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Common and differential roles of inducible NO synthase and poly (ADP-ribose) polymerase in allergen-induced inflammation and airway hyperresponsiveness: a potential connection to NO levels

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Summary

Asthma is a chronic disease of the airways characterized by inflammation, structural and functional changes that are responsible for bronchial hyperresponsiveness (AHR) and limitation in airflow. While asthma symptoms can be controlled in the majority of patients, in around 5-10% of asthmatics, the disease remains symptomatic and inadequately controlled. For these reasons, additional therapeutic approaches are urgently required to provide better life conditions for these individuals.

The present study is focused on the role of inducible Nitric Oxide Synthase (iNOS) and poly(ADP-ribose) polymerase-1 (PARP-1) in the pathophysiology of asthma and their potential interaction and cooperation.

The role of iNOS in asthma has been examined in numerous studies with conflicting results. Its potential as a viable therapeutic target for the treatment of the disease was severely hampered by the negative results of a clinical trial showing that a selective iNOS inhibitor, although reducing effectively exhaled Nitric Oxide (eNO), did not affect AHR or airway inflammation after allergen challenge in human subjects with asthma. We believe that such conclusion is premature and that more careful studies are necessary to fully understand the role of iNOS in asthma and whether the enzyme can be adequately targeted at least as an adjuvant therapy for the treatment of this disease.

In the current study I report that iNOS inhibition, pharmacologically, using the relatively selective inhibitor L-NIL or by gene knockout, in our animal experimental models provides an excellent protection against AHR upon an acute, but not upon a chronic, exposure to allergens (OVA or HDM) as assessed using full body plethysmography. These results correlate with the differential protection provided by iNOS inhibition against airway inflammation observed in a previous study.

Considering all known beneficial effects of NO through its contribution to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium, I speculate that complete inhibition of iNOS, achieved by L-NIL administration or even more efficiently by gene knockout may be deleterious for some aspects of asthma such as AHR, which highly involve one of the major targets of NO, the smooth muscle cells. The fascinating aspect of my study is that the loss of protection observed in the chronic asthma model after L-NIL treatment was reversed by NO supplementation through administration of nitrite, a moderate NO source. The anion nitrite (NO_2^-) can be reduced to NO upon a reaction with deoxyhemoglobin.

Our group, in previous studies, also established a reciprocal relationship between iNOS and PARP-1 with a special connection to oxidative DNA damage and IL-5 in the process of eosinophilia during allergen-induced lung inflammation. Such relationship is also based on the fact that PARP-1 exerts a decisive role on the interaction between p65 NF-κB and the exportin protein Crm1. PARP-1 activity, decrease the interaction between the transcription factor and Crm1, increasing the quantity of p65 NF-κB detectable in the nuclei of cells upon TLR4 stimulation. This increase leads to an upregulation of NF-κB–dependent gene expression, which includes iNOS.

PARP inhibition, pharmacologically by Olaparib (AZD2281) or by gene knockout, protects against inflammation and AHR both upon single and multiple exposures to OVA. Similar protection was observed upon chronic exposure to HDM, demonstrating that the protective effect is

not limited to the type of allergen used. Such protection may occur as a consequence that iNOS expression is markedly reduced upon PARP inhibition. However, it is interesting that PARP-1 inhibition does not completely abrogate expression of iNOS leaving the possibility that the protective effect of PARP inhibition against inflammation and AHR may be associated with the reduction but not the complete inhibition of iNOS and with consequent production of moderate levels of NO. A further confirmation of our hypothesis is that administration of iNOS inhibitor (L-NIL) to chronically HDM-exposed mice that received a PARP inhibitor (Olaparib-AZD2281) reversed the protection against AHR provided by PARP inhibition.

Overall, our results may explain why clinical studies focused on the complete inhibition of iNOS failed to protect against different aspects of asthma. However, further efforts to investigate the exact role of the enzyme in asthma may provide a clearer view on how to utilize iNOS as a therapeutic target. But what seems to be plausible is that moderate levels of NO achieved through partial inhibition of iNOS could represent a good therapeutic strategy at least against some aspect of the disease, such as AHR. Additionally, our results revealed that targeting PARP-1 may constitute a better alternative therapeutic approach. Such strategy seems to be very efficient at reducing the most important asthma traits (e.g. Th2 cytokines, mucus, and IgE production) but also partially reduces expression of iNOS with a consequent moderate production of NO that provides protections against AHR. It is important to note that the beneficial effects of PARP inhibition could be correlated with the capacity of PARP-1 to positively influence GATA3, the mast regulator of Th2 population, the paramount cells in the manifestation of the allergic disease. This is, of course, without omitting the potential impact of PARP inhibition on the T-reg population, which is responsible for the control and reduction of inflammation. Finally, it is noteworthy that L-NIL (*L*-N6-(1-Iminoethyl)lysine dihydrochloride) and AZD2281 (olaparib) are two clinically tested iNOS and PARP inhibitors, respectively, which increases considerably the clinical relevance of this study.

Riassunto

L'asma è una patologia cronica delle vie aeree caratterizzata da infiammazione e modificazioni strutturali e funzionali responsabili dell'iper-responsività bronchiale e delle marcate limitazioni respiratorie. Mentre i sintomi dell'asma possono essere ben controllati nella maggior parte dei pazienti, in circa il 5-10% delle persone affette la malattia rimane sintomatica e non controllata in maniera adeguata.
Per questa ragione, nuovi apporci terapeutici sono decisamente necessari.
Questo studio è incentrato sul ruolo di iNOS (inducible Nitric Oxide Synthase) e PARP-1 (Poly(ADP-ribose) polymerase-1) nella patofisiologia dell'asma e nella loro potenziale interazione e cooperazione per orchestrare i vari meccanismi alla base della patologia.
Il ruolo di iNOS nell'asma è stato ampiamente esaminato in diversi studi con risultati molto contrastanti. Il suo potenziale come target terapeutico per il trattamento della malattia è stato negativamente valutato in seguito ai risultati ottenuti in un trial clinico che ha mostrato che l'uso di un inibitore selettivo per iNOS, GW274150, nonostante abbia ridotto significativamente i livelli di Ossido Nitrico esalato (eNO) non è stato in grado di inibire l'iper-responsività e l'infiammazione delle vie aeree in pazienti asmatici sottoposti ad una stimolazione con un allergene.
Noi crediamo che le conclusioni raggiunte in seguito ai risultati emersi da questo lavoro siano troppo affrettate e premature. Studi più accurati sono pertanto necessari ai fini di comprendere pienamente il ruolo di iNOS nell'asma e rivalutare il suo potenziale come target terapeutico per il trattamento della malattia.
In questo studio, condotto in modelli animali, l'inibizione di iNOS, ottenuta farmacologicamente, utilizzando un inibitore relativamente selettivo, L-NIL, o geneticamente tramite knockout, ha mostrato un eccellente protezione contro l'iper-responsività bronchiale in seguito ad un esposizione acuta ma non cronica all'allergene (OVA (Ovalbumin) o HDM (House Dust Mite).
Questi risultati correlano con quelli ottenuti in un precedente studio condotto nel nostro laboratorio dove l'inibizione di iNOS ha conferito protezione contro l'infiammazione in un modello acuto di asma ma non in quello cronico.
Considerando tutti gli effetti benefici dell'Ossido Nitrico nel mantenimento dell'omeostasi del sistema cardiovascolare, attraverso l'inibizione della contrazione e crescita delle cellule della muscolatura liscia, dell'aggregazione delle piastrine e dell'adesione dei leucociti all'endotelio, abbiamo ipotizzato che la completa inibizione di iNOS potesse essere negativa tanto quanto l'iper-attivazione, per alcuni aspetti dell'asma, in particolare per l'iper-responsività delle via aeree, che appunto coinvolge uno dei più importanti target dell'Ossido Nitrico: le cellule muscolari lisce.
Il risultato più affascinante di questo studio è che la perdita di protezione osservata in un modello cronico di asma, in seguito alla somministrazione di L-NIL, viene completamente recuperata in seguito alla somministrazione di Nitriti come fonte di Ossido Nitrico. L'anione nitrito, infatti, può essere ridotto ad NO in seguito all'interazione con la deossiemoglobina.
In uno studio, precedentemente condotto dal nostro gruppo, è stata dimostrata l'esistenza di una relazione reciproca tra iNOS e PARP-1 durante il processo infiammatorio indotto da una o più esposizioni ad un allergene. Tale processo include un marcato danneggiamento del DNA mediato da stress ossidativo ed una consistente produzione di IL-5 seguita da forte eosinofilia. Questa realzione è anche basata sul fatto che PARP-1 esercita un ruolo decisivo nell'interazione tra la subunità p65 di NF-kB e Crm-1, un'esportina coinvolta nella fuoriuscita del fattore trascrizionale dal nucleo. PARP-1 attraverso la modificazione di queste proteine è in grado di ridurne l'interazione, portando ad un conseguente aumento di p65 NF-kB nel nucleo di cellule in cui è stata

attivata la via del TLR4 e ad una marcata up-regolazione della trascrizione di tutti i geni che sono sotto il suo controllo, incluso iNOS.
A questo proposito, risulta molto interessante il fatto che, l'inibizione farmacologica di PARP-1 attraverso la somministrazione di un inibitore specifico (Olaparib), o tramite knockout, sia in grado di proteggere sia dall'infiammazione che dall'iper-responsività delle vie aeree in seguito ad una singola o multipla esposizione all'allergene ovalbumina.
Risultati simili sono stati osservati anche in seguito ad un esposizione cronica all'acaro della polvere (HDM) dimostrando che l'effetto protettivo non è limitato al tipo di allergene utilizzato.
Tale protezione potrebbe essere una conseguenza del fatto che l'espressione di iNOS sia fortemente ridotta in seguito all'inibizione di PARP.
Infatti uno degli aspetti più interessanti di questo studio è che l'inibizione di PARP-1 non sia in grado di azzerare completamente l'espressione di iNOS, lasciando aperta la possibilità che l'effetto protettivo osservato contro l'infiammazione e l'AHR possa essere associato alla riduzione ma non alla completa inibizione di iNOS e conseguente moderata produzione di NO.
Un'ulteriore conferma della nostra ipotesi è che la somministrazione dell'inibitore di iNOS, L-NIL, in topi precedentemente trattati con un inibitore di PARP (Olaparib-AZD2281), ai fini di azzerare i livelli di NO, annulla la protezione ottenuta verso l'AHR.
In conclusione, i nostri risultati, che insieme supportano l'esistenza di un importante ruolo di iNOS nella patogenesi dell'asma, possono spiegare, almeno in parte, perché gli studi clinici, mirati alla completa inibizione dell'enzima, non siano stati in grado di proteggere contro i diversi aspetti dell'asma. Ovviamente, ulteriori studi, ai fini di approfondire l'esatto ruolo dell'enzima in questa patologia, potrebbero fornire una visione più chiara su come utilizzare al meglio iNOS come target terapeutico. Tuttavia, sembra esser chiaro che il raggiungimento di moderati livelli di NO, ottenuti in seguito ad una parziale inibizione di iNOS, possa rappresentare un'ottima strategia terapeutica, almeno contro alcuni sintomi dell'asma come l'AHR.
D'altra parte, i nostri risultati hanno anche rivelato che l'utilizzo di PARP-1 come target terapeutico possa rappresentare una migliore strategia. Prima di tutto perché, attraverso la sua capacità di modulare i livelli di espressione di iNOS, tramite la sua interazione con NF-kB, l'inibizione di PARP-1 è stata in grado di ridurre l'AHR. Ma anche perché è stata in grado di ridurre tutti i principali sintomi dell'asma: infiammazione, produzione di IgE ed eccessiva produzione di muco. I nostri risultati preliminari suggeriscono che questi effetti benefici possano essere correlati alla capacità di PARP-1 di interagire e regolare in maniera positiva GATA3, il fattore trascrizionale responsabile del differenziamento e mantenimento dei linfociti Th2, la sottopopolazione di linfociti T helper maggiormente coinvolta nelle patologia di tipo allergico, e probabilmente per la sua capacità di inibire la popolazione dei linfociti T regolatori (Treg) responsabili del controllo e della riduzione dell'infiammazione.
Infine, è importante sottolineare che sia L-NIL che Olaparib, rispettivamente i due inibitori di iNOS e PARP, sono stati anche precedentemente testati clinicamente. Questo aumenta considerevolmente la rilevanza di questo studio.

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List of abbreviations

iNOS: inducible Nitric Oxide Synthase

PARP-1: Poly(ADP-ribose) polymerase-1

NO: Nitric Oxide

eNO: exaled Nitric Oxide

AHR: Airway Hyperresponsivness

OVA: Ovalbumin

HDM: House Dust Mite

L-NIL: L-N6-(1-Iminoethyl)Lysine dihydrochloride

NF-kB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

Crm-1: Chromosomal maintenance 1

TLR4: Toll Like Receptor 4

Th2: Type 2 helper T cells

Ig**…**: Immunoglobulin

FEV1: Forced Expioratory Volume in one second

FVC: Forced Vital Capacity

PC20: Provocative Concentration of broncoconstrictor causing 20% fall in FEV1

ppb: part per million

PEF: Peak Expiratory Flow

DCs: Dendritic Cells

APCs: Antigen Presenting Cells

IL**…**: Interleukin

TCR: T Cell Receptor

GATA3: GATA-binding protein 3

IL-**…**R: Interleukin **…** Receptor

CD**…**: Cluster of differentiation

MBP: Major Basic Protein

TGF-β: Tumor Growth Factor beta

RORγ: RAR-related Orphan Receptor gamma

T-bet: T-box transcription factor

FOXP3: Forkhead box P3

Treg: Regulatory lymphocytes T

CXCL: (C-X-C motif) ligand

SCF: Stem Cell Factor

RANTES: Regulated on Activation, Normal T cell Expressed and Secreted

MCP-1: Monocyte Chemotactic Protein 1

TSLP: Tymic Stromal Lymphoprotein

TNFα: Tumor Necrosis Factor alpha

CCR: C-C chemokine receptor

ASM: Airway Smooth Muscle

ECM: Extracellular Matrix

TIMP-2: Tissue Inhibitor of Metalloproteinases

MMP: Matrix Metalloproteinases

VEGF: Vascular Endothelium Growth Factor

ICAM: Intercellular Adhesion Molecule

VCAM: Vascular Cell Adhesion Molecule

DRS: Dose Response Slope

MLCK: Myosin Light Chain Kinase

MLCP: Myosin Light Chain Phosphatase

WBP: Whole Body Pletismography

H4B: Tetrahydrobiopterin

CaM: Calmodulin

RNOI: Reactive Nitric Oxide Intermediate

RNOS: Reactive Nitric Oxide Species

LPS: Lipopolysaccharides

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

IFN-γ: Interferon gamma

G-CSG: Granulocite Colony Stimulating Factor

COX: Cyclooxygenase

AMD: Auto-Modification Domain

PARG: Poly (ADP-ribose) glycohydrolase

PAR: poly(ADP-ribose)

KC: Keratyinocytes Chemoattractant

ACT: Asthma Control Test

Penh: enanched Pause

H&E: Hematoxylin and Eosin

PAS: Periodic acid–Schiff

DAPI: 4',6-Diamidino-2-Phenylindole

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

PBMCs: Peripheral Blood Mononuclear Cells

WT: Wild Type

MeCh: Methacholine

IP-10**:** IFN-γ-Inducible Protein 10

i.p.: intraperitoneal

BAL: Broncho-alveolar Lavage

SD: Standard Deviation

FACS: Fluorescence-activated cell sorting

eNOS: endothelial Nitric Oxide Synthase

nNOS: neuronal Nitric Oxide Synthase

Introduction

1.Asthma disease

Asthma is a chronic inflammatory disease characterized by airway hyperreactivity (AHR), mucus overproduction, airway and vascular wall remodeling and airway narrowing [1-4]. All these symptoms lead to repeated periods of shortness of breath, wheezing and chesttightness. The incidence of the disease has drastically increased in the last few years. At the moment there are 1 in 10 children and 1 in 12 adults are affected by the disease contributing for a total of 334 million people worldwide. The United Kingdom, New Zealand, Australia, Canada, and the United States show the highest prevalence of asthma.

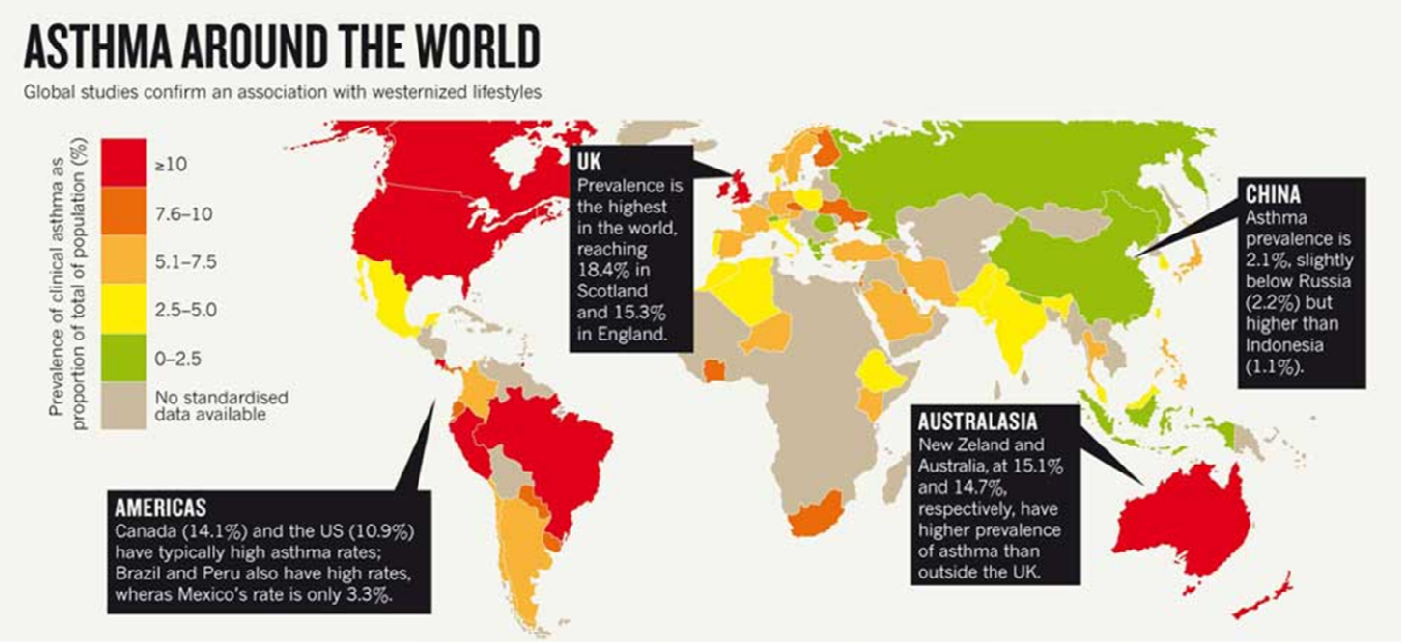


Figure 1. World map of prevalence of clinical asthma [5]

Worldwide, deaths from asthma have reached over 250.000 annually [5] (*http://www.globalasthmareport.org/resources/Global_Asthma_Report_2014.pdf)*.
This results in significant morbidity and high healthcare costs; indeed, it was estimated that both the direct and indirect cost caused by loss of productivity, has exceed 18 billion of dollar annually only in the USA.

Age-standardised mortality rates for asthma, all ages 2001 2010

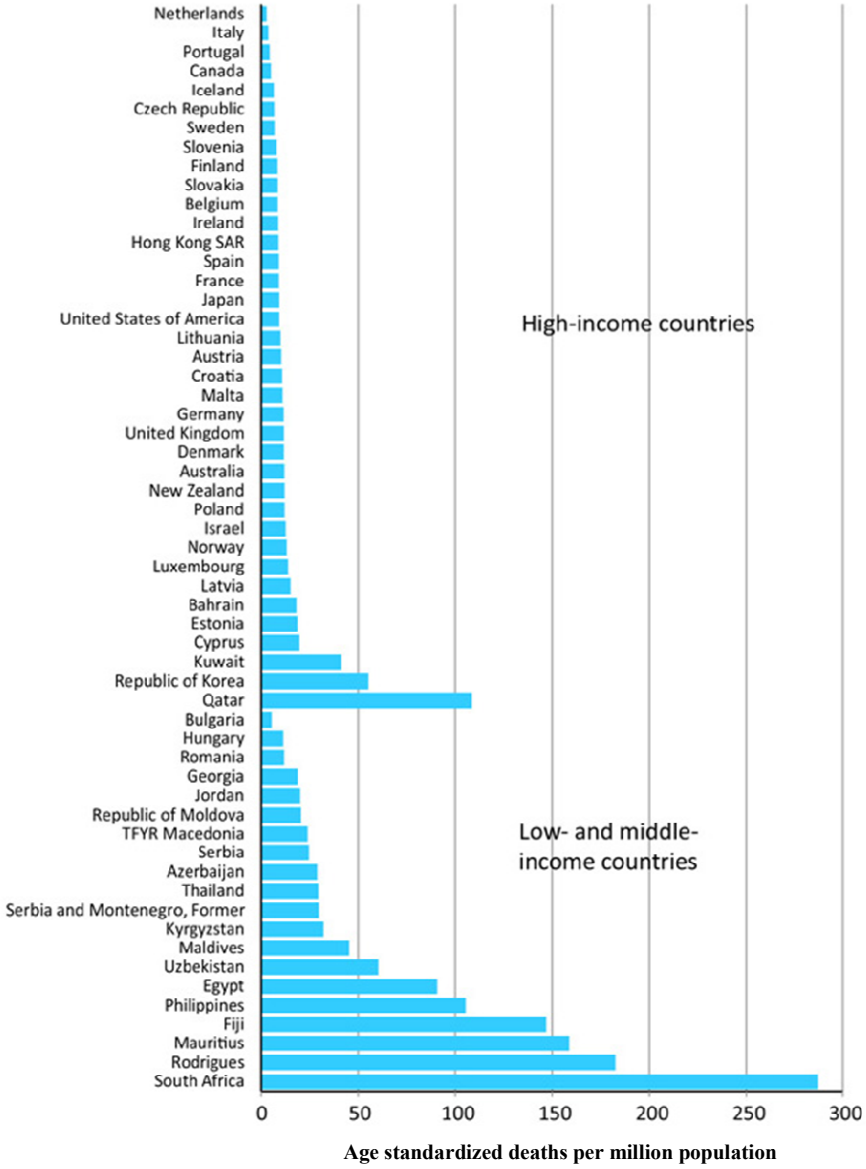


Figure 2. Age-standardised asthma mortality rates for all ages 2001-2010 from countries where asthma is separately coded as a cause of death, ordered by mortality rate and country income group (WHO Detailed Mortality Database, February 2014 update).

Asthma can be controlled by a combination of an inhaled corticosteroid (anti-inflammatory) and a short-or long-acting β2-adrenergic agonist (which open the constricting bronchial smooth muscle). However, a big percentage of patients, 5-10%, is not responding to the available therapy and although death from asthma has decreased with the regular use of inhaled glucocorticoids, the global impact of asthma remains high, and the prevalence of asthma seems to be increasing (*http://www.globalasthmareport.org/resources/Global_Asthma_Report_2014.pdf)[6-8]*. Thus, new therapies that target all or some of the symptoms of asthma are urgently needed.

Usually, two main different type of asthma are described in the clinic: the allergic asthma that affect most of the children and 50% of adults and the non-allergic or intrinsic asthma [9, 10]. In allergic asthma the disease coincides with allergic sensitization that can be defined by the presence of serum immunoglobulin E (IgE) and/or by a positive skin-prick test to the (lipo) proteins of common inhaled or ingested allergens such as house dust mite (HDM), animal dander, fungal spores, plant or tree pollen, or peanuts and other. The non-allergic disease, conversely, generally develops later in life and has neither IgE reactivity to allergens in the serum nor any obvious involvement of the adaptive immune system such as type 2 helper T cells (Th2 cells). This form of the disease is more common in women and is generally associated with chronic rhino sinusitis and nasal polyps, as well as obesity, and it is difficult to treat, often requiring long-term treatment with systemic steroids. However, this classification is oversimplified, in fact, over the last few years, more detailed studies have identified different asthma phenotypes, each with a distinct pathophysiology. These are defined as asthma endotypes. The endotypes differ in terms of genetic susceptibility, environmental risk factors, age of onset, clinical presentation, prognosis and response therapies [11-13]. Asthma is therefore increasingly seen as a syndrome rather than a single disease [13, 14]. In people with asthma who smoke, there is also considerable clinical overlap with chronic obstructive pulmonary disease.

1.1.Etiology

Several factors can be involved in the pathophisiology of asthma: host factors such as genetics, sex, obesity and environmental factors including allergens, airborne pollutants and respiratory infection are the major contributors in progression of the disease. [15-17]. The prevalence of asthma in children was found to be higher in boys than girls. But in the adulthood a greater prevalence of asthma has been reported in women [17-19]. It has been also demonstrated that obesity is highly associated with severity of asthma [20-22]; but, in this case, the phenotype appears to be predominantly non-Th2 mediated response. [21].

Exposure to allergens has been shown to be one of the strongest determinants of asthma [23-29]. Early childhood Infections with Respiratory Syncytial Virus (RSV), Human Rhinovirus (HRV), *Streptococcus pneumoniae* and *Haemophilus influenza* have been shown to be associated with development of asthma later in life [30-33]. The strong association between asthma and allergic rhinitis has suggested that patients with respiratory syndromes may be more susceptible to developing asthma *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from*:* http://www.ginasthma.org/).

Many epidemiology studies have revealed that exposure to air pollutants, such as particulate matter (PM) and ozone (O3), increases the risk of developing asthma *de novo* [34, 35] and exacerbations of established asthma [36, 37]. Also cigarette smoking is an important air pollutant risk factor for the development of asthma. Moreover, the asthma symptoms are highly severe in active smokers compared with asthmatic non-smokers [23, 38]. The children who have smoker parents or friends (i.e. passive smokers) are more likely to have asthma symptoms [38, 39].

Recent studies have shown that diet rich in vitamin E, vitamin D, zinc, selenium and iron, during pregnancy could be protective for the baby against the development of asthma [40]. Breast-fed children are also considered a protective factor against the probability to developed asthma during the childhood [41].

1.2.Diagnosis

The diagnosis of asthma is based on the respiratory signs and symptoms including cough, wheezing, chest tightness and dyspnea.

Some laboratory tests as spirometry or peak expiratory flow monitoring may also be used for diagnosis of the disease. The spirometry is a technique that evaluates airflow limitation and reversibility using a forced expiratory maneuver using a spirometer to measure forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) [42] *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: http://www.ginasthma.org/). Flow reversibility is used to characterize rapid improvements in FEV1 (i.e. minutes) following inhalation of a rapid-acting bronchodilator. FEV1 reversibility may be used to diagnose asthma; a ≥12% or 200 ml change in FEV1 from the pre-bronchodilator value is generally accepted to be indicative of an asthmatic response *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: *http://www.ginasthma.org/*). Reduction in FEV1 can be seen in many lung diseases. Therefore it is important in asthma to assess the ratio of FEV1 to FVC. The FEV1/FVC ratio is considered to be normal if it is greater than 0.75 to 0.80 in adults and greater than 0.90 in children.

Any values lower than these may suggest airflow limitation *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: *http://www.ginasthma.org/*).

Summary of Tests Used to Assess Asthma

Measurement	Methodology	Measurement Characteristics	Comments
Spirometry	Enables clear demonstration of airflow obstruction FEV_1 largely independent of effort and highly repeatable Less applicable in acute severe asthma Assesses only one aspect of the disease state	Normal ranges widely available and robust Short-term (20-minute) 95% range for repeat measure of FEV_1 <160 mL; FVC <330 mL, independent of baseline value	Good for short- and longer-term reversibility testing in subjects with preexisting airflow obstruction >400-mL increase in FEV_1 highly suggestive of asthma Less helpful in subjects with normal pretreatment values because of ceiling effect
Peak expiratory flow (PEF)	Widely available and simple Applicable in a wide variety of circumstances including acute severe asthma PEF variability can be determined from home readings in most subjects PEF effort dependent and not as repeatable as FEV_1 Assesses only one aspect of the disease state	Normal ranges of PEF are wide, and currently available normative tables are outdated and do not encompass ethnic diversity Change in PEF more meaningful than absolute value >60 L/min increase in PEF suggested as best criteria for defining reversibility Normal range of PEF variability defined as amplitude % highest <8% or <20% depending on number of readings and degree of patient coaching	Useful for short- and longer-term reversibility testing in subjects with preexisting airflow obstruction Less helpful in subjects with normal pretreatment values because of ceiling effect Little information on the use of PEF variability as an index of treatment response PEF monitoring may improve asthma control in patients with more severe disease and in those with poor perception of bronchoconstriction
Asthma Control Questionnaire	Response to 7 questions, 5 relating to symptoms, 1 rescue treatment use and 1 FEV_1 Response usually assessed over the preceding week Shortened, five-question symptom only questionnaire is just as valid	Well-controlled: ≤0.75; inadequately controlled: ≥1.5 95% range for repeat measure ± 0.36 Minimal important difference 0.5	
Mini Asthma Quality of Life Questionnaire (AQLQ)	Response to 15 questions in 4 domains (symptoms, activity limitations, emotional function, environmental stimuli) Response usually assessed over the preceding week Closely related to larger 32-item Asthma Quality of Life Questionnaire	95% range for repeat measure ± 0.36 Minimal important difference 0.5	Well-validated quality of life questionnaire Could be used to assess response to longer term treatment trials
Airway responsiveness	Responsive to change (particularly indirect challenges such as inhaled mannitol) Less of a ceiling effect Not applicable in patients with impaired lung function (i.e., FEV_1/FVC <0.7 and <70% predicted)	Normal methacholine PC_{20} >8 mg/mL 95% range for repeat measure ± 1.5-2 doubling doses	Has not been widely used to monitor disease and assess treatment responses
Exhaled nitric oxide (FeNO)	Measurements can be obtained in almost all adults Results immediately available Reasonably close relationship between FeNO and eosinophilic airway inflammation, which is independent of gender, age, atopy, and inhaled corticosteroid use Not closely related to other measures of asthma morbidity	Normal range <25 ppb at exhaled flow of 50 mL/sec. 95% range for repeat measure 4 ppb >50 ppb highly predictive of a positive response to corticosteroid therapy <25 ppb highly predictive of a poor response to corticosteroids or successful step down in corticosteroid therapy	Evidence that FeNO can be used to guide corticosteroid treatment is mixed. Protocols for diagnosis and monitoring have not been well defined, and more work is needed Low FeNO (<25 ppb) may be of particular value in identifying patients who can step down corticosteroid treatment safely
Sputum eosinophil differential count	Information available in 80-90% of patients, although immediate results not available Sputum eosinophil count not closely related to other measures of asthma morbidity	Normal range <2%; 95% range for repeat measure ± 2-3 fold	Close relationship between raised sputum eosinophil count and corticosteroid responsiveness Use of sputum eosinophil count to guide corticosteroid therapy associated with reduced exacerbations in patients with more severe disease

FEV1: forced expiratory volume in one second; FVC: forced vital capacity; PC20: provocative concentration of bronchoconstrictor (methacholine) causing a 20% fall in FEV1; ppb: part per million.

Figure 3. Summary of Tests Used to Assess Asthma

For patients with normal lung function but asthma symptoms, diagnosis can be confirmed by airway responsiveness (AHR) to direct or indirect airway challenges. Inhaled methacholine and

histamine are traditionally used for direct challenges [42, 43] *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: http://www.ginasthma.org/), while inhaled mannitol [43] or exercise are used for indirect challenges *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: http://www.ginasthma.org/) [43-45].

1.3. Treatment

To date, the most common treatment of asthma is the glucocorticosteroids, potent anti-inflammatory agents able to decrease also the airway hyperresponsiveness and to reduce frequency and severity of exacerbation with an evident improvement of lung function *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: http://www.ginasthma.org/) [46, 47].

Other classes of drug used for the treatment of the disease include the β2-agonists and anti-cholinergic agents *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: http://www.ginasthma.org/) [46, 47]. These types of treatment are considered as relieve therapy. Anticholinergics such as tiotropium bromide act as antagonists of muscarinic receptors, and block the effects of endogenous acetylcholine and consequent broncoconstriction. Leukotriene modifiers are also able to reduce the main asthma symptoms. These medications are used for mild asthma or as add-on to inhaled glucocorticosteroids *(From the Global Strategy for Asthma Management and Prevention,* Global Initiative for Asthma (GINA) 2015. Available from: http://www.ginasthma.org/) [46], particularly for patients whose symptoms are triggered by allergies. These drugs are used orally and appear to be safe. Recently Omalizumab has been licensed for use in severe allergic asthma. Omalizumab is used as an adjunctive therapy for patients up to 12 years of age who have allergies and severe persistent asthma. It is a recombinant DNA-derived humanized IgG1 monoclonal antibody that selectively binds to free and membrane-bound immunoglobulin E (IgE) antibodies [48].

While asthma symptoms can be controlled in the majority of patients using current standard therapy, in around 5-10% of people with asthma the disease remains symptomatic and inadequately controlled [49, 50]. In these patients, asthma symptoms may worsen with onset of concomitant co morbidities including rhinitis, sinusitis, gastro esophageal reflux, obesity and obstructive sleep apnea [51]. Thus, although patients with uncontrolled asthma are a minority of the total asthmatic population, they have a high risk of serious morbidity and mortality, and use the largest share of economic resources and health-care services, including emergency visits, hospitalizations and additional consumption of drugs for recurrent exacerbations [52]. Moreover, severe asthma results

in frequent absences from school and work, and patients with difficult-to-treat disease are often prone to anxiety and depression [53]. For these reasons, additional therapeutic approaches are urgently required for those individuals who have poorly controlled asthma.

1..4.Airway inflammation in asthma

Airway inflammation is the main hallmark in asthma which affects airflow through the production of mucus, release of inflammatory mediators, and by enhancing susceptibility to bronchospasm (*Guidelines for the Diagnosis and Management of Asthma.* 2007). Several cells are involved in the orchestring of asthma pathophisiology, such as dendritic cells (DCs), T lymphocytes, eosinophils, mast cells, neutrophils, and macrophages.

