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Secretory leucocyte protease inhibitor inhibits interferon-gamma-induced cathepsin S expression.

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SECRETORY LEUCOCYTE PROTEASE INHIBITOR INHIBITS INTERFERON GAMMA-INDUCED CATHEPSIN S EXPRESSION

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Running Title: SLPI inhibits Interferon Gamma

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Abstract

We have demonstrated that bronchoalveolar lavage fluid from chronic obstructive pulmonary disease patients contains higher levels of interferon gamma compared to controls. Interferon gamma is a potent inducer of various cathepsins and matrix metalloproteases. Therefore, we postulated that interferon gamma could induce protease expression by macrophages in acute and chronic lung disease. Chronic obstructive pulmonary disease patients had greater levels of cathepsin S and matrix metalloprotease-12 in their bronchoalveolar lavage fluid. Macrophages incubated with chronic obstructive pulmonary disease bronchoalveolar lavage fluid exhibited increased expression of cathepsin S and matrix metalloprotease-12, which was inhibited by the addition of interferon gamma neutralizing immunoglobulin. Human secretory leucocyte protease inhibitor is an 11.7 kDa cationic non-glycosylated antiprotease synthesized and secreted by cells at the site of inflammation. We have demonstrated that secretory leucocyte protease inhibitor can inhibit interferon gamma-induced cathepsin S production by macrophages. Pre-treatment of macrophages with secretory leucocyte protease inhibitor inhibited interferon-gamma induced inhibitor kappa B beta degradation and activation of nuclear factor kappa B.

Secretory leucocyte protease inhibitor may prove to be therapeutically important as a potential inhibitor of protease expression in chronic obstructive pulmonary disease.

Chronic Obstructive Pulmonary Disease (COPD) is a major public health problem currently ranked as the fourth leading cause of death in the world (1). It is characterized by airflow limitation and is principally associated with exposure to cigarette smoke. However, the link between cigarette smoke, bronchial inflammation and the development of airflow limitation is not completely understood, as 15-20% of smokers develop COPD. One hypothesis is that individuals who develop exhibit an exaggerated inflammatory response to cigarette smoke COPD due to an immune system that is already primed by previous infections or by the possession of inherited genetic polymorphisms.

A number of investigators have shown an increased numbers of T cells in the lungs of patients with COPD (2-4) and that disease progression is linked to lymphocyte (T cell) infiltration. CD4⁺ and CD8⁺ T cells are increased in the airways and lung parenchyma of COPD patients (4). In addition, CD4⁺ and CD8⁺ T cells are activated with increased expression of IFN- γ in COPD patients (5).

IFN- γ is produced by many cell types, including T-cells and B-cells, and is an important component of the anti-viral

response. It stimulates both macrophages and NK cells and is involved in the regulation of the immune and inflammatory responses. IFN- γ is also released predominantly by Th1 cells, and recruits leukocytes to a site of infection, resulting in increased inflammation. It activates macrophages for the expression of surface major histocompatibility class II antigens, costimulatory molecules, and inflammatory mediators for tumoricidal and antimicrobial activity. It regulates the proliferation and death of T lymphocytes, promotes the development of a type 1 T-helper cell response, and is essential for the control of many intracellular pathogens *in vivo*. IFN- γ mRNA or protein has been shown in mouse and human lung macrophages (6), spleen (7) bone marrow (8,9), resting peritoneum (10) and peritoneal exudate following sterile inflammation (11).

The primary families of proteases released into the extracellular space following cell activation include members of the serine protease, matrix metalloprotease (MMP) and cysteinyl cathepsin groups of proteases. Proteases play important roles in numerous biological processes and are capable of degrading many extracellular matrix components and as such are regarded as pivotal in the pathogenesis of COPD. MMPs are produced by a wide variety of cell types including epithelium, fibroblasts, neutrophils and macrophages. Several MMPs (including MMP-12) have been implicated in the pathogenesis of chronic lung injury, particularly in emphysema. This is shown in MMP-12 knockout mice, who do not develop air space enlargement in response to smoke exposure (12).

Macrophages also synthesize cysteinyl cathepsins, which are ubiquitous lysosomal proteases (13-15). Cytokines and microbial products have previously been demonstrated to regulate cathepsin expression and activity (16,17). IFN- γ has been shown to be a potent inducer of cathepsin S in monocytes and keratinocytes (18). IL-1 and TNF- α also induces cathepsin S activity, while exposure to IL-10 prevents conversion of pro-cathepsin S into its mature

form (16). IL-13 has been shown to induce cathepsins B, H, K, L and S (19). We have shown previously that cathepsins cleave and inactivate key innate immunity proteins including human beta defensins 2 and 3 (20), secretory leukocyte protease inhibitor (SLPI) (21) and lactoferrin (22).

SLPI is an 11.7-kD nonglycosylated protein produced at mucosal surfaces, primarily the upper respiratory tract (23) and is an important antiprotease (24). SLPI suppresses LPS-induced activation of NF- κ B, production of nitric oxide and TNF- α (25). Interestingly, IFN- γ can suppress expression of SLPI (26). In a model of acute lung injury, prior administration of SLPI attenuated pulmonary recruitment of neutrophils and decreased lung injury (27). We have recently shown that SLPI is taken up into monocytes and becomes distributed within the cytoplasm and nucleus and can bind NF- κ B DNA binding regions within the IL-8 and TNF- α promoters (28). SLPI can also diminish p65 binding, thereby attenuating inflammation in monocytes (28).

