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Cutting Edge



Cutting Edge: IL-13Rα1 Expression in Dopaminergic Neurons Contributes to Their Oxidative Stress–Mediated Loss following Chronic Peripheral Treatment with Lipopolysaccharide

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Inflammation and its mediators, including cytokines and reactive oxygen species, are thought to contribute to neurodegeneration. In the mouse brain, we found that IL-13R α 1 was expressed in the dopaminergic (DA) neurons of the substantia nigra pars compacta, which are preferentially lost in human Parkinson's disease. Mice deficient for Il13ra1 exhibited resistance to loss of DA neurons in a model of chronic peripheral inflammation using bacterial LPS. IL-13, as well as IL-4, potentiated the cytotoxic effects of t-butyl hydroperoxide and hydrogen peroxide on mouse DA MN9D cells. Collectively, our data indicate that expression of IL-13R α 1 on DA neurons can increase their susceptibility to oxidative stress-mediated damage, thereby contributing to their preferential loss. In humans, Il13ra1 lies on the X chromosome within the PARK12 locus of susceptibility to Parkinson's disease, suggesting that IL-13R α 1 may have a role in the pathogenesis of this neurodegenerative disease. The Journal of Immunology, 2012, 189: 000-000.

arkinson's disease (PD) is the second most common neurodegenerative disorder and affects ~1% of the population >60 y of age (1). Clinical symptoms are thought to arise primarily from the progressive reduction of dopamine signaling in the basal ganglia resulting from the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). Current treatments for PD focus on symptom management usually through the use of levadopa to increase brain dopamine levels. There are currently no therapies to address the underlying neurodegeneration. Genetic studies

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have identified at least nine rare, monogenic causes of PD and >13 loci (2). Most PD cases, however, remain sporadic and idiopathic with environmental factors, including exposure to pesticides and toxins, thought to contribute to disease development in these cases (3).

Recently, inflammation and its mediators, including cytokines and reactive oxygen species (ROS), were also proposed to contribute to the neuronal loss occurring in idiopathic PD (4). However, how these factors might cause the preferential loss of DA neurons observed in PD is not entirely understood. By examining the location of genes encoding immunoregulatory factors with Mendelian linkage to neurodegenerative diseases, we observed that the IL-13R α 1 gene (*Il13ra1*) lies at Xq24 within the PARK12 locus of PD susceptibility on the human X chromosome (5, 6).

IL-13R α 1 is recognized for its role in mediating allergic responses. It has been demonstrated by ablation of *Il13ra1* in mice that IL-13R α 1 is essential for allergen-induced airway hyperresponsiveness, mucus hypersecretion, and eotaxin production (7, 8). IL-13R α 1 heterodimerizes with IL-4R α , forming a complex capable of binding IL-13 or IL-4. To date, this complex is the only known signal transducer for IL-13, whereas IL-4 can also signal through an IL-4R α / γ -chain complex. IL-13 and IL-4 receptor binding results in the initiation of a gene program by phosphorylation of the transcription factor STAT6 via JAK (9).

To test the possibility that IL-13R α 1 may have a role in PD, we compared the effects of chronic peripheral inflammation with bacterial LPS on DA neuronal loss in the SNpc of mice null for IL-13R α 1 and their wild-type littermates. We also tested the effects of IL-13 and IL-4 on DA neuronal loss in vitro.

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Abbreviations used in this article: Ct, cycle threshold; DA, dopaminergic; GFAP, glial fibrillary acidic protein; LDH, lactate dehydrogenase; PD, Parkinson's disease; ROS, reactive oxygen species; SNc, substantia nigra pars compacta; tBOOH, *i*-butyl hydroperoxide; TH, tyrosine hydroxylase; VTA, ventral tegmental area; wt, wild-type.

Materials and Methods

Animals

Mouse husbandry and procedures were performed under the guidelines of the Institutional Animal Care and Use Committee. *Il13ra1*-null mice on the C57BL/6 background were obtained from Regeneron Pharmaceuticals (7). In these animals the disrupted *I13Ra1* contains a LacZ reporter cassette that can be used for β -galactosidase staining. As in humans, mouse *I13ra1* also localized to the X chromosome. All experiments were carried out on male null mice (*Il13ra^{-/Y}*) and wild-type littermates (wt or *Il13ra^{+/Y}*).

