4. Results

4.1 Release activity as a parameter of microglial activation state

The study was conducted using primary microglial culture as a model system. It is to be considered that cultured microglia show certain level of activation. Even after prolonged maintenance of isolated microglia in LPS-free media, the extensive ramification of microglial processes, as it might be seen in tissue sections or in organotypic tissue cultures (Fig. 1), is rarely achieved in the cell culture dish. Still, only following a stimulation (e.g. with LPS) microglia in culture gain a typical, fully reactive phenotype (Fig. 6).

A) **B)**

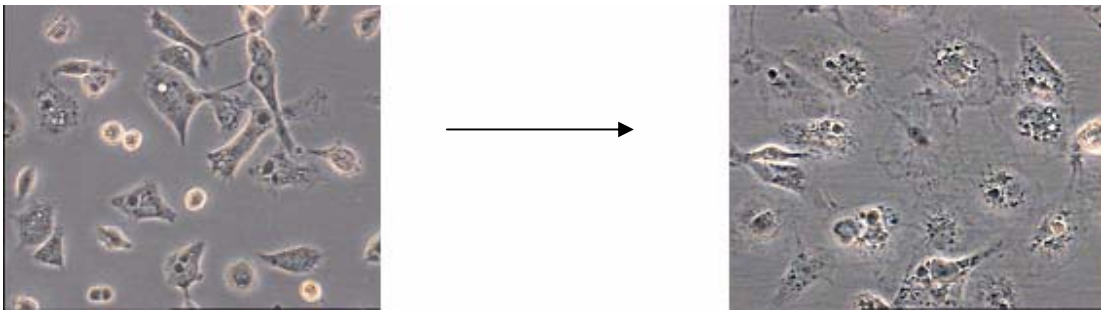


Figure 6. Microglia in primary cell culture. Microglia (A) prior to and (B) after LPS (10 ng/ml, 20 h treatment) induced stimulation.

Monitoring of cyto/chemokine and NO release activity of the microglia has been chosen as one of the most relevant and reliable markers for their activation. Therefore, levels of cytokine (TNFα, IL-1ß, IL-18, IL-6, IL-12) and chemokine (MIP-1α, KC, MIP-1ß and MCP-1) secretion as well as the release of NO were routinely determined. The immunological methods for quantification of released factors in the cell culture supernatant, i.e. ELISA, enabled high sensitivity of detection (for details see Material and Methods) and reproducibility. The release as a parameter of microglial activity is, especially in *in vitro* studies, characterized by a broad range of responses from “no” for control, unstimulated groups to “strong” for LPS, for example. Compared to the level of release evoked by LPS, a standard stimulus for macrophage like cell-types used as a

positive control, the release-inducing potential of other stimuli could be then easily compared (Hanisch et al., 2001).

To detect the gene expression profile that precedes the cytokine release from the cell, RT-PCR was performed. Treatment with LPS (100ng/ml) caused a time-dependent transcription of TNFα, IL-1ß, IL-6, IL-10 and IL-12 genes (as observed over 24h) (Fig. 7). As shown in Fig. 4, in microglia cytokine expression seems to be a very prompt event. Following activation with LPS, TNF α, IL-1ß and IL-12 mRNA could be readily detected (after 1 h of stimulation). The expression of IL-10, which is regarded as an anti-inflammatory cytokine, was not observed within 24 h of LPS treatment.

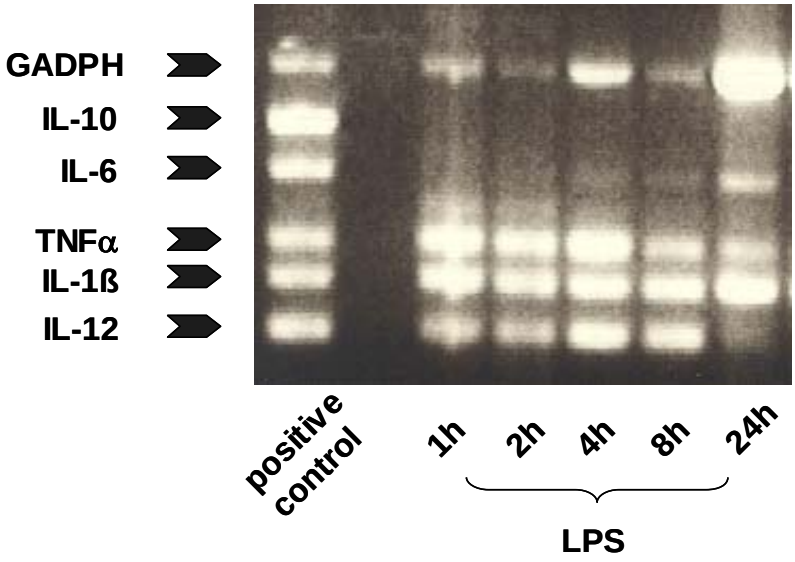


Figure 7. Cytokine expression in microglia following LPS stimulation as detected with RT-PCR. Microglial culture was stimulated with 100 ng/ml LPS for 1, 2, 4, 8 and 24 hours. RNA was isolated using Trizol reagent and subsequently complement cDNA was synthesized. The RT-PCR was carried out using a CytoXress Mouse Cytokine Sepsis Set 2 Kit. RT-PCR products were run on 2% agarose gel electrophoresis and recognized by the size (bp): 351 (TNF), 294 (IL-1ß), 237 (IL-12p40), 453 (IL-6), 538 (IL-10), and 658 (glyceraldehyde-3-phosphate dehydrogenase, GAPDH, as house-keeping gene).

In order to assess the release potency of the cells, LPS stimulation for a period of 24 h was performed. A substantial and reproducible release of TNFα, IL-6, IL-12, MIP-1α and KC was determined upon LPS (100 ng/ml) treatments (Fig 8), whereas

supernatants of unstimulated cultures were virtually devoid of any measurable amounts (data not shown).

Concentration (pg/ml)				
TNFα	**IL-6**	**IL-12**	**MIP-1α**	**KC**
7.8 ± 0.8	**30.2 ± 1.8**	**58.5 ± 4.7**	**189.6 ± 8.9**	**17.2 ± 0.8**

Figure 8. LPS-induced release of cyto- and chemokines from microglia *in vitro*. Microglial cultures were stimulated with 100 ng/ml LPS for 24 h. The resulting release was determined in the supernatants by factor-specific ELISAs. Data are given as mean ± SEM taken from two to four independent experiments (n=23-32).

4.2 Co- stimulations of microglia with Aß and cytokines

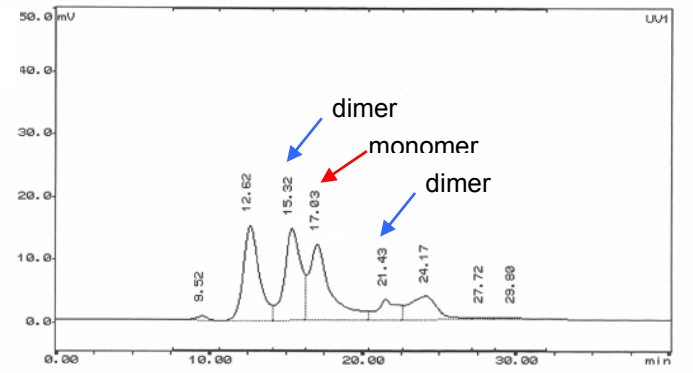
4.2.1 Amyloid beta- a weak inducer of microglial activation

4.2.1 .1. Preparation of Aß peptides and aggregates

Gel filtration of solubilized plaque components has shown that only those fractions containing Aß peptides induce neurotoxic microglia (Giulian et al., 1996). Comparison of the soluble Aß secreted by cells, soluble Aß in CSF, and insoluble Aß isolated from the AD brain has revealed that there are numerous Aß species with extensive amino and carboxyl terminal heterogenity. Quantitatively, the major 4-kDa species in human CSF is Aß 1-40 (more than 60- 70%), although some Aß 1-42 (~15%) is also present along with minor amounts of other peptides (e.g. Aß 1-28, Aß 1-33, Aß 1-34, Aß 3-34, Aß 1-37, Aß 1-38, Aß 1-39) (Suzuki et al., 1994; Seubert et al., 1992). Thus, the ratio of 5 :1 (Aß 1-40 : Aß 1-42) approximately represents the real concentration ratio of the major peptides as present in the brain. Although Aß 1-40 is the main species as being produced, the principal species deposited within the parenchyma of the AD brain is Aß 1-42 (Gravina et al., 1995; Iwatsubo et al., 1994). Immunocytochemical evidence of initial Aß 1-42 deposition in AD and Down's syndrome patients (Iwatsubo et al., 1995; Lemere et al., 1996) fits well with biochemical studies showing that the Aß 1-42 peptide,

with two additional hydrophobic residues (Ile and Ala, Fig. 2), aggregates into amyloid fibrils far more rapidly than the Aß 1-40 peptide does (Jarrett et al., 1993).

