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Novel cell-cell signaling by microglial transmembrane TNF α with implications for neuropathic pain

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Abstract

Neuropathic pain is accompanied by neuroimmune activation in dorsal horn of spinal cord. We have observed that in animal models this activation is characterized by increased expression of transmembrane tumor necrosis factor α (mTNF α) without release of soluble (sTNF α). Here we report that the pain-related neurotransmitter peptide substance P (SP) increases expression of mTNF α without release of sTNF α from primary microglial cells. We modeled this interaction using an immortalized microglial cell line; exposure of these cells to SP also resulted in increased expression of mTNF α but without any increase in expression of the TNF-cleaving enzyme (TACE) and no release of sTNF α . In order to evaluate the biological function of uncleaved mTNF α , we transfected COS-7 cells with a mutant full length TNF α construct resistant to cleavage by TACE. Co-culture of COS-7 cells expressing the mutant TNF α with microglial cells led to microglial cell activation indicated by increased OX-42 immunoreactivity and release of macrophage chemoattractant peptide 1 (CCL2) by direct cell-cell contact. These results suggest a novel pathway through which release of SP by primary afferents activates microglial expression of mTNF α , establishing a feed-forward loop that may contribute to the establishment of chronic pain.

Introduction

Acute painful stimuli are transmitted from the periphery by firing of nociceptive neurons in the DRG. Trauma to or inflammation of peripheral nerves results in sustained increased electrical activity in the undamaged C fibers [12] that leads to transcriptional and post-translational changes in second order neurons in the dorsal horn of spinal cord that are characteristic of chronic pain [41,50]. Substantial evidence indicates that peripheral nerve damage or inflammation results in activation of microglia and astrocytes in the dorsal horn that plays an important role in the pathogenesis of neuropathic pain [19,36]. In the setting of peripheral nerve damage activated glia express proinflammatory cytokines including TNF α , interleukin (IL)-1 β , and IL-6 [2,47,49]. Administration of drugs that block the effects of these cytokines [3,40,45] or that block glial activation [24,34,46] can be used prevent or reverse neuropathic pain. Pain-related effects similar to those seen in neuropathic pain can be reproduced by activation of spinal cord glia [28] or by direct intrathecal administration of proinflammatory cytokines [7] in the absence of nerve injury.

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Several lines of evidence indicate that tumor necrosis factor- α (TNF α) plays a key role in the development of chronic pain. In response to peripheral nerve crush, in toxic neuropathy or after spinal cord injury the amount of TNF α in spinal microglia and astrocytes is increased [11,23,30]. In the chronic constriction injury model of peripheral neuropathic pain, neutralizing antibodies directed against TNF α or the p55 TNF receptor (TNFR) reduce thermal hyperalgesia and mechanical allodynia [42] and intrathecal administration of the recombinant p75 soluble TNFR (sTNFR) peptide (etanercept) prior to selective spinal nerve ligation reduces mechanical allodynia [40,44]. In contrast to the classic inflammatory response that is characterized by release of sTNF α , in states of neuropathic pain created by spinal hemisection or selective spinal nerve ligation the increase in spinal TNF α mRNA correlates with an increase in the full length membrane-spanning TNF α (mTNF α) protein [18,33] without a parallel release of soluble TNF α (sTNF α). By immunohistochemistry, we found that mTNF α was localized to restricted patches in the membranes of microglia in the spinal dorsal horn [18,33].

TNF α is a member of the superfamily of type II transmembrane proteins containing an intracellular N-terminus. The full-length mTNF α (26kD) is cleaved by the inducible TNF α converting enzyme (TACE) to release the diffusible peptide (sTNF α , 17kD) that is biologically active as self-assembling non-covalent bound trimers [20]. Signaling pathways mediated by mTNF α that are distinct from those activated by sTNF α have been described in mononuclear cells [35]. In order to elucidate the mechanisms underlying the increase in expression of mTNF α without release of sTNF α in pain, we examined regulation of mTNF α expression and cleavage in microglial cells in vitro; the results of these experiments define a novel feed-forward loop initiated by the pain-related neurotransmitter peptide substance P (SP), suggesting a mechanism that may be important in the transition from acute to chronic pain.

