



Full length article

AT-RvD1 modulates the activation of bronchial epithelial cells induced by lipopolysaccharide and *Dermatophagoides pteronyssinus*

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ABSTRACT

Bronchial epithelial cells are essential to airways homeostasis; however, they are also involved in exacerbation of airway inflammatory responses of patients with conditions such as asthma. *Dermatophagoides pteronyssinus* (Dp), the most important allergen, and lipopolysaccharide (LPS), both of which are present in house dust mites (HDM), can activate immune and structural cells (such as bronchial epithelial cells) and modulate the airway inflammation in asthma patients. Resolvin D1 (RvD1) and its epimer aspirin-triggered-resolvin D1 (AT-RvD1) are lipid mediators that are produced during the resolution of inflammation and demonstrate anti-inflammatory and pro-resolution effects in several experimental models including experimental models of allergic airway inflammation. Here, we evaluated the effects of AT-RvD1 (10^{-12} – 10^{-10} M) on human bronchial epithelial cells (BEAS-2B) stimulated with LPS (2 µg/ml) or Dp (10 µg/ml). After 24 h, the C-C motif chemokine ligand 2 (CCL-2) production was increased in cells that had been stimulated with LPS and Dp compared to the control. However, AT-RvD1 (10^{-11} and 10^{-10} M) significantly reduced the concentration of CCL-2 in a manner that was dependent on the N-formyl peptide receptor 2 (FPR2/ALX) and nuclear factor kappa B (NF-κB) pathways in cells stimulated with LPS or Dp compared to controls. In addition, AT-RvD1 reduced the phosphorylation of signal transducer and activator of transcription (STAT)6 and STAT1 in cells stimulated with Dp and LPS, respectively. In conclusion, AT-RvD1 demonstrated significant anti-inflammatory effects in bronchial epithelial cells that were stimulated with LPS or Dp, which provides new perspectives for therapeutic strategies to control inflammatory airway diseases.

1. Introduction

Bronchial epithelial cells play important roles in the pathophysiology of several airway inflammatory diseases (Hammad and Lambrecht, 2008). These cells express pattern-recognition receptors such as Toll-like receptors (TLR) and protease-activated receptors (PARs), which recognize microorganisms and allergens, respectively (Kauffman, 2006; Kato et al., 2007). The activation of these receptors induces the production of chemokines and cytokines and the expression of adhesion molecules (Ebeling et al., 2007) that can influence dendritic cell maturation and T cell differentiation and consequently modulate the airway inflammation (Reibman et al., 2003; Kiss et al., 2007). Bacteria and their products (such as lipopolysaccharide (LPS)) as well as allergens (such as *Dermatophagoides pteronyssinus* (Dp)) are involved in the initiation as well as the exacerbation of airway inflammation in asthma patients (Möller et al., 2012; Kanchongkittiphon et al., 2014).

An uncontrolled immune response promotes tissue and organ damage. Lipid mediators such as resolvins and their epimers are produced in the resolution of inflammation, and these mediators play significant roles in dampening the inflammatory process (Serhan, 2010). Resolvin D1 (RvD1) and its epimer aspirin-triggered-resolvin D1 (AT-RvD1) are derived from the essential omega-3 fatty acid docosahexaenoic acid (DHA; C22:6). RvD1 and AT-RvD1 demonstrate anti-inflammatory and pro-resolution effects in several experimental models including the ovalbumin-induced allergic airway inflammation model (Rogério et al., 2012). In addition, AT-RvD1 decreases the production of C-X-C motif chemokine ligand 8 (CXCL8) and C-C motif chemokine ligand 2 (CCL-2) as well as the phosphorylation of nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 6 (STAT6) in bronchial epithelial cells that are stimulated with IL-4 (Oliveira et al., 2015). Here, we extended these results and evaluated the role of AT-RvD1 in bronchial epithelial cells stimulated with LPS or Dp.