After the antigen exposure, the resident antigen-presenting cells (APCs), such as dendritic cells, present in the airway epithelium and in the mucosa, bind the inhaled antigen and migrate to the lymphonodes to start a specific immune response. The Dendritic cells (DCs) present the antigens to naive T-cells and induce expression of selective cytokines. The presence of DCs consequentially leads to activation and differentiation of T-cells to T helper 2 (Th2) subtype and contributes indirectly to the development of airway inflammation [54].

1.5.Cytokines network in asthma

More than 50 cytokines are involved in the regulation of the asthma pathophisiology. They are responsible for the recruitment, activation and promotion of survival of several inflammatory cells in the airway. All the cytokines can be classified in different type: lymphokines, pro inflammatory cytokines, growth factors, chemokines and anti-inflammatory cytokines.

1.5.1.Lymphokines: The lymphokines include cytokines that are released predominantly from T cells, which play a critical role in orchestrating inflammation in asthma. Asthmatics patients show a substantial increase of lymphocytes $CD4^+$ T helper ($CD4^+$ Th) in the respiratory tract that are represent in high percentage by the subtype 2 (Th2) [55]. These cells are responsible for the synthesis of IL-4, IL-5, IL-9 and IL-13. In addition, IL-33, which is expressed by a wide variety of cell types, including fibroblasts, mast cells, dendritic cells, macrophages, osteoblasts, endothelial cells, and epithelial cells, has been shown as promoter of Th2 cells differentiation. IL33 is able to regulate the transcription of different genes through an effect on the chromatin structure [56]; it is also a potent chemoattractant of Th2 cells [57]. IL-4 is the cytokines that drive the differentiation of

the Th2 cells from T cells naïve and it is very important in the initial sensitization to allergens [58]. It is also crucial for the isotype switching of B lymphocyte from IgG to IgE [59] [60]. Another important cytokine involved in the asthma network is IL-13 that shares some STAT6-mediated effect, with IL-4, binding an herodimeric receptor made by the chain of the IL-4 receptor (IL-4Rα) and a specific IL-13–binding chain (IL-13Rα) [61] [62]; IL13Rα2 is a different receptor for IL-13 that may act as a decoy receptor. As IL13 is able to induce airway hyperresponsivness (AHR) and to cause airway remodeling by inducing mucus hyperproduction, airway smooth muscle proliferation and subepithelial fibrosis, this cytokine is considered an interesting potential therapeutic target for asthma [61]. Several cell (Th1 cells, lymphocyte $CD8^+$ T citotoxic subtype 2 (Tc2), NKT cells and inflammatory cells: eosiniphils, basophils and mast cells) can produce IL-13.

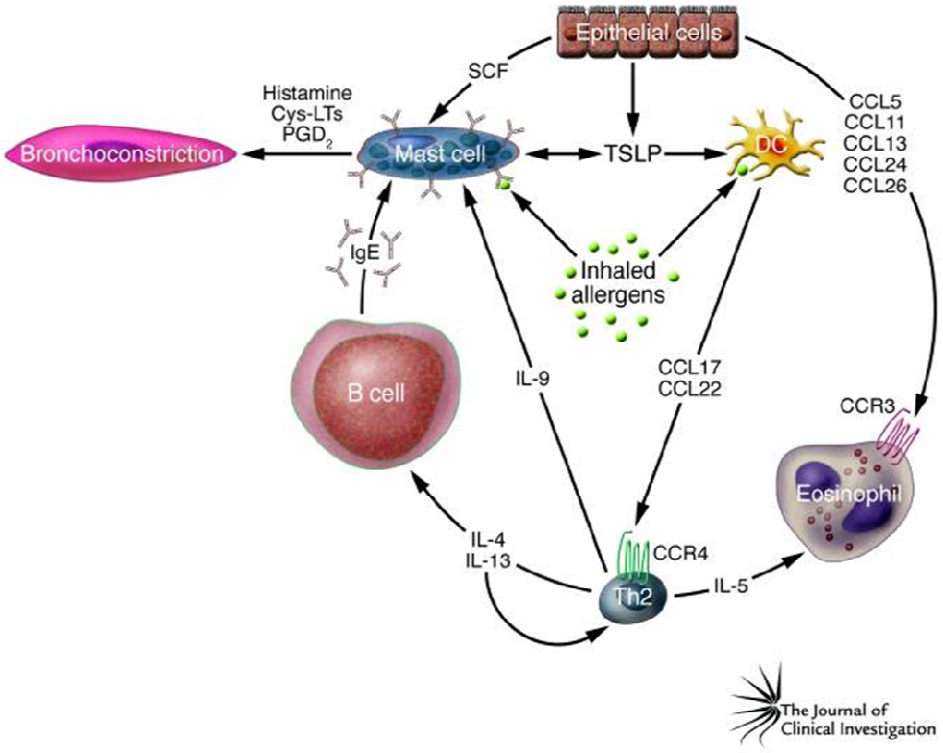


Figure 4. Cytokines involved in asthma.

Epithelial cells play an important role in orchestrating the inflammation of asthma through the release of multiple cytokines, including SCF (which maintains mast cells in the airways), TSLP (which acts on DCs to release the Th2 chemoattractants CCL17 and CCL22, which act via CCR4), and several chemokines that attract eosinophils by activating CCR3. Th2 cells orchestrate the inflammatory response in asthma through the release of IL-4 and IL-13 (which stimulate B cells to synthesize IgE), IL-5 (which is necessary for eosinophilic inflammation), and IL-9 (which stimulates mast cell proliferation). Mast cells are thus orchestrated by several interacting cytokines and play an important role in asthma through the release of the bronchoconstrictor mediators histamine, cysteinyl-leukotrienes (Cys-LTs), and PGD2. [55]

GATA3 (GATA-binding protein 3) is the transcription factor decisive for the differentiation of naïve $CD4^+$ T cells into the Th2 subtype [63]. After engagement of T Cell Receptor (TCR) and CD28 co-receptor by the antigen presenting cells (APCs), GATA3 is activated by p38 MAPK-mediated phosphorilation and translocates in the nucleus where activates the transcription of the typical Th2 genes [64]. IL-5 is another important Th2 cytokine that plays a fundamental role in eosinophils-mediated inflammation [65]. It is directly involved in eosinophils differentiation, survival and recruitment to the airway [66-71]. These eosinophils are an abundant source of granule basic proteins like major basic protein (MBP), eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin. In addition, this cell type is also capable of producing eicosanoids such as prostacyclin (PGI2) and leukotrienes, superoxide and a range of cytokines and chemokines [72].

IL-9 is emerging as an additional cytokine that induces eosinophil-mediated inflammation, mucus hyperplasia, mastocytosis, AHR, and also increased expression of other Th2 cytokines and production of IgE [73]. Because of its importance and peculiarity, this cytokine has recently been associated with a new T cells subtype (Th9) and some of these effects are mediated via IL-13 release [74]. It is important to mention that Th2 cytokines are not the only molecule involved in asthma pathogenesis. Th1 cytokines, although to a lesser extent, are important in the regulation of inflammatory process that occurs after the antigen exposure. T-bet is the transcription factor crucial for the differentiation of T-cell Naïve in Th1 subtype and for the consequent production of Th1 cytokines. In parallel with the predominant role of Th2 lymphocyte in asthma, T-bet expression is decreased in the airway of asthmatic patients compared with healthy control [75]. In fact it has been demonstrated that GATA 3 is able to inhibit the production of Th1 cytokines by inhibiting STAT-4, the main transcription factor activated by the T-bet–induced cytokine IL-12 [76].

Interferon gamma (IFNγ) is the most representative Th1 cytokine and although its levels are increased in patients with severe asthma and acute exacerbation, it is usually found at lower levels in individuals with moderate asthma [77]. Considering that IFNs (type I and type III) drive the innate immune response against viruses, it has been demonstrate that asthmatic patients, that show a reduction in the expression of IFNs, are more susceptible to rhinovirus infections and replication and consequent viral exacerbation of asthma [78, 79].

Asthmatic patients also show decreased levels of IL-12, cytokine produced by Th1 lymphocytes, activated macrophages, dendritic cells and airway epithelial cells. [80] [81]. Recently, the Th17 population, another T-cells subpopulation, has been associated with asthma inflammation. Their differentiation is regulated by RORγ and different cytokines are involved in this complex process: IL-6, IL1β, TGFβ and IL-23 [82, 83]. The correct role of these cells in asthma is still

unclear and controversial, but the levels of IL-17A, the most representative cytokine of this subpopulation, are increased in asthmatic patients [84].

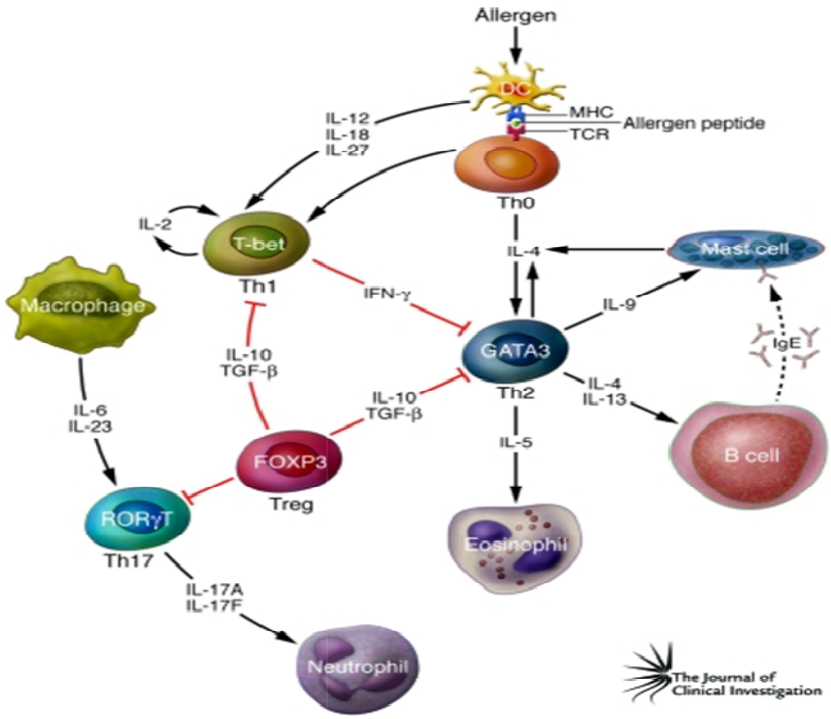


Figure 5. Th cells in airways.

Th2 cells predominate in most patients with asthma and differentiate from uncommitted precursor T cells under the influence of IL-4. Th2 cells orchestrate allergic inflammation through the release of the Th2 cytokines IL-4, IL-5, IL-9, and IL-13. Th1 cells differentiate under the influence of IL-12 and IL-27 and suppress Th2 cells through the release of IFN-γ. Th17 cells differentiate under the influence of IL-6 and IL-23. T-regs normally suppress other Th cells through the release of TGF-β and IL-10 and may have impaired function in asthma. Each Th cell type is regulated by a specific transcription factor: T-bet for Th1 cells, GATA3 for Th2 cells, retinoic acid orphan receptor-γt (RORγt) for Th17 cells, and FOXP3 for Tregs. [85]

IL-17A and IL-17F are correlated with neutrophil-mediated inflammation by induction of release of different neutrophils chemoattractants (CXCL1 and CXCL8) by airway epithelial cells and smooth muscle cells [86]. This leads us to speculate that Th17 cells play a critical role in the neutrophil-mediated inflammation of severe asthma. Mucin-coding gene expression is also increased by IL-17 [87]. On the other side, Th17 cells can produce IL-21 that is important for inhibition of FOXP3 activity, the transcriptional factor involved in the differentiation of the regulatory T cells (Treg) [88]. Another member of the IL-17 superfamily is IL-25, which is produced by Th2 cells, epithelial cells and mast cells that orchestrate the airway inflammation typical of asthmatic patients. Increased expression levels of IL-4, IL-5 and IL-13 can be considered

one of the major responsible factor for eosinophil recruitment, AHR and hyperproduction of IgE [89].

1.5.2.Non-Th2 but critical pro-inflammatory cytokines: In addition to Th2 cytokines, several pro-inflammatory cytokines were found in the sputum and BALF of asthmatics patients. TNFα, IL-1β and IL-6 are the major players for this class of cytokines. These factors are often responsible for NF-κB-activation, amplifying the expression of a variety of inflammatory genes, such as ICAM, VCAM and iNOS. TNFα can directly act on airway smooth muscle cells and increase their contractility leading to AHR [90].

The epithelial cells produce an additional cytokines, Tymic Stromal Lymphoprotein(TSLP) that works synergistic with other inflammatory cytokines to increase the release of Th2 cytokines from mast cells and to stimulate DCs to produce chemokines required for Th2 recruitment [91, 92].

1.5.3.Growth factors: Another group of cytokines is classified as growth factors being implicate in differentiation and survival of inflammatory cells or resulting in proliferation and activation of structural cells, contributing to airway remodeling. An important cytokine in this group is the Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), predominantly produced by macrophage, epithelial cells and T cells in response to inflammatory stimuli. GM-CSF is associated with the promotion of differentiation and survival of eosinophils, neutrophills and macrophages.

Nerve Growth Factor (NGF), normally involved in functionality, proliferation and survival of autonomic nerves, can be produced by immune-cells and by structural cells such as fibroblast, Smooth Muscle Cells (SMC) and epithelial cells [93, 94]. In addition, Stem Cell Factor (SCF) plays an important role in the maintenance of the typical inflammation observed in asthma. This is an important growth factor, produced by a large number of immune cells and other cells that promotes the generation of mast cells from their hematopoietic precursors and their degranulation, with the consequent increase in cytokines production due to release of tryptase and histamine [95].

Tumor Growth Factor-β (TGF-β) is one of the major factor highly involved in remodeling and fibrosis in patient with chronic asthma. TGF–β promotes fibroblast and airway smooth muscle cells proliferation , deposition of extracellular matrix and epithelial repair [96]. It is involved in the up regulation of FOXP3 and consequent activation of the Treg followed by release of IL-10, resulting in the suppression of Th1 and Th2 activity [97].

Transforming Growth Factor-α (TGF-α) can also activate the Epidermal Growth Factor Receptor (EGFR) increasing mucus production [98]. It was also reported that VEGF level, major

promoter of the vascularization, was higher in the lungs of asthmatic patients compared to non asthmatic individuals. [99] [100].

1.5.4.Chemoattractant: Cytokine network involved in asthma also includes chemoattractants, small molecules called chemokines that are responsible for the recruitment of the inflammatory cells in the inflammatory sites [101]. Important members of this group include eotaxin, RANTES and MCP-4 that bind CCR3, that is predominantly expressed on eosinophils but also on Th2 and mast cells. These molecules prolonge the permanence of eosinophils in the inflammatory sites after allergen exposure [102].

CXCL8 activates CXCR1 expressed on neutrophils [103] [104]. CXCL10 and CXCL12 bind respectively CXCR3 and CXCR4 and attract mast cells and Th2 cells [105] [106]. Monocytes, mast cells and T cells are also attracted by CX3CL1 produced after stimulation with IFNγ, IL1β and TNFα [107].

1.6.Airway epithelium

Notably, immune cells are not the only cells that govern the progression of asthma, the airway epithelium is fundamental since it represents the first physical barrier for inhaled particles, toxins and other stimuli [108, 109]. In addition, it can work as direct producer of proinflammatory cytokines in response to stimulation [109-111]. The airway epithelium also allows the selective migration of leukocytes into the airway lumen [109]. The infection of the epithelium with HRV, RSV and others viruses activates the epithelium that became the primary source of inflammatory mediators that are the major responsible of asthma exacerbations [112].

1.7.Airway smooth muscle

Airway smooth muscle (ASM) cells are the main structural cells in the bronchi [110]. These cells regulate airway resistance through contraction [113]. In some conditions, airway smooth muscle cells have been shown to synthesize cytokines, growth factors, and adhesion molecules, which may contribute to the inflammatory responses [113].

1.8.Airway remodeling in asthma

Airway remodeling is accompanied by several changes in structural cells and tissues in patients with asthma compared to healthy individuals. The Structural changes observed in the airway of asthmatic patients include epithelial alterations with consequent loss of integrity,

thickening of basement membrane, goblet cell and submucosal gland enlargement, subepithelial fibrosis, increased smooth muscle, decrease cartilage integrity and increase of the airway vascularization and edema [4, 110, 114]. All these change are correlated with an ongoing chronic inflammatory process that involves an overactivation of the immune system.

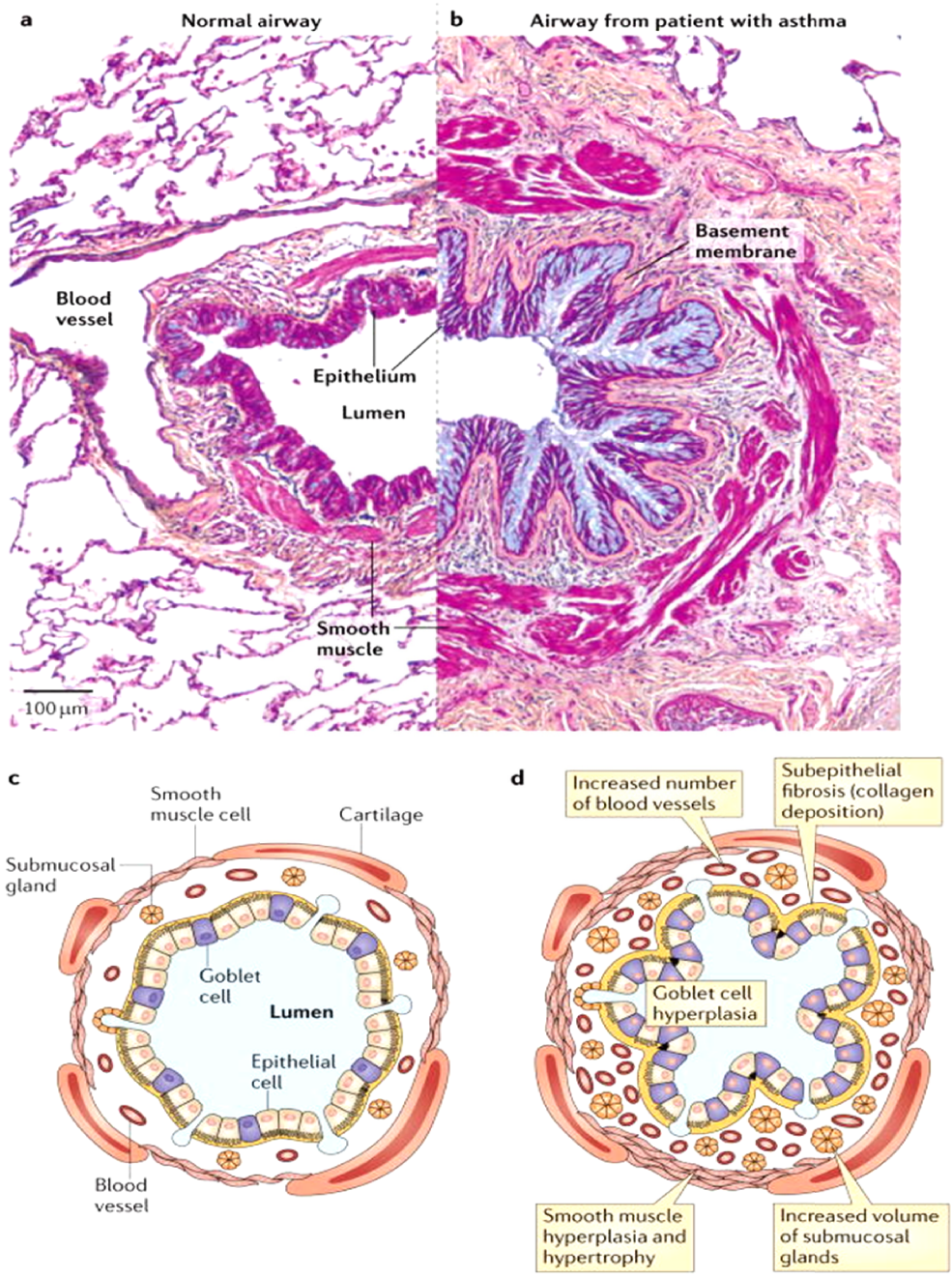


Figure 6. Airway remodeling in asthma

*Airway structures in medium-sized healthy airways (part **a**; a schematic representation is showed in part **c**) and in a patient with asthma (part **b**; a schematic representation is showed in part **d**). The airways in asthma show considerable structural remodelling, including goblet cell hyperplasia, subepithelial fibrosis and increases in smooth muscle volume. [115]*

Morphologic alterations in airway epithelium are considered to be the first step of airway remodeling in patients with asthma. The main alterations include epithelium shedding, loss of ciliated cells, goblet cell hyperplasia, increase release of cytokines and growth factor and overexpression of their receptors [110]. The loss of epithelium integrity is correlated also with a break in tight junction [110]. The epithelium layer is considered to be a physical barrier, which keeps the internal milieu of the lung safe against external factors [116, 117]. Several injuries in the airway epithelial layer allow environmental microorganisms, allergens, and toxins to have access to airways [117, 118] facilitating the worsening of the disease.

Different inflammatory mediators, involved in remodeling, such as TGF-β and chemokines, are released from repairing/damaged epithelium or in response to other inflammatory mediators [119] [120]. It has been shown that these mediators play a crucial role in the formation of subepithelial fibrosis and also increase in ASM mass [119].

Sub-epithelial fibrosis represents another structural alteration associated with airway remodeling found in asthmatics. This phenomenon occurs in the lamina reticularis situated immediately below the basement membrane and results in a thickening of the basement membrane just below the epithelium. The cells directly involved in fibrosis are the fibroblasts that increase the Extracellular Matrix (ECM) deposition. These cells are able to generate fibrosis as a result of disturbance in the balance between the inhibitor of metalloprotease (TIMP-2) and the metalloprotease (MMP2) resulting in less ECM degradation [121]. Once activated, in a proinflammatory environment, the fibroblasts differentiate to myofibroblasts that secrete pro-inflammatory mediators and ECM; they also acquired a contractile phenotype, characterized by overexpression of smooth muscle actin (SMA), participating actively to the AHR [110, 122].

Smooth muscle cells are the main structural cells in the airways. In asthmatic patients the smooth muscle mass increases for two principle reasons: an increase in the size of the cells and an increase in the number. The activated ASM are not only able to synthesize and release pro-inflammatory cytokines and chemokines, recently they have been shown to migrate to the subepithelial area [123-128]. Additionally, ASM cells express cellular adhesion molecule (CAMs), toll like receptors (TLRs), and also cytokines receptors [110]. Numerous mediators, including TNF-α, IL-1β and IFNγ, can increase expression of ICAM-1 and VCAM-1 on ASM [129]. Using these adhesion molecules, ASM cells can regulate the interaction between different inflammatory cells [110]. Recent studies have shown that interaction between activated T lymphocytes and cultured

ASM, which is mediated by ICAM-1, VCAM-1, and CD44 on ASM cells, resulted in up-regulation of cell adhesion molecules and increasing of DNA synthesis in ASM cells (hypertrophy)[130, 131].

The alteration observed in the **vessels** regarded as an increase in the size and in the angiogenesis mediated by VEGF. It is important to note that VEGF can increase the permeability of blood vessels causing edema [132].

The cartilage of the lung is also affected by remodeling. Decrease of cartilage volume and increase of proteoglycan degradation are seen in the airway of asthmatic patients [133]. These alterations may results in a powerful bronco-constriction for a given degree of ASM cells. The factors mainly involved in fibrosis, such as TGFβ, IL-11, IL-17 and histamines, have been shown as molecules that increase fibroblast proliferation and the release of connective tissue growth factor production [134-136].

Eosinophils are believed to be one of the most important cell type involved in tissue remodeling in asthma. This cell type is considered to be the main source of the pro-fibrotic cytokine TGF-β [119] [137]. In addition, eosinophils are also involved in activities such as proliferation of fibroblasts, maturation of myofibroblasts and collagen synthesis. In the airways of asthmatic subjects, eosinophils develop from CD34+ bone marrow precursor cells. IL-13, GM-CSF and eotaxins modulate their development while IL-5 increases their maturation and recruitment into the airways. Moreover, eosinophils are an abundant source of granule basic proteins, eicosanoids, cysteinyl leukotrienes, tissue-damaging reactive oxygen species, and different cytokines and chemokines.

1.9Airway hyperresponsiveness (AHR) in asthma

AHR is defined as the predisposition of the airways of asthmatic patients to narrow excessively in response to stimuli that would produce little or no effect in healthy subjects. For this reason, AHR is used as a tool in the diagnosis, classification of severity [138] and management [139, 140] of asthma. The presence of AHR is associated with increased decline in lung function [141] and its severity is associated with an increased risk of exacerbation [142], increased asthma severity as measured by symptoms [143] and an increased level of treatment required to control symptoms [144].

Traditionally measurements of airway responsiveness have been presented using two different calculations:

- the provocative concentration (or dose) of methacoline causing a 20% fall in FEV1 (Forced Expiratory Volume in 1 second) that is calculated by interpolation from the dose causing ≥ 20% fall in FEV1 and the penultimate dose on a semi-log scale.

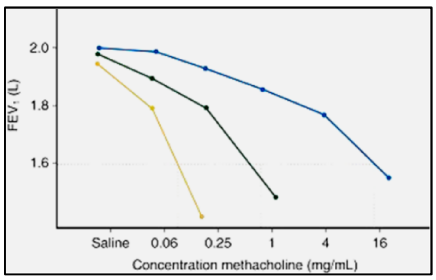


Figure 7. Airway responsiveness measured as the fall in FEV_1 after increasing inhaled concentrations of methacholine .

Severe (yellow), moderate (black), and mild (blue) airway hyperresponsiveness are shown. Note increased airway responsiveness is associated with a lower provocative concentration required to cause a 20% fall in FEV_1 (PC_{20}) (see green line), a steeper gradient, and a higher maximum % fall in FEV_1.

It is maybe not surprising that determining the actual dose delivered (PD20) appears to be a more robust measure than simply using the concentration of agonist (PC20) [145].

- The dose–response slope (or DRS), also referred to as the response dose ratio (RDR), is calculated as the slope of the dose–response curve plotted with a linear dose axis. The advantage of the DRS is that it provides a continuous measure of airway responsiveness allowing inclusion of subjects who do not reach a 20% fall in FEV1.

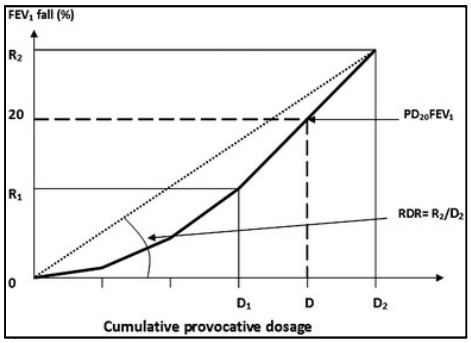


Figure 8. The dose-response slope

There are two groups of stimuli utilized in the measurement of AHR; those which were supposed to act directly on the ASM inducing bronchoconstriction, such as methacholine and

histamine, and those which indirectly cause bronchoconstriction through the release of upstream mediators. Indirect challenge tests include exercise, eucapnic voluntary hyperpnea (EVH), hypertonic saline, mannitol, adenosine 50 monophosphate (AMP) and various allergens [146, 147]. Although it is shown that administration of mannitol cause a better response under normal condition, after the allergen exposure it has been observed to increase responsiveness to methacoline [148]. Despite decades of research, there is still little consensus on the mechanisms underlying AHR in asthma. This is most likely due to the numerous pathophysiological abnormalities associated with asthma and different mechanisms or a combination of these gives rise to AHR in different patient populations. The definition of asthma as an inflammatry airways disease, characterized by exaggerated airway narrowing, immediately brings attention to the role of airway inflammation and the ASM in the manifestation of AHR.

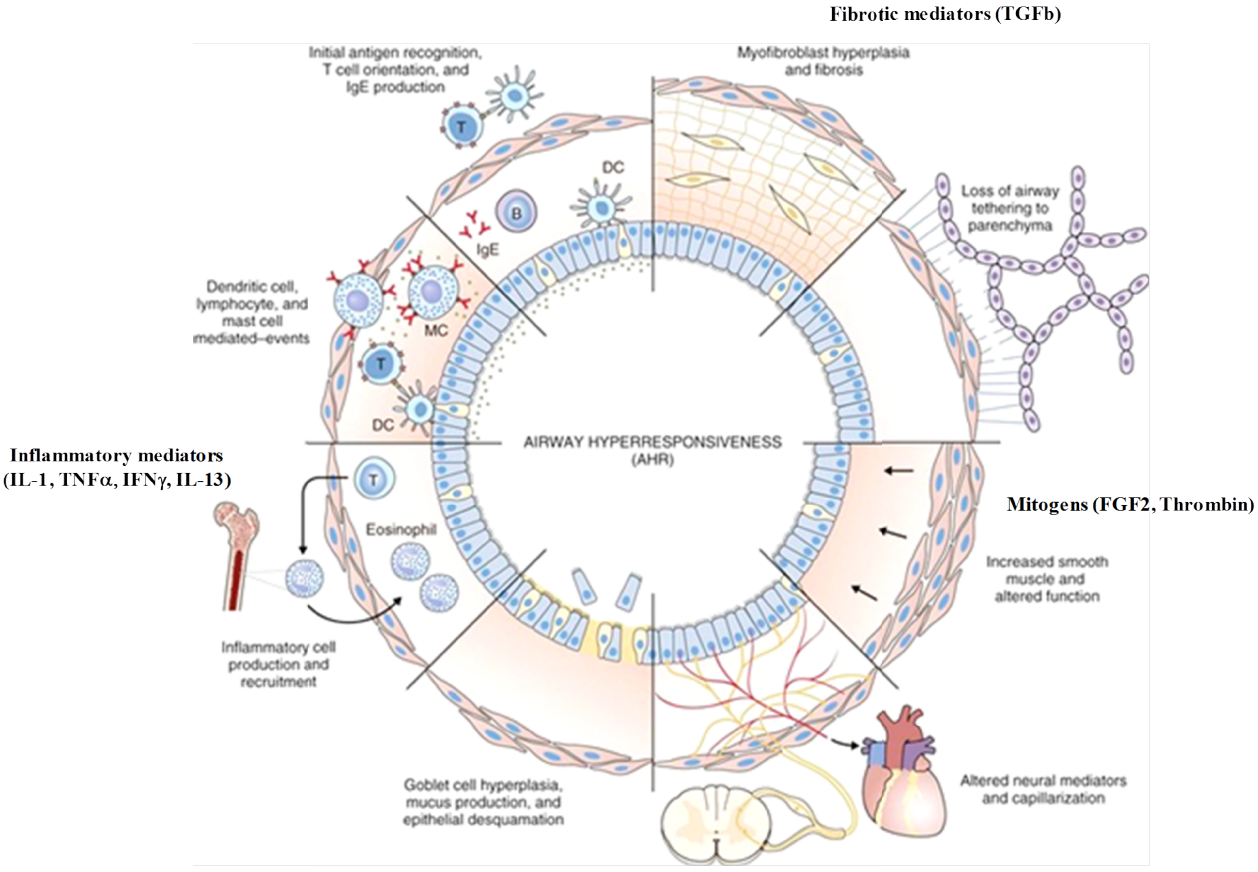


Figure 9: Potential targets for the regulation of noncontractile (proliferative and synthetic) and contractile functions of airway smooth muscle contributing to airway hyperresponsiveness.

(Courtesy Professor Mark Inman.)

1.9.1Airway inflammation: The influx of inflammatory cells in the lung of asthmatic patients contributes to AHR. A positive correlation between the severity of AHR and the number of eosinophils and metachromic cells in sputum was reported [149, 150]. Such correlation was also observed with the number of mast cells in the airways [151]. Many investigators and clinicians consider that the level of exhaled nitric oxide (eNO) as a biomarker for eosinophilic inflammation [152]. eNO levels correlate with the severity of AHR to methacholine in asthmatic subjects [153, 154]. Interestingly, the link between eNO and AHR appears driven by airway narrowing, but not airway closure [155]. Eosinophilic airway inflammation may contribute to the severity of AHR whereas airway neutrophilia may be associated (causally or coincidentally) with an alteration in the type of bronchoconstriction towards predominance of airway closure.

1.9.2.Airway smooth muscle cells: As eluded to above, bronchoconstriction is due, at least in part, to constriction of the ASM surrounding the airway. Therefore, it is not surprising that increased contractility of the ASM has long been described as a principal cause of AHR.

- ***Intrinsic factors:*** Despite considerable research, it is still unclear as to whether asthmatic ASM is intrinsically hyper-contractile and what factors are mechanistically involved.

  Recent gene expression profiling of ASM revealed four novel genes that not only differentiated asthmatic and non-asthmatic patients, but were related to the severity of AHR [156]. Furthermore, the expression of contractile proteins α-smooth muscle actin and desmin in ASM from asthmatics correlates with the severity of AHR [157] suggesting a role of intrinsic ASM dysfunction.

  Some in vitro studies have reported increased force generation of ASM from asthmatic patients [158, 159]. Increased airway narrowing could be due to an increase in the shortening velocity of ASM despite normal force generation. This would theoretically occur because a muscle that shortens quickly would produce greater airway narrowing during expiration before the dilatory effect of the proceeding inspiration [160]. Indeed, in vivo findings support an effect of ASM shortening velocity on the magnitude of ASM shortening [161].

  Airway smooth muscle contraction involves the formation of actin–myosin cross-bridges with the rate of formation dependent upon the activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). An increase in the activity of either MLCK or MLCP would lead to increased shortening velocity of ASM. Indeed, both an increased

expression of MLCK and increased shortening velocity of ASM have been reported in asthma [162]. Asthmatic ASM is also more sensitive to oxidative stress with the extent of oxidative damage within the ASM bundle correlated with the severity of AHR [163]. This relationship is in part mediated by increased NADPH Oxidase 4 (NOX4) expression as siRNA knock-down of NOX4 attenuates in vitro ASM contractility.

