Microglia activated by IL-4 or IFN-γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells

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Cell renewal in the adult central nervous system (CNS) is limited, and is blocked in inflammatory brain conditions. We show that both neurogenesis and oligodendrogenesis of adult neural progenitor cells in mice are blocked by inflammation-associated (endotoxin-activated) microglia, but induced by microglia activated by cytokines (IL-4 or low level of IFN-γ) associated with T-helper cells. Blockage was correlated with up-regulation of microglial production of tumor necrosis factor-α. The effect induced by IL-4-activated microglia was mediated, at least in part, by insulin-like growth factor-I. The IL-4-activated microglia showed a bias towards oligodendrogenesis whereas the IFN-γ-activated microglia showed a bias towards neurogenesis. It thus appears that microglial phenotype critically affects their ability to support or impair cell renewal from adult stem cell.

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Introduction

Poor recovery from acute insults or chronic degenerative disorders in the central nervous system (CNS) has been attributed to lack of neurogenesis, limited regeneration of injured nerves, and extreme vulnerability to degenerative conditions. The absence of neurogenesis was explained by the assumption that soon after birth the CNS reaches a permanently stable state, needed to maintain the equilibrium of the brain’s complex tissue network. Research during the last decade showed, however, that the brain is capable of neurogenesis throughout life, albeit to a limited extent (Morshead et al., 1994; Kuhn et al., 1996; Eriksson et al., 1998). In the inflamed brain, neurogenesis is blocked (Ekdahl et al., 2003; Monje et al., 2003). This latter finding strengthened the traditional view that local immune cells in the CNS have an adverse effect on neurogenesis. Likewise, the limited regeneration and excessive vulnerability of CNS neurons under inflammatory conditions or after an acute insult were put down to the poor ability of the CNS to tolerate the immune-derived defensive activity that is often associated with local inflammation and cytotoxicity mediated, for example, by tumor necrosis factor (TNF)-α (reviewed in Probert et al., 1997; Pettmann and Henderson, 1998) or nitric oxide (Merrill et al., 1993). More recent studies have shown, however, that although an uncontrolled local immune response indeed impairs neuronal survival and blocks repair processes, a local immune response that is properly controlled can support survival and promote recovery (Hauben and Schwartz, 2003; Schwartz et al., 2003; Butovsky et al., 2005). It was further shown that after an injury to the CNS, a local immune response that is well controlled in time, space, and intensity by peripheral adaptive immune processes (in which CD4+ helper T cells are directed against self-antigens residing at the site of the lesion) is a critical requirement for post-traumatic neuronal survival and repair (Moalem et al., 1999; Butovsky et al., 2001; Schwartz et al., 2003; Shaked et al., 2004). These and other results led our group to formulate the concept of ‘protective autoimmunity’ (Moalem et al., 1999).

The hypothesis underlying the present study was that the same immune response that causes cell loss under neurodegenerative conditions also blocks neurogenesis in the adult CNS and that an immune response that protects against cell loss also supports neurogenesis and oligodendrogenesis. Accordingly, we postulate that both neurogenesis and oligodendrogenesis can be
induced by microglia that encounter well-controlled levels of cytokines associated with adaptive immunity, but are blocked by microglia that encounter endotoxin, which is associated with an uncontrolled local immune response that impairs neuronal survival and blocks repair processes. The results of this study suggest that the hypothesis is correct.

**Results**

**Effect of microglia on neurogenesis**

Adaptive immunity, in the form of a well-controlled Th1 or Th2 response to a CNS insult, induces microglia to adopt a phenotype that facilitates neuronal protection and neuronal tissue repair (Butovsky et al., 2001; Wolf et al., 2002; Shaked et al., 2004, 2005; Butovsky et al., 2005). Here, we examined the ability of adaptive immunity, via activation of microglia (MG), to induce or support the differentiation of neuronal progenitor cells (NPCs). Neurogenesis is reportedly blocked by the inflammation caused by microglia activated with endotoxin (such as lipopolysaccharide (LPS)) (Ekdahl et al., 2003; Monje et al., 2003). We therefore compared the effects on NPCs of microglia exposed to LPS (MG(IL-PS)) with the effects of microglia exposed to the low levels of characteristic Th1 (pro-inflammatory) and Th2 (anti-inflammatory) cytokines, interferon (IFN)-γ (MG(IFN-γ)), and interleukin (IL)-4 (MG(IL-4)), respectively, found to be supportive of neural survival (Butovsky et al., 2005). We used NPCs expressing green fluorescent protein (GFP) to verify that any neural cell differentiation seen in the culture was derived from the NPCs rather than from contamination of the primary microglial culture.

