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*J Immunol* published online 18 May 2015
http://www.jimmunol.org/content/early/2015/05/16/jimmunol.1402217

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/05/16/jimmunol.1402217.DCSupplemental

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Glucocorticoid-Augmented Efferocytosis Inhibits Pulmonary Pneumococcal Clearance in Mice by Reducing Alveolar Macrophage Bactericidal Function

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Inhaled corticosteroids (ICS) increase community-acquired pneumonia (CAP) incidence in patients with chronic obstructive pulmonary disease (COPD) by unknown mechanisms. Apoptosis is increased in the lungs of COPD patients. Uptake of apoptotic cells (ACs) (“efferocytosis”) by alveolar macrophages (AMøs) reduces their ability to combat microbes, including Streptococcus pneumoniae, the most common cause of CAP in COPD patients. Having shown that ICS significantly increase AMø efferocytosis, we hypothesized that this process, termed glucocorticoid-augmented efferocytosis, might explain the association of CAP with ICS therapy in COPD. To test this hypothesis, we studied the effects of fluticasone, AC, or both on AMøs of C57BL/6 mice in vitro and in an established model of pneumococcal pneumonia. Fluticasone plus AC significantly reduced TLR4-stimulated AMø IL-12 production, relative to either treatment alone, and decreased TNF-α, CCL3, CCL5, and keratinocyte-derived chemokontact/CXCL1, relative to AC. Mice treated with fluticasone plus AC before infection with viable pneumococci developed significantly more lung CFUs at 48 h. However, none of the pretreatments altered inflammatory cell recruitment to the lungs at 48 h postinfection, and fluticasone plus AC less markedly reduced in vitro mediator production to heat-killed pneumococci. Fluticasone plus AC significantly reduced in vitro AMø killing of pneumococci, relative to other conditions, in part by delaying phagolysosome acidification without affecting production of reactive oxygen or nitrogen species. These results support glucocorticoid-augmented efferocytosis as a potential explanation for the epidemiological association of ICS therapy of COPD patients with increased risk for CAP, and establish murine experimental models to dissect underlying molecular mechanisms.

The Journal of Immunology, 2015, 195: 000–000.

therapies with inhaled corticosteroids (ICS) is central to chronic obstructive pulmonary disease (COPD) management, but it is associated in this patient population with excess cases of community-acquired pneumonia (CAP) both in multicenter clinical trials and in analyses of administrative databases (1–10). Suggestions that this risk is balanced by reduced mortality in COPD patients admitted with CAP while using ICS (11, 12) have been contested (13–15). Defining the molecular basis of this epidemiological association could lead to more precisely personalized therapies and better outcomes in COPD, currently the third leading cause of death in the United States (16).

In COPD, as in the general population, for almost three decades the organism most commonly identified in CAP has been Streptococcus pneumoniae, also known colloquially as pneumococcus (17–19). It might seem intuitively obvious that the immunosuppressive properties of ICS should increase pneumonia frequency. However, in the sole study using a murine model, glucocorticoids (GCs) alone actually reduced lung burden of pneumococcus (20). In addition, ICS therapy is extremely prevalent in asthma, but most, but not all, studies have shown no similar increased risk for CAP in asthmatics (21–24). These findings suggest that additional factors may underlie the association of increased CAP risk and ICS therapy in COPD.

Apoptotic cells (ACs) are increased in the lungs of COPD patients (25–29). Uptake of ACs, also known as efferocytosis, is a complex, incompletely understood process that is relevant to many lung diseases (30). AC uptake by alveolar macrophages (AMøs) is lower than by Møs from other organs, and is further reduced by smoking and in COPD (29, 31–37). This lung-specific suppression of AC uptake is mediated, in part, by interactions between the lung collects surfactant proteins A (SP-A) and D (SP-D) and the inhibitory Mø receptor signal regulatory protein-α (SIRPα; CD172A) (38). The reduced basal efferocytic capacity of...
Efferocytosis on host defense have been shown in vivo (39), including in a murine model of pneumococcal pneumonia (44).

We previously reported that clinically relevant doses of the potent GC fluticasone increase in vitro AC uptake by murine AMøs, via both a rapid mechanism dependent, in part, on downregulation of SIRPα and a delayed yet sustained mechanism dependent on protein synthesis (45). We term this change GC-Augmented efferocytosis (GCAE). The purpose of this study was to determine whether GCAE alters host defense against pneumococcus. Early host responses to pneumococci depend crucially on AMøs (46–48), yet relatively little is known about how AMøs responses to pneumococcus are impacted by GCs (49). Using murine AMøs analyzed in vitro and an established mouse model of pneumococcal pneumonia, we demonstrate a potent detrimental effect on AMø antimicrobial function when GC therapy is used in the presence of alveolar ACs.

Materials and Methods
Mice
C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions in the Animal Care Facility at the VA Ann Arbor Healthcare System, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were fed standard animal chow (rodent laboratory chow 5008; Purina, St. Louis, MO) and chlorinated tap water ad libitum, and were used for experiments between 8 and 16 wk of age. Animal care and experimentation were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed.) and were approved by the Ann Arbor VA Healthcare System Subcommittee on Animal Studies.

