Overexpression of Macrophage Colony Stimulating Factor Receptor on Microglial Cells Induces an Inflammatory Response*

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1 The abbreviations used are: M-CSF, macrophage colony stimulating factor; M-CSFR, macrophage colony stimulating factor receptor; Aβ, amyloid β peptide; IL-1, interleukin-1; IL-6, interleukin-6, MIP-1α, microphage inflammatory protein-1α; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; kb, kilobase pair(s); bp, base pair(s), kDa, kilodalton, LDH, lactate dehydrogenase, ELISA, enzyme-linked immunosorbent assay;

Running Title: M-CSF Receptor Overexpression in Microglia
Summary

Microglia are important in the inflammatory response in Alzheimer’s disease (AD). We previously showed that macrophage colony stimulating factor receptor (M-CSFR), encoded by the c-fms protooncogene, is overexpressed on microglia surrounding amyloid beta (Aβ) deposits in APPV717F mouse model for AD. The M-CSFR is also increased on microglia after experimental brain injury and in AD. To determine the relevance of these findings we transiently expressed M-CSFR on murine BV-2 and human SV-A3 microglial cell lines using a SV-40 promoted c-fms construct. M-CSFR overexpression resulted in microglial proliferation and increased expression of iNOS, the proinflammatory cytokines interleukin-1α, macrophage inflammatory protein 1-α, IL-6 and of macrophage colony stimulating factor (M-CSF) itself. Antibody neutralization of M-CSF showed that the M-CSFR-induced proinflammatory response was dependent on M-CSF in the culture media. Using a co-culture of c-fms transfected murine microglia and rat organotypic hippocampal slices and a species-specific real-time RT-PCR assay and ELISA, we showed that M-CSFR overexpression on exogenous microglia induced expression of IL-1α by the organotypic culture. These results show that increased M-CSFR expression induces microglial proliferation, cytokine expression and a paracrine inflammatory response, suggesting that in APPV717F mice, increased M-CSFR on microglia could be an important factor in Aβ-induced inflammatory response.
INTRODUCTION

In Alzheimer’s disease (AD) inflammation mediated by microglia may play a central role in neuronal injury and cognitive decline (1-4). Numerous activated microglia are found in clusters surrounding Aβ plaques in AD (5,6). Cytokines and reactive oxygen and nitrogen species released by microglia are thought to be toxic to neurons, as well as important in sustaining the inflammatory response through autocrine and paracrine effects. Anti-inflammatory medications reduce the risk for AD in humans and slow the progression of AD-like pathology in transgenic mice modeling AD (7-9). Identification of molecular targets involved in the initiation and maintenance of inflammation may lead to new therapeutic options for AD.

M-CSFR, expressed on cells of the monocyte-macrophage lineage, belongs to the tyrosine kinase Type III family of receptors (10,11), and interacts with multiple signal transduction proteins including src kinases, STAT1, Grb2, and phosphoinositide 3-kinase (11,12). We recently showed that microglia surrounding Aβ plaques in the AβPPV717F transgenic mouse model of AD show increased immunoreactivity for M-CSFR (13). Increased M-CSFR expression by microglia has been reported on activated microglia after ischemic and mechanical brain injury in mice (14,15). Further, the M-CSFR is increased on microglia in AD brain, suggesting that overexpression of this receptor is integral to AD pathophysiology (16).

The hematopoietic cytokine M-CSF (CSF-1) is expressed by cultured neurons, astrocytes, endothelia, and microglia (17-22). M-CSF induces proliferation, migration, and activation of microglia (23-27). M-CSF is upregulated in AD brain (28). Further,
treatment of cultured microglia with M-CSF results in a dramatic augmentation of Aβ-induced cytokine and nitric oxide production (29).

To examine biological relevance of increased M-CSFR abundance on microglia, we overexpressed M-CSFR gene, c-fms (10), in murine BV-2 and human SV-A3 microglial cell cultures. The effects of increased c-fms expression on microglial proliferation and expression of IL-1α, MIP-1α, iNOS and IL-6 were determined with real-time RT-PCR (30) and ELISA. We also used a co-culture system consisting of c-fms transfected BV-2 cells integrated into organotypic hippocampal slices to determine the paracrine effects of c-fms overexpression on cytokine expression in an organotypic environment. Our results indicate that increased expression of c-fms on microglia has powerful proliferative and proinflammatory effects that may contribute to the microglial response in AD and other neurologic disorders.
EXPERIMENTAL PROCEDURES

Microglial Cell Lines, Plasmid Transfections and Tissue culture— The c-fms expression plasmid pTK1 was a gift from Dr. Rao Tekmal, Emory University School of Medicine. The pTK1 construct contains SV-40 promoted wild-type mouse c-fms sequence that encodes the M-CSFR protein (31). Transfections were performed using mouse BV-2 and human SV-A3 microglial cell lines. The immortalized mouse BV-2 cell line has been extensively characterized (29,32-35). The human SV-A3 cell line was a gift from Dr. Robert Nelson (Pfizer Central Research, Groton, CT). To generate the SV-A3 line, mixed glial cultures were prepared from human fetal brain essentially according to McCarthy and DeVellis (36). Microglia were shaken off the confluent astrocyte monolayer at day 14 post-plating, replated and washed after 1 hour, then the cells were immortalized with SV-40 T-Ag and adenovirus E1b. The cell line was developed by dilution cloning. The cells are CD68-positive and GFAP-negative. SV-A3 cells also express cell surface receptors CD16, CD32 and CD64 (our unpublished observations). After 6 hours treatment with 1 U/ml of recombinant human IL-1α (R&D Systems, Minneapolis, MN) SV-A3 cells show a 3-fold increase in IL-1α mRNA expression. Thus, SV-A3 cells have many phenotypic features of primary human microglia (37-39).

