

Modulation of RAS and PI3-AKT pathway by Stavudine (d4T) in PBMC of Alzheimer's Disease Patients

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Abstract

Background

A β ₄₂-deposition plays a pivotal role in AD-pathogenesis by inducing the activation of microglial cells and neuroinflammation. This process is antagonized by microglia-mediated clearance of A β plaques. Activation of the NLRP3 inflammasome is involved in neuroinflammation and in the impairments of A β -plaques clearance. Stavudine (d4T) on the other hand down-regulates the NLRP3 inflammasome and stimulates autophagy-mediated A β -clearing in a TPH-1 cell line model.

Methods

We explored the effect of d4T on A β - autophagy using PBMC of AD patients that were primed with LPS and stimulated with A β in the absence/presence of d4T. We analyzed the NLRP3 inflammasome activity by measuring NLRP3-ASC complexes formation by AMNIS Flow-sight and pro-inflammatory cytokines (IL-1 β , IL-18 and Caspase-1) production by enzyme-linked immunosorbent assay (ELISA). Western blot analyses were used to measure phosphorylation and protein expression of p38, CREB, ERK and AKT, p70, LAMP 2A, beclin-1 and Bax.

Results

data showed that d4T: 1) down regulates NLRP3 inflammasome activation and the production of downstream proinflammatory cytokines even in PBMC; 2) stimulates the phosphorylation of AKT, ERK, p70 as well as LAMP2A production, but does modulate beclin-1, suggesting a selective effect of this compound on chaperone-mediated autophagy (CMA); 3) up regulates p-CREB and BAX, possibly diminishing A β -mediated cytotoxicity; and 4) reduces the phosphorylation of p-38, a protein involved in the production of proinflammatory cytokines.

Conclusions

d4T reduces the activation of the NLRP3 inflammasome and stimulates CMA autophagy as well as molecular mechanisms that modulate cytotoxicity and reduce inflammation in cells of AD patients. It might be interesting to verify the possibly beneficial effects of d4T in the clinical scenario.

Background

Alzheimer's disease (AD), the most prevalent type of dementia, is characterized by the deposition of amyloid- β (A β), the formation of neurofibrillary tangles, and neuroinflammation [1]. Thus, in AD patients A β accumulation induces the release of inflammatory mediators by the microglia; this facilitates A β deposition and neuroinflammation in a self-feeding pathogenic loop [2]. Microglia, on the other hand,

plays an important role against AD that is mediated by the generation of A β -specific antibodies, the clearance of amyloid plaques, and the recruitment of peripheral immune cells that cross the blood-brain barrier (BBB) in an attempt to remove A β aggregates [3, 4, 5]. The initiation of the inflammatory response by microglia and peripheral immune cells involves cytosolic multiprotein platforms known as inflammasomes. The NLRP3 inflammasome is the best characterized of such platforms and its formation requires multiple steps. In a priming step, transcriptionally-active signalling receptors induce the NF- κ B-dependent induction of NLRP3 itself, as well as that of the caspase-1 substrates pro-IL-1 β and IL-18 [6]; a second signal leads to the assembly of a multimolecular complex with ASC and caspase-1. At this point procaspase-1 or procaspase-8 are recruited [7], ASC specks are formed [8], caspases are activated, and cytokines are produced.

Activation of the NLRP3 inflammasome is a tightly regulated process; autophagy, in particular, an adaptive response to stress, down regulates NLRP3 inflammasome activation by a complex metabolic mechanism [9] that includes different molecular pathways [10–14]. Notably, autophagy defects in myeloid cells results in the aberrant activation of the inflammasome [15, 16], leading to development of inflammatory disorders. Different compounds can modulate the NLRP3 inflammasome activation as well. Stavudine (d4T), a nucleoside reverse transcriptase inhibitor (NRTIs), in particular was shown to hamper NLRP3 inflammasome activation as well as caspase-1 and IL-18 production both *in vivo* [17, 18] and in a TPH-1 cell line *in vitro* model [19]. Notably, in this model d4T did not have any effect on A β -phagocytosis, but it stimulated autophagy-mediated A β -clearing [19], as witnessed by its ability to modulate the ERK1/2 and AKT pathways and to upregulate LAMP-2A and p70-S6K, their downstream targets.

Given the fact that: 1) autophagy is necessary for the degradation of A β in microglia; 2) p-AKT [20] and p-ERK [21] associate with A β -accumulation and τ -phosphorylation in AD animal models [22, 23]; and 3) autophagy is modulated by d4T [19], we explored more in details the mechanisms responsible for d4T-mediated autophagy clearing of A β . To this end we used peripheral blood mononuclear cells of AD patients that were stimulated with LPS + A β in the absence/presence of d4T.

Methods

Patients

Thirteen AD patients who fulfilled inclusion criteria for a clinical diagnosis of AD were randomly selected within a large database of patients consecutively admitted between January 2017 and September 2019 by the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico in Milano, Italy. All patients were enrolled in a cognitive rehabilitation experimental program and underwent complete medical and neurological evaluation, laboratory analysis, CT scan or MRI, and other investigations (e.g., EEG, SPET scan, CSF examination, etc.) to exclude reversible causes of dementia. The clinical diagnosis of AD was performed according to the NINCDS-ADRDA work group criteria [24] and the DMS IV–R [25]. Neuropsychological evaluation and psychometric assessment were performed with a Neuropsychological

Battery that included MiniMental State Examination (MMSE) [26]. Digit Span Forward and Backward, Logical Memory and Paired Associated Words Tests, Token Test, supra Span Corsi Block Tapping Test, Verbal Fluency Tasks, Raven Colored Matrices, the Rey Complex Figure, Clinical Dementia Rating Scale (CDR) [27]; the study conformed to the ethical principles of the Helsinki Declaration.

All patients underwent lumbar puncture (LP) for quantification of A β , total tau (T-tau), and tau phosphorylated at position 181 (P-tau) in the CSF. Normality cut-off thresholds were: A β \geq 600 pg/ml; tau \leq 500 pg/ml for individuals older than 70 and \leq 450 pg/ml for individuals aged between 50 and 70 years; P-tau \leq 61 pg/ml. The Institutional Review Board of the Fondazione Cà Granda, IRCCS Ospedale Maggiore Policlinico (Milan, Italy) approved this study. All patients (or their legal guardians) gave their written informed consent for this research before entering the study.

