

Toll-Like Receptor 7/8 Ligand, S28463, Suppresses *Ascaris suum*-induced Allergic Asthma in Nonhuman Primates

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Abstract

S28463 (S28), a ligand for Toll-like receptor 7/8, has been shown to have antiinflammatory properties in rodent models of allergic asthma. The principle goal of this study was to assess whether these antiinflammatory effects can also be observed in a nonhuman primate (NHP) model of allergic asthma. NHPs were sensitized then challenged with natural allergen, *Ascaris suum* extract. The animals were treated with S28 orally before each allergen challenge. The protective effect of S28 in NHPs was assessed by measuring various asthma-related phenotypes. We also characterized the metabolomic and proteomic signatures of the lung environment and plasma to identify markers associated with the disease and treatment. Our data demonstrate that clinically relevant parameters, such as wheal and flare response, blood IgE levels, recruitment of white blood cells to the bronchoalveolar space, and lung responsiveness, are decreased in the S28-treated allergic NHPs compared with nontreated allergic NHPs. Furthermore, we also identified markers that can distinguish allergic from nonallergic or allergic and drug-treated NHPs, such as metabolites, phosphocreatine and glutathione, in the plasma and BAL fluid, respectively; and inflammatory cytokines, IL-5 and IL-13, in the bronchoalveolar lavage fluid. Our preclinical study

demonstrates that S28 has potential as a treatment for allergic asthma in primate species closely related to humans. Combined with our previous findings, we demonstrate that S28 is effective in different models of asthma and in different species, and has the antiinflammatory properties clinically relevant for the treatment of allergic asthma.

Keywords: experimental model; proteomics; metabolomics; inflammation; treatment

Clinical Relevance

Data describing the antiinflammatory effects of S28463 (S28) in rodent models of allergic asthma have been published. In this study, we assessed whether the antiinflammatory effects of S28 can also be observed in a nonhuman primate model of allergic asthma. When combined with our previous findings, we demonstrate that S28 is effective in different models of asthma and in different species, and has the antiinflammatory properties clinically relevant for the treatment of allergic asthma.

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A large family of Toll-like receptors (TLRs) exists that is specialized in the recognition of specific molecular patterns common to various pathogens. Activation of each TLR leads to specific inflammatory responses directed against the pathogens associated with the receptor's ligand. Immune modulation by targeting TLRs has been actively explored as a possible therapeutic approach for treating allergic asthma. For instance, TLR2 activating *Mycobacterium bovis* and *Mycobacterium vaccae* antigens have also been shown to prevent airway hyperresponsiveness (AHR) in a mouse model of asthma (1). TLR9 ligands, such as CpG-oligodeoxynucleosides, have shown to suppress the T-helper cell type 2 (Th2) response, induce Th1 cytokine production, and result in an expansion of regulatory T cells (2–4). In nonhuman primates (NHPs), immunostimulatory oligonucleotides can attenuate airway hyperactivity, airway remodeling, and immune cell recruitment (5).

In the present study, we explored the TLR7/8 ligand, S28463 (S28; resiquimod), as a potential candidate drug for treating allergic asthma. S28 belongs to the family of imidazoquinolines, which also includes imiquimod, clinically used for topical treatment of genital warts, basal cell carcinomas, and actinic keratosis. This group of molecules was initially demonstrated to have potent antiviral and antitumor properties, due to their ability to induce cytokines and cell-mediated immune responses via signaling through the MyD88 pathway (6–8). Several research groups have demonstrated that S28 can prevent allergic asthma phenotypes in different animal models of asthma (9–12). Using an acute mouse model of allergic asthma and a chronic rat model of allergic asthma, we have previously shown that S28 can prevent an assortment of allergic asthma phenotypes caused by allergen challenge in sensitized rodents (9–11).

Given that S28 is capable of preventing AHR, cytokine and IgE production, and recruitment of inflammatory cells in mouse and rat models, we sought to test the efficacy of this drug in an allergic asthma model induced in NHPs. In addition to exploring the classical phenotypes studied in allergic asthma models, we also aimed to capture the signature of the disease by identifying and quantifying small molecules through metabolomics and proteomics studies. These studies have the potential to identify

markers that are surrogates of the disease status and severity. Furthermore, disease-associated markers that are also corrected after drug treatment could potentially track a positive response to treatment.

To achieve this, we developed a protocol for establishing allergic asthma in NHPs by sensitization and challenge with *Ascaris suum* extract, a parasite known to naturally cause allergies in NHPs. Similar to our earlier studies, we observed that administering S28 24 hours before antigen challenge can prevent allergen-induced inflammation by inhibiting the recruitment of inflammatory cells to the bronchial fluid and the production of both Th1 and Th2 cytokines. We also witnessed a protective effect against two hallmark phenotypes of allergic asthma, AHR and IgE production.

Methods

For detailed materials and methods, please refer to the data supplement (File E1).

Briefly, NHPs were sensitized to *A. suum* antigen via subcutaneous injection three times at 2-week intervals (see Figure E1). Success of sensitization was assessed via skin prick test. After sensitization, animals were aerosol challenged with *A. suum* three times, 7 days apart. Treated animals were given S28 by nasogastric intubation 24 hours before antigen challenge. Blood samples were collected before and after treatment to assess the safety of the drug. The potential of S28 as a treatment for allergic asthma was assessed by measuring airway responsiveness to methacholine exposure, by measuring serum IgE concentration, and by quantifying the concentration of inflammatory cytokines and cells in BAL fluid (BALF), and by analysis of proteomic and metabolomics markers in the plasma and BALF.

Results

***A. suum*–induced Model of Allergic Asthma in NHPs and Safety of S28 Treatment**

Our first task was to develop an antigen sensitization and challenge protocol for inducing allergic asthma to *A. suum* in NHPs. As opposed to many other studies, which use *Macaca fascicularis* caught in the wild and already sensitized to *A. suum*, we used animals that were not skin prick test

positive to *A. suum* (Figure 1A). To mount a reaction to *A. suum* allergen, we injected the animals with *A. suum* extract every 2 weeks. The success of sensitization was monitored by skin prick testing, which was done before each sensitization and challenge, and before the final assessment of airway resistance. The results show that animals were effectively sensitized, and wheal size increased with each additional dose of antigen (Figures 1B and 1C). Aerosol challenge with *A. suum* does not cause an increase in wheal size, and sensitization is maintained for weeks after the final antigen injection (Figure 1A). However, we do observe that antigen challenge does cause an increase in both Th1 and Th2 cytokines in the BALF compared with concentrations observed before allergen challenge (Table 1). Blood biochemical and hematologic analyses were performed to assay the safety of exposing the animals to *A. suum* and treating with S28 (Table E1). The only statistically significant difference observed was an increase in blood potassium concentration, primarily caused by sensitization and challenge with *A. suum*. Nevertheless, the values observed in this parameter are still within the range observed in healthy individuals.

