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Glucocorticoids Relieve Collectin-Driven Suppression of Apoptotic Cell Uptake in Murine Alveolar Macrophages through Downregulation of SIRP α

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The lung environment actively inhibits apoptotic cell (AC) uptake by alveolar macrophages (AM ϕ s) via lung collectin signaling through signal regulatory protein α (SIRP α). Even brief glucocorticoid (GC) treatment during maturation of human blood monocyte-derived or murine bone marrow-derived macrophages (M ϕ s) increases their AC uptake. Whether GCs similarly impact differentiated tissue M ϕ s and the mechanisms for this rapid response are unknown and important to define, given the widespread therapeutic use of inhaled GCs. We found that the GC fluticasone rapidly and dose-dependently increased AC uptake by murine AM ϕ s without a requirement for protein synthesis. Fluticasone rapidly suppressed AM ϕ expression of SIRP α mRNA and surface protein, and also activated a more delayed, translation-dependent upregulation of AC recognition receptors that was not required for the early increase in AC uptake. Consistent with a role for SIRP α suppression in rapid GC action, murine peritoneal M ϕ s that had not been exposed to lung collectins showed delayed, but not rapid, increase in AC uptake. However, pretreatment of peritoneal M ϕ s with the lung collectin surfactant protein D inhibited AC uptake, and fluticasone treatment rapidly reversed this inhibition. Thus, GCs act not only by upregulating AC recognition receptors during M ϕ maturation but also via a novel rapid downregulation of SIRP α expression by differentiated tissue M ϕ s. Release of AM ϕ s from inhibition of AC uptake by lung collectins may, in part, explain the beneficial role of inhaled GCs in inflammatory lung diseases, especially emphysema, in which there is both increased lung parenchymal cell apoptosis and defective AC uptake by AM ϕ s. *The Journal of Immunology*, 2012, 189: 112–119.

Apoptotic cell (AC) uptake by phagocytes, also termed efferocytosis (1), is an essential process that promotes the resolution of injury and inflammation, facilitating tissue repair in the lung and throughout the body (2). Impaired AC uptake has been found in phagocytes from human subjects with cystic fibrosis, asthma, and chronic obstructive pulmonary disease (COPD) (3–6). Because defective AC clearance clearly contributes to autoimmunity in murine models (7, 8), and because there is growing evidence that human emphysema may have an autoimmune component (9, 10), potential therapies designed to bolster AC clearance have been proposed (11). This issue is of considerable importance, because 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 (12).

In seeming contradiction to the importance of AC clearance, the resident lung phagocyte, alveolar macrophages (AM ϕ s), bind and engulf AC less avidly than do other professional phagocytes (13–15). Reduced efferocytosis by AM ϕ results, in part, from very restricted adhesion pathway usage and markedly decreased expression of PKC β II (15, 16). Importantly, however, the normal lung environment actively suppresses the ability of AM ϕ s to ingest AC, because of the inhibitory action of specific surfactant proteins, the lung collectins surfactant protein A (SP-A) and SP-D, through their interaction with signal regulatory protein α (SIRP α ; CD172a) (17). This inhibitory effect persists in vitro for days after AM ϕ removal from the lung environment. Whether increasing the ability of AM ϕ s to ingest AC would have beneficial health effects is unproved, but better understanding of how the unique mechanisms by AM ϕ interact with AC uptake is essential to guide the development of any such future therapies.

Several pharmacological treatments can increase AC uptake in vitro. Glucocorticoids (GCs) have been shown to increase in vitro AC uptake by human blood-derived monocytes, macrophage (M ϕ) cell lines, and, in a single report, human AM ϕ s (5, 18). In human blood-derived monocytes, this increase is dependent on Merck, increased Rac phosphorylation, and altered surface sialylation (19–21). It is unclear whether GCs act via these mechanisms in other cell types such as AM ϕ s. Defining whether and how GCs and other agents increase AC uptake by murine AM ϕ is an essential step to develop murine models to test whether manipulating AC clearance improves lung health.

In this study, we report that the potent GC fluticasone increased AC uptake by murine AM ϕ in a rapid, dose-dependent fashion through downregulation of SIRP α . Our data show a novel facet of

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Abbreviations used in this article: AC, apoptotic cell; AM ϕ , alveolar macrophage; BD, Becton Dickinson Immunocytometry; BP, band-pass; COPD, chronic obstructive pulmonary disease; GC, glucocorticoid; ICS, inhaled corticosteroid; LP, long-pass; M ϕ , macrophage; PM ϕ , peritoneal macrophage; SBP, short band-pass; SIRP α , signal regulatory protein α ; SP-A, surfactant protein A; SP-D, surfactant protein D.

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GC action: a rapid decrease in the sensitivity of murine AM ϕ s to the collectin-rich, inhibitory environment of the lung, thus lifting tonic inhibition and increasing AC uptake.

Materials and Methods

Mice

We purchased C57BL/6 mice from Charles River Laboratories. Mice were housed under specific pathogen-free conditions and used for experiments between 8 and 16 wk of age. 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 Veterans Affairs Ann Arbor Healthsystem.