APO ϵ 4 genotyping

Apo ϵ genotype was determined by allelic discrimination as previously described [28].

CSF collection and A β and tau determination

CSF was collected between 8 and 10 a.m. after one-night fasting by LP in the L3/L4 or L4/L5 interspace according to standardized local procedures. CSF samples were centrifuged in 1500 rpm for 10 minutes at 4°C. The supernatants were aliquoted in polypropylene tubes and stored at -80 °C. CSF cells, glucose, and proteins were determined. CSF A β , tau and P tau were measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (Fujirebio, Ghent, Belgium).

Blood sample collection and processing

Whole blood was collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson & Co., Rutherford, NJ, USA). Peripheral blood mononuclear cells (PBMC) were separated on lympholyte separation medium (Cedarlane, Hornby, Ontario, CA) and washed twice in PBS at 1500 RPM for 10 min; viable leukocytes were determined using a TC20 Automated Cell Counter (Biorad Hercules, California, USA).

d4T Cellular toxicity

Viability of PBMC incubated with d4T was determined using the MTT 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) assay, as previously described [29].

Cell cultures

PBMC were resuspended in RPMI 1640 (PAN-Biotech GmbH, Am Gewerbepark, DE) supplemented with 10% human serum, L-glutamine (2 mM), and 1% penicillin (Invitrogen, Ltd, Paisley, UK) in 6-wells plate and incubated at 37 °C in a humidified 5% CO₂ atmosphere. Cells (7x10⁶/well) were either cultured in medium alone (Medium) or were primed with Lypopolisaccharide (LPS) (1 μ g/ml)(Sigma-Aldrich, St. Louis,

MO) for 2 hours and stimulated with A β ₄₂ (10 μ g/ml Sigma-Aldrich, St. Louis, MO) for 22 hours in the absence/presence of d4T (50 μ M)(Sigma-Aldrich) [30].

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1 β , IL-18, and activated Caspase-1 production was analyzed in supernatants of PBMC that were resuspended in medium alone or were stimulated (see above) by sandwich immunoassays according to the manufacturer's instructions (Quantikine Immunoassay; R&D Systems, Minneapolis, MN, USA).

Image Stream Analysis by FlowSight AMNIS

PBMCs (2x10⁶), stimulated as described above, were fixed with 100 μ l of PFA (1%) (BDH, UK), permeabilized with 100 μ l of Saponine (0.1%) (Life Science VWR, Lutterworth, Leicestershire, LE), and stained with FITC-anti human NLRP3 (Clone #768319, isotype Rat IgG2a, R&D Systems, Minneapolis, MN, USA) and PE-anti human ASC (clone HASC-71, isotype mouse IgG1, Biolegend, San Diego, CA, USA) for 1 h at room temperature; cells were then washed with PBS, centrifuged at 1,500 rpm for 10 min, resuspended in 50 μ l of PBS, and examined using the AMNIS FlowSight. Results were analyzed by IDEAS analysis software (Amnis Corporation, Seattle, WA, USA). The analysis of NLRP3 expression was performed by internalization feature utilizing a mask representing the whole cell, defined by the brightfield (BF) image, and an internal mask defined by eroding the whole cell mask. Apoptosis-associated speck-like protein containing CARD speck formation was analyzed using the same mask of internalization feature, differentiating diffuse or spot (speck) fluorescence inside of cells. Threshold mask was used to separate all ASC positive cells population in ASC-Speck spot cells or ASC-diffuse cells by the different diameter of the spot area: in ASC-speck, the spot shows a small area and high max pixel vice versa in cell with ASC-diffuse.

Protein extraction

Cytosol protein-extraction was performed in cultured PBMC by Cell Extraction Buffer (BioSource) containing 1mM PMSF, protease and phosphatase inhibitor cocktail (Sigma-Aldrich) (1:200 and 1:100). After incubation for 30min on ice, lysates were centrifuged at 12,000g for 10 minutes at 4°C. Protein concentration was determined by Bradford assay at 595nm.

Western Blot

Proteins (15 μ g) were separated by electrophoresis into 4-12% NuPAGE® Bis-Tris gels (Invitrogen) and blotted on nitrocellulose filter (GE Healthcare). Blots were blocked 1 hour at room temperature on a shaker in 5% fat-free dried milk in TBS-T buffer (50mM Tris-HCl pH 7.6, 200mM NaCl, 0.1% Tween 20). Blots were incubated over night on a shaker at 4°C with the following rabbit Antibody (Ab): anti-beclin1 (1:300, Cell Signaling), anti-LAMP2A (1:400, Abcam) anti-p-p38 (T180/Y182 1:350, Cell Signaling) anti-pERK1/2 (T202/Y204 1:300, Cell-Signaling), anti-pAKT (S473 1:500, Cell Signaling), anti-p-p70S6K (1:500 Cell Signaling), anti-pCREB (1:800, Biosource) and mouse anti-Bax (1:300, Chemicon). Abs were diluted in 5%

fat-free dried milk in TBS-T buffer. A mouse anti-actin Ab (1:20000, Sigma-Aldrich) was used as internal standard. A peroxidase-linked anti-rabbit/mouse (1:5,000; Sigma-Aldrich) IgG secondary Ab was incubated for 1 hour, at room temperature on an orbital shaker in TBS-T buffer containing 3% fat-free dried milk. Signals were detected by chemiluminescent reagents (ECL Plus Kit; Amersham), visualized on X-ray film, quantified using ImageJ software and expressed as the ratio between the target and the actin signals.

Statistical analysis

Quantitative data were not normally distributed (Shapiro–Wilk test) and are thus summarized as median and interquartile range. Comparisons between the different cultural conditions were made using: 1) a 2-tailed Mann–Whitney U test performed for independent samples for NLRP3 complex, cytokines and caspases production quantified by Elisa; 2) One-way repeated ANOVA test, followed by Tukey’s multiple comparison test for protein extracts evaluated by WB analysis. Data analysis was performed using the MedCalc statistical package (MedCalc Software bvba, Mariakerke Belgium). *p*-Values of less than 0.05 were considered statistically significant.