Microglia were grown in their optimal growth medium (Zielasek et al., 1992) and were then treated for 24 h with IL-4, IFN-γ (low level, Butovsky et al., 2005), or LPS. Resides of the growth medium and the cytokines were washed off, and each of the treated microglial preparations, as well as a preparation of untreated microglia (MG(−)), was freshly co-cultured with dissociated NPC spheres in the presence of differentiation medium. After 5 days in culture, GFP/β-III-tubulin cells were similar to those described above and stained with doublecortin (DCX; Fig. 2), a marker of early differentiation of the neuronal lineage. This staining revealed a similar effect of the various microglial preparations to that seen with staining for β-II-tubulin. Striking differences in the morphology of newly differentiating neurons were seen between NPCs co-cultured with MG(IL-4) and those co-cultured with MG(IFN-γ) (Fig. 2A); the former showed significant branching, whereas in the latter, the neurons were polarized and had long processes (Fig. 2A). These differences suggested that the mechanisms activated in microglia by the two cytokines are not identical. Co-expression of GFP with DCX is shown in Fig. 2B. In cultures stained for both DCX and β-II-tubulin, these two neuronal markers were found to be co-localized (Fig. 2C). In all of the above experiments, microglial viability, assayed by propidium iodide staining of live cells (Hsieh et al., 2004), was unaffected by the co-culturing conditions. Quantitative analysis of GFP/DCX−stained cells, shown in Fig. 2D, yielded similar results to those obtained when β-II-tubulin was used as the neuronal marker (Figs. 1A, B).

**Effect of microglia on oligodendrogenesis**

Next we examined whether, under the same experimental conditions, microglia would also induce NPCs to differentiate into oligodendrocytes. Under high magnification, we were able to detect newly formed oligodendrocytes. In attempting to detect possible differentiation of NPCs to oligodendrocytes, we first looked for GFP-labeled cells co-expressing oligodendrocyte progenitor marker NG2. Quantitative analysis confirmed that both MG(IL-4) and (to a lesser extent) MG(IFN-γ) induced differentiation of NG2+ cells from co-cultured NPCs (Figs. 3A, B). In both MG(−) and MG(IFN-γ) co-cultured with NPCs, significantly fewer NG2+ expressing cells were seen in the absence of insulin (Fig. 3A) than in its presence (Fig. 3B). Unlike in the case of neurogenesis (Fig. 1), MG(IFN-γ) – even in the presence of insulin – was significantly less effective than
MG(IL-4) in inducing the appearance of newly differentiating oligodendrocytes (NG2\(^+\)). A significant proportion of the NG2\(^+\) cells were also labeled for RIP [a monoclonal antibody that specifically labels the cytoplasm of the cell body and processes of premature and mature oligodendrocytes at the pre-ensheathing stage (Hsieh et al., 2004)] in both the MG(IFN-\(\gamma\)) and the MG(IL-4) co-cultures (Figs. 3A, B). In the absence of insulin, almost no GFP\(^+\)/NG2\(^+\) cells were seen in control medium containing no Fig. 1. Differentiation of NPCs into neurons can be either induced or blocked by microglia, depending on how they are activated. GFP-expressing NPCs (green) were co-cultured with differently activated microglia from mice for 5 days. Quantification of \(\beta\)-III-tubulin\(^+\) cells (expressed as a percentage of GFP\(^+\) cells) obtained from confocal images, without (−Ins) or with insulin (+Ins), is summarized in panels A and B, respectively. (C) Results of the effect of rTNF-\(\alpha\) on the number of \(\beta\)-III-tubulin\(^+\) cells, expressed as a percentage of GFP\(^+\) cells, in co-cultures of NPCs and MG(IFN-\(\gamma\)) in the presence of insulin. Error bars represent means ± SD. Data are from one of at least three independent experiments in replicate cultures. Asterisks above bars express differences relative to untreated (Control) NPCs (*\(P<0.05\); ***\(P<0.001\); ANOVA). (D) Representative confocal images of GFP-expressing NPCs (green) in the absence of insulin without microglia (Control); with untreated microglia (MG(C)); with LPS-activated microglia (MG(LPS)); with IL-4-activated microglia (MG(IL-4)); and in the presence of insulin with IFN-\(\gamma\)-activated microglia (MG(IFN-\(\gamma\)+Ins)) or IFN-\(\gamma\)-activated microglia (MG(IFN-\(\gamma\)+Ins)) and TNF. (E) GFP-expressing NPCs co-expressing \(\beta\)-III-tubulin and Nestin. (F) Newly formed neurons from NPCs are positively stained for GAD67 (\(\beta\)-III-tubulin\(^+/\)GFP\(^+/\)GAD\(^+\)). Note, confocal channels are presented separately.
in NPCs co-cultured with MG. Asterisks above bars express differences relative to untreated (control) from one of at least three independent experiments in replicate cultures. Images, without or with insulin. Error bars represent means with MG(LPS) (Fig. 3A). A dramatic increase in the numbers of DCX. (A) Representative confocal images of GFP-expressing NPCs expressing NPCs (green) were co-cultured with differently activated microglia as described in Fig. 1, and stained for the neuronal marker DCX. (C) β-III-tubulin⁺ cells co-expressing DCX. Note, confocal channels are presented separately. (D) Quantification of DCX⁺ cells (expressed as a percentage of GFP⁺ cells) obtained from confocal images, without or with insulin. Error bars represent means ± SD. Data are from one of at least three independent experiments in replicate cultures. Asterisks above bars express differences relative to untreated (control) NPCs (*P < 0.05; **P < 0.01; ANOVA).