(A)



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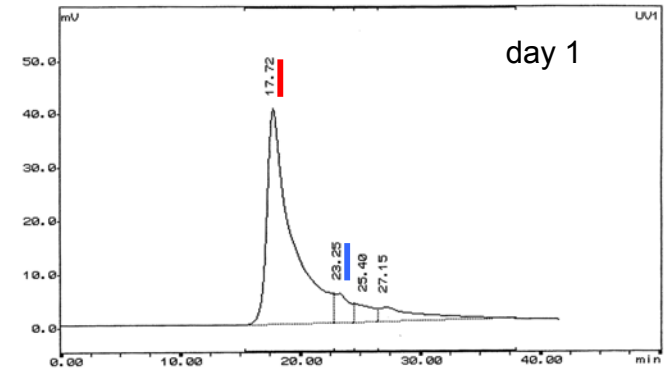

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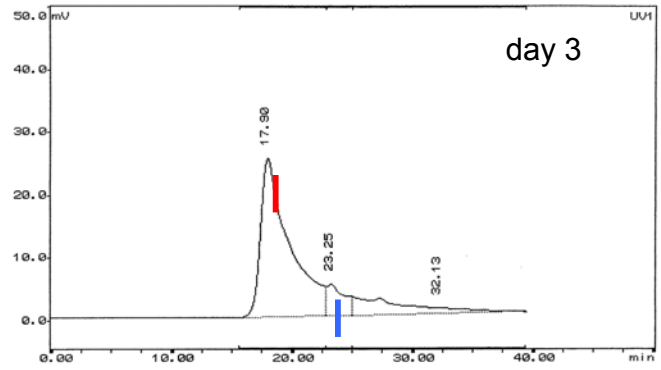

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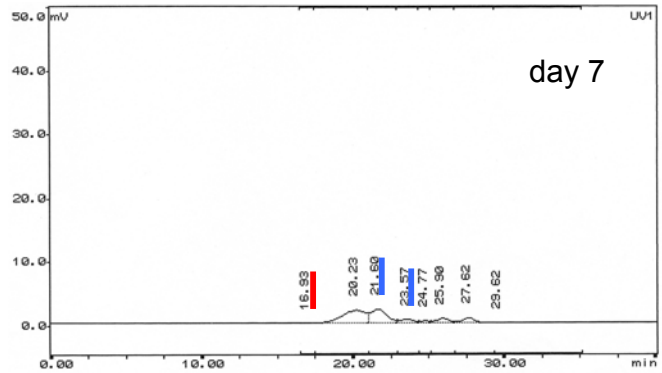


(E)

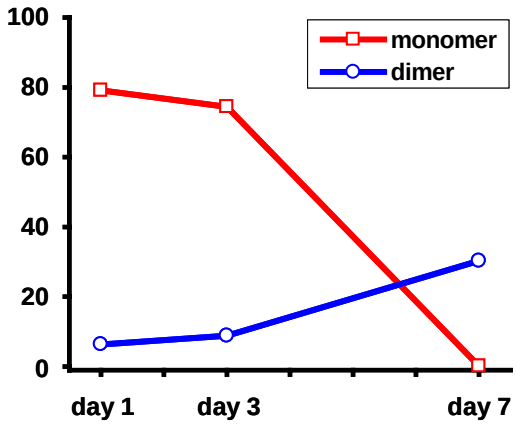


Figure 9. Amount of Aß peptide in monomeric and oligomeric form over increasing incubation time period. Size exclusion calibration column: the *arrows* point monomeric (A) and mono- and dimeric (B) Aß forms (for details see Material and Methods). Size exclusion chromatography profile of Aß samples incubated 1 (C), 3 (D), and 7 (E) days. (F) Kinetics of decline of monomeric species concentration with spontaneous formation of dimeric forms. Concentration of monomers (red) and dimmers (blue) calculated as the % in whole protein preparation, is measured after 1, 3 and 7 days of incubation.

To characterize the structural prerequisites for amyloid peptide, in order to activate microglial cells, comparative treatment of the cells with “aged” and “fresh” peptide was carried out. Fresh preparations of more soluble and “pathogenic” fibrillary forms (aggregates) of the peptide(s) were tested at various concentrations, ratios and “aging” states (as shown in Fig. 5). Furthermore, to determine the nature of peptide “species” present in Aß preparations, which would be later used for microglial treatment and eventually would be responsible for microglial activation, the “fresh” and “aged” Aß mixtures were additionaly analysed for the presence of Aß monomers and small oligomers by size exclusion chromatography (Fig. 9). On the other hand, the presence of fibrillar aggregates was assessed using electron microscopy (Fig.10).

The results of size exclusion chromatoghraphy analysis indicated a decline in the concentration of monomeric forms present in the tested preparations with time of incubation (Fig.9C-E). The strong decrease was observed, in particular, between 3 and 7 days of sample incubation, and occurs in parallel with forming dimmers (Fig.9F) and

probably other oligomers, as well as protein aggregation into the fibrils (Fig. 10). As presented in Fig.10, electron microscopy images revealed clearly the presence of fibrils in the “aged” samples after 7 days of incubation.

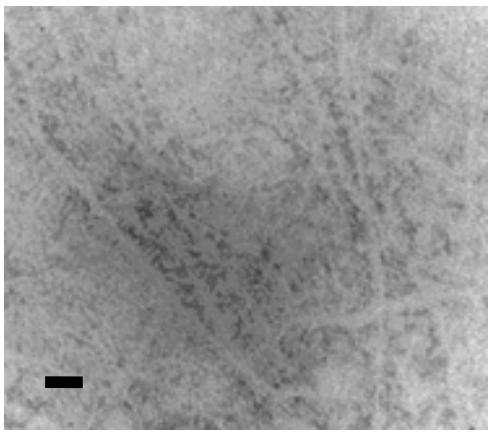


Figure 10. Fibril formation of aged Aß mixture. Aß 1-40/ Aß 1-42 mixture in the 1:1 ratio, conc. 10 µM: 10 µM, was incubated for 7 days at 37°C, thereafter samples were analysed for the presence of the amyloid fibrils by electron microscopy. Micrographs reveal a fibrillar structure of the mixture. Bar: 90 nm.

4.2.1.2. Effect of Aß preparations on microglial release activity; Fresh versus “aged” peptide preparations and Aß 1-40 / Aß 1-42 mixtures in various ratios

Microglial cultures were exposed for 24 h to “fresh” and “aged” peptides Aß 1-40 and Aß 1-42, separately, as well as mixed in ratios 1:1, 5:1 and 1:5 (in a concentration range 2.5 - 20 µM) (as shown in Fig. 8), and the level of induced release (TNFα, IL-6, IL-12, KC and MIP-1α) was quantified by ELISA.

Testing of different Aß preparations for their release-inducing activity revealed very weak stimulating potency, in general. Both Aß 1-40 and Aß 1-42 separately were very ineffective stimuli (data not shown). Aged samples induced a sligthy stronger response than corresponding fresh samples when Aß 1-40/ Aß 1-42 fresh vs. aged mixture in ratio 5:1 (Fig. 11) was applied to the culture. Further, among tested aged mixtures, Aß 1-40/ Aß 1-42 mixture in the ratio 1: 1, at the conc. 10 µM, induced a reproducibly measurable release and was selected for further experiments (Fig. 12).

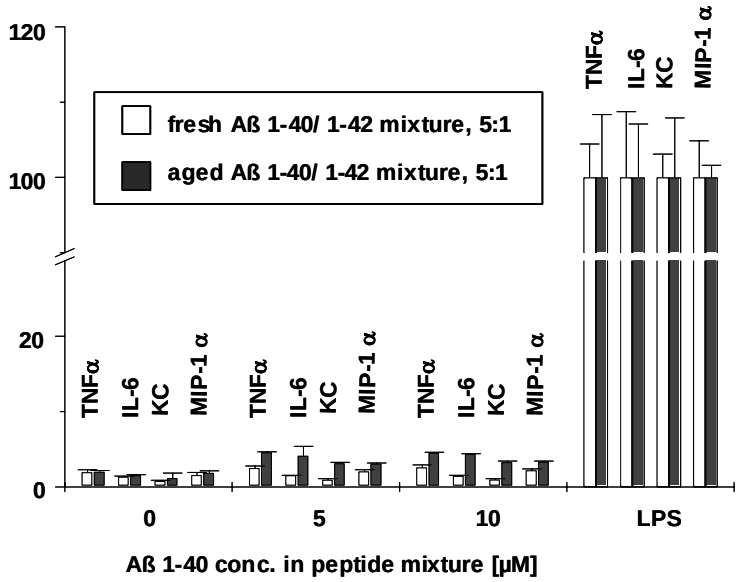
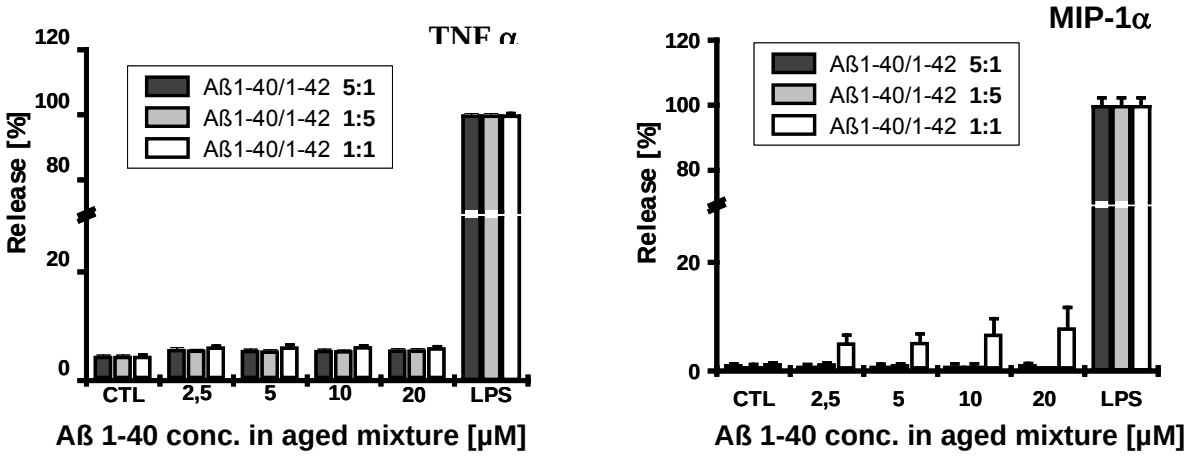


Figure 11. Inducing potency of fresh vs. aged Aß 1-40/ Aß 1-42 mixtures. Cells were stimulated with fresh or aged mixtures of Aß 1-40/ Aß 1-42 in ratio 5:1 (conc. 5µM: 1µM and 10µM: 2µM) for 24 h, and amount of the released TNFα, IL-6, KC and MIP-1α was determined in culture media by ELISA. Results are expressed as % value obtained upon LPS (100 ng/ml) stimulation and given as mean ± SEM, as summarized from 2 experiments (n = 8).