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2. Materials and methods

2.1. Cells

Bronchial epithelial cells: The human bronchial epithelial cell line BEAS-2B (ATCC, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM-F12/Gibco-Life Technologies, Carlsbad, Calif., USA) supplemented with 10% foetal bovine serum (Gibco-Life Technologies) and 1% penicillin + streptomycin (Gibco-Life Technologies, Carlsbad, Calif., USA) and incubated at 37 °C in a humidified atmosphere with 5% CO₂ and 95% ambient air.

2.2. Stimulus and treatment

AT-RvD1 was donated by Dr. David Bruce Levy of the Harvard Medical School. BEAS-2B (4×10⁴ cell/ml) cells were cultured in 96-well plates and treated with AT-RvD1 (10⁻¹²–10⁻¹⁰ M) or vehicle (0.04% ethanol) for 30 min prior to stimulation with LPS (2 µg/ml) (InvivoGen, Toulouse, France) or Dp (10 µg/ml) (FDA Allergenic, Rio de Janeiro, Brazil). In another set of experiments, we followed the same experimental procedure, except that the cells were previously (15 min before AT-RvD1) treated with BOC1 (10 µM) (MP Biomedical, Solon, France) (Oliveira et al., 2015; Zambalde et al., 2016).

2.3. CCL-2

The supernatant was collected 24 h after LPS or Dp stimulation, and the CCL-2 concentrations were measured by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (BD Pharmingen, San Diego, Calif., USA).

2.4. NF-κB, STAT1 and STAT6

The effects of AT-RvD1 on the NF-κB, STAT1 and STAT6 pathways were assessed by cytometry according to Cao et al. (2010). Briefly, 24 h after LPS or Dp stimulation, the cells were fixed with pre-warmed BD Cytofix Buffer (4% paraformaldehyde) for 10 min at 37 °C. The cells were then washed and permeabilized in ice-cold Perm/Wash Buffer (BD Biosciences Pharmingen (Phosflow, USA). For the staining with mouse monoclonal antibodies, the cells were incubated with antibodies against phospho-NF-κB (BD Biosciences Pharmingen- Phosflow, USA), phospho-STAT1 (BD Biosciences Pharmingen- Phosflow, USA), or phospho-STAT6 (BD Biosciences Pharmingen-Phosphorus, USA) diluted in cold methanol for 60 min at 10 °C in the dark. These cells were then further incubated with PE- or FITC-conjugated goat anti-mouse IgG2b for another 60 min at 10 °C in the dark. The cells were then washed, resuspended and subjected to analysis. The same protocol was carried out with the respective isotype controls. The expression of intracellular phosphorylated signalling molecules in 50,000 viable cells was performed using a FACSCalibur flow cytometer (BD Biosciences).

2.5. Statistical analysis

The results are expressed as the mean ± standard error of the mean. An evaluation of the results was performed using analysis of variance (ANOVA) followed by a Tukey post-test among the means using Graph Pad PRISM (Version 6.0; Graph Pad Software Inc., San Diego, CA, USA). P values less than 0.05 were considered statistically significant.

3. Results

3.1. AT-RvD1 reduces the concentration of the chemokine CCL-2

Our results demonstrated that either LPS or Dp increased the CCL-2 production compared to the control group (Fig. 1A and B, respectively). AT-RvD1 (10⁻¹¹ and 10⁻¹⁰ M) significantly decreased the CCL-

2 production compared to cells stimulated by LPS (Fig. 1A) or Dp (Fig. 1B). The CCL-2 production was reduced by ~66% from 653.3 ± 78.5 in the cells treated with LPS to 289.6 ± 76.96 (LPS + AT-RvD1 at 10⁻¹⁰ M) or 302.7 ± 80.25 (LPS + AT-RvD1 at 10⁻¹¹ M) (mean ± S.E.M.; n = 7; P < 0.05). In addition, the CCL2 level was reduced by ~46% from 716.6 ± 68.9 in the cells treated with Dp to 386.7 ± 82.2 (Dp + AT-RvD1 at 10⁻¹⁰ M) or 386.7 ± 82.2 (Dp + AT-RvD1 at 10⁻¹¹ M). AT-RvD1 (10⁻¹² M) did not significantly reduce the CCL2 production. The vehicle did not cause any significant difference in the responses of cells that were stimulated with LPS or Dp (data not shown). AT-RvD1 at 10⁻¹⁰ M was chosen for the subsequent experiments.

