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# EFEITOS ANTI-INFLAMATÓRIOS DO SALBUTAMOL E EPÍMERO DA RESOLVINA D1 (AT-RVD1) EM CÉLULAS EPITELIAIS BRÔNQUICAS HUMANAS ESTIMULADAS COM EXTRATO DE FUMAÇA DE CIGARRO

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Fisiológicas, Área de concentração I: Bioquímica, Fisiologia e Farmacologia, da Universidade Federal do Triângulo Mineiro, como requisito parcial para obtenção do título de mestre.

Orientador: Prof. Dr. Alexandre de Paula Rogério

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## RESUMO

O tabagismo é um dos problemas de saúde mais sérios, causando altas taxas de morbidade e mortalidade e várias doenças, como a doença pulmonar obstrutiva crônica (DPOC). A ativação das células epiteliais das vias aéreas pela fumaça do cigarro induz a produção de várias citocinas pró-inflamatórias que orquestram e influenciam as respostas imunes inatas e adaptativas. Aqui, avaliamos os efeitos do Salbutamol, AT-RvD1 e a associação de ambos nas células epiteliais brônquicas humanas (BEAS-2B) estimuladas com extrato de fumaça de cigarro (CSE; 1%; 1 cigarro) por 24 horas. A associação de salbutamol e AT-RvD1 induziu efeito aditivo na diminuição da produção de IL-1β e IL-8 e o tratamento também elevou a produção de IL-10 que possui efeitos anti-inflamatórios e imunossupressores. AT-RvD1, salbutamol e a associação de ambos reduziram a expressão de ICAM-1, uma importante molécula de adesão que medeia a interação entre leucócitos e células epiteliais brônquicas. Esses efeitos anti inflamatórios podem estar associados à regulação negativa da ativação de NF-κB demonstrada. Portanto, AT-RvD1 pode ser um tratamento alternativo potencial e um candidato em associação com beta2-agonistas para tratar a inflamação das vias aéreas causada pelo tabagismo, como em pacientes com DPOC.



Palavras-chave: Extrato de fumaça de cigarro. AT-RvD1. Salbutamol. DPOC.

## ABSTRACT

Smoking is one of the most serious health concerns causing high rates of morbidity and mortality and several diseases such as chronic obstructive pulmonary disease (COPD). The activation of airway epithelial cells by cigarette smoke and consequent oxidative stress induces the production of several pro-inflammatory mediators that orchestrate and influence the innate and adaptive immune responses. Here, we evaluated the effects of salbutamol ($10^{-5}$-$10^{-7}$M), AT-RvD1 ($10^{-7}$-$10^{-12}$M) and the association of both on human bronchial epithelial cells (BEAS-2B) stimulated with cigarette smoke extract (CSE; 1%; 1 cigarette) for 24 h in vitro. Salbutamol ($10^{-5}$ and $10^{-6}$M) or AT-RvD1 ($10^{-7}$-$10^{-11}$M), reduced the IL-1β production induced by CSE in a dose-response manner. Using the combination of a range of pharmacologically effective concentrations from each compound, except the combination of non-effective doses from both, the association AT-RvD1 ($10^{-11}$M) and salbutamol ($10^{-7}$M) potentiated the reduction of IL-1β production when compared to cells only treated with salbutamol at $10^{-7}$ M alone. AT-RvD1 ($10^{-11}$M), but not salbutamol ($10^{-7}$M), and the association of both decreased the IL-8 production and increased the IL-10 production without alter the reactive oxygen species production when compared to CSE group. These anti-inflammatory effects could be associated with the down regulation of activation of NF-κB, but not STAT3. AT-RvD1, salbutamol and the association of both reduced the ICAM-1 expression. As such, AT-RvD1 in association with salbutamol could be a potential alternative treatment to airway mucosal inflammation caused by cigarette smoking such as in COPD patients.



Keywords: Cigarette smoke extract. epithelial cells. AT-RvD1. salbutamol. COPD.

LISTA DE ABREVIATURAS E SIGLAS

AMPc - Adenosina monofosfato cíclico

AT-RvD1 - Resolvina D1 formada pela via da aspirina

BEAS-2B - Linhagem de células epiteliais brônquicas humanas

BSA - Soro albumina bovina

CSE - Cigarette smoke extract (extrato da fumaça de cigarro)

CXCL - Quimiocina Ligante Cisteína-X- Cisteína

DHA - Ácido docosahexaenoico

DPOC - Doença Pulmonar Obstrutiva Crônica

ELISA - Ensaio de imunoadsorção enzimático

EPA - Ácido eicosapentaenoico

FITC - Isotiocianato de fluoresceína

ICAM-1 - Molécula de adesão intercelular 1

IgG - Imunoglobulina G

IL - Interleucina

MCP 1 - Proteína quimioatraente de monócitos 1

NF-kB - Fator nuclear kappa B

PBS - Solução salina tamponada com fosfato

PE - Phycoerythrin

pH - Potencial hidrogeniônico

PKA - Proteína cinase A

SPMs - Specialized pro-resolving lipid mediators (mediadores de pró-resolução especializados)

STAT-3 - Sinalizador de transdução e ativação de transcrição 3

Th - Linfócito T auxiliar

TNF-α - Fator de necrose tumoral alfa

**SUMÁRIO**

## 1 INTRODUÇÃO

Os pulmões são órgãos complexos que compreendem numerosas células que são continuamente expostas a agentes infecciosos, fumaça de cigarro e poluentes. A interrupção da homeostase em resposta a níveis constantes de inalantes prejudiciais leva a alterações morfológicas e funcionais irreversíveis como câncer, enfisema pulmonar, asma e DPOC (Wang *et al*., 2018; U.S HHS, 2014).

A Doença Pulmonar Obstrutiva Crônica (DPOC) é uma síndrome caracterizada e definida por um único parâmetro fisiológico: limitação do fluxo de ar expiratório (Adam *et al*.,2015) sendo atualmente uma das principais causas de morbimortalidade em todo o mundo. A atualização mais recente (2019) da Iniciativa Global para Doença Obstrutiva Pulmonar Crônica a definiu como uma doença comum, evitável e tratável, caracterizada por sintomas respiratórios persistentes e limitação do fluxo aéreo devido a anormalidades das vias aéreas e/ou alveolares, geralmente produzidas pela maior exposição a partículas ou vapores nocivos. (GOLD, 2019). Vários mecanismos patogenéticos contribuem para o desenvolvimento da DPOC, entretanto, o principal fator de risco é o tabagismo. A fumaça de cigarro contém numerosas substâncias tóxicas e carcinogênicas que induzem a inflamação das vias aéreas (Pryor *et al*., 1993). Esse processo contínuo de inflamação conduz à destruição tecidual e estruturais no epitélio brônquico, que aumentam com a gravidade da doença e persistem mesmo após a interrupção do fumo (Lozano *et al*., 2012).