Treatment with S28 Decreases Sensitization to Antigen and Airway Responsiveness in Allergic NHPs

As stated above, exposure to *A. suum* on three occasions, each 2 weeks apart, leads to the development of a cutaneous allergic reaction. Challenge with *A. suum* tends to increase the cutaneous response, which appears to reach a plateau once sensitization is established (Figure 1). Treatment with S28 blunts the cutaneous response to *A. suum* (Figure 2A). We also observed that challenge with *A. suum* resulted in an increase in the levels of IgE compared with the group receiving PBS challenges (Figure 2B). S28 treatment in antigen-challenged animals resulted in a dramatic reduction of IgE in the plasma compared with those that were untreated, and the levels are within the range of the PBS-challenged animals.

We have previously shown that the TLR7/8 ligand S28 can protect against ovalbumin (OVA) allergen-induced increase in airway resistance in mice (9, 11). In our current model, we show that repeated treatment with *A. suum* results in AHR to methacholine exposure in monkeys (Figure 2C). Lungs of animals

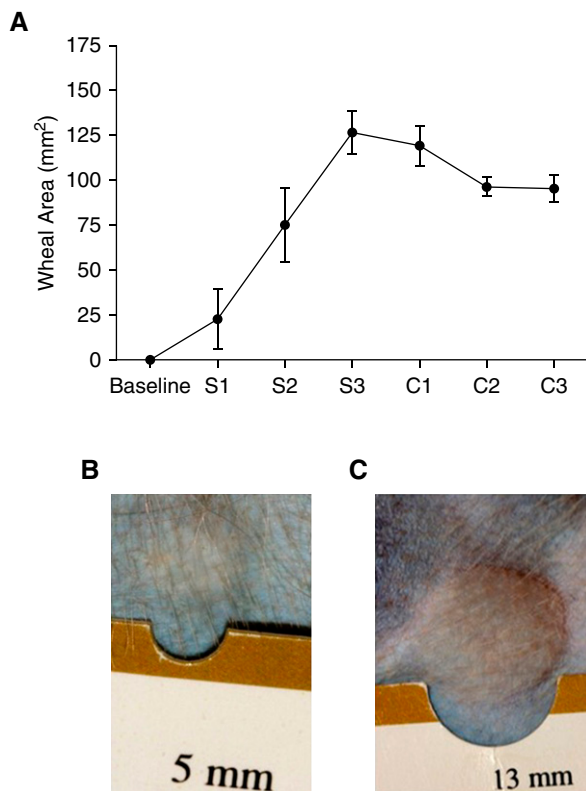


Figure 1. Development of cutaneous allergic reaction to *Ascaris suum* antigen extract. (A) Animals in all three groups were sensitized to *Ascaris suum* extract three times (S1, S2, and S3). Following sensitization, animals in positive control (POS) and S28-treated groups were challenged with *A. suum* three times (C1, C2 and C3), while animals in the PBS group were mock-challenged with PBS. Skin prick tests were done before each sensitization and challenge and 48 hours after the final challenge. Results are represented as mean ($\pm$ SEM); ($n \geq 3$ per time point). (B) Representation of wheal size after first *A. suum* sensitization. After first sensitization (S1) the wheal size was below 5 mm diameter. (C) Representation of wheal size after the third *A. suum* sensitization. After the third sensitization (S3) the wheal size reached approximately 13 mm diameter.

receiving S28 before *A. suum* challenge had a lower response when stimulated with methacholine. We also regularly monitored airway resistance during each challenge session. PBS-challenged animals depict no change in airway response over the course of the challenges; however, animals being exposed to *A. suum* (positive control; POS) demonstrate an increase in airway resistance with each challenge. Monkeys treated with S28 have a significantly lower airway resistance compared with the nontreated allergic animals (Figure 2D). Furthermore, *A. suum* aerosol challenge (POS) caused a significant increase in total cells, largely eosinophils, in the BALF compared with PBS challenge (Figures 2E and 2F, respectively). Treatment with S28 before challenge significantly reduced the total number of inflammatory cells recruited to the BALF (Figure 2E). The number of recruited eosinophils was also significantly decreased to levels observed in

PBS-challenged animals (Figure 2F). No differences were observed in the number of lymphocytes, macrophages, or neutrophils between the groups (Figures E2A–E2C).

Treatment with S28 Prevents *A. suum* Challenge-induced Increases in Both Th1 and Th2 Cytokine Levels

We assessed the concentration of cytokines in BALF collected from the lungs of the *A. suum*-induced NHP model (Figures 3A–3L). *A. suum* challenge caused significant changes in several Th2 cytokines when compared with PBS mock challenge. Many measured cytokines increased after allergen exposure, except for monocyte chemoattractant protein-1, which was higher in PBS-challenged animals (Figure 3H). Treatment with S28 before each antigen challenge significantly prevented the shift in the levels of all measured cytokines, with the exception of IL-2 and IL-5, where a nonsignificant trend was also apparent.

