RESEARCH ARTICLE

# Long-term exposure to IL-1β enhances Toll-IL-1 receptor-mediated inflammatory signaling in murine airway hyperresponsiveness

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Accepted for publication May 28, 2009

**ABSTRACT. Toll-interleukin-1 (Toll-IL-1) receptor signaling may play a key role in the development of airway hyperreactivity (AHR) and chronic airway inflammatory diseases such as asthma. Previously, we have demonstrated that pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), induce AHR. However, the underlying intracellular signaling mechanisms that lead to AHR remain elusive. In order to see if the Toll-IL-1 receptor-mediated inflammatory signal pathways are involved in the development of AHR, the present study was designed to use a real-time PCR array, a sensitive and powerful tool, consisting of 84 genes related to Toll-IL-1 receptor signal pathways. Murine tracheal segments were organ cultured for four days in the presence and absence of IL-1β. The Toll-IL-1 receptor-mediated inflammatory signal gene profile was studied using the real-time PCR-based cDNA array. The key gene expressions that were altered were verified by immunohistochemistry using confocal microscopy. Tracheal ring segment contractile responsiveness to the inflammatory mediator bradykinin was monitored using a sensitive myograph system. The results showed that after exposed to IL-1β for four days, the tracheal segments exhibited increased mRNA expression of 67 genes (out of the 84 genes in the array), although expression reached statistical significance for only 16 of these genes. There were 14 genes that showed only a tendency towards a decrease in mRNA expression following IL-1β treatment. Immunohistochemistry confirmed that protein expression for CD14, RP105, MCP-1 and phosphorylated IκB-α were increased in both the airway epithelial and smooth muscle cells. In order to link the activation of Toll-IL-1 receptor-mediated inflammatory signal mechanisms to the AHR, the anti-inflammatory drug dexamethasone, was used. Dexamethasone not only completely abolished the IL-1β-induced AHR to bradykinin, but also abrogated the increased mRNA expression for inflammatory mediators, IL-6, IFN-γ and Cox-2. In conclusion, long-term exposure of murine airway to IL-1β induces up- and down-regulation of mRNA expression for Toll-IL-1 receptor signal molecules, with a significant increase in the expression of 16 genes that contribute to the development of airway inflammation and AHR. Understanding cytokine-induced activation of the Toll-IL-1 receptor-mediated inflammatory signaling mechanisms may provide new options for the treatment of airway inflammation and AHR.**

**Keywords:** IL-1β, Toll-IL-1, receptor, signal, inflammation, AHR

Asthma is characterized by chronic airway inflammation, airway hyperresponsiveness (AHR), airway remodelling and reversible airway obstruction. Cytokines play critical roles in the development of chronic airway inflammation and AHR. Interleukin-1β (IL-1β) is a pro-inflammatory cytokine involved in the pathogenesis of asthma [1, 2]. Increased IL-1 immunostaining in the submucosa of patients with toluene diisocyanate-induced occupational asthma suggests that IL-1 is involved in human asthma development [3]. In guinea pigs, ozone exposure for three days induces IL-1-mediated airway hyperreactivity, which can be prevented by IL-1 receptor antagonist treatment [4]. IL-1β acts on type I IL-1 receptors (IL-1R1) that include the *Drosophila melanogaster* protein Toll and Toll-like receptors (TLRs) [5, 6]. Because of the similarity of IL-1RI to Toll, the conserved sequence in the cytosolic region of these proteins has been termed the Toll-IL-1 receptor (TIR) domain. This receptor superfamily represents an ancient signaling system that is a critical determinant of innate and acquired immunological inflammation [7].

Our previous studies have shown that cytokines, tumor necrosis factor-α (TNF-α) and IL-1β, induced AHR to the inflammatory mediator bradykinin, and serotonin [8, 9].

doi: 10.1684/ecn.2009.0156

The present study was designed to investigate the underlying intracellular Toll-IL-1 receptor signal mechanisms that lead to AHR. This involved a murine model of chronic airway inflammation and AHR [8, 9], using a real-time PCR-based cDNA array, a sensitive and reliable tool, consisting of 84 important genes related to the Toll-IL-1 receptor signal pathways. We demonstrated that IL-1β significantly up-regulated mRNA expression for 16 genes related to Toll-IL-1 receptor signal pathways. Immunohistochemistry verified that protein expression for CD14, RP105, MCP-1 and phosphorylated I$\kappa$B-α was increased in both the airway epithelial and smooth muscle cells. The anti-inflammatory drug dexamethasone not only completely abolished IL-1β-induced AHR, but also abrogated the increased mRNA expression for the inflammatory mediators. Taken together, this suggests that IL-1β induces up- and down-regulation of Toll-IL-1 receptor signal gene expression. This increased expression of inflammatory mediators contributes to airway inflammation and AHR.

## METHODS AND MATERIALS

### *Tissue preparation and organ culture procedures*

Tracheas from 9-10 week-old, male BALB/c J mice (MB A/S, Ry, Denmark) were dissected and placed individually into the wells of 24-well plates (Ultra-low attachment; Sigma, St. Louis, MO, USA) with 1 mL of serum-free Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) (Life Technologies, Gaithersburg, MD, USA). The tracheal segments were then incubated at 37°C, in humidified 5% $CO_2$ in air, in the presence and absence of murine IL-1β (R&D Systems, Abingdon, UK), or IL-1β plus dexamethasone (Sigma, St. Louis, MO, USA) for four days. The tracheal segments were moved into new wells containing fresh medium supplemented with IL-1β and dexamethasone each day [10]. The experimental protocols had been approved by the local Animal Ethics Committee (M124-06).

