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Differential roles of TNFR1 and TNFR2 signaling in adult hippocampal neurogenesis

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Abstract

Tumor necrosis factor alpha (TNF α) is a potent inhibitor of neurogenesis in vitro but here we show that TNF α signaling has both positive and negative effects on neurogenesis in vivo and is required to moderate the negative impact of cranial irradiation on hippocampal neurogenesis. In vitro, basal levels of TNF α signaling through TNFR2 are required for normal neural progenitor cell proliferation while basal signaling through TNFR1 impairs neural progenitor proliferation. TNFR1 also mediates further reductions in proliferation and elevated cell death following exposure to recombinant TNF α . In vivo, TNFR1^{-/-} and TNF α ^{-/-} animals have elevated baseline neurogenesis in the hippocampus, whereas absence of TNFR2 decreases baseline neurogenesis. TNF α is also implicated in defects in neurogenesis that follow radiation injury but we find that loss of TNFR1 has no protective effects on neurogenesis and loss of TNF α or TNFR2 worsened the effects of radiation injury on neurogenesis. We conclude that the immunomodulatory signaling of TNF α mediated by TNFR2 is more significant to radiation injury outcome than the proinflammatory signaling mediated through TNFR1.

Keywords

TNF α ; neural stem cells; neurogenesis; hippocampus; dentate gyrus; irradiation; inflammation

1. Introduction

Neuroinflammation is a common feature in many neurological diseases/disorders, such as Parkinson's disease, Alzheimer's disease, and stroke, etc. (Chen and Palmer, 2008; Liebig et al., 2012; Liu and Hong, 2003; Nelson et al., 2002). Previous studies have shown that microglia activation, a secondary consequence of neurological disease or injury, is detrimental to adult neurogenesis in the dentate gyrus (DG). Treatment with non-steroidal anti-inflammatory drugs can attenuate the effects on neurogenesis (Ekdahl et al., 2003; Monje et al., 2002; Monje et al., 2003); however, the detrimental cytokine(s) produced by microglia and the molecular mechanisms underlying such effects are not completely known.

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TNF α is a pleiotropic cytokine that is implicated in many immunological diseases/disorders but the precise influence of TNF α in adult neurogenesis remains controversial. Our earlier studies suggested that TNF α is a contributing factor in the inflammatory inhibition of neurogenesis (Monje et al., 2003) and Iosif et al have shown that TNFR1-mediated signaling suppresses the proliferation of adult neural stem/progenitor cells (NSCs) within the DG (Iosif et al., 2006). Keohane et al report that TNF α treatment decreases neurogenesis in cultured NSCs but has no effect on proliferation (Keohane et al., 2010). In contrast, Widera et al show that TNF α treatment promotes NSC proliferation but has no effect on differentiation in culture (Widera et al., 2006). These contrasting results suggest that the effect of TNF α signaling may be cell type-selective and/or context specific.

In the current study, we examined the effect of individual cytokines produced by activated microglia and found that exogenously applied recombinant TNF α exerts a conserved inhibitory effect on neuron production in mouse, primate and human NSC cultures but we also found that loss of TNF α or TNFR2 in vivo exacerbates the negative impact of radiation-induced inflammation on hippocampal neurogenesis while loss of TNFR1 has no significant influence on outcome. This implies that the most influential role for TNF α in chronically impaired neurogenesis that follows irradiation injury is a protective immunomodulatory effect mediated through TNFR2.

2. Materials and methods

2.1. Animals

C57BL/6J (wild-type, stock number 000664), C57BL/6-Tnfrsf1a^{tm1Imx}/J (TNFR1^{-/-}, stock number 003242), B6.129S2-Tnfrsf1b^{tm1Mwm}/J (TNFR2^{-/-}, stock number 002620), and B6.129S-Tnfrsf1Gkl/J (TNF α ^{-/-}, stock number 005540) - all on C57BL/6 background, were purchased from the Jackson laboratory (Bar Harbor, Maine). The mice were purchased as homozygous and the genotypes confirmed by PCR (data not shown). All animal protocols used in the present study have been reviewed and approved by the Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University.

2.2. Microglia culture

For microglia culture, postnatal day 2 pup brains of C57BL/6 mice (without cerebellum) were dissected out and the meninges carefully removed. The brain tissue was triturated by pipeting through a fire polished pasture pipette and filtered through a 70 μ m cell strainer. The cells were cultured in DMEM medium (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS, Life Technologies) for two weeks. On the 14th day, the culture was treated with 12 mM lidocaine (Sigma-aldrich, St Louis, MO) for 10 mins at room temperature and the flasks rocked by hand to detach the microglia. The microglia were re-plated and cultured for further analysis. To obtain conditioned medium of activated microglia, microglia were treated with 1 μ g/ml lipopolysaccharides (LPS, Sigma-aldrich) or PBS for 24 hrs. The medium was removed and the cultures rinsed once with new medium and then incubated in NSC differentiation medium over night. The conditioned medium was then collected and diluted with fresh differentiation medium (1:1) prior to adding to NSC culture.

