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Role of the type I tumor necrosis factor receptor in inflammation-associated olfactory dysfunction

Davi Sousa Garcia, MD[1,2], **Mengfei Chen, PhD**[1], **Amy K. Smith, BS**[1], **Paulo Roberto Lazarini, MD, PhD**[2], and **Andrew P. Lane, MD**[1]

[1]Department of Otolaryngology–Head and Neck Surgery, Johns Hopkins University School of Medicine, Baltimore, MD

[2]Department of Otolaryngology, Santa Casa de Sao Paulo School of Medical Sciences, Sao Paulo, Brazil

Abstract

Background—To understand mechanisms of human olfactory dysfunction in chronic rhinosinusitis, an inducible olfactory inflammation (IOI) model has been utilized to chronically express inflammatory cytokines locally, resulting in neuronal loss, diminished odorant responses, and repressed olfactory regeneration. Knockout of the minor tumor necrosis factor α receptor 2 (TNFR2) was previously shown to partially rescue these olfactory changes. The purpose of current study was to investigate the role of the major TNF receptor, TNFR1, in chronic olfactory inflammation.

Methods—Two experimental groups of mice were studied: TNFR1 knockout in IOI background and TNFR1 knockout with allergen-induced inflammation. Olfactory function was assayed by electro-olfactogram (EOG), and olfactory tissue was processed for histology and immunohistochemical staining.

Results—TNF-α was dramatically induced in IOI-TNFR1 knockout mice, but the olfactory epithelium did not show inflammation. EOG responses were normal after either 2 or 8 weeks of TNF-α expression. Ovalbumin-sensitized TNFR1 knockout mice developed markedly diminished eosinophilic inflammatory infiltration.

Conclusion—Genetic deletion of TNFR1 completely blocks TNF-α–induced inflammation and reduces allergen-induced inflammation. Preserved EOG responses suggest a TNFR1-dependent mechanism of TNF-α–induced olfactory neuron dysfunction.

Keywords

olfactory epithelium; inflammation; eosinophils; transgenic mice; TNF-alpha; TNFR1

Correspondence to: Andrew P. Lane, MD, Department of Otolaryngology–Head and Neck Surgery, Johns Hopkins University School of Medicine, 601 N Caroline St, 6th floor, Baltimore, MD 21287-0910; alane3@jhmi.edu.

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Chronic rhinosinusitis (CRS) is among the most common causes of olfactory dysfunction.[1] It is reported that up to 80% of CRS patients experience this symptom, which strongly interferes with their quality of life.[2, 3] However, the cause of olfactory loss in the setting of inflammation is not completely understood. Obstruction of airflow to the olfactory cleft may often contribute significantly in many cases, but olfactory dysfunction also occurs when the olfactory cleft is patent, suggesting that pathologic alterations in peripheral olfactory structure and physiology also play a role.[4] All forms of CRS are associated with mucosal inflammation, which can impact the olfactory epithelium (OE).[3]

Olfactory receptor neurons (ORNs) and their progenitors reside within the nasal mucosa and are thus particularly susceptible to local immune mediators in the setting of rhinosinusitis.[5] Unlike other mammalian neuronal cell populations, the OE has the ability to regenerate. Murine studies showed that this replacement occurs both as part of normal turnover and also in response to widespread ORN death.[6, 7]

Tumor necrosis factor–alpha (TNF-α) is a pleiotropic cytokine produced by a wide variety of cell types, and it is associated with most infectious and inflammatory diseases. TNF-α receptors are present on most cell types in an organism.[8–10] Among the cytokines present in sinonasal inflammatory diseases, TNF-α is particularly relevant and is universally associated with CRS, regardless of histological subtype or etiology.[11, 12] In allergic diseases, it is also known to play an important role, since it is required for both the production of T helper 2 (Th2) cytokines and the migration of Th2 cells to the sites of inflammation.[13]

The binding of TNF-α to its receptors results in recruitment of signal transducers that act on complex signaling cascades and networks. The nuclear factor κ-B (NF-κB) pathway is primarily responsible for the inflammatory response, cell differentiation, and proliferation via the mitogen-activated protein kinases (MAPK) pathway, and induction of death signaling pathway via caspase-induced cell apoptosis.[14] In olfactory tissue, TNF-α may signal through an acute proliferative phase, as well as a chronic proapoptotic phase.[7]

Two TNFRs with different molecular masses, 55 kDa (TNFR1; p55) and 75 kDa (TNFR2; p75) have been identified. TNFR1 is constitutively expressed in most tissues, but expression of TNFR2 is typically found in cells of the immune system. In the vast majority of cells, TNFR1 appears to be the key mediator of TNF signaling, whereas in the lymphoid system, TNFR2 seems to play a major role. The extracellular domains of TNFR1 and TNFR2 share homology with each other, as well as with other cell surface proteins; on the other hand, the intracellular domains show no homology, indicating that TNFR1 and TNFR2 may use different mechanisms for intracellular signaling.[1, 10, 15, 16]