We postulated that COPD is associated with a Th1 cytokine profile with IFN- γ plays a central role in activity of macrophages leading to proteolysis of lung tissue. We further propose that SLPI could dampen this protease burden not by its direct antiprotease effect but by its anti-inflammatory effects. To test this theory we investigated cytokine and protease levels in bronchoalveolar lavage fluid (BAL) fluid from control and COPD patients. Macrophage protease expression was also investigated following activation with IFN- γ stimuli and potential inhibition of protease expression induced by IFN- γ was evaluated in the presence of SLPI. The data from this study provide further evidence of an impaired protease/antiprotease balance in COPD patients due to a Th1 response that can be inhibited by SLPI.

Experimental Procedures

Culture and stimulation of monocyte cells - Myelomonocytic cells (U937) (European Collection of Cell Cultures Health

Protection Agency, Salisbury, Wiltshire, UK) (5×10^5 cells/ml) were cultured in RPMI 1640 medium (Gibco) and were differentiated to macrophage-like cells with PMA for 48 hours. The macrophage-like cells were incubated in fresh medium for a further two days before stimulation. Stimulation was performed with IFN- γ (R & D Systems, 10U/ng) for 3 or 24 hours before harvesting, for RNA or protein isolation, respectively. SLPI, Alpha-1 anti-trypsin (A1AT) and Elafin (each at 10 μ g/ml) stimulation was administered an hour prior to IFN- γ . Cells were also stimulated with BAL fluid from COPD patients for 2 hours, wash three times and resuspended in serum free medium for a further 48 hours. Mouse anti-IFN- γ (R&D Systems) (500ng/ml) or mouse IgG2A (R&D Systems) (500ng/ml) was incubated for 30 minutes at 37°C with COPD BAL fluid prior to cell stimulation. Cells were also treated for 1 hour with SN50 and its inactive control, SN50M, (Calbiochem) prior to IFN- γ stimuli to block NF- κ B activity.

Isolation of PBMCs-Mononuclear cells were isolated from heparinised venous peripheral blood obtained from healthy volunteers using Lymphoprep™ (Nycomed, Norway). Briefly, freshly collected EDTA-peripheral blood samples (7.5mls) were diluted 1:1 in 0.9% NaCl and 2 ml of 10% Dextran (Sigma) was added and incubated at RT for 15 minutes. Afterwards the upper layer containing the mixed leukocyte fraction was removed and over-layered onto 5ml of Lymphoprep™. The sample was then centrifuged at 200 x g for 10 minutes. The mononuclear cell inter-phase band was aspirated and washed three times in HBSS medium. Monocytes were purified to 97% cell purity using the EasySep human CD14 selection cocktail as recommended by manufactures (StemCell Technologies, London England). Monocytes were then cultured in RPMI containing 40% autologous serum, penicillin G (final concentration 100 U/ml), and streptomycin sulfate (final concentration 100 μ g/ml) at 37°C in a 5% CO₂ atmosphere for 9 days (29). An hour prior to stimulation, cells were

washed and incubated in serum-free medium.

BAL fluid samples - Samples of BAL fluid and serum were obtained from subjects with COPD (n=17) and healthy volunteers (n=15). Ethical approval was obtained from Beaumont Hospital Ethics Committee and informed consent from each of the patients undergoing bronchoscopy. Subjects underwent flexible fiberoptic bronchoscopy and bronchoalveolar lavage (BAL) was performed. Samples were separated into cell suspension and supernatant.

IFN- γ levels measured by sandwich ELISA - The concentration of IFN- γ was measured by ELISA using cytokine-specific Abs (R&D Systems, Abingdon, U.K.). Values are expressed as picograms per ml.

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) - After treatment with IFN- γ , cells were harvested in Tri reagent (Sigma-Ireland) and RNA was extracted as detailed in the manufacturer's protocol. Total RNA (2 μ g) was reverse-transcribed at 37°C with 1 mM deoxynucleotide mix (Promega, Southampton, UK), 1.6 μ g oligo-p[dT]15 primer (Roche, Lewes, UK) and 1 μ l M-MLV reverse transcriptase (Promega, Southampton, UK) in a 20 μ l volume as described in the manufacturer's protocol. 2 μ l of each cDNA was amplified with 1.25 U Taq DNA polymerase, 1 \times PCR buffer and 10 mM dNTPs (Promega) in a 50 μ l volume containing 100 pmol each of the following primers: 5'- ATG AAA CGG CTG GTT TGT GT-3' and 5'-CTA GAT TTC TGG GTA AGA GGG AAA GCT AGC-3' for cathepsin S; 5'- CTG GAC AAC TCA ACT CTG GCA A-3' and 5'- ATT ATA GAT CCT GTA AGT GAG GTA CCG C-3' for MMP-12; 5'- GAA GGT GAA GGT CGG AGT CA-3' and 5'- TTC ACA CCC ATG ACG AAC AT-3' for GAPDH; 5'- GCC GGG AAA TCG TGC GTG-3' and 5'- GGG TAC ATG GTG GTG CCG-3' for β -Actin PCR products were quantified densitometrically at cycle numbers between 10 and 40 to determine the appropriate cycle number at which exponential amplification of products was occurring, and to identify

