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Mast cell chymase impairs bronchial epithelium integrity by degrading cell junction molecules of epithelial cells

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[Correction added on 21 January 2019 after first online publication: The author byline has been rearranged and corrected in this version.]

Abstract

Background: An increased degree of mast cell (MC) degranulation and damage to the epithelial lining are prominent features of bronchial asthma. In asthmatic airways, it seems likely that epithelial cells will be exposed to increased concentrations of proteases from MC, though their actions on the epithelium are still not very clear.

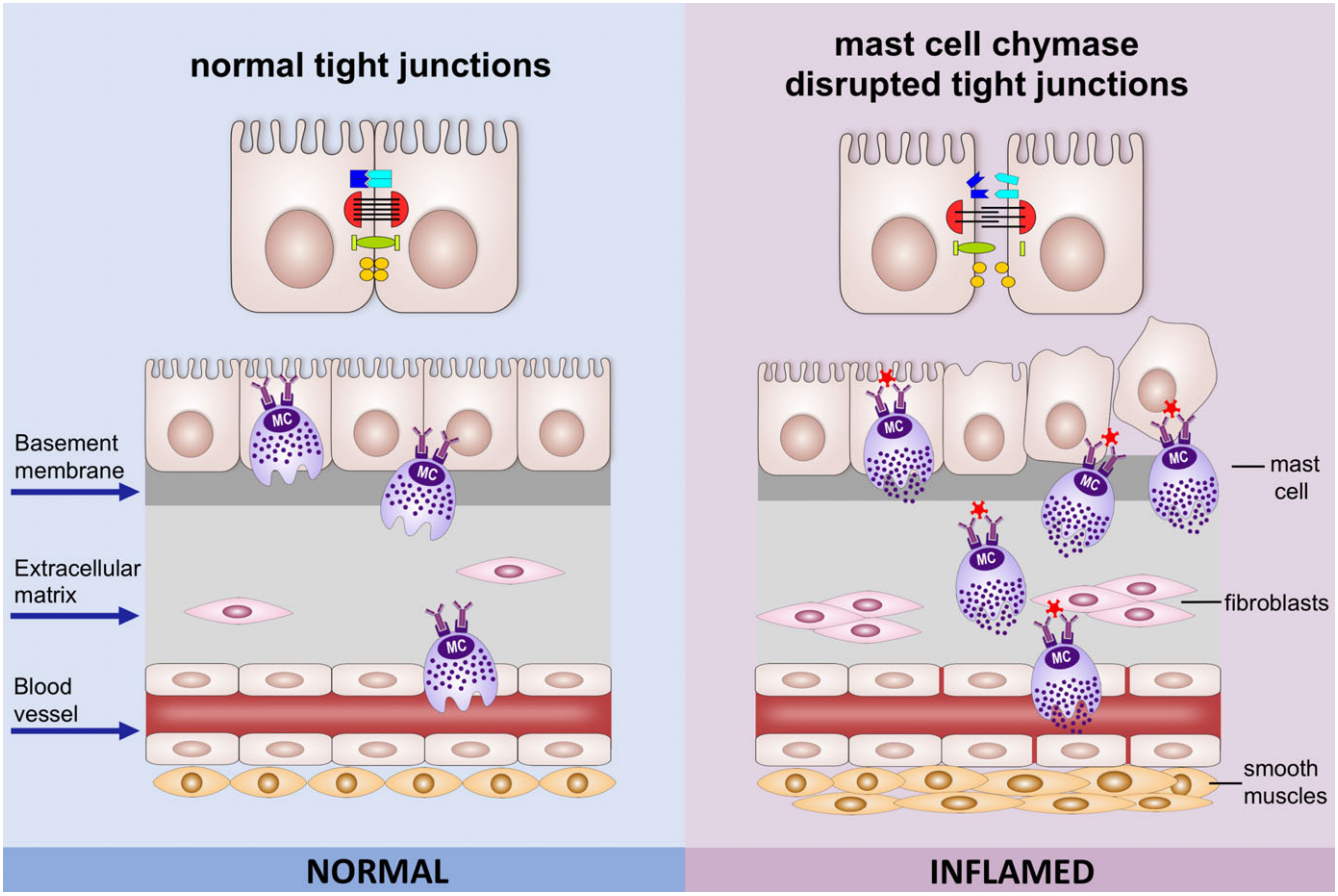
Methods: Bronchial rings from human lung tissue or 16HBE cell monolayer were incubated with MC chymase in different doses or various inhibitors. The sections of paraffin-embedded tissue were haematoxylin-eosin stained and computerized by image analysis for epithelial damage-scale-evaluation; the cell viability, proliferation, adhesion and lactate dehydrogenase activity release were assayed; the expressions of gelatinases, cell junction molecules and structure proteins of 16HBE were examined.

Results: Mast cell chymase was found to provoke profound changes in the morphology of bronchi epithelial layer. Following incubation with chymase, there was 40% reduction in the length of epithelium that was intact, with detachment of columnar epithelial cells and basal cells. Chymase reduced epithelial cell proliferation and induced cell detachment, which were associated with the changes in secretion and activation of matrix metalloproteinase-2/9. In intact epithelial cell layers, immunocytochemistry study revealed that chymase reduced the expressions of occludin, claudin-4, ZO-1, E-cadherin, focal adhesion kinase and cytokeratin. Overall data of this study indicated that MC chymase can influence tissue remodelling, disrupt epithelial cell junctions, inhibit wound healing and impair the barrier function of epithelium, resulting in dysfunction of airway wall and ECM remodelling in pathogenesis of asthma.

Conclusion: Mast cell chymase plays a key role in inducing the damage to bronchial epithelium in asthma.

KEYWORDS
bronchial epithelium, cell junction molecule, chymase, mast cells, matrix metalloproteinases

Abbreviations: AJ, adherens junctions; chy, chymase; Chymo, chymostatin; ECM, extracellular matrix; FAK, focal adhesion kinase; H & E, haematoxylin and eosin; HI, heat-inactivated; LDH, lactate dehydrogenase activity; LP, losartan potassium; Marim, Marimastat; MC, mast cell; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PT, pertussis toxin; SF, serum-free; TJ, tight junctions; ZO-1, zonula occludens-1.



GRAPHICAL ABSTRACT

Desquamation of airway epithelial cells is one of the consequences of airway inflammation in asthma. MC chymase released from MC activation contributes to desquamations of epithelium in asthmatic airway. MC chymase degrades epithelial cell junction proteins, disrupting the connections between cell-cell and cell-ECM; MC chymase decreases intracellular cell structure protein cytokeratin and FAK expressions and reduces cell viability and adhesion. Dysregulated epithelium barrier function and damages of airway wall are prominent features of chronic asthma; MC chymase inhibits wound healing by activating pro-MMP-2 and inducing ECM remodelling.

