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Reduction of Pulmonary Inflammation by pH Modifiers

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Abstract

Pulmonary inflammation is a common pathological feature of a variety of diseases, of which successful therapy with currently available anti-inflammatory drugs is limited by resistance and adverse side effects. Using the ovalbumin-induced mouse allergic asthma model, the present study shows that treatments with pH modifiers, particularly simple acids such as acetate or hydrochloric acid, effectively depleted inflammatory cells in the lungs and blood as well as hyperplastic lung tissue cells while preserving the structure of the blood vessels and lung parenchyma. The acid treatments also suppressed mucus hypersecretion. These results demonstrated pH modifiers as a new class of broad-spectrum anti-inflammatory agents with anti-proliferation and mucus suppression activities.

Introduction

Pulmonary inflammation is a common feature of many clinical conditions, most notably asthma, chronic obstructive pulmonary disease (COPD) and acute lung injury, the most severe of which is the acute respiratory distress syndrome (ARDS) of various causes such as viral or bacterial pneumonia, extra pulmonary sepsis and aspiration^{1,2}. Not only are these conditions debilitating they can also be fatal. According to a recent report, in 2019 asthma affected about 262 million people and caused 461, 000 deaths, whereas COPD ranked as the No.6 leading cause of disability and caused 3.23 million annual deaths²⁻⁴. On the other hand, ARDS patients comprised 10% of intensive care unit admissions, and almost 50% of the patients diagnosed with mild to severe ARDS succumbed to this condition⁵.

Over zealous local and systemic inflammatory responses that involve both the innate and adaptive arms of the immune system are directly responsible for tissue damages and organ failure in the various diseases with pulmonary inflammation. Therefore, while the ultimate cures and preventive measures targeting the diverse etiologies of these diseases or conditions remain unattainable in the foreseeable future, reduction of inflammation is an important strategy for managing the diseases in both emergency and long-term cares. The mainstay therapy for pulmonary inflammation is corticosteroids (CS). However, CS therapy is limited by resistance and adverse side effects⁶⁻⁹. For examples, the benefit of CS therapy of ARDS remains largely inconclusive.^{10,11} Likewise, most COPD patients respond poorly to CS, except very modest reduction of exacerbation^{6,12}. The responsiveness of asthma patients to CS therapy is dependent on the endotypes. About 50% of clinical asthma is characterized as Th2-high eosinophilic endotype^{13,14}, only a subgroup of which is CS-responsive. Over half of patients with Th2-high

asthma, particularly those with severe asthma at old ages, do not respond to CS, and Th2-low asthma endotypes are generally refractory to CS^{13,15-18}. CS therapy is further limited by side effects such as osteoporosis, hyperglycemia, increased rate of infections and viral replication¹⁹⁻²³.

Given the limitations of CS due to resistance and side effects, it is of great value to develop alternative anti-inflammatory therapy with the potential of broad applications. This report shows that altering the pH homeostasis in the lungs can effectively deplete inflammatory cells in the lung tissue and lumen while preserving the structure of the blood vessels and lung parenchyma. The findings demonstrate that pH modifiers act as a new class of broad-spectrum anti-inflammatory agents.

Results

Reduction of inflammatory cells in the bronchoalveolar lavage fluids (BALF) by pH modifiers

The current study stemmed from a serendipitous finding in the course of studying anti-inflammatory activity of plant-derived compounds. Initially, it was found that the acidic solution used to prepare the plant compounds, when instilled into the lungs of asthmatic mice, depleted inflammatory cells in the BALF without causing notable extra stress to the mice. Based on this finding, experiments were performed to compare BALF cellularity in asthmatic mice received treatments of saline or saline containing pH the modifiers HCl, HOAc or NaOH. (Figure 1). BALF from the control saline-treated mice contained large numbers of inflammatory cells (average of 3.06×10^6 /mouse). In contrast, the total inflammatory cells in the BALF of HCl- or HOAc-treated mice were dramatically reduced (averages of 1.07×10^6 and 9.01×10^5 per mouse, respectively). The total inflammatory cells in the BALF of the NaOH-treated mice (average of 2.18×10^6 /mouse) were also reduced, but not as much as the acid-treated mice. (Figure 1a and

b). Despite the significant reductions of BALF cellularity by the pH modifiers, eosinophils remained as the predominant inflammatory cell type in all treatment groups, ranging from 80-84% of total BALF cells. The percentages of macrophages and lymphocytes also showed only small variations among the treatment groups, ranging from 12.3-17.5% and 2.2-4.2% of total BALF cells, respectively. (Figure 1c).

