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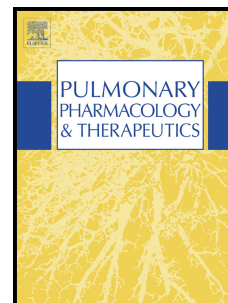


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Conserved responses to trichostatin A in rodent lungs exposed to endotoxin or stretch

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Abstract

Histone deacetylase (HDAC) isoenzymes have been suggested as possible drug targets in pulmonary cancer and in inflammatory lung diseases such as asthma and COPD. Whether HDAC inhibition is pro- or anti-inflammatory is under debate.

To further examine this clinically relevant paradigm, we analyzed 8 genes that are upregulated by two pro-inflammatory stimuli, i.e. endotoxin and mechanical stress (overventilation), in isolated rat and mouse lungs, respectively. We studied the effect of the HDAC inhibitor trichostatin A (TSA) under control conditions, in response to endotoxin and overventilation, and on the effects of the steroid dexamethasone.

TSA affected gene expression largely independent of the stimulus (endotoxin, overventilation) and the species (rat, mouse) leading to upregulation of some genes (Tnf, Cxcl2) and downregulation of others (Cxcl10, Timp1, Selp, Il6). At the protein level, TSA reduced the stimulated release of TNF, MIP-2 α and IL-6, indicating that TSA may affect protein translation independent from gene transcription. In general, the anti-inflammatory effects of TSA on gene expression and protein release were additive to that of dexamethasone, suggesting that both drugs employ different mechanisms.

We conclude that pro-inflammatory stimuli induce distinct sets of genes that are regulated by HDAC in a diverse, but consistent manner across two rodent species. The present findings together with previous *in vivo* studies suggest that the effect of HDAC inhibition in the intact lung is in part anti-inflammatory.

Key words: HDAC, biotrauma, steroids, gene transcription, mechanical ventilation, lipopolysaccharide

1. Introduction

Protein acetylation and deacetylation is an important post-translational modification involved in the control of gene transcription and is catalyzed by histone acetylase (HAT) and histone deacetylase (HDAC) isoenzymes, respectively. Histone acetylation reduces electrostatic interactions between protein and DNA, causing DNA unwinding and facilitating accessibility of the transcriptional machinery. Deacetylation reverses this process and holds transcription. Besides the histones, several other proteins such as transcription factors (e.g. NF- κ B, STAT, GATA or the glucocorticoid receptor), Hsp90, tubulin or p53 are also substrates of HAT and HDAC [1].

Recently, HDAC inhibitors (HDACi) have received attention as possible drug targets in pulmonary disorders such as cancer, COPD and asthma [2,3,4]. With respect to inflammation, it was posited that inhibition of HDAC isoenzymes (in particular HDAC2) increases histone acetylation in the vicinity of pro-inflammatory genes thus furthering pro-inflammatory gene expression [5,6]. Together with the fact that histone deacetylation was found to be decreased in steroid-resistant COPD and asthma patients [7,8], this led to the hypothesis that steroid resistance may be related to compromised HDAC activity [9].

One frequently used tool to address the pathophysiological role of histone deacetylases is trichostatin A (TSA), a fairly specific HDAC inhibitor (see discussion in ref. [10]). Up to now, most studies with TSA concerned with pro-inflammatory responses in the lung have been performed in cell lines. In keeping with the above hypothesis, the majority of these studies have reported increased gene expression (only rarely also release of the related proteins) of several cytokines in the presence of TSA: IL-8 [11-16], GM-CSF [6,17], IL-6 [12] and TGF β [18]; only three studies reported decreased cytokine expression, namely for GM-CSF [17], IL-12 [11] and VEGF-c [19]. Thus, in general and in line with the above hypothesis, cell culture studies indicate that the effects of TSA are pro-inflammatory. In addition, we have recently reported that TSA moderately increases MIP-2 α and TNF gene and/or protein expression in the intact mouse lung [10].

On the other hand, *in vivo* TSA reduced rather than enhanced the expression of IL-4, IL-5 and immunoglobulin E in an asthma model [20]. In addition, in other animal models of inflammation HDACi reduced the endotoxin-induced serum levels of TNF, IL-1, IL-6 and IFN γ in a sepsis model [21] and IL-6 and IL-1 β levels in an arthritis

model [22]. And finally, in isolated mouse lungs we had observed that TSA induced a strong decrease in IL-6 gene expression and synthesis at baseline levels and during overventilation and that this effect was additive to that of steroids [10].

Given these varying results together with the proposed relevance of HDACi for the treatment of pulmonary ailments, further information on how HDACi affect pro-inflammatory gene expression in the lung is required. Therefore, we aimed to study the response to TSA of several inflammation-related genes in the entire lung, in a model that preserves the organ anatomy on the one hand, and that allows to exclude effects of extrapulmonary cells on the other. To examine whether the findings were model and/or species specific, we used two well characterized but entirely different pro-inflammatory stimuli in two different species: mechanical ventilation in isolated mouse lungs and endotoxin administration in isolated rat lungs. Both stimuli activate what may be called the canonical inflammation pathway, but they do so by quite different mechanism: endotoxin acts on TLR4 receptors, mechanical ventilation stimulates mechanotransduction processes [23]. Here we examined the effect of TSA in these models and whether TSA was able to reverse the anti-inflammatory effects of steroids. The genes studied represent cytokines (TNF, IL-6), chemokines (MIP-2 α , MIP-1 β , IP10), adhesion molecules (P-selectin), protease inhibitors (Timp1) and extracellular matrix proteins (tenascin C). We observed very similar gene expression patterns in both models (overventilation in mice, endotoxin in rats) showing upregulation by TSA of some genes (Tnf, Cxcl2, Ccl4) and downregulation of others (Cxcl10, Timp1, Selp) and further that the direction of these effects (i.e. up- or downregulation) was similar in the presence of dexamethasone. These findings indicate that the effects of TSA on inflammatory gene transcription and translation are complex, anti- as well as pro-inflammatory, common to different stimuli and conserved within two rodent species.

2. Material and Methods

Female BalbC mice (20-25g) were obtained from FU Berlin (Berlin, Germany) and housed under standard conditions. All experiments were approved by the local authorities. Female Wistar rats (216-255g) were obtained from Charles River (Sulzfeld, Germany). Endotoxin, i.e. lipopolysaccharide (LPS) from *Salmonella minnesota*, trichostatin A and dexamethasone were obtained from Sigma (Deisenhofen, Germany).

2.1 Isolated perfused mouse and rat lung preparation

Isolated mouse [24] and rat lungs [25] were prepared as described using set-ups available from Hugo-Sachs Elektronik-Harvard Apparatus GmbH (March-Hugstetten Germany). Very briefly, both isolated rat and mouse lungs were ventilated with negative ambient pressure under pressure-controlled conditions in artificial thorax chambers. Lung mechanics were continuously calculated by applying the law of

motion: $P = \frac{1}{C} V_T + R \cdot \frac{dV}{dt}$, where P is the pressure inside the artificial thorax

chambers, C is compliance and R is resistance. Tidal volume (V_T) was derived by integration from airflow (dV/dt). Rat lungs were perfused with a modified Krebs-Henseleit buffer containing 2% albumin, 0.3% HEPES and 0.1% glucose in a recirculating fashion at about 25-30 mL/min; mouse lungs with RPMI 1640 medium supplemented with hydroxyethyl starch at 1 mL/min in a non-recirculating fashion.

Data for tidal volume and pulmonary resistance for the various groups are shown in table 1. At the end of the experiments, the lungs were disconnected, excised from the corpse and frozen in liquid nitrogen. All samples were kept at -80°C. Lungs were ground up in liquid nitrogen using a mortar and pestle and the resultant lung powder was used for subsequent analysis. For preparation of histone extracts and nuclear extracts, lung powder was thawed and washed with cold PBS.

For the mouse lungs that were analyzed in this study we have previously already reported the levels of histone acetylation and of gene expression/protein production of TNF, MIP-2 α and IL-6 [10].

2.2 The models: LPS and mechanical ventilation

Lungs were perfused under control conditions for 30 min before TSA (100 ng/mL) and/or dexamethasone (DEX, 10^{-6} or 10^{-8} M, these data were pooled) was added. At 60 min (mice) or 40 min (rats), lungs were stimulated either with overventilation (OV, mouse lungs, 22.5 cm H₂O end-inspiratory pressure) or with LPS (rats, 50 µg/mL LPS added to the perfusate) for 180 min.

