Mast cell chymase regulates extracellular matrix remodeling-related events in primary human small airway epithelial cells

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Background: Mast cells are implicated in the pathogenesis of asthma, but the underlying mechanisms are not fully elucidated. Under asthmatic conditions, mast cells can relocalize to the epithelial layer and may thereby affect the functional properties of the airway epithelial cells.

Objectives: Activated mast cells release large quantities of proteases from their secretory granules, including chymase and tryptase. Here we investigated whether these proteases may affect airway epithelial cells.

Methods: Primary small airway epithelial cells were treated with tryptase or chymase, and the effects on epithelial cell viability, proliferation, migration, cytokine output, and transcriptome were evaluated.

Results: Airway epithelial cells were relatively refractory to tryptase. In contrast, chymase had extensive effects on multiple features of the epithelial cells, with a particular emphasis on processes related to extracellular matrix (ECM) remodeling. These included suppressed expression of ECM-related genes such as matrix metalloproteinases, which was confirmed at the protein level. Further, chymase suppressed the expression of the fibronectin gene and also caused degradation of fibronectin released by the epithelial cells. Chymase was also shown to suppress the migratory capacity of the airway epithelial cells and to degrade the cell-cell contact protein E-cadherin on the epithelial cell surface.

Conclusion: Our findings suggest that chymase may affect the regulation of ECM remodeling events mediated by airway epithelial cells, with implications for the impact of mast cells in inflammatory lung diseases such as asthma. (J Allergy Clin Immunol 2022;150:1534-44.)

Key words: *Mast cells, chymase, tryptase, epithelial cells, extracellular matrix*

Mast cells (MCs) are multifaceted immune cells, contributing to a wide variety of homeostatic and pathologic settings.[1-3] In the human asthmatic lung, MCs are abundant in the airway mucosa and epithelium and are hence ideally suited to react to environmental factors. This has raised the notion that MCs can influence asthma, and indeed, MCs are now recognized as key players in asthma pathophysiology.[4-7]

A hallmark feature of MCs is their high content of secretory granules, which are densely packed with various compounds, including histamine, lysosomal hydrolases, serglycin proteoglycans, cytokines, and remarkably high amounts of MC-restricted proteases, with the latter comprising chymase, tryptase, and carboxypeptidase A3.[8-11]

Human MCs are subclassified into 2 subtypes, MC_T and MC_{TC}, based on their protease content.[12] In healthy individuals, MCs expressing tryptase only (MC_T) represent the predominant type found in the lung, whereas MCs expressing both tryptase and chymase (MC_{TC}) are rare.[12,13] However, it has been shown that there is a profound increase in the MC_{TC} subset in the small airways of individuals with severe asthma[12,14-16] (ie, pointing to an increased chymase expression under such circumstances). Further, an increase in chymase-expressing MCs in the airways has been associated with better lung function in severe asthma.[13,15] Together, these findings suggest that MC chymase can have an impact on events occurring in the small airways of asthmatic patients. However, the mechanism(s) behind such effects is currently not known.

During asthmatic conditions, MCs are prone to be activated, typically by undergoing degranulation, as a result of which the contents of their secretory granules are released.[1-3] This leads to the presence of large amounts of MC proteases in the lung environment,[8] and these will thereby have the capacity to affect cell populations that are closely situated to activated MCs. Considering that MCs are situated close to the small airway epithelium in asthmatic settings,[17-19] it is likely that proteases released from MCs could interact with the epithelial cells. Potentially, this could have a functional impact on the airway epithelial cells, which may influence the asthmatic response.

Here we have investigated this possibility by examining the effect of MC tryptase and chymase on primary human small airway epithelial cells (HSAECs). We found that MC proteases, in particular chymase, have a strong ability to influence various features of HSAECs, with a particular impact on parameters related to extracellular matrix (ECM) remodeling. MCs could thereby influence asthma outcome by regulating ECM remodeling events mediated by airway epithelial cells.

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Abbreviations used
ATCC: American Type Culture Collection
ECM: Extracellular matrix
EdU: 5-Ethynyl-2'-deoxyuridine
HSAEC: Human small airway epithelial cell
MC: Mast cell
MMP: Matrix metalloproteinase
uPAR: Urokinase plasminogen activator receptor

METHODS

Reagents

Recombinant human β-tryptase was as described.[20] The recombinant human chymase (catalog no. C8118-50UG) was from Sigma-Aldrich (St Louis, Mo).

Cell culture

HSAECs (from the American Type Culture Collection [ATCC], Manassas, Va; catalog no. PCS-301-010) were grown at 37°C (5% CO_2) in Airway Epithelial Cell Basal Medium (ATCC; catalog no. PCS-300-030) containing a Bronchial Epithelial Cell Growth Kit (ATCC; catalog no. PCS-300-040), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma-Aldrich; product no. P0781). The cells were subcultured after reaching 80% confluency, detached with trypsin-EDTA solution (Sigma-Aldrich; catalog no. T3924), and seeded at a concentration of 1×10^5 cells/mL. Cell numbers were determined by using trypan blue (Thermo Fisher Scientific, Waltham, Mass; catalog no. 15250061) staining and with an automated cell counter (Thermo Fisher Scientific, Countess II FL). Cells were obtained from 4 independent donors; similar results were obtained for the individual donors.

Cell viability assessment

The cytotoxicity of MC proteases was assessed by annexin V/DRAQ7 staining.[21]

Cell proliferation assessment

Cell proliferation was assessed by 5-ethynyl-2'-deoxyuridine (EdU) staining.[21]

Cytokine array

Human Cytokine Array C1000 (RayBio, Peachtree Corners, Ga; catalog no. AAH-CYT-1000) was used according to the manufacturer's protocol, followed by quantification and normalization the ImageJ protein array analyzer software.

ELISA

The following ELISA kits were used: uPAR (PLAUR) (Thermo Fisher Scientific, Invitrogen; catalog no. EHPLAUR), GRO alpha/CXCL1 (Thermo Fisher Scientific, Invitrogen; catalog no. BMS2122), NAP-2/CXCL7 ELISA (RayBio; catalog no. ELH-NAP2-1), IL-1RA (Thermo Fisher Scientific, Invitrogen; catalog no. KAC1181), GM-CSF (Thermo Fisher Scientific, Invitrogen; catalog no. KHC2011), IGFBP-2 (RayBio; catalog no. ELH-IGFBP2), and TIMP-2 (R&D Systems, Minneapolis, Minn; catalog no. DTM200).

