5. Discussion

5.1. Microglia *in vitro* versus *in vivo*

The study was conducted using primary microglial culture as a model system. The system is widely used for studding microglia properties both under normal and pathological conditions. It is the system of the first choice, especially convenient when direct effects of different stimuli on microglial release activity need to be assessed. System offers a high reproducibility, accessibility, is easy to handle, and does not show deviations in cell properties as observed in some cell line systems (Stohwasser et al., 2000). However, some limitations of the system exist and have to be considered. Although some reports suggest that prolonged maintenance of isolated microglia in LPS-free media can result in the formation of branched cells *in vitro,* (Gebicke-Haerter et al., 1989, Giulian et al., 1996), the extensive ramification of microglial processes, as it might be seen in tissue sections (Hailer et al., 1996; Färber and Kettenmann, 2005), organotypic tissue cultures (Fig. 1) or *in vivo* (Nimmerjahn et al., 2005), is rarely achieved in the cell culture (Fig. 6). Moreover, electrophysiological features of cultured microglia differ from characteristic electrophysiological profile of “resting” microglia in e.g. acute brain slices (Färber and Kettenmann, 2005). There are several possible explanations for observed over-activity of primary microglial cultures. First, microglial function under physiological conditions in healthy brain is likely controlled by intrinsic inhibitory factors provided by adjacent neurons (Harrison et al., 1998; Neumann et al., 1998; Hoek et al., 2000; Chang et al., 2001) and astrocytes (Hailer et al., 2001), and these calming factors apparently lack in such a simplified system. Interestingly, Eder et al. (1999) have shown that microglia cultured with astrocyte conditioned medium regain their ramified morphology. Second, cells are prepared from perinatal brain tissue at the stage when microglia is not yet in its fully resting state (for review Hanisch, 2002). Third, it has to be considered that cells undergo traumatic stress during preparation and that they are exposed to environmental changes upon cultivation, what may already cause a certain degree of stimulation. Most of the studies, which are focused on mechanisms of chronic inflammation in AD are carried out using microglial cell culture as a model. However, the results are sometimes conflicting which can result from different origin

species or use of different cell lines (Nagai et al., 2001; Dudal et al., 2004). Some authors have privilege of using human microglia cultures from freshly prepared AD brain autopsies (Lue et al., 2001b), which show some differences in comparison to rodent primary cell cultures. This has to be taken in consideration when interpreting the experimental findings and correlating them to the situation *in vivo*.

5.2 Activity through production and release of numerous soluble factors: LPS as a model inducer of microglial release activity

There are several approaches to estimate the level of microglial activation. Morphologically (as shown in Fig.1 and Fig. 6), activated microglia can be distinguished by a specific amoeboid form while resting cells are ramified with elaborated branching. However, morphology is not a parameter sufficient to determine the level of cell activation. It is difficult to quantify, and it gives little or no information about putative consequences for neighboring cells. Functional markers are naturally more relevant. The microglial activation state can also be described by electrophysiological properties of the cells. Resting microglia is characterized by inwardly rectifying potassium channels and by the lack of outward currents (Kettenmann et al., 1993) while the activation is accompanied with the induction of an outwardly rectifying K^+ channel (IK^+(OR) (Nörenberg et al., 1992), together with a concomitant suppression of the constitutively expressed inward rectifier K^+ current (Draheim et al., 1999). The typical currents are definitely a parameter that has functional meaning and also in some cases they nicely correlate with other parameters as for e.g. IL-1-like cytokine release (Ferrari et al., 1997). However, they are very difficult to quantify even through being a useful readout (Draheim et al., 1999; Prinz et al., 1999). On the other hand, release of soluble factors, i.e. inflammatory mediators and reactive oxygen species is a parameter, which can be easily quantified, thereby giving information about the extent of microglial activation. Namely, the release is, also in *in vitro* studies, characterized by a broad range of responses from “no” or minimal for non-treated groups (Haeusler et al., 2002) to “strong” for LPS, for example (Fig. 8). The release repertoire is different for various stimuli (Prinz et al., 1999) and the expression/ secretion profile is time- and dose dependent (Fig. 7, Fig. 8; Haeusler et al., 2002). The results obtained from different

stimulations provide not only information about the activation state itself, but also about functional meaning of specifically induced factors. This is because they can be produced in different amounts and ratios. Also the response can be selective for certain factors whereas there is no effect on others (Prinz et al., 2001). This all can give a hint about putative physiological consequence of a given stimulus and its potential role in the complex immunological network of the CNS (Häusler et al., 2002). The demonstration that microglia are highly secretory cells has provided a conceptional complement to the *in vivo* studies that have focused on immunophenotypic plasticity of surface molecules.

