Biochimica et Biophysica Acta 1842 (2014) 1630–1637

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

# Cigarette smoke alters IL-33 expression and release in airway epithelial cells

Elisabetta Pace [a,*], Caterina Di Sano [a], Serafina Sciarrino [a], Valeria Scafidi [a], Maria Ferraro [a], Giuseppina Chiappara [a], Liboria Siena [a], Sebastiano Gangemi [b], Patrizio Vitulo [c], Antonino Giarratano [d], Mark Gjomarkaj [a]

[a] Institute of Biomedicine and Molecular Immunology (IBIM) — National Research Council (CNR), Palermo, Italy
[b] School and Division of Allergy and Clinical Immunology, Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy
[c] Istituto Mediterraneo per i Trapianti e Terapie ad Alta Specializzazione (ISMETT), Palermo, Italy
[d] Dipartimento di Anestesia, Rianimazione e delle'Emergenze — Università degli Studi di Palermo, Palermo, Italy

ARTICLE INFO

*Article history:*
Received 18 March 2014
Received in revised form 28 May 2014
Accepted 6 June 2014
Available online 12 June 2014

*Keywords:*
Cigarette smoke
IL-33
COPD
Inflammation
Bronchial epithelial cell

ABSTRACT

Airway epithelium is a regulator of innate immune responses to a variety of insults including cigarette smoke. Cigarette smoke alters the expression and the activation of Toll Like Receptor 4 (TLR4), an innate immunity receptor. IL-33, an alarmin, increases innate immunity Th2 responses. The aims of this study were to explore whether mini-bronchoalveolar lavage (mini-BAL) or sera from smokers have altered concentrations of IL-33 and whether cigarette smoke extracts (CSE) alter both intracellular expression (mRNA and protein) and release of IL-33 in bronchial epithelial cells. The role of TLR4 in the expression of IL-33 was also explored.
Mini-BALs, but not sera, from smokers show reduced concentrations of IL-33. The expression of IL-33 was increased also in bronchial epithelium from smokers. 20% CSE reduced IL-33 release but increased the mRNA for IL-33 by real time PCR and the intracellular expression of IL-33 in bronchial epithelial cells as confirmed by flow cytometry, immunocytochemistry and western blot analysis. The effect of CSE on IL-33 expression was also observed in primary bronchial epithelial cells. IL-33 expression was mainly concentrated within the cytoplasm of the cells. LPS, an agonist of TLR4, reduced IL-33 expression, and an inhibitor of TLR4 increased the intracellular expression of IL-33. In conclusion, the release of IL-33 is tightly controlled and, in smokers, an altered activation of TLR4 may lead to an increased intracellular expression of IL-33 with a limited IL-33 release.


## 1. Introduction

Lung epithelial cells, which are positioned at the site of first exposure to many microbial pathogens and harmful inhaled substances including cigarette smoke, regulate both innate and adaptive immunity [1]. Prolonged and/or strong epithelial activation can promote the development of airway inflammatory diseases via the release of large quantities of pro-inflammatory cytokines, growth factors, and chemokines that attract inflammatory cells into the airways.

IL-33 is a cytokine belonging to the IL-1 family (IL-1α, IL-1β, IL-18) with a dual function, acting both as a traditional cytokine through activation of the ST2L receptor complex and as an intrace\llular nuclear factor with transcriptional regulatory properties [2]. IL-33 may be present in a full length and in a mature form and increases innate immunity Th2 responses. IL-33 is increased in patients with pulmonary diseases, and full-length IL-33 is the predominant form in the lungs in health and disease [3]. IL-33 is increased in the bronchial epithelium [4] and in airway smooth muscle cells of asthmatics [5], it may be considered a marker of severity, it may contribute to airway remodeling in asthma [6] and it may be associated to specific asthma phenotypes [7]. In mice cigarette smoke exposure increases IL-33 and its receptor ST2L [8] and these events lead to increased numbers of innate immunity effector cells such as neutrophils and macrophages within the airways.

The exposure to cigarette smoke, the major risk factor for chronic obstructive pulmonary disease (COPD), alters innate immune responses of the bronchial airway epithelium by increasing TLR4 expression [9–11] and reducing human beta defensin 2 expression (hBD2) [11]. TLR-mediated innate signaling pathway regulates the synthesis of IL-33 in an ocular mucosal epithelium [12]. The mechanisms regulating the expression and the release of IL-33 in the airways of smokers are largely unknown.

*Abbreviations:* CSE, cigarette smoke extracts; MoAb, monoclonal antibody; CM, complete medium

* Corresponding author at: Istituto di Biomedicina e Immunologia Molecolare, Consiglio Nazionale delle Ricerche, Via Ugo La Malfa, 153 90146 Palermo, Italy. Tel.: +39 091 680 9148; fax: +39 091 680 9122.
*E-mail address:* pace@ibim.cnr.it (E. Pace).

http://dx.doi.org/10.1016/j.bbadis.2014.06.009
0925-4439/

The aims of the present study were to test whether cigarette smoke extracts (CSEs) alter the expression and/or the release of IL-33 and to assess whether this alteration is due to an altered activation of TLR4. To this purpose, a combined ex-vivo and in vitro approach was used for assessing IL-33 levels in mini-bronchoalveolar lavage (mini-BAL) or IL-33 expression in bronchial epithelium of smokers and performing experiments with bronchial epithelial cells stimulated with CSE.

## 2. Materials and methods

### 2.1. Patient population

Non-smoking subjects (n = 7) and smoking subjects (>15 pack-year) (n = 9) both with acute respiratory failure upon surgery for abdominal or thoracic aneurysm were recruited at the ICU of the Department of Anesthesiology, Reanimation and Emergency of the University of Palermo, Italy. Patients with x-ray or clinical evidence of sepsis or pneumonia at the time of mini-BAL collection were not included. All recruited subjects required mechanical ventilation and underwent therapy with antibiotics and systemic corticosteroids (no significantly different doses among the patients included in the study). Lung tissue specimens from central bronchi from smoking (n = 5) (>15 pack-year) and from non-smoking (n = 5) subjects were also collected from patients who underwent surgery for lung cancer. These latter patients were recruited at ISMETT in Palermo, Italy. The study was approved by the Ethic Committee of Policlinico-Giaccone Hospital-Palermo Italy (7/2012) and was in agreement with the Helsinki Declaration. Informed written consent from either the patients or closest relatives was obtained.

### 2.2. mini-BAL collection and processing

Distal lung fluid samples (mini-BAL) were obtained using BAL Cath system (by Kimberly-Clark Health Care, Kent, ME19 4HA, United Kingdom) within 1 h from the intubation as previously described [13]. The protected catheter was blindly advanced through the endotracheal tube until it was wedged into a distal airway and two aliquots of 10 ml of sterile 0.9% NaCl were instilled and gently suctioned (recovered volume about 70% of the instilled volume). Mini-BAL samples were filtered through a sterile gauze and then centrifuged at 1300 rpm for 10 min to separate cells from supernatants. The supernatants were used for assessing IL-33 concentrations.