Wound closure assay

Wound closure assay was performed as described.[22]

Immunofluorescence

Cells were fixed in 2-well slides (SARSTEDT, Nümbrecht, Germany; catalog no. 94.6140.202) for 15 minutes in 4% (wt/vol) paraformaldehyde (Sigma-Aldrich; catalog no. 1.04005.1000) in PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, and blocked with 5% BSA solution (LI-COR Odyssey, Lincoln, Neb; catalog no. 927-50000) for 1 hour. This was followed by incubation overnight (at 4°C) with anti-fibronectin (1:1,000; Sigma-Aldrich; catalog no. F3648) or anti–E-cadherin (1:200, Cell Signaling, Danvers, Mass; catalog no. 3195) antibody diluted in 1% blocking buffer, followed by incubation with anti-rabbit Alexa Fluor 488–conjugated secondary antibodies (diluted 1:500 in PBS; Thermo Fisher Scientific, Invitrogen; catalog no. A32731) for 1 hour (at room temperature in dark). Nuclei were visualized by adding 10 μL of NucBlue Fixed Cell ReadyProbes Reagent (4',6-diamino-2-phenylindole; Thermo Fisher Scientific, Invitrogen; catalog no. R37606). F-actin was visualized by using ActinRed 555ReadyProbes reagent (Thermo Fisher Scientific, Invitrogen; catalog no. R37112). Extensive washes were performed with PBS after each step. Cells were mounted with 10 μL of SlowFade Diamond Antifade Mountant (Thermo Fisher Scientific, Invitrogen; catalog no. S36963). A Nikon ECLIPSE 90i fluorescence microscope was used. Primary antibodies were substituted with isotype control antibodies as negative controls.

Immunoblot and gelatin zymography

Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad, Hercules, Calif; catalog no. 4568096) and a Trans-Blot Turbo Transfer System (Bio-Rad; catalog no. 1704158) were used. Samples were reduced with 10% β-mercaptoethanol (Sigma-Aldrich; catalog no. M3148), and treated at 95°C (for 10 minutes). Blots were probed with rabbit anti-fibronectin antibody (1:500, given by Staffan Johansson, Uppsala University) or anti–E-cadherin (1:1000, Cell Signaling; catalog no. 3195) overnight (4°C). Anti-rabbit IgG, horseradish peroxidase–linked antibody (1:1500, Cell Signaling; catalog no. 7074) and ECL Prime immunoblotting detection reagent (Cytiva, Uppsala, Sweden; catalog no. GERPN2236) were used for chemiluminescence detection. Images were acquired by using the ChemoDoc MP Imaging System (Bio-Rad; catalog no. 17001402). Gelatin zymography was performed as described.[21]

Reverse-transcriptase quantitative PCR

Total RNA was isolated by using NucleoSpin RNA II (Macherey Nagel, Düren, Gemany; catalog no. 740955); reverse-transcriptase quantitative PCR analysis was performed according to the manufacturer's protocol Bio-Rad provided primers for fibronectin (qHsaCED0043611) and matrix metalloproteinase 9 (MMP9) (qHsaCID0011597); the housekeeping gene is glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward: 5′-GGATTTGGTCGTATTGGG, reverse: 5′- GGAAGATGGTGATGGGATT).

AmpliSeq transcriptome analysis using edgeR

Ampliseq transcriptome and pathway analysis were performed as described.[21]

Statistical analysis

Two-way ANOVA followed by Dunnett multiple comparisons was used. Adjusted *P* values of .05 or less were considered statistically significant. Figures were prepared with GraphPad Prism 8.0 software (GraphPad Software Inc, San Diego, Calif). Results shown are from individual experiments representative of at least 3 experiments, unless otherwise indicated.

RESULTS

MC tryptase does not affect HSAEC morphology and has a minor effect on epithelial cell viability

To assess whether MC proteases can influence HSAECs, we first investigated whether tryptase has an impact on HSAEC morphology. The morphology of HSAECs was unaffected after

treatment with recombinant tryptase at 1.25 to 100 nM and after up to 24 hours of incubation (see Fig E1, *A* in the Online Repository at www.jacionline.org). Further, we tested whether tryptase can induce HSAEC apoptosis. As judged by annexin V/DRAQ7 staining, tryptase did not induce apoptosis in the HSAECs. On the contrary, we noted that tryptase at concentrations of 1.25 to 10 nM caused a slight, yet significant increase in the percentage of viable (annexin V–negative/DRAQ7-negative) HSAECs, whereas no viability-promoting effect was seen at higher tryptase concentrations (see Fig E1, *B*).

MC chymase causes morphologic changes in HSAECs and reduces epithelial cell viability at high concentrations

Next, we assessed whether human recombinant chymase can affect HSAECs. As shown in Fig 1, *A*, chymase had no major impact on HSAEC morphology at concentrations up to 5 nM and at incubation times up to 24 hours. However, starting from concentrations of 10 nM, chymase induced morphologic alterations of the HSAECs. This was manifested as a contraction of the cells and partial cell detachment seen at chymase concentrations of 5 nM or higher. We also treated the HSAECs with a combination of chymase (5 nM) and tryptase (5 nM); the co-treated HSAECs displayed a morphology similar to that of cells treated with chymase only (data not shown). An assessment of cell viability revealed that at concentrations higher than 10 nM, chymase caused a significant increase in the populations of apoptotic (annexin V–positive/DRAQ7-negative) and necrotic/late apoptotic (annexin V–positive/DRAQ7-positive) cells (Fig 1, *B*).

MC chymase causes a modest increase in HSAEC proliferation

Next, we investigated whether the MC proteases can affect HSAEC proliferation, as monitored by EdU staining. For these analyses, we used a nontoxic concentration of tryptase (5 nM) and a range of chymase concentrations (1.25-100 nM). As seen in Fig 1, *C*, tryptase did not cause any significant effect on the percentage of EdU^+ cells, suggesting no effect on HSAEC proliferation. In contrast, chymase at 5 nM caused a slight but significant increase in the EdU^+ cell population.

MC proteases affect the output of growth factors and cytokines from primary HSAECs

To further explore the impact of the MC proteases on HSAECs, we assessed effects on the output of cytokines, growth factors, and immunoregulatory molecules. For this, we adopted an unbiased array approach (for setup, see Fig E2 in the Online Repository at www.jacionline.org). The assessment revealed that chymase causes an increased output of several proinflammatory cytokines, including macrophage migration inhibitory factor (MIF), IL-6R (soluble), CCL27, IFN-γ, neurotrophin-3 (NT-3), urokinase plasminogen activator receptor (uPAR), CXCL1, and CXCL7 (Fig 2, *A* and *B*). Chymase also stimulated the release of tissue inhibitor of metalloproteinase 2 (TIMP-2) and insulin-like growth factor-binding protein-2 (IGFBP2) and IGFBP6 (Fig 2, *A* and *B*). In addition, chymase promoted secretion of the anti-inflammatory compound IL-1 receptor antagonist (IL-1RA) (Fig 2, *B*). In addition, secretion of several proinflammatory cytokines, including GM-CSF and IL-12p70, was decreased after chymase treatment. Tryptase had less pronounced effects on the output of the corresponding compounds from HSAECs. However, similar to chymase, tryptase had an inhibitory effect on the output of IL-12p70, and the data also suggested that tryptase has suppressing effects on the output of nerve growth factor-β (β-NGF), vascular endothelial growth factor A (VEGF-A), and CXCL1. Tryptase also caused increased secretion of migration inhibitory factor, IL-1RA, CCL27, amphiregulin (AREG), CXCL11, and IGFBP-6 (Fig 2, *B*).