Reduction of inflammatory cells in the blood

The blood samples from the mice were analyzed by flow cytometry. The two distinct inflammatory cell populations of granulocytes and lymphocytes were defined by their FSC and SSC profiles. In comparison with the saline-treated mice, the percentages of both inflammatory cell populations were reduced in mice treated with the pH modifiers. The treatment with HOAc caused the greatest reductions (1.1% vs. 32.6% for granulocytes; 13.6% vs. 22.9% for lymphocytes). (Figure 2). The reduction of circulating inflammatory cells in the blood likely had contributed at least in part to the reduction of inflammation in the lungs.

Reduction of inflammatory infiltration in the lung tissue by pH modifiers

Heavy inflammatory infiltration was detected in the lungs of the saline-treated mice by H&E staining of the lung tissue sections. The inflammatory infiltration was most concentrated in the perivascular areas and to lesser degrees also in the peribronchial areas. (Figure 3a and b). In the lungs of the mice treated with i.t. instillation of HOAc or HCl, inflammation was diminished in the perivascular areas to residual levels, which also indicated that acids were in part transported to the lung tissues via blood circulation. Notably, the perivascular areas of the acid-treated mice showed a characteristic “faded” blue staining, demonstrating that most of inflammatory cells that had previously occupied the areas were depleted and only residual inflammatory cells remained. Inflammatory infiltration in the peribronchial areas was also

diminished. This reduction could be both a secondary result of the depletion in the perivascular areas because the inflammatory cells originated from the blood, and direct depletion by acids absorbed through the bronchioles. (Figure 3a). In the lungs of the NaOH-treated mice, reduction of inflammatory infiltration was prominent in the perivascular areas, but not in the peribronchial areas. (Figure 3a). This could be because NaOH was administrated by i.p. injection so that it was transported to the lungs only by blood circulation. Microscopic examination of the lung sections at high magnification further revealed that the structures of the blood vessels and the nearby alveoli and bronchioles were well preserved while inflammatory cells were effectively depleted by the pH modifiers. (Figure 3b).

Depletion of proliferating cells in the lungs

Lung tissue sections from the mice in the different treatment groups were also analyzed for the expression of the proliferating cell marker Ki-67 by immunohistochemistry. As shown in Figure 4, in the lungs of the saline-treated mice, Ki-67⁺ cells were abundant in the infiltrating inflammatory cells and in the alveolar and bronchial walls. The abundance of proliferating pneumocytes was indicative of pneumocyte hyperplasia, which was also evidenced by the thick alveolar walls consisting of multilayers of pneumocytes. By comparison, in the remaining inflammatory cells in the lungs of the HOAc- or HCl-treated mice, there were much fewer Ki-67⁺ cells, and Ki-67⁺ cells were only sporadically detected in the alveolar and bronchial walls. In contrast, in the lungs of the NaOH-treated mice, Ki-67⁺ cells were abundant in both the inflammatory cells and in the alveolar and bronchial walls. Furthermore, Ki-67 staining in the lungs of the NaOH-treated mice appeared to be stronger than that of the saline-treated mice, consistent with the notion in several other studies (manuscripts in preparation) that alkaline treatment enhances cell proliferation.

Inhibition of mucus hyper-secretion in the lungs by acid treatments

Mucus hyper-secretion is a major feature of pulmonary inflammation in allergic asthma. Lungs of the control saline-treated mice showed heavy mucus secretion as indicated by the intense Periodic acid–Schiff (PAS) staining. In contrast, lungs of the HOAc- or HCl-treated mice showed only faint PAS staining. However, no reduction of PAS staining was observed in the lungs of the NaOH-treated mice. (Figure 5).

