

Genetic and Pharmacological Evaluation of Cathepsin S in a Mouse Model of Asthma

Kathleen Deschamps¹, Wanda Cromlish², Sean Weicker¹, Sonia Lamontagne², Sarah L. Huszar¹, Jacques Yves Gauthier³, John S. Mudgett⁴, Alain Guimond⁵, Raymond Romand⁵, Nelly Frossard⁵, M. David Percival², Deborah Slipetz¹, and Christopher M. Tan¹

Departments of ¹In Vivo Sciences, ²In vitro Sciences, and ³Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada; ⁴Genetically Engineered Models Department, Merck Research Laboratories, Rahway, New Jersey; and ⁵The Mouse Clinical Institute, Strasbourg, France

Cathepsin S (Cat S) is predominantly expressed in antigen-presenting cells and is up-regulated in several preclinical models of antigen-induced inflammation, suggesting a role in the allergic response. Prophylactic dosing of an irreversible Cat S inhibitor has been shown to attenuate pulmonary eosinophilia in mice, supporting the hypothesis that Cat S inhibition before the initiation of airway inflammation is beneficial in airway disease. In addition, Cat S has been shown to play a role in more distal events in the allergic response. To determine where Cat S inhibition may affect the allergic response, we used complementary genetic and pharmacological approaches to investigate the role of Cat S in the early and downstream allergic events in a murine model of antigen-induced lung inflammation. Cat S knockout mice did not develop ovalbumin-induced pulmonary inflammation, consistent with a role for Cat S in the development of the allergic response. Alternatively, wild-type mice were treated with a reversible, highly selective Cat S inhibitor in prophylactic and therapeutic dosing paradigms and assessed for changes in airway inflammation. Although both treatment paradigms resulted in potent Cat S inhibition, only prophylactic Cat S inhibitor dosing blocked lung inflammation, consistent with our findings in Cat S knockout mice. The findings indicate that although Cat S is up-regulated in allergic models, it does not appear to play a significant role in the downstream effector inflammatory phase in this model; however, our results demonstrate that Cat S inhibition in a prophylactic paradigm would ameliorate airway inflammation.

Keywords: cathepsin S; inflammation; lung; mouse; asthma

Asthma is a heterogeneous chronic inflammatory disease of the airways that is characterized by reversible airflow limitation and recurrent episodes of coughing, breathlessness, and wheezing. The classical features of asthma include inflammatory cell infiltration into the lung, resulting in pulmonary tissue inflammation, bronchoconstriction, increased mucus production, and elevated serum IgE. These alterations are also associated with an exaggerated sensitivity to nonspecific stimuli (i.e., airways hyperresponsiveness [AHR]). Persistent inflammation in airway tissues can lead to structural airway remodeling and consequently airway obstruction that is not fully reversible and the progressive loss of lung function over time. Despite our knowledge of the pathophysiological endpoints in response to inhaled antigens, our comprehension of asthma disease pathogenesis and processes is less well appreciated.

After inhaled allergen exposure, antigen processing and presentation by major histocompatibility complex class II molecules on the surface of antigen-presenting cells (APCs) plays a critical role in the initiation of an effective pulmonary immune response. Several lysosomal proteases have been implicated at multiple stages during the presentation process (reviewed in Ref. 1). In particular, cathepsin S (Cat S), a cysteine protease belonging to the CA1 papain superfamily, is highly expressed in the spleen and is found in professional APCs, such as dendritic cells, B lymphocytes, and macrophages, as well as in epithelial cells and type II alveolar cells. The functions of Cat S in antigen processing and peptide presentation are (1) processing antigenic peptides and (2) proteolysis of the class II-associated invariant chain to confer competency for peptide loading (2). Additional findings infer an effector role for Cat S in human asthma, such as the modulation of serum Cat S protein levels in patients with asthma after methylprednisolone therapy (3). Robust evidence can also be found in pre-clinical models. In particular, Cat S mRNA and protein expression are elevated in several antigen-driven lung (and systemic) mouse models of inflammation (4–10). Mechanistically, studies indicate that Cat S expression is induced in response to proinflammatory cytokines, including IL-13, IFN γ , and IL-1 β , suggesting a downstream effector role for Cat S (11, 12). Based on the cumulative evidence, modulation of Cat S activity may represent an attractive approach to modulate the asthmatic phenotype.

We have used pharmacological and genetic tools to address the role of Cat S in allergy *in vivo*. The sulfone Compound 6 is a potent, reversible, and orally bioavailable mouse Cat S inhibitor that is highly selective versus mouse cathepsins B, K, and L. Functionally, Compound 6 inhibits antigen presentation in a mouse cell-based assay and exhibits low potential for off-target activity (13, 14). However, due to nonlinear pharmacokinetic properties of Compound 6 in rodents, we used a sulfoxide pro-drug, Compound 7, which is rapidly converted *in vivo* to the parent sulfone Compound 6 (13). After oral administration of the Compound 7, 24-hour coverage was achieved when administered as a single dose or provided to mice as rodent chow. In addition to pharmacological studies using Compound 7 to address the role of Cat S in allergic inflammation, we used genetically modified mice null for Cat S. These tools enabled us to study whether inhibition of Cat S activity (pharmacologically or genetically) would modify antigen-induced airway inflammation in prophylactic or therapeutic treatment models.

MATERIALS AND METHODS

Sensitization and Challenge with OVA: Cat S Knockout Mice

On Days 0 and 7, wild-type (WT) and Cat S knockout (KO) mice were systemically sensitized by intraperitoneal injection of 50 μ g ovalbumin (OVA) (Grade V; Sigma-Aldrich, St. Louis, MO) in 0.1 ml of a 0.5 mg/ml suspension containing 20 mg/ml aluminum hydroxide (Al(OH) $_3$;

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Correspondence and requests for reprints should be addressed to Christopher M. Tan, Ph.D., Department of In Vivo Sciences, Central Pharmacology, Merck Frosst Centre for Therapeutic Research, 16711 Trans Canada Highway Kirkland, QC, Canada, H9H 3L1. E-mail: christopher_tan@merck.com

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Sigma-Aldrich) in sterile saline (0.9%). On Days 18, 19, 20, and 21, mice were anesthetized via intraperitoneal injection with 100 μ l ketamine (50 mg/kg) (Imalgene, Merial, Lyon, France)/xylazine (3.3 mg/kg) (Rompun, 2%; Bayer, Etobicoke, ON, Canada). Anesthetized mice were intranasally administered 10 μ g OVA in saline (12.5 μ l of 0.4 mg/ml solution) or saline alone. Twenty-four hours after the final intranasal instillation (Day 22), mice were assessed for noninvasive pulmonary mechanics and killed for bronchoalveolar lavage (BAL).

Sensitization and Challenge with OVA: Cat S Pharmacological Inhibitor Studies

On Day 0, WT animals were systemically sensitized by intraperitoneal injection of 20 μ g OVA in 0.4 ml of a 5 mg/ml suspension of $\text{Al}(\text{OH})_3$ in 1 $\times$ PBS (Mediatech Inc, Laval, QC, Canada). On Day 7, animals were subjected to a second intraperitoneal sensitization of OVA (10 μ g in 0.2 ml of a 5 mg/ml $\text{Al}(\text{OH})_3$ suspension). On Days 14, 15, and 16, randomized mice were subjected to daily aerosol challenges (Devilbiss Pulmo-Aide Compressor/Nebulizer; Sunrise Medical Inc, Lasale, QC, Canada) of 0.5 $\times$ PBS or 5% OVA (diluted in 0.5 $\times$ PBS) for 20 minutes (nebulization rate, 0.5 ml/min) via whole body exposure (Whole Body Plethysmograph chambers; Buxco Electronics Inc, Wilmington, NC). Twenty-four hours after the final aerosol challenge (Day 17), mice were killed for BAL and tissue collection.