Results

1. Epidemiological, clinical and genetic characteristics of AD patients

No differences were observed across diagnostic group in age, sex, years of education, or APOE ϵ 4 carrier status (Table 1). APOE genotyping was available for all subjects and was dichotomized in being a carrier of at least one allele ϵ 4 and carrying no ϵ 4 allele.

2. Diagnostic accuracy of CSF biomarker analysis

ROC analyses were performed to test CSF biomarkers in relation to clinical diagnoses for AD. CSF biomarker variables included $A\beta_{42}$, t-Tau, and p-Tau (i.e. phosphorylated at threonine 181 or p-Tau181p) levels in ng/L, as well as ratios (t-Tau/ $A\beta_{42}$ and p-Tau/ $A\beta_{42}$). Each CSF measure was dichotomously classified as positive or negative for AD according to validated cut-off values: Cut-off thresholds of normality were: $A\beta \geq 600$ pg/ml; tau ≤ 500 pg/ml for individuals older than 70; Ptau ≤ 61 pg/ml [31]. Results are shown in Fig. 1.

3. d4T down-regulates NLRP3-inflammasome activation

d4T was shown to significantly reduce the activation of the NLRP3 inflammasome in a THP-1-derived macrophage cell line that was LPS-primed and $A\beta_{42}$ -stimulated; we verified whether the same effect could be observed in PBMC of AD patients. Results obtained when NLRP3 and apoptosis-associated speck-like protein containing CARD (ASC)-speck formation was investigated showed that ASC speck formation was significantly increased in LPS-primed and $A\beta_{42}$ -stimulated compared to unstimulated cells ($p < 0.05$) (Fig. 2a). ASC-speck positive cells were significantly decreased by d4T ($p < 0.05$) (Fig. 2b).

These results were confirmed when activated caspase-1, interleukin-1 β and IL-18 production was analyzed. Thus, IL-1 β , IL-18 and activated caspase-1 production was significantly increased in LPS + A β ₄₂ stimulated PBMC of AD patients ($p < 0.05$) (data not shown) and the production of all these proteins was reduced by d4T; the differences reached statistical significance for IL-18 ($p = 0.004$) and activated caspase-1 ($p = 0.001$) (Fig. 3. a,b,c).

4. d4T modulation of MEK-(ERK and p38) and AKT-Protein Kinase pathways

ERK, p38 and AKT phosphorylation was evaluated by WB in PBMC of AD patients that were either unstimulated (med) or had been LPS-primed and A β ₄₂ stimulated in the absence/presence of d4T. Results showed that the level of all these proteins was increased in LPS + A β ₄₂ stimulated PBMC. Addition of d4T to cell cultures significantly down-modulated p38 ($p = 0.0008$) (Fig. 4a) whereas it further up-regulated p-ERK1,2 ($p = 0.025$) (Fig. 4b) and p-AKT ($p = 0.015$) (Fig. 4c).

5. Downstream MEK and AKT pathways: CREB and Bax

Protein expression of CREB and Bax, two essential downstream mediators in the ERK and AKT-mediated pathways, as well as CREB phosphorylation (p-CREB) status were analyzed next in the same cultural conditions. Also in this case, the level of these proteins was increased in LPS + A β ₄₂ stimulated PBMC. d4T resulted in a further upregulation of both p-CREB ($p = 0.017$) (Fig. 5a) and Bax ($p = 0.0024$) (Fig. 5b).

6. Effect of d4T on autophagy-pathways

Beclin-1, LAMP2A and phospho-p70S6K signals were examined using WB analysis in protein extract of PBMCs from AD patients that were either unstimulated (med) or LPS-primed and A β ₄₂ stimulated in the absence/presence of d4T. Beclin-1, an index of the activation of p-ERK1/2- and p-AKT-mediated macroautophagy was only marginally increased by d4T in LPS-primed and A β ₄₂-stimulated-PBMCs (Fig. 6a) while p70S6Kinase, a their down-stream target, was up-phosphorylated by d4T ($p < 0.005$). Notably, both phospho-p70S6K isoforms: the 70 KDa cytosolic form and the 85KDa nuclear one, were significantly upregulated by d4T (Fig. 6b). LAMP2A, that may be also regulated by AKT signaling, plays a pivotal role in the chaperon (CMA) mediated autophagy pathway, and it was significantly increased by d4T in LPS-primed and A β ₄₂-stimulated-PBMCs ($p = 0.0007$), too (Fig. 6c).

Discussion

Microglia-driven neuroinflammation has been identified as a key player in the pathogenesis of AD; deposition of A β ₄₂ in particular is an early event in the disease that induces microglia-activation and inflammation. It has been shown that this process is highly dependent on the activation of the NLRP3/ASC-inflammasome both in microglial cells [32, 33] and in circulating peripheral monocytes [34]. Thus, systemic inflammation triggers a neuroinflammatory response that results in microglial activation with deleterious consequences for learning and memory in rodent models [35–37] and in humans [38–44]. Neuroinflammation has also convincingly been shown to down regulate autophagy [45–47]; we have

recently described how NLRP3/ASC-inflammasome activation hampers microglial clearance of A β in an *in vitro* experimental model of macrophages-derived THP-1 cell line [19]. Notably, in that experimental setting stavudine (d4T), a prototypical NRTI designed used in HIV- infection prevented active caspase-1 release but it did not restore microglial A β phagocytosis. Given this background, we investigated A β -stimulated inflammation and autophagy and the effects of d4T on these processes using peripheral blood immune cells of AD patients.

Results herein show that d4T is capable of significantly reducing NLRP3 inflammasome activation and the down stream production of proinflammatory cytokines even in PBMC of AD patients. Results obtained when autophagy was analyzed showed that p-AKT- and p-ERK as well as the expression of Bax, p-CREB, LAMP2A, and of the cytosolic and nuclear p70S6 Kinase isoforms was increased in in d4T-stimulated PBMC of AD patients, indicating that d4T up regulates the downstream AKT- and MEK-kinase pathways. In contrast Beclin-1 was modulated by d4T, suggesting that this compound mainly stimulates chaperone-mediated autophagy (CMA).

