

α -1-Antitrypsin is an endogenous inhibitor of proinflammatory cytokine production in whole blood

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Abstract: Several observations suggest endogenous suppressors of inflammatory mediators are present in human blood. α -1-Antitrypsin (AAT) is the most abundant serine protease inhibitor in blood, and AAT possesses anti-inflammatory activity in vitro and in vivo. Here, we show that in vitro stimulation of whole blood from persons with a genetic AAT deficiency resulted in enhanced cytokine production compared with blood from healthy subjects. Using whole blood from healthy subjects, dilution of blood with RPMI tissue-culture medium, followed by incubation for 18 h, increased spontaneous production of IL-8, TNF- α , IL-1 β , and IL-1R antagonist (IL-1Ra) significantly, compared with undiluted blood. Dilution-induced cytokine production suggested the presence of one or more circulating inhibitors of cytokine synthesis present in blood. Serially diluting blood with tissue-culture medium in the presence of cytokine stimulation with heat-killed *Staphylococcus epidermidis* (*S. epi*) resulted in 1.2- to 55-fold increases in cytokine production compared with *S. epi* stimulation alone. Diluting blood with autologous plasma did not increase the production of IL-8, TNF- α , IL-1 β , or IL-1Ra, suggesting that the endogenous, inhibitory activity of blood resided in plasma. In whole blood, diluted and stimulated with *S. epi*, exogenous AAT inhibited IL-8, IL-6, TNF- α , and IL-1 β significantly but did not suppress induction of the anti-inflammatory cytokines IL-1Ra and IL-10. These ex vivo and in vitro observations suggest that endogenous AAT in blood contributes to the suppression of proinflammatory cytokine synthesis. *J. Leukoc. Biol.* 85: 000–000; 2009.

Key Words: inflammation · dilution · serine protease inhibitor · interleukin

INTRODUCTION

Numerous observations demonstrate the presence of endogenous substances in blood, which suppresses the synthesis of proinflammatory molecules. For example, in health, biologically active, proinflammatory cytokines are rarely detected in blood [1]. However, diluting whole blood enhances proinflammatory cytokine production in vitro, suggesting that dilution reduces the function of cytokine inhibitors in blood. Chernoff

et al. [2] reported that whole blood dilution enhanced IL-1 β and TNF- α production. Similarly, synthesis of intracellular IFN (IFN- γ), TNF- α , IL-2, and IL-10 was optimized when blood was diluted in tissue-culture medium [3–6]. Therefore, diluting whole blood likely reduces concentrations of circulating inhibitors of cytokine production and increases cytokine synthesis per cell. Despite the common practice of diluting blood to enhance cytokine production, the identity of circulating inhibitors remains unclear.

α -1-Antitrypsin (AAT) is a 394-aa, 52-kDa glycoprotein synthesized primarily by hepatocytes [7], with smaller amounts synthesized by intestinal epithelial cells, neutrophils, pulmonary alveolar cells, and macrophages [8, 9]. AAT is the most abundant, endogenous serine protease inhibitor (Pi) in the circulation. Serum AAT concentrations in healthy subjects are 1.5–3.5 mg/mL and can increase fourfold during inflammation, indicating that AAT is an acute-phase protein [7, 10, 11]. The primary function of AAT is thought to be inactivation of neutrophil elastase and other endogenous serine proteases.

AAT has been studied extensively in the clinical setting as a result of the existence of genetic defects, resulting in abnormally low AAT concentrations in blood and referred to as AAT deficiency. More than 100 AAT alleles have been described, and the normal M-type AAT protein is designated PiM [7]. Persons with normal AAT concentrations in blood usually have two copies of the *PiM* gene (*PiMM* phenotype), and the prevalence of this phenotype is ~83% in the United States population [12]. Reduced AAT levels are typically associated with Z-type (*PiZ*) or S-type (*PiS*) AAT variants. The term “AAT deficiency” often refers to the presence of homozygous *PiZZ* AAT, where serum AAT levels approximate 10–15% of normal [13]. However, other AAT phenotypes are associated with deficient AAT levels and include *PiSS*, *PiSZ*, and the pairing of *PiZ* or *PiS* with the normal *PiM* protein (*PiMZ* or *PiMS*) [14]. AAT-deficient individuals are at increased risk for extensive and early onset pulmonary emphysema thought to result from progressive destruction of alveolar walls as a result of unopposed activity of neutrophil-derived elastase [15].

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Novel studies have expanded the link between AAT and human disease. For example, associations were shown between reduced AAT levels or abnormal AAT proteins and HIV type 1 infection [16–18], hepatitis C infection and chronic liver disease [19], atypical mycobacterial infection [20], diabetes mellitus [21], and panniculitis [22]. In mice, exogenous AAT protected islet cell allografts from rejection [23], blocked β cell apoptosis [24], prevented pulmonary emphysema [25], and inhibited angiogenesis and tumor growth [26].

Adding AAT to cultured human cells *in vitro* has revealed anti-inflammatory properties. For example, Janciauskiene et al. [27] reported that AAT inhibited LPS-stimulated synthesis and secretion of TNF- α and IL-1 β in human blood monocytes. In addition, intracellular signaling studies showed that AAT inhibited activation of NF- κ B, a transcription factor involved in the expression of several proinflammatory cytokines [18]. Importantly, some reports suggest that the anti-inflammatory properties of AAT may not require the serine Pi activity of AAT [15, 28, 29].

Investigations of AAT anti-inflammatory functions have used cell lines or PBMC-derived macrophages. As these experimental designs may not reflect AAT biological activity *in vivo*, we used an *in vitro* assay of cultured human whole blood to conduct studies that represent *in vivo* conditions more closely. In the present investigations, we addressed several issues in cytokine biology. First, in whole blood cultures, we compared cytokine synthesis in subjects with genetic AAT deficiency with cytokine synthesis in healthy subjects. Second, in whole blood from healthy subjects, we examined the effect of blood dilution on constitutive (spontaneous) and on *Staphylococcus epidermidis* (*S. epi*)-stimulated cytokine synthesis. Third, we compared spontaneous cytokine production in whole blood diluted with tissue-culture medium with blood diluted with autologous plasma. Fourth, we evaluated exogenously added AAT as an inhibitor of cytokine production in diluted and *S. epi*-stimulated whole blood cultures. Finally, a synthetic serine Pi was studied for effects on whole blood cytokine production.

MATERIALS AND METHODS

Reagents

S. epi strain 49134 was obtained from the American Type Culture Collection (Remel, Lenexa, KS, USA) and provided by Dr. Mary Bessesen (Denver Veterans Affairs Medical Center and the University of Colorado Denver, CO, USA). *S. epi* was grown overnight in suspension cultures in Luria-Bertani medium (Difco, Detroit, MI, USA). The *S. epi* suspension was heat-killed by boiling for 30 min and the protein concentration of the heat-killed *S. epi* preparation determined using the Coomassie Plus protein assay reagent (Pierce, Rockford, IL, USA). Clinical-grade human AAT (Aralast[®], 20 mg/mL stock solution, Baxter Healthcare Corp., Westlake Village, CA, USA) and clinical-grade human serum-derived albumin (250 mg/mL stock solution, ZLB Bioplasma AG, Berne, Switzerland) were used in these studies. H-Ala-Ala-Pro-Val-chloromethylketone (AAPV-CMK) was obtained from Bachem (King of Prussia, PA, USA) and solubilized in DMSO (Fisher Biotech, Fair Lawn, NJ, USA) at a stock concentration of 50 mM. RPMI-1640 medium and PBS were purchased from Mediatech (Herndon, VA, USA), and HBSS was obtained from Life Technologies (Grand Island, NY, USA).

