Tumour necrosis factor-alpha-induced neuronal loss is mediated by microglial phagocytosis

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A B S T R A C T

Tumour necrosis factor-alpha (TNF-alpha) is a pro-inflammatory cytokine, expressed in many brain pathologies and associated with neuronal loss. We show here that addition of TNF-alpha to neuronal-glial co-cultures increases microglial proliferation and phagocytosis, and results in neuronal loss that is prevented by eliminating microglia. Blocking microglial phagocytosis by inhibiting phagocytic vitronectin and P2Y6 receptors, or genetically removing opsonin MFG-E8, prevented TNF-alpha-induced loss of live neurons. Thus TNF-alpha appears to induce neuronal loss via microglial activation and phagocytosis of neurons, causing neuronal death by phagoptosis.

1. Introduction

Tumour necrosis factor-alpha (TNF-alpha) is a pro-inflammatory cytokine, central to the regulation of inflammation in body and brain. In the healthy brain, it is expressed at very low levels by a variety of brain cells including neurons [29], but microglia (brain macrophages) and astrocytes, activated by pathogens or damage via pattern recognition receptors such as Toll-like receptors (TLRs), express and release high levels of TNF-alpha [18,13,33,21]. Thus the damaged or diseased brain contains high levels of TNF-alpha, including during brain trauma, ischemia, and neurodegenerative conditions [8,22]. TNF-alpha activates microglia and astrocytes via TNF receptors 1 (TNFR1) and 2 (TNFR2) [11], which activate the phagocytic NADPH oxidase (PHOX) [20], caspase-8 [2] and NF-kappaB [5], resulting in the expression of further pro-inflammatory and phagocytic genes that increase the ability of glia to deal with pathogens and damage [31]. However, high and sustained levels of TNF-alpha can lead to neuronal damage [10], and there is evidence that TNF-alpha contributes to a variety of brain pathologies, such as ischemic stroke, Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis [8,22]. The mechanisms by which TNF-alpha is damaging to neurons is unclear, but the inflammatory activation of glia may contribute.

In culture, we have found that activation of microglial TLRs results in high levels of TNF-alpha production [13,23,15] and TNF-alpha in turn stimulates microglial proliferation [20]. We subsequently found that activation of microglia via TLR2 or TLR4 caused delayed neuronal loss via activating microglial phagocytosis of stressed-viable neurons [23]. Cell death caused by the cell being phagocytosed is called ‘phagoptosis’ with the defining characteristic that inhibition of phagocytosis or phagocytic signalling prevents death of the cell [6]. TLR activation of microglia causes: (i) release of oxidants from microglia that induce neurons to reversibly expose the ‘eat-me’ signal phosphatidylserine, (ii) release of the opsonin milk fat globule EGF factor 8 (MFG-E8) from microglia and astrocytes that binds exposed phosphatidylserine on neurons and activates their phagocytosis via the microglial vitronectin receptor (VNR), and (iii) activation of the phagocytic capacity of microglia via upregulation of Mer tyrosine kinase and other phagocytic proteins [23,24,7].

As activation of microglia can cause both TNF-alpha production and neuronal phagoptosis, we tested here whether soluble, extracellular TNF-alpha was able and sufficient to induce microglia-dependent neuronal loss by phagoptosis.
2. Materials and methods

2.1. Reagents

Rat recombinant TNF-α (Sigma), lipopolysaccharide from Salmonella typhimurium (Sigma), Alexa Fluor 488 conjugate of isoelectin B4 from Griffonia simplicifolia (Invitrogen), carboxylate-modified fluorescent microspheres (Invitrogen), cyclo(RGDfV) peptide (Bachem), recombinant MFG-E8 (R&D Systems), human soluble TNF receptor inhibitor/Fc chimera (GenScrip Corporation), and MRS2578 compound (Tocris). Cell culture reagents were from PAA. Other reagents were from Sigma.

2.2. Primary cell culture and treatment

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the Cambridge University local ethical committee. Primary mixed neuronal/glial cultures from postnatal days 5–7 Wistar rat or Mfge8−/− mice [30] cerebella were prepared as described previously [16]. Cells were plated at a density of 5 × 10^5 cells/well on poly-L-lysine coated 24-well plates. Glial cultures and pure microglial cultures were prepared as described previously [3]. Microglia cells were depleted from the cultures with L-leucine methyl ester as described previously [23]. Cells were stimulated at 7–9 days in vitro with TNF-α (50 ng/ml) or LPS (100 ng/ml), and cyclo(RGDfV) peptide (50 μM), recombinant MFG-E8 (0.4 μg/ml) or soluble TNF receptor inhibitor (100 ng/ml) were added together with TNF-α, whereas MRS2578 compound (1 μM) was added every day. Cell densities after treatment were evaluated as described previously [27]. Neurons with regular soma shape and normal nuclear Hoechst 33342 staining were counted as alive, whereas neurons staining with propidium iodide were defined as necrotic.

