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α-Linoleic Acid Enhances the Capacity of α1-Antitrypsin to Inhibit Lipopolysaccharide-Induced IL-1β in Human Blood Neutrophils

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Alpha1-antitrypsin (A1AT, SERPINA1), a major circulating inhibitor of neutrophil elastase (NE) and proteinase-3 (PR3), has been proposed to reduce the processing and release of IL-1β. Since the antiinflammatory properties of A1AT are influenced by the presence of polyunsaturated fatty acids, we compared the effects of fatty acid–free (A1AT-0) and α-linoleic acid (LA)–bound (A1AT-LA) forms of A1AT) on lipopolysaccharide (LPS)-induced synthesis of the IL-1β precursor and the release of IL-1β from human blood neutrophils. The presence of A1AT-LA or A1AT-0 significantly reduced LPS-induced release of mature IL-1β. However, only A1AT-LA reduced both steady-state mRNA levels of IL-1β and the secretion of mature IL-1β. In LPS-stimulated neutrophils, mRNA levels of TLR2/4, NFKBIA, P2RX7, NLRP3, and CASP1 decreased significantly in the presence of A1AT-LA but not A1AT-0. A1AT-0 and A1AT-LA did not inhibit the direct enzymatic activity of caspase-1, but we observed complexes of either form of A1AT with NE and PR3. Consistent with the effect on TLR and IL-1β gene expression, only A1AT-LA inhibited LPS-induced gene expression of NE and PR3. Increased gene expression of peroxisome proliferator-activated receptor (PPAR)-γ was observed in A1AT-LA–treated neutrophils without LPS stimulation, and the selective PPAR-γ antagonist (GW9662) prevented a reduction in IL-1β by A1AT-LA. We conclude from our data that the ability of A1AT to reduce TLR and IL-1β gene expression depends on its association with LA. Moreover, the antiinflammatory properties of A1AT-LA are likely to be mediated by activation of PPARγ.

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INTRODUCTION

Acute-phase proteins constitute an essential component of the innate immune response during infection or inflammation. Human alpha1-antitrypsin (A1AT) is one of the major acute-phase proteins found in the circulation. So far, the best-known and best-understood function of A1AT is rapid inhibition of neutrophil-released elastase (NE) and proteinase 3 (PR3). (1) Some studies, however, suggest that A1AT facilitates the concomitant inhibition of NE and PR3 inside and outside the cell. (2) PR3 and NE are closely related enzymes that enhance neutrophil-dependent inflammation not only by degrading components of the extracellular matrix but also by other deleterious effects, such as activation of IL-1β. (3) Hence, tight control of NE and PR3 activity by A1AT confirms its fundamental role in controlling and resolution of inflammation. More recently, A1AT has been shown to have independent antiinflammatory/immunomodulatory properties. A1AT protects against cell apoptosis, (4) inhibits neutrophil adhesion, chemotaxis, and superoxide production, (5–8) induces IL-1 receptor antagonist expression, (9) and regulates heme oxygenase-1 activity. (10) Moreover, A1AT purified from human blood modulates and/or prevents tissue injury in experimental animal models of human diseases, including graft-vs-host disease, inflammatory bowel disease, rheumatoid arthritis, acute liver failure, autoimmune

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diabetes, gouty arthritis, and ischemia-reperfusion injuries. (11–17) Despite growing reports on the antiinflammatory properties demonstrated for A1AT, the mechanism of a direct effect of A1AT on cells remains to be defined. Both *in vitro* and *in vivo* studies provide evidence that the beneficial effects of A1AT are mostly related to suppression of Toll-like receptor (TLR) agonist-induced innate immune cell activation. (18) Recent studies support the concept that some of the pleiotropic activities of A1AT are linked to the specific molecular form of the protein. For example, biological activities of A1AT can be modified due to interactions with lipid moieties, in which A1AT was found in association with cell membrane lipid rafts (19,20) and was detected in complexes with low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). (21,22) The binding of A1AT to HDL augments its protective effect in a mouse model of elastase-induced pulmonary emphysema. (22) We recently reported that A1AT purified from human plasma binds polyunsaturated fatty acids (FAs) like α-linoleic and oleic acid, and that only FA-bound forms of A1AT increase the expression and release of ANGPTL4 in human blood–adherent monocytic cells and in primary human lung microvascular endothelial cells. (23,24)

The above functional differences between FA-free and FA-bound forms of A1AT led us to hypothesize that these 2 forms may express distinct immunomodulatory activities during neutrophil activation. Neutrophils account for about 50–75 % of circulating leukocytes, and during inflammation they are the first immune defense cells to arrive and function via multiple intra- and extracellular mechanisms. (25) Antiinflammatory actions of A1AT as well as FAs in neutrophils have previously been observed. (5,6,26–28) For example, short-chain FAs reduced TNF-α production by lipopolysaccharide (LPS)-stimulated human neutrophils, (29) and also inhibited the expression of TNF-α and NO in rat neutrophils via attenuation of NF-κB activation. (30) Polyunsaturated FAs have been reported to decrease expression of adhesion molecules on the surface of neutrophils (31) and to inhibit neutrophil chemotaxis. (32) Thus, the goal of the present study was to compare effects of FA-free (A1AT-0) and α-linoleic acid (LA)–bound (A1AT-LA) forms of A1AT on LPS-induced levels of the IL-1β precursor and the release of processed IL-1β in freshly isolated human blood neutrophils *in vitro*.

MATERIALS AND METHODS

A1AT Preparations

Lipidomic analysis of A1AT Prolastin (Grifols) revealed that it consists of protein-lipid complexes, whereas A1AT (Zemaira, CSL Behring) is lipid-free, termed as A1AT-0. For experiments, A1AT-0 was mixed with α-linoleic acid (LA) (Sigma Aldrich) at a molar ratio of 1:4.8 and incubated for 3 h at 37°C in a water bath as previously described. (23) After the incubation, unbound LA was removed and A1AT-LA complexes were recovered in sterile phosphate buffered saline (PBS). A1AT-LA preparations were used immediately or were kept no longer than 48 h at 4°C.

A1AT-0 and A1AT-LA complex formation with NE and proteinase-3 (PR3) *in vitro.*

Preparations of A1AT-0 and A1AT-LA were assessed for their ability to form complexes with active pancreatic elastase (Sigma-Aldrich) and PR3 (Enzo Life Sciences). A1ATs plus NE or PR3 (both at a molar ratio of 1.2:1) were incubated at room temperature for increasing periods of time up to 5 h. The reaction was stopped by adding 2 × SDS loading buffer and boiling for 5 min. Samples were analyzed by electrophoresis on 12.5% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE).

Caspase-1 Inhibition Assay

A1AT-0 and A1AT-LA were tested for inhibition of caspase-1 activity *in vitro* by using Caspase-1 Inhibitor Drug Detection kit as recommended by the manufacturer (Abcam). In brief, increasing concentrations of A1AT-0 and A1AT-LA were pre-incubated with active caspase-1 and mixed with reaction buffer containing fluorogenic substrate, YVAD-AFC. Fluorescence readings were then taken at 37°C using 400 nm excitation and 505 nm emission wavelengths on Tecan Infinite M200 plate reader (Männedorf,) every 10 min for 90 min. The synthetic caspase-1 inhibitor z-VAD-FMK was used as a positive control for the assay.

Human Blood Neutrophil Isolation

Neutrophils were isolated from freshly obtained peripheral blood of 50 healthy donors (under 30 years of age) using Polymorphoprep (Axis-Shield PoC AS) as described previously. (6) This protocol routinely yields more than 97% cell purity as assessed by flow cytometry and Wrights-Giemsa stain (Sigma-Aldrich).

Human Neutrophil Culture and Treatments

Neutrophils (5×10^6 per well) were suspended in RPMI-1640 medium (Gibco, Life Technologies) and allowed to stand for 30–40 min in cell culture plates precoated with fetal calf serum. In some experiments, cells were pre-incubated with 10 μM GW9662 (Sigma-Aldrich), a selective irreversible PPAR-γ antagonist, for 30 min at 37°C. Neutrophils were then stimulated with 20 ng/mL LPS (*Escherichia coli* LPS 055:B5, Sigma-Aldrich) alone or in a combination with 1 mg/mL A1AT-0 or A1AT-LA at 37°C and placed in 5% CO_2. In parallel, neutrophils were incubated with PBS or LA, A1AT-0 or A1AT-LA alone. In some experiments, 1 mg/mL A1AT (Prolastin) was used alone or together with LPS. Following incubations with LPS and/or A1AT for 5 h, the supernatants and cells were analyzed for protein release and gene expression, respectively.

