Involvement of preprotachykinin A gene-encoded peptides and the neurokinin 1 receptor in endotoxin-induced murine airway inflammation

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Abstract

Tachykinins encoded by the preprotachykinin A (TAC1) gene such as substance P (SP) and neurokinin A (NKA) are involved in neurogenic inflammatory processes via predominantly neurokinin 1 and 2 (NK1 and NK2) receptor activation, respectively. Endokinins and hemokinins encoded by the TAC4 gene also have remarkable selectivity and potency for the NK1 receptors and might participate in inflammatory cell functions. The aim of the present study was to investigate endotoxin-induced airway inflammation and consequent bronchial hyperreactivity in $TAC1^{-/-}$, $NK1^{-/-}$ and also in double knockout ($TAC1^{-/-}/NK1^{-/-}$) mice.

Sub-acute interstitial lung inflammation was evoked by intranasal E. coli lipopolysaccharide in the knockout mice and their wildtype C57BL/6 counterparts 24 h before measurement. Respiratory parameters were measured with unrestrained whole body plethysmography. Bronchoconstriction was induced by inhalation of the muscarinic receptor agonist carbachol and Penh (enhanced pause) correlating with airway resistance was calculated. Lung interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) concentrations were measured with ELISA. Histological evaluation was performed and a composite morphological score was determined. Myeloperoxidase (MPO) activity in the lung was measured with spectrophotometry to quantify the number of infiltrating neutrophils/macropahges.

Airway hyperreactivity was significantly reduced in the $TAC1^{-/-}$ as well as the $TAC1^{-/-}/NK1^{-/-}$ groups. However, LPS-induced histological inflammatory changes (perivascular/peribronchial oedema, neutrophil infiltration and goblet cell hyperplasia), MPO activity and TNF-α concentration were markedly diminished only in $TAC1^{-/-}$ mice. Interestingly, the concentrations of both cytokines, IL-1β and TNF-α, were significantly greater in the $NK1^{-/-}$ group.

These data clearly demonstrated on the basis of histology, MPO and cytokine measurements that TAC1 gene-derived tachykinins, SP and NKA, play a significant role in the development of endotoxin-induced murine airway inflammation, but not solely via NK1 receptor activation. However, in inflammatory bronchial hyper-responsiveness other tachykinins, such as hemokinin-1 acting through NK1 receptors also might be involved.

Keywords:

unrestrained whole body plethysmography; enhanced pause; myeloperoxidase activity; interleukin-1β; tumor necrosis factor-α; substance P; neurokinin A

1. Introduction

Tachykinins such as substance P (SP) and neurokinin A (NKA) encoded by the preprotachykinin-A (PPTA; TAC1) gene and released from capsaicin-sensitive primary afferent sensory neurones influence a variety of effects in the airways under both normal and pathological conditions. They elicit neurogenic inflammation (plasma protein extravasation, increased microcirculation and stimulation of inflammatory cells), induce smooth muscle contraction, regulate vascular tone, mucus secretion and immune functions (Barnes, 1990; Lundberg, 1995; Maggi, 1995; Szolcsanyi, 1982). Although tachykinins are synthesized and released predominantly from sensory nerves, inflammatory and immune cells are also able to produce tachykinins in response to certain inflammatory stimuli. The cellular source of tachykinins may play an important role in inflammatory airway diseases such as asthma or chronic obstructive pulmonary disease (Groneberg et al., 2006). Consistent with this we have demonstrated that lung epithelia can express SP in response to virus infection in the mouse (Stewart et al., 2008).

Two tachykinin receptors, the neurokinin 1 (NK1) and neurokinin 2 (NK2) receptors mediate the biological actions of tachykinins in the airways, the preferred ligands for these receptors are SP and NKA, respectively (Frossard and Advenier, 1991; Regoli et al., 1994). NK2 receptors have been demonstrated to be involved in bronchoconstriction and bronchial hyperresponsiveness in several rat and guinea pig models as well as human studies, while NK1 receptors have been found to participate in neurogenic inflammation (Advenier et al., 1997; Joos et al., 2001; Lagente and Advenier, 1998). NK1 and NK2 receptors are localized on leukocytes, mononuclear cells, vascular endothelial and bronchial epithelial cells (Lundberg, 1995; Maggi, 1995; Szolcsanyi, 1996). We have previously demonstrated that in acute lung inflammation simultaneous activation of NK1 and NK2 receptors participate in neutrophil accumulation, but NK2 receptors play a predominant role in enhanced airway resistance (Elekes et al., 2007).

Since the discovery of a third preprotachykinin gene (TAC4), the number of tachykinins has doubled, several peptides have been identified in peripheral organs of many species and a broad range of actions has been revealed (Page, 2004; Page, 2005; Page, 2006). This group includes hemokinins in mice, rats and humans (HK-1, hHK-1 and hHK_{4-1}; Kurtz et al., 2002; Zhang et al., 2000), as well as endokinins in rabbits and humans (EK-1, EKA and EKB; Page et al., 2003). HKs and EKs have been shown to have remarkable selectivity and potency for the NK1 receptors and might participate in inflammatory cell functions (Page, 2006;

Groneberg et al., 2006). Predominantly peripherally expressed SP-like endokinins have been proposed to be the peripheral NK1 receptor agonists (Page, 2004), thus, the tachykinin system seems to be much more complex than it has been previously thought. Many of the tachykinin-mediated processes appear to be tissue-specific, permitting unique interactive combinations of tachykinins and receptor configurations (Page, 2006). Therefore, the aim of the present study was to investigate the involvement of the tachykinin system in endotoxin-induced airway inflammation and consequent bronchial hyper-reactivity. For this purpose TAC1, NK1 and TAC1 plus NK1 (double) gene-deleted mice were used and integrative examination was performed. Inflammatory bronchial responsiveness was measured by *in vivo* whole body plethysmography, and the inflammatory reaction was evaluated by histological, chemical and immunological techniques.