To validate these findings, selected compounds were quantified by ELISA. Indeed, ELISA measurements confirmed the upregulated output of IL-1RA, uPAR, CXCL7, and IGFBP2 in response to chymase (Fig 2, *D*, *E*, *H*, and *I*). Moreover, the downregulated output of CXCL7 and the upregulated AREG secretion in response to tryptase was confirmed by ELISA (Fig 2, *F* and *H*). In contrast, ELISA measurements did not confirm the upregulated secretion of CXCL1 and decreased GM-CSF secretion in response to chymase (Fig 2, *C* and G). The stimulatory effects of chymase on uPAR and IGFBP2 output were abolished by a chymase inhibitor, indicating that the effects of chymase are dependent on its catalytic activity (see Fig E3 in the Online Repository at www.jacionline.org).

MC chymase causes delayed migration of primary HSAECs

Considering the profound morphologic effect of chymase on HSAEC morphology, we next asked whether this can be translated to effects on cellular migration. To address this, we used a scratch migration assay. As seen in Fig 3, *A* and *B*, closure of the scratches was seen after approximately 24 hours in untreated cell cultures; a similar velocity of scratch closure was seen in cells treated with tryptase. In contrast, chymase caused a significant delay of the scratch closure, suggesting that chymase can affect the migratory properties of the HSAECs (Fig 3, *A* and *B* and see Fig E4 in the Online Repository at www.jacionline.org for the effect of extended incubation times with chymase).

MC chymase degrades fibronectin produced by primary HSAECs

The aforementioned data indicate that chymase has profound effects on airway epithelial cell morphology and migration. One conceivable explanation for these findings could be that chymase has effects on ECM compounds necessary for maintaining these features in HSAECs. To address this, we first assessed whether chymase can affect the output and/or deposition of fibronectin from the cells, based on the known ability of chymase to degrade this ECM protein,[23-25] Accordingly, we stained untreated or chymase-treated HSAECs for fibronectin. As seen in Fig 4, *A*, untreated HSAECs showed not just intracellular fibronectin staining but also abundant extracellular deposition of fibronectin (*arrows* in Fig 4, *A*). Intracellular fibronectin was also detected after treatment with chymase. However, extracellular fibronectin deposits were undetectable, suggesting that chymase has the capacity to degrade deposited extracellular fibronectin. The latter notion was also supported by immunoblot analysis, which revealed degradation of extracellular fibronectin by chymase (Fig 4, *B*). A suppressing effect of chymase on fibronectin was also seen at the mRNA level, with chymase causing a downregulation of