Compound Dosing: Cat S Pharmacological Inhibitor Studies

Compound 7 was administered via a powdered chow formulation at 0.005% and 0.05% w/w (5 and 50 mg/kg/d, respectively, based on powdered chow consumption of 5 g/d per mouse). Powdered chow formulation was prepared using geometric dilution with normal powdered chow (Teklad #2018; Harlan Teklad, Lachine, QC, Canada). WT mice were acclimated to regular powdered chow 4 days before dosing with the Compound 7 chow formulation. Mice treated with Compound 7 randomly received one of two different dosing paradigms: (1) prophylactic or (2) therapeutic. During the prophylactic paradigm, mice acclimated to powdered chow were fed chow containing Compound 7 4 days before antigen presentation (i.e., 4 d before the first intraperitoneal injection of OVA) and maintained on Compound 7 chow for the duration of the study. For the therapeutic challenge, mice presensitized with OVA were fed chow containing Compound 7 chow starting 24 hours before the first OVA aerosol challenge and maintained with Compound 7 chow until the last OVA challenge. The antiinflammatory glucocorticoid dexamethasone (dexamethasone sodium phosphate; Vetoquinol, Vineland, NJ) served as a positive control and was dosed via intraperitoneal injection at 1 mg/kg in a dosing volume of 5 ml/kg. Dexamethasone was administered to OVA presensitized mice starting 24 hours before the first OVA aerosol challenge and then 30 minutes before each of the three OVA aerosol exposures. Dexamethasone-treated mice received regular powdered chow.

Additional descriptions of the materials and methods used in this study (animals, BAL, lung function measurements, tissue preparation, and statistical analyses) are provided in the online supplement.

RESULTS

OVA-Induced Airway Inflammation and Airway Hyperreactivity: Cat S Null Mice Are Protected from OVA-Induced Inflammation but Not AHR

The OVA sensitization and challenge paradigm of the allergic lung inflammation model induces many of the key pathophysiologies associated with allergic asthma, including airway cellular recruitment, AHR to methacholine challenge, and the production of mucus in the airways.

We initially analyzed OVA-specific IgE levels in plasma from OVA-sensitized Cat S-null and WT mice exposed to PBS or OVA intranasal challenge. Intranasal PBS exposure did not elicit subsequent elevations in OVA-specific IgE in Cat S KO or WT mice and were not statistically different from each other (Figure 1). Intranasal OVA challenge in WT mice was associated with a significant increase in plasma IgE levels at each of the dilutions assessed compared with PBS-challenged WT mice

(80-dilution: 32-fold higher in OVA-versus PBS-challenged WT mice; 160-dilution: 24 $\times$; 320-dilution: 13 $\times$; each $P < 0.05$). In contrast, plasma OVA IgE levels were only marginally increased in OVA-challenged Cat S-null mice and were not statistically different relative to PBS-exposed Cat S-null mice (80-dilution: 5 $\times$; 160-dilution: 4 $\times$; 320-dilution: 3 $\times$). Two-way ANOVA revealed a significant difference in IgE levels between OVA-WT and OVA-Cat S KO mice (Figure 1).

To selectively evaluate the role of Cat S from the multiple cysteine proteases, we assessed lung inflammation endpoints in Cat S-null mice compared with WT mice in the OVA model. BAL was collected from WT and Cat S KO mice 24 hours after the final challenge with OVA or control PBS. WT mice exhibited a robust OVA-dependent increase in total airway cell infiltration. Relative to WT control mice, Cat S-null mice challenged with OVA exhibited an approximately 2-fold reduction in total airway inflammation (53% inhibition of the increase in total BAL counts above baseline) (Figure 2A). Differential cell count analysis revealed that the airways of Cat S-null mice exhibited a significant reduction in eosinophil recruitment (70% inhibition; $P < 0.05$) compared with WT mice after OVA sensitization and challenge (Figure 2B). KO mice also displayed a nonsignificant reduction in airway lymphocytes; no significant alterations in BAL macrophages were observed between WT and KO mice subjected to OVA, whereas a nonsignificant (~ 1.5 -fold) increase in neutrophils was seen in OVA-challenged KO versus WT mice.

To determine if Cat S KO mice would exhibit alterations in OVA-induced AHR, airway responsiveness to increasing concentrations of inhaled methacholine challenge in PBS- and OVA-challenged WT and Cat S KO mice was assessed. Airway reactivity to inhaled methacholine challenge was measured by whole-body noninvasive plethysmography 24 hours after the final PBS or OVA challenge. WT and Cat S-null mice challenged with PBS aerosol exhibited comparable baseline levels of methacholine-induced airway reactivity (Figure 3). WT mice challenged with OVA exhibited an approximately 2- to 2.5-fold significant increase in methacholine-induced airway reactivity. Cat S-null mice challenged with OVA aerosol exhibited comparable and significant methacholine-induced airway reactivity to methacholine (Figure 3). There was no significant difference in airway reactivity between WT and KO mice challenged with OVA, suggesting that, although the loss of Cat S in

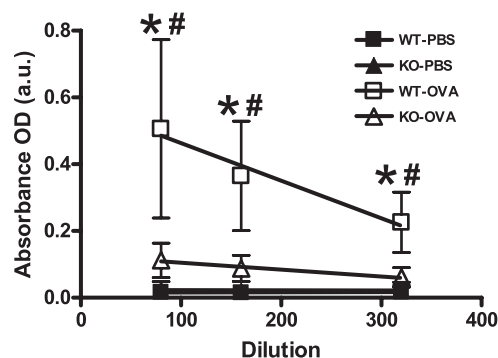


Figure 1. Ovalbumin (OVA)-specific IgE levels in plasma of wild-type (WT) or Cat S knockout (KO) mice challenged with OVA or PBS aerosol. Data are expressed as absorbance optical density (OD) values to assess relative differences in IgE between dilutions across all groups. * $P < 0.05$ compared with corresponding PBS aerosol challenge control dilution value from WT mice. # $P < 0.05$ compared with corresponding OVA aerosol challenge dilution value in KO mice. Each value represents the mean $\pm$ SEM ($n = 6$ animals per group). a.u. = arbitrary units.

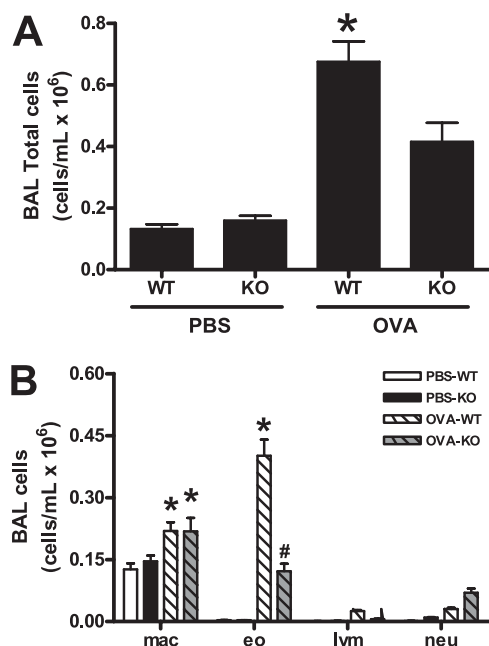


Figure 2. Cellular inflammation in bronchoalveolar lavage (BAL) fluid of WT or Cat S KO mice challenged with OVA or PBS aerosol. (A) Total and (B) differential cell counts in BAL fluid of mice challenged with PBS or OVA aerosol. * $P < 0.05$ compared with corresponding PBS aerosol challenge control. # $P < 0.05$ compared with corresponding OVA aerosol challenge. Each value represents the mean $\pm$ SEM ($n = 8$ animals per group). Eo = eosinophils; lym = lymphocytes; Mac = macrophages; neu = neutrophils.

OVA-challenged mice impairs antigen-induced eosinophil airway recruitment, it does not impair functional airway responses to inhaled stimuli, such as methacholine.

Prophylactic Dosing of a Pharmacological Cat S Inhibitor Reduces OVA-Induced Airway Inflammation

As a complementary approach to our genetic studies, we used Compound 7, an orally bioavailable, pro-drug inhibitor of Cat S. Our rationale for this pharmacological approach included (1) confirming the *in vivo* results obtained using genetically modified animals and (2) assessing whether Cat S inhibition at different time points modifies the inflammatory response. Specifically, these studies would enable us to determine if dosing naive mice with Compound 7 (relative to vehicle-dosed animals) throughout the OVA immunization/airway challenge period (i.e., prophylactic dosing) would be the pharmacological equivalent to Cat S KO mice and thus provide protection from developing an OVA-induced inflammatory response. In addition, we could ascertain the effect of therapeutic dosing of Compound 7 (i.e., before the PBS or OVA aerosol exposures only) in mice that had been preimmunized to OVA antigen. We focused on airway inflammation due to the observation that OVA-induced inflammation, and not AHR, was attenuated in Cat S KO mice versus WT mice. Specifically, these studies would allow us to evaluate at which point Cat S inhibition exerted antiinflammatory properties and if Cat S inhibition dosed therapeutically could modify downstream allergic lung pathological events.