1 | INTRODUCTION

The epithelial lining of the conducting airways forms a crucial interface between the immune system and inhaled allergens. Barrier function relies on the physical integrity of the epithelial cell layer, which is dependent on the adhesions between cell-cell/cell-matrix, the continuous action of the mucociliary transport system and the regulation of innate and adaptive immune responses by epithelial-derived mediators.[1-4] Bronchial asthma is characterized by increased fragility of the epithelial cell layers of the bronchi, as evidenced by epithelial cells shedding into patients' sputum and increased number of epithelial cells in desquamated clusters in bronchial washings.[2,5] The cellular and molecular mechanisms that underlie epithelial fragility remain poorly defined.

The cohesion of the bronchial epithelial cell layer is dependent on cell junctions, including tight junctions (TJ) sealing between the lateral boundaries of cells at their apical aspects, adherens junctions (AJ) desmosomes forming structural links between the cytoskeletons and gap junctions.[6] There are separate adhesion mechanisms that anchor the cells to the underlying basement membrane. One of the enigmas of atopic disease of the airways is how macromolecular allergens gain access to the populations of immunologically competent cells that populate the underlying bronchial mucosa and the specialized lymphoreticular structures of the airway wall and adjacent lymph nodes. Such exposures are essential to the disease persistence and exacerbations that characterize the clinical presentation of allergic asthma. While some allergens of biological origin possess intrinsic proteolytic activity, the majority of potential allergens are devoid of such enzymatic properties.

The accumulation of mast cells (MC) in the airway epithelium is a cardinal feature of the deranged mucosal immunology in allergic disease. Increased intraepithelial MC numbers are seen in association with atopy, and the highest levels are found in allergic asthma.[7] The ultrastructural and immunohistochemical features of intraepithelial MCs in human allergic asthma indicate continuous

degranulation and release of macromolecular granule contents,[7] and this is reflected in high levels of MC proteases in bronchoalveolar lavage fluid and sputum from patients with asthma.[8] Proteases represent the major constituents of MC secretary granules and include the serine proteases tryptase and chymase, and the metalloexopeptidase carboxypeptidase A3.[9] A range of functions demonstrated would be consistent with these proteases contributing to inflammation and tissue remodelling. Thus, tryptase can interact with various cell types, induce collagen secretion by fibroblasts, act as a growth factor for fibroblasts,[10] airway smooth muscle[11] and epithelial cells,[10] can stimulate release of cytokines from epithelial, endothelial and airway smooth muscle cells and can itself provoke MC degranulation.[12] MC chymase can cleave and activate matrix metalloproteinase (MMP-1, 2, 3 and 9),[13] control the bioavailability of various cytokines and convert angiotensin I to angiotensin II.[14] Human MC tryptase and chymase can both induce microvascular leakage and the accumulation of inflammatory cells,[15] but the contributions of MC chymase to airway epithelium remodelling and cell pathogenesis have not been investigated.

We hypothesized that MC protease enzymes could degrade the intercellular adhesion molecules associated with cell junction complexes in the bronchial epithelium, cause the loss of epithelial integrity and breakdown the epithelial barrier to allergens.

2 | MATERIALS AND METHODS

2.1 | Purification of MC chymase

Mast cell chymase was extracted and purified from human skin dissected from surgically amputated limbs. Prior written consent was obtained from patients by Changzhou general hospital, and the study was approved by the Changzhou University Bio-Medicine Ethics Committee. The procedure was described by McEuen and Walls.[16] The total protein concentration of purified chymase was measured by bicinchoninic acid protein assay (Sigma, Pool, UK); its purity was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and with silver staining to show the single clear band (30 kDa); its activity was determined using the substrate N-methoxysuccinyl-Ala-Ala-Pro-Val 4-nitroanilide (AAPFpNA) (Sigma) in U/mg/mL and characterized by Western blotting using human chymase-specific antibody CC1 (Immunopharmacology Group, Southampton).

2.2 | Primary bronchial tube preparation and paraffin sections

Primary bronchial tubes were explanted form pathological specimens removed from living donors at operation. Prior written consent was obtained from donors by Changzhou general hospital, and all works were undertaken with the approval of the Changzhou University Bio-Medicine Ethics Committee. Bronchial tubes were prepared and treated with chymase (0, 6 and 30 mU/mL) and 30 mU/mL heat-inactivated chymase (HI-chy30) for 16 or 24 hours (n = 6) in 5% CO_2 at 37°C before being paraffin-embedded. Bronchial ring sections were produced, and haematoxylin and eosin (H & E) staining was carried out. The in situ bronchial tube culture, slides preparation and computer image analysis were encoded by a third party to permit double blind analysis.

2.3 | Computer mapping of epithelial composition

For each coded slides, micrographs were captured (X100 objective lens) and the computer imaging software, Leica Application Suite (LAS), ensuring that the full length of epithelium present was in field. Using computer-mapping software, an algorithm was developed to permit the measurement of epithelial length. The lengths of epithelium displaying four different morphological appearances were recorded. The morphological criteria and the data were used to compare the proportions of intact, full thickness, basal cells and denuded epithelium to the total epithelial length of the bronchial ring for each slide. The slides correlating to chymase treatment conditions were then decoded after analysis.

2.4 | Cell culture and treatments

16HBE (human bronchial epithelium cell line) was seeded (5×10^5 cells/mL) in 6-, 12-, 24- or 96-well culture plate (Greiner) according to experimental design. Treatments were applied when 75% confluence; the various amounts of chymase (0, 10, 25, 50 and 100 mU/mL) or inhibitors were used in serum-free (SF) DMEM containing 1% PSG; the DMEM containing 10% foetal calf serum (FCS) as the positive control.

50 mU/mL heat-inactivated chymase (HI-chy50) was prepared at 98°C for 30 minutes until proteolytic activity was diminished. Chymostatin (Chymo, Sigma) is a chymase inhibitor. Marimastat (Marim, Tocris Bioscience, Missouri, UK) is a broad spectrum MMP inhibitor, with IC50 values of 3 and 6 nmol/L for MMP-9 and MMP-2, respectively; a final concentration of 6 nmol/L was chosen, corresponding with the lowest possible value to inhibit both MMPs. Pertussis toxin (PT, Merck, Darmstadt, Germany), an inhibitor of G protein-coupled receptors. Losartan potassium (LP, Merck), an inhibitor for angiotensin II receptors of 16HBE cells. Inhibitor (2 μmol/L; Chymo, Marim, PT or LP) was mixed with 50 mU/mL chymase (chy50) at room temperature for 30 minutes, which was named CI-chy50, Marim-chy50, PT-chy50 or LP-chy50, and the abolitions of catalytic activity were confirmed using chymase activity assay.

