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α1-Antitrypsin inhibits *Moraxella catarrhalis* MID protein-induced tonsillar B cell proliferation and IL-6 release

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Abstract

α1-Antitrypsin (AAT) is a major circulating and tissues inhibitor of serine proteinases implicated in the regulation of inflammation and host defence. There is now increasing evidence that AAT may also exhibit anti-inflammatory activities independent of its protease inhibitor function. This study was undertaken to investigate the effects of native (inhibitory) and polymerized (non-inhibitory) forms of AAT on MID (Moraxella IgD binding protein)-induced human tonsillar B cell activation in vitro. We found that 0.5 μg/ml MID induces B cell proliferation and stimulates IL-6 release ($p < 0.001$) relative to non-stimulated controls. Both native and polymerized AAT (0.5 mg/ml) inhibited MID-stimulated B cell proliferation in a similar manner (by 70%, $p < 0.001$), whereas MID-induced IL-6 release was more strongly suppressed by polymerized (9.9-fold, $p < 0.001$) as compared to native AAT (2.8-fold, $p < 0.01$). Electrophoretic analysis of cell culture media did not indicate any interaction between AAT and MID, and flow cytometry data showed no competition for the same receptor. The effects of AATs were observed whether added together with MID or 2 h after MID-addition to cell cultures. Thus, our data demonstrate that AAT inhibits MID-induced B cell activation in vitro that is unrelated to its protease inhibitory activity and is not dependent on MID binding to the cell surface.

Keywords: α1-Antitrypsin; tonsils; B cells; Inflammation; MID

1. Introduction

The inflammatory response to infection or tissue injury is characterized by an influx of inflammatory cells, followed by destruction and/or removal of foreign material. During the acute phase response, which accompanies the ongoing inflammation, a rapid rise in levels of serine protease inhibitors, such as α1-antitrypsin (AAT) (three- to four-fold above normal), is observed [1]. This constitutes an important host defence mechanism, which limits the degradative activity of serine proteases released from inflammatory cells and controls damage to host tissue. The primary function of AAT is to neutralize serine protease activity, particularly neutrophil elastase and proteinase 3, which are up-regulated in the inflammatory response. Alterations of the AAT molecule that compromise its activity and thereby reduce its functional level, may result in diseases. The most convincing example of this phenomenon is the inherited, severe Z AAT deficiency, which predisposes individuals to liver and lung diseases, not because of reduced synthesis of the AAT, but because of its polymerization and blockage of secretion which results in dramatically reduced circulating levels of the serpin [2].

There is now considerable evidence that AAT may exhibit biological activity independent of its protease inhibitory activity [3–7]. For instance AAT has been shown to inhibit neutrophil superoxide and chemokine (IL-8) production in vitro [7], to induce macrophage-derived interleukin-1 receptor antagonist release [8], and to reduce bacterial endotoxin and TNFα-induced lethality in vivo [9,10]. Recent studies provide evidence that AAT may also exert antibacterial activities. Knappstein et al. recently reported that AAT binds to the secreted enteropathogenic *Escherichia coli* proteins (EspB, EspD) and strongly reduces their mediated hemolysis of red blood cells [11]. Moreover, an interaction between AAT and *Cryptosporid-*

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ium parvum, the protozoan parasite, has been found to inhibit *C. parvum* infection suggesting a potential role for AAT in controlling cryptosporidiosis [12]. Recent work in our laboratory has demonstrated that both native (inhibitory) and polymerised or oxidised (non-inhibitory) forms of AAT strongly inhibit lipopolysaccharide (LPS)-induced human monocyte activation in vitro [13]. Moreover, we found that Prolastin, a preparation of purified pooled human AAT used for augmentation therapy, not only inhibits neutrophil elastase, but also exhibits other anti-inflammatory effects; e.g., it inhibits LPS-activated neutrophil and monocyte cytokine and chemokine release in vitro and inhibits LPS-induced nasal IL-8 release in vivo [14].