Autophagy is a complex molecular system that removes damaged organelles and proteins from the cytoplasm and is involved in programmed cell death and neurodegeneration; importantly, autophagy also represents an alternative pathway of cellular defense by removing intracellular pathogens [48]. The three main forms of autophagy are: macroautophagy-beclin-1 dependent, microautophagy endosomal mediated, and CMA the latter is associated with lysosomal degradation and is mediated by a cytoplasmic complex of chaperone proteins, including p70S6K, that interacts with LAMP2A [49, 50]. The phosphorylation of p70S6K by AKT signal is also able to interact with Atg proteins, promoting phagosome formation in mTOR-independent way [51]. All these three forms of autophagy crosstalk with each other to eliminate aberrant proteins and modulating cell apoptosis [52].

Emerging evidences show that CMA is involved in the degradation of several proteins associated with neurodegenerative diseases [53] including α -synuclein [54, 55], huntingtin [56] and amyloid precursor protein [57]. Degradation by CMA involves the selective delivery of single proteins to lysosomes upon the recognition and binding of a specific KFERQ-like pentapeptide motif to cytosolic hsp70 [58]. The chaperone-substrate complex binds the cytosolic tail of LAMP2A, triggering its assembly into a multimeric complex (protein target, KFERQ, p70S6K and LAMP2A) that mediates substrate translocation in lysosome [59, 60]. CMA is modulated by the AKT and ERK signaling pathways: p-AKT- and p-ERK; both these proteins were increased by d4T in A β -stimulated PBMC of AD patients, as were Bax, p-CREB, p-p70S6K and LAMP2A. Because active AKT can phosphorylate a variety of downstream molecules including Bax and CREB, these results indicate that d4T can up regulate this whole signaling pathways.

d4T-induced modulation of CREB is particularly important as this protein has a well-documented role in neuronal plasticity, and long-term memory formation, and, on the other hand, CREB alterations result in oxidative stress, apoptosis, and neurodegeneration [61, 62]. Importantly, p-CREB expression is reduced in PBMC of AD subjects [63]. d4T-induced Bax upregulation is potentially important as well, as Bax is a pro-apoptotic protein that promotes mitochondrial membrane permeabilization. d4T-mediated up regulation

of p-CREB and Bax could thus stimulate CMA and apoptosis to counteract A β toxicity. Notably, behavioral symptoms and progression of AD in a murine model were shown to be alleviated by the activation of PI3K/AKT signaling pathway [64]. Increased p-AKT expression and activation of Bax-mediated apoptosis were also demonstrated to improve the memory capacity of AD rats [65].

Potentially important is also the effect of d4T on p38. Thus, p38 is activated in macrophages, neutrophils, and T cells by extracellular mediators of inflammation including cytokines, chemokines, and LPS [66, 67], and phospho-p38 induces Th1 immune response and the production of proinflammatory cytokines [68]. The observed d4T down-regulation of this protein thus could be beneficial in reducing neuroinflammation in AD.

Conclusions

Efficient autophagic activity prevents the activation of inflammasomes [69], and stimulation of autophagy was shown to reduce soluble τ , as well as A β and amyloid plaques in 3xTgAD mice [70]. Herein we show results obtained in peripheral immune cells of AD patients that indicate how d4T, besides reducing NLRP3 inflammasome activation, up-regulates CMA as well as CREB phosphorylation and the pro-apoptotic protein Bax. Taken together, these findings could warrant the investigation of the use of D4T the clinical scenario.

Abbreviations

A β =amyloid beta

AD =Alzheimer's Disease

AKT = protein kinase b;

ASC =apoptosis-associated speck-like protein containing card

Bax = bcl-2-like protein 4,

BBB = blood-brain barrier

CMA = chaperone autophagy mediated

CSF= cerebrospinal fluid

CREB= camp response element-binding protein

d4T = stavudine

ELISA = enzyme-linked immunosorbent assay

ERK= extracellular signal-regulated kinases

IL-1 β = interleukin 1-beta

IL-18 = interleukin-18

LAMP2a = lysosome-associated membrane protein 2

LPS: lipopolysaccharide

NLRP3 = nod-like receptor 3

PBMC = peripheral blood mononuclear cells

WB = western blotting

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Helsinki Declaration. Informed consent was obtained from the patients. Informed consent to participate in this study was given by all subjects or their caregivers. All samples were obtained from the Neurodegenerative Disease Unit of the Fondazione Cà Granda, IRCCS Ospedale Maggiore Policlinico, University of Milan (Milan, Italy)

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during the current study are available from the corresponding author request.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' contributions

FLR, CPZ, MS and MC conceived and designed the experiments. CB, AB, EC, FP, IM performed the experiments and analyzed the data. FLR and CPZ wrote the paper, and MC revised it. CF, DG, ES and CF

are responsible for the clinical cohorts of patients. All authors read and approved the final paper.

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Tables

Table 1. Demographic, clinical and genetic characteristics of the individuals enrolled in the study.

Alzheimer's Disease patients (AD)	
N	13
Gender (M:F)	5:8
Age (years)	77.20±6.34
MMSE	17.07±4.51
A β (pg/ml)	530±70.50
Total- τ (pg/ml)	647.31±300
Phospho- τ (pg/ml)	88.62±21.89
Apo ϵ 4	30%

Data are expressed as mean \pm standard deviation.

