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

Alexandra L. McCubbrey,* Joanne Sonstein,† Theresa M. Ames,† Christine M. Freeman,†‡ and Jeffrey L. Curtis*†‡§

The lung environment actively inhibits apoptotic cell (AC) uptake by alveolar macrophages (AMøs) via lung collectin signaling through signal regulatory protein α (SIRPα). Even brief glucocorticoid (GC) treatment during maturation of human blood monocyte-derived or murine bone marrow-derived macrophages (Møs) increases their AC uptake. Whether GCs similarly impact differentiated tissue Møs 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 AMøs without a requirement for protein synthesis. Fluticasone rapidly suppressed AMø expression of SIRPα 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 SIRPα suppression in rapid GC action, murine peritoneal Møs that had not been exposed to lung collectins showed delayed, but not rapid, increase in AC uptake. However, pretreatment of peritoneal Møs 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 Mø maturation but also via a novel rapid downregulation of SIRPα expression by differentiated tissue Møs. Release of AMøs 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 AMøs. The Journal of Immunology, 2012, 189: 000–000.

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Abbreviations used in this article: AC, apoptotic cell; AMø, alveolar macrophage; BD, Becton Dickinson Immunocytometry; BP, band-pass; COPD, chronic obstructive pulmonary disease; GC, glucocorticoid; ICS, inhaled corticosteroid; LP, long-pass; Mø, macrophage; PMA, peritoneal macrophage; SBP, short band-pass; SIRPs, 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 AMs 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). AMs were adherence 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 (PMs) were adherence 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, Møs were cultured in 10% FBS, 1 mM sodium pyruvate, 0.5 mM 2-ME, 1 mM HEPES, and supplemented with penicillin, streptomycin, and L-glutamine.

During all other treatments, Møs were cultured in AIM-V (Life Technologies) without serum.

Phagocytosis and adhesion assays
We quantified AC phagocytosis and adhesion as previously described (14). For phagocytosis assays, AMs were treated with the potent GC fluticasone for 4.5 h. These conditions consistently produced 50–60% Annexin+, PI-AMo uptake of ACs, with the effect not dependent on the method used to induce apoptosis. This pro-efferocytic effect was not restricted to fluticasone, because increased AMo AC uptake could also be seen after treatment with budesonide, another potent GC used clinically (per-centage phagocytosis: untreated 16.5 ± 0.05, fluticasone 23.6 ± 0.40, budesonide 38.6 ± 0.40; phosphocytic index: untreated 0.19 ± 0.04 versus budesonide 0.51 ± 0.09; mean ± 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).

Results

Potent GC rapidly increases murine AMo uptake and binding of AC
To study the effect of GCs used clinically as inhaled corticosteroids (ICSs) on AC uptake by murine AMs, 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 AMs 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 AMo uptake of UV-killed thymocytes (Supplemental Fig. 1C, 1D), implying that the effect did not depend on the method used to induce apoptosis. This pro-efferocytic effect was not restricted to fluticasone, because increased AMo AC uptake could also be seen after treatment with budesonide, another potent GC used clinically (percentage phagocytosis: untreated 16.5 ± 0.3% versus budesonide 38.6 ± 0.4%; phosphocytic index: untreated 0.19 ± 0.04 versus budesonide 0.51 ± 0.09; mean ± 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 PMs did not increase on fluticasone treatment (Supplemental Figs. 2A, 2B), 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, 2D) or of 4-µm latex microspheres (data not shown) by murine AMs.

To study the effect of GCs on murine AMo 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 AMs to bind ACs, with the effect peaking by 6 h (Fig. 1G, 1H). The magnitude of the effect was
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 GRα, 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 AMø 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 SIRPα of the TAM family of receptor tyrosine kinases (28), CD91/LRP (29), and the negative regulator SIRPα (17). We also examined mRNA expression of the nuclear receptor PPARδ, a positive regulator of the expression of opsonins involved in bridging ACs and of Mø surface receptors including MerTK (30). Within 3 h of fluticasone treatment, MerTK mRNA significantly increased, whereas SIRPα transcripts significantly decreased (Fig. 2A). These changes are consistent with an induction by GCs of proclearance AMø phenotype, as previously described for human monocytes (31). Transcripts for Axl, LRP, and PPARδ did not change during this period of fluticasone treatment.

