IL-13-Induced Oxidative Stress via Microglial NADPH Oxidase Contributes to Death of Hippocampal Neurons In Vivo

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IL-13-Induced Oxidative Stress via Microglial NADPH Oxidase Contributes to Death of Hippocampal Neurons In Vivo

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In the present study, we investigated the effects of IL-13, a well-known anti-inflammatory cytokine, on the thrombin-treated hippocampus in vivo. NeuN immunohistochemistry and Nissl staining revealed significant loss of hippocampal CA1 neurons upon intrahippocampal injection of thrombin. This neurotoxicity was accompanied by substantial microglial activation, as evident from OX-42 immunohistochemistry results. In parallel, Western blot analysis and hydroethidine histochemistry disclosed activation of NADPH oxidase, generation of reactive oxygen species, and oxidative damage in the hippocampal CA1 area showing hippocampal neuron degeneration. Interestingly, immunohistochemical and biochemical experiments showed that intrahippocampal injection of thrombin increased IL-13 immunoreactivity and IL-13 levels as early as 8 h after thrombin, reaching a peak at 7 days, which was maintained up to 14 days. Moreover, double-label immunohistochemistry revealed IL-13 immunoreactivity exclusively in activated microglia. IL-13-neutralizing Abs significantly rescued CA1 hippocampal neurons from thrombin neurotoxicity. In parallel, neutralization of IL-13 inhibited activation of NADPH oxidase, reactive oxygen species production, and oxidative damage. Additionally, IL-13 neutralization suppressed the expression of inducible NO synthase and several proinflammatory cytokines. To our knowledge, the present study is the first to show that IL-13 triggers microglial NADPH oxidase-derived oxidative stress, leading to the degeneration of hippocampal neurons in vivo, as occurs in cases of Alzheimer’s disease. The Journal of Immunology, 2009, 183: 4666 – 4674.

Microglia are major inflammatory cells in the CNS that are activated in response to brain injury (1, 2). As activated microglia gain neurotoxic functions through the production of neurotoxic and inflammatory mediators (1, 3), brain inflammation is closely associated with the pathogenesis of various neurodegenerative diseases, such as Alzheimer’s disease (AD) (4, 5). Several lines of evidence confirm microglial activation in the brains of AD patients (6) as well as in hippocampi of AD animal models following β-amyloid (Aβ) administration (7, 8).

Activated microglia produce reactive oxygen species (ROS), such as superoxide (O2•−) and O3•−-derived oxidants, which induce or exacerbate neurotoxicity by inflicting oxidative stress on cortical (9) and dopaminergic (10, 11), as well as spinal motor neurons (12). Previous studies additionally show that microglia-derived NADPH oxidase stimulates ROS production. NADPH oxidase is a multisubunit enzyme composed of cytosolic (gp47phox, p47phox, p67phox, and GTP-binding protein P21-Rac1) and plasma membrane (gp91phox and gp22phox) subunits that catalyze the generation of superoxide radicals from oxygen (13). Activation of NADPH oxidase leads to translocation of cytosolic proteins to the plasma membrane where they assemble with other membrane-associated proteins for activity.

Thrombin activates the NADPH oxidase complex in microglia and neurons, which in turn stimulates ROS production and oxidative stress, leading to death of hippocampal neurons in vivo and in vitro (14, 15). The brains of AD patients exhibit symptoms of oxidative stress, including oxidative modifications to proteins (16), lipids (17), and DNA (18). These findings collectively suggest that NADPH oxidase-mediated oxidative stress contributes to neurodegeneration in AD (5). This hypothesis is strongly supported by the finding that NADPH oxidase is activated in AD brains, resulting in ROS formation (19).

There is growing evidence suggesting that microvascular damage is involved in the neuropathogenesis of AD (20, 21). AD patients may have increased blood-brain barrier permeability, resulting in increased serum protein accumulation within the brain extracellular space, suggesting that certain blood-derived factors are capable of inducing various pathophysiological responses (22). Among blood-derived factors, thrombin is elevated, whereas the activity of the thrombin inhibitor protease nexin I is significantly reduced in AD brains (23, 24). In addition, thrombin induces cell death in hippocampal neurons (25, 26). Collectively, these observations indicate that thrombin may act as an endogenous neurotoxin, leading to hippocampal neurodegeneration that occurs in neurodegenerative diseases such as AD.