Metabolomics and Proteomics Data

Proteomics. A total amount of 294 proteins was detected across all samples analyzed, of which 177 analytes are significantly altered after *A. suum* challenge (PBS versus POS), and 70 are altered between the two *A. suum*-challenged groups (POS versus S28) (Figure 4A). The top five markers most significantly affected by allergen challenge include fatty acid binding protein (FABP) 5, Rho GDP-dissociation inhibitor 2, peptidylprolyl isomerase A, aldo-keto reductase family 1 member A1, and leucine aminopeptidase 3. A total of 23 markers overlap between the two comparisons, and normalized to levels observed in the PBS group after S28 treatment (Figure 4B). These markers encode proteins, such as kallikrein (KLKB1), prothrombin, and FABP5. We used Gene Ontology (GO) analysis to gain an understanding of the biological processes shared by these 23 proteins. The proteins clustered into many genome ontology pathways, with the most significant biological process being “regulation of proteolysis” (Figure 4C) and molecular function being “endopeptidase regulator activity” (Figure 4D). The relationships between proteins found altered by allergen challenge alone (PBS versus POS, File E2), allergic nontreated and allergic treated groups (POS versus S28, File E3), and the overlap between PBS versus POS and POS versus S28 (File E4) were studied by protein–protein association network analysis (STRING, available from: <https://string-db.org>). In general, the STRING analysis supported data from the GO examination with major processes corrected by S28 treatment, including regulation of proteolysis, regulation of fibrinolysis, acute inflammatory response, and negative regulation of cellular protein metabolic processes (File E4).

Metabolomics. To build the metabolomics signature, we measured the level of 107 and 134 metabolites in the plasma (Figure 5) and BALF (Figure 6), respectively. Statistical evaluation by unsupervised principal component analysis can be found in Figures E3A and E3B. In both cases, clear separation of PBS and POS groups is achieved. The 20 most significant metabolites leading to the separation of the PBS and POS groups were selected by means of partial least square determinant analysis variable importance in the projection score plot (Figures E3C and E3D), for both plasma

Table 1. Changes in Expression of Inflammatory Markers in the BAL Fluid after *Ascaris suum* Allergen Exposure

Cytokine	Baseline	Final	P Value
GM-CSF	10.11 ± 2.52	13.52 ± 3.64	0.4479
TGF-α	30.72 ± 3.5	60.7 ± 13.37	0.0397*
G-CSF	10.61 ± 0.51	26.9 ± 6.47	0.0215*
IFN-γ	4.79 ± 0.86	5.98 ± 0.97	0.3777
IL-2	6.8 ± 0.69	9.3 ± 1.22	0.084
IL-10	34.18 ± 3.49	49.42 ± 5.41	0.033*
IL-15	14.02 ± 1.78	11.2 ± 2.3	0.3461
sCD40L	28.21 ± 4.59	37.91 ± 6.8	0.2506
IL-1ra	120.11 ± 20.34	192.95 ± 37.31	0.1173
IL-13	15.79 ± 1.52	28.39 ± 3.42	0.0046**
IL-4	11.71 ± 1.69	33.64 ± 7.48	0.0104*
IL-5	2.77 ± 0.38	36.03 ± 13.07	0.0183*
IL-6	43.94 ± 23.9	117.29 ± 69.07	0.2784
IL-8	199 ± 32.77	574.83 ± 257.86	0.1788
MCP-1	4,129.57 ± 92.46	2,736.33 ± 398.99	0.0037**
TNF-α	34.12 ± 5	77.88 ± 7.27	0.0006***
MIP-1β	4.01 ± 1.22	6.36 ± 1.37	0.2336
IL-12/23(p40)	15.8 ± 2.91	20.46 ± 3.09	0.2971
VEGF	171.09 ± 43.24	209.33 ± 82.84	0.666
IL-18	23.96 ± 9.87	28.36 ± 2.73	0.6974

Definition of abbreviations: G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; MCP-1 = monocyte chemoattractant protein-1; MIP-1β = macrophage inflammatory protein-1β; sCD40L = soluble CD40 ligand; TGF-α = transforming growth factor-α; VEGF = vascular endothelial growth factor.
 $n \geq 4$ per group.

* $P < 0.05$ between baseline and final.

** $P < 0.01$ between baseline and final.

*** $P < 0.001$ between baseline and final.

and BALF matrices. The markers, phosphocreatine and D-glucuronate, together with dipeptides glycine-phenylalanine and glycine-glycine, were the most significantly altered after allergen challenge, whereas glutathione, glutathione disulfide, and kynurenine levels are the most significantly altered metabolites in the BALF after challenge with *A. suum* allergen.

Through a detailed evaluation of the box plots generated from the metabolite data, we were able to identify several metabolites that were altered by *A. suum* challenge, but maintained levels similar to those observed in the PBS group when treated with S28. These metabolites include phosphocreatine and dipeptide glycine-glycine in plasma, and glutathione, glutathione disulfide, and kynurenine in BALF.

Discussion

The main purpose of our study was to determine if S28, a TLR7/8 ligand previously demonstrated as having inhibitory effects on early inflammatory responses to OVA allergen challenge in rodent models of

asthma, could also prevent the development of inflammatory reactions in allergen-exposed NHPs. We observed an inhibition of both Th1 and Th2 cytokines in the BALF, which corresponded to a reduction in the number of eosinophils and overall total number of cells in the BALF. These findings reproduce what we have previously observed in our rodent studies (10, 11). However, other studies have documented an increase in Th1 cytokines, such as IFNγ and IL-6, after TLR7 stimulation (13). These differences may be due to variations in the disease models, the TLR7 ligands used, endpoints, treatment and sample collection protocols, and variations in the kinetics of each cytokine. Type 1 IFNs, for instance, have been shown to peak at 2 hours and resolve by 6 hours (14), whereas, in our study, samples were collected 48 hours after the third allergen challenge. Furthermore, reductions in clinically relevant parameters, such as wheal and flare response after skin prick testing, blood IgE levels, and airway responsiveness to allergen methacholine exposure, were also observed in S28-treated primates. Though we did not check for the effect on allergen-specific IgE, other groups

have documented that prophylactic treatment with S28 before sensitization and challenge with birch pollen allergen can reduce the titer of allergen-specific IgE (15).

Our metabolomics and proteomics analyses identified markers that could be measured in plasma and in the BALF to facilitate asthma diagnosis and monitor the effect of treatments in patients with asthma. Several of the protein makers that we identified as being affected by *A. suum* allergen challenge and S28 treatment have previously been identified to be associated with asthma by other groups. KLKB1 has been shown to be a regulator of soluble cleaved urokinase plasminogen activator receptor, a circulating protein implicated in various diseases, including asthma (16, 17). Expression quantitative trait locus analyses have identified a single nucleotide polymorphism in the KLKB1 promoter region that affects its function and its regulation of soluble cleaved urokinase plasminogen activator receptor (18). FABP5 expression has been shown to be elevated in both the induced sputum and nasal lavage fluid samples from patients with asthma. FABP5 expression in the nasal lavage fluid correlates with the level of cysteinyl leukotrienes, which are released by eosinophils and can cause bronchoconstriction (19). We also identified prothrombin and pathways involved in fibrinolysis, proteolysis, endopeptidase regulation, acute inflammatory response, and protein metabolism from our proteomics data. Though thrombin activity and fibrin production is necessary for the repair of epithelial cells after damage, in the context of asthma it is known to be substantially increased, and can lead to the deposition of fatal clots in the airways (20–22). It would be interesting to verify if similar protein markers are identified in different models of asthma.