We previously described Compound 6 as a potent and highly selective reversible Cat S inhibitor (13, 14). *In vitro* enzyme assay experiments revealed that Compound 6 potently inhibits mouse Cat S enzyme ($IC_{50} = 0.6$ nM) and that it has a 470-fold greater selectivity for Cat S over other cysteine proteases,

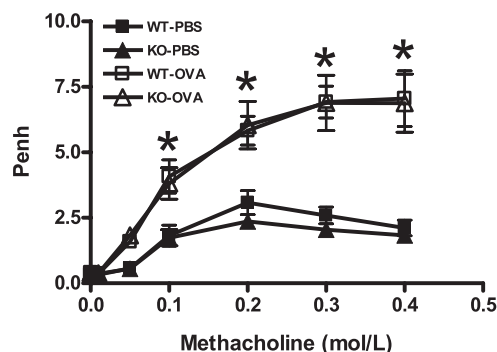


Figure 3. Airway hyperreactivity in OVA-sensitized WT or KO mice challenged with PBS or OVA aerosol. Mice were subjected to increasing concentrations of methacholine, and whole body plethysmography was performed to determine PenH. * $P < 0.05$ compared with corresponding PBS-challenged group. Each value represents the mean $\pm$ SEM ($n = 12$ animals per group).

including cathepsins B, K, and L. Because Compound 6 displays nonlinear mouse pharmacokinetic properties (13), we characterized the sulfoxide pro-drug Compound 7 (0.05%) in mouse chow. After 5 days of dosing, Compound 6 morning peak plasma levels averaged approximately 1 μ M (~ 23 -fold the mouse antigen presentation assay $IC_{50} = 44$ nM) (13, 14), ensuring adequate *in vivo* exposure. Thus, Compound 7 (0.05%) formulated in animal chow was provided to naive mice starting 4 days before initiating antigen sensitization, and animals were maintained on Compound 7 chow (or normal drug-free powdered chow) throughout the study. Mice fed drug-free powdered chow were dosed with the antiinflammatory glucocorticoid dexamethasone as a positive control via intraperitoneal injection before each OVA aerosol challenge. OVA-immunized mice were subjected to PBS or OVA aerosol challenge, and BAL was collected from mice dosed with powder chow, drug chow, and dexamethasone 24 hours after the final aerosol challenges. Consistent with our results with genetically modified mice, we observed a reduction in OVA-induced airway inflammation in mice prophylactically treated with Compound 7 compared with vehicle-treated control mice (58% reduction) (Figure 4A). By comparison, dexamethasone treatment completely inhibited OVA-induced cellular inflammation. Consistent with data obtained in KO mice challenged with OVA, differential BAL cell count analysis revealed that Compound 7 treatment significantly reduced the number of airway eosinophils after OVA challenge (71% reduction; $P < 0.05$) (Figure 4B). Drug treatment also resulted in a nonsignificant reduction in airway lymphocytes (48% inhibition); no significant alterations in neutrophils or macrophages were observed with Compound 7 dosing compared with vehicle control group. In contrast, dexamethasone potently inhibited OVA-induced airway eosinophils, lymphocytes, and neutrophils. Neither treatment modified the number of airway macrophages.

We evaluated the effect of prophylactic Compound 7 dosing on OVA-induced mucus production. Histological analysis of Alcian blue/periodic acid-Schiff-stained lung sections obtained from mice fed with normal vehicle chow and challenged with OVA aerosol revealed an increase in intensely stained, dark punctate regions indicative of mucus production in the airways, compared with control PBS aerosol-exposed mice fed with vehicle chow (Figure 5A; OVA-Veh versus PBS-Veh). In comparison, lungs sections from mice provided Compound 7 chow before OVA sensitization and OVA aerosol challenge exhibited reduced mucus staining, comparable to that observed

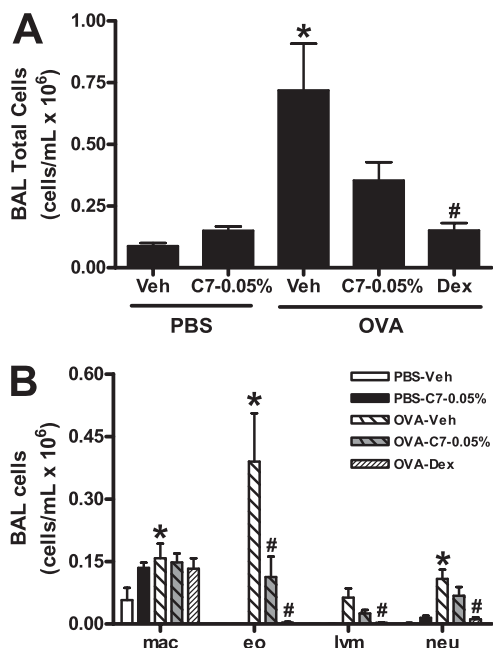


Figure 4. Cellular inflammation in BAL fluid of PBS or OVA-aerosol challenged mice provided vehicle chow (vehicle) or formulated with 0.05% Compound 7 (C7) versus mice dosed with 1 mg/kg dexamethasone (Dex). (A) Total and (B) differential cell counts in BAL fluid of mice challenged with PBS or OVA aerosol. * $P < 0.05$ compared with corresponding PBS aerosol challenge control. # $P < 0.05$ compared with corresponding OVA aerosol challenge. Each value represents the mean $\pm$ SEM ($n = 6$ –10 animals per group). Eo = eosinophils; lym = lymphocytes; Mac = macrophages; neu = neutrophils; Veh = vehicle-dosed mice. C7-0.05% = 0.05% Compound 7 in chow.

with dexamethasone treatment (Figure 5A; OVA-Veh versus OVA-C7-5, OVA-Dex). Quantitative morphometric assessment of Alcian blue/periodic acid-Schiff staining in lung sections ($n = 2$ –4 mice per group; > 15 airways per mouse per group) demonstrated an 8-fold increase in the mucus ratio in vehicle-dosed and OVA-challenged mice relative to vehicle-dosed and PBS-challenged mice. Prophylactic dosing of Compound 7 was associated with a 69% reduction in OVA-induced mucus production, a decrease in mucus ratio comparable to that observed with dexamethasone treatment (68% reduction) (Figure 5B).

To confirm that Cat S was inhibited *in vivo*, we harvested the spleens and analyzed major histocompatibility complex class II invariant chain p10 and Cat S protein levels as pharmacodynamic activity markers (Figure 6). We previously correlated inhibitor: Cat S enzyme occupancy with p10 levels in mouse splenocytes and demonstrated that p10 significantly accumulates when greater than 90% of Cat S enzyme is occupied, indicating that high fractional inhibition of Cat S is required for Ii p10 fragment accumulation (data not shown). In these studies, mice dosed with 0.05% Compound 7 showed significantly elevated levels of p10 compared with normal chow-fed mice (29-fold; $P < 0.05$) (Figure 6A), indicating potent Cat S inhibition. We used a second approach to confirm that Cat S was inhibited *in vivo*. *In vitro* studies examining Compound 6 dose-dependent inhibition of Cat S in THP-1 cells revealed that intracellular Cat S protein levels correspondingly increased in a dose-dependent fashion, consistent with our previous observations (14). In our studies, the level of Cat S protein found in spleen lysates was approximately 5 fold higher in mice treated with Compound 7 compared with vehicle-treated control mice

