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Interleukin 4 induces the apoptosis of mouse microglial cells by a caspase-dependent mechanism.

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Abstract

Microglial cells are resident macrophages in the central nervous system (CNS) and become activated in many pathological conditions. Activation of microglial cells results in reactive microgliosis, manifested by an increase in cell number in the affected CNS regions. The control of microgliosis may be important to prevent pathological damage to the brain. The type 2 cytokine IL-4 has been reported to be protective in brain inflammation. However, its effect on microglial cell survival was not well understood. In this study, we report a dual effect of IL-4 on the survival of mouse microglial cells. In a 6 h short term culture, IL-4 reduced the death of microglial cells induced by staurosporine. In contrast, in long term treatment (more than 48 h), IL-4 increased the apoptotic death of both primary mouse microglial cells and a microglial cell line N9. Mechanistic studies revealed that, in microglial cells, IL-4 increased the levels of cleaved caspase 3 and PARP, which is downstream of activated caspase 3. In addition, IL-4 down regulated the autophagy and the antiapoptotic protein Bcl-xL in microglial cells. On the other hand, the pre-incubation of microglial cells with IL-4 for 24 h, attenuated the cell death induced by the neurotoxic peptide amyloid beta 1-42 (A β 42). Our observations demonstrate a novel function of IL-4 in regulating the survival of microglial cells, which may have important significance in reduction of undesired inflammatory responses in the CNS.

Keywords

Microglia; Interleukin 4; Apoptosis; Caspases; Neuroinflammation

Introduction

Microglial cells are resident macrophages in the central nervous system (CNS) and they have been shown to survey their surrounding microenvironment (Nimmerjahn et al., 2005).

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Microglial cells have multiple functions, including maintenance functions for neurons, phagocytosis, production of growth factors and cytokines, and antigen presentation, which can either, be protective or pathogenic, depending on the disease state (Ponomarev et al., 2007). Acute activation of microglia, as a result of neural injury, could rapidly lead to reactive microgliosis, a cardinal feature manifested by the expansion of microglia in the affected CNS region (Wirenfeldt et al., 2005). Increase in microglial cell number originates in part from recruitment of myeloid cells (Wirenfeldt et al., 2005), proliferation (Hailer et al., 1999), or migration from juxtaposed regions (Rappert et al., 2004). The state of reactive microgliosis dissolves days to weeks later, according to an inherently tightly regulated schedule, which has been suggested to involve microglial apoptosis (Jones et al., 1997). The cellular population control of immigrating and resident microglia should be comparable if immigrating bone marrow (BM)-derived cells are to take part fully in regular microglial tasks. Both, parenchymal and immigrating cells may modulate reactive microgliosis resolution by secreting growth factors and cytokines that may control microglial proliferation and survival (Colton, 2009; Glass et al., 2010; Yong and Rivest, 2009).

Autophagy is a fundamental cellular homeostatic mechanism (Mizushima et al., 2008), whereby cells autodigest parts of their cytoplasm for removal or turnover. In instances of cell injury or accumulation of damaged organelles/membranes, intracellular inclusions may be transferred to the autophagic pathway, serving as homeostatic mechanism at subcellular scale. In autophagy, a double or multi-membrane-bound structure, called the autophagosome or autophagic vacuole, is formed de novo to sequester cytoplasm. Then, the vacuole membrane fuses with the lysosome to deliver the contents into the organelle lumen, where they are degraded and the resulting macromolecules recycled (Mizushima et al., 2008). Overall, autophagy constitutes a fundamental survival strategy of cells, however it has also been linked to programmed cell death (Mizushima et al., 2008). Growth factor withdrawal usually results in rapid apoptotic cell death, but recent studies in apoptotic-deficient *bax*^{-/-}, *bak*^{-/-} cells have unraveled an essential role for autophagy genes in maintaining cellular survival following IL-3 deprivation (Lum et al., 2005).

Interleukin 4 (IL-4) is a Th2 cytokine with diverse biological activities in many cell types, including co-stimulation for growth and promotion of survival of cultured T and B lymphocytes, differentiation of T lymphocytes to the Th2 phenotype, and down-regulation of inflammatory functions of monocytes and macrophages (Gordon, 2003). In addition, IL-4 has been demonstrated to be a strong anti-inflammatory cytokine (Banchereau, 1995; Chomarat and Banchereau, 1997; Hamilton et al., 2002) able to inhibit cytokine production and the expression of the chemoattractant receptor mFPR2, which mediates the chemotactic activity of Alzheimer's disease (AD)-associated A β 1-42 peptide (A β 42), by lipopolysaccharide (LPS)-activated macrophages and microglia (Iribarren et al., 2003; Kitamura et al., 2000; Szczepanik et al., 2001). Moreover, it has been reported that exposure of macrophages to IL-4 may induce an "alternative activation state" characterized by up-regulation of mannose receptor but down-regulation of nitric oxide (NO) and pro-inflammatory cytokine production (Gordon, 2003; Mosser, 2003).