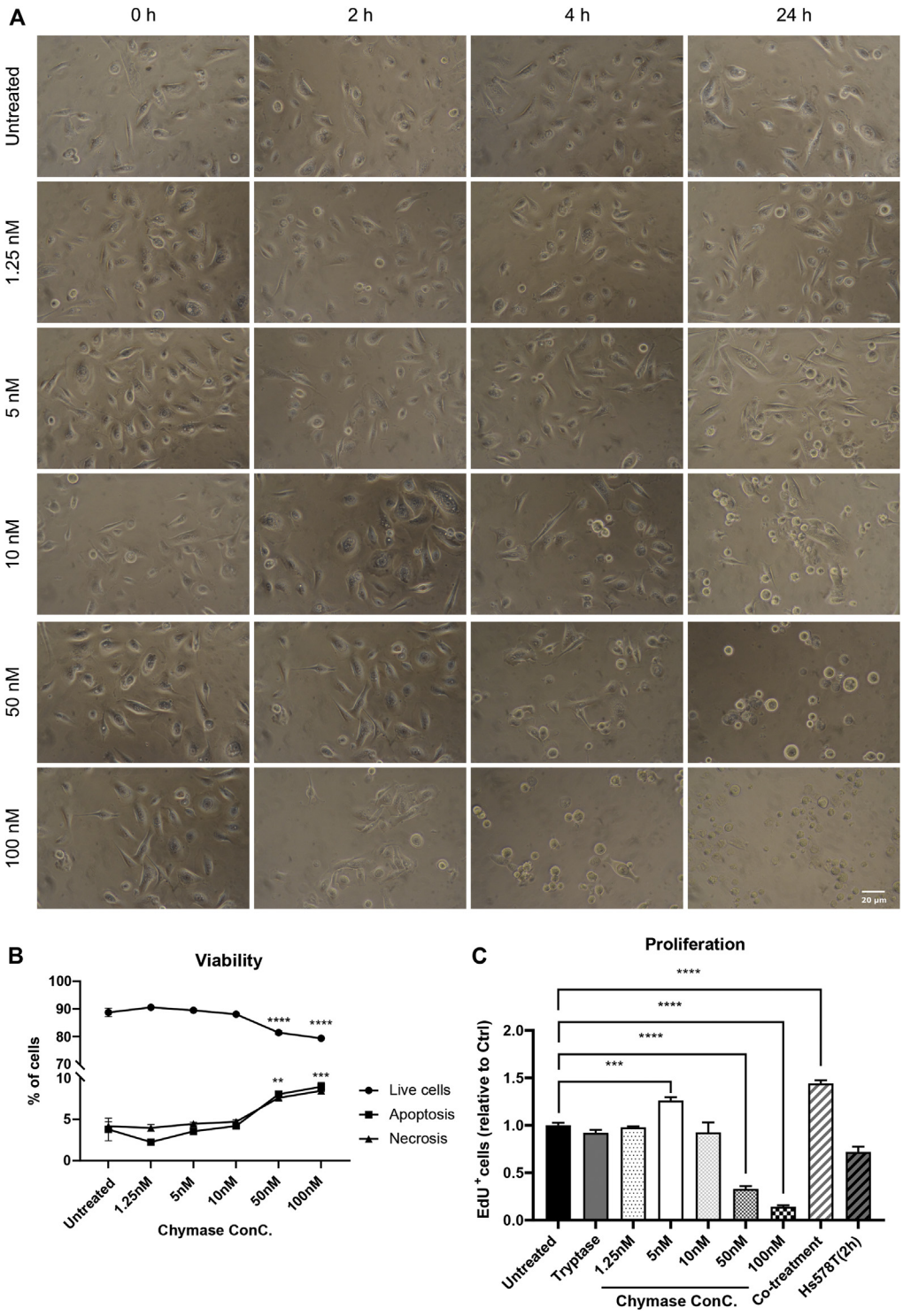


FIG 1. Chymase affects proliferation, morphology, and viability of primary HSAECs. **A**, Morphology of HSAECs (2.5×10^5 cells; 10% of the cells were sampled by flow cytometry) after treatment with chymase at the indicated concentrations (ConCs) and time points; untreated cells were used as controls (Ctrls). **B**, Viability of untreated and chymase-treated HSAECs (24 hours of treatment) as assessed by flow cytometry after staining with annexin V and DRAQ7: viable cells (annexin V–negative/DRAQ7-negative), apoptotic (annexin V–positive/DRAQ7-negative), and necrotic/late apoptotic (annexin V–positive/DRAQ7-positive) cells. Data are shown as means ± SEMs. $^{**}P \leq .01$; $^{***}P \leq .001$; $^{****}P \leq .0001$. **C**, EdU^+ HSAECs after treatment with chymase (1.25-50 nM; 24 hours of treatment), tryptase (5 nM; 24 hours) or co-treatment (5 nM tryptase, 5 nM chymase; 24 hours of treatment) relative to Ctrl (untreated) cells, with Hs578T cells as a positive Ctrl. Data are shown as means + SEMs; pooled from 2 independent experiments in triplicate. $^{**}P \leq .01$; $^{***}P \leq .001$; $^{****}P \leq .0001$.

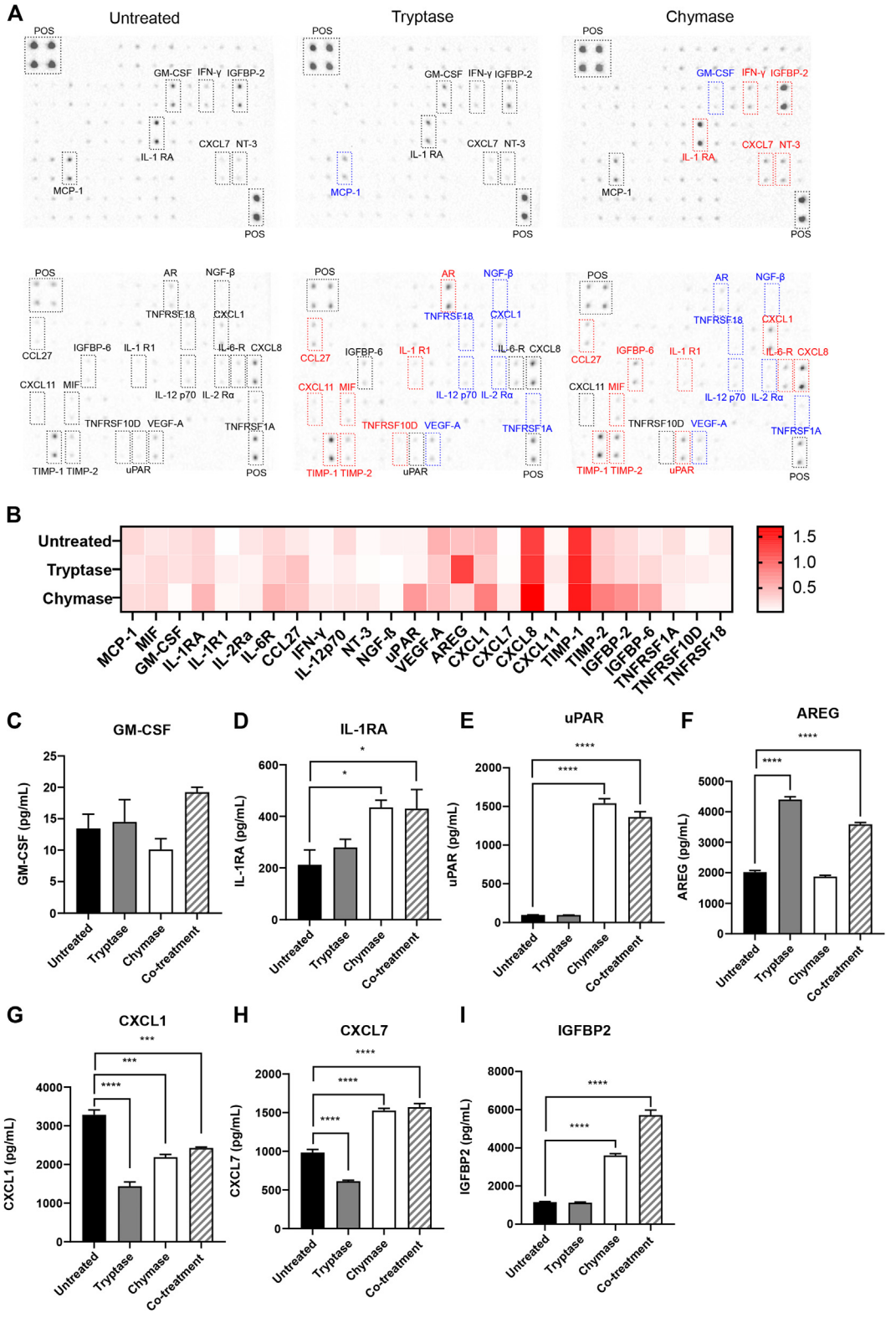


FIG 2. MC proteases affect the output of cytokines and immunoregulatory molecules from primary HSAECs. HSAECs were either untreated or treated with tryptase (5 nM; 24 hours of treatment), chymase (5 nM; 24 hours of treatment), or both (co-treatment). **A**, Cytokine profiling in conditioned medium from untreated, tryptase-treated or chymase-treated primary HSAECs. Samples were pooled from 2 independent experiments. **B**, Heatmap displaying quantification of mean pixel density from the cytokine array. **C-I**, Quantification of the release of GM-CSF, IL-1RA, uPAR, AREG, CXCL1, CXCL7, and IGFBP2 in response to tryptase, chymase, or both, as determined by ELISA. Data are shown as means + SEM. $*P \leq .05$; $***P \leq .001$; $****P \leq .0001$. Samples were collected from 3 independent donors.