No inhibition of CD4 T cell activation in BALF and MLN

Pulmonary inflammation in allergic asthma results from the activation of CD4 T cells by allergens¹⁴. To investigate whether CD4 T cells activation was inhibited by the pH modifiers, CD4 T cells in the BALF and the lung-draining lymph nodes the mediastinal lymph nodes (MLNs) were analyzed for their expression of the T cell activation markers CD44 and CD69. Most of the Foxp3⁻ CD4 T cells in the BALFs were CD44⁺, some of which co-expressed the early activation marker CD69, indicating that the CD4 T cells in the BALFs were predominantly effector or memory CD4 T cells. (Figure 6, upper panels). The percentages of CD44⁺ cells in total Foxp3⁻ CD4 T cells were comparable among saline- and acid-treated mice (67-70.2%), but higher in the NaOH-treated mice (80.6%). The percentages of CD44⁺CD69⁺ cells in total Foxp3⁻ CD4 T cells were higher in mice treated with the pH modifiers than that of the control mice treated with saline (14.1-20.2% vs. 9.79%), particularly in the acid-treated mice. This result suggests that the BALFs of the pH modifier-treated mice were being replenished with newly activated CD4 T cells after the deletion of such cells. In contrast, the percentages of CD44⁺ and CD44⁺CD69⁺ cells in Foxp3⁻ CD4 T cells in the MLNs were less variable among the different treatment groups, in the ranges of 64-75.6% and 3.52-5.8%, respectively. (Figure 6, lower panels).

No increase of Treg cell populations in BALF and MLN

Regulatory T (Treg) cells inhibit T cell activation therefore prevent pulmonary inflammation in allergic asthma²⁴. To determine whether the reduction of pulmonary inflammation was attributable to increase of the Treg cell population by the pH modifiers, Treg cell populations in the BALFs and MLNs were examined. In the BALF of HCl-, HOAc- and NaOH-treated mice, Treg cells comprised 4.45%, 7.18% and 6.97% of total CD4 T cells, respectively, compared with 7.46% in the saline-treated mice. (Figure 7, upper panels). In the MLNs of HCl-, HOAc- and NaOH-treated mice, Treg cells were 9.12%, 11.53% and 11.46% of total CD4 T cells vs. 11.80% of the saline-treated mice. (Figure 7, lower panels). Thus, treatments with pH modifiers did not increase Treg cells. This finding, particularly that of the HOAc-treated mice, is in stark contrast to previous findings that some short chain fatty acids (SCFAs) can promote extra-thymic generation of Treg cells from peripheral naïve CD4 T cells^{25,26}. The difference was apparently due to the fact that the acid treatments in the present study occurred during recall response, i.e., in the allergen challenge phase, instead of during the activation of naïve CD4 T cells in the previous studies. Since the CD4 T cells in the recall responses were already committed to effector/memory cells, they were unlikely to be diverted to Treg cell differentiation.

Death of inflammatory cells by apoptosis

Among the three pH modifiers tested in this study, HOAc was the most effective in reducing pulmonary and blood inflammation. Therefore, HOAc treatment was used as the model to further study the mechanism of inflammatory cell depletion. Having excluded the inhibition of CD4 T cell activation and induction of Treg cells as the modes of action, it appeared most likely that the depletion of inflammatory cells was by direct induction of cell death. Experiments were

conducted to test this possibility and to determine whether HOAc treatment induced apoptosis. Thus, OVA-sensitized and challenged mice received treatments with saline alone or saline containing HOAc. Shortly after the treatments, BALF cells were collected and stained for the eosinophil marker Siglec F, the macrophage marker F4/80, Annexin V and 7-AAD. The granulocyte and lymphocyte populations in the BALF were distinguished according their FSC and SSC profiles. Within the granulocyte populations, eosinophils were Siglec F^{low-high} F4/80^{negative-low}, whereas macrophages were Siglec F^{low} F4/80^{high}. (Figure 8a). In the saline-treated mice, only small fractions of the lymphocytes (3.15%), eosinophils (2.87%) and macrophages (0.71%) were apoptotic (Annexin V⁺7-AAD⁺). In contrast, the majorities of these cell populations of the HOAc-treated mice were apoptotic (85.3%, 95.2% and 56.4%, respectively). (Figure 8b).