Few studies have addressed the role of CRS-associated inflammation in functional olfactory loss. This is largely because of the inaccessibility of human olfactory tissue and the inherent difficulty of maintaining olfactory neurons in standard cell culture conditions. To study the effects of inflammation on the olfactory system in vivo, our laboratory has developed a transgenic mouse model in which the CRS-associated cytokine TNF-α is expressed by sustentacular cells in the OE. The key feature of this olfactory loss mouse model is that the expression of TNF-α is induced by an inserted transgene regulated by exposure to a drug,

the antibiotic doxycycline (DOX), in a temporally-controlled, OE-specific manner.[5] Our previous studies using this model suggested a direct effect of TNF-α on olfactory neuron function and neuroepithelial regeneration, whereas the downstream mediators and the physical infiltration of inflammatory cells contributes most critically to the histological damage. Also, there is a decrease in the electrophysiological response to odorant stimulation in these mice that occurs before neuronal loss is evident.[5–7, 17]

In TNF-α receptor knockout mice, TNF-α expression is not inhibited, but the downstream expression of cytokines initiated by activation of this receptor is. Thus, the direct effects of the knockout receptor on the olfactory system can be isolated. In an earlier study from our group, knockout of TNFR2 (inducible olfactory inflammation [IOI]-TNFR2$^{-/-}$) was shown to partially rescue the olfactory changes seen in IOI mice: less histological degeneration was observed, progenitor cell proliferation was present and the odorant responses were partially maintained.[18] In this study, we aimed to assess the importance of the TNFR1 in chronic olfactory inflammation. We hypothesize that the major TNF-α receptor plays a decisive role in the development of the olfactory loss phenotype.

Materials and methods

IOI mouse

The creation of the IOI mouse line has been described.[5] Briefly, a reverse tetracycline transactivator (rtTA) gene was inserted into the genome under the control of the olfactory sustentacular cells–specific cyp2g1 promoter. Thus, the generated cyp2g1-rtTA strain had a Tet-on genetic system controlled by cyp2g1. Another strain, in which the murine TNF-α gene is under control of the tetracycline-responsive element (TRE), was generated by cloning the murine TNF-α gene into the pTRE vector (Clontec, Mountain View, CA). True-breeding strains of TRE-TNF-α and cyp2g1-rtTA were crossed to create the IOI mouse, with the presence of both constructs determined by polymerase chain reaction. The IOI mouse line was bred to a strain deficient in the type I tumor necrosis factor receptor (TNFR1$^{-/-}$), or p55 receptor (Stock number 002818; Jackson Laboratory, Bar Harbor, ME), until homozygous progeny were achieved that were homozygous for TNFR1 knockout and carried the IOI genotype (IOI-TNFR1$^{-/-}$). Doxycycline was used on a special diet to induce TNF-α expression in adult mice between the ages of 6 and 8 weeks old.

Electro-olfactogram

Three experimental groups (wild-type, IOI-TNFR1$^{-/-}$ mice on DOX diet, and IOI mice on DOX diet) were euthanized using CO_2, according to Institutional Animal Care and Use Committee guidelines. After that, mice were decapitated and the head was bisected. One nasal cavity was used for electro-olfactogram (EOG), and the other was processed for histologic analysis. The medial surface of the olfactory turbinates was prepared for recording. Odorant solutions (Sigma-Aldrich, St. Louis, MO) were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and diluted with water to the working concentration just before EOG recording. Test odorants for air delivery were prepared at liquid concentrations of 1×10^{-3} [final DMSO concentration of 0.2% (vol/vol)], and diluted to 1×10^{-4} and 1×10^{-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 minute between 2 adjacent stimuli. Field potentials were measured with 2 electrodes, placed on either turbinate IIb or III, to acquire simultaneous recordings. Data were analyzed with Clampfit (Axon Instruments, Union City, CA). Peak amplitudes were determined from pre-pulse baseline, and data were normalized to the response to water obtained in each exam. This protocol was used in previous studies from our laboratory,[5, 17–19] and the molar concentrations were found to approximate the half-maximal effective concentration (EC50) for each odorant. Statistical comparisons were made using the Student *t* test.

Histologic analysis

One-half of the bisected heads were fixed and decalcified by immersion in TBD-2® decalcifier (Shandon, Pittsburgh, PA) solution for 24 hours. After that, the heads were embedded in paraffin, and 12-μm sections were obtained and collected on glass slides for hematoxylin and eosin (H&E) staining. Images were taken using an Axio Imager A2 microscope (Carl Zeiss Micro-Imaging, Oberkochen, Germany) with an Axio Cam MRc digital camera (Carl Zeiss Micro-Imaging, Oberkochen, Germany) at ×20 magnification. Thickness measurements were made along the same turbinate, in the olfactory portion of the nasal cavity. The measured thickness of the epithelium was from the basement membrane to the top of the olfactory knobs. All measurements were made on at least 5 sections for each mouse, using the Axio Vision software v. 4.8.1 (Carl Zeiss Micro-Imaging, Oberkochen, Germany). Values for each individual animal were averaged and statistical comparisons were made using the Student *t* test.

