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Microglia are the major source of TNF- α and TGF- β in postnatal glial cultures; regulation by cytokines, lipopolysaccharide, and vitronectin

Jennifer V. Welser-Alves and **Richard Milner**

Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

Abstract

Damage to the central nervous system (CNS) leads to increased production of TNF- α and TGF- β 1, cytokines that have pro- or anti-inflammatory actions, respectively. To define whether astrocytes or microglia express these cytokines, prior studies have used mixed glial cultures (MGC) to represent astrocytes, though these results are inevitably complicated by the presence of contaminating microglia within MGC. To clarify the cellular source of these cytokines, here we employed a recently described method of preparing microglia-free astrocyte cultures, in which neural stem cells (NSC) are differentiated into astrocytes. Using ELISA to quantify cytokine production in three types of glial culture: MGC, pure microglia or pure astrocytes, this showed that microglia but not astrocytes, produce TNF- α , and that this expression is increased by LPS, IFN- γ , and to a lesser extent by vitronectin, but decreased by TGF- β 1. In contrast, TGF- β 1 was produced by microglia and astrocytes, though at 10-fold higher levels by microglia. TGF- β 1 expression in microglia was increased by vitronectin and to a lesser extent by TNF- α and LPS, but astrocyte TGF- β 1 expression was not regulated by any factor tested. In summary, our data reveal that microglia, not astrocytes are the major source of TNF- α and TGF- β 1 in postnatal glial cultures, and that microglial production of these antagonistic cytokines is tightly regulated by cytokines, LPS, and vitronectin.

Keywords

Microglia; astrocytes; activation; TNF- α ; TGF- β 1; vitronectin

1. INTRODUCTION

Microglia and astrocytes are two abundant glial cell types in the central nervous system (CNS) that play important roles in maintaining tissue homeostasis. Microglia play a critical homeostatic role by continually sensing the environment, searching for tissue damage or

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Corresponding author: Dr. Richard Milner at: Department of Molecular and Experimental Medicine, MEM-132, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. Tel: (858) 784-8569, Fax: (858) 784-2174, rmilner@scripps.edu.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

invading microorganisms (Carson 2002). They are instrumental in removing apoptotic cells both during development (Purkinje cells) and following immune activation (T cells) (Magnus et al. 2001; Marin-Teva et al. 2004), and can also influence oligodendrogenesis in a biphasic manner (Butovsky et al. 2006). This duality of microglial function is well illustrated by the observation that microglia phagocytosing bacteria release proinflammatory cytokines, whereas removal of apoptotic cells is associated with microglial production of anti-inflammatory factors (Glezer et al. 2007; Magnus et al. 2001). Following stimulation, microglia are rapidly activated into migratory cells that phagocytose invading microorganisms and tissue debris (Hanisch and Kettenmann 2007). In this way, microglia play an important role in tissue regeneration, both by clearing away debris and by producing factors that promote tissue repair (Kreutzberg 1996; Raivich et al. 1999). However, when persistently activated, microglia cause excessive and unnecessary tissue damage, such as that observed in the autoimmune attack on myelin in multiple sclerosis (MS) (Gonzalez-Scarano and Baltuch 1999; Ransohoff 1999; Trapp et al. 1999). In contrast to microglia, which have a primary immune, phagocytic function, astrocytes play more of a supportive role, such as maintaining the barrier properties of cerebral blood vessels (Ballabh et al. 2004; del Zoppo and Milner 2006; Huber et al. 2001; Janzer and Raff 1987), and buffering excess levels of potassium ions and excitotoxic neurotransmitters such as glutamate (Nedergaard et al. 2003; Ransom et al. 2003; Ridet et al. 1997). Following tissue damage, activated astrocytes become hypertrophic, expressing increased levels of GFAP and form a reactive glial scar (Ridet et al. 1997). While current evidence suggests that the glial scar provides a useful function in limiting the extent of tissue damage, it is also apparent that the reactive glial scar impedes regeneration in the CNS, preventing both nerve regeneration (Fawcett 1997; Fawcett and Asher 1999) and remyelination (Franklin and ffrench-Constant 1996).