Microscopic Analysis of Interaction of A1AT-0 and A1AT-LA with Neutrophils

Human neutrophils (3×10^6) were plated onto glass coverslips alone or in the presence of A1AT-0 (0.5 mg/mL) or A1AT-LA (0.5 mg/mL) for 5 min and 1 h

at 37°C CO_2, or for 5 min at 4°C. Cells were then washed with PBS and fixed with 100% methanol for 10 min at –20°C. Fixed cells were washed 3 times with PBS and incubated for 1 h at room temperature without or with anti-human A1AT mouse monoclonal antibody (Santa Cruz), diluted 1:50 in PBG (PBS containing 0.5% BSA and 0.2% fish gelatin). Coverslips were washed 3 times with PBS, followed by incubation with AlexaFluor-488–labeled goat anti-mouse IgG (1:400 dilution, Thermo Fisher Scientific) for 1 h at room temperature. The cells were then washed in PBS and mounted on microscope slides using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were made using an Olympus FluorVIew 1000 scanning confocal microscope equipped with a 60 × oil immersion objective.

RNA Isolation, cDNA Synthesis and Real-Time PCR

Total RNA was prepared using RNeasy Micro kit (Qiagen Sample and Assay Technologies). For cDNA synthesis, 1 µg of total RNA was transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific). mRNA levels of selected genes (Table 1) were determined using TaqMan Gene Expression Assays (Applied Biosystems, Life Technologies) on Step One Plus real time PCR machine (Applied Biosystems). Threshold cycle (Ct) value for each sample was calculated by determining the point at which the ßuorescence exceeded a threshold limit. *GAPDH*, *HPRT*, and glucuronidase beta (*GUSB*) were used as reference genes. Relative gene expression was calculated according to the ΔΔCT method. Basal expression of *TLR2*, *TLR4*, *P2RX7*, and *SERPINA1* genes was calculated according to ΔCT method: 2^(Ct value of target gene – Ct value of reference gene).

Table 1. List of the primers. All primers were purchased from Life Technologies.

TaqMan primers	Catalogue No.
Human *IL1B*	Hs00174097_m1
Human *TLR2*	Hs01872448_s1
Human *TLR4*	Hs00152939_m1
Human *RELA*	Hs00153294_m1
Human *NFKBIA*	Hs00355671_g1
Human *NFKBIB*	Hs00182115_m1
Human *NLRP3*	Hs00918082_m1
Human *P2RX7*	Hs00175721_m1
Human *CASP1*	Hs00354836_m1
Human *ASC*	Hs01547324_gH
Human *ELANE*	Hs00975994_g1
Human *SERPINA1*	Hs01097800_m1
Human *PRTN3*	Hs01597752_m1
Human *PPARG*	Hs01115513_m1
Human *GUSB*	Hs00939627_m1
Human *GAPDH*	Hs02758991_g1
Human *HPRT*	Hs02800695_m1

Cytoplasm and Nuclei Isolation

Cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) according to the manufacturer's recommendations. Briefly, neutrophils (1.5×10^7 per well) were incubated with LPS, A1AT-0, or A1AT-LA alone and in combinations for 5 h. Afterward, cells were pelleted and cell membranes were disrupted by addition of ice-cold CER I and CER II. Cytoplasmic content was collected post centrifugation at 17 000 g for 10 min at 4°C. After recovering the intact nuclei from the cytoplasmic extract by centrifugation, the nuclei were lysed with a third reagent, NER, to yield the nuclear extract. Nuclear lysates were centrifuged at 17 000 g for 10 min and supernatants were collected. Cytoplasmic and nuclear fractions were analyzed by electrophoresis and western blot.

Electrophoresis and Western Blot Analysis

Cell pellets were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (SC-24948, Santa Cruz) for 30 min on ice. Following centrifugation at 15 000 g for 10 min, the protein concentration in the supernatants was determined by Bradford assay (Thermo Fisher Scientific). In some experiments (immunoblots for NE and PR3), neutrophils were sonicated in PBS. The sonicated preparations were centrifuged at 10 000*g* for 5 min. Following centrifugation, replicate aliquots from the supernatants were immediately analyzed. Equal amounts of protein were loaded on 7.5%, 10%, or 12.5% SDS-PAGE gels. Elecrophoretically separated proteins were stained with 0.1% Coomassie Blue R250. From the parallel gels, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) using semidry blot transfer. Blots were blocked with 5% bovine serum albumin (BSA, Calbiochem) or milk (5%, Roth) for 1 h at room temperature and then probed with specific primary antibodies (Table 2). The immune complexes were visualized with horseradish peroxidase–conjugated antibodies (DAKO A/S) and ECL western blotting substrate (Thermo Fisher Scientific). The density of the specific bands was quantified using ImageJ software (http://imagej.nih.gov/ij).

Table 2. List of the antibodies used in western blotting.

Antibody	Host species	Dilution	Company	Catalogue No.
A1AT	Rabbit	1:1000	Dado	A0012
A1AT	Mouse	1:800	Santa Cruz	SC-59438
P65	Mouse	1:200	Santa Cruz	SC-8008
IκB-α	Rabbit	1:1000	Cell Signaling	9242S
IκB-β	Goat	1:100	Santa Cruz	SC-7329
NLRP3	Rabbit	1:1000	Cell Signaling	13158
Caspase-1	Rabbit	1:200	Santa Cruz	SC-515
NE	Mouse	1:1000	Dako	MO752
PR3	Rabbit	1:200	Santa Cruz	SC-28818
Histone H2A	Rabbit	1:1000	Cell Signaling	2578
β-actin	Mouse	1:1000	Sigma-Aldrich	A5441
IL-1β	Rabbit	1:200	Santa Cruz	SC-7884

Analysis of Surface Expression of TLR2 and TLR4 by Flow Cytometry

Neutrophils (1×10^6 per condition) were incubated for 5 h with LPS and LPS plus either A1AT-0 or A1AT-LA. Neutrophils were then labeled with fluorescein conjugated monoclonal anti-human TLR2 or TLR4 (eBioscience) and the corresponding isotype controls (BD Pharmingen) for 35 min at 4°C. After labeling, cells were washed, resuspended in FACS buffer (PBS containing 0.5% BSA) and examined using FACS Calibur (Becton Dickinson) or FACS Canto (BD Biosciences). Data were analyzed using FACS Diva software (BD Biosciences).

NF-κB Pathway Array for Neutrophils

Neutrophils were incubated for 4 h with PBS, Prolastin, and 1 mg/mL LPS (10 ng/mL) separately or in combination. Total RNA was prepared using the RNeasy Mini kit (Qiagen) and quantified with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA purity was analyzed on 1% agarose gels. cDNA was synthesized from 1 μg of RNA with RT^2 First Strand kit (Biosciences, Qiagen) and analyzed for expression of NFκB-pathway related genes using Human Angiogenesis RT^2 Profiler PCR Array (Biosciences, Qiagen) according to the manufacturer's protocol. Quantitative real-time PCR was conducted on an ABI 7500 machine (Applied Biosystems). β2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, *GAPDH*, and *β-actin* were measured at the same time and used as internal controls. Relative gene expression (fold change) was determined using the ΔΔCT method on the SAB web portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Lactate Dehydrogenase Assay

Treatment-associated cytotoxicity was determined using the Cytotoxicity Detection Kit (LDH, Roche) according to the manufacturer's protocol. In brief, the assay quantifies lactate dehydrogenase (LDH) released from ruptured or dead cells into the culture supernatant by a colorimetric reaction. Neutrophils were treated according to the experimental setting and cell supernatants were collected at the end of the incubation time. Total cell lysate was used for 100% control value. For low control and background control, supernatant from untreated cells and assay medium alone were used, respectively. Absorbance of the colorimetric product of LDH reaction was measured at 490 nm using an Infinite M200 microplate reader (Tecan). Measurements were carried out in triplicate.

Myeloperoxidase Assay

For quantitative determination of supernatant myeloperoxidase (MPO), we used a commercially available ELISA kit (Human DuoSet R&D Systems). Samples were diluted 1:500 with dilution buffer and measured in duplicate using the Infinite M200 microplate reader. Detection limit was 62.5 pg/mL.