the cycle number at which sufficient discrimination was possible to accurately quantify increases or decreases in gene expression. After a hot start the amplification profile was 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C for cathepsin S, MMP-12. GAPDH and β -Actin gene amplification required only 20 cycles. RT-PCR amplification of cathepsin S, MMP-12, β -Actin and GAPDH generated products of 1000 bp, 703 bp, 307 bp and 402 bp respectively. PCR products were commercially sequenced (MWG Biotech AG, Ebersberg, Germany) to verify gene identity. PCR products were resolved on a 1% (w/v) agarose gel containing 0.5 μ g/ml ethidium bromide (Sigma). The ratio of PCR fragment intensities of each gene relative to GAPDH and β -Actin was determined by densitometry.

Presence of cathepsin S - Cathepsin S activity was determined in supernatants from macrophage-like cells 24 hours after stimulation with or without IFN- γ or BAL fluid. Cathepsin S activity was determined in 100 μ l of each BAL sample using the substrate Z-Phe-Arg-pNA (0.1 mM). The reaction buffer used for cathepsin S activity estimation was 0.2 M sodium acetate, pH 7.5, 2mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1 μ M pepstatin, and 2 mM Pefabloc. The samples were incubated with substrate for 60 min at 37°C, and fluorescence (substrate turnover) was determined by excitation at 355 nm and emission at 460 nm. Results were expressed as a change (Δ) in fluorescence units over a 60-minute period (FU).

Western blot - BAL fluid from healthy controls and COPD patients were investigated for the presence of MMP-12. Supernatant from U937 macrophages treated with IFN- γ were investigated for cathepsin S and MMP-12. BAL fluid or concentrated media from macrophage-like cells were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Sigma-Aldrich) which was probed using mouse anti-MMP-12 antibody (R&D Systems), rabbit anti-

cathepsin B antibody (Calbiochem) or mouse anti-cathepsin S antibody (Calbiochem). A rabbit anti-MMP-12 (Calbiochem, Oncogene Research Products) antibody was used to detect MMP-12 from cells treated with COPD BAL fluid plus mouse anti-IFN- γ antibody or mouse IgG2A, to minimize antibody interaction. Binding was detected using the appropriate horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Pierce).

Preparation of Subcellular Fractions - U937 cells were activated with NE and nuclear and cytoplasmic extracts were isolated. Briefly cells were washed and resuspended in 1 ml of ice-cold PBS and kept on ice for 5 min. Cells were lifted from plates with a cell scraper and pelleted by centrifugation at 10,000 rpm for 5 min at 4 °C. The supernatant was removed, and the cell pellet was resuspended in 1 ml of hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM PMSF, and 0.5 mM dithiothreitol) (Sigma). Cells were pelleted by centrifugation at 14,000 rpm for 10 min at 4 °C and then lysed for 10 min on ice in 20 μ l of hypotonic buffer containing 0.1% Igepal CA-630. Lysates were centrifuged as before, and the cytoplasmic extract was removed. The remaining nuclear pellet was lysed in 15 μ l of lysis buffer (20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF) (Sigma) for 15 min on ice. After centrifugation at 14,000 rpm for 10 min at 4 °C, nuclear extracts were removed into 35 μ l of storage buffer (10 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM PMSF, and 0.5 mM dithiothreitol). Protein concentrations of cytoplasmic and nuclear extracts were determined, and extracts were stored at -80 °C until required for use.

NF- κ B activity ELISA - The effect of IFN- γ and SLPI on NF- κ B activity was determined using the TransAM NF- κ B ELISA (Active Motif), using nuclear protein fractions.

Densitometric analysis - Gels were analysed by densitometry and compared in a semiquantitative manner using the

GeneGenius Gel Documentation and analysis system (Cambridge, UK) and GeneSnap and GeneTools software. All expression values were verified by at least three independent experiments.

Statistical analysis - Data were analyzed with the PRISM 3.0 software package (GraphPad, San Diego, CA). Results are expressed as the mean \pm standard deviation (SD) and were compared by *t* test. Differences were considered significant at $p \leq 0.05$.

RESULTS

IFN- γ in COPD and effect on Protease Expression in Macrophages: IFN- γ levels in BAL fluid were determined by ELISA and were found to be significantly elevated in COPD individuals versus controls (72.85 ± 28.61 versus 11.16 ± 6.19 pg/ml, $p=0.0484$) (Figure 1A).