In this study, bacterial LPS, a commonly used potent promoter of the macrophage and microglial activation, was used in order to induce a substantial release response. Microglia rapidly respond to LPS by transformation from resting to active states (Fig. 1 and Fig. 6), and expression and secretion of several immunoregulators (Fig. 7, Fig. 8; Häusler et al., 2002). As the results from previous investigations have shown (Prinz et al., 1999a, 1999b; Hanisch et al., 2001; Haeusler et al., 2002), and as was also partially confirmed in this study (Fig. 13), microglia are able to produce a number of cytokines: TNFα, IL-1ß, IL-18, IL-6, IL-12 and chemokines: MIP-1α, KC, MIP-1ß and MCP-1, RANTES. In addition to numerous cytokines, when stimulated with LPS, microglia produce several toxic mediators, such as nitric oxide and free radicals. We also saw a robust NO release following 48 h stimulation with LPS (Fig. 17). However, it seems that the release of cytokines (TNF-α, IL-1, IL-6) and NO upon LPS stimulation showed different time courses (Nakamura et al., 1999; Hoffmann et al., 2003). It is suggested that there are at least two different intracellular signaling pathways of LPS-induced microglial activation; one for early release of TNF-α and IL-1ß and the other for late release of IL-6 and NO, and that the transient morphological change seems to be associated with the early release (Nakamura et al., 1999). The proposed delay in the expression of IL-6 in contrast to an early expression of TNF-α and IL-1ß is also in line with cytokine gene transcription profile upon LPS stimulations as observed in the RT-PCR experiment, in this study (Fig. 7). Multiple signaling pathways are activated in microglia upon LPS exposure among which, probably as a most prominent route, is the MAPKs cascade (Bhat et al., 1998, Pyo et al., 1998) that involves a family of protein

serine/threonine kinases and includes the ERK-1 and ERK-2 (Garrington et al., 1999). This signaling cascades also lead to activation of NF-κB (Fig. 4), a homo- or heterodimeric transcription factor which regulates the expression of several cytokine genes, including TNF α, IL-6, IL-1ß, MIP-1α (Collart et al., 1990; Libermann and Baltimore 1990; Shimizu et al., 1990; Shakhov et al., 1990; Sparacio et al., 1992; Hiscott et al., 1993; Song et al., 2002) and whose function is regulated by the binding of IkB inhibitory protein. LPS is known to stimulate the degradation of one of the IκBα isoform of IκB (Muller et al., 1993), thus allowing the now transcriptionally active NF-κB protein complex to translocate into the nucleus where it can bind to various regulatory elements present in gene promoter regions (Baldwin, 1996). NF-κB does not only play a role in the regulation of cytokine genes but its binding sites are also present in the promoter of the iNOS gene. This has been shown to be involved in iNOS gene up-regulation (Xie et al., 1993). Interestingly, Aß shares a similar MAPK signaling pathway with LPS (Pyo et al., 1998). A number of in vitro studies reported involvement of p38 and p44/42 MAPKs upon exposure to fibrillar Aß (Pyo et al., 1998; McDonald et al., 1998), whereas fresh Aß seems to activate p44/42 MAPK only when CD45 is inactivated by tyrosine phosphate inhibitors (Tan et al., 2000). Cytokines like IL-1ß, which is not only produced by, but can also act on microglia, seem to overlap in several steps with the LPS signaling cascade (Sheng et al., 2001; Li et al., 2003) (see Fig.3 and Fig. 4).

5.3 Aß as a weak activator of microglial release activity in vitro

The inflammatory hypothesis of AD suggest that the silent and non-immune-mediated chronic inflammatory response, locally induced in the vicinity of accumulated senile plaques in AD, results in long–lasting injury and finally neuronal cell death. This is supported by data from various epidemiological studies, which suggest that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) could help reduce the risk or prevent the onset of the disease (Rich et al., 1995; Stewart et al., 1998; in t`Veld et al., 2002, Zandi et al., 2002). This clearly correlates with a reduced number of amyloid deposits, lower microglial activation and a significant reduction in IL1ß levels (Mackenzie et al., 1998, Lim et al., 2000). Number of evidence implicates microglia

having a crucial role in inflammatory injury in AD. Microglial interaction with amyloid plaques leads to induction of a range of proinflammatory and cytotoxic products. However, *in vitro* studies have often shown contradictory results regarding the proinflammatory potential of Aß, on its own.

As previously published by many authors, Aß is only capable of inducing a weak inflammatory response after stimulation of cultured microglia (Meda et al., 1995; Goodwin et al., 1995; Van Muiswinkel et al., 1996, Gasic-Milenkovic et al., 2003). The absence of significant microglial activation upon treatment with different Aß preparations was also observed in this study (Figs. 11 and 12). This supports the hypothesis mentioned earlier that co-stimulatory agents, possibly present in an AD brain, are required to trigger a strong inflammatory response (Meda et al. 1995; Lorton et al., 1996; Murphy et al., 1998; Yates et al., 2000; Muehlhauser et al., 2001, Gasic-Milenkovic et al., 2003). It should be mentioned that some authors report cyto/chemokine production in microglia upon stimulation with Aß alone (Lue et al., 2001a, 2001b). However, this has been observed in human microglial cultures prepared post mortem from freshly isolated AD patients' brains or non-AD elderly patients' brains, i.e. cell systems, which most likely have already been "pre-activated" to a certain extent.

5.3.1. Structure and conformation of Aß protein influence its inducing potency

Aß production is a normal physiological process *in vivo* and *in vitro* (Haass et al., 1992, Shoji et al., 1992). A soluble amyloid form is present at similar concentration (0.1-10 nM) in both normal and AD cerebrospinal fluid (Shoji et al., 1992; Seubert et al., 1992), and only its spontaneous assembly into insoluble fibrils appears to be a critical step in developing pathology. As many studies have already suggested, one of the preconditions of microglial activation by Aß is its presence in ß-sheet conformation. This type of protein secondary structure is characteristic of amyloid fibrils as present in AD plaque cores (Weldon et al., 1998, Yates et al., 2000, Muehlhauser et al., 2001). Interaction between fibrillar Aß and microglia seems to occur via CD36. This is a class-B scavenger receptor that mediates fibrillar-Aß induced secretion of cytokines, chemokines and reactive oxygen species via the Lyn and p44/42 MAPKs signal transduction pathway (El Khoury et al., 2003). However, some authors claim that