Fig. 2. Microglia activated with IFN-γ or IL-4 induce differentiation of NPCs into DCX-expressing neurons with different morphology. GFP-expressing NPCs (green) were co-cultured with differently activated microglia in the absence of insulin or with MG(IFN-γ) in the presence of insulin. (B) GFP-expressing NPCs co-expressing DCX. (C) β-III-tubulin⁺ cells co-expressing DCX. Note, confocal channels are presented separately. (D) Quantification of DCX⁺ cells (expressed as a percentage of GFP⁺ cells) obtained from confocal images, without or with insulin. Error bars represent means ± SD. Data are from one of at least three independent experiments in replicate cultures. Asterisks above bars express differences relative to untreated (control) NPCs (*P < 0.05; **P < 0.01; ANOVA).

Microglia (Fig. 3A). A few GFP⁺/NG2⁺ cells were seen in co-cultures of NPCs with MG₃₀ (Fig. 3A), but none in co-cultures with MG₅₆. A dramatic increase in the numbers of these cells was seen in co-cultures with MG₅₆ (Figs. 3A, C).

Addition of insulin to the NPC cultures did not affect the incidence of NG2⁺ cells in the absence of microglia (control; Fig. 3B). In the latter two (Fig. 4G). Interestingly, there were no significant differences between the absolute numbers of GFP⁺ cells counted in these three groups (NPCs alone: 90.2 ± 32.0; co-cultured with MG₅₆: 70.5 ± 23.0; co-cultured with MG₃₀: 66.1 ± 10.4). This raises a question: do the activated microglia, besides affecting differentiation, also affect NPC proliferation and/or survival? Table 1 records the proliferation of NPCs co-cultured with non-activated, IL-4-activated, or IFN-γ-activated microglia. Comparisons of proliferation at 24 h and 48 h of culture revealed no differences. After 72 h, a slight but non-significant difference was seen between NPCs alone and NPCs co-cultured with MG₃₀ or MG₅₆, possibly because of decreased proliferation in the culture of NPCs alone rather than any increase in the co-cultures. In the absence of insulin, there were no significant differences at any time in culture between NPCs alone and NPCs co-cultured with MG₅₆ or with
MG(IL-4). A reduction in proliferation was observed in NPCs co-cultured with MGLPS, with or without insulin. After 5 days, no proliferation was detectable in any of the co-cultures (data not shown). To identify dead or dying cells, we stained live cultures with 1 μg/ml propidium iodide, which stains dead cells, and 1 μg/ml Hoechst 33342, which stains both live and dead cells (Hsieh et al., 2004). Significant cell death was observed in NPCs co-cultured with MG(LPS) both in the absence and in the presence of insulin, whereas in NPCs cultured alone or with MG(IFN-γ) or MG(IL-4), the percentage of cell death was low and did not differ significantly from that seen in cultures of NPCs alone (Table 1). These results suggested that the primary effect of the cytokine-activated microglia on the fate of NPCs in vitro occurs via a mechanism that is instructive rather than selective.

Possible mechanism of oligodendrogenesis induction by IL-4- and IFN-γ-activated microglia

Insulin-like growth factor (IGF)-I is reportedly a key factor in neurogenesis and oligodendrogenesis (Carson et al., 1993; Aberg et al., 2000; O’Kusky et al., 2000; Hsieh et al., 2004). To determine whether the beneficial effect of the cytokine-activated microglia on the differentiation of NPCs is mediated, at least in part, by the ability of the microglia to produce IGF-I, we added neutralizing antibodies specific to IGF-I (αIGF-I) to the NPCs co-cultured with activated microglia. αIGF-I blocked the MG(IL-4)-induced effect on oligodendrogenesis (Fig. 5A), indicating that the effect of IL-4-activated microglia on oligodendrogenesis is dependent on IGF-I. Direct addition of recombinant IGF-I (rIGF-I; 500 ng/ml) to NPCs resulted in their significant differentiation to NG2-expressing cells (Fig. 5B). Such differentiation, however, was less extensive than that observed in NPCs co-cultured with MG(IL-4), suggesting that the MG(IL-4) effect is mediated through additional (possibly soluble) factors, or by cell–cell interaction, or both. αIGF-I had no effect on oligodendrogenesis induced by MG(IFN-γ) (data not shown). We also examined the effect of αIGF-I on MG(IL-4)-induced neurogenesis by assessing β-III-tubulin expression. The percentage of GFP+/β-III-tubulin+ cells was 21.9 ± 2.9% in NPCs co-cultured with MG(IL-4) and 19.7 ± 4.5% (P = 0.3) when αIGF-I was added to those co-
In light of the observed beneficial effect of αTNF-α on the outcome of MG(IFN-γ)-induced neurogenesis (Fig. 1), we examined whether neutralization of TNF-α would promote MG(IFN-γ)-induced oligodendrogenesis as well. Oligodendrogenesis was indeed enhanced by αTNF-α in NPCs co-cultured with MG(IFN-γ) (Fig. 5C). The implied negative effect of TNF-α was substantiated by direct addition of TNF-α to NPCs co-cultured with MG(IFN-γ) (Fig. 5D).