MMSE: Mini-Mental State Examination

Figures

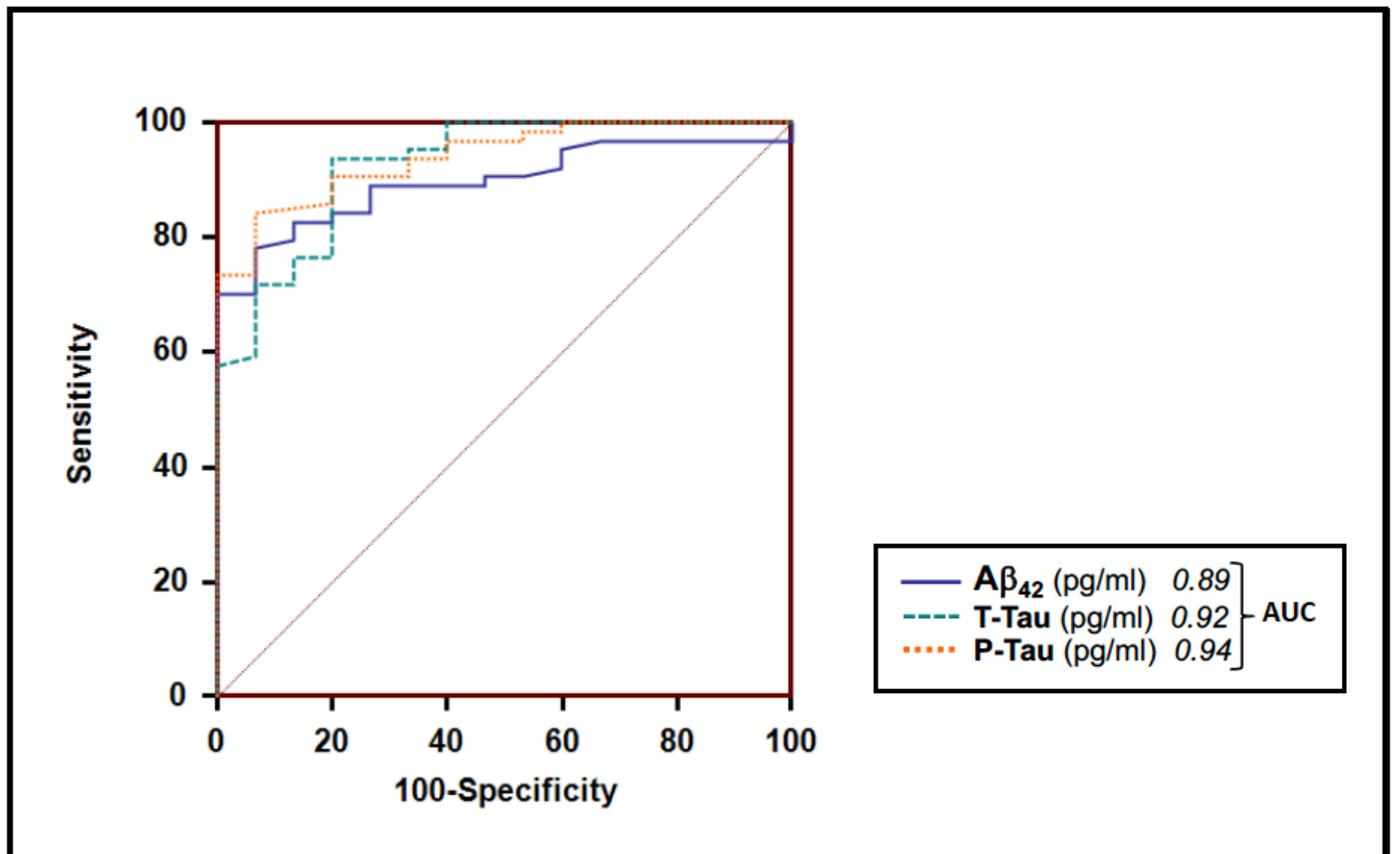


Figure 1

ROC Curves; ROC analyses were performed on CSF biomarkers to test the relation to clinical diagnoses of AD patients: amyloid- β (A β 42) with a sensitivity of 76.9% at 93.3% specificity (blue line), total tau (T-tau) with a sensitivity of 93.7% at 80% specificity (green-dotted line) and phosphorylated-tau (P-tau) with a sensitivity of 84.6% at 93.7% specificity (orange dotted-line). AUC of 0.89 (0,809 to 0,954, 95% CI) for A β 42; 0.92 (0,845 to 0,973, 95% CI) for T-tau; and 0.94 (0,864 to 0,981, 95% CI) for P-tau are shown.

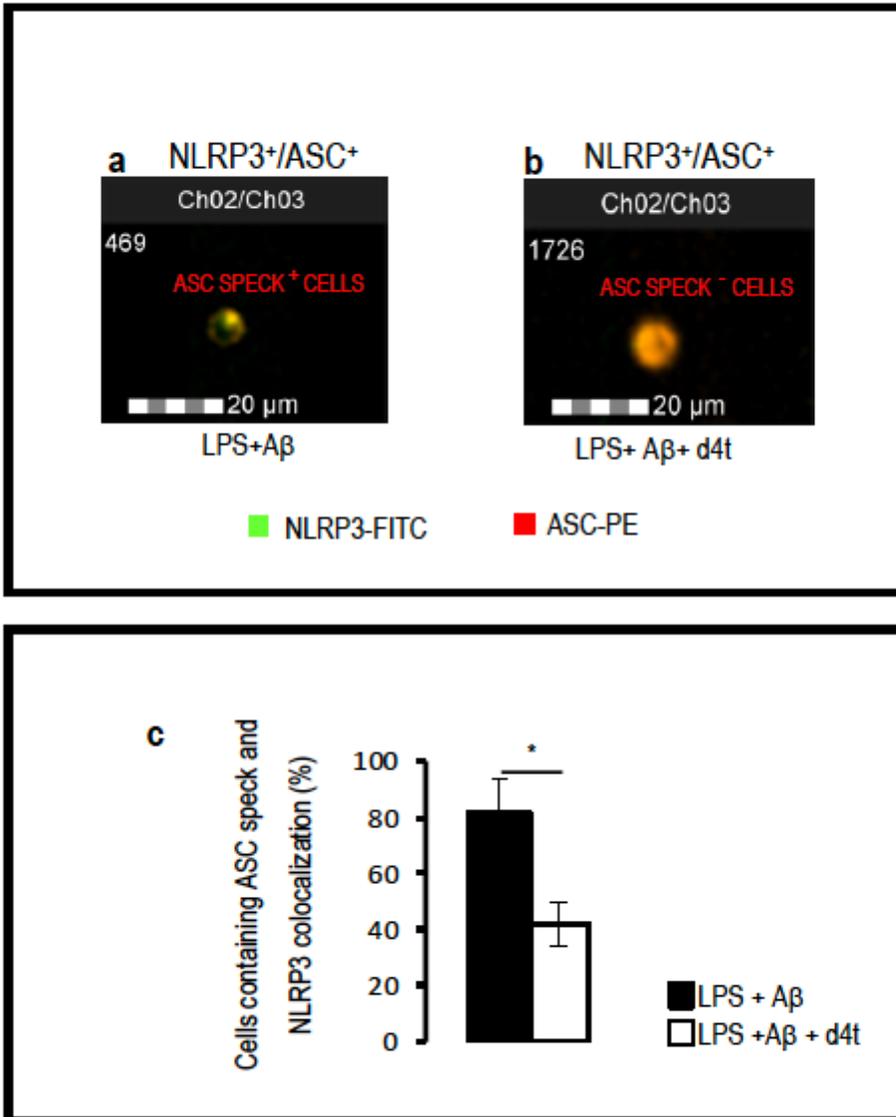


Figure 2

Inflammasome production and ASC-Speck formation. Representative images of Nod-like receptor protein 3 (NLRP3) expression and apoptosis-associated speck-like protein containing CARD (ASC)-speck