### 2.3. IL-33 concentrations

The concentrations of IL-33 (Duoset; R&D Systems, Minneapolis, MN) (range 23.4–1500 pg/ml) were determined with enzyme-linked immunosorbent assays (ELISA).

### 2.4. Immunohistochemistry

Tissue specimens from central bronchi were selected, fixed with 10% Neutral buffer formalin and embedded in paraffin wax. Three-micrometer tissue sections were attached to poly-L-lysine coated microscope slides and, after dewaxing and rehydration, were stained with hematoxylin and eosin (HE) or analyzed by means of immunohistochemistry as previously described [14]. Immunohistochemistry and image analysis were used to determine IL-33 expression using a mouse monoclonal IgG antibody anti-IL-33 (Adipogen, San Diego, CA; Clone IL33305B) (1:15; overnight) in central (internal perimeter >6 mm) airways. LSAB2 Dako kit (Code N° K0674) (Dako, Glostrup, Denmark) and Fuchsin Substrate-Chromogen System Dako were used for the staining. Nonimmune mouse (Dako) immunoglobulins were used for negative controls. The immunoreactivity was evaluated blindly by 2 independent investigators using a Leica (Wetzlar, Germany) microscope ×400 magnification.

### 2.5. Preparation of cigarette smoke extracts (CSEs)

Commercial cigarettes (Marlboro) were used in this study. Cigarette smoke solution was prepared as described previously [9]. Each cigarette was smoked for 5 min and one cigarette was used per 10 ml of PBS to generate a CSE-PBS solution. The CSE solution was filtered through a 0.22 μm-pore filter to remove bacteria and large particles. The smoke solution was then adjusted to pH 7.4 and used within 30 min of preparation. This solution was considered to be 100% CSE and diluted to obtain the desired concentration in each experiment. The concentration of CSE was calculated spectrophotometrically measuring the OD as previously described [15] at a wavelength of 320 nm. The pattern of absorbance, among different batches, showed very little differences and the mean OD of the different batches was 1.37 ± 0.16. The presence of contaminating LPS on undiluted CSE was assessed by a commercially available kit (Cambrex Corporation, East Rutherfort, New Jersey, USA) and was below the detection limit of 0.1 EU/ml.

### 2.6. Bronchial epithelial cell cultures

16-HBE, an immortalized normal bronchial epithelial cell line [16] or primary normal human bronchial epithelial (NHBE) cells (ATCC — catalog n. PCS-300-010) were used in this study. Bronchial epithelial cells were maintained in MEM (Gibco, BRL, Germany), supplemented with 10% fetal calf serum (Gibco). Cell cultures were maintained in a humidified atmosphere of 5% $CO_2$ in air at 37 °C. Cells were cultured in the presence and absence of CSE (5%, 10% and 20%) for 24 and 72 h. In some experiments cell cultures were also stimulated with LPS (Sigma-Aldrich Corporate, St Louis, MO) (1 μg/ml), with TNFα (100 ng/ml) or were incubated with an inhibitor of TLR4 (CLI-095) (InvivoGen, San Diego, CA) (1 μg/ml) 6 h before stimulation with CSE following the protocol provided with data sheet by InvivoGen. Different time points (6, 12, 24 and 48 h) for IL-33 mRNA experiments were evaluated. At the end of stimulation, cells, cell extracts, and cell culture supernatants were collected for further evaluations.

### 2.7. Flow cytometry

The expression of IL-33 in 16-HBE cells was evaluated by flow-cytometry using a FACS Calibur (Becton Dickinson, Mountain View, CA). To evaluate the expression of intracellular IL-33 before incubation with mouse monoclonal antibody, cells were treated overnight with GolgiStop (2 μM final concentration) (BD PharMingen) and fixed with PBS containing 4% paraformaldehyde for 20 min at room temperature. Fixed cells were washed twice in permeabilization buffer (PBS containing 1% FBS, 0.3% saponin, and 0.1% Na azide) for 5 min at 4 °C, then incubated with a mouse monoclonal IgG antibody anti-IL-33 (Adipogen) (1:100; for 1 h) followed by a FITC conjugated anti-mouse IgG (Dako, Glostrup, Denmark). Negative controls were performed using mouse immunoglobulin negative control (Dako). Data are expressed as percentage of positive cells.

### 2.8. Immunocytochemistry

The expression of IL-33 in 16-HBE cells was evaluated by immunocytochemistry as previously described [14] using a mouse monoclonal IgG antibody anti-IL-33 (Adipogen) (1:15; overnight). LSAB2 Dako kit (Code N° K0674) (Dako) and Fuchsin Substrate-Chromogen System Dako were used for the staining. Nonimmune mouse (Dako) immunoglobulins were used for negative controls. The immunoreactivity was evaluated blindly by 2 independent investigators using a Leica (Wetzlar, Germany) microscope ×400 magnification.

### 2.9. Real time PCR

Real time PCR was performed as previously described [10] in 16-HBE and in NHBE. The cells were stimulated with CSE (20%) for 18 h and total cellular RNA extracted using TriZol reagent (Invitrogen) was reverse-transcribed to cDNA, using M-MLV-RT and oligo(dT)$_{12-18}$ primer (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR of human IL-33 gene was carried out on Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for IL-33, Hs01125943m1, Assays on Demand, Applied Biosystems). GAPDH gene expression was used as endogenous control for normalization. Relative quantitation of mRNA was carried out with comparative CT method.

### 2.10. Western blot analysis

The expression of IL-33 was evaluated by western blot analysis as previously described [17] using a mouse IgG monoclonal antibody anti-IL-33 (1:2000; for 1 h) (Adipogen). Membranes were then stripped and incubated with goat polyclonal anti-ß-actin (Sigma). Revelation was performed with an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK) followed by autoradiography. Negative controls were performed without primary antibody or including an isotype control antibody. Data are expressed as densitometric arbitrary units by correction with the density of the bands obtained for beta-actin. To study IL-33 nuclear translocation, the protein extracts were treated to separate the cytoplasmic and nuclear protein fractions by using a commercial kit "NE-PER Nuclear and Cytoplasmic Extraction Reagents" following the manufacturer's directions (Thermo Scientific; Waltham, MA). An amount of 40 μg of total proteins was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and blotted onto nitrocellulose membranes. Membranes were then stripped and incubated with rabbit polyclonal lamin B1 antibody (#9087, Cell Signaling Technology, Danvers, MA-USA) for assessing quality control of nuclear extracts.

### 2.11. Statistics

Data are expressed as medians and inter-quartile range or as mean counts ± standard deviation. A non-parametric Mann Whitney test was applied for comparisons between the two subject groups. Comparison between different experimental conditions was evaluated by paired *t* test. $p < 0.05$ was accepted as statistically significant.

## 3. Results

### 3.1. IL-33 concentrations in mini-BAL and sera from smokers

The concentrations of IL-33 in mini-BAL and sera from smokers and from non-smokers were initially assessed. Mini-BAL (Fig. 1 A) but not sera (Fig. 1 B) from smokers showed significant reduced concentrations of IL-33 when compared to non-smokers suggesting that this reduced release of IL-33 is a phenomenon mainly compartmentalized within the airways of smokers. No correlation was found between serum and mini-BAL IL-33 concentrations (data not shown).