Pneumococcus cultivation
*S. pneumoniae* serotype 3 (clone 6303) stock culture was obtained from American Type Culture Collection (Manassas, VA). Stocks of bacteria were immediately thawed for 30 s at 37°C and cultured in 5 ml Todd Hewitt (TH) broth supplemented with 0.5% yeast extract for 3 h at 37°C and 5% CO₂. Bacterial stock was frozen in 1 ml TH broth with 10% glycerol (Sigma Aldrich, St. Louis, MO) and used for subsequent in vitro studies. Bacterial CFU counts were verified both by OD at 600 nm and by quantitative culture on SRBC/tryptose agar (Fisher Scientific, Pittsburgh, PA). To generate heat-killed *S. pneumoniae* for use in vitro, we incubated bacteria in a water bath at 56°C for 60 min. No live bacteria were detected after plating onto agar plates.

To maintain bacterial virulence for in vivo experiments, we first passaged *S. pneumoniae* serotype 3 in vivo, using our established murine pneumococcal pneumonia model (50). Untreated C57BL/6 mice received an intratracheal (IT) inoculum using the surgical technique described later, at a dose (1 × 10⁶ CFU) designed to induce bacteremia. After 24 h, mice were euthanized; spleens were harvested aseptically and processed to isolate multiple individual pneumococcal clones on blood agar plates. These clones were expanded once in TH broth and then frozen. In all subsequent in vitro experiments, these in vivo passaged pneumococcal clones were defrosted, expanded once in TH broth, and used immediately without further passage on agar plates.

Induction of thymocyte apoptosis and quantification of efferocytosis
To induce apoptosis, we treated single-cell suspensions of murine thymocytes with 10 μM dexamethasone (Sigma), as we have previously shown (51). Efferocytosis was quantified using a chamber slide-based microscopic assay, as previously described (32). Data are expressed as percentage efferocytosis, based on the number of AMøs ingesting at least one AC, and the efferocytic index, which was generated by dividing the total number of ingested AC cells by the total number of AMøs counted.

AMø isolation and culture
Murine AMøs were isolated by bronchoalveolar lavage (BAL) using 10–15 ml PBS containing 0.5 mM EDTA in 1 ml aliquots (45). BAL cells were plated in lymphocyte culture media (LCM; 10% FBS, 1 mM sodium pyruvate, 0.5 mM 2-ME, 1 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 0.292 μg/ml gentamicin in RPMI-5) for 1.5 h at 37°C and 5% CO₂, and AMøs were adhesion purified from this population, by discarding nonadherent cells. For in vitro stimulation studies, AMøs were treated with one of four conditions: media alone, 2 μl fluticasone for 3 h, ACs (at a ratio of 10 ACs/AMøs) for 2 h, or 2 μl fluticasone for 3 h followed by ACs for 2 h (Flu + AC). Without washing, LPS from *Escherichia coli* K12 (1 mg/ml; Invivogen, San Diego, CA) or heat-killed *S. pneumoniae* at a multiplicity of infection (MOI) of 10 or 100, was added for an additional 24 h. Supernatants were collected and stored at −20°C until assayed by Luminex.

Protein analysis of supernatants
We used the Luminex 200 system (Luminex Corporation, Austin, TX) running StarStation Software (Applied Cytometry, Dinnington, Sheffield, U.K.) according to manufacturer’s instructions to determine protein levels for TNF-α, IL-1β, IL-6, IL-12, CCL3, CCL5, and keratinocyte-derived chemokuant (KC)/CXCL1 (Life Technologies, Grand Island, NY).

GCAE in vivo model
For the GCAE model, mice were administered saline, fluticasone, AC, or fluticasone plus AC via the intranasal (IN) route. All mice received two IN administrations, given 4 h apart, with one of the following: saline + saline, fluticasone + saline, saline + AC, or fluticasone + AC. The dose of fluticasone varied between experiments, ranging from 100 to 10 μg; 1 × 10⁷ ACs/mouse was used in all experiments. To deliver the reagents, we anesthetized mice with isoflurane via the open-drop method and then they were held with their heads elevated. Saline, fluticasone, or AC was delivered via one nostril in a volume of 30 μl. Mice were held in the upright position for an additional 60 s after IN administration before being returned to their cages.

In vivo pneumococcal pneumonia model
At 24 h after the last IN treatment, mice were anesthetized with an i.p. injection of ketamine/xylazine at 90 and 10 mg/kg, respectively. The plane of anesthesia was assessed by lack of response to toe pinch, and mice were positioned supine on a surgical platform elevated to a 45-degree angle. To allow visualization of the trachea, a small midline skin incision was made and the neck muscles were retracted. Using a 26-gauge needle, we injected *S. pneumoniae* into the trachea (20 μl PBS containing 50,000 CFU followed by 0.1 ml air to assure deposition in the lungs). Mice were allowed to recover fully on a water-jacketed heating pad and were returned to BSL2 housing until euthanasia 48 h later by exsanguination and induction of bilateral pneumothoraces under deep anesthesia.