Materials and methods

Construction of GFP-W-TNF α plasmid

Rat spinal cord cDNA was used as template. The TNF α was amplified using the following PCR primers with BamHI site at 5' and Hind III at 3', TNF α -F: 5'-GAA TTC ACC ACC ATG GGC ACA GAA AGC ATG ATC C-3' and TNF α -R: 5'-AAG CTT TCA CAG AGC AAT GAC TCC AAA GTA G-3'. The TNF α PCR fragment was extracted by Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) once at room temperature, and 3 μ L of the gel-extracted TNF α PCR fragment ligated into PGEM-T vector using PGEM-T vector system I Kit (Promega, Madison, WI). The plasmid PGEM-T-w-TNF α was sequenced to ensure the correct reading-frame, then we subcloned the TNF α into BamH I and Hind III-cut pAcGFP1-C1 to produce GFP-w-TNF α plasmid.

Construction of PGEM-T- α crTNF α plasmid

PGEM-T- TNF α was used as template to amplify partial sequence of TNF α with a mutation in the TACE cleavage site. forward primer: 5'-GAA TTC ACC ACC ATG GGC ACA GAA AGC ATG ATC C-3' and reverse primer: 5'-CTC GAG TTT TGA GAA GAT GAT CTG ACT CTG AAG ATC TGG-3'. The PCR fragment ligated into BamH I and Xho I-cut PGEM-T-W-TNF α to get PGEM-T- α crTNF α . The plasmid was sequenced to ensure the correct reading-frame.

Construction of GFP- α crTNF α plasmid

PGEM-T- α crTNF α was cut by BamH I and Hind III. The fragment was extracted by Qiaquick Gel Extraction Kit (Qiagen) once at room temperature and cloned the fragment into BamH I and Hind III-cut pAcGFP1-C1 to produce GFP- α crTNF α plasmid.

In Vitro Protein Transcription/Translation

Using the TNT[®] SP6 High-Protein Expression System (Promega), PGEM-T-_{CR}TNF α was used as template to produce _{CR}TNF α protein. The protein product was analyzed by Western blot and enzyme-linked immunosorbent assay (ELISA).

Cell culture

Immortalized microglia (HAPI) cells derived from neonatal rat brain [5] were provided by J. R. Connor (Pennsylvania State University College of Medicine, Hershey, PA) and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Fisher, Pittsburgh, PA). HAPI cells were treated with LPS (1 μ g/ml, Sigma Aldrich, St. Louis, MO), SP or CGRP (Tocris Bioscience, Ellisville, MO) in DMEM supplemented with 1% fetal bovine serum for 6 h. COS-7 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For COS-7-HAPI cocultures, COS-7 cells were transfected with _{CR}TNF α plasmid or GFP plasmid for 6 h, followed by washing twice, then adding HAPI cells. The cocultures were maintained for 24 hr before fixation. Medium was collected for determination of TNF α and CCL2 by ELISA. The NK1 inhibitor L732138 (Sigma) and the PI3K inhibitor LY294002 (Promega) were added in individual experiments as described in the results.

Primary microglial cell culture

Primary microglial cells isolated from postnatal rat brain were obtained from ScienCell Research Laboratories (Carlsbad, CA) and grown in microglial medium supplemented with 10% fetal bovine serum (ScienCell Research Laboratories). Primary microglial cells were treated with SP (Tocris Bioscience, Ellisville, MO) in microglia medium with 1% fetal bovine serum for 6 h. Medium was collected for determination of TNF α by ELISA and TNF α in the cell pellet was detected by Western blot.

siRNA preparation and transfection

siGENOME ON-TARGET plus SMARTpool duplex directed against TACE were obtained from Dharmacon (Dharmacon, Chicago, IL). The sequences used for TACE were:

- sequence 1 sense, 5'-GGACGUAAUUGAGCGGUUUUU-3';
- sequence 1 antisense, 5'-P.AAACCGCUCAAUUACGUCCUU-3';
- sequence 2 sense, 5'-GUAUAAGUCUGAAGAUUAUCUU-3';
- sequence 2 antisense, 5'-P.GAUAUCUUCAGACUUAUACUU-3';
- sequence 3 sense, 5'-CGUCAGAGCCGAGUUGAUUAUU-3';
- sequence 3 antisense, 5'-P.UAUC AACUCGGCUCUGACGUU-3';
- sequence 4 sense, 5'-UAUGGGAACUCUUGGAUUAUU-3';
- sequence 4 antisense, 5'-P.UAAUCCAAGAGUCCCAUUAUU-3';

