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The role of TNF- α in inflammatory olfactory loss

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Abstract

Background—Despite the significant health impact of olfactory loss in chronic rhinosinusitis, the underlying pathophysiology is incompletely understood. A transgenic mouse model of olfactory inflammation induced by TNF- α has provided new insights into the cellular and molecular basis of inflammatory olfactory loss. Here, we utilize systemic corticosteroids to suppress downstream cytokine expression, in order to study the direct role of TNF- α in CRS-associated olfactory dysfunction.

Methods—Transgenic mice were induced to express TNF- α in the olfactory epithelium for 6 weeks. In a subset of mice, 1 mg/kg prednisolone was administered concurrently to inhibit downstream inflammatory responses. The olfactory epithelium (OE) was analyzed by histology and electro-olfactogram (EOG) recordings.

Results—Treatment with prednisolone successfully prevented inflammatory infiltration over significant regions of the OE. In areas where significant subepithelial inflammation was present, a corresponding loss of olfactory neurons was observed. In contrast, areas without major inflammatory changes had normal olfactory neuron layers, despite chronic local expression of TNF- α . Prednisolone partially reversed the complete loss of olfaction in the mouse model, preserving odorant responses that were significantly diminished compared to controls, but not absent.

Conclusion—The addition of prednisolone to the transgenic model of olfactory inflammation isolates the direct effects of induced TNF- α expression on the OE. The finding that prednisolone treatment prevents neuronal loss in some regions of the OE suggests that TNF- α does not directly cause neuronal apoptosis, and rather that subepithelial inflammation or other downstream mediators may be responsible. At the same time, EOG results imply that TNF- α directly causes physiologic dysfunction of olfactory neurons, independent of the inflammatory state. An understanding of the role of TNF- α and other inflammatory cytokines may suggest novel therapeutic strategies for CRS-associated olfactory loss.

Keywords

TNF- α ; olfaction; steroid; transgenic; rhinosinusitis

BACKGROUND

Olfaction has a critical impact on quality of life by contributing to enjoyment of foods and odors while also serving as a warning mechanism for dangerous environmental hazards.^{1, 2} The loss of the sense of smell is a common symptom of chronic rhinosinusitis (CRS) that

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can be very debilitating to patients.³ Despite its great clinical significance, current understanding of the pathophysiology of olfactory loss in CRS is incomplete. Two broad mechanisms analogous to conductive and sensorineural hearing loss have been proposed in CRS-induced olfactory dysfunction. Conductive olfactory loss relates to physical obstruction of odorant delivery to the olfactory cleft secondary to mucosal inflammation or abnormalities of the olfactory mucus.⁴ Sensorineural olfactory loss is caused by damage or destruction of the neuroepithelium as a result of toxic inflammatory mediators and tissue disruption from infiltrating inflammatory cells.^{5, 6} We have previously proposed an additional sensorineural mechanism in which olfactory dysfunction can occur with an intact neuroepithelium, as a consequence of direct interactions between olfactory sensory neurons and inflammatory cytokines. The olfactory epithelium normally has a remarkable capacity for regeneration, with ongoing replacement of olfactory receptor neurons occurring throughout the life of an individual. In CRS, persistent inflammation is associated with prolonged olfactory loss that may be rapidly reversible with systemic corticosteroid treatment. This reversal suggests that either the neuroepithelium is not severely damaged in CRS or that it can be rapidly reconstituted when inflammation is diminished with steroids.