Whole blood collection from healthy volunteers

Healthy subjects not taking prescribed or over-the-counter medications participated. Blood obtained following antecubital venipuncture was aspirated

into sterile glass vacuum tubes containing freeze-dried sodium heparin that resulted in a final heparin concentration of 14.3 units/mL (Becton Dickinson, Franklin Lakes, NJ, USA). Informed consent was obtained from each subject, and the Human Subject Institutional Review Board at the University of Colorado Denver approved the protocol.

Whole blood collection from AAT-deficient patients

Nine patients with AAT deficiency were studied. AAT deficiency was established using criteria defined previously, including serum AAT levels below 0.72 mg/mL (prior to initiation of replacement therapy) and the presence of Z-type AAT mutant protein by phenotype analysis. Five patients had the PiZZ mutant phenotype, two had the PiSZ phenotype, and two had the PiMZ phenotype. Eight of the nine AAT-deficient patients were treated with weekly *i.v.* infusions of 60 mg/kg Prolastin[®] (Talecris Biotherapeutics, Research Triangle Park, NC, USA), and one patient was treated with weekly infusions of 60 mg/kg Zemaira[®] (Aventis Behring LLC, King of Prussia, PA, USA). All nine AAT-deficient patients were diagnosed with chronic obstructive pulmonary disease and treated with inhaled fluticasone-salmeterol, and eight of the nine patients were treated with inhaled albuterol. Other prescribed medications included fluoxetine, tiotropium, iprotropium, lansoprazole, atorvastatin, alendronate, and fexofenadine. AAT concentrations from AAT-deficient and healthy volunteers were determined in heparinized plasma using a human AAT quantitative ELISA (GenWay Biotech, Inc., San Diego, CA, USA). Informed consent was obtained from each AAT-deficient subject, and the Human Subject Institutional Review Board at the National Jewish Medical and Research Center (Denver, CO, USA) approved the protocol.

Cytokine production in whole blood cultures from AAT-deficient persons and healthy volunteers

Heparinized venous blood obtained from healthy volunteers (controls) and from AAT-deficient donors was transferred to 12 \times 75 mm snap-cap polypropylene tubes (Becton Dickinson) under sterile conditions. Heat-killed *S. epi* (final protein concentration, 1.2 μ g/mL) was added to undiluted whole blood (1.0 mL final vol), and the cultures incubated with caps loosely applied at 37°C and 5% CO₂ for 18 h. After incubation, the culture supernatants were aspirated, transferred to new tubes, and frozen at -70°C until assayed.

Whole blood dilution studies

Freshly obtained whole blood from each of four healthy subjects was aliquoted into 12 \times 75 mm snap-cap polypropylene tubes. The blood from each subject was cultured without dilution or was serially diluted with RPMI to 1:4, 1:8, 1:16, or 1:32 in the absence or presence of 1.2 μ g/mL (final concentration) *S. epi* in a volume of 1.0 mL. The whole blood cultures were then incubated as described above.

Whole blood diluted with RPMI or autologous plasma

Donor-matched (autologous) plasma was obtained by subjecting 8.0 mL heparinized blood from each of four healthy volunteers to 400 *g* centrifugation for 10 min and aspirating the supernatant plasma. Whole blood was diluted 1:32 with RPMI or with donor-matched plasma in 1.0 mL final vol. The cultures were incubated for 18 h at 37°C and 5% CO₂ in loosely capped 12 \times 75 mm snap-cap polypropylene tubes. After incubation, the separated plasma components of the cultures were collected and frozen at -70°C until assayed.

Whole blood cultures with exogenous AAT

Whole blood from the same four healthy subjects was diluted 1:32 in RPMI only, diluted 1:32 in RPMI with *S. epi* stimulation, or diluted 1:32 in RPMI with *S. epi* stimulation in the presence of increasing concentrations of AAT or albumin (1.0–8.0 mg/mL). AAT or albumin was added 1 h prior to *S. epi* stimulation, and all final culture volumes were 1.0 mL in 12 \times 75 mm snap-cap polypropylene tubes. After 18 h of incubation with loosely applied caps, the separated plasma components of the blood cultures were aspirated and frozen at -70°C until assayed.

Whole blood cultures with exogenous AAPV-CMK

Whole blood from each of four healthy subjects was diluted 1:16 in RPMI only, diluted 1:16 in RPMI, and stimulated with *S. epi* or diluted 1:16 in RPMI and stimulated with *S. epi* in the presence of 50 μ M AAPV-CMK or 0.1% DMSO (vehicle control). AAPV-CMK or DMSO was added 1 h prior to *S. epi* stimulation, and all final culture volumes were 1.0 mL in 12 \times 75 mm snap-cap polypropylene tubes. After 18 h of incubation, the separated supernatants of the cultured blood were aspirated and frozen at -70°C until assayed.

Cytokine measurements

IL-8, IL-6, TNF- α , IL-1 β , IL-1R antagonist (IL-1Ra), and IL-10 were measured using ECL assays, as described previously [1, 30–32]. All biotinylated antibodies were obtained from eBioscience, Inc. (San Diego, CA, USA). Antibodies obtained from R&D Systems (Minneapolis, MN, USA) were rutenylated using BV-TagTM-normal human serum-ester (Bioveris Corp., Gaithersburg, MD, USA). Cytokine measurements were performed using an M8 ECL analyzer (Bioveris Corp.). The limit of detection for each cytokine ECL assay was 10 pg/mL, and cytokine levels below the assay detection limit were assigned the value 10 pg/mL.

Statistical analysis

The Mann-Whitney U test was used to compare plasma AAT concentrations and whole blood cytokine production in AAT-deficient subjects and healthy controls (see Table 1 and Fig. 1). For the whole blood dilution studies (see Fig. 2), cytokine concentrations were expressed using two methods: first, as directly measured levels (see Fig. 2, A, C, E, and G) and second, as values calculated by multiplication of the measured level by the dilution factor to obtain the amount of cytokine produced per mL of whole blood that was in the cultures (see Fig. 2, B, D, F, and H). For example, calculated levels in samples diluted 1:16 were obtained by multiplying the measured cytokine concentrations by 16. These calculations equalized the concentration of cytokine-producing leukocytes in undiluted and diluted blood cultures in each donor. For studies comparing cytokine levels in whole blood cultures in the absence or presence of dilution, *S. epi* stimulation, or AAT (see Figs. 2, 3, and 5), differences between experimental conditions were evaluated using repeated measures ANOVA with Dunnett's multiple comparison test. In the studies comparing dilution with RPMI or plasma (see Fig. 4) and in the studies comparing dilution alone with dilution and *S. epi* stimulation in the absence or presence of AAPV-CMK (see Fig. 6), group means were compared using repeated measures ANOVA with Tukey's multiple comparison test. $P < 0.05$ was defined as statistically significant.