Although a number of anti-inflammatory activities have been reported for IL-4 on activated microglia, including inhibition of the expression of mFPR2 and TNF- α , as well as other pro-inflammatory cytokines (Iribarren et al., 2005; Lyons et al., 2009; Szczepanik et al., 2001), the role of IL-4 in the control of microglial cell number has not been well defined. Evidence suggests that IL-4 may not only induce the proliferation and survival of tumor cells and B lymphocytes (Kay and Pittner, 2003; Koller et al., 2010; Pangault et al., 2010), but also enhance the death of activated microglial cells from rat (Shin et al., 2004; Yang et al., 2002). Moreover, both IL-4 and IL-13 are able to inhibit autophagy responses in murine and human macrophages, which may potentially affect cell survival (Harris et al., 2007).

The aim of the present study was to determine the mechanisms by which IL-4 regulates mouse microglial cell survival. Here we show that although IL-4 initially promotes mouse microglial cell survival, after prolonged culture it induces caspase-dependent microglial cell death with down-regulation of autophagy and Bcl-xL expression. However, pre-incubation of microglial cells with IL-4 for 24h, attenuated the A β 42-induced cell death.

Material and methods

Reagents and cells

LPS, staurosporine, propidium iodide and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse IL-4 was purchased from PeproTech (Rocky Hill, NJ). Antibodies against caspase 3, PARP, Bcl-xL and beta actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal antibody against murine IL-4 was purchased from Becton Dickinson (San Jose, CA). The caspase inhibitor zVAD-FMK was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Amyloid beta 1-42 (A β 42) was purchased from Chemicon (Temecula, CA). The murine microglial cell line N9 was a kind gift from Dr. P. Ricciardi-Castagnoli (Universita Degli Studi di Milano-Bicocca, Milan, Italy). The cells were grown in IMDM supplemented with 5% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol. Primary murine microglial cells were isolated from 90 days old C57BL/6 mice. Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals and" (NIH Publication No. 86-23, 1985). The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Our animal facility obtained NIH animal welfare assurance (assurance number A5802-01, OLAW, NIH, USA).

Isolation of primary microglial cells from adult mice

After perfusion with HBSS, brains were collected in HBSS, dispersed with scissors, resuspended in HBSS containing 0.3% collagenase D (Roche) and 10 mM HEPES buffer (Invitrogen, Carlsbad, CA), and incubated 30 minutes at 37°C. After incubation, brain homogenates were filtered in 70 μ m pore size cell strainers (Becton Dickinson, San Jose, CA), centrifuged (7 min, 1500 rpm), washed and resuspended in 70% isotonic Percoll (GE Healthcare, Fairfield, CT). Three point five ml of the cell suspension were transferred to 15 ml polypropylene conical tubes with 5 ml of 25% isotonic Percoll and 3 ml of PBS sequentially layered on top, before centrifugation (30 min, 800 g, 4°C). The 70%:25% Percoll interphase was collected and cells washed. Finally, the adherent cells, which contained more than 90 % of CD11b positive cells, were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 100 mg/l sodium pyruvate and 10 mM HEPES buffer (Invitrogen, Carlsbad, CA).

Treatment of microglial cells and microscopic evaluation of cell death

Microglial cells were washed with PBS and resuspended in medium containing 2% heat-inactivated FCS, IL-4 (20 ng/ml) or other stimuli, and then cultured for the indicated times at 37 °C. Morphological changes were observed in a contrast phase microscope. After treatment, assessment of apoptosis on live, non-fixed microglia using Hoechst 33342 staining was adapted from Davenport et al. (Davenport et al., 2010). Briefly, cells were stained with Hoechst 33342 (5 μ g/ml) for 20 min as described previously (Davenport et al., 2010) and then viewed immediately in a Nikon Eclipse TE 2000-U fluorescence microscope. Apoptotic cells possessed brightly stained pycnotic nuclei meanwhile nonapoptotic cells showed large dim stained nuclei.

Evaluation of cell death by flow cytometry

In order to analyze the frequency of hypodiploid cells after stimulation, microglial cells were stained with propidium iodide using a protocol adapted from the previous report by Moon et al. (Moon et al., 2004). Briefly, the cells were harvested after stimulation with IL-4 and other molecules, fixed in 70% ethanol on ice for 30 min and then incubated with propidium iodide (50 µg/ml) and RNase (1 mg/ml) at room temperature for 30 min. Stained cells were analyzed by flow cytometry on a FACSCanto II cytometer (Becton Dickinson, San Jose, CA).

For annexin V-7-AAD dual staining, the cells were harvested, washed twice with PBS and incubated with PE-conjugated annexin V and 7-AAD following manufacturer instructions (PE Annexin V Apoptosis Detection Kit I, Becton Dickinson, San Jose, CA). Stained cells were analyzed by flow cytometry on a FACSCanto II cytometer (Becton Dickinson, San Jose, CA).

Transmission electron microscopy

Ultrastructural features of apoptosis were studied on microglial cells by transmission electron microscopy as previously described (Rabinovich et al., 1999). Briefly, microglial cells were harvested after 48 h of incubation with IL-4, washed in PBS, fixed with 1% of glutaraldehyde in 0.1 M cacodylate buffer for 2 hours, post-fixed with osmium tetroxide at 1% in the same buffer, dehydrated and embedded in Araldite. Thin sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome and examined in a Zeiss LEO 906E electron microscope.