Neutrophils are major inflammatory cells in many inflammatory diseases, particularly those of bacterial infections such as bacterial pneumonia. However, neutrophils were scarce in the BALF of mice in the OVA asthma model. To investigate whether HOAc treatment could also induce apoptosis of neutrophils, peripheral blood samples were analyzed. Neutrophils were defined as Ly6G^{high} leukocytes in the blood. (Figure 8c)^{27,28}. In the blood of the saline-treated mice, 7.61% of neutrophils were apoptotic, whereas 38.4% of neutrophils in the blood of HOAc-treated mice were apoptotic. (Figure 8d).

Discussion

The present study explores the potential of pH modifiers as a new class of anti-inflammatory agents based on the differences between inflammatory cells and structural and functional cells of tissues in their tolerance to pH alterations. Specifically, it was found that

administering the pH modifiers HOAc, HCl, and to lesser extent NaOH, effectively depleted inflammatory cells in the lungs of asthmatic mice while preserving the structures of the alveoli, airways and blood vessels. As exemplified by HOAc treatments, acid treatments induced apoptosis of all major types of inflammatory cells including lymphocytes, eosinophils, macrophages and neutrophils. Thus, the pH modifiers can be as a new class of broad-spectrum anti-inflammatory agents that reduce inflammation regardless of what is the dominant type of inflammatory cells in an inflammatory response.

The pH in the body is maintained in a relatively narrow range of 7.35 to 7.45 with the average of 7.40²⁹. For this reason, traditionally most cellular studies do not treat pH as a variable. However, in real life, an individual can experience transient systemic or local pH fluctuations without notable adverse clinical consequences. For examples, vigorous physical exercises and grand mal seizures can cause severe systematic lactic acidosis without serious pathophysiological consequences in the aftermath^{30,31}. Fluctuation of pH can also occur locally at specific tissues and/or organs. For examples, the blood vessels can stand considerable swings of pH in either direction^{30,31}. In the lungs, the pH values of the air surface liquid (ASL) and alveolar subphase fluid (AVSF) are around 6.6 and 6.9, respectively^{32,33}. The bladder can tolerate urine pH from 4.5 to 8³⁴. Although these observations have been reported over a long period of time, there has been no investigation at the cellular level how different cells might respond differently to pH fluctuation, and how such differences and the body's tolerance to pH fluctuation can be exploited for therapeutic application. The present study is the first of such investigation.

Although the present study has investigated only three pH modifiers, it is reasonable to speculate that other pH modifiers would have the same or similar effects. Generally, pH

modifiers as anti-inflammatory agents can be any molecule or a mixture of molecules that can change or stabilize the pH of an intracellular and/or extracellular environment. In addition to acids and bases, the pH modifiers can be molecules that have pH buffering capacities, ionophores, and stimulators or inhibitors of the expression or activities of enzymes, membrane transporters or ion channels that are responsible for pH homeostasis in and/or around a cell.

The mechanisms for the differences between inflammatory cells and the structural functional cells of the lungs in their susceptibility to depletion by acids are not clear. However, it is known that multiple cross-membrane transporter systems and enzymes contribute to pH homeostasis, for examples, the monocarboxylate transporter system, the Na^+/H^+ exchanger system, the $\text{Na}^+/\text{HCO}_3^-$ cotransporter system, the carbonic anhydrase system, H^+ ATPase, H^+ channels, and the histidyl dipeptide buffering system³⁵⁻³⁸. Differences of the expression and activities of these diverse systems and enzymes may confer cells different abilities to maintain stable pH in the cells and the pericellular spaces. In addition, it must be noticed that the inflammatory cells were mostly concentrated in the perivascular and peribronchial areas of the lungs of the asthmatic mice. Such anatomical locations could also have played a part in the vulnerability of inflammatory cells to depletion because the pH modifiers appeared to be absorbed and permeated to the lung tissues from the blood vessels and bronchioles. Furthermore, large numbers of highly proliferating cells were detected in the inflammatory infiltrates. Since the proliferative status of a cell is positively correlated with its susceptibility to depletion by acids (manuscript in preparation), at least some of the inflammatory cells were more susceptible to depletion because of their relatively high proliferative statuses.