2. Materials and methods

2.1. Animals

All mice used in this study were C57BL/6J wildtypes or genetically altered ones whose background strain was C57BL/6J (BL6). All mice were 20-25 g, adult and sexually mature.

The generation of the $TAC1^{-/-}$ mice (Zimmer et al., 1998) and the $NK1^{-/-}$ mice has been previously described in details (De Felipe et al., 1998; Laird et al., 2000). The original breeding pairs of the double knockouts ($TAC1^{-/-}/NK1^{-/-}$) were generated by selective cross breeding of the above strains ($TAC1^{-/-}xNK1^{-/-}$) to produce a mouse that is null for the SP/NK1 pathway.

Screening for double knockouts. TAC1 knockout males and NK1 knockout females were bred together to produce an F1 generation that carried each of the deletions heterozygously. The F1s were then backcrossed to TAC1 knockout mice to produce an F2 generation 25% of which were homozygous for the TAC1 deletion and heterozygous for the NK1 deletion. Males and females of this genotype were then bred together and 25% of their offsprings were the double knockout genotype that we were looking for. This method of breeding greatly reduces the number of mice needed to achieve generation of a double knockout and reduces waste of genotypes that are non-desirable. This method also enhances the chances of generating a double knockout that may be physiologically challenged as the ratio is always 1 in 4 for the desired genotype as opposed to 1 in 16 if the heterozygous F1 generation were bred together. All offsprings had less than 0.5 cm of their tail removed under isoflurane

anaesthesia at 3-4% mix with oxygen. Bleeding was controlled by cauterisation and mice were ear-marked with unique identifications still under anaesthesia. DNA was extracted from the tail samples and PCR was preformed for TAC1 and NK1 separately on all samples using the following primers:

TAC1 primers:

Tac1 common (AZ95) GCC TTT AAC AGG GCC ACT TGT TTT TCA ATT

Tac1 knockout (CNKO4) ACT GTG GTT TCC AAA TGT GTC AGT T

Tac1 wildtype (AZ99) AGA CCC AAG CCT CAG CAG TTC TTT GGA TTA ATG

Wildtype band= 550bp (primers AZ95+ AZ99)
Knockout band = 220bp (primers AZ95+ CNK04)

NK1 primers:

NK-1Fwd 5'-CTGTGGACTCTGATCTCTTCC -3',

NK-1Rev 5'-ACAGCTGTCATGGAGTAGATAC -3',

NeoFwd 5'- GCAGCGCATCGCCTTCTATC -3';

Wildtype band = 344 bp (primers NK-1Fwd and NK-1Rev)

Knockout band = 298 bp (primers NeoFwd and NK-1Rev)

Breeding pairs of all knockout strains were transferred to the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pécs. They were bred on and kept there at 24-25 °C provided with standard rodent chow and water *ad libitum*.

2.2. Induction of airway inflammation

Subacute airway inflammation was evoked by 60 μl Escherichia coli (serotype: 083) LPS (167 μg/ml dissolved in sterile PBS; Sigma, St. Louis, MO, USA) applied intranasally 24 h prior to measurement (n=6-10 per group). Data showing that intranasal administration of this LPS dose evoked maximal inflammation (neutrophil accumulation and inflammatory cytokine production) 24 h after its instillation served as basis for choosing this time point (Okamoto et al., 2004). Control animals received the same volume of sterile PBS (n=6).

2.3. Determination of airway responsiveness

Airway responsiveness in conscious, spontaneously breathing animals was measured by recording respiratory pressure curves by whole body plethysmography (Buxco Europe Ltd, Winchester, UK). Aerosolized saline and then the muscarinic acetylcholine receptor agonist carbachol (carbamoylcholine; Sigma, St. Louis, MO, USA) in increasing concentrations (50 μl per mouse for 1.5 min in 5.5, 11 and 22 mM concentrations) were nebulized through an

inlet of the main chamber for 50 sec to induce bronchoconstriction 24 h after LPS administration and readings were taken and averaged for 15 minutes following each nebulization. Baseline values usually returned at the end of this period. Enhanced pause (Penh) was measured as an indicator of bronchoconstriction and consequent increase of airway resistance. Penh is a complex, calculated parameter ((expiratory time/ relaxation time)-1): (max. expiratory flow/ max. inspiratory flow), which closely correlates with airway resistance as measured by traditional invasive techniques using ventilated animals. Percentage increase of the enhanced pause (Penh) above baseline ((penh in response to the respective carbachol concentration- baseline penh/baseline penh) x 100) was calculated in each 15 min period after respective carbachol stimulations. At the end of the measurement mice were anaesthesized with ketamine (100 mg/kg i.p.; Calypsol; Richter-Gedeon Plc., Budapest, Hungary) and xylazine (10 mg/kg i.m.; Xylavet; Phylaxia-Sanofi, Veterinary Biology Co. Ltd., Budapest, Hungary), blood samples were taken by cardiac puncture, then killed by cervical dislocation and their lungs were excised (Helyes et al., 2007; Helyes et al., 2009).