### *Real-time PCR-based cDNA array*

After four days of organ culture, the tracheal segments were stored at - 80°C in RNAlater solution (QIAGEN, GmbH, Hilden, Germany) for subsequent extraction of total RNA. This was done using a PureLink™ micro-to-midi total RNA purification system (Invitrogen Corporation, Carlsbad, CA, USA) including RNase I (Invitrogen Corporation), in accordance with the manufacturer's recommendations. The quality and quantity of the extracted total RNA were assessed using spectrophotometric UV absorbance ratios of 260/280 nm and 260/230 nm, respectively. All the RNA samples meet the standards of integrity, and were free from protein, organics and genomic DNA contaminants, *i.e.* absorbance ratios at 260/280 nm were 1.98 to 2.1 and at 260/230 nm is 1.65-1.8 in Tris-HCI buffer solution (pH 7.5).

cDNA was synthesised from DNase-treated RNA by reverse transcription, in accordance with the manufacture's protocol, using first strand cDNA synthesis kits (C-02, SuperArray Bioscience Corporation, Frederick, MD, USA), which contain an effective genomic DNA elimination step and a built-in external RNA control. Briefly, total RNA (1 μg) was mixed with genomic DNA elimination mixture and incubated at 70°C for 3 min, and then immediately placed on ice for at least 1 min. Ten μl of the RT cocktail were added to each 10 μL of the genomic DNA elimination mixture to make a final volume of 20 μL, which was then incubated at 37°C for 60 min. Finally, the mixture was heated at 95°C for 5 min to degrade the RNA and to inactivate the reverse transcriptase.

The procedure for the real-time PCR-based cDNA array for the mouse Toll-IL-1 receptor signaling pathway (APMM-018A, SuperArray Bioscience Corporation) was carried out in accordance with the manufacturer's protocol. Each array was a 96-well plate containing SYBR Green-optimized primer sets corresponding to a total of 96 genes, including five housekeeping genes and three sets of negative controls. Briefly, the cDNA was diluted with RNase-free distilled water to a volume of 100 μl and added to a $RT^2$ real-time™ SYBR Green/ROX qPCR Master Mix (PA-112, SuperArray Bioscience Corporation). Aliquots of the mixture were added to a 96-well plate, where each well contained predispensed, gene-specific primer sets related to Toll-IL-1 receptor signaling pathways. The plates were then placed in an ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA), and real-time PCR was performed with heating at 95°C for 15 min for the activation of HotStart DNA polymerase, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The housekeeping genes (β-actin, GAPDH, HSPC-β, HPRT-1 and GUS-β) were used for normalization of the PCR array data, and the ROX reference dye for normalising the instrument's optics. In addition, for each reaction, "no reverse transcription control", "no template" and "genomic DNA" samples were included as negative controls. Raw data were acquired and processed with the ABI Sequence Detector System software version 1.0 (Applied Biosystems) to calculate the threshold cycle ($C_t$) value. The specificity of the PCR products were checked by examination of the dissociation curves. The relative gene expression values were subsequently determined according to the standard $^{\Delta\Delta}C_t$ method (http://www.superarray.com).

### *Immunohistochemistry*

After organ culture, the tracheal segments were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), for 3 h at 4°C. After fixation, the specimens were dehydrated in 20% sucrose with 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C, and then frozen in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, Netherlands) and stored at - 80°C. Ten μm-thick sections were cut using a cryostat. These were mounted on SuperFrost Plus slides (Menzel GMBH & COKG, Braunschweig, Germany). Immunohistochemistry

was carried out using standard protocols, *i.e.* the sections were incubated with the primary antibody overnight at 4°C and the secondary antibody for 1 h at room temperature in the dark. Primary and secondary antibodies, as well as the dilutions used were as follows: CD14 (1:100, rabbit, Abcam plc, Cambridge, UK), MCP-1 (1:100, rabbit, Abcam plc), RP105 (1:100, rabbit, ProSci Incorporated, Poway, CA, USA), bradykinin $B_1$ receptor (1:50, goat, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), bradykinin $B_2$ receptor (1:100, rabbit, Alexis Biochemical, Lausen, Switzerland), phospho-IκB-α (Ser32) (1:100, rabbit, cell signalling Technology Inc., Beverly, MA, USA). The appropriate secondary antibodies, goat anti-rabbit IgG H&L (1.200, Abcam plc), donkey anti-goat IgG H&L (1.200, Abcam plc), conjugated with fluorescein isothiocynate (FITC), were used for fluorescence microscopic imaging. In the control experiments, either the primary antibody or the secondary antibody was omitted. The stained specimens were observed under a confocal microscope (Nikon, C1plus, Nikon Instruments Inc., NY, USA). The fluorescence intensity was measured and analysed using Image J software (http://rsb.info.nih.gov/ij). For each specimen, six, randomly selected sections were studied. In each section, the fluorescence intensity was measured at six, preset areas. The mean value of fluorescence intensity was obtained from six experiments.

### *Real-time PCR*

Real-time PCR was carried out using the same procedure as used for the PCR-based cDNA array. The PCR primers for CD14, Muc13, IL-6, IFN-γ, Cox-2, and housekeeping gene β-actin (NM_007393) and hypoxanthine guanine phophoribosyl transferase 1 (Hprt1, NM_013556) were purchased from the same supplier (SuperArray Bioscience Corporation) as used for the PCR based cDNA array to ensure the same sequences and locations of PCR products as in the cDNA array assay.

