Macrophage CD40 Signaling Involving TRAF6 Releases Neurotoxic Proteins

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ABSTRACT

HIV- associated dementia (HAD) is a common central nervous disorder that occurs in the late stage of HIV-1 infection. The mechanisms of how HIV-1 induces HAD are poorly understood. Direct injury to neurons might result from viral proteins, while indirect injury to neurons could occur through toxins released by microglia, macrophages, and astrocytes. In this project we show that TRAF6 protein is a mediator of the CD40 signaling pathway in macrophages, and that products released by macrophages upon stimulation by CD40L are neurotoxic. Fractionation of supernatants from CD40L-stimulated macrophages showed that the neurotoxic factor may be between 3-30 kD, and further tests demonstrated that the factor might be a protein or protein-associated.

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BACKGROUND

HIV-Associated Dementia (HAD)

HIV-associated dementia (HAD) is a common central nervous system disorder that occurs in the late stage of HIV infection. Some of the symptoms associated with HAD are impaired short-term memory, reduced concentration, and leg weakness. In more severe cases, this disease leaves patients in a vegetative state. In the early 1990s, before the use of highly active antiretroviral therapy (HAART), about 20-30% of people infected with HIV developed this disease (Gonzalez-Scarano and Martin-Garcia, 2005). Since the use of HAART, patients develop a milder form of CNS dysfunction called minor cognitive motor disorder (MCMD). This may be due to the low-level viral replication that occurs with the use of HAART that might lead to slower neurodegeneration. MCMD is also more prevalent possibly due to the longer lifespan of the patients (Neuenburg *et al*, 2002). Moreover, antiretroviral drugs are poorly delivered to the brain and this causes treatment problems, as the virus uses the brain as a reservoir (Nottet *et al*, 1999).

Briefly, the CNS is separated from the rest of the body by the blood brain barrier (BBB), a selectively permeable cellular system composed of vascular endothelial cells and perivascular astrocytes. The BBB controls what is passed from the blood to the CNS. HIV enters the CNS early after systemic infection (An *et al*, 1999), but the early infection in the CNS may not be enough to initiate neurodegeneration. The majority of HIV enters the brain through HIV-infected monocytes that cross the BBB to replenish the population of perivascular macrophages (Gonzalez-Scarano and Martin-Garcia, 2005). Advanced HIV infection in the periphery could be the cause of the events that lead to neurodegeneration (Kaul *et al*, 2001).

Mechanisms of the Induction of HAD

Brain macrophages and microglia are the main cells infected by HIV-1 in the central nervous system. HIV enters these cells through a receptor and a co-receptor. The receptor for HIV-1 is a CD4 antigen, which is crucial for infection. The co-receptors are chemokine receptors, such as CCR5, CCR3 or CXCR4. CCR5 is the most important co-receptor for HIV infection in the brain. The infected macrophages and microglia are likely to go on to initiate the neurodegeneration, the hallmark of HAD (Gonzalez-Scarano and Martin-Garcia, 2005). Macrophages are the most frequently infected cells in HAD. Neurons, however, are not susceptible to HIV because they lack CD4, but do have chemokine receptors that function as co-receptors for HIV.

There are two theories explaining the mechanism of neuronal injury during HAD. The direct injury theory states that viral products cause neuronal apoptosis by direct interaction with neurons without any role of intermediate non-neuronal cells (Gonzalez-Scarano and Martin-Garcia, 2005). HIV proteins such as gp120 (glycoprotein 120), Tat (transcriptional transactivator), and Vpr (viral protein R) that are released by infected macrophages have been shown to be neurotoxic *in vitro* (Chang *et al*, 1997). Moreover, virions may cause neuronal damage directly by binding to CXCR4 on the neuronal surface, thereby making it feasible for viral envelope glycoprotein to regulate apoptosis in adjacent neurons (Kaul *et al*, 2001).

A theory of HIV inducing neurodegeneration indirectly, states that infected microglia and macrophages produce quinolinic and arachidonic acid, platelet activating factor (PAF), nitric oxide, and cytokines/chemokines, such as tumor-necrosis factor (TNF) which have neurotoxic effects and promote the proliferation and activation of other cells (Fabrizio *et al*, 1999). Mainly,

these factors are able to activate other monocytes and macrophages as well as astrocytes. Released chemokines may also initiate transendothelial migration of macrophages into the brain (Nottet *et al*, 1999). For instance, when stimulated, macrophages induce macrophage inhibitory protein 1α (MIP-1α), MIP-1β, and macrophage chemotractant protein 1 (MCP-1) production. These chemokines may promote macrophage recruitment into the brain (Kaul *et al*, 2001). This might explain how HIV is able to cross the BBB through infected, activated monocytes that differentiate into perivascular macrophages when in the brain and then release HIV virion. Progress has been made in the studies of these soluble regulators, but it is still unclear how they are induced, how the viral products function in this process, and the signaling pathways that lead to their release.

Nef-Gene Product Expression is Important for Chemokine Induction

Nef (Negative factor) is a viral protein that changes the cellular environment of the infected cell to help promote viral replication (Green and Peterlin, 2002). For example, it interacts with host cell signal transduction proteins to regulate the viability of infected T cells and to destroy uninfected T cells. It promotes the down-regulation of surface CD4 and MHC I expression, and inhibits apoptosis of the infected cell. Nef is also responsible for promoting the release and production of infectious virions.

Studies have shown that the expression of Nef induced production of chemokines MIP-1α and MIP- 1β in primary macrophages (Swingler *et al*, 1999). In addition, increased virus replication in macrophages was consistent with an increase in the production of MIP-1α and MIP-1β, but not RANTES, tumor necrosis factor α (TNF-α), or interleukin (IL) -1β. It has also been shown that supernatants from macrophages expressing HIV-1 Nef have chemotactic

activity for T cells, and when introduced to resting T cells (not permissive to productive infection) permitted a productive HIV-1 infection (Swingler *et al*, 1999).

CD40 Signaling Pathway

CD40 is a member of the tumor necrosis factor receptor (TNFR) family. It is found on B cells, and most importantly on antigen producing cells (APCs) such as monocytes/macrophages, and dendritic cells. Additionally, it can be found on nonleukocyte, nonprofessional APCs such as endothelial cells, vascular smooth muscle cells, and fibroblasts (Mukundan *et al*, 2005). Upon ligation of CD40 on these cells, pro-inflammatory genes are expressed. CD40 signaling in monocytes and macrophages induces transient cytokine and chemokine response as well as the induction of co-stimulatory molecules such as ICAM-1 (Mukundan *et al*, 2005).

The ability for CD40 to signal the release of pro-inflammatory molecules is dependent on Src family tyrosine kinase activity. Members of the TNFR-associated factor (TRAF) family aid in this pathway. It has previously been shown that CD40 containing a disrupted TRAF6 binding site did not induce macrophages to produce inflammatory products upon ligation. Moreover, the mutant could not activate IκB kinase (IKK) or Nuclear Factor kappa B (NF-kB) (Mukundan, *et al, 2005*).

The CD40 ligand (CD40L) is needed for cell to cell interaction between T cells and other cells of the immune system. Ligation of CD40 regulates pathways responsible for cancer, autoimmune diseases and inflammation. It was shown that direct cell to cell contact between CD40L expressing 293T cells and macrophages induced them to produce MIP-1α, MIP-1β, MCP-1, and RANTES (Kornbluth *et al*, 1998).