The extent of the stimuli (LPS or overventilation) was chosen to produce a strong effect without causing overt lung injury. In isolated blood-free perfused rat lungs, perfusion with LPS causes bronchoconstriction and formation of giant lamellar bodies, but no edema formation or structural lung damage [26]. Since in preliminary experiments to the present study, the effects of LPS on pulmonary resistance were smaller than in our previous studies, here we used 50 µg/mL rather than 10 µg/mL as before. In general, rats are quite resistant to LPS and relatively high doses are required to produce pathophysiological effects in this species [27]. Overventilation (OV) of mouse lungs between 22.5-25 cm H₂O causes robust induction of cytokine responses, but no lung injury [24,28]. We would like to emphasize that these are models for studying the pulmonary (signaling) responses to LPS or mechanical ventilation, but are not models for lung injury which for LPS or mechanical ventilation depends on the presence of blood-borne leukocytes. The concentrations of TSA and dexamethasone were chosen as before [10].

The group sizes (after pooling of the groups with different DEX concentrations) were in the perfused mouse lung model: control 9, TSA 3, OV 11, OV/TSA 3; OV/Dex 9, OV/Dex/TSA 9; in the perfused rat lung model: C 3, TSA 3, LPS 3, LPS/TSA 3, LPS/DEX 6, LPS/DEX/TSA 6.

2.3 Preparation of histone extracts

Histones were extracted from lung powder as described elsewhere [29] with slight modifications. Briefly, lung powder was washed in PBS and centrifuged. The pellet was suspended in lysis buffer (10 mM Tris/HCl, pH 6.5, 50 mM Na bisulphite, 10 mM MgCl₂, 8.6% sucrose, 0.5% NP-40, 10 mM Na-butyrate, Complete Mini) and incubated for 10 min on ice. After centrifugation (13,500 rpm, 4°C), the pellet was washed three times with lysis buffer and once with 10 mM Tris/HCl, pH 7.4, 13 mM EDTA. The pellet was then suspended in 0.2 M H₂SO₄ and incubated on ice for 1 hour with occasional mixing. The dissolved histones were separated by centrifugation

(13,500 rpm, 4°C) and precipitated with acetone at -20°C overnight. The precipitate was centrifuged and washed with ice cold acetone. The remaining pellet was air dried on ice and dissolved in 50 mM Tris/HCl, pH 7.9, 10mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol. Protein concentration was determined and the histone solution kept at -80°C.

2.4 Immunblotting of histones

Histones were separated according to their size, charge and hydrophobicity on 12% acid-urea-Triton (AUT)-polyacryl-amide gels. Briefly, histone extracts with adjusted protein concentrations were mixed with the same volume of sample buffer (8 M urea, 0.7 mM 2-mercaptoethanol, 20% Glycerol, 6% (v/v) acetic acid, pyronin Y) and incubated at 50°C for five minutes. Volumes equivalent to 10-20 µg of protein were loaded onto the gel and separated in running buffer (100 mM glycine, 0.6% acetic acid) with inverted current overnight. Gels were then equilibrated in transfer buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine, 0.1% SDS) and transferred to nitrocellulose. Transfer was checked with Ponceau S. The membrane was blocked with RotiBlock (Roth, Karlsruhe, Germany) and incubated with antibodies against acetylated histone H4 (Upstate, Millipore, Billerica, MA, USA). Fluorescently labelled secondary antibodies were used (Molecular Probes, Invitrogen, Karlsruhe, Germany, and Rockland, MolBiol, Hamburg, Germany) and acetylated histones detected on Li-Cor Odyssey Imaging System (LI-COR Biosciences, Bad Homburg, Germany). The signal of all bands was added up to total H4 fluorescence.

2.5 Cytokine concentration in the perfusate

For detection of TNF- α , IL-6 and MIP-2 α in perfusate samples, commercially available tests (OptEIA™ Set Mouse TNF- α (mono/poly), OptEIA™ Mouse IL-6 Set, BD Biosciences, San Diego, CA, USA, and Quantikine Mouse MIP-2 Immunoassay, R&D Systems GmbH, Wiesbaden, Germany) were purchased and performed exactly according to the manufacturer's instructions.

2.6 Real time RT-PCR

Total RNA of 30 mg lung powder were isolated with NucleoSpin® RNA II Kit according to manufacturer's instructions (Machery Nagel, Düren, Germany).

8 μL total RNA was completed with 2 μL Oligo-(dt)-primer (Invitrogen, Karlsruhe, Germany), mixed, centrifuged and subsequently incubated at 65°C for 10 min to linearize RNA. 2 μL of this RNA-primer-mix were added to 1 μL Superscript II™ Reverse Transcriptase (200U/ μL) (Invitrogen), 4 μL 5x Strand Puffer (250 mM Tris/HCl, pH 8.3; 375 mM KCL, 15mM MgCl_2) (Invitrogen), 2 μL DTT (0.1M) (Invitrogen), 2 μL dNTP-Mix (10mM) (peqLAB, Erlangen), 1 μL RNaseOut (40 U/ μL) (Invitrogen) and incubated for 90 min at 37 °C. Afterwards, the mixture was diluted with 30 μL H_2O and real-time (RQ)-PCR product accumulation was monitored in a Light-Cycler480 (Roche-Diagnostics) using 1 μL of total cDNA, 200 nM forward, 200 nM reverse primer and SYBR-Green I Mastermix (Roche-Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The primers are shown in Table 2.

The data were normalized first to β_2 -microglobulin as a house keeping gene and subsequently were also normalized to control lungs, and expressed as fold induction relative to controls.

2.7 Statistics

Data are given as means \pm SD. Prior to statistical analysis (JMP 7.0.1 or SAS 9.1, SAS Institute Inc., Cary, NC, USA), the data in Fig. 2 and 3 were transformed by the Box-Cox transformation to achieve homoscedasticity.

The data in Figs. 1 and 2 were analyzed by 2-way ANOVA. The data in Fig. 5 were analyzed by mixed model methods with special parametric structure on the covariance matrices as described in ref. [30]; the fixed factors were LPS and OV (PROC MIXED in SAS 9.1). Because these experiments represent a 2x2 factorial design, multiple comparison analysis was carried out, only if the interaction term was significant ($p < 0.05$).

The data in Table 1, Fig. 3 and Fig. 6 were analyzed for one factor (treatment groups) by 1-way ANOVA (Table 1, Fig. 3) or mixed model methods with special parametric structure on the covariance matrices (Fig. 6, PROC MIXED in SAS 9.1) followed by multiple comparison of the three groups [30].

The p-values from all multiple comparisons were adjusted by the *fdr* procedure. $P < 0.05$ was considered significant.

3. Results

3.1 Tidal volume

The data for tidal volume and pulmonary resistance immediately before the stimulus (termed 0 min, i.e. at 40 min (rats) or 60 min (mice) of perfusion), and 30 min and 180 min thereafter are summarized in table 1. In isolated rat lungs, LPS increased pulmonary resistance at 180 min and this was mitigated by dexamethasone, although the effect of LPS was smaller than reported before [26]. In isolated mouse lungs, after application of pressure-controlled ventilation with 22.5 cm H₂O tidal volume increased instantaneously, and subsequently decreased as described [10], while pulmonary resistance increased. These data are shown to illustrate that TSA had no effects on tidal volume, a global indicator of respiratory functions in this model; its only effect was to attenuate the LPS-induced increase in pulmonary resistance.

3.2 Histone acetylation

Treatment of isolated rat lungs with TSA enhanced acetylation at H4K8 under control conditions (Fig. 1). As before in mice, we observed several distinct bands that correspond to multiple acetylation forms [10]. Endotoxin moderately increased acetylation at H4K8 and this was unaltered by dexamethasone. In combination with either LPS or LPS plus dexamethasone, TSA increased H4K8 acetylation compared to any of these treatments alone (Fig. 1).

3.3 Gene expression

Although not significant for all the genes studied, an up-regulation by both overventilation and LPS was found for *Tnf*, *Cxcl2*, *Ccl4* and *Tnc* (Fig. 2). *Cxcl10* and *Timp1* were significantly upregulated only by LPS, and *Il6* only by overventilation. In both models, TSA had consistent effects on gene induction in the absence or presence of the stimulus (overventilation, LPS). Of eight genes studied, two were upregulated by TSA: *Tnf* (mice and rats), *Cxcl2* (MIP-2 α , rats and mice); and five genes were downregulated: *Cxcl10* (IP10, mice and rats), *Tnc* (mice), *Timp1* (mice), *Selp* (mice and rats) and *Il6* (mice), or tended to be down-regulated: *Timp1* and *IL-6* (both in rats) (Fig. 2). Only in case of *CCl4* did the pattern differ between mice and rats. Please note that the results of the 2-way ANOVA are given below the data for

each gene and that individual comparisons (indicated by stars) were performed only if the interaction term was significant.