These mRNA changes not withstanding, the rapid kinetics of increased AC uptake in murine AMøs 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 AMøs 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 SIRPα

To test the significance of the observed fluticasone-induced gene repression of SIRPα (Fig. 2A), 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. 2C, 2D).

We also tested the involvement of several pathways that have been implicated in AC uptake by other types of tissue Møs, using pharmacological inhibitors or blocking mAbs. Neither fluticasone-treated AMøs nor, as we have previously described, untreated also dose responsive; significance could be seen at doses >200 pM (Fig. 1I, 1J). To determine whether fluticasone initiated novel adhesion pathways, we pretreated AMøs with mAbs to block CD11c and CD18, which we have previously shown mediate the majority of adhesion of ACs to murine AMøs (15). Blocking either integrin subunit reduced AMø 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 PMø binding to ACs regardless of fluticasone dose (2 pM to 2 μM) 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 AMøs and not observed in a resting, fully differentiated tissue Møs 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 AMøs of other types of particles.

Fluticasone rapidly and specifically increases uptake and binding of AC by murine AMøs. (A–E) AC uptake. Adherence-purified AMøs 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 ×100 magnification under oil. (F and G) 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 ± SE of 5–8 mice assayed individually in at least two independent experiments per condition. **Statistically significant for untreated, p < 0.01 by one-way ANOVA with Bonferroni post hoc testing.
murine AMøs require CD36, αV 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 effrocytosis by murine AMøs

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 AMøs 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 AMøs 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 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 Rac-1 on AC

![FIGURE 2. Fluticasone rapidly downregulates SIRPα and increases effrocytosis without a requirement for new protein synthesis.](http://www.jimmunol.org/)

(A) Murine AMøs 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 AMøs were pretreated with 5 μM cycloheximide for 1 h followed by 2 μM 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 ×100 magnification under oil. (C and D) Surface SIRPα protein. Murine AMøs treated with 2 μM fluticasone for 6 or 24 h, then analyzed by flow cytometry for surface expression of SIRPα. Cells shown are gated CD45+CD19− TCRβ−. (C) Representative dot plot. (D) Average percentage of CD11c−SIRPα− cells within gated CD11c+ population. Data are mean ± 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.

![FIGURE 3. Azithromycin, but not simvastatin, has additive effects on effrocytosis by murine AMøs.](http://www.jimmunol.org/)

(A–D) Effect of multiagent treatment on effrocytosis. Murine AMøs were treated with 500 ng/ml azithromycin, 10 μM simvastatin, or media alone. After 18 h, 2 μM 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 ×100 magnification under oil. (A and B) Simvastatin and fluticasone. (C and D) Azithromycin and fluticasone. Data are presented as the mean ± 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 SIRPα surface expression. Azithromycin did not change SIRPα expression compared with untreated AMs, but simvastatin significantly decreased SIRPα surface expression after 24 h (Fig. 4A, 4B). However, in contrast with 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 AMs 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 AMs (Fig. 4C). These results indicate that, unlike fluticasone, both simvastatin and azithromycin do require new protein synthesis to increase AC uptake in AMs.

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

The inhibitory effect of SIRPα on AC uptake by murine AMs is tonically maintained by constant exposure to high concentrations of the lung collectins SP-A and SP-D (17). By contrast, although PMs express surface SIRPα (35), they receive limited exposure to lung collectins. These considerations led us to hypothesize that the absence of GC-augmented AC uptake by PMs is due to tonic inhibition by lung collectins, as a novel mechanism by which clinically relevant potent GCs rapidly increase AMs uptake of AC. Using primary murine AMs, 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 SIRPα 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 PMs 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.

**Discussion**

The results of this study identify downregulation of AMs of the inhibitory receptor SIRPα, which releases them from tonic inhibition by lung collectins, as a novel mechanism by which clinically relevant potent GCs rapidly increase AMs uptake of AC. Using primary murine AMs, 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 SIRPα 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 PMs 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 AMs 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 AMs 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), αv 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 Mφs, these results extend previously described mechanisms of GC actions during Mφ differentiation from precursors (18–21, 47, 48). In contrast with the early SIRPα-depen-
dent mechanism we show in mature AMs, 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 Mø precursors, but not directly relevant to the question about how ICSs might impact functions of resident AMs. 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 AMs, 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 phenotype (17, 50), particularly in terms of AC uptake. We believe that this line of investigation highlights the ability for elegant control of AM 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 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 SIRPα expression on AMs, 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 Mø 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