The transient transfections were performed with the Lipofectamine Plus® reagent (Life Technologies, Gaithersburg, MD). Mouse BV-2 and human SV-A3 microglia were grown to 60% confluency in 6-well tissues culture dishes. BV-2 cells were grown as previously described (34). SV-A3 cells were grown in DMEM/F12 (Life Technologies) supplemented with 10% fetal calf serum (Hyclone Lab Inc, Logan, UT) and 1%
penicillin/streptomycin (Sigma, St. Louis, MO). Approximately 0.2 µg of the SV 40 promoted \textit{c-fms} expression construct \textit{pTK1} was used per 5x10^5 microglial cells for transfection reaction. Lipofectamine Plus reagent alone was used as a control treatment. The \textit{pZeoSV} plasmid (Invitrogen, Carlsbad, CA) (31) used to develop the \textit{pTK1} expression construct was included as a second control. \textit{BV-2} cells were transfected with 0.2 µg of the \textit{pZeoSV} vector per 5x10^5 cells using identical procedure as described above for \textit{pTK1}. Cells were harvested after 24 hours in all experiments except for the \textit{c-fms} expression kinetics study when additional 12 and 44 hours incubation time points were included. At the end of the incubation, cells were either harvested for RNA isolation, or fixed, as described below for immunohistochemistry. Because serum contains M-CSF (40), the same serum lot was used for all experiments to minimize variation in the amount of ligand available to transfected cells.

\textit{Immunohistochemistry—}After 24 hours of transfection, cell growth medium was removed and cells were washed 4 times with 1x PBS buffer (Life Technologies), and then fixed with 4\% paraformaldehyde in 1X PBS for 20 min. at 4\(^{0}\)C. Cells were incubated with a blocking 10\% normal goat serum (Zymed Laboratories, South San Francisco, CA) and then washed 3 times for 5 minutes each in 0.5M Tris buffer, pH 7.6. Then a rabbit anti-mouse M-CSFR antiserum (Upstate, Lake Placid, NY) was added at a dilution of 1:1000 and reacted during overnight incubation at 4\(^{0}\)C. Cells were subsequently washed 3 times for 10 minutes each with 0.5M Tris buffer, pH 7.6 and then incubated for 1 hour at 37\(^{0}\)C with a 1:1000 dilution of Cy3 labeled secondary goat anti-rabbit antibody (Jackson Immuno Research, West Grove, PA). In the final step, cells were washed 4 times for 10 minutes each with 0.5M Tris buffer pH 7.6 and once with
equilibration buffer (Molecular Probes, Eugene, OR) before mounting on a glass slide using antifade reagent (Molecular Probes). Sections were examined with confocal microscopy as previously described (13).

**Western Blot Analysis of M-CSFR expression in BV-2 microglia**— Approximately $1 \times 10^6$ BV-2 cells pTK1 transfected for 24 hours were pelleted and then resuspended in lysis buffer containing 50 mM mannitol, 5 mM Hepes pH 7.4, and the enzyme inhibitors PMSF, Leupeptin and Pepsin A. The cell lysate was passed ten times through a 25 gauge needle, after which CaCl$_2$ was added to a final concentration of 10 mM. Cell homogenate was centrifuged at 15,600 g for 1 minute at $4^\circ$C, and the resulting supernatant was subjected to ultracentrifugation at 430,000g for 6 min. The pellet was resuspended in lysis buffer. All protein extractions were performed at $4^\circ$C using ice-cold reagents. Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL) before electrophoresis on a polyacrylamide 5% stacking and 8% resolving gel using 50 $\mu$g of protein per lane. After protein transfer to PVDF membrane, immunodetection was performed by overnight incubation at $4^\circ$C with a polyclonal reagent to the extracellular domain of mouse M-CSFR (Upstate; dilution 1:1000), or a polyclonal reagent to the intracellular domain of M-CSFR (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:200). Blots were than treated for 1 hour at room temperature with goat-anti rabbit IgG secondary serum conjugated to horseradish peroxidase (1:500) (Santa Cruz Biotechnologies) and visualized with diaminobenzidine as the chromogen.