IL-13, a well-known anti-inflammatory cytokine, suppresses the production of inflammatory mediators, such as TNF-α, IL-1β, and IL-6 from activated microglia in vivo and in vitro (27–31). These results were obtained after the exogenous application of IL-13 as there is no evidence of endogenous IL-13 in microglia. However, our previous studies disclose endogenous IL-13 expression in activated microglia of LPS-treated rat cerebral cortices in vivo (32). Whereas several studies have reported beneficial effects of IL-13, such as increased survival of mice in an experimental model of sepsis (33), harmful effects of IL-13 are also documented. For instance, in a mouse asthma model, allergic airway inflammation is...
inhibited by treatment with an anti-IL-13 mAb (34, 35). Additionally, in endothelial cells, IL-13 induces ROS production via activation of NADPH oxidase (36). In the present study, we examine whether thrombin neurotoxicity is associated with endogenous IL-13 expression within activated microglia of the rat hippocampus in vivo. We further investigate whether IL-13 mediates ROS generation through activation of NADPH oxidase, consequently contributing to the degeneration of hippocampal neurons in vivo.

Materials and Methods

Stereotaxic surgery and drug injection

All surgical experiments were performed in accordance with Guidelines and Polices for Rodent Survival Surgery provided by the Animal Care Committee of the Kyung Hee University. Female Sprague Dawley rats (230–250 g) were anesthetized by injection of chloral hydrate (360 mg/kg i.p.) and positioned in a stereotaxic apparatus (David Kopf Instruments). A midline sagittal incision was made in the scalp and holes were drilled in the skull over dorsal hippocampus using the following coordinates: 3.6 mm posterior to bregma and 2.0 mm lateral to the midline for intrahippocampal injections according to the atlas of Paxinos and Watson (37). The hole of the tip was directed down to 2.6 mm beneath the surface of the brain for the hippocampus. All injections were made using a Hamilton syringe equipped with a 30-gauge beveled needle and attached to a syringe pump (KD Scientific). Infusions were made at a rate of 0.2 μl/min for 1 h at room temperature in avidin-biotin complex solution (Vector Laboratories). Sections were rinsed again and incubated for 1 h at room temperature in 1/200 biotin-conjugated anti-mouse Ab in PBS containing 0.5% BSA. Sections were then rinsed in PBS and incubated for 1 h with 1/200 biotin-conjugated anti-mouse Ab in PBS containing 0.5% BSA. Sections were then rinsed in PBS containing 0.5% BSA and the following monoclonal primary Abs: OX-42 (R&D Systems) (32, 33) or nonspecific goat IgG as a control (1 mg/ml for goat Ab). Sandwich ELISA was then performed in accordance with the manufacturer’s instructions (BioSource International).

Measurement of IL-13

The production of IL-13 from rat hippocampus was determined by sandwich ELISA techniques. Tissue were homogenized in 400 μl of ice-cold radioimmunoassay precipitation buffer (60 mM NaCl, 0.1% SDS, 1% Non-Idet P-40, 0.5% sodium deoxycholic acid, and 50 mM Tris, pH 8.0) and centrifuged at 14,000 × g at 4°C for 20 min. Equal amounts of protein (150 μg) from each sample were placed in ELISA kit strips coated with the appropriate Ab. Sandwich ELISA was then performed according to the manufacturer’s instructions (BioSource International).

RT-PCR

Brain tissues from the ipsilateral hippocampus were dissected at the indicated time points after injection, and total RNA was extracted in a single step using RNAzol B (Tel-Test) following the instructions of the manufacturer. Total RNA was reverse transcribed into cDNA using Superscript II (Invitrogen) and random primers (Promega). The primer sequences used in this study were as follows: 5′-TGAGT TGC CAT TAG ACA GC-3′ (forward) and 5′-GAG GTG CTG ATG TAC CAG TT-3′ (reverse) for IL-1β; 5′-GTA GCC CAC GTC GTA GCA AA-3′ (forward) and 5′-CCC TTC TCC AGC TGG GAG AC-3′ (reverse) for TNF-α; and 5′-GCA GAA TGT GAC CAT GG-3′ (forward) and 5′-ACA TTT GAT ACA GAC TTG TTG AGC GA G-3′ (reverse) for GAPDH. The PCR amplification consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s (for IL-1β, TNF- α, and iNOS), and extension at 72°C for 90 s. PCR products were separated by electrophoresis on 1.5% agarose gels, after which the gels were stained with ethidium bromide and photographed. For semiquantitative analyses, the photographs were scanned using the Computer Imaging Device and accompanying software (Fujifilm).