To study the effects of inflammation on the olfactory system in vivo, our group has developed a transgenic mouse model in which TNF- α is expressed in a temporally-controlled, olfactory epithelium specific fashion.⁷ Continuous local production of TNF- α within the olfactory mucosa results in a progressive inflammatory infiltrate that mimics histologic features of CRS-associated olfactory loss. With this model, we have shown that chronic TNF- α -induced inflammation causes loss of mature receptor neurons, while also suppressing the normal regenerative replacement mechanism.⁸ Electrical odorant responses are lost after 5-7 weeks of inflammation, secondary to the absence of olfactory receptor neurons. Interestingly, responses to odorants become diminished after two weeks of TNF- α production prior to the neurons becoming depleted, suggesting that inflammation causes functional impairment through another mechanism. In the mouse model, expression of TNF- α initiates a downstream cascade of multiple pro-inflammatory mediators from a variety of cell types. We hypothesize that these mediators, individually or in combination, affect olfactory sensory neurons and their progenitor cells to cause initial desensitization and eventual neuronal death and suppressed regeneration. Identification of the specific cytokines and the cellular pathways they activate may lead to novel treatments for CRS-associated olfactory loss.

The ability of TNF- α to initiate inflammation is due in part to NF- κ B-mediated induction of multiple downstream cytokines.⁹ Systemic corticosteroids have significant beneficial effects in CRS and a wide range of other inflammatory diseases because they inhibit expression of pro-inflammatory mediators.¹⁰ A key feature of the olfactory loss mouse model is that the expression of TNF- α is controlled by an inserted transgene regulated by exposure to a drug, rather than by native transcription mechanisms. As a result, corticosteroid treatment in the mouse does not inhibit TNF- α expression, but only the downstream expression of cytokines initiated by TNF- α . In this study, we take advantage of this property of the mouse model to isolate the direct effects of TNF- α on the olfactory system, separate from the secondary effects of inflammatory cell infiltration and consequent inflammatory mediator production.

MATERIALS AND METHODS

Inducible Olfactory Inflammation (IOI) Mouse

The creation of the IOI mouse line has been described previously.⁷ Briefly, the reverse tetracycline transactivator gene was knocked into the olfactory-specific cyp2g1 coding region generating a cyp2g1-rtTA strain. This line was crossed with a line containing the TNF- α gene under the control of a tetracycline-responsive element (TRE-TNF- α) to

generate the IOI mouse. Doxycycline (DOX) was used to induce TNF- α expression in adult mice between the ages of 6-8 weeks old. IOI mice were induced to express TNF- α in the olfactory epithelium for 6 weeks. In a subset of mice, 1 mg/kg prednisolone was administered concurrently. As a control, a wild-type mouse was treated with DOX as well as 1 mg/kg of prednisolone for 6 weeks.

Histologic Analyses

After sacrifice by CO₂ inhalation, the mice were decapitated and the heads were fixed and decalcified by immersion in TBD2 solution (Shandon, Pittsburgh, PA) for 24 hours. The heads were embedded in paraffin, and 12 μ m sections were obtained and collected on glass slides for hematoxylin and eosin staining. For frozen section analysis, the mice were anesthetized by an intra-peritoneal injection of 100 mg/kg of xylaket (Sigma, St. Louis, MO), before intracardiac perfusion with 4% paraformaldehyde. The olfactory tissue was then dissected, post-fixed in 4% paraformaldehyde, and transferred to a solution of 30% sucrose and 250 mM of EDTA for 48 hours. The decalcified heads were then infiltrated with OCT tissue-tek compound (Miles, Elkhart, IN) and frozen on dry ice into a plastic mold. Sections of mouse olfactory tissue in OCT were cut on a cryostat (12 μ m), placed on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and dried 60 minutes before use.

Olfactory Epithelium Thickness Measurements

The thickness of the olfactory epithelium was measured on hematoxylin and eosin-stained tissue sections. Images were acquired using the Zeiss Axio Imager.A2 microscope and measurements made using the Axiovision 4.8 software (Carl Zeiss Micro-imaging, Thornwood, NJ). The measured thickness of the epithelium was from the basement membrane to the top of the olfactory knobs. All measurements were made on the same turbinate every 200 μ m from zone 4 of the olfactory epithelium on two sections from three mice from each data group. Values for each individual animal were averaged and data are represented as the mean $\pm$ SEM for each group. The range represents the thickness values for one turbinate for a representative mouse. Statistical comparisons were made using the students t-test.