Fluctuations in metabolite levels could also provide an indication of the mechanism and changes occurring during allergic asthma development. Similar to other reports, we observed that the metabolomic signature is distinct for each tissue studied (23). Kelly and colleagues (23) summarized the results of 21 metabolomics studies to identify a list of 14 metabolites that are identified as significant in multiple studies. Metabolites identified in our study as being altered by allergen challenge and overlapping with this curated list include hippurate and adenosine, an acid salt and a purine nucleoside, respectively, indicating

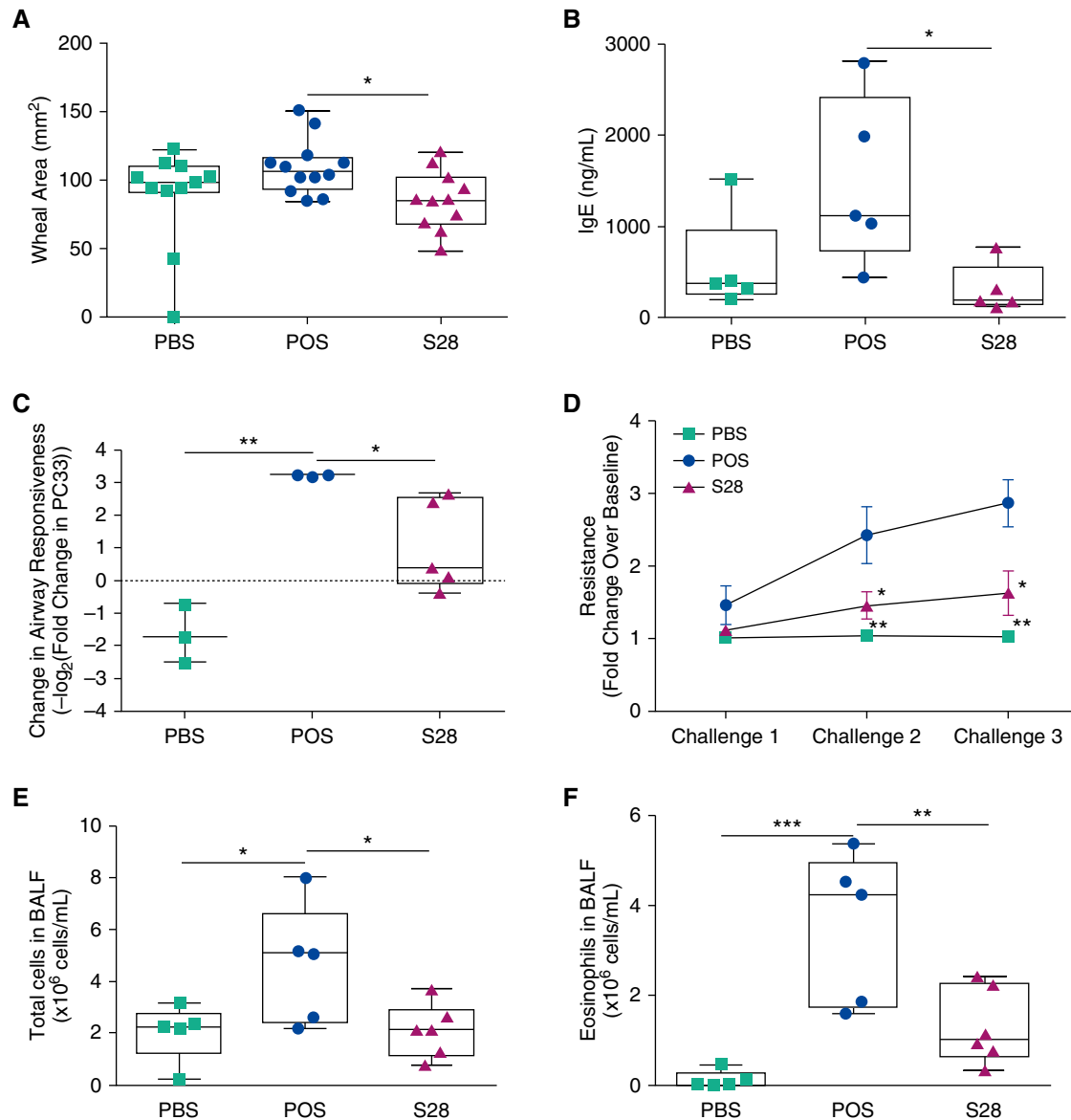


Figure 2. Treatment with S28463 (S28) blunted the development of cutaneous reaction, airway responsiveness, and inflammatory cell influx in response to *A. suum* antigen extract. Treatment with S28 24 hours before each challenge with *A. suum* prevents the increase in (A) wheal size and (B) serum IgE concentration. Consecutive challenge with *A. suum* in sensitized monkeys causes an increase in airway responsiveness to (C) methacholine and (D) *A. suum* exposure, which was reduced by treatment with S28. (E) Total number of cells is increased in the POS group compared with the PBS-challenged group. S28 treatment prevents the increase in inflammatory cell number in the BAL fluid (BALF). (F) Similar findings are also observed for the eosinophil count in the BALF. Statistical significance was calculated by the means of a one-way ANOVA, where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ ($n \geq 3$) for each group. (D) Significant results are for the PBS or S28 group compared with the POS group.

the role of adenosine and purine receptors in regulation of inflammatory response and AHR (24, 25).