Allergen sensitization and challenge

Two groups of mice (wild-type and TNFR1$^{-/-}$) were injected intraperitoneally (IP) with ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) with aluminum hydroxide (alum; Sigma-Aldrich, St. Louis, MO) in a 200-μL solution of phosphate-buffered saline (PBS). The doses and days of application were determined according to the following protocol: for males, injections on days 0 (900 μg), 5 (450 μg), and 15 (450 μg); for females, injections on days 0 (400 μg), 5 (200 μg), 15 (200 μg), and 20 (200 μg). Five days after the last IP injection, mice started to be challenged intranasally with 1500 μg OVA in a 20 μL solution for 20 consecutive days. For challenges, mice were anesthetized with 250 μL (20 mg/mL) 2,2,2-tribromoethanol (Avertin® ; Sigma, St. Louis, MO) injection. Under anesthesia, the solution was distributed on both nostrils using a pipette until no liquid was visible in the nose. After that, they were positioned on their left side until awakening.

Immunohistochemistry

Mice that underwent the allergy protocol were injected intraperitoneally with 250 to 500 μL 2,2,2-tribromoethanol and perfused transcardially with cold PBS followed by 4% paraformaldehyde (PFA; Affymetrix, Cleveland, OH) for 5 minutes each. Then, the olfactory region of the dissected nasal cavities were postfixed in 4% PFA on ice for 3 hours and decalcified in TBD2 solution overnight. After that, the tissue was equilibrated in 15% and 30% sucrose sequentially for 1 hour each. Finally, the heads were then infiltrated with

Tissue-Tek® OCT compound (Sakura Finetek, Torrance, CA) and frozen into a plastic mold, and 12-μm sections were obtained on a cryostat and placed on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). Some sections obtained from the first experimental group were deparaffinized with xylene and then washed in serial dilutions of ethanol. For immunohistochemistry, sections were microwaved at full power for 4 minutes in 0.01 M citrate buffer, pH 6.0, washed in PBS, and were blocked for 1 hour in PBS containing 10% normal donkey serum and 1% Triton (EMD Chemicals Inc, Gibbstown, NJ). Slides were incubated overnight at 4°C in 10% normal donkey serum containing primary antibody to eosinophil major basic protein (EMBP; 1:200; Santa Cruz Biotechnology, Dallas, TX), CD45 (1:200; eBioscience, San Diego, CA), F4/80 (1:800; Covance, Princeton, NJ), keratin 5 (1:800; Covance, Princeton, NJ), or Tuj1 (1:200; Millipore, Temecula, CA). Primary antibodies were detected using 1:500 dilution of fluorescent-tagged secondary antibodies (Alexa Fluor; Invitrogen, Carlsbad, CA; Dylight; Jackson ImmunoResearch, West Grove, PA). Each sample was counterstained by the nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA). Images were viewed using a LSM510 confocal microscope (Carl Zeiss Micro-Imaging, Oberkochen, Germany). Stained cells were counted in transitional OE. This area consists in the portion of the OE closer to the respiratory epithelium (RE), and was considered to begin where the first axon bundle was identified. Cell counts were made along the first 200 μm of the OE, and data were presented as number of cells per this length. Epithelium thickness was measured from the basement membrane to the top of the olfactory knobs using the Zen Lite software (Carl Zeiss Micro-Imaging, Oberkochen, Germany). Epithelium thickness measurement was made on the same turbinate spot, 100 μm away from the first axon bundle. All measurements were made on at least 5 sections from each mouse. Values were averaged and statistical comparisons were made using the Student t test.

Quantification of TNF-α expression

Prior to histological analysis, the olfactory region of one-half of the bisected heads for EOG were flushed with 500 μL of PBS and the liquid was kept at −80°C for use in an enzyme-linked immunosorbent assay (ELISA). TNF-α was quantified with an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Some mice that were subjected to the allergy protocol were euthanized by transcardial perfusion with cold PBS and had their olfactory tissue harvested. This tissue was processed for RNA extraction, reverse transcription and TaqMan quantitative real-time polymerase chain reaction (RT-qPCR) assay according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) for TNF-α (assay probe number Mm00443258_m1; Thermo Fisher Scientific, Waltham, MA) and the housekeeping gene 18S ribosomal RNA (assay probe number Mm04277571_s1; Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

All measurements and quantifications were assessed using a blinded method. Raw data were entered into spreadsheet software for statistical analysis (Excel; Microsoft Corp, Redmond, WA; and Prism 7; GraphPad Software, La Jolla, CA). Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between the groups were performed using 2-tailed t tests. Differences were considered to be statistically significant at $p < 0.05$.

Results

TNFR1 knockout prevents TNF-α–induced neuroepithelial damage

After either 2 or 8 weeks of DOX administration to IOI and IOI-TNFR1 knockout mice, TNF-α was highly expressed (>100 pg/mL) and detectable in nasal lavage fluid. In wild-type control mice, TNF-α was undetectable in the lavage fluid by ELISA (<5 pg/mL). In IOI-TNFR1 knockout mice, we found that despite of TNF-α expression there is neither a decrease in OE thickness nor prominent cell infiltration in the subepithelium, either after 2 or 8 weeks of DOX exposure. Overall the OE did not appear to have substantial changes. In IOI mice, changes were mild after 2 weeks of DOX; however, after 8 weeks of exposure to the drug, the OE was significantly thinned. Staining of CD45, a receptor-linked protein tyrosine phosphatase that is expressed on all leukocytes[20] was performed to better identify subepithelial inflammatory cell infiltration. No difference between wild-type and IOI-TNFR1$^{-/-}$ was found in terms of CD45^{+} cell count. On the other hand, there was an increase in CD45 staining in IOI mice, markedly after 8 weeks of DOX exposure (Fig. 1).