During the activation process, microglia and astrocytes show marked changes in their expression profile of cytokines. In particular, the pro-inflammatory cytokine TNF- α shows rapid induction following CNS damage, and this cytokine has been implicated in the pathogenesis of several neurological conditions, including MS (Hofman et al. 1989) and stroke (Lambertsen et al. 2005). The anti-inflammatory cytokine TGF- β 1 is also quickly upregulated following cerebral ischemia, and is thought to play an important protective role by preventing cell death of ischemic neurons and by countering some of the destructive effects of pro-inflammatory cytokines (Buisson et al. 2003; Krupinski et al. 1996). One important question to be answered is, which of these cytokines is produced by microglia or astrocytes, and how is this regulated by activating stimuli? In this regard, a large number of studies have described microglial production of both TNF- α (Gregersen et al. 2000; Lambertsen et al. 2005; Zujovic et al. 2000) and TGF- β 1 (Lehrman et al. 1998; Wang et al. 1995), but the results on astrocytes have yielded equivocal results, with some describing astrocyte expression of TNF- α (Chung and Benveniste 1990; Freyer et al. 1996), and TGF- β 1 (Morganti-Kossmann et al. 1992), but other studies not supporting these findings (Clausen et al. 2008; Gregersen et al. 2000; Lehrman et al. 1998; Morgan et al. 1993). Significantly, many in vitro studies of astrocytes have been performed using the mixed glial culture (MGC) system (Chung and Benveniste 1990; Freyer et al. 1996; Lee et al. 1993), which contain a majority of astrocytes, but also a significant population of microglia (Crocker et al. 2008; Liu et al. 2006; Saura et al. 2003). Thus, one possibility is that some of the cytokine responses described using the MGC system may have originated in microglia, not astrocytes. To specifically address this issue, we recently established a novel method of preparing microglia-free astrocyte cultures from the postnatal CNS, in which neural stem cells (NSC) are differentiated into astrocytes, and confirmed by multiple methods that this approach yields pure astrocyte cultures entirely devoid of microglia (Crocker et al. 2008). In the current study we used this approach to characterize TNF- α and TGF- β 1 production in microglia and astrocytes, and then determine how microglial or astrocyte expression of these

cytokines is regulated by other activating stimuli, including cytokines, LPS, and extracellular matrix (ECM) proteins.

2. EXPERIMENTAL PROCEDURES

2.1 Cell culture

The use of animals in this protocol was approved by the Committee on Animal Protocols, Department of Animal Resources, The Scripps Research Institute. Mixed glial cultures (MGC) were prepared from postnatal (day 0–2) C57BL6 mouse pups, as previously described (Milner and Campbell 2002; Milner and Campbell 2003). Cultures were maintained in poly-D-lysine coated T75 flasks in DMEM (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich) for 7–10 days, before being mechanically shaken to yield microglia, which were plated into 6 well plates (Nunc, Naperville, IL), previously coated with poly-D-lysine, fibronectin, laminin or vitronectin. Plates were coated by incubating with a solution containing either 5 µg/ml poly-D-lysine or 10 µg/ml of the different ECM proteins (all from Sigma) for 2 hours at 37°C. The purity of these microglial cultures was > 99% as determined by Mac-1 positivity in flow cytometry. The remaining adherent cells, consisting predominantly of astrocytes, were also plated into poly-D-lysine or ECM coated 6 well plates.

Primary cultures of neurospheres were obtained from postnatal (P0-P2) C57BL6 mouse brains as described previously (Jacques et al. 1998; Milner 2007). Briefly, spheres of neural precursors were grown in uncoated Nunc non-adherent T25 tissue culture flasks in DMEM/Hams F12 (Sigma) (50:50) supplemented with 1% B27, 20 µg/ml epidermal growth factor (EGF) and 20 µg/ml fibroblast growth factor-2 (FGF2) (all obtained from Invitrogen). Neurospheres were subsequently passaged every 5–7 days into fresh flasks. Neurospheres were differentiated into astrocytes by culturing in poly-D-lysine or ECM coated 6-well plates in DMEM containing 10% FBS, and grown to confluence. The astrocyte purity of these cultures was > 99% as determined by GFAP positivity by immunocytochemistry. Flow cytometry and immunocytochemistry demonstrated the total absence of Mac-1 positive cells in these cultures, as previously described (Crocker et al. 2008). When all three types of cell culture reached confluence, growth media was removed and replaced with serum free media containing the cytokines IFN- (10 U/ml), IL-1 (10 ng/ml), TNF- (10 ng/ml), or TGF- 1 (2 ng/ml), all from R&D Systems, Minneapolis, MN, or LPS (1 µg/ml, Sigma). All cytokines were murine except for TGF- 1, which was human. After 2 days culture, the supernatants were harvested and levels of TNF- or TGF- 1 analyzed by ELISA.

