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Sustained sensitizing effects of tumor necrosis factor alpha on sensory nerves in lung and airways

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Abstract

Tumor necrosis factor alpha (TNF α) plays a significant role in the pathogenesis of airway inflammatory diseases. Inhalation of aerosolized TNF α induced airway hyperresponsiveness accompanied by airway inflammation in healthy human subjects, but the underlying mechanism is not fully understood. We recently reported a series of studies aimed to investigate if TNF α elevates the sensitivity of vagal bronchopulmonary sensory nerves in a mouse model; these studies are summarized in this mini-review. Our results showed that intratracheal instillation of TNF α induced pronounced airway inflammation 24 hours later, as illustrated by infiltration of eosinophils and neutrophils and the release of inflammatory mediators and cytokines in the lung and airways. Accompanying these inflammatory reactions, the sensitivity of vagal pulmonary C-fibers and silent rapidly adapting receptors to capsaicin, a selective agonist of transient receptor potential vanilloid type 1 receptor, was markedly elevated after the TNF α treatment. A distinct increase in the sensitivity to capsaicin induced by TNF α was also observed in isolated pulmonary sensory neurons, suggesting that the sensitizing effect is mediated primarily through a direct action of TNF α on these neurons. Furthermore, the same TNF α treatment also induced a lingering (> 7days) cough hyperresponsiveness to inhalation challenge of NH₃ in awake mice. Both the airway inflammation and the sensitizing effect on pulmonary sensory neurons caused by the TNF α treatment were abolished in the TNF-receptor double homozygous mutant mice, indicating the involvement of TNF-receptor activation. These findings suggest that the TNF α -induced hypersensitivity of vagal bronchopulmonary afferents may be responsible for, at least in part, the airway hyperresponsiveness caused by inhaled TNF α in healthy individuals.

Keywords

asthma; airway inflammation; TRPV1; cough; mouse

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Introduction

An important role of tumor necrosis factor alpha (TNF α), a pro-inflammatory cytokine, in the pathogenesis of airway inflammatory diseases such as allergic asthma has been extensively documented [1–5]. TNF α was detected in bronchoalveolar lavage fluid, exhaled breath condensate and sputum of asthmatic patients during acute exacerbation or after antigen inhalation challenge in these patients [6,7]. TNF α is released from a variety of cell types in the airways, such as mast cells and macrophages, via the immunoglobulin E-dependent mechanism [2,5,8,9]. Once released, TNF α can exert multiple effects on a number of effector cells and induce the inflammatory reaction in the airways. Inhalation of aerosolized TNF α induced bronchial hyperresponsiveness accompanied by airway inflammation in healthy human subjects [10], but the underlying mechanism was not fully understood. One of the prominent pathophysiological features of inflammation-induced bronchial hyperresponsiveness is a heightened sensitivity of airway sensory nerves [11,12].

Previous investigators have repeatedly reported that TNF α induced a potent sensitizing effect on dorsal root ganglion (DRG) and trigeminal ganglion nociceptive neurons, leading to the development of lingering inflammatory pain in various somatic tissues [13–16]. This hyperalgesic effect was mediated through an action on the TNF receptors, TNFR1 and TNFR2, located on the cell surface, which resulted in an increase in the sensitivity and/or expression of transient receptor potential vanilloid type 1 (TRPV1) receptors expressed in these nociceptive neurons [3]. For example, in isolated DRG neurons, pretreatment with TNF α for a short-duration (60 s) immediately and reversibly increased the current amplitude evoked by activation of TRPV1 receptors, and this rapid and transient sensitizing effect of TNF α was attenuated by deletion of the TNFR2 gene [17]. On the other hand, exposure of DRG neurons to TNF α for a longer duration (48 hours) induced a pronounced increase in the proportion of DRG neurones expressing TRPV1 receptor-like immunoreactivity [18]. Interestingly, the TNF α -induced TRPV1 expression was clearly increased in the medium- and large-size neurons that normally do not exhibit TRPV1 sensitivity; and this effect of TNF α was absent in the DRG neurons isolated from mice lacking TNFR1 [18].