- ***Extrinsic factors:*** The asthmatic airway resides in a pro-inflammatory environment, which likely contributes to ASM dysfunction independent of any intrinsic abnormalities. Pro-inflammatory cytokines such as IL-4, IL-13 and tumor necrosis factor-α (TNF-α) increase ASM responsiveness in vitro, possibly via effects on calcium signaling [164]. Proteases, such as matrix metalloproteinase-1 (MMP-1), are increased within ASM bundles of asthmatics and also regulate in vitro ASM contractility [165] and structural integrity.

Additionally, the number of mast cells within the ASM correlates with the severity of AHR in asthma [166]. Although the mechanisms are not yet clear, mast cell mediators such as histamine, leucotriene D4 [167] and prostaglandin D2 [168] may contribute to increased basal ASM tone.

In vitro stimulation of human ASM with TNFα or IL-1β induces the secretion of chemokines and cytokines such as RANTES, IL-6 [169, 170] and IL-8 [171, 172]. This proliferative/secretory phenotype is associated with reduced expression of contractile proteins such smooth muscle myosin heavy chain, α-smooth muscle actin, myosin light chain kinase [172]. It is presently unclear whether the transition of ASM to the synthetic phenotype confers protection against, or further contributes to, AHR.

1.9.3.Airway epithelium: Damage to the airway epithelium, which provides an initial barrier for inhaled spasmogens, also likely contributes to AHR. Disruption of the airway epithelium would increase the amount of stimulus interacting with the ASM and thus potentiate bronchoconstriction. In addition, epithelial damage or dysregulation likely reduces the ability of the epithelium to maintain relaxation of ASM via release of epithelial-derived relaxing factor(s) [173]. For example, intratracheal administration of cationic proteins reduces both the barrier effect and control of ASM relaxation by the airway epithelium and results in AHR in animal models [174]. Additionally, damage to the epithelium may also directly contribute to airway narrowing. Recent *in vitro* findings suggest that rupture of small airway murine epithelial cells induces intracellular [Ca2+] waves and subsequent contraction in neighboring ASM [175].

1.9.4.Airway wall thickening: AHR correlates with airway wall thickening [176, 177], reticular basement membrane thickness [178] and components of the extracellular matrix. Thickening of the airway wall could contribute to excessive bronchoconstriction in two ways. Airway resistance is inversely related to airway radius such that an increase in the submucosal area would amplify the reduction in airway caliber for any given degree of ASM shortening. Increased ASM mass, due to either hypertrophy or hyperplasia, is thought to increase the total force generated by ASM and thus exaggerate airway narrowing without any alteration in ASM contractile function [179].

1.10.Pulmonary Lung Function in mice models of lung disease

It is very important to evaluate the possibility to measure the lung function and the AHR also in animal models such as mice models of the disease. These models play an important role in the translational research focused in identifying the causes and mechanisms of respiratory and allergic disease.

There are three different way to evaluate pulmonary function in mice:

-***ex vivo techniques*** that require removal of the tracheas and lungs from animals for the evaluation of the contraction of isolated airway segments after stimulation with contractile mediators. These techniques offer the possibility to study the primary variable, such as the composition of the tissue bath and the exposure time to different mediators of contraction.

-***in vivo invasive techniques*** requiring the animal be anesthetized and instrumented before airway functions can be measured. These types of analysis are performed by measurement of airway pressure-flow relationship. Different parameters can be measured with these techniques: respiratory rate, tidal volume, minute volume, airway resistance (reactivity), lung compliance and diffusion capacity.

-***In-vivo noninvasive techniques*** which do not required either anesthesia or airway surgical manipulation. These techniques have gained much interest and use as convenient and effective methods for the chronic screening of respiratory function over extended periods of time. The major advantage over the invasive techniques is that the animal is awake during all procedure and can be used again on multiple occasions. Also the broncoactive agents are administrated by non-invasive route preserving the airway completely intact. There are two different non-invasive techniques frequently used: barometric plethysmography and forced oscillation.

The barometric plethysmography is one of the most common way to measure the airway responsiveness in mice, easy to use, largely non interventional and well suited for screening large number of animals. The assessment consists of placing the unrestrained animals inside a seated chamber and measuring the pressure fluctuation that occur during the animal respiratory cycle. Changes in the nature of these pressure variations are indicators of changes in lung function.

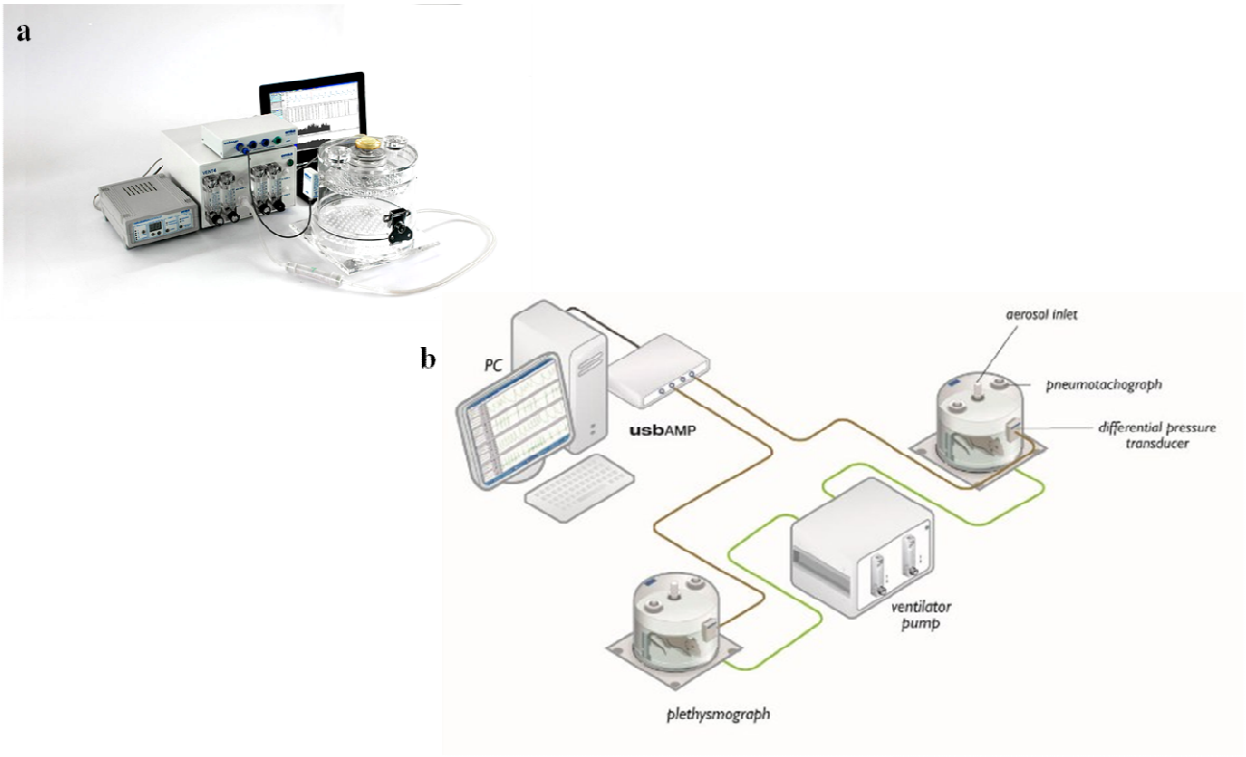


Figure 10. Whole body plethysmograph

*A plethysmograph (**a**, schematically represented in part **b**) is a close chamber in which pressure changes due to the animal's breathing are measure. Plethysmography represents a widely used technique for studying pulmonary function.*

During inspiration, tidal air is warmed and humidified as it enters the lung. These conditioning causes the air volume expands within the lung and the pressure associated with this change is reflected in the pressure within the chamber. It is generally felt that the timing of inspiration and expiration can be accurately measured using this technique. It is important that the animals have sufficient time to acclimate to the chamber and is breathing quietly in order to obtain tidal volume and ventilation from the change in pressure over the time. It is also important to provide fresh air to the chamber for ventilation and to help prevent temperature fluctuation within the chamber from the body heat of the enclosed animal.

Hamelmann and coworkers for the first time proposed whole body plethysmography (WBP) as a valid technique for assessing AHR in allergen sensitized mice. They divided expiratory period of animal ventilation into two components: an early relaxation phase and a late phase. The early

phase included the transition from inspiration to expiration. They computed respiratory pause with the equation pause=(Te-Tr)/Tr, where Te is the time of expiration and Tr is the time required for the area under the chamber pressure-time curve during expiration to decrease to 36% of its total volume. The researcher further recognized that when breathing was unobstructed, the recorder pressure signals during both expiration and inspiration had equal deflections. When breathing was obstructed, on the other hand, the expiratory pressure deflection was greater than was the expiratory inspiratory pressure deflection. So, they decided to defined a new parameter termed Penh (Penh=PEP/PIP x (Te-Tt)/Tr, where PEP and PIP represent the pick expiratory/inspiratory pressure chamber deflection.

Different studies demonstrate that the changes in Penh are closely parallel to the changes observed in pulmonary resistance and dynamic pulmonary compliance during bronchoconstriction caused by aerosolized methacholine; the same happen also after allergen sensitization of the animals. For this reason the WBP is consider valid indicator of AHR after allergic sensitization in mice. Obviously**,** although all the advantages of this technique, that include the possibility to measure the lung function in conscious mice that breath spontaneously without the necessity to use any anesthesia and also during aerosol challenge, the whole body plethysmography has several disadvantages. First of all the classic lung function parameters (lung resistance and compliance) are not available. Second, Penh, unlike other parameters, is not determined from direct measurement, but rather is an outcome indicator based entirely on empiric signal processing. As such, the absolute value of Penh has not physiologic meaning and it cannot be compared from mouse to mouse. Third, if an intervention results in a change in the respiratory pattern, than Penh can change without a true airway obstruction.

2.Nitric Oxide

For a long time, Nitric Oxide (NO) has been considered an atmospheric pollutant released by cigarette smoke [180] and vehicles that contributes to Ozone layer destruction [181]. Thirty five years ago, Furchgott and Zawadzki [182] observed for the first time the presence of a factor endothelium-dependent responsible for the arterial relaxation induced by acetylcholine. But, only 7 years later, Palmer [183] and Ingarro [184] discovered that the unknown molecule was NO. The reason why so many years passed between the first observation of NO and its effective discovered is because its half-life is extremely short, only 3-5 seconds; immediately after it's converted by oxygen and water into Nitrates and Nitrites.

Now the NO is considered one of the most important signaling molecules in the body, involved virtually in every organ system where is responsible for modulating an incredible number of effects. This small molecule can be found in higher concentration in the brain and in peripheral nervous system [185, 186] acting as a neurotransmitter in the regulation of neuroendocrine functions, blood flux and gastrointestinal motility. In the Brain it is involved in very important processes like the memory formation and the response intensity to pain stimuli [187]. It is known that NO plays a role in the regulation of skeletal musculature regulating its metabolism and muscle contractility [188]. Recently, the fundamental role of NO has been identified also in the host defense against tumors and pathogens. In fact, it has been demonstrated that, at high concentration, NO can have cytotoxic and cytostatic effects [189, 190].

The involvement of NO in nonspecific immunity and its participation in the complex mechanism of tissue injury are being particularly important in the last years. Different research groups are trying to explain the complex and controversial role of this biologic molecule in the mediation of inflammatory processes and in the regulation of apoptosis mechanisms [191].

High levels of NO are associated with different pathological conditions in humans underling that its synthesis play an important role in the host defense. In fact NO can be considered a double face molecule; it can have positive and negative effects on the physiologic homeostasis. NO has an unpaired electron, which makes its a free radical that can react with other molecules, such as oxygen, superoxide radicals, or transition metals [192] causing oxidative stress in the cells, DNA damage and consequently apoptosis.

2.1.Nitric oxide synthases

NO production begins when a group of the amino acid L-arginine is broken down by oxidative enzymatic activity [193-196], generating NO and L-citrulline [181, 189]. The L-arginine is carried inside the cells through a cationic amino acid transporter (CAT). There, it can be

metabolized by two distinct groups of enzymes: nitric oxide synthases and arginases. Classically, arginase is an enzyme that acts in the urea cycle in the liver, but it has been discovered also in other cells that do not express the complete urea cycle, including those of the lung [197, 198]. However, the most important class of enzymes involved in the generation of NO by the conversion of L-arginine to L-citrulline, using the NG-hydroxyl-L-arginine as intermediate, is the family of nitric oxide synthases (NOS). Three different nitric oxide synthase isoforms are actually known (130-160 kDa). Two of which are constitutive and Ca^{2+}/calmodulin dependent and one inducible and Ca^{2+} independent: the neuronal or type I (nNOS) [186, 199], the endothelial isoform or type III (eNOS) [24], and the inducible isoform or type II (iNOS) [200] [201, 202].

In physiological conditions, upon stimulation by specific signal molecule, NO is synthesized at low concentration. NO then binds the heme group of soluble guanylate cyclase to produce cGMP that modulate a large spectrum of molecules including various ion channels, protein kinases, phosphodiestaerase. Such modulation decreases intracellular calcium levels and causes a variety of effects including smooth muscle cell relaxation to control blood pressure [203].

	Regulatory and anti inflammatory effects of Nitric Oxide	
Tissue organ	**Fisiological action of Nitric Oxide related to inflammation**	**NOS isoforms**
Vascular endothelium	•Maintain vasodilator tone	eNOS
	•Inhibits smooth muscle cell migration and proliferation	eNOS, iNOS
	•Inhibition of blood cells-vassels wall interactions and adhesion to endothelium	eNOS
Blood cells	•Inhibition of platelet adhesion and aggregation and inhibition of microvascular thrombosis	eNOS, iNOS
	•Prevent aggregation and adhesion of white cells	iNOS
	•Mediate cytostatic and cytotoxic activity of macrophages for antimicrobial and antitumor defense	
	•Inhibition of must cells degranulation	
Heart	•Maintains coronary perfusion and regulate cardiac contractility	eNOS
	•Inhibits cardiac contractility (pathology of myocarditis)	iNOS
Lung	•Maintains ventilation/perfusion ratio and regulates bronchociliar motility and mucus secretion	?
Pancreas	•Modulate endocrine secretion	eNOS, iNOS
Intestinal system	•Modulate peristalsis and exocrine secretion	eNOS
	•Contributes to protection of mucosa	

Figure 11. Regulatory and anti-inflammatory effects of Nitric Oxide

There has been some evidence about the existence of a fourth NOS isoform, the mitochondrial NOS (mtNOS) [204-206]. This enzyme is activated by Ca^{2+} uptake to produced peroxynitrite that activates the release of Ca^{2+} working as feedback loop which prevents mitochondria Ca^{2+} overloading [207]. The NO biosynthetic enzyme, NOS, is one of the most regulated enzyme in biology because of the labile nature of NO and the potency of the gas to induce its effects. All the NOS isoforms are flavoproteins that have tetrahydrobiopterin, heme, and a portion that is homologous to cytochrome P450 reductase. They act as dioxygenases using an oxygen molecule and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) to transform the L-arginine into L-citrulline and release the nitric oxide. The tetrahydrobiopterin (BH4) is the main cofactor for all NOS isoforms, and in its absence, these enzymes can produce superoxide instead of NO [208, 209].

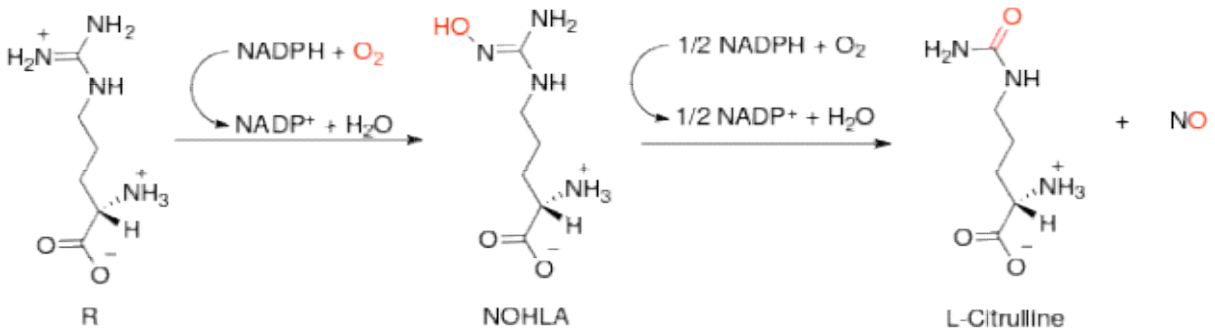


Figure 12. Nitric Oxide synthesis [210-212]

Nitric oxide synthases produce NO by catalysing a five-electron oxidation of a guanidino nitrogen of L-arginine (L-Arg). Oxidation of L-Arg to L-citrulline occurs via two successive monooxygenation reactions producing N^{ω}-hydroxy-L-arginine (NOHLA) as an intermediate. 2 mol of O_2 and 1.5 mol of NADPH are consumed per mole of NO formed.

The regulation of the two constitutive isoforms Ca^{2+}-dependent NOS is well understood and deeply investigated [212]. However, it is important to note that these NOS isoforms are responsible for only a minimum amount of NO production. Such levels of NO are mostly involved in the regulation of different physiological processes in order to control the neurotransmission and the cardiovascular system. Modulation of the inducible isoform of this enzyme remains unknown. Its involvement in different inflammatory diseases makes it critical for the pathogenesis of these conditions and perhaps an appealing therapeutic target.

2.1.1.iNOS

Some preliminary studies showed that NO that is involved in inflammatory processes is mainly produced by iNOS expressed by activated macrophages. However, it is known that iNOS

can be expressed in different cell types. The first evidence that NO could be associated with inflammation dates approximately 30 years ago [213]. But NO synthesis was initially associated with microbial metabolism [214]. However, in 1985, Stuehr and Marletta provided the first demonstration that mammalian cells (mouse macrophages) were also able to produce NO byproducts such as nitrate and nitrite after stimulation in vitro [215]. Since that time, the production of NO has been considered of primary importance in the host's antimicrobial mechanisms. iNOS is encoded by NOS2A gene and translates in a protein that shares between 50-60% sequence homology with the other two isoforms [212] (see Fig. 12).

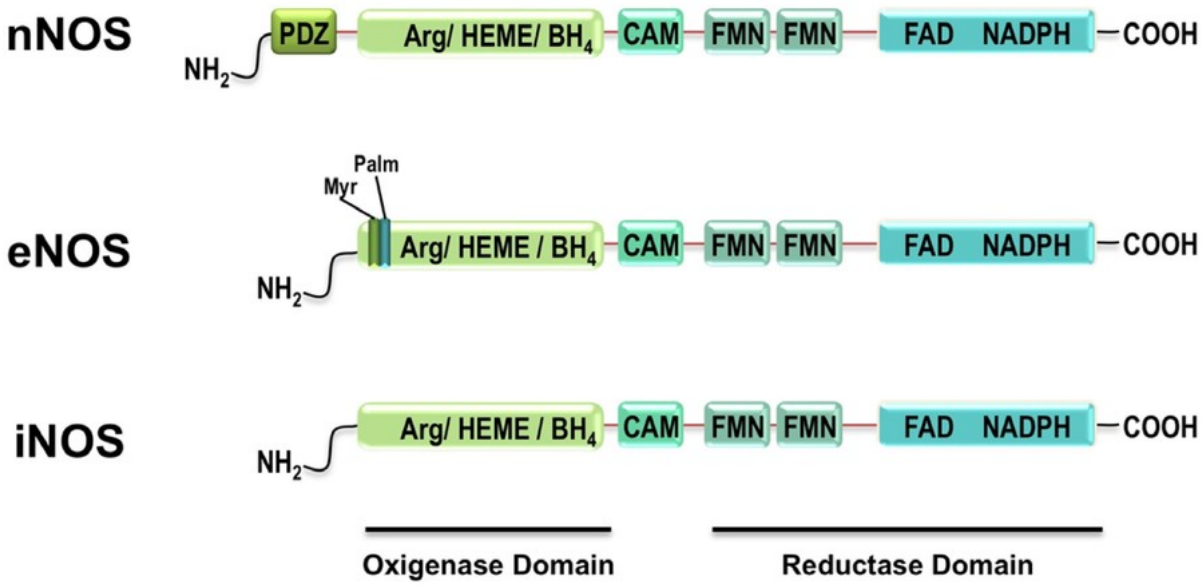


Figure 13. Schematic diagram illustrating the different isoforms of nitric oxide synthase

PDZ domain is typically present in neuronal NOS (nNos, the presence of myristoylation (Myr) and palmitoylation (Palm) sites are specific to endothelial NOS (eNos). All isoforms the oxigenase domain contains binding sites for L-arginine (Arg), Heme and tetrahydrobiopterin (BH4) while reductase domain binds to calmodulin (CAM), FMN, FAD and NADPH. iNos = induced NOS. [216]

In resting conditions, cells do not express iNOS. However, this protein is rapidly expressed upon exposure to immunostimulatory cytokines, bacterial metabolism products or infections in different cells, including endothelial cells, monocytes, mast cells, macrophages and smooth muscle cells [211, 212]. This enzyme is able to produce NO by catalysing a five-electron oxidation of a guanidino nitrogen of L-Arg. The Oxidation of L-Arg to L-citrulline occurs via two successive monooxygenation reactions producing N^{ω}-hydroxy-L-arginine (NOHLA) as an intermediate. 2 mol of O_2 and 1.5 mol of NADPH are consumed per mole of NO formed [217].

iNOS consists of a bi-domain protein in which the C-terminal half acts as a reductase domain that contains the binding sites for CaM, NADPH, FAD and FMN; and the N-terminal half of the enzyme acts as an oxygenase domain that contains the binding sites for heme, H4B, and L-arginine [218]. The CaM-binding site is situated approximately in the center of iNOS and separates the two principle domains. CaM acts as a molecular switcher, inducing a conformational change in the structure indispensable for electron flow [219] [220]. The iNOS enzyme appears to be active only in its dimeric form [212, 221]. Two monomers dimerize at the level of the oxygenase domains. iNOS dimerization is initiated by insertion of heme, resulting in rapid conformational changes. The heme is buried in the protein interior and makes extensive Van der Waals interaction with hydrophobic and aliphatic side changes. The incorporation of heme into the oxygenase domain creates the binding sites for arginine and H4B. The binding of arginine and H4B cause a transition from loose to tight NOS dimer. Zn^{2+} incorporation at the dimer interface can provide additional stabilization [219].

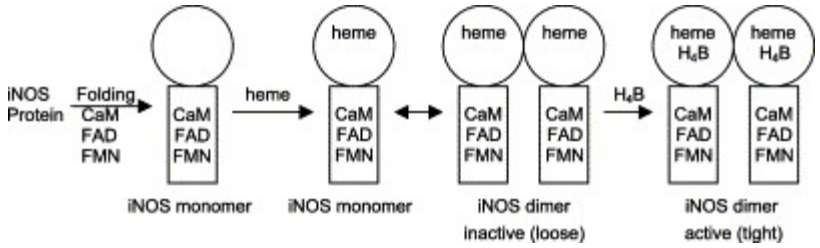


Figure 14. Inos dimerization

Model for the generation of active iNOS dimer. After the synthesis of iNOS protein, the heme free monomer that contains a functional reductase domain is generated in the presence of CaM, FAD and FMN. The heme incorporation into the oxygenase domain initiates dimerization. H_4B and Arginine generate active tight dimer iNOS form. [222]

2.2.Reactive species of NO

Whereas the moderate levels of NO produced by the two constitutive isoforms of NOS has beneficial effects, higher concentrations of NO synthesized mostly by iNOS during a variety of inflammatory diseases can interact with numerous substances generating dangerous compounds.

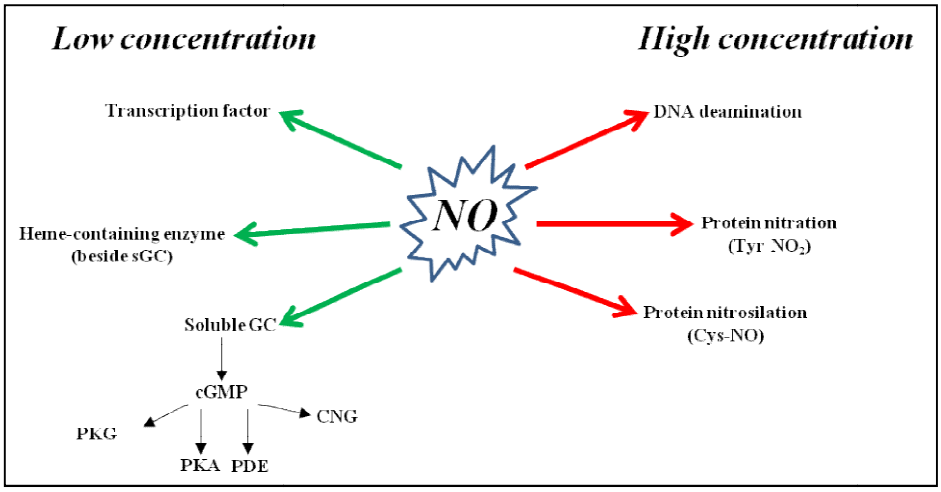


Figure 15. Nitric oxide effects.

Nitric oxide may elicit a very different set of effects depending on its concentration in the tissue. At low concentrations, those normally achieved after stimulation of constitutive enzymes (NOS1 and NOS3), nitric oxide can modulate the activity of heme-containing proteins: typically guanylyl cyclase, but also important proteins of the mitochondria like cytochromes. It is also able to regulate certain transcription factors (for instance, the hypoxia inducible factor or HIF-1). At higher concentrations, nitric oxide can nitrosylate cysteine residues or produce tyrosine nitration in different proteins. In certain cases, effects of DNA deamination have been also observed. [223]

For example in presence of high concentration of NO, it interacts with oxygen generating reactive nitrogen oxide intermediates (RNOIs) such as NO_2^-, N_2O_3 and N_2O_4 whereas, under normal conditions, NO is converted to nitrite (NO_2^-) and nitrate (NO_3^-) [224]. RNOIs can react with other biomolecules such as thiols and amines which may result in conformational changes in proteins structure. RNOIs can also nitrosylate cystein thiols involved in zinc finger structures, leading to conformational changes and modifications of functional activities of different proteins [225, 226]. Zinc finger domains are important for protein-DNA or protein-RNA interactions. Some transcription factors have zinc finger domains [227] and, therefore, NO can directly affect the transcription of a number of genes [226].

In inflammatory-mediated reactions, NO_2^- reacts with hypochlorous acid (HOCl) to form a nitryl chloride ($Cl\text{-}NO_2$). Also, myeloperoxidase in neutrophils and monocytes generates additional HOCl-derived NO metabolites [228]. Neutrophil infiltration was associated with formation of nitrotyrosine and marked apoptosis [228].

Main reactions of Nitric Oxide

Reaction with	Product
Fe^{2+}	$Fe^{2+} - NO$
O_2	$N_2O_3, N_2O_4, NO_2^-, NO_3^-, ONOO^-$
R-SH	R-S-NO
HOCl	$Cl\text{-}NO_2$

Figure 16. Main reaction of nitric oxide

NO reacts with Fe2+ within heme containing protein, with oxygen to produce reactive nitrogen oxide intermediates and peroxynitrite, and with thiols to generate nitrosothiols.

NO reacts with Fe^{2+} within heme-containing protein, with oxygen to produce reactive nitrogen oxide intermediates and peroxynitrite, and with thiols to generate nitrosothiols. NO can also act like a negative modulator of iNOS itself through the direct bound to the heme group [229]. Within the vascular system, NO may be removed by reacting with oxyhemoglobin to form nitrate, thus preventing the generation of oxidant NO metabolites and protecting against oxidative damage. At physiological concentrations, NO inhibits pro-inflammatory platelet aggregation, integrin-mediated adhesion, and pro-inflammatory-induced gene expression, factors that control vascular inflammation and oxidative injury. However, NO and NO_2^- at high concentrations can display pathogenic properties due to the production of $ONOO^-$, NO^-, and other reactive oxidizing compounds in the presence of superoxide radicals or peroxidases, causing the reversal of NO effects from protective to deleterious [230]. High levels of NO and $ONOO^-$ inhibit the activity of complexes I and II in the respiratory complex, ATP synthase, aconitase, creatine kinase, superoxide dismutase, and damage mitochondrial DNA and mitochondrial membranes [231, 232].

NO can also react with superoxide anion to form $ONOO^-$ that can interact with proteins, lipids, carbohydrates, and DNA, and with carbon dioxide to form highly reactive nitrosoperoxocarbonate. These oxidation and nitration mechanisms change the structure and function of these compounds to lead to oxidative damage of tissues [233]. Peroxynitrite can irreversibly inhibit mitochondrial respiration and damage a variety of mitochondrial components via oxidizing reactions [232]. The oxidant metabolites of NO can also inhibit the activity of antioxidant enzymes such as glutathione peroxidase and reduce the levels of some cellular antioxidants such as ascorbic acid, uric acid and plasma thiols (Han et al., 2001). Reactive nitrogen compounds and $ONOO^-$ can also modulate some enzymes in the inflammatory process and in vascular functions, including prostaglandin endoperoxide synthase, cytochrome P450 and 5-lipooxygenase metabolism [225, 233].

2.3.iNOS in inflammatory diseases

The physiological response to any kind of damaging stimuli is called inflammation. It has been demonstrate that NO is highly involved in all inflammatory processes and that the inducible NOS is the enzyme mostly involved in its production. iNOS activation can be induced by exogenous agent, as bacteria and their metabolic product, chemical irritants or endogenous mediators such as cytokines, arachidonic acid metabolites. Both pro- and anti-inflammatory cytokines can modulate iNOS activity. It has been shown that TGFβ1, an immunomodulatory cytokines, negatively regulates iNOS expression [234] how demonstrated in TGFβ KO mice that show an aberrant systemic expression of iNOS. Also IL-4 [235], IL-11 [236], IL-13 [237] and IL-10 [238] have been reported as iNOS suppressor.

A recent study show that the nitrate levels correlate with pro- and anti-inflammatory cytokines concentrations in serum of patients with severe sepsis; high levels of NO are associate with high levels of TNFα, IL-6 and IL-8 and with low concentrations of IL-10. Despite an increase of pro-inflammatory cytokine and correlated levels of NO were observed in the acute phase of sepsis , in the second phase, NO production was significantly reduced and the anti-inflammatory cytokines were predominantly present [239].

It has been demonstrate that the cytokines can play an important role in the modulation of iNOS, but it is important also mention that NO can play a direct role in cytokine modulation. For example, it is considered a potent inhibitor of cystein proteases such as IL-1β-converting enzyme [240]. NO can inhibit IL-1β and IFNγ-inducing factor (IL-18)[241].

2.4.iNOS in acute inflammatory response

iNOS expression after stimulation by bacterial endotoxin and cytokines is accompanied by the release of other mediators, such as PGE2 and prostacyclin, via the cyclooxygenase (COX) pathway [242, 243]. NO is crucial in the pathogenesis of septic shock [244-247]. The observation that iNOS-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia raised the possibility that induction of iNOS can be a homeostatic regulator for leukocyte recruitment [248]. In a murine model of hemorrhagic shock, it was found that iNOS expression and NO production caused an increase in PMN influx, activation of the transcriptional factor NF-κB, and upregulation of IL-6 and granulocyte colony stimulating factor (G-CSF) mRNA levels [249].

A critical role for the transcription factor NF-κB has been demonstrated in the transcriptional regulation of murine and human iNOS gene upon induction by LPS or cytokines in cultured cells [250-252]. It was also reported that LPS activates NF-κB in vivo, which, in turn,

induces transcription of iNOS gene and expression of iNOS protein in a rat model of septic shock [253].

iNOS transcription can be also activated by IRF-1 upon IFNγ stimulation.

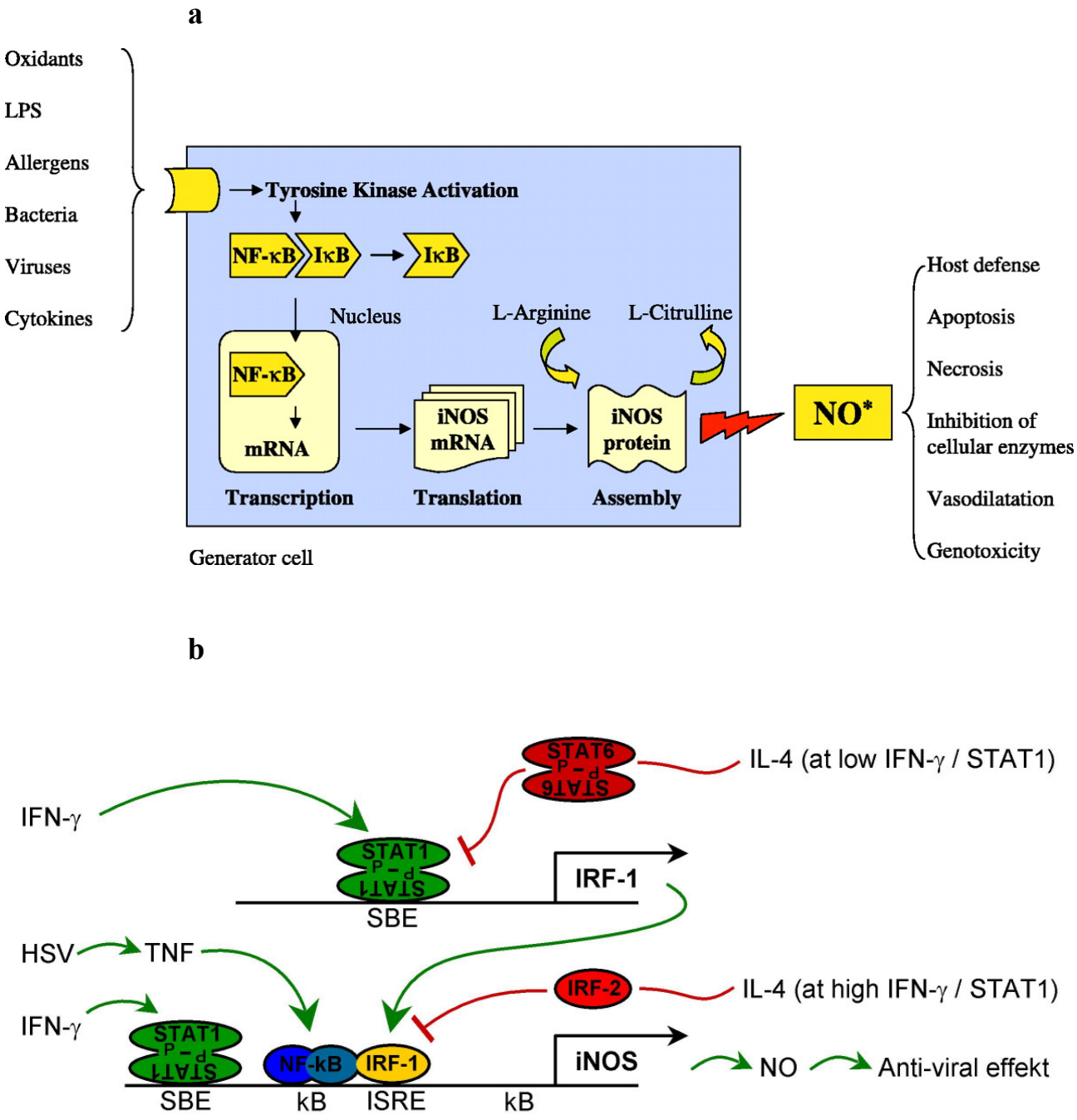


Figure 17. Regulation of iNOS induction at the molecular level.