**Cell Proliferation Assay**—BV-2 cells were grown to 40% confluency in a 6-well tissue culture dish and were subsequently transfected with c-fms expression plasmid pTK1 as described above. After 12, 24 and 44 hours, cells were collected by centrifugation and
resuspended in 0.5ml of 1x PBS. The relative cell number was measured using a Coulter particle characterization counter (Beckman Coulter, Miami, FL). The counter was gated for cell size to so as to exclude dead cells. Average values were obtained from five independent c-fms transfections, each measured in triplicate, and were compared to the values obtained for control cells (Lipofectamine Plus treatment only) that were grown in parallel.

**LDH Cytotoxicity Assay**—The CytoTox 96 kit was used to measure c-fms induced cytotoxicity in microglia (Promega Corporation, Madison, WI). This assay is based on colorimetric measurement of the cytosolic enzyme lactate dehydrogenase (LDH) that is released into culture media after cell injury. BV-2 and SV-A3 microglia were grown to 60% confluency and transfected with pTK1 plasmid as described above. After 24 hours, 50 µl of the cell growth media was removed from each sample and assayed for LDH according to the manufacturer's instructions. Colorimetric measurements were performed on a THERMOMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The number of lysed cells present in culture was determined by use of a standard curve for LDH and cell numbers. Cytotoxicity experiments were performed three times, and each sample was subsequently assayed in triplicate.

**Total RNA Isolation, ReverseTranscription and SYBR Green Real-Time Quantitative RT-PCR**—Total RNA was isolated using the TRIzol reagent following manufacturer’s instructions (Life Technologies). This yielded 10-12 µg of total RNA from 5x10⁵ microglial cells. RNA samples were diluted to a final concentration of 1µg/µl in RNase-free water and stored at –80°C until use. Synthesis of cDNA was performed using 1µg of total RNA. The 20µl reverse transcription reaction consisted of 5X first strand buffer,
0.5mM dNTP, 50nM random primers and 20U Superscript reverse transcriptase (all reagents from Life Technologies). RNA and primers were mixed and denatured by heating at 70°C for 10 minutes, and then the reverse transcription reaction was incubated for 10 minutes at 25°C, followed by 50 minutes at 42°C and then for 15 min. at 70°C. For the quantitative SYBR Green real-time PCR reaction, 250 ng of cDNA was used per reaction. Each 25 μl SYBR Green reaction consisted of 5 μl cDNA [50 ng/μl], 12.5 μl 2x Universal SYBR Green PCR Master Mix (PE Biosystems, Foster City, CA) and 3.75 μl of 50 nM forward and reverse primers. Optimization was performed for each gene-specific primer prior to the experiment to confirm that 50 nM primer concentrations did not produce non-specific primer-dimer amplification signal in no-template control tubes. Primer sequences were designed using Primer Express Software (Perkin Elmer Biosystems, Foster City, CA), and are presented in Table 1. Quantitative PCR was performed on ABI 5700 PCR Instrument (Perkin Elmer Biosystems) by using 3 stage program parameters provided by the manufacturer: 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that single DNA sequence was amplified during PCR. In addition, end reaction products were visualized on ethidium bromide stained 1.4% agarose gels. Appearance of a single band of the correct molecular size confirmed specificity of the PCR reaction. Each sample was tested in triplicate with quantitative PCR, and samples obtained from at least three independent experiments were used to calculate the means and standard deviations.
**M-CSF Neutralization Assay**—M-CSF in serum containing culture media was neutralized by the addition of 1 µg M-CSF antibody (Santa Cruz Biotechnologies) per 1ml of media. After 24 hours of incubation at 37 °C, M-CSF neutralized media was used to culture c-fms transfected and non-transfected BV-2 cells. Total RNA was harvested and gene expression was quantified using quantitative RT-PCR as described above.

**Assembly, treatment and analysis in organotypic microglia/hippocampal co-culture system.** Hippocampal organotypic cultures were prepared from 7 day old rats using the protocol of Stoppini et al. (41), and were 15 days in-vitro at the time of assembly of the co-culture with microglia. At 15 days in-vitro, inflammatory cells in organotypic culture are in a resting state (42,43). BV-2 cells were grown to 60% confluence in a 6-well tissue culture dish and were subsequently transfected with c-fms expression plasmid pTK1 as described above 24 hours prior to the addition to the co-culture. For the co-culture assembly, BV-2 cells were detached by gentle pipetting, and then overlaid on the organotypic hippocampal slice so that final cell density was 250-400 cells/mm². This density was chosen to simulate the density of activated microglia in AD (44). The co-culture was maintained in hippocampal medium containing 50% MEM, 25 % Hank’s Balanced Salt solution (both from Life Technologies), 25% Defined Equine serum (Hyclone Lab Inc.), 0.5% Glucose (Sigma), 0.5% l-Glutamine (Applied Scientific, South San Francisco, CA), 0.5% each penicillin, streptomycin, and incubated at 37°C.

**Mini-ruby labeling of mouse microglia.** To visualize integration of microglia into the organotypic slice, BV-2 cells that were labeled by the addition of 20 µg/ml Mini Ruby (Molecular Probes) 24 hours prior to co-culturing. To visualize neurons, 24 and 48 hour-old co-cultures were fixed with 4% paraformaldehyde for 30 minutes, and then
immunostained as described above using a neurofilament primary antibody (Sternberger Monoclonals, Baltimore, MD), and a Cy5-labeled secondary antibody (Jackson ImmunoResearch).

