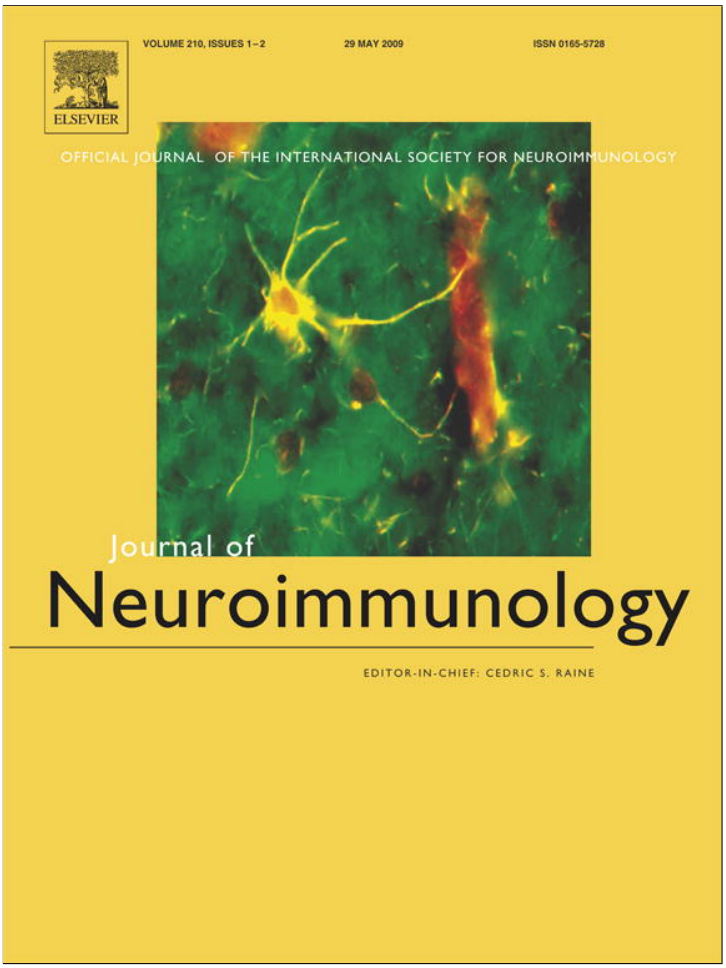

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# Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-β

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ABSTRACT

M1 and M2 are the extremes of the differentiation spectrum of activated macrophages. Since microglia are members of the same cell lineage, we have characterized their transcription profile and their phagocytic activity under different conditions. LPS or IFN-γ induce a M1-like phenotype, while IL-10 or IL-4 differentiate microglia towards a M2-deactivated or M2-alternatively-activated phenotype respectively. These differentiation processes also affect the Notch pathway. In order to study the polarization induced by Aβ, microglia was stimulated with different forms of the peptide. The oligomeric Aβ is a stronger M1-inductor than the fibrillar form. Moreover, a cytokine-induced anti-inflammatory environment reduces the microglial reactivity towards oligomeric Aβ.



## 1. Introduction

Although the ontogeny of microglial cells has long been unclear, microglia are now recognized as the tissue macrophages of the central nervous system (CNS) (Kreutzberg, 1996). Their density is comparable to that of Kupffer cells, the tissue macrophages of the liver (Lawson et al., 1990). However, microglia present a number of characteristics that distinguish them from other tissue macrophages. With their long processes, which can be maintained *in vitro* (Giulian et al., 1995), they perform a continuous surveillance of the brain parenchyma (Davalos et al., 2005; Nimmerjahn et al., 2005). They also seem to express a less mature phenotype than other tissue macrophages. Microglia easily proliferate in response to a M-CSF exposure (Alliot et al., 1991), and they express the CD34 progenitor marker (Ladeby et al., 2005). Their antigen-presenting abilities are poorly developed (Carson et al., 1998). Nevertheless, like macrophages, microgliocytes are able to react to tissue insults by a rapid differentiation process leading towards a fully activated microglial cell showing numerous similarities to any other activated macrophage (Hanisch and Kettenmann, 2007).

Cells belonging to the monocyte–macrophage lineage are heterogeneous. Macrophage heterogeneity is likely to reflect the plasticity and versatility of these cells in response to exposure to microenvironmental signals. Alternative polarized activation modes of these cells can be distinguished (Gordon, 2003; Mosser, 2003). Whereas classical activation of macrophages by microbial compounds or pro-inflammatory cytokines yields a phenotype called M1 that is hallmarked by the production of pro-inflammatory cytokines and free radicals like nitric oxide and superoxide anions, alternative activation can lead to a less well defined anti-inflammatory phenotype, labeled M2. At least two different M2 phenotypes have to be distinguished based upon their gene expression profile. IL-4 or IL-13 treated macrophages present an alternative activated phenotype hallmarked by arginase1, Found in Inflammatory Zone 1 (FIZZ1) and chitinase 3-like 3 (Ym1) expression and by the production of factors involved in tissue remodeling and wound repair (Raes et al., 2002; Song et al., 2000; Munder et al., 1999). Macrophages exposed to IL-10 or TGF-β differentiate towards a deactivated phenotype showing increased CCR2 (Sozzani et al., 1998) and scavenger receptors (Sulahian et al., 2000). Anti-inflammatory macrophages may evolve by natural neuroendocrine control mechanisms and play a role in homeostatic processes, such as dampening inflammation, scavenging debris, angiogenesis and wound healing (Goerdt et al., 1999; Gordon, 2003).

Functional polarization of macrophages into M1 or M2 cells is an operationally useful, but a simplified conceptual framework describing the plasticity of mononuclear phagocytes. This concept allows a better understanding of the numerous physiological properties of the cells belonging to this lineage. Signature genes for both extremes of the differentiation spectrum have been described for macrophages (Ghassabeh et al., 2006).

Little is known about the potentials of microglial cells to differentiate along a similar spectrum of phenotypes. While no doubt remains about the ability of microglia to react in a pro-inflammatory way towards cytokine stimulation, pattern-recognition receptor ligation or other danger signals (Block et al., 2007; Hanisch, 2002), only scarce information

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about alternative activation of these cells is available. A CNS restricted IL-4 deficiency exacerbated the clinical symptoms of EAE in a mouse model (Ponomarev et al., 2007). The same study shows that IL-4 induces the expression of the alternative activation typical gene Ym1 in brain macrophages. Microglia that encounter anti-inflammatory $T_H2$ cells or their characteristic cytokines, such as IL-4, are rendered able to support neuronal survival, oligodendrogenesis and some neurogenesis from adult neural progenitor cells (Butovsky et al., 2006). Furthermore,Komohara et al. recently documented a correlation between M-CSF production by brain tumors and the anti-inflammatory phenotype of the tumor-infiltrating brain macrophages.

The amyloid-β (Aβ) peptide, counting 40 to 42 amino-acids, has a causal role in Alzheimer's disease (AD) (Snyder et al., 2005). Aβ peptides have the capacity to self-assemble, passing from monomeric to oligomeric, and then to insoluble heavy aggregates, thus progressively forming the plaques. Oligomeric Aβ, with a molecular weight of around 56 kD, has the most marked toxic effects on the neuronal functions (Malaplate-Armand et al., 2006), notably impairing memory (Lesne et al., 2006). This form has also been shown to display the most potent effect on Long Term Potentiation inhibition (Barghorn et al., 2005). A causative link with the establishment of the later AD-typical tau pathology has also been demonstrated (Oddo et al., 2006). It thus seems well established that the oligomeric form of Aβ plays a crucial role in the disease onset and progression (Cole and Frautschy, 2006).