2.2 Immunocytochemistry

Mixed glial cultures and NSC-derived astrocytes were cultured on poly-D-lysine coated glass coverslips in DMEM containing 10% FBS. All incubations were performed at room temperature with washes in between. Cells were blocked in 5% normal goat serum (NGS) in PBS for 30 minutes then live-labeled with a Mac-1 monoclonal antibody (M1/70 clone from BD Pharmingen, La Jolla, CA) for 1 hour, then incubated with anti-rat Alexafluor 488 (Invitrogen) for 30 minutes, fixed in acid/alcohol (95:5) at –20°C for 30 minutes, then incubated with anti-GFAP-Cy3 (Sigma) for 1 hour, before being mounted in aquamount (Polysciences, Warrington, PA). The co-localization studies were performed the same way except that cultures were first fixed in acetone/methanol (50:50) at –20°C for 30 minutes. The TNF- goat polyclonal antibody was obtained from R&D Systems. The antibodies against S100 and glutamine synthetase (GS) were obtained from Sigma.

2.3 ELISA analysis of glial cytokine production

Concentrations of TNF- α and TGF- β 1 in microglial and astrocyte conditioned media were quantified by standard ELISA techniques using the DuoSet ELISA system (R&D) according to the manufacturer's instructions. Results are expressed as concentrations of cytokine (pg/ml), and represent the mean $\pm$ SEM of 4 experiments, with each sample examined in duplicate within each experiment. Statistical significance was assessed by Student's *t* test in which a value of $p < 0.05$ was defined as statistically significant.

3. RESULTS

3.1 Microglial TNF- α production is regulated by cytokines and LPS

To determine whether microglia or astrocytes express TNF- α and TGF- β 1, we prepared three different types of postnatal glial culture (Figure 1). Mixed glial cultures (MGC) represent a mixture of predominantly GFAP-positive astrocytes with a smaller though significant population of Mac-1-positive microglia (Crocker et al. 2008; Liu et al. 2006; Saura et al. 2003). Pure cultures of microglia were prepared by the well-established method of mechanical shaking MGC to harvest loosely-attached microglia (Milner and Campbell 2003) and contain only Mac-1-positive cells, with no GFAP-positive cells within. Pure astrocyte cultures were obtained by differentiating neural stem cells (NSC) into astrocytes, as recently described (Crocker et al. 2008). The advantage of this method over the traditional MGC system is that the resulting astrocyte cultures are totally microglia-free, and as illustrated in Figure 1B, contain only GFAP-positive astrocytes with absolutely no Mac-1-positive cells present. To exclude the possibility that astrocytes derived from NSC or MGC show different levels of maturation, we also examined expression of two other astrocyte markers in these cultures, S100 β and glutamine synthetase (GS). As shown in Figure 1C, astrocytes derived from both approaches expressed similar levels of S100 β and GS. Furthermore, in functional assays, both types of astrocyte culture behaved in a similar manner in causing added microglial cells to round up and become predominantly loosely-attached phase-bright cells (data not shown). Taken with our previous demonstration that astrocytes from these two sources show equivalent levels of several different adhesion molecules (four different integrins and dystroglycan), TLR4, and expression profile of matrix metalloproteinases (MMPs) (Crocker et al. 2008; Milner et al. 2001), our data indicate that these cells are virtually identical in all parameters examined. All three types of culture were grown to confluence, at which point, growth media was removed and replaced with serum-free media containing LPS or individual cytokines. After 2 days culture, cell culture supernatants were harvested and levels of TNF- α or TGF- β 1 analyzed by ELISA ($n = 4$ experiments).

As shown in Figure 2, under baseline conditions TNF- α was detected in the microglial and MGC but not the astrocyte cultures. LPS massively increased levels of TNF- α produced by microglia (from 40.6 ± 1.8 pg/ml to 1875.0 ± 36.2 pg/ml, $p < 0.0001$), and MGC (from 40.6 ± 5.4 pg/ml to 1821.9 ± 64.6 pg/ml, $p < 0.0001$), but elicited no response from astrocytes (Figure 2A). Microglial production of TNF- α was also increased by IFN- γ (from 40.6 ± 1.8 pg/ml to 162.5 ± 24.3 pg/ml, $p < 0.005$), and decreased by TGF- β 1 (from 40.6 ± 1.8 pg/ml to 14.1 ± 1.6 pg/ml, $p < 0.0005$) (Figure 2B). In sharp contrast, no TNF- α expression was detected in astrocytes under any of the conditions tested. These results suggest that in MGC, microglia are probably the source of TNF- α . To directly test this idea, we performed dual-IF on MGC with antibodies specific for TNF- α and the microglial marker Mac-1. As shown in Figure 3, this showed a very tight co-localization between Mac-1 and TNF- α , demonstrating that in MGC, TNF- α is specifically expressed by microglia.