### In vitro *pharmacology*

The cultured segments were immersed in temperature-controlled (37ºC) myographs (Organ Bath Model 700MO, JP Trading, Aarhus, Denmark), containing 5 mL of Krebs-Henseleit buffer solution, continuously equilibrated with 5% $CO_2$ in $O_2$ to obtain a stable pH of 7.4 for recording of isometric tension. A pre-tension of 0.8 mN was applied to each segment, which has been demonstrated to be optimal [10]. Each segment was then contracted with 60 mM KCl to test the contractile function. To inhibit epithelial prostaglandin release, the segments were incubated with 3μM indomethacin (Sigma, St. Louis, MO, USA) 30 min before administration of des-Arg$^9$-bradykinin or bradykinin (Neosystem SA, Strasbourg, France). Each agonist concentration-effect curve was fitted to the Hill equation using an iterative, least square method (GraphPad Prism, San Diego, USA), to provide estimates of maximal contraction ($E_{max}$) and $pEC_{50}$ values (negative logarithm of the agonist concentration that produces 50% of the maximal effect).

### *Statistical analysis*

All data were expressed as mean values ± S.E.M and analyzed using the unpaired Student's *t*-test with Welch's correction. P-values less than 0.05 were considered to be statistically significant.

## RESULTS

### *mRNA expressions for Toll-IL-1 receptor signal genes*

Eighty-four gene mRNAs related to Toll-IL-1 receptor signaling were analyzed, using a real-time PCR array, in tracheal segments following four days of organ culture in the absence (control) and presence of IL-1β. Compared with the control, IL-1β induced an up-regulation of expression of mRNA for 67 genes, although only 16 (RP105, CD14, Hspa1a, Pglyrp1, Irak2, IκB-α, MCP-1, Csf2, Csf3, IFN-γ, IL-1α, IL-6, TNF-β, Tnfaip3, Cebpb, Cox-2) of these gene mRNA expressions reached statistical significance (*table 1*, $p < 0.05$). Nine (CD14, Csf2, Csf3, IFN-γ, IL-1α, IL-6, RP105, pglyrp1 and Cox-2) of these genes exhibited a more than five-fold up-regulation, with a maximum of 36-fold increase *(table 1*, *figure 1)*. For 14 genes, mRNA expression was down-regulated by IL-1β, but none reached significance (*table 2*, $p > 0.05$). ELK1, a member of the ETS oncogene family (Elk1 (NM_007922), and TLR3 (NM_126166) remained unchanged. Interleukin-1 receptor-associated kinase 1 (Irak1, NM_008363) was not detected.

### *Immunohistochemistry*

The tracheal segments that had been organ cultured in presence of IL-1β exhibited increased protein expressions for phophorylated IκB-α *(figure 2A*, *B)*, CD14 *(figure 2D*, *E)*, RP105 *(figure 2G*, *H)*, MCP-1 *(figure 2J*, *K)*, bradykinin $B_1$ *(figure 2M*, *N)* and $B_2$ *(figure 2P*, *Q)* receptors in both epithelium and smooth muscle cells, compared to controls. The protein expressions for RP105 and CD14 were similar in both epithelium and smooth muscle, whereas MCP-1, phophorylated IκB-α and bradykinin $B_1$ receptor protein expressions were greater in the epithelium than in the smooth muscle. However, bradykinin $B_2$ receptor protein expression was predominant in the smooth muscle.

### *Effects of dexamethasone on mRNA expression of inflammatory mediators*

Dexamethasone significantly decreased IL-1β-induced enhancement of mRNA expression for IL-6, IFN-γ and Cox-2, but not Muc13 and CD14 *(table 3)*, suggesting that under IL-1β stimulation, there were inflammatory changes in the airway.

### *Dexamethasone abolished IL-1β-induced enhancement of bradykinin receptor-mediated airway contractions*

In order to focus on the role of airway smooth muscle contractility, epithelium-dependent relaxation was blocked by indomethacin. IL-1β significantly enhanced