As células epiteliais brônquicas são uma primeira linha de defesa contra agentes inflamatórios exógenos no pulmão, além da função na manutenção da condução de ar para os alvéolos, protegendo o pulmão contra patógenos, alérgenos e partículas inaladas, também modula a resposta imune natural e adaptativa determinando assim a intensidade e a qualidade da resposta inflamatória pulmonar (Lambrecht B, Hammad H, 2012).

A inflamação na DPOC é caracterizada pelo aumento do número de neutrófilos, macrófagos, linfócitos T (CD8 mais que CD4) e fibroblastos nos

pulmões. Alguns trabalhos documentaram níveis aumentados de fatores quimiotáticos de neutrófilos como a Interleucina-8 (IL-8) e proteína quimioatraente de monócitos (MCP 1) no escarro de pacientes com DPOC (Noguera *et al*., 2001; Serhan *et al*., 2008). E em resposta às repetidas exposições à fumaça do cigarro pode provocar alterações significativas na expressão de genes em células epiteliais das vias aéreas e uma ativação de uma resposta fibroblástica desregulada ocasionando depósitos excessivos de componentes da matriz extracelular podendo causar fibrose e remodelamento das vias aéreas (Noguera *et al*., 2001). Em geral, a extensão da inflamação está relacionada ao grau de obstrução do fluxo de ar, o processo inflamatório na DPOC é observado na parte inferior do trato respiratório em pacientes estáveis, os macrófagos carregados de toxinas se acumulam nos bronquíolos respiratórios e alvéolos dificultando a respiração (Eapen *et al*., 2017).

Atualmente a principal escolha farmacológica para o tratamento e manejo dos sintomas dos pacientes com DPOC em todas as fases da doença, são os broncodilatadores utilizados em combinação com algum medicamento da classe dos corticosteroides na forma inalatória (Kerstjens *et al*., 2019). No tratamento da asma, uma outra doença relacionada com inflamação das vias aéreas, a eficácia dos corticosteróides depende da dose, escolhida de acordo com a gravidade da doença, embora 500 µg de propionato de fluticasona em combinação com um broncodilatador em um inalador duas vezes ao dia sejam comumente usados para DPOC em todo o mundo, poucos estudos investigaram sistematicamente doses apropriadas para essa indicação, e efeitos colaterais têm sido associados ao seu uso prolongado, principalmente casos de pneumonias (Suissa *et al*., 2013). Um grande estudo observacional identificou um aumento na incidência de hospitalizações por pneumonia e hospitalização por pneumonia seguida de morte em 30 dias, houve correlação entre a dose de corticosteróide inalado e o risco de pneumonia, com alta taxa de pneumonia naqueles em uso de altas dose de corticosteroide inalatório, equivalente ao propionato de fluticasona a 1000 µg / d ou mais (Yawn *et al*., 2013).

Os broncodilatadores, especificamente os agonistas adrenérgicos beta, replicam as funções das catecolaminas, como adrenalina, noradrenalina e

dopamina, na produção de diferentes respostas autonômicas no corpo. Atualmente, são conhecidos três subtipos distintos de receptores para esses fármacos: β1, β2 e β3 (Billington *et al*., 2017). A ativação do receptor beta-2 adrenérgico por agonistas como o salbutamol, de curta duração, entre outros promove os efeitos broncodilatadores devido ao aumento da concentração de cAMP intracelular ativando a proteína cinase A (PKA) dependente de cAMP (Billington e Penn, 2003). Os agonistas beta-2 também demonstraram efeitos anti-inflamatórios, através da redução da molécula de adesão intercelular-1 (ICAM-1), redução da liberação de fatores estimuladores de colônias de granulócitos e macrófagos, estabilização da degranulação de mastócitos e através da inibição de múltiplas vias inflamatórias (Romberger *et al*., 2016; Dorothea, 2021)

Em um processo inflamatório comum, a resolução da inflamação aguda levando ao retorno à homeostase é iniciada por mediadores de pró-resolução especializados (SPMs), que são biossintetizados localmente, na DPOC existe um desequilíbrio desses mediadores anti-inflamatórios e de resolução em relação aos pró-inflamatórios (Serhan *et al*., 2008). Os SPMs, gerados durante a fase de resolução, inibem a infiltração de leucócitos polimorfonucleares apoptóticos e aumentam a fagocitose de células apoptóticas por macrófagos, o que difere dos corticosteróides os quais demonstram efeitos imunossupressores (Serhan *et al*., 2007).

Muitas enzimas, além de metabolizar o ácido araquidônico (derivado do ácido graxo ω-6) para formar as prostaglandinas, leucotrienos e as lipoxinas, também podem metabolizar outros ácidos graxos como os membros da família do ácido graxo ω-3, particularmente o ácido eicosapentaenoico (EPA) e o ácido docosahexaenoico (DHA). Durante as interações entre células que contêm 15-lipoxigenase e os leucócitos, o DHA é convertido primeiramente em protectina D1 e na presença dos leucócitos é convertido nas resolvinas da série D (D1-6) (Krishnamoorthy *et al*., 2012). Além das resolvinas, os seus epímeros (configuração R no carbono 17) também podem ser formados no local inflamatório, por exemplo, o epímero da resolvina D1 é denominado de AT-RvD1 (Resolvina D1 formada pela via da aspirina configuração 17 R) uma vez que, a sua produção endógena pode

ser iniciada pela ação da aspirina (via de reações dependentes da enzima ciclooxigenase-2). No entanto, sua formação também pode ocorrer na ausência da aspirina utilizando somente substratos endógenos catalisados pelo citocromo p450. Os epímeros são menos inativados localmente por enzimas que as resolvinas, demonstrando assim ações mais prolongadas e protetoras no órgão (Serhan *et al*., 2012). As resolvinas da série D (derivadas do DHA) e seus epímeros demonstram potentes efeitos biológicos em vários modelos experimentais de inflamação, como os modelos gastrointestinais, renais, vasculares, pulmonares, dentre outros (Eickmeier *et al*., 2013; Hosseini *et al*., 2021).

Nos últimos anos nosso grupo demonstrou efeitos do AT-RvD1 em vários modelos experimentais das vias aéreas tanto in vivo (asma experimental e lesão aguda pulmonar) quanto in vitro (células epiteliais brônquicas humanas, BEAS-2B, estimuladas com IL-4, *Dermatophagoides pteronyssinus* ou lipopolissacarídeos) (Rogério *et al*., 2012; Oliveira *et al*., 2015; 2017).