3.2. The inhibitory effect of AT-RvD1 on CCL2 production is FPR2/ALX receptor-dependent

As described above, LPS as well as Dp increased the CCL2 production, and AT-RvD1 reduced its production (Fig. 2A and B). Interestingly, BOC1, an FPR2/ALX antagonist, prevented the reduction in CCL-2 that was induced by AT-RvD1 in the presence of either LPS or Dp (Fig. 2A and B, respectively).

3.3. AT-RvD1 down-regulates the phosphorylation of NF-κB

LPS and Dp induced the phosphorylation of NF-κB compared to the control group (Fig. 3A and B, respectively). AT-RvD1 (10⁻¹⁰ M) significantly decreased the phosphorylation of NF-κB compared to cells that were stimulated with LPS (Fig. 3A) or Dp (Fig. 3B). NF-κB was reduced by ~90% from 0.94 ± 0.22 (LPS) to 0.094 ± 0.032 (LPS + AT-RvD1) (mean ± S.E.M.; n = 5; P < 0.05) and reduced by ~85% from 1.84 ± 0.95 (Dp) to 0.28 ± 0.14 (Dp + AT-RvD1) (mean ± S.E.M.; n = 5–8; P < 0.05).

3.4. AT-RvD1 down-regulates the phosphorylation of STAT1 and STAT6

In addition to the NF-κB pathway, the role of AT-RvD1 in the STAT1 and STAT6 activation induced by LPS and Dp, respectively, was also evaluated. LPS increased the phosphorylation of STAT1 and Dp induced phosphorylation of STAT6 compared to control group (Fig. 4A and B, respectively). AT-RvD1 (10⁻¹⁰ M) significantly decreased the phosphorylation of STAT1 (Fig. 4A) and STAT-6 (Fig. 4B) compared to cells that were stimulated by LPS or Dp, respectively. STAT1 was reduced by ~46% from 1.55 ± 0.16 (LPS) to 0.84 ± 0.15 (LPS + AT-RvD1) (mean ± S.E.M.; n = 5; P < 0.05), and STAT6 was reduced by ~45% from 2.45 ± 0.12 (Dp) to 1.37 ± 0.29 (Dp + AT-RvD1) (mean ± S.E.M.; n = 4–6; P < 0.05).

4. Discussion

In this study, AT-RvD1 demonstrated anti-inflammatory effects as indicated by a reduction in CCL-2 production by bronchial epithelial cells stimulated with LPS or Dp. These effects were dependent on the FPR2/ALX receptor and NF-κB pathways. In addition, AT-RvD1 decreased STAT1 and STAT6 activation induced by LPS and Dp, respectively.

In airway diseases and other inflammatory diseases, the balance between pro-inflammatory and anti-inflammatory mediators is shifted to favour the pro-inflammatory mediators. Resolvins and their epimers are produced during the resolution process and are known to affect the anti-inflammatory and pro-resolution processes (Marion-Letellier et al., 2015). Earlier results from our group have demonstrated that AT-RvD1 plays a significant role in dampening the airway inflammation in several experimental models, including the in vivo ovalbumin-induced experimental allergic airway model (Rogerio et al., 2012) as well as in models of in vitro allergic responses (bronchial epithelial cells