formation in LPS-primed and A β -stimulated PBMCs of AD patients in the presence/absence of d4T. ASC-speck (a) and ASC-diffuse (b) images were obtained by AMNIS FlowSight-merged (Ch02/03) NLRP3-FITC (Ch02) and ASC-PE (Ch03) fluorescences; analysis of ASC-specks and its co-localization with NLRP3 were performed by IDEAS software that provides tools to evaluate image regions (masks) and perform calculations (features). The percentage of ASC speck positive cells is shown in panel c. Statistical significance is shown (* $p < 0.05$).

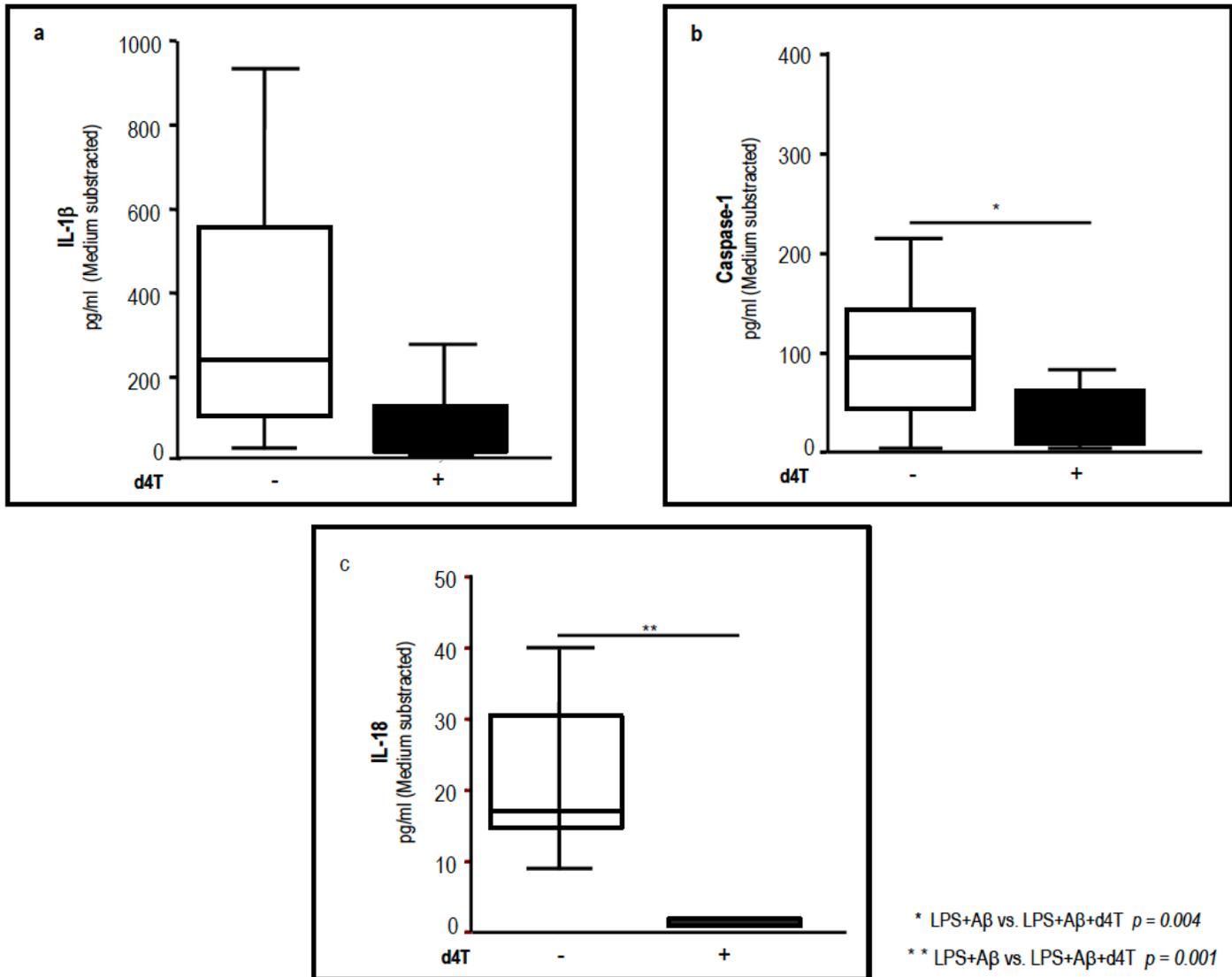


Figure 3

Caspase-1 and inflammasome effector cytokines production. Interleukin (IL)-1 β (a), Caspase-1 (b) and IL-18 (c) production by unstimulated (medium) or by LPS+A β -stimulated cells in the presence/absence of d4T. Data are expressed as median of cytokines and caspase-1 concentration (pg/ml) of stimulated cells medium subtracted. Statistical significance is shown (* $p < 0.05$).