TNFR1 knockout partially blocks TNF-α–induced loss of electrical odorant responses

Comparing EOG responses in IOI-TNFR1$^{-/-}$ and control mice, no olfactory loss was observed at either 2 or 8 weeks of DOX exposure; ie, no olfactory loss was observed. However, IOI mice showed a significant reduction in electrical responses after 2 weeks for most odorants. After 8 weeks, the functional impairment is nearly complete (Figs. 2 and 3).

Ovalbumin-sensitized TNFR1 knockout mice develop diminished inflammatory infiltration

OVA-challenged wild-type and IOI-TNFR1$^{-/-}$ mice groups expressed TNF-α in similar levels in RT-qPCR. ΔCT means normalized to 18S were (mean ± SEM) 8.58 ± 0.87 and 8.32 ± 1.11, respectively. Constitutional expression of TNF-α in nontreated wild-type mice is significantly lower ($p < 0.01$). For them, ΔCT mean normalized to 18S was 14.21 ± 0.49. In order to better identify OE areas, mainly transitional OE/RE, co-staining with anti-Tuj1, a neuron-specific marker, was performed. It was observed that eosinophil infiltration was not uniformly distributed throughout the OE. The higher amount of stained cells was found in the transitional OE, in the first 200 μm from the first identifiable axon bundle, and decreased toward the distal OE. In some animals, regardless of the group, no stained cells were identified distally. Transitional OE was chosen for cell count and epithelium thickness measurement to maximize the difference between the groups, and provided that cell infiltration in this area was a consistent finding in both groups. The use of an antibody to EMBP, a protein present in the crystalloid core of the eosinophil granule,[21] revealed eosinophil infiltration in the lamina propria, mainly in the periphery of axon bundles. No eosinophils were found in the neuronal layer. In OVA-treated wild-type mice, the number of EMBP-positive cells was significantly higher than in IOI-TNFR1$^{-/-}$ mice. F4/80 is a cell-surface molecule highly expressed on murine monocytes and tissue macrophages.[22] The difference in F4/80 labeling between the groups was not statistically significant. Co-staining with keratin 5 showed a preservation of the horizontal basal cells in both groups. In wild-types, thinning of the OE was observed, but overall thickness of IOI-TNFR1$^{-/-}$ mice was preserved (Figs. 4 and 5, and 6).

Discussion

In the current study, we demonstrated that genetic deletion of TNFR1 in a murine model of induced olfactory mucosal inflammation completely prevented histological damage and functional loss after long-term DOX induction of TNF-α expression. Compared to wild-type, eosinophilic infiltration in TNFR1 knockout mice was significantly reduced, and the neuronal layer thickness was preserved. Given these results, we hypothesize that TNFR1 plays a prominent role in development of inflammation-associated olfactory dysfunction in this model.

The mature mouse OE, in the absence of inflammation, comprises a superficial single layer of sustentacular cells overlying densely packed multiple layers of olfactory sensory neurons. Multipotent stem cells (horizontal basal cells and globose cells) lie in the most basal layer of the OE and contribute to normal turnover and injury-induced neurogenesis. Olfactory neurons are characterized by the presence of a large cell body with 1 apical process (ie, dendrite) and 1 basal process (ie, axon), which passes through the basement membrane. By joining with neighbor axons, the axons form well-demarcated bundles in the subepithelium.[23]

A transgenic mouse model of inflammation-associated olfactory loss has been used in our laboratory to study TNF-α–induced inflammation and consequent olfactory damage and regeneration. Genetic deletion of the TNFR1 receptor allows insight into the role of the TNF-α pathway. In the absence of DOX administration, the gross OE appearance of IOI and IOI-TNFR1$^{-/-}$ are normal and identical. An important feature of the IOI-TNFR1$^{-/-}$ mice is that TNF-α is equally highly expressed in the OE after DOX administration, but the effects related to TNFR1 activation are blocked in the knockout mice. Thus, it was possible to isolate the role of the major TNF-α receptor on histologic and functional TNF-α–mediated impairment on the olfactory system.

As described, the OE of the IOI mouse keeps its normal architecture during the first 14 to 21 days of DOX exposure. However, after long-term exposure, ORN layer thickness is markedly reduced and the subepithelium is populated by dense infiltration of inflammatory cells, with no discernible axon bundles.[5] When TNFR2 is knocked out (IOI-TNFR2$^{-/-}$), the olfactory neuron layer remains relatively intact after 6 weeks of DOX-induced TNF-α expression, despite a subepithelial infiltration of inflammatory cells comparable to that observed in IOI mice, and the axon bundles appear reduced in size and irregular.[18] In the present study, TNFR1 was demonstrated to be critical in mediating TNF-α effects on the OE to a greater extent than TNFR2, since the degree of OE preservation in IOI-TNFR1$^{-/-}$ mice is virtually complete despite the prolonged exposure to the cytokine. Moreover, there is no increase in inflammatory infiltrate as assessed by CD45 staining in this group. These findings suggest that TNF-α–induced inflammation damages the OE through a TNFR1-dependent mechanism, although it is not possible to determine whether these effects are direct or mediated by downstream cytokines produced by other cell types.