In this work, we have studied the signature gene expression and the phagocytic activity of microglial cells when pushed to both extremes of the differentiation spectrum M1 and M2. Since several genes implicated in the Notch pathway are affected by different inflammatory conditions, the influence of Notch pathway stimulation on the acquired phenotype is examined. The transcription profile and the behavior of microglial cells exposed to the different forms of Aβ is compared to these extremes. The influence of anti-inflammatory cytokine pathway on this activation process documents a certain degree of plasticity in the differentiation profile.

## 2. Materials and methods

### 2.1. Cell culture

For primary microglia, mixed glial cell cultures were prepared from the brains of newborn C57BL/6 mice as previously described (Morga et al., 2000). Briefly, after carefully removing meninges and large blood vessels, the brains were pooled and then minced in cold phosphate-buffered salt solution. The dissociation was completed by 10 min of incubation in 2 mM EDTA. After washes and centrifugations, the cells were allowed to attach and grow at 37 °C in DMEM supplemented with 20% fetal calf serum (FCS) (Invitrogen, Scotland), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a water-saturated atmosphere containing 5% $CO_2$. After 4 days, the cells were moved to 10% FCS-containing DMEM. After 7–10 days, when the cultures reached confluence, microglia was detached by a 30 min shaking on a rotary shaker. Detached cells, mainly microglia (>95%), were then plated in multi-well plates in the same medium.

The murine microglial cell line MMGT12 (Briers et al., 1994), a generous gift from Dr. Vanmechelen, Innogenetics, Gent (Belgium) was cultured in DMEM/F12, supplemented with 2% FCS, 1% ITS (Insulin–Transferrin–Selenium, Invitrogen) and 15% filtered conditioned medium of WEHI cells (WEHI 3, WEHI 3B and WEHI 3D, ATCC, Rockville, MD) (producer of interleukin-3 and granulocyte-macrophage-colony stimulating factor). No antibiotics were used. The cells were grown at 37 °C in a water-saturated incubator at 5% $CO_2$ and passaged twice a week. For experiments, MMGT12 cells were seeded into multi-well plates at a density of $1\times10^5$ cells/well (six-well plates) or $2\times10^4$ cells/well (48-well plates) in DMEM/F12, 10% FCS, 1% ITS (experimental medium). After 3 days of culture, the cells were activated adding different stimuli to the culture medium.

Lipopolysaccharide (LPS 055:B5 from Escherichia coli, Sigma, Belgium) was added at different doses for primary microglia (1 ng/mL) and MMGT12 (10 ng/mL) to obtain equivalent activation because of the increased sensitivity of the primary cultures compared to the cell line. IFN-γ (Hycult Biotechnology, The Netherlands) was used at 100 U/mL, IL-10 and IL-4 (Chemicon, Belgium) were at 1 ng/mL. The recombinant rat Jagged1/Fc from R&D System (Minneapolis, MN) was used at 1 μg/mL.

### 2.2. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was purified from cultured cells using the Invisorb® Spin Cell RNA Mini Kit for total RNA extractions (Invitek, Germany). RNA analysis was performed with RNA electrophoresis Experion system (Bio-Rad, CA) to check the quality of RNA and to confirm the absence of genomic contamination. RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega, CA) with 0.5 μg/reaction oligo(dT) as primer and 1 μg or 200 ng of total RNA from MMGT12 microglial cells or primary microglial cells respectively. The RNA and oligo-dT were denaturated by preheating at 70 °C for 5 min followed by an immediate chill in ice water for at least 5 min. The reverse transcription reaction was performed in a total volume of 40 μL containing ImProm-II 5X Reaction Buffer, 25 mM MgCl2, and 0.5 mM of each dATP, dTTP, dCTP, and dGTP for 1 h at 42 °C. Individual 25 μL SYBR Green real-time PCR reactions consisted of 5 μL of cDNA, 12.5 μL of 2X iQ™ SYBR Green Supermix (Bio-Rad), and 1 μL of each 12.5 μM optimized forward and reverse primers in 5.5 μL RNase-free water.

Primer sequences designed using Beacon Designer software (Bio-Rad) are described in Table 1.

The PCR was performed on a Bio-Rad iCycler (iQ5 Real Time PCR Detection System) using a 2-stage program provided by the manufacturer: 3 min at 95 °C and 40 cycles of 10 sec at 95 °C and 30 s at 54.5 °C. For standardization of quantification, L27 was amplified simultaneously.

**Table 1**
Primer list.

| | Name | PCR primer sequences, 5′–3′ | GenBank accession no. |
|---|---|---|---|
| 1 | IL-1β | GCT-TCA-GGC-AGG-CAG-TAT-C<br>AGG-ATG-GGC-TCT-TCT-TCA-AAG | NM_008361 |
| 2 | IL-6 | ACC-GCT-ATG-AAG-TTC-CTC-TC<br>CT-CTG-TGA-AGT-CTC-CTC-TCC | NM_031168 |
| 3 | TNF-α | GGT-TCT-GTC-CCT-TTC-ACT-CAC<br>T-GCC-TCT-TCT-GCC-AGT-TCC | NM_013693 |
| 4 | NOS-II | AGC-CCT-CAC-CTA-CTT-CCT-G<br>CAA-TCT-CTG-CCT-ATC-CGT-CTC | NM_010927 |
| 5 | COX-2 | GCC-TGG-TCT-GAT-GAT-GTA-TGC<br>G-AGT-ATG-AGT-CTG-CTG-GTT-TGG | NM_011198 |
| 6 | CCL2 | CAC-TCA-CCT-GCT-GCT-ACT-CAT-TC<br>GCT-TCT-TTG-GGA-CAC-CTG-CTG | NM_011333 |
| 7 | CCL20 | ATG-GGT-ACT-GCT-GGC-TCA-C<br>CT-GGA-AGG-AAG-AGG-CGT-CTG | NM_016960 |
| 8 | CCR2 | CTC-AGT-TCA-TCC-ACG-GCA-TAC<br>GA-CAA-GGC-TCA-CCA-TCA-TCG | NM_009915 |
| 9 | Arginase1 | AGA-CAG-CAG-AGG-AGG-TGA-AGA-G<br>CGA-AGC-AAG-CCA-AGG-TTA-AAG-C | NM_007482 |
| 10 | Mmr | AGT-GGC-AGG-TGG-CTT-ATG<br>G-GTT-CAG-GAG-TTG-TTG-TGG | NM_008625 |
| 11 | Ym(1/2) | CAT-TCA-GTC-AGT-TAT-CAG-ATT-CC<br>AGT-GAG-TAG-CAG-CCT-TGG | NM_009892 |
| 12 | FIZZ1 | TGG-AGA-ATA-AGG-TCA-AGG-AAC<br>G-TCA-ACG-AGT-AAG-CAC-AGG | NM_020509 |
| 13 | PPAR-γ | GCA GGA GCA GAG CAA AGA G<br>GGA CAC CAT ACT TGA GCA GAG | NM_011146 |
| 14 | Notch1 | GCA-GGC-AAG-ATG-TTA-ATG-AGT-G<br>A-GGG-CAC-ATA-GGG-CAG-TTC | NM_008714 |
| 15 | Hes1 | GCC-AAT-TTG-CCT-TTC-TCA-TCC<br>G-GTG-ACA-CTG-CGT-TAG-GAC | NM_008235 |
| 16 | L27 | ACA-TTG-ACG-ATG-GCA-CCT-C<br>GC-TTG-GCG-ATC-TTC-TTC-TTG | NM_011289 |

The change of reporter fluorescence from each reaction tube was monitored. The threshold cycle of each gene was determined as PCR cycles at which an increase in reporter fluorescence above a baseline signal was observed. The difference in threshold cycles between the target gene and reference gene (L27) gives the standardized expression level (dCt). Subtraction of dCt of a defined control condition from dCt of different conditions gives the ddCt value that was used to calculate relative expression levels of different conditions with the formula $2^{-ddCt}$. The expression levels of each gene were expressed as normalized fold expression.