CD40L stimulation of macrophages has been shown to induce transient chemokine/cytokine response which is similar to the chemokine/cytokine induction by HIV Nef (Swingler *et al*, 1999). For example, cytokines IL-6 and TARC were induced from macrophages upon expression of Nef or upon stimulation by CD40L. This indicates that the HIV Nef protein may be mediating chemokine and cytokine induction through activation of a CD40 signal transduction pathway. There is also evidence that the amyloid-beta protein, associated with Alzheimer's disease, up-regulates the expression of CD40 on microglia that, upon stimulation by CD40L, produce neuronotoxic factors (Tan *et al*, 1999). Therefore, Nef might trigger the release of neurotoxins from brain microglial cells.

PROJECT PURPOSE

Understanding the mechanisms associated with HIV-associated dementia (HAD) is the first step toward finding HIV treatments that can be delivered to the brain effectively. The aim of this project is to study the mechanisms involved in the induction of neuronotoxicity of HIV-1, and to identify what the toxin(s) could be. More specifically, based on the parallels between the effects of Nef expression in macrophages and macrophage stimulation by CD40L, the purpose of this MQP was to determine whether neurotoxins are produced after CD40 ligation in primary macrophages, to study the mechanisms of the signaling pathway in macrophages that induce the production of neurotoxins, and to identify the neurotoxic factor.

MATERIALS AND METHODS

Differentiation of Human NT2 Cells Into Neurons

NTera-2 (NT2) cells are a human teratocarcinoma cell line that can differentiate into neurons. The neurons used in this project were differentiated using two different methods.

The first method is based on culturing cells as floating spheres on a non-adherent surface (Paquet-Durand *et al*, 2003). The cells were seeded at a concentration of 10×10^6 per 100 x 15mm bacteriological petri dish in DMEM/F12 supplied with 10% fetal bovine serum (FBS). On the second day, retinoic acid (RA) was added to the medium at a final concentration of 10 µM. The medium was changed every 2-3 days. The cell suspension was centrifuged at 200xg at room temperature for 5 minutes. The cells were resuspended in 10 mL of fresh DMEM/F12 supplied with 10% FBS and RA, and plated in new petri dishes. During this period, cells formed a cluster of spherical shapes. After 7-8 days, spheres from one petri dish were seeded onto a T-75 cell culture flask and cultured for an additional 7 days. During this time, the spheres adhered to the flask and formed a very dense monolayer. The cells were trypsinized (trypsin-EDTA) and seeded in a concentration of 90-120 x 10^6 in T-175 cell culture flasks. After 1-2 days, the cells were treated with inhibitor medium (Ara C 1 µM, FudR 10 µM, Urd in 10 µM, DMEM/F12 supplemented with 5% FBS). During this culture period, the medium was changed every 2-3 days. After 7-10 days, neurons were selectively trypsinized. The neurons were plated in 96 well plates coated with poly-D-lysine and Matrigel at a density of 25,000 cells per well for neurotoxicity experiments. The harvested neurons were frozen at 5 x 10^6 cells per vial in 95% FBS in 5% DMSO.

The second method for inducing neuronal differentiation is the layer culture method (Andrews *et al* 1999), where the cells are grown in a monolayer in a tissue culture flask. This method differs from the first in that the cells are cultured in RA longer (6-8 weeks). The only replating that is required is performed a day before the differentiated cells are treated with mitotic inhibitors (7-10 days). The neurons are then harvested as previously stated. Cells differentiated using the second method are shown in Figure-1.
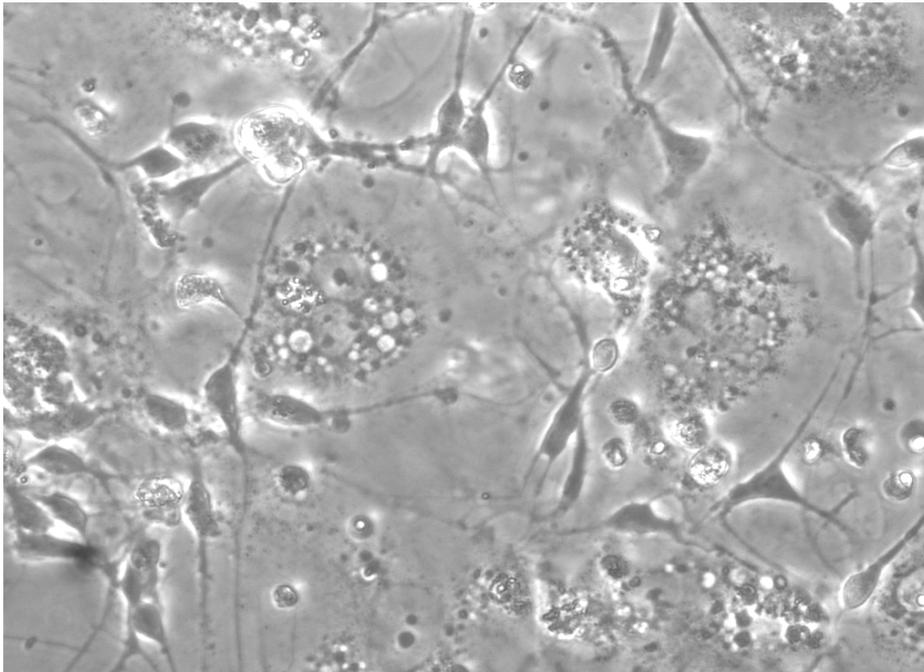
Figure 1. Neurons differentiated from NTera-2 cells just before harvesting.

Neurotoxicity Assay on NT2 Cells

Differentiated NT2 neurons seeded on 96 well plates were used 5-7 days after plating. Media was aspirated from each well and 60 μL of macrophage culture supernatants were added in triplicates. Serum-free DMEM or DMEM supplemented with 5% FBS were used as background controls. Staurosporin (1, 0.1, 0.01 μM) was used as a positive control for cell death. After 3, 24, 48, and 72 hours, caspase 3/7 activity was detected by the Caspase-Glo 3/7 Assay (Promega). Caspase activity was measured by a luminometer 1 hour after addition of the

substrate. Caspase activity is expressed in RLU (relative luminescence units). The higher the value of RLU, the more apoptosis is occurring.

Differentiation of Macrophages From Monocytes

Monocytes were obtained by leukapheresis from normal donors seronegative for HIV and hepatitis B. They were further separated by countercurrent centrifugal elutriation. The separated monocytes were cultured in medium containing MCSF (R&D Systems) for 2 days and for another 5 days in medium lacking MCSF. The monocyte derived macrophages (MDMs) were then used for silencing or stimulation experiments.

siRNA Transfection of MDMs

Monocyte-derived macrophages (MDMs) in a 24 well plate were transfected once a day for 2 days with a total of 15 pmol of a combination of three different TRAF6-specific small interfering RNAs (siRNA) (target DNA sequences: T6A, AAGGGATGCAGGTCACAAATG; T6-397, AAGCACATTGTGAGTTTGCTC; T6-399, AACCATAATCCTTGGAAAACT) or a non-specific control, scrambled siRNA (target DNA sequence: AACAGTCGCGTTTGCGACTGG) in OptiMEM (Invitrogen) and Lipofectamine (Invitrogen). The transfection mix was replaced after 3 hours by a mix of fresh and conditioned macrophage medium (DMEM supplemented with 10% FBS collected from MDMs before addition of the transfection mix) in a 1:1 ratio.

