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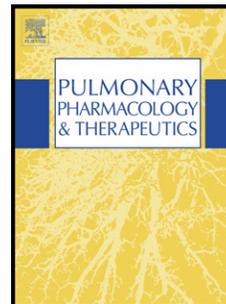
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## **Inflammation-induced airway smooth muscle responsiveness is strain dependent in mice**

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## Abstract

Different mouse strains display different degrees of inflammation-induced airway hyperresponsiveness *in vivo*. It is not known whether these variations are attributable to distinct properties of the airway smooth muscle. Therefore, tracheal ring segments from C57BL/6 and BALB/c mice were exposed to three different pro-inflammatory stimuli for 4 days while maintained under tissue-culture conditions: tumour necrosis factor  $\alpha$  (100 ng/ml), the Toll-like receptor (TLR) 3 agonist polyI:C (10  $\mu$ g/ml), and the TLR4 agonist LPS (10  $\mu$ g/ml). The contractile responses to carbachol, 5-hydroxytryptamine (5-HT) and bradykinin were assessed after culture. In addition, gene expression of TLR1-TLR9, pivotal inflammatory signal transduction proteins (jun-kinase, p38 and p65) and critical negative regulators of inflammation (A20, Itch, Tax1bp1 and RNF11) were studied in tracheal smooth muscle strips, fresh and following treatment for 4 days with LPS, from both strains. No differences between the strains were detected regarding the response of freshly isolated preparations to carbachol, 5-HT and bradykinin. After stimulation with pro-inflammatory mediators, contractions in response to 5-HT and bradykinin, but not to carbachol, were up-regulated. This up-regulation was markedly larger in BALB/c than in C57BL/6 segments and depended on the type of inflammatory stimulus. Expression of the genes investigated did not differ between the two strains. These findings indicate that strain differences in airway hyperresponsiveness can be linked to differences in the responsiveness of airway smooth muscle to pro-inflammatory mediators *per se*. These differences do not appear to be due to differential expression of TLR or common inflammatory transduction and repressor proteins.

Key words: Contractions, strains, Toll-like receptors, trachea.

## 1 Introduction

Asthma is characterized by chronic inflammation in the airways and increased airway responsiveness. A commonly used animal to investigate allergic inflammation is the mouse which can be sensitized and subsequently challenged with different allergenic agents. However, functional outcomes diverge between different mouse strains, in particular between C57BL/6 and BALB/c mice [1, 2]. After exposure to ovalbumin, both methacholine responsiveness and accumulation of collagen have been shown to be more pronounced in BALB/c mice than in C57BL/6 mice. Analysis of cytokines secreted during protocols of sensitization and challenge of ovalbumin indicated that the divergence of inflammatory responses between these two strains depended on C57BL/6 mice displaying more of a Th1-type response [3] and BALB/c a more Th2-type response [4]. The divergence between Th1- versus Th2 profiles was also demonstrated after chlamydial infection [5]. These differences bear witness to the two strains originating from different parts of the murine phylogenetic tree [6] in which the differential handling of inflammatory cell activation and recruitment is one of the organising principles [7].

In asthma, an exaggerated smooth muscle response is a major reason underlying the narrowing of the airways during exacerbations. The hyperactivity of smooth muscle cells may not only be due to an increase in the secretion of contractile mediators but also inflammation-induced alterations in the airway smooth muscle contractile phenotype [8]. In mice, studies have shown that long-term inflammatory stimulation with TNF $\alpha$ , IL-1 $\beta$  and IL-4, alters the responses to contractile agonists such as 5-hydroxytryptamine (5-HT) and bradykinin [9-13]. Moreover, smooth muscle cells can be activated directly by

stimuli of microbial origin through Toll-Like receptors (TLRs) [14, 15]. Since viral or bacterial infections are a common cause of asthma exacerbations [16], TLR activation may have an important impact on smooth muscle function [17]. In line with the varying functional outcomes of infection- and sensitization-models in different mouse strains, we hypothesise that inflammatory responses in the smooth muscle cells may be different between C57BL/6 and BALB/c mice.