**Table 1**
Genes up-regulated following IL-1β treatment

| Gene title | Symbol | GenBank No | Fold change | P-value |
|---|---|---|---|---|
| Caspase 8 | Casp8 | NM_009812 | 1.11 | 0.77 |
| **Chemokine (C-C motif) ligand 2** | **Ccl2** | **NM_011333** | **3.99** | **0.009** |
| **CD14 antigen** | **Cd14** | **NM_009841** | **5.44** | **0.001** |
| **CCAAT/enhancer binding protein (C/EBP), beta** | **Cebpb** | **NM_009883** | **2.36** | **0.007** |
| Conserved helix-loop-helix ubiquitous kinase | Chuk | NM_007700 | 1.76 | 0.35 |
| C-type lectin domain family 4, member e | Clec4e | NM_019948 | 1.76 | 0.17 |
| **Colony-stimulating factor 2 (granulocyte-macrophage)** | **Csf2** | **NM_009969** | **5.2** | **0.0003** |
| **Colony-stimulating factor 3 (granulocyte)** | **Csf3** | **NM_009971** | **36.62** | **0.0001** |
| Chemokine (C-X-C motif) ligand 10 | Cxcl10 | NM_021274 | 1.08 | 0.92 |
| Fas (TNFRSF6)-associated *via* death domain | Fadd | NM_010175 | 1.04 | 0.89 |
| FBJ osteosarcoma oncogene | Fos | NM_010234 | 1.27 | 0.37 |
| High mobility group box 1 | Hmgb1 | NM_010439 | 1.13 | 0.62 |
| Harvey rat sarcoma virus oncogene 1 | Hras1 | NM_008284 | 1.12 | 0.70 |
| HIV-1 Rev binding protein | Hrb | NM_010472 | 1.27 | 0.50 |
| **Heat shock protein 1A** | **Hspa1a** | **NM_010479** | **1.81** | **0.02** |
| Heat shock protein 1 (chaperonin) | Hspd1 | NM_010477 | 1.23 | 0.57 |
| **Interferon gamma** | **Ifng** | **NM_008337** | **20.66** | **0.04** |
| Inhibitor of kappaB kinase beta | Ikbkb | NM_010546 | 1.22 | 0.50 |
| Interleukin 10 | Il10 | NM_010548 | 1.29 | 0.69 |
| Interleukin 12A | Il12a | NM_008351 | 2.34 | 0.15 |
| **Interleukin 1 alpha** | **Il1a** | **NM_010554** | **8.06** | **0.004** |
| Interleukin 1 beta | Il1b | NM_008361 | 2.51 | 0.058 |
| Interleukin 2 | Il2 | NM_008366 | 1.37 | 0.81 |
| **Interleukin 6** | **Il6** | **NM_031168** | **36.04** | **0.0001** |
| | | | | |
| Interleukin 6 receptor, alpha | Il6ra | NM_010559 | 1.12 | 0.69 |
| **Interleukin-1 receptor-associated kinase 2** | **Irak2** | **NM_172161** | **1.76** | **0.04** |
| Interferon regulatory factor 1 | Irf1 | NM_008390 | 2.2 | 0.07 |
| Interferon regulatory factor 3 | Irf3 | NM_016849 | 1.25 | 0.42 |
| Jun oncogene | Jun | NM_010591 | 1.08 | 0.78 |
| **Lymphotoxin A** | **Lta** | **NM_010735** | **1.78** | **0.017** |
| **Mucin 13, epithelial transmembrane** | **Muc13** | **NM_010739** | **11.44** | **0.0003** |
| Lymphocyte antigen 96 | Ly96 | NM_016923 | 1.75 | 0.10 |
| Mitogen-activated protein kinase kinase 4 | Map2k4 | NM_009157 | 1.06 | 0.85 |
| Mitogen-activated protein kinase kinase kinase 7 | Map3k7 | NM_172688 | 1.38 | 0.44 |
| Mitogen-activated protein kinase 8 | Mapk8 | NM_016700 | 1.48 | 0.14 |
| Mitogen-activated protein kinase 8 interacting protein 3 | Mapk8ip3 | NM_013931 | 1.31 | 0.34 |
| Mitogen-activated protein kinase 9 | Mapk9 | NM_016961 | 1.14 | 0.59 |
| Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105 | Nfkb1 | NM_008689 | 1.82 | 0.07 |
| Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 | Nfkb2 | NM_019408 | 1.45 | 0.19 |
| **Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha** | **Nfkbia** | **NM_010907** | **2.70** | **0.007** |
| Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta | Nfkbib | NM_010908 | 1.62 | 0.06 |
| Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 | Nfkbil1 | NM_010909 | 1.27 | 0.22 |
| Nuclear factor related to kappa B binding protein | Nfrkb | NM_172766 | 1.06 | 0.86 |
| Nuclear receptor subfamily 2, group C, member 2 | Nr2c2 | NM_011630 | 1.03 | 0.96 |
| | | | | |
| Pellino 1 | Peli1 | NM_023324 | 1.19 | 0.64 |
| **Peptidoglycan recognition protein 1** | **Pglyrp1** | **NM_009402** | **6.12** | **0.0002** |
| Peroxisome proliferator-activated receptor alpha | Ppara | NM_011144 | 1.11 | 0.74 |
| Eukaryotic translation initiation factor 2-alpha kinase 2 | Eif2ak2 | NM_011163 | 1.02 | 0.98 |
| **Prostaglandin-endoperoxide synthase 2** | **Ptgs2** | **NM_011198** | **10.25** | **0.0006** |
| Reticuloendotheliosis oncogene | Rel | NM_009044 | 1.68 | 0.06 |
| V-rel reticuloendotheliosis viral oncogene homolog A (avian) | Rela | NM_009045 | 1.30 | 0.32 |
| Receptor (TNFRSF)-interacting serine-threonine kinase 2 | Ripk2 | NM_138952 | 1.6 | 0.14 |
| TANK-binding kinase 1 | Tbk1 | NM_019786 | 1.25 | 0.46 |
| Toll-like receptor adaptor molecule 1 | Ticam1 | NM_174989 | 1.15 | 0.49 |
| Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein | Tirap | NM_054096 | 1.30 | 0.51 |
| Toll-like receptor 1 | Tlr1 | NM_030682 | 1.04 | 0.95 |
| Toll-like receptor 2 | Tlr2 | NM_011905 | 1.19 | 0.63 |
| Toll-like receptor 4 | Tlr4 | NM_021297 | 1.1 | 0.76 |
| Toll-like receptor 5 | Tlr5 | NM_016928 | 1.17 | 0.80 |