TRAF6-Binding Peptide (TRAF6BP)

The peptide corresponding to the TRAF6 binding domain of CD40, was made as previously described (Mukundan, *et al,* 2005), and was obtained from J. Suttles. The sequence

of the TRAF6-BP is as follows: NH$_2$ – <u>AAVAKKOAVKKAKKAPA</u>OHOKQEPQEI-DFPDD-OH, the underlined portion being the Kaposi fibroblast growth factor signal sequence (Mukundan, *et al* 2005).

MDMs were treated with TRAF6BP at concentrations 12.5, 50, 100, 200, and 800 µM for 3 hours at 37°C.

Stimulation of MDMs with CD40 Ligand (CD40L)

Macrophages were stimulated with CD40L. The CD40L was diluted in DMEM supplemented with 5% FBS to a concentration of 10 µg/ml and 1 µg/ml of the enhancer was added. The mix was incubated at room temperature for 30 minutes. The cells were washed once with serum free DMEM and 125 µL of CD40L mix was added to every well and incubated at 37°C. After 30 minutes, the mix was aspirated, and the cells were washed twice with DMEM-serum free. Then, 1.5 mL of DMEM supplemented with 5% FBS was added to each well. The macrophage culture supernatants were collected 16 hours after CD40L stimulation.

Trypsin Treatment of MDM Supernatants

0.25% Trypsin-EDTA Solution (Sigma) was added to supernatants from MDMs stimulated and not stimulated with CD40L in a 1:1 ratio, and shaken at 37°C for 3 hours. The supernatants were then incubated at 95°C for 10 minutes to deactivate the trypsin.

Proteinase K Treatment of MDM Supernatants

Supernatants from MDMs stimulated and not stimulated with CD40L were treated with proteinase K, taken from a DNeasy kit (Qiagen). 20 µl of proteinase K was added to every 400

μl of supernatant. The supernatant was incubated at 70°C for 10 minutes. The supernatants

were then incubated at 95°C for 10 minutes to deactivate the proteinase K.

Analysis of Cytokine/Chemokine Production

Cytokine production by macrophages stimulated by CD40L was analyzed by Enzyme-

Linked ImmunoSorbent Assay (ELISA) using Quantikine ELISA (R&D Systems). For the

ELISAs, the macrophage supernatant samples were diluted accordingly.

Supernatant Fractionation

Macrophage supernatants were fractionated using Microcon Centrifugal Filter Devices

(Millipore) with sizes of 3, 10, 30, 50, and 100 KD nominal molecular weight limit. The

protocol provided by Millipore was followed. Briefly, 500 μL of the supernatant was added to

the 3 KD column, centrifuged, and the retentate was reconstituted with an equal volume of

DMEM supplemented with 5% FBS and then loaded onto the 10 KD column and so on until the

retentate of the 100 KD column was reconstituted.

Immunodepletion of Cytokines/Chemokines From MDM Supernatants

Cytokine/chemokine specific antibodies against IP-10, GRO, IL-10, TNF-α, MIP-1α,

and IL-6 (R&D Systems) were added to supernatants of CD40L stimulated and non-stimulated

MDMs along with protein A/G beads (Santa Cruz Biotechnologies) at the concentrations 2.5, 25,

2.5, 10, 50, 2.5 μg/ml, respectively. After incubation at 4°C for 16 hours, the beads were pelleted

at 2500xg for 3 minutes, replaced with fresh beads, and incubated at 4°C for another 2 hours.

Then, the supernatants were spun once more, collected, and filter-sterilized.

RNA Isolation & RT-PCR

Total macrophage RNA was purified using the RNeasy Mini Kit (Qiagen). The RNA was diluted to 2 ng/µl. RT-PCR was performed using 10 ng of total RNA per well by using the Sybr Green RT-PCR kit (Qiagen) and primers specific for TRAF6 (forward, TF6-4f: 5'-AGAAGCAGTGCAAACGCCAT-3', reverse, TF6-4r: 5'-ACCTGTCTCCTTGGACAATCCT-3') and GAPDH (forward, GAPDHf: 5'-GTCTTCACTACCATGGAGAGGG-3' and reverse, GAPDHr: 5'-TCATGGATGACCTTGGCCAG-3'). RT-PCR was performed on an ABI PRISM 7700 Sequence Detector, and cycling conditions were: 50°C for 30 min, 95°C for 15 min, followed by 35 cycles of 94°C for 20 sec, 55°C for 30 sec, and 72°C for 25 sec. For the relative quantification of TRAF6 expression, GAPDH was used as a reference.

Western Blot

The protein concentrations in the cell lysate samples were determined by the Bradford assay. The same concentration of protein was loaded in each well. The proteins were separated by electrophoresis on 10% SDS-PAGE gels. The proteins were blotted onto a nitrocellulose membrane. The blot was blocked by incubation with 5% non-fat dried milk in PBS supplemented with 0.01% Tween (PBST). The TRAF6 polyclonal rabbit antibody (Santa Cruz Biotechnologies) was diluted 1:200 in 5% milk-PBST while the actin monoclonal mouse antibody (Sigma) was diluted 1:2,000.

The western blots were detected with anti-rabbit HRP antibody and anti-mouse HRP antibody diluted 1:10,000 in 5% milk-PBST. Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer) was used as the detection reagent and the exposures were done on BioMax Light Film (Kodak).

RESULTS

Supernatants From CD40L-Stimulated MDMs Induce Neuronal Apoptosis

We stimulated macrophages with CD40L and incubated macrophage supernatants with neurons to determine whether stimulation with CD40L would induce the production of neurotoxic factors from macrophages. Indeed, this is what we found. The supernatant of stimulated macrophages induced about 8 times more caspase 3/7 than the supernatant of non-stimulated macrophages (figure 2).
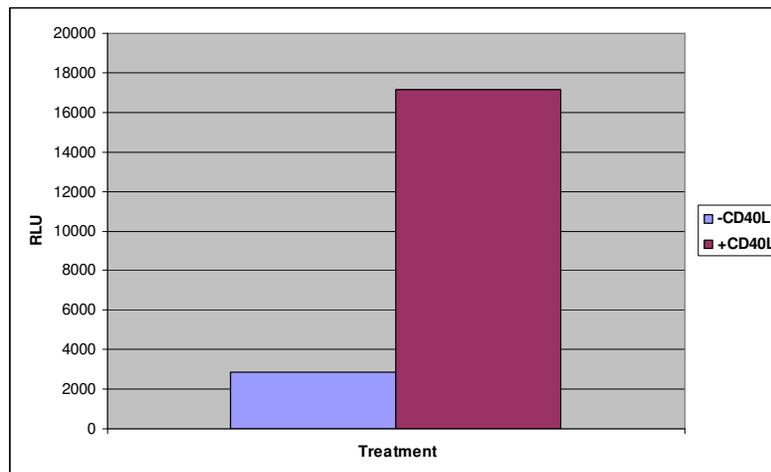
Figure 2. Induction of Caspase 3/7 in Neurons Treated With Supernatants From Macrophages Stimulated and Not Stimulated With CD40L. Monocyte-derived macrophages (MDMs) were treated with 10 µg/ml CD40L, and the supernatant was collected 6 hours after stimulation. Neurons were incubated with the supernatants for 72 hours, then apoptosis was assayed as described in *Materials and Methods*. The data shown are from a single experiment.