This study was designed to elucidate the different effects of inflammatory stimuli on airway smooth muscle function in C57BL/6 and BALB/c mice. Therefore, the contractile responses to carbachol, 5-HT and bradykinin were studied on tracheal segments, fresh or following *in vitro* exposure to TNF $\alpha$ , polyinosinic:polycytidylic acid (poly I:C) or lipopolysaccharide (LPS) for 4 days (activating TNF receptor, TLR3 and TLR4 pathways respectively) while maintained under normal tissue-culture conditions. To evaluate whether altered expression of signalling proteins contributed to differences in the inflammatory response of tracheal segments from C57BL/6 and BALB/c mice, mRNA levels of TLR, the pivotal inflammatory signal transduction proteins JNK, p38 and p65, together with the critical negative regulators of inflammation A20, Itch, Tax1bp1 and RNF11 [18, 19], were quantified in tracheal smooth muscle strips.

## 2 Method

### 2.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM), carbachol, 5-HT, bradykinin, indomethacin, atropine, poly I:C and LPS were obtained from Sigma (St. Louis, MO, U.S.A.). Penicillin and streptomycin were obtained from Life Technologies (Gathisburg, MD, U.S.A.) and recombinant murine TNF $\alpha$  was obtained from R&D Systems (Abingdon, U.K.).

### 2.2 Tissue preparation and organ culture

8-10 week old C57BL/6 and BALB/c mice (Scanbur, Sweden) were sacrificed by cervical dislocation. Tracheas were either divided into four ring segments for organ bath investigations, or used as whole segments for total RNA preparation. The tracheal segments were used fresh or placed in 200 or 1000  $\mu$ l DMEM, respectively (with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin), in the absence or presence of TNF $\alpha$  (100 ng/ml), poly I:C (10  $\mu$ g/ml), LPS (10  $\mu$ g/ml; for the whole segments), under tissue culture conditions (37°C; 5% CO<sub>2</sub> in air) for 4 days. The segments were transferred to a new well containing fresh medium every day.

### 2.3 In vitro pharmacology

The tracheal segments were mounted on two metal prongs (200  $\mu$ m diameter) for recording of isometric tension (ADInstruments Ltd., Hastings, U.K.) in myographs (Organ Bath Model 700MO<sub>3</sub>, DMT A/S, Aarhus, Denmark) containing physiological buffer solution (mM units: 144 Na<sup>+</sup>, 5.9 K<sup>+</sup>, 2.5 Ca<sup>2+</sup>, 1.2 Mg<sup>2+</sup>, 131.1 Cl<sup>-</sup>, 1.2 H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 25 HCO<sub>3</sub><sup>-</sup>, 11 D-glucose and 0.03 EDTA) continuously gassed with 5% CO<sub>2</sub> and 95%

O<sub>2</sub> at 37°C. Cumulative agonist concentration-response curves were obtained on each segment for carbachol, 5-HT and bradykinin. Indomethacin (3 µM) was present throughout all experiments and atropine (1 µM) was present during experiments with 5-HT and bradykinin to inhibit the effects of released prostanoids, or post-junctional effects of acetylcholine.

#### *2.4 RNA preparation and PCR*

Following removal of the epithelium, tracheal smooth muscle was isolated and stored in RNAlater™ (QIAGEN GmbH, Hilden, Germany) at -20°C until extraction of total RNA. Before homogenisation, tissues were pooled pair-wise and total RNA was extracted using the RNeasy Mini kit (QIAGEN). The absorption ratios of total RNA was between 1.6 and 2.0 checked spectrophotometrically (260/280 nm). Reverse transcription of total RNA to cDNA was carried out using the High-capacity cDNA™ reverse transcriptase kit (Applied Biosystems, Foster City, CA, USA) with Multi Scribe™ reverse transcriptase and random primers (Applied Biosystems). Real-time PCR with specific primers (Table. 3 and 4) was performed in an ABI Prism 7000 Sequence Detector System (Applied Biosystems) using the TaqMan Fast Universal PCR master mix and carried out with an initial set-up (95°C, 10 min) and 35 cycles of denaturation (95°C, 15 sec) followed by annealing/extension (60°C, 1 min). The relative amount of mRNA was calculated from the cycle threshold (C<sub>T</sub>) value for target gene mRNA in relation to C<sub>T</sub> values for the housekeeping gene 18S rRNA in the same sample.