In previous studies, the role of prolonged TNF-α–induced inflammation on olfactory neuron function has been investigated. In the IOI mouse, there is an approximately 60% reduction

of the amplitude of electrical responses after 2 weeks of DOX exposure, a time point prior to widespread olfactory neuron death and histological architecture disruption. At 42 days, odorant responses are nearly absent, due to the severe neuronal loss.[5] When TNFR2 is deleted in IOI mice, significant inflammation still develops, but odorant responses at 2 weeks of DOX are maintained. Long-term DOX exposure in these mice does not result in dramatic neuronal loss, and odorant responses are only diminished to a level consistent with 14-day DOX-exposed IOI mice with normal TNFR expression.[18] When IOI mice are treated with systemic steroids that inhibit the downstream expression of TNF-α–induced cytokines, the inflammatory response is prevented in portions of the OE. With this, the severe loss of olfactory function after prolonged inflammation does not occur, presumably because of preservation of neurons. However, the responses are still diminished overall.[17] The results obtained in the present study suggest similarly that TNFR1 signaling is critical to olfactory neuron dysfunction in the IOI model. Inhibition of TNF-α signaling via receptor knockout, pharmacologic TNF-α blocker, or systemic steroids leads to suppression of the overall inflammatory response, sparing olfactory neurons from dysfunction and apoptosis.[17–19] Taken together, all of these studies link the degree of olfactory loss to the severity of TNF-α–induced inflammation, rather than to the expression of TNF-α itself.

TNF-α is also known to play an important role in allergic diseases. In experimental allergic rhinitis, it is necessary for antigen-specific immunoglobulin E (IgE) production and Th2 cytokine production, and the migration of Th2 cells to the sites of allergic inflammation.[13, 21, 24] Furthermore, TNF-α modulates the expression of adhesion molecules that induce transendothelial migration of eosinophils.[25, 26]

Ovalbumin is frequently used in allergy models to avoid innate nonallergic responses to pathogen-associated allergens. Previous studies described little or no eosinophilic infiltration in olfactory regions of OVA-sensitized mice. Hussain et al.[27] used different protocols and showed that, even with the one that was considered to be the best, OVA intraperitoneal sensitization followed by aerosol OVA challenge, all eosinophil infiltration was lined by respiratory epithelium, and virtually no eosinophils were seen in areas of OE. Carr et al.,[28] with a protocol as long as 11 weeks, did not find eosinophils in OE-subjacent lamina propria. However, other changes, such as in the thickness of the OE and cellular hypertrophy, were detected uniformly throughout the nasal cavity. They hypothesized that an immune protection from inflammation by the olfactory neuroepithelium explains the marked difference between respiratory and olfactory epithelial damage.[28] On the other hand, Ozaki et al.[29] used a high concentration of OVA to challenge the mice, and showed infiltration of eosinophils, plasma cells, neutrophils, mast cells, and macrophages in olfactory mucosa, as well as an impaired olfactory function.

TNFR1 and TNFR2 seem to play antagonist roles in lower airway OVA-induced diseases. Nakae et al.[30] demonstrated that TNFR2-deficient mice developed airway hyperresponsiveness to a similar degree as wild-type mice. In contrast, both the numbers of eosinophils and levels of interleukin-5 (IL-5) in bronchoalveolar lavage fluid were significantly lower in TNFR1-deficient mice than in control mice.[30] In consonance with that, our results demonstrated a significant reduction of eosinophil infiltration and preservation of OE thickness in TNFR1$^{-/-}$ mice.

All those findings support the importance of the major TNF-α receptor in mediating TNF-α–initiated olfactory inflammation and the resulting histological and functional impairment in the IOI mouse model. Additionally, this study highlights that allergen-induced inflammation in the OE is also dependent on TNFR1. Future research is needed to elucidate the relationship between TNF-α and the initiation of downstream cytokine cascades that drive OE dysfunction in acute and chronic inflammation, as well as their influence on olfactory progenitors and neuronal regeneration.

Conclusion

Genetic deletion of TNFR1 results in complete blockage of TNF-α–induced olfactory inflammation, demonstrating that this receptor plays the central role in transducing signals that result in olfactory tissue damage. Preserved EOG responses after prolonged TNF-α expression suggest a TNFR1-dependent mechanism of inflammation-associated olfactory neuron dysfunction. Also, diminished eosinophilic infiltration in a nasal OVA-sensitization model in TNFR1 knockout mice provides evidence for a role of TNF-α or TNFR1 in development of allergen-induced olfactory inflammation.

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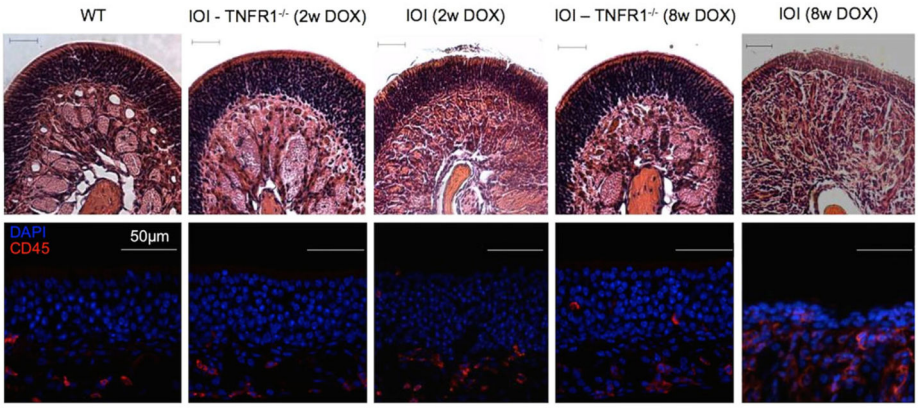

FIGURE 1.