Immunostaining

Cryostat sections were blocked for 1 hour in PBS containing 5% normal secondary serum and then incubated overnight at 4°C in 5% normal serum containing primary antibody to keratin 5 (K5, Covance, Princeton, NJ). Primary antibodies were detected using a fluorescent tagged secondary anti-body (Alexa-fluor, Invitrogen, Carlsbad, CA). Each sample was counterstained by the nuclear stain, DAPI (Vector Labs, Burlingame, CA). Images were viewed using a LSM510 confocal microscope (Carl Zeiss Micro-imaging, Thornwood, NJ) and measurements of K5 fluorescent intensity along a zone 4 turbinate were made using Axiovision 4.8 software.

Bromodeoxyuridine labeling

Mice were injected i.p. with bromodeoxyuridine (BrdU) (Sigma), 50 μ g/g of body weight, 60 minutes before death. Cryostat sections were then incubated with 3N HCl for 30 minutes and treated with proteinase K for 10 minutes before immunostaining with rat anti-BrdU antibody (Abcam, Cambridge, MA). Primary antibodies were detected using a fluorescent tagged secondary anti-body (Alexa-fluor). Each sample was counterstained by the nuclear stain, DAPI. Images were viewed using a LSM510 confocal microscope.

Electro-olfactogram (EOG)

The medial surface of the olfactory turbinates was prepared for recording after the mouse was sacrificed using CO₂. Odorant solutions (Aldrich, St. Louis, MO) were prepared in DMSO and diluted with water to the working concentration just before EOG recording. Test odorants for air delivery were prepared at liquid concentrations of 10⁻³ [final DMSO concentration of 0.2% (v/v)], and diluted to 10⁻⁴ and 10⁻⁵ M concentrations. Responses to DMSO diluent alone were measured. Odorant stimulation was delivered in the vapor phase as a 100 ms pulse by injection into the continuous stream of humidified air. The odorant stimulus pathway was cleaned by air between each stimulus presentation with a minimum interval of 1 min between two adjacent stimuli.

RESULTS

Prednisolone treatment markedly decreases TNF- α -induced inflammation, with relative preservation of large areas with olfactory epithelium neuron layers

The normal mouse olfactory epithelium (OE) consists of a superficial single layer of sustentacular cells overlying a densely packed layer of olfactory sensory neurons and their progenitors. In the most basal aspect of the neuron layer are the multipotent stem cell populations. Below the basement membrane in the subepithelium lie well-demarcated axon bundles. After six weeks of DOX-induced TNF- α expression, the OE is thinned with very few remaining mature olfactory receptor neurons, but with an intact sustentacular layer. Inflammatory cells have infiltrated the subepithelium, distorting the subepithelial architecture, and axon bundles are no longer evident. After six weeks of DOX-induced TNF- α expression concurrent with prednisolone, the OE in many areas is nearly normal with only a mild inflammatory infiltrate in the subepithelium and irregular axon bundles (Figure 1A). To control for inter-animal heterogeneity in the extent and location of inflammation, OE thickness was measured completely in the same anatomic location for all three groups. The mean OE thicknesses for the no DOX, DOX, and DOX/Prednisolone mice were 43.23 μ m (range: 28.10-51.92 μ m), 12.69 μ m (range: 7.99-28.82 μ m), and 28.03 μ m (range: 18.01-41.97 μ m), respectively. These data are graphically represented in Figure 2 and highlight the fact that prednisolone causes a significant retention of the OE neuron layer as compared to DOX-only, but there is still loss of neurons when compared to wild-type mice.