### 3.2. IL-33 expression by bronchial epithelium in smokers

IL-33 is mainly expressed by stromal cells including epithelial cells [18]. The expression of IL-33 was assessed in the epithelium of large airways from non-smokers and from smokers. In tissue samples, IL-33 immunoreactivity was more evident in basal cells of bronchial epithelium of smokers in comparison to non-smokers (Fig. 2). The immunoreactivity of IL-33 in smokers was similar to the immunoreactivity of IL-33 in an asthmatic subject used as positive control [4,5].

### 3.3. Effects of CSE on IL-33 release by bronchial epithelial cells

To clarify whether cigarette smoke alters the release of IL-33, the in vitro effects of CSE on IL-33 release by bronchial epithelial cells were explored. CSE reduced IL-33 release by bronchial epithelial cells in a dose dependent manner (Fig. 3).

### 3.4. Effects of CSE on intracellular IL-33 expression by bronchial epithelial cells

The effect of CSE on the intracellular content of IL-33 by bronchial epithelial cells was assessed. CSE at higher tested concentration (20%) increased intracellular IL-33 expression in bronchial epithelial cells at both tested time points. The expression of IL-33 was more relevant after 24 (Fig. 4A) than after 72 h (Fig. 5). The increased expression of IL-33 after 24 h of CSE exposure was confirmed also by immunocytochemistry (Fig. 4B). The immunolocalization of IL-33 is prevalent in the cell cytoplasm (see the higher magnification of Fig. 4B).

### 3.5. Effects of CSE on full length or mature IL-33 expression by bronchial epithelial cells

Further experiments were designed to clarify whether the increase of intracellular IL-33 was referred to the increase of the full-length IL-

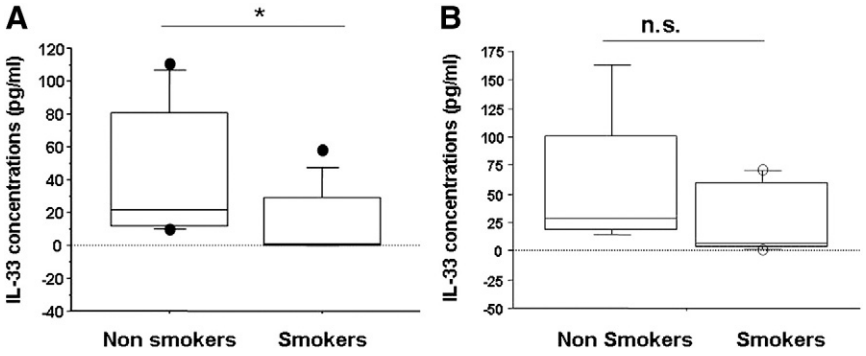


**Fig. 1.** IL-33 concentrations in mini-BAL (A) and in sera (B) from smokers. Mini-BAL supernatants were recovered from non-smoker (n = 7) and from smoker (n = 9) subjects. IL-33 concentrations were measured by ELISA as described in the "Materials and methods" section and are expressed as pg/ml. Data are expressed as median and inter-quartile range. *$p < 0.05$ vs non-smokers.

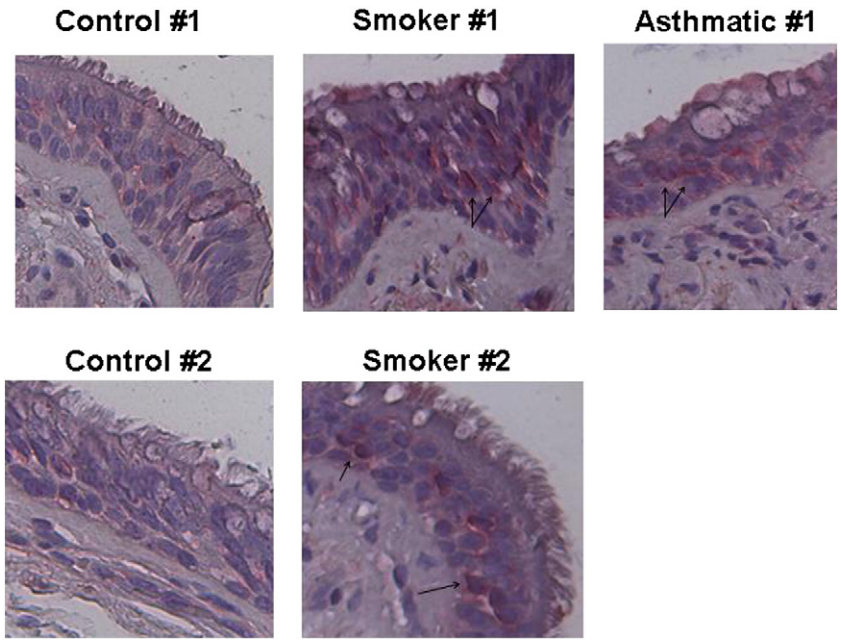


**Fig. 2.** IL-33 expression by bronchial epithelium in smokers. Immunohistochemistry for IL-33 in large airways from surgical samples of non-smokers (Controls) (n = 2), of smokers (n = 2) and of an asthmatic subject was performed. IL-33 immunostaining (red stain) at 400× magnification is shown. Arrows indicate IL-33 immunostaining.

33 by western blot analysis in cytoplasmic as well as in nuclear extracts. Full length IL-33 was increased in the cytoplasm but not in the nucleus of CSE stimulated bronchial epithelial cells (Fig. 6).

### 3.6. Effects of CSE on IL-33 mRNA expression by bronchial epithelial cells

We next assessed whether the increased expression of IL-33 due to cigarette smoke exposure was due to increased gene expression. CSE increased IL-33 mRNA expression in 16HBE (Fig. 7A) and in primary bronchial epithelial cells (Fig. 7B).

### 3.7. Effect of TLR4 on IL-33 mRNA and protein expression by bronchial epithelial cells

Cigarette smoke alters the activation of Toll like receptors [8–10]. Since the role of Toll like receptor activation on IL-33 expression is controversial [19,20], the effects of TLR4 related events on the expression of IL-33 were explored. In particular, for IL-33 mRNA expression, we tested different time points, different CSE concentrations, an agonist (LPS) and an antagonist (CLI-095) of TLR4, and included, as a positive control, TNFα [5]. IL-33 mRNA expression increased upon CSE exposure in a time and in a dose dependent manner (Fig. 8). TNFα increased, LPS reduced while TLR4 inhibition increased IL-33 mRNA in bronchial epithelial cells. The combined exposure of bronchial epithelial cells to LPS and CSE increased the expression of IL-33 mRNA in comparison to LPS alone (Fig. 8).

In terms of protein expression, the agonist of TLR4, LPS, reduced while TLR4 inhibition increased total intracellular IL-33 in bronchial epithelial cells (Fig. 9). TLR4 inhibition increased the full length IL-33 form expression in bronchial epithelial cells (Fig. 10).