_Cresyl-violet staining of hippocampal slice._ To confirm viability of neurons in the BV-2-hippocampal co-culture, cresyl violet staining was utilized on 24 and 48 hour co-cultures. Co-cultures were fixed with 1% buffered formalin acetate for 2.5 hours at room temperature, stained with cresyl violet acetate (Acros Organics, Fisher Scientific, NJ), differentiated with 95% ethanol, and mounted with aqua-mount (Lerner Laboratories, Pittsburgh, PA) on microslides.

_Quantitative PCR Species-specific Expression Analysis._ Total RNA was isolated from the organotypic co-culture after 24 hours of incubation using the Trizol reagent, and was used to prepare cDNA by reverse transcription as described above. Co-culture samples assembled with BV-2 cells overexpressing *c-fms* were compared using a rat IL-1α specific quantitative PCR assay to control samples containing either, non-transfected or transfection media only transfected BV-2 cells. Rat IL-1α specific primer sequences were designed from a unique sequence segment in rat IL-1α gene (D00403, sequence position 1625-1771) that does not produce align in a BLAST search with the mouse version of IL-1α gene (Table 1). SYBR green quantitative PCR assay was performed as described above. Amplification of cDNA derived from mouse *c-fms* transfected BV-2 cells yielded no signal for IL-1α when real time PCR was performed, demonstrating species-specificity.

_Rat IL-1α ELISA Analysis:_ IL-1α protein was measured in conditioned media from co-cultures of mouse BV-2 cells and rat hippocampal slices using a rat-specific IL-1α
ELISA kit (Endogen, Woburn, MA). Culture supernatant was collected after incubation for 48 hours from co-cultures assembled with BV-2 microglia overexpressing c-fms and from co-cultures containing non-transfected BV-2 cells. Media samples were first cleared at 2000 rpm for 5 minutes and then assayed for rat IL-1α according to the manufacturer's instructions. Concentrations of IL-1α are presented as fold change relative to levels measured in control samples. The experiment was performed five times, and each samples was assayed in triplicate.
RESULTS

Overexpression of c-fms in microglial BV-2 cells. Fig. 1A shows real time RT-PCR quantification of c-fms expression in mouse microglial BV-2 cells after transient transfection with pTK1 plasmid, whereas Fig.1B shows expression of endogenous c-fms mRNA in nontransfected cells. Increased expression of c-fms in transfected cells occurred rapidly. Twelve hours from the start of transfection, c-fms mRNA levels increased on average 40 fold, and continued to increase to 115 fold higher than baseline at 44 hours after transfection. As shown in Fig. 1B, over the same interval endogenous c-fms mRNA in BV-2 cells did not increase more than 2 fold. We also demonstrated increased expression of the M-CSFR after transfection using immunocytochemistry. As shown in Fig.1C, a strong increase in the intensity of the M-CSFR immunolabeling was observed in pTK1 transfected BV-2 cells in comparison to control cells. Finally, we confirmed overexpression of M-CSFR protein by Western blot. Figs. 1D and 1E demonstrate expression of the approximately 165 kDa M-CSFR after transfection as detected by two different M-CSFR antibodies.

Increase in proliferation of BV-2 microglia after c-fms overexpression. Non-transfected BV-2 cells divide as long as sufficient nutrients are available. However, proliferation of BV-2 microglia was increased after pTK1 transfection. As shown in Fig. 2, BV-2 proliferation rate increased between 2 and 3 fold after overexpression of c-fms.

Overexpression of c-fms does not cause extensive microglial cytotoxicity. Figure 3A demonstrates that after 24 hours of c-fms transfection there was no major change in
toxicity as measured by LDH despite an increase in \textit{c-fms} mRNA levels of over 80 fold in BV-2 cells. The two-fold increase in number of dead cells after 24 hours was most likely due to the rapid increase in BV-2 cell density that occurred after \textit{c-fms} transfection. No increase in toxicity was seen after \textit{c-fms} transfection in human SV-A3 cells.

\textit{Induction of IL-1$\alpha$, MIP-1$\alpha$, iNOS, and IL-6 in microglia overexpressing c-fms.}

Overexpression of \textit{c-fms} strongly induced expression of IL-1$\alpha$ mRNA. At 24 hours post-transfection with pTK1, the expression of IL-1$\alpha$ mRNA increased 25 fold on average in mouse BV-2 cells (Fig.4A) and approximately 17 fold in SV-A3 human microglia (Fig.4B). It should be noted that in SV-A3 cells the transfection medium itself decreased expression of IL-1$\alpha$, which may have inhibited the level of IL-1$\alpha$ expression after \textit{c-fms} transfection. Overexpression of \textit{c-fms} also increased expression of the chemokine MIP-1$\alpha$ (Fig.5A) and of iNOS, the enzyme responsible for production of nitric oxide by microglia (Fig.5 C,D). There was smaller increase of 1.9 fold in the expression of pro-inflammatory cytokine IL-6 (Fig.5B). Because transfection of macrophages with plasmid DNA can induce a proinflammatory response (45), we performed control experiments using pZeoSV vector that was originally used to make pTK1 plasmid (31). No changes in expression of the mRNAs quantified in this study were detected after pZeoSV transfection using identical conditions as those described for pTK1 (data not shown).