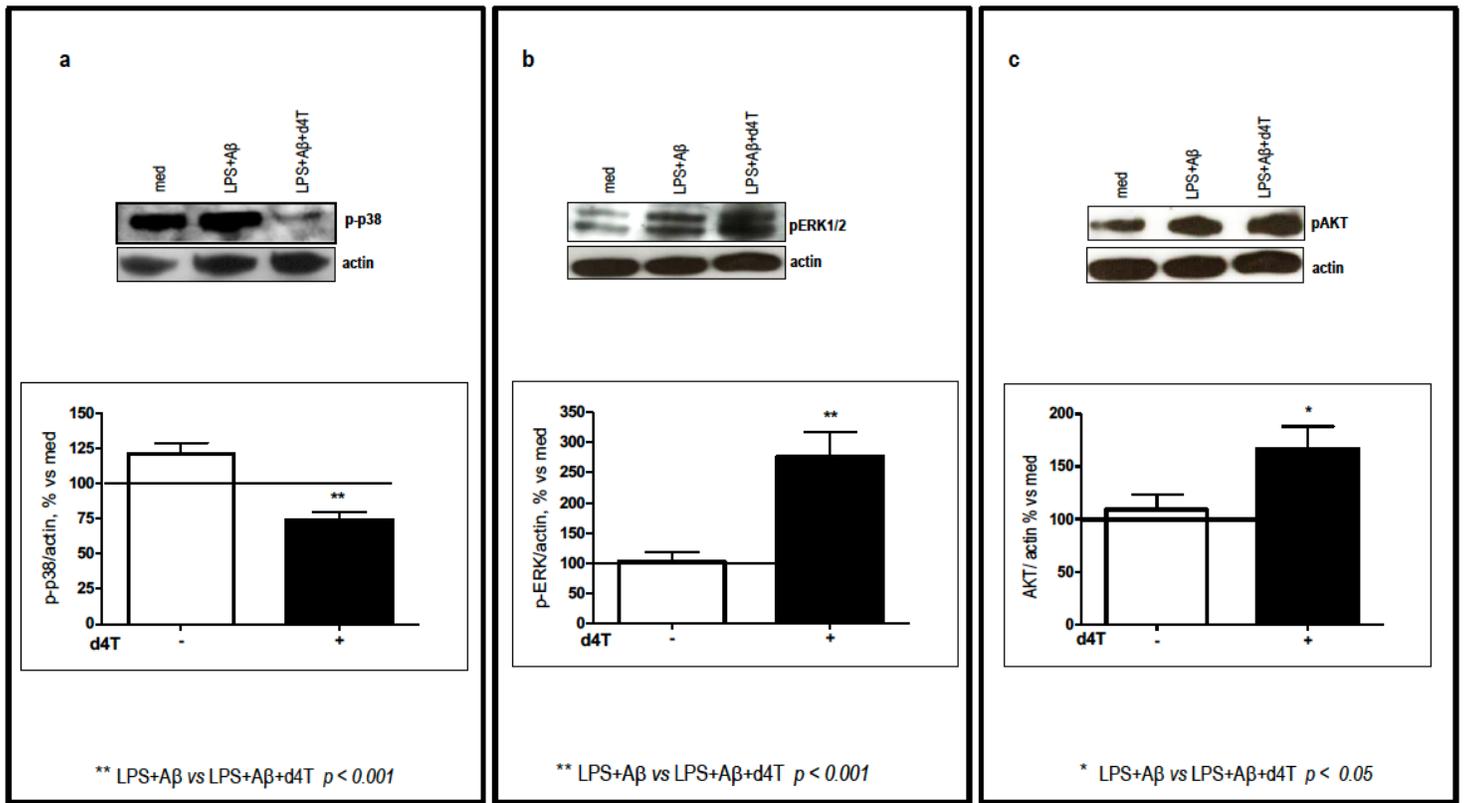


Figure 4

ERK1/2 and AKT phosphorylation. Western blot analysis (WB) of MEK-(ERK and p38) and AKT-Protein Kinase pathways in LPS-primed and A β -stimulated-PBMCs of AD patients in the presence/absence of d4T. Phosphorylation status of p38 (38KDa)(a), ERK1/2 (42-44 KDa) (b) and AKT (60KDa) (c) kinases was evaluated in cytosol protein extracts of cells. Each result is expressed as the percentage of the ratio between phosphorylated-kinase and actin versus unstimulated cells (medium, 100%, = black line). The values are expressed as percentage means \pm SD. Statistical significance is shown (** $p < 0.001$, * $p < 0.05$). Relative representative blots from single independent experiment are shown in upper part of panels a, b, and c. Actin protein was used as a loading control (42KDa).

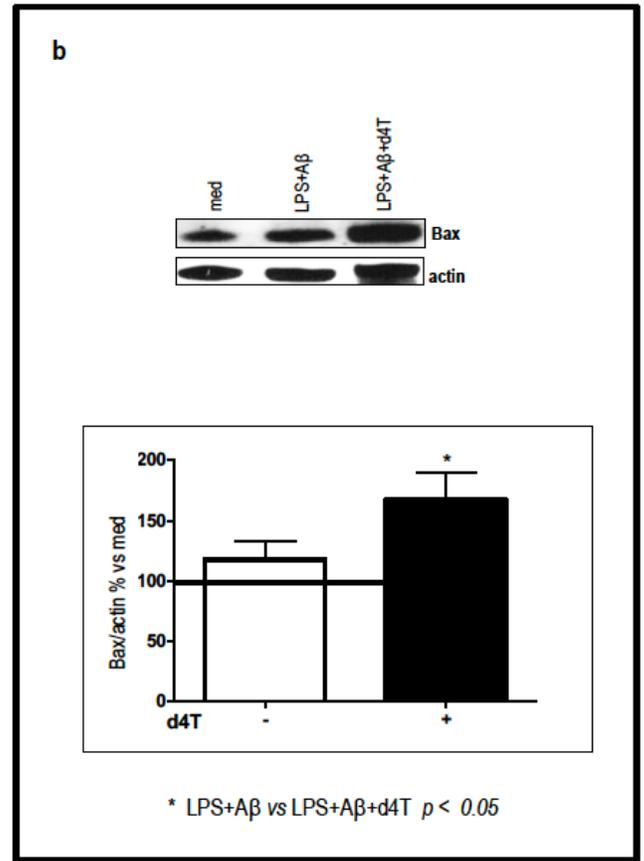
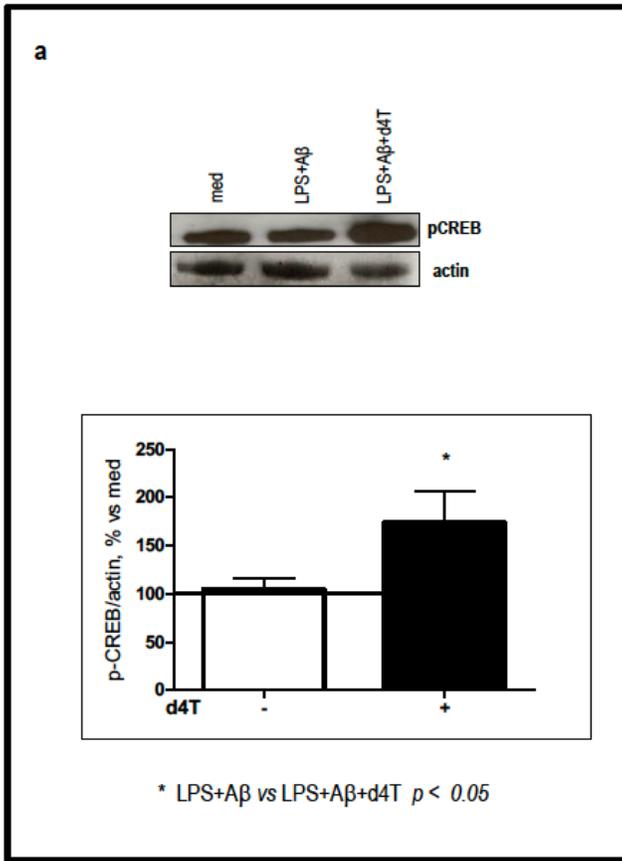