The C-fiber sensory nerves are the dominant subtype of vagal afferents innervating the entire respiratory tract in various mammalian species including mice [19–21]. Increasing evidence suggests that activation of these bronchopulmonary C-fiber afferents is responsible for the manifestation of various symptoms in the airway inflammatory diseases [11,12]. An abundant expression of TRPV1 in the neuronal soma and sensory terminals is a reliable and prominent biomarker of these C-fiber afferents [22,23]. More importantly, recent studies have shown that chronic airway inflammation upregulated the sensitivity and expression of TRPV1 in these sensory nerves innervating the airways and lungs [24,25].

In the light of these previous findings, we recently carried out a series of studies aimed to investigate if airway delivery of TNF α induced airway inflammation and elevated the TRPV1 sensitivity of bronchopulmonary sensory nerves; and if so, whether the sensitizing effect of TNF α was mediated through its action on TNFRs. Mouse was chosen as the animal species for these studies so that the involvement of TNFRs could be evaluated in TNF-receptor double homozygous mutant mice (TNF $^{-/-}$). Experimental methods, protocols and

results of these studies have been reported in details in our recent publications [26–28], and a collected summary of this series of studies is presented in this mini-review.

Airway inflammation induced by TNF α

TNF α released from mast cells, macrophages and other inflammatory cells can trigger diverse and potent inflammatory responses in the airways. Activation of TNFRs by TNF α can cause a chemotactic action, upregulate the leukocyte-endothelial cell adhesive molecules E-selectin and vascular cell adhesion molecule-1, which in turn can enhance the production of Th2 cytokines and cause infiltration and degranulation of these inflammatory cells such as neutrophils and eosinophils in the airways [5,10,29,30]. These diverse actions of TNF α can further enhance the release of various pro-inflammatory/chemotactic mediators [5,29]. We carried out this study to determine whether airway inflammation is induced by TNF α treatment in our mouse model, and if so, if this effect is mediated through the action of TNF α on TNFRs. The experiments were carried out in two groups of young male mice: wild-type (WT; B6129SF2/J) and TNF $^{-/-}$ mice (129S-*Tnfrsf1a*^{tm1Imx}*Tnfrsf1b*^{tm1Imx}/J) in which both types of TNF receptors, TNFR1 and TNFR2, were mutated.

The animal preparation and experimental methods were described in details by Lin et al [27]. Briefly, after mice were anesthetized by isoflurane inhalation, a small (~0.5 cm) mid-line incision was made on the ventral neck skin to expose the trachea. Under sterile condition, TNF α solution (10 μ g/ml; 0.03 ml) and its vehicle (Veh; phosphate buffered saline, 0.03 ml) were instilled into the trachea via a needle (28-gauge) in treated (TNF α) and control (Veh) mice, respectively; the incision was then closed. Twenty-four hours (unless noted otherwise) later, bronchoalveolar lavage fluid (BALF) was obtained for measurements of inflammatory cells and mediators from five groups of mice: 1) Naïve group (WT mice receiving no surgery or treatment); 2) Veh group (WT mice treated with Veh); 3) & 4): TNF α 1 and 7 days groups (WT mice 1 and 7 days after treatment with TNF α); and 5) TNF $^{-/-}$ +TNF α group (TNF $^{-/-}$ -mice treated with TNF α).

BALF samples were obtained from 5 mice in each group for the differential cell count, except n=7 in the TNF α (7 days) group. Results showed that the total number of leukocyte cells was significantly higher in the WT mice 1 day after a treatment with TNF α than that in each of the other four groups of mice. No significant difference was found between these four other groups [27]. More strikingly, the percentages of eosinophils and neutrophils in the TNF α (1 day) group were >15 folds higher than that in naïve, Veh, TNF α (7 days), and TNF $^{-/-}$ +TNF α groups, and no significant difference was found in either eosinophils or neutrophils between these four other groups [27].