Of the top 20 most significantly altered markers in plasma and BALF, only three markers (tetradecanoyl-L-carnitine, tetradienyl-L-carnitine, and O-decanoyl-L-carnitine) are overlapping and shifted in the same direction in allergen-challenged animals compared with PBS-challenged

animals. When comparing the top metabolites altered by allergen challenge and shifted to levels observed in the PBS group after S28 treatment, only a few markers are overlapping between the BALF and plasma. Lysine and guanosine are both altered by challenge, and reverted to baseline levels after S28 treatment. However, the direction of the change in guanosine levels is different in BALF and in plasma; although allergen

challenge causes an increase in guanosine levels in the BALF, a decrease is observed in the plasma.

Based on our initial findings, our study can be extended in many directions. Metabolomic profiles vary with the severity of the asthmatic disease, and therefore the utility of our findings from a female population of NHPs with acute asthma needs to be compared with male subjects

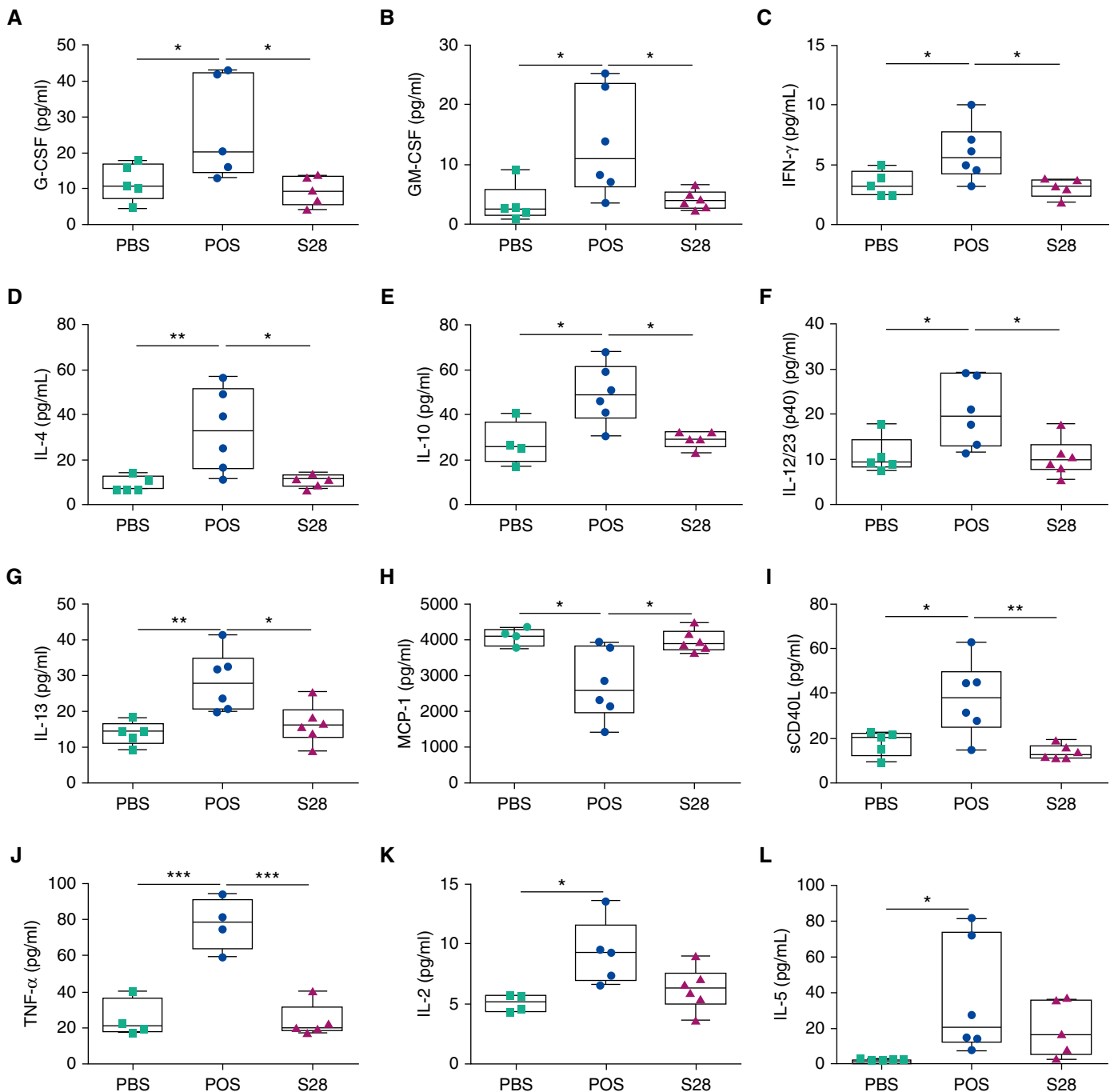


Figure 3. Assessment of cytokine concentration in BALF after *A. suum* allergen challenge in S28-treated and nontreated animals. All cytokines presented in A–L are significantly different between PBS and POS groups. In S28-treated and allergen-challenged primates (S28), excluding IL-2 (K) and IL-5 (L), all other cytokines are shifted toward values observed in PBS-challenged animals. Statistical significance was calculated by the means of a one-way ANOVA, where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ ($n \geq 4$ per group and samples were collected 48 h after the final allergen challenge). G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; MCP-1 = monocyte chemoattractant protein-1; sCD40L = soluble CD40 ligand.

and in different states of the disease (26). We also require further analysis to determine if the metabolomic markers that we have identified to be associated with *A. suum*-induced asthma are comparable

to other models created using allergens that are more relevant to humans.

Other studies characterizing the metabolomic signature in experimental models of asthma have been documented.