3.2 Microglial TNF- α production is also promoted by vitronectin

As several studies have shown that ECM proteins regulate microglial activation state (Chamak and Mallat 1991; Milner and Campbell 2003; Milner et al. 2007; Monning et al. 1995), we next investigated whether different ECM proteins influence glial production of TNF- α . All cultures were grown on 24 well plates previously coated with the ECM proteins laminin, fibronectin or vitronectin. Upon reaching confluence, growth media was removed and replaced with serum-free media. Two days later, supernatants were harvested and TNF- α and TGF- β 1 levels analyzed by ELISA, and compared with levels produced by microglia grown on poly-D-lysine coated plates (control). As shown in Figure 2C, microglial TNF- α production was significantly increased by vitronectin (from 13.3 ± 2.1 pg/ml to 35.2 ± 6.8 pg/ml, $p < 0.05$), but not affected by laminin or fibronectin. MGC failed to show any significant change in TNF- α production in response to different ECM proteins. Pure astrocytes expressed no TNF- α under any of the conditions tested.

3.3 Microglial production of TGF- β 1 is promoted by TNF- α , LPS and vitronectin

Comparison of levels of TGF- β 1 between the different culture systems showed that microglia and MGCs expressed approximately 10-fold higher levels than astrocytes (Figure 4A). In response to inflammatory stimuli, microglial production of TGF- β 1 was marginally increased by TNF- α (from 60.6 ± 7.6 pg/ml to 101.5 ± 8.2 pg/ml, $p < 0.05$) and LPS (from 60.6 ± 7.6 pg/ml to 96.8 ± 1.6 pg/ml, $p < 0.05$), but not significantly affected by the other cytokines, though IFN- γ did show a similar though non-significant trend to increase microglial TGF- β 1 levels. As shown in Figure 4B, microglial TGF- β 1 production was also significantly increased by vitronectin (from 60.0 ± 2.7 pg/ml to 185 ± 12.6 pg/ml, $p < 0.0002$), but not influenced by fibronectin or laminin. MGC failed to show any significant changes in the TGF- β 1 response to ECM proteins. TGF- β 1 levels in astrocytes showed no apparent regulation by any of the factors tested.

4. DISCUSSION

In this study we prepared pure cultures of microglia and astrocytes from the postnatal mouse CNS to quantify TNF- α and TGF- β 1 production in microglia and astrocytes, and then examined how this is regulated by pro-inflammatory cytokines, LPS and ECM proteins. To specifically examine astrocyte expression of these cytokines, we used the pure astrocyte culture system recently described (Crocker et al. 2008), so as to avoid the potentially misleading contribution of microglia that are always present in mixed glial cultures (MGC). The main findings are as follows: (i) microglia were the sole source of TNF- α ; astrocytes failed to show any production of TNF- α under any condition, (ii) microglial TNF- α production was massively increased by LPS and to a lesser degree by IFN- γ , but reduced by TGF- β 1, (iii) microglial TNF- α production was also enhanced by vitronectin, (iv) TGF- β 1 levels in microglial and MGC cultures were 10-fold higher than astrocyte cultures, (v) microglial TGF- β 1 levels were increased marginally by TNF- α and LPS, and more strongly by vitronectin. In summary, using our pure astrocyte culture system, our data demonstrates that microglia, not astrocytes are the only source of TNF- α and the major source of TGF- β 1 in postnatal glial cultures, and thus corrects the previously-held misconception that astrocytes in MGC produce TNF- α . Furthermore, we also show that microglial production of these antagonistic cytokines is tightly regulated by cytokines, LPS, and the extracellular matrix protein vitronectin.