Several inflammatory mediators and cytokines were selected in this study for measurements because their possible changes after the TNF α treatment were suggested in previous reports [2,5,30,31]. BALF samples were collected for ELISA from separate groups of mice, and the results showed that the TNF α treatment significantly elevated the levels of leukotriene (LT) B₄, LTC₄/D₄/E₄, histamine, thromboxane B₂ and interleukin 1 β in the BALF of WT mice, compared to that in the naïve, Veh and TNF $^{-/-}$ groups, and there was no significant difference in these inflammatory mediators and cytokine between these three other groups

(Fig. 1). However, we did not find any significant difference in the level of PGE₂ and IL-13 in BALF among all four groups. These measurements were not made in the TNF α (7 days) group. The total number of mice used in these groups varied: n = 10, 15, 15 and 5 in naïve, Veh, TNF α , and TNF α ^{-/-}+TNF α groups, respectively. Due to the minimum BALF sample volumes required for various ELISA tests, certain assays (e.g., LTB₄ and IL-1 β) were not performed in all 4 groups (Fig. 1). Pentraxin 3 (PTX3), a member of the pentraxin superfamily of proteins that are involved in acute immunological responses to tissue injury [32], was probably released in response to the surgical wound and needle puncture on the trachea during the intra-tracheal instillation of TNF α . There was no difference in the level of PTX3 between TNF α - and Veh-treated mice.

Some of these autacoids such as histamine, leukotrienes and thromboxanes [27] are known to exert potent stimulatory and/or sensitizing effects on vagal bronchopulmonary C-fiber afferents [11,20].

Hypersensitivity of vagal bronchopulmonary afferents to capsaicin induced by TNF α

To determine if the TNF α -induced inflammation can lead to the development of airway hypersensitivity, we studied the effect of TNF α treatment on vagal pulmonary afferents in young C57BL6/J mice [26]. TNF α and Veh were administered as described earlier. One to two days later, single-unit activities of vagal afferents were recorded from the sectioned right cervical vagus nerve in anesthetized, open-chest mice. Responses to both chemical (capsaicin) and mechanical (lung inflation and deflation) stimulations were studied in all three major types of vagal bronchopulmonary afferents that were identified based upon conventional criteria for classifications [11]: C-fibers, rapidly adapting receptors (RARs), and slowly adapting receptors (SARs). Action potentials generated from C-fiber endings are conducted in small-diameter non-myelinated afferent fibers, whereas those from RARs and SARs are carried in rapid-conducting large-diameter myelinated fibers. The general location of each receptor in the lung and airways was identified at the end of experiment.

An intravenous bolus injection of capsaicin (Cap, a selective agonist of TRPV1 [33,34]) triggered an abrupt (latency <1 s) and short-duration (3–5 s) of discharge in pulmonary C-fibers accompanied by bradycardia and hypotension (e.g., Fig. 2). The pulmonary C-fiber responses to both low (0.5 μ g/kg) and high doses (1.0 μ g/kg) of Cap were significantly higher in TNF α -treated mice than that in Veh-treated mice (Fig. 2). Pulmonary C-fibers were relatively insensitive to lung inflation, and the TNF α treatment did not increase the responses of pulmonary C-fibers to lung inflation.

In both Veh- and TNF α -treated mice, a large percentage of the RARs exhibited distinct phasic baseline activity that was synchronous with either expiratory or inspiratory phase of respiratory cycles. The remaining RARs had no detectable or very little respiratory-related baseline activity (e.g., Fig. 3). These two types of RARs were categorized as phasic RARs and silent RARs in this study. All of the SARs tested in this study exhibited distinct phasic baseline activity that reached a peak during either inspiratory or expiratory phase of the

respiratory cycles; the latter was found only in a relatively smaller number in the mouse lung [26].