Western immunoblotting

After treatment with IL-4 at the indicated time points, the N9 cells were lysed with 150 µl ice-cold lysis buffer. The cell lysates were centrifuged at 14,000 rpm and 4°C for 5 minutes. Western blotting of caspase 3, PARP or Bcl-xL was performed according to the manufacturer's instruction using specific polyclonal antibodies (Cell Signaling Technology, Inc. Beverly, MA). Briefly, proteins were electrophoresed on a 10% SDS-PAGE gel under reducing conditions, and transferred onto Immobilon-PVDF Membrane (BIO-RAD, Hercules, CA). The membranes were blocked with 5% non-fat milk, 0.1% Tween-20 in TBS overnight at 4°C and then were incubated with primary antibodies for 3 h at room temperature. After incubation with a horseradish-peroxidase conjugated secondary antibody (Cell Signaling Technology, Inc. Beverly, MA), the protein bands were detected with a Super Signal Chemiluminescent Substrate (Pierce) and BIOMAX-MR film (Eastman Kodak, Rochester, NY).

Labeling of autophagic vacuoles with monodansyl-cadaverine (MDC)

Following stimulation with IL-4, N9 cells were incubated with 0.05 mM MDC in PBS at 37°C for 10 minutes (Munafò and Colombo, 2001). After incubation, cells were washed four times with PBS and immediately MDC-labeled vesicles were observed by fluorescence microscopy using an inverted microscope (Nikon, Germany).

Statistical analysis

All experiments were performed at least three times and the results presented are from representative experiments. For all the experiments the significance of the difference between test and control groups was analyzed using the Student's t test.

Results

Prolonged incubation with interleukin 4 induces microglial cell death

Since it has been demonstrated that IL-4 is able to enhance the survival of hematopoietic cells (Illera et al., 1993; Zamorano et al., 1996), we therefore first examined if this cytokine was able to protect microglial cells from the effects of staurosporine, a classical apoptosis inducer. Similar to what it has been reported earlier (Braun et al., 2001), treatment of murine microglial cells with staurosporine for 6 h induced increased number of hypodiploid cells indicating increased apoptosis (Fig. 1 A) ($p < 0.05$). Pre-treatment of N9 microglial cells with IL-4 attenuated the staurosporine-induced cell death ($p < 0.05$) (Fig. 1 A). Unstimulated cells showed more than 96 % of euploid viable cells.

Since, it was also described (Shin et al., 2004; Yang et al., 2002) that IL-4 may potentiate rat microglial cell death after cell activation, we studied the effects of IL-4 on mouse microglial cell survival after prolonged treatment. We first evaluated the kinetics of hypodiploid cell frequency in N9 cells stimulated with IL-4. This cytokine was able to significantly increase the frequency of hypodiploid microglial cells at 48 h after stimulation ($p < 0.001$) (Fig. 1 B). Although an increase in the hypodiploid microglial cell numbers at 24 h post IL-4 treatment was observed, the difference was not statistically significant. The effect of IL-4 on microglial cell death was inhibited by a neutralizing IL-4-specific monoclonal antibody, indicating that IL-4 was responsible for microglial cells death (Fig. 2 A).

The effect of IL-4 was then tested on adult murine primary microglial cells, and here again an increase in the frequency of hypodiploid cells was observed after IL-4 treatment (Fig. 2 B). However, it required a longer culture time as compared to N9 cells (Fig. 2 B and C). On the other hand, the neurotoxic peptide A β 42 induced increased frequency of hypodiploid cells with similar kinetics in both primary microglial cells and N9 cells (Fig. 2 B and C). These results indicate that IL-4 is able to protect microglial cells from the apoptotic effect of staurosporine but it also promotes cell death two days after treatment.

IL-4 induces the apoptosis of microglial cells

Microscopic evaluation of N9 cells stimulated with IL-4 for 48 h showed the presence of cells with morphology compatible with apoptosis and late apoptosis/necrosis (Fig. 3 B). Moreover, microglial cells stimulated with IL-4 for 48 h showed increased frequency of chromatin condensation, as revealed by the presence of bright Hoechst 33342 stained and pyknotic nuclei (Fig. 3 D), compared to dim Hoechst 33342 stained nuclei in unstimulated cells (Fig. 3 C). In addition, transmission electronic microscopy studies revealed ultra-structural changes compatible with apoptosis, such as chromatin margination, cell shrinkage and blebbing in IL-4 treated cells (Fig. 3 F and H).

We further characterized the IL-4-induced microglial cell death by performing a kinetic evaluation of annexin V vs. 7-AAD staining of microglial cells by flow cytometry. IL-4 induced an increase in the frequency of both annexin V single positive cells (early apoptotic cells) and annexin V/7-AAD double positive cells (late apoptotic/necrotic cells) after 48 h of culture, (Fig. 4) ($p < 0.05$). These observations are consistent with the results obtained by measuring hypodiploidy, which confirm that IL-4 is able to induce microglial cell apoptosis after prolonged treatment.

IL-4 induces the apoptosis of microglial cells by a caspase-dependent mechanism

Caspase 3 is a key mediator of apoptosis responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Fernandes-Alnemri et al., 1994). Then, we studied the levels of caspase 3 in microglial cells after

treatment with IL-4. Microglial cells contain full length caspase 3 (35 kD) and after 48 h treatment with IL-4, the cells showed increased levels of cleaved caspase 3 (17/19 kD) (Fig. 5 A). However, IL-4 failed to increase microglial cell death in the presence of the pan caspase inhibitor zVAD (Fig. 5 B) ($p < 0.05$). As expected, zVAD alone did not affect the viability of microglial cells (Fig. 5 B).

