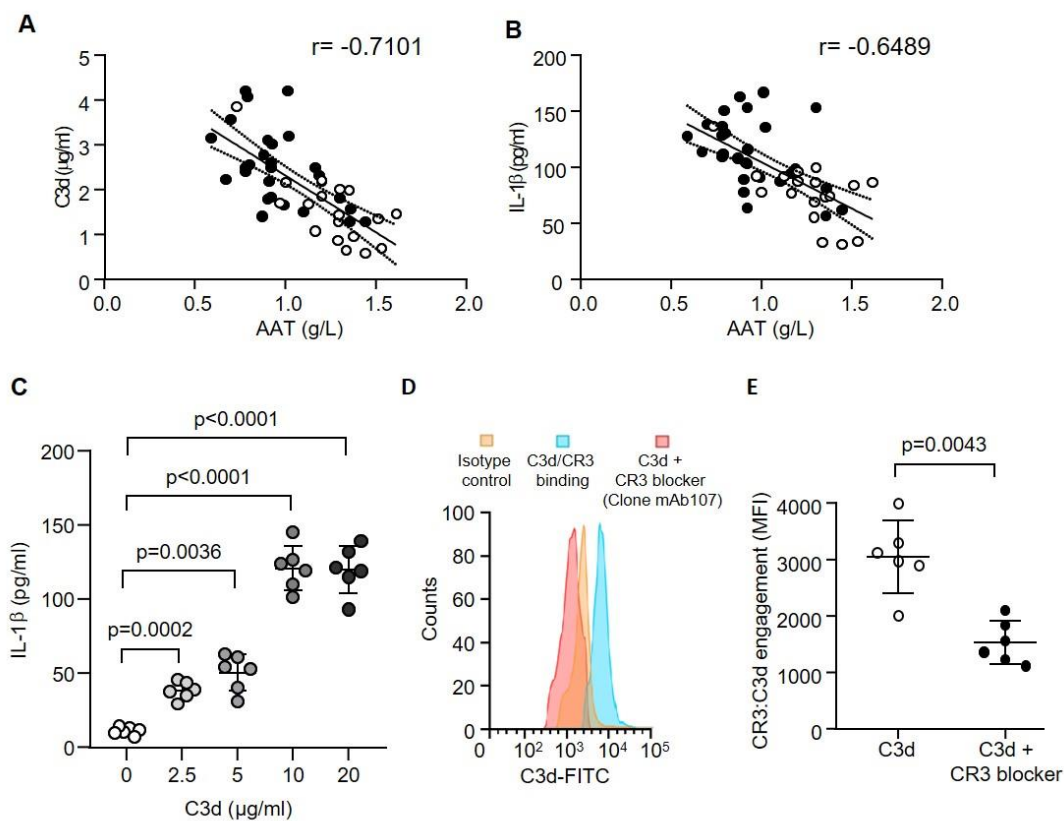# Supplementary Data

Monocyte NLRP3 inflammasome and interleukin-1β activation modulated by alpha-1 antitrypsin therapy in deficient individuals.

Debananda Gogoi, Howard Yu, Michelle Casey, Rory Baird, Azeez Yusuf, Luke Forde, Michael E. O'Brien, Jesse R. West, Tammy Flagg, Noel G. McElvaney, Edward Eden, Christian Muller, Mark L. Brantly, Patrick Geraghty, and Emer P. Reeves.

Supplementary figure 1: C3d triggers IL-1β production via CR3

(A&B) Individuals with AATD had a high level of C3d (A) and IL-1β (B) with a negative relationship detected between AAT and C3d (N=47, p<0.0001) and AAT and IL-1β (N=47, p<0.0001) ●=AATD and ○=HC. Two tailed non parametric spearman correlation test.

(C) HC monocytes were treated with increasing concentrations of C3d (0-20 µg/ml) for 16h and extracellular IL-1β production quantified by ELISA (N=6 biological repeats, statistical analysis performed by one-way ANOVA and Tukey test for multiple comparison).