Each sample was tested in triplicate PCR wells and samples obtained from three independent experiments were used to calculate the mean ± SEM.

### 2.3. Measurement of the phagocytosis capacity

Volumes of 500 µL of cell suspension (density of $2 \times 10^4$ cells/well) were seeded into 48-well microtiter plates. After 48 h at 37 °C, the cells were treated and further incubated during 24 h. Yellow-green Fluo-Spheres (1 µm, Molecular Probes, CA) were resuspended in 25 mM $Na_2HPO_4$, pH 6.0 containing 3% BSA and incubated at room temperature for 15 min with bath sonication. The sonicated suspension, containing $10^8$ microspheres in 25 µL, was added to cultured microglia. After 75 min at 37 °C, cells were washed 3 times with PBS and fluorescence was measured at 520 nm with a microplate reader (FLUOstar OPTIMA, BMG LABTECH, NC). To differentiate between phagocytosed beads and beads that non-specifically adhered to the cell surface, control cells were exposed to 0.3% azide for 10 min before the addition of microspheres. This treatment compromises microglial energetic processes and few beads are internalized as observed by fluorescent microscopy. The fluorescence of azide treated MMGT12 microglial cells was used as the negative control. Background fluorescence of MMGT12 microglial cells cultured in the absence of fluorescent microspheres was less than 10% of the fluorescence values obtained using azide treated MMGT12 microglial cells cultured in the presence of microspheres.

In the same experiments, the luminescent cell viability assay was conducted to quantify the number of cells per well. The CellTiter-Glo luminescent cell viability assay (Promega) was used to determine the number of viable cells in culture.

### 2.4. Arginase assay

Arginase activity was measured in cell lysates with slight modifications, as previously described (Corraliza et al., 1994). Briefly,

A

| | MMGT12 | | Primary microglia | |
|---|---|---|---|---|
| Genes | LPS | IFN-γ | LPS | IFN-γ |
| IL-1β | 1091.85 ± 118.07 | 0.24 ± 0.05 | 3482.76 ± 214.18 | 0.21 ± 0.05 |
| IL-6 | 2689.04 ± 207.11 | 2.90 ± 0.54 | 35936.50 ± 2734.18 | 2.19 ± 0.37 |
| TNF-α | 11.56 ± 0.56 | 2.11 ± 0.08 | 1659.40 ± 142.48 | 10.10 ± 1.86 |
| NOS-II | 581.92 ± 15.14 | 10.27 ± 1.95 | 22737.86 ± 1932.84 | 101.80 ± 17.69 |
| COX-2 | 331.53 ± 18.47 | 28.98 ± 2.59 | 3314.10 ± 197.49 | 60.24 ± 11.07 |
| CCL2 | 104.10 ± 8.97 | 31.62 ± 0.94 | 636.74 ± 54.19 | 18.63 ± 0.54 |
| CCL20 | 62.20 ± 3.49 | 0.67 ± 0.12 | 15.64 ± 2.30 | 0.69 ± 0.54 |
| CCR2 | 0.77 ± 0.04 | 1.27 ± 0.12 | 0.10 ± 0.04 | 1.75 ± 0.15 |
| Arginase1 | 0.54 ± 0.21 | 0.63 ± 0.11 | 0.29 ± 0.02 | 0.60 ± 0.10 |
| Mmr | 0.27 ± 0.04 | 0.54 ± 0.03 | 0.30 ± 0.02 | 0.58 ± 0.05 |
| Ym(1/2) | n.d. | n.d. | n.d. | n.d. |
| FIZZ1 | n.d. | n.d. | n.d. | n.d. |
| PPAR-γ | 0.06 ± 0.04 | 0.26 ± 0.14 | 0.09 ± 0.01 | 0.65 ± 0.16 |
| Notch1 | 2.50 ± 0.07 | 4.01 ± 0.39 | 3.45 ± 0.37 | 2.99 ± 0.66 |
| Hes1 | 0.08 ± 0.02 | 0.31 ± 0.03 | 0.17 ± 0.01 | 0.71 ± 0.15 |

n.d. not detectable

B

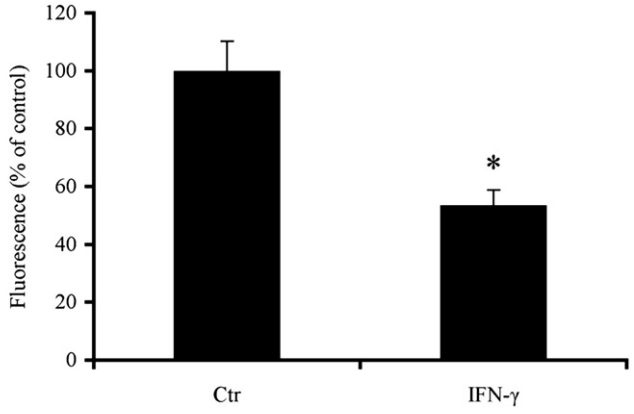


**Fig. 1.** M1-like phenotype in microglial cells. A. MMGT12 and primary microglial cells gene expression profiles. Data are representative of three independent experiments. Gene expression levels were determined by reverse transcription followed by real-time PCR and were normalized with the housekeeping gene L27. The transcription levels were analyzed after 6 h of incubation with LPS (10 ng/mL in MMGT12 cells and 1 ng/mL in primary microglia) or IFN-γ (100 U/mL). Results (value ± SD) are expressed relative to control conditions. B. Phagocytosis assay. MMGT12 microglial cells were treated with IFN-γ (100 U/mL) during 24 h. Data are corrected for the cell population values and are expressed as mean ± SEM values obtained from three experiments, each conducted in independent cultures ($n = 3$). Results are expressed relative to control (Ctr = 100%). *$p<0.05$, fluorescence levels are significantly different from the corresponding control levels.

A

| | MMGT12 | | Primary microglia | |
|---|---|---|---|---|
| Genes | IL-10 | IL-4 | IL-10 | IL-4 |
| IL-1β | 1.73 ± 0.24 | 0,77 ± 0.15 | 1.74 ± 0.15 | 0.49 ± 0.07 |
| IL-6 | 0.81 ± 0.48 | 1.23 ± 0.56 | 0.87 ± 0.25 | 0.92 ± 0.65 |
| TNF-α | 0.78 ± 0.04 | 0.35 ± 0.01 | 0.81 ± 0.03 | 0.55 ± 0.02 |
| NOS-II | 2.55 ± 1.00 | 2.27 ± 0.57 | 2.89 ± 1.75 | 1.06 ± 0.47 |
| COX-2 | 2.10 ± 0.10 | 1.01 ± 0.08 | 1.23 ± 0.08 | 1.86 ± 0.39 |
| CCL2 | 1.52 ± 0.12 | 1.81 ± 0.08 | 1.46 ± 0.12 | 4.76 ± 0.58 |
| CCL20 | 0.72 ± 0.29 | 0.87 ± 0.18 | 0.99 ± 0.07 | 1.29 ± 0.10 |
| CCR2 | 1.34 ± 0.12 | 0.60 ± 0.06 | 1.43 ± 0.17 | 0.64 ± 0.06 |
| Arginase1 | 1.27 ± 0.12 | 5.98 ± 0.75 | 1.33 ± 0.12 | 30.54 ± 2.35 |
| Mmr | 1.02 ± 0.05 | 5.25 ± 0.37 | 1.36 ± 0.08 | 3.01 ± 0.13 |
| Ym(1/2) | n.d. | 40.18 ± 4.87 | n.d. | 23.96 ± 4.63 |
| FIZZ1 | n.d. | 1.89 ± 0.03 | n.d. | 250.31 ± 67 |
| PPAR-γ | 0.93 ± 0.12 | 2.24 ± 0.51 | 0.96 ± 0.03 | 1.76 ± 0.13 |
| Notch1 | 0.99 ± 0.23 | 0.61 ± 0.14 | 1.22 ± 0.10 | 0.79 ± 0.08 |
| Hes1 | 1.04 ± 0.24 | 1.05 ± 0.23 | 0.88 ± 0.12 | 1.19 ± 0.08 |