IL-1β ELISA Assay

Cell culture supernatants and lysates from different experimental conditions were examined for IL-1β concentration using the DuoSet ELISA kit. Detection limit was 4 pg/mL.

Statistical Analysis

Statistical Package (SPSS for Windows, release 23.0) was used for statistical calculations. The differences in the means of experimental results were analyzed for their statistical significance using one-way ANOVA combined with a multiple-comparison procedure (Scheffe multiple range test), with an overall significance level of $p = 0.05$. An independent 2-sample t-test was also used.

All supplementary materials are available online at www.molmed.org.

RESULTS

IL-1β Release and IL-1β Precursor Expression Levels in LPS- and LPS/A1AT-0- or A1AT-LA-Treated Neutrophils

LPS induces the processing and release of high levels of mature IL-1β by neutrophils. (33,34) As expected, neutrophils incubated for 5 h with LPS significantly increased IL-1β release (Figure 1A) and IL-1β precursor expression (Figure 1B) relative to controls. We next investigated the capacity of non-lipidated A1AT-0 and lipidated A1AT-LA to alter the production of IL-1β by neutrophils in response to LPS stimulation. As shown in Figure 1A, LPS-stimulated IL-1β release was reduced by about 50% in both the LPS/A1AT-0 ($p = 0.006$) and LPS/A1AT-LA ($p < 0.001$) treated cells compared with LPS. The levels of IL-1β in the supernatants of control neutrophils and treated with A1AT-0 or A1AT-LA were below detection. However, mRNA expression of *IL-1β* precursor decreased significantly only in neutrophils stimulated with LPS in the presence of A1AT-LA compared with neutrophils stimulated with LPS/A1AT-0 (Figure 1B). In parallel, higher levels of mature IL-1β protein were detected in LPS- and LPS/A1AT-0 stimulated cells as compared with controls or LPS/A1AT-LA (Figure 1C). Total concentration of IL-1β in cell lysates was higher in LPS- and LPS/A1AT-0–treated than in LPS/A1AT-LA–treated cells (Figure 1D). Similarly, when human neutrophils were stimulated with LPS in the presence of commercially available lipidated A1AT (Prolastin) for 4 h, significant inhibition of LPS-induced IL-1β release and *IL-1β* mRNA levels was observed (Supplementary Figures S1A and B). LDH levels revealed no toxicity after neutrophil stimulation with LPS and A1AT preparations alone or in combinations (Supplementary Figure S2). Expression of IL-18, another proinflammatory cytokine of the IL-1 family, was undetectable (data not shown).

Expression Levels of TLRs in LPS- and LPS/A1AT-Treated Neutrophils

Human peripheral blood neutrophils express TLR2 and TLR4, albeit at lower levels than monocytes. (35) As expected, *TLR2* and *TLR4* mRNA levels were low in resting neutrophils [*TLR2* and *TLR4*, mean (SD): 0.023 (0.005), n = 9; and 0.103 (0.05), n = 15, respectively]. Commercial LPS contains TLR2-stimulating substances, lipopeptides, (36) and indeed, Figures 2A and B demonstrate an

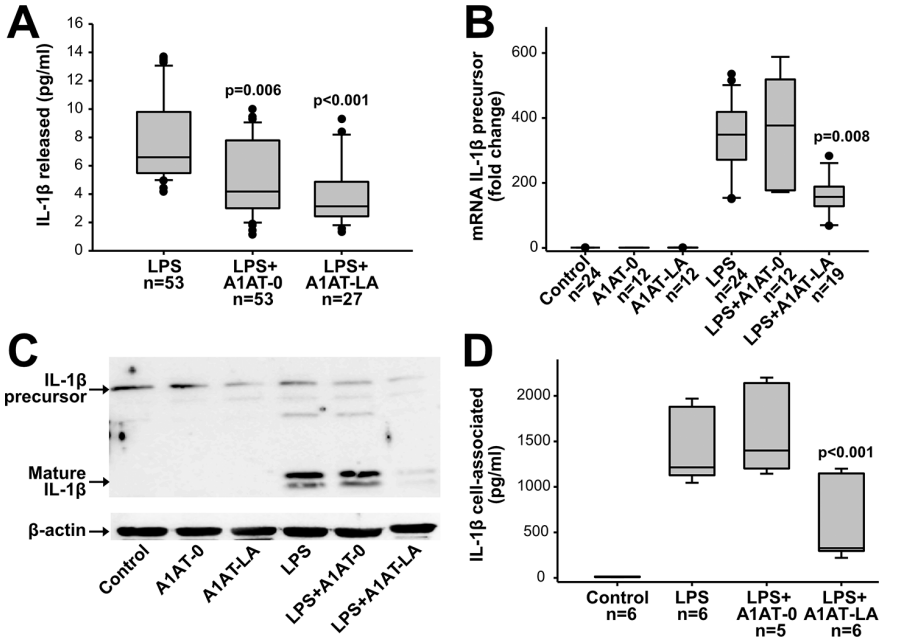

Figure 1. Effects of A1AT-0 and A1AT-LA on LPS-induced IL-1β precursor and IL-1β released. Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). Released IL-1β levels (A), *IL-1β* gene expression (B), cell-associated profile, and levels of IL-1β protein were determined by western blotting (C) and ELISA (D). *GUSB* and *GAPDH* were used as housekeeping genes in qPCR experiments. Box plots represent data from 5 individual blood donors; n = number of replicates for each experiment. Blot is representative of 3 independent donors. *P* value indicates significant differences compared with the values seen in LPS-activated cells.

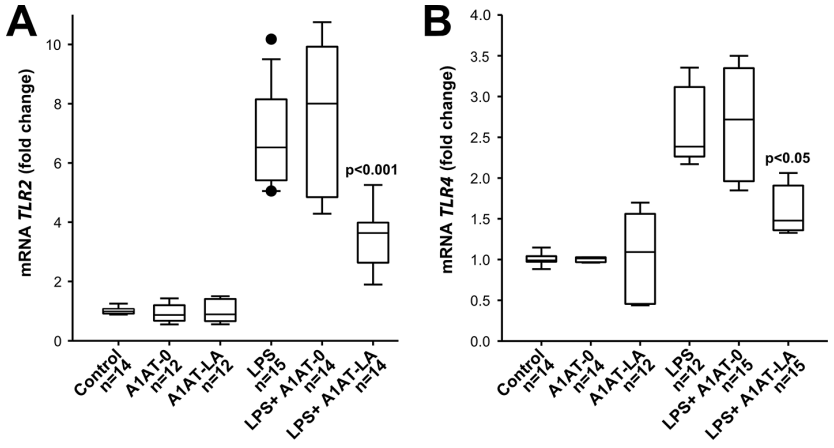

Figure 2. Effects of A1AT-0 and A1AT-L on LPS-induced *TLR2* and *TLR4* mRNA expression. Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). Expression of *TLR2* (A) and *TLR4* (B) was determined by real-time PCR as described in Materials and Methods. *GAPDH* was used as a housekeeping gene in this experiment. Box plots represent data from 4 individual donors; n = number of replicates for each experiment. *P* value indicates significant differences compared with the values seen in LPS-activated cells.

increase in *TLR2* and *TLR4* mRNA expression after LPS stimulation of neutrophils for 5 h. Stimulation of neutrophils with LPS in the presence of A1AT-0 did not change TLR mRNA expression when compared with the LPS-treated cells. In contrast, *TLR2* and *TLR4* expression was reduced by two-fold ($p < 0.001$) and 1.6-fold ($p < 0.05$), respectively, in LPS-stimulated neutrophils in the presence of A1AT-LA (Figures 2A and B). By flow cytometry, the levels of TLR2 and TLR4 expression on the cell surface were low and did not differ significantly between experimental conditions (Supplementary Figure S3).