2.3. Neural stem cell culture

Mouse NSCs were isolated from postnatal day 0 pups of wild-type, TNFR1^{-/-}, and TNFR2^{-/-} mice (all on C57BL/6 background, the Jackson Laboratory). Using methods previously described (Monje et al., 2002; Monje et al., 2003), the cerebellum and brain stem were removed from whole brains of neonatal animals and the remaining tissues enzymatically digested with a mixture of papain, neutral protease, and DNase and the debris

removed by discarding the supernatant from a 25% Percoll fractionation. Neurospheres were cultured with medium containing Neurobasal A, L-glutamine, penicillin, streptomycin, B-27 without vitamin A, 20 ng/ml FGF-2, and 20 ng/ml EGF (all from Life Technologies). Cells were passaged by dissociation with Trypsin/DNase (Worthington Biochemical Corp., Lakewood, NJ) followed by re-plating in growth medium. For proliferation assays, the spheres were first attached to poly-D-lysine/laminin-coated plates and passaged at least twice to obtain evenly distributed monolayer culture. The monolayer cells were then detached into single cell suspension by Trypsin treatment and 500,000 cells were re-plated in growth medium in uncoated flasks. Over a course of 6 days, the spheres were image-captured by a Zeiss microscope and sphere sizes measured by using the program "Image J" (NIH Image). For differentiation assays, NSCs were cultured as monolayers in basal medium without growth factors (FGF-2 and EGF) and with addition of 10 ng/ml NT-3 (Peprotech, Rocky Hill, NJ), 10 ng/ml BDNF (Peprotech), and 200 nM retinoic acid (Sigma-aldrich), with or without cytokines (recombinant murine TGF β 1, 10 ng/ml; IL6, 50 ng/ml; IL1 β , 2 ng/ml; TNF α , 20 ng/ml; MCP1, 10 ng/ml; or IFN γ , 10 ng/ml). The concentrations of the cytokines were based on previous publications (Andres et al., 2011; Blokzijl et al., 2003; Burton et al., 2011; Koo and Duman, 2008; Monje et al., 2003; Sheng et al., 2005) as well as preliminary tests (data not shown).

Human and Squirrel Monkey NSCs were isolated from 22-week old and 14-week old fetal forebrain tissues, respectively, using the same method as for mouse NSC. Human and primate NSCs were propagated as monolayer cultures on fibronectin-coated plates in DMEM/F12 medium (Life Technologies) containing 10% BIT 9500 serum substitute (Stemcell technologies, Vancouver, Canada), 20 ng/ml FGF-2, 20 ng/ml EGF, and 20 ng/ml platelet-derived growth factor-AB (PDGF-AB) (all from Life Technologies). For differentiation, FGF-2, EGF, and PDGF-AB were withdrawn and cells were cultured for 10 days in medium containing 200 nM retinoic acid, 10 ng/ml NT-3, and 10 ng/ml BDNF, with or without recombinant human TGF β 1 (10 ng/ml), IL6 (50 ng/ml), IL1 β (2 ng/ml), TNF α (20 ng/ml), MCP1 (10 ng/ml), or IFN γ (10 ng/ml) (all from peprotech).

2.4. Cell Cycle Analysis

Mouse NSCs of wild-type, TNFR1 $^{-/-}$, and TNFR2 $^{-/-}$ background were cultured as monolayer on PDL/Laminin coated plates prior to TNF α (20 ng/ml) treatment for 24 hrs. At the end of the treatment, the cells were detached with accutase and resuspended as single cell solution, followed by fixation with cold 70% Ethanol for 2 hrs at 4°C. After 3 washes with PBS, the cells were treated with 100 μ g/ml RNase for 30 mins at room temperature and incubated with 100 μ g/ml Propidium Iodide (PI, Sigma-Aldrich) for 15 mins before being subjected to flow cytometric analysis using FACSCalibur flow cytometer and CellQuest software (both from Becton Dickinson, CA, USA). The single cells were gated for cell cycle analysis based on the PI-area parameter.

2.5. TNF α Injection into the Dentate Gyrus

For TNF α injection, mice were first injected intraperitoneally with BrdU once per day for 6 days (50 mg/kg body weight, Sigma-aldrich). On the 7th day, by using a stereotactic instrument and 30 gage Hamilton syringe, 1 μ g recombinant murine TNF α (Peprotech) dissolved in saline was unilaterally introduced into the hippocampus (A/P, -0.22 cm; M/L, +0.14 cm; D/V, -0.26 cm) of mice two months of age. 1 μ l saline was injected into the contralateral dentate gyrus as a control. One month after TNF α injection, mice were perfused with 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA) in 100 mM phosphate buffer and brains were removed, post-fixed for 24 hrs in 4% paraformaldehyde, and then equilibrated in 30% sucrose prior to cryosectioning and histological evaluation.