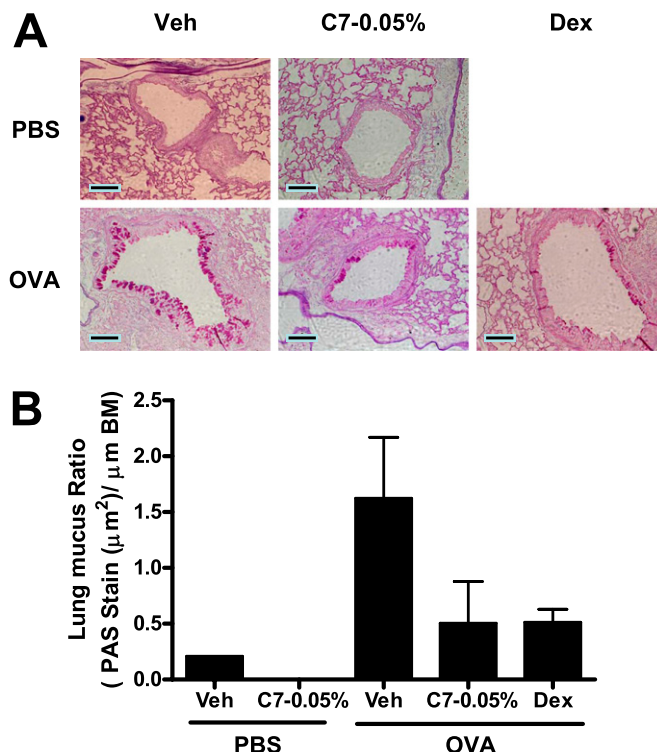


Figure 5. Mucus production in airways of PBS or OVA-aerosol challenged mice provided vehicle chow (vehicle) or formulated with 0.05% Compound 7 (C7) versus mice dosed with 1 mg/kg Dex. (A) Representative airways were assessed histologically by Alcian blue/periodic acid-Schiff (staining in lung tissue sections 24 hours after the final aerosol challenge). (B) Quantitation of mucus production ($n = 2$ –4 per group; > 15 airways per mouse per group) was performed as described. No Alcian blue/periodic acid-Schiff staining was observed in PBS-challenged mice; these mice were assigned a value of zero. C7-0.05% = 0.05% Compound 7 in chow. Bar, 50 μ m. BM = basement membrane.

($P < 0.05$ versus OVA-vehicle group) (Figure 6B), corroborating the inhibition observed after Compound 7 dosing. Finally, average morning peak plasma levels of Compound 6 in treated mice at the time of sample collection in this experiment were 2.5 μ M (~ 57 -fold the mouse antigen presentation assay IC₅₀). The activity of Compound 6 against cathepsins B, L, and S in mice *in vivo* were previously determined using a cathepsin-specific enzyme occupancy probe (14). At a dose providing plasma levels of close to 1 μ M Compound 6, Cat S was completely inhibited in lung and spleen, but there was no significant inhibition of Cat B and L in liver, spleen, lung, and kidney. In comparison, a higher dose providing plasma concentrations of approximately 30 μ M inhibited Cat B, L, and S in all tissues tested (14).

Therapeutic Dosing of a Pharmacological Cat S Inhibitor Does Not Protect Mice from OVA-Induced Airway Inflammation

We evaluated Compound 7 in a therapeutic dosing paradigm in the same OVA model to compare our results with those obtained with the prophylactic dosing paradigm. Mice that had been intraperitoneally immunized with OVA peptide antigen in the presence of adjuvant 2 weeks prior were provided with Compound 7-formulated chow (0.05 or 0.005%) or normal chow 1 day before and during the 3-day aerosol challenge. In this dosing paradigm, neither dose of Compound 7 had any effect on the OVA-induced airway inflammation as reflected by BAL total cell counts, in contrast to the potent anti-inflammatory effects of dexamethasone (90% reduction, $P < 0.05$ versus

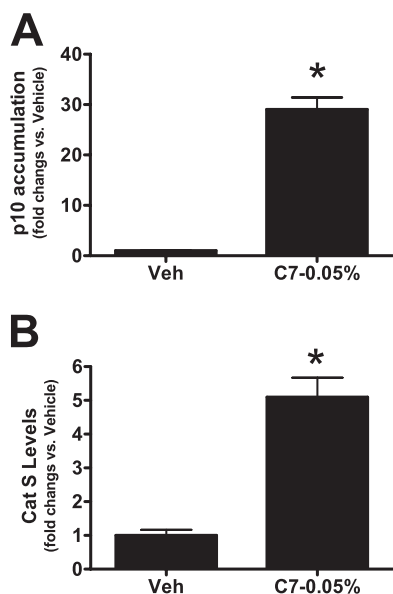


Figure 6. Splenic invariant chain p10 (A) and Cat S protein (B) levels in OVA-aerosol challenged mice provided vehicle chow (vehicle) or formulated with 0.05% Compound 7 (C7). Spleen homogenates resolved by gel electrophoresis were analyzed by immunoblotting for p10 and Cat S protein, followed by quantification with imaging software. Each value represents the mean $\pm$ SEM ($n = 6$ animals per group).

OVA-Vehicle group; see Figure E1A in the online supplement). Similar to our previous data, differential cell analysis revealed that dexamethasone significantly inhibited eosinophils, lymphocytes, and neutrophils, whereas the therapeutic dosing of Compound 7 had no effect on any airway infiltrating cell type (Figure E1B). These data are also in contrast to those observed with the prophylactic Compound 7 dosing paradigm, where a significant reduction in eosinophils was seen (Figure 4B).

Consistent with the lack of an effect on OVA-induced airway inflammation, Compound 7 in this dosing paradigm had no effect on OVA-induced mucus production. In control mice fed powdered chow, OVA aerosol challenge induced a robust increase in mucus staining compared with PBS aerosol challenge (Figure E2A; OVA-Veh versus PBS-Veh). Compound 7 therapeutic dosing had no qualitative or quantitative effect at either dose on OVA-induced mucus staining (Figures E2A and E2B; OVA-Veh versus OVA-C7-0.005%, OVA-C7-0.05%), in contrast to Compound 7 prophylactic dosing (Figure 5B).

A corresponding assessment of mouse spleen lysates and plasma Compound 7 levels confirmed that Cat S was effectively inhibited in this dosing paradigm. Dose-dependent accumulation in p10 levels and significant increases in Cat S protein were achieved with prophylactic dosing (Figure E3). Compound 6 plasma analysis confirmed adequate exposure (0.005%: 0.6 μ M; 0.05%: 3.1 μ M; $\sim$ 14-fold and $\sim$ 70-fold, respectively, the mouse antigen presentation assay IC₅₀).

DISCUSSION

Several studies have documented an increase in Cat S gene and/or protein expression in preclinical disease models, including those of pulmonary inflammation (4–10). Furthermore, using small molecule inhibitors, pharmacological inhibition of Cat S has been shown to reduce antigen-induced lung inflammation in several of these models (9, 10). Collectively, these findings demonstrate that elevated Cat S expression and function in disease can be pharmacologically modulated, which may be

beneficial for ameliorating the inflammatory response. Using genetic and pharmacologic tools, we further investigated the role of Cat S in the allergic response by using these approaches in an OVA-induced mouse lung inflammation model. Our findings demonstrate that Cat S gene knockout, or prophylactic dosing of a Cat S inhibitor in naive mice, reduces the lung inflammatory response induced by lung antigen challenge. In contrast, we found that therapeutic dosing of the Cat S inhibitor in antigen-experienced animals was ineffective in reducing OVA-induced inflammation. Collectively, the data suggest that Cat S plays a role during the initiation of the immune response to foreign antigen. Although inhibiting Cat S before the initiation of antigen lung exposure is associated with a reduced inflammatory phenotype, inhibiting Cat S during the ongoing antigenic/inflammatory response does not appear to reduce inflammation in the lungs of mice.