fibrillogenesis is not the only factor determining the toxicity of Aß preparations. For example, Aß (1-42) incubated under conditions that only allow the formation of small diffusible oligomers proved to be neurotoxic (Roher et al., 1996; Szczepanik et al., 2001). There is still not enough evidence whether small oligomers rather than large molecular aggregates mediate microglial production of nitrite, IL-6 and TNFα as induced by Aß peptides (Takata et al., 2003). The results of our comparative microglial treatment by "aged" *versus* "fresh" Aß samples speak in favour of the former statement. The resulting cyto-/ chemokine release activity of the cells, although still very weak, was higher in preparations incubated for 7 days (Fig. 11) that contained fibrillar species (as confirmed by electron microscopy, Fig. 10) than in freshly used samples, which consisted mainly of mono- and oligomeric species (as detected by size exclusion chromatography, Fig. 9). Not only the secondary but also the primary Aß protein structure affects its potency to activate cultured microglia. Aß 1-40 is believed to be the major species generated by cultured cells (Seubert et al., 1992; Dovey et al., 1993; Suzuki et al., 1994), and present in CSF (Vigo-Pelfrey, 1993). Less soluble Aß 1-42 is the predominant isoform accumulated in senile AD plaques (Iwatsubo et al., 1994,1996), and *in vitro* forms fibrils at a greater rate than shorter isoforms (Jarrett et al., 1993). Existing data on the ability of certain Aß species to provoke an inflammatory response from cultured microglia are still puzzling. Some studies showed that exposure to Aß 1-42, fibrillar Aß 1-40 as well as Aß 25-35 peptide could induce LPS-preactivated THP-1 cells to release IL-1ß (Lorton et al., 1996). Others suggested that not the whole peptide but only a so-called HHQK domain of Aß (amino-acid residues 13-16) was responsible for Aß binding to microglia and subsequent microglia-mediated neuronal toxicity (Giulian et al. 1996, 1998). In this study, Aß 1-40 and Aß 1-42 were tested separately for their potency to stimulate cyto/chemokines production in microglia. They both failed to induce a significant effect, regardless of whether they were used freshly or aged in order to enable fibril formation (data not shown). Diffuse plaques consist almost exclusively of Aß 1-42 (Iwatsubo et al., 1994), whereas neuritic plaques contain both Aß 1-40 and Aß 1-42 as well as shorter Aß peptides truncated at the N terminus (Iwatsubo et al., 1994, 1995, 1996). Thus, in order to better replicate an *in vivo* "plaque" situation, mixtures of both Aß 1-40 and Aß 1-42 peptides were examined in various peptide ratios,

both as fresh and “aged” preparations (Fig. 11 and Fig. 12). Although the 5:1 (Aß 1-40:Aß 1-42) ratio roughly mirrors the concentration ratio as present in an AD brain (Seubert et al., 1992; Suzuki et al., 1994), it was a 1:1 “aged” mixture of the peptides of all the mixture preparations tested that elicited the strongest (though still very scant) release response from treated primary microglia cultures (Fig. 12).

5.4 Putative synergistic co-stimuli of Aß microglial activation in AD

It seems that microglia has to be pre-activated to a certain extent in order to be fully responsive to Aß stimulation. This probably simulates the situation existing in the proximity of Aß clusters, where reactive (already activated) microglia interacts with Aß in the plaque. However, it is still not clear while signal primarily initiates this microglial activity. There are several known inflammatory inducers that were already tested as potential synergistic Aß co-stimuli. LPS pre-treatment of microglia was widely used prior to Aß application. LPS appears to be necessary in order to provoke IL-1ß response from Aß treated microglia cell lines (Lorton et al., 1996; Gasic-Milenkovic et al., 2003). To induce profound respiratory burst in cultured microglia, co-treatment with phorbol ester was needed (Van Muiswinkel et al., 1996). Co-stimulation with INF-γ was also shown to be a precondition for Aß induced cytokine and NO release in cultured cells (Meda et al., 1995; Muehlhauser et al., 2001). However, no administration of INF-γ or any other co-stimulatory factor was necessary *in vivo* (Muehlhauser et al., 2001). These factors are commonly used in a co-stimulating manner, but they are not of physiological importance for AD, and their presence in an AD brain is rather questionable. IFNγ, for example, is a cytokine derived from T cells. However, the presence of infiltrating T cells is not typical of AD pathology as it is for conditions of systematic inflammation, such as meningitis or multiple sclerosis (Eikelenboom et al., 1994; Kuhlmann et al., 2000). Although some data reports a certain number of detected T cells in AD brains (Itagaki et al., 1988, Togo et al., 2002), there is no clear evidence of increased IFNγ concentration in the CSF of affected patients. LPS, a component of gram-negative bacterial cell walls, is widely used as a stimulus for microglial activation, but it has no causative role in AD. Recently, however, some factors that are more relevant in AD brain have been shown to enhance Aß potency to induce cytokine production in microglia. Complement factors,

C5a and plaque-associated C1q, for instance, heighten cytokine (IL-1ß, IL-6, TNFα) release from Aß primed human monocytes and adult human microglia, respectively (O´Barr and Cooper, 2000, Veehuis et al., 2003). Macrophage colony-stimulating factor (M-CSF), which was found at increased levels in the AD brain, was shown to augment Aß induced microglial production of IL-1, IL-6 and NO (Murphy et al., 1998). In this study, the ability of LPS to enhance Aß evoked release of proinflammatory cyto- and chemokines (TNFα, IL-6, IL-12 MIP-1α, KC, MIP-1ß and MCP-1, see Fig. 13, 15, 16) as well as NO production (Fig. 17) in cultured microglia has also been confirmed. The observed augmentation could be due to a direct effect of LPS, but could also result from action of factors released upon LPS stimulation. This is because, some factors produced in microglia by LPS have already reported physiological implications in AD (e.g. IL-1). Furthermore, they also act on microglia and share similar signalling pathways with LPS.