In situ hybridization

In situ hybridization was carried out as previously described by us (10) using a digoxigenin-UTP probe kit (Roche). *Il13ra1* RNA was isolated by PCR using the following primers: forward, 5'-TTCTTGCCGACGCTGTCT-3', reverse, 5'-GGGTCC TCAAACAATAGGCA-3'.

β -galactosidase reporter assay

Mouse brains containing a *lacz*-interrupted *Il13ra1* gene were harvested, cryosectioned to 30 μ m, and placed onto slides. Sections were fixed with 0.125% glutaraldehyde, permeabilized with 0.01% sodium deoxycholate and 0.02% Nonidet P-40, and signal was detected by incubating with 1 mg/ml 5-bromo-4-chloro-3-indolyl β -D-galactoside at 30°C for 4 h while rocking. Slides were washed, dried, and mounted.

Immunohistochemistry

Mouse brains were harvested, fixed, and cryoprotected as described above. Free-floating brain sections were permeabilized with 0.2% Triton X-100, washed, and probed with primary Abs against tyrosine hydroxylase (TH; 1:1000; Millipore), Iba1 (1:300; Wako Chemicals), glial fibrillary acidic protein (GFAP; 1:300; Dako), or LacZ (1:300; MBL International) overnight at 4°C. TH DAB staining and neuronal counting was carried out as previously described by us (11, 12).

For immunofluorescence, sections were incubated with streptavidin-Alexa 596 (1:500; Invitrogen) for LacZ detection or with Alexa 488 secondary Ab (1:500; Invitrogen) for TH, Iba1, and GFAP, washed, incubated with DAPI at 1 ng/ml, washed, mounted, and visualized with a Zeiss Radiance 2100 confocal system.

Chronic inflammatory model

Mice were injected (i.p.) twice weekly with saline or bacterial LPS (100 μ g/kg from *Escherichia coli* O111:B4; Sigma-Aldrich) for 6 mo as previously described (13).

Cell viability assays

The mouse DA MN9D cells were obtained from Dr. Alfred Heller (University of Chicago) and were maintained at 37°C with 5% CO_2 and cultured in collagen-coated plates in DMEM with 10% FCS. For assessment of oxidative damage, the cells were treated with PBS or IL-13 or IL-4 at the indicated doses in the presence of PBS, H_2O_2 (80 µM), or *i*-butyl hydroperoxide (tBOOH; 10 µM). Cell viability was assayed 24 h later by the lactate dehydrogenase (LDH) assay (Roche) according to the manufacturer's instructions. Results are the average of at least three independent experiments performed in triplicate.

Real-time PCR

RNA was isolated, purified, quantified, and used as a template for cDNA as previously described (10). A dilutional analysis of the samples indicated a correlation coefficient for concentration and cycle threshold (Ct) of 0.99. Reactions were performed on a 7900HT Fast real-time PCR machine (Applied Biosystems) using SYBR Green (Invitrogen Life Technologies). The Δ Ct method was performed to determine relative concentrations, using the Ct of GAPDH as the normalizing value for calculation of relative values ($2^{\Delta Ct}$). Primers used were as follows: IL-13, forward, 5'-CTT GCC TTG GTG GTC TCG-3', reverse, 5'-CGT TGC ACA GGG GAG TCT-3'; IL-4, forward, 5'-CAT CGG CAT TTT GAA CGA G-3', reverse, 5'-CGA GCT CAC TCT CTG TGG TG-3'.

Statistical analysis

One-factor ANOVA followed by a Dunnett post hoc test was used to determine significance (p < 0.05).