Mucus hypersecretion is a major cause of airflow obstruction in both acute exacerbation of asthma and chronic severe asthma³⁹. Cytokines such as IL-9 and 13 produced by Th2 and

other inflammatory cells upon allergen exposure stimulate mucus production in the airways⁴⁰. In other muco-obstructive lung diseases such as COPD, cystic fibrosis, primary ciliary dyskinesia, and non-cystic fibrosis bronchiectasis, inflammatory responses are also key to mucus hypersecretion. Under these circumstances, an insult to the airway epithelium results in hyperconcentrated mucus, which triggers a muco-inflammatory positive feedback cycle known as “vicious cycle”, involving macrophages, epithelial cells and neutrophils, that ultimately leads to mucus hypersecretion⁴¹. In the present study, asthmatic mice treated with HOAc or HCl showed minimal production of mucus compared with mice treated with saline alone, whereas there was no reduction of mucus production in mice treated with NaOH.

Thus, pH modifiers such as acids are advantageous over other anti-inflammatory agents in that they simultaneously provide multiple reliefs of inflammation, tissue cell hyperplasia and mucus hypersecretion.

Inflammation in the local tissues is usually also manifested secondarily in the blood. In addition, there are clinical conditions caused by primary inflammation in the blood and blood vessels. For examples, in atherosclerotic cardiovascular diseases (ASCVD), inflammatory cells such as monocytes/macrophages become permanently attached to and invade the blood vessels, which together with other cells such as smooth muscle cells proliferate and ingest lipids to form atheromatous plaques⁴²; in vasculitis, the blood vessels are infiltrated by various inflammatory cells⁴³. The present study showed that inflammatory cells in the blood, like in the lungs, were depleted by the pH modifiers. Therefore, pH modifiers can also be used to treat or prevent inflammatory diseases of the blood and/or blood vessels such as the ASCVD and vasculitis.

In conclusion, the present study used an animal model of asthma to demonstrate that pH modifiers can be used as broad-spectrum anti-inflammatory agents that preferentially deplete

various types of inflammatory cells, therefore could be used to treat and prevent diseases whose pathogenesis is partially or fully attributable to inflammation.

Materials and Methods

Mice and asthma model. Balb/c mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal facility of Charles River Accelerator and Development Lab (CRADL) (Cambridge, MA, USA). Animal studies were conducted according to the protocols approved by the CRADL Institutional Animal Care and Use Committee. Asthma induction and analyses were performed essentially as previously described^{14,44}. For sensitization, grade II ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) and Alum adjuvant (Thermo Scientific, Rockford, IL, USA) were fresh mixed in PBS to make sensitization solution comprising 200µg/ml OVA and 33.4% (by volume) Alum. The mice were i.p. (intraperitoneally) injected with 100µl of the sensitization solution (20µg OVA/mouse) on day 0. On day 14, each mouse was sensitized again by i.p. injection of 500µl of fresh prepared sensitization solution (100µg OVA/mouse). Eighteen days later, each mouse was challenged with OVA together with or without pH modifiers by i.t. (intratracheal) instillation of 100µg OVA protein and 1µg OVA₃₂₃₋₃₉ peptide (Molecular Resources, Fort Collins, CO, USA) in saline every other day for a total of 3 times. On day 3 after the final challenge, mice were sacrificed for further analyses.

Treatments with pH modifiers. Treatments with HOAc or HCl and OVA challenge were carried out together. For each mouse, 60µl saline containing the OVA protein and peptide plus 175mM HOAc or HCl was administered to the mouse by i.t. instillation. Since i.t. instillation of NaOH was poorly tolerated by the mice, treatments with NaOH were carried out 1 hour after

OVA challenge by i.p. injection of 200µl saline containing 87.5mM NaOH. At the start of treatments, mice typically weighed about 25 to 32g.

BALF cellularity and cell differentials. BALF were collected using a 1cc syringe to inject and retreat 0.6ml ice cold PBS plus 1% FBS from the trachea into and from the lungs for 3 times. The cells in the BALF of each mouse were stained with Trypan Blue and counted to determine total cellularity of the BALF. For cell differentials, the BALF cells were spun onto glass slides using a StatSpin Cytofuge (Iris International, Westwood, MA). The slides were stained with hematoxylin and eosin (H&E) using the Diff-Quick Stain Set (Siemens Healthcare Diagnostics, Newark, DE). Eosinophils, macrophages, lymphocytes, and neutrophils were counted under a light microscope with mounted camera, and images were taken and processed with Motic Image Plus 2.0 software (Swift Optical Instruments, San Antonio, TX).