Western blot analysis

Brain tissues from the ipsilateral hippocampus were dissected and homogenized in ice-cold lysis buffer containing the following (in mM): 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, 0.1 mM PMSF, and protease inhibitor mixture (Sigma-Aldrich). The tissue homogenates were centrifuged at 4°C for 20 min at 14,000 × g, and the supernatant was transferred to a fresh tube. The extracts were frozen and kept at −80°C for subcellular fractionation. Protein extracts of both the cytosolic and plasma membrane fractions were prepared from the ipsilateral hippocampus at the indicated time points after injection. Tissues were gently homogenized using a glass homogenizer in ice-cold buffer consisting of the following (in mM): 20 mM HEPES, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl2, 2 mM EDTA, and protease inhibitor mixture (Sigma-Aldrich).

Homogenates were centrifuged at 550 × g for 10 min at 14,000 × g, and the supernatants were collected and centrifuged for 20 min at 130,000 × g at 4°C. The pellets were further centrifuged for 1 h at 100,000 × g at 4°C, and the resulting supernatants and pellets were designated as the cytosolic and plasma membrane fractions, respectively. Equal amounts of protein (50 μg) were mixed with loading buffer (0.125 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% 2-ME, and 0.002% bromophenol blue), boiled for 5 min, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore) using an electrophoretic transfer system (Bio-Rad). The membranes were washed with TBS solution and then blocked for 1 h in TBS containing 5% skim
milk. The membranes were then incubated overnight at 4°C with one of the following specific primary Abs: rabbit anti-iNOS (1/1000; Upstate Biotechnology), mouse anti-p65<sub>phox</sub> (1/500; BD Biosciences), and rabbit anti-p47<sub>phox</sub> (1/200; Santa Cruz Biotechnology). After washing, the membranes were incubated for 1 h at room temperature with secondary Abs (1/2000; Amersham Biosciences) and washed again. Finally, the blots were developed with ECL detection reagents (Amersham Biosciences). The blots were reprobed with Abs against β-actin (1/4000; Santa Cruz Biotechnology).

To determine the relative degree of membrane purification, the membrane fraction was subjected to immunoblotting for calnexin, a membrane marker, using a rabbit polyclonal Ab against calnexin (1/4000; Santa Cruz Biotechnology). Detection of protein oxidation

The extent of protein oxidation was assessed by measuring protein carbonyl levels with an OxyBlot protein oxidation detection kit (Chemicon International) according to the protocol of the manufacturer as described previously (15). Protein samples were prepared from rat brains harvested 48 h after injection. Subsequently, protein samples (15 μg) were mixed in a microcentrifuge tube with 5 μl of 12% SDS and 10 μl of 1× 2,4-dinitrophenylhydrazine (DNPH) solution. Ten microliters of 1× neutralization solution (a kit component) was added instead of the DNPH solution as the negative control. Tubes were incubated at room temperature for 15 min and then mixed with 7.5 μl of neutralization solution. Next, the samples were mixed in equal volumes of SDS sample buffer and separated by SDSPAGE. After electrophoresis, proteins were transferred to polyvinylidenefluoride membranes (Millipore). The membranes were then blocked for 1 h at room temperature in TBS containing 0.1% Tween 20 and 1% BSA. Membranes were incubated overnight at room temperature with the anti-DNPH Ab (1/150) and then incubated at room temperature for 1 h with secondary Abs (1/300). Blots were developed using ECL reagents (Amer-

sham Biosciences). Proteins that underwent oxidative modification (i.e., carbonyl group formation) were identified as a band in the samples deri-

vated with DNPH. The OD of the bands was measured using the Comuter Imaging Device and accompanying software (Fujifilm). Levels of protein carbonyls were quantitated and expressed as the fold increase vs untreated controls.

