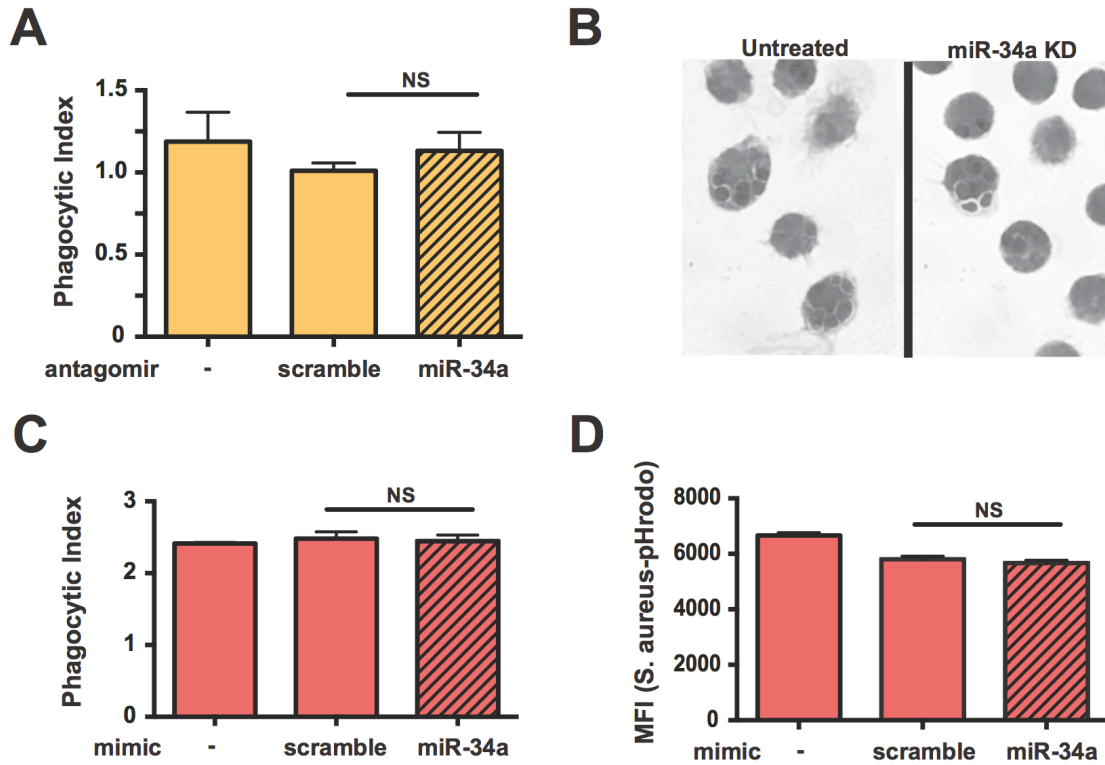
Figure 4.5. MiR-34a does not regulate Fc-mediated uptake. Mø were plated in chamber slides and transfected using RNAiMAX lipofectamine containing no construct (-), control scramble construct (scramble), or miR-34a antagomir/mimic (miR-34a). 24 h after transfection with antagomir or 48 h after transfection with mimic, opsonized targets were added. A-C. Ig-opsonized-sheep red blood cell (SRBC) uptake. 10:1 opsonized-SRBC were added for 1 h. Slides were washed and stained using H&E, then ingested SRBC were counted at 100X magnification under oil. A. Opsonized SRBC uptake by murine AMø. B. Representative photos of opsonized SRBC uptake by murine AMø. C. Opsonized SRBC uptake by murine PMø. D. Opsonized heat-killed Staphylococcus aureus uptake by murine PMø. Opsonized pHrodo-labeled *S. aureus* was added for 1h. Mø were harvested and internalized *S. aureus* was detected by flow cytometry. Data are mean ± SE of 3-6 mice assayed individually in two independent experiments. Significance was calculated by One-Way ANOVA with Bonferroni post-hoc testing.
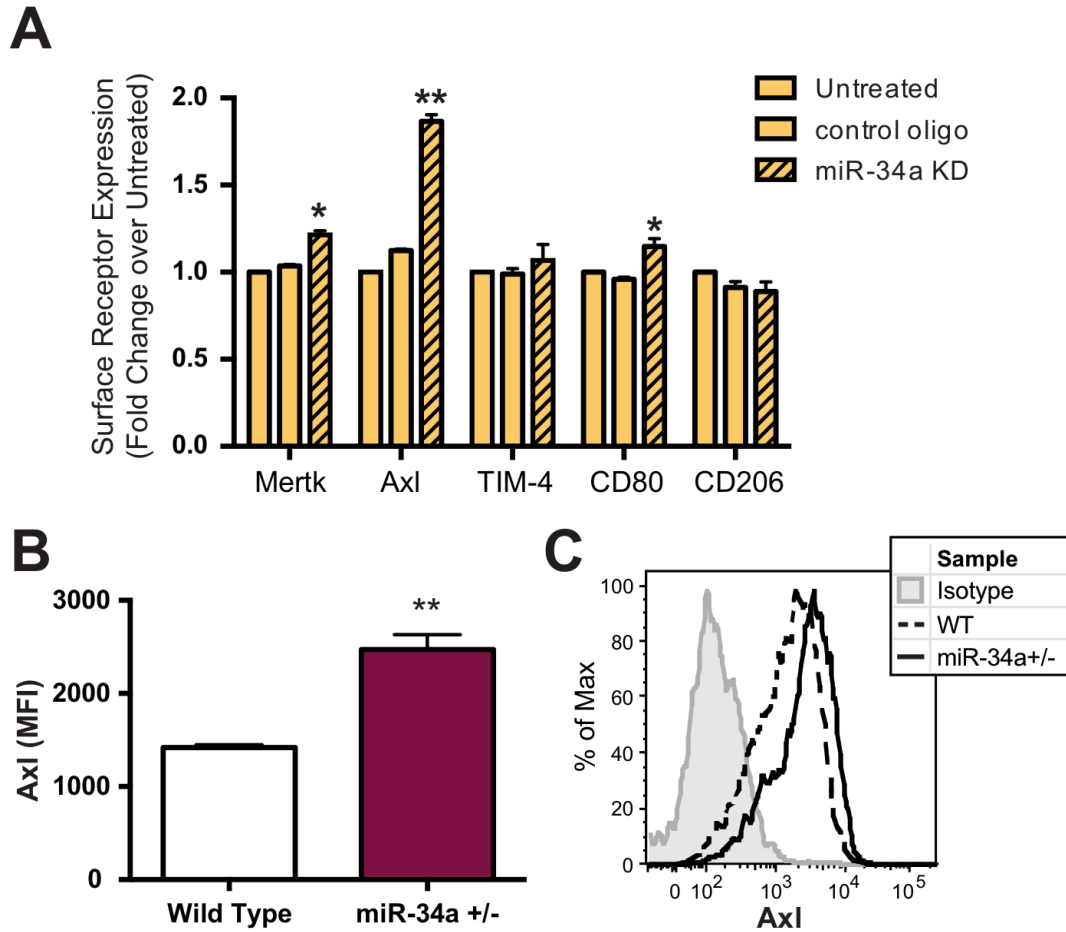
Figure 4.6. MiR-34a inhibits expression of target gene Axl and to a small extent, Mertk and CD80. A. AMø were transfected using RNAiMAX lipofectamine containing no construct (untreated), control scramble construct (control oligo), or miR-34a antagomir (miR-34a KD). 24 h after transfection, AMø were collected and stained for flow cytometry. Cells were gated on CD45+CD11c+ cells. Surface expression is shown as fold change above MFI of untreated. B,C. Surface Axl expression of BMDMø from WT and miR-34a+/- mice was measured by flow cytometry. Cells were gated on CD45+CD11c+ cells. B. Quantified change in Axl MFI. C. Representative histogram of Axl. Data are mean ± SE of 3-6 mice assayed individually in one-two independent experiments. *, statistically significant, $p < 0.05$ and **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
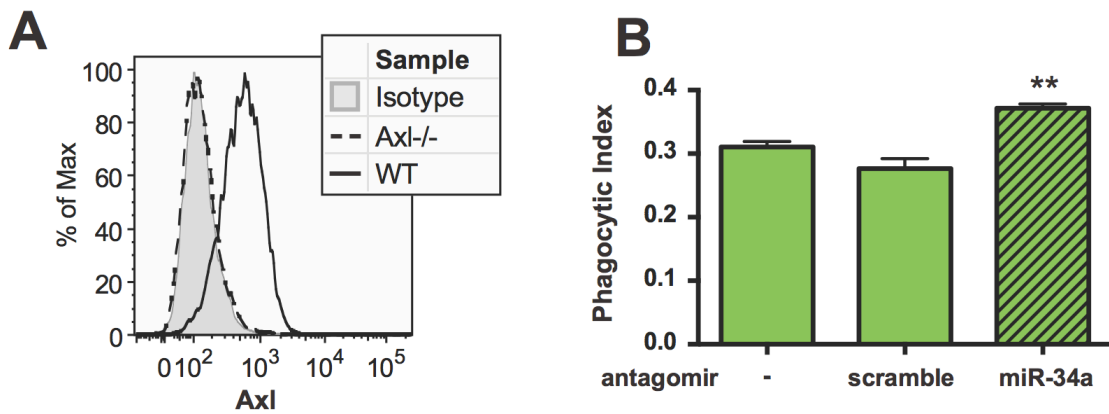
Figure 4.7. Axl downregulation is not required for the effect of miR-34a on AC uptake. Bone marrow from Axl-/- and WT mice was differentiated into BMDMø using GMCSF. A. Axl expression in BMDMø measured by flow cytometry, gated on CD45+ cells. B. AC uptake by Axl-/- BMDMø.   BMDMø from Axl-/- mice were plated in chamber slides and transfected using RNAiMAX lipofectamine containing no construct (-), control scramble construct (scramble), or miR-34a antagomir (miR-34a). 24 h after transfection, 10:1 AC were added for 1.5 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. Data are mean ± SE of BMDMø isolated from two mice and transfected in duplicate. **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
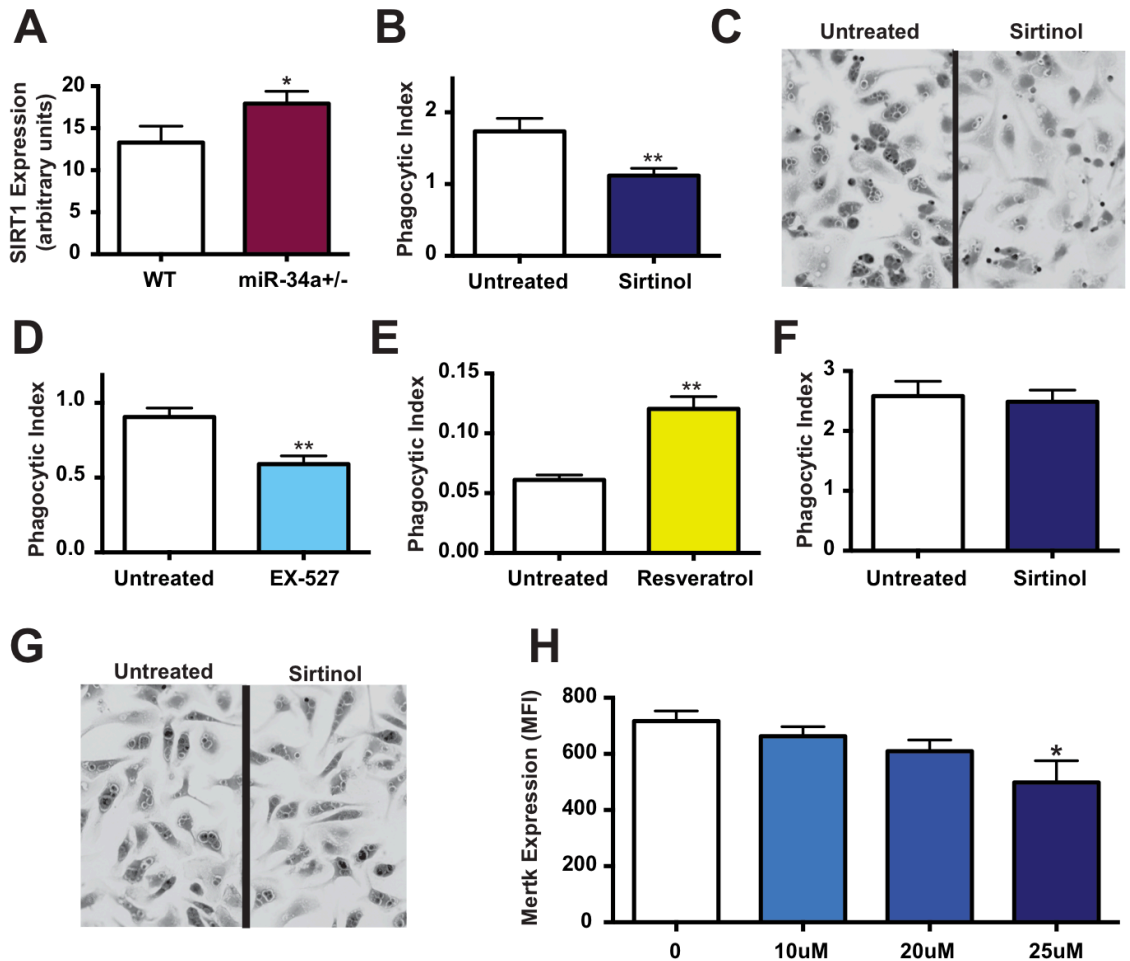
Figure 4.8. MiR-34a target SIRT1 is a novel regulator of AC uptake. A. SIRT1 expression in WT and miR-34a+/- AMø. SIRT1 levels were quantified by RT-PCR shown as arbitrary units SIRT1/GAPDH. B-E. AC uptake by Mø after treatment with SIRT1 agonists and antagonist. Mø were plated in chamber slides and 10:1 AC were added for 1.5 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. B. AC uptake by PMø after treatment with Sirtinol. C. Representative AC uptake by PMø after treatment with Sirtinol. D. AC uptake by PMø after treatment with