Blood sample collection. After the mice were sacrificed, 200µl blood was drawn from the heart with a 26G needle attached to a 1cc syringe, and immediately transferred to a microcentrifuge tube with 800µl Hank's Balanced Salt Solution (HBSS) plus 1mM EDTA. After centrifuge, red blood cells were removed by resuspending the cells in 500µl ddH₂O for 15 seconds, followed by addition of 500µl 2x PBS. The cells were then centrifuged and resuspended in 1x PBS plus 1% FBS and kept on ice for further analyses.

Analyses of tissue inflammatory infiltration and mucus secretion. After BALF collection, lungs were harvested and fixed in 10% neural formalin (Fisher Scientific, Kalamazoo, MI). The fixed lungs were paraffin embedded and sectioned on a Microtome (Leica Biosystem, Buffalo Grove, IL). The tissue sections were stained with hematoxylin and eosin (H&E) to determine inflammatory infiltration. Periodic acid-Schiff (PAS) staining was also performed with the lung tissue sections to determine mucus secretion in the lungs. The tissue slides were

examined under a light microscope with mounted camera, and images were taken and processed with Motic Image Plus 2.0 software (Swift Optical Instruments, San Antonio, TX, USA), or alternatively the slides were scanned and images were processed with QuPath software (<https://qupath.github.io>).

Immunohistochemistry staining of Ki-67. Proliferating cells in the lungs were detected by immunohistochemical staining of Ki-67 as previously described⁴⁵. Briefly Paraffin-embedded lung tissue sections were first deparaffinized and rehydrated. Antigens in the tissue sections were unmasked by heating in 10mM Sodium Citrate Buffer, pH 6.0, to boiling for 2 minutes in a microwave oven for 3 times, followed by washing with PBS and incubation in 10% H₂O₂ at room temperature for 15 minutes. After washing with PBS, the tissue sections were blocked with 5% BSA. Afterwards, the tissue sections on the slides were incubated with rabbit anti-mouse Ki-67 antibody (GB13030-2, Servicebio, Wuhan, China) in PBS at 4°C overnight. After washing with PBST (PBS plus 0.1% Tween 20), the tissue sections were incubated with Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit antibodies (G21234, Invitrogen, Carlsbad, CA) at room temperature for 1 hr. After washing the slides, color deposits were developed using the DAB Substrate Kit (Cat. 34002, ThermoFisher, Waltham, MA) according to the manufacturer's instruction. After color was developed, the slides were washed with ddH₂O, and counter-stained with hematoxylin. The slides were scanned, and images were processed with QuPath.

Detection of apoptosis and flow cytometry. BALF and blood cells were washed twice with plain PBS and once with Annexin V binding buffer (10mM HEPES, pH7.4, 140mM NaCl, 2.5mM CaCl₂) after surface antigen staining, and incubated with fluorochrome-conjugated Annexin V (eBioscience/ThermoFisher Scientific, San Diego, CA) in Annexin V binding buffer

for 15min at room temperature. The cells were washed twice in Annexin V binding buffer and resuspended in Annexin V binding buffer, and 7-AAD was added to the cells before analyzed by flow cytometry. Fluorochrome-conjugated Avidin and anti-mouse antibodies against CD4, CD8a, CD44, CD69 and TCR β were purchased from eBioscience/ThermoFisher Scientific (San Diego, CA). Foxp3 buffer kit, Siglec F-Biotin, Ly6G-Biotin and fluorochrome-conjugated anti-mouse antibodies against CD19 and F4/80 were purchased from Biolegend (San Diego, CA). Cells were stained for surface antigens on ice. For Foxp3 staining, after surface antigen staining, cells were fixed and permeabilized using the Foxp3 buffer kit. After wash, the cells were stained with fluorochrome-conjugated anti-Foxp3 antibodies in the permeabilization buffer. Stained and unstained cells were analyzed on FACS Canto (BD Bioscience, San Jose, CA). Data analyses were carried out using FlowJo X.