IFN- γ -induced protease mRNA expression was examined in U937 macrophages. Macrophages were exposed to 25 ng/ml IFN- γ for 3 hours. Cathepsin S and MMP-12 mRNA expression levels were investigated by RT-PCR (Figure 1B). IFN- γ significantly increased expression of cathepsin S and MMP-12 in U937 macrophages ($p=0.0147$ and 0.0049 respectively). The effect of IFN- γ stimulation on release of cathepsin S and MMP-12 from U937 macrophages was investigated. Elevated cathepsin S activity was observed when cells were stimulated with IFN- γ , using the cathepsin substrate Z-Phe-Arg-AMC, at pH7.5 (Figure 1Ci). A statistically significant difference for cathepsin S activity between the control and cells stimulated with 10 ng/ml IFN- γ was observed ($p=0.0013$). This was further confirmed by Western blot, which demonstrated the increased production of cathepsin S by IFN- γ stimulated U937 macrophages (Figure 1Cii).

Western blot demonstrated that there was increased MMP-12 release from IFN- γ stimulated U937 macrophages (Figure 1Cii). Increased MMP-9 activity was observed in U937 macrophages but was

most likely due to the presence of small quantities of PMA in the cell medium (data not included). A change in MMP-9 activity was not observed when using similar experiments with PBM macrophages (data not included).

IFN- γ induced Protease Expression: Neutrophil elastase (NE), cathepsin and MMP levels were measured in COPD and control BAL fluid. NE activity was not detected in COPD or control samples, however elevated cathepsin S activity was observed in COPD compared to control BAL fluid (Figure 2A, $p=0.0268$). Western blot demonstrated the presence of MMP-12 in COPD, but not control BAL fluid (Figure 2B).

Levels of cathepsin S and MMP-12 from U937 macrophages exposed to COPD BAL fluid were also evaluated. Elevated cathepsin S activity was observed when cells were stimulated with COPD BAL fluid compared to untreated cells (Figure 2C) ($p=0.0011$). An anti IFN- γ neutralizing antibody significantly inhibited this effect ($p=0.0073$) (Figure 2C). A mouse IgG2A antibody (isotype control) was also added to COPD BAL fluid and then to cells and was shown to have no inhibitory effects on the COPD BAL fluid induced cathepsin S production ($p=0.8495$). The increase in cathepsin S by COPD BAL fluid was further demonstrated by Western blot (Figure 2Di). COPD BAL fluid exposure also increased MMP-12 protein levels, demonstrated by Western blot (Figure 2Dii). The anti-IFN- γ antibody had the same inhibition effect on MMP-12 expression in macrophages exposed to COPD BAL fluid.

Effect of SLPI on IFN- γ induced Protease Expression: IFN- γ -induced protease mRNA expression was further examined by the pretreatment of macrophages with SLPI prior to IFN- γ stimulation. U937 and PBM macrophages were exposed to 10 μ g/ml SLPI followed by 10ng/ml IFN- γ . Cathepsin S and 12 mRNA expression levels were investigated by RT-PCR from U937 macrophages (Figure 3A). IFN- γ

significantly increased expression of cathepsin S and MMP-12 even at 10ng/ml IFN- γ . SLPI significantly decreased the gene expression of cathepsin S ($p= 0.0061$). A reduced level of cathepsin S activity was observed when U937 (Figure 3B) and PBM (Figure 3D) macrophages were incubated firstly with SPLI follow by IFN- γ , using the cathepsin substrate Z-Phe-Arg-AMC, at pH7.5. A statistically significant decrease in cathepsin S activity was observed between cells stimulated with 10 ng/ml IFN- γ and cells incubated with SLPI and IFN- γ ($p=0.0229$ and 0.023 for U937s and PBM, respectively). Exposing U937 macrophages to A1AT or elafin prior to IFN- γ stimulation had no significant effect on IFN- γ -induced cathepsin S activity (Figure 3B). No reduction in MMP-12 was observed when U937 (Figure 3C) and PBM macrophages (Figure 3D) were incubated firstly with SPLI follow by IFN- γ .

Effect of SLPI on IFN- γ signalling pathways in Macrophages: SLPI inhibited IFN- γ -induced I κ -B β degradation (Figure 4A). Similarly NF- κ B activation was observed after 3 hours in the presence of IFN- γ (Figure 4B) and IFN- γ -induced NF- κ B nuclear translocation was increased 2-fold compared with control. IFN- γ -induced NF- κ B activation was also inhibited by SLPI.

SN50, a cell-permeable peptide that inhibits NF- κ B nuclear translocation downstream of IKK, and its mutant peptide, NF- κ B SN50M, were used to investigate whether inhibition of NF- κ B could reduce IFN- γ -induced protease expression. SN50 was able to inhibit IFN- γ -induced cathepsin S (Figure 4C, $P=0.022$) demonstrating that IFN- γ signals via NF- κ B to induce cathepsin S expression in macrophages.

DISCUSSION

Proteases have been implicated in COPD as a cause of airway inflammation and parenchymal destruction (2,30). Although a prominent role for NE has been postulated in COPD for many years, recent work has demonstrated that cathepsins and matrix metalloproteases, produced primarily by

macrophages, are also important (31). In addition to macrophages and neutrophils as modulators of inflammation in COPD, an increasing number of authors have described an important role for lymphocytes (2-4,32). We postulated that increased IFN- γ observed in COPD would induce expression of these proteases (cathepsins and MMPs). This study demonstrates significantly elevated levels of IFN- γ , cathepsin S and MMP-12 in COPD BAL fluid compared to control BAL fluid. We have further demonstrated that incubation of macrophages with IFN- γ leads to increased cathepsin S and MMP-12 expression and that the neutralization of IFN- γ activity in COPD BAL fluid decreases COPD BAL fluid-induced cathepsin S and MMP-12 expression by macrophages. Finally, we have demonstrated that SLPI can down-regulate IFN- γ -induced protease production, via inactivation of the NF- κ B signaling pathway. Therefore, these studies provide molecular and clinical data to support the view that IFN- γ up-regulation leads to expression of proteases responsible for lung tissue damage in COPD and that this is remediable by the anti-inflammatory effects of SLPI.