*Transcription factors controlling induction of the iNOS gene. Activated NF-Kb alone (**a**), after stimulation of TLR4, or in collaboration with STAT1 (**b**), after stimulation with IFNγ, induces transcription of iNOS genes. IRF-1 interacts physically with NF-κB, binds to the distal κB-binding site of the iNOS promoter region, and stimulates transcription. Only when NF-κB is absent, IRF-2 can bind to the ISRE site and block transcription. Stimulatory pathways are indicated by green arrows (→), and inhibitory pathways are drawn in red. [254]*

2.5.Nitric Oxide in Chronic Inflammation

Release of high quantity of NO represents a common characteristic of inflammatory responses initiated by microbial products or autoimmune reactions. In inflammatory response generation, the defensive machinery of the immune system is based mainly on effector cells activity, such as T lymphocytes, macrophages and neutrophils. The intensity of such activation determines the intensity and duration of the inflammatory response.

Based on cytokine secretion pattern of CD4+ helper T lymphocytes, two main subsets of T helper cells are defined [255]: T helper type I (Th-1) that produce mostly IL-2, IFN-γ and TNF-α, and T helper type II (Th2) that produce IL-4, IL-5, IL-6, IL-10 and IL-13. The former are more involved in cell-mediated immune reactions, macrophage activation and production of opsonizing antibodies while the others are key players in humoral immunity and activate mast cells and eosinophils [255, 256].

Different studies showed the controversial role of iNOS in T lymphocyte proliferation of. For example a study by Taylor-Robinson et al. showed that in antigen-stimulated mice, Th1 cells produce high levels of NO that result in a concomitant reduction of IL-2 secretion and lymphocyte proliferation; this is reversed by addition of recombinant IL-2 [257]. Other groups demonstrated that NO can increase the secretion of IL-4 by Th2 cells [258]. The question of whether NO differentially affects T lymphocyte functions merits special attention, because the outcome of numerous diseases appears to depend critically on the Th1/Th2 balance [256] [259] [260] [261] [262]. In asthma, the increased proliferation of Th2 lymphocytes and consequent cytokine production is considered one of the most important factors responsible for the exacerbation of the disease. The involvement of NO production by iNOS in important autoimmune diseases, such as immunologically induced diabetes, inflammatory arthritis and graft versus host disease (GVHD) is widely documented [259, 263].

Understanding the beneficial or detrimental roles of NO in the different diseases onset is more difficult than expected. One possible explanation for these often contradictory results is that iNOS inhibition is detrimental to the host during priming of pathogenic T-cell responses in the periphery, but largely protective at the site of disease [264]. Another factor that can modify NO effect is the time during which the enzyme remains active and the quantity of NO produced in the different pathological conditions. More recently, inhibition of allergic airway inflammation was observed in mice lacking iNOS [265] [121, 266].

Other relevant immunologic effects attributed to NO are the inhibition of lymphokine-activated killer-cell induction by triggering apoptosis of cytolytic lymphocyte precursors [267], inhibition of Major Histocompatibility class II molecule expression on mouse peritoneal

macrophages and antigen presentation by lung dendritic cells and tumor-induced immunosuppression. Despite its direct involvement in a large number of inflammatory pathology, it is not clear whether the effector molecule is NO itself or RNOS, such as peroxynitrite or nitroxyl anion.

Disease	
Rheumatic disease	•Systemic lupus erythematosus •Vasculitis •Reumathoid arthritis •Osteoarthritis
Inflammatory airway disease	•Asthma •Respiratory tract infections •Idiopathic pulmunary fibrosis •Bronchiectasis
Gastrointestinal system	•Inflammatory blowel disease (Ulcerative colitis and Crohn's disease) •Diverticulitis •Necrotizing enterocolitis •Celiac disease •Helicobacter pylori-associated chronic gastritis
Kidney	•Glomerulonephritis •Lupus nephritis
Pancreas	•Diabetes •Pancreatitis
Liver	•Chronic hepatitis
Bladder	•Infectious and noninfectious cystitis
Central and periperal nervous system	•Parkinson's disease •Multiple sclerosis •Severe AIDS dementia •Vasculitic and optic neutropathy
Skin	•Psoriasis •Cutaneus Lupus erithematosus •Systemic sclerosis •Dermatitis
Atherosclerosis	
Periapical periodontitis	
Sjogren's syndrome	

Figure 18. Nitric oxide in human autoimmune and chronic inflammatory diseases

The hypothetical role of peroxynitrite as inflammatory mediator has been suggested after detection of nitrotyrosine in animal models of endotoxemia [268-270], lung injury [271, 272], ileitis [273, 274], experimental autoimmune encephalomyelitis [275, 276], myocardial ischemia-reperfusion injury [277, 278], myocardial dysfunction [279], and glomerulonephritis [280], as well as in human atherosclerotic plaques [281, 282], adult respiratory distress syndrome [283], airways of asthmatic patients [284], multiple sclerosis [285, 286], and human sepsis and myocarditis [287].

2.6.Inducible NOS in Chronic Inflammatory Diseases

Chronic inflammation is characterized by a proliferation of fibroblasts and small blood vessels, as well as an influx of chronic inflammatory cells (lymphocytes, plasma cells, macrophages). Human iNOS expression has been found in chronic inflammatory diseases of airways, vessels, bowels, kidney, heart, skin and apex of teeth [288], strongly indicating that NO plays an important role in the pathogenesis of chronic inflammation.

Nitric oxide stimulates TNFα production by synoviocytes and its catabolic effects on chondrocyte functions promote the degradation of articular cartilage implicated in certain rheumatic diseases [289, 290]. Several studies indicate that NO is at least partly responsible for IL-1β-induced suppression of glycosaminoglycan and collagen synthesis [291]. In human chondrocytes, IL-18 has been identified as a cytokine that regulates chondrocyte responses and contributes to cartilage destruction through stimulation of the expression of several genes, including iNOS, inducible COX, IL-6, and stromelysin [292]. Furthermore, in a model of osteoarthritis (OA) in dogs, inhibition of iNOS reduced the progression of cartilage lesions and the production of metalloproteinases and IL-1β [289].

Administration of a natural IL-12 antagonist, which suppressed the progression of islet inflammation and concomitant upregulation of iNOS [293], and overexpression of the anti-apoptotic gene A20, which abrogated cytokine-induced NO production and protected both human and rat islet cells against apoptosis [294], suggests possible strategies for therapeutic intervention against NO-mediated toxicity in islet inflammation. Moreover, NO also contributes to mucosal damage in inflammatory bowel disease [295, 296] and the beneficial effects of iNOS inhibitors in reducing intestinal inflammation is observed in various models of colitis [297-299]. Furthermore, NO also is reported to promote mucosal integrity [300].

Defective NO production by eNOS, together with iNOS and superoxide anions generated by inflammatory cells, are detrimental events that may cause apoptosis and injury to both endothelium and myocytes, and possibly lead to plaque rupture in atherosclerosis. In this way, the balance between the possible protective effects of NO and the deleterious effects of RNS may be

disturbed [301]. In endothelial cells, NO prevents apoptosis [240, 302]; whereas, it induces apoptosis in smooth muscle cells [303, 304]. The presence of iNOS in atherosclerotic plaques suggests a role for NO in atherosclerosis [305, 306], but its exact role is still unknown. Interestingly, in a recent study of advanced human atherosclerotic plaques, high expression levels of the anti-inflammatory cytokine, IL-10, is associated with significant decreases in iNOS expression and cell death [307]. Additionally, TGF-β1 and its signaling system are perturbed in atherosclerosis [308]. These findings suggest that a balance between iNOS-inducing and iNOS-suppressing mediators might modulate the expression of iNOS in atherosclerosis.

The expression of iNOS is also described in allergic asthma [309], foreign body-induced granulomatous lung inflammation [310], as well as in radiation pneumonitis and fibrosis in rats [311]. The exhaled NO levels are increased in patients with asthmatic flares, bronchiectasis, and active tuberculosis, and are considered as a marker of inflammatory injury; however, the precise role of NO in lung inflammation is still under debate [312, 313]. Given the increasing evidence that viruses are a major cause of acute exacerbation of asthma, the cytototoxic and potent antiviral properties of inducible NO may be beneficial [312]. The interaction of NO with the transcription factor NF-κB, which is activated by diverse inflammatory stimuli, is causally linked to respiratory cell inflammation and pulmonary disease [314]. It should be noted that high concentrations of NO are capable of killing *Mycobacterium tuberculosis* and this may be significant for the control of infection in the lung [315].

2.7.iNOS in asthma

Despite the alarming increased rate in prevalence and morbidity of asthma worldwide, the complexity this chronic inflammatory disease remains a serious hindrance to establishing a clear understanding [316]. Because nitrosative stress affects respiratory health, it has been hypothesized that genetic variants of NOS2A could be associated with asthma incidence and lung function growth during adolescence. Furthermore, because the formation of peroxynitrite is dependent on the availability of ROS, it is associated with genetic variants of determinants of oxidative stress (GSTM1, GSTP1, CAT and HMOX1) that previously have been reported to be associated with lung function growth or asthma pathogenesis in a specific cohort of patients. [317, 318] [39]. An increasing number of conflicting reports have demonstrated the detrimental, protective and sometimes neutral roles for iNOS in the pathogenesis of asthma [319, 320]. However, it is undoubtedly established that iNOS is expressed in lungs of asthmatics with a subsequent production of NO and generation of the reactive metabolite as $ONOO^-$ [321-324]. It appears that expression of

iNOS is even higher in sputum cells from asthmatics compared to those from patients with controlled disease or healthy individuals [325].

It is well accepted that the increased amount of NO in exhaled air in individuals with asthma reflects disease severity [319, 320]. On the other hand, NO is considered a major beneficial player in airway function, as it controls vascular and bronchial tone and neuroendocrine regulation of airway mediator release [320]. NO rapidly oxidizes sulfhydryl groups and mediates nitration and hydroxylation of aromatic compounds including tyrosine and guanosine [321] after combining with superoxide to form the highly reactive peroxynitrite ($ONOO^-$). The $ONOO^-$ participates not only in cell killing and tissue injury in airway inflammation [321, 322], but also influences the function and/or expression of many inflammatory factors [323].

iNOS is rapidly induced upon exposure to a variety of inflammatory agents including allergens, oxidants, or cytokines [322, 323, 326]. It is important to note that arginases compete for L-arginine, which may influence the function of iNOS and the outcome of NO production [327]. The role of arginases in asthma remains unsettled, as several studies reported that inhibition of these enzymes could exert either anti- or proinflammatory effects [328-330].

The potential of iNOS as a therapeutic target for the treatment of the disease was severely hampered by the negative results of a clinical trial showing that a selective iNOS inhibitor did not affect AHR or airway inflammation after allergen challenge in steroid-naive human subjects with asthma, although it effectively reduces exhaled Nitric Oxide (NO). However, the association between asthma protection or susceptibility with polymorphisms in the iNOS gene maintains its viability as a potential therapeutic target and the importance of this gene in the pathogenesis of the disease. So, we believe that such conclusion is premature and that more careful studies are necessary to fully understand the role of iNOS in asthma and whether the enzyme can be adequately targeted at least as an adjuvant therapy for the treatment of the disease.

Our laboratory recently showed that iNOS deletion was associated with a reduction in eosinophilia, mucus hypersecretion and Th2 cytokine production (IL-5 and IL-13) upon an acute (single) exposure to ovalbumin (OVA). Such protection was completely abolished upon repeated (chronic) exposure to OVA. Interestingly, pulmonary fibrosis observed in wild-type mice under the chronic protocol was completely absent in $iNOS^{(-/-)}$ mice despite persistent IL-5 and IL-13 production, suggesting that these cytokines were insufficient for pulmonary fibrosis. Such protection was associated with reduced collagen synthesis and indirect but severe TGF-beta modulation as confirmed using primary lung smooth muscle cells. Although activation of matrix metalloproteinase-2/-9 exhibited little change, the large tissue inhibitor of metalloproteinase-2 (TIMP-2) increase detected in wild-type mice was absent in the $iNOS^{(-/-)}$ counterparts. The

regulatory effect of iNOS on TIMP-2 may be mediated by peroxynitrite, as the latter reversed TIMP-2 expression in iNOS$^{(-/-)}$ lung smooth muscle cells and fibroblasts, suggesting that the iNOS-TIMP-2 link may explain the protective effect of iNOS-knockout against pulmonary fibrosis. These results suggest that iNOS inhibition may be protective against some aspects of asthma but not others.

Our laboratory also established a reciprocal relationship between iNOS and Poly(ADP)-ribose polymerase 1 (PARP-1). However, PARP inhibition, pharmacologically or by gene knockout, protected against inflammation and AHR (as shown in the present study) both in acute and chronic asthma models. It is interesting that PARP-1 inhibition does not completely abrogate expression of iNOS leaving the possibility that the protective effect of PARP inhibition against inflammation and AHR may be associated with the reduction but not the complete inhibition of iNOS and associated production of moderate levels of NO.

3.PARP-1

PARP-1 is the most characterized member of its family that consists of 17 members.

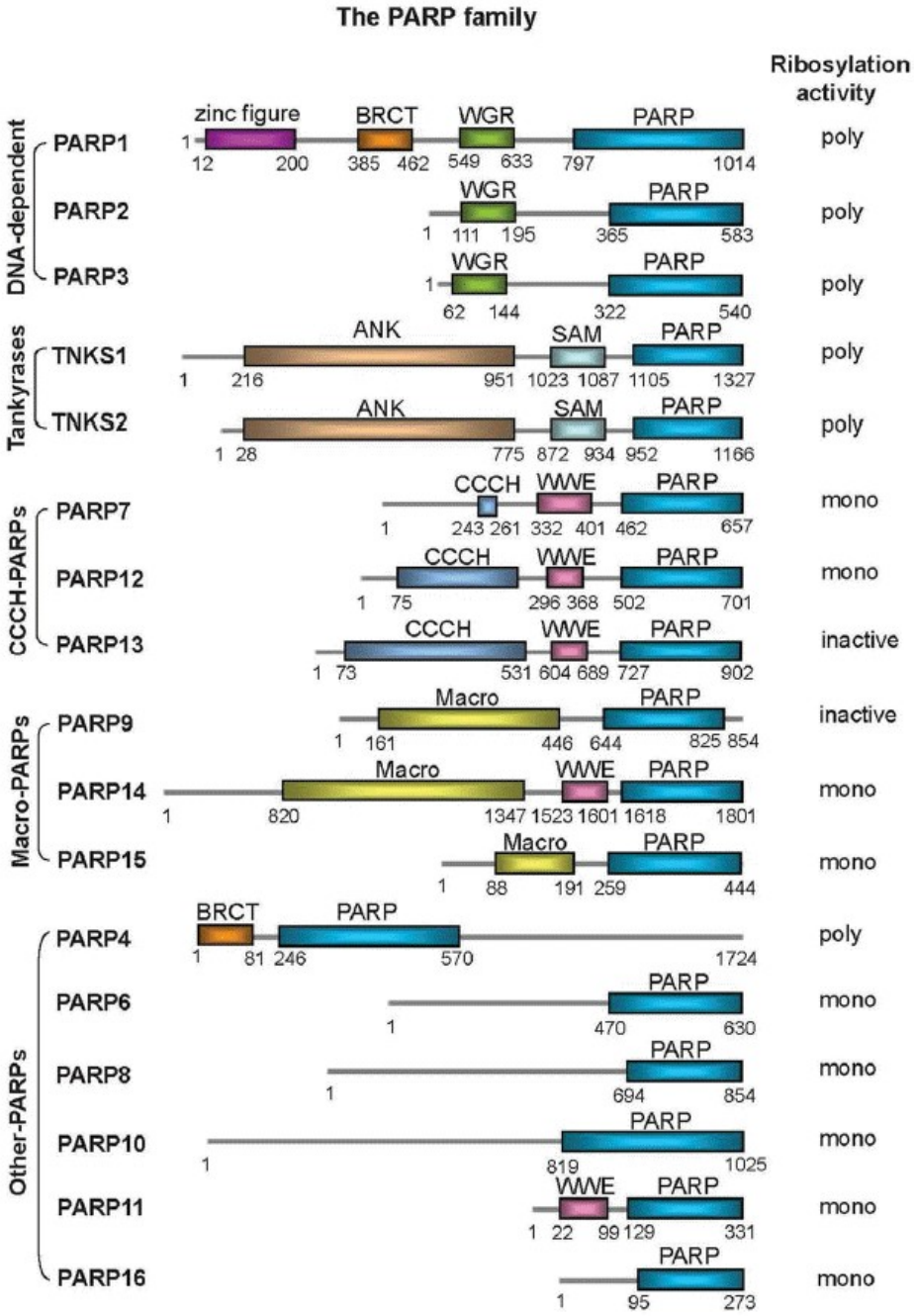


Figure 19. The PARP superfamily

The PARP domain is the catalytic domain and is required for NAD^+ binding and PAR synthesis. The zinc finger domain is an DNA-binding domain. The BRCA1 C terminus (BRCT), ankyrin repeat (ANK) and sterile α-motif (SAM) domains are protein-protein interaction domains. The CCCH domain is a Cys-Cys-Cys-His zinc finger domain. The WGR is a functionally unknown domain. Macro and WWE are PAR-binding domains.

PARP-1 account for at least 85% of the cellular PARP activity. This enzyme is highly involved in DNA repair, as it is responsible for the maintenance of the genome integrity and

increases cell survival as a consequence. PARP-1 catalyzes the synthesis and attachment of highly negatively charged PARs to target proteins, including histones, topoisomerases, DNA helicases, and single-strand break repair and base excision repair factors; and facilitates relaxation of the chromatin superstructure, protein-protein interaction, and DNA-binding ability of the members of the DNA repair machinery. In addition, the importance of poly(ADP-ribose) synthesis has been established in many other cellular processes, such as chromatin replication, transcriptional regulation, and cell death. PARP-1 is also the enzyme responsible for one of the most essential protein modification, the Poly(ADP-ribosyl)ation. Polymers of ADP-ribose (PAR) are formed from donor NAD^+ molecules and covalently attached via an ester linkage to glutamic acid and less commonly to aspartic acid or lysine of target protein.

In general, the PAR-binding motif localizes near a functional domain of the protein, such as a Protein or DNA-binding domain in the way that the (PAR)ylation alter the functional property of the target.

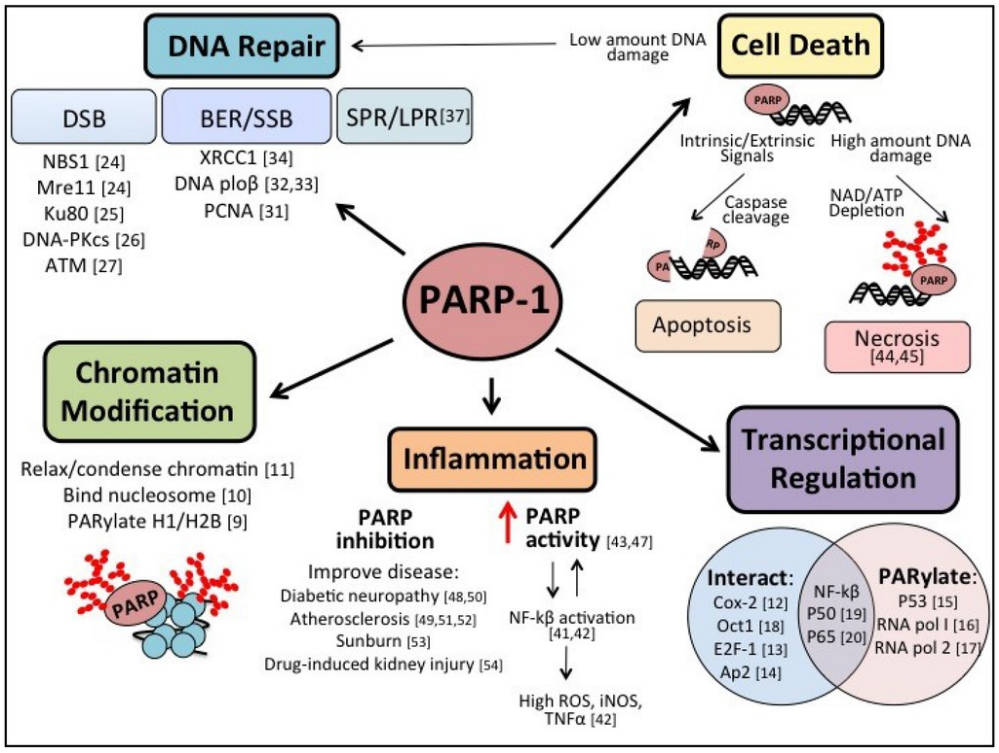


Figure 20. Schematic delineating the multifaceted nature of Poly(ADP) Ribose Polymerase (PARP): DNA repair, Chromatin Modification, Inflammation, Transcriptional Regulation, and Cell Death. [331]

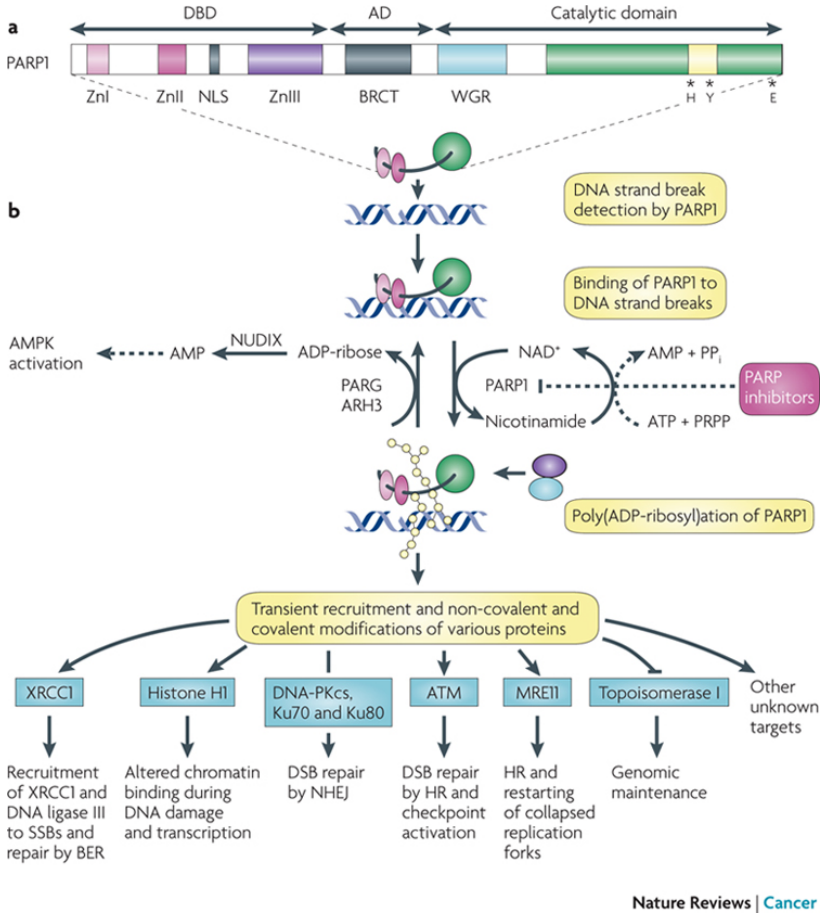


Figure 21. Consequences of PARP-1 activation by DNA damage.

Although not shown to simplify the scheme, PARP1 is active in a homodimeric form. PARP1 detects DNA damage through its DBD. This activates PARP1 to synthesize poly(ADP) ribose (pADPr; yellow beads) on acceptor proteins, including histones and PARP1. Owing to the dense negative charge of pADPr, PARP1 loses affinity for DNA, allowing the recruitment of repair proteins by pADPr to the damaged DNA (blue and purple circles). Poly(ADP-ribose) glycohydrolase (PARG) and possibly ADP-ribose hydrolase 3 (ARH3) hydrolyse pADPr into ADP-ribose molecules and free pADPr. ADP-ribose is further metabolized by the pyrophosphohydrolase NUDIX enzymes into AMP, raising AMP:ATP ratios, which in turn activate the metabolic sensor AMP-activated protein kinase (AMPK). NAD^+ is replenished by the enzymatic conversion of nicotinamide into NAD^+ at the expense of phosphoribosylpyrophosphate (PRPP) and ATP. Examples of proteins non-covalently (pADPr-binding proteins) or covalently poly(ADP-ribosyl)ated are shown with the functional consequences of modification. It is important to note that many potential protein acceptors of pADPr remain to be identified owing to the difficulty of purifying pADPr-binding proteins in vivo. PARP inhibitors prevent the synthesis of pADPr and hinder subsequent downstream repair processes, lengthening the lifetime of DNA lesions. ATM, ataxia telangiectasia-mutated; BER, base excision repair; BRCT, BRCA1 carboxy-terminal repeat motif; DNA-PKcs, DNA-protein kinase catalytic subunit; DSB, double-strand break; HR, homologous recombination; NHEJ, non-homologous end joining; NLS, nuclear localisation signal; PP_i, inorganic pyrophosphate; SSB, single-strand break; Zn, zinc finger [332].

PARP-1 is a protein composed of 1014 aa (MW ~116 kDa). It has three main domains: a DNA-binding domain (DBD), an auto modification domain and the catalytic domain that bind

NAD+. The N-terminal 46-kDa DBD contains three zinc-finger motifs, of which the first two recognize DNA damage and direct PARP-1 binding to the damaged DNA [333, 334]. More recently, it was reported that the third zinc-finger motif in the DBD facilitated interdomain contact and assembly of the DNA-activated conformation of PARP-1 [335]; therefore, it was considered essential for DNA-dependent PARP-1 activity. [335-337]. The automodification domain (AMD) has a MW of 22 kDa. It is rich in glutamic acid residues that are suggested to be a site for covalent binding with poly (ADP-ribose) on PARP-1 activation. This looks like a mechanism of auto control for the PAR synthesis. The AMD is considered an important factor for protein-protein interaction between PARP-1 and the members of the DNA repair and gene transcriptional machinery, as well as for PARP-1 homodimerization or heterodimerization with PARP-2 [338-340]. Dimerization is assumed to be a prerequisite for PARP-1 activation [341]; however, the AMD deletion mutant is catalytically active, indicating that this segment is not indispensable for PARP-1 activity[342].

Moreover, the C-terminal region has a molecular weight of 54 kDa and represent the most conserved part of the enzyme. It consists of an NAD^+-binding domain and executes the catalytic function of PARP-1, synthesizing PARs by using NAD^+ as a substrate. After its binding to DNA, PARP-1 catalyzes the formation of PARs. The most abundant poly (ADP-ribosyl)ated protein in the cell is PARP-1 itself, and the accumulation of PAR on PARP-1 leads to its repulsion and dissociation from DNA strands[343, 344].

The PARs are rapidly degraded by PARG [345, 346], and PARG has both endoglycosidase and exoglycosidase activities (endoglycosidase being greater than exoglycosidase), producing free PAR and mono(ADP-ribose) [347, 348]. The amount of PAR formation and its attachment to other proteins are controlled by PARP-1 and PARG.

Our laboratory has shown the involvement of poly(ADP-ribose) polymerase (PARP)-1 in tissue injury and its implication in several conditions associated with oxidative stress and inflammation including allergic airway inflammation [349-352]. In addition to its effects on cell and tissue homeostasis through nicotinamide adenine dinucleotide (NAD^+) metabolism, PARP-1 is thought to participate in inflammation by regulating, directly or indirectly, the expression of several inflammatory factors including iNOS (reviewed [353-355]. Such activity has been associated with the ability of PARP-1 to regulate signal transduction events that result in the activation of nuclear factor (NF)-κB [356, 357] and in increasing its permanence in the nucleus, trough reduction of interaction with the exportine Crm-1, to activate the transcription of different pro-inflammatory genes such as iNOS, ICAM VCAM and others.

Our laboratory also conducted a study that investigated the role of iNOS in ovalbumin-induced eosinophilia from the perspective of its relationship with PARP-1 and oxidative DNA damage purposing the following mechanism model.

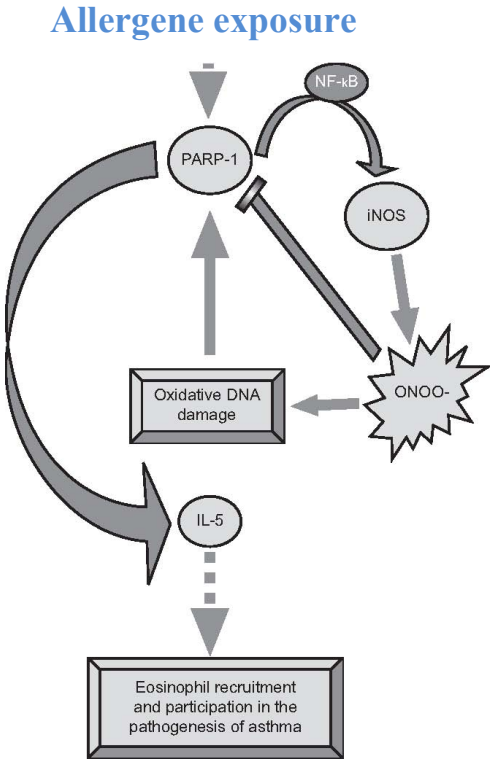


Figure 21. Model for the potential reciprocal regulation of inducible nitric oxide synthase (iNOS) and poly(ADP-ribose) polymerase-1 (PARP-1) during allergen-induced airway inflammation.

Upon allergen exposure that involves a number of intricate processes, PARP-1 participates in the process of iNOS expression potentially through nuclear factor (NF)-κB-mediated signal transduction and interleukin (IL)-5 production through a yet-unknown mechanism. IL-5, in addition to several important cytokines, promotes the recruitment of inflammatory cells such as eosinophils to the lung. iNOS produces high levels of nitric oxide, which can be converted into peroxynitrite ($ONOO^-$) after its interaction with superoxide. $ONOO^-$ causes oxidative tissue damage as manifested by protein nitration and induces DNA strand breaks which are potent activators of the nicotinamide adenine dinucleotide-utilising enzymatic activity of PARP-1. Nitration of PARP-1 renders the enzyme inactive, representing a potential regulatory mechanism by which iNOS modulates PARP-1 enzymatic activity. Inhibition of PARP-1 may represent an attempt by the cells to control the inflammatory response. [358]

Upon allergen exposure that involves different complicated processes, PARP-1 participates in the process of iNOS expression potentially through nuclear factor (NF)-κB-mediated signal transduction and interleukin (IL)-5 production through an unknown mechanism. IL-5 promotes the recruitment of inflammatory cells such as eosinophils in the lung. The hyper-activation of iNOS produces high levels of nitric oxide, which can be converted into peroxynitrite ($ONOO^-$) after its interaction with superoxide. $ONOO^-$ causes oxidative tissue damage as manifested by protein

nitration and induces DNA strand breaks which are potent activators of the nicotinamide adenine dinucleotide-utilising enzymatic activity of PARP-1. Nitration of PARP-1 inactivates the enzyme, representing a potential regulatory mechanism by which iNOS modulates PARP-1 enzymatic activity. Inhibition of PARP-1 may be an attempt of the cells to control the inflammatory response.

3.1.PARP-1 beyond DNA repair

The pathological over-activation of PARP-1, due to reactive oxygen and nitrogen species formation, promotes cell death, and stimulates pro-inflammatory mediator production [359]. PARP inhibitors have been shown to exert tissue protective and anti-inflammatory effects in animal models of ischemia-reperfusion injury, circulatory shock, and various forms of inflammation [359, 360]. It has also been recently suggested that PARP-1 and PARP-2 (a minor isoform of PARP enzyme family) play important role in regulating metabolic functions in rodents (e.g. mitochondrial function/biogenesis, and adipogenesis) in various organ systems, at least in part via modulation of NAD+ levels and consequently sirtuin 1 activity [361, 362] [363]. Furthermore, PARP inhibition has recently been shown to improve mitochondrial function (respiration, enzyme activity, reactive oxygen species defense) in both C. elegans worms and in AML12 hepatocyte cell line, and promoted longevity in worms [364].

Recent studies have linked PARP-1 activation and up-regulation to the production of pro-fibrotic markers such as connective tissue growth factor (CTGF) [365] and TGF-β [366] in kidney tubular epithelial and vascular smooth muscle cells. A study conducted in our laboratory unveiled a pathogenic role of PARP-1 in liver inflammation, metabolism and fibrosis, and identify the potential therapeutic utility of PARP inhibitors for liver inflammatory diseases and fibrosis induced by chronic CCl4 exposure [367]. PARP-1 activation and the pro-fibrotic gene expression such as TGF-β and CTGF have been documented in vascular smooth muscle cells and renal proximal tubular epithelial cells in vitro [365, 366].