In mice, metabolites, urea-1-carboxylate and ornithine, have been shown to positively and negatively correlate with AHR, respectively (27). Pathways influenced by allergen challenge include purine

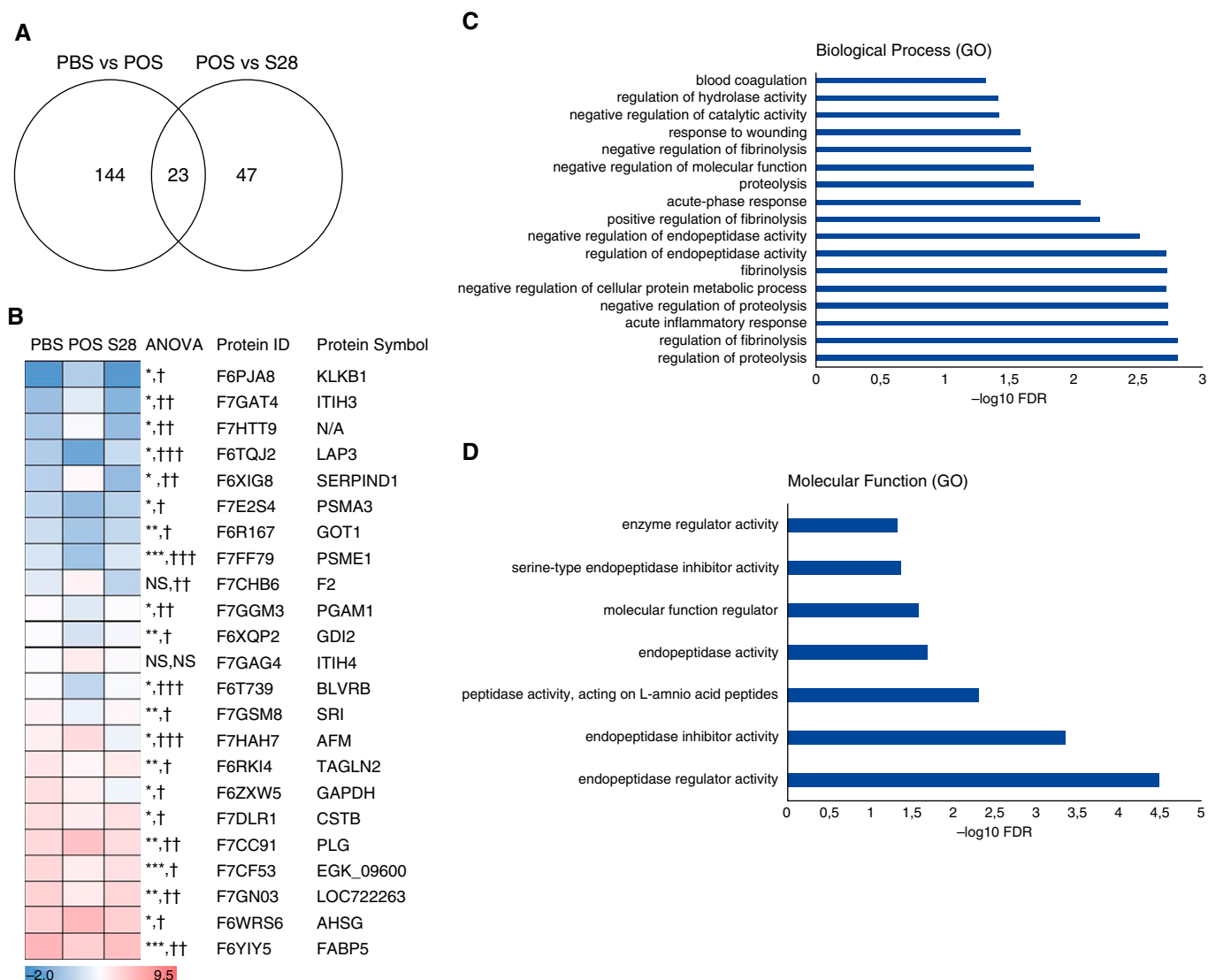


Figure 4. Protein changes in BALF associated with allergic asthma. (A) The levels of 167 proteins were changed in the BALF 48 hours after challenge with *A. suum* compared with the nonallergic group (PBS). The levels of 70 proteins were significantly different between vehicle-treated allergic nonhuman primates (NHPs; POS) and S28-treated allergic NHPs (S28). (B) A total of 23 proteins overlap between PBS versus POS and POS versus S28 comparisons representing proteins that are altered by *A. suum* challenge and corrected by S28 treatment. Data are presented as heat map of log-transformed values. (C) Gene Ontology (GO) enrichment analysis (biological process) of 23 proteins overlap between the two comparisons (PBS versus POS and POS versus S28). (D) GO enrichment analysis (molecular function) of 23 proteins overlap between the two comparisons (PBS versus POS and POS versus S28). Statistical significance was calculated by the means of *t* test when comparing two groups, and ANOVA followed by Bonferroni correction when comparing three groups. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 between PBS and POS groups, and †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.001 between POS and S28 groups (*n* ≥ 4 per group). FDR = false discovery rate; NS = not significant.

metabolism, sphingolipid metabolism, arginine and proline metabolism, glycerophospholipid metabolism, and neurotrophin signaling pathway (27, 28). Recent studies have also demonstrated that, as for many other types of markers, the time at which sample was collected after allergen challenge can cause variations in the metabolomic signature (27). We profiled the metabolites at 48 hours after final allergen challenge; however, studying

earlier time points, such as 6 and 24 hours, as done by Quinn and colleagues (27), in a mouse model of experimental asthma might provide us with additional valuable information on the disease. Furthermore, metabolomics analysis was done in both the BALF and the plasma in parallel, largely to identify overlapping markers in proximal versus systemic fluids. We found that majority of markers are differently expressed in these two materials. There are

also many differences between expression of markers in the two sources after allergen challenges, so the difference in the expression associated with the drug treatment needs to be assessed, comparing the same type of fluids in each experimental group.

Interestingly, results of combined omics analyses were complementary. Both proteomics and metabolomics signatures indicate the involvement of protein degradation/metabolism pathways, as