n.d. not detectable

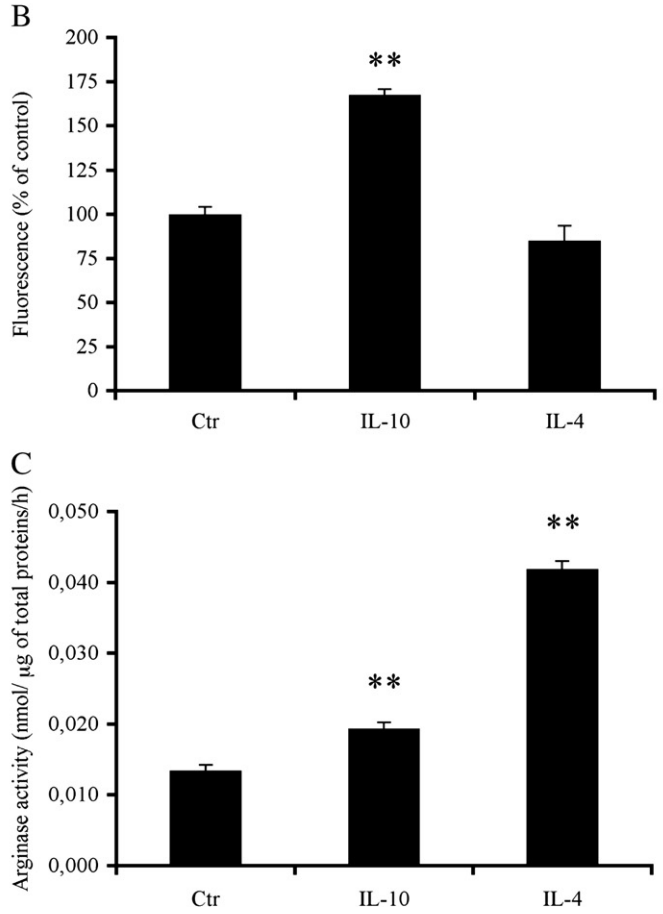


**Fig. 2.** M2-like phenotype in microglial cells. A. MMGT12 and primary microglial cells gene expression profiles. Data are representative of three independent experiments. Gene expression levels were determined by reverse transcription followed by real-time PCR and were normalized with the housekeeping gene L27. The transcription levels were analyzed after 6 h of incubation with IL-10 (1 ng/mL) or IL-4 (1 ng/mL). Results (value ± SD) are expressed relative to control conditions. B. Phagocytosis assay. MMGT12 microglial cells were treated with IL-10 (1 ng/mL) or IL-4 (1 ng/mL) during 24 h. Data are corrected for the cell population values and are expressed as mean ± SEM values obtained from three experiments, each conducted in independent cultures ($n = 3$). Results are expressed relative to control (Ctr = 100%). $^{**}p < 0.01$, fluorescence levels are significantly different from the corresponding control levels. C. Arginase assay. MMGT12 microglial cells were treated with IL-10 (1 ng/mL) or IL-4 (1 ng/mL) during 24 h and arginase activity was determined. Data are expressed as mean ± SEM values obtained from three experiments, each conducted in independent cultures ($n = 3$). Results are expressed relative to control (Ctr = 0.013 nmol/µg of total proteins/h). $^{**}p < 0.01$, arginase activity is significantly different from the corresponding control level.

cells were lysed with 150 µL of 0.1% Triton X-100 (Sigma). After 30 min on a shaker, 50 µL of 50 mM Tris–HCl, 10 mM $MnCl_2$ (pH 7.5) were mixed with 50 µL of lysate and the enzyme was activated by heating for 10 min at 56 °C. Arginine hydrolysis was conducted by incubating the lysate with 50 µL of 0.5 M L-arginine (pH 9.7) at 37 °C for 120 min. The reaction was stopped with 400 µL of $H_2SO_4$ (96%)/$H_3PO_4$ (85%)/

$H_2O$ (1/3/7, v/v/v). The urea concentration was measured after addition of 25 μL 9% α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 100 °C for 45 min. Absorbance was measured at 550 nm in a microplate reader (TECAN, Austria). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per min.

### 2.5. Aβ preparation and Thioflavine T assay

Aβ1-42 (Bachem, Germany) was dissolved at a concentration of 20 mg/mL (5 mM) in dimethyl sulfoxide. The stock was diluted to 2 mg/mL (0.5 mM) in DMEM/F12 containing 1% antibiotics. The oligomeric-Aβ (ol-Aβ) was added directly to cultures at a final concentration of 5 μM. The fibrillar-Aβ (f-Aβ) was obtained incubating the oligomeric form during 2 days at 37 °C and then added to cultures at the same final concentration.

For the Thioflavine T assay, the stock of Aβ1-42 was diluted to 0.2 mg/mL (50 μM) in PBS and then 100 μL of this solution were mixed with 900 μL of 3 μM Thioflavine T (in 50 mM potassium phosphate buffer, pH 6.0). The solution was added to a 96-well plate at 100 μL/well ($n=6$). Fluorescence was read at 485 nm using a microplate reader (FLUOstar OPTIMA).

### 2.6. Statistical analysis

For comparison of means between two different treatments, statistical analysis was done by Student's *t*-test (two-sample assuming equal variances). For comparison of multiple treatments, results were analyzed by an ANOVA followed by a Fisher's exact test.

## 3. Results

### 3.1. Characterization of the M1-like state in microglial cells

In an attempt to analyze different pro-inflammatory phenotypes, microglial cells were exposed to LPS (10 ng/mL or 1 ng/mL for MMGT12 or primary microglial cells respectively, according to their response levels) or IFN-γ (100 U/mL). Both stimuli induced high expression levels of the inflammatory cytokines, IL-6 and TNF-α, as well as the chemokine CCL2 (Fig. 1A). The expression of genes encoding for the enzymes responsible for the production of free radicals (NOS-II) or prostaglandins (COX-2) was also enhanced. In contrast to LPS, IFN-γ down regulated IL-1β and CCL20 transcription levels in MMGT12 and primary microglial cells compared to the control levels. Moreover, LPS inhibited the M2 signature gene CCR2, while IFN-γ increased it. Arginase1 and Mmr expression levels were reduced by LPS as well as by IFN-γ.