PARP inhibitors also exert beneficial effects in preclinical and clinical models of cancers via multiple mechanisms involving attenuation of cancer cell proliferation and migration, decrease of angiogenesis, modulation of the tumor pro-inflammatory environment, and promotion of cancer cell demise. The selective promotion of apoptotic cell death in cancer, but not in normal cells by PARP inhibitors is based on the novel approach of “synthetic lethality” in cancer therapy, because in certain cancers with selective defects in homologous recombination repair (cancer cells frequently harbor defects in DNA repair pathways leading to genomic instability) inactivation of PARP-1, and possibly other minor isoforms of PARP, directly causes cell death as in the case of BRCA-deficient breast cancer. Because of this, several classes of ultrapotent PARP inhibitors are

currently in clinical trials for the experimental therapy of various malignancies, including triple-negative breast and ovarian cancers [368].

3.2.PARP-1 and NF-κB connection

We previously demonstrated a critical role of PARP-1 during inflammation, in part, through its relationship with NF-κB, the transcription factor responsible for regulation of expression of iNOS as well as of other inflammatory genes including MCP-1, VCAM, and ICAM. For a long time, the mechanism by which PARP-1 affects NF-κB activation has been completely unknown . Our group showed that PARP-1 inhibition by gene knockout, knockdown, or pharmacologic blockade prevented p65 NF-κB nuclear translocation in smooth muscle cells upon TLR4 stimulation. NF-κB DNA-binding activity and subsequent inflammatory gene expression were reversed by reconstitution of PARP-1 expression. PARP-1 was dispensable for LPS-induced IκBα phosphorylation and subsequent degradation; however, it was required for p65 NF-κB phosphorylation. The perinuclear p65 NF-κB localization in LPS-treated PARP-$1^{-/-}$ cells was associated with a defect in the exportins rather than importins system. Whereas PARP-1 deficiency did not modify expression of importin-alpha3 and alpha4 and their cytosolic localization, the cytosolic levels of exportin (Crm)-1 were increased. Interestingly, p65 NF-κB poly (ADP-ribosyl)ation decreased its interaction with Crm1 in vitro and pharmacologic inhibition of PARP-1 increased p65 NF-κB-Crm1 interaction in LPS-treated smooth muscle cells. [369] These results suggest that p65 NF-κB poly (ADP-ribosyl)ation may be a critical determinant for the interaction with Crm1 and its nuclear retention upon TLR4 stimulation. These results provided novel insights into the mechanism by which PARP-1 promotes NF-κB nuclear retention, which ultimately can influence NF-κB-dependent gene regulation.

Aim of the study

Asthma can be controlled by a combination of an inhaled corticosteroid (anti-inflammatory) and a short or long-acting acting β_2-adrenergic receptor agonists in the majority of the case. However, 5-10% of the patients do not respond to the available therapies (*http://www.globalasthmareport.org)*. In these patients, asthma symptoms may worsen with onset of concomitant co-morbidities including rhinitis, sinusitis, gastroesophageal reflux, obesity and obstructive sleep apnea [51]. Thus, although patients with uncontrolled asthma are a minority of the total asthmatic population, they have a high risk of serious morbidity and mortality, and use the largest share of economic resources and health-care services, including emergency visits, hospitalizations and additional consumption of drugs for recurrent exacerbations [52]. Moreover, severe asthma results in frequent absences from school and work. Those patients are more susceptible to anxiety and depression [53]. For these reasons, additional therapeutic approaches are urgently required for those individuals who have poorly controlled asthma.

Although a clinical trial conducted in a group of steroid-naive patients showed that inhibiting iNOS failed to block inflammation and AHR in asthmatics [370], we believe that such conclusion is premature and that we should explore new avenues to take advantage of such an important clinical target (i.e. iNOS). Our assessment stems from the complexity and the lack of a clear understanding of the role iNOS in inflammatory diseases and asthma in particular. Accordingly, an important *aim of this study was to shed light on the role of iNOS in acute and chronic asthma by focusing primarily on the role of the protein in AHR manifestation*. Based on previous data from our group showing that PARP-1 inhibition-associated protection against different aspects of asthma including inflammation and AHR potentially trough the partial inhibition of iNOS, another aim of this study was to investigate the relationship between iNOS and PARP-1 in the manifestation of asthma-related AHR. Different strategies were used to conduct the study including clinically tested specific iNOS inhibitor (L-NIL) and a PARP inhibitor (AZD2281 or olaparib). $iNOS^{-/-}$ and $PARP\text{-}1^{-/-}$ mice were also used in the studies to determine specificity of the observed effects. The ultimate aim of this study was to renew the potential of iNOS as an appealing therapeutic target for the treatment of asthma either directly or through inhibiting PARP-1.

Overall, the results of the present study suggest that the amount of iNOS and NO are critical determinants in the modulation of AHR by selective iNOS inhibitors and renew the potential of iNOS as being a potential therapeutic target for treatment of asthma.

Materials and methods

Human subjects

Lung histopathology:

Paraffin-embedded tissue sections from two de-identified lung from two individuals who died from severe asthma were acquired from the LSUHSC Pathology Department (kindly provided by Dr. C. Espinoza). The sections were subjected to removal of paraffin with xylene, re-hydrated using ethanol gradient, and then assessed for inflammatory cells using hematoxylin and eosin (H&E) stain. Mucus secretion was quantified from sections stained with periodic acid-Schiff (PAS) using standard protocols. All images were taken on Olympus microscope using AxioVision software**.**

Immunofluorescence.

Paraffin-embedded tissue sections from two de-identified lung from two individuals who died from severe asthma and a normal lung (verified by a pathologist from the SSSCC Molecular Histopathology and Analytical Microscopy Core) from one individual who died from asthma-unrelated conditions were acquired from the LSUHSC Pathology Department (kindly provided by Dr. C. Espinoza). The sections were subjected to removal of paraffin with xylene, re-hydrated using ethanol gradient, and then incubated in Citrate buffer to retrieve the antigen. After, sections were incubated over night with primary antibodies to PAR, INOS and CD68 at room temperature. Antibody–antigen complexes were detected with Alexa-546-conjugated secondary antibody for 1 h at room temperature. The sections were thoroughly washed after each incubation and then counterstained with DAPI. Section were examined under a Leica DMRA2 fluorescence microscope using a 40× objective lens (Leica, Buffalo Grove, IL).

Immunohistochemistry.

Paraffin-embedded tissue sections from two de-identified lung specimens from individuals who died from severe asthma were subjected to removal of paraffin with xylene, re-hydrated using ethanol gradient, and then incubated in Citrate buffer to retrieve the antigen. Endogenous peroxidase activity was neutralized by incubation of the sections for 20 min in Methanol containing 20% H_2O_2. After several washes with PBS, the sections were incubated for 2 hours with 5% normal horse or goat serum for staining that require mouse and rabbit antibodies respectively and then overnight at room temperature with the mouse monoclonal antibody to PAR (ENZO Life Sciences, Inc NY) and iNOS (Abcam MA) or the rabbit polyclonal antibody to Nitrotyrosine (EMD Millipore CA). The sections were washed again with PBS, after which

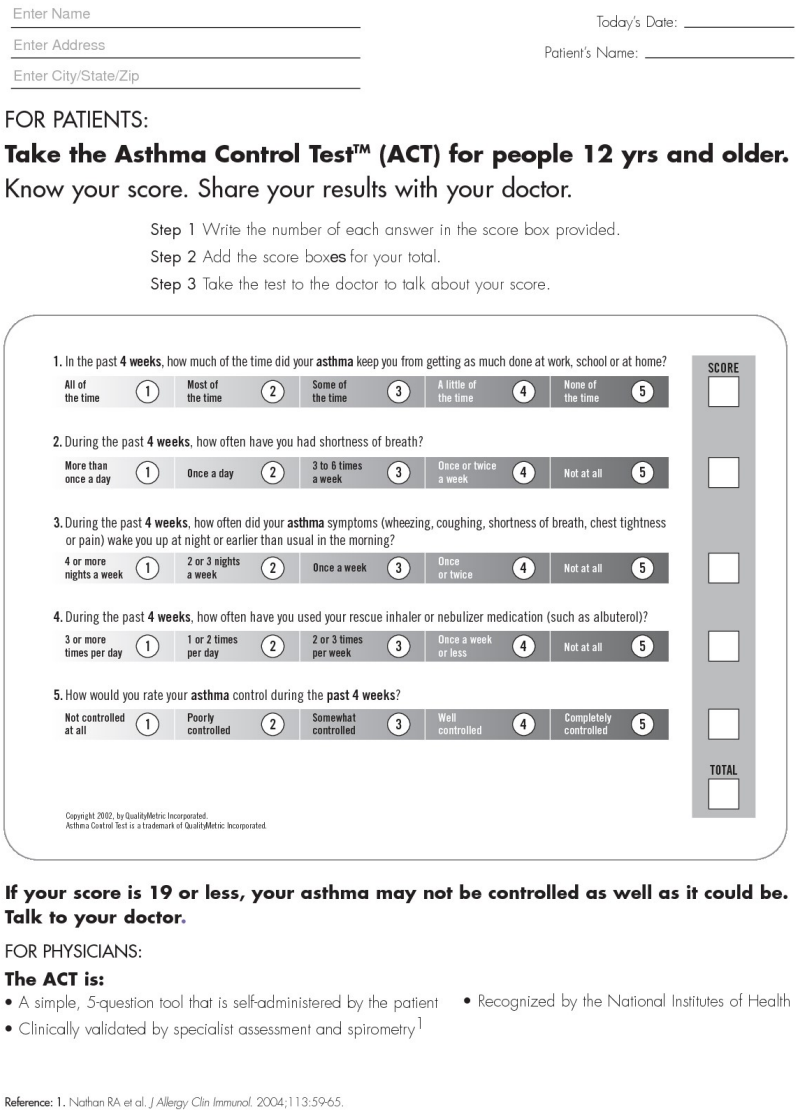
they were exposed consecutively to biotinylated horse/goat secondary antibodies to mouse and rabbit immunoglobulin G, avidin-conjugated horseradish peroxidase, and peroxidase substrate (ABC kit; Vector Laboratories CA). The sections were then counterstained with hematoxylin for 20 s, and re-blue in ammonia water, exposed to a graded series of ethanol solutions to dehydrate and clear in xylene for 1 hour. In the end the slides were mount in permount, covered with coverslips, and examined by light microscopy.

Immunoblot.

Healthy and asthmatic individuals were recruited under a protocol (#8450) approved by the LSUHSC institutional review board. Subjects were included if they were ≥18 years of age with a physician diagnosis of asthma. Exclusion criteria were a diagnosis of another lung disease other than asthma, active malignancy or inflammatory condition, or ≥10 pack-years of smoking. Human PBMCs isolated from peripheral blood of healthy donors or asthmatic individuals were subjected to protein extractions followed by immunoblot analysis with antibodies to Nitrotyrosine (EMD Millipore CA), the recombinant fragment corresponding to Human iNOS aa 997-1058 (Abcam MA) and GAPDH (Abcam MA) and PAR (ENZO Life Sciences, Inc NY).

ACT score

The Asthma Control Test Score was evaluated for all the patients. It consists in a questionnaire-based tool that can be used to standardise review of asthma symptoms.



Enter Name
Enter Address
Enter City/State/Zip

Today's Date: _____
Patient's Name: _____

FOR PATIENTS:

Take the Asthma Control Test™ (ACT) for people 12 yrs and older.

Know your score. Share your results with your doctor.

Step 1 Write the number of each answer in the score box provided.
Step 2 Add the score boxes for your total.
Step 3 Take the test to the doctor to talk about your score.

									SCORE
1. In the past **4 weeks**, how much of the time did your **asthma** keep you from getting as much done at work, school or at home?	All of the time	1	Most of the time	2	Some of the time	3	A little of the time	4	None of the time 5	
2. During the past **4 weeks**, how often have you had shortness of breath?	More than once a day	1	Once a day	2	3 to 6 times a week	3	Once or twice a week	4	Not at all 5	
3. During the past **4 weeks**, how often did your **asthma** symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?	4 or more nights a week	1	2 or 3 nights a week	2	Once a week	3	Once or twice	4	Not at all 5	
4. During the past **4 weeks**, how often have you used your rescue inhaler or nebulizer medication (such as albuterol)?	3 or more times per day	1	1 or 2 times per day	2	2 or 3 times per week	3	Once a week or less	4	Not at all 5	
5. How would you rate your **asthma** control during the **past 4 weeks**?	Not controlled at all	1	Poorly controlled	2	Somewhat controlled	3	Well controlled	4	Completely controlled 5	
									TOTAL	

Copyright 2002, by QualityMetric Incorporated.
Asthma Control Test is a trademark of QualityMetric Incorporated.

If your score is 19 or less, your asthma may not be controlled as well as it could be. Talk to your doctor.

FOR PHYSICIANS:

The ACT is:

- A simple, 5-question tool that is self-administered by the patient
- Clinically validated by specialist assessment and spirometry[1]
- Recognized by the National Institutes of Health

Reference: 1. Nathan RA et al. *J Allergy Clin Immunol.* 2004;113:59-65.

Figure 23. Asthma Control Test

The ACT asks 5 questions related to a patient's asthma control over the previous 4 weeks. Scores range from 5 to 25 and higher scores indicate better control. If a patient scores 19 or less, the asthma is considered not well controlled, on the basis of validation studies.

Human $CD4^+$ T cell proliferation assay

T-cell proliferation was tested by CFSE (Molecular Probes–Invitrogen, Eugene, OR, USA) following the vendor's recommendations. Briefly CD4+ T cells were re suspended in complete RPMI 1640 medium at concentration of 10^7cells/mL. Carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA) was added at a final concentration of 5 μM, and the cells were incubated for 10 min at 37 °C in 5% CO_2. The labeling was

quenched using 5 times the volume of ice-cold complete RPMI 1640 for 5 min and excess dye was washed away 3 times with cold RPMI-1640. CFSE-labeled cells were then stimulated with anti-CD3/ anti-CD28 antibodies as previously described. After 5 days of culture, Cells were harvested and the CFSE fluorescent staining was analyzed by flow cytometry and analyzed using FlowJo software.

RT-PCR analysis.

Human and mouse CD4+-enriched T cells were purified using EasySep™ Human/ Mouse CD4+ T Cell Isolation Kit following the vendor's recommendations. Cells were then stimulated in the presence or absence of 1 or 5 μM olaparib with immuno-immobilized anti-CD3 plus anti-CD28 antibodies followed by RNA extraction as described [371]. The extracted RNA was reverse transcribed into cDNA using reverse transcriptase III (Invitrogen), the resulting cDNA was subjected to conventional or quantitative PCR using primer sets (IDT, San Jose, CA, USA) specific for human *gata-3*, *t-bet*, IL-4 or *gapdh*.

Gene	Primer sequence
Human *Gata -3*	5'-TGTCTGCAGCCAGGAGAGC-3' 5'-ATGCATCAAACAACTGTGGCCA-3'.
Human *T-bet*	5'-GATGTTTGTGGACGTGGTCTTG-3' 5'-CTTTCCACACTGCACCCACTT-3'
Human *gapdh*	5'-GCGAGATCCCTCCAAAATCAA-3' 5'-GTTCACACCCATGACGAACAT-3'

Animals

OVA/HDM challenge and airway hyper responsiveness.

Six-eight week old C57BL/6J male mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). C57BL/6 iNOS$^{-/-}$ and PARP-1$^{-/-}$ mice were bred at the LSUHSC vivarium and allowed unlimited access to sterilized chow and water. Husbandry, experimental protocols, and procedures were all approved by the LSUHSC Animal Care & Use Committee.

Mice were sensitized to chicken OVA (Sigma-Aldrich, St. Louis MO) or HDM (*Dermatophagoides pteronyssinus*) extract (Greer Labs, Lenoir, NC). Then, mice were challenged by placing them in groups of eight in a Plexiglas chamber (14 x 14 x 9 cm) and exposing them for 30 minutes to aerosolized OVA (3% OVA in saline) for three time for one week (acute asthma model) or three time a week for three weeks (chronic asthma model) or with a single or multiple administration. The OVA aerosol was generated by a Bennett nebulizer (Figure23).

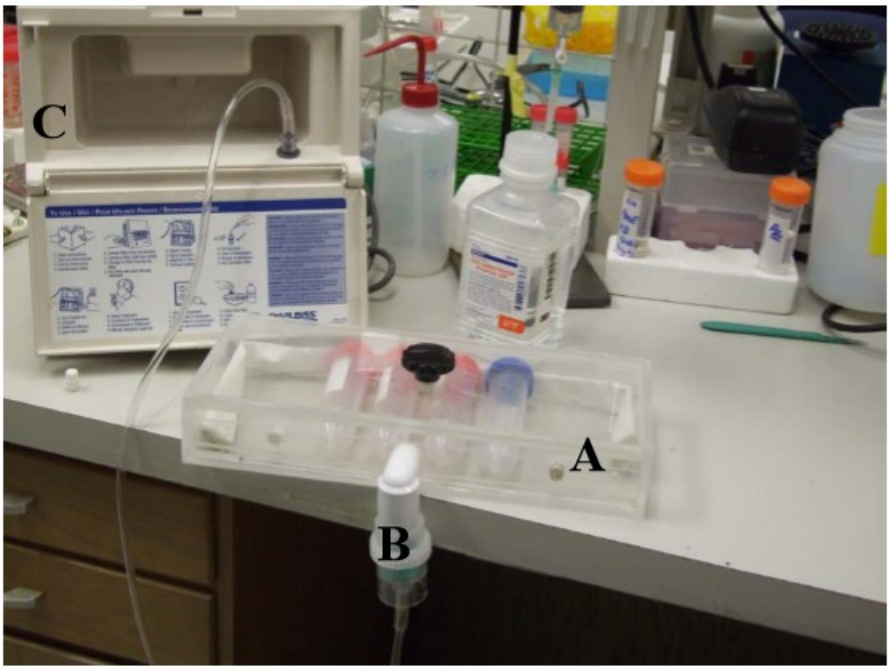


Figure 24. Nebulizer system to the OVA challenge model.

This is the apparatus used to challenge the mice in the asthma model. A) The mice are placed in chamber to direct them towards the airflow. B) OVA dissolved in saline is placed in this chamber. C) The nebulizer aerosolizes the dissolved OVA to deliver it to the mice.

Other groups of animals were challenged, under isofluoran anesthesia, intranasally with 1.25 μg/kg whole HDM extract, 3 times per week for a week (acute asthma model) or 3 times per week for 4 weeks (chronic asthma model). Another model is the one used in the PARP-1

inhibition experiments. Were the Mice were challenge intranasally with 25 µL of saline or 1 mg/ml whole house dust mite (HDM; *Dermatophagoides pteronyssinus*) extract (Greer Laboratories, Lenoir, NC) 3 times per week for 5 weeks. Control groups were not sensitized or challenged. Additional challenged groups of mice received *i.p.* 1/5/10 mg/kg Olaparib (Selleckchem, TX, USA) and/or 5 mg/kg *L*-N6-(1-Iminoethyl)lysine dihydrochloride (L-NIL-Sigma-Aldrich, St. Louis MO) in saline 30 minutes after each challenge. Plus two additional group received i.p or i.n. (data don't show) 20 µg/kg of Nitrite ($NaNO_2$) as a NO source. Each administration has been performed 30 min after each challenge.

AHR to inhaled methacholine was measured in unrestrained, conscious mice 24 h after the last challenge by recording "enhanced pause" (Penh) by whole body plethysmography (EMKA Systems, Falls Church, VA). In brief, the base-line readings were taken and averaged for 3 min after animals were placed in a barometric plethysmographic chamber. Normal saline or increasing concentrations of aerosolized methacholine (12.5–100 mg/ml) were nebulized, and readings were taken and averaged for 3 min after each nebulization and enhanced pause (Penh) representing AHR was calculated.

Mice were sacrificed 48 h after the last challenge for bronchoalveolar lavage (BAL), lung fixation and lung RNA and protein extraction.

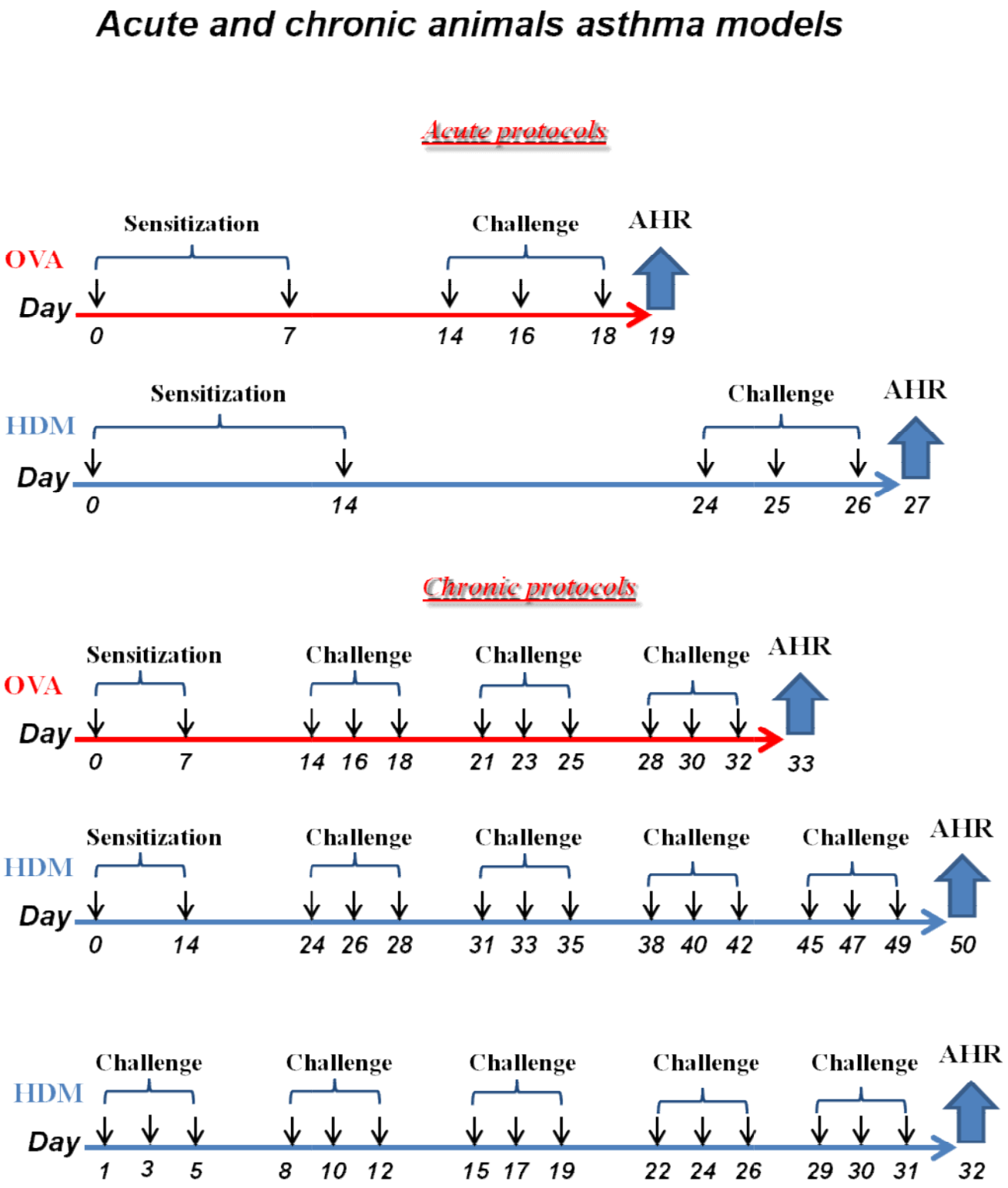


Figure 25. Animal asthma models

Circulating immunoglobulin measurement in serum and BALF.

The levels of OVA specific IgE were measured in serum by sandwich ELISA (Serotec, NC, USA).

Lung histopathology.

Animals were euthanized 48h after the last exposure by CO2 asphyxiation and lungs were inflated and fixed with neutral buffered, 10% formalin (Sigma, St. Louis, MO). Fixed lungs were dehydrated, paraffin-embedded, and sectioned at 4 μm onto slides. Sections were assessed for inflammatory cells using hematoxylin and eosin (H&E) stain. Mucus secretion was quantified from sections stained with periodic acid-Schiff (PAS) using standard protocols. All images were taken on Olympus microscope using AxioVision software**.**

Organ recovery, staining, cytokines assessments.

Lungs from sacrificed mice were fixed with formalin then sectioned and stained by hematoxylin and eosin (H&E) using standard protocols. Some lungs were subjected to bronchoalveolar lavage (BAL). The BAL fluids and the serum samples were assesses for inflammatory cytokine production (IL-4, IL-5 and IL-13), using a Bio-Plex system (Bio-Rad laboratories, CA, USA).

Broncho alveolar fluid cellularity and cytokines measurement.

Broncho alveolar lavage (BAL) fluid was collected 48h after the last allergen exposure. Lungs from all experimental groups were subjected to bronchoalveolar lavage (BAL), briefly, chest was opened, an incision was made in the trachea and a small cannula was inserted. through which 1 ml of sterile phosphate buffered saline (PBS, PH=7.4; lifetechnologies, USA) was injected into the lung and collected into tubes, the first ml is stored immediately at -20 C for cytokine assessment, the process is repeated 5 more times to collect and prepare the alveolar cells for microscopy by cytospin using shandon cytocentrifuge (Thermo Fisher Scientific, MA, USA). Total cell counts were obtained, and cells were spun onto glass slides (one slide/animal sample) then inflammatory cells were stained and differentiated by Diff-Quick stain (Siemens Medical Solution, PA). Cells were counted and results were compared and analyzed

Cytokines levels were measured in cell-free BAL fluid using Bio-Plex system (Bio-Rad laboratories, CA, USA). All kits were used according to manufacturer's protocol. Briefly, 96-

well filter plates were pre-wetted and 50 µl of beads are added and washed twice with Bio-Plex buffer. Then, 50 µl of standards and samples are added, incubated for 30 minutes and washed 3 times. Detection antibodies (25 µl) were incubated for 30 minutes followed by 50 µl of streptavidin-PE for 10 minutes. The beads were re-suspended in 125 µl of buffer and read in a Bio-Plex array reader (BIO-RAD). The following cytokines were assayed: Eotaxin, M-CSF, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IP-10 and IL-17, IL-22, KC, GM-CSF. Raw data were plotted against a standard curve using a 5-parameter logistic regression to interpolate unknowns.

Cell culture, Real Time PCR.

Mouse embryonic fibroblast wild type and PARP-1 knock out were treated, after 24 hour of starvation, with 1 µg/ml LPS and 10 ng/ml IFNγ for 6 hours. Endothelial and epithelial cells were treated for six hours with 1µg/ml LPS for 6 hours.

After the cells were collected and the RNA was extracted using the RNeasy mini kit (QIAGEN- Hilden, Germany). The extracted RNA was reverse transcribed into cDNA using reverse transcriptase III (Invitrogen), the resulting cDNA was subjected to PCR using primer sets (IDT, San Jose, CA, USA) specific designed for iNOS (F 5'-GTG TTG CAA GCT GAT GGT CA-3' and R 5'-TGT TGT AGC GCT GTG TGT CA-3') or β-actin (F 5'-TAC AGC TTC ACC ACC ACA GC-3' and R 5'-TCT CCA GGG AGG AAG AGG AT-3').

Spleen recovery and T-helper cells skewing, Th1 and Th2 cytokines assessment.

Animals were killed by CO2 asphyxiation and spleens were isolated.

$CD4^+$ T cells were purified from a single cell suspension procured from spleens of OVA-sensitized wild-type or PARP-1 KO mice by negative selection with the Easy Sep kit from Stem Cell Technologies (Vancouver, Canada). All $CD4^+$ T cells were stimulated on coated plates (1 µg/ml anti-CD3 and 0.5ug/ml anti-CD28). $CD4^+$ subsets were generated by culture under the following conditions: Th1, IL-12 (10 ng/ml; R&D Systems), and anti–IL-4 (10 µg/ml; clone 11B11); Th2, IL-4 (100 U/ml; R&D Systems) and anti–IL-12 (10 µg/ml; clone C17.8). Some groups of wild-type CD4+ subsets were treated with AZD2281 an inhibitor of PARP-1. On day 3 post-stimulation, cells were replated with IL-2 alone for an additional 2 days, also the AZD2281 were added. The extracted total RNA from the cells was used for the generation of cDNA using reverse transcriptase III (Invitrogen) and analyzed by real-time PCR using primers specific for mouse *gata-3*, *Il*-4*, t-bet*, *Ifn*-g and *il-17*.

Gene	Primer sequence
Mouse *Gata-3*	5′-TCTCACTCTCGAGGCAGCATGA-3′ 5′-GGTACCATCTCGCCGCCACAG-3′
Mouse *Il-4*	5′-CCCCAGCTAGTTGTCATCCTG-3′ 5′-CGCATCCGTGGATATGGCTC-3′
Mouse *Ifn-g*	5′-TCAAGTGGCATAGATGTGGAAGAA-3′ 5′-TGGCTCTGCAGGATTTTCATG-3′
Mouse *T-bet*	5′-GCCAGGGAA-CCGCTTATATG-3′ 5′-GACGATCATCTGGGTCACATTGT-3′
Mouse *β-actin*	5′-CGGTTCCGATGCCCTGAGGCTCTT-3′ 5′-CGTCACACTTCATGATGGAATTGA-3′
Mouse *il-17*	5′-GGTCAACCTCAAAGTCTTTAACTC-3′ 5′-TTAAAAATGCAAGTAAGTTTGCTG-3′

Data analysis

All data are expressed as means_SD of values from at least six mice per group unless stated otherwise. PRISM software (GraphPad, San Diego, CA, USA) was used to analyze the differences between experimental groups by one way ANOVA followed by Dunnett's multiple comparison tests.

Results

Expression of iNOS, subsequent protein nitration and activation of PARP are elevated in lung tissues of asthmatics individuals.

To increase the clinical significance of our studies on the role of iNOS and PARP in asthma pathogenesis, it is imperative to demonstrate that iNOS expression and PARP-1 activation are actually present in human asthma. It is also important to correlate iNOS expression with protein nitration as a result of $ONOO^-$ production. To this end we examined whether PARP activation, iNOS expression, and protein nitration are detected in lung tissue of two de-identified individuals who died from asthma and the lack thereof in tissue from two de-identified individuals who died from an asthma-unrelated cause but with normal lungs (verified by a pathologist from the Stanley Scott Cancer Center Molecular Histopathology and Analytical Microscopy Core). Figures 26A and 26B show the typical eosinophilic inflammation and extensive mucus production in the lung of the asthmatic individual as assessed by H&E and PAS staining, respectively. Figures 26C-D-E show a marked activation of PARP-1 and the high expression of iNOS in lung from asthmatics compared to the non-asthmatic individuals as assessed by immunofluorescence using antibody to PAR or iNOS. Figure 27A-B-C confirm the consistent activation of PARP-1 and the over expression of iNOS with consequent protein nitration in lung tissue of the asthmatics individual as assessed by immunohistochemistry with antibodies to PAR, iNOS or nitrotyrosine and give us an idea about their distribution in the lung. It is clear from the immunohistochemistry data that endothelial, epithelial and a rather large portion of immune cells highly express iNOS. Given the fact that macrophages are major sources of iNOS in the lung, I conducted a dual immunofluorescence labeling using antibodies to CD68, a marker of macrophages, and to iNOS. Figure 27D shows that, indeed, iNOS was highly expressed in CD68-positive macrophages.

iNOS expression and protein nitration and PARP-1 activation in human asthmatics PBMCs.

PBMCs collected from asthmatics or healthy individuals were subjected to immunoblot analysis with antibodies to nitrotyrosine, iNOS, PARP-1 or GAPDH. Figure 28 shows that iNOS is highly expressed in PBMCs from asthmatics compared to cells from healthy individuals. However, the expression of iNOS did not strictly correspond with protein nitration. Indeed, some PBMCs exhibited high levels of iNOS but showed protein nitration

levels comparable to those detected in cells from non-asthmatics. Conversely, PBMCs that exhibited extensive protein nitration displayed low levels of iNOS. Interestingly, the two samples (5 and 6) that displayed high levels of protein nitration were collected from patients whose asthma was under control according to their ACT tests (≥20). However, samples from uncontrolled asthma (5 and 7 with ACT scores=16) displayed levels of protein nitration comparable to those from non-asthmatics but with high levels of iNOS. Overall, these results exemplify the known complexity of the relationship between iNOS, protein nitration, and asthma in humans. On the other hand Figure 29A shows that PARP-1 is more activated in PBMC from asthmatic patients and its level of expression reflect the severity of the disease as shown in figure 29B . These results demonstrate qualitatively for the first time that PARP is activated in human asthma and clarify the expression of iNOS and subsequent protein nitration in asthmatics.

Nevertheless, it is noteworthy that the presence of these factors does not necessarily mean a critical function in the disease but results from our laboratory and many others have suggested a potentially direct role for PARP and iNOS in some or most aspects of asthma.

Differential protection of iNOS inhibition against AHR manifestation upon acute and chronic exposure to OVA in mice.

Our laboratory has shown that iNOS gene deletion is protective against airway inflammation upon acute, but not chronic, exposures to OVA [121]. Interestingly, such gene deletion prevented lung fibrosis in the chronic model of the disease. Given the potential connection between and the co-existence of lung fibrosis and AHR in chronic asthma [372], we explored the possibility that administration of L-NIL, a clinically tested iNOS inhibitor, may be protective against AHR upon both acute and chronic exposures to OVA in mice. L-NIL is a selective and long acting inhibitor of iNOS with an IC50=3.3 μM for mouse iNOS [373]. A clinical trial conducted by Barnes group [370] showed that administration of 200 mg of L-NIL reduced exhaled NO in patients with mild to moderate asthma to levels lower than those detected in placebo-administered healthy subjects as early as 30 min post-administration. Mice were subjected to the acute or chronic model of asthma as described in Fig. 25 followed by an assessment of AHR using full body plethysmography. Figure 30A shows that L-NIL administration at a dose of 5 mg/kg was very effective in blocking the manifestation of AHR upon acute exposure to OVA. Similar results were achieved using $iNOS^{-/-}$ mice that were sensitized and acutely challenged to OVA (Fig. 24). Surprisingly, however, the protection

achieved by L-NIL administration was completely lost when mice were chronically exposed to OVA (Fig. 30B). The effects of iNOS inhibition on AHR were similar to the differential protection conferred by iNOS gene deletion against acute vs. chronic airway inflammation reported by us [121].