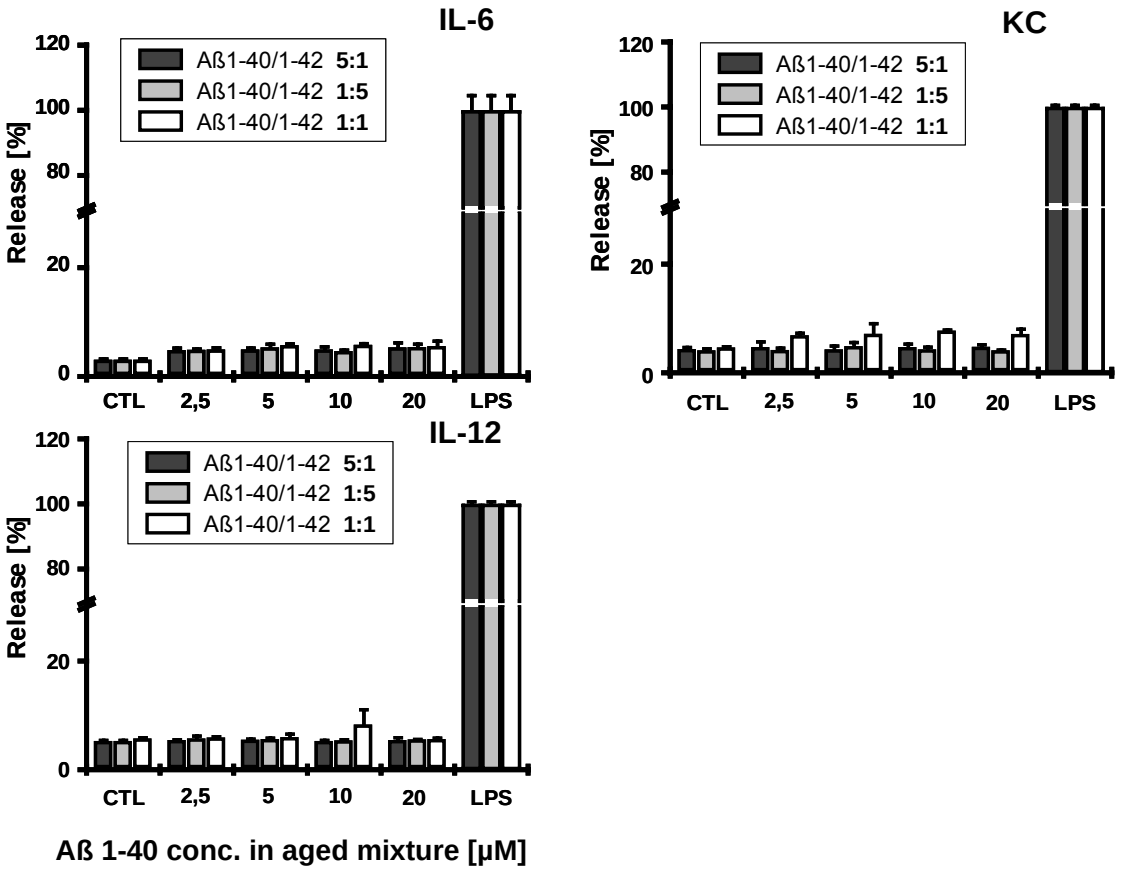
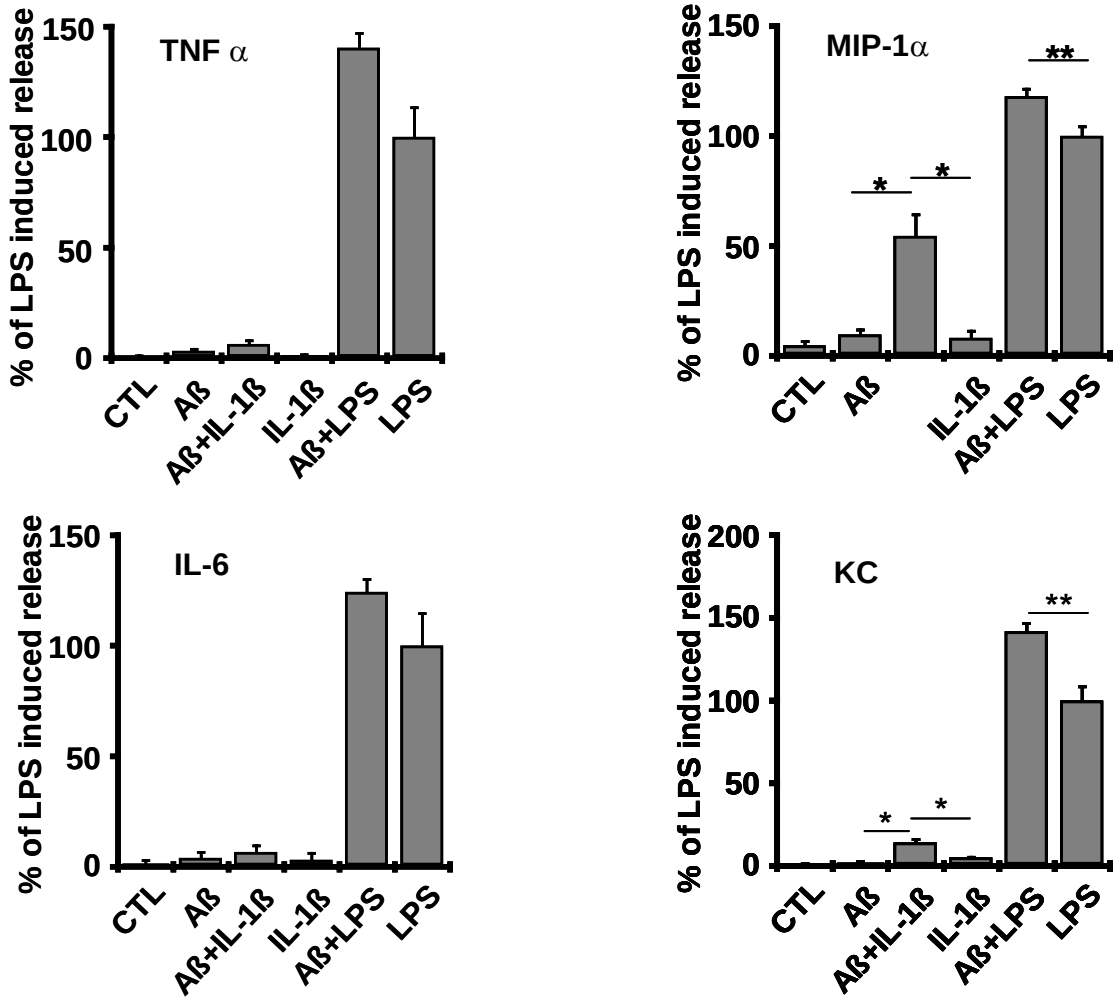


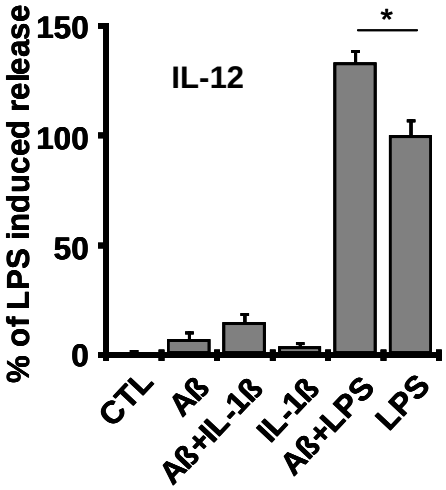
Figure 12. Cyto/chemokine release from microglia upon exposure to different ratios of aged Aß 1-40/ Aß 1-42 mixture. Cells were stimulated with various aged mixtures of Aß 1-40/ Aß 1-42 (in ratio 5:1, 1:5, 1:1) for 24 h, and amount of the released factors was determined in culture media by ELISA. Results are expressed as % value obtained upon LPS (100 ng/ml) stimulation and given as mean ± SEM, as summarized from 2 experiments (n = 8).

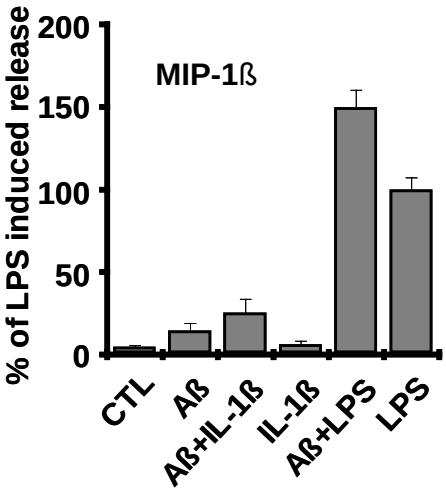
4.2.2. Costimulation with Aß and IL-1ß, but not IL-18, results in supra-additve cyto/chemokine release

The Aß preparation alone was very weak inducer of microglia release activity (Fig. 11 and Fig. 12). In order to enhance the Aß potential to activate microglia, we incubated the cells with selected Aß mixture preparation (Aß 1-40/ Aß 1-42 in 1:1 ratio, conc. 10 µM: 10 µM, aged for 7 days) in the presence of LPS (10 ng/ml) for 48 h. This co-incubation resulted in a release, which was more then only additive (KC, TNFα, IL-6) (Fig. 13). As shown in Fig. 3 and Fig. 4, LPS and IL-1ß share a similar

signaling pathway. Therefore, role of IL-1ß as a potential co-inducer involved in Aß activation of microglial cyto/ chemokine release was further investigated. The Aß peptide mixture was applied to the cells under the same conditions as described for LPS, but in combination with 10 ng/ml recombinant IL-1ß. After 48 h, concentration of cytokines: TNFα, IL-6, IL-12, and chemokines: MIP-1α, KC, MIP-1ß and MCP-1 was measured in cell supernatants by ELISA. Aß preparation on its own did not induce a significant release of the measured factors in reference to the control. The IL-1ß-induced response was comparably weak. However, the potency of Aß to provoke microglial release activity increased when IL-1ß was added (Fig.13). Compared to the values obtained in Aß- or IL-1ß-only stimulations, the resulting release was at least additive, and in some cases as for chemokines MIP-1α, KC and MCP-1 the effect was synergistic (Fig.13, Fig.14). Interestingly, this synergistic upregulation was more pronounced for some chemokines (MCP-1, MIP-1α, KC) than for cytokines (IL-6, TNFα, IL-12) (Fig. 14).