2.5 | Cell morphology and time-lapse photography

The cells with various treatments of chymase and others described above were monitored continuingly up to 72 hours using time-lapse photography, and the images were taken at 0, 6, 12, 18, 24, 48 and 72 hours at the same position within the culture well by a Leica X10 objective lens and the computer imaging software, Leica Application Suite.

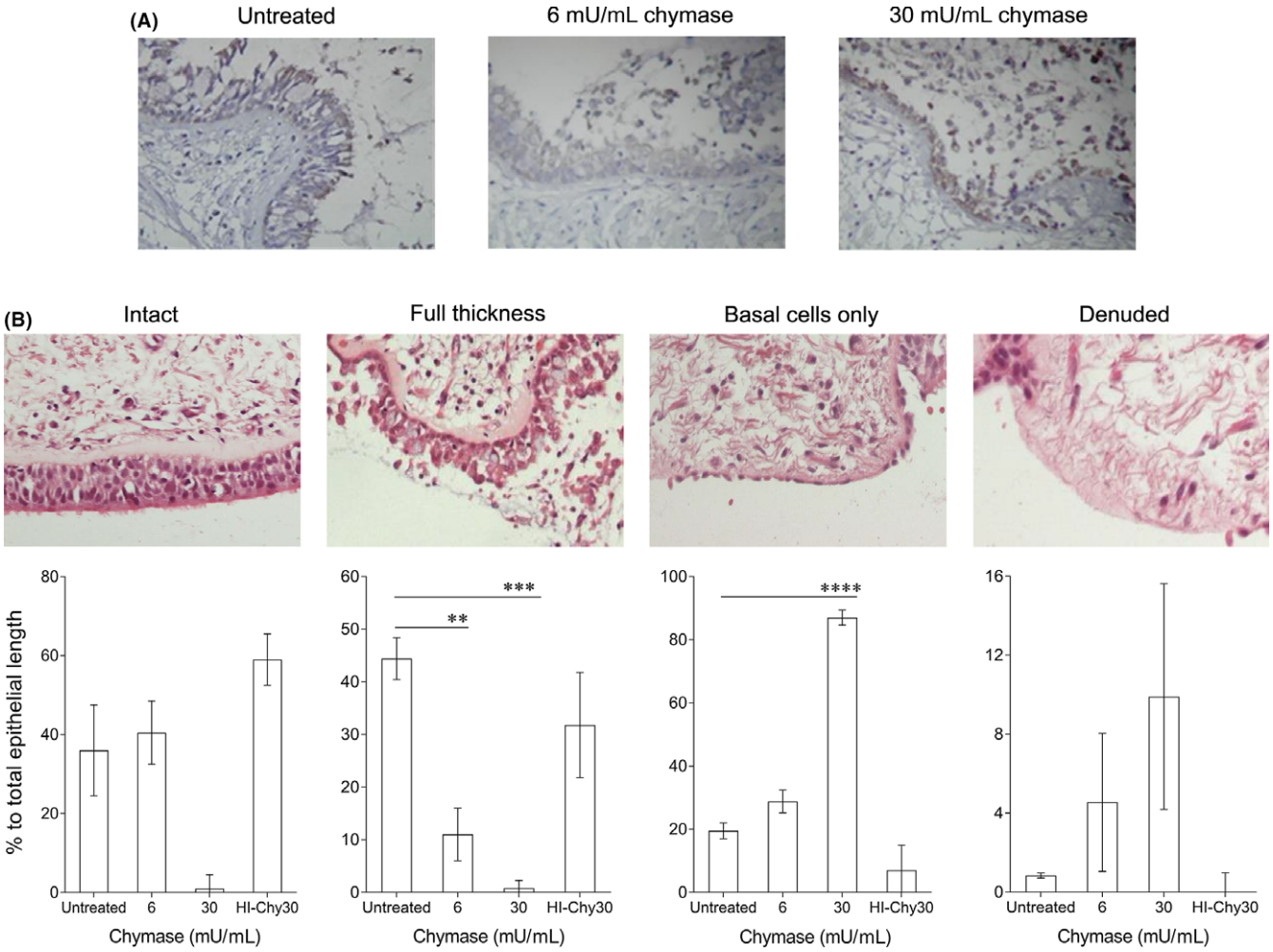
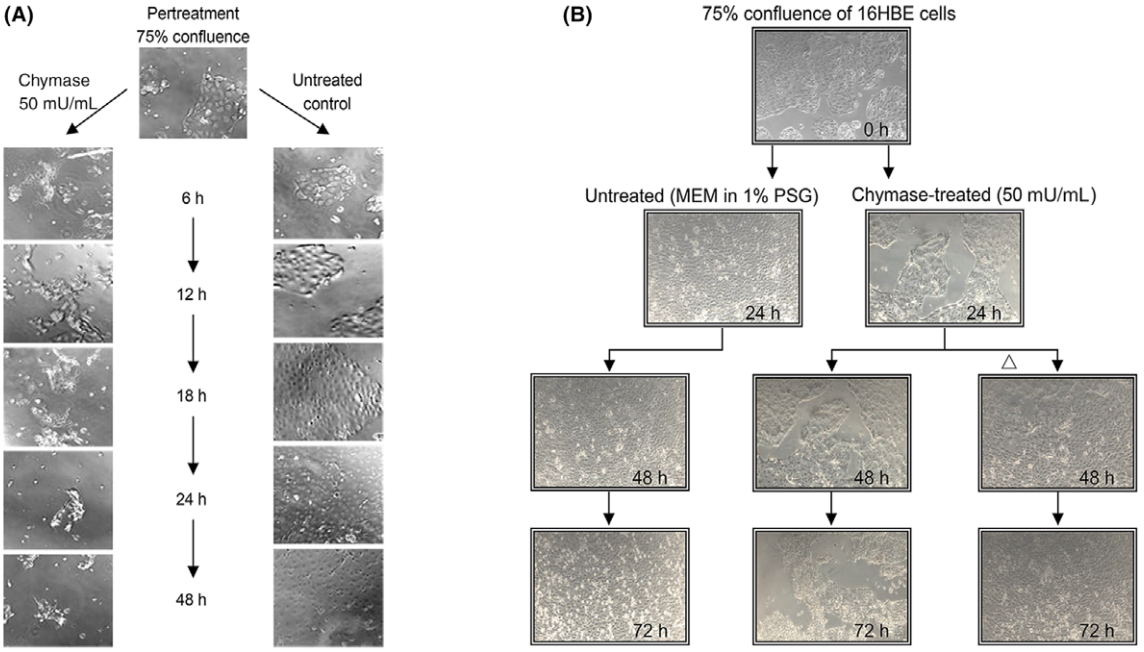