RESULTS

Cytokine production is increased in whole blood from AAT-deficient patients

We compared cytokine production in stimulated whole blood from nine subjects with genetic AAT deficiency to production in 10 healthy controls. **Table 1** depicts characteristics of the two groups of participants. Blood from the AAT-deficient donors was collected immediately prior to infusion of Prolastin[®] or Zemaira[®] (clinical formulations of AAT) so that blood AAT levels were at their respective nadirs. Plasma AAT concentrations were significantly lower ($P=0.004$) in the AAT-deficient patients compared with the healthy volunteers (median levels of 1.67 mg/mL and 2.73 mg/mL, respectively). The blood for these studies was cultured without dilution to maintain endogenous AAT concentrations. Whole blood from AAT-deficient and control subjects was stimulated with *S. epi* and assessed for cytokine production after 18 h of incubation. As shown in **Figure 1A**, healthy controls produced a median 4.7 ng/mL

TABLE 1. Characteristics of AAT-Deficient Subjects and Healthy Controls

Characteristic	AAT-deficient ($n = 9$)	Healthy controls ($n = 10$)
Age in years ^a	58 (46–72)	37 (24–49)
Gender (M/F)	6/3	6/4
AAT phenotype: PiZZ	five patients	ND
PiSZ	two patients	
PiMZ	two patients	
AAT dose	60 mg/kg ^b	NA
Plasma AAT concentration in mg/mL ^a	1.67 (1.43–2.61) ^c	2.73 (1.81–4.32)

^aData shown as median (range). ^bDosing frequency = 1 i.v. infusion per week. ^cLevel determined immediately prior to AAT infusion; $P = 0.004$. ND = Not determined. NA = Not applicable.

IL-8, whereas blood cultures in the AAT-deficient donors produced a median 67.6 ng/mL (14.4 times the level in healthy donors, $P < 0.0005$). Median IL-6 and IL-1Ra production were also increased significantly in the AAT-deficient group (3.4 and 8.4 times the median level in healthy controls, respectively, Fig. 1, B and E). However, no significant difference in median TNF- α or IL-1 β production was observed in AAT-deficient patients compared with controls (Fig. 1, C and D). IL-10 levels were elevated in the AAT-deficient group (median IL-10 in the AAT-deficient group was 1.8 times that of the healthy controls), but this difference was not statistically significant (Fig. 1F).

Diluting whole blood with RPMI increases spontaneous cytokine production

To assess whether blood contains inhibitors of cytokine synthesis, whole blood was collected from four healthy donors and incubated for 18 h, undiluted or diluted with RPMI, to final blood concentrations 1:4, 1:8, 1:16, or 1:32. **Figure 2, A, C, E, and G**, shows spontaneous cytokine production presented as measured concentrations. In Figure 2, B, D, F, and H, cytokine levels are depicted after multiplication by the dilution factors to obtain the calculated cytokine concentrations in the undiluted blood component of the cultures. These calculations were designed to assess alterations in cytokine produced per white blood cell in blood as a result of dilution. In Figure 2, A and B, undiluted blood (dilution=0) produced 97.8 ± 56.4 pg/mL IL-8 (mean \pm SEM). After multiplying by the dilution factor, blood diluted to final concentrations 1:4, 1:8, 1:16, and 1:32 contained IL-8 levels of 93.9 ± 28.4 pg/mL, 118.9 ± 13.1 pg/mL, 192.7 ± 21.9 pg/mL, and 398.2 ± 26.1 pg/mL, respectively (Fig. 2B). This represented a maximum mean 3.1-fold increase in IL-8 levels in blood diluted 1:32 compared with undiluted blood. In the same whole blood cultures, we measured TNF- α (Fig. 2, C and D), IL-1 β (Fig. 2, E and F), and IL-1Ra (Fig. 2, G and H). As observed for IL-8, dilution of blood with RPMI resulted in significant and dose-dependent escalation in spontaneous TNF- α , IL-1 β , and IL-1Ra after adjustment for dilution. We observed maximum increases of 40-fold, 299-fold, and 18-fold in cytokine levels adjusted for dilution compared with undiluted blood, respectively (Fig. 2, D, F, and H). IL-6 and IL-10 concentrations in the same

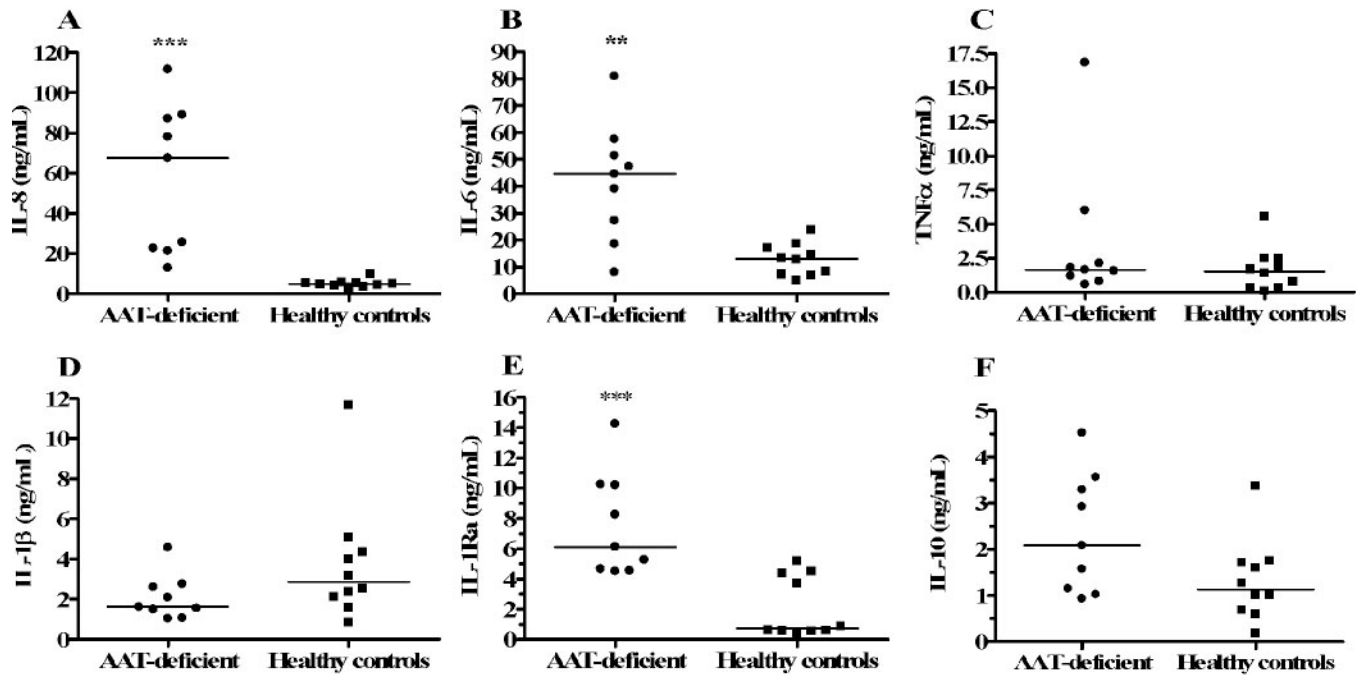


Fig. 1. Effect of AAT deficiency on cytokine production in stimulated whole blood. Cytokine production was measured in undiluted, *S. epi*-stimulated blood obtained from nine AAT-deficient patients (●) and from 10 healthy controls (■). After 18 h of stimulation, supernatants were removed for cytokine assays, including IL-8 (A), IL-6 (B), TNF- α (C), IL-1 β (D), IL-1Ra (E), and IL-10 (F). Horizontal bars indicate median levels. **, $P < 0.005$, and ***, $P < 0.0005$, compared with healthy controls.

cultures were below the limit of assay detection for all conditions tested (data not shown).

To determine if increased spontaneous cytokine production was dependent on RPMI constituents, we repeated the blood dilution experiments, except that blood was diluted in PBS or HBSS instead of RPMI. In blood diluted 1:16 or 1:32 in PBS or HBSS, IL-8, TNF- α , IL-1 β , and IL-1Ra were increased to similar extents, as observed for dilution in RPMI (data not shown). These results indicate that cytokine increases were dilution-dependent and not a result of RPMI components.