TRAF6 Silencing in MDMs Reduces CD40L-Induced Chemokine Production

It has been shown previously that the stimulation of MDMs with CD40L induces transient production of chemokines (Swingler et al, 1999). We wanted to examine the possible role of TRAF6 in this process. We transfected macrophages with a combination of 3 different TRAF6 siRNAs or a scrambled (scr) siRNA then analyzed the silencing of TRAF6 by

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performing RT-PCR and a western blot at day 1 and 2 after 2nd siRNA transfection (Figure 3).

Experiment 1 (blue histobar) shows 71% silencing of TRAF6 on day 1 (figure 3, a). Each

histobar is normalized relative to scrambled siRNA. Experiment 2 (purple histobars) shows 41%

silencing on day 1 and 45% silencing on day 2. The western blots also show TRAF6 silencing

when compared to scrambled (scr) siRNA (figure 3, b).

a)
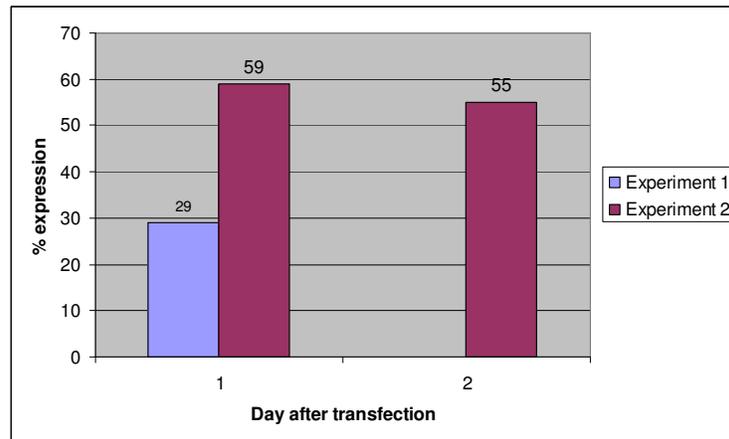
b)
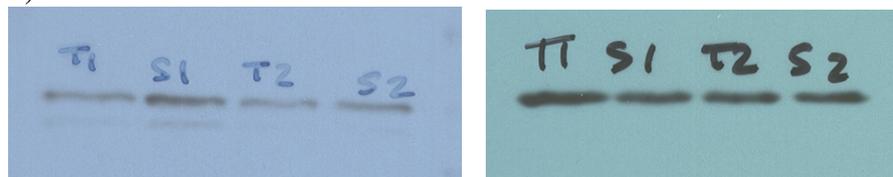
Figure 3. (a) TRAF6 Silencing Detected by Sybr Green RT-PCR on Total RNA Collected from MDMs Transfected With TRAF6 siRNA. MDMs were transfected on day 7 after plating. A second transfection followed the next day. The samples were normalized to GAPDH and samples treated with scrambled siRNA. **(b) TRAF6 Silencing Detected by Western Blot.** To the left, expression of TRAF6 monitored by a polyclonal rabbit antibody. To the right, expression of actin monoclonal mouse antibody. Protein was collected on day 1 and days 2 after the second transfection. T, TRAF6 siRNA; S, scr siRNA. The numbers represent days after transfection.

Once TRAF6 silencing had been confirmed by RT-PCR and western blot, we performed

ELISAs on supernatants from macrophages stimulated with CD40L 1 day after siRNA

transfection to monitor the production of cytokines/chemokines TNF-α and MIP-1β. The

supernatant of the TRAF6 siRNA treated cells contained less than twice as much TNF-α than the

control scrambled (scr) siRNA treated cell supernatant (figure 4, a). MIP-1β ELISA showed

that the TRAF6 siRNA treated macrophages produced less MIP-1β than the scr siRNA treated

ones (figure 4, b), although the decrease is not as pronounced as with TNF-α.

a)   b)
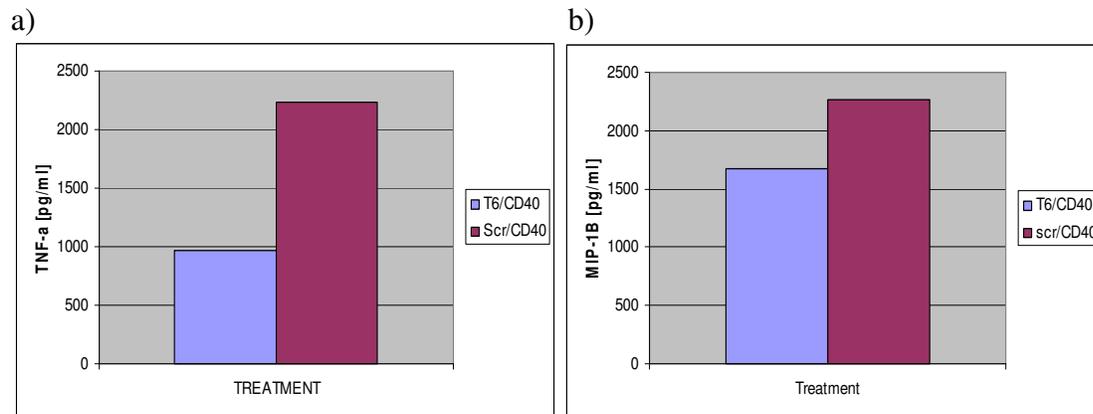
Figure 4. (a)TNF-α and (b) MIP-1β Concentrations in the Supernatants from TRAF6 and scr siRNA Transfected Macrophages. MDMs were transfected twice with either TRAF6 (T6) or scrambled (Scr) siRNA and then stimulated with CD40L (10 μg/ml) the day after the second transfection. Supernatant was collected 16 hours after simulation with CD40L. The concentrations of TNFα and MIP-1β in the supernatants were detected with an ELISA assay.   The data shown are from a single experiment.

TRAF6 Silencing in MDMs Decreases CD40L-Induced Neurotoxin Production

To determine the effect of TRAF6 silencing on CD40L-induced production of

neurotoxins by macrophages, neurons were incubated with the MDM supernatants for 72 hours.

We observed that the induction of neuronal apoptosis by the supernatant from TRAF6 siRNA

treated macrophages was less than that of the scr siRNA treated supernatant (figure 5).

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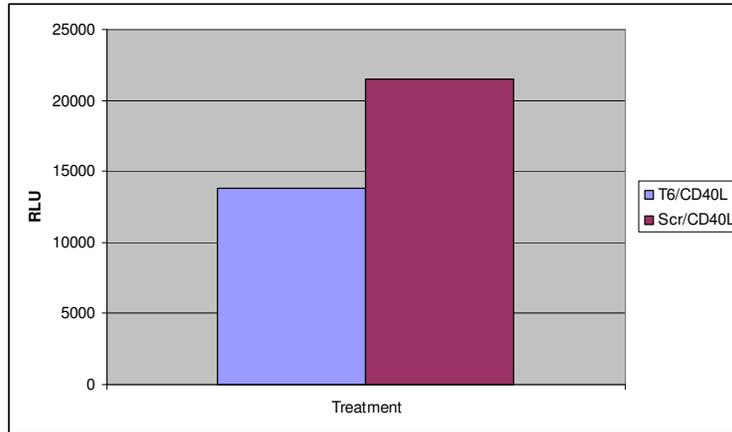
Figure 5. Induction of Caspase 3/7 in Neurons Treated With Supernatant From Macrophages Transfected With TRAF6 (T6) or scr siRNA. Supernatants from MDMs transfected with TRAF6 siRNA (T6) or scr siRNA (Scr) were placed on neurons, and apoptosis was measured by assaying for caspases 3 and 7 72 hours after the addition of MDM supernatants. The data shown are from a single experiment.