2.6. Limited field cranial irradiation

One month old adult male mice were anesthetized with ketamine and xylazine and exposed to cranial irradiation using a Philips orthovoltage X-ray system operated at 200 kV and 20 mA. On day 1, a single dose of 10 Gy was delivered to a 1 cm vertical column centered over the hippocampal formations of each mouse. Dosimetry using TLD dosimeters (K & S Associates, Nashville, Tennessee) buried in the hippocampi of euthanized mice confirmed a total of 10 Gy dose at hippocampal depth. The dose rate was approximately 78.0 cGy/minute. Sham-irradiated controls for all experiments received anesthesia only.

2.7. Tissue Preparation and Immunohistochemistry

Free floating 40 μ m sections were collected on a freezing microtome and stored in tissue cryoprotectant solution at -20°C until used. Immunostaining was performed on brain sections or fixed cell cultures as previously described using the following primary antibodies and working concentrations. Rat anti-BrdU ascites (1:1000, Accurate Chemical & Scientific Corp, Westbury, NY), mouse anti-NeuN (1:500, Chemicon, Billerica, MA), goat anti-Doublecortin (Dcx, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), guinea pig anti-GFAP (1:1000, Advanced Immunochemicals, Long Beach, CA), mouse anti-Nestin (1:500, BD Bioscience, San Jose, CA), rabbit anti-ki67 (1:500, Novocastra, Newcastle, UK), rabbit anti-p65 (1:500, Santa Cruz), and rabbit anti-cleaved caspase 3 (1:500, Cell Signaling Technology, Boston, MA). Donkey-anti mouse, rat, goat, or rabbit antibodies conjugated to FITC, Cy3, Cy5 or biotin were each used at 1:500 (Jackson Immuno Research, West Grove, PA). ABC kits were used for DAB stained samples (Jackson Immuno Research, West Grove, PA).

2.8. Confocal Microscopy

All confocal microscopy was performed using a Zeiss 510 Meta confocal microscope. Appropriate gain and black level settings were determined on control tissues stained with secondary antibodies alone. Upper and lower thresholds were always set using the range indicator function to minimize data loss through under or over saturation. Upper and lower thresholds were then held constant for all samples when scoring tissues or cells for a given experiment.

2.9. Cell Quantification and Unbiased Stereology

Quantification of cultured cells was performed using confocal microscopy with at least 300 DAPI-positive nuclei systematically scanned and the percentage of cells positive for Dcx, GFAP, p65, Nestin, Caspase 3, or Ki67 was scored. For tissue sections, all counts of endogenous neural stem/progenitor cells were limited to the hippocampal granule cell layer proper and a 50 μ m border along the hilar margin that included the neurogenic subgranular zone. The proportion of BrdU-positive cells displaying a lineage-specific phenotype was determined by scoring the co-localization of cellular phenotype markers with BrdU using confocal microscopy. Split panel and z-axis orthogonal projections were used for all counting to minimize false positives. The counts were performed using multi-channel configuration with a 40 $\times$ objective and digital zoom of 2. When possible, 100 or more BrdU-positive cells were scored for each marker per animal. Each cell was manually examined in its full "z"-dimension and only those cells for which the nucleus was unambiguously associated with the lineage-specific marker were scored as positive. The total number of BrdU-positive cells per hippocampal granular cell layer and subgranular zone was determined using DAB immunodetection and unbiased stereology using Microbrightfield Stereo Investigator software. Estimates of cell number were determined using the di-sector method with Abercrombie corrections based on average object diameter

and section thickness. All analyses were performed by investigators blinded to sample identity and treatment group.

2.10. Statistics

Differences between more than two groups were tested with parametrical one-factor analysis of variance (ANOVA), using Bonferroni-Dunnnett corrections as appropriate. Differences between two groups were tested by using Student's *t* test. The level of significance was set at $p < 0.05$.

3. Results

3.1. TNFR1- and TNFR2-mediated Signaling Affects Proliferation and Differentiation of Neural Stem Cells

We have previously shown that microglial activation inversely correlates with neuron production from grafted as well as endogenous NSCs (Chen et al., 2011; Monje et al., 2002; Monje et al., 2003). The combined impact of cytokines released by acutely activated microglia is illustrated in Figure 1A. Primary microglia were isolated from neonatal pups of C57BL/6 mice. Following treatment with 1 $\mu\text{g/ml}$ LPS, the microglial culture was rinsed with fresh medium to remove LPS and then incubated in NSC differentiation medium over night. The conditioned medium was collected and applied to NSCs. After differentiation for 72 hrs, the percentages of Dcx-positive cells were scored and compared with sham-treated control NSCs. Treatment with conditioned medium from activated microglia significantly reduced the fraction of Dcx-positive cells in the differentiation culture ($t=3.28$; $df=4$; $p=0.03$).