Prednisolone treatment greatly diminishes the loss of horizontal basal cells associated with prolonged TNF- α expression

Our previous studies have shown a surprising absence of proliferation during active TNF- α -induced inflammation, despite progressively severe neuronal depletion. The reason for this failure of regeneration is not understood. Horizontal and globose basal cells lie in the basal layer of the OE and contribute to normal turnover and injury-induced neurogenesis.¹¹ It has been shown that horizontal basal cells (HBC), which are normally quiescent, undergo a proliferative burst to repopulate all olfactory epithelial cell compartments when there is extensive olfactory neuronal depletion.¹² HBC specifically express keratin 5 (K5),¹³ which can be used as a marker for their identification by immunohistochemistry. The basal aspect of the OE prior to TNF- α expression shows a uniform layer of HBC directly above the basement membrane (Figure 1B, left side). After six weeks of TNF- α expression, there is no visible expression of K5 in the thinned OE, suggesting that the HBC population has either been destroyed, lost its expression of K5, or possibly depleted itself through extensive proliferation (Figure 1B, central panel). With concurrent prednisolone, K5 expression is maintained in areas where the olfactory neuron layer is preserved, although reduced in intensity when compared to untreated mice (Figure 1B, right panel). Densitometric analysis revealed a mean intensity of 763 $\pm$ 17 Grey levels in untreated mice, whereas the DOX-prednisolone mouse had a mean intensity of 575 $\pm$ 43 Grey levels ($p < 0.03$). In areas of

inflammation and neuronal loss, K5 staining is absent in the prednisolone-treated mice. This suggests that the loss of K5-positive HBC is not caused directly by TNF- α , which is expressed throughout the entire OE, but rather occurs only when there is robust inflammation and widespread death of olfactory receptor neurons.

The preservation of large areas of intact olfactory neuroepithelium with prednisolone is not consequent to increased proliferation

The majority of cell division during OE regeneration occurs in the basal compartment where the progenitor cells are located. In order to assess the effect of TNF- α and its downstream mediators on proliferation, we labeled dividing cells with BrdU and used an anti-BrdU antibody to visualize dividing cells in the OE. In an adult mouse, in the absence of OE injury, neuronal turnover is relatively slow, with 1-2 BrdU positive cells observed per high-power field in the OE. We have previously reported the absence of BrdU positive cells during active DOX-induced TNF- α expression. The lack of proliferation is seen prior to the widespread loss of neurons and continues as long as the TNF- α is expressed, but there is a dramatic wave of cell division and regeneration that begins once the DOX is stopped. In concurrent DOX-prednisolone administration, areas of preserved neuroepithelium have 1-2 BrdU positive cells per high power field, similar to wild-type mice, but in areas with thinned neuroepithelium, there is no increase in BrdU positive cells in response to neuronal loss (Figure 3). This suggests that the preservation of OE thickness and the olfactory neuron layer with prednisolone administration does not occur through active repopulation by progenitor cells, but rather through prevention of neuronal loss. This is consistent with our previous hypothesis that TNF- α directly inhibits progenitor cell proliferation.¹⁴

Prednisolone partially reverses the loss of electrical odorant responses

We have previously demonstrated that prolonged TNF- α induced inflammation of the OE results in loss of electrical odorant responses. Shorter durations of TNF- α expression result in a diminished amplitude of odorant response, even prior to the histologic loss of olfactory neurons. In order to better understand the specific role of TNF- α in modulating olfactory neuron function, we performed electro-olfactogram (EOG) recordings in mice treated with DOX alone and with DOX-prednisolone. After six weeks of DOX-induced TNF- α expression the EOG responses even at the highest odorant concentrations are absent, correlating with the extremely depleted neuronal layer. With concurrent prednisolone, there is preservation of odorant responses, but they are significantly reduced in amplitude compared to littermates not treated with DOX (Figure 4). The preservation of EOG amplitude is not proportional to the much greater retention of olfactory neurons with prednisolone treatment.