2.4. Histological studies and scoring

Lung specimens were fixed in 4% formaldehyde for 8 h, embedded in paraffin, sectioned with a microtome (5-7 μm) and routinely stained with hematoxylin and eosin. The periodic acid-Schiff reaction was used to visualize mucus producing goblet cells. Semiquantitative scoring of the inflammatory changes was performed by an expert pathologist blinded from the study as described by Zeldin et al. (2001) on the basis of the presence or abundance of the following: (1) perivascular oedema (0: absent; 1: mild to moderate, involving fewer than 25% of the perivascular spaces; 2: moderate to severe, involving more than 25% but less than 75% of perivascular spaces; 3: severe involving more than 75% of perivascular spaces); (2) perivascular/peribronchial acute inflammation (0: absent; 1: mild acute inflammation in the perivascular oedematous space with fewer than 5 neutrophils per high-power field (hpf); 2: moderate acute inflammation in the perivascular spaces extending to involve the peribronchial spaces with more than 5 neutrophils per hpf in these regions; 3: severe acute inflammation in the perivascular and peribronchial spaces with numerous neutrophils encircling most bronchioles; (3) goblet cell hyperplasia of the bronchioles (0: absent; 1: few goblet cells present in one or two bronchioles; 2: large number of goblet cells present); (4) macrophages in the alveolar spaces (0: absent; 1: present in fewer than 25% of alveolar spaces, 2: >25% of alveolar spaces). The score values for these individual parameters were added to form a composite inflammatory score (ranging from 0 to 10). From every specimen (8-10 mice in

each group) 4-5 sections were taken from different depths to give a representative appreciation of the whole lung. Mean scores were determined from the different sections of the individual animals and composite score values of the different experimental groups were calculated from these mean scores.

2.5. Measurement of myeloperoxidase (MPO) activity in the lung

Accumulation of granulocytes, especially neutrophils, was determined from the frozen lung samples by assessment of MPO activity. Lung pieces were thawed and chopped into small pieces then homogenized in 4 ml 20 mM potassium-phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000g at 4 °C for 10 min and supernatant was removed for somatostatin radioimmunoassay (see below). The pellet was resuspended in 4 ml 50 mM potassium-phosphate buffer containing 0.5% hexadecyl-trimethyl-ammonium-bromide (pH 6.0) and centrifuged again. Neutrophil accumulation was assessed by comparing MPO enzyme activity of the samples to a human standard MPO preparation (Sigma, St. Louis, MO, USA). MPO activity was assayed from the supernatant using H_2O_2-3,3',5,5`-tetramethyl-benzidine (TMB/H_2O_2) (Sigma, St. Louis, MO, USA). Reactions were performed in 96-well microtitre plates in room temperature. The optical density (OD) at 620 nm was measured at 5 min intervals for 30 min, using a microplate reader (Labsystems) and plotted. The reaction rate (Δ OD/time) was derived from an initial slope of the curve. A calibration curve was then produced, with the rate of reaction plotted against the standard samples.

2.6. Measurement of inflammatory cytokine concentrations in the lung

Lung samples were frozen in liquid nitrogen and stored at -80 °C. Homogenization was performed in 450 μl RPMI 1640 buffer (Biochrom Ltd., Berlin, Germany) containing 50 μl phenyl-methyl-sulphonyl-fluoride (PMSF) (Sigma, St. Louis, MO, USA) with a polytrone homogenizer (Kika Lab Techniques) at 13.500 rpm for 2 min. The homogenates were centrifuged for 10 min at 10.000 rpm and 4 °C. The concentrations of two inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), were determined by specific ELISA techniques (BD Sciences Eastern Europe, Heidelberg, Germany).

2.7. Statistical analysis

Percentage increase of Penh above baseline ((Penh in response to the respective carbachol concentration- baseline Penh/baseline Penh) x 100), MPO acitivity values and cytokine

concentrations are expressed as the mean±SEM of n=8-10 mice in each group and analyzed with two way ANOVA followed by Newman-Keuls post test. Histological inflammatory score values are demonstrated in box plots showing the median, upper/lower quatile and maximum/minimum values, statistical evaluation was performed with Kruskal-Wallis followed by Dunn's post test. In all cases $p<0.05$ was considered to be significant.

2.8. Ethics

All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied with the recommendations of the Helsinki Declaration. The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments and licence was given (licence No.: BA 02/2000-11-2006).

3. Results

3.1. Inflammatory airway hyperresponsiveness in TAC1, NK1 and double knockout mice

Baseline Penh significantly increased 24 h after intranasal LPS treatment in all groups compared to the respective PBS-treated control mice. Inhalation of increasing concentrations (5.5, 11 and 22 mM) of the muscarinic receptor agonist carbachol evoked a concentration-dependent bronchoconstriction as shown by the Penh curves (Figure 1). In wildtype mice responses demonstrated as percentage increase of Penh above baseline were markedly enhanced in the LPS-treated group compared to the respective non-inflamed controls which supports the development of inflammatory bronchial hyperresponsiveness.

In TAC1 and TAC1/NK1 gene-deficient mice the LPS-induced airway hyperreactivity was markedly reduced, particularly at the highest carbachol concentration. LPS-evoked airway hyperreactivity remained practically unchanged in the NK1 receptor-deleted group (Figure 1). LPS-evoked airway hyperreactivity was decreased in the NK1 receptor-deleted group, but it was not statistically significant.