TRAF6BP Treatment on MDMs Decreases CD40L-Induced Neurotoxin Production

To further evaluate the role of TRAF6 from a different angle, we treated macrophages with TRAF6-binding peptide (TRAF6BP) to see which inflammatory responses are TRAF6 dependent. TRAF6BP incubation with the cells should block the recruitment of TRAF6 to CD40. We treated macrophages with different concentrations of TRAF6BP for 3 hours, and then stimulated them with 1 μg/ml of CD40L and collected supernatants 16 hours after stimulation. We then assayed for the following cytokines and chemokines: TNF-α, MIP-1β, MIP-1α, and IL-1β by ELISA.

The increase in TRAF6BP peptide concentration decreased MDM production of TNF-α in a dose-dependent manner (figure 6, a). There was no correlation in concentration of either MIP-1α (figure 6, b) or MIP-1β (figure 6, c) in response to TRAF6BP concentration, though MIP-1β concentration decreased a little with treatment of 800 μM TRAF6BP.

The IL-1β concentrations detected in MDM supernatants were very low in each sample. No dose response was observed in this case (data not shown).
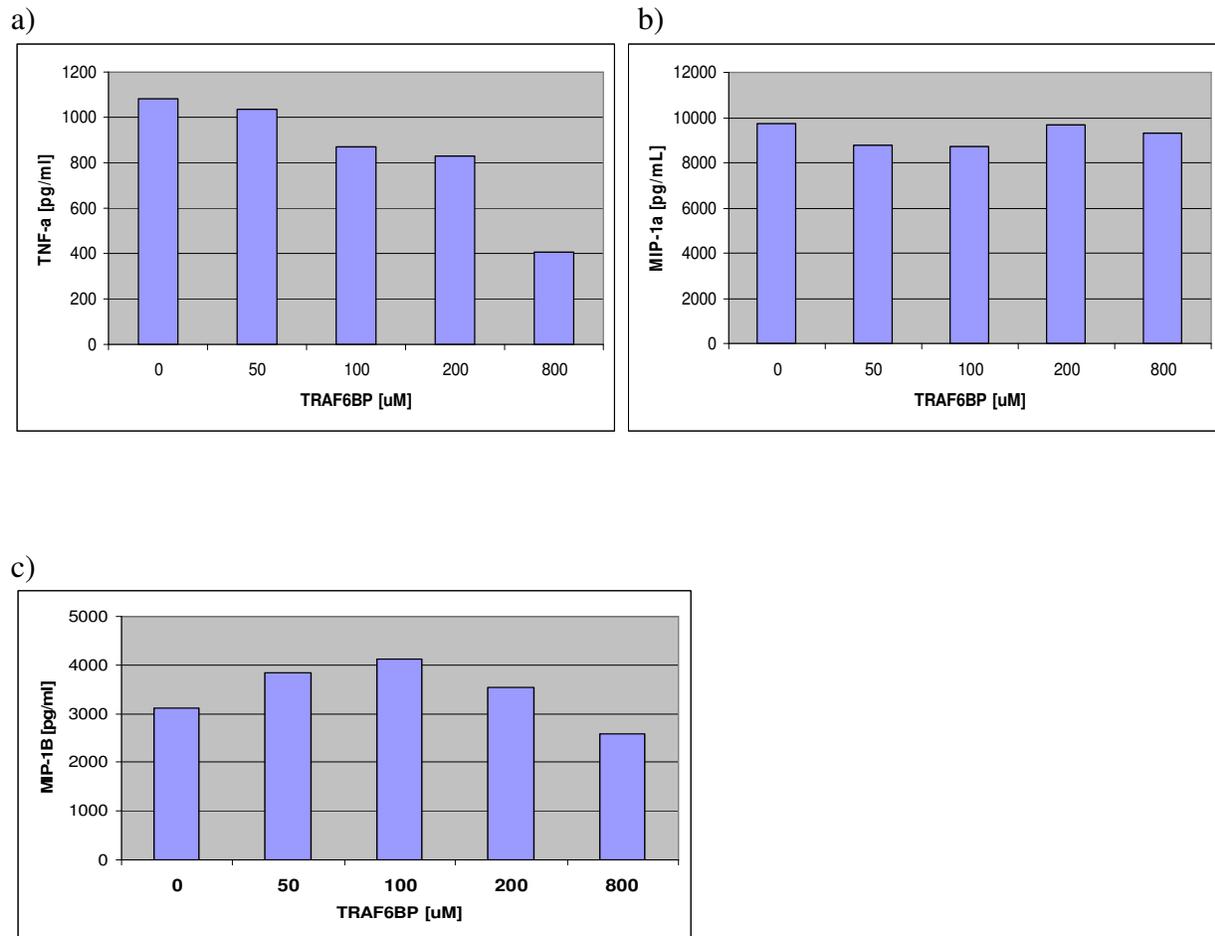
a)

b)

c)

Figure 6. (a)TNFα, (b) MIP-1α, and (c) MIP-1β Concentrations in Supernatants from Macrophages Treated with TRAF6BP. MDMs were incubated with TRAF6BP (0, 50, 100, 200, 800 μM) for 3 hours and stimulated with1μg/ml CD40L in serum free DMEM, as described in *Materials and Methods* for 16 hours. Supernatants were assayed for TNFα, MIP-1α, and MIP-1β by ELISA.   The data shown are from individual experiments.

TRAF6BP Treatment on MDMs Decreases Neurotoxic Activity

To determine the effect of TRAF6BP on CD40L-induced production of neurotoxins by

macrophages, neurons were incubated with the MDM supernatants for 48 hours. Some dose

response to TRAF6BP was observed (figure 7), there was a decrease in neuronal apoptosis

induction with increasing peptide concentration except for the supernatant from the cells treated

with 200 μM TRAF6BP. There was more than a two-fold decrease of caspase 3/7 induction

between 50 μM and 100 μM treatments with the peptide.
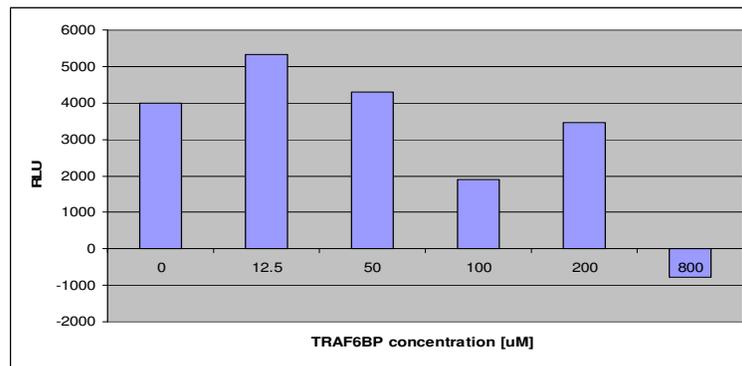
Figure 7. Induction of Caspase 3/7 in Neurons Treated With Supernatant From Macrophages Treated With TRAF6BP. MDMs were incubated with TRAF6BP (0, 12.5, 50, 100, 200, 800 μM) for 3 hours and stimulated with1μg/ml CD40L in serum free DMEM, as described in *Materials and Methods* for 16 hours. Apoptosis was measured with a caspase 3/7 assay after 48 hours of incubation of neurons with MDM supernatants as described in *Materials and Methods*. The data shown are from a single experiment.