We observed that mice null for Cat S and WT mice dosed with Compound 7 before antigen exposure were protected from antigen-induced inflammatory responses. These animals exhibited diminished OVA-specific IgE, reduced cellular eosinophil recruitment to the lungs, and mucus secretion in the airways in response to OVA challenge. Despite a reduced inflammatory response, OVA-sensitized/OVA aerosol-challenged Cat S-null mice retained OVA-dependent changes in airway resistance to inhaled methacholine indistinguishable from WT mice. The findings suggesting a dissociation between lung inflammation and airway resistance should be tempered to acknowledge inherent limitations of Penh as a measure of airway hyperreactivity (15, 16). In spite of these caveats, dissociation between antigen-induced airway eosinophilic inflammation and methacholine-induced airway resistance has been observed in the characterization of several mechanistic pathways (17–20). For example, studies by Henderson and colleagues (21) demonstrated that CysLT1 receptor blockade was ineffective at modulating airway hyperreactivity despite reducing OVA-induced airway eosinophilia, cytokine production, and mucus hypersecretion. Thus, similar to other mechanisms, Cat S may exert a more predominant role in the development of airway inflammation, relative to airway responsiveness, in this mouse lung model. Alternatively, although Cat S-null mice or prophylactic Compound 7 dosing significantly decreased OVA-mediated airway eosinophils, airway cellular recruitment was not fully abrogated and OVA-induced airway neutrophilia was marginally increased in Cat S KO mice. It is therefore possible that the residual cellular populations (e.g., eosinophils, lymphocytes, and neutrophils) and reactive lung milieu remain sufficient to drive airway hyperresponsiveness in OVA-challenged Cat S-null mice. In addition to these other cell types, airway neutrophils have been documented to correlate with AHR (22–24). Consistent with this, it has been shown that prophylactic dosing of Cat S inhibitor can diminish ozone-induced airway neutrophils and reduces methacholine-induced AHR and BAL cytokines, such as IL-6 and TNF- α (10).

The results are consistent with a proximal role for Cat S in the allergic response. Our *in vivo* genetic and prophylactic pharmacological results are in accord with the comprehensive mechanistic *in vivo* studies by Reise and colleagues (9) demonstrating the proximal role of Cat S in the immune response. Impairment of OVA antigen processing and presentation correlated with decreased OVA-induced eosinophilia in the airways of mice dosed with the vinyl sulfone cysteine protease inhibitor morpholinurea-leucine-homophenylalanine-vinylsulfone phenyl (LVHS). Similarly, our studies with prophylactic Compound 7 dosing demonstrated potent Cat S inhibition during the initial immunization period. As a result, Cat S-inhibited animals display decreased OVA antigen-induced eosinophil recruitment

in the airways and a concomitant reduction in airway mucus secretion. These findings underscore the critical involvement of Cat S in the early stages of the immune response.

There is evidence that Cat S can modify the allergic response. Cat S is known to function as a secreted protease with elastolytic activity (1, 6, 8) and can directly contribute to the development of airway pathologies, such as tissue remodeling and destruction (11, 12). For example, in a tissue-targeted over-expression model of IFN- γ -induced lung injury, IFN- γ was shown to robustly induce Cat S expression. The demonstration that genetic and pharmacological inhibition of Cat S attenuated IFN- γ -induced DNA injury, apoptosis, lung alveolar remodeling, pulmonary emphysema, and inflammation highlights Cat S as a downstream effector, presumably via its elastolytic activity on tissue (12). Similarly, Cat S KO neonate mice are resistant to hyperoxia-induced lung damage and inflammation, in part attributable to the decreased elastolytic activity of Cat S (8). Therefore, because Cat S can directly contribute to airway pathologies, we ascertained if inhibiting Cat S therapeutically, in this pulmonary antigen-dependent allergic inflammation model, could similarly attenuate the inflammatory response. We found that pharmacological inhibition of Cat S in a therapeutic dosing paradigm did not attenuate OVA-induced inflammation or mucus production, in contrast to the antiinflammatory effect observed with prophylactic dosing of Compound 7. Immunoblot analysis of spleens harvested from mice dosed with Compound 7 in therapeutic and prophylactic modes revealed significant accumulation in p10 as well as Cat S protein, consistent with our previous findings (14) and confirming that Cat S was indeed inhibited in both dosing paradigms, relative to control mice dosed with powder chow. Our results suggest that inhibiting Cat S immediately before antigen challenge in mice previously exposed to that same antigen is ineffective at blocking the inflammatory response. Collectively, the findings are consistent with a predominant role during the initiation phase of the immune reaction, and not during the effector phase, at least in this antigen-driven lung model, where elastolytic activity may not be as critical. However, it is possible that therapeutic Cat S inhibitor dosing may be efficacious under conditions where its protease activity is more essential (e.g., in Cat S-dependent tissue injury models).

In summary, we used genetic and pharmacological approaches to illustrate that Cat S is critically required for antigen-induced lung inflammation. The combined use of these approaches extends the mechanistic understanding of Cat S in allergy and its therapeutic applicability. The results illustrating protection from developing OVA-induced eosinophilic inflammation when using Cat S-null mice or prophylactic dosing of Compound 7 highlight its role primarily during the initiation of the immune response to antigen. In parallel, the assessment of Compound 7, when dosed therapeutically, and the lack of a protective effect on OVA-induced lung responses indicate that Cat S does not appear to play a significant role in the downstream effector inflammatory response, at least in this model. To our knowledge, these are the first preclinical data to describe the effect of a Cat S inhibitor on antigen-induced lung inflammation when dosed therapeutically. Nonetheless, our data illustrate that prophylactic dosing is effective at reducing antigen-induced eosinophilic airway inflammation and thus constitutes an appealing mechanism to target prophylactically.