Figure 5

CREB and Bax protein production. WB analysis of the phosphorylation status of CREB (43 KDa) (a) and Bax (21KDa) (b) production in cytosol protein extracts of LPS-Primed and Aβ-Stimulated-PBMCs from AD patients in presence/absence of Stavudine (d4T). Each result is expressed as the percentage of the ratio between phosphorylated-kinase and actin versus unstimulated cells (medium, 100%, = black line). The values are expressed as percentage means±SD. Statistical significance is shown (* $p < 0.05$). Relative representative blots from single independent experiment are shown in upper part of panels a and b. Actin protein was used as a loading control (42KDa).

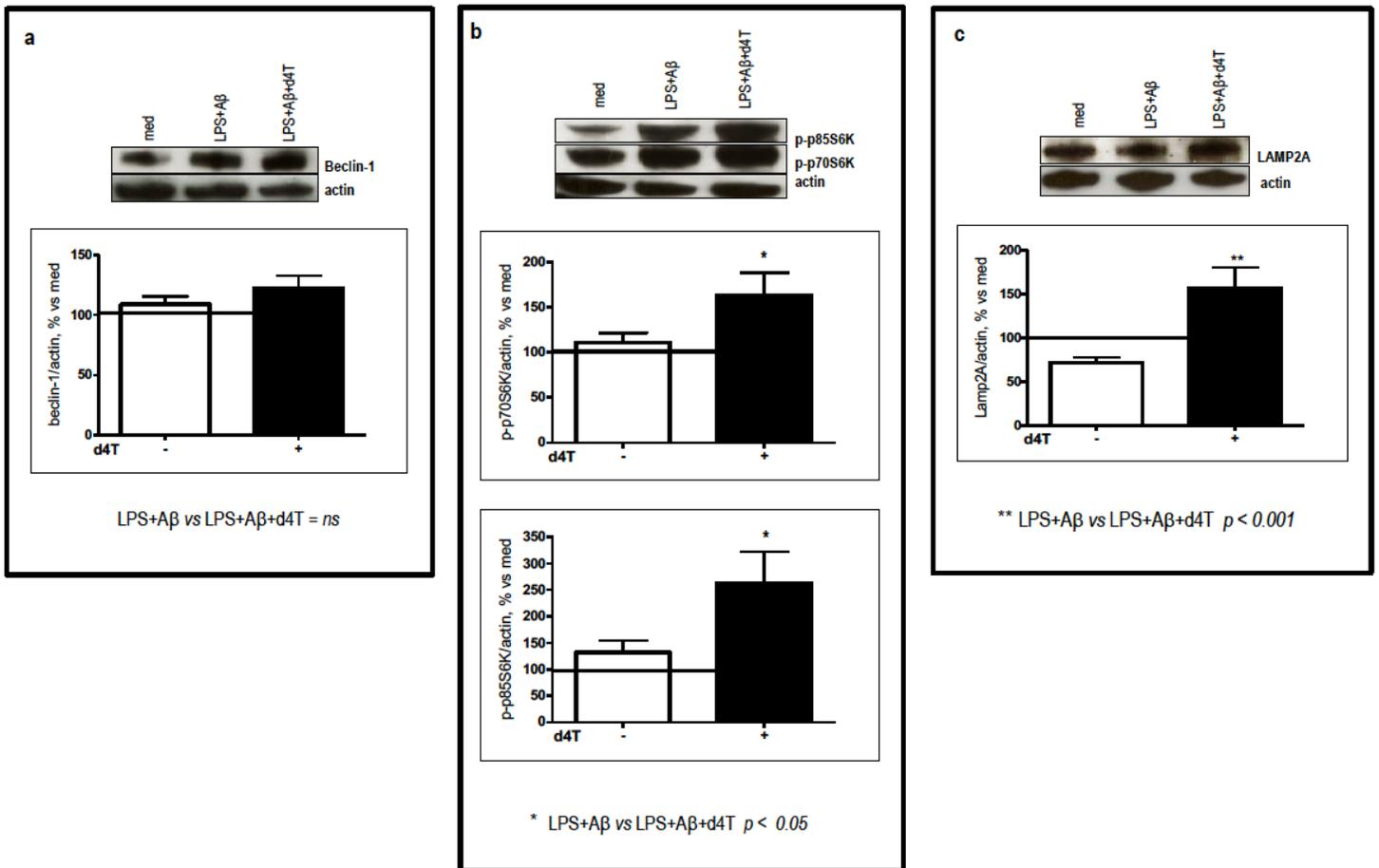


Figure 6

Beclin-1 and down-stream CMA-mediated autophagy protein production. The determination by WB of Beclin-1 (a, 52KDa) and downstream targets that are involved in CMA-autophagy as phospho-p70S6K, phospho-p85S6K (b, 70 and 85 KDa) and Lamp2A (c, 100KDa), in cytosol protein extracts of LPS-Primed and Aβ-Stimulated-PBMCs from AD patients in presence/absence of Stavudine (d4T). Each result is expressed as the percentage of the ratio between phosphorylated-kinase and actin versus unstimulated cells (medium, 100%, = black line). The values are expressed as percentage means±SD. Statistical significance is shown (** $p < 0.001$, * $p < 0.05$). Relative representative blots from single independent experiment are shown in upper part of panels a,b and c. Actin protein was used as a loading control (42KDa).