Effect of A1AT on LPS-Induced NF-κB Pathway Gene and Protein Expression

The main NF-κB subunits RelA/p65 and c-Rel/p50 and the inhibitor (I) κB, an NF-κB-inactivating factor, are constitutively expressed in neutrophils. (37) Scheibel *et al.* (38) demonstrated that the transcription of IL-1β depends on a positively acting p65-c-Rel-IκB complex. As expected, *RelA*/p65, *NFKBIA*, and *NFKBIB* mRNA levels were significantly enhanced in the LPS-stimulated neutrophils in comparison to controls (Figures 3A, B, and C). LPS-stimulated expression of these genes was not significantly different from neutrophil-stimulated LPS in the presence of A1AT-0. In contrast, in neutrophils treated with A1AT-LA, LPS-induced expression of *RelA*/p65 and *NFKBIA* but not *NFKBIB* was reduced (*RelA*/p65 was reduced by 1.48-fold, $p < 0.05$, and *NFKBIA* by three-fold, $p < 0.001$) (Figures 3A, B, and C). Lipid-containing A1AT (Prolastin) added to neutrophil cultures for 4 h also lowered LPS-induced expression of *RelA*/p65 and other genes of the NF-κB pathway (Table 3). However, after 5 h profiles of cytosolic p65, IκBβ, and IκBα proteins did not differ markedly between LPS and LPS/A1ATs. In the nuclear fraction, only IκBα protein was detected but did not differ between treatments (Figures 3D, E, and F).

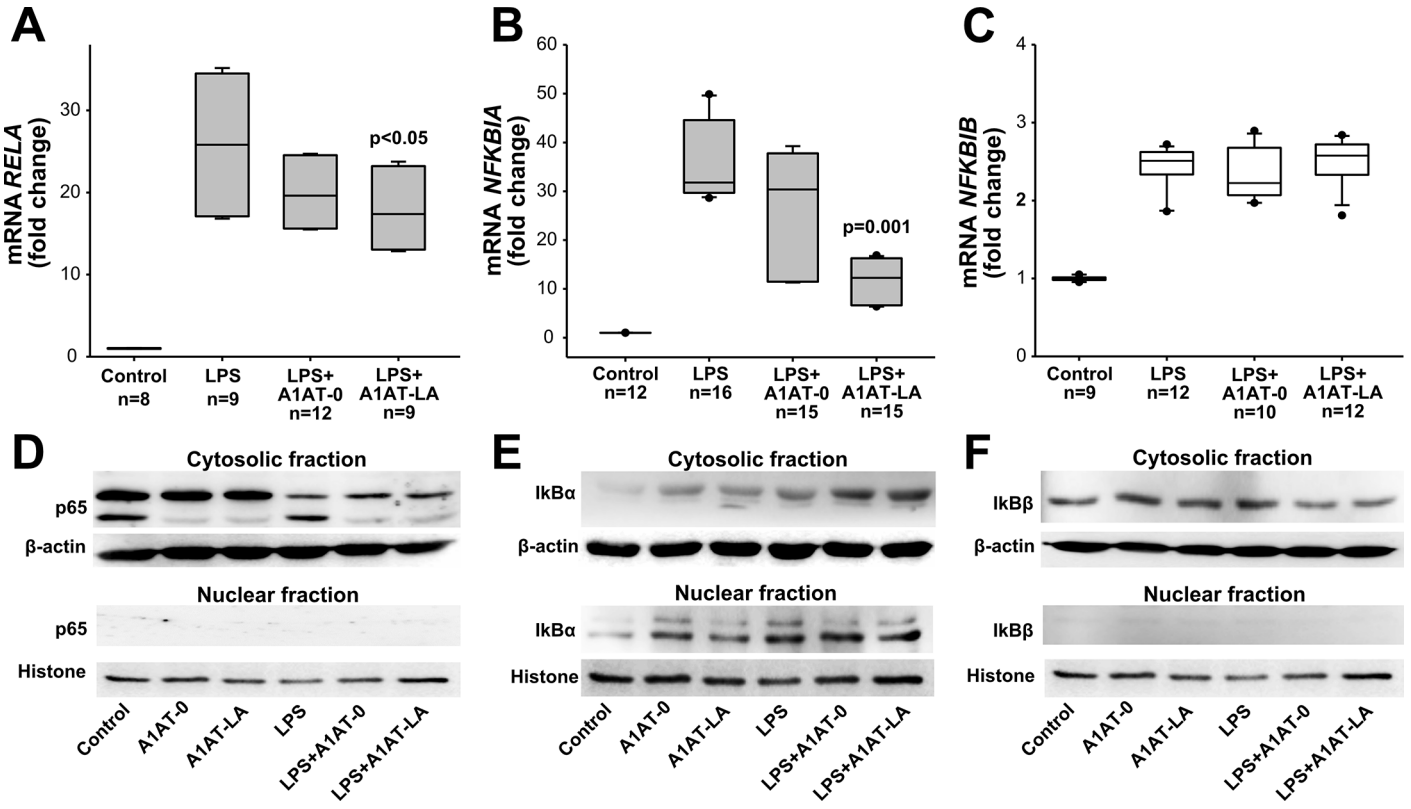

Figure 3. Effects of LPS and LPS/A1AT-0 and LPS/A1AT-LA on *RelA*/p65, *NFKBIA*/IκBα and *NFKBIB*/IκBβ expression and protein levels. Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). mRNA expression of *RelA*/p65 (A), *NFKBIA*/IκBα (B) and *NFKBIA*/IκBβ (C) genes was analyzed as described in Materials and Methods. *GAPDH* and *GUSB* were used as housekeeping genes in qPCR experiments. Box plots represent data from 3 to 5 individual donors; n = number of replicates for each experiment. *P* value indicates significant differences compared with the values seen in LPS-activated cells. Subcellular fractions were analyzed by western blotting with anti-p65 (D), anti-NFKBIA/IκBα (E) and anti-NFKBIA/IκBβ (F) antibodies. The blots were reprobed with anti-β-actin and anti-histone H1 antibodies as loading controls for cytosolic and nuclear fractions, respectively. Each blot is representative of 3 independent donors.

A1AT-LA Inhibits the Ability of LPS to Upregulate *NLRP3* and *CASP-1* mRNA Levels

Neutrophil IL-1β processing and secretion in response to LPS requires activation of the inflammasome containing the components NLRP3, pro-caspase-1, and apoptosis-associated speck-like protein containing a *CARD* (ASC), encoded by the *PYCARD* gene. Human peripheral blood neutrophils constitutively contain NLRP3 and ASC protein, and *NLRP3* gene expression also increases following TLR2 stimulation. (39) After 5 h, neutrophils treated with LPS significantly increased *NLRP3* mRNA expression (Figure 4A). A1AT-LA but not A1AT-0 lowered LPS-induced *NLRP3* mRNA. Under the same experimental conditions, we found a marginal increase in the level of NLRP3 protein in LPS-stimulated neutrophils, but this was not observed in LPS/A1AT-LA or LPS/A1AT-0 treatments (Figure 4B). The expression levels of *ASC* in LPS-treated neutrophils were low, and A1AT-LA and A1AT-0 had no specific effect [0.21-fold (0.1) (LPS), 0.17-fold (0.04) (LPS + A1AT-0), and 0.17-fold (0.04) (LPS + A1AT-LA) against the controls, n.s.] (data not shown). The expression of caspase-1 (*CASP-1*) was also significantly increased by LPS treatment compared to controls (Figure 5A). Treating neutrophils with A1AT-0 did not alter LPS-induced *CASP-1* expression compared with LPS. However, treatment with A1AT-LA reduced LPS-induced *CASP-1* mRNA levels (by 1.4-fold, $p = 0.032$) compared with LPS (Figure 5A). The molecular forms of caspase-1 protein were lower in neutrophils treated with A1AT-LA or A1AT-0 relative to controls. Furthermore, neutrophils stimulated with LPS plus A1AT-0 or A1AT-LA showed a shift toward the precursor form (45 kDa) of caspase-1 compared with neutrophils stimulated with LPS alone (Figure 5B). As illustrated in Figure 5C, neither A1AT-0 nor A1AT-LA inhibited caspase-1 activity *in vitro*.

Table 3. Fold changes in NF-κB pathway mRNA gene expression following neutrophil treatment with LPS and LPS/A1AT (Prolastin®) for 4 h.