In order to evaluate the downstream events of caspase 3, we examined the levels of PARP-1 in microglial cells after IL-4 treatment. Untreated microglial cells contained mainly the full length PARP1 (116 kD) (Fig. 5 C). IL-4 was able to induce increased levels of cleaved PARP-1 (89 kD) in a dose dependent manner after 48 h of treatment (Fig. 5 C). These results suggest that IL-4 is able to induce microglial cell death by a caspase-dependent mechanism.

IL-4 decreases survival factors in microglial cells

Autophagy is involved in removing damaged mitochondria and other organelles, in degrading intracellular pathogens, and in degrading protein aggregates too large to be removed by the ubiquitin-proteasomal system. These functions of autophagy could promote cellular survival during aging, infectious diseases, and neurodegenerative processes. We therefore evaluated the effects of IL-4 on the constitutive autophagy in microglial cells. We found that IL-4 down-regulated the constitutive autophagy in N9 cells observed as decreased frequency of cells containing MDC-labeled vesicles and decreased number of MDC-labeled vesicles per cell (Fig. 6 A). In addition, IL-4 down-regulated the protein levels of Beclin-1, a molecule involved in the induction of autophagy (Fig. 6 B). Similar to the effect of IL-4, treatment of N9 cells with 3-methyladenine (3-MA), a specific inhibitor of early stages autophagy, induced increased frequency of hypodiploid cells (Fig. 6 C).

We next evaluated the effects of IL-4 on the expression of the anti-apoptotic protein Bcl-xL. N9 cells constitutively expressed Bcl-xL (Fig. 6 D) and treatment with IL-4 down-regulated the level of Bcl-xL protein (Fig. 6 D). In addition, LPS, a well known down-regulator of Bcl-xL in macrophages (Ramana et al., 2007), also decreased the levels of this anti-apoptotic protein.

These data indicate that the effect of IL-4 inducing microglial cell death is associated with a decrease in two survival factors, constitutive autophagy and Bcl-xL expression.

The effects of IL-4 on the apoptosis of activated microglial cells

Both LPS and A β 42 are molecules able to initially activate and later induce apoptosis of microglial cells (Jang et al., 2005; Jung et al., 2005; Yazawa et al., 2001). We next studied the effects of IL-4 on LPS- and A β 42-induced apoptosis of microglial cells. Cotimulation of N9 cells with IL-4 plus LPS or A β 42, did not modify the pro-apoptotic effects of LPS or A β 42 alone (data not shown). Moreover, we evaluated the pro-apoptotic effects of LPS and A β 42 in cells previously incubated with IL-4 for 24 h. The pre-incubation with IL-4 did not modify the pro-apoptotic effects of LPS (data not shown). However, IL-4 was able to attenuate the A β 42-induced death of both primary microglial and N9 cells (Fig. 7).

These results suggest that pre-incubation with IL-4, a cytokine that initially promotes cell survival but later induces apoptosis of microglial cells, may selectively attenuate the pro-apoptotic effects of A β 42 in these cells.

Discussion

We have shown that in short term culture, IL-4 was able to attenuate staurosporine-induced microglial cell death; however this type 2 cytokine was also able to induce microglial cell

death after 48 h of treatment. To our knowledge, this is the first demonstration that IL-4 alone exerts a pro-apoptotic effect on mouse microglial cells, by activating a caspase-dependent pathway, and down-regulating spontaneous autophagy and Bcl-xL. On the other hand, when microglial cells were pre-incubated with IL-4 for 24 h, the A β 42-induced cell death was attenuated.

The ramified microglia in normal central nervous system display a typical resting phenotype characterized by lack of endocytic activity and low levels of activation markers that are expressed by tissue macrophages and peripheral blood monocytes (Kreutzberg, 1996). However, microglia rapidly respond to endogenous (e.g., damaged cells, cytokines and tumors), as well as exogenous (infectious agents and endotoxin) stimuli, and actively participate in immune responses, inflammation and tissue repair in the central nervous system (Aloisi, 2001; Iribarren et al., 2002). During these processes, microglia acquires markers shown on differentiated macrophages and display effector functions including secretion of pro-inflammatory and neurotoxic mediators (Bezzi et al., 2001). Activated microglial cells also accumulate at sites of inflammation in central nervous system diseases, including multiple sclerosis (Kreutzberg, 1996), AIDS encephalitis (Streit et al., 1999), prion disease and AD (Boddeke et al., 1999). On the other hand, apoptosis has been suggested as an important mode of microglial population control, however, through a nonclassic pathway that does not culminate with condensed fragmented DNA (Jones et al., 1997). Wrenfeldt et al., demonstrated that apoptosis does play a role in the control of microglial population in brain regions displaying a dense anterograde axonal and terminal degeneration in both resident and immigrating microglia as investigated by identification of phosphatidylserine externalization by annexin V binding (Wrenfeldt et al., 2007). Furthermore, the authors showed that microglia can undergo apoptosis in a classic sense with apoptotic morphology, condensed fragmented DNA, and cytoplasmic expression of activated caspase-3 (Wrenfeldt et al., 2007). In our study, we also observed that IL-4 was able to induce death of microglial cells with features of classical apoptosis such as hypodiploid DNA content, appearance of annexin V single positive cells, cell shrinkage, blebbing, chromatin margination and caspase 3 activation. Moreover, IL-4 reduced the constitutive levels autophagy and the survival protein Bcl-xL.