Results

Il13ra1 is expressed by DA neurons in the SNc

To map the expression of *Il13ra1* in the brain, we performed in situ hybridization of Il13ra1 using RNA antisense and sense control probes on wt and Il13ra1 knockout mouse brain sections (Fig. 1A, 1B). Il13ra1 mRNA was detected in the SNc and the ventral tegmental area (VTA), but not in the glomerular layer of the olfactory bulb containing DA neurons or in the locus coeruleus containing noradrenergic neurons (Supplemental Fig. 1A, 1B). This distribution was similar to that for Il13ra1 reported in the Allen Brain Atlas (http:// www.brain-map.org). An RNA probe for TH, the first and rate-limiting enzyme in the synthesis of catecholamines, was used as a marker for DA neurons (Fig. 1C). We observed that Il13ra1 and TH labeling showed similar patterns at comparable levels of the ventral midbrain, indicating that Il13ra1 mRNA is expressed in the DA neurons of the SNc and VTA. No detectable *Il13ra1* signal was found in the SNc of $Il13ra^{-/Y}$ mice by in situ hybridization carried out with the antisense probe (Fig. 1D). Expression of Il13ra1 in the SNc was also confirmed by utilizing an Il13ra1 knockout mouse containing a Lacz reporter cassette expressed within the Il13ra1 gene locus. Brain sections from these mice assayed for β-galactosidase activity showed staining in the SNc and VTA (Fig. 1E). To confirm the expression of IL-13Ra1 in DA neurons in the SNc, double immunohistochemistry was



FIGURE 1. Mapping *Il13ra1* expression in SNc and VTA. (**A**) In situ hybridization of *Il13ra1* mRNA using antisense RNA probes in representative wt (*Il13ra^{+/Y}*) mouse brains shows that expression of *Il13ra1* is restricted to the SNc and VTA. (**B**) Representative in situ hybridization of *Il13ra1* mRNA sense probe on a brain section from a wt mouse used as a control. (**C**) Representative in situ hybridization with TH antisense probe used as a marker for DA neurons on a brain section from a wt mouse. (**D**) In situ hybridization of *Il13ra1* mRNA using antisense RNA probes in a representative *Il13ra^{-/Y}* mouse brain shows no detectable *Il13ra1* expression. (**E**) β-galactosidase assay for the detection of LacZ reporter in a brain from a *Il13ra^{-/Y}* mouse reveals SNc- and VTA-restricted expression of *Il13ra1*. (**F**) The *Il13ra1* reporter, LacZ, localizes to DA neurons in the SNc of *Il13ra^{-/Y}* mice. Representative confocal microscopy images shown were obtained by immunohistochemistry: TH, green; LacZ, red; merge, nuclei stained with DAPI, blue. Scale bars, 200 µm in (A)–(E), 50 µm in (F).



FIGURE 2. $ll13ra^{1}$ enhances neuronal loss in vivo and in vitro. (**A**) $ll13ra^{-N}$ mice show decreased DA neuron loss in a chronic peripheral inflammatory model of PD. Histogram of the total number of TH⁺ neurons in the SNc of $ll13ra^{-N}$ and $ll13ra^{+N}$ mice chronically injected (i.p.) with LPS (100 µg/kg) twice weekly for 6 mo versus control mice injected with saline used as vehicle (n = 11 mice/group). Values (means ± SEM) were normalized to the $ll13ra^{+N}$ Isaline group. *p < 0.05 versus all other groups. (**B**) Representative images showing immunohistochemical labeling of DA neurons using

performed on the *Il13ra^{-/Y}* mice containing the LacZ reporter using Abs against TH and LacZ (Fig. 1F, Supplemental Fig. 2). More than 80% of the TH neurons were found positive for LacZ. A three-dimensional rendering produced from Z-stacks of confocal images demonstrating colocalization in one cell is shown in Supplemental Fig. 2A. Conversely, LacZ expression did not colocalize with the microglial marker, Iba1, or the astrocyte marker, GFAP (Supplemental Fig. 2B, 2C).