Except for *Cxcl2* all genes were downregulated by dexamethasone treatment (Fig. 3). The effect of TSA in steroid-treated lungs was in most cases similar between rats and mice. Of the genes that were downregulated by TSA alone, *Cxcl10* in mice, *Selp1* in mice and rats, and *IL-6* in rats showed a statistically significant additive downregulation with dexamethasone plus TSA. A significant increase of gene expression by TSA in dexamethasone-treated lungs was noted for *Cxcl2* (MIP-2 α) in mice and – although on a small scale – for *Tnc* in rats.

One way to examine whether the gene expression patterns to mechanical stress in mice and to LPS in rats were similar is to analyze the correlation between gene expression in these species under the different conditions of this study. Ideally, if both the direction and the extent of the expression were similar the regression line would not differ from unity. In total we analyzed 48 different data sets for each species (96 sets in total): 8 genes each with 6 different treatments (Fig. 4). As each single point in Fig. 4a represents the mean, this analysis summarizes a total of 544 gene expression measurements. It indicates that the gene expression patterns in rat lungs with LPS as the stimulus and mouse lungs with overventilation as the stimulus were similar under all conditions studied. The coefficient of correlation was 0.83 and the slope of the regression line was not different from unity ($p=0.14$). Cluster analysis of these data (Fig. 4b) showed that the pattern of *Cxcl2* and *Tnf* gene expression deviated from that of the other genes, which clustered closer together.

3.4 Cytokines

In our previous study we had reported the cytokine levels in the mouse subset of our data [10] (see also table 3). Here we report the findings for rat lungs perfused with LPS and TSA. TSA alone had no effect on the unstimulated release of *IL-6* (Fig. 5a) or *TNF* (Fig. 5b), and caused a small increase of MIP-2 α (Figure 5c). LPS induced the release of *IL-6* (Fig. 5a), *TNF* (Fig. 5b), and MIP-2 α (Fig. 5c) and this increase was always abated by TSA.

Again in rat lungs, treatment with dexamethasone reduced the LPS-induced release of all three cytokines (Fig. 6). This effect of dexamethasone was further enhanced by TSA in the case of *IL-6* (Fig. 6a), while it had no effect on *TNF* (Fig. 6b) and MIP-2 α (Fig. 6c) release under these conditions.

4. Discussion

A striking finding of this study was the excellent correlation of the gene expression in two different models of inflammatory lung disease: the pattern of gene expression was nearly identical in rat and mouse lungs both in the absence and presence of TSA and/or steroids, despite the fact that two completely different stimuli (mechanical stress, LPS) were used and despite the fact that tidal volume and resistance readings were quite different between the models. This study suggests that inflammatory stimuli – such as mechanical stress or LPS – induce distinct sets of genes that are regulated by HDAC in a diverse, but consistent manner across two rodent species. And finally, although the gene expression of TNF and MIP-2 α was elevated, this was not translated into increased release of these proteins into the perfusate. These findings have several implications.

First, the findings further corroborate our previous conclusion that the pulmonary responses to overventilation and LPS are remarkably similar [31,32], which is most likely explained by their activating a canonical inflammation pathway [33]. As a corollary of this, the genes studied herein are likely part of this canonical pathway.

Second, our findings indicate that TSA has characteristic effects on this canonical inflammation pathway, which are independent of the two tested stimuli (LPS, mechanical stress) and at least in part independent of the lung physiology. These characteristic effects of TSA also argue for some specificity of TSA. Such specificity is also indicated by the fact that a more thorough analysis of gene expression in cell lines showed that TSA modifies gene expression of selected genes only: 2% (8/340) of the genes studied in lymphoid cell lines [34]; upregulation of 4% (93/2400) and downregulation of 1% (27/2400) of the genes studied in a human gastric cancer cell line [35].

Third, several genes studied here were downregulated while others were upregulated by TSA. This clearly indicates that different checkpoints must exist for the gene expression of Cxcl10 (IP10), Tnc, Timp1, Selp and Il6 on the hand and of Cxcl2 (MIP-2 α) and possibly also Tnf on the other.

Fourth, as summarized in table 3, there was a discrepancy between the expression of TNF and MIP-2 α at the gene and the protein level. While TSA increased the

expression of both genes, at the same time it decreased the production of these cytokines in the presence of LPS or overventilation. The latter observation is in line with *in vivo* data showing that HDACi reduce the release of TNF, IL-6 and MIP-2 α proteins in mice treated with LPS intraperitoneally [21]. This may indicate that parts of the protein translation or secretion machinery belong to the substrates of HDACs. One exception from this general pattern was MIP-2 α in overventilated lungs, where also protein levels were increased by TSA (Table 3). However, MIP-2 α does frequently stand out among the chemokines, as is also illustrated in Fig. 4b. For instance, in the murine peritonitis model, the effect of TSA on MIP-2-release was dose-dependent, as lower HDACi concentrations reduced and higher concentrations increased MIP-2 α production [21]. In this regard it may be of relevance that MIP-2 α differs from other chemokines, because it can be stored inside cells and thus its release need not always depend on *de novo* synthesis [36].

Fifth, at least in the present rodent models it seems uncertain whether a major part of the anti-inflammatory effects of steroids is to be explained by their recruiting HDAC isoenzymes to the sites of pro-inflammatory gene transcription as it has been proposed [9]. Despite the fact that the pattern of H4K8 acetylation changed as expected in the presence of TSA and dexamethasone (Fig. 1, [10]), TSA was additive rather than antagonistic to dexamethasone for several genes studied.

As pointed out in the introduction, the majority of cell culture studies performed with lung cells has suggested that the effects of TSA in the lung are predominantly pro-inflammatory. Most of these studies have focussed on IL-8, the function of which in mice and rats is at partly assumed by MIP-2 α . However, to our knowledge did only one study [13] also measure IL-8 protein (after 24h of 100 ng/mL TSA IL-8 levels increased, although others have reported that TSA may be effective for at most 15h [37]). In contrast, comprehensive evidence from experiments with isolated lungs and those with intact animals suggest that on the protein level the overall effect of TSA *in vivo* tends to be anti-inflammatory. This conclusion is corroborated by the anti-inflammatory action of HDACi in experimental models of acute lung injury (this study), asthma [20], sepsis [21], arthritis [22,38], autoimmune encephalomyelitis [39], colitis [40] and concanavalin-A-induced hepatitis [21]. From the present study we suggest that the discrepancies between the *in vivo* and perfused lung studies on the one hand, and the cell culture studies on the other may be ascribed to the focus on

relatively few genes in the *in vitro* studies and to the differential regulation of gene expression versus protein production by TSA.

At present, the molecular basis for the mitigating effects of HDACi on the expression of pro-inflammatory genes and the release of the related proteins is far from clear. During inflammation, acetylation of histone H4 at the lysine residues 8 (H4K8) and 12 (H4K12) – known to be elevated in the promoter and transcribed regions of active genes [41] – is strongly increased, e.g. by IL-1 treatment in A549 cells [6], by H₂O₂ in BEAS 2B cells [16], by mechanical ventilation in mouse lungs [10] and by LPS in rat lungs (this study). However, histone acetylation appears to correlate poorly with expression of the genes studied herein. Possibly, the up- or down-regulation of genes by TSA could result from hyperacetylation of regulatory factors that control gene transcription either positively or negatively. Similar mechanisms might also apply to proteins involved in protein translation and/or secretion. An alternative mechanism to explain the effects of HDACi on gene expression at the gene level, would be acetylation of transcription factors, which are also affected by HDACi (see introduction). A preliminary analysis of potential transcription factors involved in the regulation of the genes studied here (P-MATCH 1.0, gene-regulation.com), however, has not revealed a pattern that correlated with the current findings (data not shown). For instance, one transcription factor that has been discussed as possibly being related to the effects of TSA on pro-inflammatory genes is NF- κ B, that is involved in the transcription of TNF, MIP-2 α , IL-6, P-selectin, tenascin C and IP10 [42]. However, as these genes were affected quite differently by TSA, it appears rather unlikely that the effects of TSA on gene transcription are related to this transcription factor. This is in line with studies in A549 cells suggesting that promoter occlusion or HDAC recruitment is unlikely to account for the GR-mediated repression of NF- κ B action [43].