EX-527. E. AC uptake by AMø after treatment with Resveratrol. F,G. Opsonized Sheep red blood cell (SRBC) uptake by PMø after treatment with Sirtinol. Mø were plated in chamber slides and 10:1 Opsonized-SRBC were added for 1 h. Slides were washed and stained using H&E, then ingested SRBC were counted at 100X magnification under oil. F. Quantified SRBC uptake. G. Representative SRBC uptake. H. Mertk expression on PMø following Sirtinol treatment. PMø were treating with increasing doses of Sirtinol for 24 h, then stained for flow cytometry. Mertk MFI is shown for cells gated on CD45+CD11b+. Data are mean ± SE of 5-6 mice assayed individually in two independent experiments. *, statistically significant, $p < 0.05$ and **, statistically significant, $p < 0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
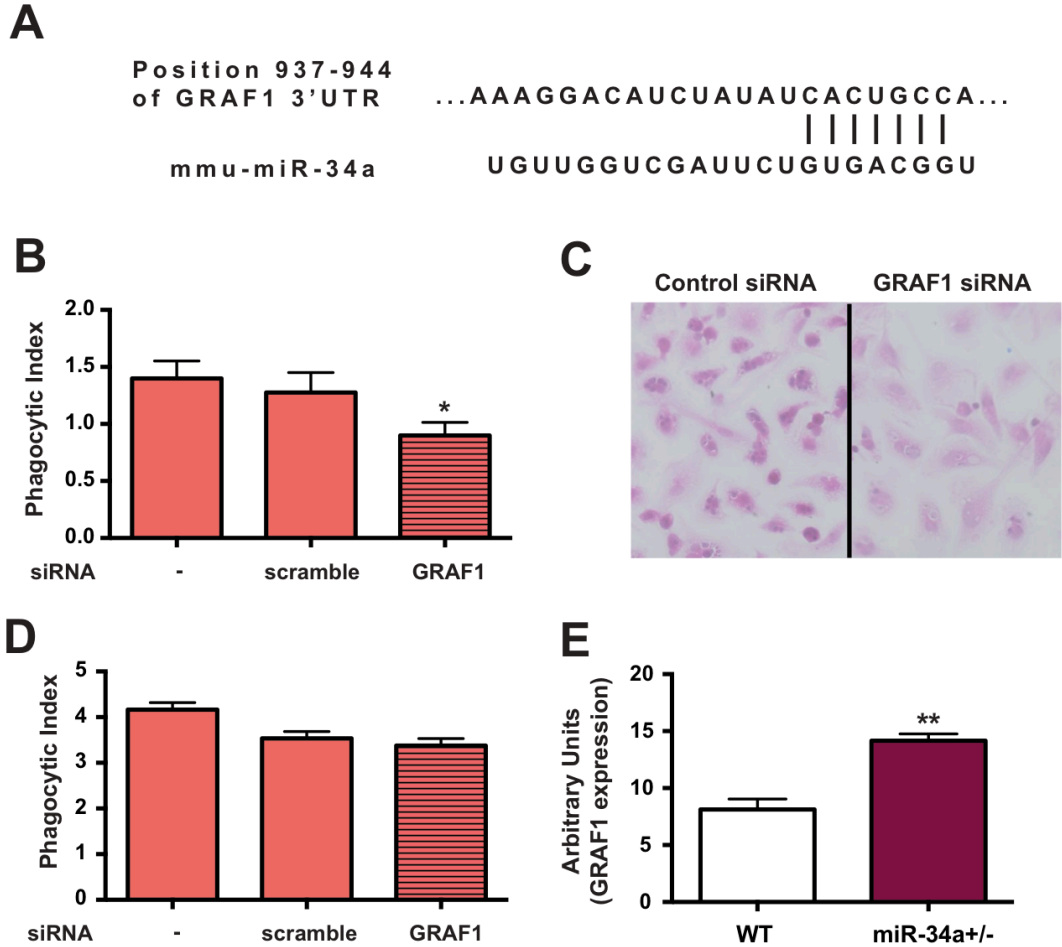
Figure 4.9. MiR-34a target GRAF1 is a novel regulator of AC uptake. A. Predicted binding site of miR-34a within the 3'-UTR of GRAF1. Targetscan 6.2 calculates a PCT of 0.9 for this interaction, indicating a high probability that this site is conserved due to selective maintenance of miRNA targeting. B,C. AC uptake by PMø following GRAF1 knockdown. PMø were transfected using RNAiMAX lipofectamine containing no construct (-), control scramble construct (scramble), or GRAF1 siRNA (GRAF1). 24 h after transfection, 10:1 AC were added for 1.5 h. Slides were washed and stained using H&E, then ingested AC were counted at 100X magnification under oil. C. Quantified AC uptake. D. Representative AC uptake. D. Opsonized Sheep red blood cell (SRBC) uptake by PMø following GRAF1 knockdown. PMø were transfected using RNAiMAX lipofectamine containing no construct (-), control scramble construct (scramble), or GRAF1 siRNA (GRAF1). 24 h after transfection, 10:1 opsonized SRBC were added for 1 h. Slides were washed and stained using H&E, then ingested SRBC were counted at 100X magnification under oil. E. GRAF1 expression in WT and miR-34a+/- AMø. GRAF1 levels quantified by RT-PCR, shown as arbitrary units GRAF1/GAPDH. Data are mean ± SE of 5-6 mice assayed individually in two independent experiments. *, statistically significant, $p<0.05$ and **, statistically significant, $p<0.01$ by One-Way ANOVA with Bonferroni post-hoc testing.
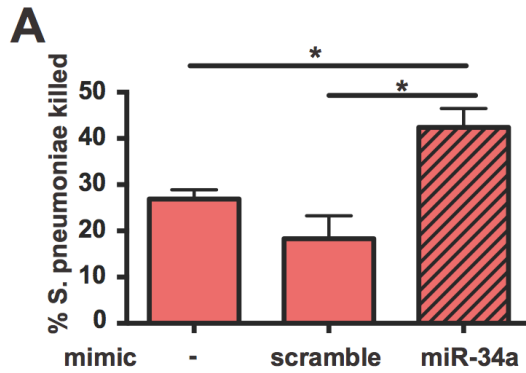
Figure 4.10. MiR-34a enhances killing of *Streptococcus pneumoniae*. PMø were plated in duplicate 96-well plates and transfected with RNAiMAX lipofectamine containing no construct (untreated), control scramble construct (control oligo), or miR-34a mimic (miR-34a). 24 h after transfection, live *S. pneumoniae* was added to each plate for 20 minutes. After washing to remove external bacteria, Mø from one plate were lysed and placed at 4C (T0). Mø from the second plate were incubated 2 h to allow killing, then lysed (T120). Both plates were returned to the incubator to allow bacterial replication. Bacteria was detected by MTT reaction and bacterial killing was calculated by determining Dbacteria between T0 and T120 plates. Cytochalasin D pre-treated wells were used as controls. Data shown is mean ± SE from a single experiment of three pooled mice performed with six replicate wells per condition. *, statistically significant, $p < 0.05$ by One-Way ANOVA with Bonferroni post-hoc testing.