Declaration of Conflict of Interest

Wei-ping Zeng is the inventor of patent application based in part on some data described in this manuscript.

Author contribution

Wei-ping Zeng conceived, designed and carried out the study, and wrote the manuscript.

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Figure Legends

Figure 1. Depletion of inflammatory cells in the lumens of inflamed lungs by pH modifiers. (a) Representatives of microscopic views of the H&E-stained BALF cells of mice in the different treatment groups. (b) The averages of total number of BALF cells per mouse in the different treatment groups. Statistical significance of the differences between saline and other treatment groups was determined by Student t test; * $p < 0.05$; ** $p < .001$. (c) BALF cell differentials. Graphs show the averages of the percentages of eosinophils, macrophages and lymphocytes in the BALF cells.

Figure 2. Reduction of inflammation in the blood by pH modifiers. The upper panels are flow cytometric pseudocolor plots of the RBC-depleted blood samples of mice in the different treatment groups, showing the populations of granulocytes and lymphocytes. The lower panel is a graph showing the percentages of the granulocytes and lymphocytes in the pseudocolor plots.

Figure 3. Depletion of inflammatory cells in the lung parenchyma by pH modifiers. (a) Microscopic views at low magnification of H&E-stained lung tissue sections of mice in the

different treatment groups, showing the depletion of inflammatory cells or loss of blue staining in the perivascular and/or peribronchial areas of the lungs of the pH modifier-treated mice. (b) Microscopic views at high magnification of the lung tissue sections, showing the depletion of inflammatory cells at the perivascular areas, but the preservation of structures of the blood vessels, the nearby alveoli and bronchioles in the mice treated with the pH modifiers. Blood vessels (BV) and bronchioles (Br) are indicated.

Figure 4. Effects of pH modifiers on proliferating cells in the lungs. Shown are microscopic views of lung tissue sections of mice in the different treatment groups that were stained for Ki-67 (brown) with haematoxylin counter staining (blue). Blood vessels (BV) and bronchioles (Br) are indicated.

Figure 5. Suppression of mucus hypersecretion in the lungs by acid treatments. Shown are Periodic acid–Schiff (PAS) staining of lung tissue sections of mice in the different treatment groups.

Figure 6. CD4 T cell activation statuses in the BALF and MLNs. Left panels are flow cytometric pseudocolor plots of BALF and MLN cells of mice in the different treatment groups, showing the expression of the T cell activation markers CD44 and CD69 within the Foxp3⁻ CD4 T cells. Right panels are graphs showing the percentages of CD44⁺ and CD44⁺CD69⁺ cells in the Foxp3⁻ CD4 T cells in the pseudocolor plots.

Figure 7. Foxp3⁺ Treg cells in the BALF and MLNs. Left panels are flow cytometric pseudocolor plots of BALF and MLN cells of mice in the different treatment groups, showing the expression of CD4 and Foxp3. Right panels are graphs based on the pseudocolor plots, showing the percentages of Foxp3⁺ Treg cells in total CD4 T cells.

Figure 8. Induction of apoptosis of inflammatory cells by HOAc. After the third OVA challenge of the OVA-sensitized mice, the mice were treated with saline or saline containing HOAc by i.t. instillation. About three hours later, apoptosis in BALF (a and b) or peripheral blood (c and d) cells was analyzed. (a) Flow cytometric pseudocolor plots showing the lymphocyte (Lym) and granulocyte (Gran) populations in the BALF cells (left panel), and the eosinophil and macrophage populations within the granulocytes based on their expression patterns of F4/80 and Siglec F (right panel). (b) Flow cytometric pseudocolor plots showing the profiles of Annexin V and 7AAD staining in the lymphocytes, eosinophils and macrophages as defined in (a). (c) Flow cytometric pseudocolor plot of peripheral blood cells showing the neutrophil population based on their high expression of Ly6G. (d) Flow cytometric pseudocolor plots showing the profiles of Annexin V and 7AAD staining in the neutrophil (Ly6G^{hi}) populations as defined in (c). Numbers in the plots are percentages of the cell populations in their respective quadrants.

Figure 1

Figure 2

a)

Saline

HCl

HOAc

NaOH

400μm

Figure 3a

b)

Saline

HCl

HOAc

NaOH

50µm

Figure 3b

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8