Flow cytometry
The pulmonary vasculature was perfused via right-heart injection of PBS until the effluent was clear; then the lungs were excised and mechanically disaggregated without enzyme treatments, which we have shown efficiently produces single-cell suspensions of high viability (52). After washing, lung cells were stained, fixed, and run on an LSR II flow cytometer using FACSDiva software (version 6.1.3; BD Biosciences) with automatic compensation, and data were analyzed using FlowJo software (Tree Star, Ashland, OR) as previously described (45). We stained for the following Abs, using anti-murine Abs (clone): CD1d (1B1), CD11b (M1/70), CD45 (30-F11), CD45R/B220 (RA3-6B2), CD103 (2E7), Ly6G (1A8), anti-GalCer/CD1d complex (L363) (Biologend, San Diego, CA); CD3 (145-2C11), CD4 (GK1.5), CD11c (N418), MHC Class II (NIR4-5), NK1.1 (PK136) (eBioscience, San Diego, CA); and Ly6C (AL-21) (BD Biosciences, San Jose, CA).

We analyzed acidification of AMøs phagolysosomes by measuring the pH-dependent change in fluorescence of pHrodo. AMøs were plated at 40,000 AMøs per well in LCM and adherence purified for 1.5 h; then media were removed and fluticasone (2 μM) in RPMI-5 (or RPMI-5 alone) was added and plates were cultured for 22 h at 37°C in 5% CO₂. Next, 400,000 ACs (10:1 ratio of ACs/AMøs) in RPMI-5 (or RPMI-5 alone) were added and plates were incubated at 37°C in 5% CO₂ for another 2 h. Then pHrodo Zymosan Bioparticles (Life Technologies) were added according to manufacturer’s instructions and incubated at 37°C in 5% CO₂ for 90 min. As a control, one well in each condition received media alone and not Bioparticles. Samples were stained with CD45 and analyzed by flow
cytometry. To exclude the possibility of treatment-induced differences in particle ingestion, we performed a similar experiment using FITC-Zymosan Bioparticles (Life Technologies).

**Bacterial killing assay**

AMøs were plated in 2 different 96-well polystyrene tissue-culture plates (Corning Incorporated, Corning, NY) at 40,000 AMøs/well in LCM. One plate was designated the time 0 min (T0) control, to quantify live bacteria within AMøs after phagocytosis but before killing could have time to occur; the other plate was designated the time 120 min (T120) plate, to quantify ingested bacteria remaining viable 2 h after ingestion. AMøs on both plates were adherence purified for 1.5 h; then media were removed by suctioning and fluticasone (2 μM) in RPMI-5 (or RPMI-5 alone) was added and plates were cultured for 22 h at 37°C in 5% CO2. Next, 400,000 ACs (10:1 ratio of ACs/AMøs) in RPMI-5 (or RPMI-5 alone) were added and plates were incubated at 37°C in 5% CO2 for another 2 h. Then viable *S. pneumoniae*, which had been opsonized by incubation with normal rat serum (10% in HBSS) for 1 h at 37°C with constant shaking, were added to all wells at 2×106 CFUs/well. Plates were incubated for 20 min at 37°C and 5% CO2 and then centrifuged for 5 min at 250×g. Supernatants were aspirated and discarded; then 100 μl RPMI-5 was added to each well. Plates were again centrifuged for 10 min at 250×g and at this point the two plates were treated differently. The T0 plate received 20 μl 5% saponin per well and was incubated for 1 min at room temperature. Then 100 μl TH broth was added; the plate was covered with parafilm and stored overnight at 4°C. By contrast, the T120 plate was incubated at 37°C in 5% CO2 for 2 h before addition of saponin and then TH broth, followed by storage overnight at 4°C.

The following day, both the T0 and the T120 plates were incubated at 37°C and 5% CO2 for 2 h. After addition of 15 μl of 5 mg/ml MTT (Sigma) and an additional 20-min incubation at 37°C in 5% CO2, absorbance was measured at 570 nm using a microtiter plate reader. The CFUs in the T0 and T120 plates were extrapolated from a standard curve. Bacterial killing was calculated as a percentage, using the following equation: 

\[
\text{Bacterial killing}(%)) = \left( \frac{\text{T}_{0} \text{ CFU} - \text{T}_{120} \text{ CFU}}{\text{T}_{0} \text{ CFU}} \right) \times 100.
\]

**Analysis of reactive oxygen species and reactive nitrogen species**

To detect reactive oxygen species (ROS) and reactive nitrogen species (RNS), we plated AMøs in 96-well polystyrene tissue-culture plates (Corning Incorporated, Corning, NY) at 40,000 AMøs/well in LCM. AMøs were adherence purified for 1.5 h; then media were removed by suctioning.

To assay ROS, we added fluticasone (2 μM) in RPMI-5 (or RPMI-5 alone) and cultured plates for 22 h at 37°C in 5% CO2. Next, 400,000 ACs (10:1 ratio of ACs/AMøs) in RPMI-5 (or RPMI-5 alone) were added and plates were incubated at 37°C in 5% CO2 for another 2 h. Media were suctioned off and cells were washed with warm HBSS. A 50-μm solution of 2′,7′-dichlorodihydrofluorescein diacetate (Life Technologies) was prepared in RPMI-5 without phenol red and then added to each well. Cells were incubated at 37°C in 5% CO2 for 1 h. The plate was washed with HBSS, then read at an excitation of 492 nm and emission of 522 nm using an FLx800 fluorescent plate reader (BioTek, Winooski, VT). Then viable *S. pneumoniae* were added at 2×105 CFUs/well and incubated at 37°C in 5% CO2. The plate was read every 15 min for a total of 90 min and returned to the incubator after each reading.