ON-TARGET plus sicontrol non-targeting pool siRNA (Dharmacon) was used as control. We used Dharmafect siRNA transfection reagent 4 (Dharmacon) for siRNA transfection. In one tube, 10 μ l of siRNA was mixed in 240 μ l antibiotic-free cultured medium, while a second tube contained 5 μ l Dharmafect siRNA transfection reagent 4 (Dharmacon) and 245 μ l antibiotic-free cultured medium and each was incubated at room temperature for 5 min before the two solutions were combined and allowed to incubate for a further 20 min at room temperature for complex formation. 500 μ l of the entire mixture/well was added in a 6-well plate with 1.5 ml antibiotic free complete medium. HAPI cells were incubated for a further 48 h before cell lysis for RT-PCR.

Enzyme-Linked ImmunoSorbent Assay

The amount of TNF α and CCL2 released from the HAPI cells were determined using Quantikine ELISA kit for TNF α and CCL2 (R&D Systems, Minneapolis, MN).

Western blot

Proteins from cytosolic extracts were separated on 12% SDS-PAGE gels and then transferred onto a polyvinylidene difluoride membrane (Millipore, Medford, MA). Immunoblots were probed with primary antibody to anti-TNF α (Millipore, Billerica, MA), anti-AKT, anti-pAKT, anti-TNFR1, anti-TNFR2 and anti- NK1 (Santa Cruz Biotechnology, Santa Cruz, CA); and anti- β -actin (Sigma, St. Louis, MO) then incubated with HRP-conjugated secondary antibody, followed by enhanced chemiluminescence detection (Amersham Biosciences, Arlington Heights, IL). Quantification of Western blots was done from chemiluminescence values obtained (BioRad ChemiDoc). A ratio of the intensity of the band of interest to the appropriate internal control was determination of; the statistical significance of the difference between control and experimental groups determined using one-way ANOVA (SPSS 10 software). Each experiment was repeated 4 times. Data presented as mean $\pm$ SEM.

Immunocytochemistry

Cells were fixed, blocked, and probed with anti-TNF α (1:1000; Millipore) or OX42 (1:50, Millipore). The secondary antibodies utilized were fluorescent anti-rabbit IgG Alexa Fluor 594 or anti-mouse IgG Alexa Fluor 594 (1:2,000; Molecular Probes, Eugene, OR). HAPI cell nuclei were detected by Hoechst staining. Images were captured using a Zeiss LSM 510 META confocal microscope, a 40X objective with a numerical aperture of 1.4, an LSM 510 camera (Zeiss) and AIM acquisition software (Zeiss) at room temperature with water immersion.

Semiquantitative RT-PCR analysis

Total RNA was isolated from cells via TRIzol (Invitrogen). cDNA prepared from mRNA isolated from HAPI cells was amplified using following primer sets: β -actin-forward (5'-CAG TTC GCC ATG GAT GAC GAT ATC-3') and β -actin-reverse (5'-CAC GCT CGG TCA GGA TCT TCA TG-3') for β -actin, TNF α -forward (5'-TCC GAG ATG TGG AAC TGG CAG AG-3') and TNF α -reverse (5'-GAG CAA TGA CTC CAA AGT AGA CCT GC-3') for TNF α . The levels of TACE was determined by its specific primers: TACE-forward (5'-AGT GTG AAG TGG CAG GAC TTC-3') and TACE-reverse (5'-TCA CAC TCT TCT CCT TCG TCC-3') for TACE. All reactions involved initial denaturation at 94°C for 5 min followed by 28 cycles for β -actin and TNF α , 30 cycles for TACE (94°C for 30 sec, 68°C for 3 min) and 1 cycle 68°C for 8 min using a GeneAmp PCR 2700 (Applied Biosystems, Foster City, CA). Each *in vitro* experiment was repeated 4 times. Data presented as mean $\pm$ SEM.