Inhibition of iNOS by L-NIL failed to protect against AHR induced by a chronic exposure to HDM, which is reversed upon NO supplementation by nitrite administration.

Despite the success of the OVA asthma model in mechanistic studies, it still has many limitations including the development of tolerance during chronic exposure and the requirement for systemic priming with adjuvant. A better model that more closely resembles human asthma is inhaled delivery of HDM, a major allergen for humans. This model involves mucosal sensitization and following intranasal challenges within the lungs with a true allergen to induce Th2-driven inflammation.

To examine whether iNOS inhibition also is protecting against AHR manifestation upon acute but not chronic exposure to HDM, mice were sensitized and then subjected to intranasal exposures to the allergen either acutely constituted by simultaneous daily exposures for 3 days or chronically by challenging the animals three times a week for four weeks as described in Figure 25. One group of mice was administered *i.p.* 5mg/kg L-NIL 30 min after each intranasal challenge with the allergen. Figure 31A shows that similar to the acute OVA model, L-NIL administration was extremely efficient in blocking HDM-induced AHR; in fact, AHR of HDM-treated mice that received the drug was identical to animals that were not exposed to HDM. Contrary to the acute HDM exposure model, iNOS inhibition by L-NIL did not provide a significant protection against AHR upon a chronic exposure to HDM (Fig. 31B) These results also demonstrate that the role of iNOS in AHR manifestation is not specific to a given model and may be considered as a general phenomenon.

A variety of studies have suggested that chronic overexpression of iNOS is detrimental because the production of large amounts of NO and its subsequent interaction with the ROS to generate $ONOO^-$ [374]. $ONOO^-$, in turn, promotes the nitration of a large number of enzymes and structural proteins as well as oxidative DNA damage. Protein nitration is well known for its ability to inhibit the enzymatic activity of target proteins and/or promotes their degradation [375]. Inhibition of iNOS may be very critical in blocking these detrimental changes. The other

facet of NO is its ability to preserve endothelial and smooth muscle function. However, such beneficial trait is always associated with low levels of the gas. Accordingly, I speculated that there must be a very important balance in the level of NO to achieve protection during asthma. I surmised that if I supplement NO with a donor such as nitrite [376], then I may achieve protection against AHR when iNOS is inhibited. To this end, a group of mice was subjected to HDM exposure and L-NIL administration as described above but received a dose of 20 μg/kg i.p. after each challenge.

Figure 31B shows that administration of small dose of nitrite was able to significantly protect against AHR manifestation in response to increasing doses of methacholine in L-NIL-treated mice chronically exposed to HDM. These results suggest that NO levels are an important determinant for the protection against AHR.

PARP-1 inhibition partially reduces iNOS expression in MEF LPS/INFγ–treated and in mouse heart endothelial cells transfected with human PARP-1.

Our laboratory established an interesting relationship between PARP-1 and iNOS. PARP-1 appears to regulate the expression of iNOS by controlling NF-κB binding activity [369]. Reciprocally, iNOS may control PARP-1 activity by nitration. Exposure of recombinant PARP-1 to $ONOO^-$ completely abrogates its enzymatic activity [358]. The most fascinating aspect of the connection between PARP-1 and iNOS is the fact that PARP-1 inhibition does not completely inhibit expression of iNOS. Figures 32A-B show that, indeed, PARP-1 gene deletion reduces expression of iNOS in MEF LPS/INFγ-treated and in mouse heart endothelial cells transfected with human PARP-1 but not completely.

These results suggest that the beneficial effect of PARP-1 inhibition on AHR may be associated with the partial reduction in iNOS expression, which presumably lead to a production of moderate levels of NO. However, the relevance of this hypothesis to an acute allergen exposure may be not so clear given the fact that iNOS inhibition is protective against AHR in this model.

PARP-1 inhibition-mediated protection against AHR in chronically HDM-exposed mice is lost upon L-NIL administration.

We have demonstrated that PARP inhibition, genetically by gene deletion or pharmacologically by olaparib, protects against asthma manifestation including airway inflammation and AHR. We also have shown that PARP-1 regulates iNOS expression in both the animal model of asthma and cells cultured system [358]. Considering that PARP-1 inhibition decrease the INOS expression, we hypothesized that the beneficial effect of PARP-1 inhibition on AHR may be associated with the partial reduction in iNOS expression, which presumably lead to a production of low to moderate levels of NO. Accordingly, complete inhibition of iNOS by L-NIL treatment was predicted to abrogate the protective effect of PARP inhibition. To test this hypothesis, mice were chronically exposed to HDM and then administered a combination of 5 mg/kg olaparib and 5 mg/kg L-NIL after each challenge. Figure 32C shows that olaparib treatment provided an excellent protection against AHR, which was significantly reduced by L-NIL administration. These results reveal that the low expression of iNOS observed upon PARP inhibition was protective.

Olaparib blocks airway eosinophilia, mucus and IgE production, and AHR upon a single or repeated challenge with OVA in a mouse model of asthma.

Figure 33A shows that a single administration of olaparib at the 1 mg/kg dose almost completely prevented the elevation of OVA-specific IgE production in BAL fluids (BALF) but not sera collected from OVA-sensitized and challenged mice. A slightly higher dose of 5 mg/kg was sufficient to cause a significant reduction in the sera levels of OVA-specific IgE. As expected, PARP-1 gene deletion provided similar protection. The blockade in IgE production coincided with a significant reduction in the total number of inflammatory cells recruited to the lung of treated animals with a prominent effect on eosinophils, neutrophils, and lymphocytes (Figure 33B). Figure 33C shows an example of the inflammatory cell infiltration into the lungs of OVA-challenged mouse and the effective protection against such infiltration by treatment with 5mg/kg olaparib as assessed by H&E staining. Treatment with olaparib also reduced mucus production as assessed by Periodic acid–Schiff (PAS) staining (Figure 33D). Figure 33E shows that administration of 5mg/kg olaparib almost completely prevented AHR manifestation to increasing doses of methacholine. The effects of olaparib administration were similar to those observed in OVA-challenged PARP-1$^{-/-}$ mice.

The protective effect of olaparib against a single OVA challenge does not necessarily mean that the drug would maintain its anti-inflammatory efficacy upon multiple challenges. Accordingly, mice were challenged daily for three consecutive days and received increasing doses of olaparib 30 min after every challenge. Figure 34A shows that olaparib maintained a remarkable efficacy in reducing OVA-specific IgE production with a maximal protection conferred by the 5 mg/kg dose of the drug. At this dose, the drug exerted a pronounced protection against the inflammatory burden induced by repeated OVA challenges including eosinophilia (Figures 34B-C), mucus production (Figure 34D), and AHR (Figure 34E) in a manner similar to that conferred by PARP-1 gene deletion.

Olaparib treatment differentially affects production of Th1 and Th2 cytokines.

Figure 35A shows that both single and multiple OVA challenge induced considerable levels of several Th2 cytokines including eotaxin, IL-4, IL-5, IL-6, IL-13, and M-CSF, and that olaparib administration suppressed production of these cytokines. It is important to note that in the single OVA challenge model, olaparib at 1 mg/kg provided a remarkable reduction in the production of the before mentioned cytokines most notably eotaxin, IL-4, and M-CSF. Upon repeated OVA challenges, the lowest dose of olaparib only reduced the levels of IL-5 and IL-6. However, the 5 mg/kg dose was sufficient to almost completely block the production of all measured cytokines. It is worth mentioning that the effect of PARP inhibition either pharmacologically or by gene knockout on IL-2 production was marginal in both the single and repeated OVA challenge models (Fig. 35B). Figure 35C shows that the levels of IFN-γ were reduced upon a single or repeated challenge with OVA. Such decrease was prevented by administration of the PARP inhibitor. Interestingly, the levels of IFN-γ were markedly lower in control PARP-$1^{-/-}$ mice and, unlike in olaparib-treated animals, OVA challenge did not cause an elevation of the cytokine in the knockout animals.

PARP inhibition by olaparib or gene knockout blocks asthma-like manifestation in a chronic HDM asthma model.

We next examined whether PARP inhibition pharmacologically by olaparib or genetically by gene knockout blocks asthma-like manifestation upon *i.n.* administration of HDM. Figure 36A shows that a single administration of olaparib at the end of the HDM exposure protocol

was highly effective in decreasing recruitment of eosinophils and macrophages as well as overall cellularity in the lungs. However, the increase in the number of lymphocytes was not affected. A remarkable protection was achieved upon two additional administrations of the drug including a reduction in the number of lymphocytes. Similar results were observed in HDM-exposed PARP-1$^{-/-}$ mice, which provide evidence for the specificity of such protective effects. Interestingly, repeated administration of olaparib provided significantly better reduction in recruitment of the total number of inflammatory cells, eosinophils and macrophages, than that provided by PARP-1 gene deletion. The manifestation of AHR upon chronic HDM exposure was modestly affected by a single administration of olaparib; a more pronounced reduction in AHR required two additional administration of the drug (Fig. 36B). PARP-1 gene deletion and repeated olaparib administration provided a similar protection against AHR (Fig. 36B).

PARP inhibition by olaparib or gene knockout reduces Th2 cytokine production without a prominent effect on IFN-γ or IL-10.

Figure 37A shows that HDM-induced lung eosinophilia was accompanied with an increase in production of a number of Th2 cytokines in BALF collected from the treated animals such as eotaxin, IL-4, IL-5, and IL-13. These cytokines were markedly reduced in BAL fluids of mice that received a single or triple administration of olaparib. Similar reduction was observed in HDM-exposed PARP-1$^{-/-}$ mice. Although PARP inhibition pharmacologically or by gene knockout reduced production of the Th1 cytokines IL-2 and IP-10 in HDM-treated mice, the IFN-γ levels either slightly increased or remained unaffected by PARP inhibition (Fig. 37B). Interestingly, while the levels of the anti-inflammatory cytokine IL-10 were not affected by olaparib treatment, the levels of the cytokine in HDM-exposed PARP-1$^{-/-}$ mice remained lower than those detected in BALF of HDM-exposed WT mice.

PARP inhibition inconsistently increases IL-17 production without a concomitant increase in IL-17-associated factors but with an increase in the percentage of T-reg cells in vitro and in HDM-exposed mice.

Several recent studies have reported conflicting findings on the relationship between PARP and IL-17. While some reports show an increase in IL-17 production [377], others showed either

no change [378] or a decrease in the cytokine upon PARP inhibition [379-381]. It therefore became imperative to examine whether olaparib or PARP-1 gene deletion affects the levels of the cytokine in our experimental models. Figure 38A shows chronic HDM exposure induced a slight increase in IL-17 only in sera but not in BALF of treated mice at the end of the exposure protocol. In BALF of HDM-exposed mice, the level of the cytokine modestly increased upon repeated, but not upon a single, administration of olaparib. IL-17 did not increase in BALF of HDM-exposed PARP-$1^{-/-}$ mice. In sera of HDM-exposed mice, the level of IL-17 also increased upon repeated, but not upon a single, administration of olaparib. Unlike in BALF, IL-17 increased in sera of HDM-exposed PARP-$1^{-/-}$ mice. The increase in IL-17 upon PARP inhibition was confirmed in CD4+ T cells derived from PARP-$1^{-/-}$ mice that were activated with antibodies to CD3/CD28 (Figure 38B). In CD3/CD28-activated human CD4+ T cells, olaparib treatment only increased IL-17 production at the 5 μM concentration and only after an extended time of treatment (Figure 38C). Figure 38D shows that the increase in IL-17 in CD3/CD28-stimulated PARP-$1^{-/-}$ CD4+ T cells occurred at the mRNA level. Surprisingly, the PARP inhibition-associated increase of IL-17 was accompanied with a decrease, rather than an increase, in known IL-17-dependent cytokines such as IL-22, keratinocyte chemoattractant (KC) and GM-CSF in HDM-treated mice (Figure 38D). Furthermore, such increase in IL-17 but decrease in IL-22 coincided with a substantial increase in the percentage of CD4+/CD25+/Foxp3+ T-reg cell population in spleens of HDM-exposed animals (Figure 30F). HDM exposure did not increase the percentage of T-reg cells in spleens of WT mice (Figure 30F), which is consistent with the report by Kim et al. [382]. The T-reg cell population was also increased in PARP-$1^{-/-}$ mice without any HDM exposure. Consistent with these results, olaparib treatment also promoted a significant increase in the T-reg cell population in CD3/CD28-activated human CD4+ T cells but only in response to the 5μM concentration of the drug (Figure 30G).

Effects of PARP inhibition by olaparib on human $CD4^+$ T cell proliferation and Th1/ Th2 populations.

Figure 39A shows that olaparib treatment is very effective in reducing expression of *gata-3*, the master regulator of the *Il4/Il5/Il13* cytokine locus [383], in human CD4+ T cells upon TCR stimulation as assessed by real-time PCR. Interestingly, the decrease in *gata-3* mRNA upon PARP inhibition is not accompanied by an increase in the expression of *t-bet* in TCR-stimulated cells. These findings show that olaparib modulates the function of $CD4^+$ Th2 cells

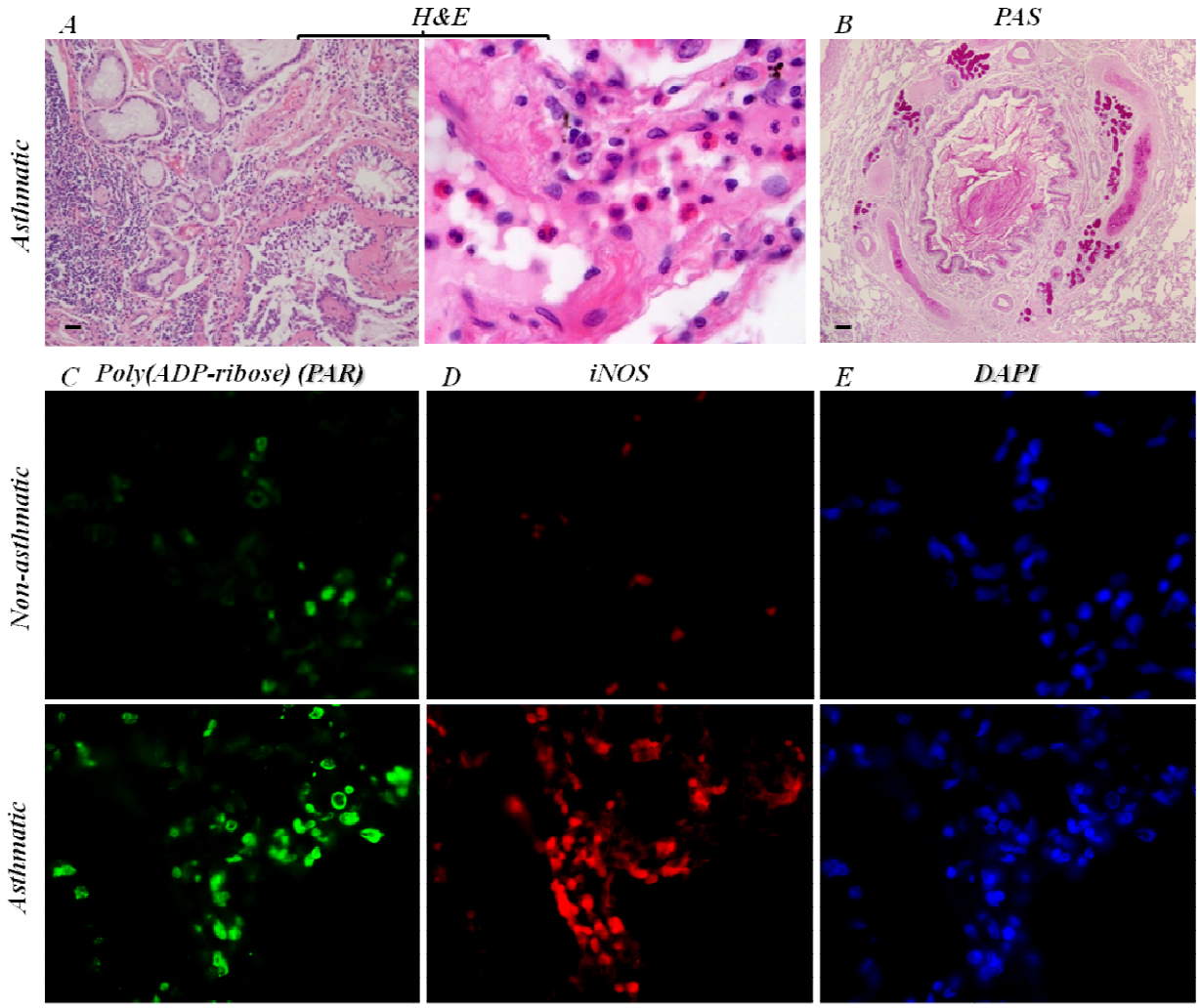
by modulating the expression of *gata-3* without a prominent effect on their proliferation (Fig. 39A-B)

PARP-1 inhibition reduced the differentiation of Th2 cells in mice under specific skewing condition

Another interesting aspect of my work concerns the role of PARP-1 and iNOS in skewing of CD4+ Th cells into Th1/Th2 phenotypes. Our results show that PARP-1 inhibition blocks expression of Th2 cytokines upon acute and chronic exposures to OVA or HDM. However, iNOS gene deletion blocks Th2 cytokines upon acute, but not chronic, exposures to allergens [121]. These set of results suggest that the differential yet related roles of iNOS and PARP-1 in AHR and inflammation may be connected to their role in CD4+ T cell function. We examined whether the effect of olaparib on Th2 and Th1 cytokines was by controlling mRNA expression of key transcription factors that regulate the expression of these cytokines focusing primarily on *gata-3, t-bet, IL-4,* and *IFN-γ.* To this end, CD4+ T cells were skewed toward a Th1 or Th2 phenotype and stimulated with anti-CD3/CD28 antibodies in the presence or absence of 5 μM olaparib. Figure 40 shows that olaparib markedly reduced CD3/CD28-stimulated GATA-3 mRNA expression with a concomitant reduction in IL-4 mRNA expression. Interestingly, olaparib treatment caused an elevation in T-bet and IFN-γ mRNA expression in Th1-skewed CD4+ T cells.

Figures

Figure 26

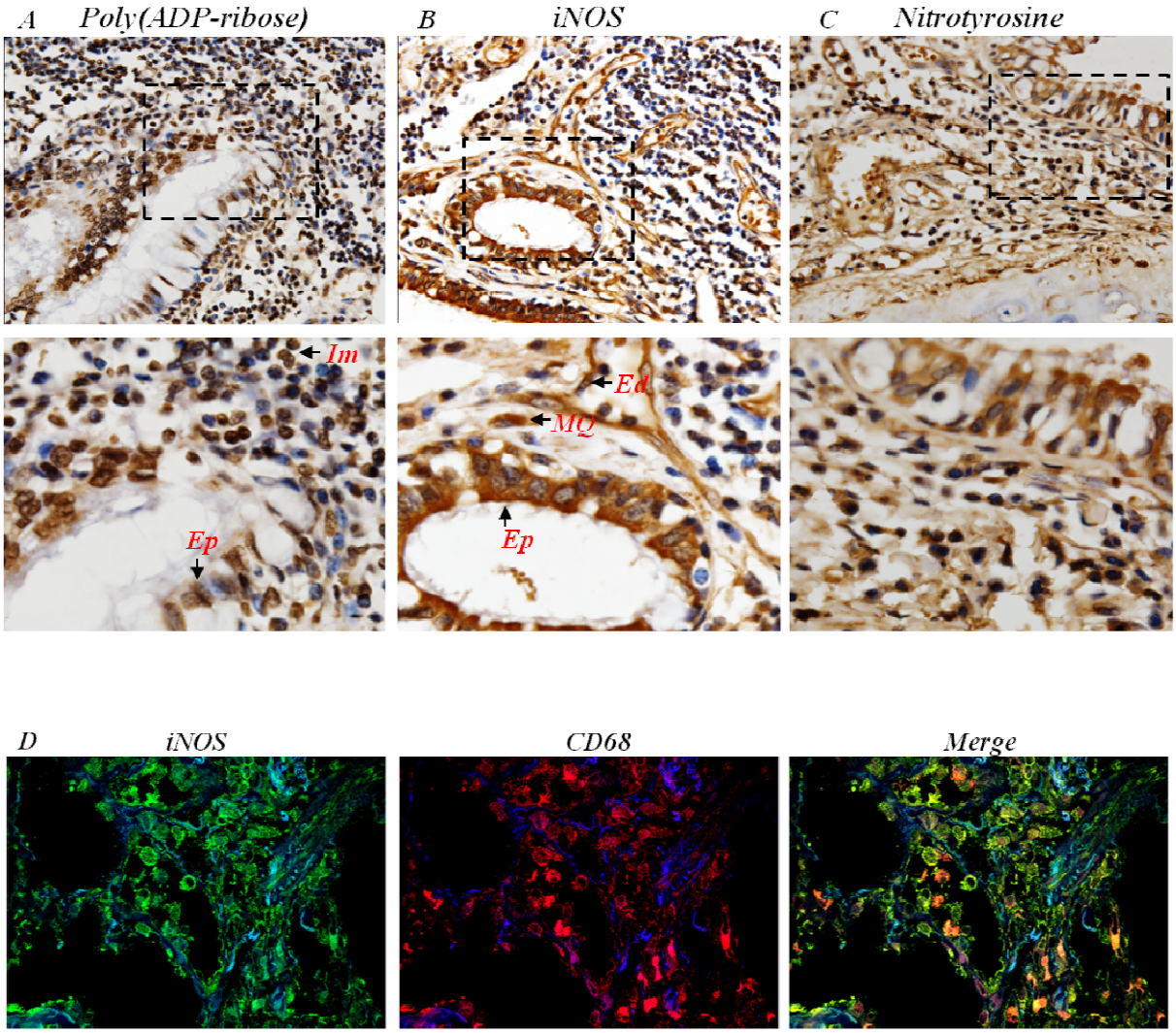


PARP-1 activation and iNOS expression in human asthmatics lungs.

Lung sections from a de-identified individuals who died from severe asthma were subjected to H&E and PAS staining to show the typical inflammation (A) and mucus Hyperproduction (B) respectively.

Other lung sections from two de-dentified individuals who died from severe asthma and from an asthma-unrelated causes but with normal lungs (verified by a pathologist from the Stanley Scott Cancer Center Molecular Histopathology and Analytical Microscopy Core) were subjected to immunofluorescence staining with antibodies to PAR (C), iNOS (D) than counterstained with DAPI (E) followed by visualization with a Leica DMRA2 fluorescence microscope using a 40× objective lens.

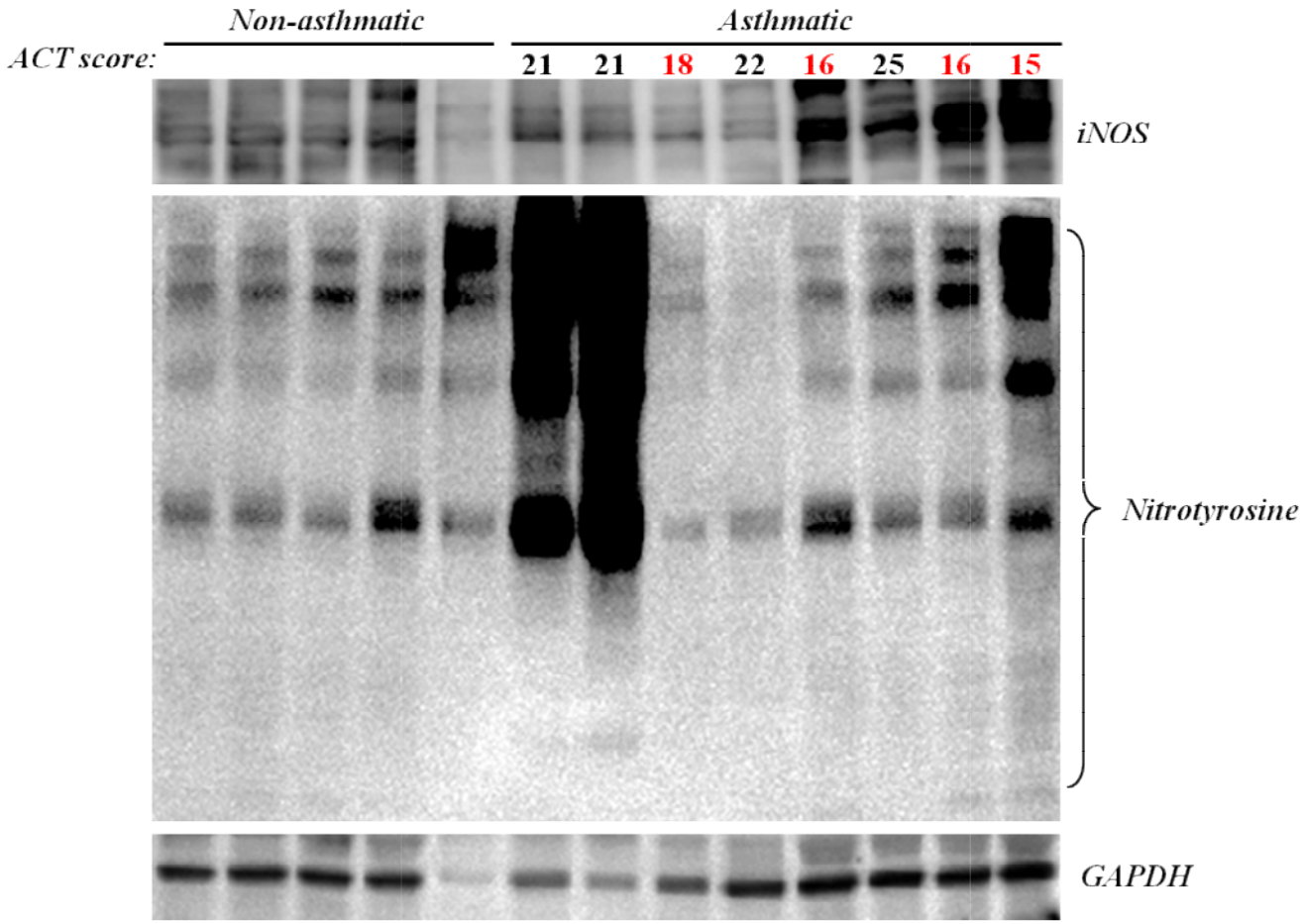
Figure 27



Tissue distribution of PARP -1 activation, iNOS expression, and protein nitration in the asthmatic lung.

Lung sections from a de-identified individuals who died from severe asthma were subjected to immunohistochemistry with antibodies to PAR (A), iNOS (B), or nitrotyrosine (C) followed by visualization with light microscopy. The lower panels represent magnifications of the boxed areas. The abbreviations are: Ed, Endothelial cells; Ep, Epithelial cells; Im, Immune cells; MQ, Macrophages. (D) Lung sections were also subjected to immunofluorescence staining using antibody to CD68 and INOS, followed by visualization with a Leica DMRA2 fluorescence microscope using a 60× objective lens, to confirm that the immune cells that highly express INOS are effectively Macrophages.

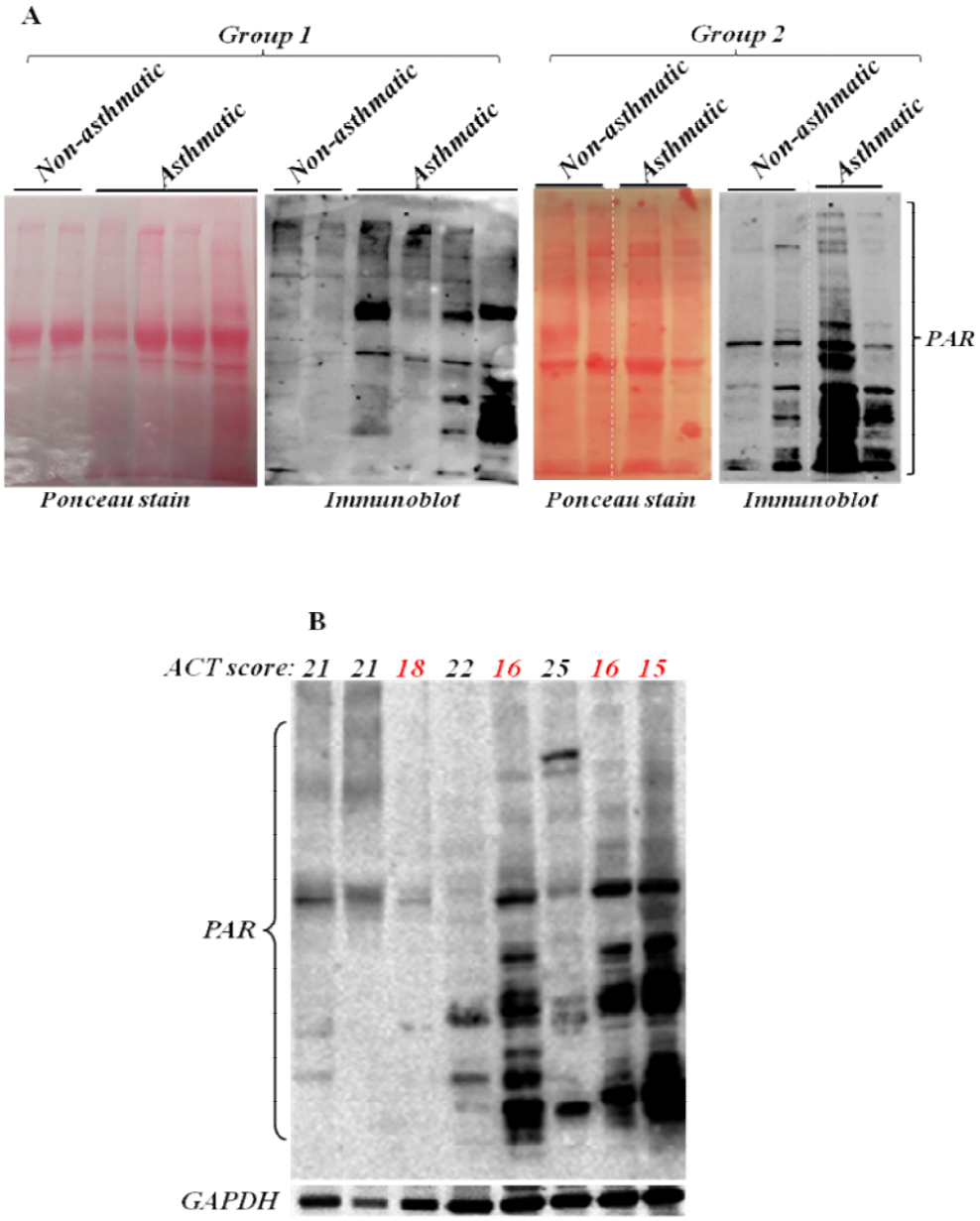
Figure 28



iNOS expression and protein nitration in human asthmatics PBMCs.

PBMCs collected from asthmatics or healthy volunteers were subjected to protein extraction followed by immunoblot analysis with antibodies to human iNOS, nitrotyrosine and GAPDH. The ACT score was evaluated for all asthmatic patient

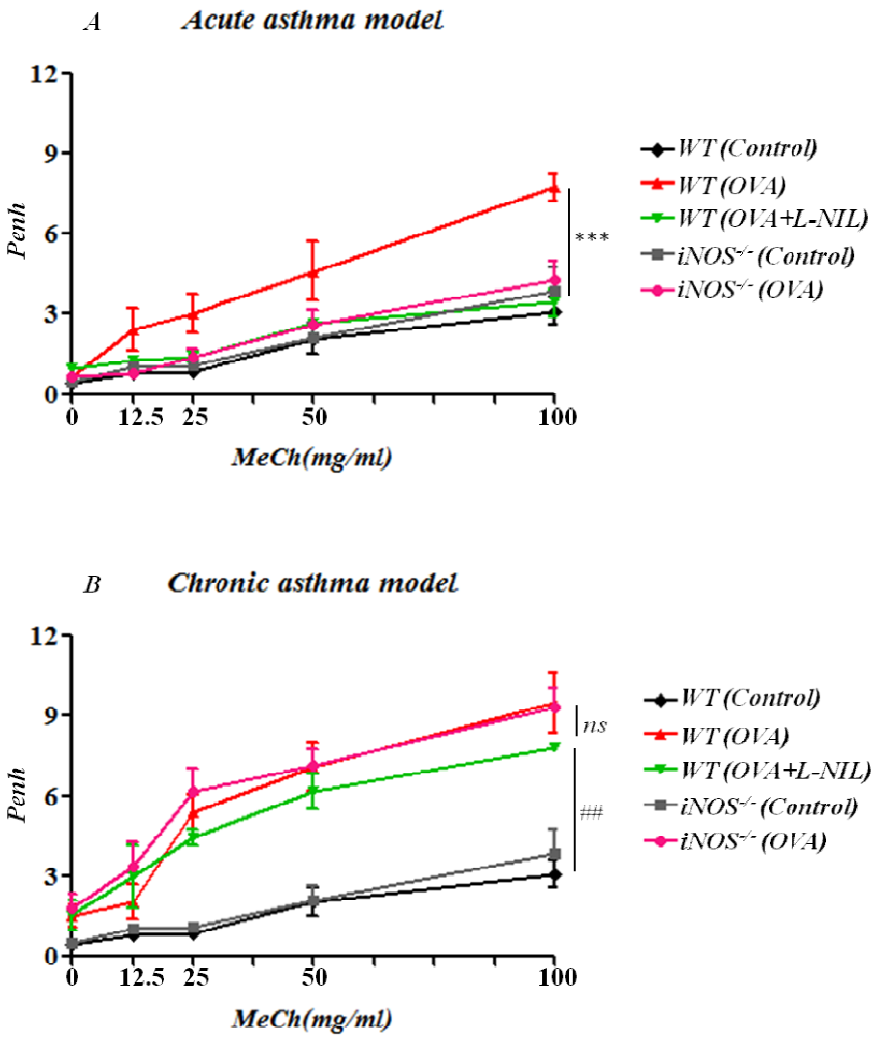
Figure 29



PARP-1 activation in human asthmatics PBMCs.