**Counting of hippocampal CA1 neurons**

The number of CA1 neurons was assessed at three levels of the dorsal hippocampus. Specifically, alternate sections were obtained at 3.3, 3.6, 4.16, and 4.3 mm posterior to the bregma and two regions from each level (n = 8 regions for each animal) were used to count cells in the CA1 region. The number of neurons within the CA1 layer was counted using a light microscope (Olympus Optical) at a magnification of ×400 and expressed as the number of CA1 neurons per millimeter of linear length as described previously (15). To maintain consistency across animals, a rectangular box (0.5 × 0.05 mm) was centered over the CA1 cell layer beginning 1.5 mm lateral to the midline. Only neurons with normal visible nuclei were counted. The mean number of CA1 neurons per millimeter for both hemi-

spheres was calculated for each treatment group.

**Statistical analysis**

All values are expressed as mean ± SEM. Statistical significance (p < 0.05 for all analyses) was assessed by ANOVA using Instat 3.05 (Graph-Pad Software) followed by Student-Newman-Keuls analyses.

**Results**

**Thrombin induces neurodegeneration in the hippocampus in vivo**

Thrombin (20 U/μl) or vehicle (PBS) control was unilaterally injected into the CA1 layer of the rat hippocampus. Seven days later, brains were removed and coronal sections were processed for NeuN and Nissl immunostaining. Neurons were labeled with an Ab against a nuclear protein (NeuN). Significant loss of NeuN-immunopositive (NeuN-ip) neurons was evident in the thrombin-

pered CA1 layer of the hippocampus (Fig. 1B) compared with the PBS-treated CA1 layer (Fig. 1A). Thrombin-induced neurodegeneration was further confirmed by staining for Nissl using sections adjacent to those used for NeuN immunostaining. Consistent with NeuN immunostaining data, a dramatic reduction in Nissl-stained cells was evident in the CA1 area of the hippocampus in thrombin-treated animals (Fig. 1D) compared with PBS-treated animals (Fig. 1C).

**Microglial activation and expression of IL-13 in thrombin-injected hippocampus**

Commercial thrombin contains impurities responsible for microglial activation (40, 41). Hirudin, a thrombin inhibitor, blocks thrombin-induced neuronal death in rat mesencephalic (39), hippocampal (supplemental Fig. 1E), and human fetal neu-

rons (42) in cultures deprived of microglia and enhances the survival of hippocampal neurons after ischemia (26). The data suggest that thrombin induces neuronal death, which indirectly triggers microglial activation (11, 43, 44). Therefore, it is logical to infer that activation of microglia as a consequence of neuronal injury represents a risk factor that triggers the onset of a cascade of events leading to the ongoing neurodegeneration of hippocampal neurons in vivo. Accordingly, we examined microglial activation following neuronal degeneration in the CA1 area of the hippocampus injected with thrombin in vivo (supplemental Fig. 2). Rat brain sections were obtained 24 h after injection and immunostained with OX-42 Abs recognizing complement receptor 3. In the PBS-injected CA1 layer of the hippocampus, OX-42-immunopositive microglia exhibited the typical ramified morphology of resting microglia (Fig. 2, A and B). In contrast, in the thrombin-injected hippocampus, the majority of OX-42-immunopositive microglia displayed activated morphology, including larger cell bodies with short, thick, or no processes (Fig. 2, C and D).

Next, we investigated whether thrombin might induce IL-13 protein expression in the hippocampal CA1 layer. Immunohis-


tochemical analysis revealed high expression of IL-13 (Fig. 2, 


<sup>4</sup> The online version of this article contains supplemental material.
E–H) as early as 8 h after thrombin injection, which was maintained up to 14 days, in the hippocampal CA1 layer (Fig. 2. E–H). The IL-13 level was measured after thrombin injection using the sandwich ELISA technique. Consistent with immunohistochemical evidence, IL-13 levels in the hippocampus reached a peak at 7 days and remained high for up to 14 days after thrombin. At 7 days postinjection, the IL-13 level was ~13.8-fold (Fig. 2f; \( p < 0.001 \)) higher than that in PBS-injected (24 h postinjection) and intact (undetectable; data not shown) hippocampus controls.