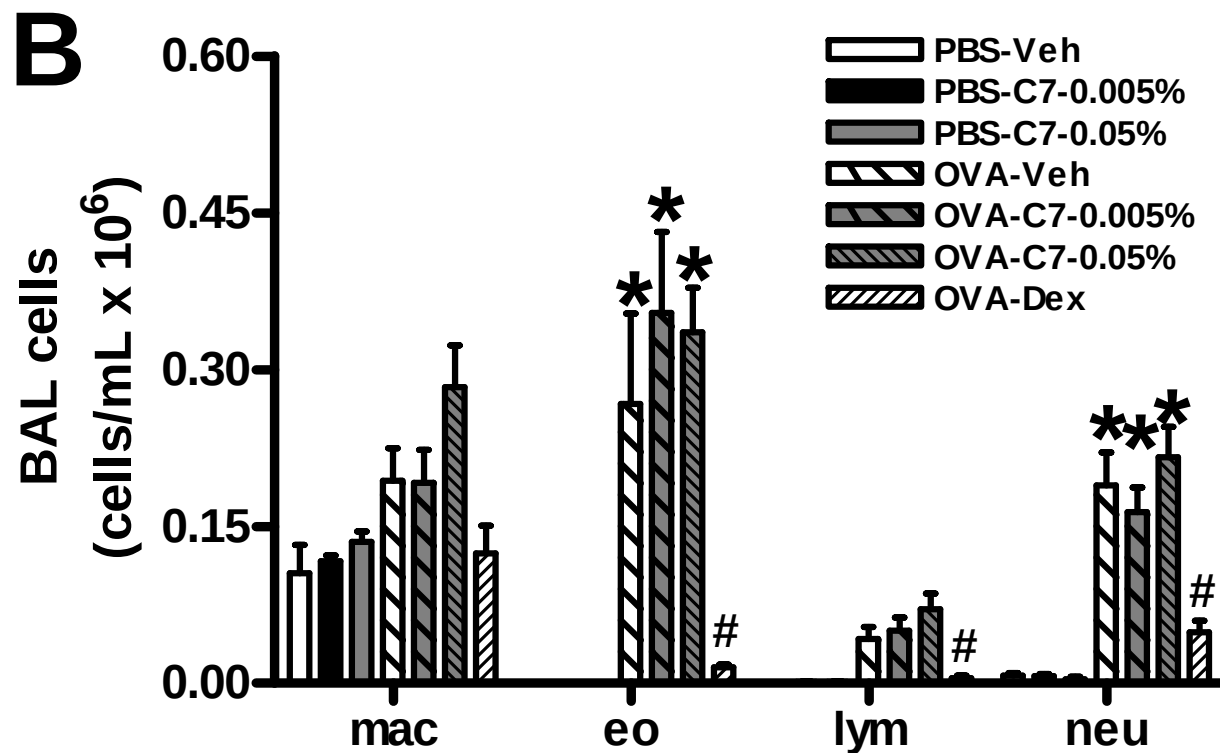
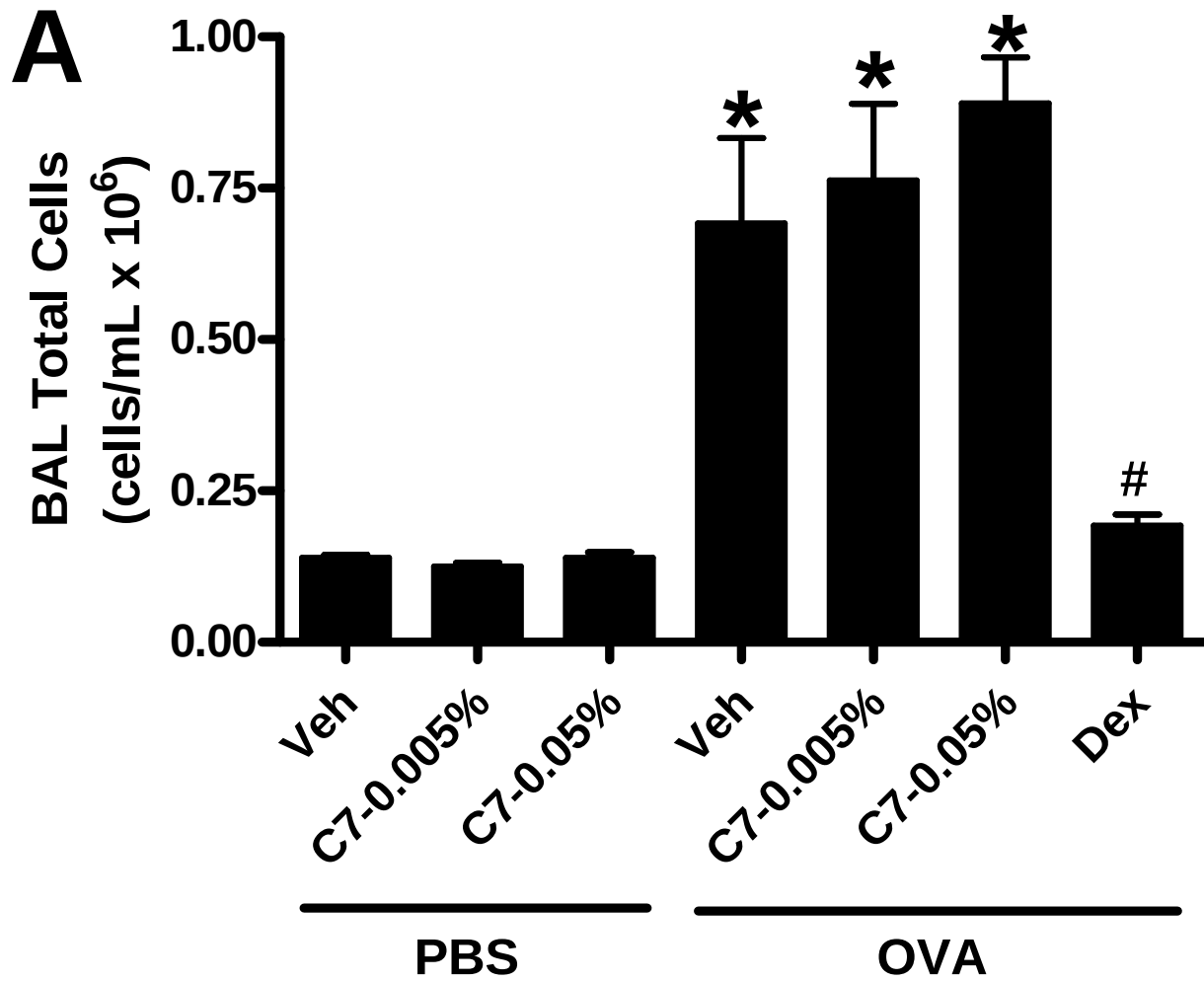
Author disclosure: W.C. is a full-time employee of Merck Frosst Canada and owns stock for \$1,001 to \$5,000. K.D. is a full-time employee of Merck Frosst Canada and owns stock for more than \$100,001. N.F. has received industry-sponsored grants from AB Science for \$1,001–\$5,000; 4SC, NewThera, and Polyphor Ltd. for \$10,001–\$50,000 each. N.F. has also received sponsored grants from CNMRT for \$10,001–\$50,000; ANR and VLM for more than \$100,001. J.Y.G. is a full-time employee of Merck and owns stock up to \$1,000. M.D.P. is a full-time employee

