

PARP inhibition treatment in a nonconventional experimental mouse model of chronic asthma

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Abstract Allergic asthma is an immunological disease that occurs as a consequence of aeroallergen exposure. Inhibition of poly(ADP-ribose) polymerases (PARPs) in conventional models of asthma-like reaction has emerged as an effective anti-inflammatory and airway remodeling intervention. In a house dust mite (HDM) exposure mouse model, we investigated the impact of PARP inhibition on allergic airway inflammation, sensitization, and remodeling. Mice were intranasally exposed to a HDM extract for 5 days per week for up to 5 weeks. Mice were administered, or not, by PARP inhibitors 3-aminobenzamide (3-ABA) or 5-aminoisoquinolinone (5-AIQ) during the last 2 weeks of HDM treatment. Mice treated with PARP inhibitors after HDM stimulation showed a significant decrease in the number of total cells and eosinophils detectable in the bronchoalveolar lavage fluid as compared with the HDM-stimulated ones. In vitro HDM-stimulated splenocyte culture produced considerable amounts of the Th2 cytokines that were not affected by treatment with PARP inhibitors. Immunoglobulin levels in the serum were also unchanged. In the lung tissue, collagen deposition was decreased, whereas α -smooth muscle actin thickening was not significantly affected. Moreover, in HDM-stimulated PARP inhibitor-treated groups, we found a downregulation in the activation of signal transducer and activator of transcription-6 (STAT-6) and a significant decrease in the mRNA levels of C-

C motif chemokine 11 (CCL11). In this mouse model of chronic asthma PARP inhibition treatment, although it does not affect sensitization, it effectively reduces the allergic airway inflammation and affects the remodeling through a mechanism involving STAT6 and CCL11.

Keywords Model of chronic asthma · House dust mite · PARP inhibition · Airway inflammation · Sensitization · Remodeling

Introduction

Poly(ADP-ribosyl)ation is a process catalyzed by a family of ubiquitous enzymes named poly(ADP-ribose) polymerases (PARPs). Baseline PARP activity in normal cells is thought to be necessary for DNA repair and signaling of DNA damage, regulation of genomic stability in cells under stress conditions, and transcriptional regulation (D'Amours et al. 1999). PARPs are dramatically activated by an extended DNA damage leading to a rapid depletion of NAD⁺ that induces energetic failure of cells, ultimately resulting in cell suicide (Bürkle 2005; Yu et al. 2002). PARP activation can be triggered by various environmental stimuli and by free radicals produced during an inflammatory response (Cuzzocrea 2005). Moreover, PARP activation causes an increased expression of pro-inflammatory factors, such as tumor necrosis factor- α (TNF- α), RANTES, and C-C motif chemokine 11 (CCL11), supplying the inflammatory response (Jagtap and Szabo 2005).

Asthma disease is characterized by chronic airway inflammation and oxidative stress; it occurs in response to allergens, pollutants, infections, or drugs. Inflammatory cells in the airways release reactive oxygen species (ROS) causing airway epithelium injury by DNA damage (Holgate 2002; MacNee 2001).

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Whereas the activation of PARP-1 is for a long time considered a key event in the molecular processes leading from acute asthma attack to leukocyte recruitment, chronic inflammation, and airway remodeling (Boulares et al. 2003; Virag et al. 2004; Cuzzocrea et al. 2002), only recently, Mehrotra et al. (2013) demonstrated the important role of PARP-14 to facilitate the differentiation of T cells toward Th2 phenotype in allergic airway disease. Many separate studies investigated the inhibition/deletion of this enzyme in conventional models of acute allergic airway disease employing the surrogate allergen, ovalbumin (OVA). Collectively, they have shown that PARP-1 deficiency protects against inflammation when administered before the allergen challenge, decreasing cellular migration, reducing myeloperoxidase activity, tyrosine nitration, and TNF- α secretion in the bronchoalveolar lavage fluids (BALFs), delaying the onset of respiratory abnormalities, and preventing eosinophilic infiltration (Virag et al. 2004; Suzuki et al. 2004; Boulares et al. 2003; Oumouna et al. 2006). Also, in the case in which inhibitors were administered as a post-allergen challenge, their protective effects occur through the inhibition of eosinophil recruitment and mucus secretion (Naura et al. 2008). Recently, in a conventional model of asthma using guinea pigs, it was shown that PARP-1 inhibition reduces bronchial or alveolar abnormalities and decreases smooth muscle or goblet cell hyperplasia and collagen deposition affecting airway damage and remodeling (Lucarini et al. 2014). However, conventional model using OVA present limitation because this model of asthma does not lead to most of the structural changes associated with chronic asthma. Differently to OVA, house dust mite (HDM) extract belongs to environmental antigens, and it elicits a chronic inflammatory response characterized by systemic Th2 immunity and airway remodeling (Cates et al. 2007; Johnson et al. 2004).