	A1AT (Prolastin®)		10 ng/mL LPS			A1AT (Prolastin®) + LPS		
Gene	N	Mean(SD)	N	Mean(SD)	P	N	Mean(SD)	P*
IKBKB	3	0.9 (0.28)	3	2.4 (0.53)	0.04	3	2.3 (0.73)	NS
IKBKE/*IKKε*	3	1.2 (0.61)	3	12.5 (6.5)	0.03	3	8.1 (1.1)	NS
IKBKG	3	0.77 (0.5)	3	0.8 (0.47)	NS	3	0.7 (0.1)	NS
NFKB1/p50	3	0.87 (0.34)	3	9.5 (0.46)	0.001	3	8.1 (2.6)	0.004
NFKB2/p52	3	0.59 (0.32)	3	5.9 (2.8)	0.023	3	4.0 (0.9)	NS
NFKBIB/*IkB-β*	3	0.76 (0.31)	3	1.8 (0.54)	0.002	3	1.6 (0.1)	0.017
NFKBIA/*IkB-α*	3	0.99 (0.13)	3	9.4 (1.2)	0.001	3	7.4 (1.6)	NS
NFKBIE/*IkB-ε*	4	0.86 (0.52)	3	5.3 (0.74)	0.036	3	4.0 (2.5)	NS
REL/*c-Rel*	4	0.63 (0.21)	3	4.2 (0.41)	0.001	3	3.1 (0.92)	NS
RELA/p65	4	0.8 (0.28)	3	2.8 (0.7)	0.004	3	1.6 (0.2)	0.009
RELB	5	0.57 (0.33)	3	2.6 (0.94)	0.02	3	1.1 (0.4)	NS

N, number of experiments; SD, standard deviation; NS, not significant; P value when comparing LPS to A1AT and P* value when comparing LPS to LPS + A1AT.

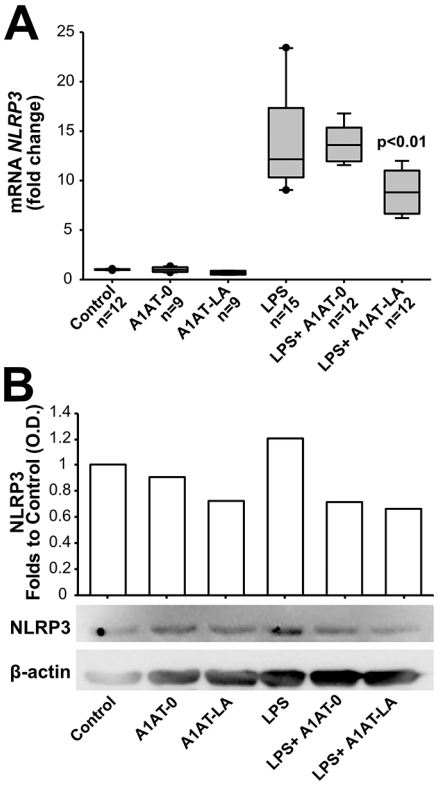

Figure 4. Effects of A1AT-0 and A1AT-LA on LPS-induced *NLRP3* expression. Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). mRNA expression of the *NLRP3* gene (A) was analyzed by real-time qPCR as described in Materials and Methods. *GAPDH* and *GUSB* were used as housekeeping genes. Box plots represent data from 4 individual donors; n = number of replicates for each experiment. *P* value indicates significant differences compared with the values seen in LPS-activated cells. Total cell lysates were analyzed for NLRP3 protein levels by western blotting (B). For loading control, blots were reprobed with antibodies to β-actin. Each blot is representative of 3 independent donors with similar results.

A1AT-LA Reduces LPS-Induced P2RX7 Expression in Human Neutrophils

P2RX7 expression is reported in human neutrophils (40) and found to be involved in NLRP3 inflammasome activation and IL-1β secretion. The basal expression of *P2RX7* (relative to *GUSB*, used as a housekeeping gene) was low in neutrophils [mean (SD): 0.068 (0.02), n = 15]. However, as shown in Figure 6, in LPS-stimulated neutrophils, *P2RX7* mRNA levels increased by 30-fold, $p < 0.001$, compared with unstimulated cells. Compared with A1AT-LA– but not A1AT-0–treated neutrophils, LPS-induced *P2RX7* mRNA levels were reduced by 45%, $p < 0.01$ (Figure 6).

Effects of A1AT-LA and A1AT-0 on LPS-Induced Expression of *SERPINA1*, *ELANE*, and *PRTN3* Genes

As expected, gene expression of *SERPINA1* was significantly induced following LPS treatment as compared with control neutrophils [mRNA relative expression, fold changes, mean (SD): 5.0 (0.98) vs 1.0 (0.13), respectively, n = 18, $p < 0.001$]. However, treatment of neutrophils with A1AT-0 or A1AT-LA did not significantly alter LPS-induced *SERPINA1* expression as compared with LPS-treated neutrophils in the absence of LPS stimulation [five-fold (1.4) and 4.2-fold (0.9), respectively, n = 15 vs five-fold (0.98) LPS] (data not shown). As illustrated in Figures 7A and B, LPS stimulated expression of *PRTN3* and *ELANE* remained enhanced in A1AT-0–treated neutrophils (*PRTN3*, $p < 0.001$; *ELANE*, $p = 0.008$) but not in A1AT-LA–treated cells. Analysis of cell lysates by western blots using specific antibodies against human A1AT (as a control) and PR3 detected multiple forms of high-molecular-size bands representing enzyme-inhibitor complexes in A1AT-0 or A1AT-LA–treated samples but not in controls or LPS-treated cells (Figures 7C and D). Concomitantly, the level of PR3 protein alone (29 kDa) was lower in A1AT- and LPS/A1AT-treated cells relative to controls and LPS-stimulated cells. Regarding NE, similar profiles of protein were detected in all experimental conditions (Figure 7E). In support, A1AT-0 and A1AT-LA formed similar complexes with pancreatic elastase and PR3 *in vitro* (Supplementary Figures S4A and B). Under these experimental conditions, neither A1AT-0 nor A1AT-LA had a significant effect on LPS-induced MPO release (Supplementary Figure S5).

Interaction of A1AT-LA and A1AT-0 with Neutrophils

To compare cellular localization of exogenous AAT-0 and A1AT-LA, neutrophils were incubated with each form

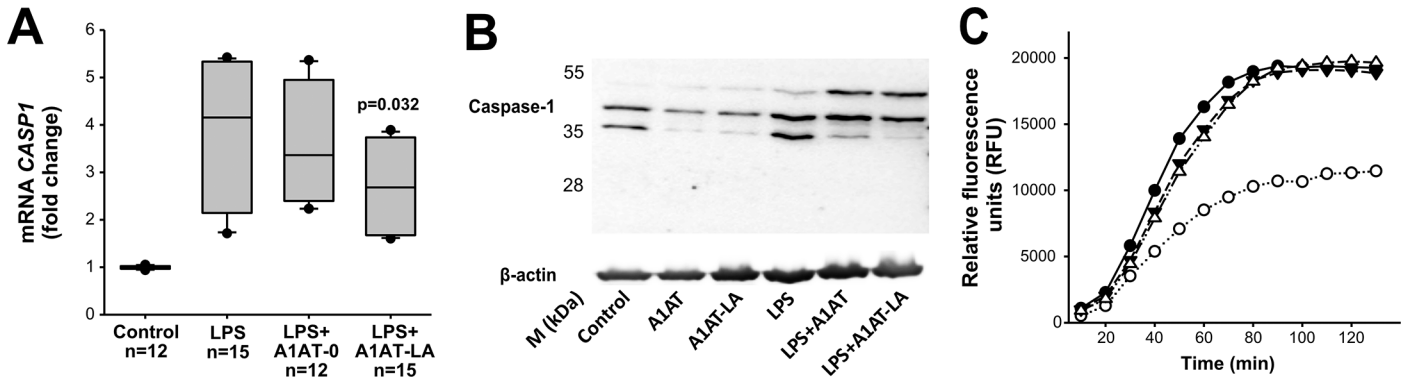

Figure 5. Effect of A1AT-0 and A1AT-LA on LPS-induced caspase-1 expression and activity. Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). mRNA expression of the *CASP1* gene (A) was analyzed by real-time qPCR as described in Materials and Methods. GAPDH and HPRT were used as housekeeping genes. Box plots represent data from 4 individual experiments; n = number of replicates for each experiment. *P* value indicates significant differences compared with the values seen in LPS-activated cells. Total cell lysates were analyzed for the caspase-1 protein profile characterization by western blotting (B). For loading control, blots were reprobed with antibodies to β-actin. The blot is representative of 3 independent donors with similar results. Caspase-1 activity (C) was analyzed *in vitro* by using caspase-1 fluorogenic tetrapeptide substrate (YVAD-AFC). Data represents caspase activity (in relative fluorescence units) in the presence of either 1 mg/mL A1AT-0 or A1AT-LA or 20 μM z-VAD-FMK (generic caspase inhibitor). Measurements were taken every 10 min. Each curve represents data from 2 independent experiments, each performed in triplicate.