To further dissect which cytokine(s) mediate the inhibitory effect, we examined the effects of several well recognized proinflammatory cytokines that are released by microglia in response to tissue injury - TGF β 1, IL6, IL1 β , TNF α , and MCP1. Mouse, human, and monkey NSCs were evaluated in parallel for changes in the abundance of Dcx-positive neurons after differentiation. Although there were variations between species for some cytokines, TNF α showed the most consistent reduction in the abundance of new neurons across all the three species (Figs. 1B–1D).

To more fully explore TNF α signaling and its influence on neurogenesis, NSC growth and differentiation were evaluated in NSCs isolated from wild-type, TNFR1 $^{-/-}$, and TNFR2 $^{-/-}$ animals. To evaluate the potential effects of basal signaling through each receptor, single cell suspensions were allowed to form neurospheres and cultures monitored for changes in neurosphere size over 6 days. Spheres formed in all cultures but the TNFR1 $^{-/-}$ spheres grew faster and TNFR2 $^{-/-}$ more slowly than did wild-type NSCs (Figs. 2A and 2B and Table 1), suggesting that endogenous TNF α signaling through TNFR2 promoted growth and/or survival while signaling through TNFR1 inhibited growth and/or survival.

Elevating TNF α signaling by the addition of 20 ng/ml recombinant TNF α to the medium greatly inhibited sphere formation and growth in wild-type and TNFR2 $^{-/-}$ NSCs but had no effect on the growth of TNFR1 $^{-/-}$ NSCs, suggesting that the negative effects of TNF α signaling through TNFR1 are dominant over those of TNFR2 when TNF α signaling is amplified with exogenously added TNF α . To explore whether the change in cell growth under basal conditions was due to alterations in cell cycle, NSCs of each genotype were stained for Propidium Iodide (PI) and subjected to flow cytometric analysis (Figs. 2C–2H). Deficiency of endogenous TNFR1 significantly reduced the proportion of cells at G1 phase (Fig. 2F, $F(5,12)=114.7$, $p<0.0001$) whereas increased those at G2/M phases (Fig. 2H, $F(5,12)=44.3$, $p<0.0001$). In contrast, knockout of endogenous TNFR2 up-regulated the proportion of G1 and decreased that of G2/M phases. Following treatment of exogenous

TNF α at 20 ng/ml for 24 hrs, wild-type and TNFR2 $^{-/-}$ NSCs displayed a higher fraction of cells at G1 phase while a lower proportion at S phase (Fig. 2G, F(5,12)=42.2, $p<0.0001$), suggesting that exogenous TNF α treatment can trigger a cell cycle arrest between G1 and S phase.

To evaluate the effects of TNFR1 and TNFR2 on differentiation of newborn neurons, cells were plated as monolayers on poly-D-lysine/laminin coated coverslips and allowed to differentiate in the presence or absence of recombinant TNF α for 5 days and the fraction of cells positive for Dcx or glial fibrillary acidic protein (GFAP) was determined (Figs. 2I and 2J). Approximately 5% of cells in wild-type NSC cultures differentiated sufficiently to express Dcx after 5 days. Loss of TNFR1 increased the abundance of Dcx-positive cells to approximately 12% under control conditions. Loss of TNFR2 also led to an increase in the percentage of Dcx-positive cells. Exogenously added TNF α reduced the abundance of Dcx+ neurons in wild-type and TNFR2 $^{-/-}$ cultures, but had no effect in TNFR1 $^{-/-}$ cultures (Fig. 2I, F(5,12)=55.9, $p<0.0001$).

The abundance of GFAP+ cells generated under control conditions was reduced in TNFR1 $^{-/-}$ relative to wild-type cultures. Loss of TNFR2 had no effect on the abundance of GFAP positive cells and exogenously added TNF α had no significant impact on GFAP+ cell abundance in any of the NSC cultures. (Fig. 2J, F(5, 12)=78.3, $p<0.0001$).

3.2. NF- κ B and Caspase 3 Pathways are Activated in Different Cell Populations Following Exogenous TNF α Treatment

TNF α receptor signaling is known to engage both NF- κ B and caspase activation pathways (MacEwan, 2002). To determine the relative importance of each pathway, SN50 was used to block the translocation of NF- κ B from cytoplasm to nucleus, and caspase 3, or caspase 9-selective inhibitors were used to block TNF-evoked apoptosis. The addition of SN50 attenuated the inhibitory effect of exogenous TNF α on neuron production. Caspase 3 inhibitor was also protective but blocking caspase 9 was not protective (Figs. 3A and 3B). This indicated that both NF- κ B signaling and the direct apoptotic pathway, rather than mitochondria-mediated apoptotic pathway, contributed to TNF α -induced neuron reduction.