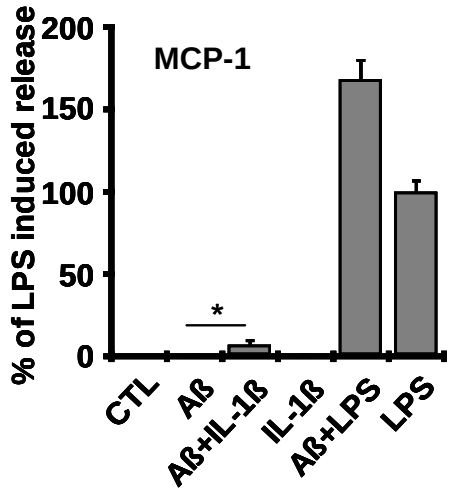


Figure 13. Chemokine and cytokine release upon Aß + IL-1ß co-stimulation of microglia. Cells were treated with Aß (10 µM, Aß 1-40/Aß 1-42 mixture, ratio 1:1, aged) alone or in combination with IL-1ß (10 ng/ml), as well as in combination with LPS (10 ng/ml), for 48 h. The values of released cytokines: TNFα, IL-6 and IL-12 and chemokines: MIP-1α, KC, MIP-1ß, MCP-1 were determined in the cell culture supernatants by specific ELISAs, taken from three separate experiments (each carried out in triplicate). The values are recalculated as a percentage according to the release values obtained in LPS (10 ng/ml) stimulation and are expressed as mean ± SEM. (P*< 0.05 and P**< 0.01).

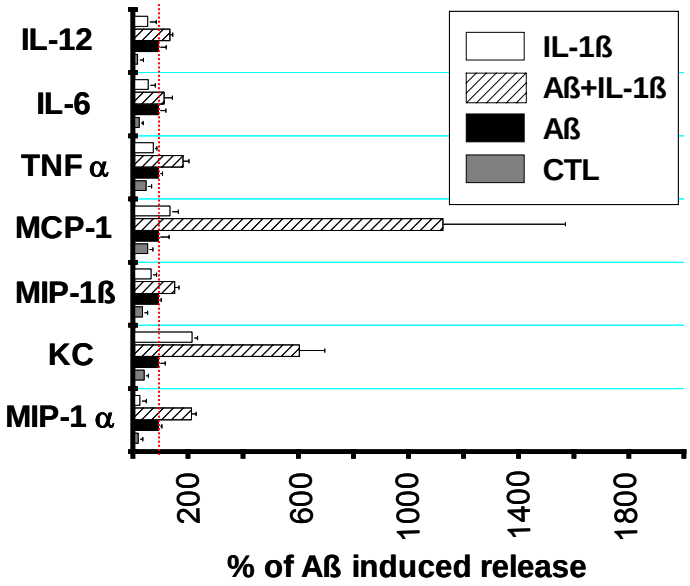


Figure 14. IL-1ß enhances Aß induced cyto/chemokine release Cells were treated with Aß mixture alone (black bars), IL-1ß (10 ng/ml) (white bars) or with combination of both (striped bars), over 48 h. Medium only treated sample was considered as a control (gray bars). The values of released factors were determined in the cell culture medium by ELISA,

taken from three separate experiments (each carried out in triplicate). In order to compare enhancing effect of IL-1ß for distinct factors results are presented as % of Aß mixture (alone) induced release, for each factor and expressed as mean ± SEM.

Rather small up-regulation in cyto/ chemokine release-inducing potency was observed when Aß was tested in combination with another member of IL-1 family, IL-18 (Fig. 15). As already seen in previous experiments, Aß evoked only slight chemokine and hardly detectable cytokine release. IL-18 alone induced low production of MIP-1α and even lower of KC and no detectable cytokine (TNFα, IL-6) release.

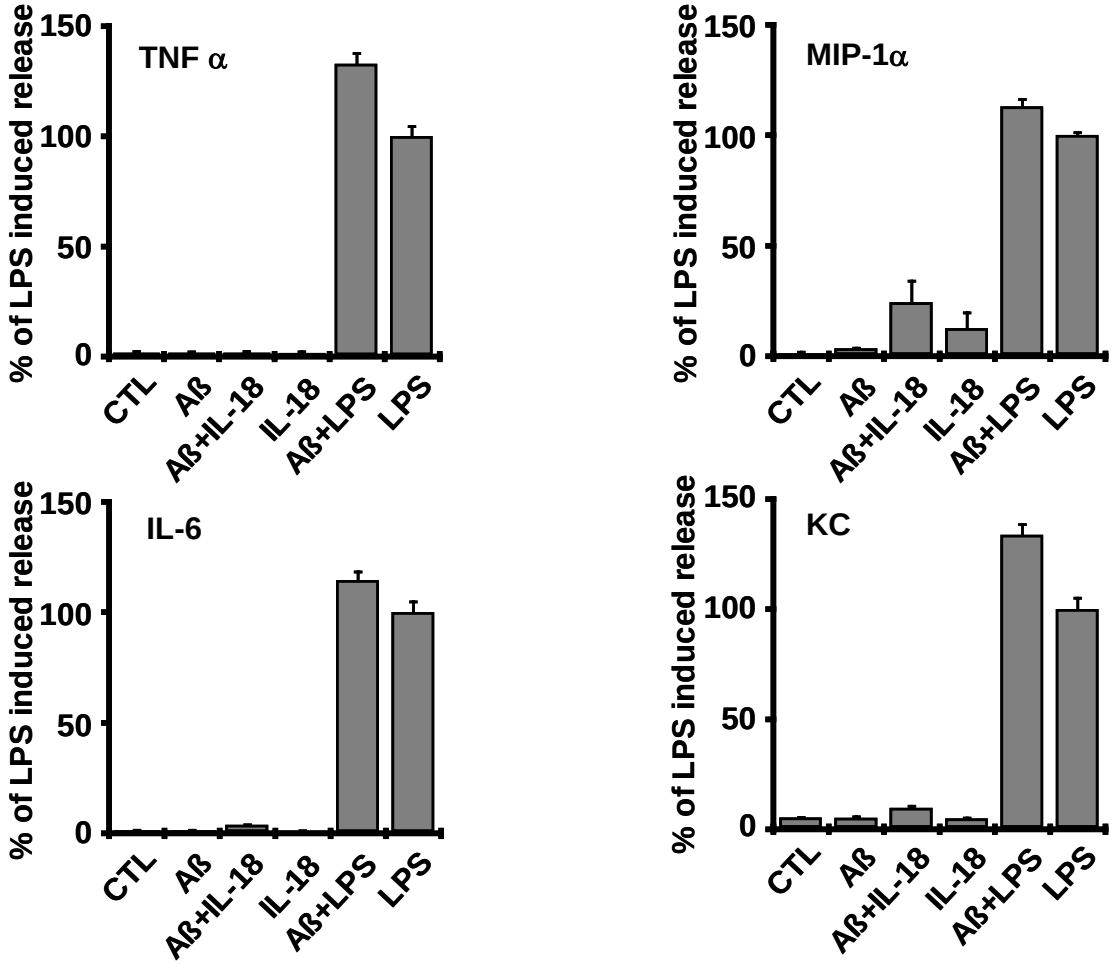


Figure 15. Cyto/chemokine release upon Aß + IL-18 co-stimulation of microglia. Aß preparation (10 µM, Aß 1-40/ Aß 1-42 mixture, ratio 1:1, aged) combined with IL-18 (10 ng/ml) or LPS (10 ng/ml), was added to the cells for 48 h. The amount of IL-6, TNFα, MIP-1α and KC released in the cell culture supernatants was measured by specific ELISAs. The values, taken from three separate

experiments (each carried out in triplicate), are presented as a percentage in reference to the values of LPS (10 ng/ml) induced release. Data are expressed as mean ±SEM.

4.2.3 Co-stimulation with IL-6 does not enhance Aß release-inducing potency

The IL-6, another cytokine associated with AD neuropathology was tested in the same way for its putative potency to enhance Aß stimulation of microglia. IL-6 alone did not induce a detectable release of measured factors (Fig. 16). Combined stimulation of microglia with Aß peptide mixture and recombinant IL-6 protein did not result in significant changes in the response, compared to basal release (control sample-medium only treated cells)