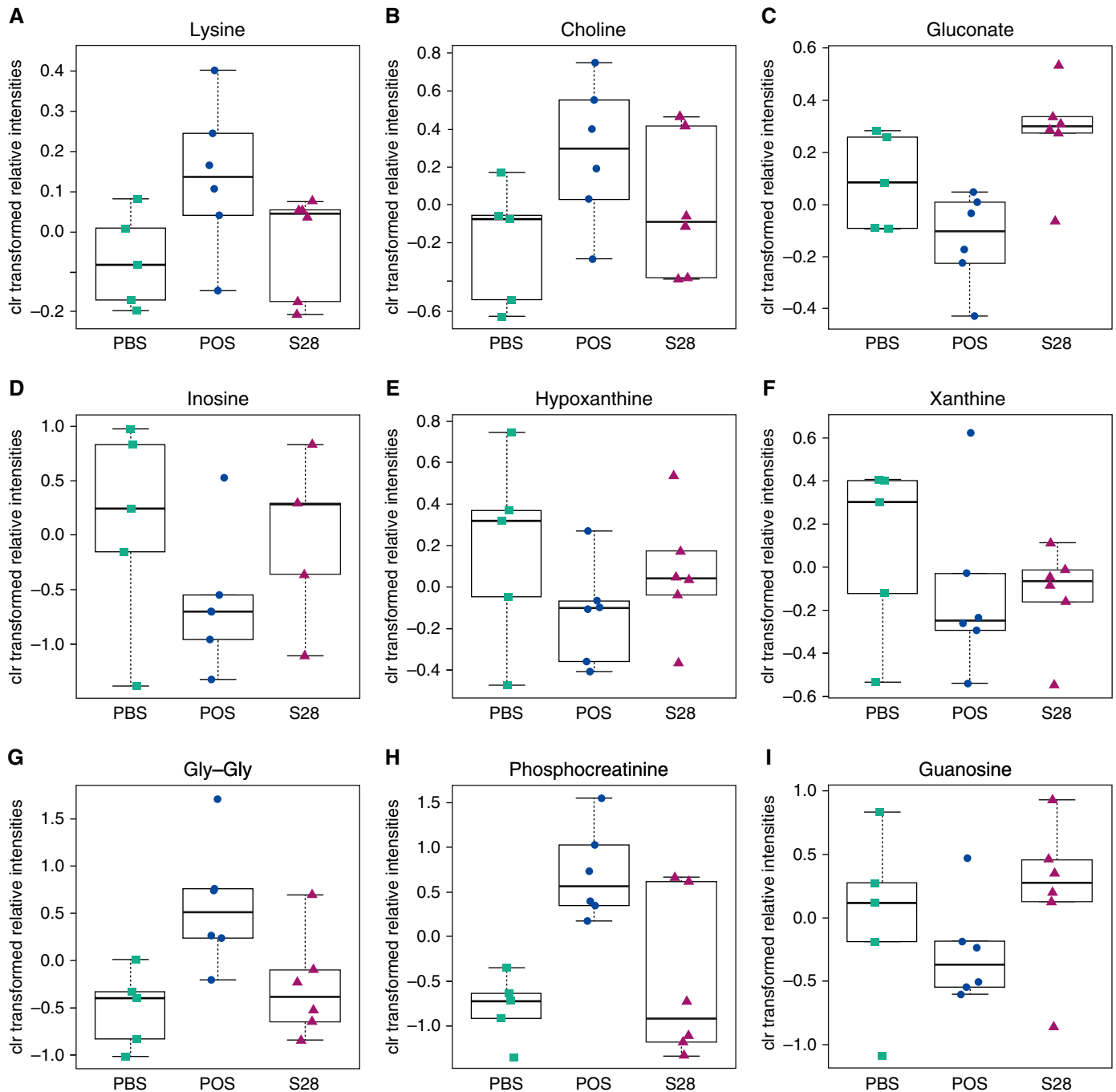


Figure 5. Metabolites altered by allergen exposure and corrected by S28 in plasma. Box plots of centered log ratio-transformed and mean centered metabolites changed by the aerosol challenge with *A. suum* antigen (POS) and returned toward the physiological levels (PBS) by treatment (S28) in plasma samples. Metabolites presented are lysine (A), choline (B), gluconate (C), inosine (D), hypoxanthine (E), xanthine (F), glycine-glycine (gly-gly) (G), phosphocreatinine (H), and guanosine (I). Data were transformed by centered log ratio (clr) transformation and mean centered ($n \geq 5$ per group and samples were collected 48 h after the third allergen challenge).

suggested by STRING and GO analysis of differential protein expression and appearance of protein degradation metabolites, such as dipeptides glycine-phenylalanine and glycine-glycine. Whereas in BALF, glutathione and glutathione disulfate levels were dramatically decreased

following allergen challenges. Decrease in the levels of these two metabolites important for oxidative stress homeostasis was prevented in allergen-challenged animals treated with S28.

Other asthma models have been developed in rhesus or cynomolgous monkeys using house dust mite and birch

pollen allergen (29–31). These models would be useful in determining if S28 can also confer protection against common human allergens. *In vitro*, studies involving blood lymphocytes from allergic patients have indicated that a protective effect may also be observed in patients. $CD4^+$ T cells