Dilution of blood augments *S. epi*-induced cytokine production

We extended the dilution studies to assess the effect of dilution on whole blood cultures stimulated with heat-killed *S. epi*, which is a well-described cytokine inducer in vitro, and *S. epi*-exposed blood reflects cytokine responses following activation of the TLR-2 [33]. Blood was cultured undiluted, undiluted and stimulated with *S. epi*, or diluted and stimulated with *S. epi*. As shown in **Figure 3A**, undiluted blood (dilution=0; far-left bars) and undiluted blood stimulated with *S. epi* (dilution=0, second bars from left) produced 97.8 ± 56.4 pg/mL and 4.2 ± 0.3 ng/mL IL-8 after 18 h of incubation, respectively. Blood stimulated with *S. epi* and diluted 1:4, 1:8, 1:16, or 1:32 with RPMI produced 88.6 ± 9.5 ng/mL, 163.7 ± 27.9 ng/mL, 233.6 ± 50.7 ng/mL, and 108.8 ± 18.1 ng/mL IL-8, respectively. Diluting *S. epi*-stimulated blood 1:16 produced a maximum mean 55-fold IL-8 increase ($P < 0.01$) compared with blood exposed to *S. epi* in the absence of dilution. In the same whole blood cultures, we measured IL-6 (Fig. 3B), TNF- α (Fig. 3C), IL-1 β (Fig. 3D), IL-1Ra (Fig. 3E), and IL-10 (Fig. 3F). As

observed for IL-8, cultures stimulated with *S. epi* and diluted with RPMI demonstrated significant increases in cytokine concentrations compared with undiluted, *S. epi*-stimulated cultures. Combined *S. epi* stimulation and RPMI dilution resulted in maximum IL-6, TNF- α , IL-1 β , IL-1Ra, and IL-10 levels that were increased 1.3-fold ($P < 0.05$), 8.1-fold ($P < 0.01$), 1.3-fold ($P < 0.01$), 5.6-fold ($P < 0.01$), and 1.2-fold ($P = \text{not significant}$) compared with levels observed with *S. epi* stimulation alone (no dilution), respectively.

Effect of diluting blood with autologous plasma on cytokine production

To determine if increased cytokine production in diluted blood was a result of reduced concentration of inhibitory substances in plasma, we examined the effect of blood dilution with autologous plasma. Blood was collected from four healthy donors and incubated for 18 h undiluted, diluted with RPMI to a final blood concentration of 1:32, or diluted 1:32 in autologous plasma. Cytokine levels were not multiplied by the dilution factor (1:32) to directly compare the cytokine levels in undiluted blood (dilution=0) and in plasma-diluted blood. Compared with blood diluted in RPMI, dilution in plasma suppressed TNF- α , IL-1 β , and IL-1Ra production (**Fig. 4, B–D**, respectively). IL-8 production was decreased in RPMI and plasma-diluted samples (Fig. 4A). Although IL-8 measured in the plasma-diluted cultures was increased compared with the RPMI-diluted cultures, the difference was not statistically significant. We also measured IL-6 and IL-10 in these cultures, and levels were below the detection limit (10 pg/mL) of the ECL assays in all conditions tested (data not shown).

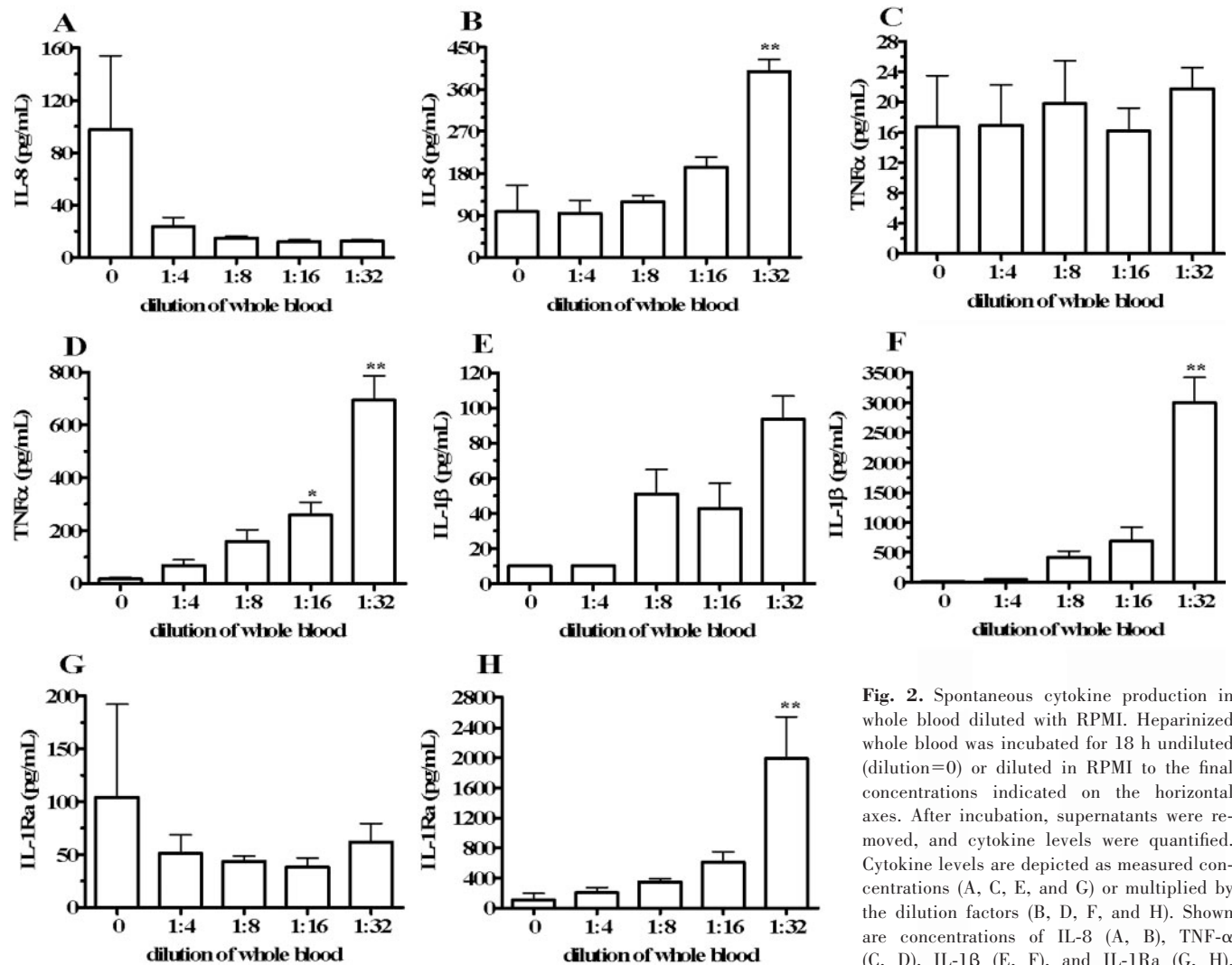


Fig. 2. Spontaneous cytokine production in whole blood diluted with RPMI. Heparinized whole blood was incubated for 18 h undiluted (dilution=0) or diluted in RPMI to the final concentrations indicated on the horizontal axes. After incubation, supernatants were removed, and cytokine levels were quantified. Cytokine levels are depicted as measured concentrations (A, C, E, and G) or multiplied by the dilution factors (B, D, F, and H). Shown are concentrations of IL-8 (A, B), TNF- α (C, D), IL-1 β (E, F), and IL-1Ra (G, H).