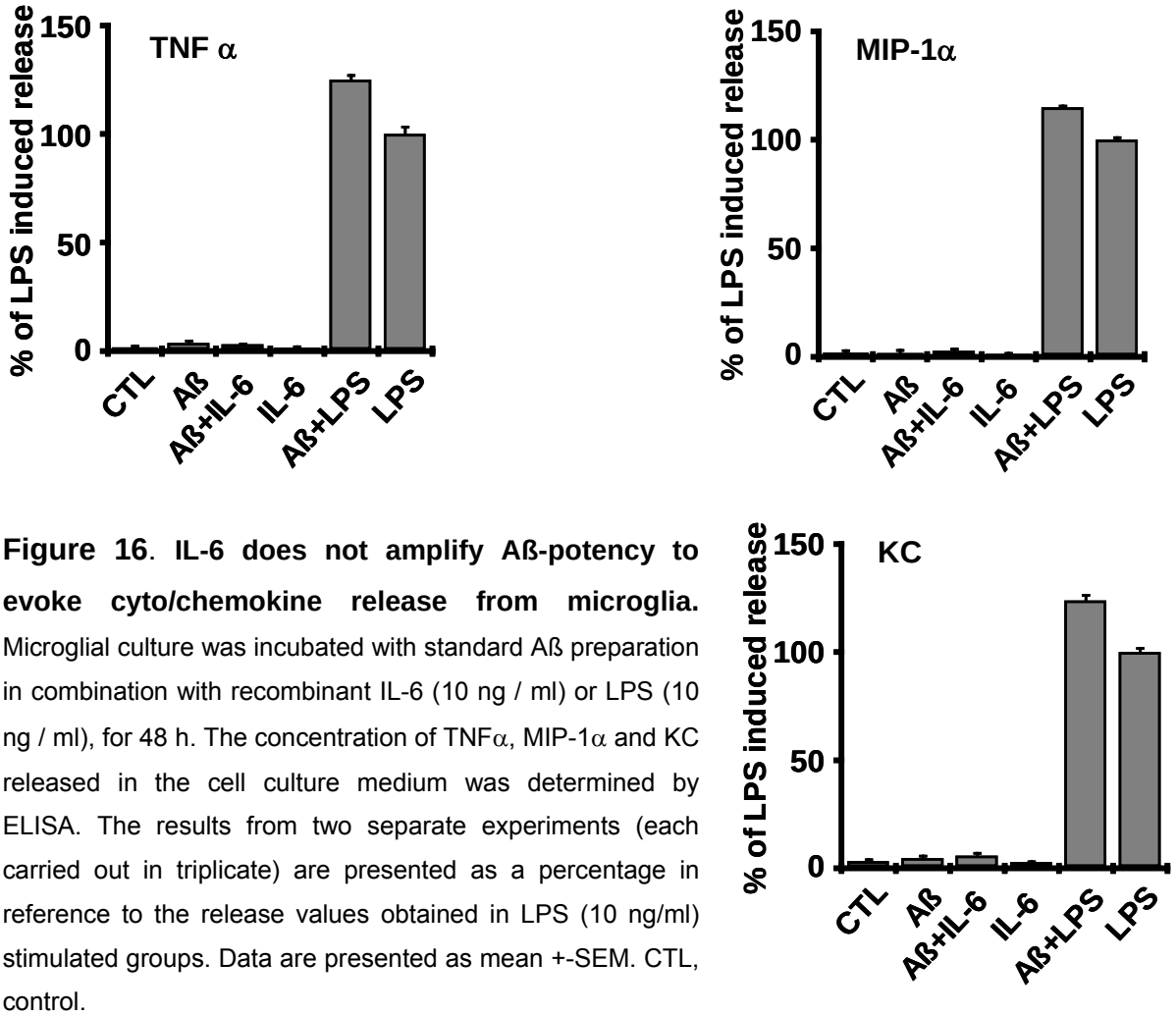


Figure 16. **IL-6 does not amplify Aß-potency to evoke cyto/chemokine release from microglia.** Microglial culture was incubated with standard Aß preparation in combination with recombinant IL-6 (10 ng / ml) or LPS (10 ng / ml), for 48 h. The concentration of TNFα, MIP-1α and KC released in the cell culture medium was determined by ELISA. The results from two separate experiments (each carried out in triplicate) are presented as a percentage in reference to the release values obtained in LPS (10 ng/ml) stimulated groups. Data are presented as mean +-SEM. CTL, control.

4.2.4. Consequences for other properties of microglia (NO release)

Beside the cyto/chemokine release, release of nitric oxide, the known neuroregulatory as well as neurotoxic mediator is a typical marker of the microglia activation state and an important event in the whole inflammatory network occurring in AD. NO was measured in the samples taken from differently treated groups. Production of NO and induction of cyto/ chemokines are both challenged by activating microglia, but have also some differences in the mechanisms. As shown in Fig. 17, compared to non-treated cells as a control, Aß induced only a weak NO release from the treated cells. Challenge with IL-1ß or IL-18 alone, had almost no effect on NO secretion. In contrast to chemokine production (MIP-1α for instance), when Aß and IL-1ß in combination induced a synergistic response, no increase or additive effect on NO generation was detected. The same was observed when Aß was combined with IL-18. On the other hand, together with LPS (10 ng/ml) Aß evoked a significantly higher release than that induced by LPS (10 ng/ml) alone.

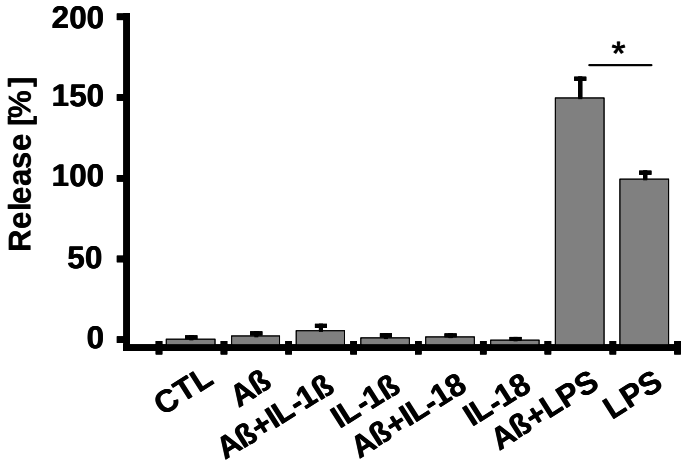


Figure 17. NO release following microglial exposure to Aß alone and in combination with IL-1ß, IL-18 or LPS. Cells were incubated with the aged Aß mixture (10 µM) alone or combined with IL-1ß (10 ng/ml), IL-18 (10 ng/ml) or LPS (100 ng/ml), for 48h. Contol group represents medium only treated cells. NO concentration was determined in the cell culture supernatants by Griess assay. Results are expressed as percentage of the value obtained in LPS treated group. Data are mean ±bSEM as summarized from 2 independent experiments (n=8). (P*$<$ 0.05 and P**$<$ 0.01).

4.3. Strong inducing effect of α2M on microglial release activity

Before testing for its potency to augment Aß-induced microglial response, the effect of α2M alone on microglial release activity was measured. Results showed that substantial release followed α2M treatment of cultures. As determined by specific ELISAs, in microglia exposed to native α2M (10 nM, 100 nM, 250 nM, 500 nM) for 48 h, the level of the cytokines IL-6 and TNF-α as well as the chemokines KC and MIP-1 α was dose-dependently increased (Fig. 18). The release evoked by α2M was comparable to that observed in cultures exposed to LPS (10 ng/ml). Furthermore, when microglia was challenged with concentrations of α2M higher than 100 nM the release of the factors was increasing, even surpassing the LPS-induced TNFα and KC response (as at concentration of 500 nM).

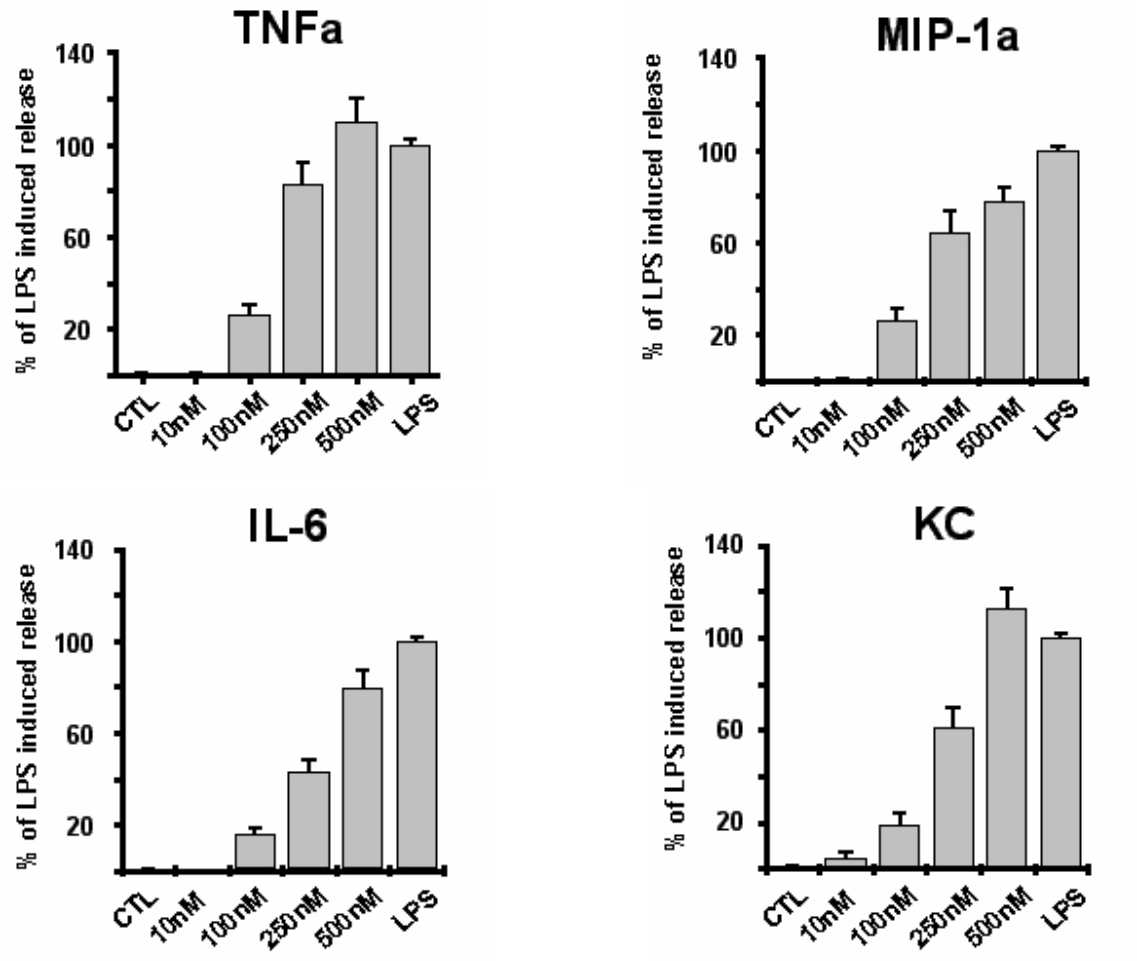
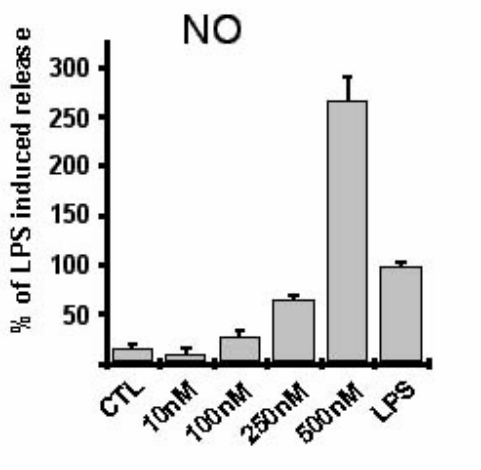


Figure 18. α2M strongly induces cyto-/chemokine release from microglia. Cells were incubated with various concentrations of α2M (10 nM-500 nM) and LPS (10 ng/ml), for 48 h. Control

group represents cells incubated with medium only. The concentrations of IL-6, TNF-α, MIP-1α and KC were determined in the cell culture supernatants by ELISA. Release is expressed as percentage of the value obtained in LPS treated group. Data are mean ± SEM as summarized from 3 independent experiments (n=36).



A similar effect was detected when NO release was measured in the culture medium. α2M provoked significant release of NO after 48 h of incubation, in particular at doses higher than 250 nM (Fig. 19).

Figure 19. Induction of microglial NO production by α2M. Microglia were exposed to α2M (10 nM - 500 nM) and LPS (10 ng/ml) for 48 h, after which the medium was removed for nitrite determination. Results are presented as a percentage in reference to positive control (LPS treated group). Data taken from 3 separate experiments (n=18) and are expressed as mean ± SEM. CTL, control.