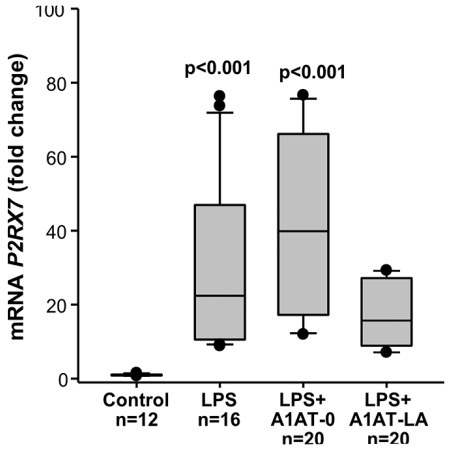

Figure 6. Effects of A1AT-0 and A1AT-LA on LPS-induced *P2RX7* expression Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). mRNA expression of the *P2RX7* gene was analyzed by real-time qPCR as described in Materials and Methods. *GAPDH* and *GUSB* were used as housekeeping genes. Box plots represent data from 4 individual donors; n = number of replicates for each experiment. *P* value indicates significant differences compared with the values seen in control cells.

of A1AT for 5 min at 4°C or 37°C, or for 1 h at 37°C, and then subjected to immunofluorescence microscopy. Cells without A1AT treatment were used as controls (Figure 8A). Microscopic analysis revealed a marked difference in fluorescence intensity. To appreciate A1AT distribution in the samples with the weaker signal, such as the control and 4°C-incubated cells, the settings were optimized for the weak fluorescence, resulting in overexposed signals in the cells incubated for 1 h at 37°C. As expected, A1AT-treated cells showed enrichment of the A1AT-positive signal at the cell surface, as compared with control cells (Figure 8). Notably, on cells incubated at 4°C, which inhibits A1AT uptake, finely dispersed immunoreactivity was observed, whereas cells incubated at 37°C revealed large A1AT-positive granules or aggregates, especially prominent after 1 h of incubation. As shown in Figure 8C, if compared with A1AT-LA–treated neutrophils, cells treated with AAT-0 formed larger granules with stronger fluorescence. Apart from that, neutrophils incubated with AAT-0 or A1AT-LA, in general, showed a similar pattern of A1AT distribution on the cell surface and/or within the cells (Figure 8). Analysis of cytoplasmic A1AT-0 and A1AT-LA by western blots confirmed the similar pattern between both forms of A1AT (Supplementary Figure S6).

Effects of A1AT-LA and A1AT-0 on Antiinflammatory Transcription Factor PPAR-γ

As demonstrated in Figures 9A and B, neutrophils incubated with A1AT-LA or lipid-containing A1AT (Prolastin) significantly increased *PPAR-γ* gene expression relative to untreated cells. AIAT-0 had no effect on *PPAR-γ* mRNA levels. Similarly, treatment with A1AT-LA but not A1AT-0 strongly enhanced LPS-induced *PPAR-γ* mRNA levels compared with LPS-treated cells without A1AT (Figures 9 C and D). Neutrophils treated with GW9662 reversed the ability of A1AT-LA to inhibit LPS-induced *IL-1β* mRNA levels (Figure 9E).

DISCUSSION

IL-1β is a highly inflammatory cytokine and is implicated in several pathological conditions. (41) Neutrophils are

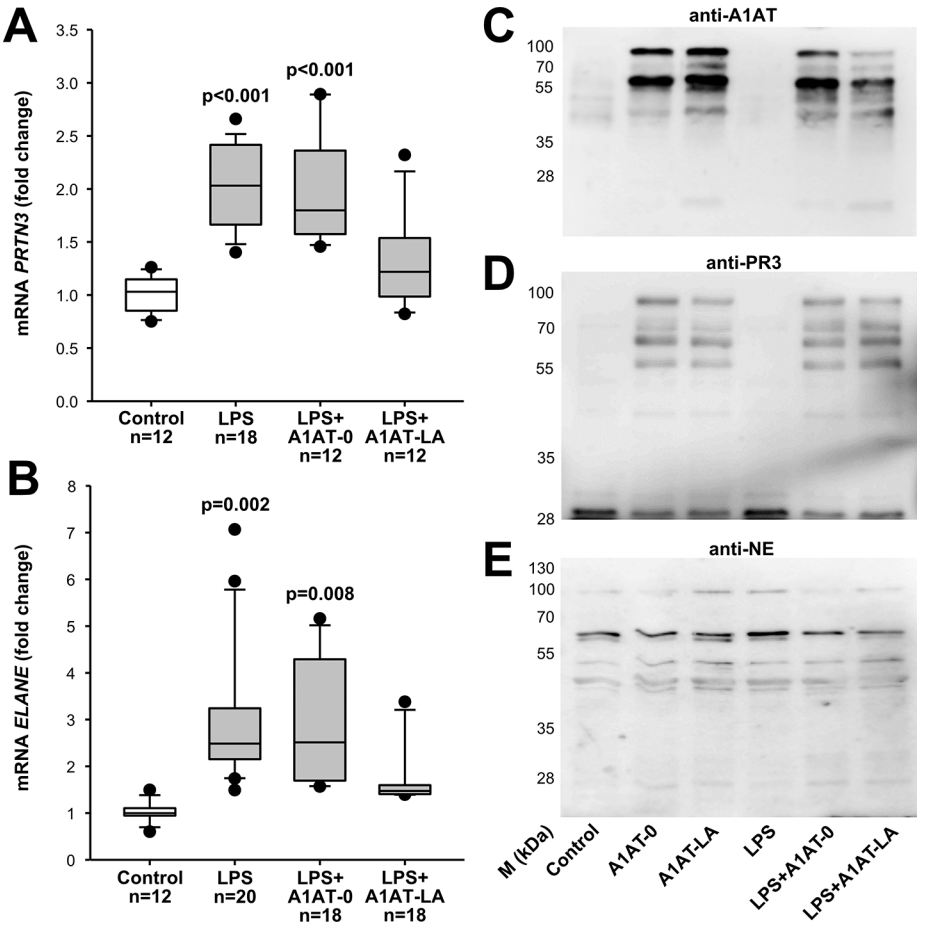

Figure 7. Effects of A1AT-0 and A1AT-LA on LPS-induced NE and PR3 expression and protein profile. Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). mRNA expression of *PRTN3* (A) and *ELANE* (B) were analyzed by real-time qPCR as described in Materials and Methods. *GAPDH* and *HPRT* were used as housekeeping genes. Box plots represent data from 5 individual donors; n = number of replicates for each experiment. *P* value indicates significant differences compared with the values seen in control cells. Cell lysates were analyzed for A1AT (C), PR3 (D) and NE (E) protein profile characterization by 12.5% SDS-PAGE following western blotting. M-molecular size marker (kDa). Each blot is representative of 3 independent donors.

a major source of IL-1β (39,42,43); moreover, neutrophil-derived IL-1β mediates further neutrophil recruitment and activation. Synthesis of the IL-1β precursor is considered as a priming step, whereas intracellular cleavage of the IL-1β precursor and secretion of active IL-1β is a key step in the inflammatory response. Therefore, understanding the molecular regulation of these steps is of critical importance for the design of IL-1β-based modulatory therapies.

Human-plasma-purified A1AT used as therapy not only inhibits neutrophil elastase and proteinase 3 but also expresses broad antiinflammatory and immunomodulatory effects, including modulation of IL-1β levels. (11,39–41) This latter observation prompted us to investigate the effect of currently used preparations of A1AT in treating humans on processing secretion of active IL-1β in LPS-activated human blood neutrophils *in vitro*. It is important to point out that therapeutic preparations of human A1AT may contain lipid-free (38) and polyunsaturated fatty acid (LA) bound forms of A1AT. (42) Therefore, we investigated whether lipid-free (A1AT-0) and LA-bound (A1AT-LA) forms of A1AT differ in their effects on LPS-induced IL-1β.