2.3. Microglial phagocytosis of beads

Phagocytic capacity of microglial cells was evaluated as described previously [27]. In short, pure microglial culture was treated with 50 ng/ml TNF-α for 24 h before 3 μl of 1:10 dilution of 1 μm fluorescently labelled carboxylate-modified microspheres were added, and cells were incubated for 2 h at 37 °C, 5% CO₂. The medium was removed, and the culture was washed several times to remove excess beads. Microglia cells were then labelled with Alexa Fluor 488-tagged isoelectin B4 (2 μg/ml) and bead number per cell was evaluated in >50 cells per condition.

2.4. Microglial phagocytosis of neurons

Glial cultures were treated ± 50 ng/ml of TNF-α for 24 h. Microglia from untreated and treated flasks were detached from other glia by shaking the flask. 10⁵ microglia (untreated and treated) were added to each well (in a 24 well plate) of a mixed neuronal/glial culture, which had previously been stained for 15 min with TAMRA (red fluorescence) and washed. Phagocytosis was assayed in a medium half from a glial culture and half from a mixed neuronal/glial culture. Phagocytosis of neurons by microglia was evaluated by microscopy at 6 h after adding microglia as the number of microglia per field containing red fluorescent debris. Cells were also stained with Hoechst 33342 (for nuclei) and green fluorescent isoelectin B4 (for microglia).

2.5. Statistical analysis

For all experiments, each condition/treatment was repeated at least in duplicate, and each experiment was replicated in at least three independent cultures – except the experiment on microglial phagocytosis of neurons, which was repeated in quadruplicate but on one culture. Statistical analysis was performed using IBM SPSS Statistics v20 software. Normality of data was verified by Shapiro–Wilks test. Means were compared by one-way ANOVA, and the significance of the difference between each treatment mean and the control or TNF-α treatment mean was quantified by post-hoc Bonferroni tests. All such significant changes are reported in the Figures – those not reported as significant are not significant. P values < 0.05 were considered as significant. Numbers of alive, apoptotic and necrotic neurons were compared separately. All data presented are expressed as mean ± standard error of the mean (S.E.M.).

3. Results and discussion

TNF-α (50 ng/ml, equivalent to 3 nM of monomer) caused microglial proliferation (Figs. 1A and 2A), stimulated microglial phagocytosis of beads (Fig. 1B), and increased the phagocytosis of neurons by added microglia (Fig. 1C). However, in mixed neuronal–glial cultures, a single dose of TNF-α was not sufficient to cause significant neuronal loss (Fig. 2B). As extracellular TNF-α is

![Figure 1](image-url)
rapidly removed/degraded [15], we tested whether significant neuronal loss could be induced by a second bolus of 50 ng/ml TNF-α added 24 h after the first dose, and cultures were incubated for a further 2 or 6 days (in total 3 or 7 days treatment, respectively). Two doses of TNF-α were sufficient to induce significant neuronal loss after 3 days of treatment (Fig. 2A and B). There was no further loss of neurons for up to 7 days (Fig. 2B), even though prolonged treatment with TNF-α increased microglial densities by up to ten times (Fig. 1A). This is in accordance with previously published data demonstrating that phagoptosis induced with different stimuli is maximal 2–3 days after culture stimulation [23,27]. Higher concentration (100 ng/ml) of TNF-α did not further increase microglial numbers or neuronal loss (data not shown). Adding soluble TNF receptor inhibitor to chelate extracellular TNF-α prevented the neuronal loss induced by TNF-α (Fig. 3B), indicating that loss was indeed due to TNF-α rather than some contaminant such as endotoxin.

Treatment with TNF-α did not increase the number of apoptotic or necrotic neurons in the neuronal glial cultures (Fig. 2A and B), suggesting that TNF-α was not directly toxic to the neurons. Therefore we tested whether eliminating microglia from the cultures by treating them with l-leucine methyl ester [23] would prevent TNF-α-induced neuronal loss. There was no neuronal death or loss in microglia-deficient cultures stimulated with TNF-α (Fig. 3A), indicating that TNF-α was not directly toxic to neurons, and the neuronal loss required the presence of microglia.

Microglia-induced loss of neurons may be mediated via variety of mechanisms: microglial release of glutamate to induce excitotoxicity [14], microglial neurotoxic mediators (such as reactive oxygen species) [19] that could cause neuronal apoptosis or detachment from the culture, or neuronal death executed by microglial phagocytosis. We have previously shown that microglial phagocytosis of stressed-but-viable neurons depends on neuronal exposure of phosphatidylserine [23,27]. Exposed phosphatidylserine is bound by the soluble opsonin milk fat globule EGF factor-8 (MFG-E8), which is in turn recognized by the microglial vitronectin receptor (VNR, an αvβ3 or αvβ1 integrin) [28,12,26]. To investigate whether microglia might contribute to TNF-α-induced neuronal loss by phagocytosing viable neurons, we targeted the phagocytic MFG-E8/VNR pathway. Inhibition of phagocytosis with specific VNR inhibitor cyclo(RGDfV) peptide prevented the loss of neurons induced by TNF-α (Fig. 4A), indicating that neuronal loss induced by TNF-α was dependent on VNR.