Since a modulatory role of the Notch pathway during macrophage activation has been documented (Monsalve et al., 2006), two components of this pathway have been undertaken as M1 or M2 markers. As shown in our previous results (Grandbarbe et al., 2007), under inflammatory conditions (LPS or IFN-γ) Notch1 mRNA was raised, while Hes1 was strongly reduced. In the same conditions, PPAR-γ expression levels, known to be modulated during inflammatory responses (Bernardo and Minghetti, 2006), were strongly reduced (Fig. 1A).

As the microglial phagocytic activity is influenced by different inflammatory stimuli (Koenigsknecht-Talboo and Landreth, 2005), the study of the behavior towards this parameter was deepened. IFN-γ decreased the MMGT12 phagocytic activity by 46.5% (Fig. 1B), while LPS had no influence on the capacity to ingest microspheres (data not shown).

### 3.2. Characterization of the M2-like state in microglial cells

At the opposite side of the spectrum of phenotypes, in order to study their different anti-inflammatory states, microglial cells were exposed to IL-10 (1 ng/mL) or IL-4 (1 ng/mL).

Microglial cells treated with IL-10 were characterized by a slight increase of IL-1β, NOS-II, COX-2, and CCL2 expression levels (Fig. 2A). TNF-α mRNA was decreased by IL-10 compared to the control levels. IL-10 was also able to slightly enhance the M2 signature genes, CCR2 and arginase1. The Notch components and PPAR-γ were not influenced by IL-10 treatment.

IL-4 reduced IL-1β and CCR2 expression levels compared to the control conditions. As IL-10, IL-4 reduced TNF-α mRNA. The transcription of the typical "alternative" genes, arginase1, Mmr, Ym(1/2) and FIZZ1 was strongly enhanced after IL-4 treatment in MMGT12 and primary microglial cells. The Notch receptor transcription level was decreased by IL-4, while PPAR-γ was enhanced (Fig. 2A).

The phagocytosis ability was strongly enhanced by IL-10 (168%), but was not influenced by IL-4 (Fig. 2B). The arginase assay confirmed that IL-10, and more potently IL-4, shift microglial cells towards an increase of the arginase activity (Fig. 2C).

### 3.3. Jagged1-induced polarization

Since Notch ligands are expressed by neurons and astrocytes (Elyaman et al., 2007; Sestan et al., 1999), the effect of Notch stimulation on microglial cells by the soluble ligand Jagged1 was analyzed. Stimulation of MMGT12 microglial cells with the soluble ligand Jagged1

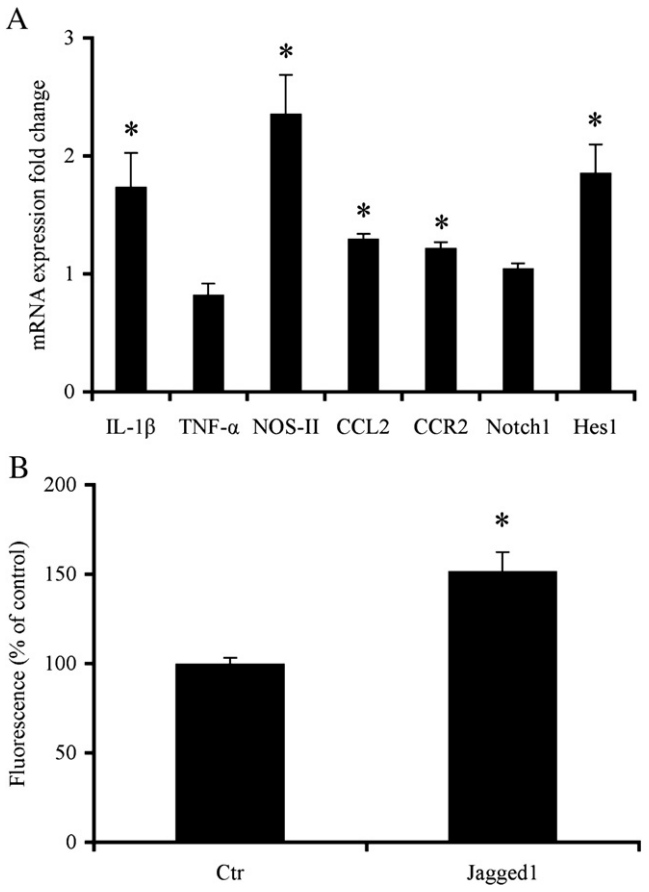


**Fig. 3.** Notch stimulation affects microglial cells behaviour. A. MMGT12 microglial cells were treated with Jagged1 (1 μg/mL) and RNA extraction was realized after 6 h of incubation. Real-time RT-PCR results were normalized using L27 as a reference gene and are shown as average expression fold change (±SEM) values obtained from three experiments, each conducted in independent cultures ($n=3$), respective mRNA in control conditions. *$p<0.05$, mRNA levels are significantly different from the corresponding control levels. B. Phagocytosis assay. MMGT12 microglial cells were treated with Jagged1 (1 μg/mL) during 24 h. Data are corrected for the cell population values and are expressed as mean ± SEM values obtained from three experiments, each conducted in independent cultures ($n=3$). Results are expressed relative to control (Ctr = 100%). *$p<0.05$, fluorescence levels are significantly different from the corresponding control levels.

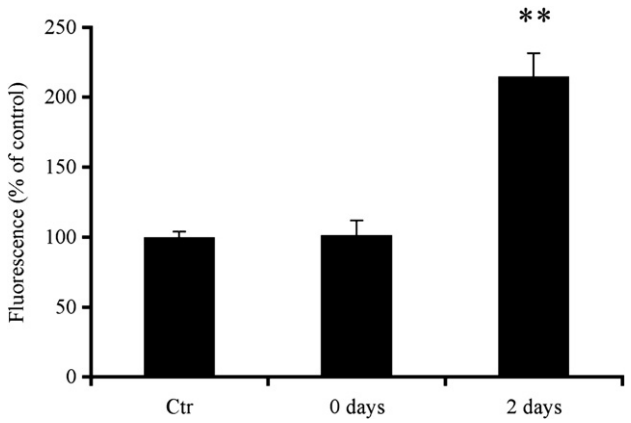


**Fig. 4.** Verification of the Aβ form by the Thioflavine T assay. The two forms of Aβ (ol-Aβ and f-Aβ) were prepared as described (see Materials and methods). The Thioflavine T assay was performed to assess the fibrillization of Aβ after a pre-incubation at 37 °C of the oligomeric form during 2 days. Data are expressed as mean ± SEM values obtained from three experiments, each conducted in independent assays ($n = 3$). **$p < 0.01$, fluorescence levels are significantly different from the corresponding control levels.

(1 μg/mL) enhanced IL-1β, NOS-II, and to a lesser extent CCL2 and CCR2 expression levels (Fig. 3A). The increase of Hes1 expression level after Jagged1 treatment confirmed that the Notch pathway was indeed stimulated by the Notch ligand (Fig. 3A).

The phagocytic ability was increased by the soluble ligand Jagged1 compared to the control levels (Fig. 3B).

### 3.4. Oligomeric versus fibrillar Aβ-induced polarization

In order to compare the behavior of microglial cells in the presence of Aβ to the characterized M1- or M2-like phenotypes, two different Aβ forms have been analyzed. The ol-Aβ was obtained without previous incubation, while the f-Aβ was obtained by an incubation of the oligomeric form at 37 °C during 2 days (see Materials and methods). The fibrillization of the Aβ was confirmed by the thioflavine T assay (Fig. 4).