Histopathological features of the OE in the WT, IOI-TNFR1$^{-/-}$, and IOI mice. (Top) Gross appearance of the OE by hematoxylin-eosin staining in the same turbinate of WT and IOI-TNFR1$^{-/-}$ mice both after 2 and 8 weeks of DOX exposure reveals similarity among the 3 groups. In IOI mice, impairment after 2 weeks is mild, but thickness of OE after 8 weeks is dramatically diminished. Images are at ×20 magnification. (Bottom) An antibody to the CD45 common leukocyte antigen was used to visualize nucleated cells of hematopoietic origin within the olfactory turbinate, to better demarcate the inflammatory cell infiltrate. Number of stained cells is approximately the same in WT and IOI-TNFR1$^{-/-}$, either after 2 or 8 weeks, and uniformly distributed throughout the olfactory regions. There is an increase in CD45 staining in IOI after 2 weeks and a very high staining after 8 weeks. Images are at ×40 magnification. Scale bars = 50 μm. Data are representative of at least 6 mice from each group. 2w = 2 weeks; 8w = 8 weeks; DAPI = 4′,6-diamidino-2-phenylindole; DOX = doxycycline; IOI = inducible olfactory inflammation; OE = olfactory epithelium; TNFR1 = tumor necrosis factor α receptor 1; WT = wild-type.

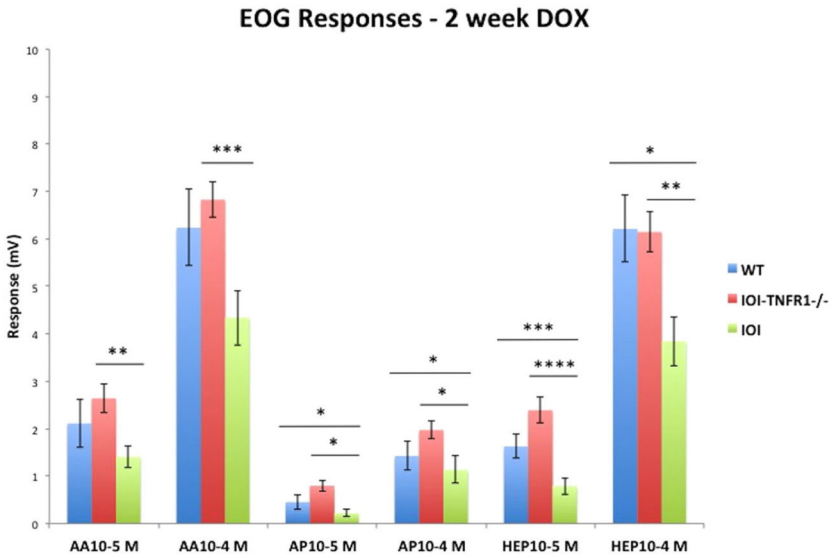

FIGURE 2.

Effect of TNFR1 knockout on functional odorant responses in TNF-α–induced olfactory inflammation with DOX for 2 weeks. The bars represent EOG recordings after exposure to vapor phase of 1×10^{-4} and 1×10^{-5} AA, AP, and HEP, respectively. The quantitative assessment of responses shows no statistically significant ($p < 0.05$) difference between WT and IOI-TNFR1$^{-/-}$ mice for all odorants. There is significant reduction of amplitudes in IOI mice for all odorants when comparing to IOI-TNFR1$^{-/-}$ mice. Data reflect a minimum of recordings from 4 animals. Error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$. AA = amyl acetate; AP = acetophenone; DOX = doxycycline; EOG = electro-olfactogram; HEP = heptaldehyde; IOI = inducible olfactory inflammation; SEM = standard error; TNFR1 = tumor necrosis factor α receptor 1; WT = wild-type.

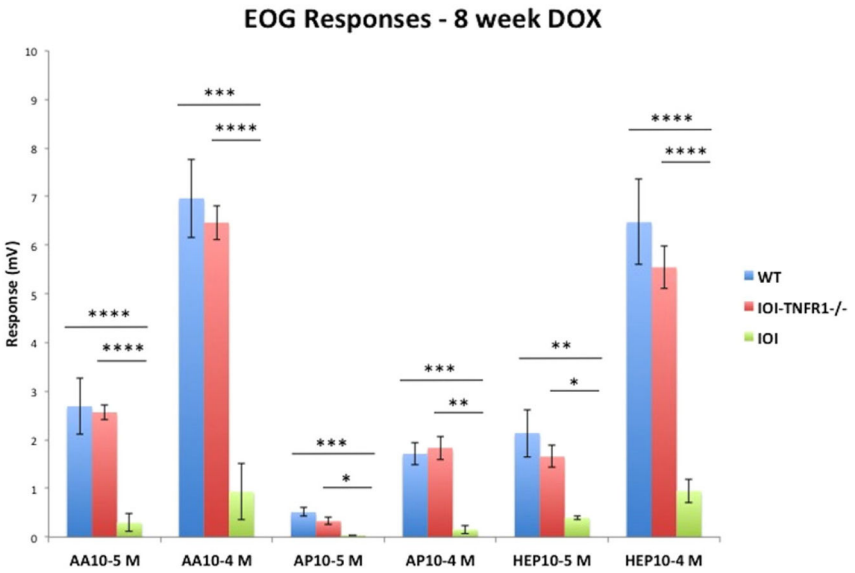

FIGURE 3.