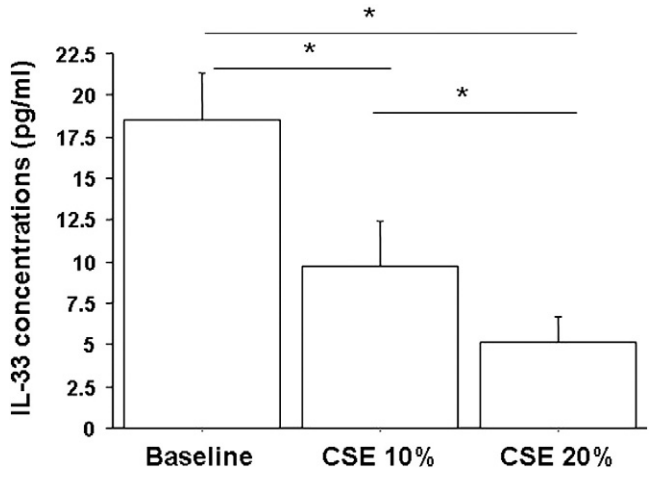


**Fig. 3.** Effects of CSE on IL-33 release by bronchial epithelial cells. Bronchial epithelial cells (16-HBE, n = 5) were cultured in the presence and in the absence of CSE (10% and 20%) for 24 h. The supernatants were collected and used for evaluating IL-33 concentrations by ELISA. Results are expressed as mean ± SD. *p < 0.05.

### 3.8. Effect of TLR4 on IL-33 release by bronchial epithelial cells

Finally, the effects of TLR4 on IL-33 release by bronchial epithelial cells were explored. TNFα increased while CSE 20% and CLI-095 reduced IL-33 release by bronchial epithelial cells. LPS alone did not have any significant effects on IL-33 release. The combined exposure of LPS and CSE increased the release of IL-33 in comparison to CSE alone (Fig. 11).

## 4. Discussion

Cigarette smoke is a profound pro-inflammatory stimulus and is the principal cause of chronic obstructive pulmonary disease (COPD), a disorder characterized by airway inflammation and bronchial obstruction.

IL-33 is a cytokine belonging to IL-1 family (IL-1α, IL-1β, IL-18) with a dual function, acting both as a traditional cytokine through activation of the ST2L receptor complex and as an intracellular nuclear factor with transcriptional regulatory properties. As epithelial cells are the first line of defense against foreign noxae, the response of normal epithelial cells to smoke has been extensively studied. The present study demonstrates for the first time that in smokers IL-33 levels are reduced within the lumen of the airways and that IL-33 expression is increased within the bronchial epithelium. Cigarette smoke is responsible for the reduced IL-33 release as well as for the intracellular cytoplasmic accumulation of the full length forms of IL-33 in bronchial epithelial cells. The role of

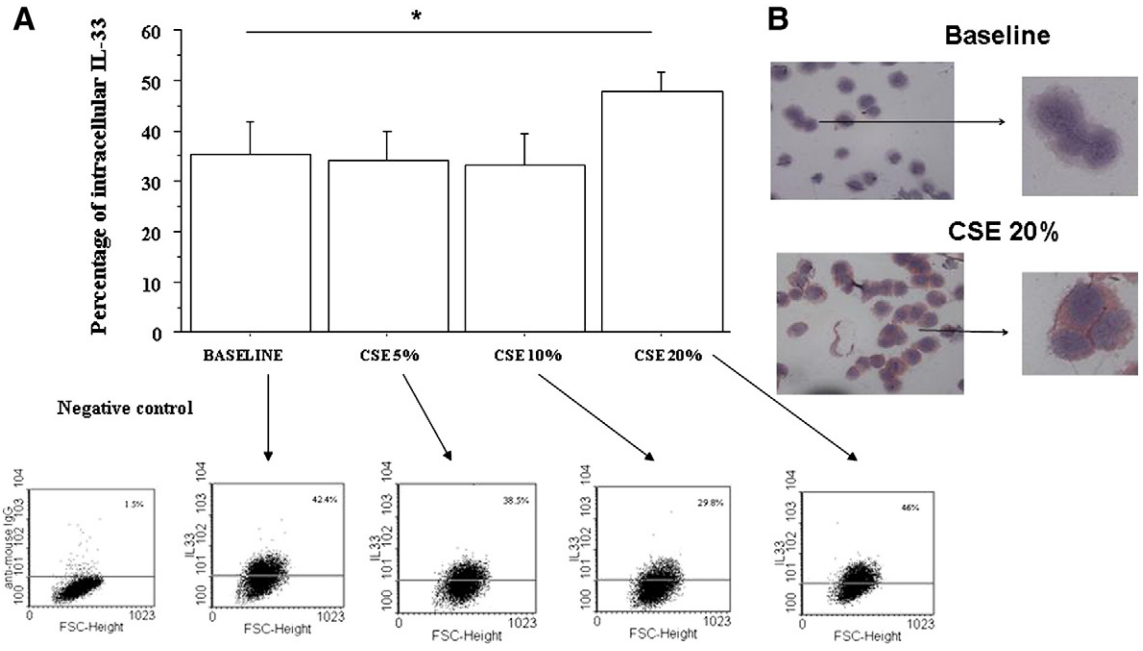


**Fig. 4.** Effects of CSE on intracellular IL-33 expression by bronchial epithelial cells (24 h). Bronchial epithelial cells (16-HBE, n = 5) were cultured in the presence and in the absence of different concentrations of CSE (5%, 10% and 20%) for 24 h. A. The intracellular expression of IL-33 was assessed by flow-cytometry and the results are expressed as percentage of positive cells (mean ± SD). *p < 0.05 vs baseline. Representative dot plots are shown. B. 16-HBE were cultured in the presence and in the absence of CSE 20% and IL-33 expression was assessed by immunocytochemistry. Representative images of IL-33 expression from 3 experiments are shown.

TLR4 in regulating the synthesis of IL-33 within bronchial epithelial cells is also described.

IL-33 is mainly expressed by stromal cells, such as epithelial and endothelial cells, and has been suggested to act as an "alarmin" orchestrating protective antiviral cytotoxic T lymphocyte responses [21] or increasing the activity of the innate type 2 lymphoid cells. These latter cells are involved in the initiation of the type 2 immune response (secretion of IL-5 and IL-13) during parasitic infection and allergic diseases such as bronchial asthma [18]. IL-33 mRNA and protein as well as IL-33 lumen secretion are all increased within the airways of asthmatics [4,5] suggesting that bronchial epithelium and other airway resident cells express elevated levels of IL-33 in asthma and may also be subjected to conditions favoring its release.

In the present study IL-33 levels are significantly reduced within the lumen of the airways but not in sera of smokers suggesting that specific signals present in the lung compartment are responsible for the reduced release of this alarmin, a signal crucial to alert the host innate immune system.