DISCUSSION

In this study, we have used the olfactory inflammation mouse model to study the effects of prolonged in vivo exposure to TNF- α on the appearance and function of the olfactory epithelium. We exploited the ability of prednisolone to inhibit downstream expression of inflammatory cytokines to isolate the direct effects of TNF- α . These experiments demonstrate that systemic corticosteroids significantly prevent the profound inflammatory infiltrate from developing over large areas of the mouse olfactory epithelium. In regions where the olfactory epithelium is not inflamed, the neuronal layer is largely preserved, the horizontal basal cell progenitors remain intact, and there is no change in neuronal proliferation. However, while the olfactory receptor neurons appear to be intact histologically, electrical odorant responses are diminished. Taken together, these findings suggest that the widespread loss of olfactory receptor neurons in the inflamed state is not due directly to TNF- α , but rather results from other inflammatory mediators or physical

crowding by the inflammatory cellular infiltrate itself. Similarly, the disappearance of the horizontal basal cell population is also not due to TNF- α alone, since these cells are preserved in the basal compartment of normal-appearing regions. An expanded analysis of the transition areas between adjacent normal and thinned neuroepithelium in the future may yield a better understanding of the relationship between subepithelial inflammation and neuroepithelial loss. The fact that electrical odorant responses are present, but greatly reduced, may point to a direct role of TNF- α in desensitizing olfactory receptor neuron function. This fits with our previously reported observation in this mouse model that the functional olfactory loss precedes the onset of dramatic histologic changes.⁷

Systemic corticosteroids are the only consistently effective treatment for CRS-associated olfactory loss in humans, and their cellular and molecular effects have been studied extensively.¹⁵ In CRS, corticosteroids greatly reduce expression of a wide variety of immune mediators and cytokines, including TNF- α , leading to resolution of inflammation. While baseline levels of TNF- α may be greater in CRSsNP than CRSwNP, a significant reduction in TNF- α levels in polyps is effected by steroids.^{16, 17} In addition to their anti-inflammatory activity, steroids have also been hypothesized to act directly on olfactory receptor neurons. Glucocorticoid receptor mRNA and protein can be found in the olfactory mucosa.¹⁸ Glucocorticoids increase the expression of cyclic nucleotide-gated (CNG) channels, which are key components in olfactory receptor signal transduction, and can modulate the olfactory Na-K-ATPase.^{19, 20} In our mouse model, the loss of mature olfactory receptor neurons is greatly ameliorated by prednisolone treatment. While we believe that this occurs via inhibition of inflammatory cells and their cytokine products, it is conceivable that prednisolone may act directly on olfactory neurons to block TNF- α -induced apoptosis.

The specific mechanisms through which TNF- α affects olfactory neuron function have not been fully characterized. mRNA for TNF- α and TNF- α receptors are found in normal olfactory epithelium.²¹ In vitro, TNF- α been shown to induce cell death in olfactory epithelial explants.²² In non-olfactory neurons, Zhang et. al. have reported TNF- α -induced hypersensitivity of sensory neurons, and Soliven et. al. have demonstrated TNF- α modulation of sympathetic neuron calcium currents.^{23, 24} These two studies highlight direct effects of TNF- α on neurons that are independent of downstream inflammation and mediator expression. We postulate that through similar intracellular mechanisms, prolonged TNF- α exposure can result in a desensitized state in olfactory receptor neurons. Future studies utilizing TNF- α receptor knockout strains will be useful to further address the specific role of TNF- α versus other downstream inflammatory cytokines.

CRS-associated olfactory loss is a troublesome health condition that remains difficult to treat. While the process can be transiently responsive to systemic corticosteroids, the side effects and long-term risks of this therapy are prohibitive.¹⁵ Endoscopic sinus surgery and removal of nasal polyps can greatly improve many CRS symptoms, but generally result in limited sustained improvement in olfactory function.^{25, 26} This likely reflects the limitation of surgical approaches to address only the conductive component of olfactory loss, without directly impacting the sensorineural dysfunction caused by local neuroepithelial inflammation. To that point, patients with allergic rhinitis can have hyposmia even in the absence of significant olfactory cleft obstruction on computed tomography scan,²⁷ and viral upper respiratory infections can result in olfactory dysfunction that continues after resolution of nasal airway blockage.^{28, 29} Further research is needed to fully elucidate the role of TNF- α and other inflammatory cytokines in causing persistent olfactory dysfunction in the setting of chronic rhinosinusitis, where a mixture of conductive and sensorineural factors likely contribute.