In Veh-treated mice, injections of Cap did not cause significant stimulatory effect on silent RARs (e.g., Fig. 3A). However, the responses of these silent RARs to Cap injections were elevated by >10 folds in TNF α -treated mice (Figs. 3B & 3C). Furthermore, the number of silent RARs that were activated by Cap injections, judged by the criterion that the increase in fiber activity was > 1.0 imp/s, was markedly higher in TNF α -treated mice: e.g., none of the 9 silent RARs tested were activated by the low dose of Cap (0.5 μ g/kg) in the Veh group, whereas 6 of the 8 silent RARs were stimulated in the TNF group (Fig. 3D). In contrast, there was no significant difference in the responses of silent RARs to either lung inflation ($P>0.05$) or deflation ($P>0.05$) between Veh- and TNF α -treated mice (Figs. 3E & 3F). In addition, the treatment with TNF α did not cause any potentiating effect on the responses to the same Cap injection, lung inflation and deflation in phasic RARs or SARs [26].

It is well recognized that the afferent activity generated by these C-fiber endings plays an important role in eliciting the pulmonary defense reflexes in both healthy and disease conditions [11,20]. Activation of these afferent endings by either inhaled chemical irritants or endogenous inflammatory mediators can generate respiratory sensations such as airway irritation and urge to cough [11]. In addition, it elicits powerful reflex responses including airway smooth muscle contraction, bronchial vasodilation and mucous hypersecretion mediated through the cholinergic reflex pathways [20,35]. Intense and/or sustained stimulation of these afferents causes “neurogenic inflammation” in the tracheobronchial tree: extravasation of macromolecules and inflammatory cell chemotaxis, which involves the local “axonal reflex” and release of several potent bioactive tachykinins from these sensory endings [36,37]. Thus, it seems logic to postulate that when the excitability of C-fiber afferents is enhanced by endogenous TNF α , these airway reflex responses to a given stimulus will be intensified.

After the TNF α treatment, a distinct sensitivity to Cap injection emerged in the silent RARs. This is an interesting finding because these receptors rarely exhibited any Cap sensitivity in control mice. However, very little information is available regarding the electrophysiological properties (e.g., conduction velocity, ion channels, etc.) and reflex functions of these silent RARs in mice. The firing behavior of these silent RARs exhibited certain features resembling the “high-threshold A δ vagal afferents” described by Yu and coworkers [38]; for example, they displayed a very mild sensitivity to Cap and other chemical stimulants of C-fibers (e.g., sulfur dioxide; data not shown). Based upon our observations in a follow-up cough study (described later), it is also possible that some of these RARs are the cough receptors that have been identified in other species [39,40].

Hypersensitivity of isolated pulmonary sensory neurons induced by TNF α

Because of the diverse actions of TNF α on a number of other target cells in the airways such as neutrophils, eosinophils and endothelial cells [5], it was impossible to determine if the sensitizing effect is mediated through a direct action of TNF α on these neurons in a whole-animal preparation. Therefore, this in-vitro study was carried out to determine the direct

effect of TNF α in isolated mouse pulmonary sensory neurons [26], using the Ca²⁺ imaging technique [41].

Pulmonary sensory neurons were isolated and cultured from the jugular-nodose ganglia complex in mice [21], and identified by retrograde labeling with DiI, a fluorescent dye, as previously described [42]. The changes in the intracellular Ca²⁺ concentration, [Ca²⁺]_i, in response to three concentrations of Cap (30, 100 and 300 nM) were measured in these neurons by the ratiometric method as described in our previous report [43].

Two matching dishes of pulmonary jugular-nodose ganglia neurons isolated and cultured from the same mouse were incubated with vehicle (Veh group) and 50 ng/ml of TNF α (TNF group) in culture medium for 24 h. In the WT neurons incubated with TNF α , Cap challenge evoked a rapid and transient increase in the [Ca²⁺]_i in a concentration-dependent manner; this Cap-evoked increase in Ca²⁺_i was significantly greater than that incubated with Veh, particularly at the higher concentrations of Cap (100 nM & 300 nM; Fig. 4). In another study series, the Cap-evoked response was markedly attenuated in the TNF^{-/-} neurons when the responses of [Ca²⁺]_i to increasing concentrations of Cap were compared between pulmonary sensory neurons isolated from WT mice and TNF^{-/-} mice after both groups of neurons were incubated with TNF α (50 ng/ml) for ~24 h. These results suggest the involvement of TNFR receptors in the TNF α -induced hypersensitivity of these neurons.