Supplementing MDM With Serum Improves CD40L-Mediated Production of Cytokines/Chemokines

We performed CD40L stimulation experiments on MDMs with serum free DMEM as well as DMEM supplemented with 5% FBS to see whether the serum had an effect on chemokine production. We found that the cells that were supplemented with serum showed an increased response to CD40L stimulation. The TNF-α concentrations in supernatants of macrophages incubated with serum decreased as the TRAF6BP concentrations increased (figure 8, a). Without serum, however, there was not as an abundant production of TNF-α and also no clear dose response was observed. The MIP-1β concentration in the supernatants of macrophages incubated with serum was a little higher than the ones without serum (figure 8, b). No clear dose response of both TNF-α and MIP-1β to increasing TRAF6BP concentrations was observed for serum free macrophage supernatant.
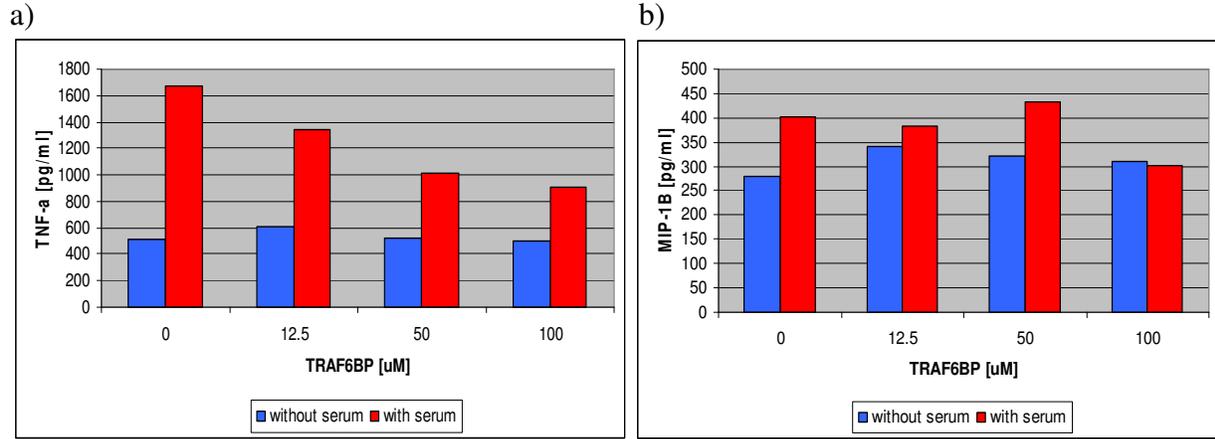
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a)

b)

Figure 8. (a)TNF-α and (b) MIP-1β Concentrations in Supernatants From Macrophages Treated With TRAF6BP. MDMs were treated with TRAF6BP as previously stated and then stimulated with 1µg/ml CD40L. Either DMEM supplemented with 5% FBS or serum free DMEM was used after stimulation. Supernatants were collected 16 hours after stimulation. The data shown are from a single experiment.

The MDM supernatants were then used to treat neurons. This experiment was done to observe the effects of serum in MDM supernatants on the production of neurotoxins. It was observed that the MDM supernatants containing serum (red histobars) produced more neurotoxins than those without serum (blue histobars) (figure 9). In the presence of the serum (red histobars), treatment with the peptide resulted in decreased induction of neuronal apoptosis
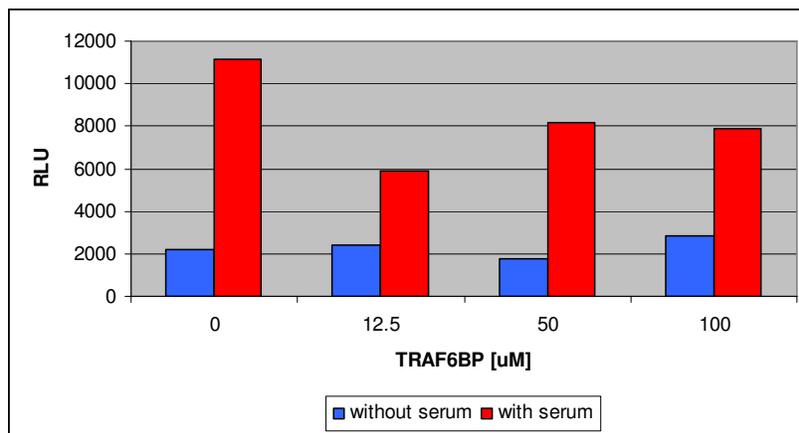
Figure 9. Induction of Caspase 3/7 in Neurons Treated With Supernatant From Macrophages Treated With TRAF6BP in the Presence or Absence of Serum. MDMs were incubated with TRAF6BP (0, 12.5, 50, 100, 200, 800 µM) for 3 hours and stimulated with 1µg/ml CD40L, as described in *Materials and Methods* for 16 hours. Neurons were incubated with MDM supernatants. Supernatants contained DMEM serum free or DMEM

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supplemented with 5% FBS. Apoptosis was measured with a caspase 3/7 assay after 48 hours as described in *Materials and Methods*.

The Neurotoxic Factor is Protein or Protein-Associated

In order to determine whether the neurotoxic factor is a protein or protein-associated, we treated CD40L-stimulated and non-stimulated MDM supernatants with trypsin, and tested their apoptotic activity on neurons. We found that the trypsin-treated CD40L supernatant showed a reduction in neurotoxic activity (figure 10). The supernatant that was not stimulated, however, retained neurotoxic activity, indicating that the trypsin treatment was specific for CD40L induced neurotoxic products.
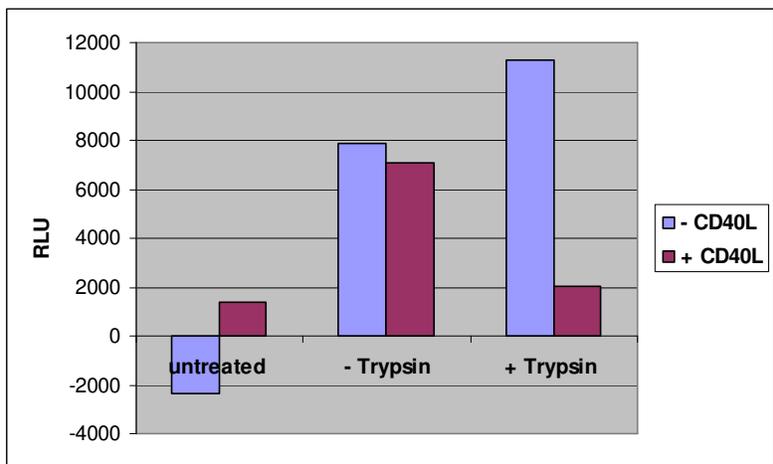
Figure 10. Induction of Caspase 3/7 in Neurons Treated With Trypsin-Treated MDM Supernatants. CD40L stimulated (+CD40L) and non-stimulated (-CD40L) MDM supernatants were treated with trypsin in a 1:1 ratio as described in *Materials and Methods*. Trypsin controls were diluted in a 1:1 ratio of serum-free DMEM and treated in the same manner. Neurons were incubated with the supernatants for 72 hours, upon when apoptosis was assayed. The data shown are from a single experiment.