Cytokine concentrations are indicated on the vertical axes as means \pm SEM in four separate donors. *, $P < 0.05$; **, $P < 0.01$, compared with dilution = 0.

Exogenous AAT inhibits cytokine production in whole blood cultures

As AAT deficiency resulted in greater cytokine production in whole blood cultures (Fig. 1), and other studies report anti-inflammatory properties of AAT [7, 10, 11, 27, 34], we examined the effect of exogenous AAT on stimulated cytokine production in whole blood. As shown in **Figure 5A**, whole blood cultures diluted 1:32 with RPMI and stimulated with *S. epi* (AAT=0) induced a mean 163.7 ± 30.9 ng/mL IL-8. The addition of AAT reduced IL-8 in diluted and *S. epi*-stimulated whole blood by a maximum of 99% using 8 mg/mL AAT compared with cultures conducted in the absence of AAT. In these cultures, we also measured IL-6 (Fig. 5B), TNF- α (Fig. 5C), IL-1 β (Fig. 5D), IL-1Ra (Fig. 5E), and IL-10 (Fig. 5F). Dose-dependent IL-6, TNF- α , and IL-1 β suppression was observed in the presence of AAT, with maximum mean reductions of 97%, 91%, and 47%, respectively, compared with cultures without AAT, which did not affect the levels of stimulated IL-1Ra and IL-10 significantly (Fig. 5, E and F).

To assess the specificity of AAT inhibition of stimulated whole blood cytokine production, we used human serum-de-

rived albumin as a protein control. Whole blood was diluted 1:32 with RPMI and stimulated with *S. epi* in the absence (control) or presence of 1–8 mg/mL albumin. In three separate experiments, albumin did not affect diluted and *S. epi*-stimulated whole blood production of any cytokine tested (IL-8, IL-6, TNF- α , IL-1 β , IL-1Ra, and IL-10; data not shown).

Effect of AAPV-CMK, a synthetic serine Pi, on cytokine production

As AAT is the prototypical serine Pi in the circulation, we surmised that AAT-induced suppression of whole blood proinflammatory cytokine production was a result of inhibition of serine proteases. Therefore, we tested AAPV-CMK, a small-molecule synthetic inhibitor of serine proteases in whole blood cytokine production [18]. Blood was collected from healthy donors and diluted 1:16 with RPMI (control), diluted 1:16 with RPMI and stimulated with *S. epi*, or diluted 1:16 with RPMI and stimulated with *S. epi* in the presence of 50 μ M AAPV-CMK (added 1 h prior to *S. epi*). After 18 h of incubation, the mean IL-8 and IL-6 levels in blood diluted and stimulated with *S. epi* were 163.8 ± 30.4 ng/mL and 33.7 ± 1.1 ng/mL,

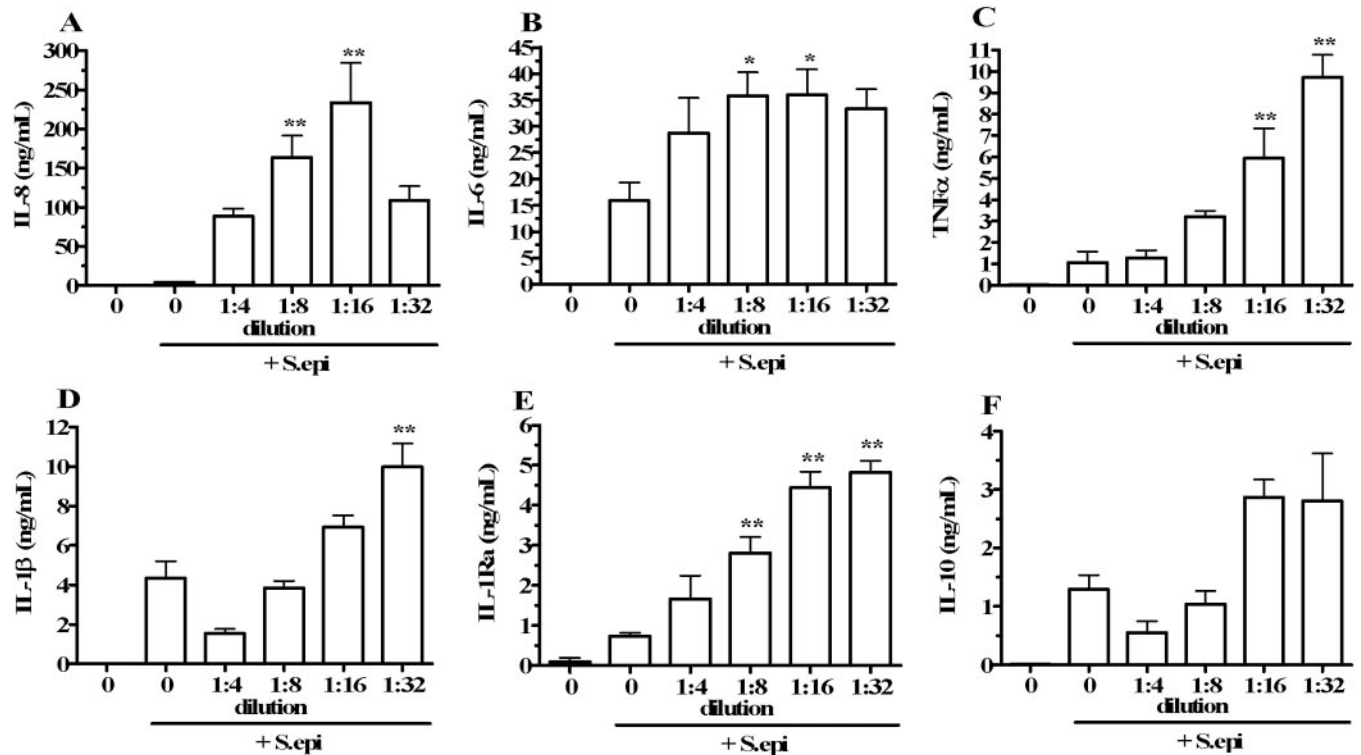


Fig. 3. Effect of whole blood dilution on *S. epi*-stimulated cytokine production. Heparinized whole blood was incubated for 18 h undiluted (dilution=0, far-left bars), incubated undiluted with heat-killed *S. epi* as a stimulus (dilution=0, second bars from left), or with RPMI dilution to the levels indicated on the horizontal axes and with *S. epi* stimulation. After incubation, cytokine concentrations were measured and expressed as concentration per mL of blood (multiplied by dilution factor) for IL-8 (A), IL-6 (B), TNF- α (C), IL-1 β (D), IL-1Ra (E), and IL-10 (F). Cytokine concentrations are indicated on the vertical axes as means \pm SEM in four separate donors. *, $P < 0.05$, and **, $P < 0.01$, compared with cultures stimulated with *S. epi* in the absence of dilution (dilution=0, second bars from left).

respectively (**Fig. 6, A and B, *S. epi***). Compared with diluted and *S. epi*-stimulated blood, AAPV-CMK exposure resulted in a statistically significant increase in IL-8 (234.3 ± 12.4 ng/mL, $P < 0.05$) and IL-6 (45.0 ± 3.5 ng/mL, $P < 0.05$) production.