To assay RNS, we removed media and prepared fluticasone (2 μM) in a fluorescein amine methyl ester staining solution, as directed by the manufacturer (Cayman Chemical, Ann Arbor, MI). Wells that were not treated with fluticasone received the fluorescein amine methyl ester staining solution alone. Plates were cultured for 22 h at 37°C in 5% CO2. Next, 400,000 ACs in the staining solution (or staining solution alone) were added and plates were incubated at 37°C in 5% CO2 for another 2 h. Then viable *S. pneumoniae* were added at 2×105 CFUs/well and incubated at 37°C in 5% CO2 for 2 h. Media were suctioned off and Hoechst Dye staining solution was prepared and added to each well, as directed by the manufacturer (Cayman Chemical). Plates were incubated for 10 min at 37°C. After a final wash, cells were analyzed using an FLx800 fluorescent plate reader. NO staining intensity was measured with excitation and emission wavelengths of 485 and 535 nm, respectively. Cell number density, indicated by the Hoechst Dye, was detectable at excitation and emission wavelengths of 355 and 465 nm, respectively.

**Statistics**

Statistical analyses were performed using GraphPad Prism 6.0.1 (GraphPad Software, La Jolla, CA) on a Macintosh Quad-Core Intel Xeon computer running OS X 10.10.3 (Apple, Cupertino, CA). To test for significant differences between groups, we used either ANOVA with appropriate post hoc testing (Dunn test for comparison with a single control condition, Fisher least significant difference testing for multiple comparisons) or the analogous nonparametric Kruskal–Wallis test with appropriate post hoc testing (Dunn for comparison with single control group, Holm-St¨adker for multiple comparisons). A p value <0.05 was considered to indicate significance.

**Results**

**GCAE reduced production of inflammatory mediators by murine AMøs**

To test for suppression of host defenses by GCAE, we first studied its effect on stimulated production of inflammatory cytokines by murine AMøs after in vitro pretreatment with either fluticasone, AC, or fluticasone followed by AC. AMø production of all three chemokines and especially IL-6, which have all been shown to be essential to combat pneumococci in vivo (54–56), and the chemokines CCL3 (MIP-1α), CCL5 (RANTES) (57), and KC, a murine functional homolog of human CXCL1.

Exposure of AMøs to fluticasone plus AC significantly reduced AMø elaboration of IL-12 relative to the other three conditions, and also significantly decreased secretion of TNF-α relative to AMøs pretreated with AC alone or with medium alone (Fig. 1A–C). Fluticasone plus AC treatment also led to significantly reduced secretion of CCL3 and KC (Fig. 1D–F) in comparison with the unpretreated AMøs or those that received AC alone. Fluticasone alone strongly inhibited AMø production of all three chemokines and especially of IL-6, so that IL-12 was the only analyte that was significantly lower in AMøs pretreated with fluticasone plus AC than in those pretreated with fluticasone alone; although for TNF-α, CCL5, and KC, there were nonsignificant trends toward lower production in response to the combined stimuli. We also measured IL-1β in these experiments, but secretion was near or below the limit of detection for all conditions (data not shown). These results imply that GCAE could reduce the ability of murine AMøs to secrete inflammatory mediators crucial for recruitment of other leukocyte subsets during early pneumococcal pneumonia. Accordingly, we next set about to test that possibility in vivo.

**GCAE inhibited in vivo clearance of *S. pneumoniae* in a murine model**

To establish a murine model of GCAE, we first needed to verify that a physiological dose of fluticasone altered murine AMø effectorcytosis in vivo. Mice were pretreated with fluticasone by IN inoculation for 6 h, then received ACs by the IN route. After an additional 2 h, we harvested AMøs by BAL and quantified ingested ACs. Results showed that fluticasone pretreatment significantly increased uptake of ACs by murine AMøs in vivo in a dose-dependent manner (Fig. 2).

In the next set of experiments, mice were pretreated with saline, fluticasone, ACs, or fluticasone followed by ACs (all by IN route); then all groups received *S. pneumoniae* (50,000 CFU/mouse by the IT route). After 48 h, lungs were harvested and total CFUs per lung were calculated (Fig. 3A). Pretreatment by fluticasone followed by ACs significantly reduced pneumococcal clearance from the lungs, relative to all other treatment groups (Fig. 3B). Thus, short-term treatment leading to GCAE in vivo had an adverse effect during bacterial pneumonia.
Dendritic cells (DCs) as low autofluorescent, MHC II+, CD11c+dim high side scatter cells abundantly expressing Ly6G. We defined negative for CD3, CD19, and Ly6G. Neutrophils were identified as (type II) NKT cells (CD1d+ anti-GalCer+) or nonclassical numbers or relative composition of lung inflammatory cells.