Chapter 5

Discussion

Summary

The data we have presented demonstrate that AMø AC clearance is tightly controlled by negative regulation. Altering this negative regulation impacts immunity. In Chapter 2 we show that GC enhance AC clearance by AMø. In chapter 3, we go on to show that this augmented engulfment (GCAE) has a negative impact on immunity, decreasing TLR responses and the ability of AMø to kill *S. pneumoniae* in vitro and in vivo. In Chapter 4 we demonstrate that miR-34a, highly expressed in AMø, is a master-regulator of AC clearance. We identify two targets of miR-34a that are novel components of efferocytosis: SIRT1 and GRAF1. Further, independent of a role in regulating efferocytosis, miR-34a overexpression enhances killing of *S. pneumoniae* in vitro. Collectively, this work supports a reconsideration of the physiological roles of AMø, in which limited efferocytosis - rather than something to be "corrected" - is an important component of the AMø identity and is integral to maintaining appropriate responses to potential lung pathogens.

Reflections on therapeutic efferocytosis

We show that GC are a rapid, effective method to enhance efferocytosis by murine AMø and human AMø. Enhancing AC clearance in murine models has been

highly effective at reducing inflammation (103, 145-149), but it remains unclear how these results might translate to therapeutic interventions in human inflammatory disease. Unintentional enhanced efferocytosis likely occurs in many patients with chronic airways diseases as a result of the side effects of widely prescribed medications such as steroids (65, 169) and macrolide antibiotics (136, 137). However, no research has separated the direct anti-inflammatory or antimicrobial target effects of these agents from their pro-efferocytic side effect although it is known that efferocytosis causes significant changes to the anti-inflammatory and antimicrobial response (125). Whether enhanced efferocytosis contributes to improved clinical outcomes or unanticipated negative side effects following the use of steroids and macrolides is uncertain. Individuals with increased disease-associated cell death would be more susceptible to any positive or negative side effects caused by enhancing efferocytosis. Further study of this issue will allow for a better-informed clinical decision when prescribing pharmaceutical agents with the ability to enhance AC clearance.