To determine whether NF- κ B and caspase 3 signaling pathways are co-activated and/or operate selectively within separate subpopulations of cells, we first differentiated the mouse NSCs for 3 days in the presence of retinoic acid (Neurobasal + B27 + glutamine + retinoic acid + NT-3 + BDNF) to generate mixed populations of undifferentiated cells and committed Dcx-positive neuronal progenitor cells. The localization of p65, a subunit of NF- κ B, was evaluated in untreated control cultures and 30 or 60 mins after the addition of 20 ng/ml TNF α . Cultures were also stained for activated caspase 3 8 hrs after TNF α exposure (Figs. 3C–3G). Prior to TNF α treatment, p65 displayed very faint cytoplasmic staining in all cells (Fig. 3C). After exposure to TNF α , p65 became more abundant and translocated to the nucleus in a sub-population of cells (Fig. 3C). Co-staining of p65 with Nestin and Dcx revealed that NF- κ B translocation occurred in the Dcx-negative subset of nestin-expressing cells (Figs. 3D and 3E). After 8 hr of exposure cleaved caspase 3 was observed primarily in Dcx-positive cells (Figs. 3F and 3G). These results suggest that exogenous TNF α selectively activates NF- κ B signaling in undifferentiated progenitors but preferentially evokes caspase 3 cleavage in newly generated neurons.

3.3. Infusion of recombinant TNF α into the dentate gyrus inhibits neurogenesis

To examine the role of TNF α -mediated signaling in vivo, we unilaterally introduced recombinant murine TNF α (1 μ g in 1 μ l saline) into the DG of adult mice following 6 Bromodeoxyuridine (BrdU) injections to label the endogenous neural stem/progenitor cells.

One month after TNF α injection, the mice were sacrificed for analysis (Fig. 4A). Quantification of BrdU-positive cells by using stereology revealed that proliferation and/or survival of endogenous NSCs within the DG had been reduced in mice with TNF α administration (Fig. 4B, $t=3.12$, $df=7$, $p=0.016$). In addition, the number of new neurons (BrdU-labeled Dcx- and/or NeuN-positive cells) was also significantly decreased by TNF α treatment (Fig. 4C, $t=27.8$, $df=7$, $p<0.0001$). The results suggest that exogenous TNF α can suppress endogenous neurogenesis within the DG in adult brain.

3.4. Effects of TNFR1- or TNFR2-mediated Signaling on hippocampal neurogenesis in radiation-induced inflammation

In our previous studies, we have shown that attenuating inflammation following radiation can promote the partial recovery of neurogenesis and our studies had implicated TNF α in this process (Monje et al., 2002; Monje et al., 2003). To determine the roles of TNF α , TNFR1 or TNFR2 in this model, neurogenesis was evaluated at baseline and following limited-field X-irradiation of the hippocampal formations in wild-type, TNFR1 $^{-/-}$, TNFR2 $^{-/-}$, and TNF α $^{-/-}$ mice.

To evaluate TNFR1- and TNFR2-dependant signaling effects on baseline neurogenesis, wild-type, TNFR1 $^{-/-}$, TNFR2 $^{-/-}$, and TNF α $^{-/-}$ mice 2 months of age were injected with 50 mg/kg BrdU i.p. once per day for 6 days to label the endogenous neural progenitor cells. 4 weeks later, the mice were sacrificed for analysis (Fig. 5A) and the total number of BrdU-labeled cells and total number of BrdU-labeled neurons (Dcx- and/or NeuN-positive cells) was determined (Figs. 5B and 5C). Loss of TNF α or TNFR1 both resulted in an increase in BrdU-positive cells while loss of TNFR2 resulted in a decrease in BrdU-labeled cells (Fig. 5D, $F(3,28)=117.2$, $p<0.0001$). Within the BrdU-labeled population, there was a net increase in the total number of neurons in TNFR1 $^{-/-}$ and TNF α $^{-/-}$ animals and net decrease in TNFR2 $^{-/-}$ animals (Fig. 5E, $F(3,28)=94.7$, $p<0.0001$).

To evaluate the loss-of function effects following irradiation, animals 4 weeks of age received a single 10 Gy dose of irradiation limited to a 1 cm diameter column centered over the hippocampal formations. 4 weeks later, the mice were given 6 daily i.p. injections of BrdU. Two months after irradiation (1 month after BrdU injection), the mice were sacrificed for analysis (Fig. 5A). Consistent with our prior observations (Monje et al., 2002; Monje et al., 2003), irradiation significantly reduced the number of dividing cells in each genotype (Fig. 5D). Although we anticipated that loss of TNFR1 would protect neurogenesis, we found that loss of TNFR1 had no significant effect on outcome. In contrast, loss of either TNFR2 or TNF α substantially reduced the number of new neurons produced in the chronic inflammatory context that results from irradiation (Fig. 5E, $F(3,28)=50.1$, $p<0.0001$). These data suggest that TNFR2-mediated signaling is protective in the irradiation-induced chronic inflammation model while loss of TNFR1 has no significant additional benefits beyond those naturally provided by TNFR2.