| Gene title | Symbol | GenBank No | Fold change | P-value |
|---|---|---|---|---|
| Toll-like receptor 6 | Tlr6 | NM_011604 | 1.10 | 0.81 |
| Tumor necrosis factor | Tnf | NM_013693 | 1.59 | 0.07 |
| **Tumor necrosis factor, alpha-induced protein 3** | **Tnfaip3** | **NM_009397** | **2.45** | **0.01** |
| Toll interacting protein | Tollip | NM_023764 | 1.44 | 0.36 |
| TNF-RSF1A-associated *via* death domain | Tradd | NM_001033161 | 1.15 | 0.58 |
| TNF-receptor-associated factor 6 | Traf6 | NM_009424 | 1.28 | 0.51 |
| Ubiquitin-conjugating enzyme E2N | Ube2n | NM_080560 | 1.01 | 0.97 |
| Ubiquitin-conjugating enzyme E2 variant 1 | Ube2v1 | NM_023230 | 1.17 | 0.59 |

Isolated tracheal segments were cultured in the absence (control) and presence of IL-1β (10 ng/mL) for four days. The Toll-IL-1 receptor signaling gene profile was analyzed using a real-time PCR-based cDNA array. Each data point is derived from three experiments. P-values were calculated using two-tailed, unpaired Student's *t*-test with Welch's correction. Text in bold indicates that the altered genes expressions are statistically significance.

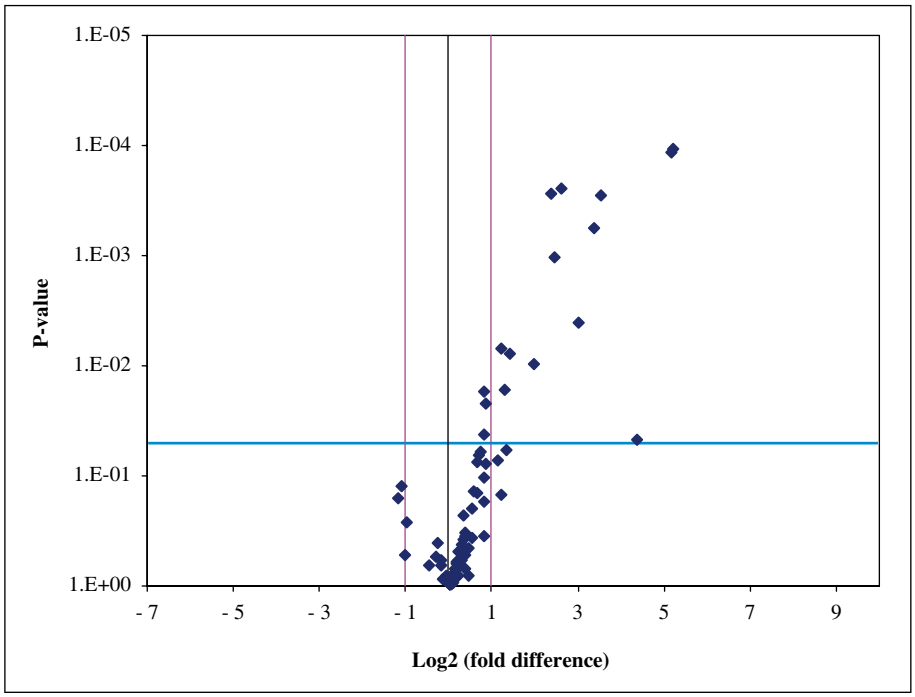


**Figure 1**
A volcano plot of the Toll-IL-1 receptor signaling gene profile. Isolated tracheal segments were cultured in the absence (control) and presence of IL-1β (10 ng/mL) for four days. The gene profile was analyzed using a real-time PCR-based cDNA array. The pink lines mark a two-fold change in gene expression level induced by IL-1β treatment (n = 3). P-values were calculated using two-tailed, unpaired Student's *t*-test with Welch's correction. The blue line represents p-value = 0.05.

**Table 2**
Genes down-regulated following IL-1β treatment

| Gene title | Symbol | GenBank No | Fold change | P-value |
|---|---|---|---|---|
| Bruton agammaglobulinemia tyrosine kinase | Btk | NM_013482 | 0.47 | 0.12 |
| CD80 antigen | Cd80 | NM_009855 | 0.95 | 0.84 |
| CD86 antigen | Cd86 | NM_019388 | 0.81 | 0.53 |
| Interferon beta 1, fibroblast | Ifnb1 | NM_010510 | 0.49 | 0.52 |
| Interleukin 1 receptor, type I | Il1r1 | NM_008362 | 0.85 | 0.40 |
| Lymphocyte antigen 86 | Ly86 | NM_010745 | 0.44 | 0.16 |
| Mitogen-activated protein kinase kinase 3 | Map2k3 | NM_008928 | 0.96 | 0.83 |
| Mitogen-activated protein kinase kinase kinase 1 | Map3k1 | NM_011945 | 0.96 | 0.79 |
| Myeloid differentiation primary response gene 88 | Myd88 | NM_010851 | 0.89 | 0.58 |
| Toll-like receptor adaptor molecule 2 | Ticam2 | NM_173394 | 0.92 | 0.86 |
| Toll-like receptor 7 | Tlr7 | NM_133211 | 0.95 | 0.87 |
| Toll-like receptor 8 | Tlr8 | NM_133212 | 0.51 | 0.26 |
| Toll-like receptor 9 | Tlr9 | NM_031178 | 0.72 | 0.65 |
| Tumor necrosis factor receptor superfamily, member 1a | Tnfrsf1a | NM_011609 | 0.89 | 0.64 |

Each data point is derived from three experiments. P-values were calculated using two-tailed, unpaired Student's *t*-test with Welch's correction.