Primary microglial cells exposed to ol-Aβ (5 μM) were characterized by high expression levels of IL-1β, IL-6, TNF-α, NOS-II, COX-2, and CCL2 (Fig. 5A, B). On the contrary, f-Aβ increased the pro-inflammatory parameters to a much lesser extent compared to the oligomeric form. CCR2, arginase1, and Mmr were more decreased by ol-Aβ than by f-Aβ. Like in classical pro-inflammatory conditions (LPS and IFN-γ), Notch1 expression level was increased (also if not in a significant manner), while Hes1 and PPAR-γ were reduced by the two forms of Aβ (Fig. 5C). Moreover, the phagocytosis capacity was decreased in the presence of both forms of the peptide compared to the untreated cells (Fig. 5D).

### 3.5. The effect of oligomeric Aβ treatment on M2-like microglia

Compared to ol-Aβ alone, the pre-treatment with the anti-inflammatory cytokine IL-10 (1 ng/mL) 1 h before ol-Aβ (5 μM)

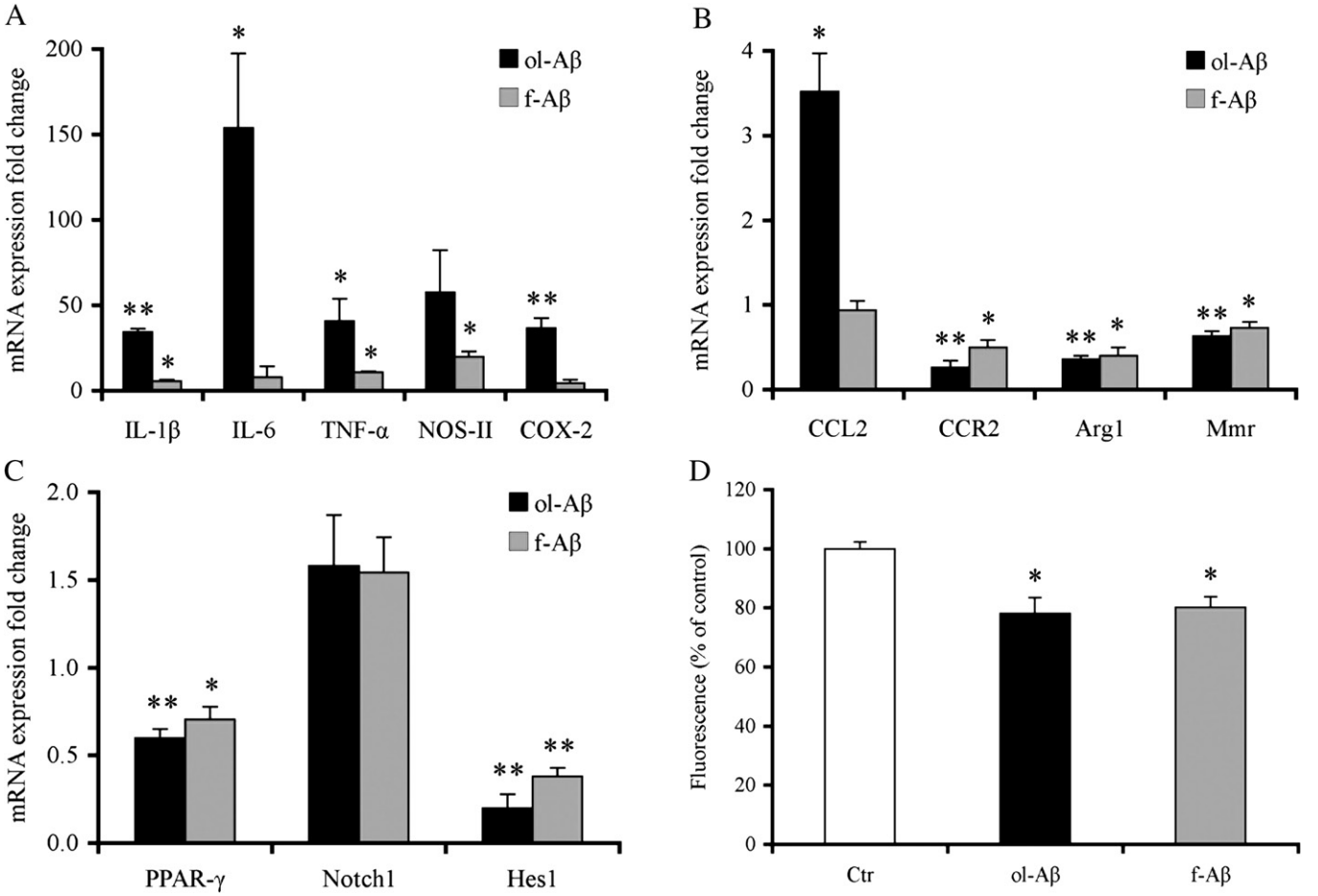


**Fig. 5.** Aβ induces a M1-like polarization, but the levels differ between ol-Aβ and f-Aβ. A, B, C. Primary microglial cells were treated with ol-Aβ (5 μM) or f-Aβ (5 μM) and RNA extraction was realized after 6 h of incubation. Real-time RT-PCR results were normalized using L27 as a reference gene and are shown as average expression fold change (± SEM) values obtained from three experiments, each conducted in independent cultures ($n = 3$), respective mRNA in control conditions. **$p < 0.01$, *$p < 0.05$, mRNA levels are significantly different from the corresponding control levels. D. Phagocytosis assay. MMGT12 microglial cells were treated with ol-Aβ (5 μM) or f-Aβ (5 μM) during 24 h. Data are corrected for the cell population values and are expressed as mean ± SEM values obtained from three experiments, each conducted in independent cultures (n = 3). Results are expressed relative to control (Ctr = 100%). *$p < 0.05$, fluorescence levels are significantly different from the corresponding control levels.