Localization of IL-13 within activated microglia in the hippocampus in vivo

To identify cells expressing IL-13 in the hippocampal CA1 layer, double immunofluorescence staining with tomato lectin (Fig. 3A) for microglia and IL-13 Abs (Fig. 3B), or glial fibrillary acidic protein (Fig. 3D) for astrocytes and IL-13 Abs (Fig. 3E), was performed at 24 h after thrombin injection. The fluorescence image from each channel was merged from the same double-labeled section (Fig. 3, C and F). These results indicate that thrombin-induced IL-13 expression is localized exclusively within activated microglia (supplemental Fig. 3).

**IL-13 contributes to neurodegeneration in the hippocampus in vivo**

To establish the role of IL-13 in thrombin-induced neurodegeneration in vivo, we investigated whether IL-13-neutralizing Ab (NA) might affect the neurodegenerative effects of thrombin in the hippocampal CA1 layer. NeuN immunohistochemistry (Fig. 4, A, C, and E) and Nissl staining (Fig. 4, B, D, and F) were performed 7 days after intrahippocampal coinjection of thrombin and IL-13 NA. A combination of IL-13 NA (Fig. 4, E and F) and thrombin partially protected neurons in the hippocampal CA1 layer against thrombin-induced toxicity (Fig. 4, C and D). Estimation of the percentage of NeuN-ip cells on the ipsilateral vs the contralateral side disclosed that treatment with IL-13 NA increased the number of NeuN-ip neurons in the CA1 layer of the hippocampus by ~40% (\( p < 0.001 \); Fig. 4G). As a control, injection of nonspecific goat IgG with thrombin had little effect, similar to that observed with injection of thrombin only.

**IL-13 contributes to the expression of proinflammatory cytokines and iNOS in the hippocampus in vivo**

We previously demonstrated that thrombin induces microglia-derived proinflammatory cytokines, such as TNF-\( \alpha \), IL-1\( \beta \), and iNOS, in the hippocampus (15). Accumulating evidence (including our results) shows that activated microglia-derived proinflammatory and/or cytotoxic factors, such as TNF-\( \alpha \), IL-1\( \beta \), and iNOS, contribute to neurodegeneration in vivo (39, 45–47). Accordingly, we examined whether thrombin-induced expression of IL-1\( \beta \), TNF-\( \alpha \), and iNOS in the hippocampus and, consequently, neuronal
survival might be affected by IL-13 NA. For this purpose, animals were killed at 8 h after intrahippocampal injection and brain tissues were dissected and prepared for RT-PCR analysis. We selected the 8-h postthrombin time point since thrombin-induced expression of TNF-α, IL-1β, and iNOS in the hippocampus was evident at this time (15). RT-PCR data showed that IL-13 NA attenuated thrombin-induced mRNA expression of TNF-α by 52% \( (p < 0.01) \), IL-1β by 60% \( (p < 0.01) \), and iNOS by 51% \( (p < 0.001) \) in the hippocampus (Fig. 5, A and B). Additional Western blot data showed that at 12 h after hrombin, IL-13 NA inhibited thrombin-induced expression of iNOS by 75% \( (p < 0.001) \) in the hippocampus (Fig. 5, C and D). To establish the cellular localization of TNF-α, IL-1β, and iNOS, brain sections were examined by double immunofluorescence staining using the appropriate Abs and to-

**FIGURE 4.** IL-13 mediates thrombin-induced neuronal death in the hippocampus in vivo. A–F, PBS (A and B), thrombin (C and D; 20 U/4 μl) only, or thrombin plus IL-13 NA (E and F; 1 μg/μl) was unilaterally injected into the hippocampus. Animals were sacrificed 7 days after injection. The brain sections were processed for NeuN immunostaining (A, C, and E) or Nissl staining (B, D, and F). Scale bar, 1 mm. Insets show magnified photomicrographs of the area in the CA1 layer marked by dotted rectangles. G, Number of NeuN-ip neurons in the CA1 layer of hippocampi treated with thrombin in either the absence or presence of IL-13 NA (1 μg/μl) or nonspecific IgG. Error bars represent the means ± SEM from six samples per group. *, \( p < 0.001 \) compared with PBS and ***, \( p < 0.001 \) compared with hippocampi treated with thrombin only (ANOVA and Student-Newman-Keuls analyses).