Thus, the observed significant differences in lung pneumococcal CFUs during pneumococcal pneumonia could not be attributed to disparity in inflammatory cell recruitment to the lungs during pneumonia. Mice were treated exactly as in the previous experiment, but at 48 h, lungs were harvested without previous BAL and total cells were stained for flow-cytometric analysis. All cell populations were initially gated on viable CD45+ cells. We defined AMs as high autofluorescent, CD11c+ cells that were negative for CD3, CD19, and Ly6G. Neutrophils were identified as high side scatter cells abundantly expressing Ly6G. We defined dendritic cells (DCs) as low autofluorescent, MHC II+, CD11c+dim cells that were negative for CD3, CD19, and Ly6G; two DC subsets were distinguished, CD11b+ CD103+ (which we and others have shown are located predominately in parenchymal lung interstitium) and CD11b− CD103+ (which have been shown to reside largely in airways) (58, 59). Similarly, exudate Møs were identified as low autofluorescent, CD11bhigh cells, among which we distinguished two subsets by their expression of Ly6C. We also identified CD4+ T cells, which have recently been recognized to contribute importantly and acutely to host defense during pneumococcal pneumonia (60, 61). Finally, we searched for NK cells and NKT cells, using staining for NK1.1, CD1d, CD3, and anti-GalCer.

There were no significant differences between the four in vivo treatments for any of these inflammatory cell populations at this time point, whereas Smyth et al. (4) expressed as absolute number of cells per mouse lung (Fig. 4) or as percentage of each cell type among all CD45+ lung cells (data not shown). Although we identified lung NK cells (NK1.1+, CD3−, CD1d−, anti-GalCer−), we found no evidence of classical (type I) NKT cells (NK1.1+, CD1d+ anti-GalCer+) or nonclassical (type II) NKT cells (CD1d− anti-GalCer−). Interestingly, for all cell types except the CD11b−, CD103+ DC subset, there was a nonsignificant trend toward higher absolute numbers in the MHC II− mice. Pretreatment with either saline, fluticasone, ACs, or fluticasone followed by ACs resulted in a modest decrease in bacterial killing, relative to each of the three other conditions (Fig. 6A). Fluticasone pretreatment also resulted in a modest decrease in bacterial killing, which was significantly different from the saline control. Hence, an additive reduction in AMs killing of pneumococci is one means by which GCAE impairs host defense against pneumonia. Numbers of CFU at T0 were significantly increased in AMs pretreated with fluticasone plus AC, relative to the untreated and AC-only groups (Fig. 6B). This result implies that the observed GCAE-induced deficit in killing by T120 did not result from reduced initial phagocytosis of pneumococcus.

**GCAE did not alter inflammatory cell recruitment to the lungs during pneumococcal pneumonia**

As a first step toward determining the mechanism by which GCAE had this adverse effect on lung host defenses, we used a separate cohort of mice to analyze inflammatory cell recruitment to the lungs during pneumonia. Mice were treated exactly as in the previous experiment, but at 48 h, lungs were harvested without previous BAL and total cells were stained for flow-cytometric analysis. All cell populations were initially gated on viable CD45+ cells. We defined AMs as high autofluorescent, CD11c+ cells that were negative for CD3, CD19, and Ly6G. Neutrophils were identified as high side scatter cells abundantly expressing Ly6G. We defined dendritic cells (DCs) as low autofluorescent, MHC II+, CD11c+dim cells that were negative for CD3, CD19, and Ly6G; two DC subsets were distinguished, CD11b+ CD103− (which we and others have shown are located predominately in parenchymal lung interstitium) and CD11b− CD103+ (which have been shown to reside largely in airways) (58, 59). Similarly, exudate Møs were identified as low autofluorescent, CD11bhigh cells, among which we distinguished two subsets by their expression of Ly6C. We also identified CD4+ T cells, which have recently been recognized to contribute importantly and acutely to host defense during pneumococcal pneumonia (60, 61). Finally, we searched for NK cells and NKT cells, using staining for NK1.1, CD1d, CD3, and anti-GalCer.

There were no significant differences between the four in vivo treatments for any of these inflammatory cell populations at this time point, whereas Smyth et al. (4) expressed as absolute number of cells per mouse lung (Fig. 4) or as percentage of each cell type among all CD45+ lung cells (data not shown). Although we identified lung NK cells (NK1.1+, CD3−, CD1d−, anti-GalCer−), we found no evidence of classical (type I) NKT cells (NK1.1+, CD1d+ anti-GalCer+) or nonclassical (type II) NKT cells (CD1d− anti-GalCer−). Interestingly, for all cell types except the CD11b−, CD103+ DC subset, there was a nonsignificant trend toward higher absolute numbers in the MHC II− mice. Pretreatment with either saline, fluticasone, ACs, or fluticasone followed by ACs resulted in a modest decrease in bacterial killing, relative to each of the three other conditions (Fig. 6A). Fluticasone pretreatment also resulted in a modest decrease in bacterial killing, which was significantly different from the saline control. Hence, an additive reduction in AMs killing of pneumococci is one means by which GCAE impairs host defense against pneumonia. Numbers of CFU at T0 were significantly increased in AMs pretreated with fluticasone plus AC, relative to the untreated and AC-only groups (Fig. 6B). This result implies that the observed GCAE-induced deficit in killing by T120 did not result from reduced initial phagocytosis of pneumococcus.