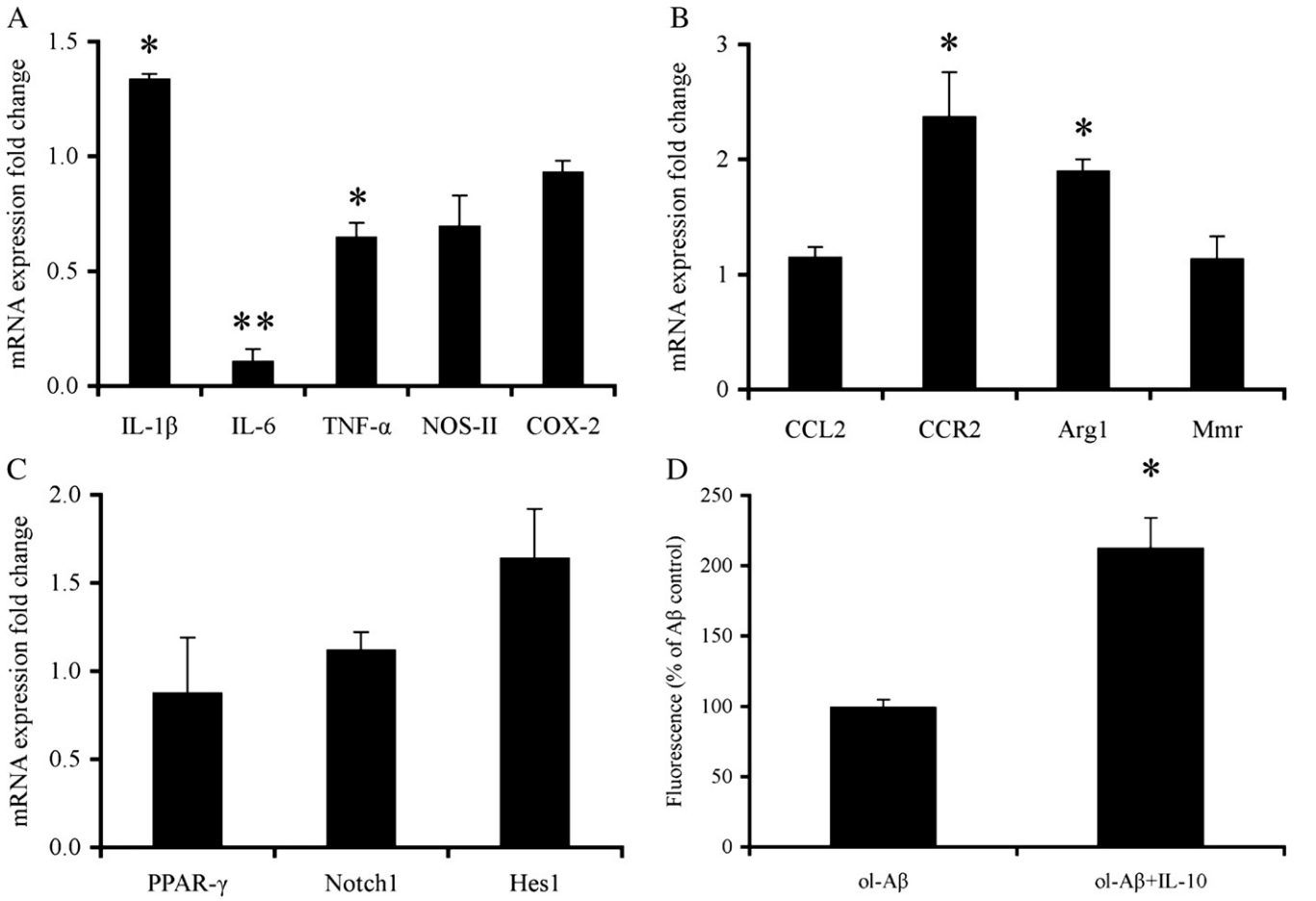


**Fig. 6.** The effect of IL-10 on ol-Aβ-induced microglial activation. A, B, C. Primary microglial cells were treated with IL-10 (1 ng/mL) 1 h before ol-Aβ (5 μM) activation and RNA extraction was realized after 6 h of ol-Aβ incubation. Real-time RT-PCR results were normalized using L27 as a reference gene and are shown as average expression fold change (±SEM) values obtained from three experiments, each conducted in independent cultures ($n=3$), respective mRNA in ol-Aβ treated cells. **$p<0.01$, *$p<0.05$, mRNA levels are significantly different from the corresponding control levels. D. Phagocytosis assay. MMGT12 microglial cells were treated with IL-10 (1 ng/mL) 1 h before ol-Aβ (5 μM) activation during 24 h. Data are corrected for the cell population values and are expressed as mean ± SEM values obtained from three experiments, each conducted in independent cultures ($n=3$). Results are expressed relative to ol-Aβ treated cells (ol-Aβ = 100%). *$p<0.05$, fluorescence levels are significantly different from the corresponding control levels.

activation slightly enhanced IL-1β expression levels, strongly reduced those of IL-6, and decreased this of TNF-α (Fig. 6A). The transcription levels of CCR2 and arginase1 were increased (Fig. 6B). Notch1 and PPAR-γ expression levels were not modified by the pre-treatment of IL-10 1 h before ol-Aβ activation compared to ol-Aβ alone, while Hes1 was enhanced (also if not in a significant manner) (Fig. 6C). Moreover, in the same conditions, the phagocytosis ability was strongly increased (Fig. 6D).

IL-4 (1 ng/mL) pre-treatment 1 h before ol-Aβ (5 μM) activation reduced IL-1β, IL-6, and TNF-α transcription levels, while raised this of NOS-II compared to ol-Aβ treatment alone (Fig. 7A). In the same conditions, mRNA "alternative" genes arginase1, Ym(1/2), and FIZZ1 were increased (Fig. 7B). Moreover, Notch1 expression level was decreased, while PPAR-γ mRNA was enhanced (Fig. 7C). IL-4 did not affect the decrease of the phagocytic ability induced by Aβ (data not shown).

## 4. Discussion

Microglia, the standby cells for immune defense in the CNS, have a reputation for exacerbating the neural damage that occurs in neurodegenerative diseases (Kreutzberg, 1996). However, research over the past few years has established that microglia do not constitute a single, uniform cell population, but rather comprise a family of cells with diverse phenotypes, some that are beneficial and others that the CNS can hardly tolerate and that are therefore destructive (Schwartz et al., 2006; Butovsky et al., 2006; Butovsky et al., 2005).

In the present study, we have analyzed the phenotypes of microglial cells induced by different stimuli using the terminology that is well accepted for the description of different states of activation in macrophages/microglia, M1 and M2 being the extremes of a spectrum of phenotypes (Mantovani et al., 2004; Mantovani et al., 2002).

In the first part of the study, our transcription profile analysis clearly shows that MMGT12 and primary microglial cells exposed to LPS or IFN-γ are characterized by a pronounced pro-inflammatory phenotype that is comparable to the classical M1-like state in macrophages. Moreover, the phagocytic capacity is dramatically decreased. On the other hand, microglial cells treated with IL-10 or IL-4 are characterized by anti-inflammatory states, called respectively the M2-deactivated state and the M2-alternatively activated. Phagocytosis is clearly enhanced by an IL-10 treatment. These different states of microglial differentiation reflect the complexity of these cells and their capacity to differentiate towards a multitude of phenotypes depending on their surrounding environment.

In order to complete the list of signature genes useful to understand the phenotype that is acquired by microglia during the progression of neurodegenerative diseases, we have included two novel genes pertaining to the Notch pathway. As shown in our previous results (Grandbarbe et al., 2007), under pro-inflammatory conditions (LPS or IFN-γ), Notch1 transcription is raised, while that of the correspondent effector Hes1 is diminished, suggesting that Notch1 and Hes1 could be seen as a M1 and M2 signature genes respectively. Furthermore, for the first time, our data