#### *2.5 Analysis*

All data were expressed as mean values  $\pm$  SEM. Agonist concentration-response data were fitted to the Hill equation (GraphPad Prism, San Diego, U.S.A) for estimates of  $E_{\max}$  (maximal contraction) and  $pEC_{50}$  (-log M units). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's post-test or t-test.  $n$  indicates the number of animals used. The null hypothesis was rejected when  $P < 0.05$ .

### 3 Results

#### 3.1 Contractile responses to carbachol, 5-HT and bradykinin in fresh and cultured tracheal segments

In fresh segments, the contractions to carbachol in C57BL/6 and BALB/c mice showed no statistically significant differences, neither with respect to  $E_{\max}$  nor  $pEC_{50}$  (Fig. 1A). In the fresh segments from both strains, contractions to 5-HT approached 5% of carbachol without any significant differences (Fig. 1B). Due to the negligible responses to bradykinin in both C57BL/6 and BALB/c mice, fully-defined concentration-response curves could not be determined in all experiments (Fig 1C).

Following 4 days of tissue culture, both the maximal contraction and potency towards carbachol were decreased for C57BL/6 mice, whereas no differences were observed in BALB/c mice (Table 1). In segments from C57BL/6 mice, the  $E_{\max}$  to 5-HT was slightly increased, after culture without any change in  $pEC_{50}$  (Table 1). No changes in response to 5-HT were seen after culture of segments from BALB/c mice. Bradykinin-induced responses ( $E_{\max}$ ) increased marginally in segments from both strains (Table 1).

### 3.2 Contractile responses to carbachol, 5-HT and bradykinin following culture with TNF $\alpha$ , LPS or poly I:C for 4 days.

None of the inflammatory mediators had any effect on the concentration-response relationship to carbachol in either strain (Fig 2A and B; Table 1). By contrast, the  $E_{\max}$  (5-HT), but not  $pEC_{50}$ , was markedly increased after TNF $\alpha$  treatment in segments from both strains. However, the increase observed in BALB/c segments was larger ( $8.3 \pm 0.5$ -fold; Fig. 1H) than in C57BL/6 segments ( $2.6 \pm 0.5$ -fold;  $p < 0.0001$ ; Fig. 2C and D). Treatment with poly I:C only increased the maximal contraction to 5-HT in segments from BALB/c mice, and to a lesser extent ( $3.5 \pm 0.3$ -fold) than both TNF $\alpha$  and LPS ( $p < 0.05$ ). Segments cultured for 4 days with LPS showed the same pattern of 5-HT-induced responses as those cultured with TNF $\alpha$  in that a greater increase was observed in BALB/c mice ( $6.3 \pm 0.9$ -fold) than C57BL/6 mice ( $2.7 \pm 0.6$ -fold;  $p < 0.01$ ).

A marked increase in bradykinin-induced contractions was apparent in both strains when segments were cultured with TNF $\alpha$  (Table 1). Again,  $E_{\max}$  was higher in BALB/c segments ( $7.3 \pm 0.5$ -fold; Fig. 2F) compared to C57BL/6 segments ( $4.1 \pm 0.7$ -fold;  $p < 0.05$ ; Fig 2E). Poly I:C failed to alter responses to bradykinin in C57BL/6 segments but clearly increased them in BALB/c segments ( $3.7 \pm 0.4$ -fold), albeit to a lower extent than following culture with TNF $\alpha$  or LPS ( $p < 0.0001$ ). Treatment with LPS only increased the maximal contraction to bradykinin in segments from BALB/c mice ( $7.2 \pm 0.3$ -fold).