\textit{Effects of c-fms transfection are dependent on presence of M-CSF.} M-CSF is present in serum used for tissue culture media. To demonstrate that the effects of \textit{c-fms} transfection were due to an interaction of M-CSFR with its ligand, we performed antibody neutralization experiments by reacting transfection media with an M-CSF antibody. As shown in Fig.6A, neutralization of M-CSF partially blocked the effects of \textit{c-fms}
transfection on IL-1α expression. It should be noted that despite pre-neutralization of M-CSF in transfection media, c-fms transfection resulted in increased expression of M-CSF by microglia (Fig. 6B), which likely interacted with overexpressed M-CSFR via autocrine and paracrine effects. Hence, blocking was incomplete.

*Induction of IL-1α in organotypic hippocampal slices after co-culture with microglia overexpressing c-fms.* To demonstrate that c-fms-activated microglia can affect inflammatory gene expression in neighboring cells, we assembled an organotypic co-culture consisting of rat hippocampal slice and pTK1 transfected mouse BV-2 microglia. Fig. 7A is a confocal image demonstrating integration of BV-2 cells into the hippocampal culture after 24 hours after co-culture assembly. Of note, the co-culture of BV-2 cells overexpressing c-fms and hippocampal slice did not result in neuronal loss. As shown in Fig. 7B, cresyl violet staining of the slice performed 48 hours after combining with pTK1 transfected BV-2 cells shows preservation of the neuronal cell layer. Rat-specific real-time RT-PCR showed that addition of nontransfected BV-2 cells resulted in a 2.5 fold increase in rat IL-1α expression by the slice culture. However, 24 hours after the addition of c-fms transfected BV-2 microglia, rat IL-1α mRNA was induced 9.01 fold compared to controls (Fig 7C). To confirm induction of rat IL-1α on a protein level, we measured release into conditioned media using a rat IL-1α ELISA. Measurements were performed 48 hours after assembly of the co-culture to allow for paracrine activation of the slice culture. As shown in Fig. 7D, rat IL-1α protein levels were higher 15.5 fold higher in co-culture samples with c-fms-transfected microglia than slice cultures without BV-2 cells. In comparison with co-cultures in which BV-2 cells were treated with transfection media only, c-fms transfected cells resulted in a 7.7 fold induction of IL-1α
by the slice cultures (Fig. 7D). This value is in agreement with the 6.48 fold increase of rat IL-1α mRNA due to c-fms in the co-culture system.
DISCUSSION

These results demonstrate that overexpression of \textit{c-fms} in cultured mouse and human microglia induces proliferation, cytokine expression, and nitric oxide production. Further, microglia overexpression \textit{c-fms} initiates a paracrine inflammatory response in organotypic hippocampal slices in a co-culture system. We recently demonstrated increased expression of M-CSFR, encoded by \textit{c-fms}, on activated microglia in the APP\textsuperscript{V717F} transgenic mouse model for AD (13). Expression of M-CSFR is also increased on microglia in AD brain (16), and after experimental ischemic or traumatic brain injury (14,15). Our results suggest that increased expression of \textit{c-fms} may be an important component of the molecular pathway leading to microglial proliferation, cytokine expression, and nitric oxide production in these diverse forms of neurologic insult.

Inflammation mediated by activated microglia may have a central role in the pathology and progression of AD (3,46). However, the molecular events initiate and sustain this response are unknown. Because inflammatory cells and effects are found near A\textsubscript{β} deposits in AD (5,6), and because A\textsubscript{β} can induce cytokine expression by cultured microglia (47), it is often hypothesized that A\textsubscript{β} initiates the inflammatory response in AD. Yet, the magnitude of cytokine induction in by A\textsubscript{β} in microglia is rather modest, approximately 2 to 3-fold above control values unless augmenting factors are present (48-50). We previously demonstrated that the cytokine M-CSF dramatically augments the effects of A\textsubscript{β} on cultured microglia (29). The present results show that increased expression of the M-CSFR alone without A\textsubscript{β} can cause a marked proliferative and proinflammatory response in both mouse and human microglia.
Increased numbers of activated microglia can be visualized with a variety of techniques in AD brain and in various transgenic models for AD (6,13,51,52). It is unknown whether this results from proliferation, migration to affected brain regions, increased expression of markers used for detection in tissue sections, or a combination of these effects. Recent studies using the APP23 transgenic mouse model for AD suggest that microglia proliferate in the vicinity of Aβ deposits (52). If microglia proliferation occurs in AD brain, then increased M-CSFR expression could be a driving force. Aggregates of microglia surrounding Aβ deposits could also be due to migration. MIP-1α is expressed by microglia and results in microglial chemotaxis (53,54). Prior work has shown that Aβ can induce microglial MIP-1α expression (55). However, the present results demonstrate that increased M-CSFR expression is also a stimulus for MIP-1α expression in the absence of Aβ. Hence, MIP-1α expression in AD could be the result of increased M-CSFR signal transduction, direct Aβ effects, or both.