(D) Flow cytometry analysis of CR3 membrane-bound C3d on isolated HC neutrophils (blue) and in the presence of the CR3 blocker, clone mAb 107 (1µg/ml). C3d (5µg) binding was evaluated using C3d mouse monoclonal IgG1 (1µg/ml). The level of CR3/C3d binding was reduced by inclusion of clone mAb 107 (red).

(E) Analysis of the ability of exogenous C3d to bind monocyte membrane CR3 examined by flow cytometry. Cells exposed to CR3 blocking antibody (clone mAb 107) displayed a significant reduction in median fluorescence intensity (MFI) (N=6 biological repeats, Student's t-test).
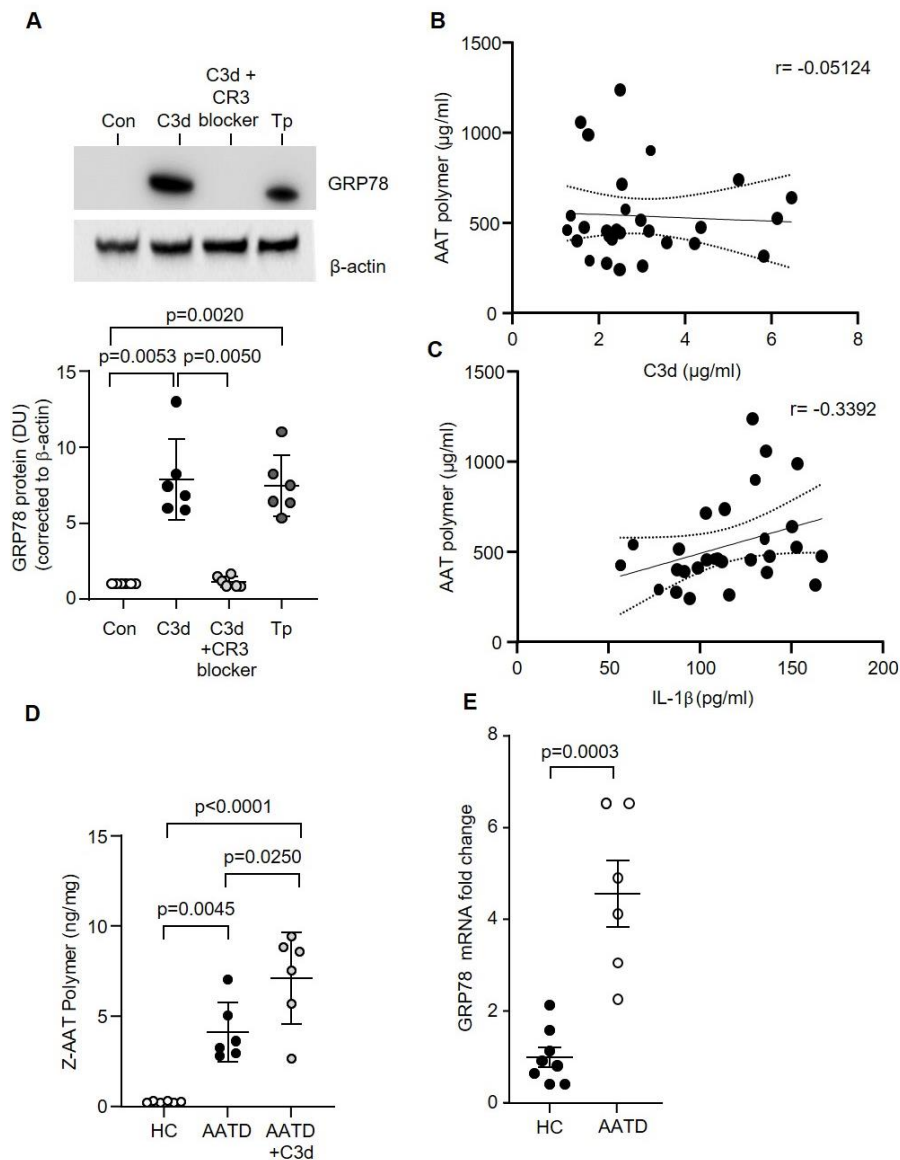
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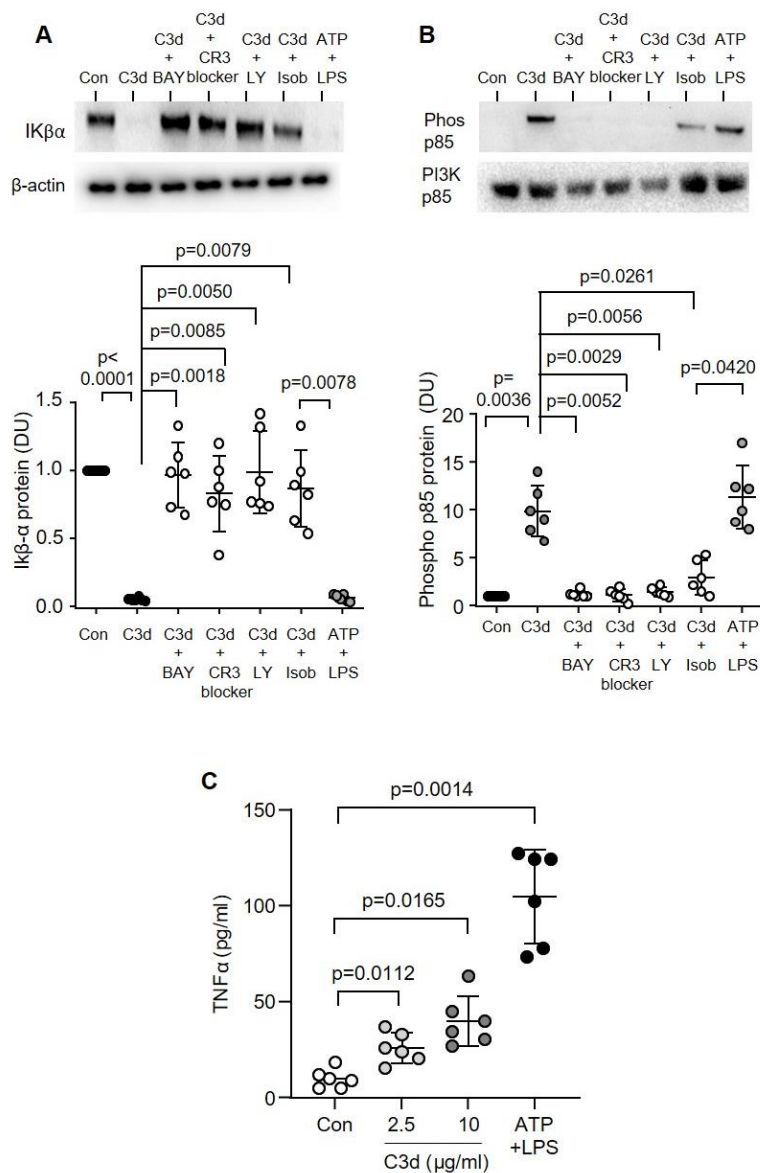
Supplementary figure 2. C3d activation of the PI3K/Akt-NFκβ pathway.
(A&B) Expression levels of IKβα (A) or phosphorylation of p85 (B) post C3d ($10\mu g/1x10^5$ cells) treatment was measured by Western blot analysis of monocyte whole cell lysates. Controls included pre-incubation with BAY 11-7082 (NFκβ inhibitor, 5 μg/ml), CR3 blocker (clone mAb 107, 1 μg/ml), LY-294002 (LY) PI3 kinase