After *Haemophilus influenzae* and *Streptococcus pneumoniae*, *Moraxella catarrhalis* is the third most common bacterial agent in acute otitis media in children. *M. catarrhalis* is a gram-negative human mucosal pathogen causing middle ear infections in infants and children and lower respiratory tract infections in adults, particularly in those with predisposing conditions, such as chronic obstructive pulmonary disease (COPD) [15,16]. Both *H. influenzae* and *M. catarrhalis* express outer membrane surface proteins that activate immunoglobulin D (IgD^+) B lymphocytes by cross-linking IgD [17–19]. MID (Moraxella IgD binding protein) is an IgD-binding surface protein which is known to induce a strong proliferative response in human peripheral IgD^+ B lymphocytes [19]. In addition to its specific affinity for IgD, MID functions as an adhesin and binds to human epithelium [20,21].

In the present study, we aimed to investigate B cell responses elicited by MID in the presence or absence of native (inhibitory, active) or polymeric (temperature inactivated, non-inhibitory) form of AAT. In some experiments, the C-terminal fragment (C-36 peptide) of AAT was used as a negative control. Our findings demonstrate that both native and polymerized AAT significantly inhibit MID-induced tonsillar B cell proliferation and IL-6 release, in vitro.

2. Materials and methods

2.1. Reagents

The purification of the *M. catarrhalis* outer membrane protein MID has been described previously [22]. Monoclonal mouse-anti human HLA-DR-FITC and CD3-RPE antibodies (Immunotech, Marseille, France) were used to determine B cell purity.

2.2. α1-Antitrypsin (AAT) preparations

Purified human AAT ($M_w = 52\,000$, purity by SDS-PAGE > 98%, containing minor contamination of human plasma albumin, inhibitory activity > 85%) was obtained from the Department of Clinical Chemistry, Malmö University Hospital, Sweden. Native AAT was diluted in phosphate buffered saline (PBS), pH 7.4. To ensure the removal of endotoxins, AAT was subjected to Detoxi-Gel AffinityPak columns according to instructions from the manufacturer (Pierce, IL, USA). Purified batches of AAT were then tested for endotoxin contamination with the Limulus amebocyte lysate endochrome kit (Charles River Endosafe, SC, USA). Endotoxin levels were less than 0.1 enzyme units/mg protein in all preparations used. The concentrations of AAT in the endotoxin-purified batches were determined according to the Lowry method. Polymeric AAT was produced by incubation at 60 °C for 10 h. Polymers were confirmed on non-denaturing 7.5% PAGE gels. The polymerized AAT was tested for ability to form complex with pancreatic elastase (EC 3.4.21.36) (Sigma, USA). Samples of polymerized or native AAT were digested with pancreatic elastase at a 1.2:1 molar ratio for 15 min at room temperature. The reaction was stopped by adding SDS sample buffer, mixtures were analysed by 7.5% SDS-PAGE and stained with Coomassie Blue. Synthetic C-terminal peptide of AAT (C-36) corresponding to residues 358–394, greater than 98% purity, was obtained from Saveen Biotech AB (Sweden). The peptide was reconstituted in sterile medium at a concentration of 2 mg/ml and then diluted immediately, prior to use. In all experiments peptide was used at a concentration of 0.03 or 0.06 mg/ml.

2.3. One percent agarose electrophoresis

Cell supernatants with and without AAT and MID alone or their combinations were analysed by a 1% agarose electrophoresis as described [23]. Briefly, a 1% (w/v) suspension of agarose in barbital buffer, pH 8.6, containing 2 mM calcium lactate, was heated and then spread on a glass plate. After a firm gel was formed, the samples were applied and the electrophoretic separation was performed with a potential gradient of 20 V/cm for about 50 min (samples migrated towards the anode). After the separation, gels were fixed in picric–acetic acid solution, dried and stained with Coomassie Blue.