Results

Substance P (SP) increases expression of mTNF α

In order to investigate the role of neuropeptides commonly released in the dorsal horn by primary nociceptor afferents on the activation of microglia, we exposed primary rat microglial cells isolated from post-natal rat brain to SP (1 μ M for 6 h). Exposure to SP resulted in a significant and substantial increase in mTNF α without detectable release of sTNF α from the cells (Fig. 1A,B). In order to investigate the mechanism in detail, we moved to an immortalized microglial cell line. Immortalized microglial cells *in vitro* were exposed to increasing concentrations of SP or calcitonin gene related peptide (CGRP). SP treatment resulted in a dose dependent increase in mTNF α protein and mRNA (Fig. 2A,C). Similar concentrations of SP produced no change in TACE mRNA (Fig. 2C), and as a result there was no increase in the amount of sTNF α released into the medium by the treated cells (Fig. 2E). These biological

influences of SP on TNF α expression and protein processing were distinct from those of CGRP which did not induce expression of TNF α or TACE mRNA and protein in microglial cells (Fig. 2B,D,E).

SP induces expression of mTNF α through activation of PI3K-AKT

In order to elucidate the mechanism by which SP regulates TNF α expression, we studied the presence of its receptor in immortalized microglial cells. Two naturally occurring variants of neurokinin 1 (NK1) receptor have been described; a full length receptor and a truncated form lacking a 96 amino acid sequence in the intracellular C-terminus. The full length (53 kD) NK1 receptor was detected by Western blot using an antibody recognizing the C- terminal intracellular domain in primary microglia and in the immortalized microglial cells (data not shown). Treatment with the NK1 inhibitor L732138 (0.8 μ M; Sigma-Aldrich) for 30 minutes prior to exposure to 1 μ M SP for 6 hrs prevented the increase in mTNF α that would otherwise result from exposure to SP (Fig. 2F). Activation of NK1 receptor by SP resulted in the phosphorylation of AKT (Fig. 2G); inhibition of PI3K activity by 10 μ M LY294002 blocked the phosphorylation of AKT and prevented the increase in mTNF α caused by exposure to SP (Fig. 2H).

Immortalized microglial cells activated by LPS release TNF α and CCL2

Immortalized rat microglial cells retain many of the characteristics of primary microglia cells isolated from the central nervous system [5], and in the conditions used in these experiments are activated by inflammatory stimuli in a manner similar to primary dissociated microglial cells. We exposed the immortalized microglial cells to 1 μ g/ml LPS for 6 hr. Exposure to LPS resulted in an increase in TNF α mRNA (Fig. 3A), and an increase in full-length mTNF α protein with a MW 26 kD (Fig. 3B). Simultaneous induction of TACE expression by LPS was confirmed by mRNA determination (Fig. 3A). This led to the release of a substantial amount of sTNF α into the medium (Fig. 3C). The LPS-activated immortalized microglial cells also released CCL2 (Fig 3D).

Inhibition of TACE results in failure to release sTNF α while increasing levels of mTNF α

Soluble TNF α is a product of cleavage by the metalloproteinase TACE, also known as ADAM 17, from the full length TNF α . Because TACE-mediated cleavage is required to release sTNF α after LPS stimulation, we anticipated that the difference in TNF α release between LPS-treated and SP-treated cells might be due to the divergent activation of TACE expression by these two agents. We used two methods to explicitly test the role of TACE in the release of sTNF α from LPS-stimulated microglial cells. Pretreatment with the TACE inhibitor TAPI-2 for 30 min prior to exposure to LPS prevented the release of sTNF α caused by LPS stimulation even though mTNF α mRNA and protein were increased by exposure LPS (Fig. 4A,B,C). Treatment with TAPI-2 had no effect on the amount of TACE (Fig. 4C). In a second series of experiments we constructed an siRNA to block expression of TACE mRNA in microglial cells. Treatment of the cells with TACE siRNA but not with scrambled siRNA reduced TACE mRNA in transfected cells (Fig 5A), and exposure of the TACE siRNA-transfected microglial cells to LPS resulted in the expected increase in mTNF α mRNA (Fig. 5B) and protein (Fig. 5C) without any increase in TACE mRNA (Fig. 5B); in fact, TACE mRNA was reduced below control levels in the siRNA-treated cells. Microglial cells transfected with the TACE siRNA and exposed to LPS showed a marked reduction in release of sTNF α compared to untransfected cells or cells transfected with a scrambled siRNA (Fig. 5D). Under both conditions of reduced TACE activity there was additional accumulation of mTNF α in the cells beyond that induced by LPS reflecting the lack of processing of the transmembrane protein.