## 2 JUSTIFICATIVA

Várias doenças estão associadas ao tabagismo e causam elevadas taxas de morbidade e mortalidade, representando uma das mais sérias preocupações na área da saúde, uma delas é a doença pulmonar obstrutiva crônica DPOC. O principal tratamento de manejo dos sintomas é a associação de broncodilatadores e corticosteroides inalatórios, mas estudos contestam a segurança na utilização dos corticosteróides devido à falta de pesquisa em uma dose-resposta efetiva e também aos efeitos colaterais advindos do uso prolongado desses fármacos. Nos últimos anos, nosso grupo demonstrou efeitos do AT-RvD1 em vários modelos experimentais das vias aéreas tanto in vivo quanto in vitro. Uma possível substituição para os corticosteróides seria o epímero da resolvina AT-RvD1, um mediador de pró-resolução especializado com potente ação anti-inflamatória e de pró-resolução.

# 3 OBJETIVOS

## 3.1 OBJETIVO GERAL

Avaliar a ação do salbutamol e do epímero da resolvina AT-RvD1 e suas associações na modulação da ativação das células epiteliais brônquicas humanas estimuladas com extrato da fumaça do cigarro.

## 3.2 OBJETIVOS ESPECÍFICOS

3.2.1 Avaliar a produção de IL-8, IL-1β, ROS e IL-10 nas células epiteliais brônquicas humanas (BEAS-2B) estimuladas com o extrato de fumaça de cigarro.

3.2.2 Avaliar a expressão dos fatores NF-kB e STAT-3 nas células epiteliais brônquicas humanas (BEAS-2B) estimuladas com o extrato de fumaça de cigarro.

3.2.3 Avaliar a expressão da molécula de adesão ICAM-1 nas células epiteliais brônquicas humanas (BEAS-2B) estimuladas com o extrato de fumaça de cigarro.

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## APÊNDICE A - ARTIGO SUBMETIDO

### Anti-inflammatory effects of salbutamol and resolvin D1 epimer (AT-RvD1) on human bronchial epithelial cells stimulated by cigarette smoke extract

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## ABSTRACT

Smoking is one of the most serious health concerns causing high rates of morbidity and mortality and several diseases such as chronic obstructive pulmonary disease (COPD). The activation of airway epithelial cells by cigarette smoke and consequent oxidative stress induces the production of several pro-inflammatory mediators that orchestrate and influence the innate and adaptive immune responses. Here, we evaluated the effects of salbutamol ($10^{-5}$-$10^{-7}$M), AT-RvD1 ($10^{-7}$-$10^{-12}$M) and the association of both on human bronchial epithelial cells (BEAS-2B) stimulated with cigarette smoke extract (CSE; 1%; 1 cigarette) for 24 h in vitro. Salbutamol ($10^{-5}$ and $10^{-6}$M) or AT-RvD1 ($10^{-7}$-$10^{-11}$M), reduced the IL-1β production induced by CSE in a concentration-response manner. Using the combination of a range of pharmacologically effective concentrations from each compound, except the combination of non-effective concentrations from both, the association AT-RvD1 ($10^{-11}$M) and salbutamol ($10^{-7}$M) potentiated the reduction of IL-1β production when compared to cells only treated with salbutamol at $10^{-7}$M alone. Combination treatment of both decreased the release of the pro-inflammatory neutrophil chemokine, IL-8 and reciprocally increased the production of the anti-inflammatory cytokine, IL-10 without altering the reactive oxygen species production when compared to the CSE group. These anti-inflammatory effects could be associated with the down regulation of activation of NF-κB, but not STAT3. AT-RvD1, salbutamol and the combination of both reduced the ICAM-1 expression. As such, AT-RvD1 in association with salbutamol could be a potential alternative treatment to airway mucosal inflammation caused by cigarette smoking such as in COPD patients.


Keywords: Cigarette smoke extract, epithelial cells, AT-RvD1, salbutamol, COPD.

# 1 INTRODUCTION

Smoking is one of the most serious health concerns causing high rates of morbidity and mortality. Several diseases are associated with smoking such as chronic obstructive pulmonary disease (COPD) (Agustí and Hogg, 2019; Eisner, 2005), which is characterized by chronic lung inflammation and progressive and irreversible airflow obstruction. Airway epithelial cells play a significant role in the regulation of airway inflammation in COPD patients (Lange, 2015). The activation of these cells by cigarette smoke induces the production of several pro-inflammatory mediators such as cytokines (Salvi et al. 2014) that orchestrate and influence the innate and adaptive immune responses (Zhang et al. 2012). Salbutamol, a short acting β2 agonist (SABA), is a bronchodilator used in treatment of bronchial asthma and COPD diseases to improve the forced expiratory flow (Culpitt et al. 1999). In addition, salbutamol could also demonstrate anti-inflammatory activities such as the reduction of TNF-α, IL-6, and IL-8 in vitro (macrophages) in vivo (experimental acute lung injury) models (Keränen et al. 2016; Tanaka et al. 2010; Bosmann et al. 2012). The asthma and COPD treatment are managed out using corticosteroids which demonstrate significant anti-inflammatory effects but also present significant adverse effects by long-term use, notably pneumonia (Suissa et al. 2013; Brode et al. 2017; Crim et al. 2019). Hence, there is a need to find and develop new anti-inflammatory drugs with fewer side effects (Calverley et al. 2011; Ernst et al. 2015). The resolvins, lipids mediator of resolution, are a family of mediators derived from the omega-3 fatty acids eicosapentaenoic acid (i.e., E-series resolvins) and docosahexaenoic acid (i.e., D-series resolvins) (Serhan et al. 2015). Distinct from corticosteroids, resolvins have been considered as natural agonists for the resolution of pulmonary inflammation (Uddin & Levy). These specialized pro-resolving mediators have also been shown to exhibit anti-inflammatory effects without causing any immunosuppressive effects whilst facilitating the rapid clearance of bacteria in the lungs (Wang et al. 2017). Our group have demonstrated the effects of D-series resolvins and mainly its aspirin-triggered epimer AT-RvD1 in various experimental airway models both in vivo (experimental asthma and acute

lung injury) and in vitro (human bronchial epithelial cells, BEAS-2B, stimulated with IL-4, *Dermatophagoides pteronyssinus* or lipopolysaccharides) (de Oliveira et al. 2017, 2015; Rogerio et al. 2012).

Here we have evaluated, in an in vitro model, the anti-inflammatory effects of salbutamol and/or AT-RvD1 in the human bronchial epithelial cells stimulated by cigarette smoking extract (CSE) in vitro.

# 2 MATERIALS AND METHODS

## 2.1 Cell culture

The human bronchial epithelial cell line (BEAS-2B; ATCC) was kept in culture flasks containing DMEM/F-12 culture medium plus fetal bovine serum (10%) and 1% penicillin + streptomycin (Gibco-Life Technologies) in an atmosphere of 5% CO2 at 37° C (de Oliveira et al. 2015).