### 3.3 Expression levels of TLR in the tracheal smooth muscle layer

To investigate the basal capacity of the smooth muscle to react to TLR agonists, the expression of mRNA for TLR1-TLR9 was quantified in fresh tracheal smooth muscle

from C57BL/6 and BALB/c mice. TLR expression was found to be markedly similar in both strains with TLR4 being the most prominently expressed receptor (Fig. 3, Table 2). Expression levels of TLR3 were approximately one third of those of TLR-4 and similar to those of TLR2 and TLR5. The expression of the other TLR was more than 10-fold lower (Table 2).

### *3.4 Expression levels of enzymes involved in signal-transduction of the inflammatory response*

To explore how the tracheal smooth muscle layer responds to pro-inflammatory stimuli, the expression of mRNA for p38 and p65 (transducers of inflammation) and A20, Itch, Tax1bp1 and RNF1 (repressors of inflammation) were measured, both in fresh tissue and following 4 days of LPS treatment. LPS was chosen due to its strong effects on the functional responses of both strains. The order of expression for enzymes of the inflammatory signal transduction pathway was p65>p38=JNK both in fresh and LPS-treated smooth muscle layers (Table 3) but there were no significant differences between expression in C57BL/6 and BALB/c mice. The order of magnitude for expression of the repressing enzymes was not identical in the two strains, but generally levels of Tax1bp1 and RNF1 were highest both in fresh and LPS-treated smooth muscle layers, followed by Itch and, thereafter, A20 (Table 3). However, no significant differences were observed between the strains.

## **4 Discussion**

The most important conclusions to be drawn from this study are that airway smooth muscle from C57BL/6 and BALB/c mice differs with respect to the activation of

inflammatory reactions, and furthermore, that these differences cannot be explained by differences in the expression of TLRs, or key enzymes involved in inflammatory signal transduction.

In both C57BL/6 and BALB/c mice, the strong contractions induced by carbachol and negligible responses to 5-HT and bradykinin, were similar in fresh segments and those cultured for 4 days in the absence of other treatments. The small but significant decrease in both  $E_{\max}$  and  $pEC_{50}$  for carbachol, caused by culture for 4 days of segments from C57BL/6 mice, could be due to the fact that this strain is more sensitive to the current tissue culture procedure. The results in BALB/c mice were in accordance with earlier studies [9, 10]. The procedure of maintaining mouse tracheal segments for 4 days under tissue culture conditions does not alter gene expression by more than 5%, as shown previously [9]. Thus, the similarity of the responses between basal (fresh) and control (following 4 days of tissue culture) segments indicates that the smooth muscle phenotype remained largely unaltered in both strains with similar numbers of muscarinic, 5-HT and bradykinin receptors as well as signalling pathways and contractile proteins.

No differences in  $E_{\max}$  or  $pEC_{50}$  to carbachol were seen in either strain after exposure to the inflammatory mediators. This is in agreement with earlier studies of tracheal segments from BALB/c mice cultured for 4 days in the presence of  $TNF\alpha$ , poly I:C and LPS [9, 14]. Interestingly, the absence of an exaggerated inflammation-induced response to carbachol contrasts with methacholine hyperresponsiveness *in vivo* after sensitization and challenge to allergens [1, 2]. This might be due the fact that the allergen-induced

responses *in vivo* do not measure tracheal effects, or because there are different inflammatory mechanisms underlying the two experimental approaches. Allergen exposure clearly causes a recruitment of inflammatory cells together with the secretion of a multitude of cytokines and chemokines [4], whereas the deliberate aim of this assay was to be able to study the specific action of a single inflammatory substance at the level of the smooth muscle.