FIGURE 1 Mast cell (MC) chymase disturbed human airway epithelium. A, Morphology of the epithelium of human bronchial tube exposed to MC chymase in low and high doses for 16 h. B, Morphology of the epithelium of human bronchial tube exposed to MC chymase in at different conditions (mU/mL): untreated, 6, 30, heat-inactivated chymase (HI-chy30) for 24 h followed by paraffin embedding and H & E staining. The lengths of epithelium displaying four different morphological appearances. Intact: the intact epithelium is still attached to the basal membrane, and the cilia can be clearly seen. No visible changes to epithelial structure. Full thickness: the full thickness of epithelial layer is present but appears damaged. Basal cells: ciliated columnar cells have been lost. Denuded: all epithelial cells are absent. Morphological criteria for computer mapping of epithelial length by image analysis software. The data collected were to compare the proportions of intact, full thickness, basal cells and denuded epithelium to the total epithelial length (%) for each slide (×100 magnification, n = 4-6). $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$

2.6 | Fluorescence immunocytochemistry

Cells cultured with or without chymase in a 4-well glass chamber slide (NalgeNunc International, Rochester, New York, USA), after 24-hour treatment (SF), were fixed with pre-cooled methanol −20°C for 10 minutes and permeated with 0.1% Triton X-100 (Sigma) in PBS for 15 minutes at room temperature and then washed and blocked with 1% bovine serum albumin (BSA) in PBS. All primary antibodies against occludin, claudin, zonula occludens-1 (ZO-1), E-cadherin, focal adhesion kinase (FAK) or cytokeratin (Abcam, Cambridge, UK) were

FIGURE 2 Mast cell chymase reduced cell viability, adhesion and proliferation of 16HBE. A, Time-lapse photography and the images taken at different time point at same area (mid-zone) in the cell culture wells. B, Micrographs captured at 24, 48 and 72 h following application of treatments Leica (10X). △: chymase-treated cells had their culture medium replaced with complete DMEM at 24 h. C, Lactate dehydrogenase activity (LDH) released in the supernatants of 16HBE incubated with a range of treatments; expressed as a percentage of the total releasable LDH. D, Cell viability of 16HBE incubated with a range of chymase treatments, and 50 mg/mL heat-inactivated chymase (HI-chy50), 50 mU/mL chymostatin-inhibited chymase (CI-chy50), chymostatin alone (MTT assay). E, Cells proliferation rate of 16HBE exposed to 50 mU/mL chymase (chy50) alone and mixed with Marimastat (Marim-chy50), pertussis toxin (PT-chy50), losartan potassium (LP-chy50); also Marimastat, pertussis toxin and losartan potassium only (H3 incorporation assay) (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with untreated. $^{\#}P < 0.05$ compared with cells treated with chymase (50 mU/mL)


(A)
Pertreatment
75% confluence
Chymase
50 mU/mL
Untreated
control
6 h
12 h
18 h
24 h
48 h
(B)
75% confluence of 16HBE cells
0 h
Untreated (MEM in 1% PSG)
Chymase-treated (50 mU/mL)
24 h
24 h
48 h
48 h
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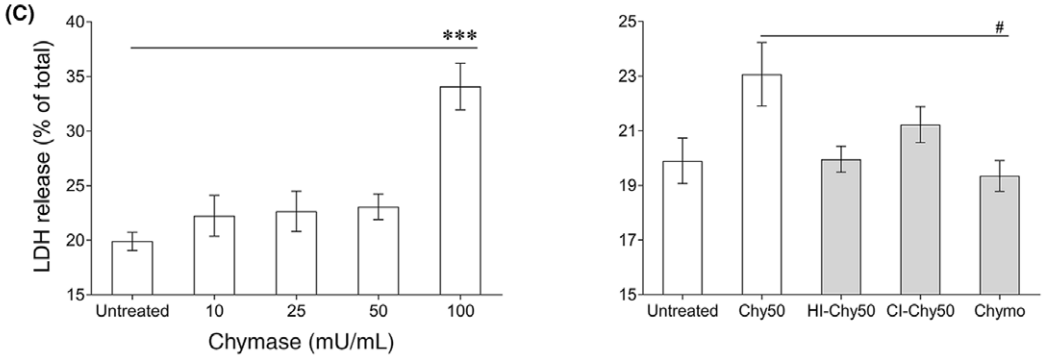
72 h
72 h
72 h


(C)
LDH release (% of total)
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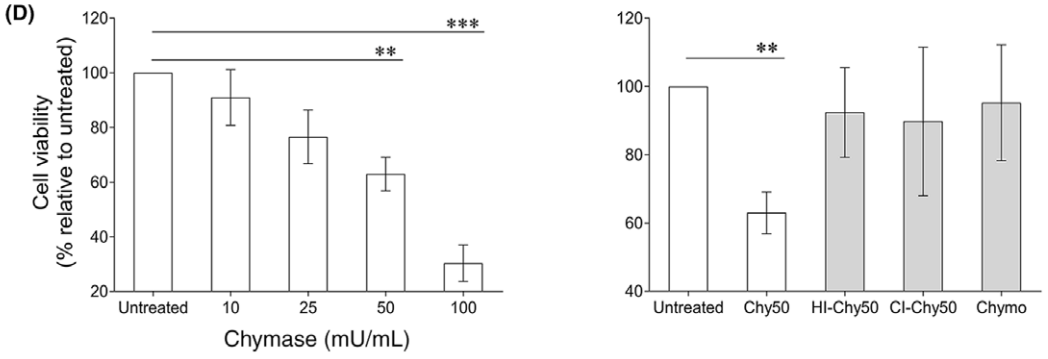
(D)
Cell viability
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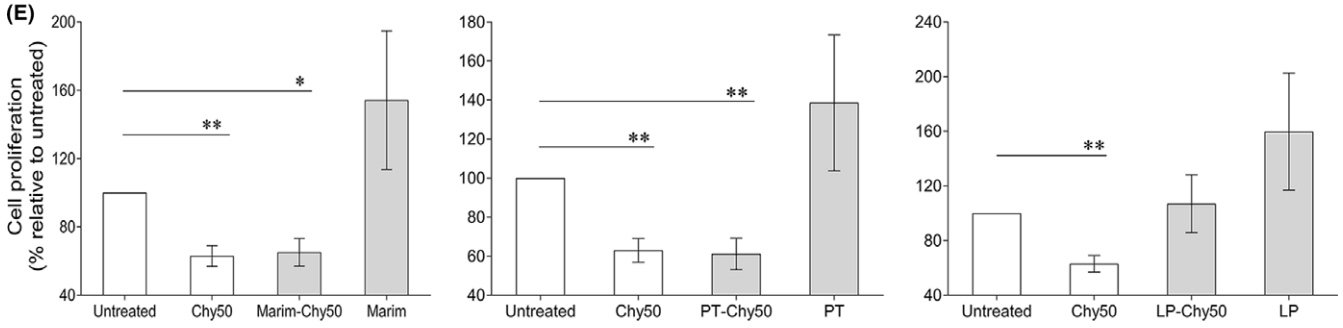
(E)
Cell proliferation
(% relative to untreated)
*
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Untreated
Chy50
Marim-Chy50
Marim
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Untreated
Chy50
PT-Chy50
PT
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Untreated
Chy50
LP-Chy50
LP