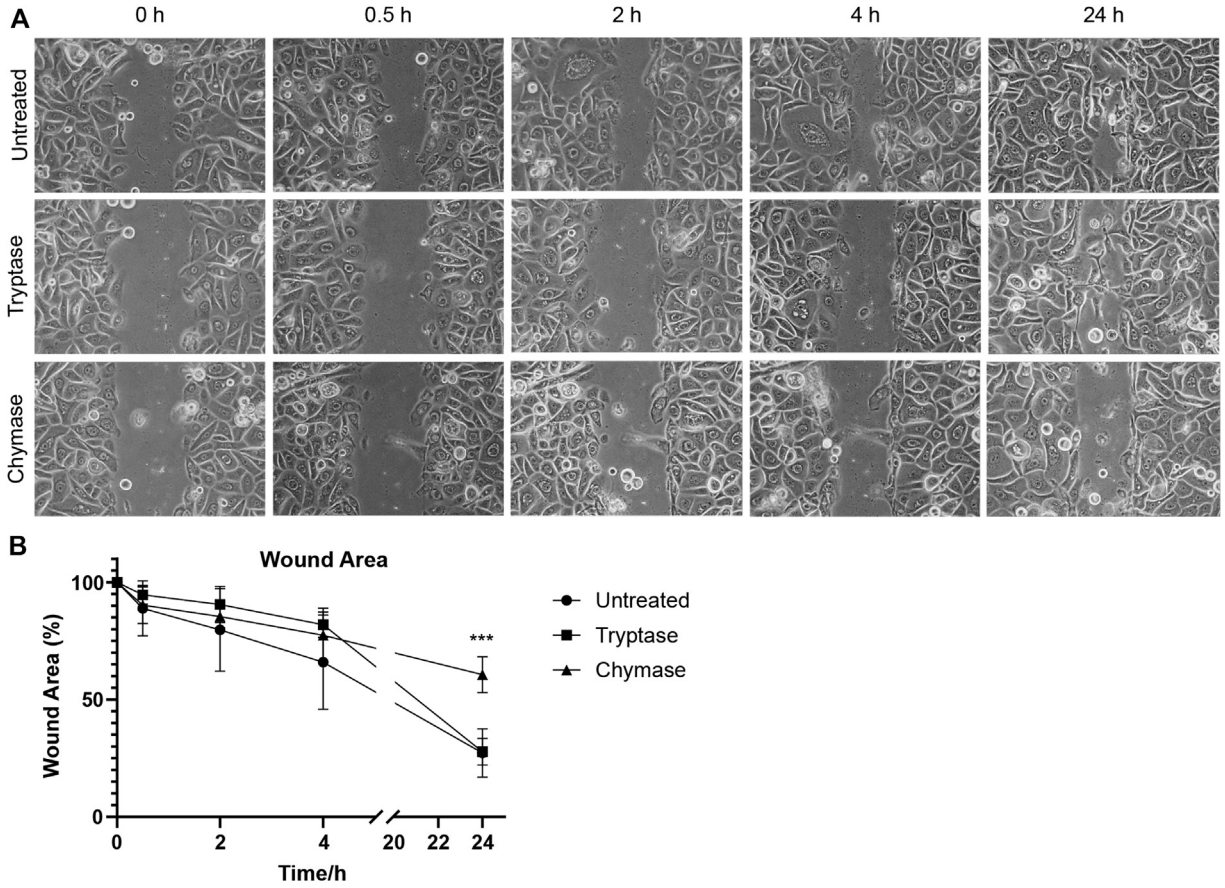


FIG 3. Chymase causes delayed migration of primary HSAECs. **A**, Representative images of scratch wounds acquired at different time points in untreated, tryptase-treated (5 nM; 24 hours of treatment), and chymase-treated (5 nM; 24 hours of treatment) HSAECs. **B**, Quantification of wound area. Data are given as means ± SEMs; pooled from 3 independent experiments with triplicates per group; $***P \leq .001$.

fibronectin gene expression (Fig 4, *D*). Another potential explanation for the observed effects could be that chymase affects the ability of the cells to interact with their environment (eg, through effects on cell-cell contacts, a notion that is supported by previous findings).[26-28] To address this possibility, we assessed the impact of chymase on E-cadherin. Indeed, chymase was shown to cause E-cadherin degradation, as indicated by immunofluorescence staining and also by immunoblot analysis (Fig 4, *A* and *C*). We also assessed whether chymase affects the actin network in HSAECs. However, both the untreated and chymase-treated HSAECs exhibited an intact actin network (Fig 4, *A*).

MC proteases affect the transcriptome of HSAECs

To provide further insight into how the MC proteases affect HSAECs, we assessed their effects at the transcriptome level. For this purpose, HSAECs were treated with either tryptase, chymase, or a combination of tryptase plus chymase (co-treatment), followed by AmpliSeq transcriptome analyses. As depicted by a multidimensional scaling plot (Fig 5, *A*), cells treated with either tryptase or chymase clustered such that they were clearly separated from each other and from the untreated cells. Notably, in comparison with tryptase-treated cells, chymase-treated cells clustered at a greater distance from the untreated cells, suggesting that chymase had a more profound overall impact on the HSAEC transcriptome than tryptase did. This notion is reinforced by the finding that the cells representing the co-treatment clustered closely together with the chymase-treated samples (Fig 5, *A*). As judged by the volcano plot analyses, chymase and to a lesser extent, tryptase had predominantly suppressing effects on gene expression in HSAECs, with relatively few genes being upregulated (Fig 5, *C-E*; for an enlarged volcano plot view of the effects of chymase, see Fig E5 in the Online Repository at www.jacionline.org). The co-treatment produced effects similar to those produced by chymase alone (Fig 5, *C-E*). In comparison with the untreated cells, 10, 60, and 79 genes were found to be differentially expressed in the tryptase-treated, chymase-treated, or co-treated cells, respectively (see Table E1 in the Online Repository at www.jacionline.org). To further dissect the impact of the MC proteases on the HSAEC transcriptome, we performed Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses. These analyses indicated a striking preponderance of pathways related to ECM remodeling and cell migration among the differentially expressed genes, as manifested by the following categories: metalloendopeptidase activity (*MMP2, MMP9, ADAM19, ADAMTS15,* and *FAP*), protease binding (*FAP, FN1, SERPINB3*, and *SERPINB4*), integrin binding (*FAP, FGF1, FN1*, and *SEMA7A*), cell adhesion (*AMTN, CDH6, COL1A1, COL7A1, FAP, FN1, NEDD9*, and *POSTN*), collagen metabolism (*COL1A1, COL7A1, MMP2*, and *MMP9*)