The pro-inflammatory cytokine IL-1 is thought to play a key role in neuronal injury in AD. IL-1 is increased in the brain in AD (56), and is associated mainly with activated, phagocytic microglia near plaques (57). IL-1 also potentiates βAP-induced inflammatory cytokine release by glial cells, and may potentiate βAP toxicity (57). IL-1 also induces astrocyte and microglial proliferation (58). Finally, IL-1 induces iNOS expression by microglia (59). Recently, polymorphisms in the IL-1α and IL1β genes have been associated with risk for AD (60-62). Our data suggest that overexpression of M-CSFR on microglia in AD is directly involved in the multiple actions of IL-1 in AD pathogenesis.
The role of microglial NO in the pathogenesis of AD is controversial. Evidence exists for injury to nerve cells in AD via the toxic NO derivative peroxynitrite (63,64), but iNOS immunoreactivity in AD brain has been localized to neurons and astrocytes, not to microglia (65,66). Microglial expression of iNOS could occur at an earlier stage of AD than is typically obtained at autopsy, but conflicting results have been obtained regarding iNOS expression by cultured human microglia (22,67-69). BV-2 cells, like other rodent microglia, show a large increase in iNOS expression when treated with Aβ and M-CSF (29). Our data show that increased M-CSFR expression results in iNOS expression in mouse BV-2 cells. Interestingly, c-fms overexpression also resulted in an increase in iNOS expression by SV-A3 human microglia. Possibly, increased expression of c-fms is necessary for iNOS expression by cultured human microglia.

Overexpression of c-fms increased expression of IL-6, another major pro-inflammatory cytokine found to be expressed at increased levels in AD. (29,70,71). However, the increase was smaller than that for IL-1α and MIP-1α, even though BV-2 cells readily express high levels of IL-6 under different conditions (29). In AD increased microglial expression of IL-6 may require factors in addition to overexpression of M-CSFR.

Antibody neutralization experiments showed that the effects of c-fms transfection on cultured microglia was dependent on M-CSF in serum in the culture media. Serum is a rich source of M-CSF (40), and M-CSF is essential for dimerization and signal transduction by the M-CSFR (72). Our antibody neutralization experiments indicate that interactions between M-CSFR and its ligand form the basis for transfection-induced effects on microglia. Although antibody neutralization did not completely block the
effects of \textit{c-fms} transfection, this may have been due to the increased expression of M-CSF that occurred in the transfected BV-2 cells, resulting in autocrine and paracrine interaction of newly synthesized M-CSF with microglial M-CSFR.

Studies of human microglia are difficult due to the scarcity of appropriate fetal material. Even when cultures are established, the small number of cells limits experimental options. The SV-A3 human microglial cell line shows similarities to primary human microglia, including IL-1 induction of IL-1 expression, cell surface Fc-\(\gamma\) receptor expression, and CD68 expression (37-39). In our experiments with \textit{c-fms} transfection, the SV-A3 cells responded similarly to mouse BV-2 microglia. Further, the SV-A3 cells are readily transfected, thus providing a means for genetically manipulating human microglia. This cell line may provide a means to expand research on the biology of human microglia.

We quantified gene-expression using highly sensitive real-time SYBR Green RT-PCR. Due to high sensitivity, real-time RT-PCR is more likely to detect low-abundance mRNAs and small changes in gene-expression that could be missed using traditional RNA analysis tools such as Northern blots or ribonuclease protection assay. Unlike traditional “end-point” RT-PCR where the amount of amplified product can be greatly affected by primer concentrations and amplification efficiency, real-time RT-PCR measures mRNA during the exponential growth phase, and hence is considerably more accurate (30).

It is important to show that increased numbers of M-CSFR on microglia result in proliferation and expression of inflammatory effectors, but it more important to show that these changes have functional consequences. To address this issue we developed a co-
culture system of \textit{c-fms} transfected mouse BV-2 cells and organotypic hippocampal cultures. The BV-2 microglia readily integrated themselves into the hippocampal cultures, where they induced expression of rat IL-1\(\alpha\) by endogenous cells of the organotypic slice. These results suggest that microglial cells with high levels of M-CSFR as are found in AD brain can induce paracrine expression of proinflammatory factors by neighboring cells to expand and perpetuate the inflammatory state.