In order to test whether the VNR-specific opsonin MFG-E8 was required for TNF-α-induced neuronal loss, we isolated neuronal-glial cultures from the cerebella of Mfge8−/− mice [30]. The amount of necrotic neurons in these untreated cultures was higher (Fig. 4B). Addition of TNF-α induced no neuronal loss in these cultures lacking MFG-E8 (Fig. 4B). However, TNF-α-induced neuronal loss in Mfge8−/− culture was reconstituted by adding recombinant MFG-E8 protein together with TNF-α, whereas adding MFG-E8 alone had no effect (Fig. 4B). Thus MFG-E8 is required for TNF-α-induced neuronal loss.

The microglial P2Y6 receptor is required for microglial phagocytosis of neurons, as UDP released from damaged neurons induces formation of the phagocytic cup via activating microglial P2Y6.
receptors is phagocytic. The focal/local extracellular concentration of TNF-α and Mfge8 in inflammation (0.4 ng ml in rat experimental meningitis [4]). How does damage due to microglial activation. Here we demonstrated that at the time of their phagocytosis, i.e. the neurons died by phagocytosis, indicating that the neurons must have been viable and not necrotic or apoptotic neurons. Whereas we found that P2Y6 itself is not directly neurotoxic [1], it may contribute to neuronal damage due to microglial activation. In addition, it has been previously established that TNF-α induces neuronal loss that is mediated by microglial phagocytosis of otherwise viable neurons recognized via the MFG-E8/VNR pathway.

Importantly, inhibition of P2Y6 and lack of MFG-E8 prevented neuronal loss without statistically significant increases of the number of apoptotic or necrotic neurons in the culture treated with TNF-α (Fig. 4). If microglia had been phagocytosing dead or dying neurons then inhibition of their phagocytosis would have left necrotic or apoptotic neurons. Whereas we found that inhibition of phagocytosis prevented neuronal loss and left viable neurons, indicating that the neurons must have been viable at the time of their phagocytosis, i.e. the neurons died by phagocytosis. These data demonstrated that TNF-α induces neuronal loss that is mediated by microglial phagocytosis of otherwise viable neurons recognized via the MFG-E8/VNR pathway.

It has been previously established that TNF-α activates microglia and triggers NADPH oxidase activation [9,13]. Even though TNF-α itself is not directly neurotoxic [1], it may contribute to neuronal damage due to microglial activation. Here we demonstrated that TNF-α induced strong microglial proliferation (Fig. 1A), which is often used as an indicator of microglial activation. In addition, TNF-α increased the microglial phagocytic capacity (Fig. 1B), thus promoting microglial uptake of neurons. Under inflammatory conditions TNF-α may be released by either activated microglial cells or astrocytes. While it has been previously shown that microglia cells produce TNF-α when activated with such triggers as LPS [23] or amyloid β [13], whether TNF-α levels released by microglia are sufficient to induce phagocytosis has not yet been demonstrated. TNF-α concentrations that were used in this study to induce phagocytosis were significantly higher than those found in cerebrospinal fluid of patients with neurodegenerative diseases (0.8 ng/ml in Alzheimer’s disease; [32]) or animal models of brain inflammation (0.4 ng ml in rat experimental meningitis [4]). However, it is difficult to extrapolate from these studies to what might be focal/local extracellular concentration of TNF-α in diseased brain. The relevance of TNF-α for phagocytosis in vivo is yet to be determined.

Treatment with TNF-α alone was sufficient to induce microglia-dependent neuronal loss (Fig. 2A and B). Furthermore, this loss of neurons was prevented by the VNR inhibitor cyclo(RGDfV) peptide and in Mfge8−/− cultures, indicating that neuronal loss was mediated by the MFG-E8/VNR phagocytic pathway of microglial cells (Fig. 4A and B). Inhibition of P2Y6 also prevented neuronal loss indicating that this neuronal loss also required the UDP/P2Y6 phagocytic pathway (Fig. 4C). While pharmacological treatments used in this study may potentially have had side effects unrelated to phagocytosis (such as changes in microglial secretome), it is unlikely that such effects could have blocked neuronal death or promoted neuronal survival.

Altogether, the data presented here revealed that TNF-α induces neuronal death by microglial phagocytosis. Consequently the delayed neuronal loss that occurs in many pathologies accompanied by inflammation may be due to TNF-α-induced phagocytosis, and might be prevented by blocking TNF-α production or function, its receptor, microglial activation, MFG-E8, the vitronectin receptor or the P2Y6 receptor.

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