3.2. Inflammatory histopathological changes in the lung of TAC1, NK1 and double knockout mice

LPS induced marked peribronchial/perivascular oedema formation, neutrophil accumulation around the bronchi, infiltration of activated recruited macrophages/lymphocytes into the alveolar spaces and moderately increased number of mucus producing goblet cells in the wildtype group (Fig. 2A,B). Meanwhile, in mice lacking SP and NKA due to the deletion of the TAC1 gene, the extent of the oedema and the number of the oedematous structures, neutrophil accumulation as well as macrophage infiltration, but not goblet cell hyperplasia were significantly less intense, therefore the composite inflammation scores calculated from these parameters were also markedly lower (Fig. 2C). In contrast, these inflammatory parameters were not altered by NK1 receptor deletion (Fig. 2D). Surprisingly, a lower oedema intensity and neutrophil accumulation observed in case of missing SP and NKA was not seen in mice that did not express the NK1 receptor (Fig. 2E; Fig. 3 A,B). We have not observed any changes in the control mice in any of the three knockout groups.

3.3. Myeloperoxidase activity in the lung homogenates

Endotoxin induced more than 2-fold elevation of MPO activity in the lung one day after intranasal administration. This quantitative marker of accumulated neutrophils in the inflamed tissue was significantly decreased in TAC1 gene-deficient mice, but not in the NK1 and the TAC1/NK1 knockout animals (Fig. 4).

3.4. Inflammatory cytokine concentrations in the lung

Lung IL-1β and TNF-α levels markedly increased 25 h after intranasal LPS administration compared to the concentrations measured in the lungs of PBS-treated control animals. The absolute concentration of the latter inflammatory cytokine was about ten-fold less. Lung TNF-α, but not IL-1β concentration was significantly lower in the $TAC^{-/-}$ group. In contrast, LPS-induced IL-1β and TNF-α production was elevated in NK1 receptor-deficient animals. No change was observed, however, in cytokine concentrations in the pulmonary tissues of the double knockout mice (Fig. 5).

4. Discussion

With the help of genetically manipulated mice the present results provide clear evidence that deletion of the TAC1 gene and consequent lack of both SP and NKA inhibits endotoxin-induced inflammatory changes and hyperresponsiveness. In contrast, NK1 receptor deficiency does not influence bronchial hyperreactivity, MPO activity and the extent of the inflammatory response, but surprisingly, enhances cytokine production. Interestingly, the anti-inflammatory actions observed in TAC1 gene-deficient mice are counteracted by deleting the NK1 receptor gene.

Intranasally administered endotoxin induces a subacute interstitial inflammation and hyperreactivity with a well-defined mechanism (Lefort et al., 2001). LPS is a primary activator of macrophages through Toll-like receptor activation and elicits cytokine release with consequent granulocyte activation (Rocksen et al., 2003; Savov et al., 2002; Vargaftig, 1997). Neutrophils are recruited to the subepithelial regions, generate reactive oxygen species, proteases, cytokines and chemokines (Kraneveld and Nijkamp, 2001), which attract and stimulate further macrophages and lymphocytes. Several other inflammatory mediators, such as leukotriens, prostaglandins, bradykinin, are released from these cells which can directly activate afferent terminals in the airways or can induce epithelial damage resulting in the exposure of sensory nerves (Barnes, 2001; Kraneveld and Nijkamp, 2001). Tachykinins released from these nerves in turn influence the inflammatory process by acting at receptors localized on these peripheral nerve terminals themselves, vascular endothelial, bronchial epithelial and inflammatory cells. They have recently been shown to increase nNOS expression in inflammatory and reticulo-endothelial cells (Prado et al., 2008). Furthermore, tachykinins also induce bronchoconstriction (Lundberg, 1995), therefore, they might be mediators of bronchial hyperresponsiveness acting directly on the smooth mucle cells (Colasurdo et al., 1995; Ladenius et al., 1995).

We have previously shown with radioimmunoassay that SP-like immunoreactivity (SP-LI) significantly increases in the lung one day after the same dose of intranasal endotoxin administration in the mouse. This elevation is abolished after selective destruction of the capsaicin-sensitive nerve endings, but basal SP-LI is not altered (Elekes et al., 2007). This supports the concept that SP is mainly released from capsaicin-sensitive sensory nerve terminals during the inflammatory reaction, but there is also a considerable amount of these peptides in other non-neural cells, such as airway epithelial cells (Hastings and Hua, 1995; Li et al., 2004; Rennick et al., 1992), pulmonary neuroendocrine cells and immune cells (Nelson

and Bost, 2004; Springer et al., 2003). Since TAC4 gene-derived peptides, hemokinins and endokinins, give an immunological cross-reaction with SP, they cannot be differentiated with radioimmunoassay (Page, 2004). Therefore, in our mouse model the measured lung SP-LI might have reflected to HK-1 content.

The present data support our earlier results that in the same model NK2 receptors play a predominant role in bronchial hyperreactivity, since the NK2 receptor antagonist SR 48968 inhibited this response (Elekes et al., 2007). NK2 receptors were shown to be involved in bronchoconstriction and bronchial hyperresponsiveness in several other animal models and human studies as well (Advenier et al., 1997; Joos et al., 2001; Lagente and Advenier, 1998). The inflammatory signs were, however, only inhibited by combined administration of NK1 and NK2 receptor antagonists (Elekes et al., 2007). Other groups have also described that only the combination of NK_1 and NK_2 receptor antagonists resulted in a significant reduction of neutrophil influx and matrix metalloproteinase-9 activity in a similar LPS-induced mouse model of airway inflammation (Veron et al., 2004). Activation of NK1+NK2 receptors localized on the surface of endothelial cells and granulocytes is involved in the expression of adhesion molecules and consequent leukocyte adhesion and accumulation (DeRose et al., 1994). A potent triple tachykinin NK1, NK2 and NK3 receptor antagonist more effectively inhibited the inflammatory reaction in the respiratory tract than selective tachykinin receptor antagonists (Tsuchida et al., 2008).