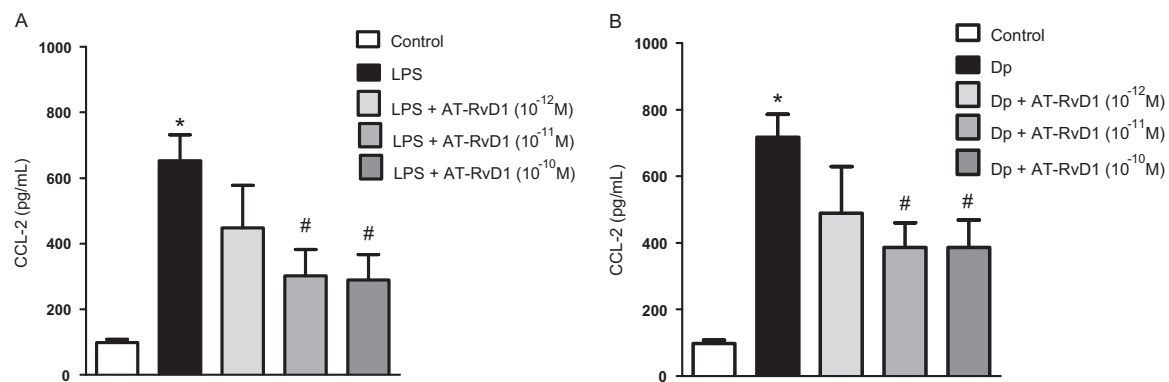


Fig. 1. AT-RvD1 reduced the production of CCL-2 in bronchial epithelial cells stimulated with LPS (A) or Dp (B). BEAS-2B cells were stimulated with LPS (2 $\mu\text{g}/\text{ml}$) or Dp (10 $\mu\text{g}/\text{ml}$) in the presence or absence of AT-RvD1 (10^{-12} – 10^{-10} M). After 24 h, the culture supernatants were collected and the CCL-2 concentrations were measured using an ELISA kit. The data are reported as the means $\pm$ S.E.M. (n = 7/group). *P < 0.05 versus the control group, #P < 0.05 versus the LPS- or Dp-treated group.

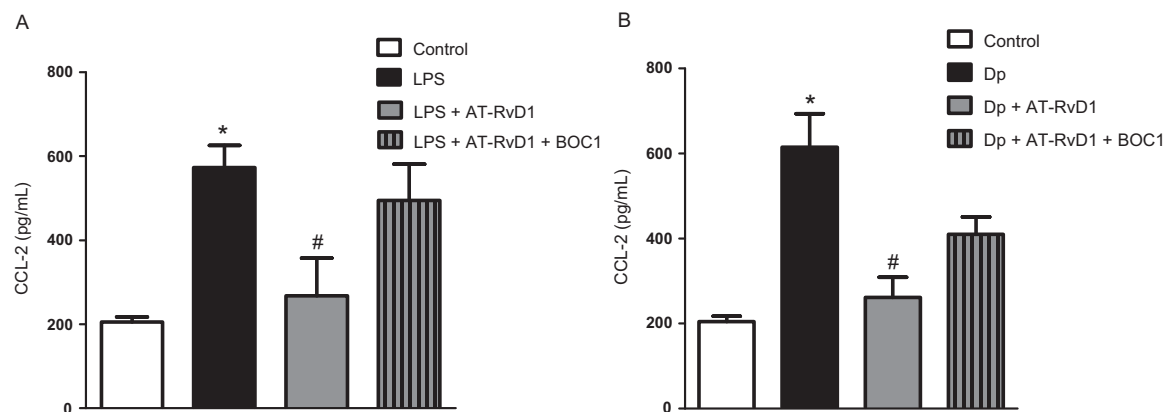


Fig. 2. CCL-2 production in BEAS-2B cells stimulated with LPS (A) or Dp (B) was reduced by AT-RvD1 through FPR2/ALX receptor activation. BEAS-2B cells were stimulated with LPS (2 $\mu\text{g}/\text{ml}$) or Dp (10 $\mu\text{g}/\text{ml}$) in the presence or absence of AT-RvD1 (10^{-10} M) or in combination with BOC1 (10 μM), an FPR2/ALX-selective antagonist. After 24 h, the culture supernatants were collected and the CCL-2 concentrations were measured using an ELISA kit. The data are reported as the means $\pm$ S.E.M. (n = 5/group). *P < 0.05 versus the control group, #P < 0.05 versus the LPS- or Dp-treated group.