Mice lacking Il13ra1 are protected in a chronic inflammatory model of DA neuron loss

We next examined whether IL-13Ra1 affects DA neuron survival in vivo. We chose the chronic peripheral inflammatory model of DA neuron loss (13). We used 8-wk-old Il13ra^{-/Y} and $Il13ra^{+/Y}$ mice (n = 11/group) that were injected twice weekly for 6 mo with saline or 100 μ g/kg LPS (i.p.). Out of 23 animals treated with LPS, 1 *Il13ra*^{-/Y} mouse died 2 wk before termination of the study. DA neurons of the SNc were then labeled by immunohistochemistry using a TH Ab and counted (Fig. 2A, 2B). Vehicle-treated Il13ra-/Y mice and their wt littermates used in the study showed a similar number of SNc TH⁺ neurons (10,132 \pm 192 for wt animals treated with vehicle [saline], versus 9,626 \pm 346 for *Il13ra^{-/Y}* mice treated with vehicle; n = 11/group, NS). Similar to what was originally described (13), 6 mo LPS treatment induced a 44% reduction in SNc DA neurons in wt mice whereas the number of SNc DA neurons was reduced by only 10% in Il13ra^{-/Y} mice (5711 \pm 330 for wt mice receiving LPS, and 8511 \pm 476 for *Il13ra* ^{-/Y} mice treated with LPS; n = 11/group, p <0.05) (Fig. 2A, 2B).

Semiquantitative RT-PCR performed on RNA extracted from the SNc of mice treated with LPS showed that compared with the level of saline-treated animals, arbitrarily fixed to 1 (±0.19), levels were 0.62 (±0.26), 1.23 (±0.58), and 2.5 (±0.99) at 0.5, 1, and 2 h, respectively (p < 0.05 at 2 h). This is similar to other studies showing that peripheral LPS treatment elevated the transcription of IL-13 centrally (14, 15). No IL-4 transcript was detected in the same samples.

IL-13R α 1 signaling increases the sensitivity of DA cells to oxidative stress

The above results led us to employ a DA cell culture model to more closely examine IL-13R α 1-mediated death. We used the mouse DA line MN9D, which expresses both IL-13R α 1 and IL-4R α as determined by PCR (not shown), and measured cell viability by the LDH assay following treatment with IL-13 or IL-4 alone or in the presence of oxidative stress.

Neither IL-13 or IL-4 alone induced cellular loss. Instead, they decreased cell mortality in a dose-dependent manner during 24 h (Fig. 2C, 2D). Because neuroinflammation results in the production of neurotoxic factors that include ROS, we

a TH Ab in *Il13ra^{-/Y}* and *Il13ra^{+/Y}* mice injected with saline or LPS as indicated. Scale bar, 200 μ m. (**C**) IL-13 and IL-4 potentiate oxidative stress-induced cell death in MN9D cells. Cells were treated with saline, IL-13, or IL-4 at the doses indicated followed by H₂O₂. Cell viability was assessed by the LDH assay 24 h later. Values (means ± SEM) are normalized to the PBS-only group and are from three independent experiments. (**D**) Similar experiments were carried out in the presence of tBOOH. **p* < 0.05 versus saline or 80 μ M H₂O₂ group.

then tested the effects of IL-13 and IL-4 on cell survival in the presence of two different oxidative agents, H₂O₂ and tBOOH. Based on dose curve responses (not shown) we chose to use the maximal dose of agent that caused minimal damage (80 μ M for H₂O₂ and 10 μ M for tBOOH). Under these conditions, both cytokines potentiated oxidative stress-induced cytotoxicity. In the presence of H₂O₂, toxicity increased in a dose-dependent manner and at the maximal dose of cytokine tested (10 ng/ml) reached 33 and 49% for IL-13 and IL-4, respectively (Fig. 2C). The dose curve response was U-shaped for both cytokines in the presence of tBOOH and was highest at the smallest and at the highest doses tested with a maximum of 58 and 31% increase for IL-13 and IL-4, respectively (Fig. 2D). These differences were larger when compared with the effects of the cytokine alone. These data indicate that IL-13 and IL-4 can contribute to the loss of DA neurons by increasing susceptibility to oxidative damage and that this phenomenon may underlie the resistance of $Il13ra^{-/Y}$ DA neurons to chronic inflammation observed in vivo.