2.4. Cell preparations

Human tonsils were obtained from children and adults undergoing tonsillectomy at Malmö University Hospital (approved by Ethical Committee Lund University, LU 486-01). Briefly, tonsils were minced and cell suspension filtered through 70 μm nylon cell strainer (Becton Dickinson, NJ, USA) before Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. B lymphocytes were isolated by a negative selection using human B Cell isolation kit II and thereafter anti-CD3-conjugated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to eliminate remaining CD3 positive T lymphocytes. A VarioMACS magnetic cell sorter (Miltenyi Biotec) was used for B cell isolation according to the manufacturer's instructions. Negative selection of B cells allows a purification protocol without the addition of B cell specific mAbs. The purity of negatively selected B cells was routinely >97% $HLA\text{-}DR^+$.

2.5. Cell proliferation assay

Purified tonsillar B cells (2×10^5) were incubated in RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 10% FCS, 50 μg/ml gentamicin and 200 U/ml penicillin (culture medium) and cultured in 96-well round-bottom plates

(Nunc, Roskilde, Denmark) in triplicates in a final volume of 200 μl culture medium. Cell proliferation was measured routinely after 3 days by [*methyl*-^{3}H]thymidine incorporation (5 μCi/well; Amersham Biosciences) using an 18 h pulse period.

2.6. Biotinylation of MID

MID was conjugated with biotin using a FluoReporter® Mini-Biotin-XX protein labeling kit (Molecular Probes, USA) according to the manufacturer's instructions. For analysis of MID-binding to membrane IgD on tonsillar B cells, 25 μg/ml of MID-biotin was incubated with 4×10^5 cells for 20 min on ice. The B cells were washed twice with 0.5% PBS-BSA and thereafter incubated for 20 min with streptavidin-RPE as a secondary step. After two washes in 0.5% PBS-BSA, the binding of MID-biotin to the B cells was analysed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

2.7. Detection of IL-6 by ELISA

IL-6 production was measured from cell supernatants harvested after 48 h of incubation. In brief, ELISA plates (Maxisorp, Nunc) were coated with 50 μl of a solution containing rat anti-IL-6 Ab (2 μg/ml; BD Pharmingen) diluted 1/1500 in 0.1 M Na_2HPO_4, pH 9.0. Standards and supernatants were diluted in phosphate buffered saline (PBS)/Tween 20 (0.05%). Biotinylated rat anti-IL-6 Ab (1 μg/ml; BD PharMingen) was used as the secondary antibody, and thereafter HRP-conjugated avidin was added. Finally, plates were developed and absorbance measured at 405 nm.

2.8. Statistical analysis

The differences in the means of experimental results were analysed for their statistical significance with the one-way ANOVA combined with a multiple-comparisons procedure (Scheffe multiple range test), with an overall significance level of $\alpha = 0.05$. Statistical Package (SPSS for Windows, release 12.0) was used for the statistical calculations.

3. Results

3.1. MID binds and activates tonsillar B cells

Flow cytometry analysis was performed on eight donors to demonstrate the binding of MID to purified tonsillar B cells. Our observations show that on average 30–40% of the tonsillar B cells express IgD (data not shown). B cells were incubated with MID-biotin and streptavidin-PE mAb as a secondary step. The mean value for the MID binding to the cells was calculated to be 19% ($n = 8$). In one of the analyzed donors, MID was found to bind to 28% of the tonsillar B cells (Fig. 1A). We have previously shown that MID induces B cell proliferation in vitro [19,21]. Here we used MID over a concentration range of 0.1–5.0 μg/ml in order to investigate whether MID induces B cell proliferation in a concentration-dependent manner. As illustrated in Fig. 1B, MID induces maximal tonsillar B cell proliferation at a concentration of 0.5 μg/ml compared to controls.