HDACi have been suggested as drugs for the treatment of several inflammatory disorders including asthma, COPD, lupus erythematosus or arthritis [3,4,20,44,45], as well as cancer [2]. Given the contradictory effects of TSA and other HDACi on the expression of many genes, their use as potential drugs is expected to have difficult-to-predict effects and side effects. The present study indicates that in rodents the HDACi trichostatin affects a distinct set of pro-inflammatory genes independent of the stimulus (mechanical, immunological) leading to upregulation of some genes (Tnf,

Cxcl2) and downregulation (Cxcl10, Timp1, Selp, Il6) of others. At the protein level, however, under most conditions TSA reduced the stimulated release of TNF, MIP-2 α and IL-6, suggesting that *in vivo* the overall effects of TSA may be anti-inflammatory. In most cases the anti-inflammatory effect of TSA was additive to that of dexamethasone, indicating that these two drugs may affect gene transcription by separate mechanisms. The molecular mechanisms to explain the anti-inflammatory effects of TSA and other HDACi are not known and deserve further study.

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Reference List

- [1] Ito K, Charron CE, Adcock IM. Impact of protein acetylation in inflammatory lung diseases. *Pharmacol Ther* 2007;116:249-265.
- [2] Schrump DS, Nguyen DM. Targeting the epigenome for the treatment of thoracic malignancies. *Thorac Surg Clin* 2006;16:367-77.
- [3] Bhavsar P, Ahmad T, Adcock IM. The role of histone deacetylases in asthma and allergic diseases. *J Allergy Clin Immunol* 2008;121:580-584.
- [4] Adcock IM. Histone deacetylase inhibitors as novel anti-inflammatory agents. *Curr Opin Investig Drugs* 2006;7:966-973.
- [5] Adcock IM, Ito K, Barnes PJ. Histone deacetylation: an important mechanism in inflammatory lung diseases. *COPD* 2005;2:445-455.
- [6] Ito K, Barnes PJ, Adcock IM. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1 β -induced histone H4 acetylation on lysines 8 and 12. *Mol Cell Biol* 2000;20:6891-6903.
- [7] Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM *et al*. Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med* 2005;352:1967-1976.
- [8] Cosio BG, Mann B, Ito K, Jazrawi E, Barnes PJ, Chung KF *et al*. Histone acetylase and deacetylase activity in alveolar macrophages and blood monocytes in asthma. *Am J Respir Crit Care Med* 2004;170:141-147.
- [9] Barnes PJ. Corticosteroid effects on cell signalling. *Eur Respir J* 2006;27:413-426.
- [10] Dombrowsky H, Uhlig S. Steroids and histone deacetylase in ventilation-induced gene transcription. *Eur Respir J* 2007;30:865-877.

- [11] Iwata K, Tomita K, Sano H, Fujii Y, Yamasaki A, Shimizu E. Trichostatin A, a histone deacetylase inhibitor, down-regulates interleukin-12 transcription in SV-40-transformed lung epithelial cells. *Cell Immunol* 2002;218:26-33.
- [12] Keslacy S, Tliba O, Baidouri H, Amrani Y. Inhibition of tumor necrosis factor- α -inducible inflammatory genes by interferon- γ is associated with altered nuclear factor-kappaB transactivation and enhanced histone deacetylase activity. *Mol Pharmacol* 2007;71:609-618.
- [13] Gilmour PS, Rahman I, Donaldson K, MacNee W. Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L533-L540.
- [14] Schmeck B, Lorenz J, N'Guessan PD, Opitz B, van L, V, Zahlten J et al. Histone acetylation and flagellin are essential for *Legionella pneumophila*-induced cytokine expression. *J Immunol* 2008;181:940-947.
- [15] Slevogt H, Schmeck B, Jonat C, Zahlten J, Beermann W, van L, V et al. *Moraxella catarrhalis* induces inflammatory response of bronchial epithelial cells via MAPK and NF- κ B activation and histone deacetylase activity reduction. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L818-L826.
- [16] Tomita K, Barnes PJ, Adcock IM. The effect of oxidative stress on histone acetylation and IL-8 release. *Biochem Biophys Res Commun* 2003;301:572-577.
- [17] Kagoshima M, Wilcke T, Ito K, Tsaprouni L, Barnes PJ, Punchard N et al. Glucocorticoid-mediated transrepression is regulated by histone acetylation and DNA methylation. *Eur J Pharmacol* 2001;429:327-334.
- [18] Lee KY, Ito K, Hayashi R, Jazrawi EP, Barnes PJ, Adcock IM. NF- κ B and activator protein 1 response elements and the role of histone modifications in IL-1 β -induced TGF- β 1 gene transcription. *J Immunol* 2006;176:603-615.

- [19] Imre G, Gekeler V, Leja A, Beckers T, Boehm M. Histone deacetylase inhibitors suppress the inducibility of nuclear factor- κ B by tumor necrosis factor- α receptor-1 down-regulation. *Cancer Res* 2006;66:5409-5418.
- [20] Choi JH, Oh SW, Kang MS, Kwon HJ, Oh GT, Kim DY. Trichostatin A attenuates airway inflammation in mouse asthma model. *Clin Exp Allergy* 2005;35:89-96.
- [21] Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P *et al.* The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proc Natl Acad Sci USA* 2002;99:2995-3000.
- [22] Lin HS, Hu CY, Chan HY, Liew YY, Huang HP, Lepescheux L *et al.* Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents. *Br J Pharmacol* 2007;150:862-872.
- [23] Uhlig S, Uhlig U. Pharmacological interventions in ventilator-induced lung injury. *Trends Pharmacol Sci* 2004;25:592-600.
- [24] von Bethmann AN, Brasch F, Nüsing R, Vogt K, Volk D, Müller KM *et al.* Hyperventilation induces release of cytokines from perfused mouse lung. *Am J Respir Crit Care Med* 1998;157:263-272.
- [25] Uhlig S, Wollin L. An improved setup for the isolated perfused rat lung. *J Pharm Tox Meth* 1994;31:85-94.
- [26] Uhlig S, Brasch F, Wollin L, Fehrenbach H, Richter J, Wendel A. Functional and fine structural changes in isolated rat lungs challenged with endotoxin ex vivo and in vitro. *Am J Pathol* 1995;146:1235-1247.
- [27] Opal SM. The value of animal models in endotoxin research. 1999;809-816.
- [28] Uhlig U, Drömann D, Goldmann T, Dombrowsky H, Vollmer E, Zabel P *et al.* Pulmonary responses to overventilation in late multiple organ failure. *Anesthesiology* 2006;105:1192-1200.

- [29] Ito K, Adcock IM. Histone acetylation and histone deacetylation. *Mol Biotechnol* 2002;20:99-106.
- [30] Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. *SAS for Mixed Models*. 2nd ed. Cary, NC: SAS Inst. Inc.; 2006.
- [31] Held HD, Boettcher S, Hamann L, Uhlig S. Ventilation-induced chemokine and cytokine release is associated with activation of NF- κ B and is blocked by steroids. *Am J Respir Crit Care Med* 2001;163:711-716.
- [32] Dolinay T, Kaminski N, Felgendreher M, Kim HP, Reynolds P, Watkins SC *et al*. Gene expression profiling of target genes in ventilator-induced lung injury. *Physiol Genomics* 2006;26:68-75.
- [33] Meier T, Lange A, Papenberg H, Ziemann M, Fentrop C, Uhlig U *et al*. Pulmonary cytokine responses during mechanical ventilation of non-injured lungs with and without end-expiratory pressure. *Anesth Analg* 2008;107:1265-1275.
- [34] Van LC, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr* 1996;5:245-253.
- [35] Lee H, Lee S, Baek M, Kim H-Y, Jeoung DJ. Expression profile analysis of trichostatin A in human gastric cancer cells. *Bioch Lett* 2002;24:377-381.
- [36] Isowa N, Keshavjee SH, Liu M. Role of microtubules in LPS-induced macrophage inflammatory protein-2 production from rat pneumocytes. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1075-L1082.
- [37] Siavoshian S, Segain JP, Kornprobst M, Bonnet C, Cherbut C, Galmiche JP *et al*. Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. *Gut* 2000;46:507-514.
- [38] Nasu Y, Nishida K, Miyazawa S, Komiyama T, Kadota Y, Abe Net *al*. Trichostatin A, a histone deacetylase inhibitor, suppresses synovial

- inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model. *Osteoarthritis Cartilage* 2008;16:723-732.
- [39] Camelo S, Iglesias AH, Hwang D, Due B, Ryu H, Smith K *et al.* Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2005;164:10-21.
- [40] Glauben R, Batra A, Fedke I, Zeitz M, Lehr HA, Leoni F *et al.* Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *J Immunol* 2006;176:5015-5022.
- [41] Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S *et al.* Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* 2008;40:897-903.
- [42] Ahn KS, Aggarwal BB. Transcription factor NF- κ B: a sensor for smoke and stress signals. *Ann N Y Acad Sci* 2005;1056:218-233.
- [43] Nissen RM, Yamamoto KR. The glucocorticoid receptor inhibits NF- κ B by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev* 2000;14:2314-2329.
- [44] Mishra N, Reilly CM, Brown DR, Ruiz P, Gilkeson GS. Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *J Clin Invest* 2003;111:539-552.
- [45] Choo QY, Ho PC, Lin HS. Histone deacetylase inhibitors: new hope for rheumatoid arthritis? *Curr Pharm Des* 2008;14:803-820.