Cell isolation and culture conditions

We isolated alveolar cells by bronchoalveolar lavage using 10 ml PBS containing 0.5 mM EDTA (14). AM ϕ s were adhesion purified from this population; nonadherent 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- to 2-ml aliquots. Peritoneal macrophages (PM ϕ s) were adhesion purified from this depleted population; nonadherent cells were discarded after 45 min of culture.

All culture was performed in a 5% CO₂ environment at 37°C. During adhesion purification, phagocytosis, and adhesion assays, M ϕ s were cultured in 10% FBS, 1 mM sodium pyruvate, 0.5 mM 2-ME, 1 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 0.292 mg/ml L-glutamine in RPMI. During all other treatments, M ϕ s were cultured in AIM-V (Life Technologies) without serum.

Induction of thymocyte apoptosis and SRBC opsonization

To induce apoptosis, in most experiments, we treated single-cell suspensions of murine thymocytes with 10 μ M dexamethasone (Sigma) for 4.5 h. These conditions consistently produced 50–60% Annexin⁺, PI⁺ thymocytes, as we have previously shown (22). In selected experiments, thymocyte suspensions were UV-irradiated using a gel box (FOTO/ultraviolet 15; Fotodyne, Hartland, WI) on high power for 15 min and 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, as previously described (22).

Phagocytosis and adhesion assays

We quantified AC phagocytosis and adhesion as previously described (14). In brief, M ϕ s were cultured in 8-well Permaxox chamber slides (Nunc, Thermo Fisher Scientific) at 10⁵ cells/well. We added target cells to M ϕ s at a ratio of 10:1 to measure phagocytosis after 2 h or at 100:1 to measure adhesion after 20 min. Cells were stained with H&E, and at least 200 M ϕ s were counted at \times 100 magnification.

Fluticasone propionate, budesonide, azithromycin dihydrate, and simvastatin (Sigma) were all rehydrated according to the manufacturers' instructions and used at the concentrations described. Simvastatin was activated before use by treatment with NaOH in ethanol as described previously (23).

For certain adhesion assays, we treated AM ϕ s with mAbs 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 μ g/ml final concentration, for 30 min before the addition of AC. For certain phagocytosis assays, AM ϕ s were treated with 2 μ g/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 pretreated AM ϕ s with 5 μ M cycloheximide (Sigma) for 1 h, then washed before the addition of fluticasone, simvastatin, or azithromycin. For some experiments, we treated AM ϕ s with indomethacin (Sigma) for 30 min and then washed before addition of fluticasone. For other experiments, PM ϕ s were treated with 20 μ M recombinant murine SP-D (R&D Systems, Minneapolis, MN) for 4 h and then washed before the addition of fluticasone. After all treatments, M ϕ s were washed with warm media before the addition of AC.

RNA isolation and real-time RT-PCR

We isolated total RNA from murine AM ϕ s and PM ϕ s 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). All reagents and kits were used according to the manufacturer's instructions. 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), SIRP α (Mm00455928_m1), LRP (Mm00464608_m1), and PPAR δ (Mm00803184_m1).

Flow cytometry

AM ϕ s were cultured in 48-well tissue culture plates at 10⁵ cells/well with 2 μ M fluticasone, 10 μ M simvastatin, 500 ng/ml azithromycin, or control media for 6–24 h. Cells were released from culture plates using the dissociation enzyme TrypLE (Invitrogen) and stained after Fc-block with a panel of fluorochrome-conjugated Abs as previously described (24). The following anti-murine Abs were used (clone; source): CD45 (30-F11; BD), TCR β (57-597; BD), CD19 (MCA1439F; AB Serotec), CD11c (N418; eBioscience), CD11b (M1/70; eBioscience), and SIRP α (P84; BD).

Experiments were performed on an LSR II flow cytometer (BD Bioscience, 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).

Statistical analyses

We calculated significance using one-way ANOVA with Bonferroni post hoc testing or using Student *t* test where appropriate using GraphPad Prism4 (La Jolla, CA) on an Intel iMac computer. Results were considered significant at *p* < 0.05.

Results

Potent GC rapidly increases murine AM ϕ uptake and binding of AC

To study the effect of GCs used clinically as inhaled corticosteroids (ICSs) on AC uptake by murine AM ϕ s, we first performed in vitro phagocytosis assays after treatment with the potent GC fluticasone (Fig. 1A, Supplemental Fig. 1A, 1B). Pretreatment with fluticasone significantly increased the ability of murine AM ϕ s to ingest AC after only 3 h, with peak effect by 6 h (Fig. 1B, 1C). The magnitude of the effect was dose responsive, increasing with higher doses of fluticasone; significance could be seen at 2 nM (Fig. 1D, 1E). Fluticasone treatment also increased AM ϕ uptake of UV-killed thymocytes (Supplemental Fig. 1C, 1D), implying that the effect did not depend on the method used to induce apoptosis. This profferocytic effect was not restricted to fluticasone, because increased AM ϕ AC uptake could also be seen after treatment with budesonide, another potent GC used clinically (percentage phagocytosis: untreated 16.5 \pm 3.4% versus budesonide 38.6 \pm 4.0%; phagocytic index: untreated 0.19 \pm 0.04 versus budesonide 0.51 \pm 0.09; mean \pm SE of 3 mice assayed individually in two independent experiments; both measurements significant, *p* < 0.05 by one-way ANOVA with Bonferroni post hoc testing). In contrast, AC uptake by resident murine PM ϕ s did not increase on fluticasone treatment (Supplemental Figs. 2A, 4B), even on treatment up to 6 h (data not shown). In addition, fluticasone did not increase Fc-mediated clearance of IgG-opsonized SRBC (Supplemental Figs. 2C, 4D) or of 4- μ m latex microspheres (data not shown) by murine AM ϕ s.