4.3.1 α2M as a potent co-stimulatory partner of Aß

As described earlier, α2M is an important factor involved in the pathogenesis of AD. In order to reveal whether α2M could influence microglia activation in a synergistic context with already present Aß, it was used as a co-stimulus in a similar experimental paradigm as described above for cytokines. Namely, microglial were treated with Aß 1-40/ Aß 1-42 mixture (ratio 1:1, 10 µM, aged) alone or combined with native α2M (10 nM-500 nM) for 48 h and subsequently concentrations of the relevant factors, released in cell culture medium, were measured.

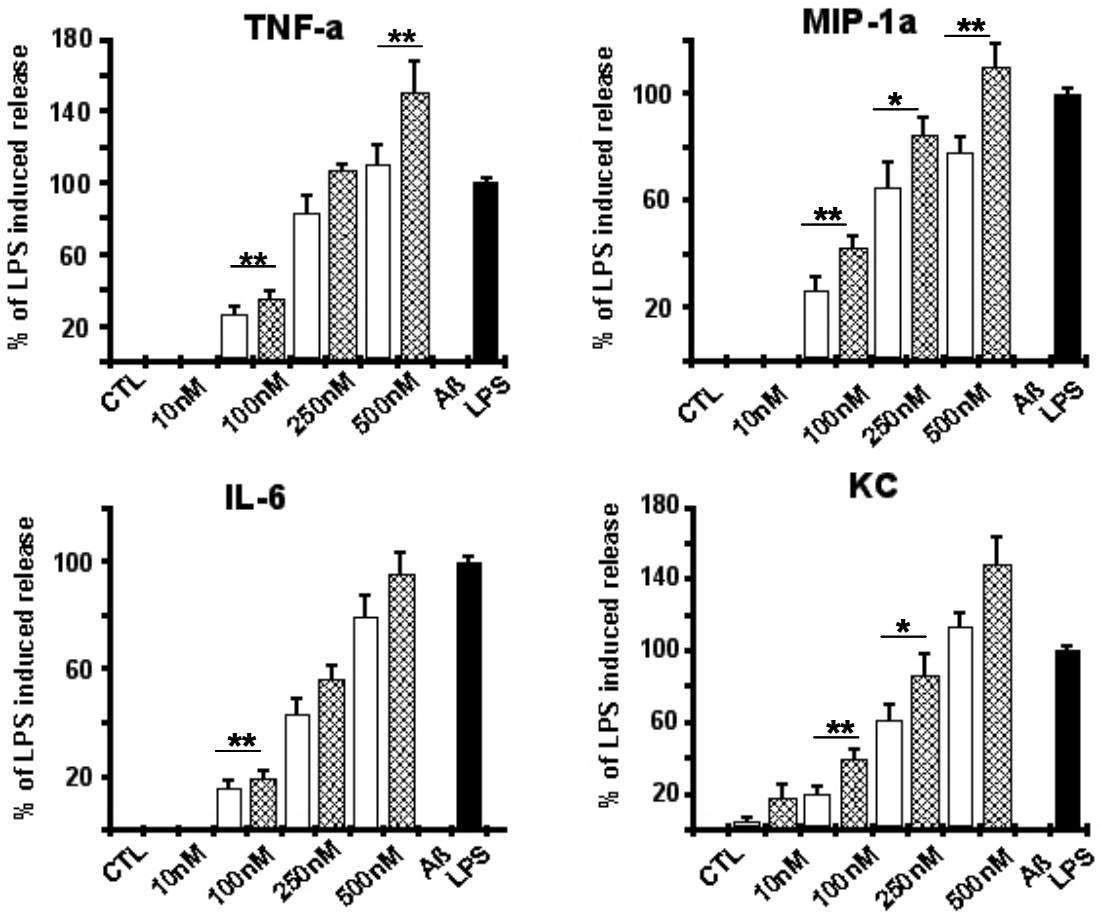


Figure 20. Enhanced cyto/ chemokine release upon α2M + Aß co-stimulation of microglia. Cells were stimulated with α2M (10 nM- 500 nM) alone and in combination with Aß sample (10 μM Aß 1-40/ Aß 1-42 mixture, ratio 1:1, aged) for 48 h, after which concentrations of IL-6, TNFα, MIP-1α and KC were determined in the cell culture supernatants by ELISA. Results are expressed as percentage of the values obtained in stimulation with LPS (10 ng/ml; used as a positive control). Open bars present groups stimulated with α2M alone, and hatched results of α2M + Aß stimulated groups. Data are mean +-SEM as summarized from 4 independent experiments (n=32). CTL, control. Asterisks indicate statistical significance (P*< 0.05 and P**< 0.01).

While Aß alone had no direct stimulatory effect on microglial cyto/ chemokine production (*in vitro)*, there was significant release measured when combined with α2M preparation. Bearing in mind that Aß alone induces barely measurable release of the factors it appears that combined together, α2M and Aß have a clear synergistic effect

on treated cells (Fig. 20). For concentrations higher than 100 nM, this additional effect was even more prominent. When α2M (100 nM, 250 nM and 500 nM) was combined with Aß, measured TNFα and IL-6 values were in average ~30% increased compared to release induced by corresponding concentrations of α2M alone, whereas for the chemokines, MIP-1α and KC, this effect was even stronger, reaching 30-60% and 30-90% (respectively) of the effect induced by α2M alone (Fig. 21).

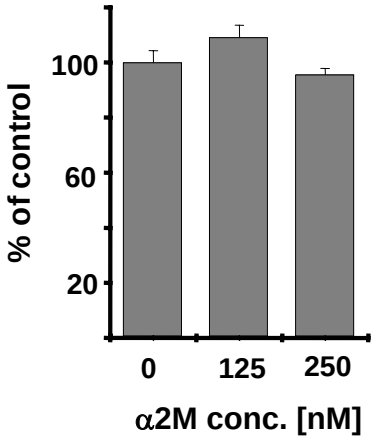
α2M +Aß	IL-6	TNF-α	MIP-1α	KC
100 nM	+23.9%	+32.7%	+61.7%	+90.1%
250 nM	+31.1%	+28.9%	+31.1%	+40.8%
500 nM	+20.3%	+35.8%	+40.8%	+30.9%

Figure 21. Synergistic effect of α2M+Aß co-stimulation. The increase presented as percentage over the release induced by α2M alone.

4.3.2 Microglial proliferation rate does not change following exposure to α2M

Unlike most cells in the mature central nervous system, microglia are easily induced to proliferate which makes them susceptible to labeling with markers of dividing cells, such as 5-bromo-2'-deoxyuridine (BrdU). Microglial proliferation is usually triggered by some disturbance in central nervous system homeostasis and represents one of the typical markers of activation. α2M is known as an inducer of cell proliferation in other cell systems (Misra and Pizzo 2002). In order to clarify whether the effect seen on release activity is accompanied by increased proliferative activity, BrdU incorporation into cellular DNA upon α2M (100nM, 250nM) was evaluated. Non-treated (medium only incubated) cells served as a control (Fig. 22). Results showed that α2M did not significantly affect cell proliferation, compared to control.

Figure 22. α2M stimulation does not result in microglial proliferation. Cells were stimulated with 100 nM and 250 nM α2M and co-incubated with BrdU labeling solution for 18 h. The concentration of incorporated BrdU was detected in the cells using a monoclonal antibody against BrdU and measured by a fluorochome-conjugated secondary antibody. Results are expressed as percentage of the values obtained in control group (incubated only with medium). Data are mean ± SEM as summarized from 2 experiments (n=12).



4.3.3. α2M itself, and not some bound compound, is the microglial activator

4.3.3.1. High temperature inactivation of α2M release-inducing potency

Bearing in mind the size and an extraordinary binding ability of α2M molecule, the nature of the microglial activator was examined. The possibility that some bound compound but not α2M itself is responsible for stimulating effect on microglia was taken into consideration. To confirm that α2M molecule, in fact, is a cause of the observed microglial activation, a first step was to confirm the protein nature of the activator. Therefore, a α2M preparation (250 nM) was challenged by high temperature treatment. Indeed, the inactivation of α2M — or the release-inducing factor associated with it — was time-dependent as well as temperature-dependent. In a first experiment, α2M was incubated at 100°C for different periods (15 sec to 32 min) and such treated α2M was further used for stimulation of the cells (Fig. 23a). Also to define the temperature range in which the "activator" becomes inactivated, α2M was incubated at different temperatures (40°C-100°C) for 15 min (Fig. 23b).