5.4.1 Interleukin-1ß as a co-stimulatory partner of Aß in microglia mediated chronic inflammation

The ability of LPS to augment Aß-induced microglial activation and release of proinflammatory cyto- and chemokines could at least partially be assigned to the action of factors that are normally released following LPS. Among numerous factors that are secreted in the LPS treated microglia, IL-1 is probably the key candidate for such an effect. Its signaling cascade, which involves members of the MAPKs family and leads to NFkB transcription factor activation, significantly overlaps with the cascade that follows LPS binding to its “binding complex” (Fig. 3 and 4). Moreover, the IL-1 potency to modulate Aß provoked cytokine response has already been shown in astrocytic cell line. Whereas freshly prepared and aged Aß only stimulated IL-6 and IL-8 release moderately, aged but not fresh Aß markedly potentiated cytokine secretion in the presence of IL-1ß (Gitter et al., 1995). Here we show that IL-1ß potentiats the ability of fibrillar Aß to activate microglia. An augmentation of the resulting release, which was more than just additive, was observed in particular with chemokine production (MCP-1, KC, MIP-1α and MIP-1ß) (Fig. 13, Fig. 14). It has been shown that a tyrosine phosphorylation cascade plays an important role in Aß-induced microglial activation

(McDonald et al., 1998; Combs et al., 1999). Combs and colleges (2001) have found that although Aß-induced tyrosine kinase activity was stimulated after activation of Src family members such as Lyn, transduction of the signalling required for TNFα and IL-1ß release was mediated by nonreceptor tyrosine kinase, Syk. They also reported that in parallel an NF-κB-dependent pathway was required for increased cytokine production. On the other hand, findings from the studies carried in the fibroblast cell line show that Syk is required for the IL-1-induced chemokine production through the association with TRAF-6 (Yamada et al., 2001). IL-1 signaling cascade also leads to the activation of NF-κB. Thus, sharing similar signaling pathways Aß and IL-1ß co-action could result in a potentiated effectory signal.

However, NO production (Fig. 17) was not altered when Aß was combined with IL-1ß. This may be surprising since NF-κB is also suggested as a key factor in the induction of iNOS gene transcription (Colasanti et al., 1995). It could be that a factor other than IL1ß is responsible for enhancement of NO production by LPS. In fact, it has already been suggested in some reports that TNFα could mediate this effect (Colasanti et al., 1995).

In contrast to IL-1ß, the effect of IL-18 (the second member of the IL-1 family tested) on the ability of Aß to provoke microglial release response was not significant. (Fig.15). It could be explained by differences in signaling pathways by IL-1ß and IL-18. Lee et al. (2004) have shown that although several biological properties overlap for these cytokines, in endothelial cells IL-18 signal transduction is primarily via the MAPK p38 pathway rather than NF-κB. Similar explanation could be also applied for IL-6, which although suggested as one of relevant factors in AD failed to induce an increased response when combined with Aß (Fig. 16). Namely, IL-6 mediated activity seems not to include NF-κB which seem to be a key factor in integrating signals from multiple inflammatory mediators in given co-stimulations (Daffada et al., 1994; Schuringa et al. 2000)

5.4.2 α2M as a microglial activator and an Aß's partner in synergistic stimulation of microglia

Many genetic, immunohistochemical and biochemical studies have clearly shown implication of α_2M and its receptor LRP in AD. However, the exact role of α_2M in the

pathophysiology of the disease is still unknown. Both neuroprotective and neurotoxic mechanisms have been suggested. Some authors reported that α_2M by specific binding to Aß could mediate Aß clearance (Qiu et al., 1999) and/ or interfere with Aß aggregation into the fibrils (Du et al., 1998; Hughes et al., 1998), suggesting its beneficial role. On the other hand, α_2M may directly increase Aß-mediated neurotoxicity (Fabrizi et al., 1999). While most of the reports have focused on α_2M-Aß interactions and action of α_2M on neurons, the potential involvement of α_2M in inflammatory processes that occur in the AD development has not yet been investigated. In general, α_2M is not often considered an immunoregulatory protein. This is because linking α_2M to inflammation is often limited to the ability of α_2M to bind some proinflammatory cytokines and thereby indirectly influence an inflammatory response, e.g. the induction of nitric oxide (NO) and NO-dependent cytotoxicity due to binding and thereby inhibition of anti-inflammatory TGFß (Lysiak et al., 1995; Webb et al., 1996). Although some direct inflammatory-related actions of α_2M (e.g. induction of cyclooxygenase-2, Misra et al., 2001) were demonstrated in macrophages, no evidence of such a direct effect in microglia has yet been reported. Marzolo et al. (2000) showed the presence of α_2M receptor- LRP in microglial cells. It is not clear if α_2M binding to LRP could lead to intracellular signaling events downstream, or if LRP function is limited to its known “scavenger” role. Despite there being some evidence for the presence of the “signaling receptor” for α_2M in peripheral macrophages, investigations on microglia have not yet been performed. In this study we show the ability of native α_2M preparations to induce dose-dependent cyto- and chemokine production and release in primary microglial culture (Fig. 20). α_2M also provoked substantial NO release (Fig. 21). Considering such a molecule with an extraordinary binding potency, and having in mind that α2M preparation (from Calbiochem) originates from human plasma, one has to be aware of the possibility that some impurity might be present and that some bound material, but not α2M itself, might be responsible for the observed effects on microglia. Here we show that the high molecular weight fraction of the α2M preparation is the cause of the inducing activity (Fig. 28). FPLC analysis of the α2M preparation only showed the presence of the expected HMW molecule fraction – none other – in the molecular size range ~720 kD (Fig. 27). However, in the release profiles of the time dependent high