In accordance with our results, a recent study showed markedly diminished systemic endotoxin-induced liver, lung and renal injury in TAC1 gene-deleted mice as evidenced by tissue MPO activities, plasma alanine aminotransferase, aspartate aminotransferase levels and histological examination. Significantly decreased chemokine, proinflammatory cytokine and adhesion molecule levels are produced in TAC1 knockout animals, which suggests that SP and NKA are critical proinflammatory mediators in endotoxinaemia and the associated multiple organ injury (Ng et al., 2008). The significance of TAC1 gene expression and SP production in the airways is supported by its induction in several cells in the respiratory tract such as tracheal, bronchiolar and alveolar epithelial cells and macrophages one day after viral infection, earlier than the observable inflammation and the expression of pro-inflammatory chemokines (Stewart et al., 2008).

Data concerning the role of NK1 receptors in the respiratory tract are rather divergent and seem to be dependent on the inflammatory mechanisms. Its activation contributes to acute ozone inhalation-induced epithelial injury and subsequent proliferation, a critical component of repair (Oslund et al., 2008). NK1 receptor stimulation triggers chronic cigarette smoke-

induced metalloelastase MMP-12 synthesis in activated pulmonary macrophages, which plays a key role in the pathogenesis of emphysema (Xu et al., 2008). Oil smoke exposure significantly increases the production of reactive oxygen species in the lung and the size of experimentally induced thrombi through NK1 receptors (De Swert et al., 2009; Ping-Chia et al., 2009). In contrast, there are also data suggesting a protective role of NK1 receptor in the lung. Oxidative lung injury, inflammatory cell accumulation and oedema induced by 90% oxygen are markedly worse in mice lacking the NK1 receptors (Dib et al., 2009). The present data also suggest an inhibitory action of NK1 receptors on inflammatory cytokine production in our model. The findings that most decreased inflammatory parameters and hyperactivity observed in the TAC1 gene-deleted mice were counteracted in the double knockouts might be explained by the predominantly inhibitory effect mediated through NK1 receptors on neutrophils and macrophages. SP and TAC4-derived tachykinins both bind to the NK1 receptor, but they have distinct binding sites and different signaling pathways (Page, 2004; Page 2005; Page 2006). Gs protein-coupled processes have been described following NK1 receptor activation leading to increased cAMP production and decreased cytokine production and release (Maggi and Schwartz, 1997; Page, 2006). We propose that this mechanism is likely to operate when both SP and HK-1 bind to the NK1 receptor in wildtype animals. In contrast, in case of SP deficiency, when only HK-1 binding can activate NK1 receptors, G_q receptor-coupled processes might be present resulting in increased Ca^{2+} concentration and enhanced cytokine release from the inflammatory cells.

In conclusion, the present data clearly demonstrate on the basis of histological evaluation, MPO and cytokine measurements that the lack of TAC1 gene-derived tachykinins, SP and NKA, play a predominant role in the development of endotoxin-induced murine airway inflammation and inflammatory bronchial hyperresponsiveness, but not via NK1 receptor activation.

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Figure Legends

Fig. 1. Inflammatory airway hyperresponsiveness. Bronchoconstriction was evoked by inhalation of increasing concentrations of carbachol measured in freely moving, unrestrained mice with whole body plethysmography. Responses are shown as percentage increases of the mean enhanced pause (Penh) responses above baseline (saline) within a 15-min period after each carbachol stimulation. Each data point represents the mean±S.E.M. of n=6-10 mice per group; *p<0.05, **p<0.01 vs. the respective PBS-treated non-inflamed mice; #p<0.05, ##p<0.01 vs. the LPS-treated wildtype group (one-way ANOVA followed by Bonferroni's modified t-test).

Fig. 2. Representative histopathological pictures of the lung of (A) PBS-treated control and (B) LPS-treated inflamed wildtype mice. Peribronchial/perivascular oedema formation, neutrophil accumulation around the bronchi, infiltration of activated recruited macrophages/lymphocytes into the alveolar spaces and moderately increased number of mucus producing goblet cells can be seen in response to intranasal LPS administration.
The lower three panels show the light micrographs of the pulmonary tissues of LPS-treated (C) TAC1 gene-deleted, (D) NK1 receptor deficient and (E) TAC1/NK1 knockout mice. Decreased oedema, less oedematous structures, and reduced neutrophil/macrophage recruitment were seen in the TAC1 gene-deficient mice, but not in the other two groups compared to wildtypes.
Periodic-acid-Schiff staining, 200X magnification; a: alveoli, b: bronchioles, v: veins, oed: oedema.

Fig. 3. Semiquantitative histopathological evaluation of LPS-induced inflammatory changes in the lung. (A) Morphological scores of the four evaluated parameters: perivascular oedema formation, perivascular/peribronchial granulocyte accumulation, alveolar infiltration of activated macrophages and goblet cell hyperplasia. (B) Composite inflammation score values of the lung samples determined from the four parameters demonstrated in panel A.
Box plots represent the medians with upper and lower quartiles of n= 6-8 mice; *p<0.05 compared to the LPS-treated wildtype group determined by Kruskal-Wallis (non-parametric ANOVA) followed by Dunn's post test.