Mammalian target of rapamycin (mTOR) is a major integration site for nutrient responses in eukaryotic cells. For instance, upstream of mTOR, class I PI3K/Akt signaling molecules link receptor tyrosine kinases to mTOR activation, thereby inhibiting autophagy in response to insulin-like and other growth factor signals. A class III PI3K complex including Beclin 1/Atg6/hVps34 is able to control autophagosome formation. In this paper we demonstrated that IL-4 reduced the levels of both Beclin-1 and autophagosomes in microglial cells leading to decreased constitutive autophagy, which may predispose the cells to suffering apoptosis.

Interleukin 4 act as a co-mitogen for B cells (Howard et al., 1982). Although not being a growth factor by itself for resting lymphocytes, IL-4 substantially prolongs the survival of T and B lymphocytes in culture (Hu-Li et al., 1987) and prevents apoptosis of myeloid lines that express IL-4 receptors (Minshall et al., 1997). On the other hand, both IL-4 and IL-13 have also been shown to induce death of rat activated microglia, which will prevent tissue damage caused by chronic inflammation (Yang et al., 2002). Moreover, previous studies have shown that interleukin 13 (IL-13), an anti-inflammatory cytokine, enhances cyclooxygenase-2 (COX 2) expression and production of PGE2 and 15-deoxy- $\Delta^{12,14}$ -PGJ2 (15d-PGJ2) in rat microglia activated by LPS, which in turn may induce the death of activated microglia, and this may lead to the termination of the process of brain inflammation (Yang et al., 2006). In another study performed in rats, Shin et al. (Shin et al., 2004) have shown that intracerebral injection of LPS in rats induced the expression of IL-13 followed by substantial loss of microglia, which was inhibited by an IL-13 neutralizing

antibody, suggesting that this cytokine may control brain inflammation in rats by inducing the death of activated microglia.

It has been reported (Liu et al., 2010) that IL-13, but not IL-4 or IL-10, enhanced the expression of ER stress markers such as, phospho eIF2 α , GRP94, GRP78, and GADD153 and apoptosis in LPS-activated microglia. These findings imply that anti-inflammatory cytokines may have selective reactivity in response to microglial activation during brain inflammation. On the other hand, Yang et al. have shown that both IL-13 and IL-4 are able to induce the death of activated microglia (Yang et al., 2006; Yang et al., 2002).

We have previously demonstrated that host-derived anti-inflammatory cytokines also have the capacity to maintain microglial cell homeostasis by reducing their responses to pro-inflammatory stimulants (Iribarren et al., 2007). For instance, we have reported that IL-4 is able to “disrupt” the microglial response to A β 42 promoted by both LPS and TNF α signaling, through the induction of the phosphatase PP2A, which may rapidly dephosphorylate MAPKs activation in microglia (Cui et al., 2002; Iribarren et al., 2005; Shanley et al., 2001). Our further studies revealed that PI3K plays a key role in IL-4-triggered signaling events that rendered microglial cells resistant to LPS (Iribarren et al., 2003). Thus, in an AD murine model, IL-4 is reduced in mice brains (Abbas et al., 2002), and it appears to maintain the homeostasis of the CNS limiting microglial activation in response to pro-inflammatory stimulants. Interestingly, here we showed that pre-incubation of microglial cells for 24 h with IL-4 reduced the A β 42- but not LPS-induced cell death. One possibility is that IL-4, as we previously demonstrated (Iribarren et al., 2003; Iribarren et al., 2005), was able to reduce the expression of mFPR2, one of the A β receptors, therefore limiting microglial cell death. However, complex cross talk between signaling transduction pathways might also account for this effect of IL-4. These issues will be further evaluated in future studies.

Recent studies suggest that regulation of microglial cell survival may be important in limiting CNS disease progression. For instance, inhibiting p53 mediated microglial apoptosis prevented microglial neurotoxicity suggesting that targeting p53-mediated pathways in microglia may have therapeutic benefit in AD (Davenport et al., 2010). Moreover, microglial apoptosis caused by toxic lysophosphatidylcholine containing very-long-chain fatty acids might constitute an early pathogenic change in cerebral X-linked adrenoleukodystrophy (X-ALD) (Eichler et al., 2008).

In our study, we provided evidence indicating that the effects of IL-4 on the regulation of neuroinflammation may be due to controlling the microglial cell number by induction of caspase-dependent cell death. Elucidating the mechanisms by which IL-4 controls microglial cell activation and expansion should be beneficial for the design of therapeutic approaches to neurodegenerative diseases in which inflammatory responses exacerbate the pathogenic processes.