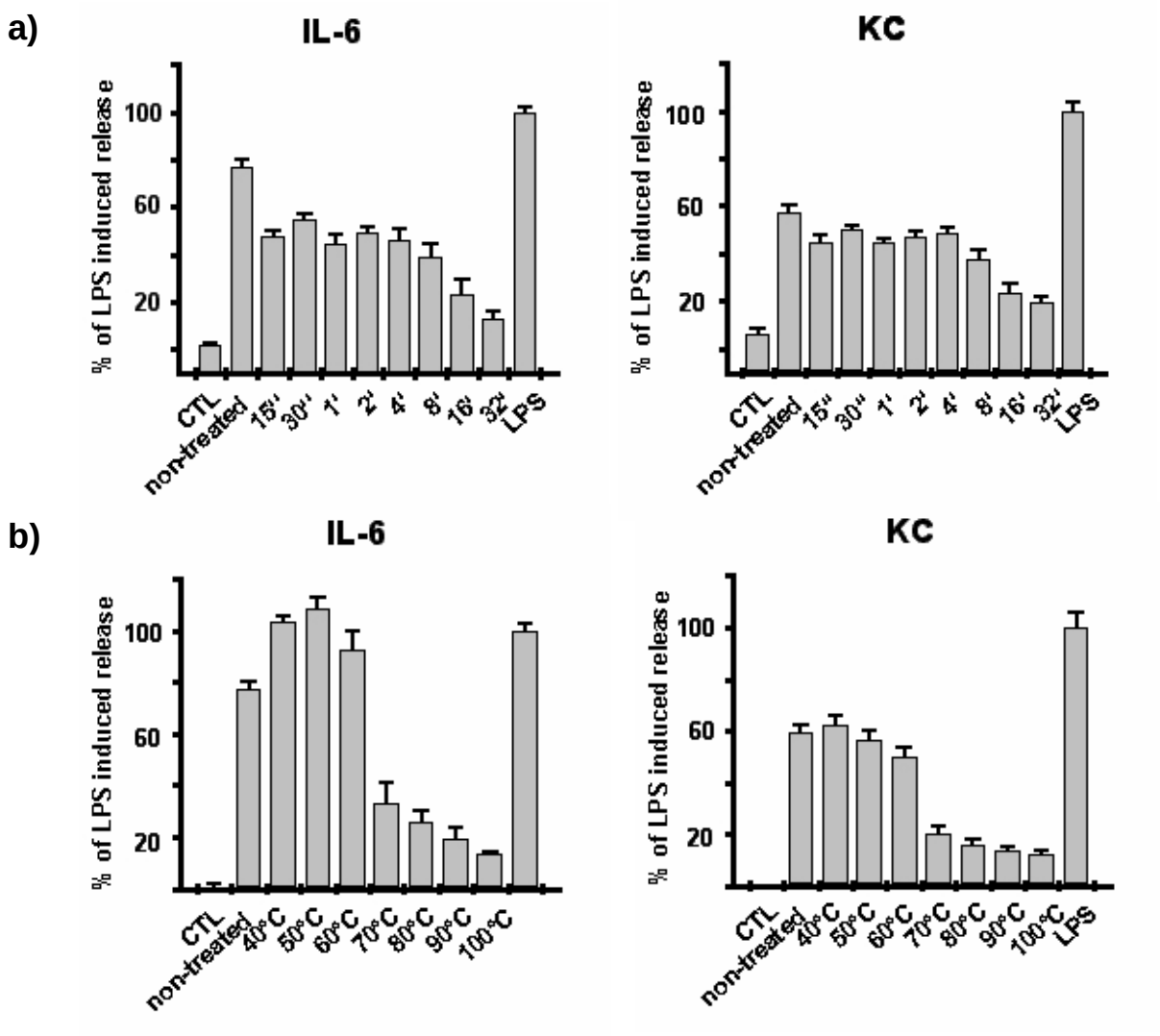


Figure 23. Loss of α2M release-inducing activity upon treatment with high temperatures indicates protein nature of the activator. α2M was incubated at a) 100°C for various periods of time (15 sec, 30 sec, 1 min, 2 min, 4 min, 8 min, 16 min and 32 min) or at b) 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C for 15 min, and subsequently added to the cells for 48 h incubation. The concentrations of IL-6 and KC were measured in the cell culture medium. Release is expressed as percentage of the values obtained for stimulation with LPS (10 ng/ml) as a positive control. Data are mean ± SEM as summarized from 2 independent experiments (n=16). CTL, control.

As shown in figure 23, for IL-6 and KC as examples, release was significantly inhibited when α2M was treated at temperatures higher than 70°C. In both release profiles of the

time dependent inactivation, there was a first instant drop in activity upon 15 sec of high temperature treatment, followed by second decrease after 8 minutes. This temperature dependency indicates a protein nature of the responsible agent, but may suggest more complex unfolding kinetics or the existence of two components. Followed up to 30 min of incubation at 100°C, the release-inducing capacity declined to $<$ 20%. It should be added that the experimental procedure was designed in a way to allow for proper heating of the α2M samples. The protein was incubated in small volume to ensure rapid heating of the sample. Only subsequently, the treated α2M was further diluted in culture medium. Moreover, this procedure also avoided the heat treatment of the medium itself, avoiding undesired effects on medium components.

As for the time course of inactivation, both factors showed a similar drop in release when α2M preparation was denaturated at 70°C. Again there was a residual induction activity of $<$ 20%, even upon the 100°C treatment. Interestingly, for IL-6, moderate heating of the α2M preparation was even enhancing the released amounts.

4.3.3.2. Partial neutralization of α2M activity upon immunoprecipitation with anti-α2M

To confirm further whether α2M, indeed, is the protein responsible for microglial cyto-/chemokine release, α2M (250 nM) was immunoprecipitated with anti-human-α2M antibody, and the immunoprecipitate was removed prior to adding the preparation to the cells. In parallel, a α2M sample treated in the same way, but in the absence of anti-α2M, was used as an internal control.

Compared to the release induced by non-treated α2M (as measured in previous experiments) this control sample induced the response in the expected range (expressed as % of LPS-induced release). Results of the cyto-/ chemokine release indicated that the activity was at least partially neutralized by the specific antibody (Fig. 24). An efficiency of α2M neutralization is certainly dependent on the used concentration of the anti-α2M antibody, as a limiting factor. However, a possibility that the remaining activity is not an “unbound” α2M activity but that some other factor also contributes the microglial stimulation still exists.

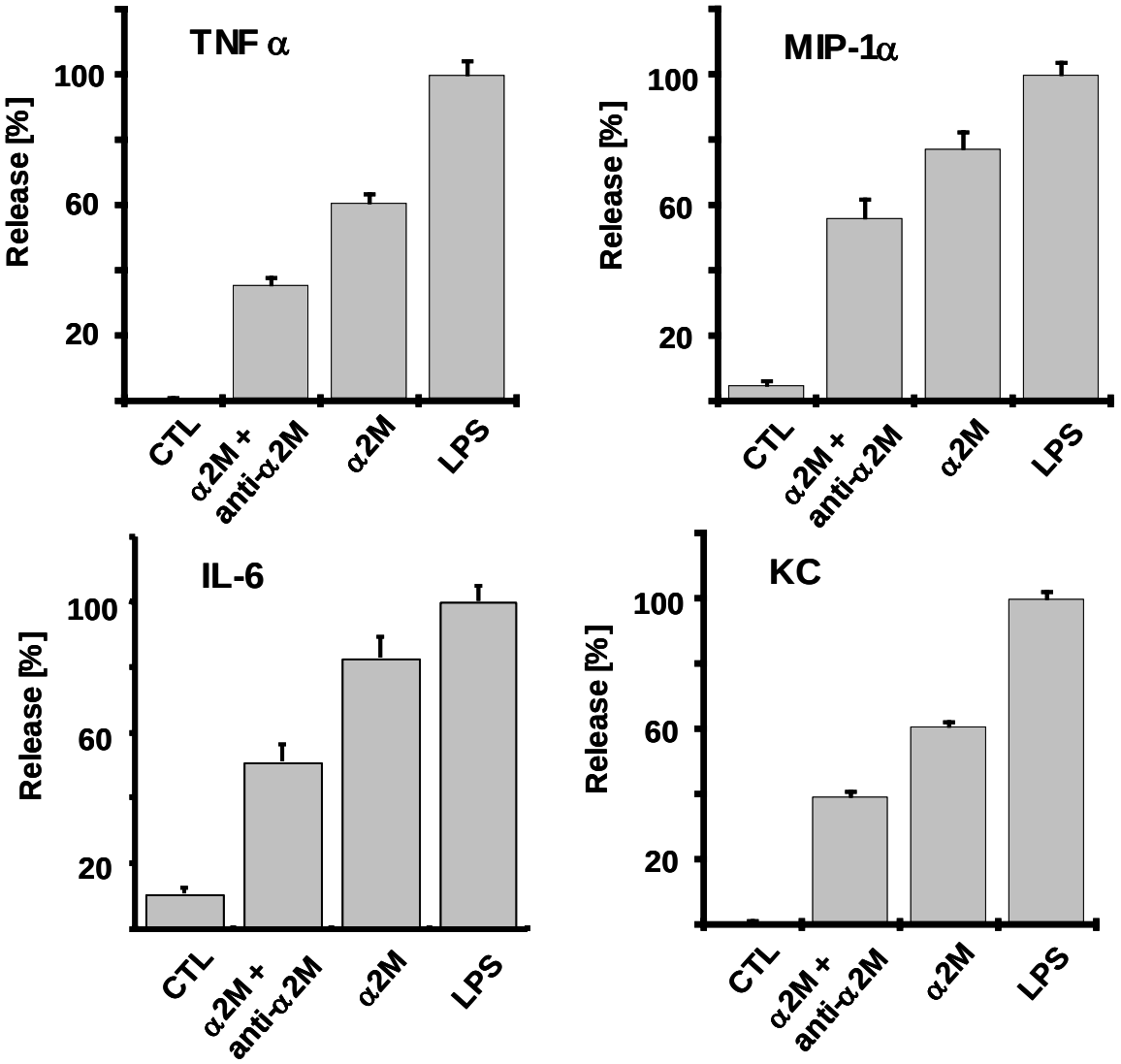


Figure 24. Immunoprecipitation of α2M with anti-human-α2M antibody results in diminished inducing activity. 250 nM α2M was incubated with antibody against human α2M for 1 h (for details see Material and Methods). The immunoprecipitate was removed and cells were stimulated with the solution for 48 h. The concentrations of IL-6, TNF-α, MIP-1α and KC were determined in the cell culture supernatants by ELISA and expressed as percentage of the values obtained for stimulation with LPS (10 ng/ml) as a positive control. Medium only treated cells served as a negative control (CTL). Data are mean ± SEM as summarized from 3 independent experiments (n=16-40).