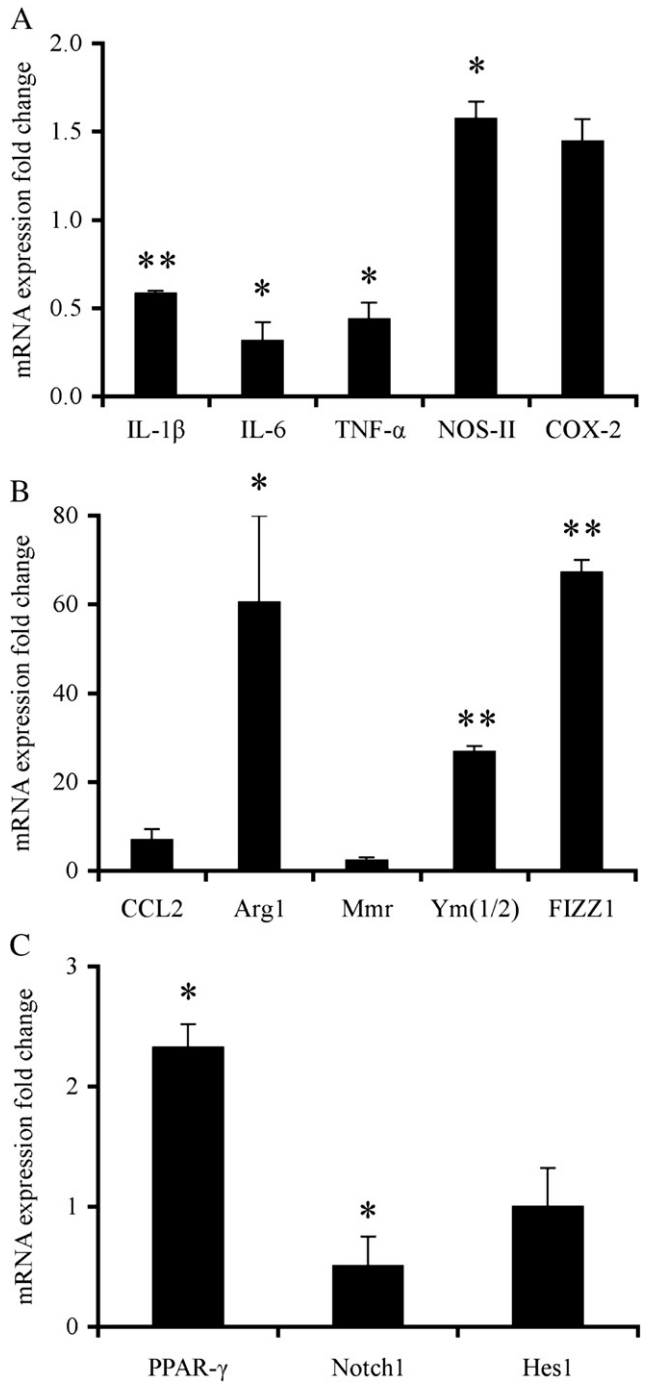


**Fig. 7.** The effect of IL-4 on ol-Aβ-induced microglial activation. A, B, C. Primary microglial cells were treated with IL-4 (1 ng/mL) 1 h before ol-Aβ (5 μM) activation and RNA extraction was realized after 6 h of ol-Aβ incubation. Real-time RT-PCR results were normalized using L27 as a reference gene and are shown as average expression fold change (±SEM) values obtained from three experiments, each conducted in independent cultures ($n=3$), respective mRNA in ol-Aβ treated cells. $^{**}p<0.01$, $^{*}p<0.05$, mRNA levels are significantly different from the corresponding control levels.

show that IL-4 decreases Notch1 expression in MMGT12 and primary microglial cells. Since an up-regulation of Notch1 is associated with pro-inflammatory events (Grandbarbe et al., 2007; Monsalve et al., 2006), the alternatively activated cells confirm a phenotype that is at the opposite side of a pro-inflammatory differentiation.

Since Hes1 is affected by a pro-inflammatory exposure, the effect of the soluble Notch1-ligand Jagged1 on microglial cell differentiation has been compared to the M1 and M2 phenotypes. Our data show that MMGT12 microglial cells stimulated with the ligand present a gene expression profile that is only slightly different from a resting state and that, for the expression of some genes, resembles that characterized for IL-10. Moreover, in support of these results, the phagocytic activity is strongly enhanced after Notch stimulation, suggesting that the treatment of microglial cells with Jagged1 steers them towards a similar M2-like state. In a normal brain, neurons can communicate with microglia through the release of "calming inputs" (e.g. fractalkine produced by neurons and its receptor on microglial cells) (Cardona et al., 2006). Since microglial cells are tightly integrated into a dense tissue, it seems reasonable that cell-contacts between microglial cells and other cells of the CNS could be a means to inform themselves of their state and their surrounding environment. The expression of Jagged1 by neurons or astrocytes (Elyaman et al., 2007; Sestan et al., 1999) could serve to maintain microglial cells in an anti-inflammatory state. It has been shown that astroglial expression of Jagged1 is modulated by inflammatory cytokine signaling (Elyaman et al., 2007; John et al., 2002). An increased expression of Jagged1 on astrocytes will thus influence the microglial phenotype towards a deactivated M2-like differentiation.

In the second part of the study, we have analyzed the phenotype that is acquired by microglial cells primed with two different forms of the Aβ peptide. Our results show that microglia exposed to oligomeric-Aβ (ol-Aβ) present a pro-inflammatory M1-like phenotype that closely resembles that induced by LPS treatment. The fibrillar form induces a similar response, also if with much lower amplitudes in gene expression. It thus appears that the most toxic form of Aβ for neurons, the oligomers (Lesne et al., 2006), is also the most potent activator of microglial cells. Supporting the idea that Aβ generates a M1-like state, the phagocytic capacity is decreased in the presence of both oligomeric and fibrillar forms. This diminished phagocytic ability, together with the pro-inflammatory gene expression profile acquired by microglial cells, could explain the vicious circle that characterizes AD progression and the subsequent Aβ accumulation. This self-sustained pro-inflammatory environment will thus decrease the clearance of Aβ by microglial cells and simultaneously increase the neuronal production of the peptide (Sastre et al., 2006).

In the last few years, several groups have focused on the understanding of microglial cell phenotypes during AD progression. Recently, Jimenez (Jimenez et al., 2008) shows evidence of a switch in the activated microglia phenotype from alternative, at the beginning of Aβ pathology, to a classical at advanced stage of the disease in a mice model of AD. Colton and collaborators (Colton et al., 2006) demonstrates that, in AD patients and in mice models of AD and of cerebral amyloid angiopathy, microglial cells exhibit a hybrid activation state that includes characteristics of classical and alternative activation. Although these results are conflicting, they show that during AD progression, it is highly likely that microglial cells change their activation state as a function of the disease and time. It is well known that macrophages may become refractory to inflammatory stimulation and shift towards an anti-inflammatory state after prolonged exposure (Mosser, 2003). Emerging evidence indicates that the same phenomenon can occur in microglia. Persistent activation of cultured rat microglia with LPS induces significant alterations in the signaling network downstream from the LPS receptor, and progressive down-regulation of TNF-α and nitric oxide (NO) production (Ajmone-Cat et al., 2003; Cacci et al., 2008). A similar process could occur in the presence of Aβ because this peptide, when aggregating into fibrils, shares receptors and intracellular signaling cascades with LPS (Fassbender et al., 2004). Therefore, while inducing a M1-like state immediately in their early oligomeric form, Aβ in their aggregating form will skew microglial cells towards a refractory, or less activated phenotype. Thus, our results are in line with those of Colton and co-workers, who describe the co-existence of these two phenotypes in the in vivo situation.

Finally, we were interested on the phenotype of microglial cells pre-treated with anti-inflammatory cytokines before ol-Aβ activation. It is known that microglia respond differently to the same stimulus

depending on whether other stimuli precede or follow it, suggesting that behavior of microglia is dictated by an acquired phenotype rather than stereotypically (Schwartz et al., 2006). It has been shown that IL-10 or IL-4 are able to reduce the microglial pro-inflammatory state induced by Aβ (Lyons et al., 2007; Szczepanik et al., 2001). Our data show that microglia activated with even low doses of IL-10 or IL-4 remain affected by these treatments even when they were exposed to a threatening environment in the form of the more potent ol-Aβ. Since the normal aging of the human brain is characterized by an increase in the expression of pro-inflammatory cytokines (Ye and Johnson, 1999) along with a decrease of anti-inflammatory cytokines, such as IL-10 (Ye and Johnson, 2001) and IL-4 (Maher et al., 2005; Nolan et al., 2005), it is to be expected that senescent microglial cells will be more easily skewed towards a M1-like phenotype in the presence of newly synthesized oligomers of Aβ than young ones.

Taken together, our results clearly emphasize that the variability of microglial activity is not merely a reflection of stimulus strength or persistence. Rather, it is determined largely by the nature and context of the stimuli and the intracellular signal transduction pathways that they activate.

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