Of particular concern is the documented potential for enhanced AC clearance to increase susceptibility to bacterial infection (108), the focus of Chapter 3. Our data, though preliminary, give credence to the hypothesis that enhanced AC clearance in the lungs of COPD patients as a result of ICS use contributes to the observed increase in community-acquired pneumonia (223-233): a potentially fatal infection.

Increased susceptibility to infection is a potential barrier for adapting any method of enhanced efferocytosis for human use. Additional concerns about therapeutic targeting of AC clearance stem from the observation that AC can induce both immunogenic and tolerogenic responses depending on the environmental context (12).

Both the context in which AC are engulfed and the phagocyte which engulfs them impact subsequent immunity (12, 13).

Murine models have been effective at harnessing the anti-inflammatory effects of AC clearance to induce a tolerogenic response in the lung to accelerate the resolution of various inflammatory insults (103, 145-149). It is a mark in favor of therapeutic efferocytosis that it is beneficial in murine models when initiated following the inflammatory insult and thus could be used as an intervention for patients already experiencing symptoms rather than a preventative measure. However, significant work is required before we can accurately predict the safety of such interventions in humans. Important issues to clarify include: identifying which phagocytes engulf administered AC and how to target AC to specific phagocytes; clarifying whether administration of AC at varying times following infection alters to efficacy of intervention, particularly whether intervention that is "too early" or "too late" either increases inflammation or favors immunogenic presentation of self-antigens; and testing whether susceptibility to secondary infection is increased. Another issue of interest is how the adaptive immune response will be altered. Cytokines produced following DC clearance of AC can polarize naïve T cells towards Th17 (424, 425). This may be beneficial, as Th17 improve IgA response and host defense (426, 427), but may also be detrimental, as Th17 cells enhance allergic airway inflammation (428-430). Thus, while therapeutic efferocytosis remains an attractive goal, further work is required to determine the long-term safety of enhancing a process that can cause susceptibility to lung infection and induce immunogenic responses.

Directed clearance: protection from autoimmunity and infection?

Cross-presentation of self-antigen has the well-described ability to induce context-dependent immunogenic and tolerogenic responses (12, 121). The factors by which the immune system compartmentalizes immunogenic versus tolerogenic responses to AC are poorly understood. The concept of directed AC clearance is a recent theory that suggests maintaining tolerance requires, in part, restricting the phagocytes involved in efferocytosis. In brief, the idea is that some phagocytes are not "meant" to clear AC; if these phagocytes clear AC they will cross-present self-antigen in an immunogenic manner and induce autoimmunity (124, 273). There is strong evidence to support that this is true for certain phagocyte populations, particularly in the 12/15-LO-/- mice (124). AC are rapidly engulfed in these mice, but this clearance is misdirected. AC are engulfed by inflammatory monocytes rather than resident Mø in the peritoneal space, these monocytes cross-present self-antigen and this leads to autoimmunity.

The concept of directed clearance is interesting for AMø. The double-layer of negative regulation through SIRPα and miR-34a suggests that low AMø AC uptake provides an evolutionary advantage. However, we suggest that the major advantage for AMø is an enhanced antimicrobial response rather than diminished auto-reactivity. Although preventing autoimmunity is clearly of evolutionary value, there is considerably more evolutionary pressure to fight childhood respiratory infections than to prevent autoimmunity. There is documented potential for efferocytosis to block crucial phagocyte defensive functions including bacterial killing by AMø (108). We observed a similar inhibition of host-defense by inducing GCAE. We hypothesize that low basal AC clearance in AMø protects the ability of AMø to respond efficiently to pathogens.

However, we do not rule out the possibility that low AC clearance in AMø also benefits the maintenance of self-tolerance. Although early publications reported that AMø were ineffective antigen presenting cells (214, 431), recent work has shown that AMø can efficiently cross-present antigens, although activation of naïve T cells is inhibited while AMø are within the lung environment (126, 432). However, the ability of AMø to present antigen to T cells may be irrelevant; AMø constitutively traffic antigen to the B-cell rich regions of draining lymph nodes rather than the T-cell rich zone (433). AMø may provide trafficked antigen to B-cells within the lymph node (433), a function that has been described for other Mø types (434, 435). Providing self-antigen in this manner could initiate an auto-reactive response; inhibiting clearance of AC by AMø may prevent subsequent induction of auto-reactivity.

Our data support the interpretation that directed clearance occurs in the lung involving SIRPα/SP-A/SP-D and miR-34a suppression of AC clearance by AMø. However, we suggest that directed AC clearance, particularly within the lung, is about more than segregating potential auto-antigens. We hypothesize that directed AC clearance protects certain phagocyte subsets from the immunomodulatory activities of AC in order to maintain efficient pathogen response and effective host defense.

SIRPα and MiR-34a: insights into the negative regulation of AC clearance

SIRPα/SP-A/SP-D and miR-34a are unique among known negative regulators of efferocytosis. Other inhibitory pathways predominantly function to discriminate live cells from dead, as in the case of homophilic CD31 binding and SIRPα recognition of CD47, and inhibit the engulfment of any bound live cells (63, 67). The function of

CD300a is less clear, as it actually recognizes phosphatidylethanolamine (PE) and PS, both of which are expressed on the surface of AC (66). However, PS can also be upregulated on viable cells during activation and differentiation (436-438); it is possible that CD300a acts as a safety mechanism to prevent clearance of live cells, similar to CD31 and SIRPα/CD47. The ITIM domain of CD300a may be sufficient to inhibit the positive engulfment signals sent by receptors such as TIM-4 and Mertk when only small amounts of PS are exposed. Importantly, CD300a, CD31 and SIRPα/CD47 all inhibit the engulfment of a specific bound cell (i.e. a viable cell), and thus their involvement in altering AC clearance is in segregating cargo rather than regulating the total capacity of a phagocyte for efferocytosis.

In contrast, SIRPα/SP-A/SP-D and miR-34a reduce basal efferocytosis; they are not involved in discrimination of cargo but rather function to regulate the efficiency of efferocytosis. This suggests two important points: 1) AMø, with active SIRPα/SP-A/SP-D signaling and high miR-34a, have intense negative regulation of efferocytosis and 2) both secretion of SP-A/SP-D and altered transcription of SIRPα and miR-34a are mechanisms by which efferocytosis can be dynamically regulated during infection and inflammation.

Is efferocytic capacity a dynamic state?

There is growing evidence that recruited Mø are polarized toward one of two distinct functional states by their environment: M1 (classic or inflammatory) or M2 (alternatively activated or pro-resolution) (316, 439-444). Although we find that resting AMø do not fall within the confines of either M1 or M2, the overall concept of dynamic

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polarization in Mø is interesting. Other groups have suggested that AMø can be polarized by factors including IFN-gamma, IL-4, IL-33, and PGE2 (445-447). However, based on the methods used for purification in these studies of inflamed lungs, "AMø" are often a mixture of cells that includes some tissue resident AMø, but is mainly populated by recruited Mø (448). As such, this data is not fully congruent with our work where we have studied true AMø from uninflamed lungs.