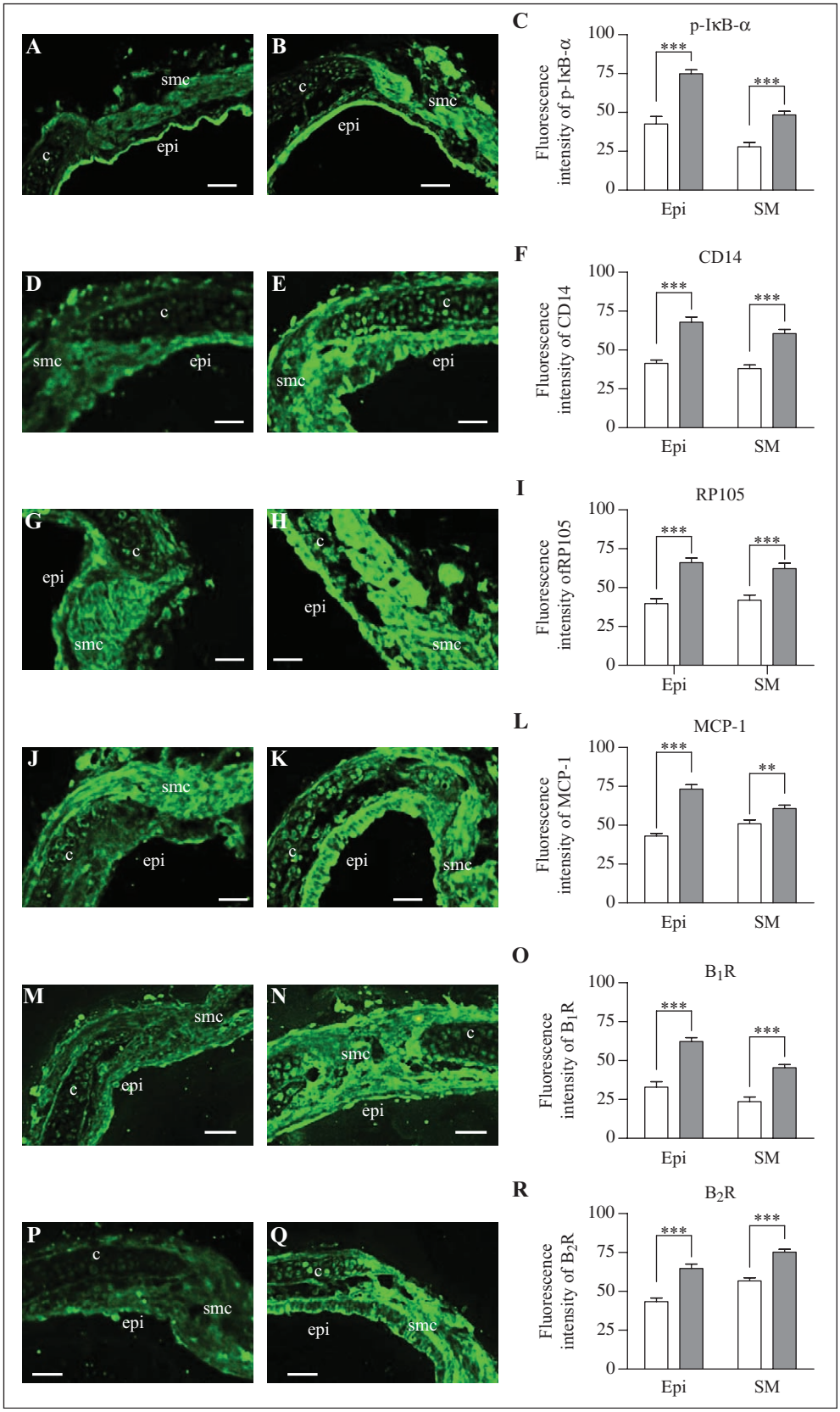


**Figure 2**

Immunohistochemical representation of key genes, phophorylated IκB-α (p-IκB-α), CD14, RP105, MCP-1, bradykinin $B_1$ ($B_1R$) and $B_2$ ($B_2R$) receptor proteins in isolated murine airway segments cultured without (control, **A**, **D**, **G**, **J**, **M**, **P**) and with (**B**, **E**, **H**, **K**, **N**, **Q**) IL-1β (10 ng/mL) for four days. The intensity of fluorescence was quantified using Image J software (**C**, **F**, **I**, **L**, **O**, **R**). Empty bars are controls, and dashed bars denote IL-1β groups. Each data point is derived from six experiments. P-values were calculated using two-tailed, unpaired Student's *t*-test with Welch's correction.

* Control *versus* IL-1β, ** $p < 0.01$, *** $p < 0.001$.

Epi: epithelium; SMC: smooth muscle, C: cartilage.