CONCLUSION

Treatment with systemic corticosteroids in a mouse model of inflammation-induced olfactory loss sheds new light on the underlying pathophysiology. The present study suggests that while TNF- α directly alters olfactory neuron function and suppresses neuroepithelial regeneration, it is downstream mediators and the physical infiltration of inflammatory cells that contributes most critically to the olfactory loss phenotype in this model. As the impact of individual cytokines and inflammatory mediators on olfactory neuron desensitization and cell death are identified, the potential will exist for development of targeted therapies to restore function even in the face of ongoing inflammation.

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Figure 1.

Olfactory epithelial changes during TNF- α expression. **A.** With no doxycycline (No Dox), the olfactory epithelium has a normal appearance with intact sustentacular cells (SUS), multi-layered olfactory neurons (ORN), and intact axon bundles. With 6 weeks of dox-induced TNF- α expression (Dox), the olfactory neuronal layer is significantly thinned with significant subepithelial infiltration of inflammatory cells and lack of discernable axon bundles. 6 weeks of dox concurrent with prednisolone (Dox/Prednisolone) results in a relatively normal olfactory epithelium with decreased axon bundle diameter and some infiltration of inflammatory cells in the subepithelium. Slides are stained with hematoxylin and eosin and images were taken at 20x magnification. Scale bar represents 20 μ m. **B.** The basal aspect of the olfactory epithelium prior to TNF- α expression shows a uniform layer of horizontal basal cells through the contiguous layer of K5 immunostaining (red). After six weeks of TNF- α expression, there is no visible expression of K5 in the basal layer of thinned olfactory epithelium. With concurrent prednisolone administration, the basal layer has a slight reduction in K5 expression when compared to untreated mice. Wild-type mouse had a mean intensity of 763 ± 17 Grey levels whereas the DOX-prednisolone mouse had a mean intensity of 575 ± 43 Grey levels ($p < 0.03$). . Scale bar represents 10 μ m.

Figure 2.

Prednisolone partially inhibits olfactory neuronal loss in mice expressing TNF- α . The mean olfactory epithelium thicknesses for the wild-type (No DOX), doxycycline induced TNF- α expression (DOX) and concurrent doxycycline/prednisolone administration (DOX/PRED) mice are 42.23 μm (range: 28.10-51.92 μm), 12.69 μm (range: 7.99-28.82 μm), and 28.03 μm (range: 18.01-41.97 μm). Error bars represent the SEM. * $p < 0.02$, ** $p < 0.05$.

Figure 3.

Proliferation is not increased with concurrent prednisolone administration.

Bromodeoxyuridine (BrdU) staining (red) was used as a measure of proliferation. At baseline (No Dox), there are 1-2 BrdU positive cells per high-power field in the neuroepithelium. With doxycycline-induced TNF- α expression for six weeks (Dox), there are no visible BrdU positive cells even in the small areas of preserved neuroepithelium. In concurrent doxycycline-prednisolone administration (Dox/Prednisolone), areas of preserved neuroepithelium have a similar staining as the wild-type mouse. Scale bar represents 10 μ m.

Figure 4.

Functional loss secondary to TNF- α expression. The quantitative assessment of electro-olfactogram (EOG) responses shows essentially no response after 6 weeks of dox-induced TNF- α expression even at the highest odorant concentrations. With concurrent prednisolone (pred), there is slight retention of responses but still markedly decreased when compared to control responses. Data reflects a minimum of 4 independent recordings. Error bars represent SEM.