Our data demonstrate a significant inhibitory effect of A1AT-LA and A1AT-0 on LPS- induced IL-1β release from human blood neutrophils *in vitro*. Regarding *IL-1β* mRNA and protein levels, the findings were unexpected. When compared with LPS, there was a marked decrease of *IL-1β* mRNA and IL-1β immunoreactivity in cell lysates following LPS/A1AT-LA stimulation. However, under the same experimental conditions, A1AT-0 did not affect LPS-induced IL-1β gene expression and protein levels. This latter finding illustrates a fundamental role of the lipid content in A1AT therapeutic preparations. We therefore investigated the mechanism for the effects of the 2 forms of A1AT on LPS-induced inflammatory gene expression. To generate activated neutrophils, we treated them for 5 h with a low concentration of LPS (20 ng/mL) in the absence or presence of A1AT (1 mg/mL). LDH release as a measure of cytotoxicity confirmed that our experimental settings did not affect the viability of neutrophils.

In neutrophils, LPS triggers synthesis of the IL-1β precursor through the TLR-NFκB pathway (44) and induces release of mature IL-1β. (33,34) As expected, LPS-treated neutrophils showed higher expression of NF-κB pathway genes compared with nontreated controls and A1AT-treated cells. Concomitantly, we observed a several-fold increase in IL-1β precursor and supernatant levels of IL-1β. Compared with neutrophils treated with LPS, treatment with LPS/A1AT-LA but not A1AT-0 significantly reversed LPS-induced *TLRs*, *RelA*/p65, and *IκBα* mRNA levels. Interestingly, A1AT-LA had no significant effect on LPS-induced *IκBβ* expression. Considering protein levels, we found no differences in cytosol-nuclear profiles of RelA/p65, IκBα, and IκBβ between LPS and LPS/A1AT- or

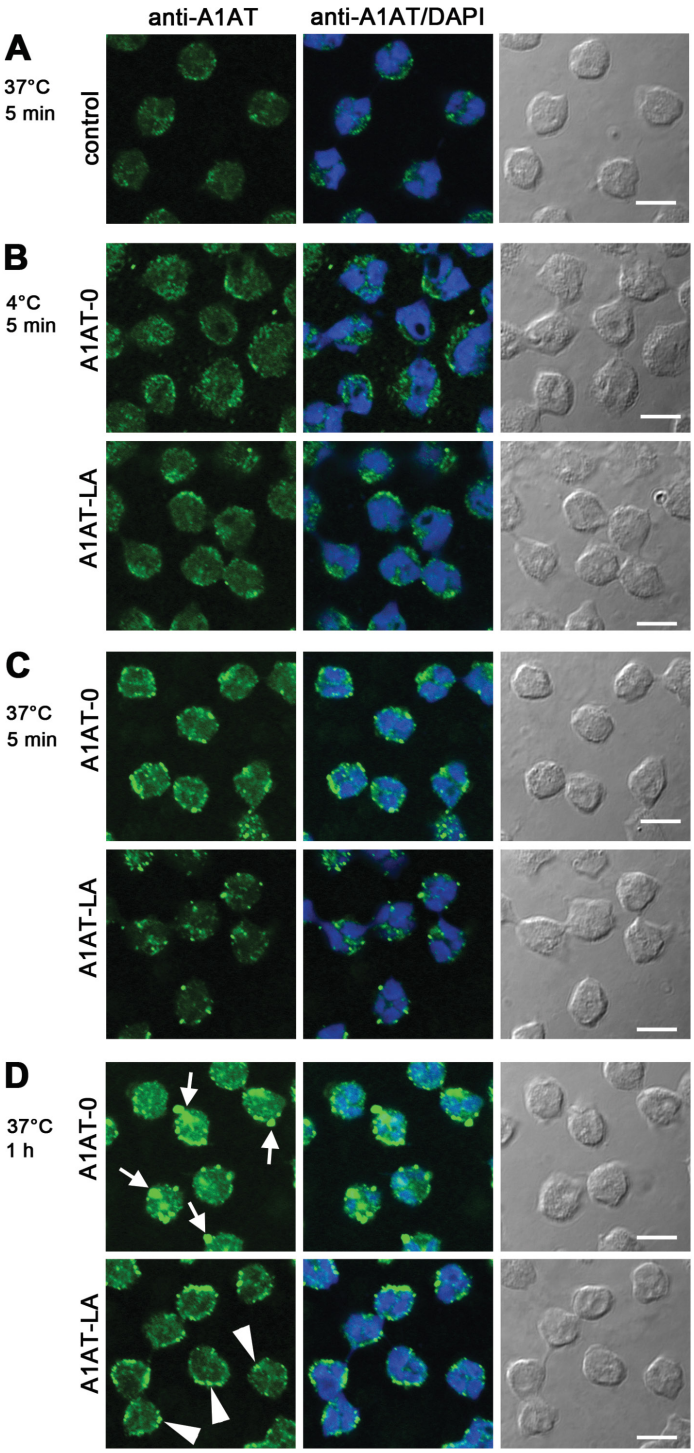

Figure 8. Immunofluorescence microscopy analysis of A1AT-0 and A1AT-LA interaction with neutrophils. Neutrophils were incubated alone as controls for 5 min at 37°C (A), or with either A1AT-0 or A1AT-LA for 5 min at 4°C (B), or for 5 min and 1 h at 37°C (C). Cells were stained with anti-A1AT antibody (green) and DAPI (blue). Arrows point to the larger A1AT-reactive granules formed upon A1AT-0 treatment as compared with those of A1AT-LA (arrowheads). Images were acquired using Olympus FluorView 1000 scanning confocal microscope equipped with a 60 × oil immersion objective. Scale bar: 10 μm.

LPS/A1AT-LA–treated neutrophils. As all experiments were performed within 5 h of incubation time, we avoid making firm conclusions based on the protein data. Different incubation periods may be required to monitor changes at the protein level. Several studies provide evidence that in neutrophils, the relationship between gene transcription and translation is far from linear. (42,45,46) Notably, we did not detect p65 and IκBβ proteins in the nuclear fraction. This observation is in line with the finding that the IκBα-NF-κB complex shuttles between cytoplasm and nucleus while the IκBβ-NF-κB complex is cytosolic and does not undergo shuttling at all. (47–49) IκBα and IκBβ are cytoplasmic isoforms of IκB responsible for keeping NF-κB inactive (50,51); however, signaling that proceeds through these proteins has unique implications for target gene expression. (52) For example, in response to LPS, IκBβ is required for IL-1β transcription, (53) whereas IκBα is associated with caspase-3 activation and increased apoptosis. (54)

A detailed investigation of how A1AT-LA affects LPS-induced expression of IκB isoforms was beyond the scope of this work. However, the data suggest that A1AT-LA reduces LPS-induced synthesis of the IL-1β precursor by impairing the ability of LPS to upregulate the TLR-NFκB pathway.

Recent findings have revealed the pivotal role of NLRP3 in IL-1β production by human peripheral blood neutrophils. (55) Human neutrophils constitutively express NLRP3, whereas NF-κB activation downstream of TLRs can increase the expression of *NLRP3* required to activate caspase-1. (39) Thus, in neutrophils, LPS alone can upregulate caspase-1 and generate a release of small amounts of IL-1β. (34,56,57) Therefore, we next investigated NLRP3 inflammasome components in neutrophils stimulated with LPS compared with neutrophils treated with LPS and A1AT combinations. The mRNA and protein expression of *NLRP3* and *CASP1* increased following LPS treatment as compared with cells not