Table 1: Pulmonary resistance and compliance

Rat							
	Time [min]	C (n=3)	C/TSA (n=3)	LPS (n=3)	LPS/TSA (n=3)	LPS/Dex (n=6)	LPS/Dex/TSA (n=6)
V_T [mL]	0	1.94±0.03	1.91±0.06	1.88±0.03	1.96±0.09	1.92±0.05	1.89±0.06
	30	1.78±0.02	1.73±0.12	1.69±0.03	1.77±0.14	1.75±0.09	1.73±0.06
V_T [mL]	180	1.40±0.05*	1.26±0.33	0.90±0.21	1.18±0.37	0.98±0.23	1.28±0.14
R_L [cmH ₂ O.s.mL ⁻¹]	0	0.279±0.004	0.269±0.00	0.281±0.007	0.285±0.006	0.275±0.004	0.272±0.015
	30	0.280±0.003	0.270±0.003	0.285±0.009	0.278±0.005	0.277±0.004	0.274±0.006
R_L [cmH ₂ O.s.mL ⁻¹]	180	0.291±0.007*	0.284±0.015	0.338±0.039	0.300±0.015*	0.312±0.019*	0.287±0.009†
Mouse							
		C (n=9)	C/TSA (n=3)	OV (n=11)	OV/TSA (n=3)	OV/Dex (n=9)	OV/Dex/TSA (n=10)
V_T [mL]	0	0.28±0.02*	0.26±0.01*	0.30±0.03	0.27±0.01	0.28±0.03*	0.29±0.01
	30	0.27±0.02*	0.24±0.01	0.70±0.08	0.75±0.02	0.68±0.10	0.70±0.06
V_T [mL]	180	0.24±0.02*	0.23±0.01	0.37±0.07	0.38±0.09	0.38±0.07	0.36±0.09
R_L [cmH ₂ O.s.mL ⁻¹]	0	0.29±0.06	0.39±0.02	0.33±0.08	0.37±0.06	0.34±0.07	0.32±0.05
	30	0.30±0.06	0.38±0.01	0.25±0.03	0.26±0.02	0.26±0.07	0.24±0.04
R_L [cmH ₂ O.s.mL ⁻¹]	180	0.37±0.07*	0.44±0.06	1.65±0.44	1.86±0.72	1.32±0.26	1.53±0.47

Data are mean±SD. *, p<0.05 vs LPS or OV; †, p<0.05 vs LPS/Dex or OV/Dex. V_T , tidal volume, R_L , pulmonary resistance.

The following 5 comparisons were made (treatment was LPS or OV): C vs treat, C vs TSA, treat vs treat/TSA, treat vs treat/Dex and treat/Dex vs Treat/Dex/TSA

Table 2: Primers used for the RQ-PCR

	forward primer	reverse primer
RAT		
B2m	5'-TGACCGGCTTGTATGCTATC-3'	5'-CAGTGTGAGCCAGGATATAG-3'
Ccl4	5'-CACCAATAGGCTCTGACC-3'	5'-TGCCTGCCTTTTTTGGTCA-3'
Cxcl10	5'-TCCTGTCCGCATGTTGAG-3'	5'-CTTCTTTGGCTCACCCT-3'
Cxcl2	5'-AATGCCTGACGACCCTACCA-3'	5'-GTTAGCCTTGCCTTTGTTTCAG-3'
Il6	5'-CCAGCCAGTTGCCTTCTTG-3'	5'-AGTGCATCATCGCTGCTGTTTCATA-3'
Selp	5'-AGTGCTTTGATCTCTGAGC-3'	5'-AAGTCTGTGAAGTGCTCT-3'
Timp1	5'-CTAAGATGCTCAAAGGATTCG-3'	5'-CTGCGTTTCTGGGACTTGT-3'
Tnc	5'-TGCTCAGAACCTCACAGTATC-3'	5'-TCAGGTTCTTTGCGTGTGGA-3'
Tnf	5'-GGGGCCACCACGCTCTTCTGTCTA-3'	5'-CCTCCGCTTGGTGGTTTGTCTACG-3'
MOUSE		
B2m	5'-TGACCGGCTTGTATGCTATC-3'	5'-CAGTGTGAGCCAGGATATAG-3'
Ccl4	5'-AAACCTAACCCCGAGCAACA-3'	5'-CCATTGGTGCTGAGAACCT-3'
Cxcl10	5'-GCCGTCATTTTCTGCCTCAT-3'	5'-GCTTCCCTATGGCCCTCATT-3'
Cxcl2	5'-AGTGAAGTGCCTGTCAATGC-3'	5'-AGGCAAACTTTTGGACCGCC-3'
Il6	5'-CCAGAGATACAAAGAAATGATGG-3'	5'-ACTCCAGAAGACCAGAGGAAA-3'
Selp	5'-CAACACCACCTGGGAAGCTTT-3'	5'-CCAGGGATTGGAACAGTTCATT-3'
Timp1	5'-AGGTATTTGATGCTCGGGAC-3'	5'-GATGGCTGAACGAAGATTGC-3'
Tnc	5'-CTTCATTCGTGTGTTGCGCA-3'	5'-ATCCCACTCTACTTCCACAG-3'
Tnf	5'-TCTCATCAGTTCTATGGCCC-3'	5'-GGGAGTAGACAAGGTACAAC-3'

Table 3: Effect of TSA on three genes and their proteins induced by LPS and overventilation.

		TSA					
		no Dexamethasone			Dexamethasone		
		LPS/IPL	LPS <i>in vivo</i>	OV/IPL	LPS/IPL	OV/IPL	
TNF	gene	↑	—	↑	↔	↔	
	protein	↓	↓	↔	↔	↔	
MIP-2 α	gene	↑	—	↑	↔	↑	
	protein	↓	↑/ ↓	↑	↔	↔	
IL-6	gene	↓	—	↓	↓	↔	
	protein	↓	↓	↓	↓	↓	

TSA, trichostatin A; LPS, lipopolysaccharide; IPL, isolated perfused lung; OV, overventilation.

The arrows indicate induction/increase, no effect or decrease of gene induction or protein concentrations in the effluate from the isolated perfused lungs (IPL) or in the systemic circulation (*in vivo*). The data are from the present study and from the references [10] and [22].

Legends to the figures

Figure 1. Histone H4 acetylation. Lungs were perfused under control conditions before TSA (100 ng/mL) and dexamethasone (10^{-6} or 10^{-8} M DEX, these data were pooled) were added after 30 min, and LPS (50 μ g/mL) after 40 min. Lung extracts were probed by immunoblotting with antibodies against acetylated lysine 8 on histone H4 (H4K8) and visualized by fluorescence labelled secondary antibodies. **a)** Shown is one typical experiments for each group **b)** Summary data. Shown are the experiments with (black bars) and without TSA (white bars) in the absence (two left bars, n=3) and presence of dexamethasone (two right bars, n=6). Because there was considerable variation in the absolute signal levels from gel to gel we used the following normalization procedure: One set of data was always analyzed on the same gel and the data normalized to the difference between control perfused lungs (control) and lungs perfused with TSA alone (dotted line). Analysis by 2-way ANOVA (factors being TSA and DEX) showed that the effect of TSA was significant ($P=0.003$), while the effects of dexamethasone and the interaction term were not ($p>0.2$). Data are shown as mean \pm SD.