## 2.2 Preparation of cigarette smoke extract (CSE)

Commercial cigarettes (Marlboro®) were used in this study. The extract of the smoke from one cigarette was prepared as described (Zhang et al. 2012). The smoke from a cigarette bubbled into a Bunsen filter flask containing 25 mL of PBS using a vacuum. The smoke extract from one cigarette was adjusted to pH 7.4 and then filtered through a 0.22 μm filter. This extract was considered as 100%and further diluted for the subsequent experiments. The absorbance of the diluted samples measured by spectrophotometry at 320 nm absorbance to standardize the concentration of the extract in each independent experiment.

## 2.3 Treatment and stimulus

Dr. David Bruce Levy of Harvard Medical School (Boston, USA) donated the AT-RvD1. The bronchial epithelial cells (1 x $10^5$ cells / mL) were incubated in 96-well plates, treated with salbutamol ($10^{-5}$-$10^{-7}$M) (Zhang et al. 2007), AT-RvD1 ($10^{-7}$-$10^{-12}$ M) (Zambalde et al. 2016) and their combinations and stimulated thirty minutes after with the CSE (1%) for a period of 24 hours.

## 2.4 Quantification of IL-1β, IL-8 and IL-10

The detection of cytokines in the epithelial cell culture supernatant was carried out by immunoenzymatic assay (ELISA), according to manufacturer's specifications (BD Biosciences).

## 2.5 ICAM-1 expression

ICAM-1 expression was performed according to Xie et al. (2012). After this period of stimulation by CSE, the cells recovered from the culture were washed twice in PBS. Cell suspensions in PBS were stained at 4°C for 30 min with antibodies to anti-CD54 (BD Biosciences). The goat IgG1 isotype control conjugated to PE was used as a negative control. The cells were then washed 3 times with 0.5 mL of PBS and resuspended in 0.5 mL of 2% paraformaldehyde in PBS for fixation. The cells were washed, resuspended and subjected to analysis. The expression of signaling molecules in 50,000 viable cells was analyzed by flow cytometry (FACSCalibur; BD Biosciences Pharmingen). All data obtained was analyzed with the aid of the FlowJo software.

2.6 NF-κB and STAT-3 expression

The NF-κB and STAT-3 signaling pathways were assessed by cytometry according to Oliveira et al. (2015). After CSE stimulation, the cells were fixed with 4% paraformaldehyde for 10 min at 37°C. After centrifugation, the cells were permeabilized in ice-cooled methanol for 30 minutes and then stained with monoclonal antibodies. Anti-phospho-NF-κB and anti-phospho-STAT-3 (BD Biosciences Pharmingen) or its corresponding isotype for 60 minutes followed by incubation with the secondary antibody labeled with FITC or PE for another 45 minutes in the dark. The cells were then washed, resuspended and subjected to analysis. The expression of phosphorylated intracellular signaling molecules in 50,000 viable cells was analyzed by flow cytometry (FACSCalibur; BD Biosciences Pharmingen). All data obtained was analyzed with the aid of the FlowJo software.

2.7 ROS assay

After 24 h of stimulus, cells were incubated with the diluted fluoroprobe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at 37°C with slight shaking for 5 min. After washing with a serum-free culture medium, the cells were examined under a fluorescence microscope (exc: ~485nm/ em: ~535nm).

2.8 Statistical analysis

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

# 3 RESULTS

## 3.1 Concentration-response effects of salbutamol and AT-RvD1 in the inhibition of IL-1β production induced by CSE in bronchial epithelial cells.

In the first set of experiments, we analyzed the effect of different concentrations of salbutamol and AT-RvD1 on IL-1β production in BEAS-2B cells stimulated by CSE. The IL-1β production was increased in cells stimulated by CSE when compared to control group (Fig 1A and 1B). Salbutamol ($10^{-5}$ and $10^{-6}$M), but not at $10^{-7}$M, reduced the concentration of IL-1β when compared to CSE-stimulated cells. IL-1β production were decreased by 56.03% and 53.64% from 777.19 ± 85.41 (CSE) to 341.76 ± 75.59 (salbutamol at $10^{-5}$M) and to 360.30 ± 67.51 (salbutamol at $10^{-6}$M) (mean ± SEM), respectively. AT-RvD1 ($10^{-7}$-$10^{-11}$M), but not at $10^{-12}$M, reduced the concentration of IL-1β when compared to CSE-stimulated cells. IL-1β production were decreased by 57.47%, 55.99%, 60.24%, 64.37% and 65.14% from 777.19 ± 85.41 (CSE) to 330.51 ± 75.26 (AT-RvD1 at $10^{-7}$M), 342.02 ± 112.99 (AT-RvD1 at $10^{-8}$M), 309.01 ± 104.73 (AT-RvD1 at $10^{-9}$M), 276.86 ± 90.03 (AT-RvD1 at $10^{-10}$M) and 270.88 ± 68.74 (AT-RvD1 at $10^{-11}$M) (mean ± SEM), respectively. No alteration on IL-1β production was observed in cells only treated with each compound at higher concentration (salbutamol at $10^{-5}$M and AT-RvD1 at $10^{-7}$M) separately when compared to control (data not shown).

We have chosen the minimal effective concentration on IL-1β production (salbutamol at $10^{-6}$M and AT-RvD1 at $10^{-11}$M) as well as the non-effective concentration dose (salbutamol at $10^{-7}$M and AT-RvD1 at $10^{-12}$M) from each substance to evaluate the effect of combination of both.

## 3.2 The combination of salbutamol and AT-RvD1 potentiated reduction of IL-1β production induced by CSE in bronchial epithelial cells.

As demonstrated earlier, CSE induced the IL-1β production in bronchial epithelial cells. In addition, salbutamol at $10^{-6}$M, but not at $10^{-7}$M, or AT-RvD1 at $10^{-11}$M, but not at $10^{-12}$M, reduced the IL-1β production induced by CSE (Fig 2A and 2B). The combination of salbutamol ($10^{-6}$M or $10^{-7}$M) and AT-RvD1 ($10^{-11}$M) reduced

IL-1β production when compared to CSE group. IL-1β productions were decreased by 80.07% and 76.36% from 657.03 ± 171.04 (CSE) to 126.75 ± 75.75 (salbutamol at $10^{-6}$M and AT-RvD1 at $10^{-11}$M) and to 155.29 ± 42.51 (salbutamol at $10^{-7}$M and AT-RvD1 at $10^{-11}$M) (mean ± SEM), respectively. In addition, the association of salbutamol ($10^{-7}$M) and AT-RvD1 ($10^{-11}$M) potentiated the decrease in IL-1β production when compared to cells stimulated by CSE and treated with salbutamol at $10^{-7}$M. In view of these results, we have chosen the combination of salbutamol ($10^{-7}$M) and AT-RvD1 ($10^{-11}$M) to carry out the subsequent experiments.