Differently, IL-33 immunoreactivity is more evident in basal cells of bronchial epithelium of smokers in comparison to non-smokers. A more accurate estimation of the differences between smokers and non-smokers could be provided by analysing a greater number of

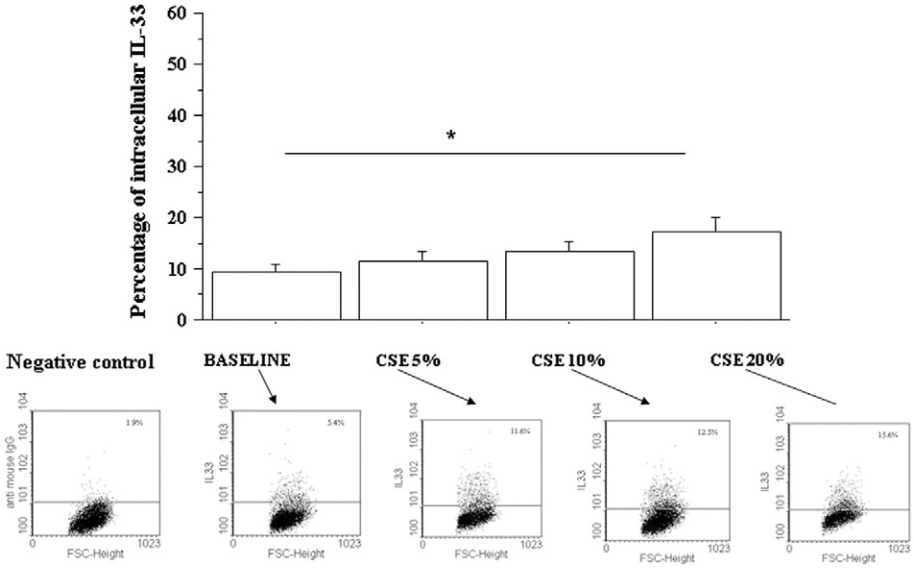


**Fig. 5.** Effects of CSE on intracellular IL-33 expression by bronchial epithelial cells (72 h). Bronchial epithelial cells (16-HBE, n = 5) were cultured in the presence and in the absence of different concentrations of CSE (5%, 10% and 20%) for 72 h. The intracellular expression of IL-33 was assessed by flow-cytometry. A. The results are expressed as percentage of positive cells (mean ± SD). *p < 0.05 vs baseline. B. Representative dot plots are shown.

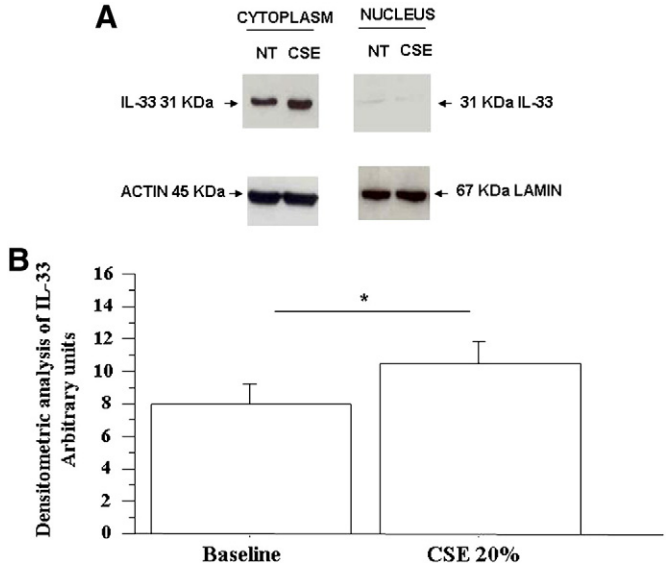


**Fig. 6.** Effects of CSE on full length IL-33 expression by bronchial epithelial cells. Bronchial epithelial cells (16-HBE, n = 3) were cultured in the presence and in the absence of CSE 20% for 24 h. A. Cytoplasmic and nuclear proteins were extracted and the full length expression of IL-33 was assessed by western blot analysis using a mouse IgG monoclonal antibody anti-IL-33. Membranes were then stripped and incubated with goat polyclonal anti-ß-actin and with rabbit polyclonal anti-lamin B1. Representative western blots are shown (NT = not treated/baseline). B. Signals corresponding to cytoplasmic IL-33 expression on the various western blots were semiquantified by densitometric scanning, normalized, and expressed after correction with the density of the band obtained for beta-actin. Data are expressed as arbitrary units ± SD. *$p < 0.05$ vs baseline.

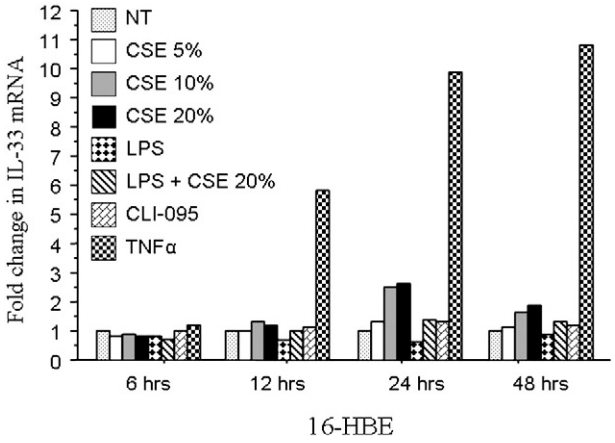


**Fig. 8.** Effects of different CSE concentrations and of TLR4 on IL-33 mRNA expression by bronchial epithelial cells. Bronchial epithelial cells (16-HBE) were cultured in the presence and in the absence of CSE (5%, 10% and 20%), of LPS (1 μg/ml), of an inhibitor of TLR4 (CLI-095) and of TNFα (100 ng/ml) for 6, 12, 24 and 48 h. Total RNA was extracted and real time PCR was used to assess IL-33 gene expression. GAPDH gene expression was used as endogenous control for normalization. Relative quantitation of mRNA was carried out with comparative CT method. Results are reported as relative unit and normalized to non-treated control (baseline).

samples. To better elucidate the underlining molecular mechanisms, in vitro experiments with CSE stimulated bronchial epithelial cells were designed. Cigarette smoke in bronchial epithelial cells reduced IL-33 release while it increased the IL-33 mRNA as well as the intracellular content of the full length form of Il-33. IL-33 may be released to effect paracrine signaling not only exclusively during cellular necrosis, as initially thought, but also by membrane-bound cytoplasmic vesicles during biomechanical overload [22]. The impairment in the release of IL-33 may be linked to the finding that IL-33 mRNA could be translated without a signal sequence for secretion [23]. It is conceivable that CSE in bronchial epithelial cells could induce this IL-33 mRNA without signal sequence for secretion and further experiments are needed to clarify this point. Alternatively CSE might induce post-transcriptional alterations on IL-33 that in turn lead to the reduced secretion. Furthermore, in the present study CSE did not increase the nuclear translocation of IL-33 in bronchial epithelial cells. Taken together these findings suggest that CSE may promote the accumulation of full length IL-33 within the cytoplasm of bronchial epithelial cells via multiple mechanisms: 1) increasing IL-33 gene expression and 2) negatively interfering with the extracellular release, and 3) not promoting the nuclear translocation.