Fig. 4. Myeloperoxidase (MPO) activity in the lung. MPO activity, as a quantitative indicator of the number of accumulated granulocytes determined from homogenized lung samples one day after intranasal LPS administration. Results are means ± S.E.M. of n= 6-8 mice; *p<0.05, **<0.01 compared to the LPS-treated inflamed group (one-way ANOVA followed by Bonferroni's modified t-test).

Fig. 5. Inflammatory cytokine concentrations in the lung. (A) Interleukin-1β (IL-1β) and (B) TNF-a concentrations of the lung samples 25h after the induction of the pulmonary inflammation by intranasal LPS administration. Results are means±S.E.M. of n=6-8 mice per group; *p<0.05compared to the LPS-treated control group (one-way ANOVA followed by Bonferroni's modified t-test).

Figure 1

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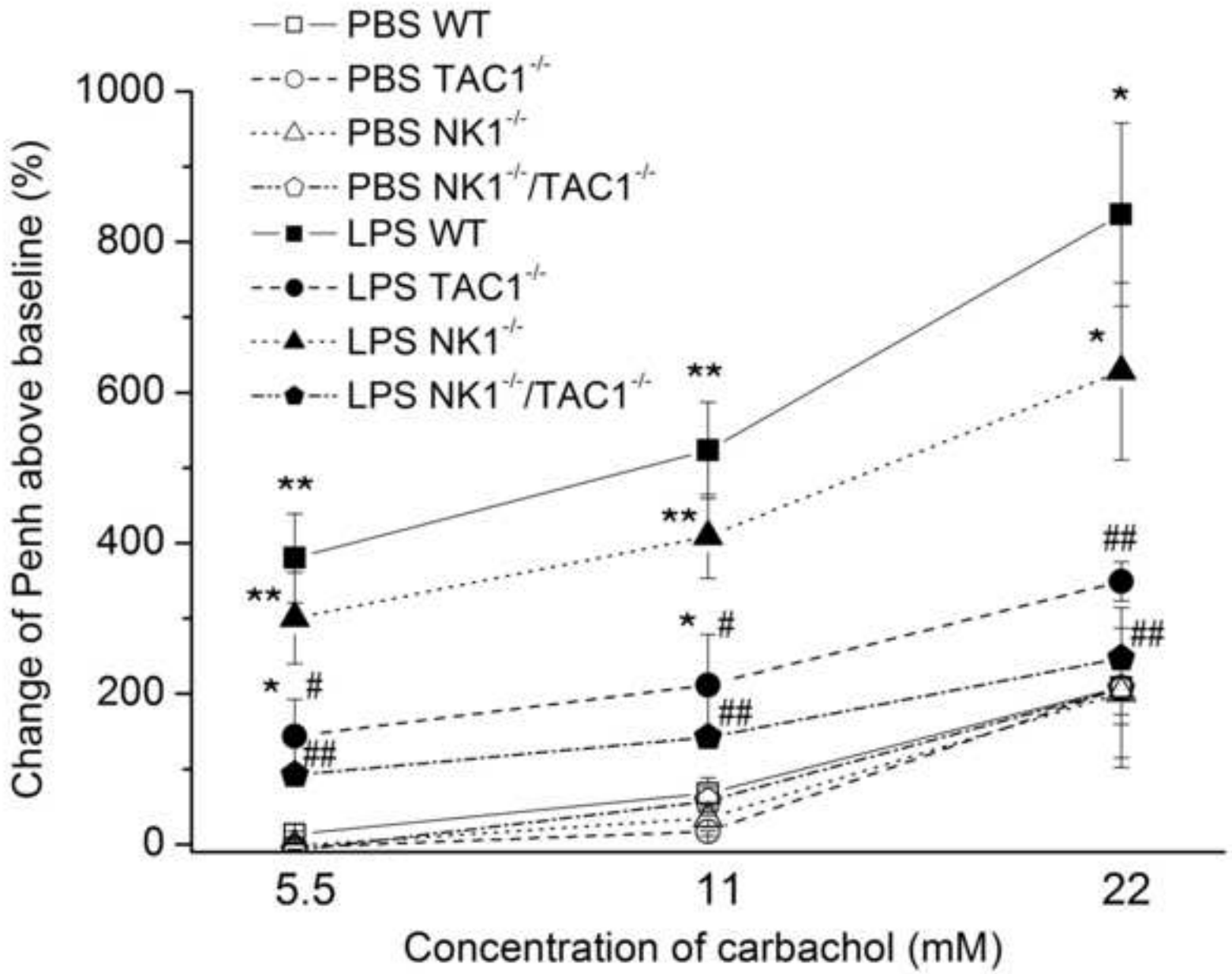