stimulated with IL-4) (Oliveira et al., 2015). Here, we extended these previous studies and demonstrated that AT-RvD1 effectively reduced the production of CCL-2 in bronchial epithelial cells that were stimulated with LPS or Dp and that this process involved the FPR2/ALX receptor. These results are consistent with previous studies by our group (Rogerio et al., 2012; Oliveira et al., 2015). CCL2 plays a significant role in asthma pathogenesis (Rose et al., 2003), and its reduction by AT-RvD1 could favour the control and/or prevention of the development of LPS- or Dp-induced asthma or limit its severity

(Möller et al., 2012; Thomas, 2015).

NF- κ B, a significant therapeutic target for the control of inflammatory processes in asthma (Barnes and Adcock, 2009), regulates the production of CCL2 as well as other inflammatory mediators (Thompson and Van Eldik, 2009; Rose et al., 2003). Interestingly, AT-RvD1 reduced the phosphorylation of NF- κ B that was induced by either LPS or Dp. Therefore, the effect of AT-RvD1 on CCL2 production was related to inhibition of the NF- κ B activation. Thus, AT-RvD1 demonstrated a potential anti-inflammatory effect that could control

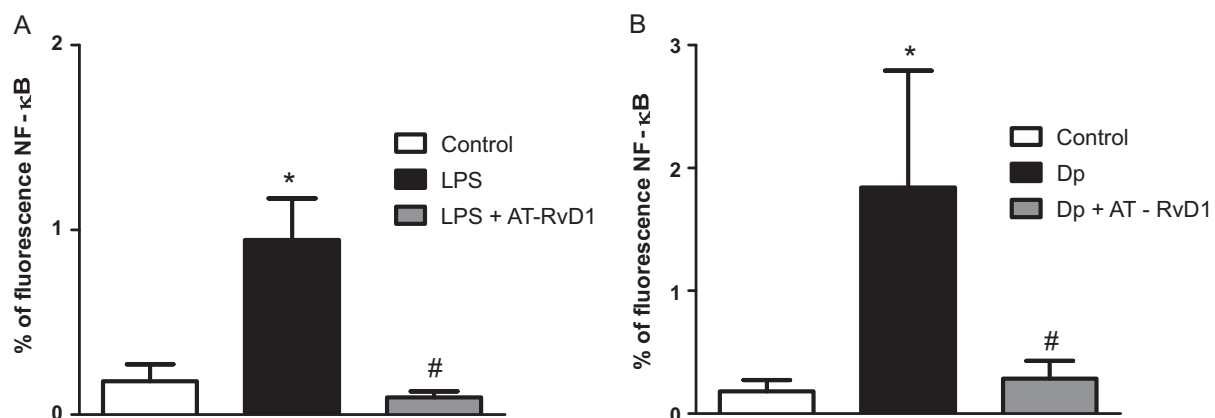


Fig. 3. AT-RvD1 downregulates the NF- κ B pathway in bronchial epithelial cells stimulated with LPS (A) or Dp (B). BEAS-2B cells were stimulated with LPS (2 $\mu\text{g}/\text{ml}$) or Dp (10 $\mu\text{g}/\text{ml}$) in the presence or absence of AT-RvD1 (10^{-10} M). After 24 h, the cells were recovered and the percentage of fluorescence in 50,000 cells was analysed using cytometry. The data are reported as the means $\pm$ S.E.M. (n = 5–8/group), from three independent experiments. *P < 0.05 versus the control group, #P < 0.05 versus the LPS- or Dp-treated group.