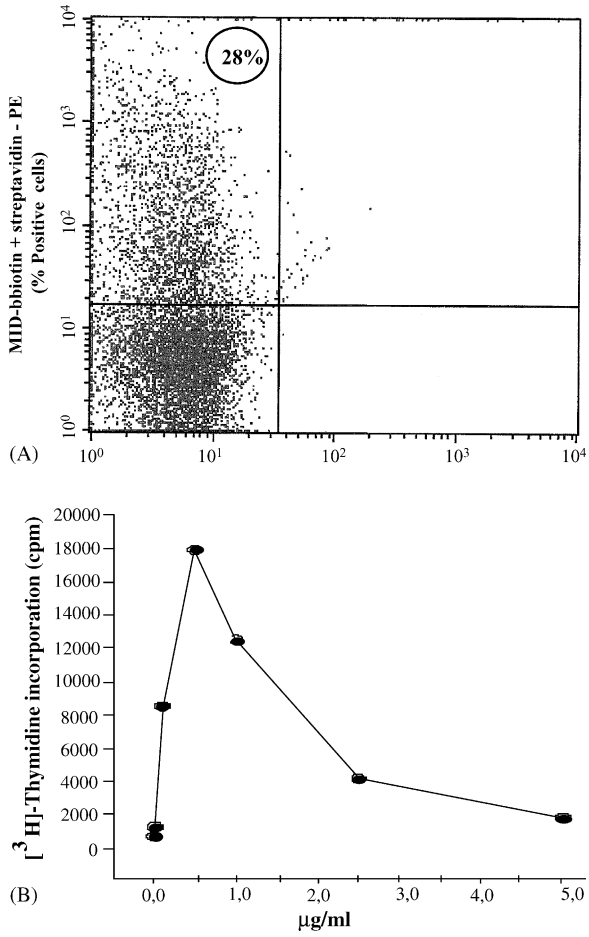

Fig. 1. Tonsillar B cells bind and proliferate in response to the *M. catarrhalis* MID protein. Purified tonsillar B cells (4×10^5 cells/ml) were incubated with biotinylated MID (25 μg/ml) and streptavidin-PE as a secondary step, and analysed by flow cytometry. The figure shows % of MID-binding cells from one donor out of eight performed. Purified tonsillar B cells (1×10^6 cells/ml) were incubated in culture medium with or without various concentrations of MID (0.1–5.0 μg/ml). Cell proliferation was measured by [*methyl*-^{3}H]-thymidine (5 μCi/well) uptake after 3 days of culture. Results are presented as one representative experiment out of two performed.

3.2. MID-induced B cell proliferation is inhibited by native and polymeric AAT

Next, we aimed to investigate whether MID-induced B cell proliferation can be affected by native or polymerized AAT. B cells were cultured with MID (0.5 μg/ml) either alone or in combination with AATs (0.5 mg/ml) for 72 h. Five individual donors were analysed, and as shown in Fig. 2 MID strongly increased the B cell proliferation. Combinations of MID and AAT, however, significantly suppressed B cell proliferation compared to MID alone ($p < 0.001$). The C-terminal fragment of AAT (C-36) was used as a negative control and showed no significant effects (Fig. 2). Native or polymerized AAT alone had no effect on B cell proliferation compared to basal levels (data not shown).

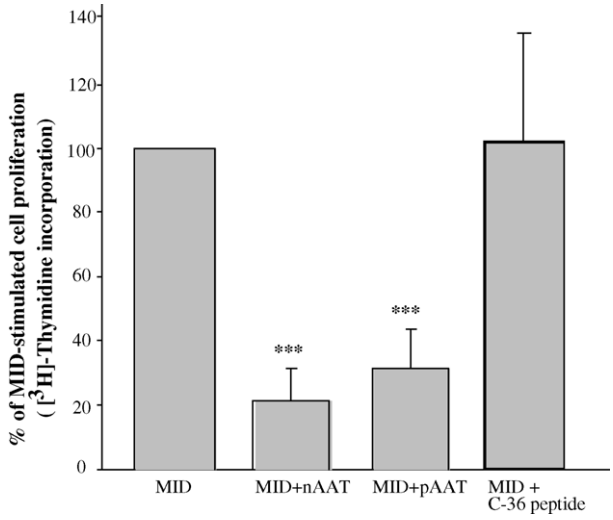

Fig. 2. MID-induced proliferation of tonsillar B cells is inhibited by native and polymeric AAT. Purified tonsillar B cells (1×10^6 cells/ml) were incubated in culture medium alone or with MID (0.5 μg/ml), nAAT (0.5 mg/ml), pAAT (0.5 mg/ml) or C-36 peptide of AAT (0.06 mg/ml) or MID–AATs combinations. Cell proliferation was measured by [*methyl*-^{3}H]-thymidine (5 μCi/well) uptake after 3 days of culture. Results are presented as % of max response as measured from MID-stimulated cells, and show mean values ±S.D. from five donors.