In addition to trypsin, we also incubated MDM supernatants with proteinase K (PK). PK treatment removed nearly all of the neurotoxic activity but did not seem to be specific for CD40L supernatants (figure 11). Both of the samples that were treated with PK decreased greatly in

neurotoxic activity.
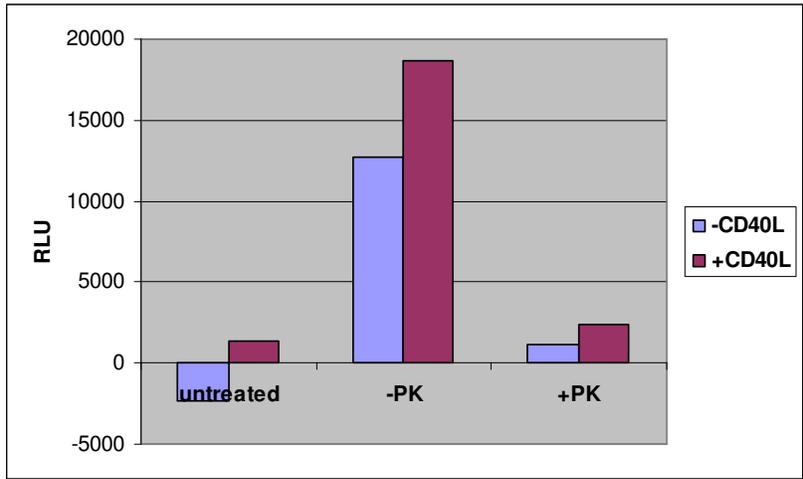
Figure 11. Induction of Caspase 3/7 in Neurons Treated with Supernatants of Proteinase K (PK)-Treated MDMs. MDM CD40L stimulated (purple histobars) and non-stimulated (blue histobars) supernatants were treated with proteinase K. Neurons were incubated with the supernatants for 72 hours and then assayed for caspases 3 and 7.

Furthermore, we heat-treated the supernatants to see how the heat denaturation affects the

neurotoxic effects of the protein. We observed that heating of the samples to 99˚C increased the

induction of apoptosis nonspecifically, but also CD40L specific induction of apoptosis was

largely decreased. This could indicate that neurotoxic function is altered when the protein loses

its native structure (figure 12).
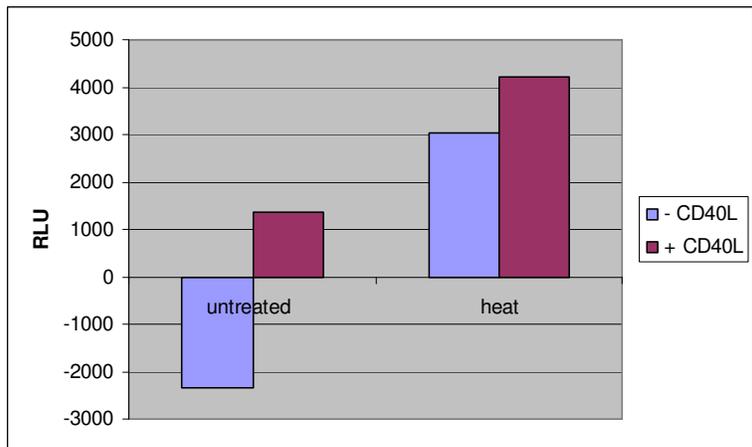
Figure 12. Induction of Caspase 3/7 in Neurons Treated With Heat-Treated MDM Supernatants. CD40L stimulated (+CD40L) and non-stimulated (-CD40L) supernatants were incubated at 99˚C for 10 minutes. The

supernatants were placed on neurons. Apoptosis was measured 72 hours after treatment with the supernatants as described in *Materials and Methods*.

The 3-10 kD and 10-30 kD Supernatant Fractions Contain the Neurotoxic Factor Produced by CD40L Stimulated MDMs

To determine what size the neurotoxic factor could possibly be, we performed size-fractionation of the CD40L stimulated and non-stimulated MDM supernatants. The fractions were incubated with neurons for 72 hours. We observed that the fractions 3-10 kD and to a lesser extent, 10-30 kD, retained the specific neurotoxic activity. The CD40L 3-10 kD fraction showed a two-fold increase in caspase 3/7 induction compared to the supernatant from the non-stimulated cells (figure 13). The CD40L 10-30 kD fraction showed a little less than 2-fold increase. The rest of the fractions showed nonspecific or low caspase-induction.
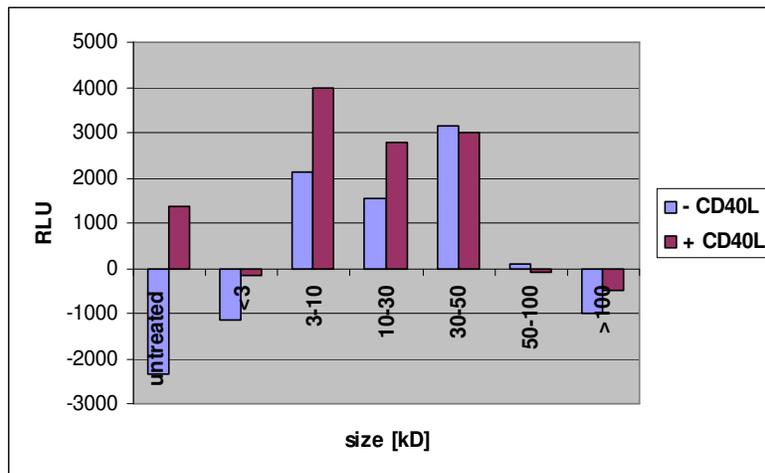
Figure 13. Induction of Caspase 3/7 in Neurons Treated With Fractionated Macrophage Supernatant Samples. Macrophage supernatants were fractionated as described in *Materials and Methods*. The macrophages were incubated in serum-free DMEM to prevent possible clogging of the columns by serum. Neurons were incubated with the supernatant fractions for 72 hours and then apoptosis was measured. The data shown are from a single experiment.

Immunodepletion of TNF-α Removes the Neurotoxic Activity of MDM Supernatants

We next performed cytokine/chemokine immunodepletion experiments on MDM

supernatants that were stimulated or not with CD40L (in the presence of serum). We thought to

test whether any of the major cytokines/chemokines produced by CD40L stimulated MDMs may

play a role in the neurotoxic activity. Immunodepletion of IL-6, IL-10, IP-10, Gro, and MIP-1α,

did not affect the neurotoxic activity of the supernatants (figure 14, b, c, d). However,

immunodepletion of TNF-α greatly decreased the induction of apoptosis in neurons (figure 14,

a).