**FIGURE 5.** IL-13 contributes to the expression of proinflammatory cytokine and iNOS in vivo. A, RT-PCR analysis showing mRNA expression of TNF-α, IL-1β, and iNOS. Total RNA was isolated from the ipsilateral hippocampus at 8 h after PBS, thrombin (20 U/4 μl), or coinjection of thrombin with IL-13 NA (1 μg/μl). B, Graphic representation of the mean ± SEM of six samples. *, \( p < 0.001 \) compared with control; †, \( p < 0.01 \); and #, \( p < 0.001 \) compared with thrombin-only treated samples according to ANOVA and Student-Newman-Keuls analyses. C, Western blot analysis of iNOS expression. Tissue lysates from the ipsilateral hippocampus were prepared at 12 h after injection with the same combination adjacent to A. D, Graphic representation of the mean ± SEM of five samples. *, \( p < 0.001 \) compared with control and †, \( p < 0.001 \) compared with hippocampi treated with thrombin only (ANOVA and Student-Newman-Keuls analyses). E–G, Double immunohistochemistry showing the localization of TNF-α (E), IL-1β (F), and iNOS (G) in activated microglia stained with tomato lectin (green) at 12 h after thrombin injection. Scale bar, 20 μm.

IL-1β (Fig. 5F), and iNOS (Fig. 5G) were localized in tomato lectin-positive microglia at 12 h after thrombin injection.

**IL-13 contributes to ROS production via NADPH oxidase in the hippocampus in vivo**

Previously, we demonstrated that thrombin induced ROS production through NADPH oxidase activation, and this resulted in neurodegeneration in the rat hippocampus and substantia nigra (15, 45). A number of studies have shown that IL-13 triggers ROS production via NADPH oxidase activation in endothelial cells (36, 48). Accordingly, we assessed whether IL-13 might contribute to ROS generation via activation of NADPH oxidase. Consistent with an earlier report by our group (15), in situ analysis of ROS production using hydroethidine histochemistry revealed a significant increase in the fluorescent oxidized hydroethidine products (i.e., ethidium; red fluorescence) 48 h after thrombin injection (Fig. 6B) compared with PBS-injected controls (Fig. 6A). In contrast, IL-13 neutralization led to a dramatic reduction in the accumulation of thrombin-induced ethidium (Fig. 6C). The results indicate that IL-13 plays a role in ROS generation in the hippocampus in vivo.
NADPH oxidase is a major source of ROS in microglia, particularly in the hippocampus (15). Next, we analyzed whether IL-13-induced ROS generation is mediated by activation of microglial NADPH oxidase in the thrombin-treated hippocampus in vivo. Specifically, we monitored the translocation of NADPH oxidase cytosolic subunits, p67phox and p47phox, from the cytosol to the plasma membrane. Western blot analyses revealed that IL-13 neutralization led to reduced levels of p67phox and p47phox in both the cytosolic and plasma membrane fractions compared with thrombin-injected samples (Fig. 6D), indicating that translocation and activation of the NADPH oxidase complex is mediated by IL-13. Quantification and expression as a ratio of the membrane fraction to total protein disclosed that IL-13 NA inhibits expression of p67phox by 62% and p47phox by 38% (Fig. 6E). In line with our recent findings (15), double immunofluorescence staining and confocal microscopy showed that immunoreactivity to p67phox (Fig. 6, F–H) and p47phox (Fig. 6, I–K) localized to activated microglia within the CA1 layer of the hippocampus 24 h after treatment with thrombin.

To determine the extent of oxidative damage to proteins, we analyzed the protein carbonyl levels after thrombin injection, with or without IL-13 NA, into the hippocampus (15, 49). Carbonyl levels were assessed by Western blotting, and band intensities were compared. The increased protein carbonyl level in thrombin-injected samples was inhibited by 56% within 48 h after the intrahippocampal injection of IL-13 NA (Fig. 6C). Further evidence for the involvement of IL-13 in oxidative stress-related neurotoxic events was obtained from data showing that an IL-13-neutralizing Ab (NA) reduced neuronal loss in the hippocampus in vivo by inhibiting NADPH oxidase activity in the hippocampus in vivo is accompanied by IL-13 up-regulation within activated microglia. Microglia-derived IL-13 activates NADPH oxidase, resulting in ROS production and subsequent oxidative modification of proteins (supplemental Fig. 4). Further evidence for the involvement of IL-13 in oxidative stress-related neurotoxic events was obtained from data showing that an IL-13-neutralizing Ab (NA) reduced neuronal loss in the hippocampus in vivo by inhibiting NADPH oxidase activation and ROS production, thus affording eventual attenuation of oxidative damage. In addition, IL-13 mediated microglia-derived expression of proinflammatory cytokines and iNOS. The present results are the first to demonstrate that endogenous IL-13 contributes to neurodegeneration. Moreover, this neurotoxicity is mediated by oxidative stress via activation of microglia-specific NADPH oxidase, proinflammatory cytokines, and iNOS in the hippocampus.