3.3 Effects of salbutamol, AT-RvD1 and the combination of both in the IL-10, IL-8 and ROS in BEAS-2B cells stimulated by CSE.

Next, we have evaluated the effect of salbutamol, AT-RvD1 and the combination of salbutamol ($10^{-7}$M) and AT-RvD1 ($10^{-11}$M) in IL-10, IL-8 and reactive oxygen species (ROS) production. CSE increased the IL-10, IL-8 and ROS production when compared to control group (Fig 3A, 3B and 3C, respectively). AT-RvD1, but not salbutamol, and the combination of both increased the IL-10 production and reduced the IL-8 production when compared to CSE group. IL-10 production was increased by 34.11% and 31.82% from 858.31 ± 12.24 (CSE) to 1,302.76 ± 99.50 (AT-RvD1 at $10^{-11}$M) and to 1,258.97 ± 70.22 (salbutamol at 10-7M and AT-RvD1 at $10^{-11}$M) (mean ± SEM), respectively (Fig 3A). IL-8 production was decreased by 35.23% and 29.82% from 1,422.26 ± 46.21 (CSE) to 921.19 ± 236.36 (AT-RvD1 at $10^{-11}$M) and to 998.07 ± 280.90 (salbutamol at $10^{-7}$M and AT-RvD1 at $10^{-11}$M) (mean ± SEM), respectively. No significant effect was observed in ROS production by treatments when compared to CSE group (Fig 3C).

3.4 Effects of salbutamol, AT-RvD1 and the combination of both in the STAT-3 and NF-kB activation in BEAS-2B cells stimulated by CSE.

STAT-3 and NF-kB phosphorylation is critical for IL-1β and/or IL-8 production. In this regard, we analyzed the effect of salbutamol, AT-RvD1 and the combination of salbutamol ($10^{-7}$M) and AT-RvD1 ($10^{-11}$M) in these signaling pathways. CSE increased the activation of NF-kB and STAT3 in bronchial epithelial cells when

compared to control group (Fig 4A and 4B, respectively). AT-RvD1, but not salbutamol, and the combination of both decreased the phosphorylation of NF-kB, but not STAT-3, when compared to CSE group.

3.5 Effects of salbutamol, AT-RvD1 and the combination of both in the ICAM-1 expression in BEAS-2B cells stimulated by CSE.

CSE increased the ICAM-1 expression on BEAS-2B cells when compared to control group (Fig 5). AT-RvD1, salbutamol and the combination of both decreased the ICAM-1 expression when compared to CSE group.

# 4 DISCUSSION

Airway epithelial cells play an important role in innate immune functions in the airways through the production of pro-inflammatory chemokines and cytokines (Barnes, 2017). However, dysregulation of this epithelium may trigger the onset of chronic airway inflammation associated with the pathophysiology of COPD (Wang et al. 2018). Cigarette smoking causes a continuous oxidative insult to the airway inflammatory process, airways remodeling and pulmonary emphysema in COPD patients (Caramori et al. 2016). In such chronic inflammatory airways diseases, there is an imbalance between pro-inflammatory and anti-inflammatory mediators in favor of pro-inflammatory mediators (Caramori et al. 2011; Hogg and Timens, 2009). The cigarette exposure in the airways environment causes an imbalance of endogenous ALX/FPR2 receptor agonists favoring the increase of pro-inflammatory agonists such as SAA (Bozinovski et al., 2012), in detriment of protective anti-inflammatory and pro-resolution agonists, such as lipoxin A4 and AT-RvD1 (; Kim et al. 2016). The anti-inflammatory and pro-resolving actions of AT-RvD1 are widely known in several experimental models including the airway models as in vivo (Chen et al. 2014; Eickmeier et al. 2013) as in vitro (Cox et al. 2015; de Oliveira et al. 2015).

Beta 2 adrenergic agonists are administered to COPD patients as a reliever therapy, primarily for their bronchodilatory effects, relaxation of smooth muscle cells, thereby providing critical symptomatic relief (Bourbeau et al. 2007; Choy et al. 1999; Theron et al. 2013). In addition, they may also exert potential anti-inflammatory effects on all cell types (monocytes/macrophages, basophils, eosinophils, mast cells, neutrophils, T lymphocytes), as well as those of airway structural cells (epithelial cells, fibroblasts, smooth muscle cells) by several distinct mechanisms (Hanania and Moore, 2004; Miyamoto et al. 2004). The effects of β2-agonists on various immune and inflammatory cells are considered to be associated with inhibition of leukocyte recruitment, production of cytokines and chemokines and other mediators (Sabatini et al. 2003; Nijhuis et al. 2014). These effects improve their efficacy in the management of airways inflammatory diseases. However, the

monotherapy with beta2-agonists has also been associated with poor asthma and COPD control and their administration with other anti-inflammatory drugs could improve asthma and COPD control and prevent airway exacerbations (Bourbeau et al. 2007). In this regard, we evaluated the anti-inflammatory effects of a beta2-agonist, salbutamol, and a pro-resolution mediator, AT-RvD1, and the combination of both drugs in bronchial epithelial cells lineage (BEAS-2B) stimulated with CSE.

IL-1β is a pro-inflammatory mediator involved in the enhancement of the expression cytokines, chemokines and adhesion molecules (Pinkerton et al. 2017). AT-RvD1 was more effective than salbutamol in inhibiting IL-1 release from CSE-stimulated BEAS-2B. These results agree with previous studies where salbutamol reduced the IL-1 production in an air pouch model of inflammation in rats in vivo (Eteraf-Oskouei et al. 2017) and AT-RvD1 decreased IL-1β production in macrophages exposed to H2O2 and ATP (Cox et al. 2015). Of note, the combination of both salbutamol and AT-RvD1, using the least effective and non-effective concentration, reinforced their anti-inflammatory effects of reducing the IL-1 production with an additive effect when compared to cells only treated with salbutamol alone ($10^{-7}$M i.e a non-effective concentration). These results demonstrated the anti-inflammatory effects of combination of AT-RvD1 and salbutamol and suggest a reduction of some deleterious effect caused by regular use of short-acting beta2-agonists alone on COPD (Lin et al. 2012; Chiarella et al. 2014).

IL-8, acting via CXCR2 is a potent pro-inflammatory chemokine that induces trafficking of neutrophils to the lungs and contributes to the pathogenesis of COPD through regulating neutrophilic airway inflammation (Larsson, 2008; Pedersen et al 2018). AT-RvD1 reduced the IL-8 in A549 cells or BEAS-2B cells stimulated by IL-1β or IL-4 (Rogerio et al. 2012; Eickmeier et al. 2013), respectively. In keratinocytes stimulated by trypsin, salbutamol inhibited the production of IL-8, however in another study using 16HBE cells, salbutamol increased the IL-8 production (Lindén, 1996). AT-RvD1 ($10^{-11}$M), but not salbutamol ($10^{-7}$M), and the combination of both reduced

the IL-8 production induced by CSE. These results could favor the reduction of excessive neutrophil traffic to airways which is associated with disease progression, GOLD (Global Initiative for Chronic Obstructive Lung Disease) status and exacerbations of COPD (Singh et al. 2019).