In an immunohistochemical study, we have previously demonstrated the presence of both TNFR1 and TNFR2 on the cell membrane of rat vagal pulmonary sensory neurons [44]. Activation of these TNFRs by TNF α can trigger several signaling pathways that are involved in a wide range of immunological responses and inflammatory reactions [45–47]. TNF α is initially produced as a membrane-anchored precursor protein, and subsequently cleaved into free proteins, which form biologically active homotrimers and interact with these TNFRs. Activation of TNFR1 is known to activate nuclear factor-kappa B [48] and mitogen activated protein kinases (MAPK) pathways, including the extracellular signal regulated kinase (ERK) [49]. Indeed, activation of p38 MAPK pathway increased TRPV1 expression in peripheral nociceptor neurons in a transcription- independent manner [50]. Furthermore, it has been shown that incubation of DRG neurons with TNF α for 24-48 h significantly increased the proportion of DRG neurons expressing TRPV1 receptor-like immunoreactivity via TNFR1 and the ERK activation [18]. Interestingly, a recent study has demonstrated the co-localization of TNFR1 and TRPV1 in the same DRG neurons in rats [16].

Intracellular Ca²⁺ is an important signal transduction molecule and plays a significant role in the regulation of neuronal membrane excitability, synaptic activity and neurotransmitter release [51,52]. Transient changes in [Ca²⁺]_i are known to contribute to short- or long-term alterations in ion channels and gene expression, and can modulate the overall function of these afferents [53,54]. Because Ca²⁺ is one of the main ion species that is conducted through TRPV1, the TNF α -induced increase in Ca²⁺ transient in response to Cap may result from an increase in the TRPV1 expression and/or excitability. On the other hand, the TNF α -induced sensitization of vagal C-fiber neurons should lead to a greater degree of subthreshold depolarization of the neuronal membrane and/or a larger number of action

potentials in response to a given level of chemical stimulation, which may in turn generate a higher level of Ca^{2+} influx via voltage-dependent Ca^{2+} channels [26].

Cough hyperresponsiveness induced by $\text{TNF}\alpha$ in awake mice

One of the most important functions of these vagal bronchopulmonary afferents is to protect the lung and the rest of body against inhaled irritants and harmful gases in the environmental air. When the bronchopulmonary C-fibers are activated by irritant gases inhaled into the respiratory tract, it can trigger vigorous airway reflex responses such as cough, bronchoconstriction and airway secretion; these reflex responses can expel and/or reduce the penetration of these harmful gases into deeper regions of the lung. Furthermore, a subset of RARs have been identified and characterized as cough receptors in other species by previous investigators; these receptors are located in the extrapulmonary airways and quiescent during eupneic breathing [39,40]. Based upon these background information, we hypothesized that the hypersensitivity of these afferents induced by $\text{TNF}\alpha$ elevated the cough responses to inhaled irritant gases in mice. We chose ammonia (NH_3) as the irritant gas to test this hypothesis because NH_3 is generated by nature as part of the nitrogen cycle in the environment, and is produced in large quantity for industrial use (e.g., agriculture fertilizers and household cleaning products); NH_3 at high concentrations (e.g., during occupational exposure or chemical accident) can cause lung injury and other detrimental health effects [55].

$\text{TNF}\alpha$ and Veh were administered by i.t. instillation in two separate groups of young C57BL/6/J mice as described earlier [27], and cough responses to NH_3 inhalation challenges were measured on days 2, 3, 6 and 7 after the $\text{TNF}\alpha$ or Veh treatment. Because the cough reflex is suppressed by general anesthesia, this study was carried out in awake mice. The methodology was described in details in a recent report [28]. Briefly, during the experiment, mice moved freely in a plexiglass recording chamber (volume 160 ml). To prevent CO_2 accumulation and hyperthermia, room air was drawn through the chamber at a constant flow rate (200 ml/min) by a negative pressure. The pressure change in the chamber (P_{cham}), audio and video signals of the mouse movements were recorded simultaneously and used collectively for analyzing the cough frequency (Fig. 5A). Because breathing is inherently unstable in awake free-moving mice, the accuracy of the cough analysis was further verified by comparing with the changes of the intra-pleural pressure (P_{ip}) (e.g., Fig. 5A), which was measured directly by surgically implanting the tip of a telemetry sensor in the intra-pleural space [28].