It remains unclear whether actual tissue resident AMø were polarized during lung inflammation or if the measured changes in M1/M2 markers reflect changes in the polarization of recruited monocyte-derived Mø. Recruited Mø outnumber AMø more than three to one during inflammation (64, 448) and recruited Mø phenotype and function is undoubtedly important in immunity. However, AMø are very long-lived cells and at the resolution of inflammation when recruited Mø have emigrated or died, the majority of AMø remain (448, 449). Thus, long after recruited Mø polarization becomes irrelevant, AMø polarization remains fantastically important in ongoing tissue repair and biasing subsequent immune responses. Understanding how tissue resident AMø may be polarized in phenotype and function is of extreme interest.

Although efferocytosis is not a common outcome measured in studies of Mø polarization, there has been a connection made between M2 Mø and a high capacity for AC engulfment (397-399). No evidence to support an M2-efferocytosis connection exists within the lung. In the lung, sterile inflammation through instillation of SRBC resulted in recruited Mø that engulfed AC poorly (130). However, inducing inflammation through the administration of LPS, a classic M1 stimuli, led to recruited Mø that could avidly engulf AC (64). Importantly, efferocytosis by resident AMø was also increased (64).

This finding suggests that repression of efferocytosis in AMø is dynamic and diminished in response to certain types of inflammation. Most interestingly, miR-34a is downregulated by LPS (353). Based on our data identifying miR-34a as a negative regulator of AC clearance, we suggest that downregulation of miR-34a may be one mechanism by which LPS can increase AC clearance. Changes in SP-A/SP-D and SIRPα following LPS exposure could also contribute to enhanced AC uptake by AMø. SP-A and SP-D are upregulated following LPS (450) and SIRPα expression is decreased (451). Thus, LPS likely downregulates both negative regulators within AMø. This supports the idea that AMø undergo a natural polarization during inflammation that involves downregulation of miR-34a and SIRPα to enhance AMø efferocytosis and favor the resolution of inflammation and tissue repair.

Although we suspect that similar regulatory models exist throughout the body, we find these data particularly interesting in terms of AMø. We suggest a model in which this shift relates to the waves of apoptosis that occur following lung inflammation as first neutrophils, then monocytes, then lymphocytes expand and contract (64, 448, 452-454) (**Fig. 5.1**). In early waves of cell death, AMø play a minor role in AC clearance (64); we hypothesize that over time, as miR-34a and SIRPα are downregulated, the role of AMø increases. As inflammation resolves, the signals that led to downregulation of miR-34a and SIRPα wane, miR-34a and SIRPα expression increases, AMø efferocytosis decreases, and the system returns to homeostasis. This would be congruent with other AMø functions; AMø are essential first-response cells to inhaled pathogens (455-457) and AC clearance too early in inflammation would suppress their ability to recruit inflammatory leukocytes and compromise their ability kill bacteria. However,

appropriate initiation of inflammation must be balanced against the deleterious effect of prolonged inflammation, particularly within delicate tissues such as the lung where inflammation inhibits gas exchange (458-460). Thus, it is likely advantageous for AMø to transiently enhance efferocytosis and assist in returning the alveolar space to homeostasis.

We hypothesize that enhanced AMø efferocytosis peaks following the first wave of neutrophil death and clearance. MiR-34a in hepatocellular carcinoma cells is downregulated by TGF-β (364), which would be released by other phagocytes following clearance of apoptotic neutrophils. We are interested in testing whether LPS and TGF-β inhibit miR-34a in AMø and other Mø subtypes and understanding whether decreased expression of miR-34a is responsible for the increased efferocytosis that has been observed following LPS (353) and TGF-β (364).

Future directions for understanding the role of miR-34a in AC clearance

Our data thoroughly demonstrate the ability of miR-34a to inhibit AC uptake. However, we suspect that we have only begun to identify the pathways through which miR-34a can impact Mø function. Even Axl, SIRT1 and GRAF1 – the three genes we have identified as downstream miR-34a targets that regulate efferocytosis – may be only three of many. CD44, which is not involved in the uptake of apoptotic thymocytes but can recognize apoptotic neutrophils (276), is another miR-34a target (461, 462). In addition, there is evidence that miR-21, the only other miRNA that has been connected to AC uptake (318), is regulated by miR-34a (367). Expression of CD44, miR-21 and the

miR-21 target PTEN should be measured in our miR-34a knockdown AMø or AMø from our miR-34a+/- mice.

We can also use in vitro and in vivo techniques to test our proposed model of dynamic regulation of efferocytosis by miR-34a AMø during inflammation. We should begin by measuring miR-34a expression in AMø after in vitro stimulation with LPS and TGF-β to confirm the previously published observation that these stimuli suppress miR-34a (353, 364). We can perform supernatant transfer experiments, collecting supernatants from Mø exposed to AC and transferring them to untreated AMø; we hypothesize these supernatants will suppress miR-34a. We could track resident AMø in vivo following LPS injury by labeling AMø with the florescent lipophilic dye PKH-26 (130, 463, 464) prior to instillation of LPS, allowing for discrimination of resident and recruited phagocytes by flow cytometry. AMø could then be flow sorted by PKH-26-positivity at various time points during inflammation and both miR-34a level and efferocytic capacity could be assessed. We would expect AMø to express the lowest amount of miR-34a and possess the highest ability for AC uptake following the apoptosis of neutrophils and concomitant release of TGF-β by inflammatory recruited Mø in response to clearing apoptotic neutrophils.

Future directions for studying the effects of AC clearance on bacterial infection

Over the course of this work we have developed the hypothesis that the unusually low rate of AC clearance by AMø is caused by evolutionary pressure of childhood respiratory infections selecting for active inhibition of AC clearance in order to favor AMø antimicrobial response. We theorize that high AC clearance by AMø would

prevent key initial bacterial clearance, resulting in lingering bacterial burden instead of resolution, supported by data involving intranasal administration of AC prior to *S. pneumoniae* infection (108). Our in vitro and in vivo studies of GCAE support this hypothesis and show that following co-exposure to GC and AC, bacterial killing is inhibited. Our GCAE studies along with other published in vitro assays (108) demonstrate that AC clearance inhibits AMø bactericidal capacity, however, it is unclear whether AMø are the primary phagocyte responsible for the in vivo defect in bacterial clearance. Importantly, bronchial epithelial cells – not AMø – perform the critical majority of AC clearance during allergic inflammation when exogenous AC are added to the lung (134). Deletion of Rac in epithelial cells was used to prevent AC clearance in this study. Such an approach is not conducive for the study of bacterial clearance as Rac is required for normal cell motility and phagocytosis of bacteria (465). Thus, other model systems are required to parse the in vivo effects of AC exposure to the different phagocyte subsets in terms of bactericidal capacity. Further, as normal AC clearance by AMø is already low, a model where AC clearance is enhanced would be superior to a model with further knockdown of efferocytoic function. Our miR-34a-flox/flox LysMcre mice, in which AC clearance by Mø is increased, provide a useful model of enhanced AC uptake with which to study the effects of AC clearance on host defense.

MiR-34a deletion under control of the LysM-cre promotor targets both Mø and granulocytes such as neutrophils (466, 467). There is also deletion in some dendritic cell compartments, but not in bronchial epithelial cells or in lymphocyte populations (466, 467). It will be important to establish whether miR-34a deletion in neutrophils and dendritic cells also enhances AC uptake in these subsets. This would not in any way

detract from the utility of the miR-34a-flox/flox LysMcre mice but would be important in interpreting the data as infection causes recruitment of neutrophils to the lung. Finding that efferocytosis by phagocytes other than Mø is regulated by miR-34a would further strengthen our finding that miR-34a acts as a master-regulator of AC clearance. The minority alveolar epithelial population of AE2 cells also express LysM (468-470). It is difficult to predict how deletion of miR-34a in these cells may be important; how AE2 cells may normally contribute to AC clearance and what functions miR-34a may have in AE2 cells has not been studied. If miR-34a affects the production of surfactant by AE2 cells this could strongly affect both host defense and AC clearance by AMø (57, 62, 64, 200). Comparing the surfactant of WT and miR-34a-/- mice will be important before beginning in vivo studies.