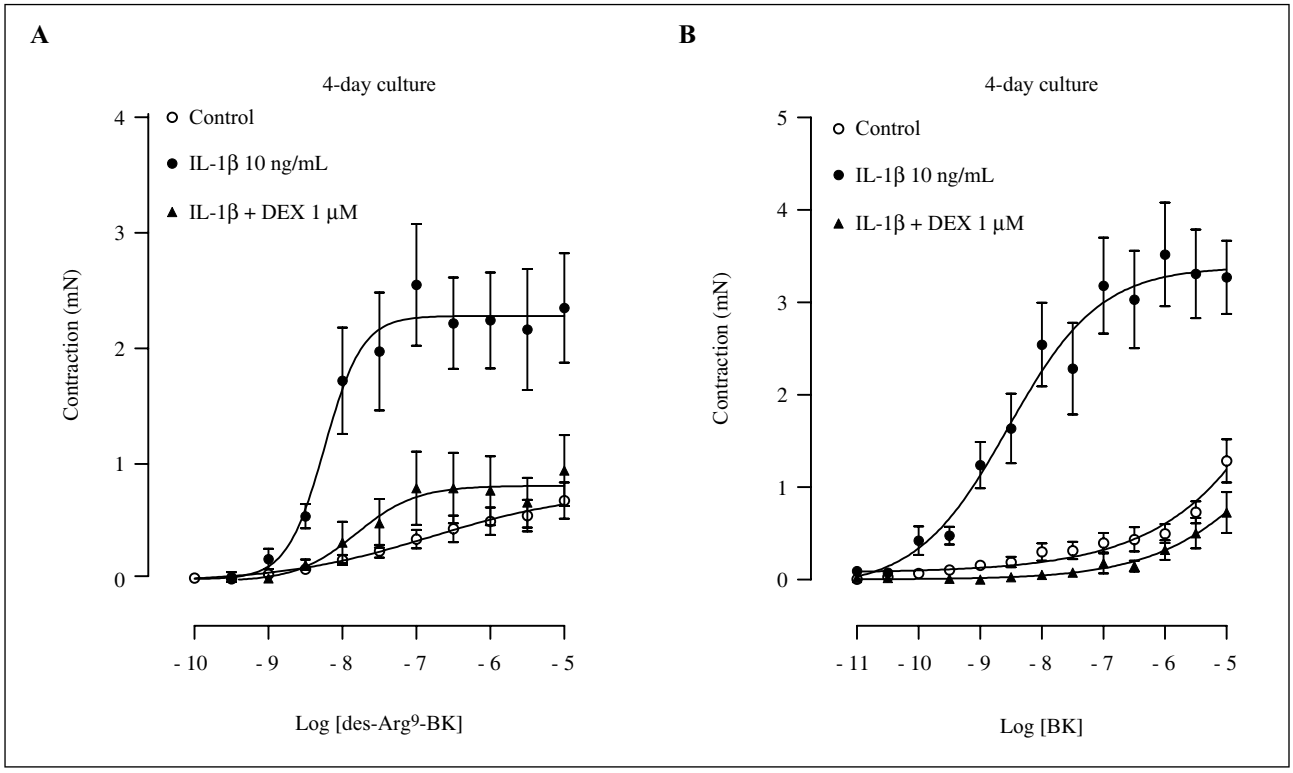


**Figure 3**
Concentration response curves for des-Arg$^9$-bradykinin (**A**) and bradykinin (**B**). The tracheal segments were cultured for four days in the absence (control) and presence of IL-1β (10 ng/mL) or IL-1β (10 ng/mL) plus dexamethasone (DEX, 1 μM). Each data point is derived from six-nine experiments and presented as mean ± SEM.

the airway contractile responses to des-Arg$^9$-bradykinin ($B_1$-agonist) and bradykinin ($B_2$-agonist), with maximal contractions ($E_{max}$) that increased from $0.77 \pm 0.19$ to $2.32 \pm 0.42$ mN and from $1.62 \pm 0.22$ to $3.53 \pm 0.33$ mN, respectively (*figure 3A, B*, $p < 0.05$). In addition, IL-1β shifted the concentration effect curves leftwards for both des-Arg$^9$-bradykinin (control $pEC_{50} = 6.78 \pm 0.21$ *versus* IL-1β $pEC_{50} = 8.12 \pm 0.16$, $p < 0.05$) and bradykinin (control $pEC_{50} = 5.16 \pm 0.38$ *versus* IL-1β $pEC_{50} = 8.26 \pm 0.38$, $p < 0.05$). The increased bradykinin $B_1$ and $B_2$ receptor-mediated airway contractions were in parallel with IL-1β-induced increased protein expressions for bradykinin $B_1$ *(figure 2M, N)* and $B_2$ *(figure 2P, Q)* receptors in the airway smooth muscle cells. As expected, dexamethasone (1 μM) almost completely abolished the IL-1β-induced increase in contractile responses to des-Arg$^9$-bradykinin and bradykinin (*figure 3A, B*; $p < 0.001$).

## DISCUSSION

IL-1 is involved in the development of AHR in man [3] and in guinea pigs [4]. Previously, we have demonstrated that pro-inflammatory cytokines induced AHR to inflammatory mediators [8, 9]. However, the underlying cytokine, intracellular signal mechanisms that lead to the AHR remain elusive. The present study used a real-time PCR array, a sensitive and reliable tool, consisting of 84 genes related to the Toll-IL-1 receptor signal pathways, and observed that after long-term exposed to IL-1β, the airway exhibited increased mRNA expression for 67 genes related to Toll-IL-1 receptor signaling pathway, although only 16 of these reached statistical significance. Protein expressions for CD14, RP105, MCP-1, phosphorylated IκB-α, bradykinin $B_1$ and $B_2$ receptors were enhanced by IL-1β in both the tracheal epithelium