4.1 Microglia are the major source of TNF- α and TGF- β 1

In this study of postnatal glial cells, we have demonstrated that microglia, not astrocytes are the source of TNF- α . Using pure astrocyte cultures, we failed to detect any TNF- α under any condition tested, including stimulation by LPS or several pro-inflammatory cytokines,

including IFN- γ and IL-1 β . Previous studies, both in vivo and in vitro, support the idea that microglia are the major producers of TNF- α , with many of these describing an absence of TNF- α expression in astrocytes (Gregersen et al. 2000; Lamberts et al. 2005; Zujovic et al. 2000). However, a number of reports have described astrocyte expression of TNF- α (Chung and Benveniste 1990; Freyer et al. 1996), though it should be pointed out that most of these studies employed MGC as an “enriched” astrocyte culture. Our results demonstrate that while pure postnatal astrocytes do not express TNF- α under any condition tested, MGC show a pattern remarkably similar to microglia. As MGCs contain microglial cells (Crocker et al. 2008; Liu et al. 2006; Saura et al. 2003), this suggests that the TNF- α response in MGC comes from microglia within these cultures, and not from astrocytes, and indeed our Mac-1/TNF- α co-localization studies of MGC confirmed this. While our data show that pure astrocyte cultures do not express TNF- α under any of the conditions tested in our study, how predictive is this of in vivo astrocyte biology? It is difficult to extrapolate in vitro into in vivo behavior as gene expression profiles start to change immediately cells are removed from intact tissue. However, as astrocytes in vitro generally show a higher state of activation compared to their in vivo counterparts, and we found no evidence of TNF- α production by pro-inflammatory cytokines in vitro, this would tend to argue against astrocytes producing TNF- α in vivo. But while our in vitro evidence suggests that astrocytes do not make TNF- α , we cannot exclude the possibility that under certain conditions, for instance in the more complex CNS environment in vivo, or in response to stimuli not tested in our study, that astrocytes may produce TNF- α . Consistent with previous data (Lehrman et al. 1998; Lindholm et al. 1992; Morganti-Kossmann et al. 1992; Wang et al. 1995), we found that both microglia and astrocytes produce TGF- β 1, though microglia produced this cytokine at approximately 10-fold the level seen in astrocytes. Interestingly, while microglial TGF- β 1 levels were regulated by TNF- α and LPS, these were very modest changes (approximately 1.5-fold) when compared with the large changes in microglial TNF- α induced by IFN- γ (5-fold) or LPS (50-fold).

4.2 Different roles for microglia and astrocytes in the damaged CNS

The expression profiles of TNF- α and TGF- β 1 we have described for microglia and astrocytes are entirely consistent with the described roles for these two cell types. Microglia are rapidly activated cells and form the first line of defense in the CNS (Carson 2002; Hanisch and Kettenmann 2007; Raivich et al. 1999). Thus it makes sense that microglia quickly ramp up the pro-inflammatory cytokine TNF- α , in attempt to prepare the CNS for an immunological challenge. In contrast to microglia, astrocytes do not play a major role in launching the immunological response or in the subsequent phagocytic removal of cellular and tissue debris. Rather, astrocyte activation follows a longer time-course, more consistent with a role in suppressing the inflammatory response, and restructuring the damaged CNS (Fawcett and Asher 1999; Ridet et al. 1997). Thus, as the rapid-response element, microglia upregulate expression of pro-inflammatory cytokines, but also increase expression of anti-inflammatory cytokines, such as TGF- β 1. Our data supports this role for microglia, because microglial TNF- α production was strongly increased by the pro-inflammatory factors LPS and IFN- γ , but reduced by TGF- β 1. Interestingly, of the three ECM proteins tested, only vitronectin increased microglial TNF- α expression. This is consistent with our previous data showing that vitronectin is the most potent ECM substrate for inducing microglial activation and expression of the matrix metalloproteinase MMP-9 (Milner and Campbell 2003; Milner et al. 2007). Significantly, TNF- α and TGF- β 1 influenced microglial production of each other, though while TGF- β 1 reduced microglial TNF- α release, TNF- α actually promoted TGF- β 1 release. This suggests the presence of an overall inhibitory/braking mechanism to prevent excessive microglial activation.

Overall, microglial and MGC expression profiles of TNF- α and TGF- β 1 were remarkably similar. However, with the exception of the TNF- α response to LPS, these responses were always attenuated in MGC. This implies one of two things; either the number of microglia within MGC is lower than pure microglial cultures, or that astrocytes actively suppress microglial activation responses. The last interpretation would be consistent with an emerging theme for astrocytes in “policing” the microglial response, and preventing microglia from getting excessively activated (Min et al. 2006; Tichauer et al. 2007). This is in line with our previous finding that contact with astrocytes maintains microglia in a loosely attached, non-activated morphology (Milner and Campbell 2002). In future experiments we plan to further investigate this regulation.