Comparative RT-PCR analyses of microglial mRNA disclosed, as was recently shown (Butovsky et al., 2005), that in the absence of activation the microglia produced both IGF-I and low levels of TNF-α (Fig. 6 A). Analysis of TNF-α and IGF-I production as a function of time revealed that IFN-γ, unlike IL-4, caused a transient increase in TNF-α production and down-regulation of IGF-I (Fig. 6B). At the protein level, quantitative immunocytochemical analysis also disclosed up-regulation of the expression of IGF-I by MG(IL-4). LPS completely blocked the production of IGF-I (Fig. 6C).

**Discussion**

The results of this study strongly suggest that certain specifically activated microglia can induce neural cell renewal in the adult CNS. The findings showed that microglia can determine the fate of differentiating adult NPCs. Both neurogenesis and oligodendrogenesis were induced in NPCs co-cultured with MG(IL-4) and MG(IFN-γ), whereas both were blocked by MG(LPS), in line with reports that inflammation associated with LPS blocks adult neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). NPCs co-cultured with MG(IL-4) showed a bias towards oligodendrogenesis, whereas NPCs co-cultured with MG(IFN-γ) were biased towards neurogenesis.

**Local immune response in the damaged CNS**

Defense mechanisms in the form of activated microglia commonly operate in acute and chronic neurodegenerative conditions, but often the CNS is poorly equipped to tolerate them (Dijkstra et al., 1992; Merrill et al., 1993). As a result, activated microglia have generally been viewed as a uniformly hostile cell population that causes inflammation, interferes with cell survival (Popovich et al., 2002), and blocks neurogenesis (Monje et al.,...
Recent studies have shown, however, that whether the effect of activated microglia on injured or inflamed CNS will be positive or negative is determined by the type of activation, and that just as activated microglia can be inimical to cell survival in some instances, they can be protective in others (Schwartz et al., 2003; Butovsky et al., 2005; Shaked et al., 2005). Thus, for exam-

### Table 1

<table>
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<tr>
<th>Treatment</th>
<th>+Insulin</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>% BrdU^+ cells out of GFP^+ cells</th>
<th>Treatment</th>
<th>+Insulin</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>% PI^- cells out of GFP^+ cells</th>
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<tr>
<td>Control</td>
<td></td>
<td>5.8 ± 0.6</td>
<td>5.4 ± 1.5</td>
<td>1.4 ± 0.9</td>
<td>Control</td>
<td>3.3 ± 0.9</td>
<td>1.7 ± 0.4</td>
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<tr>
<td>MG_(IL-4)</td>
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<td>6.2 ± 0.5</td>
<td>6.9 ± 1.9</td>
<td>5.6 ± 1.4</td>
<td>MG_(IL-4)</td>
<td>4.0 ± 0.5</td>
<td>2.8 ± 0.7</td>
<td>2.8 ± 0.9</td>
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<tr>
<td>MG_(IFN-γ)</td>
<td>+</td>
<td>5.8 ± 1.5</td>
<td>7.1 ± 2.5</td>
<td>5.6 ± 1.1</td>
<td>MG_(IFN-γ)</td>
<td>5.9 ± 0.6</td>
<td>4.2 ± 1.5</td>
<td>3.6 ± 2.1</td>
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<tr>
<td>MG_(LPS)</td>
<td>+</td>
<td>3.1 ± 0.8</td>
<td>1.7 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>MG_(LPS)</td>
<td>14.0 ± 0.1***</td>
<td>7.3 ± 1.9</td>
<td>4.1 ± 0.2</td>
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<tr>
<td>Control</td>
<td>-</td>
<td>4.1 ± 0.6</td>
<td>3.2 ± 0.5</td>
<td>1.2 ± 0.7</td>
<td>Control</td>
<td>3.7 ± 0.2</td>
<td>2.3 ± 0.8</td>
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<td>MG_(IL-4)</td>
<td>-</td>
<td>6.5 ± 1.5</td>
<td>3.5 ± 0.7</td>
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<td>MG_(IL-4)</td>
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<td>MG_(IFN-γ)</td>
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<td>6.2 ± 1.1</td>
<td>5.4 ± 1.9</td>
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<td>MG_(IFN-γ)</td>
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<tr>
<td>MG_(LPS)</td>
<td>-</td>
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<td>1.7 ± 0.5</td>
<td>1.1 ± 0.6</td>
<td>MG_(LPS)</td>
<td>15.8 ± 1.9*</td>
<td>7.0 ± 5.0</td>
<td>4.8 ± 0.8</td>
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Cultures of untreated NPCs (control) or NPC’s co-cultured with MG_(IL-4) or MG_(IFN-γ) or MG_(LPS), with or without insulin, were analyzed for proliferation and cell death 24, 48, or 72 h after plating. For the proliferation assay, a pulse of BrdU was applied 12 h before each time point. Numbers of BrdU^+ cells are expressed as percentages of GFP^+ cells (mean ± SEM from three independent experiments in duplicate) and analyzed by ANOVA. Cell death with and without insulin was determined by live staining with 1 μg/ml propidium iodide and 1 μg/ml Hoechst 33342 (mean ± SEM from two independent experiments in duplicate; *P < 0.05; ***P < 0.001; ANOVA).
ple, microglia that encounter well-controlled adaptive immunity (in the form of CD4+ T cells) were shown to correlate with a protective phenotype (Butovsky et al., 2001). Among the cytokines that are produced by such T cells are IFN- γ and IL-4, characteristic of Th1 and Th2 cells, respectively.