Both CD4⁺ and CD8⁺ T cells are increased in the COPD lung, with CD8⁺ cells being predominant (33) evidence that COPD is predominantly a Th1 driven condition. In general, Th1 responses preferentially stimulate genes that are involved in apoptosis and tissue injury (34). The Th1 cells observed in COPD are the major potential source of IFN- γ in the lungs.

It has been shown that some of the mediators involved in the inflammatory process observed in emphysematous tissues can directly induce emphysema. IFN- γ is one of those candidates. Transgenic over-expression of IFN- γ in the adult murine lung results in pulmonary emphysema (35). BAL fluids from the over-expressing IFN- γ mice have increased neutrophil and lymphocyte levels and an exaggerated number of macrophages compared with controls (35). Elevated levels of IFN- γ alter the pulmonary protease/antiprotease balance in a

proteolytic direction via the induction of MMPs and a variety of cathepsins (35). Production of high amounts of IFN- γ (~20 ng/ml) has been reported with dendritic cell populations after stimulation with IL-12/IL-18 (7,36,37).

In this study cathepsin S and MMP-12 gene expressions and protein activity were increased in macrophages exposed to IFN- γ . This increase in proteases can have a major impact on innate host defences in the lung, as we have previously shown that cathepsin S can cleave and inactivate human β -defensin 2 and 3 (21), SLPI (21) and lactoferrin (23). The cleavage of SLPI by cathepsin S results in the loss of the active site of SLPI and the inactivation of SLPI anti-neutrophil elastase capacity, further worsening the potential protease burden. IFN- γ also down-regulates SLPI (26,38) via CCR-5 and we have previously shown decreased levels of SLPI in the COPD lung mediated in part by cathepsin degradation (21). Cathepsin S has been identified as the dominant endocytic protease that is specifically upregulated under inflammatory conditions in the presence of IFN- γ (39). Cathepsin S upregulation in COPD is extremely relevant in the context of recent data showing that cathepsin S inhibition (and a null mutation of cathepsin S) decreases IFN- γ -induced DNA injury, apoptosis, emphysema, inflammation, and protease accumulation in a murine pulmonary emphysema modeling system (40).

MMP-12 has also been implicated the pathogenesis of chronic lung injury, particularly emphysema. MMP-12 is elevated in the lungs of COPD patients (31). Interestingly, MMP-12 knockout mice do not develop air space enlargement in response to smoke exposure, implicating MMP-12 in development of cigarette smoke induced emphysema (12). The increased expression of this important MMP in our study driven by IFN- γ is therefore of interest.

SLPI possesses anti-inflammatory activity (41). LPS hyporesponsive cells transcribe SLPI and transfection of macrophages with SLPI suppresses LPS-induced activation of NF- κ B, production of nitric oxide and TNF- α by an unknown mechanism (25). IFN- γ can suppress expression of SLPI and restore LPS responsiveness to SLPI-producing cells (25). We have previously shown that SLPI can inhibit LPS and lipoteichoic acid (LTA)-induced NF- κ B activation in monocytic cells by preventing degradation of key regulatory proteins, like I κ B α and I κ B β (42,43). SLPI prevents the degradation of I κ B α and β without affecting I κ B α phosphorylation and ubiquitination (42). SLPI inhibition of IFN- γ -induced I κ B β degradation and NF- κ B activation is due to the anti-inflammatory activity of SLPI (28). SLPI's ability to inhibit IFN- γ -induced NF- κ B activation also resulted in decreased cathepsin S expression but not MMP-12 production. SLPI's inability to inhibit IFN- γ -induced MMP-12 may be due in part to activation of MMP-12 expression via another transcription factor pathway other than NF- κ B. Recently, Churg *et al.* have demonstrated that another anti-protease, A1AT, can inhibit cigarette smoke induced MMP-12 and TNF- α via inactivation of PAR-1 by inhibiting thrombin and plasmin (44). This further demonstrates the important anti-inflammatory functions of classically described anti-proteases. Further investigation of the interaction of SLPI with the IFN- γ pathway would be beneficial.

In conclusion, we have demonstrated that a Th1 response leads to increased production of a spectrum of proteases driven by IFN- γ . Inhibition of IFN- γ in COPD BAL fluid inhibits this process. Therefore, by minimizing the IFN-induced protease expression, SLPI is of potential therapeutic relevance in the treatment of COPD.