**GCAE INHIBITS MURINE AMø ANTIPNEUMOCOCCAL DEFENSES**

To test whether bacterial killing by AMs was inhibited by GCAE and could contribute to the observed increase in bacterial burden in vivo, we performed an in vitro bacterial killing assay. AMs from normal C57BL/6 mice were adherence purified before pretreatment with either saline, fluticasone, ACs, or fluticasone followed by ACs. Pretreatment with fluticasone plus ACs significantly reduced AMs-mediated bacterial killing, relative to each of the three other conditions (Fig. 6A). Fluticasone pretreatment also resulted in a modest decrease in bacterial killing, which was significantly different from the saline control. Hence, an additive reduction in AMs killing of pneumococci is one means by which GCAE impairs host defense against pneumonia. Numbers of CFU at T0 were significantly increased in AMs pretreated with fluticasone plus AC, relative to the untreated and AC-only groups (Fig. 6B). This result implies that the observed GCAE-induced deficit in killing by T120 did not result from reduced initial phagocytosis of pneumococcus.
An effect of ACs on in vitro killing of *K. pneumoniae* by rat AMøs was previously shown in experiments using opsonization by specific IgG (44). Accordingly, we investigated whether the GCAE-induced decrement in killing of pneumococcus by murine AMøs that we showed using complement-opsonized bacteria would also be seen with specific IgG opsonization. We compared killing of pneumococci opsonized by the two methods by untreated murine AMøs or AMøs treated in vitro with ACs plus fluticasone. Results showed a similar reduction in bacterial killing by GCAE regardless of the method of opsonization (Supplemental Fig. 1), although the difference from untreated AMøs did not attain statistical significance in this single experiment.

**FIGURE 2.** Fluticasone increased in vivo uptake of ACs by resident AMøs. (A) C57BL/6 mice received two IN administrations, of either various doses of fluticasone (100–10,000 ng/ml) or saline control, followed 6 h later by an IN administration of 1 × 10⁷ ACs. One hour later, AMøs were collected by BAL, cytospins were stained with H&E and ingested ACs were counted under oil at ×1000 final magnification. (A) Representative cytospins showing in vivo AC uptake after in vivo fluticasone treatment with either saline (top panel) or 1000 ng fluticasone. Arrows point to ingested AC. Top panel, Percentage of AMøs ingesting at least one AC; lower panel, efferocytic index. (B) Data are mean ± SEM from three mice in a single experiment and are representative of results of three independent experiments. Statistical testing using one-way ANOVA with Dunnett’s post hoc testing for multiple comparisons relative to saline-only control group.

GCAE and fluticasone impaired AMø killing of *S. pneumoniae* in part by reducing phagolysosome acidification

We performed several types of experiments to begin to define the possible mechanisms by which GCAE impairs in vitro killing of *S. pneumoniae* by murine AMøs. Bacterial killing by murine macrophages (Møs) often involves production of ROS by the NADPH oxidase complex and of RNS by inducible NO. However, exposure of murine AMøs to fluticasone, ACs, or both did not significantly affect ROS production in response to ingestion of viable pneumococci (Supplemental Fig. 2A). Similarly, none of these treatments impacted production of RNS (Supplemental Fig. 2B).

Finally, we examined the effect of GCAE on phagolysosome acidification. We assayed AMø phagolysosome acidification by quantifying the change in fluorescence of the pH-sensitive dye pHrodo using flow cytometry. Pretreatment with either fluticasone or fluticasone followed by ACs significantly impaired phagosome acidification.

**FIGURE 3.** GCAE specifically reduced clearance of viable *S. pneumoniae* from the lungs in a murine model. C57BL/6 mice received two IN administrations, given 4 h apart, of either saline (indicated by minus sign [−]; fluticasone followed by saline, saline followed by ACs, or fluticasone followed by ACs. All mice were infected via the IT route 24 h after the final IN treatment using 50,000 CFUs *S. pneumoniae* serotype 3. Lungs were collected 48 h later to assay total CFU by serial dilution on blood agar plates. Data are derived from two to three mice per condition assayed individually in each of three independent experiments (total *n* = 34). (A) Log lung CFUs of individual mice; symbols denote mice from different experiments. (B) Fold-change in lung CFUs, relative to the group pretreated twice with saline before infection, the geometric mean of which was set to 1. Data are shown as median, 25% and 75% (box) and 5%, 95% CI (whiskers), with outliers shown individually. *p* < 0.5, **p** < 0.01, by Kruskal–Wallis nonparametric ANOVA with Dunn’s post hoc testing for multiple comparisons to saline-only control group.
pretreatment and intra-alveolar ACs led to significantly greater lung bacterial burdens in a murine model of pneumococcal pneumonia, without altering inflammatory cell recruitment to the lungs. GCAE also decreased in vitro killing of pneumococci by murine AMs, but not their uptake, in association with a decrease in phagolysosome acidification. These data provide one mechanism for the increased susceptibility to CAP when COPD patients are treated with potent ICS therapy.