Effect of TNFR1 knockout on functional odorant responses in TNF-α–induced olfactory inflammation for 8 weeks. The bars represent EOG recordings after exposure to vapor phase of 1×10^{-4} and 1×10^{-5} AA, AP, and HEP, respectively. The quantitative assessment of responses shows no statistically significant ($p < 0.05$) difference between WT and IOI-TNFR1$^{-/-}$ mice for all odorants. Reduction of amplitudes in IOI group is substantial. Data reflect a minimum of recordings from 4 animals. Error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$. AA = amyl acetate; AP = acetophenone; DOX = doxycycline; EOG = electro-olfactogram; HEP = heptaldehyde; IOI = inducible olfactory inflammation; SEM = standard error; TNFR1 = tumor necrosis factor α receptor 1; WT = wild-type.

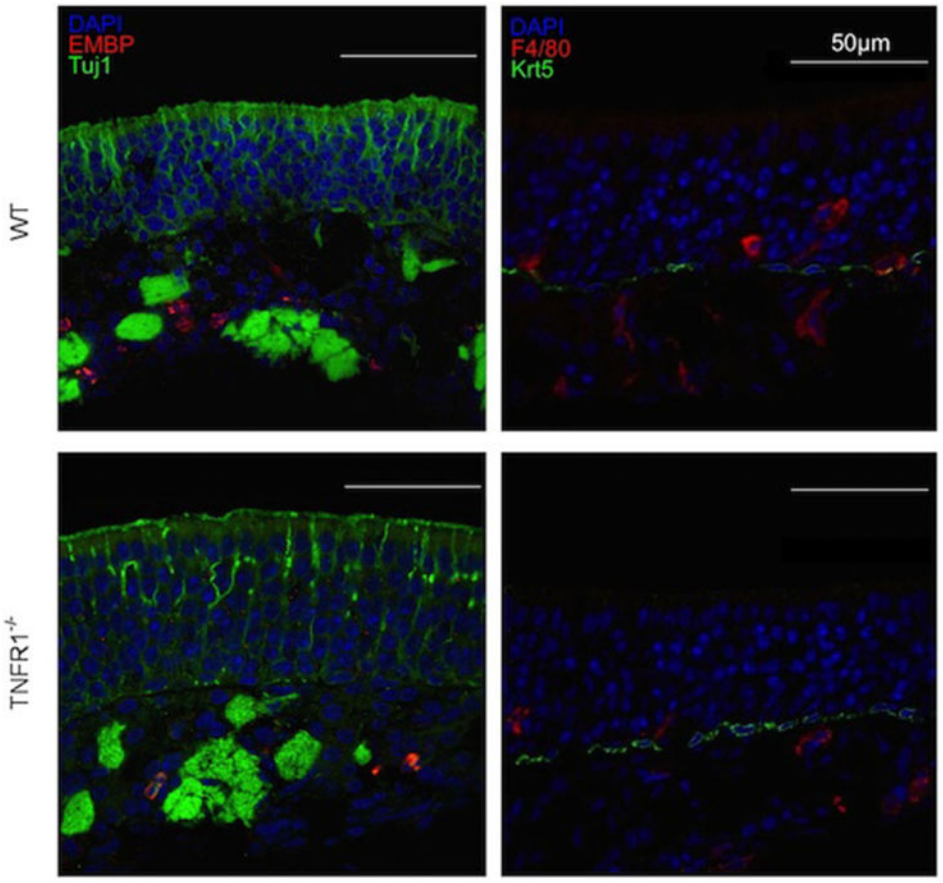

FIGURE 4.

Effect of TNFR1 knockout in allergen-induced olfactory inflammation. (Left column) TNFR1 knockout mice developed markedly diminished eosinophilic inflammatory infiltration in comparison to WT. In general, eosinophils surround axon bundles in subepithelium and do not invade the neuronal layer. (Right column) A trend toward decreased macrophage infiltration is observed in TNFR1 knockout mice. Transition regions to respiratory areas show more robust eosinophil and macrophage infiltration. Images are at ×40 magnification. Scale bars = 50 μm. Data are representative of at least 5 mice from each group. DAPI = 4′,6-diamidino-2-phenylindole; EMBP = eosinophil major basic protein; Krt5 = keratin 5; TNFR1 = tumor necrosis factor α receptor 1; WT = wild-type.