4. Discussion

In this study, we extend our earlier findings to show that elevated TNF α signaling has a robust and consistent inhibitory effect on neuron production in vitro. In testing a panel of cardinal pro-inflammatory cytokines on NSCs from mouse, monkey and human brain tissues, we found that the effects of several cytokines were dependent on the species of origin. For example, IL1 β generated a statistically significant reduction in neuron abundance in human or monkey, but not mouse NSCs. IL6 treatment had significant effects in monkey NSCs but only showed a trend toward a decrease in Dcx-positive human and mouse cells. TGF β 1 had the most significant inhibitory effects on mouse NSCs but not on monkey or

human cells. Of the cytokines tested, exogenously applied TNF α inhibited neurogenesis in all species and generated the largest effects.

Previous studies have shown that NSCs express TNFR1 and TNFR2 (Bernardino et al., 2008; Keohane et al., 2010). In our studies, loss of TNFR1 resulted in a substantial increase in the basal growth rate of neurospheres, increased the relative abundance of neurons, and prevented the negative effects of exogenous TNF α on neurogenesis (Fig. 2). Loss of TNFR1 also reduced the abundance of GFAP-positive cells in differentiating NSC cultures, suggesting that baseline TNFR1 signaling may have both selective and instructive influences that decrease the production and/or survival of young neurons and promote the accumulation of GFAP-positive cells. We also found that loss of TNFR2 decreased sphere growth as well as reduced the net yield of new neurons in vivo suggesting that TNFR2 is required for normal NSC growth and/or survival under baseline conditions (in the absence of exogenously added TNF α).

The application of exogenous TNF α decreased neurogenesis in vitro. In subsequent experiments we also tested whether direct injection of recombinant TNF α into the mouse hippocampus inhibited neurogenesis and found a small but significant reduction in total BrdU labeling and substantial reduction in the abundance of BrdU-labeled newborn neurons in TNF α injected animals relative to saline injected controls (Fig. 4). This suggests that high levels of TNF α can cause an acute decrease in neurogenesis and our in vitro data suggest that both NF- κ B signaling and caspase 3-mediated apoptotic pathway differentially contribute to the decrease in neuron abundance by acting selectively in undifferentiated progenitors vs. neuronal precursors, respectively (Fig. 3).

Contrary to our expectations, we found that endogenous TNF α signaling was protective in the irradiation-induced chronic inflammation model. Localized irradiation causes persistent microglial activation within the DG and our earlier work showed that attenuating inflammation with non-steroidal anti-inflammatory drugs partially restored neurogenesis. TNF α is one of several proinflammatory cytokines that is down-regulated by NSAIDs and we expected that the loss of TNF α or TNFR1 might be protective in this model. Instead, we found that the absence of TNF α had the opposite effect and resulted in a more pronounced loss of neurogenesis (Fig. 5E). Loss of TNFR2 yielded similar detrimental effects while the absence of TNFR1 had no measurable positive or negative effect on outcome. While it is clear that TNF α provides an important TNFR2-mediated protective effect, the specific roles of TNF α , TNFR1, and TNFR2 in vivo are more complex than the mechanisms that operate intrinsically within NSCs in vitro.

The in vitro data using TNFR1 $-/-$ and TNFR2 $-/-$ NSCs and the in vivo data using knockout mice indicate that TNFR1 and TNFR2 may have competing influences on NSC proliferation with endogenous levels of TNF α acting through TNFR1 to inhibit growth or acting through TNFR2 to promote growth. In adult infarct myocardium, TNFR1-mediated signaling is also deleterious and TNFR2-mediated signaling protective (Kishore et al., 2011). TNFR1 is also a key mediator of TNF α -induced sickness behavior in mice (Palin et al., 2009; Palin et al., 2007). Our data within the uninjured hippocampus is consistent with TNFR1 signaling providing a mild inhibitory effect and TNFR2 providing a mild stimulatory effect on neurogenesis (Figs. 5D and 5E). Loss of TNF α should affect both pathways. The net result yields a small but statistically significant increase in the number of BrdU-positive cells and abundance of new neurons, suggesting that TNFR1-mediated signaling may play a dominant role under baseline conditions. Golan et al. (Golan et al., 2004) also report that TNF α $-/-$ mice show improved performance in learning and memory tasks, which may correlate with the enhanced neurogenesis as observed in TNF α $-/-$ mice in our study. After injury and chronic microglial activation is induced in the irradiation model,