It is interesting that over-expression of miR-34a enhances in vitro killing of S. pneumoniae without the addition of AC. Based on this result, we expect that miR-34a+/- and miR-34a-/- mice will be more susceptible to bacterial infection than WT mice. We expect that this susceptibility will be exacerbated by the addition of AC prior to infection due to enhanced AC uptake by miR-34a+/- and miR-34a-/- AMø suppressing the capacity for bacterial killing. This would support our hypothesis that enhancing AC clearance will enhance susceptibility to bacterial infection.

It is possible that the ability of miR-34a to enhance bacterial killing will obscure the importance of enhanced efferocytosis on bacterial killing when miR-34a is deleted. However, we hypothesize that bacterial killing by miR-34a+/- and miR-34a-/- mice will be inhibited to a greater degree than WT mice by exposure to AC. If we are unable to parse the effects of miR-34a from the effects of enhanced efferocytosis on bacterial

killing, finding that miR-34a+/- and miR-34a-/- mice are more susceptible to bacterial infection would still provide anecdotal support for our hypothesis that AMø are biased away from efferocytic function to preserve host defense.

In addition to studying the effect of miR-34a-deletion on bacterial clearance, it would be interesting to see whether Mø or DC from miR-34a+/- mice have an increased ability to cross-present self-antigen and whether miR-34a+/- or miR-34a-/- mice show unsorted AC clearance in various tissues and models of inflammation, such as has been observed in 12/15-LO (124) and A20 (273) deficient mice. We hypothesize that miR-34a+/- mice will be simultaneously more susceptible to AC immunosuppression (due to increased AC uptake) and posses a greater ability to induce autoreactive immune responses (due to uptake of AC by unusual phagocytes capable of immunogenic cross-presentation). Thus, miR-34a+/- mice may provide an interesting model in which to study how the regulation of AC clearance, particularly through directed clearance, is a balance between autoimmunity and host defense.

Final thoughts

AC clearance is an essential biological function that dramatically shapes the immune system. Significant progress in the last twenty-five years has identified dozens of proteins involved in efferocytosis, many of which are conserved throughout eukaryotic organisms. However, questions remain, including how AC clearance impacts host defense and why engulfment capacity varies so dramatically between phagocyte populations such as AMø and PMø, both mechanistically and evolutionarily. The data presented within in thesis provides evidence that negative regulation, particularly by

miR-34a but also by SIRPα/SP-A/SP-D in the alveolar space, explains much of the varied engulfment capacity of Mø subtypes. Further, reversing negative regulation through pharmacologic or genetic means inhibits host defense against S. pneumonia, suggesting that the evolutionary pressure of preventing infection may drive the negative regulation of efferocytosis in many phagocytes, particularly AMø. Both miR-34a and SP-A/SP-D improve bacterial phagocytosis and killing independent of their inhibitory effect on AC clearance. Combined with the further inhibitory effect of AC clearance on bacterial killing, we hypothesize that AC clearance and bacterial killing, while both essential processes for human health, are interconnected and conflicting functions in Mø. The necessity of a strong host defense within the alveolar space selects for AMø with an avid capacity for bacterial killing at the expense of efficient AC clearance. How this bias may be dynamically regulated in response to infection and inflammation will be an important avenue of further study.
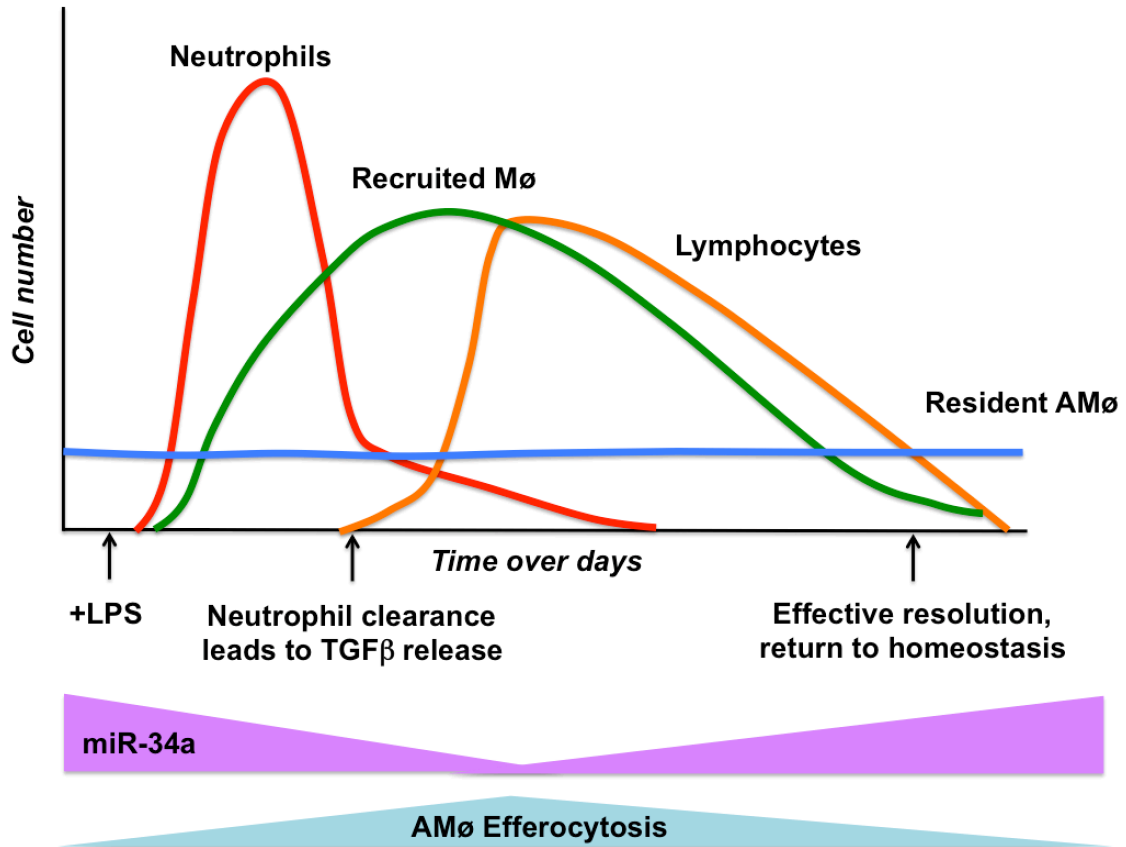
Figure 5.1 Hypothesis regarding SIRPα and miR-34a-driven regulation of dynamic AMø efferocytosis. In homeostasis, AMø efferocytosis is low and miR-34a expression is high. Upon LPS injury, miR-34a and SIRPα are downregulated in AMø. Simultaneously, a wave of recruited neutrophils enters the lung followed by a wave a recruited Mø. As the neutrophils die, their clearance leads to further depression of miR-34a in AMø by TGF-β signaling. Due to low miR-34a and SIRPα, AMø transiently increase their capacity for AC clearance; this assists with clearance of recruited Mø and lymphocytes. As inflammation resolves, the factors suppressing miR-34a and SIRPα abate and their expression returns, shutting off AMø efferocytosis and returning the system to homeostasis.