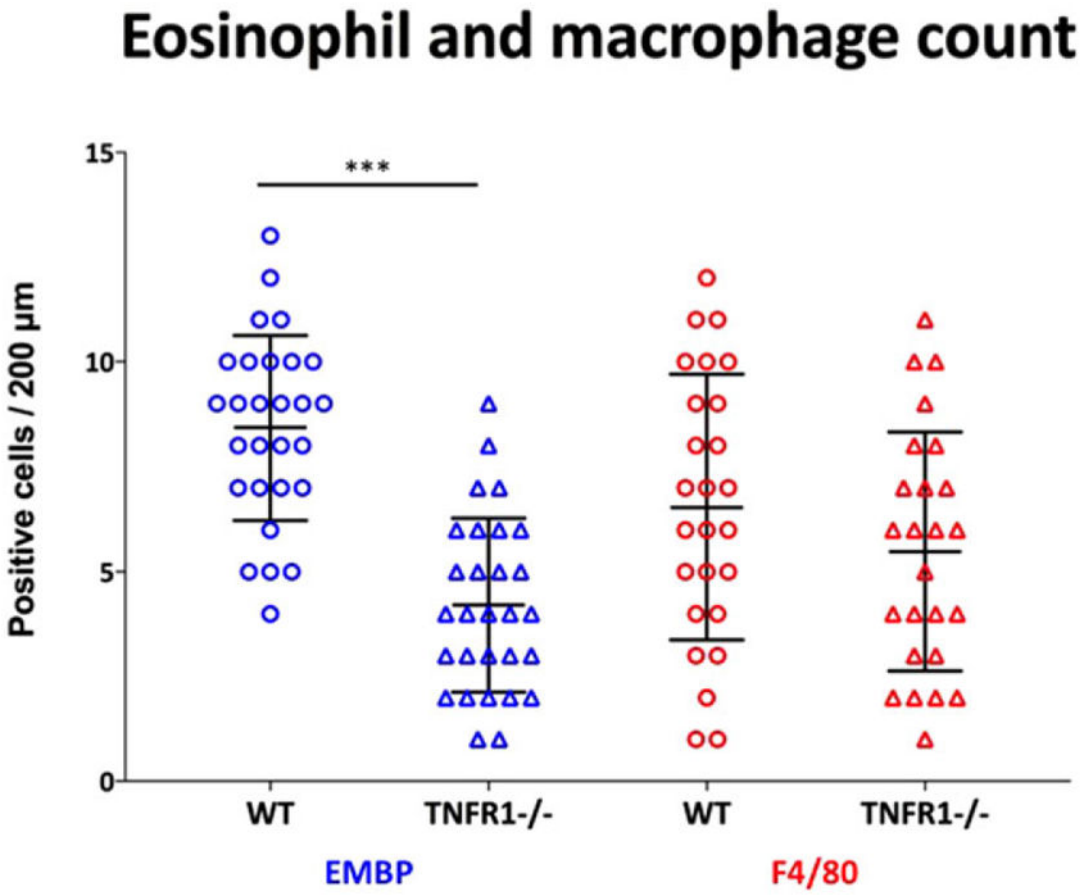

FIGURE 5.

Scatter plot for quantification of eosinophil and macrophage staining in WT and TNFR1$^{-/-}$ mice. Raw values are indicated by circular markers for WT and triangular markers for TNFR1$^{-/-}$; blue markers indicate EMBP (eosinophils) and red markers indicate F4/80 (macrophages). Bars represent the mean and the SEM. A reduction in EMBP^{+} cells in TNFR1$^{-/-}$mice suggests that TNFR1 plays an important role in eosinophilic allergen-induced inflammation in the olfactory tissue. On the other hand, the number of F4/80^{+} cells in both groups was not significantly different. Data includes measurements of at least 5 mice from each group. ***$p < 0.001$. EMBP = eosinophil major basic protein; SEM = standard error; TNFR1 = tumor necrosis factor α receptor 1; WT = wild-type.

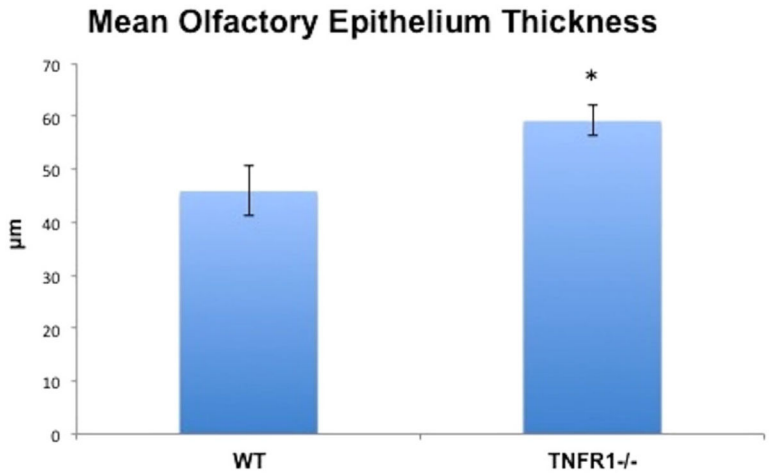

FIGURE 6.

TNFR1 knockout inhibits olfactory neuronal loss in OVA-challenged mice. The mean olfactory epithelium thickness for wild-type (WT) and TNFR1$^{-/-}$ are 45.89 μm (range, 27.85 to 70.00 μm) and 59.15 μm (range, 49.38 to 76.92 μm) in transitional olfactory epithelium. Data represent a minimum of 5 measurements from each mouse. At least 5 mice from each group were analyzed. Error bars represent SEM. *$p < 0.05$. SEM = standard error; TNFR1 = tumor necrosis factor α receptor 1; WT = wild-type.