temperature inactivation the remarkable reduction in the stimulating activity occurred in two steps (Fig. 24a). There was a first instant drop, followed by second decrease. This result indicates not only a protein nature of the inducing factor that can be inactivated by heat denaturation, but also a more complex unfolding kinetics or the existence of two components. Interestingly, there was some residual induction activity, even upon the 100°C treatment (Fig. 24). Contamination by LPS can be a source of such heat-resistant residual activities. Alternatively, heat – resistant protein aggregates may represent a stimulus for these cells. In the immunoadsorption assay anti-α2M antibodies failed to completely neutralize α2M release-inducing activity (Fig. 26). A reason for this could be an insufficient concentration of the antibodies that was used. There is also a possibility that in our experimental design a contact between the antibodies and the corresponding epitopes on the huge α2M molecules was not fully enabled. However, a possibility that the effect, which has been seen in microglia, might be only partially assigned to α2M itself and that factors carried by α_2M may serve the microglial activation, rather than the carrier, can not be absolutely excluded.

Native α2M can be transformed into the activated state, either by treatment with endoproteases, such as chymotrypsin, physiological ligands and inducers of its activity, or by modification with small nucleophiles, such as methylamine. The “active” form of the molecule is thought to bind cellular receptors. A particular activation state of the α_2M molecule seems to be relevant when interactions with cytokines are examined. Some cytokines, including IL-6, bind preferentially to the native secreted form of α_2M (LaMarre J et al., 1991). In contrast, TGF-ß, TNF-α and IL-1ß prefer to bind to forms of α_2M that have been modified by proteinases. It has been observed that cytokines bound to native α_2M retain much of their biological activity in various bioassays, whereas cytokines bound to "activated" α_2M have decreased activity in some cell systems. For instance, the biological activity of IL-2 was not disturbed when it was bound to the native α_2M, but interaction with activated α_2M lead to a loss in activity (Legres et al., 1995). This could be a reason for the attenuation of the α_2M-stimulating potency as seen upon a treatment with chymotrypsin (Fig. 28), which firstly appeared as an unexpected result, as protease-activation of α2M was assumed to rather augment the cellular

consequences. Some authors even suggest that disorders resulting from an apparent change in the production of one or several different cytokines might represent changes in either the production of α_2M "cytokine scavenger" or their α_2M-receptor-mediated clearance mechanisms. This would mean that activated α_2M or augmentation of α_2M-receptor-dependent cytokine clearance might be a strategy for preventing the harmful systemic or local effects of excess cytokines such as TGFß and TNFα *in vivo* as for instance suggested by James and colleagues (1992). It was found that the activated form of α_2M stimulates cell proliferation in macrophages (Misra and Pizzo 2002). When tested for their potency to induce microglial cell proliferation, our preparations of native α_2M did not affect proliferation rate of microglia in comparison to non-treated samples (Fig 23). Therefore, it can be concluded that the stimulatory effect of α_2M on the release of cyto/chemokines is not influenced by an increased number of cells formed through treatment as a result of induced cell division.

α_2M is able to bind specifically with Aß, regardless of its activation state (Du et al., 1997). When microglia were exposed to a combination of Aß and α_2M, the resulting release was clearly synergistic (Fig. 22). This might mean that α_2M present in the neuritic plaques could also contribute to ongoing inflammation, in addition to its previously suggested contribution to Aß-mediated neurotoxicity. The protective function of α_2M to promote Aß clearance might be relevant in earlier phases of disease, before compact plaques are formed. This is because α_2M is specifically associated with the neuritic plaques, but not to immature amyloid ones. In contrast to it, IL-1 shows co-localisation with diffuse plaques and can therefore already be implicated in the early phases of AD development. Furthermore, the amyloid plaques that show IL-6 immunoreactivity show simultaneously an increase in α2M (Strauss et al 1992, Bauer et al., 1991, Thal et al., 1997). This finding supports the idea of functional link between IL-6 and α2M, which has consequences for the development of AD. It is well known that IL-6 serves as an important initiator and modulator of the action of acute phase proteins. IL-6 has been shown to upregulate α_2M in neuroblastoma cells (Ganter et al., 1991). On the other hand, IL-1ß induces synthesis and release of α_2M in human astroglia (Gao et al., 2002). Upregulated IL-1ß and IL-6 in AD lesions could thus intensify production of

α_2M in neurons and astrocytes, which in turn, in combination with the ever-present Aß, might evoke further release of the cytokines from microglia and therefore take part in the positive loop that contributes to the maintenance of chronic inflammation (Fig. 28). α_2M could also contribute to NO overproduction by binding TGFß that attenuates iNOS synthesis. This is because both microglia and astrocytes produce and respond to TGFß. TGFß has been shown to inhibit dose-dependent LPS-induced iNOS and NO synthesis (Lieb et al., 2003).