4.3.3.3. Protease treatment leads to a decline in α2M stimulating activity

α2M molecule exists in two different activation forms, i.e. native and activated. To reveal how a transformation from an inactive into the active form of the molecule would affect the α2M-induced cyto/ chemokine release, the cells were exposed to α2M (100 nM, 250 nM) previously incubated with 25 nM chymotrypsin for 1 h. Chymotrypsin was chosen among other proteases since certain other proteins, e.g. thrombin or trypsin, are known to have receptors on microglia, which could influence the resulting release. The values of cyto/chemokines measured in supernatant upon microglial incubation with such treated

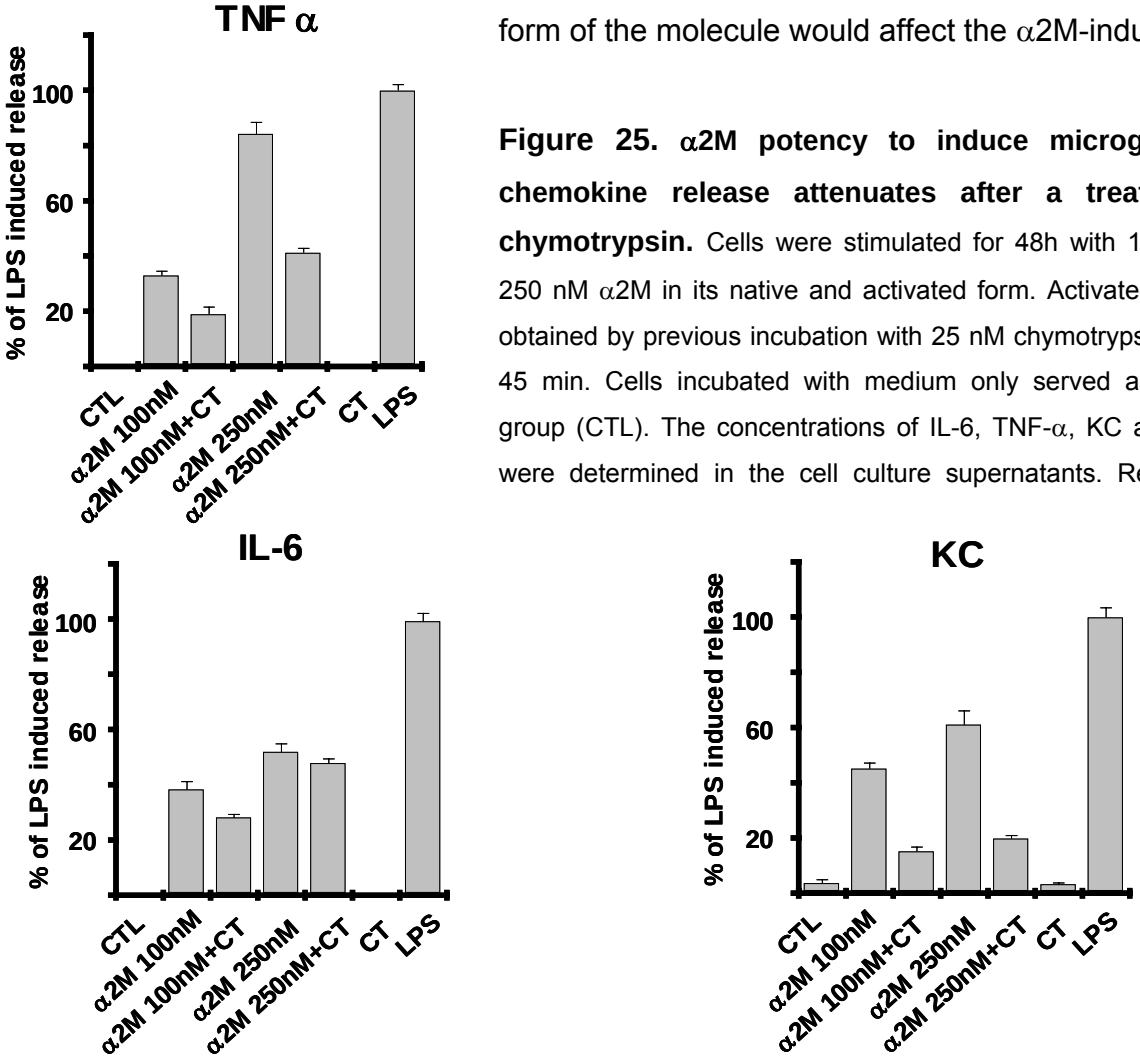


Figure 25. α2M potency to induce microglial cyto-chemokine release attenuates after a treatment by chymotrypsin. Cells were stimulated for 48h with 100 nM and 250 nM α2M in its native and activated form. Activated α2M was obtained by previous incubation with 25 nM chymotrypsin (CT), for 45 min. Cells incubated with medium only served as a control group (CTL). The concentrations of IL-6, TNF-α, KC and MIP-1α were determined in the cell culture supernatants. Release was expressed as percentage of the value obtained in stimulation with LPS. Data are mean ± SEM as summarized from 2 independent experiments (n=8).

α2M showed that the release-inducing activity of native α2M was significantly inhibited compared to non-treated α2M samples (Fig. 25). The release of TNF-α and KC, for example, was reduced by about 50%. This was an unexpected result, as protease-activation of α2M was assumed to rather augment the cellular consequences. However, protease-activated α2M is also target of LPR "scavenger" activity. LPR receptors, which are shown to be present on microglia, could serve in the clearance of α2M and thereby prevent its further actions.

4.3.3.4. Fast Performance Low Chromatography (FPLC) of α2M preparations

To prove purity of α2M preparations used in stimulatory experiments, the sample was additionally analysed by FPLC. As shown in figure 26, gel chromatography of the α2M preparation revealed an apparently single large protein eluting within the expected MW range of α2M (~720kD).

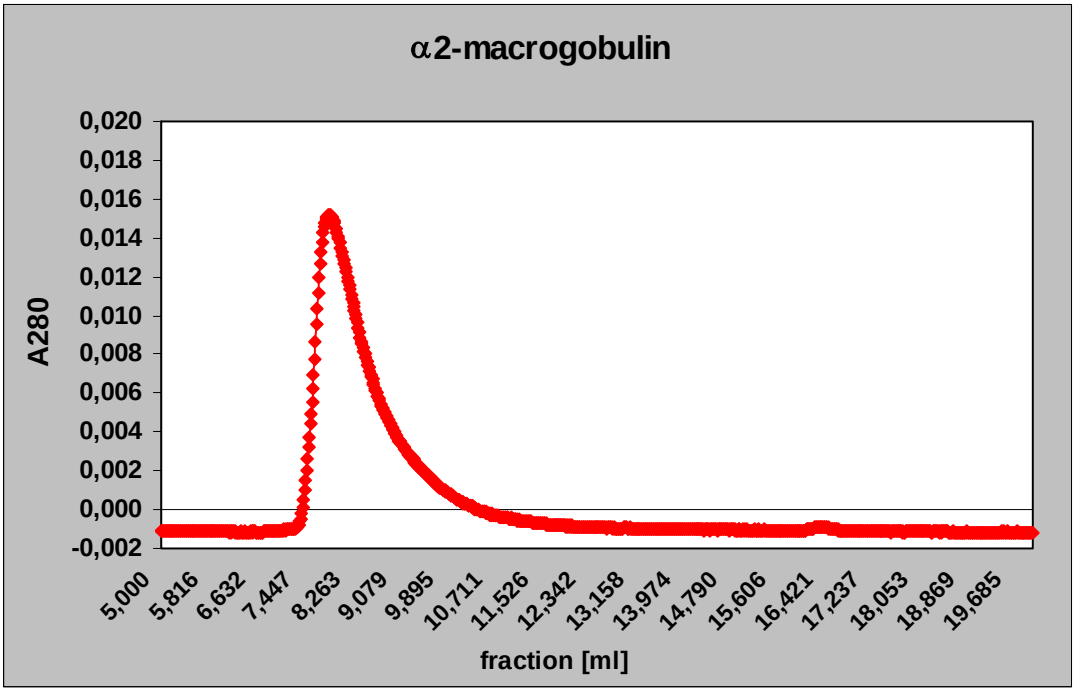


Figure 26. Purity of α2M as assessed by FPLC. α2M preparation (10 μM) was fractioned using a Pharmacia LCC501 Plus (for details see Material and Methods), with a flow rate 0.5 ml/min. The peak was identified at ~720kD.

4.3.3.5. HMW co-isolates are carriers of α2M stimulating activity

α2M is a high molecular weight (HMW) compound with a molecular weight (MW) of ~720 kD. The molecule has an extraordinary capability to bind different ligands. To reveal whether some ligand or "impurity" of lower molecular weight instead of α2M itself was responsible for the observed stimulating effect on microglia, the native α2M (Calbiochem) (250 nM) was washed on filters with a 100kD MW cut off (Microcon-Centrifugal Filter Devices for concentration and purification of biological samples, Millipore, Bedford, MA, USA). Thereby potential factors of low molecular weight (LMW) were set apart. Both the LMW and the retained high molecular weight (HMW) fractions were later used for cell stimulation. After 48 h of incubation, cell culture medium was collected and IL-6 and KC were measured by ELISA. Results showed that the inducing activity was completely carried by material with a molecular weight higher than 100 kD (Fig. 27).

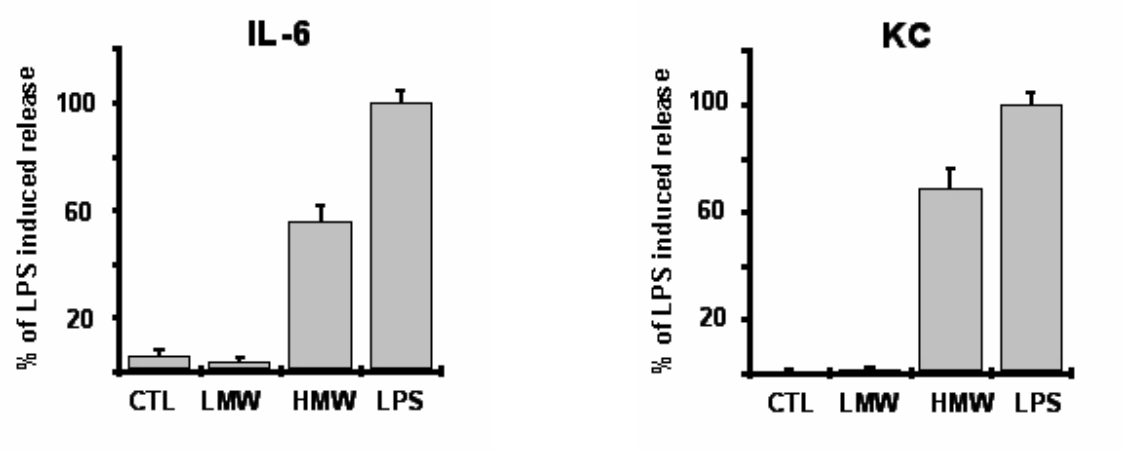


Figure 27. Microglia stimulation with α2M filtration fractions confirmed that activity is carried by high molecular weight material. α2M preparation was separated to low molecular (LMW) and high molecular (HMW) weight fraction by filtration with 100 kD cut off Centrifugal Filter Devices. Microglia was stimulated with the fractions for 48 h and subsequently, the concentration of IL-6 and KC secreted into cell culture medium, was measured by ELISA. The release is presented as % of release evoked by LPS (10 ng/ml). Data are given as mean ±SEM as summarized from 2 experiments for KC (n=24) and 1 experiment for IL-6 (n=12). CTL, medium only treated groups.