diluted in 1% BSA/PBS and incubated for 1 hour at room temperature, and fluorescent conjugated secondary antibodies were applied. Nuclear counterstaining was performed (PI, 1:2000) prior to mounting under coverslips with an antifade fluorescent mounting (Prolong Glod Invitrogen, Carlsbad, California, USA). Micrographs were taken using Zeiss Axiocam (X40) and KS400 software.

2.7 | MTT cell viability assay and lactate dehydrogenase activity (LDH) assay

100 μL of 10^5 cells/mL per well in 96-well plate was incubated for 6 hours and then washed twice with PBS; various treatments with chymase (0, 10, 25, 50 or 100 mU/mL), HI-chy50 or various inhibitors (Chymo, Marim, PT or LP) in SF condition were applied in triplicates for 24 hours. The supernatants were collected for gelatine zymography assay and LDH assay (LDH cytotoxicity kit, Roche, Basel, Switzerland) following manufacturer's instructions. The cells were further incubated with MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, 5 mg/mL) for another 4 hours. DMSO was added to dissolve the formazan crystals, and the optical densities (OD) were read at 570 nm by Bio-Rad microplate reader. Microplate Manager®6 software (Bio-Rad, Berkeley, California, USA) was used for analysis; the cell viability (%) relative to untreated cells was calculated (n = 6).

2.8 | Cell adhesion assay

Cells were resuspended in DMEM and diluted to 10^5 cells/mL in tubes; the treatments as above were applied immediately and gently agitated for 10 minutes. 100 μL of cell suspension from each tube was seeded in triplicates into a 96-well plate and incubated for 2 hours. Supernatants were discarded, plate was washed, and MTT cell counting assay was performed. Cell adhesion rates were calculated in percentage relative to untreated condition (n = 6).

2.9 | Tritiated thymidine incorporation assay

100 μL of 10^5 cells/mL per well in 96-well plates reached 75% confluence; chymase treatments (SF) were applied in triplicates for 24 hours. 10 μL of 50 μCi/mL H^3-thymidine was added to each well (0.5 μCi/mL) 6 hours before cells were fixed (100% pre-cooled methanol) and dissociated by 0.25 mol/L sodium hydroxide plus 0.2% sodium dodecyl sulphate (SDS). The cells were harvested from each well and transferred into a well of scintillation plate with 50 μL of scintillator, and the radiation count per minute (cpm) was measured for each well using the TopCount radioactive counter coupled with computer software (Perkin Elmer, UK) (n = 6); the cell proliferation rate (%) relative to untreated cells was calculated.

2.10 | Gelatin zymography analysis

The cell supernatants were mixed with zymography sample buffer. 10% SDS-PAGE copolymerized with 1 mg/mL gelatin was used for protein separation. The gels were washed in 2.5% Triton X-100 (3 × 10 minutes) at room temperature with agitation and incubated at 37°C overnight with proteolysis buffer. The gels were stained with coomassie blue G-250 for 30 minutes with agitation. Destaining was in tap water until clear gelatinolytic bands were visible. Zymograms were captured by Bio-Rad GS-800 densitometer coupled with Quantity One software package. The band intensity was quantified using ImageLab software (Bio-Rad, Berkeley, California, USA), and the relative quantity of each band was expressed as a percentage to untreated condition.

2.11 | Statistical analysis

Graphpad Prism 7.0 software (GraphPad Software Inc., San Diego, California, USA) was used for statistical analysis and graph preparations. Data were presented as the means ± standard error of the mean (SEM). The Mann-Whitney non-parametric test was used to compare differences between groups. $^*P < 0.05$ was taken as significant.

3 | RESULTS

3.1 | MC chymase disturbs human airway epithelium

To address whether MC chymase has direct effects on human airway epithelium, the epithelium of human bronchial tube in situ model was used. Relating to untreated controls, after exposure to chymase, fragmentation of the extracellular matrix (ECM) and dissociation of epithelial cells from the basement membrane can be observed. The morphology staining (Figure 1A) demonstrated epithelial cells were sloughed into airway lumen and the damage of subepithelial tissue by chymase was also observed dose-dependently. The morphological criteria of computer mapping of epithelial length on the sections from 4 to 6 different human bronchial tubes were analysed, and the mean of levels in loss of epithelium was scored as four degree, comparing to the untreated human bronchial tube; the intake epithelium and full thickness were reduced 40% and 43%, while the basal cell only and denude were increase by 60% and 9% after 24 hours incubation with high dose of MC chymase (30 mU/mL) (Figure 1B).