*Streptococcus pneumoniae* is a near-obligate human pathogen that causes more deaths globally than any other organism, principally because of infections in children <5 y old in the developing world (62). However, pneumococcal pneumonia also continues to be a very significant health problem in industrialized nations, despite vaccines that have efficacy even in elderly patients with COPD (63). Moreover, the lethality of pneumococcal pneumonia remains considerable even when appropriate antibiotic therapy is initiated promptly (64). Providing appropriate antibiotic therapy against pneumococcus is becoming more difficult, as high-grade resistance to multiple antibiotics is already prevalent in many regions, especially Asia. Although antibiotic stewardship may delay the global spread of resistance, the profound ability of pneumococcus to undergo DNA transformation virtually assures that the trend will continue. Hence investigating the immunological basis of susceptibility to pneumococcal pneumonia will remain important.

Our studies used mice, a well-accepted model of human pneumococcal pneumonia, provided appropriate attention is given to pneumococcal serotype, murine strain, and anesthesia method (46, 65, 66). We used the encapsulated serotype 3, which in humans remains associated with both common nasopharyngeal carriage and frequent pneumonia with relatively high mortality (67–69), perhaps recently in part because of its lower immunogenicity relative to other strains in polyvalent vaccines (70). We (50, 71, 72) and others (44, 73–85) have used variations of this murine model extensively in wild-type and transgenic mice to define the molecular mechanisms of lung host defense against pneumococcus. Together with established murine models mimicking the pathogenic changes of COPD, either employing or independent of cigarette smoke exposure (86, 87), the tools are now available to determine whether the beneficial effects of ICS can be dissociated from this and other adverse effects.

The demonstration that GCAE was associated with both significantly greater lung bacterial burdens and reduced killing in vitro is important because resident AMs are so crucial to defend against pneumococcal pneumonia (88). Part of their role depends on killing pneumococci, which for AMs occurs almost entirely intracellularly. AMs require opsonization to ingest encapsulated strains, which are associated with most episodes of CAP in humans. Accordingly, we used the encapsulated serotype 3 and performed serum opsonization in all experiments. Ingestion depends on several Mø receptors, including FcγR and the scavenger receptors SR-A and MARCO (89) and (primarily for unopsonized pneumococci) mannose receptor. Our data indicate GCAE and fluticasone alone increased ingestion of pneumococcus by murine AMs in vitro, which literature searching leads us to conclude is a novel finding. The reason for this increase is uncertain. Our previous demonstration that fluticasone induced downregulation by murine AMs of the inhibitory receptor SIRPα (45) together with the finding that alveolar lining fluid (which contains SP-A and SP-D, the collectin ligands for SIRPα) impaired phagocytosis by murine AMs (90) provides a possible explanation. However, increased phagocytosis of pneumococcus was induced in human neutrophils by both SP-A and SP-D (91), and in murine AMs by SP-A (via surface localization of SR-A), but not by SP-D (92). Hence further study will be needed to es-

**FIGURE 4.** GCAE did not alter inflammatory cell recruitment to the lungs during pneumococcal pneumonia. Mice were pretreated by the IN route and infected by the IT route with 50,000 CFUs/mouse *S. pneumoniae* serotype 3, exactly as described in the legend to Fig. 3, except that 48 h postinfection, lungs were harvested and processed individually for flow cytometry. Hematopoietic cells were gated using light-scatter parameters and CD45 staining as described in the Results. Data are expressed as the absolute number of cells per lung for each cell type on the vertical axis (note differences in scales), as mean ± SEM of two to three mice per condition assayed individually in each of two independent experiments (total n = 21). There were no statistically significant differences between treatment groups by ANOVA with Fisher’s LSD post hoc testing.

Discussion

The results of this study demonstrate that by the process we term GCAE, the combination of the potent GC fluticasone and subsequent AC exposure impairs the ability of resident AMs to defend against pneumococcus more greatly than either stimulus alone. GC pretreatment in vitro significantly reduced proinflammatory production by murine AMs in response to TLR4 stimulation, although the effect was less marked using heat-killed pneumococci under the conditions tested. The combination of steroid acidification in vitro (Fig. 7A, 7B). To exclude the possibility that this difference was artifactual, and instead reflected treatment-induced differences in particle ingestion, we performed a similar experiment using FITC-Zymosan Bioparticles. That control experiment showed that the combination of fluticasone followed by ACs slightly but significantly increased AMs ingestion, which was avid for all treatments (Fig. 7C).
tablish that mechanism. Importantly, the calculation of percentage bacterial killing takes into account difference in bacterial uptake, so this effect does not explain the GCAE-induced killing defect we show.

Unlike the better studied neutrophil, which eliminates pneumococci using multiple extracellular and intracellular mechanisms, precisely which elements are essential for AMs to kill ingested pneumococci remains incompletely defined. Our finding that neither GCAE nor fluticasone alone affected ROS production extends results of Marriott and colleagues (93), who used gp91phox−/− mice to show that ROS production was dispensable in a subclinical pneumonia model. GCs do reduce phagocyte generation of superoxide at least in part via reduced eicosanoid signaling (94), but that effect is seen at micromolar concentrations irrelevant to GCAE, which occurs at nanomolar concentration identical to those achieved clinically during ICS therapy. Further investigation is needed to define the roles of ROS and RNS in AMø killing of pneumococcus, but our results show that changes in these mediators cannot explain the defect induced by GCAE.