3.3. MID-induced IL-6 release is inhibited by AATs

To further investigate the effects of AATs on MID-induced B cell activation, IL-6 release was measured in cell supernatants after exposure for 48 h to MID alone or in combination with AATs. In our earlier studies, using B cell isolation method based on a positive selection with anti-CD19 conjugated beads, we found that MID on its own does not stimulate IL-6 release but rather enhances the IL-6 release induced by CD40L, IL-2 and IL-4 [19]. Recently we used negative selection for the isolation of B cells [21] and show that, indeed, MID on its own stimulates IL-6 secretion from tonsillar B cells (Fig. 3). In contrast, co-incubation of B cells with MID and native or polymerized AAT inhibited the release of IL-6 compared to cells treated with MID alone. Interestingly, MID-induced IL-6 release was more strongly suppressed by the polymerized form (9.9-fold, $p < 0.001$) compared to native AAT (2.8-fold, $p < 0.01$). The C-36 fragment of AAT had no significant effect on MID-induced IL-6 secretion (Fig. 3).

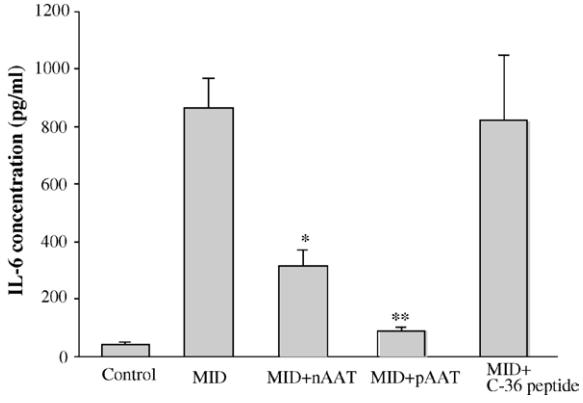

Fig. 3. MID-induced IL-6 secretion from tonsillar B cells is inhibited by native and polymeric AAT. Purified tonsillar B cells (1×10^6 cells/ml) were stimulated in culture medium with or without MID (0.5 μg/ml), or in combinations of MID with nAAT, pAAT or C-36 peptide. Supernatants were harvested after 48 h and analysed for IL-6 release by ELISA. Results are presented as the mean value ±S.D. of IL-6 secretion from three experiments performed.

3.4. Analysis of MID and AAT interaction

For investigating a possible binding between MID and AATs, tonsillar B cells were incubated for 24 h with or without MID (0.1 and 0.5 μg/ml), or in different combinations of MID and native AAT. Cell supernatants were analysed for AAT-MID interaction on 1% agarose gel electrophoresis, at pH 8.6. Fig. 4A compares the electrophoretic pattern produced by AAT alone and in combination with MID. It is evident that AAT alone or in combination with MID shows the same electrophoretic mobility towards the anode, therefore, indicating no direct interaction between MID and AAT.

Furthermore, the inhibition of IL-6 secretion from B cells stimulated with combinations of MID and AATs was observed when AATs were added simultaneously with MID or 2 h after the MID protein (Fig. 4B). This suggests that direct AAT–MID interaction is unlikely to explain the anti-inflammatory effects of AATs.