inhibitor, 10 μM) or isobavachalcone (Isob) (AKT inhibitor, 5mM) for 30 min prior to C3d challenge. Degradation or phosphorylation levels were normalized to respective total protein. Results are expressed as DU, with representative Western blots presented (N=6 biological repeats, one-way ANOVA, followed by Tukeys' post-hoc multiple comparison). (C) HC monocytes were treated with C3d (2.5 or 10 μg/ml) for 16h and extracellular TNF-α production quantified by ELISA. Data are represented as mean ±SD (N=6 biological repeats, one way ANOVA, followed by Tukey's post hoc multiple comparison)
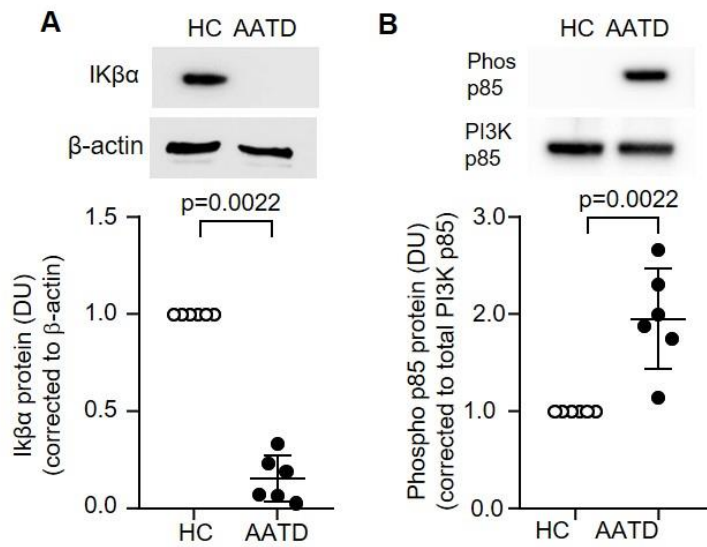
Supplementary figure 3. **The CR3:C3d signalling pathway is increased in the monocytes of AATD patients.**
Expression levels of IKβα (A) or phosphorylation of p85 (B) in AATD or HC subjects measured by Western blot analysis of monocyte whole cell lysates. Degradation or phosphorylation levels were normalized to respective total protein. Results are expressed as DU, with representative Western blots presented (N=6 subjects per group, non-parametric Mann-Whitney U test).