IL-10 has anti-inflammatory and immunosuppressive effects, limiting inflammation (Ogawa et al. 2008). In sputum from COPD and in smokers, the IL-10 concentration is reduced (Takanashi et al. 1999). Salbutamol increased the IL-10 production in bone marrow derived dendritic cells (Posso et al. 2018). AT-RvD1 lowered the production of IL-10 in PBMCs from patients with severe asthma stimulated with LPS (Zambalde et al. 2016). However, in another study using a murine model of cigarette smoke-induced emphysema, AT-RvD1 increased IL-10 concentration in bronchoalveolar lavage fluid (Hervé et al. 2016). In our study, AT-RvD1, but not salbutamol, and the combination of both increased the IL-10 concentration when compared to BEAS-2B cells stimulated by CSE. The increased IL-10 production could contribute to reducing the local inflammation and tissue damage favoring the immune control of pathogenesis of COPD.

ICAM-1 plays an important role in leukocyte (neutrophils and macrophages) trafficking mediating the interaction between these cells and bronchial epithelial cells (Ramos et al. 2014). ICAM-1 expression is up-regulated in smokers and COPD patients (Scott and Palmer, 2002). AT-RvD1 reduced the ICAM-1 expression on A549 cells (Eickmeier et al. 2013) or BEAS-2B cells (Rogerio et al. 2012) stimulated by IL-1β or IL-4, respectively. Using a mouse model of lipopolysaccharide-induced acute kidney injury, AT-RvD1 reduced the ICAM-1 and VCAM-1 expression in the kidney (Chen et al. 2014). Salbutamol reduced the ICAM-1 expression in epithelial cells (BEAS-2B) stimulated by aqueous extracts of organic dust derived from hog CAFOs (Romberger et al. 2016). Our results agree with these findings, AT-RvD1, salbutamol and the combination of both reduced the ICAM-1 expression induced by CSE in BEAS-2B cells. These results, in association with the IL-8 results, confirm the anti-inflammatory effect of combination treatment of AT-RvD1 with salbutamol.

ROS can lead to epithelial cell injury through oxidative stress and can contribute to COPD pathophysiology (Boukhenouna et al. 2018). Salbutamol inhibited the ROS production in RAW264.7 macrophages stimulated by LPS (Wang et al. 2020). In a murine model of CS-induced emphysema, AT-RvD1 reduced the ROS production (Hervé et al. 2017). Our results demonstrated no effect on inhibition of ROS by salbutamol, AT-RvD1 or their combination, therefore are in disagreement with the previous studies.

NF-κB and STAT-3 signaling are up-regulated in COPD patients and play a significant role in the airway epithelium in regulating cigarette smoking-induced inflammation (Schuliga, 2015; Samavati et al. 2009). AT-RvD1 reduced the phosphorylation of NF-κB in BEAS-2B cells stimulated with IL-4 (de Oliveira et al. 2015), lipopolysaccharide or Dermatophagoides pteronyssinus (de Oliveira et al. 2017). In addition, the inhibition of NF-κB by AT-RvD1 also occurred in a murine self-limited model of hydrochloric acid-induced acute lung injury (Kim et al. 2016) and a murine experimental model of asthma induced by ovalbumin (Rogerio et al. 2012). Using a mouse model of lipopolysaccharide-induced acute kidney injury in vivo, AT-RvD1 also reduced the STAT-3 expression in the kidney (Chen et al. 2014). Salbutamol inhibited the activation of NF-κB in bone marrow derived dendritic cells stimulated by LPS (Hervé et al. 2013). The activation of NF-κB induced by CSE on BEAS-2B cells was reduced by AT-RvD1, but not salbutamol, and the combination of both, however no modulation of activation of STAT-3 was observed by these treatments. The anti-inflammatory effects of AT-RvD1 and the combination of AT-RvD1 and salbutamol could be associated with the down regulation of NF-κB activation which plays significant role in airway inflammatory mechanisms in COPD pathogenesis and is critical for IL-1β and IL-8 production and ICAM-1 expression.

The discrepancies of some results found in this study from the others using AT-RvD1, but mainly with salbutamol, could be associated with a number of experimental factors, including using different cells (for example, bronchial epithelial cells vs macrophages), models (in vitro vs in vivo) and concentration of the compounds (low vs high).

## 5 CONCLUSION

AT-RvD1 could be a potential alternative treatment and a candidate in association with beta2-agonists to treat airway inflammation caused by cigarette smoking in patients with COPD.

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## Declaration of competing interest

MU is currently an employee of AstraZeneca and holds share in the company.

All other authors declare that they have no conflict of interest.