mTNF α -expressing cells activate microglia through cell-cell contact to express OX42 and release CCL2

In order to critically test the role of mTNF α independent of sTNF α we constructed a cleavage-resistant ($_{CR}$) TNF α -expressing plasmid containing the rat full length TNF α sequence with the substitution of the four amino acids corresponding to the TACE cleavage site (TLTL) by FSAH, fused with the GFP reporter gene (GFP- $_{CR}$ TNF α , Fig 6A). A similar plasmid expressing GFP was used as control. Transfection of COS-7 cells with the $_{CR}$ TNF α plasmid resulted in expression of the GFP fusion cleavage-resistant mTNF α protein in the cell membrane with no detectable sTNF α in the medium (Fig. 6B,C). By immunocytochemical staining mTNF α was found localized along the plasma membrane of COS-7 cells transfected with the $_{CR}$ TNF α construct (Fig. 6D).

Coculture of microglial cells with COS-7 cells transiently transfected to overexpress GFP- $_{CR}$ TNF α resulted in activation of the microglial cells assessed by increased expression of OX42 (Fig. 7A), and resulted in the release of substantial amounts of CCL2 (Fig. 7B). We confirmed that the effect of coculture was not related to factors released into the medium of the transfected COS-7 cells, because exposure of microglial cells to culture medium from the control and transfected COS-7 cells ("conditioned medium") did not stimulate CCL2 release (Fig. 7C). These results indicate that microglial activation depends on cell-cell contact through mTNF α . Although in immune cells mTNF α preferentially activates and signals through TNF α RII, mTNF α can also bind TNF α RI [13,16]. Both primary microglia and the immortalized microglial cells express TNF α receptors RI and RII detected by Western blot (data not shown). Interestingly, overexpression of $_{CR}$ TNF α in either COS-7 (Fig. 7A) or microglial cells (Fig. 7D) resulted in the elaboration of long processes by these cells.

Recombinant mTNF α protein increases of mTNF α expression but not TACE and results in CCL2 release

In order to confirm that activation of microglial cells in the coculture experiments was caused by contact with mTNF α -expressing cells and subsequent activation of the TNF α receptor, full length $_{CR}$ TNF α was produced by a SP6 high-protein expression system and purified recombinant $_{CR}$ TNF α was added to immortalized microglial cells in vitro. Treatment with the recombinant $_{CR}$ TNF α protein (Fig. 8A; 50 ng/ml) for 6 hrs resulted in an increase in mTNF α mRNA and protein but no change in TACE (Fig. 8B-D) and an increase in OX42 and CCL2 release into the culture media (Fig. 8E,F).

Discussion

TNF α sits at a crucial intersection in the activation of many immune pathways, and several different lines of evidence implicate spinal expression of TNF α in central and peripheral neuropathic pain. Immunocytochemical staining for spinal TNF α is increased in rats with chronic constriction injury [8] or selective L5 spinal nerve ligation [51], and transgenic mice engineered to overexpress TNF α in astrocytes show significantly enhanced mechanical allodynia after selective L5 spinal nerve ligation [9]. The critical role of TNF α is supported by animal studies demonstrating that intrathecal administration of the soluble TNF α RII fragment (etanercept) reduces mechanical allodynia in the spinal nerve ligation model of neuropathic pain [44] and gene transfer of a soluble fragment of TNF α RI to DRG from skin delivery using an HSV-based vector reduces mechanical allodynia in the spinal nerve ligation and spinal cord hemisection models of peripheral and central neuropathic pain [18,33]. TNF α RNA is increased in the chronic constriction model of neuropathic pain in the rat [25] and administration of minocycline, an inhibitor of microglial activation, reduces pain behaviors coincident with reduced expression of TNF α mRNA [24].

While there are many studies that demonstrate increased TNF α mRNA or TNF α protein by immunocytochemistry, none of those reports demonstrate release of sTNF α in the spinal cord in models of persistent pain. In previous studies of below level pain in the spinal hemisection model of spinal cord injury [33], in spinal nerve ligation [18] and in inflammatory pain caused by injection of formalin into the paw [53], we have found by Western blot that there is an increase in full-length mTNF α without detectable sTNF α in the spinal cord. TNF α is synthesized as a preprotein (mTNF α) that when cleaved by TACE [29] releases the diffusible peptide sTNF α [14]. The observations that knockdown of TACE, inhibition of TACE with TAPI-2, and mutation of the TACE cleavage site all block release of sTNF α from activated microglial cells support the interpretation that TACE plays a key role in the release of sTNF α from microglia.