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References

- Abbas N, et al. Up-regulation of the inflammatory cytokines IFN- γ and IL-12 and down-regulation of IL-4 in cerebral cortex regions of APP(SWE) transgenic mice. *J Neuroimmunol.* 2002; 126:50–7. [PubMed: 12020956]

- Aloisi F. Immune function of microglia. *Glia*. 2001; 36:165–79. [PubMed: 11596125]
- Banchereau J. Converging and diverging properties of human interleukin-4 and interleukin-10. *Behring Inst Mitt*. 1995;58–77. [PubMed: 7575353]
- Bezzi P, et al. CXCR4-activated astrocyte glutamate release via TNF α : amplification by microglia triggers neurotoxicity. *Nat Neurosci*. 2001; 4:702–10. [PubMed: 11426226]
- Boddeke EW, et al. Cultured rat microglia express functional beta-chemokine receptors. *J Neuroimmunol*. 1999; 98:176–84. [PubMed: 10430051]
- Braun JS, et al. Apoptosis-inducing factor mediates microglial and neuronal apoptosis caused by pneumococcus. *J Infect Dis*. 2001; 184:1300–9. [PubMed: 11679919]
- Colton CA. Heterogeneity of microglial activation in the innate immune response in the brain. *J Neuroimmune Pharmacol*. 2009; 4:399–418. [PubMed: 19655259]
- Cui YH, et al. Bacterial lipopolysaccharide selectively up-regulates the function of the chemotactic peptide receptor formyl peptide receptor 2 in murine microglial cells. *J Immunol*. 2002; 168:434–42. [PubMed: 11751990]
- Chomarat P, Banchereau J. An update on interleukin-4 and its receptor. *Eur Cytokine Netw*. 1997; 8:333–44. [PubMed: 9459612]
- Davenport CM, et al. Inhibiting p53 pathways in microglia attenuates microglial-evoked neurotoxicity following exposure to Alzheimer peptides. *J Neurochem*. 2010; 112:552–63. [PubMed: 19895660]
- Eichler FS, et al. Is microglial apoptosis an early pathogenic change in cerebral X-linked adrenoleukodystrophy? *Ann Neurol*. 2008; 63:729–42. [PubMed: 18571777]
- Fernandes-Alnemri T, et al. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J Biol Chem*. 1994; 269:30761–4. [PubMed: 7983002]
- Glass CK, et al. Mechanisms underlying inflammation in neurodegeneration. *Cell*. 2010; 140:918–34. [PubMed: 20303880]
- Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003; 3:23–35. [PubMed: 12511873]
- Hailer NP, et al. Proliferation of microglia and astrocytes in the dentate gyrus following entorhinal cortex lesion: a quantitative bromodeoxyuridine-labelling study. *Eur J Neurosci*. 1999; 11:3359–64. [PubMed: 10510203]
- Hamilton TA, et al. Regulation of chemokine expression by antiinflammatory cytokines. *Immunol Res*. 2002; 25:229–45. [PubMed: 12018462]
- Harris J, et al. T helper 2 cytokines inhibit autophagic control of intracellular *Mycobacterium tuberculosis*. *Immunity*. 2007; 27:505–17. [PubMed: 17892853]
- Howard M, et al. Identification of a T cell-derived b cell growth factor distinct from interleukin 2. *J Exp Med*. 1982; 155:914–23. [PubMed: 6977612]
- Hu-Li J, et al. B cell stimulatory factor 1 (interleukin 4) is a potent costimulant for normal resting T lymphocytes. *J Exp Med*. 1987; 165:157–72. [PubMed: 3098893]
- Illera VA, et al. Apoptosis in splenic B lymphocytes. Regulation by protein kinase C and IL-4. *J Immunol*. 1993; 151:2965–73. [PubMed: 8376764]
- Iribarren P, et al. The role of dendritic cells in neurodegenerative diseases. *Arch Immunol Ther Exp (Warsz)*. 2002; 50:187–96. [PubMed: 12098934]
- Iribarren P, et al. IL-4 down-regulates lipopolysaccharide-induced formyl peptide receptor 2 in murine microglial cells by inhibiting the activation of mitogen-activated protein kinases. *J Immunol*. 2003; 171:5482–8. [PubMed: 14607954]
- Iribarren P, et al. Interleukin 10 and TNF α synergistically enhance the expression of the G protein-coupled formylpeptide receptor 2 in microglia. *Neurobiol Dis*. 2007; 27:90–8. [PubMed: 17544285]
- Iribarren P, et al. IL-4 inhibits the expression of mouse formyl peptide receptor 2, a receptor for amyloid β 1–42, in TNF- α -activated microglia. *J Immunol*. 2005; 175:6100–6. [PubMed: 16237106]
- Jang MH, et al. Melatonin attenuates amyloid β 25–35-induced apoptosis in mouse microglial BV2 cells. *Neurosci Lett*. 2005; 380:26–31. [PubMed: 15854745]