5.5. Relevance of factors secreted from activated microglia for AD pathology

Many microglia-released factors, which have been measured in this study as the parameters of cell activation, are thought to play a role in a complex network of inflammation-related events that finally lead to neuronal injury and death in AD. According to the inflammatory hypothesis, neuronal injury results both from the direct effects of inflammatory factors (cytokines or activated complement), and indirect effects of neurotoxic Aß, which is over-produced in response to the inflammatory stimuli (Rogers et al., 1996). For instance, Aß can be overproduced in response to IL-1 and IL-6 (Goldgaber et al. 1989; Vandenabeele et al., 1991; Ringheim et al., 1998). Thus, therapies could target either Aß as a proiflammatory enhancer, inhibit its formation through ß- or γ- secretase inhibitors, or block primary proinflammatory signals by using NSAIDs. The inflammatory hypothesis has developed in parallel with the oxidative injury hypothesis, that NO and its derivatives play a role in neuronal cell injury and death (Miranda et al., 2000). Peroxynitrite is though to be a principal candidate whereby NO can be responsible for the neurotoxicity (Beckmann et al., 1996). A certain body of evidence points to the presence of peroxynitrite-induced nitration of neuronal proteins in the AD brain (Good et al., 1996). Reactive oxygen and nitrogen species, which affect neurons in AD, seem to be derived both from glia (Meda et al., 1995) and neurons when they are in conditions of oxidative stress such as Aß toxicity (Behl et al., 1994).

5.5.1. Cytokines (TNF-α, IL-6)

Both cytokines whose release was found heightened upon Aß+IL-1 and Aß+α2M co-stimulations (TNFα and IL-6) (Figs. 13, 14, 20, 21) have been detected elevated in

brains and sera of AD patients compared to controls (Fillit et al., 1991, Strauss et al 1992; Wood et al., 1993). Among other factors, an increase in TNFα has been observed after exposure of microglia from rapid AD brain autopsies to pre-aggregated Aß 1-42 (Lue et al., 2001). It was shown that production of TNFα following Aß activation of microglia and monocytes can lead to the further production of NO by induction of iNOS (Colasanti et al., 1995; Combs et al., 2001). Also in astrocytes, Aß induces iNOS production that seems to be IL-1ß and TNFα dependent (Akata et al., 2000). In microglia-neuronal co-cultures Aß induced neurotoxicity that was attributed to microglia derived TNFα and reactive nitrogen intermediates (Meda et al., 1995). In this way TNFα could act indirectly, via NO. Although beneficial for killing bacteria and further activating the immune system, NO production contributes to significant tissue damage by enhancing vascular permeability and potentially compromising the BBB (Calingasan et al., 1998). TNFα-inducing iNOS expression can be responsible for peroxynitrite production, and subsequent neuronal cell death (Beckman et al., 1996). Thus, suppression of NO production may be fundamental for survival of neurons. Namely, neurotoxicity provoked by exposure to Aß was blocked by a non-specific iNOS inhibitor in mixed astrocytes/ microglia/ neuronal co-cultures (McMillian et al., 1995). It was also shown that TNFα potentiats cell death of cholinergic neurons *in vitro* and *in vivo*, possibly via retrograde axonal damage (Zassler et al., 2003). The induction of TNFα death domain signalling pathways seems to be present in the early stages of neuronal degeneration in AD (Zhao et al., 2003). Interestingly, α2M provoked strong production of TNFα (Fig. 18), comparable to that induced by LPS. In previous studies it was demonstrated that protease-activated α2M may bind TNFα and thereby aid its removal by the α2M-receptor pathway, affecting TNFα activity and distribution *in vivo* (Wollenberg et al, 1991). Here, we show that native α2M in microglia can enhance TNFα production and thereby participate in regulating the complex cytokine network in the brain. Furthermore, data from Lysiak et al. (1995) has demonstrated that both native and activated α2M can increase macrophage NO synthesis and profoundly affect cellular function without gaining entry into the cell and without binding specific plasma membrane receptors. This effect was, however, assigned to the TGFß-carrier and not

receptor-binding α2M activity. Namely, a TGFß-neutralizing antibody mimicked the activity of α2M to increase NO synthesis. We speculate that such a mechanism could also be present in AD lesions, and together with augmented TNFα-mediated NO induction could contribute to tissue damage caused by excessive amounts of NO and its toxic derivatives.

Transgenic mice with overexpression of IL-6 show age-related deficits in avoidance learning (Heyser et al., 1997) and reduced long-term potentiation in the hippocampus (Bellinger et al., 1995), supporting the role of IL-6 in the pathogenesis of memory-related disorders. Together with IL-1, IL-6 and its IL-6/sIL-6R complex are implicated in modulation of APP synthesis (Vandenabeele et al., 1991; Ringheim et al., 1998). Histological studies have demonstrated IL-6 immunoreactivity co-localisation with diffuse, but not with compact, neuritic plaques (Hull et al., 1996), suggesting that IL-6 probably precedes neurotic changes rather than appearing after neuronal degeneration. This is in similar to IL-1, which also appears to be involved in the early phases of plaque formations and pathogenesis. There is some evidence of a link between IL-6 and α2M in AD pathology. For instance, IL-6 immunoreactive plaques show a co-localized increase in α2M (Strauss et al 1992, Bauer et al., 1991, Thal et al., 1997). Among other cytokines, α2M-carrier activity also includes IL-6. It was demonstrated that IL-6 binds preferentially to the native secreted form of α2M (LaMarre et al., 1991). Importantly, cytokines bound to native α2M retain much of their biological activity as shown in various bioassays, whereas cytokines bound to the "activated" α2M show decreased activity. IL-6 may act on neurons, inducing α2M expression (Ganter et al., 1991). We show that α2M, on the other hand, can provoke significant IL-6 release from microglia and that this induction can even be enhanced when Aß is present (Fig. 20, 22). Furthermore, IL-1ß can challenge astrocytes to produce IL-6, and also together with Aß can stimulate microglia to release IL-6. This excess of IL-6 could further promote α2M production in neurons in a positive feedback manner (Fig.28).