In conclusion, our results show that overexpression of M-CSFR on mouse and human microglia by \textit{c-fms} transfection results microglial proliferation and increased expression of iNOS and the proinflammatory cytokines IL-1\(\alpha\) and MIP-1\(\alpha\). Further, \textit{c-fms} transfected microglia have a paracrine proinflammatory effects when co-cultured with organotypic hippocampal slices. In AD brain microglia overexpressing M-CSFR may propagate a proinflammatory signal to nearby resting microglia and astrocytes, which could augment the overall inflammatory response and ultimately lead to neuronal toxicity. Microglia, however, may have neuroprotective functions. As endogenous macrophages of the brain, these cells are capable to removing cellular debris and A\(\beta\) via phagocytosis \((73,74)\). Phagocytosis of A\(\beta\) may by microglia may be the means whereby A\(\beta\) vaccination results in clearance of plaque deposits in transgenic mice modeling AD \((75)\). Future studies will be designed to determine if increased expression of M-CSFR on microglia has proinflammatory neurotoxic effects or scavenging neuroprotective functions, or both.
Figure Legends

Figure 1. Overexpression of c-fms in microglial cells. A. Kinetics of c-fms mRNA expression. BV-2 cells were transiently transfected with SV40 promoted c-fms plasmid and c-fms mRNA was measured by real-time quantitative RT-PCR at the time intervals indicated. For comparison of expression among samples, values were normalized using GAPDH and are shown as expression fold change relative to the endogenous c-fms mRNA level determined in non-transfected control cells. Mean values with standard deviations from three independent experiments are presented, each quantified in triplicate by RT-PCR. B. Expression of the endogenous c-fms mRNA measured in parallel in non-transfected cells. C. Immunohistochemical visualization of c-fms transfected (right) and non-transfected (left) BV-2 microglia with M-CSFR antibody. An increase in M-CSFR immunoreactivity is apparent after transfection. D. Western blot showing M-CSFR induction in c-fms transfected BV-2 microglia harvested after 24 hours detected with antibody to M-CSFR intracellular domain. Lane 1 shows signal from nontransfected cells, whereas lane 2 shows M-CSFR expression by transfected cells. E. Western blot showing results with an antibody to the extracellular domain of the M-CSF. Lanes 1 and 2 are from two separate sets of nontransfected BV-2 cells, whereas lanes 3 and 4 are from two separate sets of c-fms transfected BV-2 cells.

Figure 2. Increased proliferation of microglia after overexpression of M-CSFR.
BV-2 cells were transfected with c-fms expression plasmid pTK1, and proliferation rate was measured by counting the number of viable cells using a Coulter counter. Shown are the average values obtained from two independent experiments, each performed in

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triplicate. R² values represent correlation coefficient of the experimental data to the best-fit polynomial function. Standard deviation values were lower than 5% for both experiments.

**Figure 3. LDH cytotoxicity assay in mouse BV-2 and human SV-A3 microglia overexpressing M-CSFR.** LDH measurements showed no cytotoxicity in SV-A3 cells conditioned media from SV-A3 cells 24 hours after c-fms transfection. The increase in LDH in conditioned media after c-fms transfection of BV-2 cells was likely due to high density after c-fms-induced proliferation. Average values from three independent experiments are presented with standard deviations. Values for non-transfected control cells and cells treated with Lipofectamine PLUS-containing media (M) only are also presented.

**Figure 4. Increased expression of IL-1α mRNA in microglia overproducing M-CSFR.** Mouse BV-2 (A) and human SV-A3 (B) cells were transfected with c-fms plasmid pTK1 for 24 hours. Shown are results of real-time quantitative RT-PCR using mouse (A) and human (B) IL-1α specific primers. Mean fold change in expression (with standard deviations) are shown for cells treated with Lipofectamine PLUS-containing media (M) or with pTK1. Values based on three independent experiments with triplicate measurements for each.

**Figure 5. Expression of MIP-1α, IL-6 and iNOS mRNAs in microglia overexpressing M-CSFR.** Mouse BV-2 microglia were transfected for 24 hours with
Figure 6. A. Neutralization of exogenous M-CSF inhibits increased expression of IL-1α by microglia overexpressing c-fms. Fresh BV-2 culture medium was treated with M-CSF neutralizing antibody for 24 hours prior to addition to BV-2 cells. Following c-fms transfection cells were maintained for 24 hours in M-CSF-neutralized media, and subsequently expression of IL-1α mRNA was measured using real-time quantitative RT-PCR. Data obtained for M-CSF neutralized samples were compared to c-fms transfected samples without antibody neutralization. B. Overexpression of c-fms in BV-2 microglia increases M-CSF expression. Shown are results from real-time quantitative RT-PCR measured after 24 hours transfection with pTK1. Average values from three independent experiments are plotted, with triplicate RT-PCR assays for each.