4. Discussion

MID, a protein from *M. catarrhalis,* is a strong B cell stimulator by its unique ability to target IgD [19,21,22]. No binding of MID was detected to IgG, -M, -A or -E and MID was not found to bind or activate human peripheral blood T cells and monocytes [19,24]. These properties of MID make it an important tool to study IgD-targeted activation of B cells. In this study, we have addressed the question of whether serine protease inhibitor, AAT, could affect the MID-induced tonsillar B cell responses. Our data indicate that both native (inhibitory) and heat inactivated (polymerized) forms of AAT strongly suppress MID-stimulated B cell proliferation and IL-6 secretion. It is important to point out, that polymerized AAT, a protein without antiproteinase activity, not only retained but also enhanced its ability to inhibit MID-induced B cell activation. Thus, the data presented here clearly show that inhibitory activity of AAT is not required for its anti-MID effects. Similarly, studies by She et al. have shown that heating of AAT at 65 °C for 30 min markedly increases its stimulatory effects on human fibroblast DNA synthesis and phosphorylation of p42/44 mitogen-activating protein kinases, compared to native (inhibitory) form of AAT [25]. It is presently unclear why AAT is more active after treatment at 60 °C, however, one can speculate that temperature-induced conformational changes of AAT molecule may favor the optimal interaction with B cell surface and triggering of anti-inflammatory mechanisms. To prove this idea further studies are needed.

The specificity of AAT effects was demonstrated by failure of a C-terminal fragment of AAT (C-36 peptide) to elicit any biological activity in our experimental model and the failure of

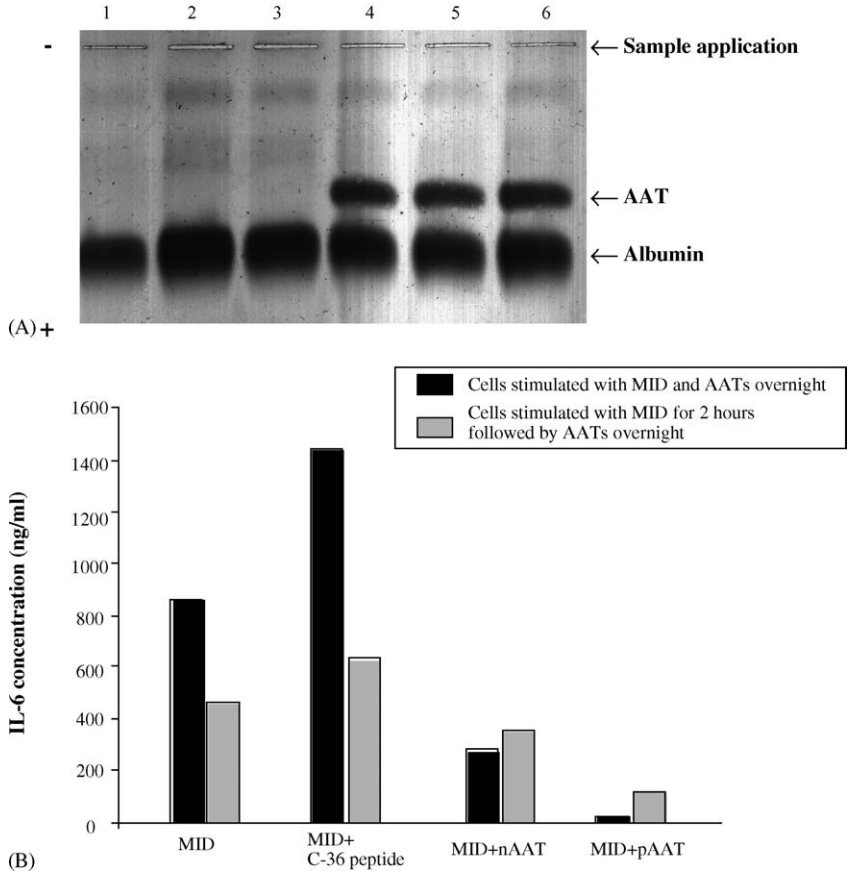

Fig. 4. No interaction between MID and AATs can be demonstrated. (A) Analysis of cell culture medium by 1% agarose electrophoresis, at pH 8.6. The anode is at the bottom of the figure. B cells were cultured alone or in the presence of 0.1 and 0.5 μg/ml MID or 0.5 mg/ml nAAT or their combinations for 24 h. The cell supernatants were collected and analysed by electrophoresis. Samples were loaded as follows: lane 1, cell medium alone; lanes 2 and 3, cell medium + 0.1 and 0.5 μg/ml MID, respectively; lane 4, cell medium + nAAT; lanes 5 and 6, cell medium + 0.1 and 0.5 μg/ml MID and 0.5 mg/ml nAAT, respectively. (B) Purified tonsillar B cells (1×10^6 cells/ml) were stimulated in culture medium with MID (0.5 μg/ml), or MID in combinations with nAAT, pAAT or C-36 peptide. Cells were either exposed to MID and AATs simultaneously overnight, or exposed first for 2 h to MID and after ttreated with MID–AATs combinations overnight. Supernatants were harvested and analysed for IL-6 production by ELISA. Results are presented as one experiment out of two performed.