5.5.2. Chemokines (MCP-1, MIP-1α, MIP-1ß, KC)

In the present study a particularly significant augmentation after co-treatment with Aß and AD-related proteins (IL-1ß, α2M) has been observed for chemokine (MCP-1, MIP-

1α, MIP-1ß, KC) production. Although AD is not a disorder characterized by an excessive leukocyte infiltration into the CNS (Akiyama et al., 2000), like some other known neurological diseases (multiple sclerosis, infections, stroke, HIV associated dementia), an upregulation in chemokines and chemokine receptors expression in the AD brain is evident. It is known that Aß- provoked microglial activation is associated with chemotactic responses, consistent with the extensive clustering of reactive microglia at sites of Aß deposition (Terry and Wisniewski, 1975). At the same time chemokines serve in important communication between microglia and neurons (Coughlan et al., 2000; Streit et al., 2001). In this study we focus on MIP-1α (CCL3), MIP-1ß (CCL4), MCP-1 (CCL2) - all members of CC chemokine subfamily and KC (CXCL1), member of the α (CXC) family.

MCP-1

There is an increasing body of data supporting MCP-1 as an AD-related factor. Elevated levels of MCP-1 were found in plasma and CSF (Sun et al., 2003), and immunohistochemically in mature senile plaques and reactive microglia in AD patients' brain tissue (Ishizuka et al., 1997). MCP-1/ CCR2 receptors are expressed constitutively on neurons and astrocytes and to a lesser extent on activated microglia (Banisadr et al., 2002), meaning that MCP-1 released from reactive microglia in the vicinity of the plaque may influence neighboring cell populations.

MIP-1α/ß

Although there is no direct evidence of an elevated concentration of MIP-1 in CSF or plasma of AD patients, overexpression of the MIP-1 receptors CCR3 and CCR5 has been demonstrated on reactive microglia in AD (Xia et al., 1998). Furthermore, microglia cultured from rapid autopsies of AD patients exhibit significant, dose-dependent increases in both MCP-1 and MIP-1α after exposure to pre-aggregated Aß (Lue et al., 2001). Unstimulated microglia expressed minimal levels of MIP-1α, MIP-1ß and MCP-1. In response to LPS, all three chemokines are induced at the mRNA and protein levels in fetal human microglia (McManus et al., 1998). In our study we also see clear inducibility of the chemokine release following exposure to LPS (Fig.8, Fig. 13). *In*

vitro data show the ability of Aß to stimulate the production of MIP-1α, MIP-1ß, and MCP-1 from human monocytes (Fiala et al., 1998). Using primary monocyte-derived macrophages and primary adult astrocytes as a model, Aß 1-42 was able to stimulate the production of MIP-1α and MIP-1ß in macrophages, and MCP-1 in astrocytes and oligodendrocytes (Smits et al., 2002; Johnstone et al., 1999). However, as it was seen for the release of cytokines, in our primary microglial cell culture Aß preparation was only a weak inducer of microglial MIP-1α/ß release potency. The release was clearly enhanced when Aß was combined with IL-1ß or α2M (Figs. 13, 14 and Figs. 20, 21).

GROα/KC

Among the chemokine receptors detected on neurons, CXCR2, the receptor for GROα/KC, was most strongly expressed and also upregulated in a subpopulation of neuritic plaques (Horuk et al., 1997; Xia et al., 1997; Xia et al., 2002). Recently, GROα/KC was detected immunohistochemicaly in neurons in normal, and found upregulated in some AD brains (Xia et al., 2002). The same authors showed that it could induce the ERK1/2 and PI-3 kinase pathways, as well as hyperphosphorylation of tau in neurons, implicating its putative involvement in AD pathologenesis. However, the role of KC in microglia-mediated inflammatory processes in AD was not yet studied. As yet there is no data as to whether Aß has an effect on microglial KC production. It was demonstrated that KC could be induced in mouse microglia cultures by LPS (Prinz et al., 1999; Häusler et al., 2002) and microglia and astrocytes by IL-1ß and TNFα (Janabi et al., 1999). In this study we report a significant, dose-dependent KC release after stimulation with α2M (Fig. 18), as well as augmentation of such an induced release when α2M and Aß were combined (Fig. 20). The KC release induced by Aß alone, however, was scarcely measurable. Some authors report KC release upon IL-1ß treatment (Janabi et al., 1999), but we observed only minimal, barely detectable KC induction when IL-1ß was used alone. After co-incubation with Aß, the resulted release was more than just additive (Fig. 13). However, the mechanisms underlying this stimulating effect on KC production still have to be elucidated.