TNFR2-mediated signaling appears to take precedence over TNFR1 signaling. Other neuroinflammatory models may show similarities in this regard. For example, loss of both TNFR1 and TNFR2 leads to an aggravated pathology in an Alzheimer's disease mouse model (Montgomery et al., 2011), and infusion of TNF α antibody impairs the survival of stroke-generated neuroblasts in adult rat brain (Heldmann et al., 2005). These observations are consistent with a predominant protective role for TNFR2 signaling during recovery from neurological injury. In other inflammatory contexts, reducing TNF α can be beneficial. For example, blocking TNF α attenuates loss of dopaminergic neurons in a rat Parkinson's disease model (McCoy et al., 2006).

The contrasting effects of TNF α in different contexts may be related to known differences in signaling mechanisms between TNFR1 and TNFR2. TNFR1 can be activated by both soluble and membrane-bound TNF α while TNFR2 is fully activated only by membrane-bound TNF α (MacEwan, 2002). Although TNF α is mainly produced by glia or microglia in the central nervous system, NSCs are also able to synthesize TNF α (Covacu et al., 2009). Therefore, when NSCs differentiate in culture to generate mixed neuronal and glial lineages, low levels of both soluble and membrane-bound endogenous TNF α may be present and activate both TNF receptors. However, recombinant TNF α added in cell culture or infused into the DG is unlikely to efficiently activate TNFR2 and in both cases, would presumably act predominantly through TNFR1 to reduce the abundance of newborn neurons.

The in vitro data showing protection from exogenous TNF α in TNFR1 $^{-/-}$ but not TNFR2 $^{-/-}$ cells is consistent with a majority of the detrimental effects on neurogenesis being mediated by TNFR1. Nevertheless, it is not possible to exclude a potential detrimental effect from TNFR2-mediated signaling since loss of TNFR2 resulted in a significant increase in the fraction of cells that adopted a neuronal fate (Fig. 2I). Further studies applying engineered TNF α that can specifically activate TNFR2 (Fischer et al., 2011) may be helpful in elucidating the exact roles of TNFR1 vs. TNFR2 signaling intrinsic to the neural stem and progenitor cells as well as the more complex signaling that occurs in an injury context in vivo. Given the contrasting roles for TNFR1 and TNFR2 in these models, therapeutic strategies that target TNF α should be approached with caution and will need to consider the disease context as well as the relative importance of TNFR1 and TNFR2 signaling in the underlying disease or injury process.

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Highlights

We find that the immunomodulatory signaling of TNF α mediated by TNFR2 is more significant to radiation injury outcome than the proinflammatory signaling mediated by TNFR1.

Fig. 1. TNFα treatment decreases neuron production in mouse, human and monkey NSC cultures

(A) Treatment with conditioned medium of LPS-activated primary mouse microglia culture significantly inhibited differentiation of mouse NSCs to Dcx-positive cells. The percentage of Dcx-positive cells was normalized to sham-treated control culture. $t=3.28$; $df=4$; $p=0.03$. Ctl, control; MG-CM, conditioned medium from microglia culture. (B) Mouse NSCs were treated with individual cytokine in differentiation medium for 5 days. The proportion of cells positive for Dcx was scored. $F(5,12)=15.8$; $p<0.0001$. (C–D) Human (C) and Monkey (D) NSCs were cultured in differentiation medium with or without individual cytokines for 10 days and the fractions of cells positive for Dcx or Ki67 were scored. (C), Dcx: $F(5,34)=30.3$, $p<0.0001$; Ki67: $F(5,34)=4.5$, $p=0.002$; (D), Dcx: $F(5,17)=11.9$, $p<0.0001$; Ki67: $F(5,17)=11.3$, $p<0.0001$. (B–D), Dunnett comparison with control groups. * $p<0.05$; # $0.05<p<0.1$.

Fig. 2. Differential roles of TNFR1- and TNFR2-mediated signaling in vitro

(A) Wild-type, TNFR1^{-/-}, and TNFR2^{-/-} NSCs were grown as neurospheres with or without TNFα treatment and sphere sizes over a course of 6 days were plotted. The results of statistical analysis were listed in Table 1. (B) Representative pictures of NSCs with or without TNFα treatment on day 6. Scale bar: 50μm. (C–H) Deficiency of TNFR1 or TNFR2 alters cell cycle progression. NSCs of the different genotypes were subjected to flow cytometric analysis. (C) Cell debris was excluded on the FSC versus SSC plot. FSC, forward scatter; SSC, side scatter. (D) The gated cells were further analyzed on the PI-Area versus PI-Width plot to select for the singlets. (E) Standard cell cycle analysis was performed based on the histogram showing the proportion of cells at the G1, S, and G2/M phases. A

representative sample from the wild-type NSCs without TNF α treatment was shown. (F–H) The fractions of cells at G1 (F), S (G), and G2/M (H) phases were compared between each genotype with or without TNF α treatment. (I–J) NSCs were cultured as monolayer and differentiated for 5 days with or without TNF α treatment. The proportions of cells positive for Dcx (I), or GFAP (J) were scored. n=5 in figure A; n=3 in figures F–J. *p<0.05, compared between the indicated groups; ^ap<0.05, compared with vehicle-treated wild-type.