3.2 | Morphological, viability and proliferation impairment of 16HBE by MC chymase

Time-lapse photography was used to assess the behaviour of 16HBE during culture period with MC chymase (50 mU/mL); the morphology and growth were monitored up to 72 hours, and the images were taken at different time point at same area in the cell culture wells (Figure 2A). The cells were dissociated, the cell growth was impaired, and the morphology was changed gradually after exposure to chymase. The recoveries of cell regrowth and re-association were observed after removed chymase and replaced with fresh completed DMEM in cells (Figure 2B). The viability of

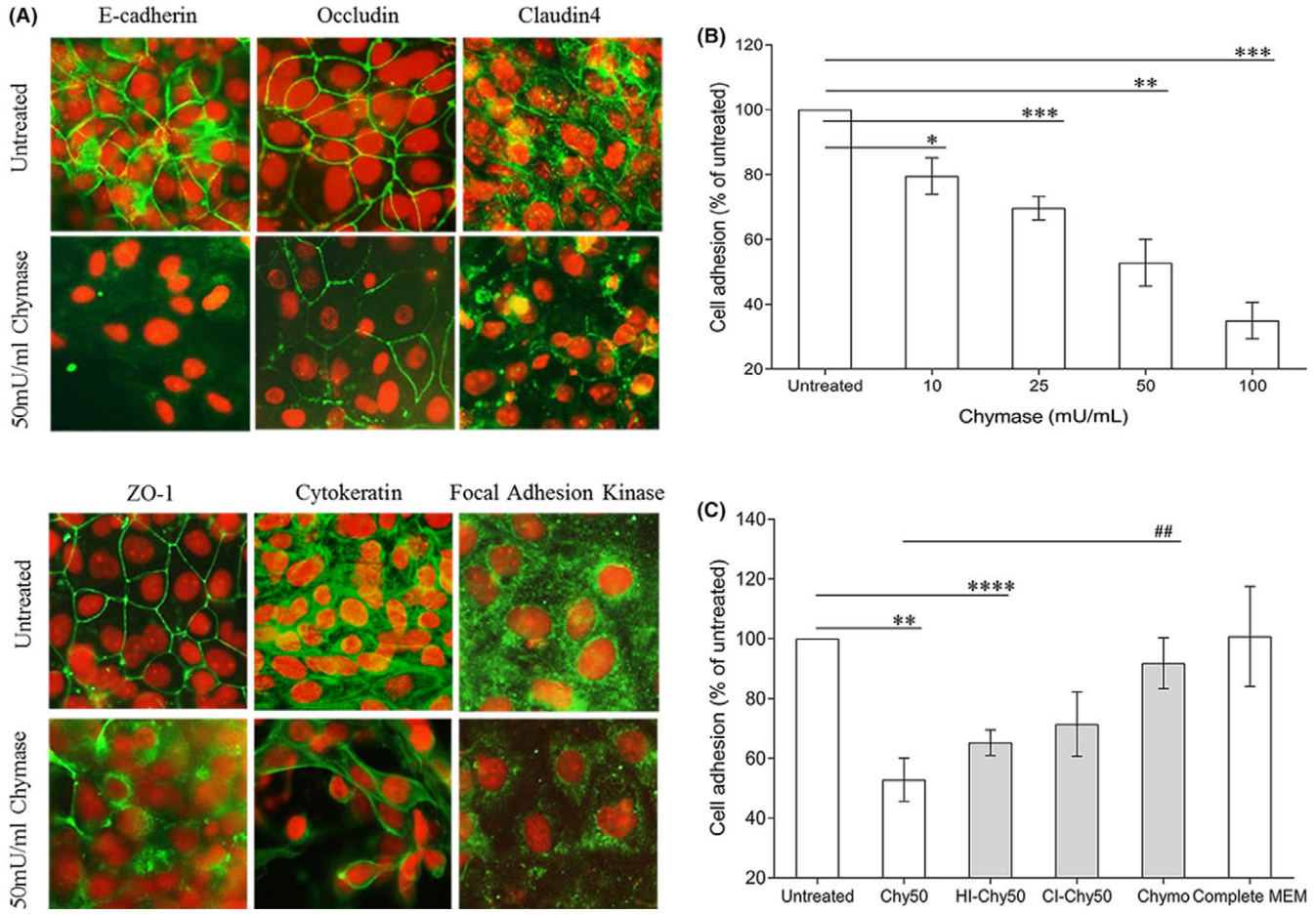

FIGURE 3 Mast cell chymase reduced the expressions of cell junction proteins and altered extracellular matrix components. A, Images using immunofluorescent microscopy (X40): the expressions of E-cadherin, occludin, claudin 4, ZO-1, cytokeratin and FAK of 16HBE cultured with or without chymase (50 mU/mL) for 24 h. B, The changes in 16HBE cell adhesion rates following 2-h treatment with chymase at various activities. C, 16HBE cell adhesion rate following 2-h treatments of 50 mU/mL chymase (chy50), 50 mg/mL heat-inactivated chymase (HI-chy50), 50 mU/mL chymostatin-inhibited chymase (CI-chy50), chymostatin (chymo) alone and DMEM with serum in triplicate (n = 6); data were expressed as a percentage of untreated, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared with untreated. $^{\#\#}P < 0.01$ compared with cells treated with chymase (50 mU/mL)

16HBE was reduced while LDH activity was increased by MC chymase compared with untreated cells, and the cells exposed to HI-chy50, CI-chy50 and Chymo only (Figure 2C). With high dose of chymase (100 mU/mL), the cell viability was decreased by 43%, in parallel, LDH activity was elevated by 26% (Figure 2D). In H3-thymidine incorporation assay, Marim and PT did not block chymase action on the reduction in cell proliferation rate, but LP rescued cells from chymase damage by 50%, although it is not significant statistically (Figure 2E).

3.3 | MC chymase disturbs cell junctions and ECM components

The data from immunocytochemistry illustrated the diminished expressions of cell adhesion proteins (E-cadherin, occludin, claudin 4 and ZO-1), FAK and cytokeratin of 16HBE after chymase treatment (Figure 3A). The cell shedding from culture plated occurred as time extended, indicating the interruption in cell-matrix association that could lead to cell death. E-cadherin, occludin and claudin 4 were all seen to show structural disorganization with gaps appearing in the continuity of proteins expressed on the cell surface membrane. ZO-1 expression was altered in that the organized pattern of expression around the cell surface membrane has been lost. The intermediate filament cytokeratin with the extracellular mesh-like structure was lost between cells. There was also an apparent reduction in the expression of FAK by chymase in cells. Cell adhesion assay indicated the loss of 16HBE adhesion to matrix was chymase induced dose-dependently (Figure 3B) and partially effected by HI-chy50 or CI-chy50 (Figure 3C).