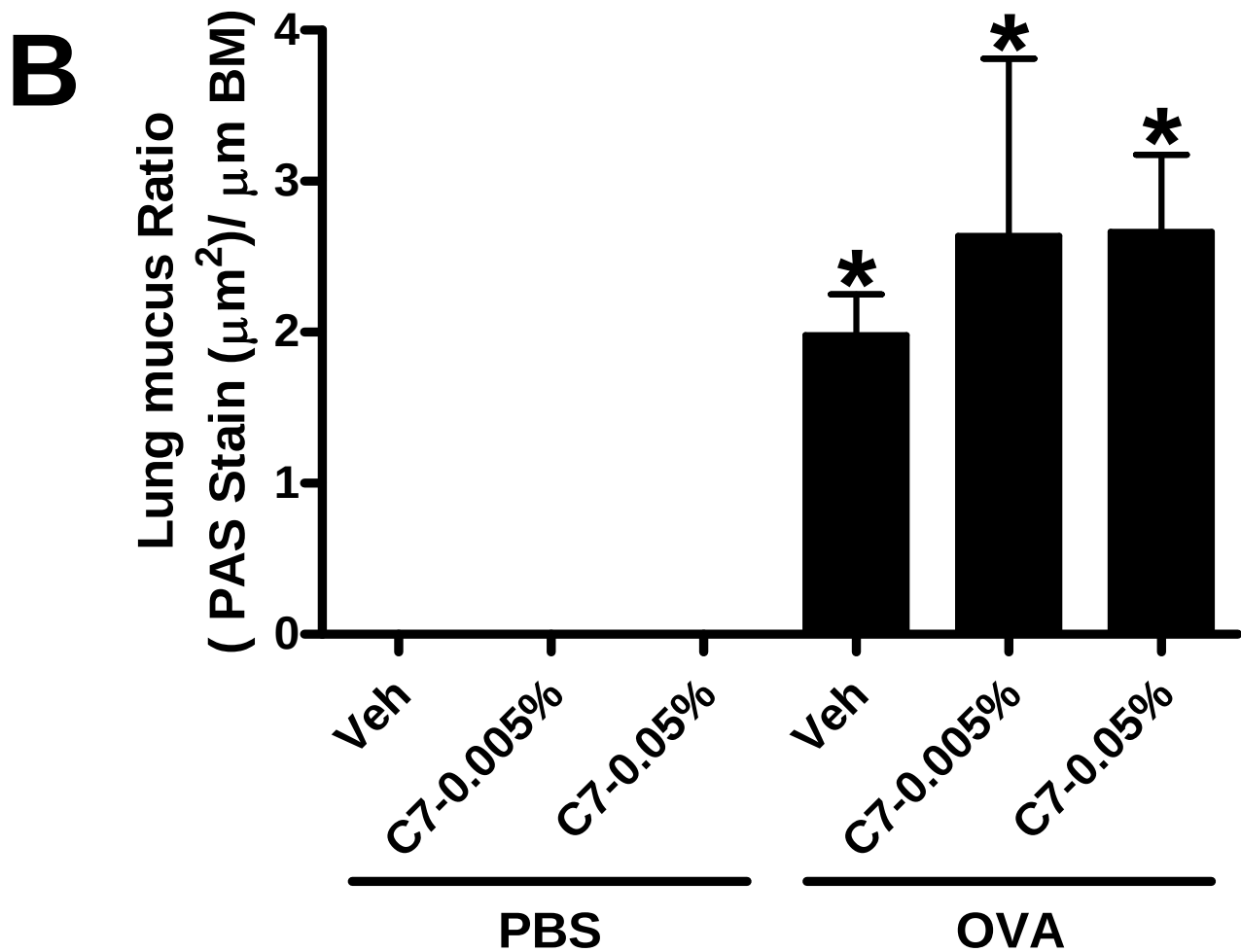
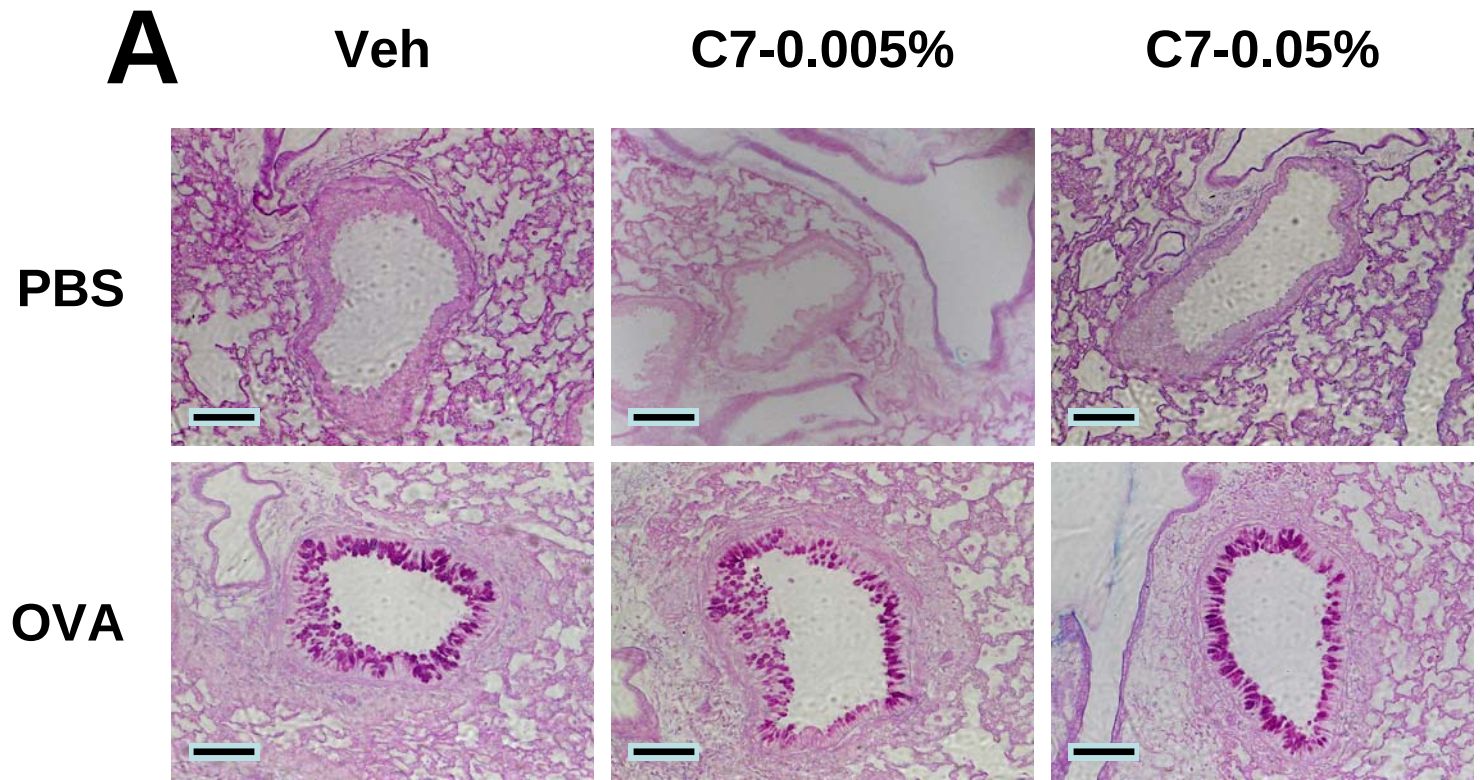
of Merck Frosst Canada and owns stock for \$50,001–\$100,000. C.T. is a full-time employee of Merck and Co. and owns stock for \$10,001–\$50,000. S.W. is a full-time employee of Pfizer Canada. J.S.M. is a full-time employee of Merck and Co. and owns stock for more than \$100,001. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

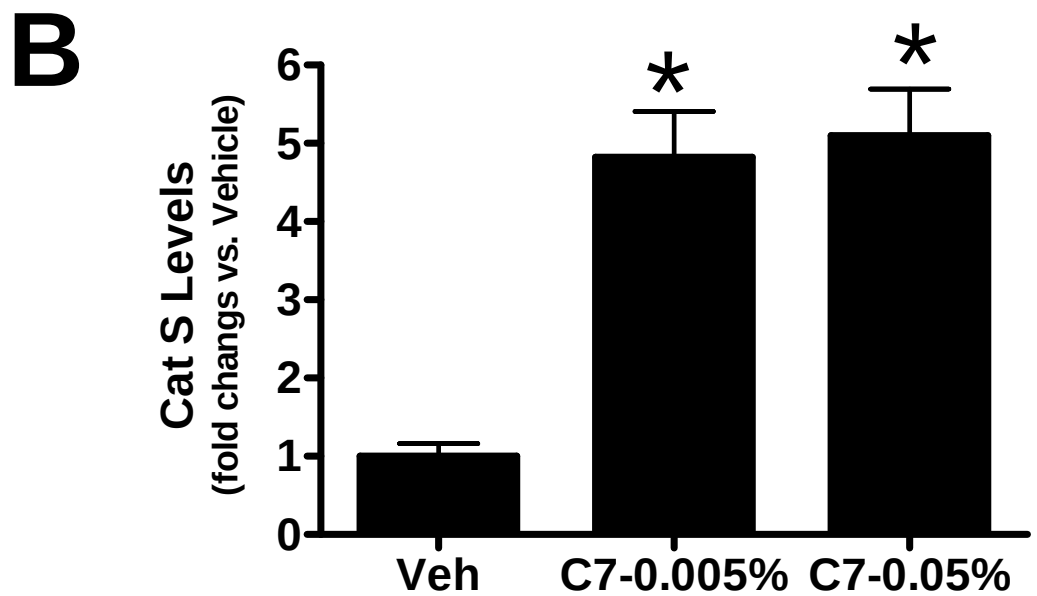
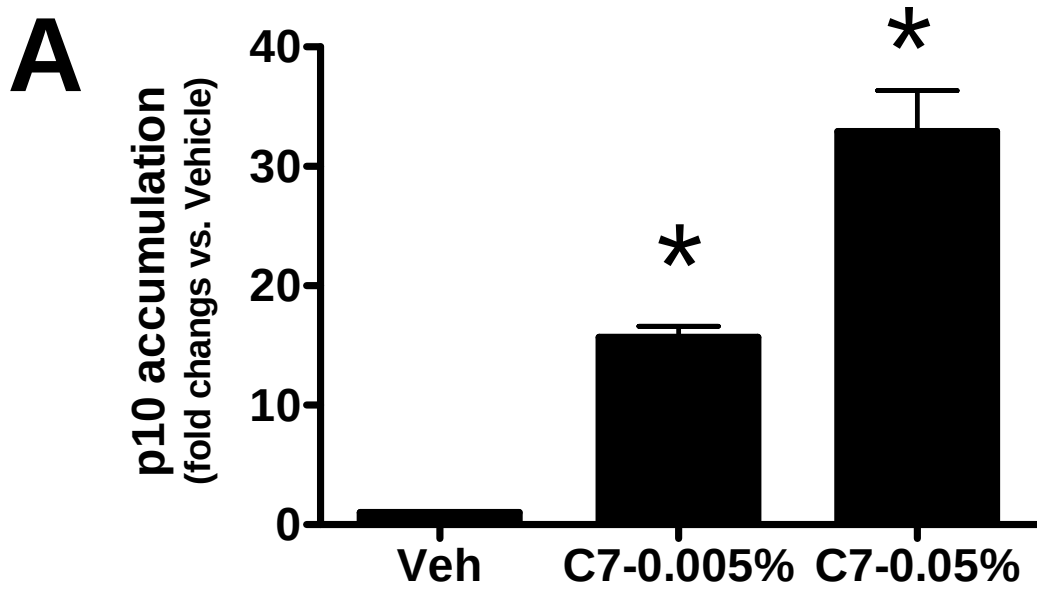
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Online Data Supplement

Genetic and Pharmacological Evaluation of Cathepsin S in a Mouse Model of Asthma

Kathleen Deschamps*, Wanda Cromlish[†], Sean Weicker*[‡], Sonia Lamontagne[†], Sarah L. Huszar*^{###}, Jacques Yves Gauthier[¶], John S. Mudgett[♢], Alain Guimond^{¶¶}, Raymond Romand^{¶¶}, Nelly Frossard^{¶¶}, M. David Percival[†], Deborah Slipetz*^{††}, and Christopher M. Tan*[§]

Methods and Materials

Animals

Wild type male BALB/c mice (20-25g) were purchased from Charles River Laboratories (St-Constant, Quebec). Mice were housed under a 12-hour light/dark cycle and were allowed access to food and water *ad libitum*. All animal protocols were reviewed and approved by the institutional Animal Care Committees at the Merck Frosst Centre for Therapeutic Research and the Mouse Clinical Institute. Cat S knockout mice (15) bred onto the BALB/c background were generously provided by Dr. Harold A Chapman (UCSF) for *in vivo* studies at the Mouse Clinical Institute.