To our knowledge, corticosteroids have not previously been shown in any mammalian phagocyte to affect phagolysosome acidification, a particularly crucial step in the killing process (95). An acidic intraluminal pH is necessary for optimal activity of cathepsins, which contribute to pneumococcal clearance in murine models both by inducing AMø apoptosis linked to bacterial killing (83) and, at least in neutrophils, by direct antibacterial activity (96). In addition, acidification of the maturing phagolysosome is important to counteract three potentially detrimental effects of the oxidative burst that would otherwise increase pH: 1) consumption of protons as superoxide undergoes dismutation to hydrogen peroxide; 2) leakage of H+ into the cytoplasm caused by membrane oxidation; and 3) impaired recruitment to the phagosome of V-ATPase, which is essential to complete acidification (97). Because the effect on acidification was equivalent in AMøs treated with fluticasone without or with subsequent ACs, this mechanism cannot explain the even greater reduction in killing induced by GCAE, relative to fluticasone alone. Considerable additional investigation will be required to define the molecular basis for this GCAE-induced defect, but these results advance the field by excluding defective ROS or RNS generation.

In experimental models, AMøs also contribute to defense against pneumococcal pneumonia by production of cytokines and chemokines, which are particularly crucial to activate and recruit other cell types in response to larger inocula (98). The disparity in results of stimulating AMøs in vitro with purified LPS versus with heat-killed pneumococci does not necessarily detract from the relevance of GCAE shown by our lung CFUs and in vitro killing data because neither experiment fully simulates the indirect contribution of AMøs to host defense during pneumococcal pneumonia. For example, production of CCL5 by murine Møs in response to pneumococcus requires pneumolysin-dependent escape from phagolysosomes that likely does not occur efficiently using heat-killed organisms (99, 100). However, the absence of any significant difference between treatment groups in recruitment of inflammatory cell subsets implies that in vivo, either AMø production or recruitment signals was not essential under the conditions tested or that other factors compensated. We suspect that the disparity in our results regarding neutrophil recruitment from the decreases seen by Medeiros and colleagues in response to AC alone (44) relate to differences from our experiments in pneumococcal inoculum (10^6 CFUs versus 5 × 10^7 CFUs in this study) (98). Moreover, it is likely that GCAE-induced failure of early pneumococcal eradication in our study led to compensatory changes by other cell populations that culminated in increased neutrophil recruitment to the lungs by the time point we studied.

In adults, COPD is one of the strongest risk factors for development of CAP. COPD is also a pervasive and rapidly increasing problem worldwide, due to the juxtaposition in much of the developing world of rapidly increasing cigarette consumption and air pollution, particularly by indoor use of biomass fuel or coal. Although COPD is also increasing as a directly attributable cause of death in industrialized nations, its true burden is likely vastly underestimated. COPD is underdiagnosed, especially in women (101). Hence defining precisely why there is an association between ICS therapy, which clearly has beneficial effects in COPD, and potentially lethal hospitalizations for CAP should be of significant public health interest. Our data imply that the risk of GCAE contributing to CAP might be greatest in those with emphysema, which among the several pathological processes contributing to COPD phenotypes is the one most closely associated with excessive apoptosis of parenchymal lung cells. To date, none of the studies on the association of CAP and ICS usage has included the imaging data necessary to address that possibility. Indirect support for that possibility comes from the recent identification of the emphysematous subset as being at elevated risk for acute exacerbations of COPD (102), a largely infectious syndrome that blends clinically with CAP.
Based on our in vitro findings that budesonide also induced GCAE in murine AMs (45), we suspect that the effect we have shown in this study using fluticasone is a class effect of all potent ICS medications. However, we recognize that there is controversy on this important clinical point. A retrospective observational trial using administrative health data found lower pneumonia event rates in COPD patients using budesonide compared with fluticasone (10). An individual-subject data meta-analysis relying heavily on adverse event reporting and including studies of relatively short duration found no difference in incidence of CAP from placebo among users of budesonide (103). However, this conclusion has also been contested (104, 105). Verifying whether specific ICS agents have different risks for infection or other adverse effects is a highly significant question, but one that will likely require large clinical trials or very careful epidemiological approaches. Importantly for our hypothesis that GCAE might explain the increased risk for CAP among COPD patients using ICS therapy, asthma is not associated with apoptosis of alveolar epithelial and endothelial cells that can interact with AMs.

In summary, we have extended our previous identification of GCAE as a property of resident tissue Mφs (45) (i.e., not only a process relevant during maturation of blood monocytes) by showing its impact on a clinically relevant bacterial pathogen in vitro and in vivo. Our results support GCAE as a potential explanation for the epidemiological association between ICS therapy of COPD patients and an increased risk for CAP, although further support for that possibility is required, including studies using human AMs that we have under way. These findings also establish murine in vitro and in vivo experimental models to dissect the underlying molecular mechanisms by which GCAE impacts lung host defense.
GCAE INHIBITS MURINE AMø ANTIPNEUMOCOCCAL DEFENSES