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FOOTNOTES

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Abbreviations: COPD – Chronic Obstructive Pulmonary Disease, SLPI – Secretory leucocyte protease inhibitor, BALF – Bronchoalveolar Lavage Fluid, IFN- γ – interferon gamma, MMP – matrix metalloprotease, A1AT – alpha 1 antitrypsin, I κ B β – inhibitor kappaB beta, NF- κ B – nuclear factor kappaB

FIGURE LEGENDS

Fig. 1. IFN- γ levels in control and COPD patients and IFN- γ effect on protease production in U937s. (A) IFN- γ levels in COPD and controls. (B) RT-PCR was carried out on mRNA from differentiated U937 cells treated with IFN- γ to amplify regions of the cathepsin S, MMP-12, GAPDH and β -Actin genes. The quantification of the expression of cathepsin S and MMP-12 was assessed compared to β -Actin and GAPDH from U937 macrophages. (C) Cathepsin S activity was determined using the Z-Phe-Arg-AMC substrate (pH 7.5). (D) Western blot analysis for cathepsin S (36 kDa) and MMP-12 (45 kDa) levels in supernatants recovered from U937 macrophages exposed to IFN- γ . Protein loading was corrected for sample protein concentrations. Experiments were performed at least 3 times and representative data and SE are shown. A statistical significance is highlighted with its appropriate p value.

Fig. 2. Protease profile from control and COPD BAL fluid and macrophages exposed to COPD BAL fluid. (A) Cathepsin S activity from control and COPD BAL fluid was determined using the Z-Phe-Arg-pNA substrate (pH 7.5). (B) MMP-12 Western blot of control and COPD BAL fluid. The 22 kDa bands represent the catalytic domain of human MMP-12. (A) Cathepsin S activity was determined after 24 hours following a 2-hour stimulation with COPD BAL fluid or COPD BAL fluid plus anti IFN- γ or isotype control. A statistical significance is highlighted with its appropriate p value. (D) Western blot analysis for cathepsin S (i) and MMP-12 (ii) levels in supernatants recovered from U937 macrophages exposed to COPD BAL fluid or COPD BAL fluid plus anti IFN- γ /isotype control antibody. Protein loading was corrected for sample protein concentrations. Experiments were performed at least 3 times and representative data and SE are shown.

Fig. 3. Inhibitory effects of SLPI on IFN- γ induced protease gene expression and protein release. (A) RT-PCR was carried out on mRNA from differentiated U937 cells treated with SLPI, IFN- γ and SLPI followed by IFN- γ to amplify regions of the cathepsin S, -12, β -Actin and GAPDH genes. The quantification of the expression of cathepsin S and -12 was assessed compared to β -Actin and GAPDH from U937 macrophages. (B) Cathepsin S activity was determined after 24 hours following incubation of U937s with IFN- γ , antiprotease (SLPI, elafin or A1AT) or pre-incubation with an antiprotease followed by IFN- γ . (C) MMP-12 levels were examined after

incubation of U937s with IFN- γ , SLPI or SLPI followed by IFN- γ . (D) Cathepsin S activity was determined 24 hours after incubating PBM-derived macrophages with IFN- γ , SLPI or SLPI followed by IFN- γ . (E) MMP-12 levels were examined after incubating PBM-derived macrophages with IFN- γ , SLPI or SLPI followed by IFN- γ . Protein loading was corrected for sample protein concentrations. Experiments were performed at least 3 times and representative data and SE are shown.

Fig. 4. SLPI inhibits IFN- γ -induced NF- κ B activation. (A) I κ -B β degradation was analyzed by Western blot using an anti-I κ -B β antibody and cytosolic extracts (10 μ g) from control (-), IFN- γ - treated, SLPI-treated or IFN- γ /SLPI-treated macrophages (10 ng/ml IFN- γ , 180 minutes). (B) NF- κ B activation was measured using the TransAM NF- κ B activity ELISA in nuclear extracts (2 μ g) from control (-) and IFN- γ -treated macrophages (10 ng/ml, 180 minutes) +/- SLPI (10 μ g/ml). (C) Cathepsin S activity was determined using the Z-Phe-Arg-AMC substrate 24 hours after stimulation with IFN- γ . Cells were treated for 60 minutes with SN50 (1 μ g/ml) and its inactive control (SN50M) (Calbiochem), prior to treated macrophages with IFN- γ (10 ng/ml), to block NF- κ B activity. Protein loading was corrected for sample protein concentrations. Experiments or analyses of results were performed at least 3 times and representative data and SE are shown.