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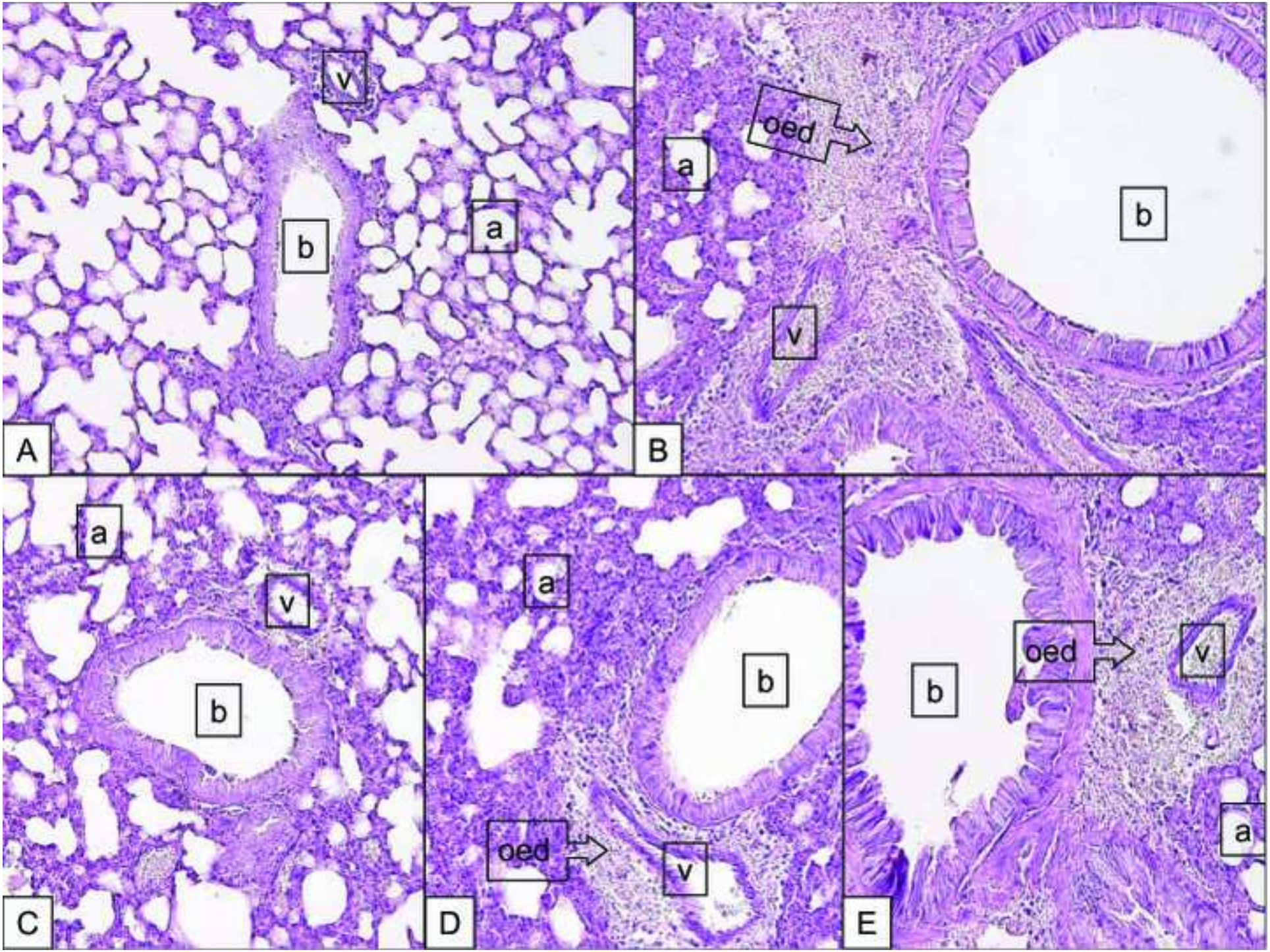

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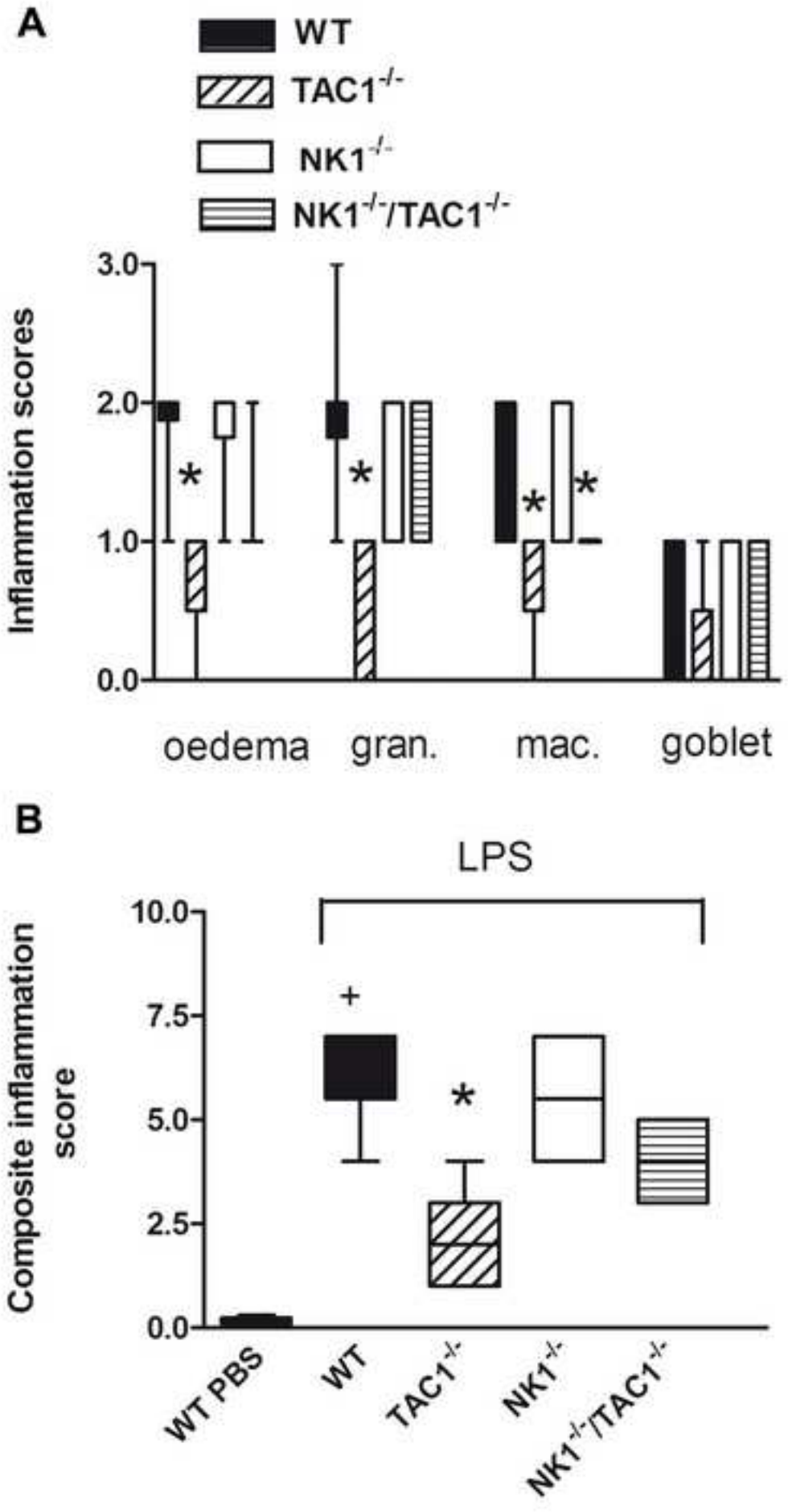