PBMCs collected from asthmatics or healthy volunteers were subjected to protein extraction followed by immunoblot analysis with antibodies to human PAR and GAPDH. The ACT score was evaluated for all asthmatic patients.

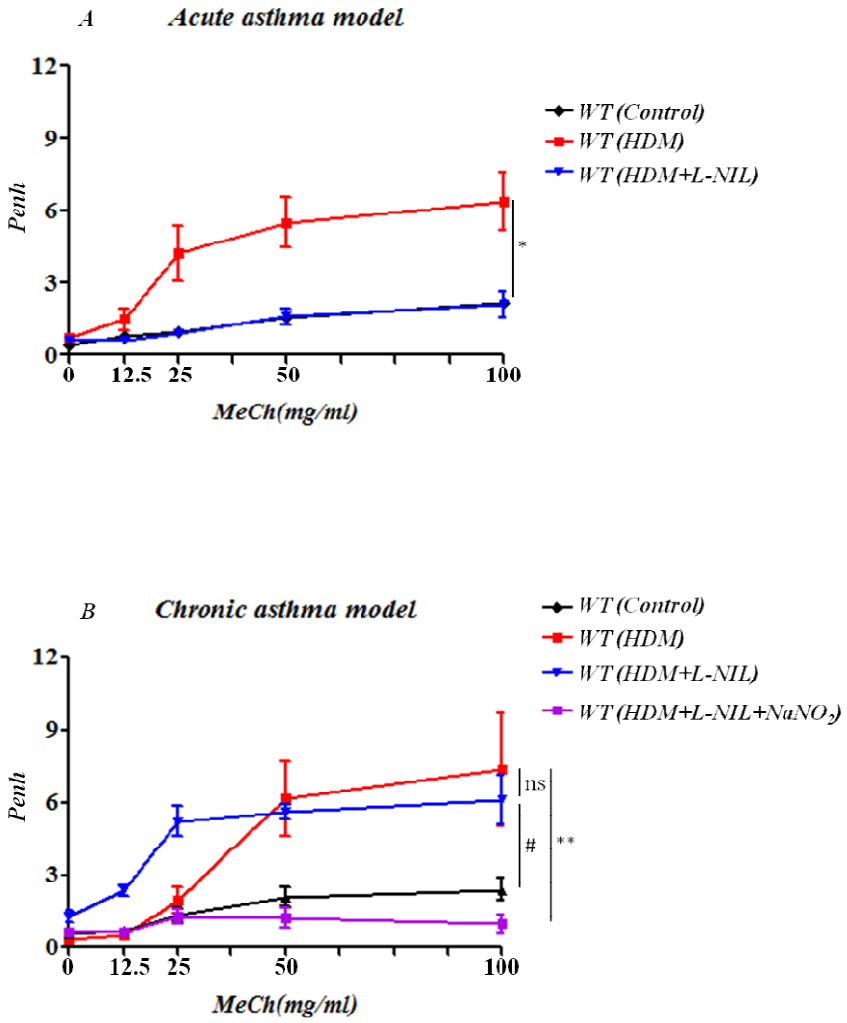
Figure 30



Effect of iNOS inhibition by L-NIL on AHR manifestation upon acute or chronic exposure to OVA in mice.

WT and iNOS$^{-/-}$ C57BL/6 mice were subjected to OVA sensitization followed by an acute (A) or chronic (B) challenge to aerosolized OVA or left unchallenged. A group of WT mice were administered i.p. 5 mg/kg of the specific iNOS inhibitor (L-NIL) or saline 30 min after each OVA challenge. *Penh* was recorded 24 h after the last challenge using a whole body plethysmograph system before and after the indicated concentrations of aerosolized methacholine (MeCh). Results are plotted as maximal fold increase of *Penh* relative to baseline (0 mM MeCh) and expressed as mean ± SEM where n=5 mice per group. *, difference from HDM challenged mice; #, difference from control unchallenged mice $p < 0.05$.

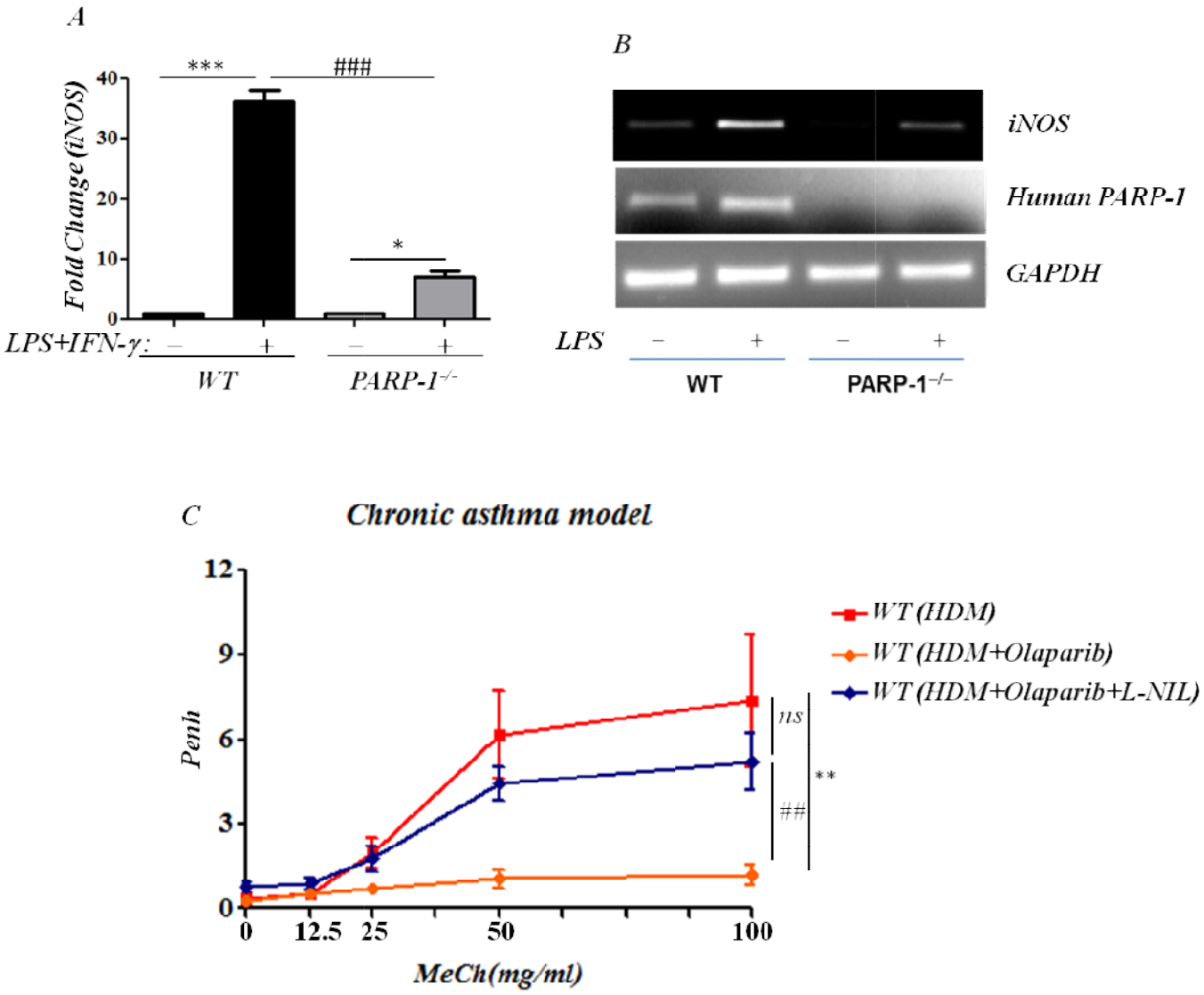
Figure 31



Effect of iNOS inhibition by L-NIL on AHR manifestation upon exposure to HDM in mice and the influence of NO supplementation by nitrite administration.

C57BL/6 mice were subjected to HDM sensitization followed by acute (A) or chronic (B) intranasal challenge with HDM or were left unchallenged. Challenged mice were administered *i.p.* 5 mg/kg L-NIL with or without 20 mg/kg of nitrite ($NaNO_2$) as a NO source 30 min after each HDM challenge. *Penh* was recorded 24 h after the last challenge before in response to increasing doses of MeCh. Results are plotted as maximal fold increase of *Penh* relative to baseline and expressed as mean ± SEM where n=5 mice per group. *, difference from HDM challenged mice; #, difference from control unchallenged mice; $p < 0.05$.

Figure 32

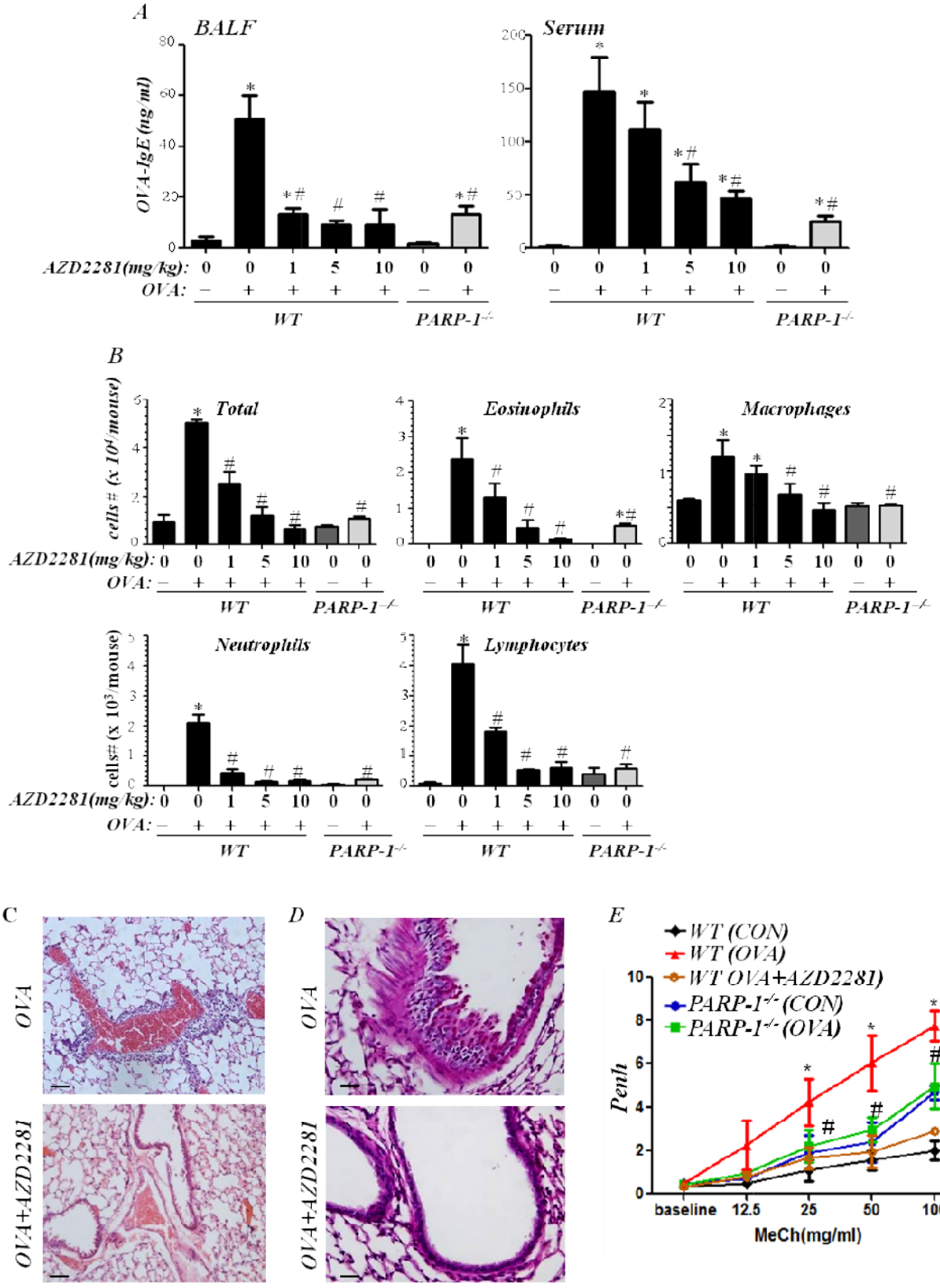


Effects of PARP inhibition on iNOS expression in LPS/IFN-g-treated cells and of iNOS inhibition on the protection conferred by the PARP inhibitor olaparib against AHR in chronically HDM-exposed mice.

MEFs derived from WT or PARP-1$^{-/-}$ mice and heart endothelia l mice cells transected with human PARP-1 were treated with a combination of 1 mg/ml LPS and 10 ng/ml IFNγ or with 1mg/ml of LPS alone respectively. RNA was isolated after 6 hours of treatment and reverse transcribed to generate cDNA, which was subjected to RT quantitative and conventional PCR with primers specific to mouse iNOS , PARP-1 or *b*-actin. The data is expressed as fold change normalized to levels of b-actin. *, difference from non-stimulated cells; #, difference from LPS/IFNg-treated WT cells; p <0.01. (D) WT mice were subjected to HDM sensitization followed by a chronic *i.n.*

challenge with HDM or left unchallenged. Challenged mice were administered *i.p.* 5 mg/kg olaparib with or without 5 mg/kg of L-NIL 30 min after each HDM challenge. *Penh* was recorded 24 h after the last challenge in response to increasing doses of MeCh. Results are plotted as maximal fold increase of *Penh* relative to baseline and expressed as mean ± SEM where n=5 mice per group. *, difference from HDM challenged mice; #, difference from the group that received 5 mg/kg olaparib; $p < 0.05$.

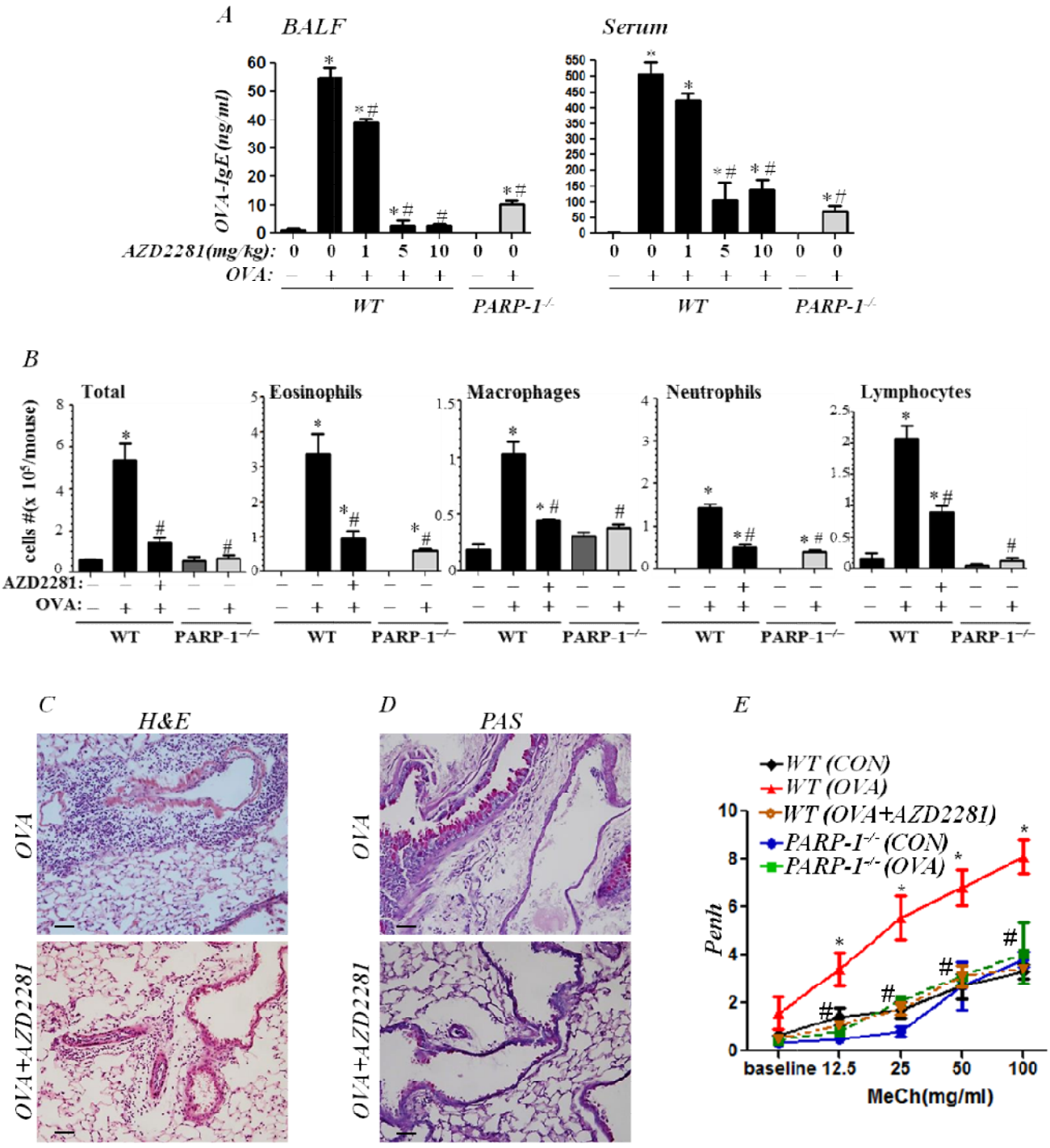
Figure 33


A
BALF
Serum
OVA-IgE (ng/ml)
AZD2281(mg/kg): 0 0 1 5 10 0 0
OVA: – + + + + – +
WT
PARP-1-/-
B
Total
Eosinophils
Macrophages
Neutrophils
Lymphocytes
cells# (x 10⁴/mouse)
cells# (x 10³/mouse)
C
OVA
OVA+AZD2281
D
E
WT (CON)
WT (OVA)
WT OVA+AZD2281
PARP-1-/- (CON)
PARP-1-/- (OVA)
Penh
baseline 12.5 25 50 100
MeCh(mg/ml)

Olaparib blocks airway eosinophilia, mucus and IgE production, and AHR upon a single challenge with OVA in a mouse model of asthma.

C57BL/6J WT or PARP-1$^{-/-}$ mice were subjected to OVA sensitization followed by a single challenge to aerosolized OVA or left unchallenged. WT mice were administered, *i.p.*, 1 mg/kg, 5 mg/kg or 10 mg/kg of olaparib or saline thirty minutes after OVA challenge. Mice were sacrificed 48 h later and lungs were subjected to formalin fixation or BAL. (A) Assessment of BALF or sera collected from the different experimental groups 48 h after OVA challenge for OVA-specific IgE using sandwich ELISA. (B) Cells of BALF were differentially stained, and total cells, eosinophils, macrophages, lymphocytes, and neutrophils were counted. Data are expressed as total number of cells per mouse. Data are means ± SD of values from at least six mice per group. (C) Lung sections from OVA-challenged mice that were treated with either saline or olaparib were subjected to H&E or (D) PAS staining. (E) Mice were sensitized and challenged with OVA as described above. A group of WT mice received an injection of 5 mg/kg of olaparib. Penh was recorded 24 h later using a whole body plethysmograph system before and after the indicated concentrations of aerosolized methacholine (MeCh). Results are plotted as maximal fold increase of Penh relative to baseline and expressed as mean ± SEM where n=6 mice per group. For A, B, and E: *, difference from control unchallenged mice, $p < 0.01$; #, difference from OVA-challenged mice; $p < 0.01$. For C and D: bar= 5mm.

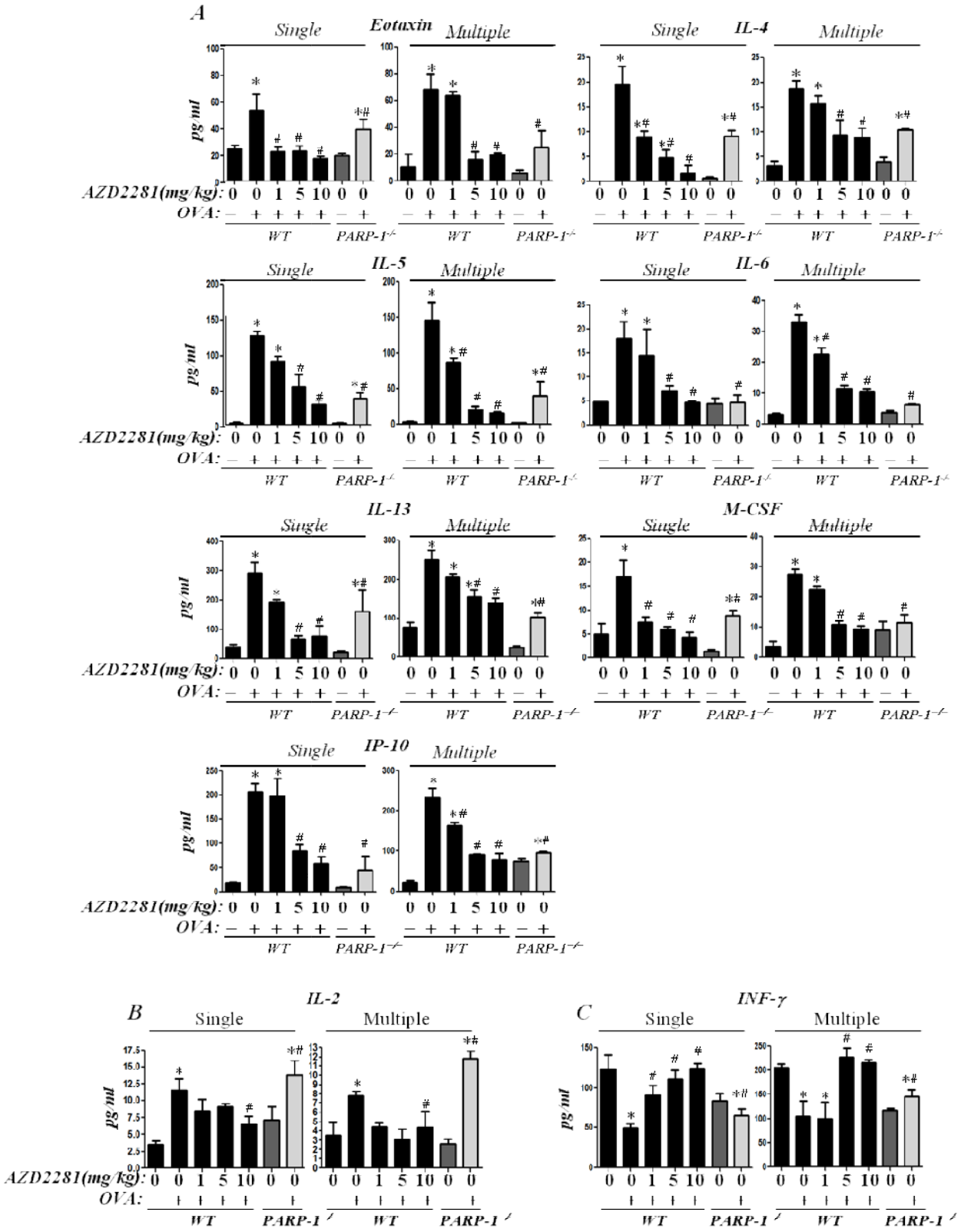
Figure 34


A
BALF
Serum
OVA-IgE (ng/ml)
AZD2281(mg/kg): 0 0 1 5 10 0 0
OVA: – + + + + – +
WT
PARP-1-/-
B
Total
Eosinophils
Macrophages
Neutrophils
Lymphocytes
cells #(x 10⁵/mouse)
AZD2281:
OVA:
WT
PARP-1-/-
C
H&E
D
PAS
OVA
OVA+AZD2281
E
WT (CON)
WT (OVA)
WT (OVA+AZD2281)
PARP-1-/- (CON)
PARP-1-/- (OVA)
Penh
baseline 12.5 25 50 100
MeCh(mg/ml)

Olaparib blocks airway eosinophilia, mucus and IgE production, and AHR upon a multiple challenge with OVA in a mouse model of asthma.

WT or PARP-1$^{-/-}$ mice were subjected to OVA sensitization followed by triple challenge (Multiple) or left unchallenged. WT mice were administered, *i.p.*, 1 mg/kg, 5 mg/kg or 10 mg/kg of olaparib or saline thirty minutes after each challenge. Mice were sacrificed 48 h later and lungs were subjected to formalin fixation or BAL. (A) Assessment of BALF or sera collected from the different experimental groups 48 h after the last challenge for OVA-specific IgE. (B) Cells of BALF were differentially stained, and total cells, eosinophils, macrophages, lymphocytes, and neutrophils were counted. Data are means ± SD of values from at least six mice per group and are expressed as total number of cells per mouse. Lung sections from multiple OVA-challenged mice that were treated with either saline or olaparib were subjected to H&E (C) or PAS (D) staining. (E) Mice were sensitized and challenged with OVA as described above. A group of WT mice received an injection of 5 mg/kg of olaparib. Penh was recorded 24 h later using a whole body plethysmograph system before and after the indicated concentrations of aerosolized methacholine (MeCh). Results are plotted as maximal fold increase of Penh relative to baseline and expressed as mean ± SEM where n=6 mice per group. For A, B, and E: *, difference from control unchallenged mice, $p < 0.01$; #, difference from OVA-challenged mice; $p < 0.01$. For C and D: bar= 5mm.

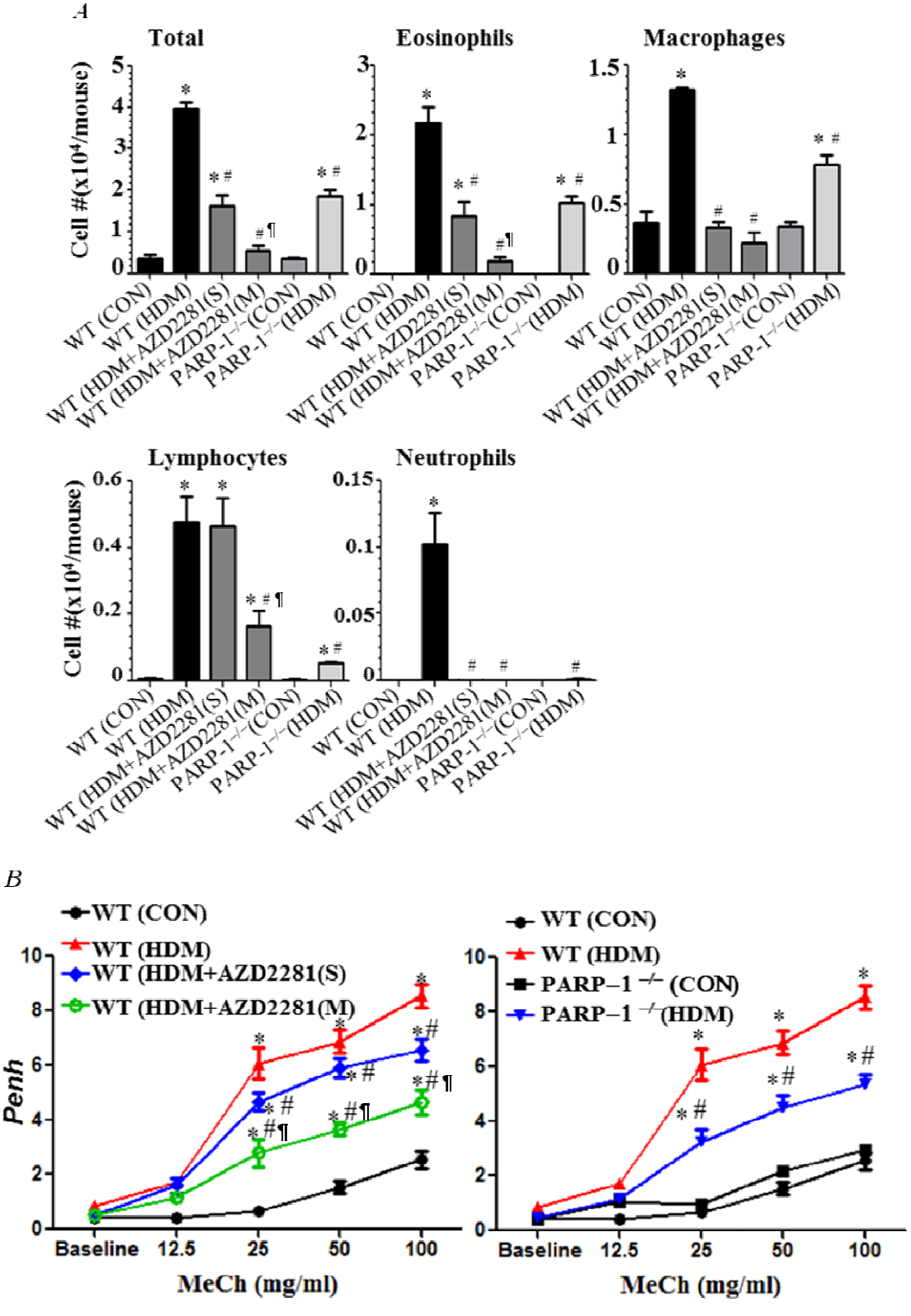
Figure 35


A
Eotaxin
IL-4
IL-5
IL-6
IL-13
M-CSF
IP-10
Single
Multiple
pg/ml
AZD2281(mg/kg): 0 0 1 5 10 0 0
OVA: – + + + + – +
WT
PARP-1-/-
B
IL-2
C
INF-γ
PARP-1 -/-

Olaparib treatment differentially affects production of Th1 and Th2 cytokines.

WT or PARP-$1^{-/-}$ mice were subjected to OVA sensitization followed by a single or triple challenge (Multiple) or left unchallenged. WT mice were administered *i.p.* 1 mg/kg, 5 mg/kg or 10 mg/kg of olaparib or saline thirty minutes after each challenge. Mice were sacrificed 48 h later and lungs were subjected to BAL. Assessment of BALF from the different groups for Th2 cytokines eotaxin, IL-4, IL-5, IL-6, IL-13, or M-CSF (A), IL-2 (B) or INF-g (C). Data are means ± SD of values from at least six mice per group. *, difference from control unchallenged mice, $p < 0.01$; #, difference from OVA-challenged mice; $p < 0.01$.

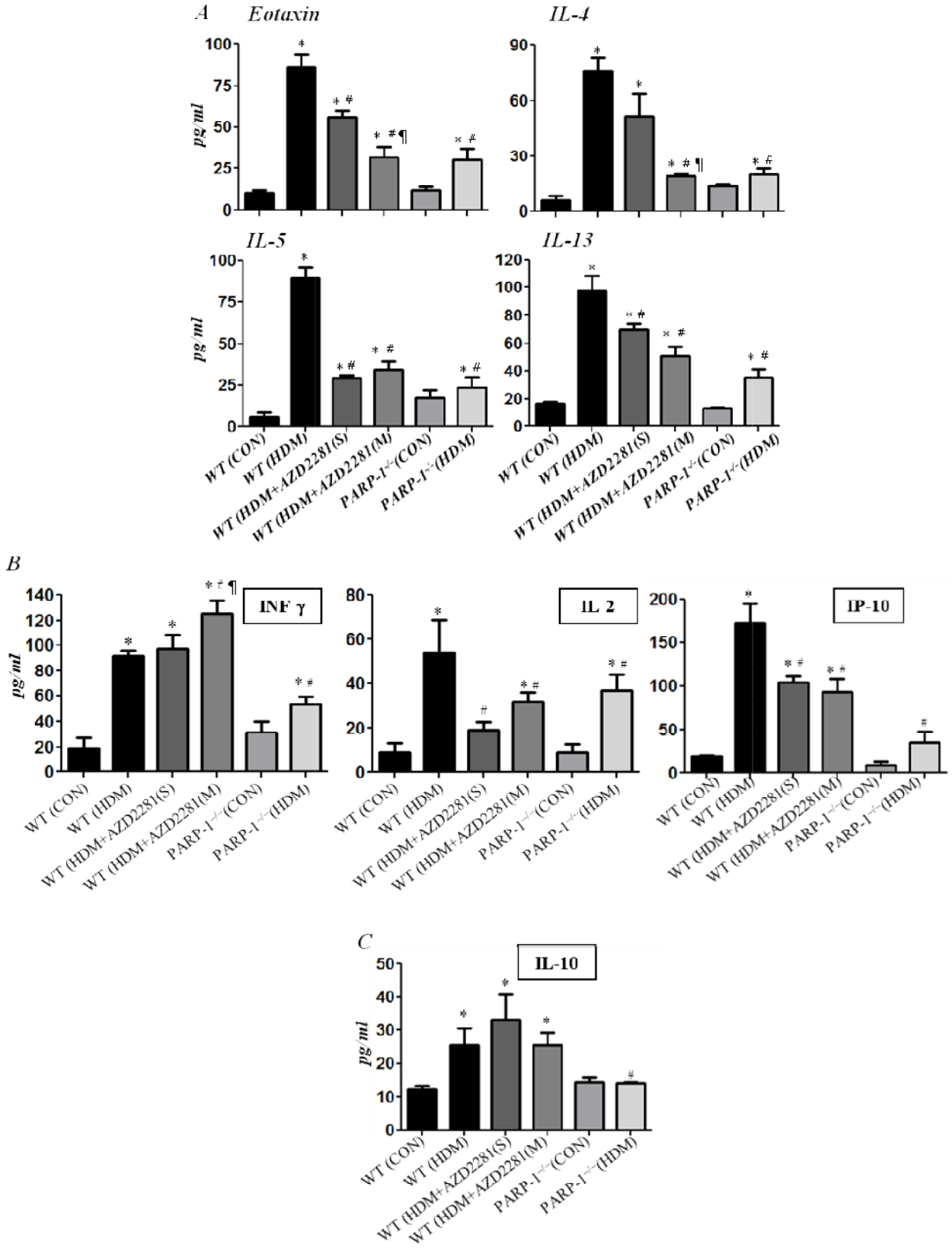
Figure 36


A
Total
Eosinophils
Macrophages
Lymphocytes
Neutrophils
Cell #(x10⁴/mouse)
WT (CON)
WT (HDM)
WT (HDM+AZD2281(S))
WT (HDM+AZD2281(M))
PARP-1⁻/⁻(CON)
PARP-1⁻/⁻(HDM)
B
WT (CON)
WT (HDM)
WT (HDM+AZD2281(S))
WT (HDM+AZD2281(M))
PARP–1 ⁻/⁻ (CON)
PARP–1 ⁻/⁻(HDM)
Penh
Baseline
12.5
25
50
100
MeCh (mg/ml)

PARP inhibition by olaparib or gene knockout blocks asthma-like traits in chronically HDM-exposed mice.