To study the effect of GCs on murine AM ϕ binding of AC, we next performed adhesion assays (Fig. 1F). Similar to the effect on AC engulfment, 4-h treatment with fluticasone significantly increased the ability of murine AM ϕ s to bind ACs, with the effect peaking by 6 h (Fig. 1G, 1H). The magnitude of the effect was

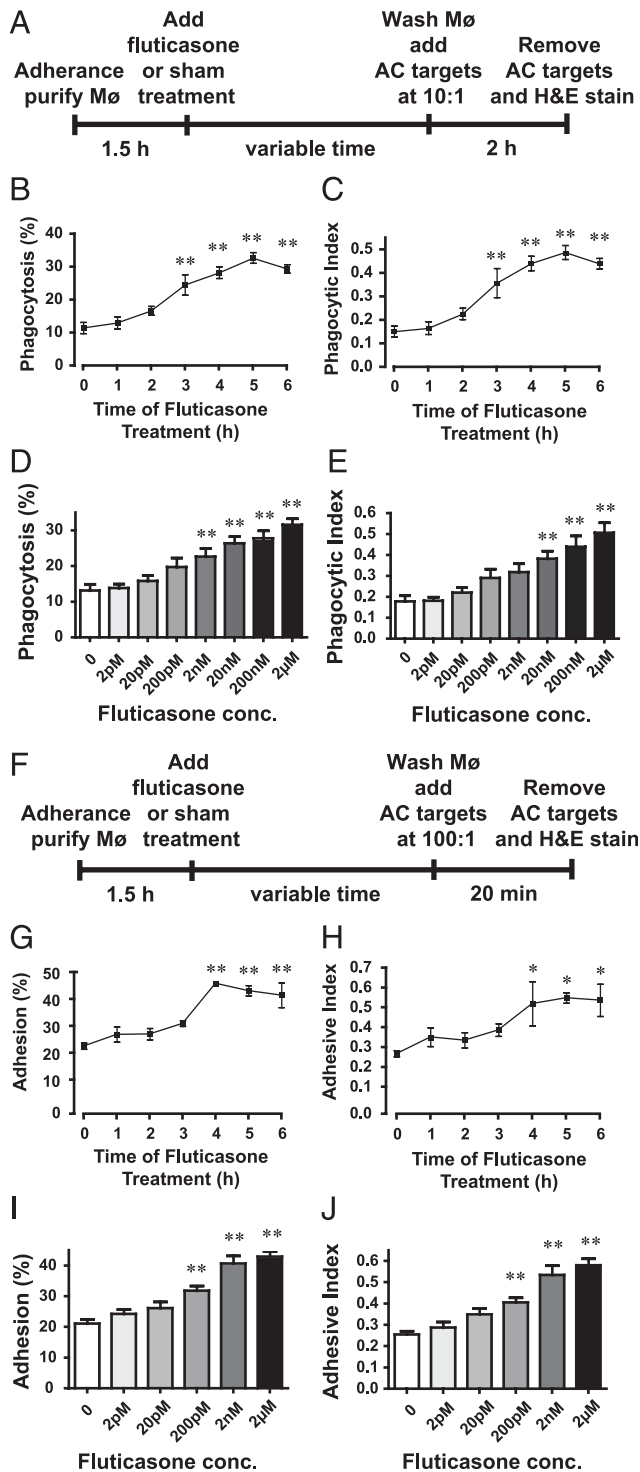


FIGURE 1. Fluticasone rapidly and specifically increases uptake and binding of AC by murine AM ϕ s. (**A–E**) AC uptake. Adherence-purified AM ϕ s from normal C57 BL/6 mice were treated in chamber slides with fluticasone (2 nM unless indicated) for 0–6 h; then ACs were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E; then ingested ACs were counted at $\times 100$ magnification under oil. (**A**) Graphic timeline of a phagocytosis assay. (**B** and **C**) Kinetics of GC-augmented AC uptake. (**D** and **E**) Dose response of GC-augmented AC uptake. (**F–J**) AC binding. Adherence-purified AM ϕ s from normal C57 BL/6 mice were treated in chamber slides with fluticasone (2 nM unless indicated) for 0–6 h; then ACs were added at a 100:1 ratio for 20 min. Slides were washed and stained using H&E; then surface-bound ACs were counted at $\times 100$ magnification under oil. (**F**) Graphic timeline of a binding assay. (**G** and **H**) Kinetics of GC-augmented AC binding. (**I** and **J**) Dose response of GC-augmented AC binding. Data are mean \pm SE of 5–8 mice assayed individ-