![FIGURE 5.](http://www.jimmunol.org/content/data/6/steroids-relieve-suppressed-ac-uptake-by-murine-amos/figure5.jpg)

**FIGURE 5.** SP-D activates SIRPα pathway in PMøs and makes PMøs sensitive to fluticasone-driven increase in AC clearance. (A–C) Surface SIRPα protein. Murine PMøs were treated with 2 μM fluticasone for 6 or 24 h and then analyzed by flow cytometry for surface expression of SIRPα. Cells shown are gated CD45–CD19–TCRβ–. (A) Representative dot plot. (B) Average percentage of CD11b–SIRPα– cells within gated CD11b+ population. (C) Average mean fluorescence index of SIRPα on gated CD11b+ cells. (D) Fluticasone rescues SP-D inhibition of AC uptake. Murine PMøs were treated with 25 μg/ml SP-D for 4 h, followed by control media or 2 μM 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 ×100 magnification under oil. Data are mean ± 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.

![FIGURE 6.](http://www.jimmunol.org/content/data/6/steroids-relieve-suppressed-ac-uptake-by-murine-amos/figure6.jpg)

**FIGURE 6.** Model of GC regulation of SIRPα-mediated control of murine AMø efferocytosis. (A) In untreated AMøs, 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 ACs by AMøs, even though SP-A and SP-D can also bind ACs. (B) Treatment with fluticasone (triangles) reduces SIRPα surface expression, in part via transrepression of SIRPα by ligand-occupied GRα 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).

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 (17, 50), 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 functions in the alveolar space: modulating basal AMø 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 SIRPα expression on AMøs, 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 Mø 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 AMs 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 SIRPα on PMs 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). Although our results strongly imply that SIRPα signaling is not active in resident PMs harvested from untreated mice, they do suggest that increased circulating levels of lung collectins could contribute to reduced effectorcytosis throughout the body during acute lung injury. Moreover, signaling via SIRPα also suppresses Mψ phagocytosis mediated by FcγRs 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 AMs 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 AMs and previous findings in human AMs strongly suggest that GC treatment, by increasing AC uptake, will enhance AC-mediated immunosuppression of AMs. 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 AMs effectorcytosis 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 AMs. We provide evidence that this rapid increase 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 AMs and can be activated in PMs after exposure to SP-D.

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Disclosures

The authors have no financial conflicts of interest.

References


SUPPLEMENTAL FIGURE LEGENDS

**Supplemental Figure 1.** Fluticasone increases AC uptake by resident murine AMø. Adherence-purified AMø from normal C57 BL/6 mice were treated in chamber slides with control media or various doses of 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 then photographed or counted at 100X magnification under oil. A, B. Representative photomicrographs; A, control medium, B, fluticasone 2 µM; arrows denote ingested AC targetys. C, D. Apoptosis of murine thymocytes was induced by UV treatment, as described in Materials & Methods, rather than dexamethasone treatment. Data are mean ± SE of 8 mice assayed individually in three independent experiments per condition. *, statistically significant, p<0.05 and **, statistically significant, p<0.01 by One-Way ANOVA with Bonferroni post-hoc testing.

**Supplemental Figure 2.** Fluticasone does not rapidly increase AC uptake by PMø or Fc-mediated uptake by AMø. A, B. AC uptake by 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.

**Supplemental Figure 3.** Fluticasone does not alter AMø dependence on CD11c and CD18 for AC binding. Adherence-purified AMø from normal C57 BL/6 mice were 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, 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 6 mice assayed individually in two independent experiments per condition. One-Way ANOVA with Bonferroni post-hoc testing was performed to assess significance.

**Supplemental Figure 4.** Fluticasone increases AC uptake by murine AMø independent of CD36, alphaV integrin or autocrine prostanoid signaling. A, B. 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. C, D. AlphaV 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 RGDS or control peptide RGES for 30 min, then AC were added at a 10:1 ratio for 2 h. E, F. 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 mice assayed individually in at least two independent experiments per condition. One-Way ANOVA with Bonferroni post-hoc testing was performed to assess significance.
Supplemental Figure 2

A) Phagocytosis (%) for Alveolar and Peritoneal cells in the presence of different treatments.

B) Phagocytic Index for Alveolar and Peritoneal cells in the presence of different treatments.

C) Phagocytosis (%) for Untreated and Fluticasone treated cells.

D) Phagocytic Index for Untreated and Fluticasone treated cells.