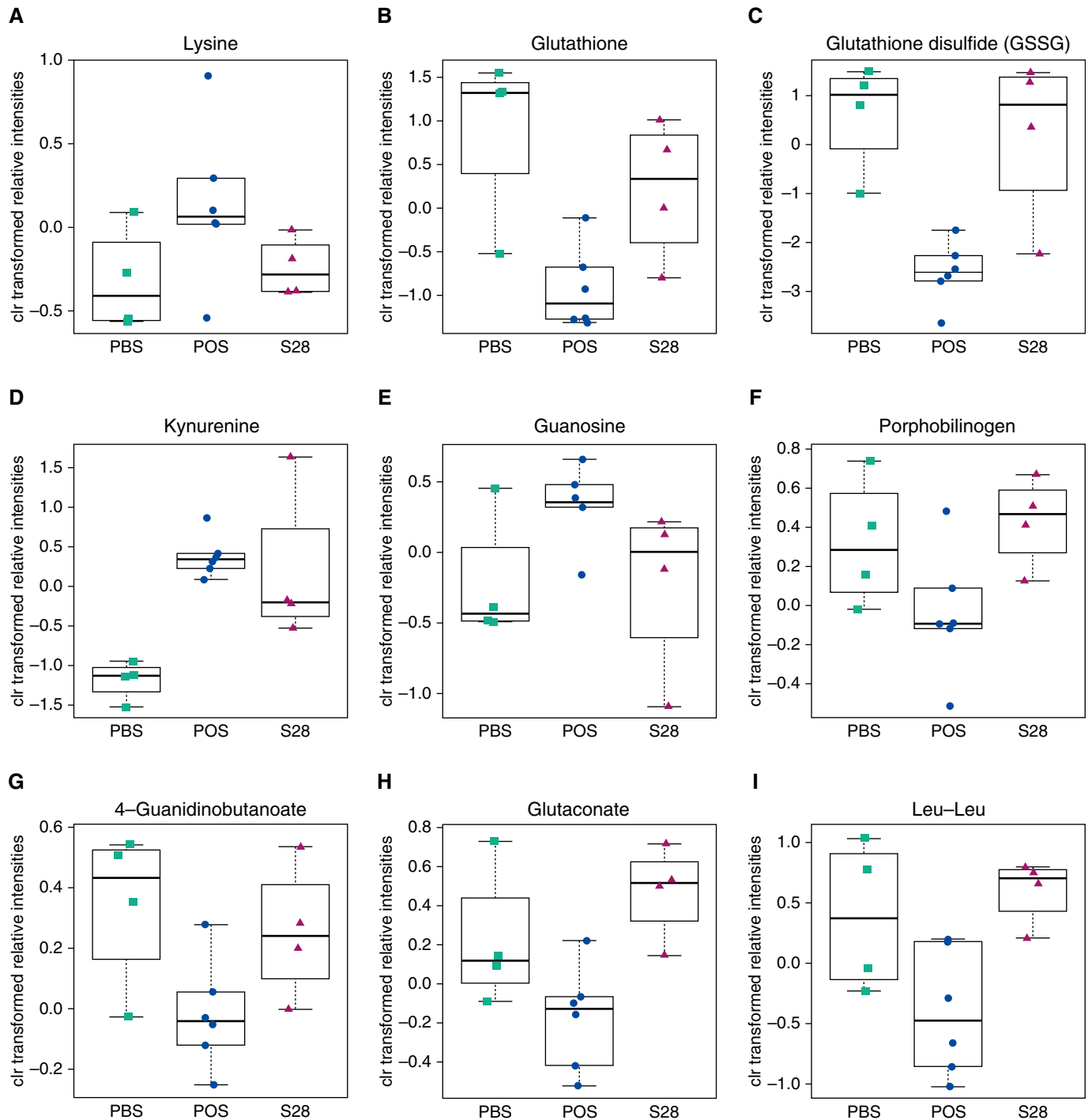


Figure 6. Metabolites altered by allergen exposure and corrected by S28 in BALF. Box plots of centered log ratio-transformed and mean centered metabolites changed by the aerosol with *A. suum* antigen (POS) and returned toward the physiological levels (PBS) by treatment (S28) in BALF samples. Metabolites presented are lysine (A), glutathione (B), glutathione disulfide (GSSG) (C), kynurenine (D), guanosine (E), porphobilinogen (F), 4-guanidinobutanoate (G), glutaconate (H), and leucine-leucine (Leu-Leu) (I). Data were transformed by clr transformation and mean centered ($n \geq 4$ per group and samples were collected 48 h after the third allergen challenge).

specific for ampicillin or house dust mite antigens from peripheral blood of allergic donors treated with S28 are induced to produce IFN γ , whereas their ability to

make IL-4 is decreased or eliminated, shifting the overall cytokine profile from Th2 to a Th0 or Th1 (32). Similarly, PBMN cells isolated from patients with atopic

dermatitis and allergic rhinitis stimulated with CD40 and IL-4 showed a decrease in IgE production after S28 treatment, an effect mediated in an IFN γ -dependent

manner (33). In our study, we observe a decrease in IFN γ after S28 treatment, and this difference could be due to study type and design, tissue tested and time point of analysis after allergen exposure.

S28 has also been explored as a possible adjuvant in specific immunotherapy. Epicutaneous immunization with recombinant birch pollen allergen combined with S28 induces IFN γ producing CD4⁺ T cells in lymph nodes and allergen-specific IgG2 antibodies. Prophylactic epicutaneous immunization before allergen sensitization and challenge can prevent the production of allergen-specific IgE, in addition to lung inflammation and hyperresponsiveness (15).

Although safe, TLR7 agonists, such as S28, are limited by their systemic release and dispersion of proinflammatory cytokines, which, in turn, cause unwanted side effects. In a clinical study involving S28 as a treatment of hepatitis C infection, the dose of S28 used induced Th1 cytokines which often caused fever, fatigue, headache, shivering, and influenza-like symptoms (34). In both our previous studies involving mice and rats as well in this study, the dose of S28 used decreased the level of Th1 and Th2 cytokines. No fever or other side effects were observed in the S28-treated asthmatic monkeys involved in our study in which the drug was administered orally, and none of the NHPs were infected with hepatitis C virus. Using a mouse model of allergic asthma, we have also previously established that S28 treatment is

efficacious, regardless of the route of the treatment (oral, intranasal, and systemic delivery [intraperitoneal]; data not shown). Systemic side effects could potentially be reduced in infected animals by localizing the action of the drug. Various mouse and rat models of OVA-induced asthma have demonstrated that S28 can be delivered intranasally or intratracheally to confer protection against asthma, by decreasing Th2 cytokine production, and decreasing airway remodeling and AHR (12, 35, 36). Delivery of synthetic TLR7 agonist 4-[6-amino-8-hydroxy-2-(2-methoxyethoxy)purin-9-ylmethyl]benzaldehyde coupled to mouse serum albumin to the lungs, causes a prolonged local cytokine production at values 10 times greater than what is measured in the serum (37).

In our studies, we demonstrated that various phenotypes are ameliorated when the allergen-sensitized animals are treated 24 hours before each allergen challenge. However, this prophylaxis treatment method may not be feasible for patients with asthma when they cannot predict the onset of an allergen challenge. Therefore, it is also important to determine the duration of the protective effects of S28, and to determine the treatment interval that would be sufficient to protect sensitized subjects against allergic asthma symptoms, including shortness of breath, AHR, infiltration of inflammatory cells into the lungs and mast cells degranulation triggered by excessive IgE levels. However, studies have

shown that TLR7 stimulation 30 minutes after OVA exposure can also prevent AHR measured 24 hours after allergen challenge, implying its potential as a rescue medication and not only as a prophylactic one (38). Furthermore, the attenuation of inflammatory cell recruitment after TLR7 stimulation by active synthetic derivatives can persist for up to 40 days after treatment, even with repeated allergen challenge (39).

Based on our studies done in mice, rats, and NHPs, the culmination of our work on S28 and allergic asthma have demonstrated that S28 can prevent phenotypes of acute and chronic asthma. These antiinflammatory effects of S28 can be observed against asthma induced by multiple different types of allergens. Additional experiments exploring the effect of S28 against human relevant allergens and as a rescue medication would provide a better understanding of the clinical potential of this drug. ■

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