also dose responsive; significance could be seen at doses >200 pM (Fig. 1I, 1J). To determine whether fluticasone initiated novel adhesion pathways, we pretreated AM ϕ s with mAbs to block CD11c and CD18, which we have previously shown mediate the majority of adhesion of ACs to murine AM ϕ s (15). Blocking either integrin subunit reduced AM ϕ binding to ACs, regardless of treatment with fluticasone (Supplemental Fig. 3). In contrast, similar to the lack of effect on engulfment, fluticasone treatment did not increase PM ϕ binding to ACs regardless of fluticasone dose (2 pM to 2 μ M) or duration of treatment to 6 h (data not shown).

Thus, GC pretreatment is associated with rapidly increased AC binding and engulfment that is specific to AM ϕ s and not observed in a resting, fully differentiated tissue M ϕ from another mucosal surface. Furthermore, the ability to increase AC uptake appears to be a class effect of potent GC, which, however, does not alter phagocytosis by murine AM ϕ s of other types of particles.

Fluticasone initiates reprogramming toward a proclearance phenotype and increases AC uptake without a requirement for new protein synthesis

GCs alter expression of large numbers of target genes, for the most part via the specific GC receptor GR α , a member of the ligand-regulated family of nuclear receptors (25), but also by incompletely understood translation-independent mechanisms (26, 27). To begin to define how fluticasone upregulates murine AM ϕ uptake of ACs, 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 (28), CD91/LRP (29), and the negative regulator SIRP α (17). We also examined mRNA expression of the nuclear receptor PPAR δ , a positive regulator of the expression of opsonins involved in bridging ACs and of M ϕ surface receptors including Mertk (30). Within 3 h of fluticasone treatment, Mertk mRNA significantly increased, whereas SIRP α transcripts significantly decreased (Fig. 2A). These changes are consistent with an induction by GCs of proclearance AM ϕ phenotype, as previously described for human monocytes (31). Transcripts for Axl, LRP, and PPAR δ did not change during this period of fluticasone treatment.

These mRNA changes notwithstanding, the rapid kinetics of increased AC uptake in murine AM ϕ led us to postulate that fluticasone may act on a short-lived inhibitor. To test that possibility, we blocked new protein synthesis using cycloheximide. Treatment of AM ϕ s with cycloheximide before an additional 5-h fluticasone treatment did not abrogate the increase in AC uptake (Fig. 2B). Thus, although Mertk and likely other AC recognition molecules were significantly increased by fluticasone treatment, translation-dependent increases in Mertk or any other protein are not required for the rapid (<5 h) effect of fluticasone.

Fluticasone decreases protein expression of SIRP α

To test the significance of the observed fluticasone-induced gene repression of SIRP α (Fig. 2A), we examined protein expression of SIRP α . Using flow cytometry, we found that surface expression of SIRP α was decreased within 6 h of fluticasone treatment, with statistical significance reached by 24 h (Fig. 2C, 2D).

We also tested the involvement of several pathways that have been implicated in AC uptake by other types of tissue M ϕ s, using pharmacological inhibitors or blocking mAbs. Neither fluticasone-treated AM ϕ s nor, as we have previously described, untreated

usually in at least two independent experiments per condition. **Statistically significant for untreated, $p < 0.01$ by one-way ANOVA with Bonferroni post hoc testing.