In BALB/c mice, the response to both 5-HT and bradykinin increased after exposure to the three inflammatory substances. In C57BL/6 mice, TNF $\alpha$  increased the responses to both 5-HT and bradykinin, and LPS increased the response to 5-HT, whereas poly I:C had no effect on responses to either 5-HT or bradykinin. Moreover, the effects of TNF $\alpha$  and LPS were clearly reduced in C57BL/6 mice compared to BALB/c mice. Confirming earlier data [9, 10, 14], all three inflammatory substances increased the maximal contractions to 5-HT and bradykinin in BALB/c mice. These experiments therefore show for the first time that similar pathways exist in C57BL/6 mice but that they are activated to a lesser degree than in BALB/c mice. This similarity is of particular interest, since the up-regulation of the responses to the two contractile agonists is at least partly due to separate mechanisms, transcriptional and non-transcriptional, as described above.

The reduced responses to the different inflammatory mediators observed in C57BL/6 compared to BALB/c mice could be due to a lower expression of TLR3 and TLR4 in C57BL/6, but the results of our experiments would suggest otherwise. Expression of all TLRs investigated was remarkably similar in the two mice strains studied. This could

indicate a similar tracheal smooth muscle phenotype in the mouse species as a whole. The fact that expression of TLR4 was greater than TLR3 in both strains probably explains why LPS generally caused a higher degree of contraction towards 5-HT and bradykinin than poly I:C. Thus, mechanisms located downstream of the inflammatory receptors are most likely responsible for the observed strain-dependent difference.

We also investigated the expression of JNK (involved in the TNF $\alpha$ - and LPS/poly I:C-induced up-regulation of bradykinin-response [11, 14]), p38 (involved in the IL-4-induced up-regulation of des-arg<sup>9</sup>-bradykinin [10]) and p65 (involved in the LPS/poly I:C-induced up-regulation of bradykinin [14]). Our expectation was that smooth muscle from BALB/c mice would express higher levels of inflammatory pathway proteins than C57BL/6 smooth muscle. However, no differences between the strains were found in either fresh segments or segments that had been treated with LPS for 4 days. Therefore, other factors need to be investigated to explain the differences. It has been reported that A20, which acts together with Itch, Tax1bp1 and RNF11 in a ubiquitin-editing protein complex [20], inhibits both TNF- and TLR-induced responses [18, 19]. For this reason we hypothesised anew that these genes were expressed to a greater extent in C57BL/6 compared to BALB/c mice but again, no significant differences could be detected before or after LPS treatment. Recently, 880 of 1654 investigated SNPs were found to differ between C57BL/6 and BALB/c mice [6]. This would indicate not only that expression of different genes might be of importance, but also possible differences in the enzymatic activity of each protein, caused by for example phosphorylation. Indeed, a database search (<http://phenome.jax.org>) revealed three differences in the amino acid sequence for

A20, which could indicate that the activity of this protein is strain-dependent and that phosphorylation of A20 and its down-stream proteins may be important for future investigations regarding strain-differences.

Several aspects of the airway inflammatory response *in vivo* cannot be mimicked during the *in vitro* exposure to inflammatory modulators. However, the model enables us to specifically examine individual factors that affect isolated airway smooth muscle. The results suggest that the difference in airway inflammatory responses between C57BL/6 and BALB/c mice can in part originate in a difference in responses of the airway smooth muscle cells *per se*. The strain-dependent differences in both the smooth muscle reactivity and the earlier reported divergence recruitment of inflammatory cells might suggest that genetic differences can affect airway responsiveness at different levels.

In conclusion, this study demonstrates for the first time that intrinsic inflammatory responses of the airway smooth muscle are higher in BALB/c mice than in C57BL/6 mice. This difference between the two mouse strains indicates that in experimental studies of airway responsiveness, BALB/c mice is a more suitable strain than C57BL/6 mice for assessment of smooth muscle reactions towards inflammation is of interest. It should also be noted that several of the genetically modified mouse strains are derived from a C57BL/6 background which may limit the possibility to explore responsiveness of their airway smooth muscle to inflammatory substances. Although our study clearly shows a difference in smooth muscle reactivity between the mouse strains, we have not been able to specifically identify the underlying genetic cause, which seems to be located

in signaling pathways downstream of the inflammatory receptors. Further comparative studies may identify the factor (or factors) causing this strain-dependent difference.