Figure 2. Effect of TSA on gene expression stimulated by overventilation in mouse lungs and by LPS in rat lungs. Lungs were perfused under control conditions for 30 min before TSA (100 ng/mL) was added. At 60 min (mice) or 40 min (rats), lungs were stimulated either with overventilation (OV, mouse lungs, 22.5 cm H₂O end-inspiratory pressure) or with LPS (rats, 50 μ g/mL LPS) for 3h. At the end of the experiment, lungs were frozen in liquid nitrogen, RNA prepared from lung powder and analyzed by quantitative real time PCR for expression of tumor necrosis factor (Tnf), MIP-2 α (Cxcl2), MIP1 β (Ccl4), IP10 (Cxcl10), tenascin C (Tnc), tissue inhibitor of metalloproteinase 1 (Timp1), selectin P (Selp) and interleukin 6 (Il-6). mRNA levels were normalized to β_2 microglobulin and expressed relative to control (C) levels. The groups sizes were in the mouse model: control 9, TSA 3, OV 11, OV/TSA 3; in the rat model: C 3, TSA 3, LPS 3, LPS/TSA 3. The results of the 2-way ANOVA are shown below the graphs. Because these experiments represent a 2x2 factorial design, multiple comparison analysis was carried out, only if the interaction term was significant: *, $p<0.05$ vs. control; †, $p<0.05$ vs OV. Data are shown as mean + SD.

Figure 3. Effect of dexamethasone together with TSA on gene expression stimulated by overventilation in mouse lungs and LPS in rat lungs. Lungs were perfused under control conditions for 30 min before TSA (100 ng/mL) and/or dexamethasone (10^{-6} or 10^{-8} M, these data were pooled) was added. At 60 min (mice) or 40 min (rats), lungs were stimulated either with overventilation (OV, mouse lungs, 22.5 cm H₂O end-inspiratory pressure) or with LPS (rats, 50 µg/mL LPS) for 3h. At the end of the experiment, lungs were frozen in liquid nitrogen, RNA prepared from lung powder and analyzed by quantitative real time PCR for expression of tumor necrosis factor (Tnf), MIP-2α (Cxl2), MIP1β (Ccl4), IP10 (Cxl10), tenascin C (Tnc), tissue inhibitor of metalloproteinase 1 (Timp1), selectin P (Selp) and interleukin 6 (Il-6). mRNA levels were normalized to β₂ microglobulin and expressed relative to control (C) levels. The groups sizes were in the mouse model: OV 11, OV/Dex 9, OV/Dex/TSA 9; in the rat model: LPS 3, LPS/DEX 6, LPS/DEX/TSA 6. *, p<0.05 vs. control; †, p<0.05 vs OV/Dex. Data are shown as mean + SD.

Figure 4. Summary of the gene expression data in this study. a) Correlation between relative gene expression in isolated mouse lungs and in isolated rat lungs under various experimental conditions. The slope of the regression line was not different from 1 (p=0.14), the coefficient of correlation was 0.83. The insert shows the magnified area between 0 and 2 on the x-axis. The different genes are represented by different colors: CCl2 dark green; Il6 dark blue, Tnf, light blue, Tnc, light green, Ccl4 red, Cxl10 black, Selp orange, Timp1, violet. Each color is shown six times, representing the conditions control (open circles), TSA (open squares), stimulus (filled circles), DEX/stimulus (open diamonds), TSA/stimulus (open triangles), TSA/DEX/stimulus (stars). The stimulus was overventilation in mice and LPS in rats. **b)** Cluster analysis (Single linkage, JMP 7.0) of the conditions studied after the rat and mouse data had been pooled.

Figure 5. Effect of TSA on cytokine release stimulated by LPS in rat lungs. Lungs were perfused under control conditions for 30 min before TSA (100 ng/mL) was added. At 40 min, lungs were stimulated with LPS (50 µg/mL LPS) for 3h. Perfusate samples were taken at regular intervals and analyzed for IL-6 (**a**), TNF (**b**)

and MIP-2 α (**c**). The groups were control (n=3, open circles, solid line), TSA (n=3, open square, dotted line), LPS (n=3, filled circle, solid line) and LPS/TSA (n=3, filled square, dotted line). These time course data were analyzed by mixed model methods with special parametric structure on the covariance matrices as described in ref. [30]. Because the interaction term between the two factors LPS and TSA was always significant ($p<0.01$), multiple comparisons were performed. If $p<0.05$ this was indicated in the graphs: *, LPS vs. control; †, LPS vs TSA/LPS; \$, TSA vs control. Data are shown as mean \pm SD.

Figure 6. Effect of dexamethasone together with TSA on cytokine release stimulated by LPS in rat lungs. Lungs were perfused under control conditions for 30 min before TSA (100 ng/mL) and/or dexamethasone (10^{-6} or 10^{-8} M, these data were pooled) was added. At 40 min, lungs were stimulated with LPS (50 μ g/mL LPS) for 3h. Perfusate samples were taken at regular intervals and analyzed for IL-6 (**a**), TNF (**b**) and MIP-2 α (**c**). The groups LPS (n=3, filled circle, solid line), dexamethasone/LPS (n=6, open diamond, solid line) and dexamethasone/LPS/TSA (n=6, filled diamond, dotted line). These time course data were analyzed by mixed model methods with special parametric structure on the covariance matrices as described in ref. [30]. Subsequently multiple comparisons were performed: *, $p<0.05$ vs LPS; †, $p<0.05$ vs dexamethasone/LPS Data are shown as mean \pm SD.

ACCEPTED MANUSCRIPT

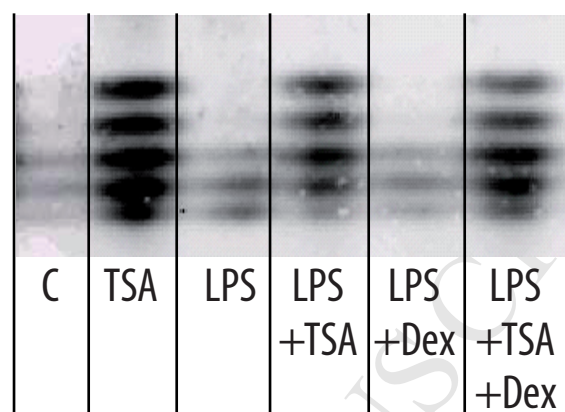
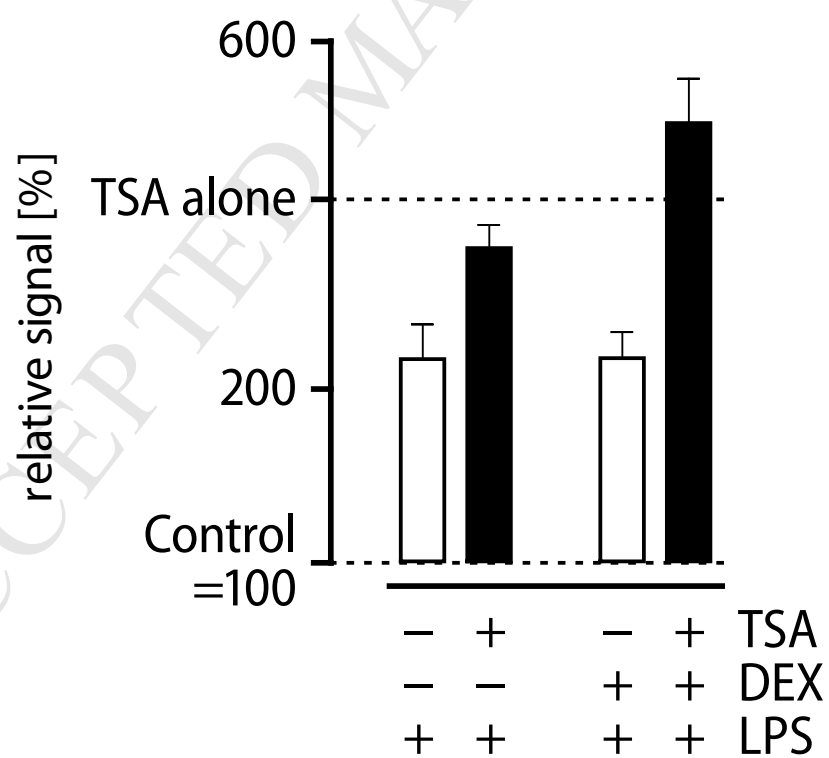
Fig. 1**a****b**