AATs to inhibit CD3/CD28 stimulated IL-2 receptor expression or proliferation in T-cells which lack TLR4 and CD14 (unpublished data). Furthermore, the significant effects of AATs were achieved at concentrations below those reported in vivo (i.e., at 0.5 mg/ml which is equivalent to about 50% of plasma AAT levels). Together these data imply that AAT is a potent and rather specific inhibitor of MID-induced tonsillar B cell activation. Moreover, our data indicate that full length AAT either in its inhibitory active or inactive, polymerized form is needed to express the anti-inflammatory activity.

To exclude the possibility that the inhibitory effects of AAT on MID-stimulated B cell responses are due to a sequestration of the MID, two experimental protocols were followed. First, cells were incubated with AAT or MID separately or in combination and cell culture medium was subjected to electrophoretic analysis. However, no changes in the electrophoretic mobility of the AAT/MID mixture compared to AAT alone were observed. Second, B cells were pre-incubated with MID prior to addition of AATs, but again this made no specific contribution to anti-inflammatory effects of AATs. These results led us to conclusion that the inhibitory effects of AAT towards MID-induced B cell activation do not appear to involve a direct AAT–MID interaction.

These latter findings prompted us to investigate whether AAT interferes with MID binding to the B cell receptors. However, flow cytometer analysis of cells stained with MID-biotin did not show reduced MID-binding when cells were pre-incubated with AATs, indicating that there is no competition for the receptor binding between MID and AAT (data not shown). Therefore, we conclude that neither direct AAT–MID interaction nor AAT interference with the MID cellular receptor(s) binding are likely mechanisms for the observed anti-inflammatory effects of AATs. Further studies are needed to clarify the mechanisms responsible for the observed anti-inflammatory effects of AAT.

Finally, it should be noted that both native (inhibitory) and temperature inactivated, polymeric (non-inhibitory) forms of AAT are effective modulators of MID-stimulated B cell activation suggesting that observed properties of AAT are independent on its serine protease inhibitory activity. This strengthens the idea that in vivo AAT may not only limit the destructive capacity of serine proteases released from inflammatory cells, but also may act as a protein with broader anti-inflammatory

properties and constitute an important part of host defensive mechanisms.

A strong association between inherited severe Z AAT deficiency (basal levels of AAT are reduced by 90%) and COPD led to the hypothesis that a protease–antiprotease imbalance is an important factor for disease development [26]. It became widely accepted that the primary role of AAT in vivo is to minimize proteolytic injury to host tissue at sites of inflammation, infection and injury by inhibiting the activity of neutrophil-released serine proteases. However, current studies demonstrate that a short-term regimen of AAT augmentation therapy not only restores airway concentrations of AAT to normal, but also reduces the incidence of lung infections in patients with AAT deficiency and reduces levels of chemoattractants, such as leukotriene B4 [27,28]. Cantin and Woods have also reported that aerosolized AAT suppresses bacterial proliferation in a rat model of chronic *Pseudomonas aeruginosa* lung infection [29].

It is known that individuals with inherited AAT deficiency are more susceptible to bacterial infections [30,31], and that combinations of environmental factors, such as smoking and air pollution, bacterial infections and AAT-deficiency may result in COPD development in the early years of life [32,33]. *M. catarrhalis*, a common and important human respiratory tract pathogen, is one of the leading causes of exacerbations in patients with COPD [34]. Thus, our findings that AAT inhibits *M. catarrhalis* MID protein-induced B cell activation in vitro, further support the notion that AAT is a multifunctional protein and provide a basis for exploring as yet uninvestigated anti-inflammatory properties of AAT in vitro and in vivo.

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