Bronchoalveolar lavage

Twenty four hours after the last PBS or OVA challenge, mice are deeply anesthetised via i.p. administration of sodium pentobarbital (120mg/kg i.p.). Animals were tracheostomized and bronchoalveolar lavage (BAL) was performed using 0.5mL 1X PBS. Total cell numbers in the recovered volume (~70-80% recovery) were determined via automated cell count (Celldyn 3500). Cytospin slides of BAL cells were prepared using a cytocentrifuge (Cytospin; Shandon, Pittsburgh, PA) and stained with May-Grunwald-Giemsa stain. Total and differential cell counts were determined by light microscopy from a count of up to 400

cells. Remaining BAL supernatant was frozen individually in aliquots and stored at -80°C.

Lung function Measurement: Cat S knockout mice

Twenty-four hours after the last PBS or OVA challenge, airway reactivity to methacholine in conscious, unrestrained wild type or Cat S knockout mice was assessed by a whole body noninvasive plethysmograph (Buxco Electronics, Troy, NY). This system estimates total pulmonary airflow in mice using a dimensionless parameter known as enhanced pause (Penh). Pressure differences were used to calculate Penh values, which are a function of the sum of the airflows in the upper and lower respiratory tracts during a respiratory cycle. Following an acclimatization period, baseline Penh was determined by exposing mice to nebulized saline (3 minutes). The mice were then exposed to doubling concentrations of aerosolized methacholine dissolved in saline for 3 minutes, and then Penh values were recorded and averaged for 5 minutes following each nebulization.

Plasma OVA-specific IgE determination in wild type or Cat S knockout mice

Twenty four hours after the last PBS or OVA challenge, blood was collected and plasma isolated to assess for OVA-specific IgE levels. Briefly, plasma from each

animal within the study groups were individually diluted 80-, 160-, and 320-fold using fresh mouse plasma. Diluted samples were evaluated using a commercially available ELISA kit (insert vendor) as per the manufacturer's instructions and results for each dilution plotted as optical density (OD) absorbance values for relative differences in IgE between dilutions, for each of the groups. Linear regression and two-way ANOVA analysis was performed.

Lungs preparation

Twenty four hours after the last PBS or OVA challenge, the left lung was ligated, excised, and snap-frozen in liquid nitrogen. Mice were exsanguinated and systemically perfused with cold saline followed by 1% buffered-neutral formalin. Trachea, heart and right lung block was removed *en toto* and the right lung was formalin-inflated by continuous infusion via the tracheal cannula. Mid-trachea longitudinal paraffin (5µm) sections were stained with hematoxylin and eosin (H&E) followed by periodic acid-Schiff (PAS) and Alcian blue (AB) staining to quantify mucus-producing goblet cells. Under light microscope, the total amount of AB/PAS staining was quantified and normalized to the total airways basement length using the AxioVision imaging software (Empix Imaging Ltd, Mississauga, ON, Canada).

Sample homogenization

Twenty four hours after the last PBS or OVA challenge, frozen mouse spleens were homogenized in T-Per solubilization buffer (Pierce, Rockland IL) containing Complete protease cocktail inhibitors (Roche, Laval QC) using a MagNalyser (2 X 30sec at 6500 rpm). Samples were centrifuged at 10 000 rpm for 5 minutes at 4°C and supernatants were collected.

Invariant Chain p10 and Cathepsin expression by Western blot analysis

For analysis of p10 accumulation, samples (10-30 µg per lane) were separated on 10-20% tricine gels (Invitrogen, Burlington ON) and transferred to 0.05 µm nitrocellulose membranes. For detection of cathepsin S or cathepsin B levels, 50 µg samples were separated on 12% tris-glycine gels (Invitrogen) and transferred to 0.45 µm nitrocellulose membranes. The membranes were blocked with 5% milk in TBST (TBS, 0.1% tween-20) for 1 hour at room temperature, probed with either a 1:100 dilution of rat anti-mouse CD74 (BD Biosciences, Palo Alto CA), 1:200 dilutions of anti-cathepsin S (M-19) or anti-cathepsin B (S-12) (Santa Cruz Biotechnology, Santa Cruz CA) antibodies in 3% milk TBST for 1 hour, then washed 3 X 10 minutes with TBST. The blots were incubated for 1 hour in 3% TBST containing 1:3000 dilution of anti-rat HRP-conjugated antibody (Amersham, Baie d'Urfe QC) for p10 or with 1:3000 dilution of anti-goat HRP conjugated antibody for the cathepsins, then washed 3 X 10 minutes with TBST. Signals were detected using ECL Plus reagents (Amersham) and quantified using Quantity One or Image Gauge software.

Statistical analysis

Endpoint analysis between experimental groups were performed using one-way or two way analysis of variance (ANOVA) followed by Bonferroni's Multiple comparison post tests. In the case of mucus quantitation, if no visible mucus was observed in lung histological sections stained with AB/PAS in mice challenged with 0.5% PBS, sections were assigned an arbitrary value of 0 for the purpose of data presentation. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, California, U.S.A.).

Figure Legends

Figure 1. Cellular inflammation in BAL fluid of PBS or OVA-aerosol challenged mice provided vehicle chow (vehicle) or formulated with 0.05% or 0.005% Compound 7 (C7) versus mice dosed with 1 mg/kg Dexamethasone (Dex). (A) Total and (B) differential cell counts in BAL fluid of mice challenged with PBS or OVA aerosol. * $P < 0.05$ compared with corresponding PBS aerosol challenge control. # $P < 0.05$ compared with corresponding OVA aerosol challenge. Each value represents the mean $\pm$ SEM ($n=6-10$ animals per group). Mac, macrophages; eo, eosinophils; lym, lymphocytes; neu, neutrophils.

Figure 2. Mucus production in airways of PBS or OVA-aerosol challenged mice provided vehicle chow (vehicle) or formulated with 0.05% or 0.005% Compound 7 (C7) versus mice dosed with 1 mg/kg Dexamethasone (Dex). Representative airways (A) were assessed histologically by Alcian Blue/Periodic Acid-Schiff (AB/ PAS) staining in lung tissue sections 24 hours after the final aerosol challenge. Quantitation (B) of mucus production ($n = 2-3$ per group; > 15 airways per mouse per group) was performed as described. No AB/PAS staining was observed in PBS-challenged animals and was assigned a value of zero. C7-0.005%, 0.005% Compound 7 in chow; C7-0.05%, 0.05% Compound 7 in chow; Bar, 50 μ m.

Figure 3. Splenic invariant chain p10 (A) and Cat S protein (B) levels in OVA-aerosol challenged mice provided vehicle chow (vehicle) or formulated with 0.05% or 0.005% Compound 7 (C7). Spleen homogenates resolved by gel electrophoresis were analyzed by immunoblotting for p10 and Cat S protein followed by quantification with imaging software. Each value represents the mean $\pm$ SEM ($n=6$ animals per group). C7-0.005%, 0.005% Compound 7 in chow; C7-0.05%, 0.05% Compound 7 in chow.