**Table 3**
Dexamethasone inhibited IL-1β-induced gene up-regulation

| Gene title | Symbol | GenBank No | Fold change | P-value |
|---|---|---|---|---|
| CD14 antigen | Cd14 | NM_009841 | 0.78 | 0.61 |
| Mucin 13, epithelial transmembrane | Muc13 | NM_010739 | 0.45 | 0.23 |
| **Interleukin 6** | **Il6** | **NM_031168** | **0.01** | **0.0002** |
| **Interferon gamma** | **Ifng** | **NM_008337** | **0.15** | **0.001** |
| **Prostaglandin-endoperoxide synthase 2** | **Ptgs2** | **NM_011198** | **0.02** | **0.0006** |

Isolated tracheal segments were cultured in the presence of IL-1β (10 ng/mL) with/without dexamethasone (1 μM) for four days. Real-time PCR was performed. Each data point is derived from six experiments. P-values were calculated using two-tailed, unpaired Student's *t*-test with Welch's correction. Text in bold indicates genes whose expressions are statistically significant.

and smooth muscle cells. Dexamethasone, a well-known anti-inflammatory drug, completely abolished IL-1β-induced up-regulation of IFN-γ, IL-6 and Cox-2 mRNA expression, and abrogated the increased bradykinin $B_1$ and $B_2$ receptor-mediated airway contractions. Our results suggest that Toll-IL-1 receptor-mediated inflammatory signaling changes in the airways might be an underlying mechanism for AHR.

CD14 acts as a co-receptor, along with TLR4 and MD-2. RP105 is a Toll-like receptor signalling molecule for TLR4 [7]. Activation of TLR4 intracellular inflammatory signalling subsequently increases production of inflammatory cytokines [11-14]. Here, we observed that IL-1β increased gene expression for CD14 and RP105, at both mRNA and protein levels. This paralleled increased mRNA expression for inflammatory mediators (Csf2, Csf3, IFN-γ, IL-1α, IL-6 and Cox-2), suggesting that IL-1β induced activation and enhancement of TLR4 inflammatory signaling, the most important signaling pathway involved in immunological responses to gram negative bacterial invasion. In addition, increased mRNA and protein expression for MCP-1 was seen in the airway epithelial and smooth muscle cells after exposure to IL-1β. This is in agreement with a recent report that demonstrates that airway epithelial cells actively participate in airway inflammation and immune defence against pathogens, by producing regulatory molecules in response to house dust mites [14]. Our data suggest that airway epithelial and smooth muscle cells participate in Toll-IL-1 receptor-mediated airway inflammation [15-17].

NF-κB is a pleiotropic transcription factor that is regulated by the inhibitory protein that normally presents in the cytosol complex bound to NF-κB dimers. The dimer binding prevents nuclear localization of NF-κB, ensuring low basal transcriptional activity [18, 19]. Activation of NF-κB by IκB-α phosphorylation and degradation causes amplified expression of several genes encoding a plethora of pro-inflammatory mediators [20, 21]. The present study demonstrated that IL-1β induced phosphorylation (activation) of IκB-α and increased mRNA expression of multiple inflammatory mediators, Csf2, Csf3, IFN-γ, IL-1α, IL-6, TNF-β, Cox-2, Hspa1a, Pglyrp1 and Cebpb. Dexamethasone suppressed IL-1β-enhanced mRNA expression of the inflammatory mediators, IFN-γ, IL-6 and Cox-2, and abolished IL-1β-induced AHR, demonstrating that increased transcription of inflammatory mediators is involved in IL-1β-induced AHR.

Organ culture of isolated airways has been used to study the role of G-protein-coupled receptors in airway inflammation and AHR [8, 9]. Evidence from *in vivo* studies has shown that bradykinin $B_1$ and $B_2$ receptors were involved in allergen-induced AHR in rats [22] and in asthmatic patients [23]. On the other hand, bradykinin can induce airway relaxation *via* bradykinin $B_1$ and $B_2$ receptors [24]. In mice, this relaxation is epithelium-dependent and mediated *via* the release of prostacyclin, which in the present study, was blocked by administration of indomethacin, an inhibitor of cyclooxygenase enzymes (COXs), in order to examine bradykinin receptor-mediated contractions. In addition, bradykinin receptors located on airway epithelium not only mediate relaxation of airway tissue, but also induce airway secretion and oedema [2, 25]. Thus, IL-1β-induced up-regulation of bradykinin $B_1$ and $B_2$ receptors in the epithelium, may also contribute to the development of airway oedema and hypersecretion.

Dexamethasone is well-known to be the most effective anti-inflammatory drug for reducing AHR in asthmatic airways [26-28]. It also inhibits the inducible, bradykinin receptor expression in cultured human airway fibroblast and smooth muscle cells [29, 30]. Previously, we have demonstrated that dexamethasone can inhibit the TNF-α-induced enhancement of the airway contractile response to bradykinin [31]. Here, we demonstrated that IL-1β-induced AHR was completely abolished by dexamethasone. In conclusion, Toll-IL-1 receptor-mediated inflammatory signaling changes in the airways might be an underlying mechanism for AHR. Further investigation of the changes in airway inflammatory mediators and the underlying Toll-IL-1 receptor-mediated intracellular inflammatory signaling mechanisms, may provide new pharmacological targets for the treatment of AHR and asthma.

***Acknowledgments.*** *This study was supported by the Swedish Medical Research Council, the Swedish Heart Lung Foundation, the Swedish Association for Allergology and the Swedish Foundation for Health Care Science and Allergic Research.*

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