IL-33 may be present in a full length and in a mature form. Unlike IL-1β and IL-18, full-length IL-33 is biologically active independently of caspase-1 cleavage [24] and processing by caspases results in IL-33 inactivation [25]. In our model, also at higher cigarette smoke concentration (20%) we did not observe any significant caspase-3 activation (data not shown). Neutrophil serine proteases, cathepsin G and elastase can cleave full-length human IL-33 and generate mature forms IL-33 that are biologically active and have a 10-fold higher activity than full-length IL-33 in cellular assays [24]. Intracellular full length IL-33 was found to possess transcriptional repressor properties [26]. Although in mouse the two isoforms share some activities, such as lymphocyte and neutrophil increasing infiltration, pulmonary eosinophilia and goblet cell hyperplasia are promoted by mature IL-33 [2]. IL-33 expression increases, together with an increased infiltration of neutrophils and macrophages and to increased expression of inflammatory markers, in the lung tissues of mice exposed to cigarette smoke and all these inflammatory events are reverted in cigarette smoke exposed animals by the

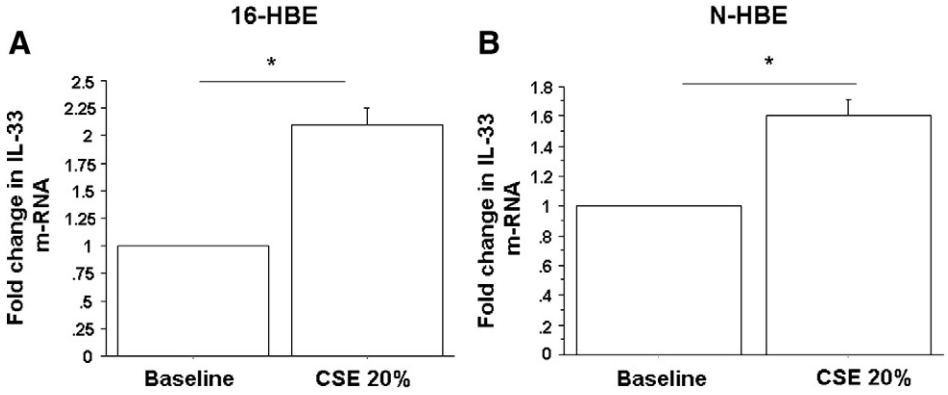


**Fig. 7.** Effects of CSE on IL-33 mRNA expression by bronchial epithelial cells. Bronchial epithelial cells (16-HBE, n = 3) (A) and primary normal bronchial epithelial cells (NHBE, n = 3) (B) were cultured in the presence and in the absence of CSE 20% for 18 h. Total RNA was extracted and real time PCR was used to assess IL-33 gene expression. GAPDH gene expression was used as endogenous control for normalization. Relative quantitation of mRNA was carried out with comparative CT method. Results are reported as relative unit and normalized to non-treated control (baseline). Data are expressed as mean ± SD. *$p < 0.05$ vs baseline.

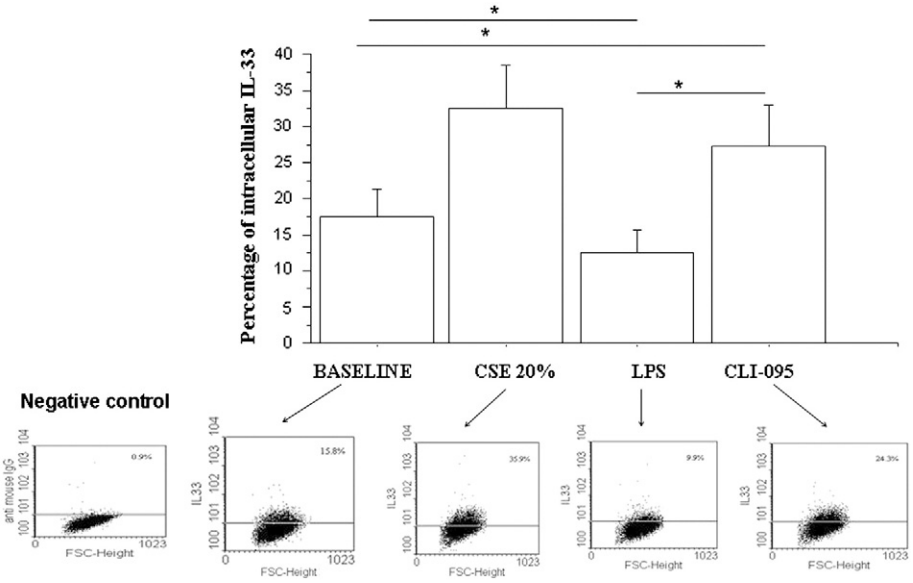


**Fig. 9.** Effect of TLR4 on intracellular IL-33 expression by bronchial epithelial cells. Bronchial epithelial cells (16-HBE, n = 3) were cultured in the presence and in the absence of CSE 20%, of LPS (1 μg/ml), and of an inhibitor of TLR4 (CLI-095) for 24 h. The intracellular expression of IL-33 was assessed by flow-cytometry. A. The results are expressed as percentage of positive cells (mean ± SD). *p < 0.05. B. Representative dot plots are shown.

intranasal administration of an anti-IL-33 antibody [5]. Infiltration of neutrophils could be due to the release of chemokines including IL-8. In this regard, it has been demonstrated that IL-33 mediates IL-8 production by epithelial cells via ERK activation [27]. IL-8 up-regulation is an event associated to cell senescence and IL-33 may be associated to senescence mechanisms since it increased in elderly in comparison to young mice [28].

IL-33 acts as a transcriptional regulator of the p65 subunit of NF-κB [29] and mediates the expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in endothelial cells both basally and in response to tumor necrosis factor-a-treatment [30]. It is not known whether IL-33 increases adhesion molecule expression also in airway epithelial cells.

IL-33 is a marker of endothelial cell quiescence, is coexpressed with the cyclin-dependent kinase inhibitor p27(Kip1) [31], and is induced by Notch 1 [3]. Notch 1 significantly suppresses TLR4 mediated inflammatory responses in macrophages [32]. In smokers with or without COPD although TLR4 expression is increased, the activation of this receptor is altered [10]. Indeed, the exposure of CSE in bronchial epithelial cells blocks the induction of HBD2 mRNA generated by exposure to IL-1 beta [10] and negatively interferes with the LPS-induced activation of NFkB pathway [8,9]. In the present study a crucial role of TLR4 in the