4.3 Summary

Using an improved technique to prepare microglia-free cultures of astrocytes, we have clarified the microglial and astrocyte expression profiles of TNF- α and TGF- β 1 in postnatal glial cultures. Our data demonstrate that microglia, not astrocytes, are the only source of TNF- α and the major source of TGF- β 1, and that microglial production of these antagonistic cytokines is tightly regulated by proinflammatory cytokines, LPS, and vitronectin. These results significantly clarify the cellular source of the pivotal cytokines TNF- α and TGF- β 1 in the different cell types in postnatal glial cultures. In light of the clear differences observed between MGC and pure astrocyte cultures, most notably that TNF- α was never present in pure astrocyte cultures, some of the findings made in previous studies using MGC can now be re-examined using pure glial cell populations. In future studies it will be important to determine whether these differences in microglial/astrocyte expression of TNF- α and TGF- β 1 are also apparent in glial cultures derived from the adult brain, or indeed within microglia and astrocytes resident in the intact CNS.

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Figure 1.

Characterization of the three different types of glial culture. Mixed glial cultures (MGC) and pure cultures of astrocytes or microglia were cultured on poly-D-lysine coated plates as described in Materials and Methods. A. Phase contrast images. Scale bar = 100 μ m. Note that phase-bright microglia were present in the MGC, but not in the pure astrocyte cultures. B. Cultures were analyzed by immunofluorescence for expression of the astrocyte marker GFAP (Cy3, red), or the microglial marker Mac-1 (Alexafluor 488, green). Scale bar = 100 μ m. Note that MGC contain both astrocytes and microglia, while in contrast, pure astrocytes contain only astrocytes, and pure microglia contain only microglia. C. Astrocyte cultures were also analyzed for expression of the astrocyte markers S100 and glutamine

synthetase (GS). Note that both types of astrocyte culture (MGC and pure NSC-derived astrocytes) showed similar staining patterns of S100 and glutamine synthetase (GS).

Figure 2. Quantification of TNF- α production in MGC, astrocytes, or microglia. Mixed glial cultures (MGC) and pure cultures of astrocytes (Astro) or microglia (MG) were cultured on poly-D-lysine (A and B) or ECM-coated (C) plates as described in Materials and Methods. Upon reaching confluence, growth media was removed and replaced with serum free media containing individual cytokines or LPS. After 2 days culture, the supernatants were harvested and TNF- α levels analyzed by ELISA. All points represent the mean $\pm$ SEM of four experiments. Note that under resting conditions, TNF- α was produced by MGC and microglia but not by astrocytes, and that this expression was massively increased by LPS, (* $p < 0.0001$). Microglial TNF- α production was also increased by IFN- γ (* $p < 0.005$) but

decreased by TGF- β 1 (* $p < 0.005$). Furthermore, microglial TNF- α production was also increased by vitronectin (* $p < 0.05$), but not by fibronectin or laminin.

Figure 3. Within MGC, TNF- α production is microglial-specific. Mixed glial cultures were prepared as described in Materials and Methods, and then sub-cultured on poly-D-lysine coated glass coverslips. Cultures were treated with LPS (1 μ g/ml) for two days before being analyzed by dual immunofluorescence with antibodies against Mac-1 (Alexafluor 488, green) and TNF- α (Cy3, red). Scale bar = 100 μ m. Note that TNF- α showed strong co-localization with Mac-1 positive microglia, identifying microglia as the source of TNF- α in MGC.

Figure 4. Quantification of TGF-β1 production in MGC, astrocytes, and microglia. Mixed glial cultures (MGC) and pure cultures of astrocytes (Astro) or microglia (MG) were cultured on poly-D-lysine (A) or ECM-coated (B) plates as described in Materials and Methods. Upon reaching confluence, growth media was removed and replaced with serum free media containing individual cytokines or LPS. Two days later the supernatants were harvested and TGF-β1 levels analyzed by ELISA. All points represent the mean ± SEM of four experiments. Note that under resting conditions, MGC and MG expressed approximately 10-fold higher levels of TGF-β1 than astrocytes, and that microglial TGF-β1 production was

increased by TNF- and LPS (* $p < 0.05$). In addition, microglial TGF- β 1 production was also increased by vitronectin (* $p < 0.002$), but not by fibronectin or laminin.