Neuropeptides, SP and CGRP, co-localize in C fiber sensory afferents in the dorsal horn and release of substance P leading to activation of the NK1 receptor [4,21]. We found that SP activates the NK1 receptor in microglial cells to increase expression of mTNF α mRNA and protein without increasing the expression of TACE mRNA, resulting in cells that express mTNF α in the membrane without releasing sTNF α . Both microglia and astrocytes have previously reported to express the NK1 receptor [17,31], and we confirmed expression of the full-length NK1 receptor message and protein, but we did not observe expression of the truncated form of NK1 receptor protein, consistent with previous reports regarding receptor expression in spinal cord dorsal horn [1].

In the immune system full-length mTNF α plays a distinct role, mediating the activation of monocytes by contact with T cells [32,35]. Using COS-7 cells transfected with a mutated TNF α construct that cannot be cleaved by TACE cocultured with microglia, we found that microglia can similarly be activated by contact with cells expressing mTNF α . The specificity of this interaction was confirmed by: 1) the absence of detectable sTNF α in the culture medium; 2) the inability of the supernatant to elicit the same; and 3) activation of microglia by direct application of $\text{crTNF}\alpha$. Activation of microglia by coculture with $\text{crTNF}\alpha$ -expressing COS-7 cells were demonstrated by increased expression of OX42 and by release of CCL2. While the former is a marker of unknown significance, the release of CCL2 is potentially important as the release of CCL2 by spinal cord astrocytes has been shown to contribute to neuropathic pain facilitation by enhancing excitatory synaptic transmission in the spinal cord [15].

The data from the current experiments should not be interpreted to imply that TNF α acts solely at the spinal level. Soluble TNF α induces hyperalgesia in normal rats [43] and increases allodynia and spontaneous pain behavior in nerve injured rats [38], an effect that correlates with increased expression of p38 MAP kinase in the DRG [40]. Application of soluble TNF α to the acutely dissociated neurons from either normal DRG or chronically compressed DRG increases the sensitivity of those cells to depolarization [26], enhances tetrodotoxin resistant sodium currents [22] and increases ectopic activity in sensory neurons [39]. Local perfusion of DRG with sTNF α induces cutaneous hyperalgesia [52]. Nonetheless, the results of the current experiments suggest a potential mechanism for a spinal feed-forward loop that could contribute to the establishment of a state of chronic pain. Continued activation of peripheral nociceptors resulting in release of SP in the spinal cord might activate microglia to express mTNF α , without any increase in TACE. Through cell-cell interactions the activated microglia expressing mTNF α would activate neighboring microglia, leading to the release of CCL2 among other substances to potentiate excitatory synaptic transmission in the spinal cord.

The mechanisms underlying chronic pain are poorly understood. Peripheral nerve injury results in a barrage of electrical activity that corresponds to acute pain, but over time adaptive changes in the spinal cord lead to a state of sensitization with both spontaneous pain and hypersensitivity in which normal activation of peripheral sensory neurons is perceived as painful [6]. There is

abundant evidence to suggest that one important element involved in this transition is neuro-immune activation of microglia and astrocytes the spinal cord [10,27,37,48]. While the full details of these interactions remain to be established, the results of the current investigation provide an important insight into the pathogenesis of chronic pain and another target that may be addressed in our efforts to interrupt chronic pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Exposure of primary microglial cells to SP (1 μ M for 6 h) resulted in an increase in TNF α protein (A) but no detectable release of sTNF α from the treated cells (B).

Figure 2.

Immortalized microglial cells were treated with SP or CGRP for 6 h. Exposure to SP (A,C) but not CGRP (B,D) resulted in increased TNFα mRNA and protein; neither exposure to SP nor treatment with CGRP increased TACE mRNA (A,B) or led to the release of sTNFα from the treated cells (E). (F) Exposure to 1 μM SP in the presence of the NK1 antagonist L732,138 (0.8 μM) resulted in no increase in mTNFα protein. (G) Addition of 1 μM SP *in vitro* induced phosphorylation of AKT (S473). (H) The effect of exposure to SP on mTNFα levels was blocked by 10 μM LY294002.