Differential effects of IFN-γ-activated and IL-4-activated microglia on neurogenesis and oligodendrogenesis

Both MG(IL-4) and MG(IFN-γ) induced neurogenesis and oligodendrogenesis in this study, but apparently through different mechanisms. Under the same conditions, cultured NPCs were unaffected by the direct addition (for 24 h) of these cytokines. The effects of the cytokine-activated microglia on oligodendrogenesis were blocked by αIGF-I, indicating that the hormonal growth factor IGF-I, which these activated microglia produce (Butovsky et al., 2005), participates in their effect on oligodendrogenesis. In contrast, the effect of MG(IL-4) on neurogenesis in vitro was not affected by the addition of αIGF-I. The fact that IGF-I, although produced by MG(IL-4), did not mediate the effect of these cells on differentiation of NPCs to neurons suggests that other factors produced by MG(IL-4) might be operating in this connection. The production of neurotrophic factors and additional growth factors by microglia is well documented (Elkabes et al., 1996; O’Donnell et al., 2002). As for MG(IFN-γ), their effect on oligodendrogenesis was weak, unlike their effect on neurogenesis (as manifested by the number of newly differentiated neurons and by the length of the neurites emerging from them). It was also interesting to note the difference in morphology of the new neurons formed in response to co-culturing of NPCs with these two types of cytokine-activated microglial preparations.

Beneficial effect of IFN-γ in conferring microglia phenotype supportive of neural tissue is restricted to a low range of concentrations (Butovsky et al., 2005). This explains why IFN-γ, a proinflammatory cytokine, has received a bad reputation and has been associated only with pathology. In line with this contention, the effects of MG(IFN-γ) on neurogenesis and on oligodendrogenesis, found in the present study, were significantly stronger when αTNF-α was added to the co-cultures. In a complementary experiment, addition of TNF-α to these co-cultures resulted in impairment of both oligodendrogenesis and neurogenesis induced by MG(IFN-γ). These results indicate that the beneficial effect of MG(IFN-γ) on NPCs might be limited to a narrow range of IFN-γ concentrations, above which the benefit might be counteracted by the microglial production of TNF-α; increasing level of IFN-γ results in up-regulation of TNF-α (Butovsky et al., 2005; Shaked et al., 2005). Notwithstanding the well-known negative effect of TNF-α, the effect of this cytokine in general, and on neuronal

Fig. 6. IFN-γ, unlike IL-4, transiently induced TNF-α and reduced IGF-I expression in microglia. (A) Microglia treated with IL-4 (10 ng/ml), IFN-γ (20 ng/ml), or LPS (100 ng/ml) for 24 h were analyzed for TNF-α and IGF-I mRNA by semi-quantitative RT-PCR. Representative results of one of three independent experiments are shown. (B) Time courses of TNF-α and IGF-I mRNA expression by MG(IL-4) and MG(IFN-γ) PCR at each time point was performed with the same reverse-transcription mixtures for all cDNA species. Values represent relative amounts of amplified mRNA normalized against β-actin in the same sample, and are represented as fold of induction relative to control (means ± SD). The linear working range of amplifications was ascertained before the experiments were carried out. Each sample was tested in three replicates, and similar results were obtained in three different microglial cultures. (C) Statistical analysis of IGF-I expression demonstrates fluorescence intensity per cell, calculated as a percentage of increased intensity relative to MG(IL-4) control (means ± SD; obtained in two independent experiments, each repeated four times). Note, relative to the untreated control, MG(IL-4) showed a significant increase in IGF-I. Asterisks above bar express differences relative to MG(IL-4) (*P < 0.05; **P < 0.001; two-tailed Student’s t-test).
survival in particular, is largely dependent on the context, timing, and dosage of its activity (Lenzlinger et al., 2001).

Neither neurogenesis nor oligodendrogenesis induced by MG(IL-4) in this study was affected by iTNF-α. This was not surprising, as IL-4 down-regulated the microglial production of TNF-α. The adverse effect of TNF-α is in line with the negative effect of LPS on neurogenesis, found in this study and also previously reported (Ekdahl et al., 2003; Monje et al., 2003). Nitric oxide acts as an important negative regulator of cell proliferation and neurogenesis in the adult mammalian brain (Packer et al., 2003). It is tempting to suggest that the observed blockage of neurogenesis and oligodendrogenesis by LPS results from the production by microglia, after their encounter with LPS, of excessive amounts of TNF-α and nitric oxide (Boje and Arora, 1992), as well as from downregulation of their IGF-I production (Butovsky et al., 2005).