Chapter 6

Methods

Mice. For all experiments with wild type mice, C57BL/6 mice were purchased from Charles River Laboratories. Mice were housed under specific pathogen-free conditions and used for experiments between 8 and 16 weeks of age. For generation of miR-34a+/- mice, miR-34a flox/flox mice on C57BL/6 background (471) (Jackson) were crossed with LysM Cre mice (Jackson). The F1 generation of miR34a+/fl-LysMcre mice was genotyped following Jackson protocols and used for all experiments with non-littermate, age-matched C57BL/6 mice as wild-type controls.

Animal care and experimentation were conducted in accordance with U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the Animal Use Committee at VA Ann Arbor Healthsystem.

Medias. For most experiments, Mø were cultured in LCM: 10% FBS, 1 mM sodium pyruvate, 0.5 mM 2-Mercaptoethanol, 1 mM HEPES, 100 u/ml penicillin, 100 u/ml streptomycin, 0.292 mg/ml L-Glutamine in RPMI-1640 (GIBCO). For certain experiments, Mø were cultured in AIM-V (GIBCO) without serum. During transfection, reagents were diluted using OptiMEM (Invitrogen). To differentiate bone marrow-derived macrophages (BMDMø), bone marrow cells from the femurs of mice were

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cultured in LCM with 20% FBS: 20% FBS, 1 mM sodium pyruvate, 0.5 mM 2-Mercaptoethanol, 1 mM HEPES, 100 u/ml penicillin, 100 u/ml streptomycin, 0.292 mg/ml L-Glutamine in RPMI-1640 (GIBCO). GMCSF or MCSF was also added during bone marrow differentiation at 25 ng/mL. For microglia isolation and culture, cells were cultured in glia media: DMEM +10% FBS +1x L-Glutamine + Pen-strep + OPI (1vial/1L). GMCSF was also added to glia media at 5 ng/mL.

Isolation of primary macrophages. Alveolar cells were collected by bronchoalveolar lavage using 10 mL PBS containing 0.5 mM EDTA (65). AMø were adhesion purified from this population; non-adherent cells were discarded after 1.5 h of culture. Unstimulated peritoneal cells were isolated by peritoneal lavage using 7-10 mL PBS containing 0.5 mM EDTA, administered in 1-2 mL aliquots. PMø were adhesion purified from this population; non-adherent cells were discarded after 45 min of culture. All culture was performed in a 5% CO_2 environment at 37C.

Isolation of bone marrow derived macrophages and microglia. For BMDMø (472), fibulas and tibias were collected from 8-16 week old mice, skin and musculature was removed, and bones were disinfected, briefly, with ethanol. Bone marrow cells were collected by flushing bone marrow from bones with 10mL RPMI-1640/mouse using a 21 gauge needle. Bone marrow was disaggregated to a single cell suspension using the same 21 gauge needle and a 70uM strainer. Bone marrow from one mouse was resuspended in 125 mL LCM with 20% FBS. 25mL of bone marrow suspension was plated/15cm non-tissue culture treated petri dish. GMCSF or MCSF was added at 25 ng/mL. On day 4,

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10mL fresh LCM with 20% FBS was added to each plate as well as fresh GMCSF or MCSF at 25 ng/mL. On day 7, non-adherent cells were discarded and adherent cells (BMDM) were detached by incubating the dishes in cold PBS at 4C. BMDM were counted and plated in LCM as needed for experiments.

For microglia (473, 474), brains were collected from newborn pups, minced, trypsinized and filtered through a 70uM strainer to achieve a single cell suspension. Cells were plated in T150 flasks containing Glia media with 5ng/mL GMCSF. On day 7, media was aspirated to remove non-adherent cells and replaced with fresh Glia media with 5ng/mL GMCSF. On day 10, microglia were detached from adherent cells using overnight shaking at 200rpm; supernatants were collected, counted and plated in Glia media with 5ng/mL GMCSF as needed for experiments.

In vitro treatments. Fluticasone propionate, budesonide, azithromycin dihydrate, and simvastatin (Sigma) were all rehydrated according to the manufacturers' instructions and used at the concentrations described for each experiment. Simvastatin was activated before use by treatment with NaOH in ethanol (170). During treatments with these chemicals, Mø were cultured in AIM-V (GIBCO) without serum.

For certain adhesion assays, we treated AMø with mAb against anti-CD11c (HL3; Becton Dickinson Immunocytometry (BD), Mountain View, CA), anti-CD18 (GAME-46; BD), hamster IgG (eBioscience, San Diego, CA), or rat IgG (BD), all at 5 µg/mL final concentration, for 30 min before the addition of AC. For certain phagocytosis assays, AMø were treated with 2 µg/mL anti-CD36 (JC63.1; Cayman, Ann Arbor, MI) or mouse IgA (eBioscience) for 30 min before addition of AC. For other phagocytosis

assays, we pre-treated AMø with 5 µM cycloheximide (Sigma) for 1 h, then washed prior

to the addition of fluticasone, simvastatin or azithromycin. For some experiments, we

treated AMø with indomethacin (Sigma) for 30 min, then washed before addition of

fluticasone. For other experiments PMø were treated with 20 µM recombinant murine

SP-D (R&D, Minneapolis, MN) for 4 h, then washed prior to the addition of fluticasone.

During these treatments, Mø were cultured in LCM. For other experiments, PMø were

treated with 10 µM sirtinol (Cayman) or 10 µM EX-527 for 24 h before the addition of

AC or AMø were treated with 10 µM Resveratrol for 24 h before the addition of AC.

Following all treatments, Mø were washed with warm media before the addition of AC or

other targets in LCM.

Transient transfection of primary macrophages. AMø, PMø, microglia and BMDMø

were all transiently transfected using Lipofectamine RNAiMAX (Invitrogen) based on

manufacturer's instructions. Cells were adhesion purified at least 3 h for AMø and at

least 6 h for PMø, microglia, and BMDMø. RNAiMAX and siRNA/antagomir/mimic

were diluted separately in OptiMEM (Invitrogen). Diluted RNAiMAX and diluted

siRNA/antagomir/mimic were then combined at 1:1 with a final concentration of 1 µL

lipofectamine and 5 pmol siRNA/antagomir/mimic in 50 µL. The RNAiMAX/construct

complex was incubated at room temperature for 15 minutes. RNAiMAX/construct

complex was added to cells, 50 µL/well in chamber slides, 100 µL/well in 24-well plates.

At this point cells were incubated for five minutes, then 3x LCM was added to each well

for a final volume of 200 µL/well in chamber slides and 400 µL/well in 24-well plates.

Cells were incubated 24-48 h to complete transfection. Reagents used: miR-34a-5p

antagomir and negative control A (Exiqon), miR-34a-5p mimic or mimic negative control #1 (Invitrogen), GRAF1 siRNA B and medium GC negative control #2 (Invitrogen).

Preparation of phagocytic targets. For the production of apoptotic cells, in most experiments, we treated single cell suspensions of murine thymocytes with 10 μM dexamethasone (Sigma) for 4.5 h to induce apoptosis. These conditions consistently produced 50-60% Annexin+, PI- thymocytes. In selected experiments, thymocyte suspensions were UV-irradiated using a gel box (FOTO/UV 15, Fotodyne, Hartland, WI) on high power for 15 min, then were incubated a further 4 h to allow apoptosis to progress. SRBC (Colorado Serum Company, Denver, CO) were opsonized with anti-SRBC (Sigma) for 1 h (475). *Staphylococcus aureus* (Invitrogen) was opsonized with *S. aureus* opsonizing reagent (Invitrogen) or rat serum (Sigma) for 1h at 37C, vortexing every 10 minutes. Following induction of apoptosis or opsonization, apoptotic cells, opsonized SRBC, and opsonized bacteria were all washed with PBS.