3.4 | MC chymase activates MMP-2 and MMP-9 secreted from 16HBE

To evaluate the effects of chymase on MMP-2/9 expression and activity of 16HBE, gelatine zymography analysis of the supernatants collected from 16HBE 24-hour post-treatment was used

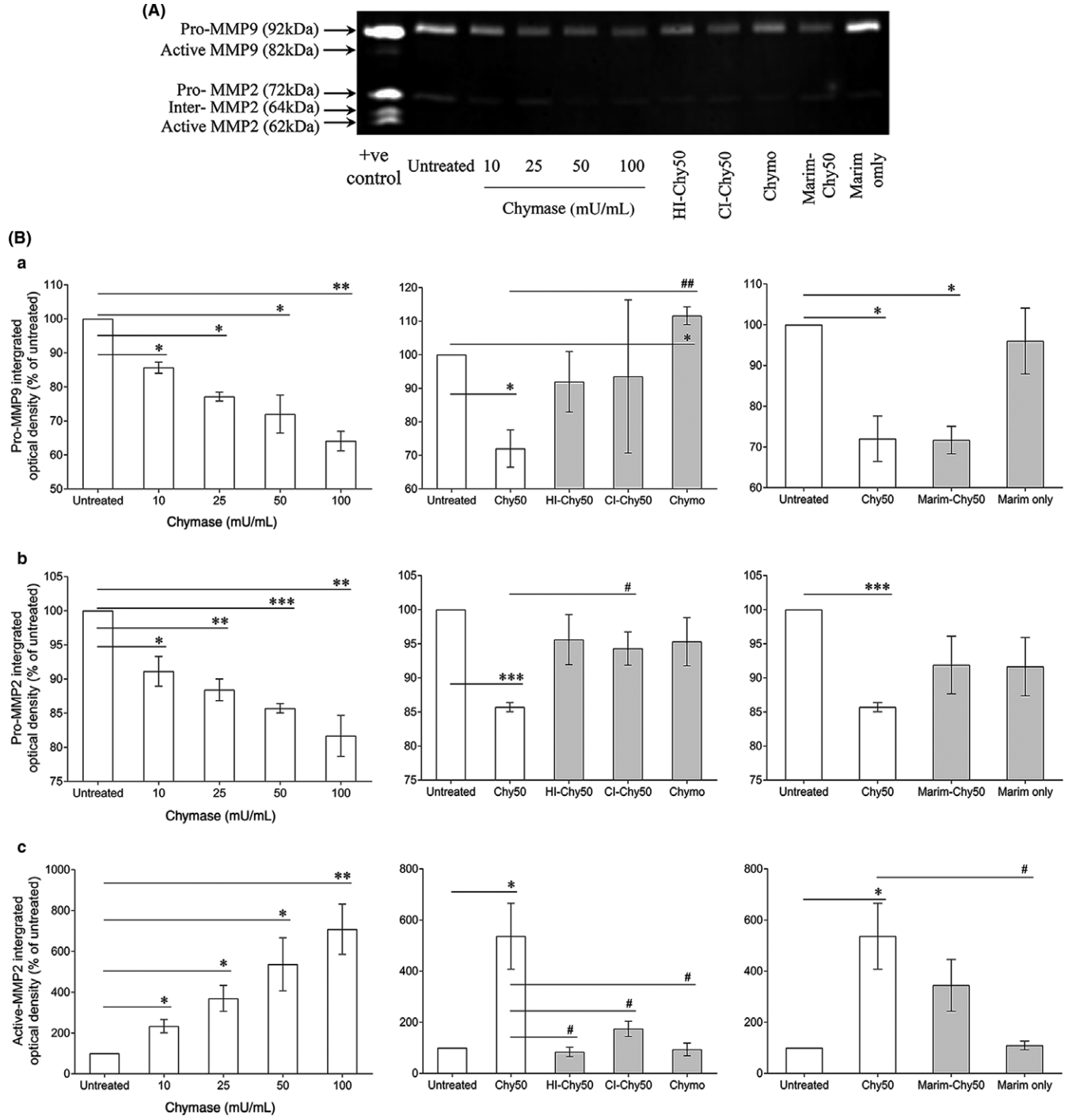

FIGURE 4 Mast cell chymase induced pro-MMP-2 activation and reduced pro-MMP-9 expression of 16HBE. A, Gelatin zymography images. B, Band intensity quantization by Image Lab software (Bio-Rad) of pro-MMP-9 band (92 kDa, B-a); pro-MMP-2 band (72 kDa, B-b); active-MMP-2 band (62 kDa, B-c), expressed as the percentage to untreated cells (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with untreated. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with cells treated with chymase (50 mU/mL)

(Figure 4A) and the relative quantitation (%) of the band intensity to untreated cells was analysed (Figure 4B). The increased chymase activity reduced the intensity of the bands for pro-MMP-2/9, but increased that of active-MMP-2, whereas HI-chy50 and CI-chy50 blocked chymase activity on pro-MMP-2/9, but Marim did not. To differ about the effects between MC chymase and MMP-2/9 secreted by 16HBE cells on gelatine digestion, and to understand the interactions between MC chymase and MMP-2/9, the different types of cell mediums with or without chymase addition and chymase alone were analysed. The results showed that chymase alone could not digest gelatine detected by zymography (Figure 5B-a,b) and Western blotting (Figure 5B-c).

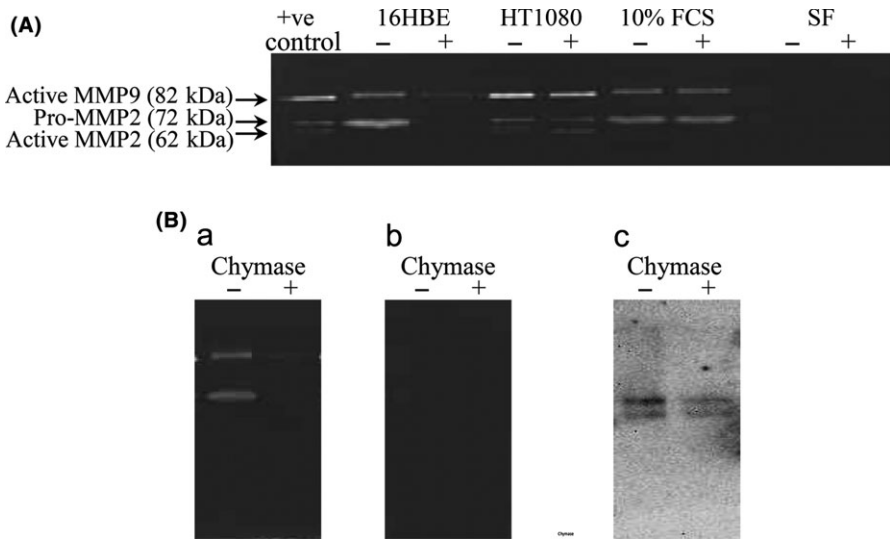

FIGURE 5 Differed effects of chymase on gelatine or on MMP-2. The culture medium (SF) of HT-1080 (positive control) or 16HBE with or without chymase incubated for 2 h. A, Zymography to detect MMP-2/9 activity in different conditions. B-a, Gelatin zymography image of 16HBE cell culture medium with (+) or without (–) chymase (50 mU/mL); B-b, Gelatin zymography image of chymase (50 mU/mL) only (+) or none (–) in serum-free medium. B-c, Western blotting image of MMP-2 expressions by 16HBE cells cultured with (+) or without (–) chymase (50 mU/mL)