To determine whether the cytokine-activated microglia, in addition to their instructive effect on NPCs in vitro, affected proliferation and survival, we counted both the proliferating cells and the dead cells in the different co-cultures. Proliferation during the first 3 days of culture was induced to a limited extent only (<10%), and the rates of proliferation in the cytokine-activated microglial co-cultures did not differ significantly. It is therefore unlikely that the effect of these microglia on NPCs was attributable to their proliferative effect. The findings that the two tested cytokine-activated microglia showed different effects on differentiation but not on proliferation or on cell death further argue in favor of instructive mechanisms.

After 10 days in co-culture, MG(IL-4) and MG(IFN-γ) showed distinctly different effects on both neurogenesis and oligodendrogenesis, but their inductive effects on differentiation into astrocytes (GFAP+ cells) were similar. These findings suggested that the two cytokine-activated microglia affect the three neural lineages in distinctive ways.

The relevance of adaptive immunity for neurogenesis in vivo

The cellular source of IFN-γ and IL-4 in vivo is likely to be T cells. Accordingly, it is possible that microglia residing in the vicinity of the proliferating stem/progenitor cells are normally in a quiescent state, and become activated to support neurogenesis when needed (for example, when the individual needs to adjust to an enriched or other type of novel environment). The known constitutive patrolling of the healthy CNS by memory T cells might be one way to ensure lifelong accessibility of adaptive immunity when needed (Hickey et al., 1991; Flugel et al., 2001). T cells might enter the CNS by extravasating across the endothelium of the choroid plexus into the cerebrospinal fluid (Ransohoff et al., 2003), thus gaining access to sites of stem cell proliferation. This hypothesis is supported by several lines of evidence demonstrating that a T cell-mediated immune response is an integral part of the body’s mechanism for neuroprotection (Yoles et al., 2001; Kipnis et al., 2002) and that interaction of T cells with microglia and macrophages in the injured spinal cord correlates with enhanced neuronal survival (Butovsky et al., 2001). The observation that cell renewal in the CNS is stimulated by injury (Parent et al., 1997; Arvidsson et al., 2002; Nakatomi et al., 2002) further supports this notion.

Possible implications for CNS repair

Cell renewal serves as the basis for repair mechanisms in most tissues of the body. It was long believed that in the brain such processes do not occur, and therefore that any loss of neurons, being irreplaceable, would result in functional deficits with more or less debilitating effects. Because an insult to the CNS, whether acute or chronic, is often followed by the post-injury spread of neuronal damage, much research has been devoted to finding ways to minimize this degeneration by rescuing as many damaged neurons as possible.

The results of the present study lead to some intriguing conclusions. First, the microglia that lead to neuronal loss under pathological conditions (when cell renewal is critical) are the selfsame microglia that block cell renewal. Secondly, this apparently paradoxical situation can be remedied by a well-controlled adaptive immunity, which can shape the microglia in such a way that their activity is not cytotoxic but is both protective of the cells threatened by spreading degeneration and conducive to cell renewal. These findings suggest that in those cases in which protective autoimmunity leads to improved recovery (Moalem et al., 1999; Schwartz et al., 2003) not only will survival increase, but also both neurogenesis and gliogenesis are likely to be promoted. Furthermore, our results might explain why, in cases of autoimmune disease (when the abundance of autoimmune cells at the site of a lesion might result, for example, in profuse production of TNF-α induced by an excess of IFN-γ), the microglia are incapable of supporting either neurogenesis or oligodendrogenesis. As indicated above, in these cases, the beneficial effect of IFN-γ would be overridden by the negative effects of TNF-α. The results can also explain why IL-4 promotes recovery in cases of destructive autoimmune (Furlan et al., 2001) and why the use of steroids to treat neurodegenerative conditions is not helpful in the long term (Qian et al., 2000; Steinsapir et al., 2000; Matsumoto et al., 2001; Short, 2001), as their anti-inflammatory activity wipes out not only the destructive but also the beneficial effects of adaptive immunity. This notion is supported by the observation that remyelination requires T cells (Bieber et al., 2003). In a mouse model of experimental autoimmune encephalomyelitis, systemically injected NPCs were found to migrate to damaged sites (Pluchino et al., 2003). It is possible that the local immune response, mediated by Th1-associated cytokines, plays a role in such migration.

Further in vivo studies are needed to determine whether cell renewal in the context of acute or chronic neurodegenerative conditions requires the presence of Th2 cells only, or of both Th1 and Th2 cells, and if the latter, whether these two subpopulations are needed at different stages of the renewal process or at the same time. If they are needed at different stages, it could be speculated that Th1 cells would be the first to arrive and would support neurogenesis, whereas Th2 cells would ensure myelination of the newly formed neurons.