The action of cough recorded in these mice consisted three distinct but tightly connected phases as characterized by the synchronized changes in both P_{cham} and P_{ip} : it was initiated by an inspiratory effort followed by a chest compression, and then a forced expiration that coincided with a weak but detectable cough sound. Expiratory reflex, similar to that described in other species [56,57], were also frequently found in awake mice; in comparison, the expiration reflex consisted only a chest compression, with no clear or significant preceding inspiratory effort, and followed by a forced expiration (Fig. 5B). In addition, ~15% of the responses could not be easily distinguished between cough and expiration reflexes in our experiments [28]. Because both coughs and expiration reflexes are airway

defense responses, they were pooled for data analysis in the evaluation of the overall cough responses in this study (Fig. 6).

In this study, the mouse was placed in the recording chamber for >30 min of adaptation period before each experiment. During the irritant gas inhalation challenge, ammonia (NH₃; 0.1% in air) was drawn into the chamber at the same flow rate as during baseline for 8 minutes; the cough frequency was analyzed for the 5-min baseline, 8-min challenge and 5-min recovery in each animal. The cough frequency was near zero at the baseline (before the NH₃ inhalation challenge) in both Veh- and TNF α -treated mice, which did not change significantly after either Veh or TNF α treatment (Fig. 6). However, 48 h after the TNF α treatment, the cough frequency during the NH₃ inhalation challenge was elevated to more than five folds of that before the TNF α treatment, and the enhanced cough responsiveness to the NH₃ inhalation challenge persisted even after 7 days (Fig. 6). In sharp contrast, there was no significant change in the cough response to NH₃ at any of these time points after the Veh treatment.

The cough reflex elicited by NH₃ was probably elicited by activation of C-fibers and/or rapidly adapting receptors (RARs) located in the lung and airways [11,22,58] and/or tracheal cough receptors [40]. Indeed, using a single-fiber recording technique, we have shown that the NH₃ (0.1% concentration) inhalation challenge stimulated both these two types of vagal bronchopulmonary afferents in anesthetized mice (unpublished data). However, we cannot evaluate the relative role(s) of these different afferents in the TNF α -induced increase in cough response to inhaled NH₃ in this study.

NH₃ has been shown to stimulate both TRPV1 and transient receptor potential ankyrine type 1 (TRPA1) receptors [59]. Both TRPV1 and TRPA1 receptors are ligand-gated non-selective cation channels [34], and known to play important roles in the inflammation-induced hypersensitivity of vagal bronchopulmonary afferents [11,12]. Our earlier studies have demonstrated that the same TNF α treatment induced pronounced airway inflammation and elevated sensitivities of both C-fibers and RARs in the lungs. Together, these results seem to suggest that the increased cough responses to NH₃ was caused by the TNF α -induced hypersensitivity of these bronchopulmonary afferents.

Conclusion

These studies demonstrated that intra-tracheal instillation of TNF α induced pronounced airway inflammation as illustrated by infiltration of eosinophils and neutrophils and the release of inflammatory mediators and cytokines in the lung and airways. Coinciding with the time of these changes, the TNF α treatment induced a distinct increase in the Cap sensitivity in vagal pulmonary C-fibers and silent RARs. Furthermore, the increased sensitivity to Cap was also present in the isolated pulmonary sensory neurons, suggesting that the sensitizing effect was mediated primarily through a direct action of TNF α on these neurons. Indeed, the same TNF α treatment also induced a lingering (> 7days) cough hyperresponsiveness to inhalation challenge of NH₃ in awake mice, further illustrating the heightening sensitivity of these bronchopulmonary afferents.