**Discussion**
In the present study, we show that thrombin-induced neurotoxicity in the hippocampus in vivo is accompanied by IL-13 up-regulation within activated microglia. Microglia-derived IL-13 activates NADPH oxidase, resulting in ROS production and subsequent oxidative modification of proteins (supplemental Fig. 4). Further evidence for the involvement of IL-13 in oxidative stress-related neurotoxic events was obtained from data showing that an IL-13-neutralizing Ab (NA) reduced neuronal loss in the hippocampus in vivo by inhibiting NADPH oxidase activation and ROS production, thus affording eventual attenuation of oxidative damage. In addition, IL-13 mediated microglia-derived expression of proinflammatory cytokines and iNOS. The present results are the first to demonstrate that endogenous IL-13 contributes to neurodegeneration. Moreover, this neurotoxicity is mediated by oxidative stress via activation of microglia-specific NADPH oxidase, proinflammatory cytokines, and iNOS in the hippocampus.

![Image](http://www.jimmunol.org/)

**FIGURE 6.** IL-13 contributes to activation of NADPH oxidase and generation of ROS. A–C, In situ visualization of $O_2^-$ and $O_2^{-}$-derived oxidant production. Animals were injected with hydroethidine 48 h after intrahippocampal injection of PBS (A) or thrombin (20 U/4 μl; B) in the absence or presence of IL-13 NA (1 μg/μl). Confocal micrographs show ethidium fluorescence (red). Nuclei were counterstained with Hoechst 33258 (blue). Scale bar, 50 μm. D, NADPH oxidase is activated by thrombin, as evidenced by the translocation of the p67phox and p47phox subunits from the cytosol to the plasma membrane; this translocation was inhibited by IL-13 NA. Tissue lysates from the ipsilateral hippocampus were prepared 24 h after intrahippocampal injection of PBS or thrombin (20 U/4 μl) in the absence or presence of IL-13 NA (1 μg/μl). Fractionated proteins were analyzed by SDS-PAGE and subjected to immunoblotting with anti-p67phox and p47phox Ab. The blots were reprobed with Abs against the calnexin membrane protein as loading controls and to exhibit fractionation efficiency. E, The histogram shows quantitation of p67phox and p47phox levels expressed as the ratio of membrane fraction:total. The results represent the means ± SEM of four to five separate experiments. *, p < 0.001, compared with control; †, p < 0.001, compared with thrombin only (ANOVA and Student-Newman-Keuls analyses). F–K, Localization of p67phox and p47phox immunoreactivity within activated microglia in thrombin-treated rat hippocampus. The sections of hippocampus were prepared 24 h after thrombin injection (20 U/4 μl) and stained with an Ab against p67phox (G) or p47phox (J) and tomato lectin (T.L.; F and I) for microglia. Confocal images were captured from the same area and merged (H and K). Scale bar, 25 μm. L, Animals were decapitated 48 h after intrahippocampal injection of thrombin (20 U/4 μl) in the absence or presence of IL-13 NA (1 μg/μl). Samples were analyzed by Western blotting for protein carbonyls as markers of oxidatively modified proteins. M, Bars represent the means ± SEM of four separate samples. *, p < 0.001, compared with control and †, p < 0.01, compared with hippocampi treated with thrombin only (ANOVA and Student-Newman-Keuls analyses).
IL-13, a well-known anti-inflammatory cytokine, suppresses the production of inflammatory mediators, including ROS, NO, IL-1β, IFN-γ, and TNF-α, from activated microglia in vivo and in vitro (27–31). Moreover, human rIL-13-secreting cells inhibited the development of experimental autoimmune disease in a demyelinating disease model of the CNS (28). In an experimental model of sepsis, IL-13 contributed to survival of mice by suppressing the excessive production of inflammatory cytokines and chemokines, including TNF-α and MIP-1α (33). IL-13 additionally controlled brain inflammation by reducing the iNOS and TNF-α levels in LPS-injected rat cerebral cortices in vivo, resulting in enhanced neuronal survival (32).