On the basis of our present findings, we suggest that impairment of spontaneous neurogenesis in the adult brain (for example, in age-related dementias and other acute or chronic neurodegenerative conditions) reflects, at least in part, the nature of the innate local immune activity and its dialog with adaptive immunity. It follows that harnessing of a well controlled adaptive immunity, rather than induction of immunosuppression, is the path to choose in designing ways to promote cell renewal in the CNS. In this way, it will be feasible to bypass the technically challenging problems arising from the fact that the conditions impairing neuronal survival and hence creating a critical need for new cells are, paradoxically, the very conditions that stand in the way of cell renewal and repair.
Experimental methods

Animals

Neonatal (P0–P1) C57Bl/6J mice were supplied by the Animal Breeding Center of The Weizmann Institute of Science. All animals were handled according to the regulations formulated by the Weizmann Institute’s Animal Care and Use Committee.

Reagents

Lipopolysaccharide (LPS) (containing <1% contaminating proteins) was obtained from Escherichia coli 0127:B8 (Sigma-Aldrich, St. Louis, MO). Recombinant mouse tumor necrosis factor (TNF)-α and insulin-like growth factor (IGF)-I (both containing endotoxin at a concentration below 1 EU per μg of cytokine), recombinant mouse interferon (IFN)-γ and interleukin (IL)-4 (both containing endotoxin at a concentration below 0.1 ng per μg of cytokine), goat anti-mouse neutralizing anti-TNF-α antibodies (αTNF-α; containing endotoxin at a concentration below 0.001 EU per μg of Ab), and goat anti-mouse neutralizing anti-IGF-I (aIgf-I; containing endotoxin at a concentration below 0.1 EU per μg of Ab) were obtained from R&D Systems (Minneapolis, MN).

Neural progenitor cell culture

Coronal sections (2 mm thick) of tissue containing the subventricular zone of the lateral ventricle were obtained from the brains of adult C57Bl6/J mice. The tissue was minced and then incubated for digestion at 37°C, 5% CO₂ for 45 min in Earle’s balanced salt solution containing 0.94 mg/ml papain (Worthington, Lakewood, NJ) and 0.18 mg/ml of l-cysteine and EDTA. After centrifugation at 110 × g for 15 min at room temperature, the tissue was mechanically dissociated by pipette trituration. Cells obtained from single-cell suspensions were plated (3500 cells/cm²) in 75-cm² Falcon tissue-culture flasks (BD Biosciences, Franklin Lakes, NJ), in NPC-culturing medium [Dulbecco’s modified Eagles’s medium (DMEM)/F12 medium (Gibco/Invitrogen, Carlsbad, CA) containing 2 mM l-glutamine, 0.6% glucose, 9.6 μg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.02 mg/ml insulin, 0.1 mg/ml transferrin, 2 μg/ml heparin (all from Sigma-Aldrich, Rehovot, Israel), fibroblast growth factor-2 (human recombinant, 20 ng/ml), and epidermal growth factor (human recombinant, 20 ng/ml; both from Peprotech, Rocky Hill, NJ)]. Spheres were passaged every 4–6 days and replated as single cells. Green fluorescent protein (GFP)-expressing neural progenitor cells (NPCs) were obtained as previously described (Pluchino et al., 2003).

Primary microglial culture

Brains from neonatal (P0–P1) C57Bl/6J mice were stripped of their meninges and minced with scissors under a dissecting microscope (Zeiss, Stemi DV4, Germany) in Leibovitz-15 medium (Biological Industries, Beit Ha-Emek, Israel). After trypsinization (0.5% trypsin, 10 min, 37°C/5% CO₂), the tissue was trituated. The cell suspension was washed in culture medium for glial cells [DMEM supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, Rehovot), l-glutamine (1 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml)] and cultured at 37°C/5% CO₂ in 75-cm² Falcon tissue-culture flasks (BD Biosciences) coated with poly-d-lysine (PDL) (10 mg/ml; Sigma-Aldrich, Rehovot) in borate buffer (2.37 g borax and 1.55 g boric acid dissolved in 500 ml sterile water, pH 8.4) for 1 h, then rinsed thoroughly with sterile, glass-distilled water. Half of the medium was changed after 6 h in culture and every 2nd day thereafter, starting on day 2, for a total culture time of 10–14 days. Microglia were shaken off the primary mixed brain glial cell cultures (150 rpm, 37°C, 6 h) with maximum yields between days 10 and 14, seeded (10⁵ cells/ml) onto PDL-pretreated 24-well plates (1 ml/well; Corning, Corning, NY), and grown in culture medium for microglia [RPMI-1640 medium (Sigma-Aldrich, Rehovot) supplemented with 10% FCS, l-glutamine (1 mM), sodium pyruvate (1 mM), β-mercaptoethanol (50 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml)]. The cells were allowed to adhere to the surface of a PDL-coated culture flask (1 h, 37°C/5%/CO₂), and non-adherent cells were rinsed off.