AAPV-CMK did not affect stimulated TNF- α (Fig. 6C) or IL-1Ra significantly (Fig. 6E). In contrast, IL-1 β was reduced in diluted and *S. epi*-stimulated cultures containing AAPV-CMK compared with diluted and *S. epi*-stimulated cultures

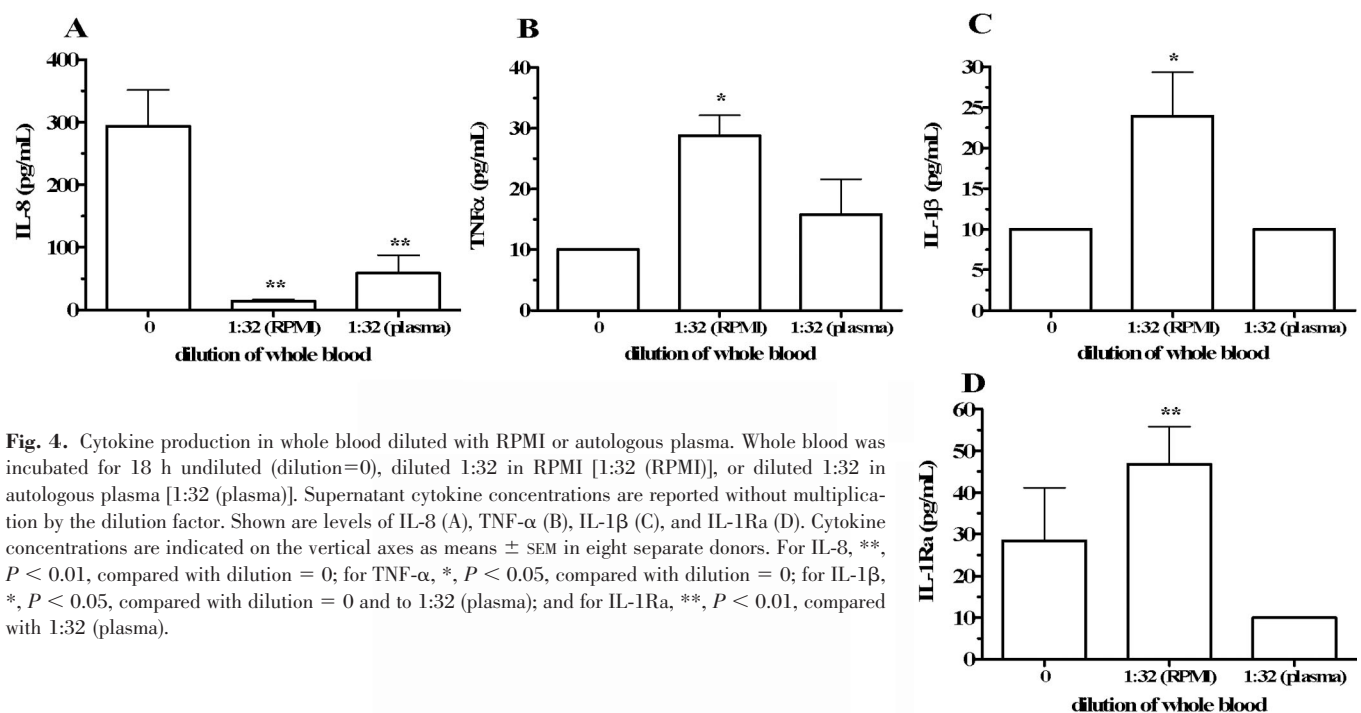


Fig. 4. Cytokine production in whole blood diluted with RPMI or autologous plasma. Whole blood was incubated for 18 h undiluted (dilution=0), diluted 1:32 in RPMI [1:32 (RPMI)], or diluted 1:32 in autologous plasma [1:32 (plasma)]. Supernatant cytokine concentrations are reported without multiplication by the dilution factor. Shown are levels of IL-8 (A), TNF- α (B), IL-1 β (C), and IL-1Ra (D). Cytokine concentrations are indicated on the vertical axes as means \pm SEM in eight separate donors. For IL-8, **, $P < 0.01$, compared with dilution = 0; for TNF- α , *, $P < 0.05$, compared with dilution = 0; for IL-1 β , *, $P < 0.05$, compared with dilution = 0 and to 1:32 (plasma); and for IL-1Ra, **, $P < 0.01$, compared with 1:32 (plasma).

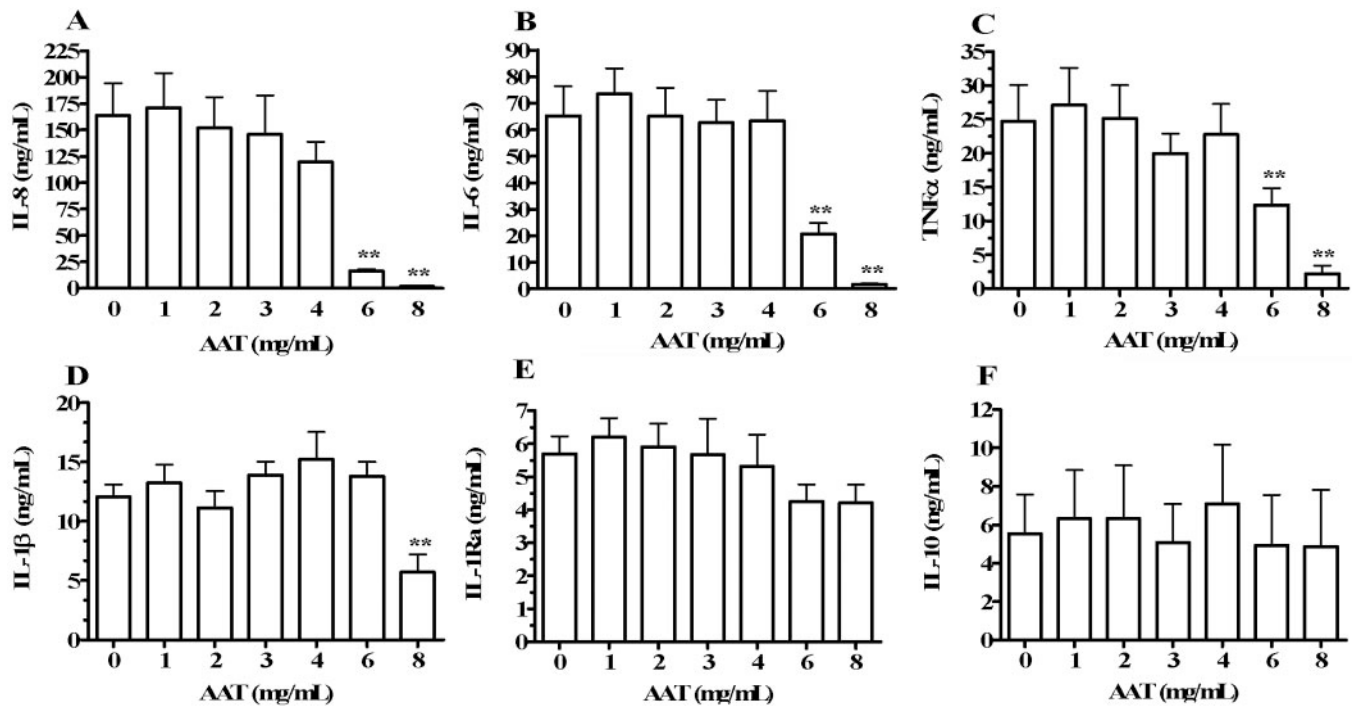


Fig. 5. Effect of exogenous AAT on cytokine production in diluted whole blood with *S. epi* stimulation. Whole blood was diluted 1:32 in RPMI and stimulated with heat-killed *S. epi* in the absence (AAT=0) or presence of AAT added 1 h prior to *S. epi*. Final AAT concentrations are shown on the horizontal axes. Supernatant cytokine concentrations were measured and expressed as concentrations per mL of blood (multiplied by dilution factor). Shown are levels of IL-8 (A), IL-6 (B), TNF- α (C), IL-1 β (D), IL-1Ra (E), and IL-10 (F). Cytokine concentrations are shown on the vertical axes as means \pm SEM in cultures from four separate donors. **, $P < 0.01$, compared with AAT = 0.