Notably, harmful effects of IL-13 have been reported. In a mouse asthma model, treatment with an anti-IL-13 mAb prevented progression of inflammation by inhibiting the expression of cytokines and chemokines, such as TNF-α and MIP-1α (34, 35). In addition, in endothelial cells, IL-13 induced NADPH oxidase activation and consequent production of ROS (36), possibly leading to oxidative damage and eventual cell death. The data imply that IL-13 contributes to neurotoxicity by stimulating NADPH oxidase activity for ROS production. This hypothesis is strongly supported by our current data showing that IL-13 neutralization inhibits ROS generation in thrombin-treated hippocampi by limiting oxidative damage caused by microglia-derived NADPH oxidase, eventually promoting neuronal survival.

Microglia-derived proinflammatory cytokines and cytotoxic factors are involved in thrombin-induced neurotoxicity. The levels of IL-1β (50) and TNF-α (51) are up-regulated in AD brains. Moreover, NO formation catalyzed by iNOS may be involved in the pathological processes of AD (52). Recent reports show the involvement of such factors, including TNF-α, IL-1β, and iNOS, in thrombin-induced neurodegeneration in substantia nigra (45, 53) and hippocampus (15) in vivo. Moreover, inhibition of TNF-α, IL-1β, and iNOS leads to neuronal survival. In the present study, we show that IL-13 neutralization inhibits expression of iNOS and proinflammatory cytokines after thrombin injection. Our results collectively suggest that blockade of IL-13 reduces proinflammatory cytokine expression, which results in neuronal survival in the hippocampus in vivo. Our data are strongly supported by earlier reports that treatment with an anti-IL-13 mAb inhibits expression of cytokines and chemokines, such as TNF-α and MIP-1α, in a mouse asthma model (34, 35).

It seems noteworthy that these results might be irrelevant for our previous report showing IL-13 effects on cell death of microglia, resultant neuronal survival (32). This study showed that IL-13 possibly controls brain inflammation by inducing death of activated microglia, resulting in the reduction of iNOS and TNF-α expression and eventual enhancement of neuronal survival in the LPS-treated cortex in vivo. In contrast, the current
data show that IL-13 mediates the production of proinflammatory cytokines (TNF-α and IL-1β), iNOS, and NADPH oxidase-derived ROS, leading to neurodegeneration in the hippocampus in vivo. Although the underlying mechanisms are yet to be clarified, these apparent discrepancies may be attributed to the use of different stimuli (LPS vs thrombin) and/or target areas (cortex vs hippocampus). This interpretation is further supported by our unpublished observation that IL-13 expression within activated microglia was observed in both thrombin-treated cortex and LPS-treated hippocampus in a time-dependent manner. As previously reported (32), cell death of microglia was only detected in LPS-treated cortex, as evident from OX-42 immunocytochemical staining (data not shown).

To our knowledge, the present study is the first to demonstrate the possible involvement of IL-13 expressed endogenously in activated microglia after thrombin injection in the degeneration of hippocampal neurons. A number of studies, including ours, confirm the in vivo and in vitro neurotoxicity of thrombin on human fetal (42), dopaminergic (39, 45), cortical (54), and hippocampal neurons (14, 15, 26). Importantly, thrombin is increased, whereas the activity of the thrombin inhibitor protease nexin I sharply reduced in the brain of AD patients (24, 55, 56). Moreover, thrombin modulates the production of amyloid protein precursors and their cleavage into fragments detected in the amyloid plaques of AD brains (57, 58). The current findings, in combination with thrombin neuromodulation, neurotoxicity and microglial activation, suggest that the deleterious effects of microglia-derived endogenous IL-13 are involved in oxidative stress-mediated neurodegenerative diseases, such as AD.

Disclosures

The authors have no financial conflict of interest.

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