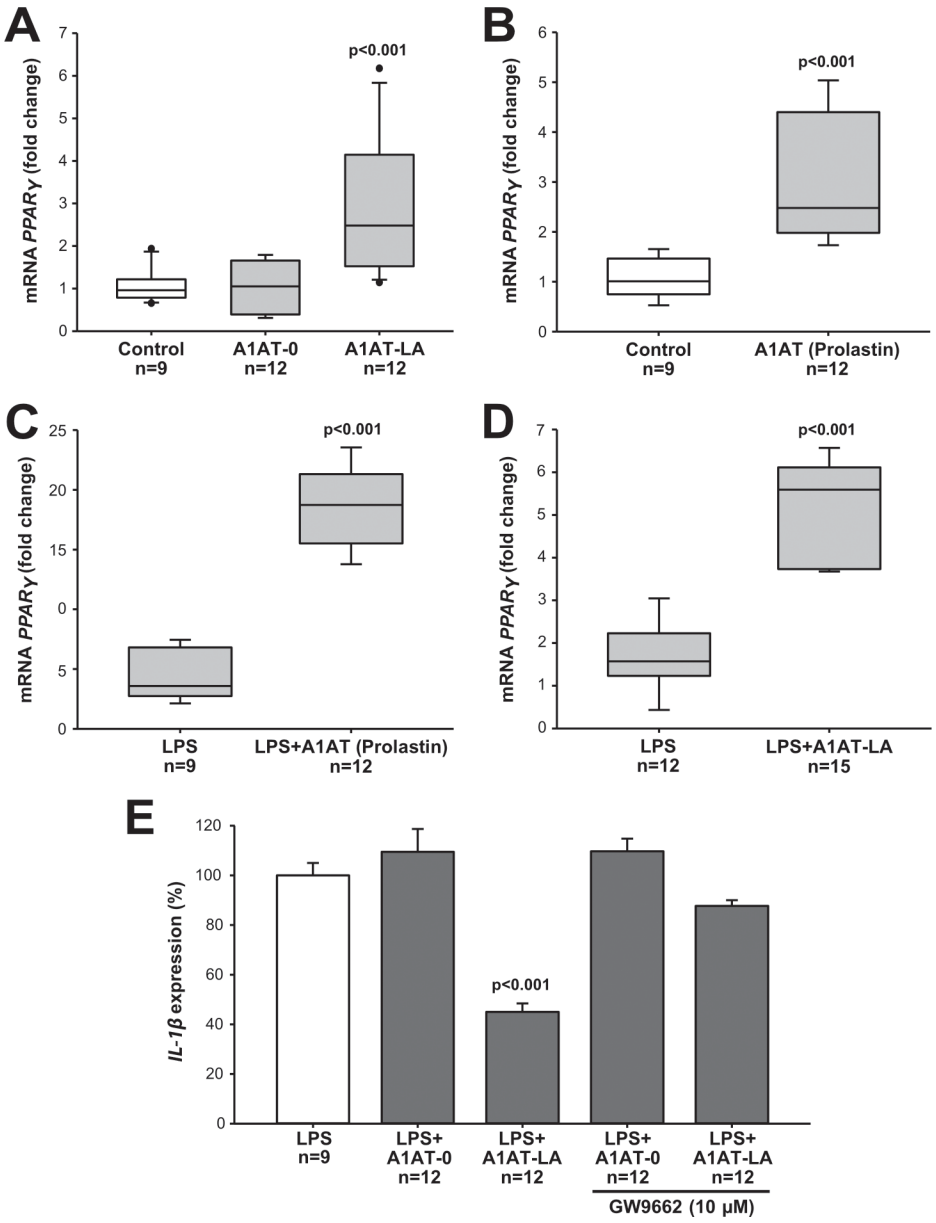

Figure 9. A1AT and LPS/A1AT induce PPAR-γ expression; effect of GW9662 on A1AT-LA ability to inhibit LPS-induced IL-1β expression Human neutrophils (5×10^6) were incubated for 5 h in either medium alone, medium containing A1AT (Prolastin, 1 mg/mL) or LPS (20 ng/mL) in the absence or presence of A1AT-0 (1 mg/mL) or A1AT-LA (1 mg/mL). Expression of *PPAR-γ* (A–D) was analyzed by real-time qPCR as described in Materials and Methods. *GAPDH* and *HPRT* were used as housekeeping genes. Box plots represent data from 3 individual donors; n = number of replicates for each experiment. *P* value indicates significant differences compared with the values seen in control cells (A and B) or LPS (C and D). (E) Cells were pretreated for 30 min with 10 μM GW9662, an irreversible PPAR-γ antagonist, prior to addition of LPS or LPS/A1ATs. Data are expressed as percentage of *IL-1β* gene expression in cells activated with LPS (100%) and show means (SD) of 3 independent donors; n = number of replicates for each experiment. $P < 0.001$ represents significant difference compared with the values seen in LPS-activated cells.

exposed to LPS. Under the same experimental conditions, A1AT-LA but not AAT-0 significantly lowered LPS-induced *NLRP3* and *CASP1* mRNA levels. Protein levels of NLRP3 were marginally lower in LPS/A1AT and LPS/A1AT-LA compared with LPS-treated cells. ASC acts as a bridge to recruit NLRP3 using the PYD-PYD interaction, and to recruit caspase-1 via the CARD-CARD interaction. (58) In the present experimental setting, in which neutrophils were treated for 5 h, the levels of *ASC* mRNA did not change in response to LPS and LPS/A1AT-LA. Indeed, earlier studies on ASC reported a transient induction (at 2 h of LPS stimulation). (59–61) Ionotropic P2RX7 expression on neutrophils is required for NLRP3 inflammasome assembly and IL-1β secretion. (39) In the present experimental model, basal expression of *P2RX7* was low but increased after treatment with LPS. When neutrophils were treated with LPS in the presence of A1AT-LA, no significant induction of *P2RX7* expression was observed. These data further support the notion that A1AT-LA but not A1AT-0 inhibits LPS-induced synthesis of the IL-1β precursor by reducing gene expression of the TLR/inflammasome pathway.

Proteolytic Cleavage of the IL-1β precursor Is Required for Its Activity and Secretion

Caspase-1–mediated cleavage of the IL-1β precursor following inflammasome complex formation is suggested as the major mechanism responsible for the secretion of IL-1β. (62,63) Previous studies reported conflicting results regarding the ability of A1AT to inhibit caspase-1. (64–66) Data from our *in vitro* experiments show that neither A1AT-0 nor A1AT-LA inhibits active caspase-1. Neutrophil-derived serine proteases can activate TLR4 (67) and can also be responsible for processing the IL-1β precursor. (68–73) A mouse model of arthritis identified that IL-1β processing by neutrophils is independent of caspase-1 but is dependent on NE. (74) Some

studies, however, suggest that PR3 has a greater role than NE in IL-1β processing and secretion. (3) PR3 is localized in the same lipid raft domains (75,76) as A1AT (5,20) and LA (77). Bilayer-bound PR3 has a reduced catalytic efficiency, whereas its inhibition by A1AT is more important than that observed for the soluble form of the enzyme. (78) We previously published results showing that A1AT-0 and A1AT-LA have similar inhibitory activity against elastase. (23) Here we confirm that A1AT-LA and A1AT-0 can form complexes equally well with NE and PR3 *in vitro* and in neutrophil lysates. In support of this, both forms significantly inhibited LPS-induced IL-1β release, although the effect of A1AT-LA was more pronounced relative to A1AT-0. Noticeably, when compared with LPS, treatment with A1AT-LA but not A1AT-0 inhibited *ELANE* and *PRTN3* mRNA levels. This further favors A1AT-LA as a more effective inhibitor of LPS-induced IL-1β release. Assuming that NE and PR3 play a critical role in LPS-induced IL-1β release, the above results would explain why both A1AT-LA and A1AT-0 inhibited LPS-induced IL-1β release.

Taken together, A1AT complexed with unsaturated fatty acids such as LA reduces LPS-induced synthesis of the IL-1β precursor and release of active IL-1β, whereas the fatty acid–free form of A1AT only reduces LPS-induced release of IL-1β. The molecular mechanism behind the observed differences between the 2 forms of A1AT appears to be dependent on PPARs as sensors for fatty acids. (79) We previously found that A1AT-LA requires the activity of PPARs to induce expression of *ANGPTL4* and related genes in adherent peripheral blood mononuclear cells. (23) PPAR-γ is expressed in neutrophils, and the ligand-dependent activation of this receptor results in suppression of cytokine production. (80) The present data confirm that A1AT-LA independent of LPS stimulation upregulates PPAR-γ mRNA in neutrophils, while A1AT-0 has no effect. Furthermore, neutrophil pretreatment with GW9662, a specific inhibitor of PPAR-γ, markedly diminished the ability of A1AT-LA to inhibit LPS-induced synthesis of the IL-1β precursor. Hence, A1AT-LA–induced PPAR-γ appears to control synthesis of the IL-1β precursor in activated neutrophils.

CONCLUSION

We speculate that LA in complex with A1AT results in a novel form of A1AT having a strong ability to regulate inflammation-induced synthesis, processing, and release of active IL-1β. These observations may be of value in evaluating the biological effects and therapeutic efficiency of A1AT preparations.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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