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## 7 Figure legends

Figure 1. Concentration-response curves to (A) carbachol, (B) 5-HT and (C) bradykinin in fresh segments from either C57BL/6 (closed symbols) or BALB/c mice (open symbols) ( $n=6-8$ ).

Figure 2. Concentration-response curves to (A and B) carbachol, (C and D) 5-HT and (E and F) bradykinin in segments maintained under tissue culture conditions for 4 days with TNF $\alpha$  (closed squares), LPS (closed circles)(D-I) and poly I:C (closed triangles) from either C57BL/6 (A, C and E) or BALB/c (B, D and F) mice ( $n=6-8$ ); control tissues (open circles) received vehicle during this period.

Figure 3. mRNA expression for TLR3 and TLR4 in tracheal segments from C57BL/6 and BALB/c mice. Each point represents the mean of all segments tested with error bars representing SEM from 6-8 animals in pools of 2 for each sample.

Table 1. Responses to carbachol (Cch), 5-HT and bradykinin in tracheal segments from C57BL/6 and BALB/c mice; fresh or cultured for 4 days in the absence and presence of TNF $\alpha$ , poly I:C and LPS

Treatments		C57BL/6 mice		BALB/c mice		
Carbachol	<i>n</i>	$E_{\max}$ (mN)	pEC <sub>50</sub>	<i>n</i>	$E_{\max}$ (mN)	pEC <sub>50</sub>
Fresh	8	5.89±0.66*	6.89±0.06***	6	4.94±0.68	6.98±0.09
4 days	8	3.53±0.47	6.60±0.02	6	5.02±0.40	6.77±0.14
4 days TNF $\alpha$	6	3.28±0.58	6.52±0.07	6	4.64±0.45	6.64±0.12
4 days poly I:C	7	4.39±0.32	6.58±0.07	6	4.86±0.35	6.85±0.09
4 days LPS	7	3.47±0.46	6.59±0.08	6	5.21±0.51	6.70±0.12
5-HT		(% of Cch)		(% of Cch)		
Fresh	8	3.6±1.4*	6.66±0.17	6	8.2±2.7	6.50±0.24
4 days	8	9.7±1.9	6.88±0.15	6	7.0±0.41	6.84±0.10
4 days TNF $\alpha$	6	25.5±4.9*	6.62±0.07	6	57.8±3.8***	6.50±0.11
4 days poly I:C	7	13.8±2.3	6.81±0.05	6	24.8±2.1*	6.35±0.08
4 days LPS	7	26.1±6.0*	6.51±0.08*	6	44.7±6.5***	6.68±0.19
Bradykinin		(% of Cch)		(% of Cch)		
Fresh	8	0.7±0.1***	n.d.	6	3.9±0.17**	n.d.
4 days	8	5.9±1.0	7.82±0.28	6	10.8±2.2	7.39±0.29
4 days TNF $\alpha$	6	24.0±4.3***	8.15±0.31	6	65.9±4.1***	7.98±0.30
4 days poly I:C	7	6.4±1.0	7.68±0.26	6	33.0±3.5***	7.18±0.11
4 days LPS	7	12.4±3.9	8.50±0.13	6	64.8±2.8***	7.69±0.28

One-way ANOVA with Dunnett's post-test comparing all vs 4 days without treatment;

\*P<0.05, \*\*P<0.01 and \*\*\*P<0.001.