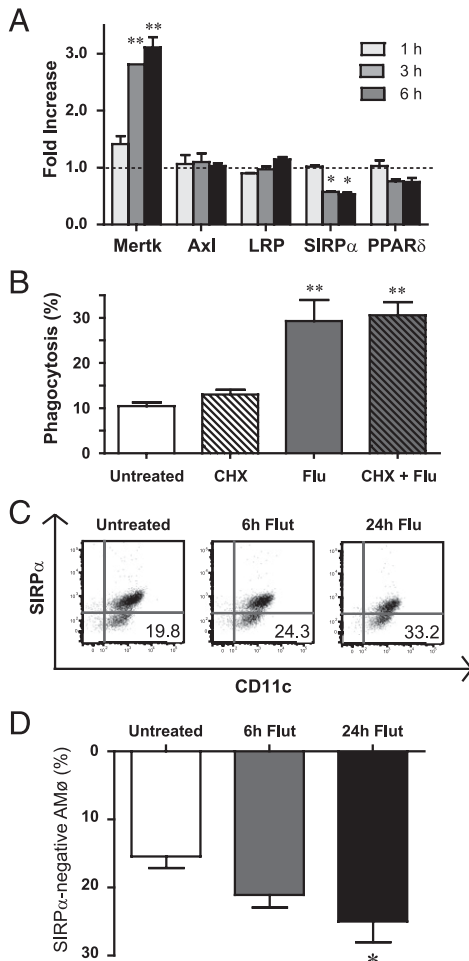


FIGURE 2. Fluticasone rapidly downregulates SIRP α and increases efferocytosis without a requirement for new protein synthesis. **(A)** Murine AM ϕ s were treated with 2 nM fluticasone for 0, 1, 3, or 6 h. RNA was collected at each time point and analyzed by real-time RT-PCR with GAPDH as the housekeeping gene; results are displayed as fold increase from untreated. **(B)** Murine AM ϕ s were pretreated with 5 μ M cycloheximide for 1 h followed by 2 μ M fluticasone for 5 h; then ACs were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E; then ingested ACs were counted at $\times 100$ magnification under oil. **(C and D)** Surface SIRP α protein. Murine AM ϕ s treated with 2 μ M fluticasone for 6 or 24 h, then analyzed by flow cytometry for surface expression of SIRP α . Cells shown are gated CD45⁺CD19⁻TCR β ⁻. **(C)** Representative dot plot. **(D)** Average percentage of CD11c⁺SIRP α ⁻ cells within gated CD11c⁺ population. Data are mean \pm SE of five to seven individual mice assayed individually in at least two independent experiments per condition. *Statistically significant from untreated, $p < 0.05$; **statistically significant from untreated, $p < 0.01$ by one-way ANOVA with Bonferroni post hoc testing.

murine AM ϕ s require CD36, α V integrin, or autocrine prostanoid signaling for AC uptake (Supplemental Fig. 4A–F). These results complement those in which we blocked CD11c and CD18 (Supplemental Fig. 3) 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 GCs, AC uptake is known to be increased by other commonly prescribed pharmaceuticals including statins and macrolides (32–34). To study interactions between these medications,

we treated murine AM ϕ s with combinations of fluticasone, simvastatin, and azithromycin, 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. 3A, 3B). By contrast, treatment of AM ϕ s with azithromycin and fluticasone was additive, resulting in near doubling of uptake capacity over either treatment alone (Fig. 3C, 3D).

Simvastatin affects AC uptake via the SIRP α 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 SIRP α signaling; because RhoA antagonizes the essential action of Rac-1 on AC

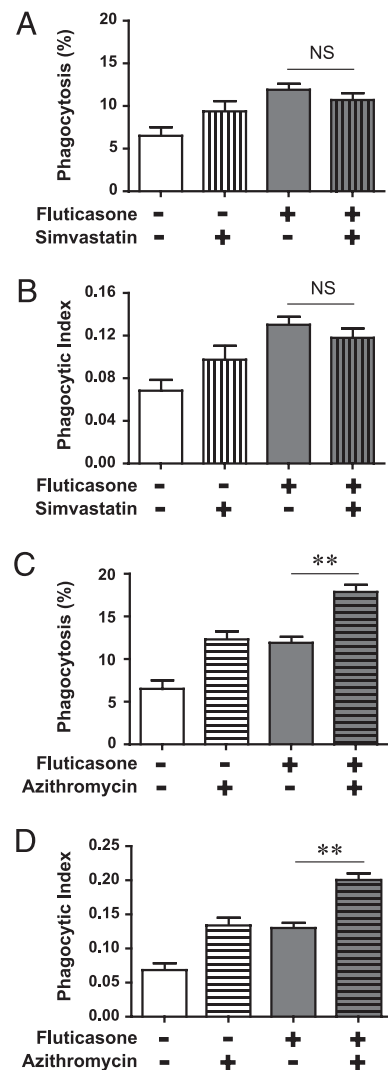


FIGURE 3. Azithromycin, but not simvastatin, has additive effects on efferocytosis by murine AM ϕ s. **(A–D)** Effect of multiagent treatment on efferocytosis. Murine AM ϕ s were treated with 500 ng/ml azithromycin, 10 μ M simvastatin, or media alone. After 18 h, 2 μ M fluticasone was added for a further 6 h; then ACs were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E; then ingested ACs were counted at $\times 100$ magnification under oil. **(A and B)** Simvastatin and fluticasone. **(C and D)** Azithromycin and fluticasone. Data are presented as the mean \pm SE of seven mice assayed individually in three independent experiments. **Statistically significant compared with fluticasone alone, $p < 0.01$ by one-way ANOVA with Bonferroni post hoc testing.

uptake, the net effect is increased efferocytosis (23). We used flow cytometry to test whether either simvastatin or azithromycin also affected SIRP α surface expression. Azithromycin did not change SIRP α expression compared with untreated AM ϕ , but simvastatin significantly decreased SIRP α surface expression after 24 h (Fig. 4A, 4B). However, in contrast with fluticasone, simvastatin did not change SIRP α 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 AM ϕ s with cycloheximide before 24 h of treatment with simvastatin or azithromycin blocked the ability of either agent to increase AC uptake over that of untreated AM ϕ s (Fig. 4C). These results indicate that, unlike fluticasone, both simvastatin and azithromycin do require new protein synthesis to increase AC uptake in AM ϕ s.