Fig. 2

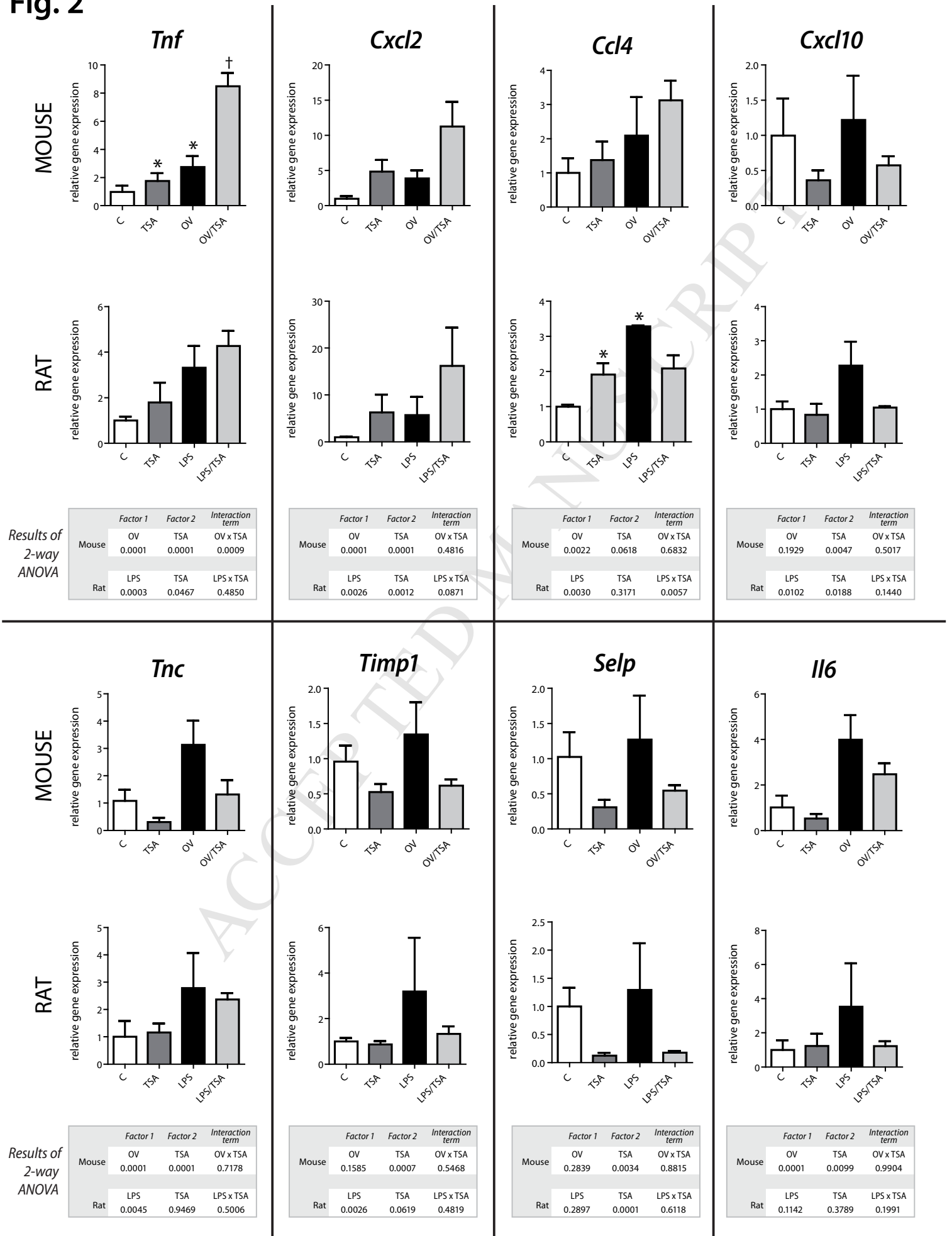


Fig. 3

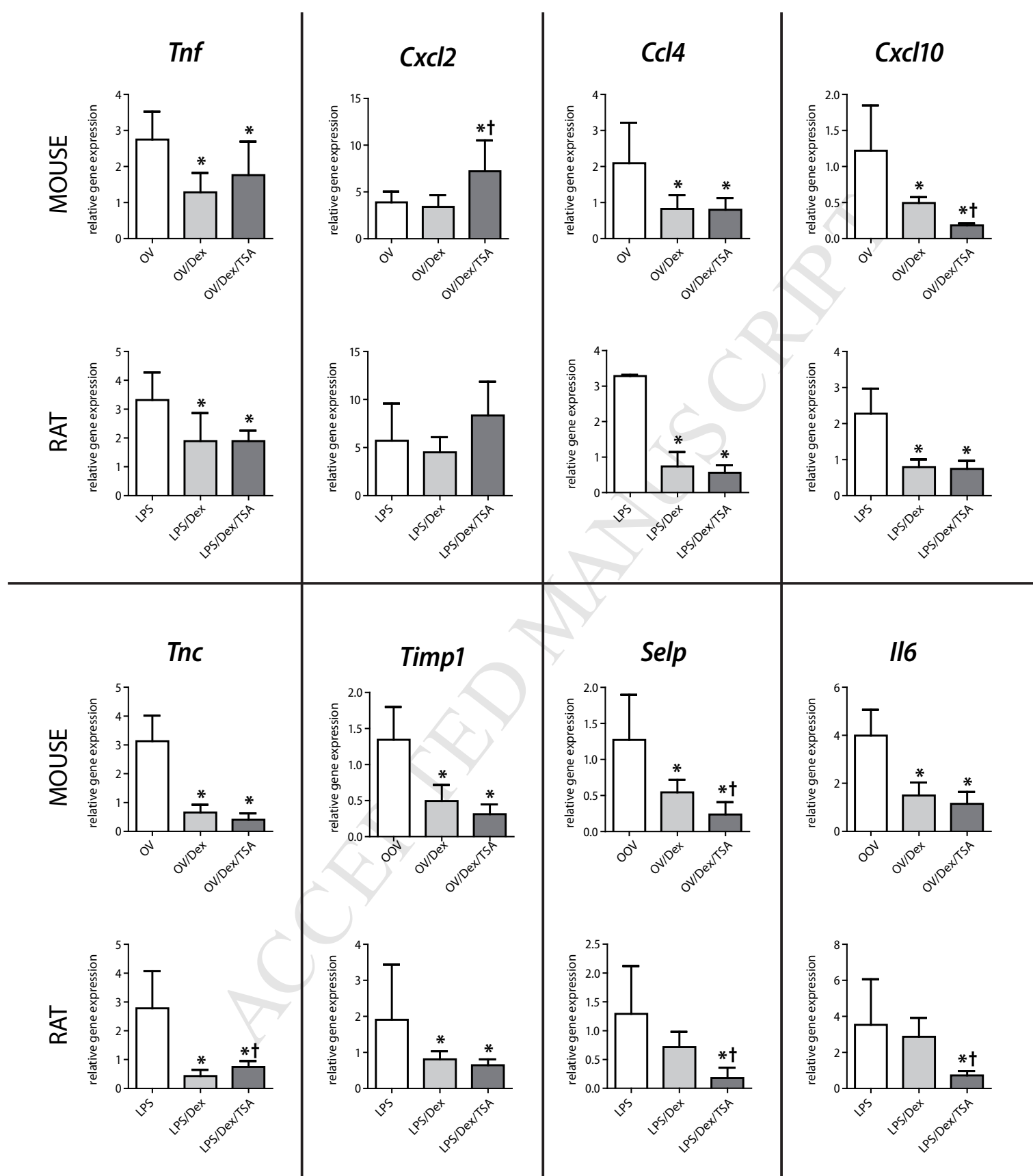


Fig. 4

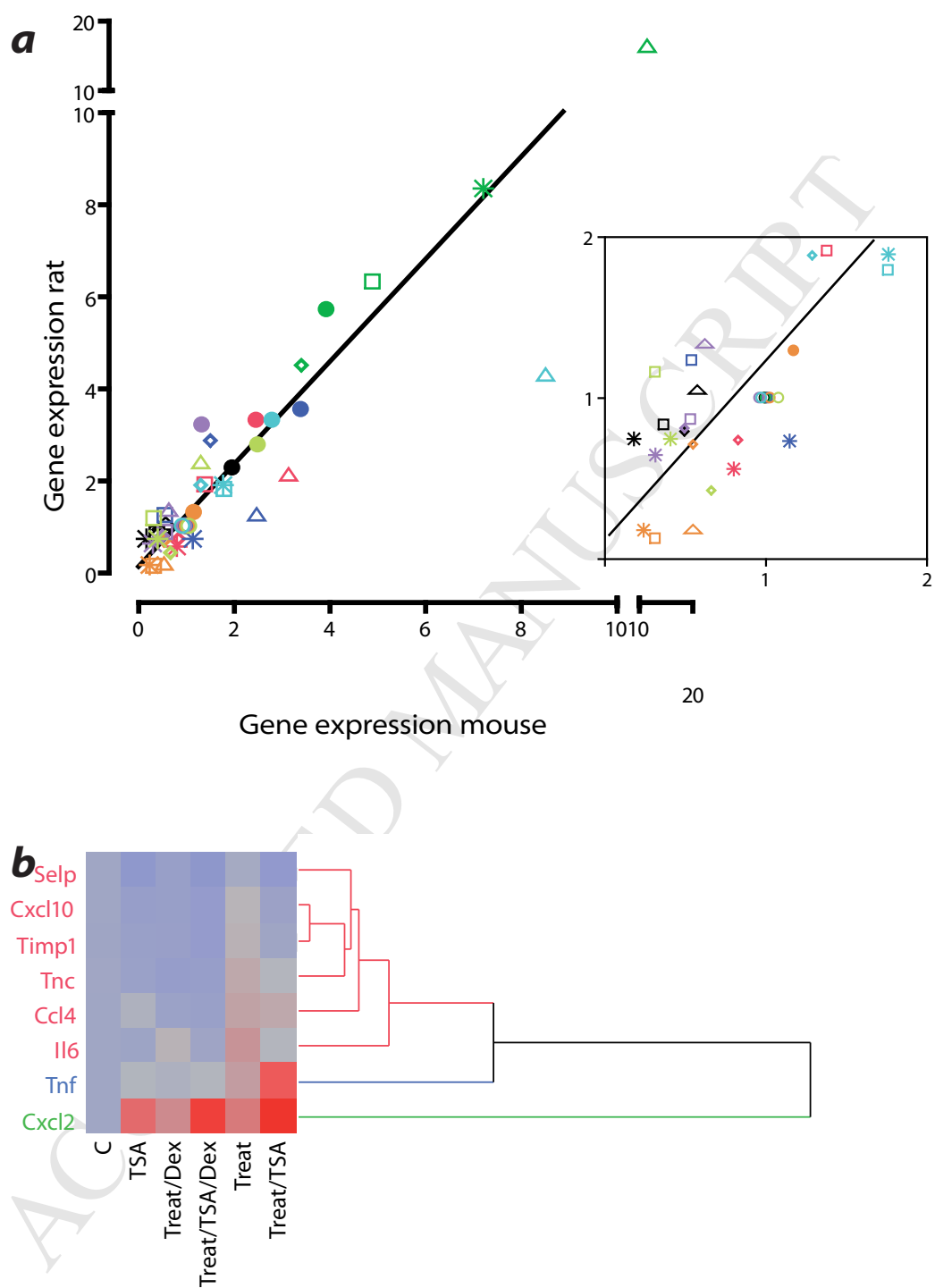