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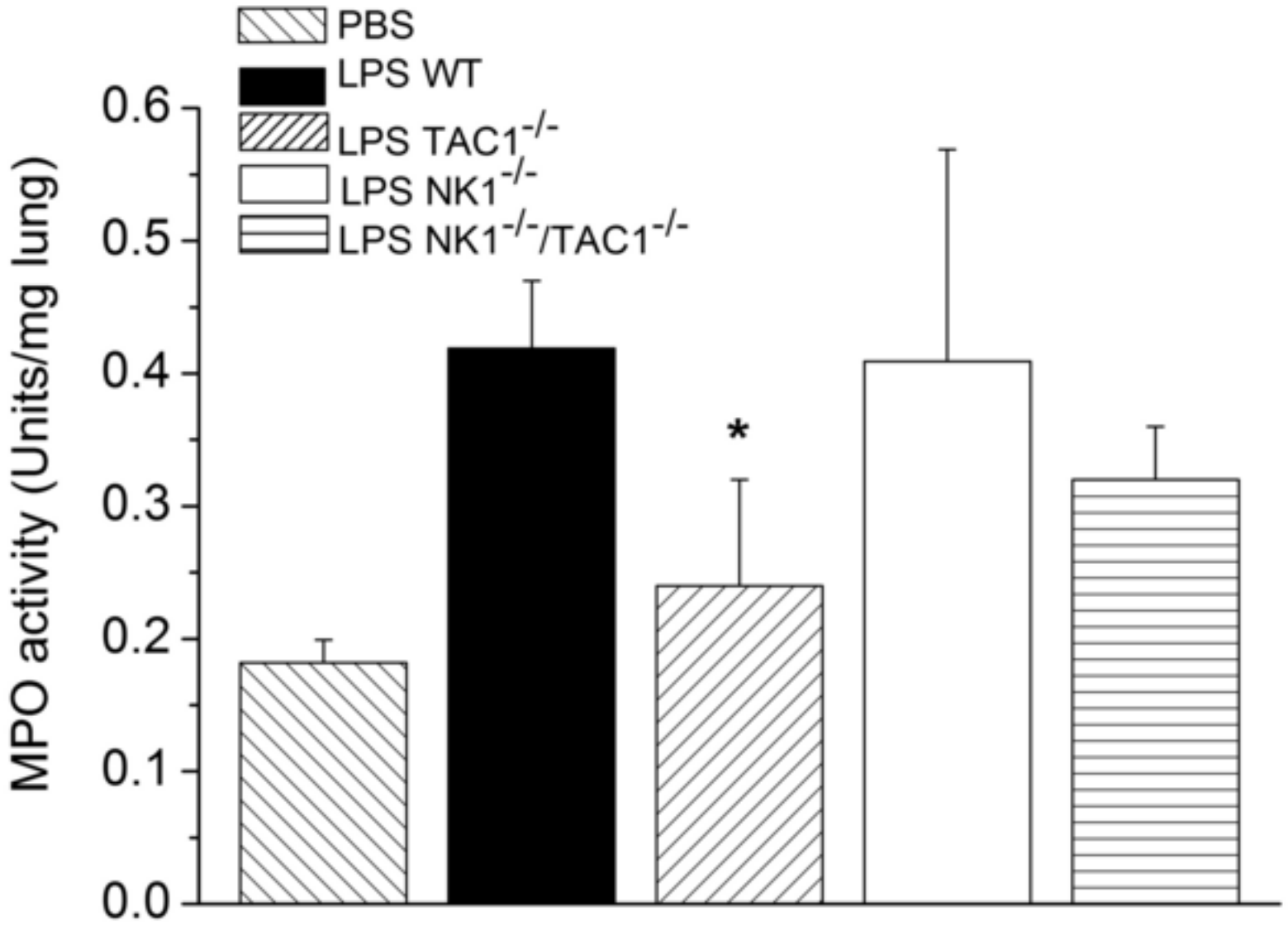

Figure 5

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