SP-D treatment inhibits AC uptake by PM ϕ , which is reversed with fluticasone treatment

The inhibitory effect of SIRP α on AC uptake by murine AM ϕ s is tonically maintained by constant exposure in the alveolar space to high concentrations of the lung collectins SP-A and SP-D (17). By contrast, although PM ϕ s express surface SIRP α (35), they receive limited exposure to lung collectins. These considerations led us to

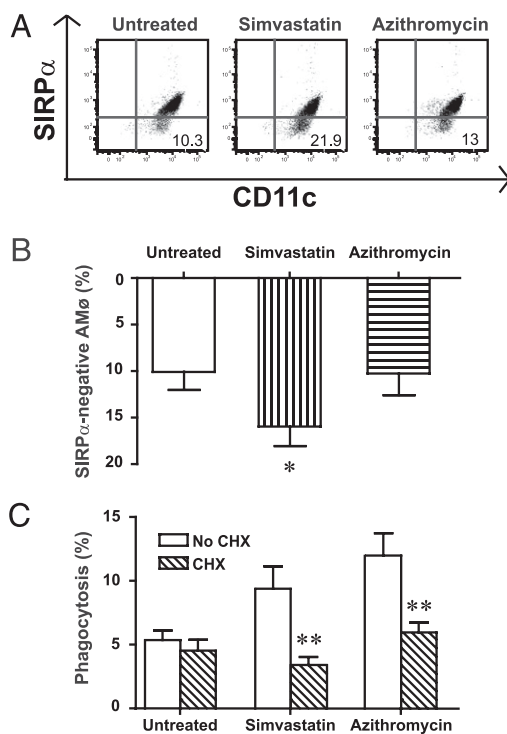


FIGURE 4. Simvastatin downregulates SIRP α expression, whereas azithromycin does not. **(A and B)** Surface SIRP α protein. Murine AM ϕ s treated with 10 μ M simvastatin or 500 ng/ml azithromycin for 24 h, then analyzed by flow cytometry for surface expression of SIRP α . Cells shown are gated CD45⁺CD19⁻TCR β ⁻. **(A)** Representative dot plot. **(B)** Average percentage of CD11c⁺SIRP α ⁻ cells within gated CD11c⁺ population. *Statistically significant from other conditions, $p < 0.05$ by one-way ANOVA with Bonferroni post hoc testing. **(C)** Murine AM ϕ s were pre-treated with 5 μ M cycloheximide (CHX) for 1 h followed by 10 μ M simvastatin or 500 ng/ml azithromycin for 24 h; then ACs were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E; then ingested ACs were counted at $\times 100$ magnification under oil. Data in **(B)** and **(C)** are mean \pm SE of five to seven mice assayed individually in at least two independent experiments. **Statistically significant from no cycloheximide, $p < 0.01$ by one-way ANOVA with Bonferroni post hoc testing.

hypothesize that the absence of GC-augmented AC uptake by PM ϕ (Supplemental Fig. 2A, 2B) might reflect limited activation of SIRP α in the peritoneal cavity, which unlike the alveolar spaces, does not contain significant concentrations of SP-A or SP-D. To test this possibility, we first used flow cytometry to test whether SIRP α expression on resident murine PM ϕ s was altered by fluticasone treatment in vitro. Similar to AM ϕ s, 24 h of fluticasone treatment significantly decreased PM ϕ expression of SIRP α surface protein, whether expressed as percentage positive relative to isotype control or mean fluorescence index (Fig. 5A–C). Next, by pre-incubating PM ϕ s with the SIRP α ligand SP-D, we investigated whether activation of SIRP α could repress AC uptake by murine PM ϕ s. SP-D significantly inhibited AC uptake by PM ϕ s within 4 h (Fig. 5D). Finally, we tested whether fluticasone treatment could rescue decreased PM ϕ AC uptake after SP-D treatment. Although treatment with SP-D alone again significantly inhibited AC uptake, subsequent incubation with fluticasone for 5 h completely reversed this inhibition (Fig. 5D). These results provide a proof-of-concept that the rapid effect of GC on AC uptake by tissue M ϕ s is mediated by release of collectin-induced repression acting via surface SIRP α expression (Fig. 6), and does not depend on GC modification of other features of the AM ϕ phenotype.

Discussion

The results of this study identify downregulation on AM ϕ s of the inhibitory receptor SIRP α , which releases them from tonic inhibition by lung collectins, as a novel mechanism by which clinically relevant potent GCs rapidly increase AM ϕ uptake of AC. Using primary murine AM ϕ s, we found that treatment with fluticasone or budesonide increased both binding and uptake of ACs within 2–3 h, without apparent induction of novel adhesive pathways. The effect did not require new protein synthesis, although its magnitude continued to increase through 5–6 h in association with significantly increased Mertk surface expression. Reduced SIRP α surface expression would be fully anticipated to prevent the previously described downstream activation of RhoA, and hence Rho kinase, to inhibit Rac (17), on which AC ingestion crucially depends (36, 37). Although fluticasone treatment of resting murine PM ϕ s did not show the same effect on AC uptake, brief treatment with SP-D induced a significant reduction in their AC uptake that was rapidly reversed by fluticasone. These findings emphasize the importance of the unique lung environment and, thus, more globally, of studying primary phagocytes isolated from mucosal surfaces in attempting to understand host defense of specific organs.