Co-culturing of neural progenitor cells and mouse microglia

Microglia were treated for 24 h with cytokines (IFN-γ, 20 ng/ml; IL-4, 10 ng/ml) or LPS (100 ng/ml). Cultures of treated or untreated microglia were washed twice with fresh NPC-differentiation medium (same as the culture medium for NPCs but without growth factors and with 2.5% FCS) to remove all traces of the tested reagents, then incubated on ice for 15 min, and shaken at 350 rpm for 20 min at room temperature. Microglia were removed from the flasks and immediately co-cultured (5 × 10⁴ cells/well) with NPCs (5 × 10⁴ cells/well) for 5 or 10 days on cover slips coated with Matrigel (BD Biosciences) in 24-well plates, in the presence of NPC differentiation medium, with or without insulin. The cultures were then fixed with 2.5% paraformaldehyde in PBS for 30 min at room temperature and stained for neuronal and glial markers. Cell proliferation rates and cell survival in vitro were determined by staining with 5-bromo-2’-deoxyuridine (BrDU, 2.5 μM; Sigma-Aldrich, St. Louis). For quantification of live and dead cells, live cultures were stained with 1 μg/ml propidium iodide (Molecular Probes, Invitrogen, Carlsbad, CA) and 1 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis), and cells were counted using Image-Pro (Media Cybernetics, Silver Spring, MD), as described (Hsieh et al., 2004).

Immunocytochemistry

Cover slips from co-cultures of NPCs and mouse microglia were washed with PBS, fixed as described above, treated with a permeabilization/blocking solution containing 10% FCS, 2% bovine serum albumin, 1% glycine, and 0.1% Triton X-100 (Sigma-Aldrich, Rehovot), and stained with a combination of the mouse or rabbit anti-tubulin β-III-isofrom C-terminus antibodies (β-III-tubulin; 1:500), rabbit anti-NG2 chondroitin sulfate proteoglycan (NG2; 1:500), mouse anti-RIP (RIP; 1:2000), mouse anti-galactocerebroside (GalC; 1:250), mouse anti-glutamic acid decarboxylase 67 (GAD; 1:1000), mouse anti-nestin (Nestin; 1:1000), rat anti-myelin basic protein (MBP; 1:300) (all from Chemicon, Temecula, CA), goat anti-double cortin (DCX; 1:400; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-glial fibrillary acidic protein (GFAP; 1:100; Sigma-Aldrich, St. Louis). For labeling of microglia, we used either rat anti-CD11b (MAC1; 1:50; BD–Pharmingen, NJ) or FITC-conjugated Bandeiraea simplicifolia isoelectin B4 (IB4; 1:50; Sigma-Aldrich, Rehovot).
Expression of IGF-I was detected by goat anti-IGF-I (1:20; R&D Systems).

**RNA purification, cDNA synthesis, and reverse-transcription PCR analysis**

Cells were lysed with TRI reagent (MRC, Cincinnati, OH), and total cellular RNA was purified from lysates using the RNase kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Residual genomic DNA was removed during the purification process by incubation with RNase-free DNase (Qiagen). RNA was stored in RNase-free water (Qiagen) at –80°C. RNA (1 µg) was converted to cDNA using SuperScript II (Promega, Madison, WI), as recommended by the manufacturer. The cDNA mixture was diluted 1:5 with PCR-grade water.

We assayed the expression of specific mRNAs using semi-quantitative reverse transcription PCR (RT-PCR) with selected gene-specific primer pairs, using Oligo v6.4 (Molecular Biology Insights, Cascade, CO). The primers used were: TNF-α, sense 5'-GGGACAGTGACCTGGACTGT-3', antisense 5'-AGGCTGTGCATTGCACCTCA-3'; beta-actin, sense 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'; and -351 bp). The RT-PCR reactions were carried out using 1 µg of cDNA, 35 nmol of each primer, and ReadyMix PCR Master Mix (ABgene, Epsom, UK) in 30-µl reactions. PCR reactions were carried out in an Eppendorf PCR system with 30 cycles (usually 25–30) of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and then kept at 4°C. As an internal standard for the amount of cDNA synthesized, we used beta-actin mRNA. PCR products were subjected to agarose gel analysis and visualized by ethidium bromide staining. Signals were quantified using a Gel-Pro analyzer 3.1 (Media Cybernetics). In all cases one product was observed with each primer set, and the observed product had an amplicon size that matched the size predicted from published cDNA sequences.

**Quantification**

For microscopic analysis, we used a Zeiss LSM 510 confocal laser scanning microscope (40× magnification). For experiments in vitro, we scanned fields of 0.053 mm² (laser scanning microscope (40× magnification)). In all cases one product was observed with each experimental group. For each marker, 500–1000 cells were counted. Cells co-expressing GFP and beta-3-tubulin, DCX, NG2, RIP, GalC, and GFAP were counted.

**Statistical analysis**

The results were analyzed by the Tukey–Kramer multiple comparisons test (ANOVA) and are expressed as means ± SD (unless differently indicated).

**References**


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