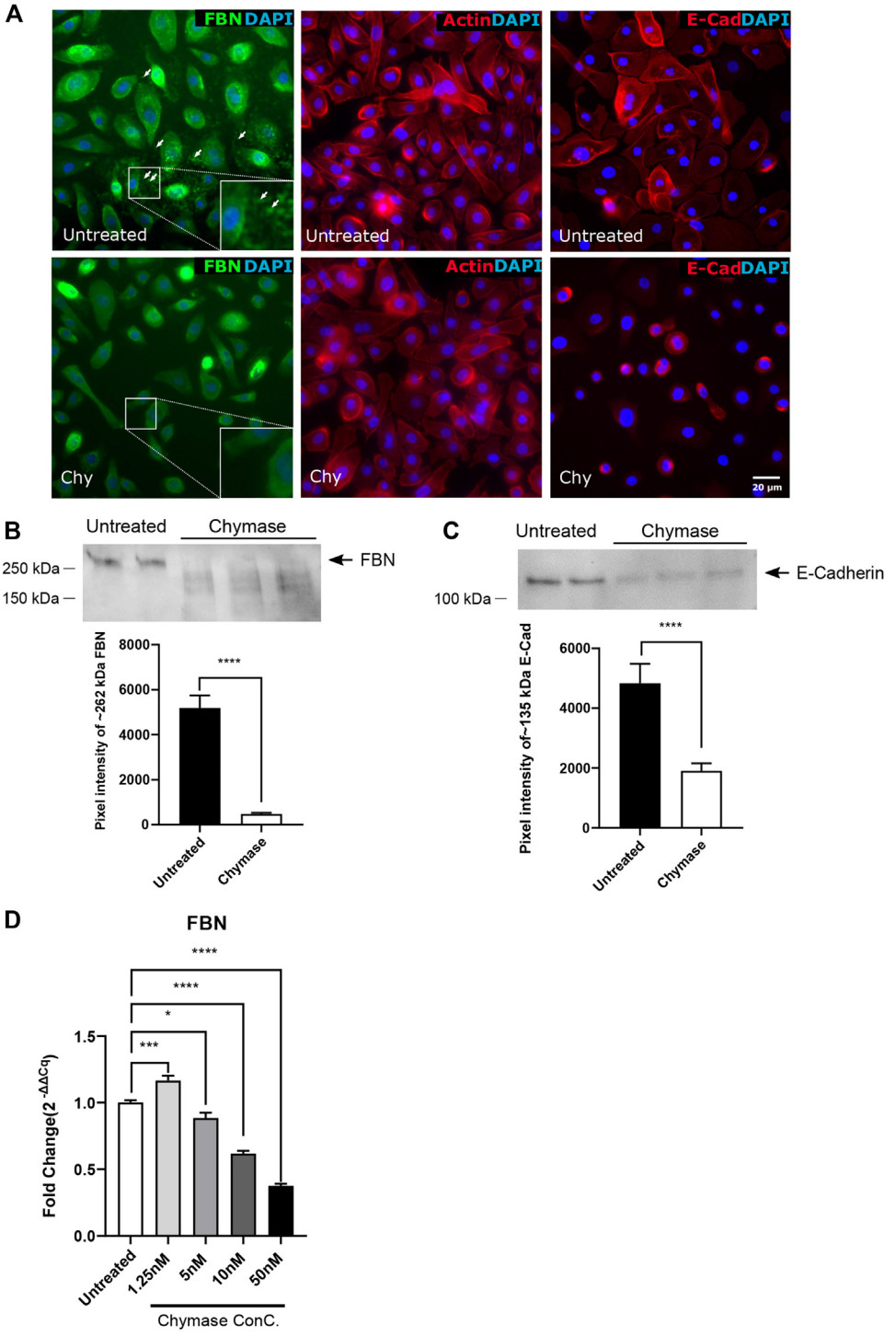
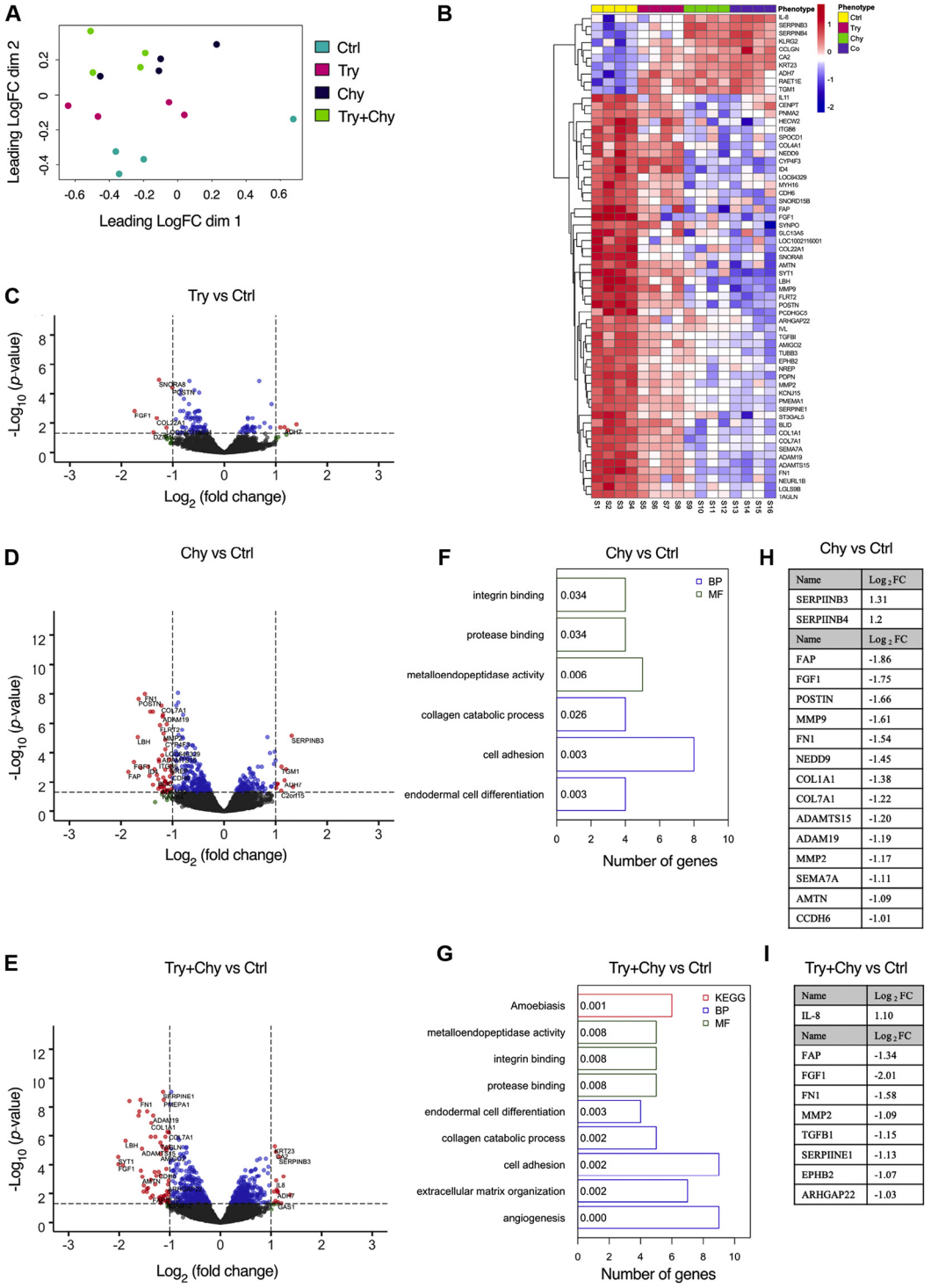


FIG 4. Chymase affects HSAEC-produced fibronectin and E-cadherin. **A,** HSAECs were treated with chymase (5 nM; 24 hours of treatment) or left untreated before fixation and staining for fibronectin (FBN), actin, and E-cadherin (E-cad) as indicated. 4′,6-Diamino-2-phenylindole (DAPI) staining was used for visualization of nuclei (×20 magnification). **B** and **C**, Immunoblot analysis of HSAEC-conditioned medium for FBN (**B**) and E-cad (**C**); quantification of the data is shown in the lower part of the panel. Conditioned media were obtained from untreated and chymase-treated HSAECs, as indicated. **D**, Quantitative PCR analysis of fibronectin gene expression in response to chymase. Data are given as means + SEM. pooled from 4 biologic replicates. The 2-tailed Student *t* test was used for statistical analysis. $*P \leq .05$; $***P \leq .001$; $****P \leq .0001$. *ConC*, Concentration.