Fig. 5

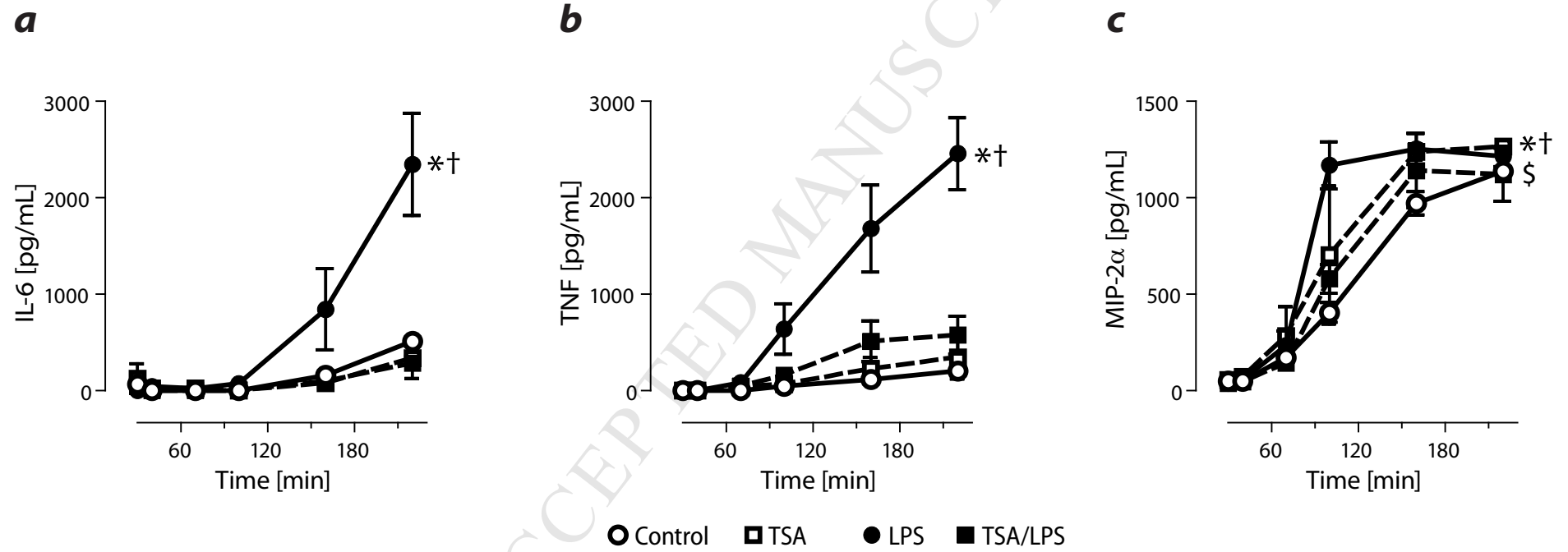


Fig. 6

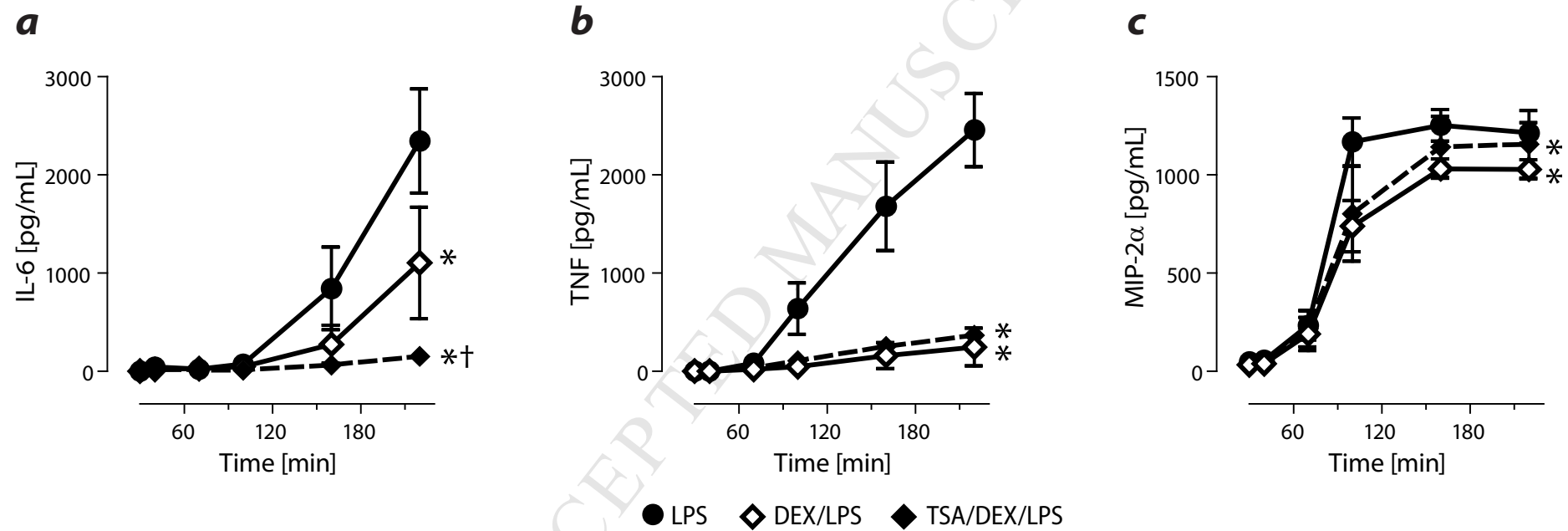


Fig. 7