4 | DISCUSSION

The increased number of MCs infiltrated into bronchial epithelium has been observed in the airways of allergic asthma patients.[17-19] Recent clinical study using network analyses (BNA and TDA, IRIS 2.0 software) in large patients cohorts data has found the strong associations of asthma severities with MC mediator levels and shown the evidences that MC activation is a key role in severe asthma especially.[20] Previous reports have demonstrated that tryptase and cytokines derived from MC have inflammatory effects on bronchial epithelial cells.[21-24] However, how MC shapes the local airway inflammation, interacting with bronchial epithelium during allergic asthma development is still a hot spot for the research interests.

Airway epithelial barrier is an important part of the innate immune system against outer invading. Dysregulated epithelium barrier immunity and the damage to the airway epithelium are prominent features of chronic asthma. The increased reports demonstrated the epithelial disruption and mucosal damage in the asthmatic bronchial epithelium includes gap openings, partial denudation and loss of ciliated cells.[25-28] The susceptibility of epithelial barrier to the action of different inhaled component from environment determines the asthmatic phenotype.[27] But the underline mechanisms were not clear. Our data from in situ bronchial tube model and computer-mapping analysis provided the evidences that MC chymase provoked profound changes in the morphology of the epithelial layer. Following incubation with MC chymase, there was a reduction of 40% in the length of epithelium that was intact, with detachment of columnar epithelial cells and even of basal cells, exhibiting the similar damage features, such as gap openings, partial denudation and loss of ciliated cells, indicating that the overactivation of accumulated MCs at basal and intra-epithelium released great amount proteases including chymase, which degraded the connections between ECM-cells and cell-cell, resulting in different levelled epithelium damages, and these damages determine the severities of asthma, respectively.

Cell junctions consist of multiprotein complexes that provide contacts between neighbouring cells or between cells to ECM. They also build up the paracellular barrier of epithelia and control the paracellular transport. Cell junctions are especially abundant in epithelial tissues; TJ and AJ, both linked to perijunctional actin filaments.[29,30] TJ (claudin 1, occludin) regulate paracellular transport of ions and small molecules; AJ (E-cadherin) are for initiation and maintenance of cell-cell adhesion.[31,32] Epithelial TJ and AJ bind to cytoplasmic proteins (ZO family) which link to the actin cytoskeleton and cell structure protein cytokeratin and FAK, involving in numerous signal transductions for cell growth or death, and building apical-basal epithelial cell polarity and epithelium integrity.[33,34] Increased epithelial permeability is a hallmark of epithelium mucosal inflammation. Any insults that result in epithelial cell death or detachment from basement membrane will lead to increased permeability. More subtle exposures also increase leakiness of the epithelial barrier by affecting cell junctional complex structure and function without causing cell death. Our present study using immunocytochemistry staining illustrated the disruptions of cell junction proteins (claudin 1, occludin, E-cadherin, ZO-1) and structure proteins (cytokeratin and FAK) by chymase in vitro indicating that chymase disrupts epithelial barriers by degrading cell junction proteins and that the local MC accumulation and activation are responsible to the leakage of epithelial during asthma development.

Epithelial cell proliferation and adhesion are important for local wound healing after damage. MMPs are the components of ECM and associated with cell proliferation and adhesion by mediating the degradation of ECM. The images from gelatin zymography demonstrated that chymase reduced MMP-2/9 expressions of 16HBE and clearly illustrated that chymase activated/degraded MMP-2, but had no effects on gelatin digestion, indicating that during ECM remodelling, gelatin is not the substrate for chymase, whereas the MMP-2/9 could be the substrates for chymase. Previous study reported that chymase activated latent TGF-β1 to active TGF-β1 which leads to activate epithelial cells.[10] Our data showed

that in vitro chymase, at low concentration, directly stimulated an increase in proliferation of epithelial cells, but at higher concentrations of chymase-treated epithelial cells, there were decreases in cell numbers, adhesion rates, MMP-2/9 expressions and an increase in LDH activities in dose-dependent manner, which were associated with the disruption in the expressions of adhesion molecules, cytokeratin and FAK. The interesting observations on the applications of various inhibitors mixed with same amount of chymase and inhibitors alone were that Marim, PT and LP did not significantly inhibit chymase proteolysis function on the reduction in 16HBE proliferations, although LP had a little recovery effect to rescue cells from chymase damage; while the chymase mixed with Chymo (a chymase inhibitor) and the heat-inactivated chymase, both reduced the chymase-caused damage on cell growths, respectively, indicating that the main function of chymase in this case is proteolysis, cleaving the contacts between cells and cell-ECM by disrupting cell junctions, MMP expressions and cell proliferation/adhesion, resulting in the damage of epithelium integrity, reduction in cell viability and the inhibition of wound healing of epithelial cell layer in airway inner wall. Our present study might have revealed the one of the underline mechanisms for epithelial desquamation and apoptosis reported in several publications.[4,5,28,35-37]

In conclusion, our findings indicate the proteolysis function of MC chymase on epithelium ECM remodelling and cell lining damages, which can explain the overall associations of disturbed cell adhesion junctions, cell monolayer shedding and the disruptions of epithelium integrity of bronchial tube with the inhibited wound healing (caused by reduced rates in epithelial proliferation and adhesion) by chymase derived from activated MCs in asthmatic airway epithelium. Desquamation of airway epithelial cells is one of the consequences of airway inflammation in asthma, resulting from the action of inflammatory mediators, whereas MC chymase has displayed the contributions to the desquamative epithelium of bronchi tube in this study, suggesting that MC chymase impairs the role of epithelial barrier in nature defence and induces the dysfunction of the airway wall ECM remodelling in pathogenesis of asthma, and such as a pharmaceutical target needs to have well attention.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Prof. Xiaoying Zhou contributed to overall experimental plan, support and guidance; Miss Tao Wei contributed to cell culture works; Mr Christopher W Cox and Miss Yuan Jiang contributed to computer mapping and image analysis; Dr. Andrew F Walls and Prof. William R Roche contributed to support and advice.

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