(Fig. 6D) with a mean reduction of 62.5% compared with blood-diluted 1:16 and stimulated with *S. epi* ($P < 0.01$).

As AAPV-CMK was solubilized in DMSO, we examined the DMSO effect in whole blood cytokine production. Whole blood was diluted 1:16 with RPMI and *S. epi*, or blood was diluted 1:16 with *S. epi* and the equivalent volume of DMSO used in the AAPV-CMK experiments (added 1 h prior to *S. epi*). After the whole blood cultures were incubated for 18 h, the presence of DMSO did not

significantly affect diluted and *S. epi*-stimulated production of any cytokine tested (IL-8, TNF- α , IL-1 β , and IL-1Ra; data not shown).

DISCUSSION

Cytokine production in disease is studied commonly to elucidate pathogenesis or to quantify disease severity. Although

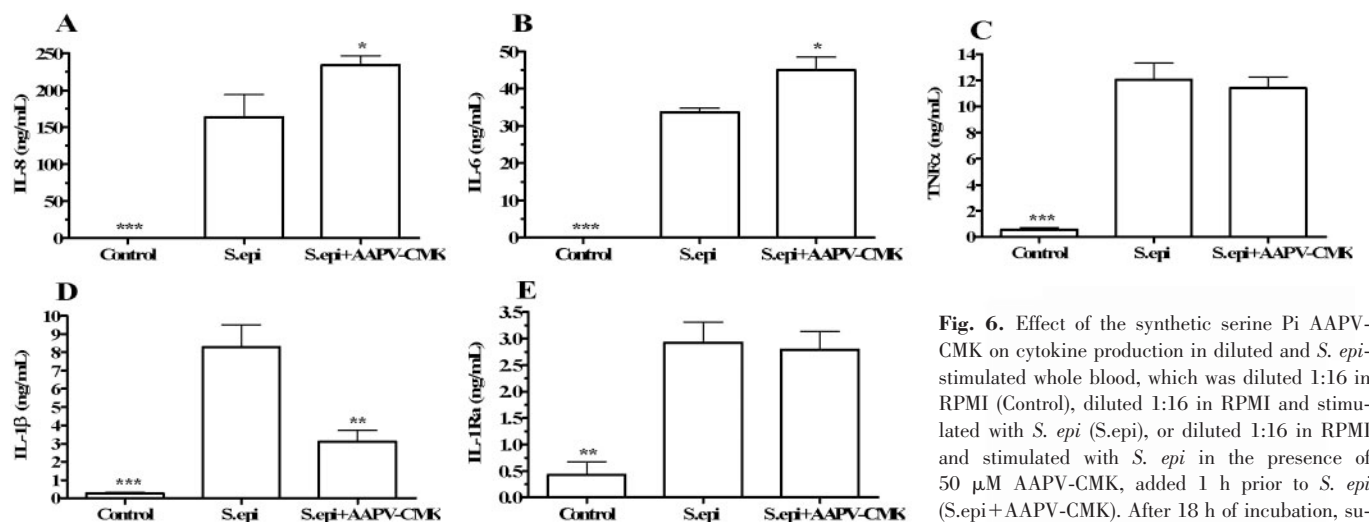


Fig. 6. Effect of the synthetic serine Pi AAPV-CMK on cytokine production in diluted and *S. epi*-stimulated whole blood, which was diluted 1:16 in RPMI (Control), diluted 1:16 in RPMI and stimulated with *S. epi* (S.epi), or diluted 1:16 in RPMI and stimulated with *S. epi* in the presence of 50 μ M AAPV-CMK, added 1 h prior to *S. epi* (S.epi+AAPV-CMK). After 18 h of incubation, supernatant cytokine concentrations were determined and expressed as concentration per mL of blood (multiplied by dilution factor). Shown are levels of IL-8 (A), IL-6 (B), TNF- α (C), IL-1 β (D), and IL-1Ra (E). Cytokine concentrations are shown on the vertical axes as means \pm SEM in four separate donors. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared with *S. epi*.

cytokines are measured routinely in serum, circulating cytokine levels are transient and reflect production, renal clearance, hepatic metabolism, and binding to soluble cytokine receptors or natural anticytokine antibodies [35–38]. Several studies have shown that cytokine RNA expression in freshly isolated whole blood is low or absent [39–41]. In the case of IL-1 β , low gene expression and absence of the IL-1 β precursor protein have been reported [40, 41]. In contrast, IL-18 gene expression and the biologically inactive IL-18 precursor protein are present in the circulation of healthy individuals [41, 42].

Although PBMC or monocyte-derived macrophages are commonly used to study in vitro cytokine production, the isolation procedures are time-consuming and can result in nonspecific stimulation of cytokine production [38]. In addition, PBMC populations do not reflect the ratios of cellular components in circulating blood [3, 43]. For example, in PBMC preparations, there are few or no neutrophils, and monocytes are three to five times more abundant than in the circulation. For these reasons, incubation of whole blood cultures for the analysis of cytokine production and regulation emulates in vivo conditions more closely. However, dilution of blood is necessary to maximize cytokine synthesis in whole blood cultures [2–6], suggesting that in vitro dilution of whole blood reduces the concentration of factors present in the plasma that suppress cytokine production. In the present study, we assessed the role of AAT in cytokine production in whole blood cultures.

AAT deficiency is a genetic condition that increases the risk of early onset and severe emphysema, chronic bronchitis, bronchiectasis, and liver disease [7, 13, 15]. To examine AAT anticytokine activity under in vivo-like conditions, we assessed cytokine production in blood obtained from AAT-deficient persons and from healthy controls. The whole blood was not diluted to maintain endogenous AAT concentrations. *S. epi*-stimulated blood collected from AAT-deficient individuals demonstrated significantly greater IL-8, IL-6, and IL-1Ra production compared with blood from healthy donors (Fig. 1). These findings suggest that reduced AAT blood levels in AAT-deficient subjects are associated with increased cytokine production, implicating AAT as a cytokine-suppressive factor in whole blood cultures.

These studies likely underestimate the cytokine-suppressive effects of AAT. As our AAT-deficient patients received chronic AAT replacement therapy, the difference in AAT levels between AAT-deficient patients and healthy controls was narrowed. Despite AAT replacement therapy, the median AAT level was reduced significantly in the AAT-deficient patients (1.67 mg/mL) compared with healthy controls (2.73 mg/mL). This difference was sufficient to result in significantly higher IL-8, IL-6, and IL-1Ra production in the AAT-deficient group (Fig. 1). It is possible that larger reductions in AAT (for example, in AAT-deficient persons not receiving AAT supplementation) would result in significantly enhanced levels of other cytokines compared with healthy persons with normal amounts of AAT.