The well-described distinctive characteristics of resident AM ϕ s include a low capacity for AC binding and uptake (13–15). This feature may be of evolutionary value by preventing AC-induced immunosuppression, thus maintaining AM ϕ s as sentinel immune responders. Uptake of AC activates multiple anti-inflammatory pathways within phagocytes, notably through upregulation of SOCS1 and SOCS3, and subsequent inhibition of Jak-STAT signaling (38). The in vivo relevance of such AC-induced suppression on host defense has been shown in a murine model, in which intrapulmonary administration of AC reduced phagocytosis and killing of *Streptococcus pneumoniae*, and impaired leukocyte recruitment through PGE₂-EP2-dependent signaling (39). Conversely, the induction of lupus-like autoimmunity in mice by deletions of genes including C1q (40), MFG-E8 (41), α_v integrins (42, 43), and the TAM receptors (7, 44–46) argue for concurrent evolutionary pressures to fine-tune AC clearance.

By defining a rapid, translation-independent effect on fully differentiated tissue M ϕ s, these results extend previously described mechanisms of GC actions during M ϕ differentiation from precursors (18–21, 47, 48). In contrast with the early SIRP α -depen-

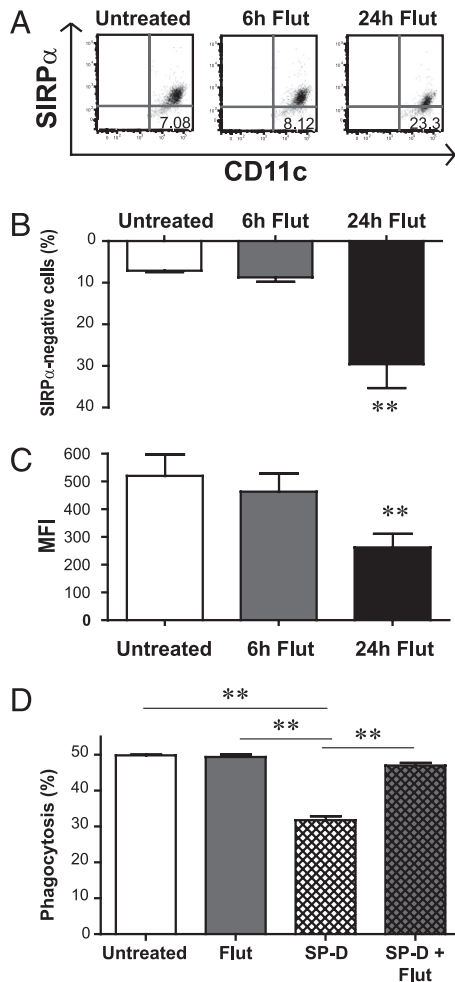


FIGURE 5. SP-D activates SIRP α pathway in PM ϕ s and makes PM ϕ s sensitive to fluticasone-driven increase in AC clearance. (**A–C**) Surface SIRP α protein. Murine PM ϕ s were treated with 2 μ M fluticasone for 6 or 24 h and then analyzed by flow cytometry for surface expression of SIRP α . Cells shown are gated CD45⁺CD19⁻TCR β ⁻. (**A**) Representative dot plot. (**B**) Average percentage of CD11b⁺SIRP α ⁻ cells within gated CD11b⁺ population. (**C**) Average mean fluorescence index of SIRP α on gated CD11b⁺ cells. (**D**) Fluticasone rescues SP-D inhibition of AC uptake. Murine PM ϕ s were treated with 25 μ g/ml SP-D for 4 h, followed by control media or 2 μ M fluticasone for 5 h; then ACs were added at a 10:1 ratio for 2 h. Slides were washed and stained using H&E; then ingested ACs were counted at $\times 100$ magnification under oil. Data are mean \pm SE of five to eight 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.

dent mechanism we show in mature AM ϕ s, results in those studies required new protein synthesis and more prolonged treatment, maximal when GC was added 3–5 d earlier. Thus, these studies were informative of the effects of systemic steroid treatments on M ϕ precursors, but not directly relevant to the question about how ICSSs might impact functions of resident AM ϕ s. Similarly, two groups have used microarray technology to define the effects of GC on gene regulation during in vitro differentiation of human monocytes (31, 49). 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 IFN regulatory family genes. Our finding of upregulation of Mertk transcripts is compatible with the initiation by GCs of such a more prolonged multigene program in AM ϕ s,