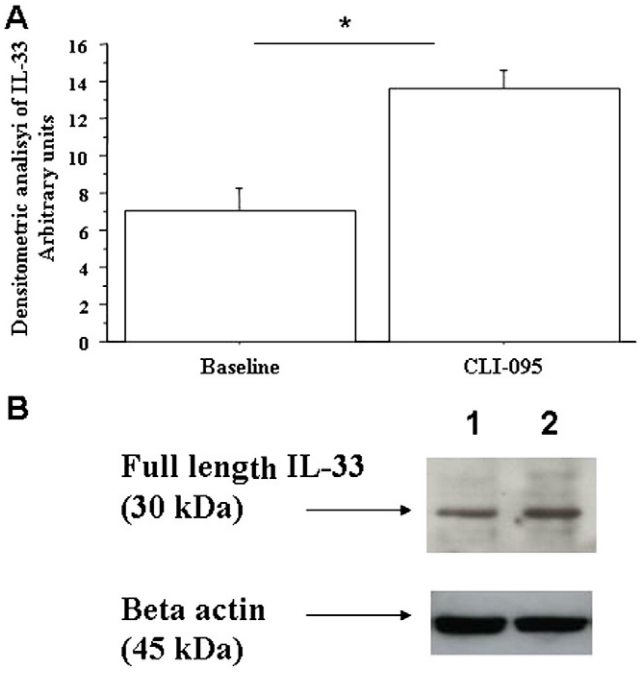


**Fig. 10.** Effects of TLR4 on full length IL-33 expression by bronchial epithelial cells. Bronchial epithelial cells (16-HBE, n = 3) were cultured in the presence and in the absence of an inhibitor of TLR4 (CLI-095) for 24 h. Total proteins were extracted and the expression of IL-33 was assessed by western blot analysis using a mouse IgG monoclonal antibody anti-IL-33. Membranes were then stripped and incubated with goat polyclonal anti-ß-actin. Signals corresponding to IL-33 on the various western blots were semiquantified by densitometric scanning, normalized and expressed after correction with the density of the band obtained for beta-actin. A. Data are expressed as arbitrary units ± SD. *p < 0.05 vs baseline. B. Representative western blots are shown. Lane 1 = baseline; lane 2 = CLI-095.

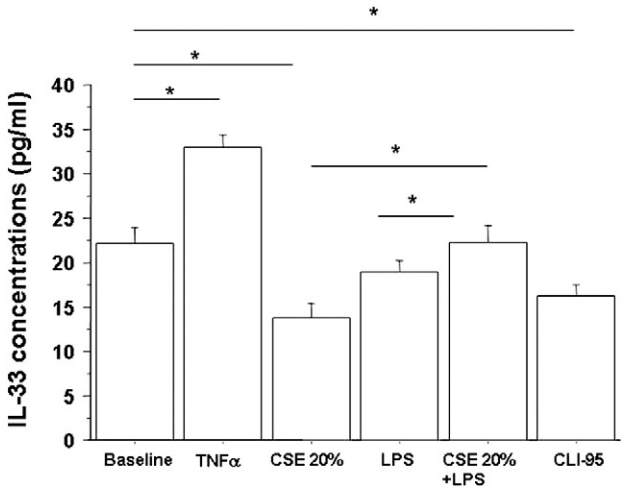


**Fig. 11.** Effects of TLR4 on IL-33 release by bronchial epithelial cells. Bronchial epithelial cells (16-HBE, n = 5) were cultured in the presence and in the absence of CSE (20%), of LPS (1 μg/ml), of an inhibitor of TLR4 (CLI-095), and of TNFα (100 ng/ml) for 24 h. The supernatants were collected and used for evaluating IL-33 concentrations by ELISA. Results are expressed mean ± SD. *p < 0.05.

expression and release of IL-33 was demonstrated since: 1) the release of IL-33 was increased inhibiting TLR4; 2) LPS reduced and a TLR4 inhibitor increased both the IL-33 mRNA and constitutive full length form of IL-33. A previous paper demonstrates that IL-33 in bronchial epithelial cells may be induced by IFNγ but not by LPS [19]. On the other hand, LPS induces IL-33 mRNA in murine macrophages [19] and IL-33 can significantly contribute to reduce innate immunity responses in skin since it decreases the expression of hBD2 in acute eczematous reaction [33]. Furthermore, since CSE may contain LPS, in our experimental model the addition of the LPS alone to bronchial epithelial cells represents an internal control to discriminate the effect of LPS and of CSE extracts.

Taken together these findings support the concept that within the airways of smokers a defective TLR4 activation could lead to increased IL-33 levels in bronchial epithelial cells and further support previous published data on asthma showing an association between IL-33 and toll like receptor pathway molecules [7].

In conclusions, in bronchial epithelial cells the expression and the release of IL-33 are tightly controlled. Cigarette smoke, within the airways, altering the innate immune responses could promote the accumulation of IL-33 in bronchial epithelial cells. Further studies aimed to clarify the functional consequences of the increased expression of IL-33 in cigarette smoke stimulated cells are needed.

## Acknowledgements

This work was supported by the Italian National Research Council.

Elisabetta Pace designed the study, performed the statistical analysis of the data, wrote the manuscript and declares that she has had access to and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Caterina Di Sano, Serafina Sciarrino, Valeria Scafidi, Maria Ferraro, Giuseppina Chiappara, Liboria Siena performed all the experiments of the study and participated to the interpretation of the data.

Patrizio Vitulo and Antonino Giarratano contributed to the patient selection, collected and managed biological samples.

Sebastiano Gangemi and Mark Gjomarkaj contributed to the interpretation of the data and to writing out the manuscript.

## Declaration of interest

The authors report no conflicts of interest.

## References

[1] Edith Puchelle, Jean-Marie Zahm, Jean-Marie Tournier, Christelle Coraux, Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease, Proc. Am. Thorac. Soc. 3 (2006) 726–733.

[2] J.E. Sims, D.E. Smith, The IL-1 family: regulators of immunity, Nat. Rev. Immunol. 10 (2010) 89–102.

[3] I.G. Luzina, E.M. Pickering, P. Kopach, P.H. Kang, V. Lockatell, N.W. Todd, J.C. Papadimitriou, A.N. McKenzie, S.P. Atamas, Full-length IL-33 promotes inflammation but not Th2 response in vivo in an ST2-independent fashion, J. Immunol. 189 (2012) 403–410.

[4] D. Préfontaine, J. Nadigel, F. Chouiali, S. Audusseau, A. Semlali, J. Chakir, J.G. Martin, Q. Hamid, Increased IL-33 expression by epithelial cells in bronchial asthma, J. Allergy Clin. Immunol. 125 (2010) 752–754.

[5] D. Préfontaine, S. Lajoie-Kadoch, S. Foley, S. Audusseau, R. Olivenstein, A.J. Halayko, C. Lemière, J.G. Martin, Q. Hamid, Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells, J. Immunol. 183 (2009) 5094–5103.

[6] Z. Guo, J. Wu, J. Zhao, F. Liu, Y. Chen, L. Bi, L. Dong, S. Liu, IL-33 promotes airway remodeling and is a marker of asthma disease severity, J. Asthma (2014 May 6) (Electronic publication ahead of print).

[7] O.E. Savenije, J.M. Mahachie John, R. Granell, M. Kerkhof, F.N. Dijk, J.C. de Jongste, H. A. Smit, B. Brunekreef, D.S. Postma, K. Van Steen, J. Henderson, G.H. Koppelman, Association of IL33-IL-1 receptor-like 1 (IL1RL1) pathway polymorphisms with wheezing phenotypes and asthma in childhood, J. Allergy Clin. Immunol. (2014), http://dx.doi.org/10.1016/j.jaci.2013.12.1080 (pii: S0091-6749[14]00059-1).

[8] C. Qiu, Y. Li, M. Li, M. Li, X. Liu, C. McSharry, D. Xu, Anti-interleukin-33 inhibits cigarette smoke-induced lung inflammation in mice, Immunology 138 (2013) 76–82.

[9] E. Pace, M. Ferraro, L. Siena, M. Melis, A.M. Montalbano, M. Johnson, M.R. Bonsignore, G. Bonsignore, M. Gjomarkaj, Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells, Immunology 124 (2008) 401–411.