a)

b)

c)

d)
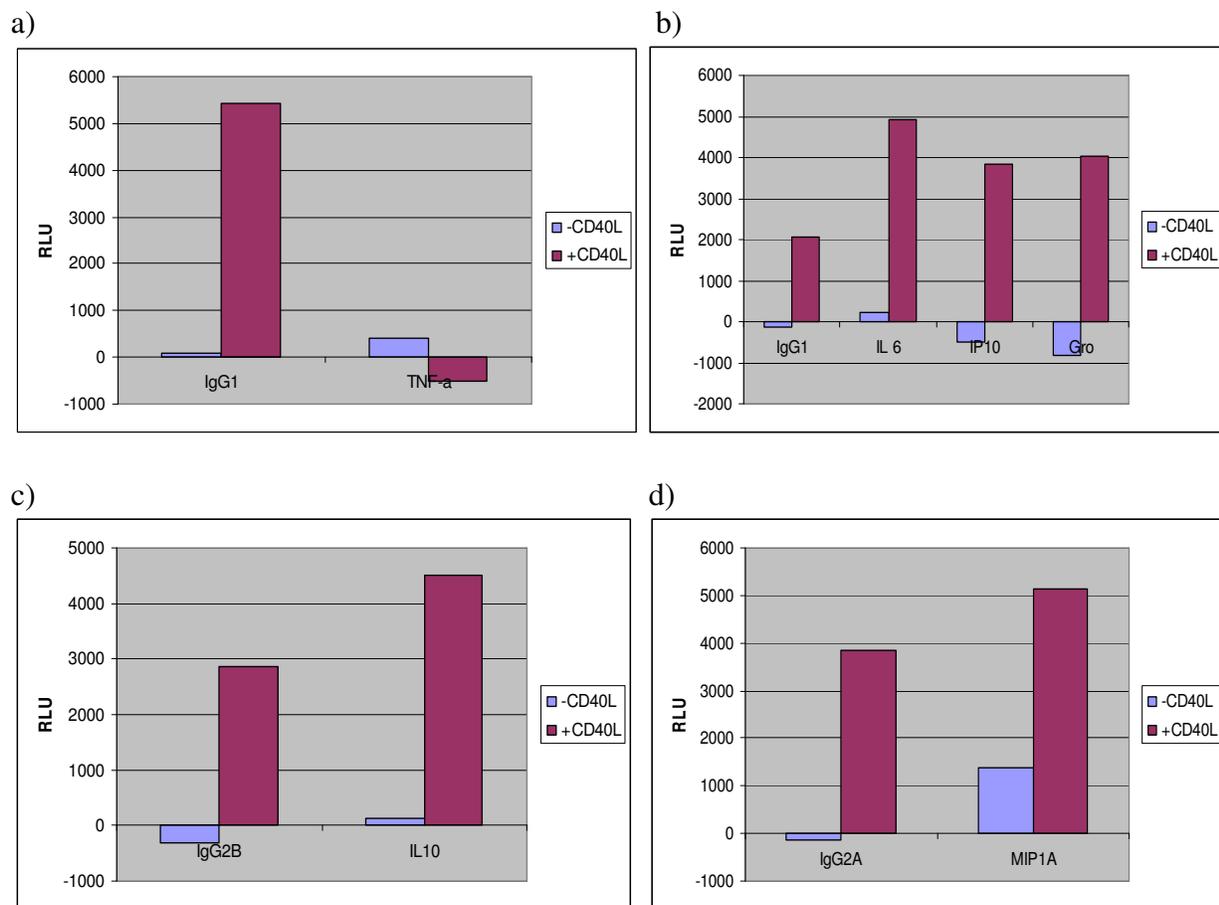
Figure 14. Induction of Caspase 3/7 in Neurons Treated With Immuodepleted MDM Supernatants.
Cytokine/chemokine specific antibodies were used in immunodepletion. IgG1 was used as a control for **(a)** TNF-α,
(b) IL-6, IP-10, and Gro. IgG2B was a control for **(c)** IL-10 and IgG2A was a control for **(d)** MIP-1α. 10 µg/ml
CD40L was used for stimulation. Neurons were incubated with MDM supernatant for 72 hours and then apoptosis
was measured as described in *Materials and Methods*. The data shown are from a single experiment.

DISCUSSION

It has been known that macrophages play an important role in the onset of neurodegeneration seen in HIV-associated dementia (HAD). As macrophages are likely the main source of viral replication, understanding the mechanism of how neurotoxic factors are produced by them, as well as the signaling pathways that regulate these processes, is integral to find a way to treat HAD. It is also crucial to identify what the neurotoxic factor is. The goals of this MQP were to explore these signaling pathways and to find out what neurotoxic factors are being produced by macrophages.

First, we have shown that CD40L stimulation of primary macrophages induced production of neurotoxic factors in the supernatants. We observed that these factors induced apoptosis in NT2 neurons. The neuronal apoptosis was mediated by caspases 3 and 7.

We found that TRAF6 and CD40 are part of a pathway that induces the production of neurotoxins. In this paper, we used a TRAF6 peptide to block the TRAF6 binding site of CD40. We also used TRAF6 siRNA to decrease the production of TRAF6 protein. Both methods showed that TRAF6 does play an important role in mediating the CD40L signaling pathway. This was characterized by the decrease of TNF-α (figure 6, a) as well as the decrease in induction of neuronal apoptosis (figure 5). This confirms the research of Mukundan *et al* (2005) where they showed that TRAF6 mediates CD40-activated proinflammatory signaling pathways in monocytes and macrophages.

It was not yet clear whether the neurotoxic factor being produced by stimulated macrophages was a protein or protein-associated. After the trypsin treatment, the neurotoxic effects of the supernatant from CD40L stimulated macrophages were shown to be mostly inactivated (figure 10). Moreover, when the samples were treated with proteinase K, there was

almost a total inactivation of neurotoxicity in both supernatants from non-stimulated and stimulated macrophages (figure 11). The proteinase K treatment might have removed other neurotoxic factors in the supernatant, but not those specific for CD40L stimulation. As a result of the two experiments previously mentioned, the neurotoxic factor might be a protein or protein associated. An interesting observation we made was that when the supernatants were heat treated, the heat increased the neurotoxicity of the supernatants (figure 12). This might be because the neurotoxic function of the protein is altered when it loses its native structure. The protein somehow becomes more neurotoxic. More evidence of this phenomenon can be seen in the trypsin-treated and PK-treated supernatants that were heat inactivated after treatment. Apart from this nonspecific heat-induced increase in neurotoxicity, the heat denaturation did also specifically decrease the neurotoxicity of supernatants from CD40L stimulated macrophages.

Determination of the size was another important step in the process of identification of the potential neurotoxic factor. Our results suggest that the neurotoxic factor might be in the range of 3-30 kD (figure 13).

Finally, we showed that depletion of TNF-α, but not of IL-6, IL-10, IP-10, Gro, or MIP-1α completely abrogated the neurotoxic effects of the CD40L stimulated MDM supernatants (figure 14, a). It was reported that TNF-α mRNA increased with severity of dementia in the brain of HAD patients (Griffin, 1997). Another study showed that TNF-α inhibitors reduced neuronal damage in the murine model of HAD (Persidsky and Gendelman, 2002). Given the striking similarity between the effects of CD40 ligation and HIV Nef expression on macrophages, the next step will be to set up similar experiments using macrophage HIV infection model and compare the outcome with our results on CD40 ligation presented here.

The experiments done in this project have only been done on neurons, which is a disadvantage to fully understanding the mechanisms of neurotoxicity induced by HAD. There are many more cell types involved in the process of neurodegeneration that include neurons, macrophages, microglia, astrocytes, and oligodendrocytes. Theses cells also produce multiple factors upon stimulation that need to be taken into consideration as well. In future experiments, it will be important to validate our findings in a model that would more closely mimic the *in vivo* environment of the CNS.

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