Fig. 3. TNFα reduces neuron production through activating NF-κB pathway in Nestin-positive cells as well as induced apoptosis of Dcx-positive cells

(A) NSCs were differentiated for 6 days in the presence of 20 ng/ml TNFα in combination with Caspase 3 inhibitor (2 μM, 6 days), or Caspase 9 inhibitor (2 μM, 6 days), or following SN50 treatment (25 μg/ml) for 30 mins. Treatment with SN50 or Caspase 3 inhibitor could reverse the inhibitory effect of TNFα on neuron production. $F(4,15)=7.7$; $p=0.0014$. Casp3 In, Caspase 3 inhibitor; Casp9 In, Caspase 9 inhibitor. (B) Representative pictures of Dcx-positive cells. (C) NSCs were differentiated for 3 days prior to addition of TNFα (20 ng/ml) for 30 or 60 mins. After TNFα treatment, NF-κB translocated from cytoplasm to nucleus, as evidenced by p65 staining. (D, E) Co-labeling of p65 with Nestin and Dcx revealed that

Nestin- but not Dcx-positive cells responded to TNF α treatment by activating NF- κ B signaling cascades. $F(2,6)=14.5$; $p=0.005$. (F, G) NSCs were differentiated for 3 days followed by treatment with TNF α for 8 hrs. The proportions of Dcx-, and Nestin-positive cells co-labeled with cleaved Caspase 3 were scored. TNF α treatment resulted in increased apoptosis of Dcx-positive cells. $t=4.93$; $df=4$; $p=0.008$. A representative picture of cells with TNF α treatment for 8 hrs was shown in F. * $p<0.05$, compared between indicated groups in (A) or with respective control groups in (E) and (G). $n=3-4$ in figures A, E and G. Scale bars: 20 μ m.

Fig. 4. TNFα infusion into the DG reduces neurogenesis

(A) Schematic representation of the experimental paradigms in vivo. (B) TNFα infusion into the DG significantly reduced the number of BrdU-labeled cells as well as (C) the number of BrdU-labeled cells that had turned into Dcx- and/or NeuN-positive neurons. * $p < 0.05$, compared with control groups. $n = 4-5$ in figures B and C.

Fig. 5. Effects of TNFR1- or TNFR2-mediated Signaling on hippocampal neurogenesis in radiation-induced inflammation

(A) Schematic representation of the experimental paradigm. (B, C) Representative pictures of Dcx-positive alone (B), Dcx/NeuN-double positive (C), and NeuN-positive alone (C) cells. (D) The numbers of BrdU-labeled cells in the DG were scored using stereology. Under control conditions without irradiation, TNFR1^{-/-} and TNFα^{-/-} mice showed an increased number of BrdU-positive cells and TNFR2^{-/-} mice exhibited a reduced number of BrdU-positive cells, compared with that of wild-type mice. In the irradiated mice, no significant difference was observed in the total number of BrdU-positive cells between the genotypes. (E) The numbers of BrdU-labeled cells that had turned into neurons (Dcx- and/or NeuN-positive cells) were scored. Under control conditions without irradiation, more new neurons in TNFR1^{-/-} and TNFα^{-/-} and fewer new neurons in TNFR2^{-/-} mice were observed. In the irradiated groups, TNFR2^{-/-} and TNFα^{-/-} mice exhibited a reduced number of new neurons, whereas TNFR1^{-/-} mice did not show a measurable change, compared to that of wild-type mice. *p<0.05, compared with naïve wild-type groups in figures D and E; ^ap<0.05, compared with irradiated wild-type group in figure E. n=8 in figures D and E. Scale bar: 10 μm.

Table 1

	Day 1	Day 2	Day 3	Day 4	Day 6
WT vs. R1-/-	*	ns	*	*	*
WT vs. R2-/-	ns	ns	ns	ns	*
WT vs. WT +TNFα	ns	*	*	*	*
R1-/- vs. R1-/- +TNFα	ns	ns	ns	ns	ns
R2-/- vs. R2-/- +TNFα	ns	*	*	*	*

Note: One way ANOVA followed by Bonferroni comparison.

* p<0.05;

ns, non-significant; WT, wild-type; R1-/-, TNFR1-/-; R2-/-, TNFR2-/-.