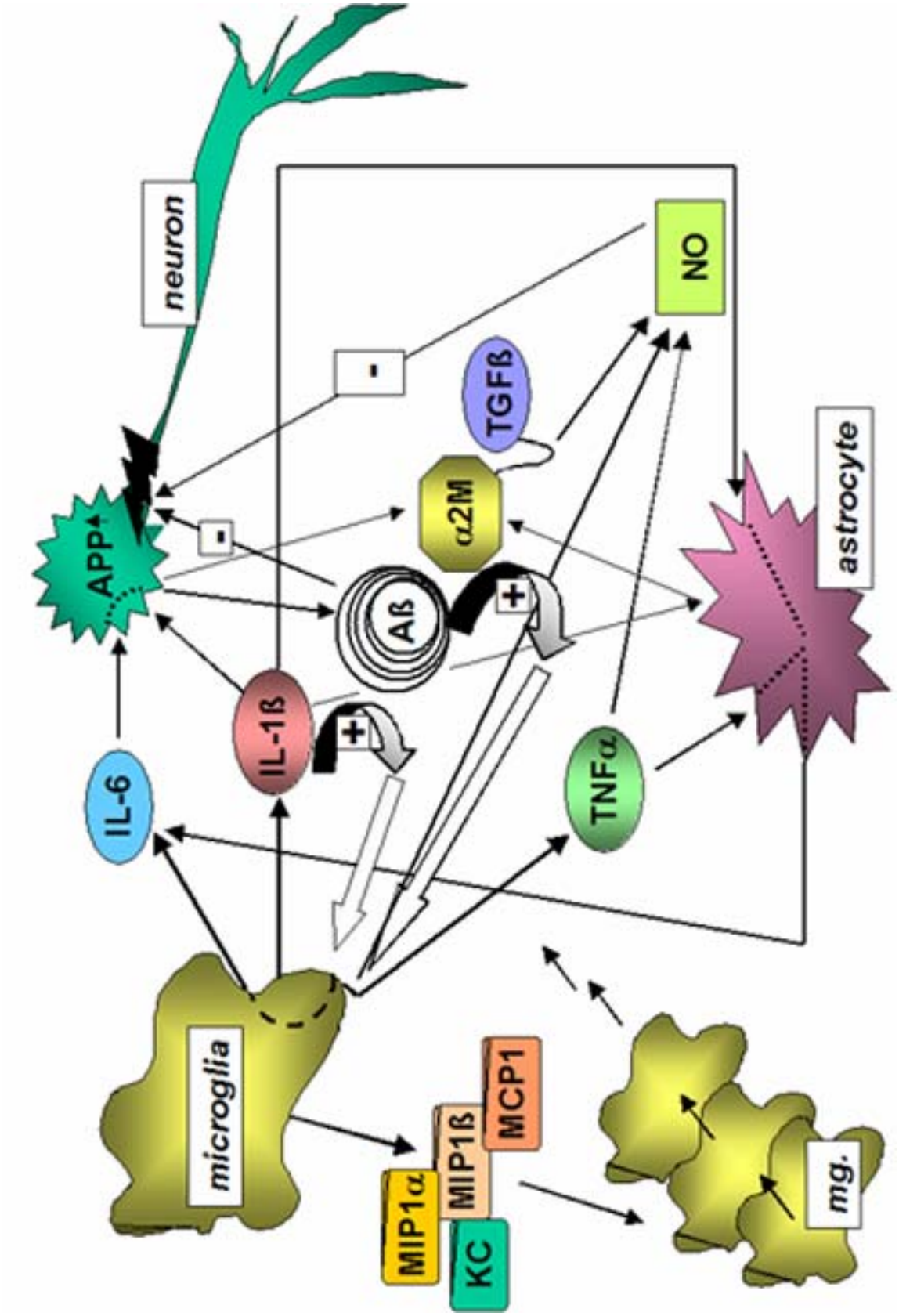

Figure 28. Inflammatory network in the proximity of amyloid deposits. Complex interactions between Aß- and AD-relevant proteins result in production of numerous factors. They, in turn, themselves contribute the complicated intercellular communication in the vicinity of the plaques.

5.6. Inflammatory mediators as therapeutic target- use of NSAIDs

In support of the inflammatory hypothesis of AD there are results of several studies showing a beneficial effect of NSAIDs on pathogenesis and pathology of AD. So far suggested targets of NSAIDs include cyclooxygenase, NF-κB, and peroxisome proliferator-activated receptors (PPARs) (McGeer et al., 2006). The first pilot trial of a classical NSAID in the treatment of AD was the study on effect of indomethacin (Rogers et al., 1993). Investigation of its effects on rat microglia *in vitro* has demonstrated that the mechanism by which indomethacin might be beneficial in treatment of AD could be due to its inhibition of IL-1, TNFα and NO production by microglia (Du et al., 1999). Another tested drug tepoxalin showed to markedly inhibit IL-1ß-induced IL-6 and anti-chimotrypsin synthesis in astrocytes and the synthesis of IL-1ß and IL-6 in LPS-stimulated microglial cells. The mechanism is apparently the inhibition of NF-κB activation, which is mediated by preventing I-κB-α degradation (Fiebich et al., 1999). Similarly, ibuprofen treatment caused significant reductions in final IL-1ß levels, which was accompanied with a diminution in the number and total area of Aß deposits (Lim et al., 2000, Morihara et al., 2005). Alternative NSAIDs, primarily tested in order to avoid toxicity caused by excessive use of classical NSAIDs (those targeting Cox-1), as e.g. anti-oxidant compound curcumin from the curry spice turmeric, also significantly lowered IL-ß and oxidized proteins in the brains of AD transgenic mouse model (Lim et al., 2001).