Stimulation of bronchopulmonary C-fiber afferents can also elicit bronchoconstriction and airway hypersecretion via the cholinergic reflex pathways [20,35]; intense stimulation can further generate neurogenic inflammation in the airways mediated through local axonal reflex mechanism [36,37]. Hence, when the excitability of C-fiber afferents is enhanced by endogenous TNF α , these airway reflex responses to a given stimulus is expected to be intensified. The finding of the TNF α -induced hypersensitivity of bronchopulmonary C-fiber afferents in these studies may explain, at least in part, the airway hyperresponsiveness and inflammation caused by Inhalation of TNF α in healthy humans [10].

Both the airway inflammation and the sensitizing effect on pulmonary sensory neurons caused by the TNF α treatment were abolished in the TNF $^{-/-}$ mice. These results further suggested the involvement of TNFR receptors. We believe that the activation of TNFRs by TNF α may activate several signaling pathways, trigger chemotactic actions, and enhance the release of inflammatory mediators and cytokines in the airways. Together, these TNFRs-mediated actions may upregulate the sensitivity and/or expression of TRPV1 in the bronchopulmonary sensory nerves. Although the mechanism(s) underlying the potentiating effect of TNF α on these vagal bronchopulmonary sensory nerves is still not fully understood, these studies have established the evidence of this sensitizing effect of TNF α in mice, which should facilitate further investigations of the potential involvements of certain TNFR-mediated signaling pathways in transgenic and knockout mouse models.

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Fig. 1.

Effect of TNF α treatment on inflammatory mediators in bronchoalveolar lavage fluid (BALF). Naïve group (open bars; WT mice receiving no surgery or treatment); Veh group (shaded bars; WT mice pretreated with vehicle PBS); TNF α group (closed bars; WT mice pretreated with TNF α); TNF $^{-/-}$ + TNF α group (hatched bars; TNF $^{-/-}$ mice pretreated with TNF α). BALF was collected at 24 hours after the pretreatment of TNF α or vehicle. LTC₄ and LTC₄/D₄/E₄: leukotrienes B₄ and C₄/D₄/E₄, respectively; PTX3: pentraxin 3; PGE₂: prostaglandin E₂; TXB₂, thromboxane B₂; IL-13 and IL-1 β : interleukins 13 and 1 beta, respectively. Each data point represents means $\pm$ SEM of the measurements; the total number of mice used in these groups varied: n = 10, 15, 15 and 5 in naïve, Veh, TNF α , and TNF $^{-/-}$ + TNF α groups, respectively. Thus, some tests (e.g., LTB₄, IL-1 β) were only performed in two or three groups of mice. *, significantly different from naïve group ($P < 0.05$). #, significantly different from Veh group ($P < 0.05$). (Modified from reference [27])

Fig. 2.

A) Experimental records illustrating the effect of TNF α on the responses of pulmonary C fibers to capsaicin in anesthetized, open-chest and artificially ventilated mice. Vehicle (Veh; 0.03 ml of PBS) and TNF α (10 μ g/ml, 0.03 ml) were administered by intratracheal instillation into the lungs of Veh and TNF α mice (weights: 27.8 g & 25.9 g), respectively, 24 h earlier. Arrows were added to mark the time when capsaicin (Cap; 1.0 μ g/kg) was injected intravenously as a bolus. Both receptor locations were in the right lung. P_T, tracheal pressure; AP, action potential; ABP, arterial blood pressure. B) Group data of pulmonary C fiber responses to the Cap injection ~24 h after the Veh (open bars) and TNF α (closed bars) treatments. FA: the increase in fiber activity (FA; impulses/s) from baseline (averaged over the 10-s period preceding the injection) to the peak response (averaged over 2-s duration) within the first 5 s after the injection. The number in each bar represents the number of fibers studied under that condition. * significantly different from the low dose of Cap (0.5 μ g/kg) ($P < 0.05$). # significantly different from the Veh group ($P < 0.05$). Data are means $\pm$ SEM. (Modified from reference [26])

Fig. 3.