Name	Log$_2$FC
SERPIINB3	1.31
SERPIINB4	1.2
Name	Log$_2$FC
FAP	-1.86
FGF1	-1.75
POSTIN	-1.66
MMP9	-1.61
FN1	-1.54
NEDD9	-1.45
COL1A1	-1.38
COL7A1	-1.22
ADAMTS15	-1.20
ADAM19	-1.19
MMP2	-1.17
SEMA7A	-1.11
AMTN	-1.09
CCDH6	-1.01

Name	Log$_2$FC
IL-8	1.10
Name	Log$_2$FC
FAP	-1.34
FGF1	-2.01
FN1	-1.58
MMP2	-1.09
TGFB1	-1.15
SERPIINE1	-1.13
EPHB2	-1.07
ARHGAP22	-1.03

FIG 5. Effects of chymase (Chy) and tryptase (Try) on the primary HSAEC transcriptome. HSAECs were untreated or treated with Chy, Try, or both, followed by transcriptome analysis. **A,** Multidimensional scaling plot displaying the clustering of samples. **B**, Heatmap displaying hierarchic clustering of differentially expressed genes (DEGs) (*red represents upregulation and blue downregulation*). Original data were scaled to have a zero mean. **C-E**, Volcano plots of $\log_2$ fold changes (FCs) of gene expression after treatment of HSAECs with Try (C), Chy (**D**), or both (**E**) compared with untreated cells. DEGs with a *P* value less than .05 and absolute FC greater than 2 are indicated in red. Vertical dashed lines represent the $\log_2$ FC of –1 or 1; horizontal dashed lines denote *P* cutoff values of .05. **F** and **G**, Gene Ontology (GO) (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) pathway enrichment analyses for DEGs in Chy-treated (**F**) and Try plus Chy (co-treated) (**G**) versus untreated cells (*P* values are indicated within the bars). GO and KEGG pathway enrichment analyses indicate the number of genes that were significantly enriched in the biologic process (BP) and molecular function (MF) categories. All *P* values were adjusted by using the Benjamini-Hochberg procedure. **H** and **I**, List of genes showing the highest extent of differential expression in response to Chy or Chy plus Try (co-treatment). *ConC*, Concentration; *Ctrl*, control.

and endodermal cell differentiation (*COL7A1, FN1, MMP2*, and *MMP9*) (Fig 5, *F* and *H*). Co-treatment affected pathways similar to those affected by chymase, with the addition of the angiogenesis category (*ARHGAP22, EPHB2, FAP, FGF1, FN1, IL8, MMP2, SERPINE1*, and *TGFBI*) (Fig 5, *G* and *I*). The impact of chymase on genes implicated in ECM-related processes was also highlighted by a heatmap analysis (Fig 5, *B*). In line with the marginal overall effect of tryptase on gene expression, we did not observe any enrichment of genes in either the biologic processes or molecular function categories in response to tryptase.

Chymase causes decreased output of pro-MMP2 and pro-MMP9, and increased TIMP-2 secretion from primary HSAECs

As we have already described, the transcriptome analysis suggested that chymase regulates the expression of genes related to ECM remodeling, including *MMP2* and *MMP9*. MMPs are considered to be major players in ECM modeling processes[29]; we therefore sought to provide extended insight into how chymase affects these enzymes. For this, we used gelatin zymography, which revealed that HSAECs secreted both MMP2 and MMP9, as proenzymes (pro-MMP2 and pro-MMP9 [Fig 6, *A*]). Notably, HSAECs that had been subjected to chymase treatment (either alone or in combination with tryptase) secreted lower amounts of both pro-MMP2 and pro-MMP9 than did untreated or tryptase-treated cells (ie, supporting the notion that chymase suppresses expression of these enzymes [Fig 6, *A*]). For MMP9, the levels of both the proenzymes and active enzymes were reduced after chymase treatment. In contrast, although the levels of pro-MMP2 were reduced in response to chymase, active MMP2 was seen only after chymase treatment. The latter finding is thus in agreement with the known ability of chymase to activate pro-MMP2.[21,27,30] A downregulatory impact of chymase on MMP9 was also confirmed at the mRNA level (Fig 6, *C*). MMPs are subject to regulation by various TIMPs,[31] and a plausible scenario could thus be that the active forms of the MMPs (eg, MMP2 and/or MMP9) are suppressed by TIMPs secreted by HSAECs. As judged by the cytokine array approach (see Fig 2, *A* and *B*), chymase can in fact cause increased secretion of TIMP-2, pointing to the possibility that MMPs released by the HSAECs could be subject to regulation by endogenously produced TIMPs. To provide further proof in support of the ability of chymase to regulate TIMP-2 secretion, we used ELISA. Indeed, the ELISA measurements confirmed that chymase induces a slight enhancement of TIMP-2 secretion (Fig 6, *B*).

DISCUSSION

Our findings reveal that tryptase has a relatively minor impact on HSAECs. This is in some contrast to the findings of previous studies in which tryptase has been shown to be mitogenic for airway epithelial cells.[32-34] In contrast to the modest effects of tryptase, chymase was found to have far more pronounced effects on the HSAECs. First, chymase was shown to slightly increase their rate of proliferation, a finding that is in contrast with those of a recent study on bronchial epithelial cells.[33] Further, chymase suppressed the migratory capacity of the HSAECs, which is also in some contrast to the findings of a recent study.[33] A plausible explanation for these apparent discrepancies could be that the present study focused on primary airway epithelial cells, whereas previous studies utilized bronchial epithelial cell lines.

Further insight into how the MC proteases affect HSAECs came from unbiased secretome analysis, in which chymase was shown to cause the release of several proinflammatory factors, including uPAR, CXCL7, and IL6R. However, chymase also induced release of the anti-inflammatory factor IL-1RA. Considering that IL-1 signaling has been implicated in asthma,[35,36] IL-1RA induction by chymase could have a regulatory role by downregulating the effects of IL-1 under such circumstances. Hence, chymase induces both proinflammatory and anti-inflammatory compounds in HSAECs, and whether the effects of chymase on the airway epithelial cells result in an altogether proinflammatory or anti-inflammatory outcome is thus not certain. Although tryptase had an overall modest impact on the epithelial cell secretome, we noted that tryptase caused a markedly upregulated output of amphiregulin, which is an EGF receptor ligand. Hence, a plausible scenario would be that tryptase-induced upregulation of amphiregulin may cause autocrine effects on the epithelial cells, mediated by an amphiregulin/EGF receptor axis.

