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

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1. Introduction

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine, expressed in many brain pathologies and associated with neuronal loss. We show here that addition of TNF-α 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-α induced loss of live neurons. Thus TNF-α appears to induce neuronal loss via microglial activation and phagocytosis of neurons, causing neuronal death by phagoptosis.

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A B S T R A C T

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine, expressed in many brain pathologies and associated with neuronal loss. We show here that addition of TNF-α 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-α induced loss of live neurons. Thus TNF-α appears to induce neuronal loss via microglial activation and phagocytosis of neurons, causing neuronal death by phagoptosis.

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

**Abbreviations:** cRGD, cyclic peptide arginine–glycine–aspartate–D-phenylalanine–valine; LPS, lipopolysaccharide; MFG-E8, milk fat globule EGF factor-8; MRS, MR52578 compound; TNF-α, tumour necrosis factor-α; VNR, vitronectin receptor

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2. Materials and methods

2.1. Reagents

Rat recombinant TNF-α (Sigma), lipopolysaccharide from *Salmonella typhimurium* (Sigma), Alexa Fluor 488 conjugate of isolecitin 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 × 10⁵ 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 isoecliotin B₄ (2 μg/ml) and bead number per cell was evaluated in >50 cells per condition.

2.4. Microglial phagocytosis of neurons

Glia 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. 10⁶ 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 isoecliotin 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
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 αvβ 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(RGDIV) 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 lower (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 also prevented neuronal loss mediated by the MFG-E8/VNR phagocytic pathway of microglial cells (Fig. 4A and B). Inhibition of P2Y<sub>6</sub> also prevented neuronal loss indicating that this neuronal loss also required the UDP/P2Y<sub>6</sub> 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 P2Y<sub>6</sub> receptor.

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