Figure 3.

Exposure of immortalized microglial cells to LPS (1 µg/ml for 6 h) resulted in an increase in TNFα and TACE mRNA (A) and mTNFα protein (B) in the cells, with release of sTNFα into the culture medium determined by ELISA (C). Exposure to LPS also resulted in release of CCL2 from the treated HAPI cells (D).

Figure 4.

Immortalized microglial cells were treated with TAPI-2 for 30 min, followed by exposure to LPS (1 μ g/ml) for 6 h. HAPI cells exposed to LPS showed an increase in mTNF α , an effect that was augmented in cells pretreated with TAPI-2 (A). The release of sTNF α induced by LPS was prevented by TAPI-2 treatment (B). The effect of LPS on expression of TNF α and TACE mRNA was not blocked by exposure to TAPI-2 (C).

Figure 5.

Immortalized microglial cells treated with siRNA-TACE or siRNA-con for 24 h were then exposed to LPS (1 μ g/ml) for 6 h. siRNA-TACE significantly reduced TACE mRNA but not TNF α mRNA (A,B). siRNA-TACE treated cells exposed to LPS showed a greater amount of mTNF α protein than cells treated with siRNA-con exposed to LPS (C). Release of sTNF α induced by exposure to LPS was inhibited by pretreatment with siRNA-TACE (D).

Figure 6.

In order to evaluate the potential role of mTNF α in microglial activation, we constructed a plasmid to express a GFP- $\text{crTNF}\alpha$ fusion protein (GFP- $\text{crTNF}\alpha$) with the substitution of 4 amino acids (TLTL for SFAH) (A). We confirmed by Western blot that COS-7 cells transiently transfected with GFP- $\text{crTNF}\alpha$ express $\text{crTNF}\alpha$ fusion protein (B). Cells transfected with a plasmid expressing a GFP- $\text{crTNF}\alpha$ fusion protein (GFP- $\text{crTNF}\alpha$) released very little sTNF α into the culture medium (ELISA, C). $\text{crTNF}\alpha$ in the transfected cells localized to the cell membrane by immunocytochemistry (D).

Figure 7.

(A) COS-7 cells transiently transfected with the GFP-crTNF α plasmid and co-cultured with microglial cells resulted in increased expression of OX42 by the microglia. Bar = 20 μ m. (B) Coculture of immortalized microglial cells with COS-7 cells transiently transfected with GFP-crTNF α (6 hrs prior to coculture; 24 hrs of coculture) resulted in release of CCL2. (C) There was no release of CCL2 from microglial cells exposed to the supernatant from GFP-crTNF α transfected COS-7 cells for 24 h. (D) Immortalized microglial cells were transfected with GFP or GFP-crTNF α for 24 hr. Cells transfected with GFP-crTNF α developed a stellate morphology characterized by extensive processes not seen in untransfected or GFP-transfected cells. Bar = 10 μ m.

Figure 8.

The identity of $crTNF\alpha$ recombinant protein was confirmed by Western blot (A). Exposure of immortalized microglial cells to $crTNF\alpha$ protein (50 ng/ml) for 6 h increased the level of $TNF\alpha$ mRNA (B) and protein (C) in the cells, but had no effect on the level of $TACE$ mRNA (D). (E) Exposure of microglial cells to $crTNF\alpha$ recombinant protein (50 ng/ml for 6 h) stimulated release of CCL2 from the treated cells. (F) Immortalized microglial cells exposed to $crTNF\alpha$ recombinant protein (50 ng/ml) for 6 h showed increased expression of OX42.

Figure 9. Schematic summary. (A) Resting microglial cells express low levels of mTNF α . (B) Activation of the TLR4 receptor by an inflammatory stimulus such as LPS results in increased expression of both mTNF α (purple arrows) and TACE (blue arrows) with cleavage of mTNF α by TACE resulting in the release of soluble fragments of TNF α . (C) Activation of the NK1 receptor by SP results in an increase in mTNF α , but no increase in TACE and consequently no release of sTNF α . (D) Microglia activated by SP after nerve injury express mTNF α . mTNF α -expressing microglia activate adjacent microglia through cell-cell contact mediated by mTNF α , and release of CCL2 induced by mTNF α .