- Jones LL, et al. Population control of microglia: does apoptosis play a role? *J Neurocytol.* 1997; 26:755–70. [PubMed: 9426172]
- Jung DY, et al. TLR4, but not TLR2, signals autoregulatory apoptosis of cultured microglia: a critical role of IFN-beta as a decision maker. *J Immunol.* 2005; 174:6467–76. [PubMed: 15879150]
- Kay NE, Pittner BT. IL-4 biology: impact on normal and leukemic CLL B cells. *Leuk Lymphoma.* 2003; 44:897–903. [PubMed: 12854886]
- Kitamura Y, et al. Interleukin-4-inhibited mRNA expression in mixed rat glial and in isolated microglial cultures. *J Neuroimmunol.* 2000; 106:95–104. [PubMed: 10814787]
- Koller FL, et al. Epithelial interleukin-4 receptor expression promotes colon tumor growth. *Carcinogenesis.* 2010; 31:1010–7. [PubMed: 20176658]
- Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 1996; 19:312–8. [PubMed: 8843599]
- Liu SH, et al. IL-13 downregulates PPAR-gamma/heme oxygenase-1 via ER stress-stimulated calpain activation: aggravation of activated microglia death. *Cell Mol Life Sci.* 2010; 67:1465–76. [PubMed: 20221786]
- Lum JJ, et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell.* 2005; 120:237–48. [PubMed: 15680329]
- Lyons A, et al. Decreased neuronal CD200 expression in IL-4-deficient mice results in increased neuroinflammation in response to lipopolysaccharide. *Brain Behav Immun.* 2009; 23:1020–7. [PubMed: 19501645]
- Minshall C, et al. IL-4 and insulin-like growth factor-I inhibit the decline in Bcl-2 and promote the survival of IL-3-deprived myeloid progenitors. *J Immunol.* 1997; 159:1225–32. [PubMed: 9233617]
- Mizushima N, et al. Autophagy fights disease through cellular self-digestion. *Nature.* 2008; 451:1069–75. [PubMed: 18305538]
- Moon EY, et al. Reactive oxygen species induced by the deletion of peroxiredoxin II (PrxII) increases the number of thymocytes resulting in the enlargement of PrxII-null thymus. *Eur J Immunol.* 2004; 34:2119–28. [PubMed: 15259009]
- Mosser DM. The many faces of macrophage activation. *J Leukoc Biol.* 2003; 73:209–12. [PubMed: 12554797]
- Munafò DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J Cell Sci.* 2001; 114:3619–29. [PubMed: 11707514]
- Nimmerjahn A, et al. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science.* 2005; 308:1314–8. [PubMed: 15831717]
- Pangault C, et al. Follicular lymphoma cell niche: identification of a preeminent IL-4-dependent T(FH)-B cell axis. *Leukemia.* 2010
- Ponomarev ED, et al. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. *J Neurosci.* 2007; 27:10714–21. [PubMed: 17913905]
- Rabinovich GA, et al. Granulocyte-macrophage colony-stimulating factor protects dendritic cells from liposome-encapsulated dichloromethylene diphosphonate-induced apoptosis through a Bcl-2-mediated pathway. *Eur J Immunol.* 1999; 29:563–70. [PubMed: 10064072]
- Rappert A, et al. CXCR3-dependent microglial recruitment is essential for dendrite loss after brain lesion. *J Neurosci.* 2004; 24:8500–9. [PubMed: 15456824]
- Shanley TP, et al. The serine/threonine phosphatase, PP2A: endogenous regulator of inflammatory cell signaling. *J Immunol.* 2001; 166:966–72. [PubMed: 11145674]
- Shin WH, et al. Microglia expressing interleukin-13 undergo cell death and contribute to neuronal survival in vivo. *Glia.* 2004; 46:142–52. [PubMed: 15042582]
- Streit WJ, et al. Reactive microgliosis. *Prog Neurobiol.* 1999; 57:563–81. [PubMed: 10221782]
- Szczepanik AM, et al. IL-4, IL-10 and IL-13 modulate A beta(1–42)-induced cytokine and chemokine production in primary murine microglia and a human monocyte cell line. *J Neuroimmunol.* 2001; 113:49–62. [PubMed: 11137576]

- Wirenfeldt M, et al. Reactive microgliosis engages distinct responses by microglial subpopulations after minor central nervous system injury. *J Neurosci Res.* 2005; 82:507–14. [PubMed: 16237722]
- Wirenfeldt M, et al. Population control of resident and immigrant microglia by mitosis and apoptosis. *Am J Pathol.* 2007; 171:617–31. [PubMed: 17600121]
- Yang MS, et al. Interleukin-13 enhances cyclooxygenase-2 expression in activated rat brain microglia: implications for death of activated microglia. *J Immunol.* 2006; 177:1323–9. [PubMed: 16818793]
- Yang MS, et al. Interleukin-13 and -4 induce death of activated microglia. *Glia.* 2002; 38:273–80. [PubMed: 12007140]
- Yazawa H, et al. Beta amyloid peptide (A β 42) is internalized via the G-protein-coupled receptor FPRL1 and forms fibrillar aggregates in macrophages. *FASEB J.* 2001; 15:2454–62. [PubMed: 11689470]
- Yong VW, Rivest S. Taking advantage of the systemic immune system to cure brain diseases. *Neuron.* 2009; 64:55–60. [PubMed: 19840549]
- Zamorano J, et al. IL-4 protects cells from apoptosis via the insulin receptor substrate pathway and a second independent signaling pathway. *J Immunol.* 1996; 157:4926–34. [PubMed: 8943397]

Highlights

> In 6 h culture, IL-4 reduced the death of microglial cells induced by staurosporine. > In contrast, in long term treatment IL-4 increased the apoptotic death. > IL-4 increased the levels of cleaved caspase 3 and PARP. > IL-4 down regulated the autophagy and Bcl-xL in microglial cells.

Fig. 1.

The effects of IL-4 on microglial cell survival. (A) N9 cells were incubated with 20 ng/ml IL-4 for 1 h at 37°C, followed by addition of 0.5 μ M staurosporine (St) for 6 h. (B) N9 cells were cultured in the presence 20 ng/ml IL-4 for 12, 24 or 48 h. Cells were fixed with ethanol, stained with PI and percentages of hypodiploid cells were determined by flow cytometry. The figure shows histograms of PI red fluorescence and the numbers indicate the frequency of hypodiploid cells. The bar graph represents average of three separated experiments. * Indicates statistically significant (p < 0.01) increase of hypodiploid cell frequency compared to unstimulated cells.