Further understanding of how the MC proteases affect HSAECs was derived from a transcriptomic analysis. Overall, chymase had a generally dampening effect on gene transcription in the HSAECs, whereas tryptase had less pronounced effects. When examining the gene expression profile induced by chymase, we noted that chymase generally affects genes associated with ECM remodeling, including the downregulation of several proteolytic enzymes of the metalloendopeptidase family (MMP2, MMP9, ADAM12, ADAMTS15, and ADAM19). Importantly, the downregulated expression of MMP2 and MMP9 was verified at the protein level, as well as by mRNA analysis. Chymase was also shown to enhance the secretion of the MMP inhibitor TIMP-2, and the increased levels of this compound could thus contribute to a reduction of metalloprotease levels in tissues.

Altogether, our findings suggest that chymase may cause a reduction in the levels of ECM-modifying enzymes in the vicinity of the HSAECs, thereby playing a potential regulatory role in ECM remodeling processes that occur in asthma.[37] Possibly, such chymase-mediated effects on ECM remodeling may account, at least partly, for the protective effects of chymase seen in mouse asthma models[38-40] and for the association of chymase positivity with preserved lung function in asthmatic individuals.[13,15] An effect of chymase on ECM remodeling is also supported by the suppression of genes coding for collagens (*COL1A1, COL4A1, COL7A1*, and *COL22A1*) and profibrotic growth factors (*TGFB1* and *FGF1*). In line with these findings, chymase was also shown to enhance the secretion of IGFBP2 and IGFBP6, both of which have the ability to regulate IGF-mediated responses. Notably, IGF regulation has been shown to dampen asthma-associated fibrosis,[41,42] and it is thus conceivable that chymase-mediated enhancement of IGFBP2/6 secretion could serve to suppress fibrosis-associated ECM remodeling. In further agreement with the notion of chymase having an effect on ECM remodeling, we noted that chymase can degrade fibronectin deposited by the HSAECs and also suppress fibronectin gene expression. Intriguingly, proteolytic fibronectin fragments can have biologic activities distinct from those of the intact protein,[43] and it is thus possible that fibronectin fragments formed by the proteolytic action of chymase could contribute to the effects of chymase on the epithelial cells.

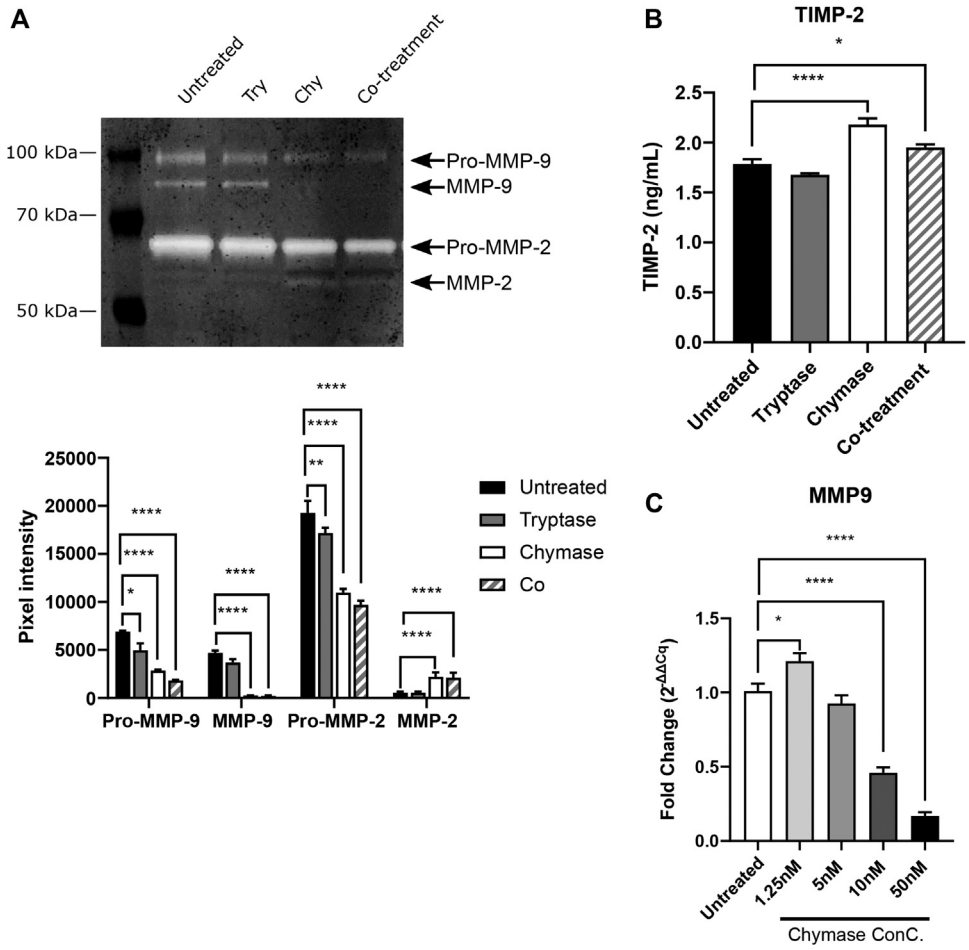


FIG 6. Chymase (Chy) affects the output of MMP9, MMP2, and TIMP-2 from primary HSAECs. Primary HSAECs were either untreated or treated with tryptase (Try) (5 nM; 24 hours of treatment), Chy (5 nM; 24 hours of treatment), or both (co-treatment). **A,** Conditioned medium was recovered and analyzed by gelatin zymography. Bands corresponding to pro-MMP9, MMP9, pro-MMP2, and MMP2 are indicated. The bar plot below shows the quantification of band intensities. **B,** Quantification of the release of TIMP-2 in response to Try, Chy, or both as determined by ELISA. **C**, Quantitative PCR analysis of MMP gene expression in response to Chy. Data are given as means + SEMs; pooled from 4 biologic replicates. Two-tailed Student *t* tests were used for statistical analysis. $*P \leq .05$; $**P \leq .01$; $****P \leq .0001$.

An important point is whether the chymase and/or tryptase concentrations used in this study are of physiologic relevance. Although the *in vivo* concentrations of these proteases at sites of MC degranulation are not known, calculations based on the extraordinary high content of tryptase and/or chymase in human MCs[44] suggest that the concentrations used in this study are in a range similar to what can be expected *in vivo*.

Altogether, our study indicates that chymase has a complex impact on HSAECs, particularly by affecting compounds involved in ECM remodeling. However, future studies will be required to assess the relevance of these findings in more complex systems, including in MC-epithelial cell coculture settings and in human asthma pathology.

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