C57BL/6J WT or PARP-$1^{-/-}$ mice were subjected to HDM challenge or left untreated. HDM-challenged WT mice received 5 mg/kg of olaparib or saline once (S) thirty minutes after the last HDM challenge or once daily for three days (M). All mice were sacrificed 48h later and BAL fluids and organs were collected. (A) Cells of BAL fluids were differentially stained, and total, eosinophils, macrophages, lymphocytes, and neutrophils were counted. Data are expressed as total number of cells per mouse. (B) WT mice were subjected to HDM challenge followed by an *i.p.* injection of saline (▲), single (♦) or multiple administrations of 5 mg/kg olaparib (○). Control mice were not sensitized or challenged (●). PARP-$1^{-/-}$ mice were also subjected to HDM challenge (▼) and control mice were left unchallenged (■). Penh was recorded 24 h later using a whole body plethysmograph system before and after the indicated concentrations of aerosolized methacholine (MeCh). Results are plotted as maximal fold increase of Penh relative to baseline and expressed as mean ± SEM where n=6 mice per group. *, difference from control WT mice, $p < 0.01$; #, difference from HDM-challenged WT mice, $p < 0.01$.

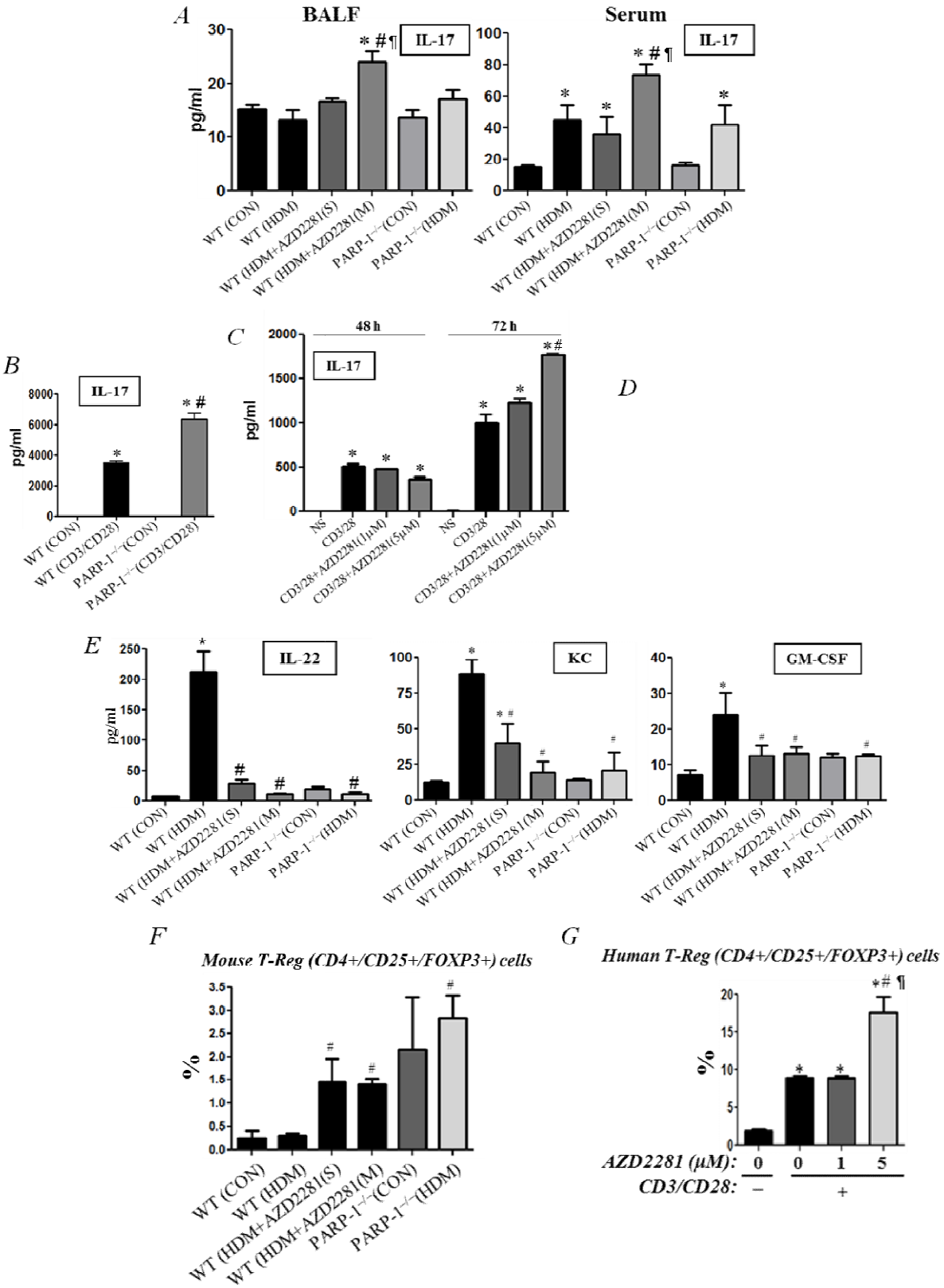
Figure 37


A
Eotaxin
IL-4
IL-5
IL-13
pg/ml
WT (CON)
WT (HDM)
WT (HDM+AZD2281(S)
WT (HDM+AZD2281(M)
PARP-1-/-(CON)
PARP-1-/-(HDM)
B
INF γ
IL 2
IP-10
C
IL-10

PARP inhibition by olaparib reduces Th2 cytokine production without a prominent effect on IFN-γ or IL-10.

WT and PARP-$1^{-/-}$ mice were subjected to chronic HDM challenge or left untreated. HDM challenged WT mice received a single dose of 5 mg/kg of olaparib or saline thirty minutes after the last challenge or given multiple administrations of olaparib every 24 h for a total of three times. All mice were sacrificed 48 h later and lungs were subjected to BAL. Assessment of BAL fluid from different groups for Th2 cytokines; eotaxin, IL-4, IL-5, or IL-13 (A), IFN-γ IL-2 or IP-10 (B) and IL-10 (C). Data are means SD of values from at least six mice per group. *, difference from control mice, $p < 0.01$; #, difference from HDM challenged WT mice; $p < 0.01$.

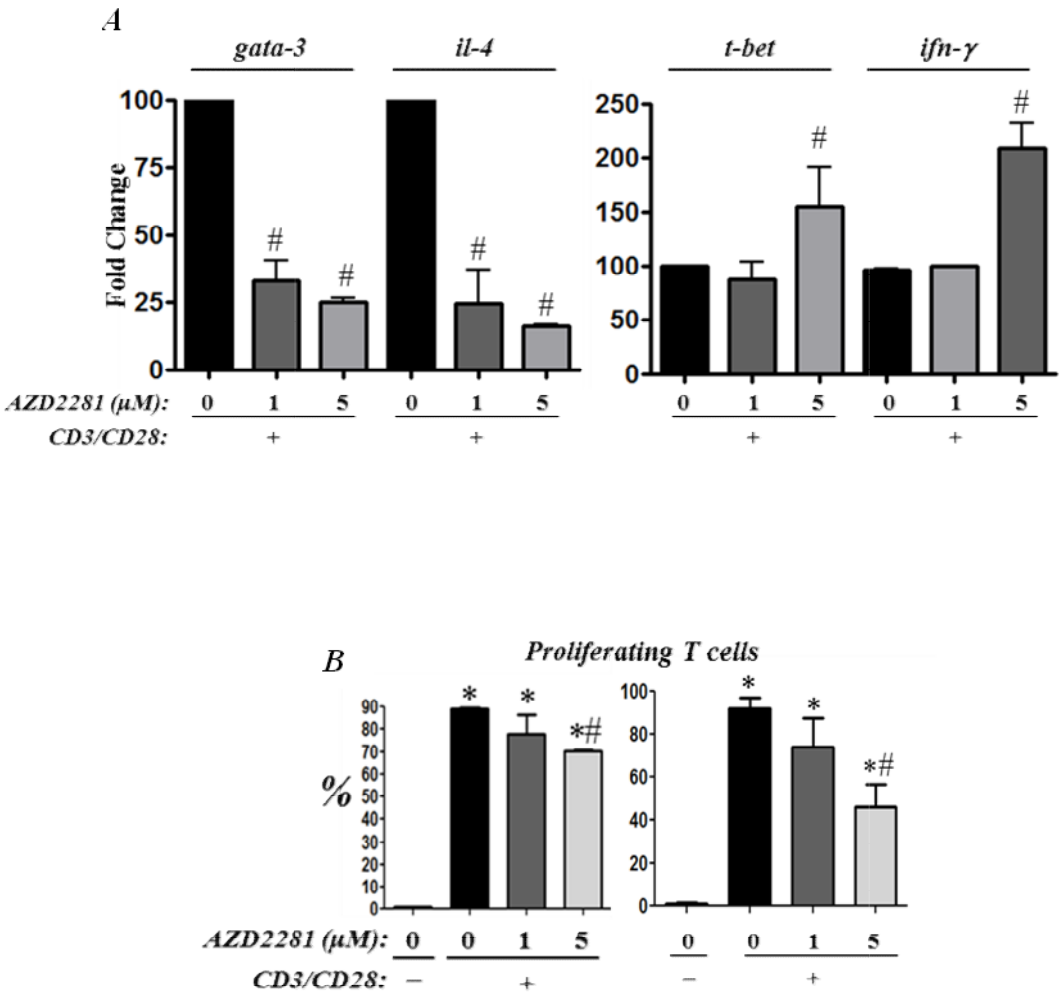
Figure 38


A
BALF
IL-17
pg/ml
Serum
IL-17
WT (CON)
WT (HDM)
WT (HDM+AZD2281(S)
WT (HDM+AZD2281(M)
PARP-1-/-(CON)
PARP-1-/-(HDM)
B
IL-17
pg/ml
WT (CON)
WT (CD3/CD28)
PARP-1-/-(CON)
PARP-1-/-(CD3/CD28)
C
48 h
72 h
IL-17
pg/ml
NS
CD3/28
CD3/28+AZD2281(1μM)
CD3/28+AZD2281(5μM)
D
E
IL-22
pg/ml
KC
GM-CSF
F
Mouse T-Reg (CD4+/CD25+/FOXP3+) cells
%
G
Human T-Reg (CD4+/CD25+/FOXP3+) cells
%
AZD2281 (μM): 0 0 1 5
CD3/CD28: – +

PARP inhibition inconsistently increases IL-17 production in HDM-exposed mice and CD3/CD28-activated human or mouse CD4+ T cells without a concomitant increase in its regulated factors.

WT or PARP-$1^{-/-}$ mice were subjected to HDM challenge or left untreated. HDM-challenged WT mice received a single dose or multiple doses of olaparib (5 mg/kg) or saline as described above. Mice were sacrificed 48 h later. (A) BAL fluid and sera of the sacrificed mice were assessed for IL-17. *, difference from control WT mice, $p < 0.01$; #, difference from HDM-challenged WT mice, $p < 0.01$; ¶, difference from HDM-challenged WT mice subjected a single olaparib administration. (B) Splenic CD4+ T cells isolated from naïve WT and PARP-$1^{-/-}$ mice were activated with antibodies to CD3/CD28. After an incubation of 96 hours, culture supernatants were assessed for IL-17. *, difference from non-stimulated cells, $p < 0.01$; #, difference from anti-CD3/CD28-stimulated cells; $p < 0.01$. (C) Negatively selected human $CD4^{+}$T cells from healthy donors were stimulated with antibodies to CD3/CD28 in absence or presence of 1 or 5 μM of olaparib. Cell supernatant was tested for IL-17 production after 48h and 72h. *, difference from non-stimulated cells, $p < 0.01$; #, difference from anti-CD3/CD28-stimulated cells; $p < 0.01$. (D) mRNA was isolated from WT or PARP-$1^{-/-}$ splenic CD4+ T cells that were activated with anti-CD3/CD28 antibodies for 12h and 24 h. mRNA was reverse-transcribed and the resulting cDNA was subjected to conventional PCR with primer sets specific to mouse il-17 or b-actin. (E) BAL fluids from all the experimental groups were tested for IL-22, KC and GM-CSF production. *, difference from control WT mice, $p < 0.01$; #, difference from HDM-challenged WT mice, $p < 0.01$. (F) HDM-challenged WT mice received a single dose or multiple doses of olaparib (5 mg/kg) or saline. Spleens were processed to generate single cell suspensions, which were analyzed by FACS for CD4+/CD25+/Foxp3+ cells. #, difference from HDM-challenged WT mice, $p < 0.05$.

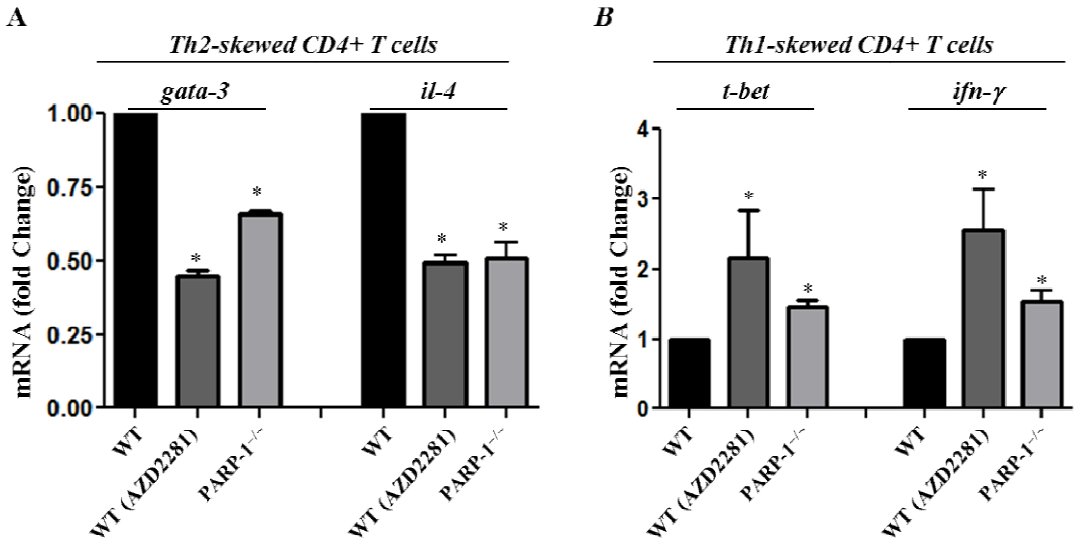
Figure 39



Effects of PARP inhibition by olaparib on human CD4$^+$T cell proliferation and Th1/ Th2 populations.

Negatively selected human CD4$^+$T cells from healthy donors were stimulated in triplicates with antibodies to CD3/CD28 in absence or presence of 1 or 5 μM olaparib. (A) CD3/CD28-stimulated and control CD4$^+$T were subjected to RNA extraction after an incubation of 12 h. RNA was then reverse-transcribed and the resulting cDNA was subjected to PCR with primer sets specific to human *gata-3*, *t-bet* or *gapdh;* (B) Cell proliferation was assessed by CFSE staining. *, difference from non-stimulated cells, $p < 0.01$; #, difference from CD3/CD28-stimulated cells; $p < 0.01$.

Figure 40



PARP-1 inhibition reduced the differentiation of Th2 cells in mice under specific skewing condition.

WT or PARP-$1^{-/-}$ mice were subjected to OVA sensitization followed by a single or triple challenge (Multiple) or left unchallenged. WT mice were administered *i.p.* 5 mg/kg of olaparib or saline thirty minutes after each challenge or left untreated. All mice were sacrificed 48 h later. Spleens from the different experimental groups were used to generate single-cell suspensions. Purified $CD4^{+}$ T cells procured from spleens of OVA-sensitized mice were stimulated with anti-CD3 and anti-CD28 antibodies and then skewed into a Th1 (B) or Th2 (A) phenotype in the presence or absence of 5 μM olaparib. RNA was extracted then used to generate corresponding cDNA followed by quantitative PCR with primer sets specific for mouse *gata-3*, *il-4*, *t-bet*, or *ifn-g* or b-act, difference from CD3/CD28-stimulated cells; $p < 0.01$.

Discussion and Conclusions

The role of iNOS in asthma has been examined in numerous studies with conflicting results. Its potential as a viable therapeutic target for the treatment of the disease was severely hampered by the negative results of a clinical trial showing that a selective iNOS inhibitor although it effectively reduces exhaled NO, it did not affect AHR or airway inflammation after allergen challenge in steroid-naïve human subjects with asthma [384]. We believe that such conclusion is premature and that more careful studies are necessary to fully understand the role of iNOS in asthma and whether the enzyme may be adequately targeted at least as an adjuvant therapy for the treatment of the disease.

Our laboratory has shown that iNOS inhibition is protective against airway inflammation upon acute, but not chronic, exposures to OVA [121]. These effects on chronic inflammation were consistent with those reported by Singh et al. using a selective iNOS inhibitor (GW274150). More importantly, iNOS inhibition protects against lung fibrosis despite the persistence of inflammation, potentially by preventing TIMP-2 expression, suggesting that iNOS inhibition may be protective against some aspects of asthma but not others. The current study represents a continuation of previous efforts of our laboratory to understand the role of iNOS in asthma and to explore new mechanisms by which the enzyme can be targeted for therapy of the disease.

High levels of iNOS and $ONOO^-$, the reactive byproduct of NO, are undoubtedly deleterious and participate to the pathology of asthma [121, 385, 386] as well as many chronic inflammatory diseases [321-324]. Sugiura et al. showed that patients with refractory asthma have even higher levels of iNOS and protein nitration in cells collected from their sputum than that from patients with well-controlled asthma [387]. This connection was recently strengthened by a study demonstrating that an iNOS-Dual oxidase-2-thyroid peroxidase metabolome is the basis of nitrogen radicals and subsequent protein nitration in human severe asthma [388]. Our results show that the elevated iNOS expression is also observed in PBMCs of asthmatics and potentially even higher in cells from asthmatics with uncontrolled disease. Surprisingly, this is the first report examining the levels of iNOS and protein nitration in PBMCs of asthmatics. Our results suggest that the role of iNOS in the pathogenesis of the disease may stem from circulating cells in addition to those in the lung. Although the cohort size is small in the present study, our results show a potential disconnection between expression of iNOS and protein nitration within the same cells suggesting that PBMCs may not be the major target of NO and its byproducts. This disconnection does not appear to exist in cells derived from either bronchial lavage fluids or sputum as shown by Sugiura et al. and Yamamoto et al., respectively [325, 387]. Altogether, the results of these studies and many

others including ours [121, 358] predict that iNOS may be an ideal target for the treatment of asthma. Surprisingly, as mentioned before, targeting this enzyme has been unsuccessful [384]. We speculated that our understanding of the role of NO and iNOS in asthma may not be sufficient to allow a better approach to achieve the desirable clinical outcomes using selective iNOS inhibitors.

Here, I show also that iNOS inhibition, pharmacologically using the relatively selective inhibitor, L-NIL, or by gene knockout provided an excellent protection against AHR upon an acute, but not upon a chronic, exposure to allergens as assessed using full body plethysmography. These results are rather similar to the differential protection provided by iNOS inhibition against airway inflammation. At this stage it is not clear whether the protection and loss of protection in the acute and chronic models, respectively, are associated with the status of inflammation as manifestation of AHR. But, given the fact that NO has several beneficial effects, we hypothesized that the loss of protection in the chronic asthma models, may be associated with the complete inhibition of iNOS and consequent reset of NO levels that, as an excessive production, may have deleterious effects. The first interesting result that supports our hypothesis is that the protection was recovered in the chronic OVA/HDM asthma model after NO supplementation by nitrite administration. The dose of nitrite used in this study was shown to deliver low to moderate levels of NO and protects against several oxidative stress-related conditions including hypoxic vasodilation, ischemia of the heart and liver and postoperative ileus. The anion nitrite (NO_2^-) forms as a consequence of NO oxidation may be reduced to NO during hypoxia and acidosis. Recent studies demonstrate that nitrite are reduced to NO by reaction with deoxyhemoglobin under physiologic conditions. It is important to acknowledge that the failure of iNOS inhibition to protect against AHR in chronic asthma may be related to other factors besides NO; however, the level of the gas may constitute a major determinant in the protection against AHR. It is also important, at this point, to acknowledge that one of the limitations of the current study is that the levels of NO upon nitrate supplementation were not measured since the method used to detect NO is based on nitrite levels. I am currently searching for an alternate but accurate method to assess NO in my experimental models. We predict that these levels might not have reached those in OVA/HDM-exposed mice; otherwise, we would expect a failure of protection against AHR manifestation. An additional limitation is the quantification of Penh using whole body plethysmography to measure AHR. Although measuring lung resistance is regarded as a better

means to assess lung function, our previous studies demonstrate, for instance, a similar protection against AHR by PARP inhibitors using either method.

These first set of results allow to belive that there may be a strict correlation between the levels of NO and the protection agains AHR manifestations.

Our laboratory also established a reciprocal relationship between iNOS and PARP-1[358]. PARP-1 activity and expression are required for iNOS expression. Such regulation is linked to the dependence of iNOS gene on NF-κB and control of the activity as well as the subcellular trafficking of the transcription factor by PARP-1 [389]. Interestingly, PARP inhibition, pharmacologically or by gene knockout, protected against inflammation and AHR both upon acute and chronic exposures to OVA. Similar protection was observed upon chronic exposure to HDM demonstrating that the protective effect is not limited to OVA. Such protection may be correlated with the fact that iNOS expression is markedly reduced upon PARP inhibition. It is interesting indeed, that PARP-1 inhibition does not completely abrogate expression of iNOS. This partial inhibition may represent the basis for the persistent protection conferred by PARP inhibition even after a chronic exposure to allergens.

Different groups have shown that excessive activation of iNOS can be detrimental since NO can interact with ROS generating $ONOO^-$ and consequently causing DNA damage. This oxidative DNA damage obviously leads to a hyper-activation of PARP-1, which may participate to additional amplification of the inflammatory responses or causes necrosis of cells complicating the condition even more. The role of PARP in tissue damage and cell death is very well established and our laboratory extensively contributed to the long effort in deciphering the role of PARP in the before mentioned pathological processes [390].

The high level of NO correlate with increased nitration of different proteins and consequent modification of their enzymatic or structural activities. Furthermore, protein nitration is an irreversible modification after which the modified proteins may be targeted for degradation. Our laboratory showed that nitration can negatively regulate the activity of PARP-1 [358]. Accordingly, it is possible to speculate that an over-expression of iNOS and an elevation in $ONOO^-$ lead to an inhibition rather than over-activation of PARP-1. However, I believe that the process is more complex. It is important to subdivide the inflammatory responses to separate phases, which include initiation of the process followed by an amplification phase leading to tissue injury. During the early stages of an asthmatic response, the role of PARP-1 in the signal transduction of NF-κB and subsequent induction of target genes such as iNOS may not involve an extensive activation of PARP-1. Preliminary results from our laboratory

suggest that PARP-1 activity in signaling may not even involve DNA damage. Once iNOS protein is expressed, large amounts of NO may be generated. NO then interact with ROS to form high quantities of $ONOO^-$. This process may be late in the inflammatory response and at this stage an inhibition of PARP-1 may participate to the death process as a result of the inability of injured cells to repair their DNA and thus die by apoptosis complicating the inflammatory process. We need to keep in mind that, for instance, if PARP-1 is nitrated and targeted for degradation, the PARP-1 gene is constitutive and remains producing mRNA that can be translated to fresh unmodified enzyme leading to an amplification of the inflammatory response. The cumulative effects of the two first phases may lead to the third injury stage.

In conclusion, also these second set of results, provide additional support to my hypothesis that the amount of iNOS and NO are critical determinants in asthma pathogenesis and in the modulation of AHR and may also help explain why clinical studies on asthma, that aimed to complete inhibition of NO production by iNOS, using specific inhibitors, failed to observe beneficial effects [384]. It is noteworthy that the levels of exhaled NO achieved upon administration of the inhibitor reached amounts lower than that observed in healthy nonsmoker and nonatopic subjects as comprehensively reviewed by Dweik et al.[391]. Furthermore, the drug was delivered orally, which may be another limitation of the study. Continuing efforts to investigate the exact role of the enzyme in asthma may provide a clearer view on how to utilize iNOS as a therapeutic target. Additionally, targeting PARP-1 to modulate iNOS during asthma may constitute a better alternative.

The severe reduction in the levels of NO in the study by Singh et al. may be detrimental and as such prevention of AHR might not have been possible. These speculations and the positive results of our study on the role of iNOS and the critical influence of NO levels on AHR should lead to a reevaluation of the benefit of selective iNOS inhibitors in blocking AHR in human asthmatics, especially those with uncontrolled disease.

Figure 40 display some preliminary result that show that PARP-1 may be involved in the Th2 skewing of CD4+ T-helper lymphocytes. This is demonstrated by a down-regulation of GATA-3 mRNA expression and its target gene (IL-4) following treatment with the PARP inhibitor Olaparib in response to T cell receptor stimulation (CD3/CD28). I am currently attempting to correlate the role of PARP-1 in T cell differentiation and function with iNOS expression and enzymatic activity. Given that is know that NO can skew the TH1/TH2 balance into the direction of a TH2 response by enanching IL4 production [392], I will examine whether the results observed with PARP-1 inhibition in mice and human can be caused by its

interaction/relationship with iNOS. Additional studies on the relationships between these two proteins may contribute to the clarification of the intricate roles of iNOS in asthma pathogenesis and may help in the design of new therapeutic strategies for the treatment of the condition.

We expanded our studies on the role of PARP in the function of TCR-stimulated CD4+-enriched T cells derived from human PBMCs. The effects of PARP inhibition by Olaparib were more associated with a reduction in the ability of Th2 cells to produce Th2 cytokines rather than through their differentiation. This conclusion stems from the fact that PARP inhibition marginally affected overall proliferation of T cells and the percentage of CD4+/IL-4+ Th2 cells upon CD3/CD28 activation. The marginal effect of PARP inhibition on T cell proliferation is consistent with that reported by Saenz et al. [393]. It is noteworthy that although PARP inhibition reduced IL-4 production by anti-CD3/CD28-activated human Th2 cells, the effect was insufficient to cause a drastic reduction in differentiation of the cells. The effect of PARP inhibition of Th2 cytokine production upon TCR stimulation may be directly linked to a modulation of *gata*-3 expression. Currently, the mechanism by which PARP-1 regulates the expression of *gata-3* is not clear. It is however established that *gata-3* is regulated by NF-κB [394] and STAT-6 [395], both of which can be regulated by PARP-1 as shown in our previous studies [369, 389]. Studies are underway to decipher the exact role of PARP-1 in the regulation of the NF-kB and STAT-6 pathways. The role of PARP in regulating the expression of Th1 cytokines is rather intriguing as inhibition of the enzyme reduced some but not all Th1 cytokines despite the lack of clear effect on expression of *t-bet*. For instance, PARP inhibition did not affect expression of IFN-γ or IL-2 but decreased expression of IL-12(p70) and IP-10 in anti-CD3/CD28-activated human CD4+ T cells. The differential effects on IFN-γ and IP-10 in human CD4+ T cells was consistent with those observed in HDM-based animal model of asthma. Obviously, more experimentation is necessary to clarify such differential effects and identify the exact factors that can be regulated by PARP. It is important to note that sometimes PARP inhibition by Olaparib provided a more pronounced effect compared to that achieved by gene knockout. For instance, the effect of multiple administration of Olaparib on lung eosinophilia and total BALF cell count at the 5 mg/kg dose was better than that observed in PARP-$1^{-/-}$ mice. The enhanced effect may be associated with the ability of Olaparib to inhibit both PARP-1 and PARP-2.

In these studies, after have demonstrated that PARP is actually activated in human asthma, we provide convincing evidence that Olaparib, a PARP inhibitor that is currently being tested in cancer clinical trials, efficiently blocked established asthma-like traits including production of Th2 cytokines, mucus and AHR in both OVA and HDM-based asthma models. Some of these effects seems to be correlate with iNOS, in particular the protection against AHR. But others, in particular the those correlate with reduction of the typical airway inflammation associated with asthma, seems to be correlate with the capacity of PARP-1 to interact and modify directly different proteins involved in the inflammatory process. The anti-inflammatory effects conferred by PARP inhibition seemed to be aided by an increase in T-reg cells. Finally, we show that PARP inhibition inconsistently increased IL-17 production. Interestingly, the factors that may be influenced by IL-17 were decreased rather than increased upon PARP inhibition in both HDM mouse model and in stimulated human CD4+-enriched T cells. We thus propose PARP inhibition as a viable strategy in targeting at least some aspects of human asthma and that the therapeutic strategy merits a consideration in clinical trials.

The modulatory effects of PARP inhibition by Olaparib or by gene knockout on eosinophilia and IL-4 are indicative of a potential reduction in IgE. Indeed, PARP inhibition pharmacologically or by gene knockout blocks IgE production in both OVA- and HDM-based mouse models [352, 396]. We also show that olaparib very efficiently reduces OVA-specific IgE levels in BALF and sera of challenged mice.

Recently published works analyze the role of IL-17 in different inflammatory disease.

Considering the important role of PARP-1 in asthma pathophysiology emerged from our studies, we decide to investigate the potential connection between PARP-1 and IL-17.

Several reports showed conflicting results on the ability of PARP inhibition to modulate IL-17 in animal models of inflammatory diseases and in *in vitro* systems. Indeed, a report by Nasta et al. [378] showed that PARP-1 deficiency does not affect production of IL-17 in response to multiple stimuli including CD3 and CD28. In contrast, PARP inhibition was shown to reduce IL-17 production in adjuvant-induced arthritis mouse model [380] and carrageenan-induced lung inflammation in mice [381]. However, recently, Zhang et al. [377] showed that in TGF-β and IL-6-treated CD4+ T cells, PARP inhibition can in fact increase IL-17. Using both the chronic HDM exposure mouse model and CD3/CD28-activated CD4+ T cells, we showed that the association between PARP inhibition and modulation of IL-17 production is inconsistent. PARP appears to regulate IL-17 at the level of mRNA in activated CD4+ T cells. Surprisingly, the increase in IL-17 in both the animal and cell culture models was accompanied

by a decrease rather than an increase in factors that can be influenced by IL-17 such as IL-22, KC, IL-6, and GM-CSF. It is interesting that despite the increase in IL-17 in our experimental systems, the net effect was anti- rather than pro-inflammatory. The reduction in KC and GM-CSF can be unrelated to the effect on IL-17 as PARP inhibition, pharmacologically or by gene knockout, reduces expression of the two inflammatory factors in response to LPS ([11] and unpublished observation). It is important to note that if IL-17 is highly increased by PARP inhibition and that this increase exerts some pathology then using olaparib for the treatment of asthma in humans constitutes an important limitation of the strategy. Obviously, further investigation is necessary to decipher the exact role of PARP inhibition-induced increase in IL-17 production in our experimental models of asthma.

An emerging role for PARP in inflammation is its potential influence on T-reg cells [378-380, 397]. This role is associated with the stabilization of the transcription factor Foxp3 [379]. Our results show that PARP inhibition by olaparib or gene knockout increased T-reg cell population. This appears to be independent of HDM exposure as naïve PARP-$1^{-/-}$ mice displayed higher levels of T-reg cells compared to the WT counterparts. In human CD4+ T cells, the increase in T-reg cell population was only observed upon an exposure to the high dose (5 μM) but not to the low dose (1 μM) of olaparib. These results and those of aforementioned published reports strongly suggest that the role of PARP-1 in T-reg differentiation and function is complex; additional studies are required to reach a better understanding of the relationship. Nevertheless, an increase in T-reg cell population may be an added protective trait achieved upon PARP inhibition in the control of asthma-associated inflammation.

Overall, the results of the present study may explain why clinical studies focused on the completely inhibition of iNOS failed to protect against different aspect of asthma. However, further efforts to investigate the exact role of the enzyme in asthma may provide a clearer view on how to utilize iNOS as a therapeutic target. It's assured that reach moderate levels of NO playing with the partial inhibition of iNOS could represent a good therapeutic strategy at least against some aspects of the disease, such as AHR.

On the other hand, our results revealed that targeting PARP-1 may constitute a better alternative therapeutic approach for asthma. First of all because, through its capacity to modulate NF-kB-mediated iNOS expression, PARP-1 inhibition was able to reduce AHR. Another reason is that PARP-1 inhibition significantly reduced the most important asthma symptoms: inflammation, IgE production and mucus hyper-secretion. Our preliminary results show that these beneficial effects could be correlated probably with the capacity of PARP-1 to

positively interacts with GATA3, the master regulator of Th2 population, the Th subtype most involved in the allergic disease, and probably, with its ability to inhibit Treg population, responsible for the control and reduction of inflammation.

It is noteworthy that *L*-N6-(1-Iminoethyl)lysine dihydrochloride (L-NIL) and AZD2281 (olaparib), are two clinically tested iNOS and PARP inhibitors, respectively. This increases considerably the relevance of this study.

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Under rewiew papers

- **Salome' V. Ibba**, Mohamed A. Ghonim, Kusma Pyakurel, Matthew R. Lammi; Anil Mishra, A. Hamid Boulares - ***Potential of inducible NO synthase as a therapeutic target for allergen-induced airway hyperresponsiveness: A critical connection to NO levels and PARP-1 activity*** - Clinical Science

- Matthew Lammi, Mohamed Ghonim, Kusma Pyakurel, Amarjit Naura, **Salome Ibba**, Christian Davis, Samuel Okpechi, Kyle Happel, Bennett de Boisblanc, Judd Shellito, and Hamid Boulares- ***Treatment with intranasal iloprost reduces disease manifestations in a murine model of previously established COPD***