Table 2. PCR analysis investigating the expression of TLR1-TLR9 in fresh tracheal segments before organ culture

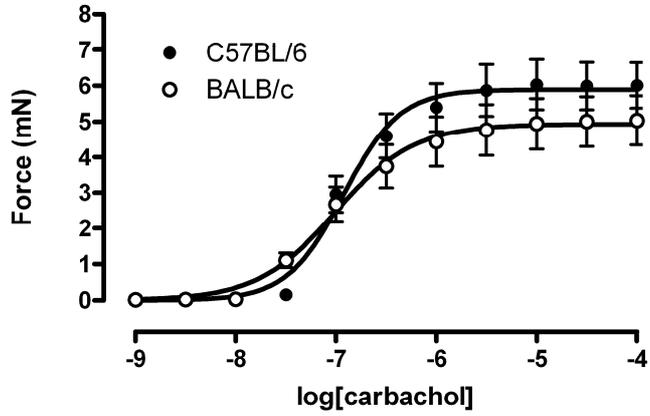
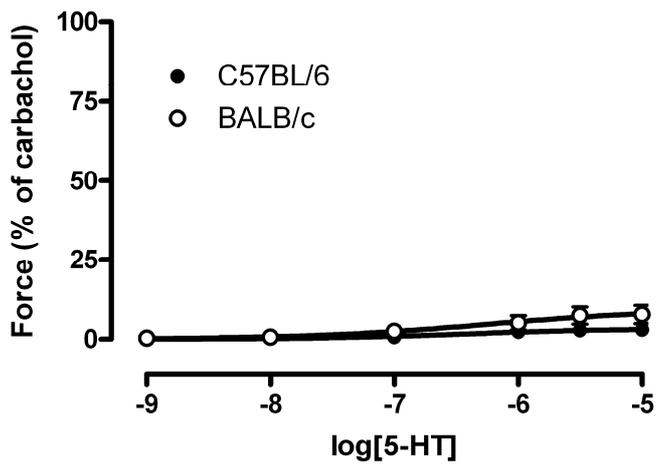
	Assay ID	C57BL/6	BALB/c
TLR 1	Mm00446095_m1	7.4±1.1	3.1±1.0
TLR 2	Mm00442346_m1	24.1±13.1	42.9±9.9
TLR 3	Mm00446577_g1	40.0±2.7	46.1±13.4
TLR 4	Mm00445274_m1	133.5±20.1	134.7±29.5
TLR 5	Mm00546288_s1	38.3±6.1	40.4±11.5
TLR 6	Mm02529782_s1	0.7±0.4	0.4±0.3
TLR 7	Mm00446590_m1	2.1±1.0	3.4±2.6
TLR 8	Mm01157262_m1	0.6±0.3	1.3±1.1
TLR 9	Mm00446193_m1	1.1±0.6	1.9±1.5

Expression values are expressed in relation to 100,000 mol 18S. No significant differences between C57BL/6 and BALB/c mice were found using T-tests (6-8 animals in pools of 2 in each sample).

Table 3. PCR analysis investigating the expression of inflammatory signal transduction proteins (JNK, p38 and p65) and negative regulators of inflammation (A20, Itch, Tax1bp1 and RNF11) in fresh tracheal segments and segments treated with LPS during 4 days culture

	Assay ID	C57BL/6	BALB/c	C57BL/6	BALB/c
		Fresh	Fresh	LPS-treated	LPS-treated
p65	Mm00501346_m1	2769±1714	628±519	99±38	427±30
JNK	Mm00442497_m1	317±173	213±179	23±8	36±8
p38	Mm00489514_m1	396±133	288±220	30±12	32±80
A20	Mm01188374_m1	144±97	21±15	12±7	141±1
ITCH	Mm00492683_m1	1827±560	851±696	14±6	18±5
Tax1bp1	Mm01326379_m1	14428±10359	2080±1857	35±17	200±106
Rnf11	Mm00450015_m1	4568±1963	3147±2812	84±45	150±68

Expression values are expressed in relation to 100,000 mol 18S. No significant differences between C57BL/6 and BALB/c mice were found using T-test (4-14 animals in pools of 2 in each sample).

**A****B****C**