Fig. 2.

IL-4 induces increased frequency of hypodiploid N9 and primary microglial cells. (A) IL-4 (20 ng/ml) was preincubated with an IL-4-specific monoclonal antibody (α IL-4) (10 μ g/ml) or control IgG (10 μ g/ml) for 1 h at 37°C before being added to N9 cells, which were then cultured for 48 h at 37°C. * Indicates statistically significant ($p < 0.01$) increase of hypodiploid cell frequency compared to unstimulated cells. ** Indicates statistically significant ($p < 0.02$) decrease of hypodiploid cell frequency compared to IL-4-stimulated cells. Primary microglia (B) or N9 cells (C), cultured in the presence of IL-4 (20 ng/ml) or A β 42 (10 μ M) for 48 or 72 h, were fixed with ethanol and then examined for PI staining by flow cytometry. The figure shows bar graphs representing average of hypodiploid cell

frequencies of three separated experiments * Indicates statistically significant ($p < 0.01$) increase of hypodiploid cell frequency compared to unstimulated cells. ** Indicates statistically significant ($p < 0.05$) increase of hypodiploid cell frequency compared to unstimulated cells.

Fig. 3.

The morphological features of IL-4-induced microglial cell death. N9 cells were cultured in the presence or the absence of IL-4 (20 ng/ml) for 48 h and were observed with a phase contrast microscope (A and B), stained with Hoechst 33342 and observed with a fluorescence microscope (C and D) or processed and examined in an electron microscope (E - H). (B) Black arrows indicate chromatin condensation and cell degeneration. (D) Bright Hoechst 33342 staining of condensed chromatin (white arrows) and pyknotic nuclei (arrowheads) was observed. (F) Black arrows indicate chromatin condensation and margination. Arrowheads indicate cell vacuolization. (H) Black arrows indicate cell blebbing.

Fig. 4.

IL-4 increases the frequency of annexin V stained microglial cells. N9 cells were incubated with medium or IL-4 (20 ng/ml) for 24 or 48 h at 37°C. Later on the cells were co-stained with annexin V-PE and 7-AAD and analyzed by flow cytometry. Numbers in dot plots represent percentage of cells in each quadrant. Data are representative of three independent experiments with similar results.

Fig. 5.

IL-4 activates caspase 3 and induces caspase-dependent microglial cell death. N9 cells were cultured in the presence of IL-4 (5, 10 or 20 ng/ml) for the indicated time points at 37°C. The cells were lysed and caspase 3 (A) or PARP (C) were examined by western immunoblotting. (B) N9 cells were cultured in the presence of zVAD (20 μM) (a pancaspase inhibitor) or DMSO for 1 h at 37°C, then stimulated with IL-4 (20 ng/ml) for 48 h. Cells were fixed with ethanol, stained with PI and percentages of hypodiploid cells were evaluated by flow cytometry. The bar graph represents average of three separated experiments. * Indicates statistically significant ($p < 0.01$) decrease of hypodiploid cell frequency compared to IL-4-stimulated cells.

Fig. 6.

The effects of IL-4 on constitutive autophagy and Bcl-xL expression. (A) N9 cells were incubated with medium or IL-4 (20 ng/ml) for 48 h at 37°C. Later on the cells were stained with 0.05 mM monodansyl-cadaverine (MDC) in PBS at 37°C for 10 minutes, and immediately MDC-labeled vesicles were observed by fluorescence microscopy. The bar graph represents average of the frequency of cells presenting MDC-labeled vesicles and the average of number of stained vesicles per cell. Data are representative of three independent experiments with similar results. (B) N9 cells were cultured in the absence or the presence of IL-4 (20 ng/ml) for 24 and 48 h at 37°C. The cells were lysed and Beclin-1 was examined by western immunoblotting. (C) N9 cells were stimulated with IL-4 (20 ng/ml) or 3-methyladenine (3-MA) (specific inhibitor of early stages of autophagy) for 48 h. Cells were fixed with ethanol, stained with PI and percentages of hypodiploid cells were determined by flow cytometry. The bar graph represents average of three separated experiments. * Indicates statistically significant ($p < 0.01$) increase of hypodiploid cell frequency compared to unstimulated cells. (D) N9 cells were cultured in the presence of IL-4 (20 ng/ml) or lipopolysaccharide (LPS) for 48 h at 37°C. The cells were lysed and Bcl-xL was examined by western immunoblotting.

Fig. 7.

The effects of IL-4 on the apoptosis of activated microglial cells. N9 cells (A) or primary microglial cells (B) were cultured in the presence or the absence of 20 ng/ml IL-4 for 24 h at 37°C, followed by addition of A β 42 (10 μ M) for 48h. Cells were fixed with ethanol, stained with PI and percentages of hypodiploid cells were determined by flow cytometry. The figure shows histograms of PI red fluorescence and the numbers indicate the frequency of hypodiploid cells. The bar graph represents fold increase of hypodiploidy in treated cells vs. unstimulated control group of three separated experiments. * Indicates statistically significant ($p < 0.01$) increase of hypodiploid cell frequency compared to unstimulated cells. ** Indicates statistically significant ($p < 0.05$) decrease of hypodiploid cell frequency compared to A β 42-stimulated cells.