[10] E. Pace, M. Ferraro, C.G. Uasuf, S. La Grutta, G. Liotta, A. Giarratano, M. Johnson, M. Gjomarkaj, Cilomilast counteracts the effects of cigarette smoke in innate responses of airway epithelial cells, Cell. Immunol. 268 (2011) 47–53.

[11] E. Pace, M. Ferraro, M.I. Minervini, P. Vitulo, Pipitone, G. Chiappara, L. Siena, A.M. Montalbano, M. Johnson, M. Gjomarkaj, Beta defensin-2 is reduced in central but not in distal airways of smoker COPD patients, PLoS One 7 (2012) e33601.

[12] L. Zhang, R. Lu, G. Zhao, S.C. Pflugfelder, D.Q. Li, TLR-mediated induction of pro-allergic cytokine IL-33 in ocular mucosal epithelium, Int. J. Biochem. Cell Biol. 43 (2011) 1383–1391.

[13] E. Pace, A. Giarratano, M. Ferraro, A. Bruno, L. Siena, S. Mangione, M. Johnson, M. Gjomarkaj, TLR4 upregulation underpins airway neutrophilia in smokers with chronic obstructive pulmonary disease and acute respiratory failure, Hum. Immunol. 72 (2011) 54–62.

[14] E. Pace, V. Scafidi, D. Di Bona, L. Siena, G. Chiappara, M. Ferraro, S. La Grutta, S. Gallina, R. Speciale, A. Ballacchino, C. Bachert, J. Bousquet, M. Gjomarkaj, Increased expression of IL-19 in the epithelium of patients with chronic rhinosinusitis and nasal polyps, Allergy 67 (2012) 878–886.

[15] F. Luppi, J. Aarbiou, S. van Wetering, I. Rahman, W.I. de Boer, K.F. Rabe, P.S. Hiemstra, Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells in vitro: role of glutathione, Respir. Res. 6 (2005) 140.

[16] A.L. Cozens, M.J. Yezzi, M. Yamaya, D. Steiger, J.A. Wagner, S.S. Garber, L. Chin, E.M. Simon, G.R. Cutting, P. Gardner, A transformed human epithelial cell line that retains tight junctions post crisis, In Vitro Cell Dev. Biol 28A (1992) 735–744.

[17] E. Pace, M. Ferraro, C.H. Mody, M. Melis, A. Bonanno, M. Profita, A. Giarratano, M. Gjomarkaj, Pleural mesothelial cells express both BLT2 and PPARa and mount an integrated response to pleural $LTB_4$, J. Immunol. 181 (2008) 7292–7299.

[18] K.R. Bartemes, H. Kita, Dynamic role of epithelium-derived cytokines in asthma, Clin. Immunol. 143 (2012) 222–235.

[19] R.T. Chustz, D.R. Nagarkar, J.A. Poposki, S. Favoreto Jr., P.C. Avila, R.P. Schleimer, A. Kato, Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells, Am. J. Respir. Cell Mol. Biol. 45 (2011) 145–153.

[20] S.K. Polumuri, G.G. Jayakar, K.A. Shirey, Z.J. Roberts, D.J. Perkins, P.M. Pitha, S.N. Vogel, Transcriptional regulation of murine IL-33 by TLR and non-TLR agonists, J. Immunol. 189 (2012) 50–60.

[21] W.V. Bonilla, A. Fröhlich, K. Senn, S. Kallert, M. Fernandez, S. Johnson, M. Kreutzfeldt, A.N. Hegazy, C. Schrick, P.G. Fallon, R. Klemenz, S. Nakae, H. Adler, D. Merkler, M. Löhning, D.D. Pinschewer, The alarmin interleukin-33 drives protective antiviral $CD8^+$ T cell responses, Science 335 (2012) 984–989.

[22] R. Kakkar, H. Hei, S. Dobner, R.T. Lee, Interleukin 33 as a mechanically responsive cytokine secreted by living cells, J. Biol. Chem. 287 (2012) 6941–6948.

[23] W. Zhao, Z. Hu, The enigmatic processing and secretion of interleukin-33, Cell. Mol. Immunol. 7 (2010 Jul) 260–262.

[24] E. Lefrançais, S. Roga, V. Gautier, A. Gonzalez-de-Peredo, B. Monsarrat, J.P. Girard, C. Cayrol, IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 1673–1678.

[25] A.U. Lüthi, S.P. Cullen, E.A. McNeela, P.J. Duriez, I.S. Afonina, C. Sheridan, G. Brumatti, R.C. Taylor, K. Kersse, P. Vandenabeele, E.C. Lavelle, S.J. Martin, Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases, Immunity 31 (2009) 84–98.

[26] V. Carriere, L. Roussel, N. Ortega, D.A. Lacorre, L. Americh, L. Aguilar, G. Bouche, J.P. Girard, IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo, Proc. Natl. Acad. Sci. U. S. A. 104 (1) (2007 Jan 2) 282–287.

[27] A. Yagami, K. Orihara, H. Morita, K. Futamura, N. Hashimoto, K. Matsumoto, H. Saito, A. Matsuda, IL-33 mediates inflammatory responses in human lung tissue cells, J. Immunol. 185 (2010) 5743–5750.

[28] H.W. Stout-Delgado, S.E. Vaughan, A.C. Shirali, R.J. Jaramillo, K.S. Harrod, Impaired NLRP3 inflammasome function in elderly mice during influenza infection is rescued by treatment with nigericin, J. Immunol. 188 (2012) 2815–2824.

[29] S. Ali, A. Mohs, M. Thomas, J. Klare, R. Ross, M.L. Schmitz, M.U. Martin, The dual function cytokine IL-33 interacts with the transcription factor NF-κB to dampen NF-κB-stimulated gene transcription, J. Immunol. 187 (4) (2011 Aug 15) 1609–1616.

[30] Y.S. Choi, J.A. Park, J. Kim, S.S. Rho, H. Park, Y.M. Kim, Y.G. Kwon, Nuclear IL-33 is a transcriptional regulator of NF-κB p65 and induces endothelial cell activation, Biochem. Biophys. Res. Commun. 421 (2012) 305–311.

[31] A.M. Küchler, J. Pollheimer, J. Balogh, J. Sponheim, L. Manley, D.R. Sorensen, P.M. De Angelis, H. Scott, G. Haraldsen, Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation, Am. J. Pathol. 173 (4) (2008 Oct) 1229–1242.

[32] Q. Zhang, C. Wang, Z. Liu, X. Liu, C. Han, X. Cao, N. Li, Notch signal suppresses Toll-like receptor-triggered inflammatory responses in macrophages by inhibiting extracellular signal-regulated kinase 1/2-mediated nuclear factor κB activation, J. Biol. Chem. 287 (9) (2012 Feb 24) 6208–6217.

[33] A. Alase, J. Seltmann, T. Werfel, M. Wittmann, Interleukin-33 modulates the expression of human β-defensin 2 in human primary keratinocytes and may influence the susceptibility to bacterial superinfection in acute atopic dermatitis, Br. J. Dermatol. 167 (2012) 1386–1389.