Chamber slide adhesion and phagocytosis assays. Mø were plated at 1-2 x 10^5 cells/well in 8-well Permavox chamber slides (Nunc, Thermo Fisher Scientific). Target apoptotic cells or opsonized SRBC were added to Mø at 10:1 or 20:1 ratio for phagocytosis or 100:1 ratio for adhesion. Slides were collected by removing gaskets and washing in PBS after 20 minutes to remove unbound targets, or after 1-2 h to remove unengulfed targets. Slides were dried, then stained with H&E. To quantify phagocytosis and adhesion at least 200 macrophages were counted at 100x magnification.

TAMRA and pHrodo phagocytosis assays. For TAMRA-labeled apoptotic cells, 50 x 10^6 Apoptotic thymocytes were labeled with 75μg TAMRA (Invitrogen) in 1 mL PBS and 1mL LCM for 15 minutes at 37C (45, 383, 476). Labeled thymocytes were washed two times with PBS to remove excess TAMRA. *S. aureus* pre-labeled with pHrodo-green (Invitrogen) was opsonized as described above in preparation of phagocytic targets. Mø were plated at 3 x 10^6 cells/well in 24-well plates. Some Mø were pretreated with cytochalasin D (Sigma) for 1 h, prior to addition of targets, as a negative control. TAMRA-labeled AC or pHrodo-green labeled *S. aureus* targets were added to Mø for 1 h, then wells were washed with cold PBS to remove unengulfed targets. Mø were detached from culture dishes using TrypLE (Invitrogen) for 10 minutes followed by the addition of cold Marker Buffer and incubation at 4C for 15 minutes, then pelleted in flow tubes. Cells were either surface stained or run immediately on an LSRII Flow Cytometer.

Antibodies and Flow Cytometry. Cells were released from culture plates using the dissociation enzyme TrypLE (Invitrogen) and stained after Fc-block with a panel of fluorochrome-conjugated Abs. The following anti-murine Abs were used (clone; source): CD45 (30-F11; BD), TCRb (57-597; BD), CD19 (MCA1439F; AB Serotec), CD11c (N418; eBioscience), CD11b (M1/70; eBioscience), SIRPα (P84; BD), Axl (R&D), Mertk (R&D), CD80 (16-10A1; BioLegend), CD206 (C068C2; BioLegend), TIM-4 (RMT4-54; eBiosceince), CD4 (L3T4; eBioscience), CD8 (CD8b.2; Biolegend), Ly6G (1A8; BD), Ly6C (AL-21; BD).

Experiments were performed on an LSR II flow cytometer (BD Bio- science, San Jose, CA), equipped with the following lasers (numbers) and their associated filter sets

(letters): (1) 488 nm blue (primary laser), (a) 550 nm long-pass (LP), 530/30 nm short band-pass (SBP), (b) 685 nm LP, 695/ 40 nm SBP; (2) 405 nm violet laser, (c) 505 nm LP, 530/30 nm band-pass (BP), (d) 450/50 nm PB; (3) 633 nm red HeNe laser, (e) 735 nm LP, 780/60 nm SBP, (f) 685 nm LP, 710/50 nm SBP, (g) 660/20 nm BP; and (4) 561 nm yellow-green laser, (h) 735 nm LP, 780/60 SBP, (i) 685 nm LP 710/50 nm SBP, (j) 635 nm LP, 610/20 nm SBP, (k) 581/15 nm BP. In all experiments, we used isotype-matched controls and collected a minimum of 10,000 CD45$^+$ viable events per sample. Data were collected on an HP XW4300 Workstation (Hewlett-Packard, Palo Alto, CA) using FACSDiva software (version 6.1.3; BD Biosciences) with automatic compensation and were analyzed using FlowJo software (Tree Star, Ashland, OR) on an Intel iMac computer (Apple, Cupertino, CA).

RNA Isolation and RT-PCR. We isolated total RNA from murine AMø, PMø, BMDM and microglia using the RiboPure Kit (Ambion) and removed DNA contamination using the TURBO DNA-free kit (Ambion). cDNA was prepared from total RNA using the RETROscript kit (Ambion). For microRNA amplification, specific cDNA was prepared from total RNA using the TaqMan MicroRNA Reverse Transcription kit. All reagents and kits were used according to the manufacturer's instructions. For mRNA we performed real-time RT-PCR using TaqMan Gene Expression Master Mix with TaqMan primer-probe sets from Applied Biosystems for GAPD (4352932), Axl (Mm00437221_m1), Mertk (Mm00434920_m1), SIRPalpha (Mm00455928_m1), LRP (Mm00464608_m1) and PPARgamma (Mm00803184_m1). For miRNA we performed RT-PCR using TaqMan Gene Expression Master Mix with TaqMan primer-probe sets

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from Applied Biosystems for RNU6B (001093), sno142 (001231), miR-29c (000587), let-7i (002221), miR-34a (000426).

Secreted Cytokine Measurements. Cells were plated in 24-well plates and stimulated with 1ng/mL LPS or 50mg/mL PolyI:C in 300 μL LCM for 24 h. Cell culture supernatants were collected and cytokine profiles were measured by Luminex (Invitrogen) as per the manufacturer's instructions.

Streptococcus pneumoniae growth and CFU calculation. *S. pneumoniae* serotype 3, ATCC 6303 (American Type Culture Collection, Manassas, VA), was grown in Todd Hewitt Broth Media (BD) at 37°C in 5% CO_2. Bacteria were washed twice in PBS and the concentration of bacteria was obtained by measuring optical density (A_{600}) and confirmed by plating serially diluted bacteria on blood agar plates (Fisher). The virulence of this organism was maintained by culturing bacteria obtained from the spleens of mice rendered bacteremic 24 h after an intratracheal (i.t.) challenge with 10^6 CFU *S. pneumoniae* (288).

In vitro Streptococcus pneumoniae killing assay. Macrophages were plated in duplicate 96-well plates at 40,000 Mø/well. Following adhesion purification and desired treatments, 2x10^6 bacteria were added in 100 μL RPMI-/- and incubated for 20 minutes. The plates were washed. RPMI-/- was added to both plates. Saponin was additionally added to plate T0 to lyse Mø; plate T0 was stored at 4oC and plate T120 was returned to 37oC. After 2 h, saponin was added to plate T120 to lyse Mø. Both plates were

incubated a further 2 h to allow bacterial replication. MTT was then added to wells and

optical density was measured using a spectrophotometer. Values were plotted against a

previously determined standard curve to determine CFU. ΔCFU between the T0 and

T120 plates was calculated to determine killing (288, 477).

Intranasal administration of fluticasone and apoptotic cells. Mice were anesthetized

with inhaled Isoflorane. 50 µL of PBS alone or containing 0.1-10 µg fluticasone or 10^7

apoptotic thymocytes was instilled via intranasal administration. Mice were held at a 45-

degree angle during administration to favor aspirating over swallowing.

In vivo Streptococcus pneumoniae infection. Mice were anesthetized using Ketamine.

Fur was cleaned with 70% ethanol and iodine. A small incision was made in the throat,

forceps were used to move the vocal chords and access the trachea. 50,000 CFU of live

S. pneumoniae were injected in a 30 µL volume (288). The incision site was sealed using

3M Vetbond surgical glue (3M Animal Care Products). Mice were kept warm following

surgery to support post-operative recovery.

Statistical Analysis. We calculated significance using one-way ANOVA or two-way

ANOVA with Bonferroni post hoc testing or using Student t test where appropriate using

GraphPad Prism5 (La Jolla, CA) on an Intel iMac computer. Results were considered

significant* at p, 0.05 and highly significant** at p, 0.01

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