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FIGURES

Figure 1

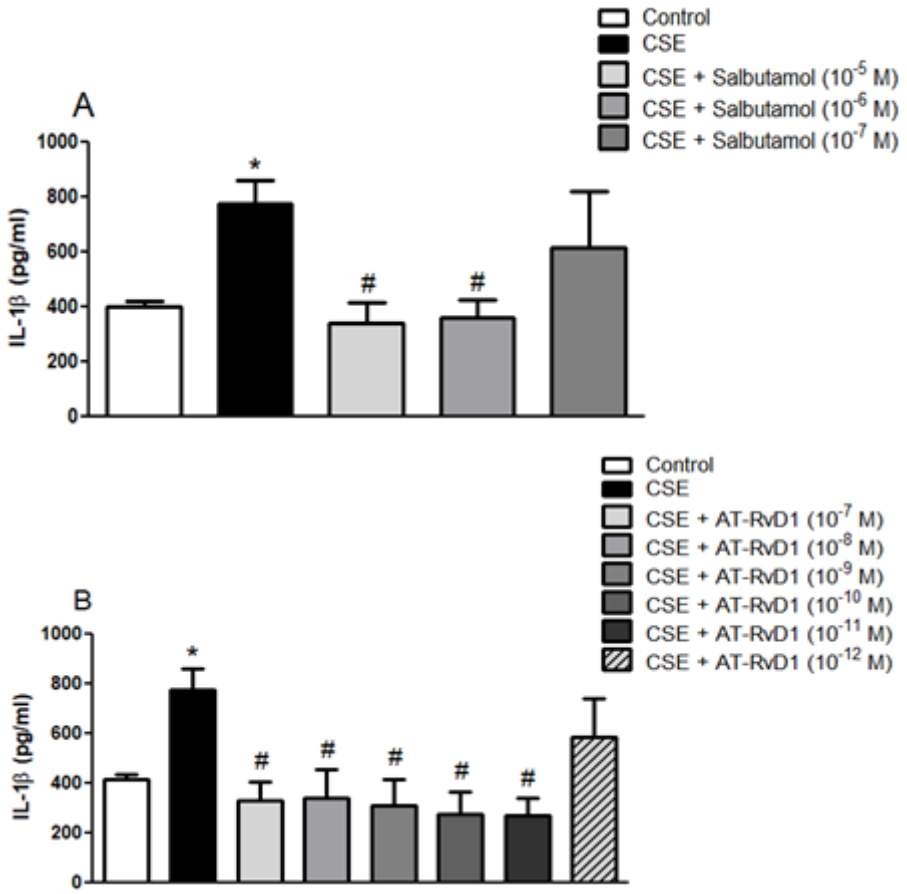


Figure 1: Anti-inflammatory effects of salbutamol (A) and AT-RvD1 (B) in the IL-1β production on BEAS-2B cells stimulated cigarette smoke extract. BEAS were treated for 30 min with salbutamol or AT-RvD1 prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n=4 experiments. * $P < 0.05$ versus the control group, # $P < 0.05$ versus the CSE group.

Figure 2

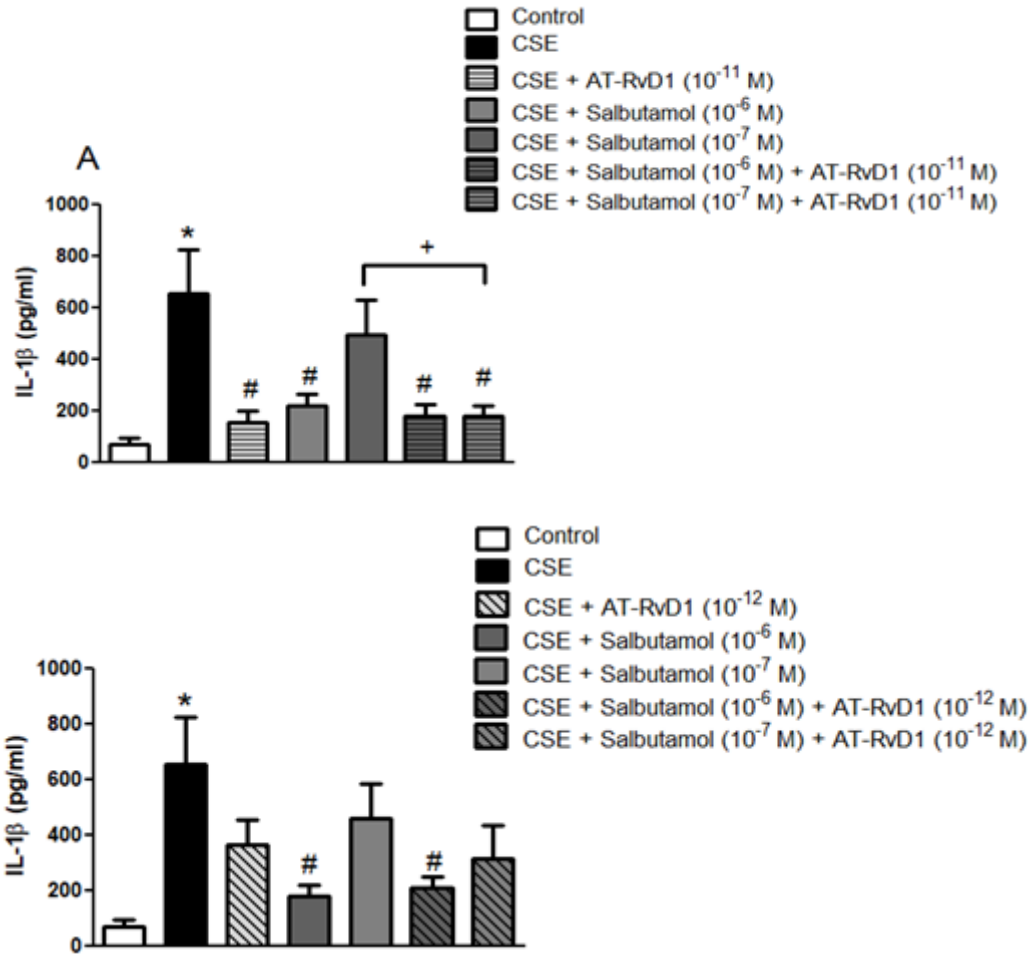


Figure 2: Effect of combination of salbutamol and AT-RvD1 in the IL-1β production on BEAS-2B cells stimulated cigarette smoke extract. BEAS were treated for 30 min with salbutamol or AT-RvD1 prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n=4 experiments. * $P < 0.05$ versus the control group, # $P < 0.05$ versus the CSE group.