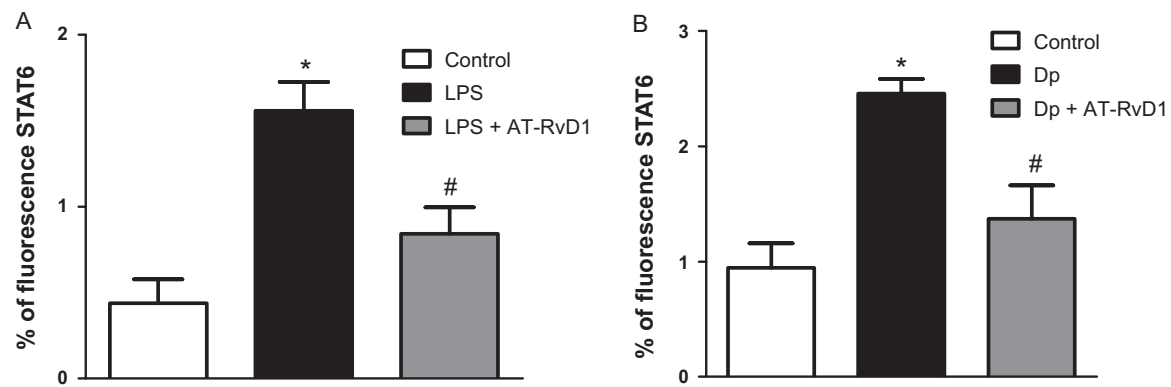


Fig. 4. AT-RvD1 down-regulated the STAT1 (A) and STAT6 (B) pathways in bronchial epithelial cells stimulated with LPS (A) or Dp (B), respectively. BEAS-2B cells were stimulated with LPS (2 μ g/ml) or Dp (10 μ g/ml) in the presence or absence of AT-RvD1 (10^{-10} M). After 24 h, the cells were recovered and the percentage of fluorescence in 50,000 cells was analysed using cytometry. The results are expressed as the means $\pm$ S.E.M. (n = 4–6/group), from three independent experiments. *P < 0.05 versus the control group, #P < 0.05 versus the LPS- or Dp-treated group.

inflammation in the airways especially in asthma patients.

STAT1 plays a significant role in the inflammatory process (Feng et al., 2014; Gambin et al., 2013). Quarcoo et al. (2004) used inhibition by a STAT1-specific decoy oligonucleotide to demonstrate the role of STAT-1 in eosinophilic airway inflammation as well as in airway hyperactivity in an experimental model of ovalbumin-induced allergic airway inflammation. In our study, AT-RvD1 down-regulated the activation of STAT1 in bronchial epithelial cells that were stimulated by LPS. This result is consistent with another study that demonstrated that the incubation of inflamed obese visceral adipose tissues and human macrophages with RvD1 (a diastereomer, differing from AT-RvD1 only in stereochemistry at carbon 17) blocked STAT1 activation among other inflammatory parameters (Titos et al., 2016). The disruption of STAT-1 signalling by AT-RvD1 might inhibit expression and production of pivotal factors for the development of airway inflammation as well as the LPS-stimulated exacerbations of airway hyper-reactivity in asthma patients.

STAT6 has been demonstrated to regulate many pathologic features of lung inflammatory responses, including Th2 cell differentiation, airway eosinophilia, epithelial mucus production and smooth muscle changes (Goenka et al., 2006; Hoppenot et al., 2015). Interesting, AT-RvD1 down-regulated the activation of STAT6 in bronchial epithelial cells that had been stimulated by Dp, which is consistent with a previous study of our group (Oliveira et al., 2015). This finding shows that AT-RvD1 is a potential anti-inflammatory therapeutic agent for allergic responses in the airways.

Taken together, these results demonstrate the potential of AT-RvD1 to modulate the airway microenvironment and consequently the airway inflammation particularly in asthma patients in whom Dp and LPS are the main cause of asthma exacerbations.

5. Conclusion

Our results demonstrated that AT-RvD1 modulates the activation of bronchial epithelial cells induced by LPS and Dp and suggest that AT-RvD1 is a potential therapeutic agent for the treatment of airway inflammatory diseases such as asthma.

Conflict of interest

The authors declare that they have no conflict of interest.

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