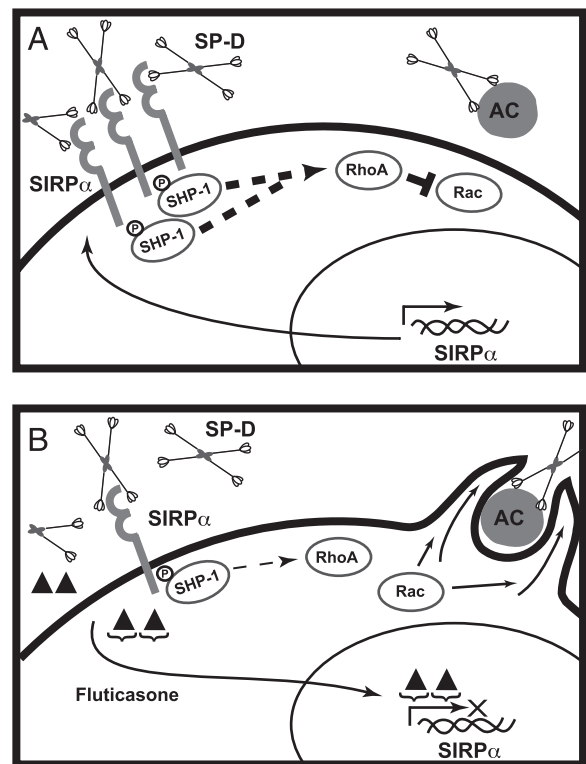


FIGURE 6. Model of GC regulation of SIRP α -mediated control of murine AM ϕ efferocytosis. (**A**) In untreated AM ϕ s, which express high amounts of SIRP α , lung collectins SP-D and SP-A (not shown) signal constitutively through SIRP α , activating SHP-1 and leading to downstream activation of RhoA. By inhibiting Rac-dependent mobilization of actin, the lung collectins tonically impede efficient uptake of ACs by AM ϕ s, even though SP-A and SP-D can also bind ACs. (**B**) Treatment with fluticasone reduces SIRP α surface expression, in part via transrepression of SIRP α by ligand-occupied GR α homodimers (brackets). The consequent decreased activation of SHP-1 relieves inhibition of Rac, permitting efficient AC uptake. Based on data in this study, in addition to previously published data (17, 36, 37, 50).

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 AM ϕ phenotype (17, 50), particularly in terms of AC uptake. We believe that this line of investigation highlights the ability for elegant control of AM ϕ function by altered expression of key receptors rather than by disruption of this fragile environment. SP-A and SP-D serve at least three functions in the alveolar space: modulating basal AM ϕ signaling in the absence of AC, binding directly to AC to increase their uptake, and as opsonins of multiple lung pathogens (51). Transgenic mice deficient in SP-A or SP-D have increased susceptibility to multiple viral, bacterial, and fungal infections (reviewed in Ref. 52). Deficiency of SP-D can also lead to chronic, low-grade pulmonary inflammation and fibrosis (53). We speculate that regulating SP-A and SP-D signaling by altering SIRP α expression on AM ϕ s, rather than directly by modulation of lung collectin levels, permits the continuation of other signaling and particularly opsonic functions of the lung collectins.

Increased AC uptake by inflammatory M ϕ in the alveolar spaces occurs in mice treated with intratracheal LPS (17) and has been shown in various phagocytes in vitro using a number of pharmacological agents including GCs, statins, and macrolides. To our knowledge, this is the first report to describe 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 (54). The mechanism for the positive effect of azithromycin on AC uptake remains undefined and will require considerable additional investigation; our results imply that azithromycin does not act on RhoA. Decreased AC uptake has been found in AM ϕ s from individuals with COPD (6) and asthma (5) when compared with healthy control subjects, 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 it 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 SIRP α on PM ϕ merits discussion in relation to acute lung injury, in which plasma concentrations of SP-A and SP-D increase significantly and correlate with clinical outcomes (55–57). 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 (58, 59). Although our results strongly imply that SIRP α signaling is not active in resident PM ϕ s harvested from untreated mice, they do suggest that increased circulating levels of lung collectins could contribute to reduced efferocytosis throughout the body during acute lung injury. Moreover, signaling via SIRP α also suppresses M ϕ phagocytosis mediated by Fc γ Rs and complement receptors (60, 61). 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 GCs affect AM ϕ s is particularly important as a result of the widespread prescribing of ICSs for the treatment of lung disease. Multiple clinical trials have noted that receiving ICSs is associated with increased hospitalization of COPD patients with pneumonia compared with COPD patients receiving nonsteroidal treatment, suggesting ICS treatment results in increased susceptibility to infection (62). In contrast, mice pretreated 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 (63). Our findings in murine AM ϕ s and previous findings in human AM ϕ s strongly suggest that GC treatment, by increasing AC uptake, will enhance AC-mediated immunosuppression of AM ϕ s. It would be interesting to test whether increased immunosuppression from ACs within the lung may explain these opposing results between COPD patients and model systems regarding ICS use and pneumonia, particularly for emphysema patients where lung destruction generates large numbers of ACs. Our finding that murine AM ϕ efferocytosis is increased after 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 GCs increase AC uptake by murine AM ϕ s. We provide evidence that this rapid increase is caused by disruption of

collectin-SIRP α signaling through downregulation of SIRP α transcript and surface protein, a novel GC mechanism. Finally, we demonstrate that regulation of AC uptake by SIRP α is not restricted to AM ϕ s and can be activated in PM ϕ s after exposure to SP-D.

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Disclosures

The authors have no financial conflicts of interest.

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