Figure 3

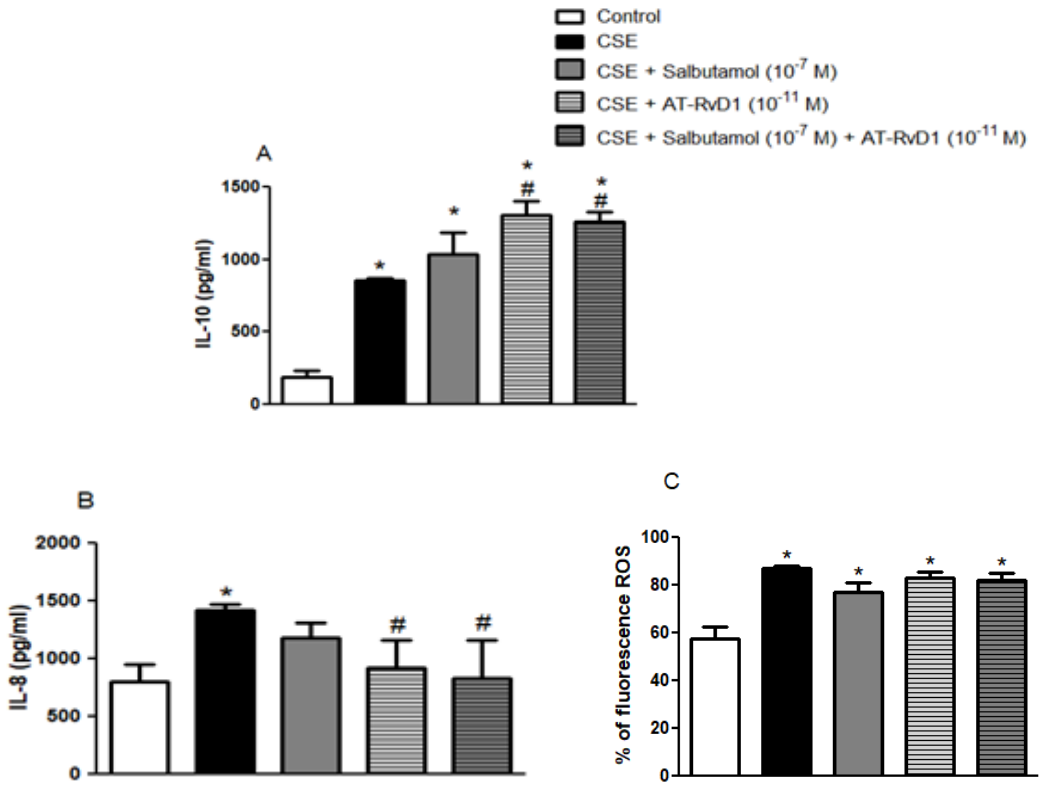


Figure 3: Effect of combination of salbutamol and AT-RvD1 in the IL-10 (A), IL-8 (B), ROS (C) production on BEAS-2B cells stimulated cigarette smoke extract. BEAS were treated for 30 min with salbutamol or AT-RvD1 prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n=4 experiments. * $P < 0.05$ versus the control group, # $P < 0.05$ versus the CSE group.

Figure 4

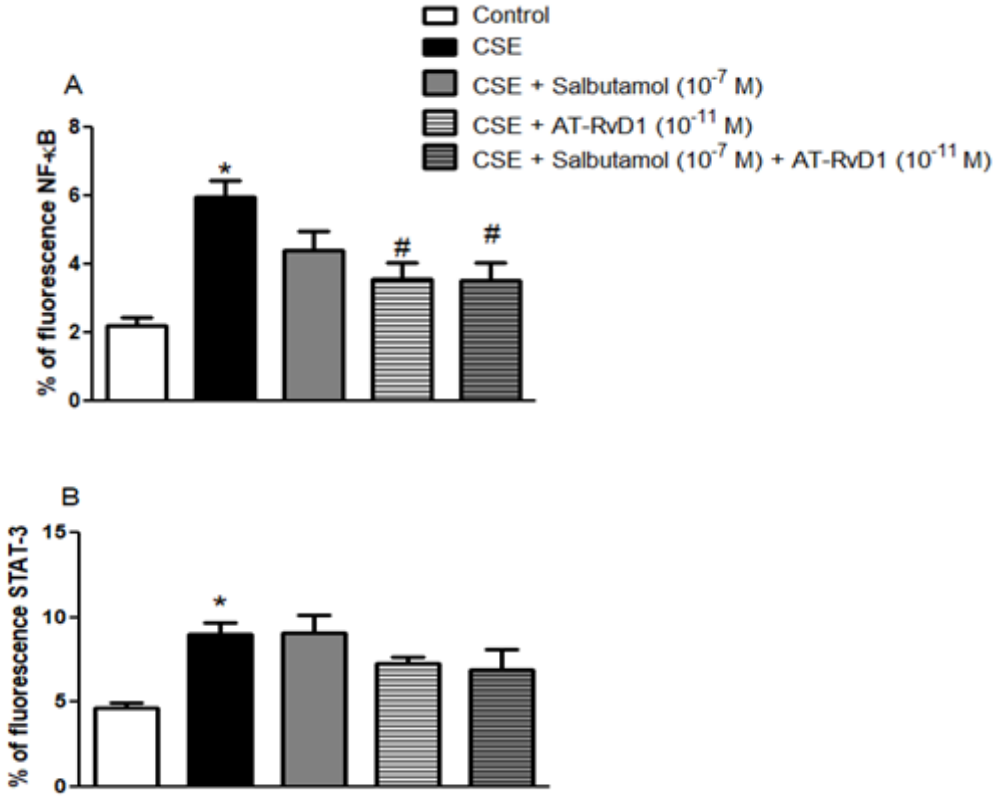


Figure 4: Effect of combination of salbutamol and AT-RvD1 in the NF-κB (A) and STAT-3(B) pathways on BEAS-2B cells stimulated cigarette smoke extract. BEAS were treated for 30 min with salbutamol or AT-RvD1 prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n=3 experiments. *P < 0.05 versus the control group, #P < 0.05 versus the CSE group.

Figure 5

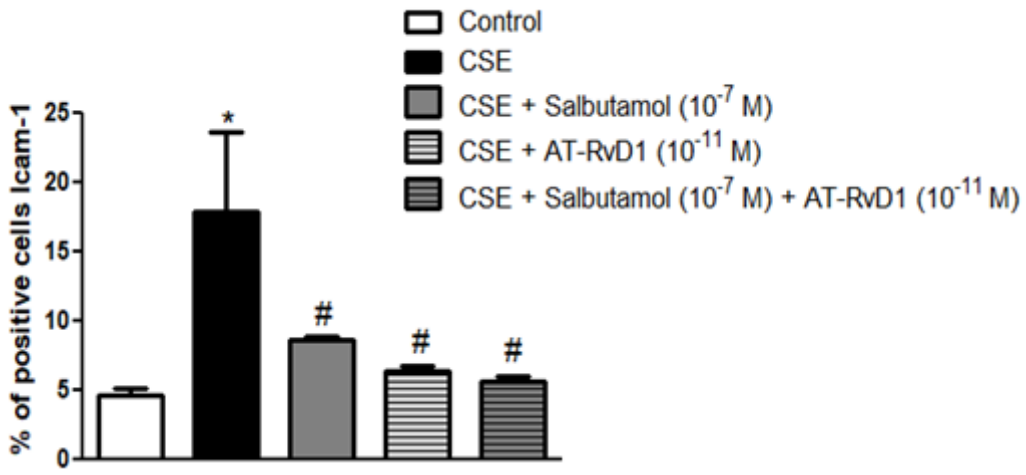


Figure 5: Effect of combination of salbutamol and AT-RvD1 in the ICAM-1 expression on BEAS-2B cells stimulated cigarette smoke extract. BEAS were treated for 30 min with salbutamol or AT-RvD1 prior to incubation with CSE (1%) for 24 h. The data are reported as the means ± S.E.M of n=3 experiments. *P < 0.05 versus the control group, #P < 0.05 versus the CSE group.

## APÊNDICE D - PARTICIPAÇÃO DOS COAUTORES NO ARTIGO

Dr. Bruce David Levy

Cedeu o mediador lipídico AT-RvD1 para a realização dos experimentos.

Henrique Ismarsi de Sousa

Ajudou na padronização e realização do ELISA.

Bruno Sada Salermo

Ajudou na padronização e realização do ELISA.

Ma. Aline Beatriz Mahler Pereira

Ajudou na padronização e realização da citometria.

Dr. Paulo Roberto da Silva

Disponibilizou alguns dos aparelhos e reagentes para realização de nosso projeto.

Dr. Mohib Uddin

Auxiliou na correção e ortografia do artigo.