Figure 1

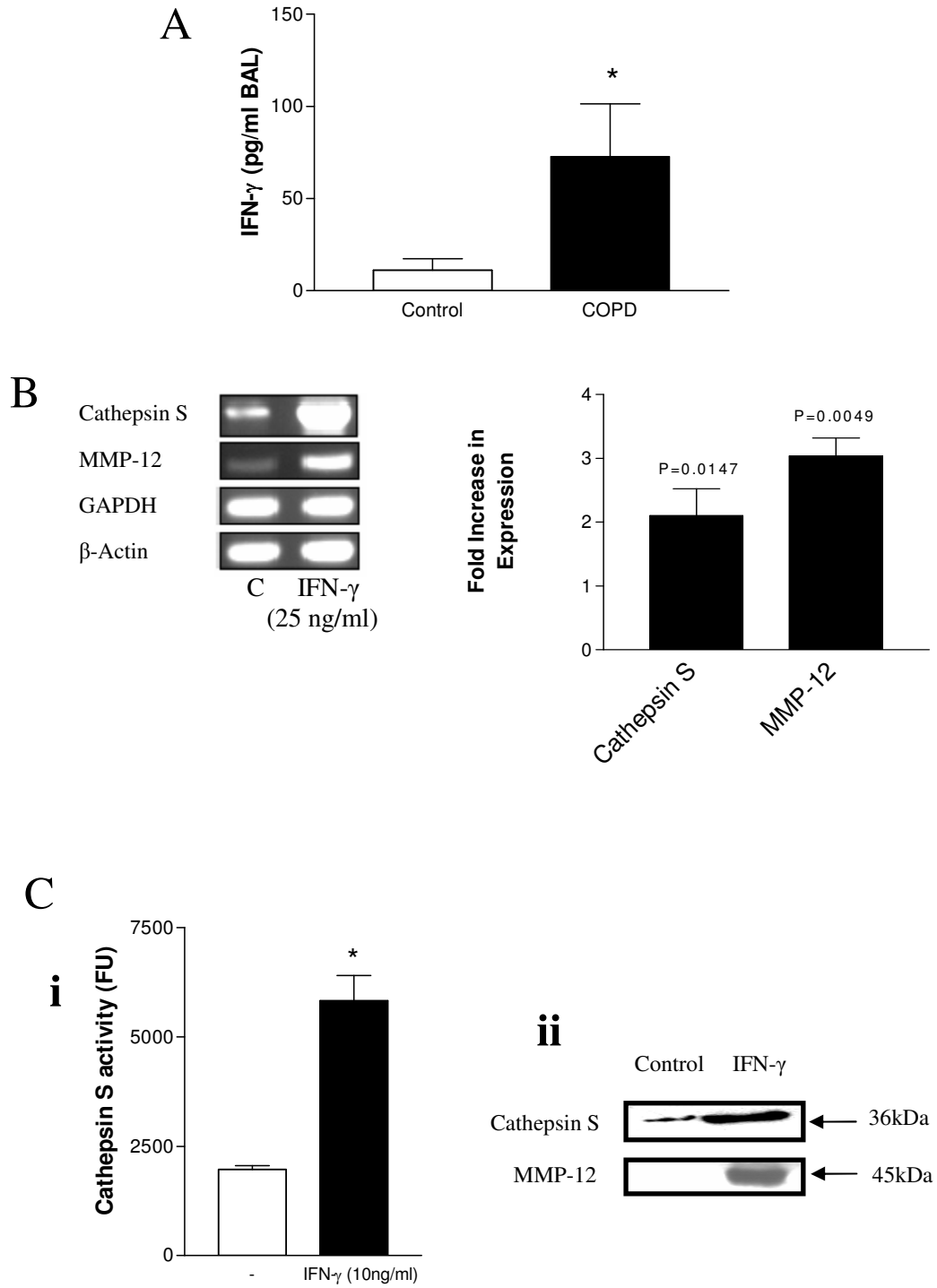


Figure 2

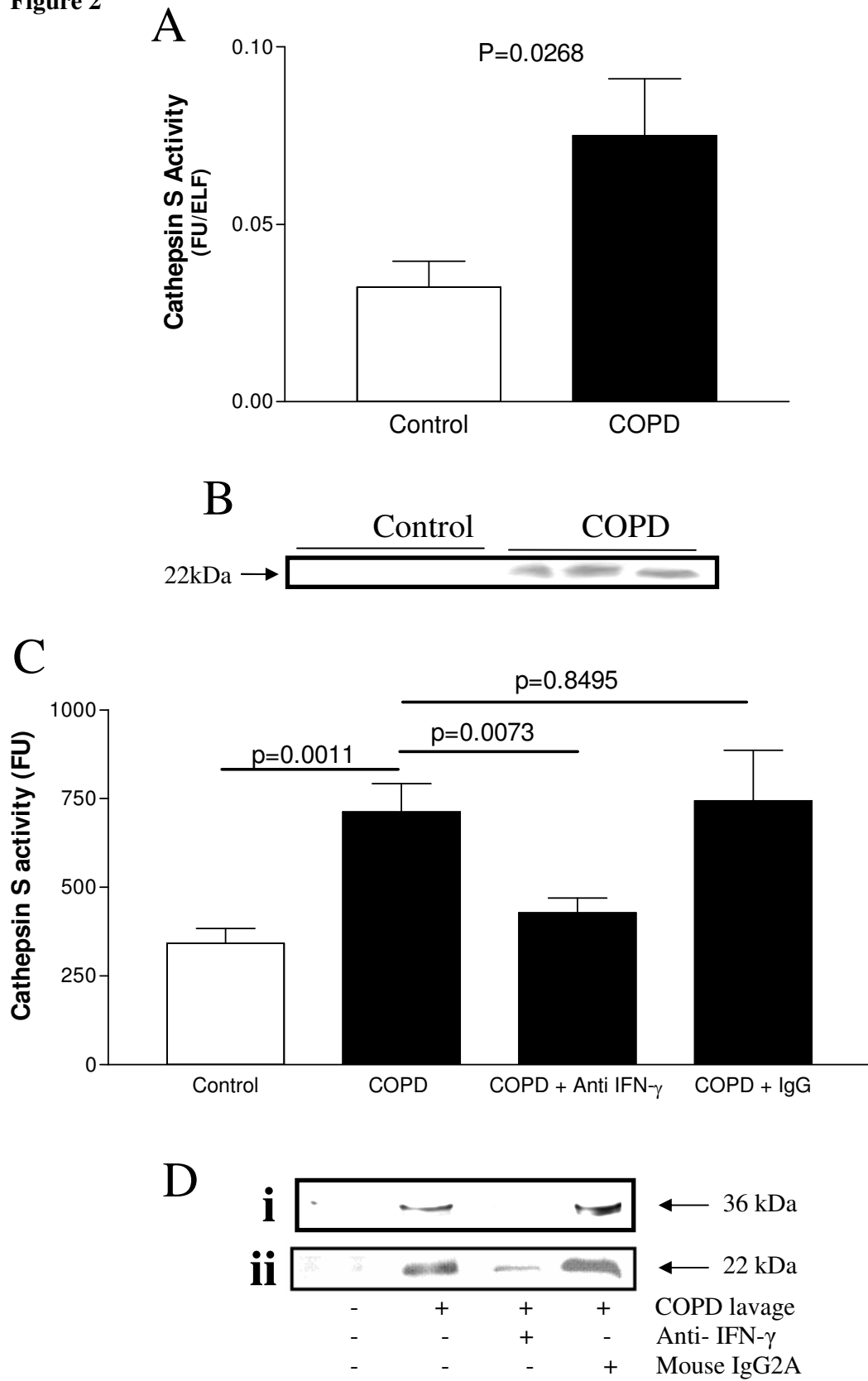
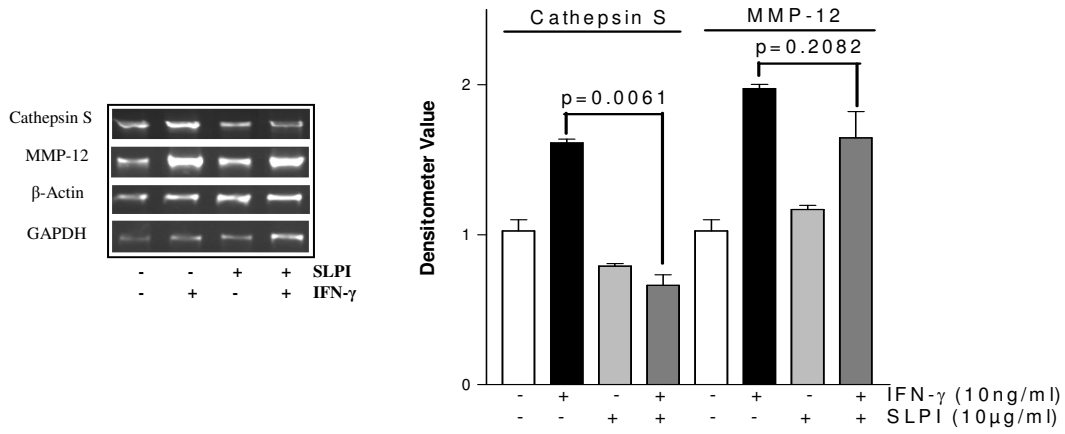
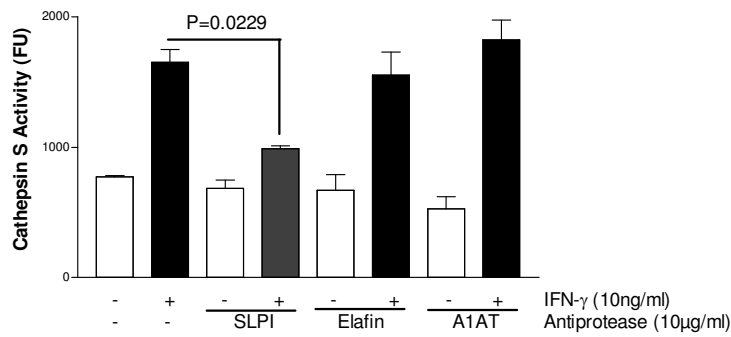


Figure 3

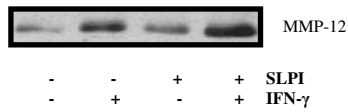
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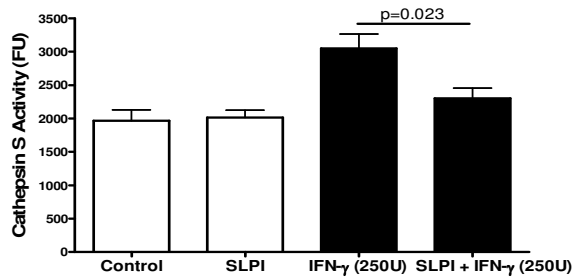
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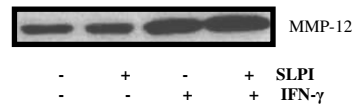


Figure 4