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Supplemental material

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Supplementary figure 4. CR3 inhibition modulates C3d-induced GRP78 ER stress in AATD monocytes

(A) Western blot and densitometry analysis of GRP78 expression in control (Con) untreated monocytes from healthy individuals (HC) or following treatment with C3d (10 µg/ml) for 16h. Negative and positive controls included pre-incubation with CR3 blocker (clone mAb 107, 1 µg/ml) and treatment with the ER stress inducer thapsigargin (Tp) (100 nM), respectively, (N=6 biological repeats per group, one-way ANOVA). (B) No correlation between C3 activation product C3d and plasma Z-AAT polymers (p=0.7957 by two tailed non parametric spearman correlation test). (C) No correlation between plasma IL-1β and Z-AAT polymers (p=0.0775 by two tailed non parametric spearman correlation test). (D) Z-AAT polymer levels in whole cell lysates of HC or AATD purified monocytes (N= 6 biological repeats per group, one way ANOVA using Tukey's post hoc multiple comparison test). (E) GRP78 qRT-PCR of HC or AATD monocyte RNA revealed significantly increased expression in AATD (N=6 subjects per group, non-parametric Mann-Whitney U test).

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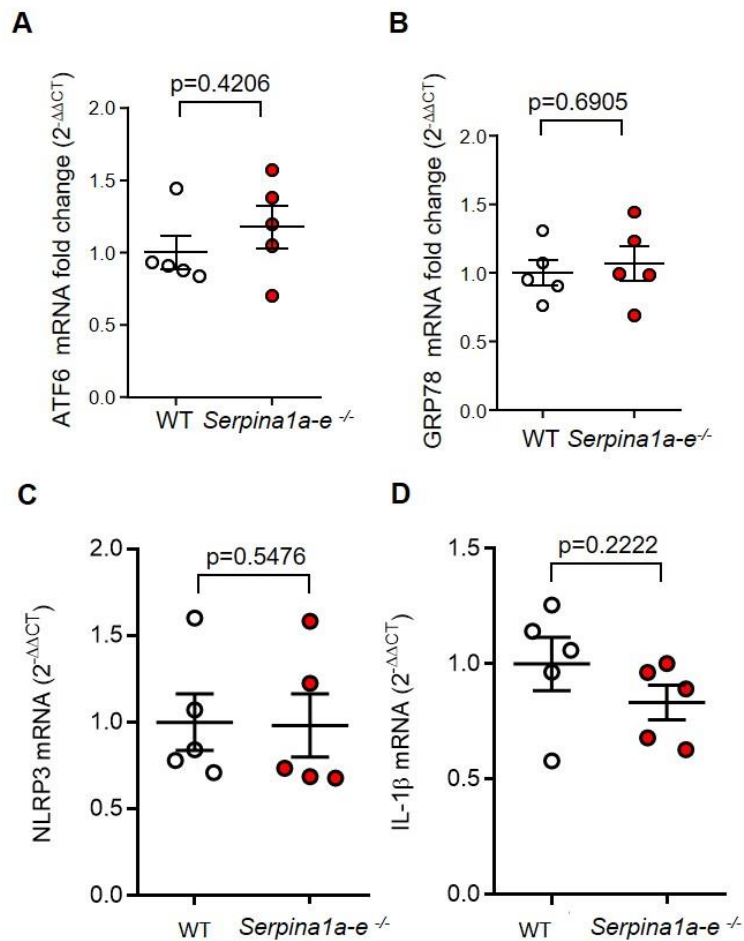
Supplementary figure 5. Levels of ER-stress markers and NLRP3 activation are unchanged in *Serpina1a-e$^{-/-}$* mice.