Figure 7. Induction of IL-1α in organotypic hippocampal slice after co-culture with c-fms transfected BV-2 microglia. A. Confocal visualization of c-fms-transfected BV-2 microglia after integration into hippocampal co-culture. BV-2 cells are labeled with Mini Ruby (red), whereas neurons are labeled with a neurofilament antibody (yellow).
B. Whole microglial-hippocampal co-culture, 48 hours old, stained with cresyl violet to demonstrate intact neuronal layer. C. Real-time quantitative RT-PCR analysis showing increased expression of rat IL-1α mRNA in hippocampal slice (Hcs) after co-culturing with c-fms transfected or Lipofectamine PLUS (M) treated mouse BV-2 microglia. D. Rat-specific IL-1α ELISA demonstrating increased release of IL-1α into medium 48 hours after co-culture of c-fms transfected mouse BV-2 cells and rat organotypic hippocampal slice.
### Table 1. Real-time RT-PCR Primers

| Gene name | Genbank Accession Number | Primer orientation | Nucleotide Sequence (from 5’ to 3’) | Starting sequence position | Size of the PCR amplicon (bp) |
|-----------|--------------------------|-------------------|------------------------------------|---------------------------|-----------------------------|
| M GAPDH   | M32599                   | Forward Reverse   | TGAAGCAGGATGCTGAGGGCGAAGGTGGAAGAATGGAGAG | 825 926                   | 102 bp                      |
| M c-fms   | X06368                   | Forward Reverse   | TCCACCGGGACGTAGCACCCATGAAAAGTCCCCAATCT | 2393 2467                 | 75 bp                       |
| M IL-1α   | NM_010554                | Forward Reverse   | CACAATGTCTGGAGCCGGCTTGTGTTTTCGGCAAACCTTTCT | 269 337                   | 69 bp                       |
| M MIP-1α  | X12531                   | Forward Reverse   | CGTTCCTCACCCCCCATTGTGCAATCGTATGTTTGTCATCAT | 605 695                   | 91 bp                       |
| M M-CSF   | X05010                   | Forward Reverse   | CCCATATGGCGACACGGAAAGCAGGAATCGGAAACGAGG | 1380 1447                 | 68 bp                       |
| M iNOS    | NM_010927                | Forward Reverse   | GCCTTTCATCCCGCCCCATCCCCATCATGTTGTCATCAT | 2200 2271                 | 72 bp                       |
| M IL-6    | X54542                   | Forward Reverse   | CGTCAAGAGACTTCCATCCAGTTGAAGTACGGGAAACGAGG | 45 114                     | 70 bp                       |
| R IL-1α   | D00403                   | Forward Reverse   | ACATCCCGGTGAGGCTCTTTTACAACATGGAAGGAGTGTGACAGACATGACATGACATT | 1625 1711                 | 87 bp                       |
| R GAPDH   | AF106860                 | Forward Reverse   | AAGAGAGAGCGCCCTCAGTGCGTATGAGGTGACGTCAATGTCATG | 1892 1964                 | 73 bp                       |
| H IL-1α   | X02531                   | Forward Reverse   | CATGGGCCATCCAAAATTGTATAAGGGTTGAGGCCCC | 707 782                     | 76 bp                       |
| H iNOS    | L09210                   | Forward Reverse   | GAGATCAACATGCTGATGACTCATAGCACGGACGGGTATCATT | 1455 1569                 | 115 bp                      |
| H 18s rRNA| M10098                   | Forward Reverse   | CGGCTACCACTCCAAAGGAACTGGAATTACCGCCGT | 551 737                     | 187 bp                      |
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Figure 1A, B

A.

![Graph A: Transfection time vs. expression fold change for c-fms mRNA](image)

- Expression Fold Change:
  - 0.00
  - 40.04
  - 74.90
  - 115.48

- Transfection time (h):
  - 0
  - 12
  - 24
  - 44

B.

![Graph B: Time vs. expression fold change for endogenous c-fms mRNA](image)

- Expression Fold Change:
  - 1.00
  - 2.09
  - 1.22
  - 0.89

- Time (h):
  - 0
  - 12
  - 24
  - 44
Figure 2

![Graph showing cell number (x10^5) over transfection time (h) for BV-2 (-) control and BV-2/c-fms cells.](image)

- BV-2 (-) control:
  - R^2 = 0.99

- BV-2/c-fms:
  - R^2 = 0.98
Figure 3

[Bar graph showing the number of non-viable cells for different cell lines: BV-2, BV-2 + M, BV-2 + M + c-fms, SV-A3, SV-A3 + M, SV-A3 + M + c-fms. The y-axis represents the number of non-viable cells, ranging from 0 to 25000.]
Figures 4A, B

A.

![Graph showing expression fold change for mouse IL-1 mRNA in BV-2, BV-2 + M, and BV-2 + M + c-fms conditions.]

B.

![Graph showing expression fold change for human IL-1 mRNA in SV-A3, SV-A3 + M, and SV-A3 + M + c-fms conditions.]

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Figures 5A, 5B

A.

B.
Figures 5C, D

C.

![Graph showing expression fold change for iNOS mRNA.

D.

![Graph showing expression fold change for h iNOS mRNA.]
Figures 6A, B

A.

![Bar graph showing expression fold change for IL-1 mRNA.](image)

B.

![Bar graph showing expression fold change for M-CSF mRNA.](image)
Figures 7A, B, C, D

A.

B.

C.

D.
Overexpression of macrophage colony stimulating factor receptor on microglial cells induces an inflammatory response
Olivera M. Mitrasinovic, Grace V. Perez, FeiFei Zhao, Yuen Ling Lee, Clara Poon and Greer M. Murphy, Jr

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