When blood was diluted with RPMI tissue-culture medium, spontaneous, proinflammatory cytokine production per mL of blood was increased significantly (Fig. 2). Mean IL-8, TNF- α , IL-1 β , and IL-1Ra levels were increased 3.1-, 40-, 299-, and

18-fold compared with levels observed in undiluted blood. Diluted whole blood cytokine levels that were not adjusted for dilution demonstrated a dramatic reduction in IL-8, which was not present for any other cytokine tested (Fig. 2A). In fact, blood dilution resulted in increased, unadjusted TNF- α and IL-1 β concentrations, and IL-1Ra concentrations were only slightly reduced. Although the reason for this anomalous IL-8 observation is uncertain, it may be relevant that of the cytokines measured, only IL-8 is produced by polymorphonuclear neutrophils (PMN). It is possible that blood dilution reduced PMN concentrations in the cultures to the point that unadjusted IL-8 levels declined precipitously. Alternatively, as cell–cell contact between monocytes and T cells has been shown to enhance IL-8 production [44], larger dilutions of blood may limit the intercellular contact necessary for efficient IL-8 production. It is noteworthy that dilution-induced diminution in monocyte concentrations did not result in similar reductions in the monocyte-derived cytokines TNF- α , IL-1 β , and IL-1Ra (Fig. 2, C, E, and G). However, despite dilution-induced reductions in unadjusted IL-8 levels, adjusted IL-8 concentrations (multiplication of IL-8 concentrations by the dilution factor) increased with dilution (Fig. 2B).

We also examined the effect of dilution on cytokine production in blood stimulated with *S. epi* (Fig. 3). Dilution-induced increases in cytokine production were observed for each *S. epi*-stimulated cytokine tested (IL-8, IL-6, TNF- α , IL-1 β , IL-1Ra, and IL-10). These results show that dilution increased cytokine levels in stimulated whole blood beyond levels observed with *S. epi* stimulation alone. In contrast to all other measured cytokines, IL-8 production was not maximal at the highest dilution but peaked at 1:16 dilution and decreased at the 1:32 dilution (Fig. 3A). It is not clear why IL-8 production did not continue to increase with higher dilution. It is possible that in the presence of *S. epi* stimulation and high (1:32) dilution, the IL-8 contribution by PMN decreased, as described for Figure 2A.

Three possibilities may explain increases in dilution-induced cytokine production in the presence of *S. epi* stimulation as observed in Figure 3: i) Dilution decreased cell–cell interaction, which may enhance cytokine production; ii) the concentration of cells was decreased in diluted samples, resulting in an increase in the total amount of stimulus (*S. epi*) molecules per cell; and iii) cytokine production was enhanced by reducing concentrations of natural inhibitors in the blood. As a dilution-induced reduction in cell–cell interaction would be expected to decrease the levels of cytokines produced [45], this likely cannot explain the increase in cytokine production associated with dilution (Figs. 2 and 3). Furthermore, as the fluid-phase concentration of *S. epi* was identical in each diluted blood culture, the increased, total amount of *S. epi* per cell in the cultures would not alter the magnitude of stimulation per cell. Therefore, the hypothesis that soluble plasma inhibitors are depleted by dilution is the most likely explanation.

To confirm the presence of cytokine inhibitors in the plasma component of circulating blood, we diluted whole blood 1:32 in RPMI or autologous plasma (Fig. 4). Dilution in plasma did not increase levels of TNF- α , IL-1 β , and IL-1Ra compared with levels observed in undiluted blood. In no case did plasma dilution increase cytokine synthesis significantly, as observed

for blood diluted in RPMI. These results support the contention that dilution-induced cytokine synthesis is a result of reduced concentrations of suppressive factors in plasma (as shown in Fig. 2). Unlike the other cytokines we tested, IL-8 decreased in response to dilution (Fig. 4A). As Figure 4 data are presented as levels unadjusted for dilution, the results are similar to the data shown in Figure 2A. As described in the text above that refers to Figure 2, only IL-8 levels declined substantially with dilution. We surmise this is a result of diminishing PMN contribution to IL-8 production, which by each PMN, may not respond to dilution with increased IL-8 synthesis to the extent that the monocyte-derived cytokines increase with dilution.

Major protein components of plasma include albumin, Igs, α 2-macroglobulin, and AAT. Several studies have suggested that AAT possesses anti-inflammatory function [10, 23, 27, 34, 46–48], raising the possibility that AAT contributes to proinflammatory cytokine suppression in whole blood. As shown in Figure 1, we demonstrated enhanced cytokine production in stimulated cultures of whole blood obtained from AAT-deficient patients compared with whole blood from healthy subjects. These data identified AAT as a likely cytokine-suppressive factor in blood. To determine if AAT inhibited cytokine production directly in whole blood cultures, we added exogenous AAT to whole blood that was diluted with RPMI and stimulated with *S. epi* (Fig. 5). AAT (6–8 mg/mL) suppressed IL-8, IL-6, TNF- α , and IL-1 β production significantly by 99%, 97%, 91%, and 47%, respectively (Fig. 5, A–D). Relatively high concentrations (6–8 mg/mL) of exogenous AAT were required for substantial cytokine suppression in these experiments. Although AAT levels of this magnitude can occur during the acute-phase response, it is noteworthy that other molecules in the circulation besides AAT suppress cytokines. For example, α 2-macroglobulin contributes to inflammatory response modulation by binding and sequestering cytokines [49]. Therefore, significant cytokine suppression in diluted whole blood likely requires high exogenous AAT concentrations to compensate for the reduced levels of other (non-AAT) inhibitors. Also, high AAT levels may have been necessary to overcome the large cytokine induction effect provided by the combination of dilution and *S. epi* stimulation.

Unexpectedly, exogenous AAT at these same levels did not suppress IL-1Ra and IL-10 significantly (Fig. 5, E and F), two cytokines with anti-inflammatory activities. This suggests that AAT preferentially inhibits proinflammatory cytokines, many of which are regulated through the NF- κ B pathway [50, 51], and AAT has been shown to inhibit NF- κ B activation [18, 46]. In contrast, IL-10 is stimulated through cAMP, and AAT has been shown to increase cAMP synthesis and IL-10 production in human monocytes in vitro [34, 52]. These opposing, AAT-induced, intracellular signaling effects may explain why AAT suppressed pro- but not anti-inflammatory cytokines in our in vitro studies.

We used a synthetic inhibitor of serine proteases, AAPV-CMK, to determine if serine protease blockade is the mechanism by which AAT suppresses cytokine production in whole blood (Fig. 6). AAPV-CMK (50 μ M) did not inhibit dilution and *S. epi*-stimulated TNF- α or IL-1Ra production, suggesting that serine

protease blockade does not necessarily inhibit these cytokines in whole blood. Interestingly, AAPV-CMK increased IL-8 and IL-6 production to a significant extent, which contradicts the hypothesis that serine protease inhibition is the mechanism by which AAT suppressed cytokine production in these investigations. Unlike the other cytokines assessed, IL-1 β production was inhibited significantly by AAPV-CMK added to stimulated whole blood (Fig. 6D). Of the cytokines we tested, only IL-1 β is secreted following processing by the caspase-1 inflammasome [53]. It is possible that inhibition of caspase-1 activity by AAPV-CMK blocked IL-1 β processing and prevented mature IL-1 β release into the culture supernatant. Collectively, these AAPV-CMK results further suggest that serine protease inhibition cannot completely account for AAT suppression of proinflammatory cytokines, an observation noted by others [15, 28, 29].

These studies suggest that AAT is an endogenous inhibitor of proinflammatory cytokine production in whole blood, and AAT may participate in containing an aggressive innate immune response to an inflammation-inducing stimulus. Furthermore, AAT activities separate from serine protease inhibition likely participate in proinflammatory cytokine suppression. Administration of exogenous AAT to patients with disease characterized by excessive cytokine synthesis and inflammation may provide therapeutic benefit.

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