A) & B): Experimental records illustrating the effect of TNF α on the responses of silent RARs to capsaicin, inflation and deflation of the lung in two anesthetized, open-chest and artificially ventilated mice. A) Veh (0.03 ml of PBS) and B) TNF α (10 μ g/ml, 0.03 ml) were administered ~24 h earlier by intratracheal instillation into the lungs of Veh and TNF α mice (weights: 31.8 g & 25.9 g), respectively. In both A) and B), left panels: responses to constant pressure (30 cmH₂O) hyperinflations of the lungs for 8 s when the ventilator was turned off; middle panels: responses to lung deflations when the expiratory line of the ventilator was exposed to atmospheric pressure; right panels: responses to intravenous bolus injections of capsaicin (1.0 μ g/kg) at arrows. Both receptor locations were in the right lung. From C) to F): group data of silent RARs after treatments with Veh (open bars) and TNF α (closed bars). C): responses to Cap injections (0.5 & 1.0 μ g/kg); see the legend of Fig. 2B for explanations. D): percentages of silent RARs that exhibited Cap sensitivity (FA > 1.0 imp/s after Cap injection). E): response to lung hyperinflation (P_T=30 cmH₂O); FA: the difference in FA between the first second of lung inflation and the baseline (averaged over 10 s). F): response to lung deflation; FA: the difference between the FA averaged over the

8-s duration of deflation and the baseline (averaged over 10 s). * significantly different from the Veh group ($P < 0.05$). (Modified from reference [26])

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Fig. 4.

A) Representative experimental records illustrating the change in $[Ca^{2+}]_i$ evoked by increasing concentrations of capsaicin in vagal pulmonary sensory neurons. *Left panel:* a pulmonary neuron (diameter: 24 μ m) isolated from a wild-type (WT) mouse; *Right panel:* a pulmonary neuron (diameter: 22 μ m) isolated from a TNF-receptor double homozygous mutant (TNF $^{-/-}$) mouse. Both WT and TNF $^{-/-}$ neurons had been incubated with TNF α (50 ng/ml in culture medium) for ~24 h. Cap was applied for 30 s each, and KCl solution (60 mM, 20 s) was applied to test cell vitality at the end of each experiment. B) Group data showing a comparison of the responses of $[Ca^{2+}]_i$ to increasing concentrations of Cap between Veh- and TNF α -treated neurons isolated from wild-type (WT) mice. C) Group data illustrating the effect of TNF α treatment on the $[Ca^{2+}]_i$ responses to increasing concentrations of Cap in neurons isolated from WT and TNF $^{-/-}$ mice. In both B) & C), *: significantly different from the corresponding response to 30 nM capsaicin ($P < 0.05$). # in B): significantly different from the corresponding data in Veh- treated neurons ($P < 0.05$); in C): significantly different from the corresponding data in TNF $^{-/-}$ neurons ($P < 0.05$). Data are mean $\pm$ SEM. (Modified from reference [26])

Fig. 5.
A): Schematic diagram of the experimental set-up. Mic: microphone for recording audio signal; Temp: air temperature in the chamber. B) Representative experimental records illustrating the characteristic features of pressure changes and the audio signal generated by a single cough (*Left*) and a single expiration reflex (*Right*) in an awake mouse. P_{cham} : pressure inside the chamber; P_{ip} : intra-pleural pressure recorded via the telemetry sensor. (Modified from reference [28])

Fig. 6.

Effects of A) Vehicle (Veh, 0.03 ml of PBS; n=5) and B) TNF α (10 μ g/ml, 0.03 ml; n=5) treatments on the cough response to inhalation challenge of NH₃ (0.1%) in awake mice at different time points after the treatment. The cough frequency count included both cough and expiration reflex. Data (means $\pm$ SEM) represent the group responses averaged over the 5-, 8- and 5-min durations of “Baseline” “During NH₃” and “Recovery”, respectively. *, significantly ($P < 0.05$) different from the baseline data (open bars). #, significantly ($P < 0.05$) different from the corresponding data obtained before the Veh or TNF α treatment.