Discussion

In this study we showed that IL-13R α 1 is expressed in DA neurons and that mice deficient for IL-13R α 1 have increased resistance to loss of SNc DA neurons in a chronic model of peripheral LPS inflammation.

Expression of IL-13R α 1 was detected in DA neurons of the VTA and the SNc and was not found in noradrenergic neurons of the locus coeruleus or in the glomerular DA neurons of the olfactory bulb, although we cannot exclude that expression may occur at a lower level also in these cells. Because preferential loss of SNc neurons and, to a minor extent, of VTA neurons (16) is a hallmark of PD, we tested the hypothesis that IL-13R α 1 may contribute to the vulnerability of these cells.

Neuronal degeneration in PD includes mitochondrial dysfunction, oxidative stress, and protein misfolding (17-21). Recently, inflammatory processes mediated by glial or infiltrating T cells have also been proposed to contribute to PD by generating toxic mediators (4, 22-25). These include cytokines as well as ROS that can sustain inflammation but lack specificity for DA neurons. Thus, we used a mouse model in which chronic inflammation induced by peripheral injection of LPS was previously shown to induce neuroinflammation, oxidative stress, and the eventual loss of DA neurons (13). We chose to use LPS over the toxin-based PD models of 1methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 6-hydroxydopamine because although inflammation occurs in these toxin-based models the specificity for DA neuronal loss is conferred by the neurotoxins (26), and because the LPS model mimics a condition in which a peripheral insult and a nonneuronal-specific insult induce CNS damage. Thus, this type of model may be highly relevant toward understanding the correlation between environmental factors and idiopathic PD. Our finding that using this model IL-13Ra1-deficient mice showed significantly decreased loss of DA neurons in the SNc suggests that IL-13Ra1 may be a factor contributing to the selective loss of these cells.

In the brain, IL-13 was demonstrated previously in the microglia of LPS-treated mice and in the cerebrospinal fluid (27–30). A role for this cytokine in modulating neuro-inflammation is being investigated by other groups with a

focus on the ability of IL-13 to contribute to the death of activated microglia cells, thus reducing the contribution of these cells to neuronal loss. Indeed, both IL-13 and IL-4 are considered anti-inflammatory and can lower the production of several proinflammatory cytokines, including some previously shown to contribute to the inflammation-associated loss of DA neurons (31-37). Thus, our finding that mice devoid of IL-3Ra1 have increased rather than reduced resistance to loss of TH⁺ neurons in the SNc appears at first paradoxical. However, despite its ability to ultimately reduce inflammation, IL-13 and possibly IL-4 may act directly on neurons expressing IL-13Ra1 to cause damage. This damage might occur only under pathological conditions and in an inflammatory environment, as neither IL-13 nor IL-4 alone diminished MN9D cell survival but both significantly potentiated the toxic effects of H₂O₂ and tBOOH. These findings are in agreement with other studies showing that IL-13 and IL-4 can exert cytotoxic effects primarily by enhancing the action of other factors such as LPS, IFN-y, and thrombin (27, 29, 38-40). However, the mechanisms mediating this phenomenon remain to be determined.

The chromosomal localization of ll13Ra1 supports the possibility that it may be involved in human PD. In fact, human ll13Ra1 lies on the X chromosome at Xq24 within the Xq21–q25 region found to contain the PARK12 locus of PD susceptibility (5, 6). Although PARK12 is sizable, this locus is of particular interest because an X-linked factor helps explain the observation that the incidence of PD in men is about twice that in women (41). Finally, further studies could determine whether IL-13Ra1 may also be an important factor in the hypothesized link between allergic rhinitis and increased susceptibility to PD (42).

In summary, our study shows that neuronal IL-13R α 1 can increase the susceptibility to oxidative damage and that its expression in DA neurons may contribute to their preferential loss. IL-13R α 1, IL-13, IL-4, and other regulators of their respective signaling pathways may represent novel therapeutic targets for PD.

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Disclosures

The authors have no financial conflicts of interest.

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