Monocytes were isolated from wild type and *Serpina1a-e* knockout mice. qPCR analysis for gene expression of ATF6 (A), GRP78 (B), NLRP3 (C) and IL-1β (D) demonstrated that monocytes from *Serpina1a-e$^{-/-}$* mice have similar expression of ER stress markers and possess similar gene expression levels of NLRP3 and IL-1β, when compared to wild-type mice (N=5 animals/group. No significant difference was observed between WT and *Serpina1a-e$^{-/-}$* mice for ATF6, GRP78, NLRP3 or IL-1β expression. Non-parametric Mann-Whitney U test was used for statistical analysis.
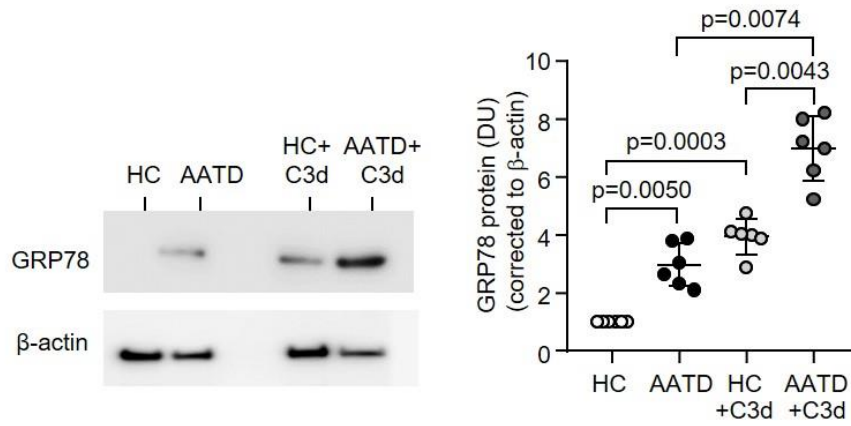
Supplementary figure 6. C3d-induced ER stress in AATD monocytes
Western blot and densitometry analysis of GRP78 expression in resting HC monocytes compared to AATD cells, and following C3d challenge (N=6 subjects per group, one-way ANOVA, followed by Tukeys' post-hoc multiple comparison test).
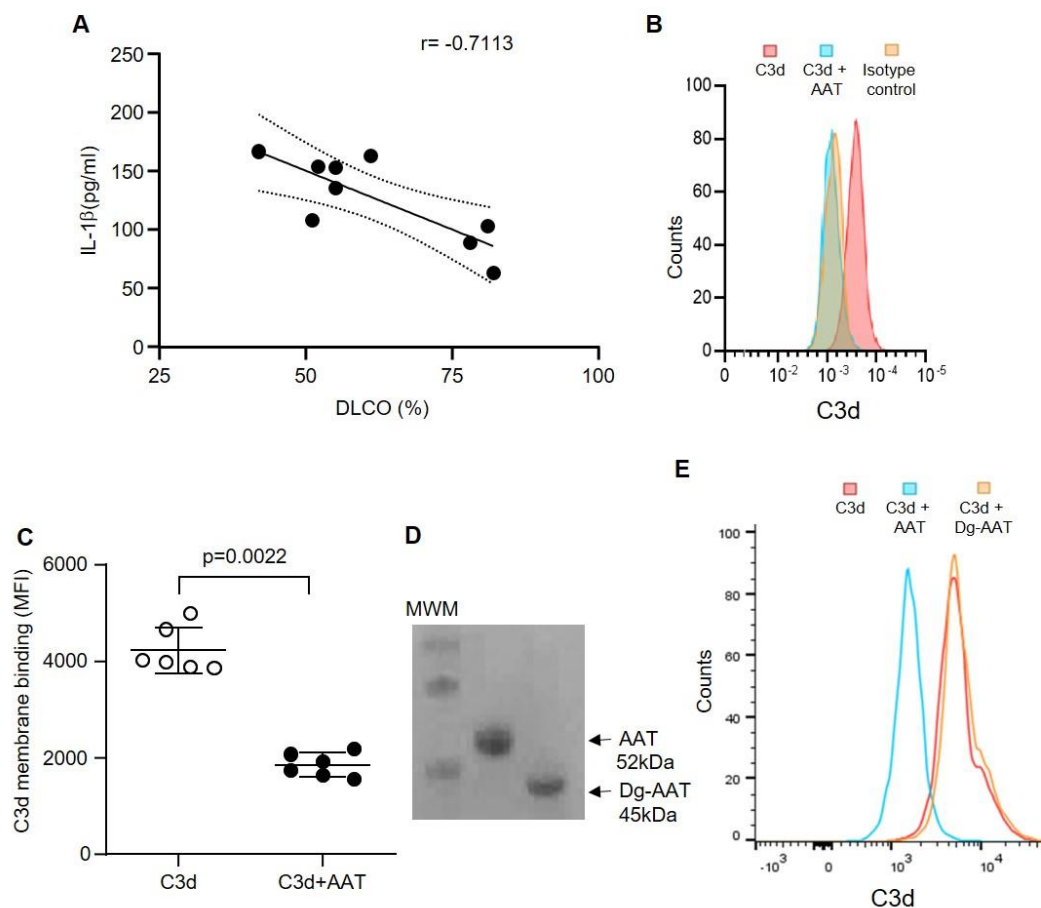
Supplementary figure 7. The addition of human serum purified AAT modulates monocyte IL-1β production *in vitro* by inhibiting C3d membrane engagement.

(A) Negative correlation between IL-1β and DLCO (N=9, p=0.0371, statistical test by two tailed non parametric spearman correlation test).

(B & C) Flow cytometry analysis of C3d (5 µg) monocyte membrane interaction in the presence of exogenous AAT (1 mg/ml). C3d binding was evaluated using C3d mouse monoclonal IgG1 (N=6 biological repeats, statistical analysis performed by non-parametric student's t-test)

(D) SDS-PAGE analysis of deglycosylated AAT post PNGase treatment (1µg/ml). Molecular mass markers (MWM, kDa) are included in left-hand lane.

(E) Representative flow cytometry analysis of C3d (5 µg) monocyte membrane interaction in the presence of glycosylated or deglycosylated AAT (dg-AAT) (1 mg/ml).

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