The central aim of this study was to investigate the effect of PARP inhibition, using the inhibitors 3-aminobenzamide (3-ABA) and 5-aminoisoquinolinone (5-AIQ), on allergic airway inflammation, sensitization, and remodeling in an experimental mouse model of chronic asthma induced by respiratory exposure to a common environmental allergen, HDM.

Methods

Animals

Female Balb/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Montreal, Canada). The mice were housed in a specific pathogen-free environment under a 12-h light, 12-h dark cycle. All experiments described were

approved by the Animal Research Ethics Board of McMaster University.

Antigen and PARP inhibitor administration

Mice were exposed to purified HDM extract (Greer Laboratories, Lenoir, NC) intranasally (25 μ g of protein in 10 μ l of saline) for 5 days/week for up to five consecutive weeks.

For the inhibition of PARP-1, we used 3-ABA and 5-AIQ intraperitoneally at a concentration of 40 mg/kg the former and 0.5 mg/kg the latter, respectively, for the last two consecutive weeks. Mice were divided into six experimental groups:

- Group 1 ($n = 6$): naïve mice not treated
- Group 2 ($n = 6$): animals received intranasally 10 μ l of saline for five consecutive weeks and 3-ABA intraperitoneally for the last 2 weeks
- Group 3 ($n = 6$): animals received intranasally 10 μ l of saline for five consecutive weeks and 5-AIQ intraperitoneally for the last 2 weeks
- Group 4 ($n = 8$): animals received intranasally HDM extract dissolved in 10 μ l of saline for five consecutive weeks
- Group 5 ($n = 8$): animals received intranasally HDM extract dissolved in 10 μ l of saline for five consecutive weeks and 3-ABA intraperitoneally for the last 2 weeks
- Group 6 ($n = 8$): animals received intranasally HDM extract dissolved in 10 μ l of saline for five consecutive weeks and 5-AIQ intraperitoneally for the last 2 weeks

Groups 2 and 3 are referred as control groups not sensitized with HDM but only treated with PARP inhibitors and saline. All treatments were administered to isoflurane-anesthetized mice and ended 72 h before sacrifice.

Collection and measurement of specimens

Seventy-two hours after last exposure to HDM and PARP inhibitors, mice were killed; the lungs and BALF were collected and evaluated as previously described (Stampfli et al. 1998). Serum was obtained by centrifugation after incubating whole blood for 30 min at 37 °C.

Splenocyte culture

Spleens were harvested into tubes containing sterile Hanks' balanced salt solution (HBSS) (Invitrogen Life Technologies, Burlington, Canada) at 4 °C. Tissue was triturated into HBSS through nylon mesh (Becton Dickinson, Mississauga, Ontario, Canada) using the plunger from a 5-ml syringe. Red blood cells were lysed with ACK lysis buffer (0.5 M NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA (pH 7.2–

7.4). The remaining splenocytes were washed twice with HBSS and then resuspended in RPMI supplemented with 10 % v/v FBS (Life Technologies Inc.), 1 % v/v L-glutamine, 1 % v/v penicillin/streptomycin, and 0.1 % v/v β -mercaptoethanol.

Isolated splenocytes were cultured in medium alone or with 3 μ g HDM per well, at a concentration of 8×10^5 cells per well in a flat-bottom, 96-well plate (Becton Dickinson). After 120 h of culture, supernatants were harvested for cytokine measurements.

Quantification of cytokines

Measurement of in vitro cytokines were performed with ELISA kits for mouse interleukin (IL)-4, IL-5, IL-13, and interferon- γ (IFN- γ) that were purchased from R&D Systems (Minneapolis, MN). Thresholds of detection of these assays are <2, 1.5, 7, and 2 pg/ml, respectively.

Measurement of HDM-specific IgG1, IgG2a, and total IgE

Ig's measurement was performed as previously described (Cates et al. 2004). Briefly, 96 MaxiSorp plates (Nunc, VWR Canlab, Mississauga, Canada) were coated overnight at 4 °C with 100 μ l of a 4 μ g/ml solution of HDM in carbonate-bicarbonate buffer pH 9.6 (Sigma-Aldrich Canada, Oakville, Canada). Coated wells were blocked with 1 % bovine serum albumin (BSA) solution for 2.5 h at room temperature. ELISA was performed using four serial dilutions of each serum sample and biotinylated goat anti-mouse for IgG1 and IgG2a. Alkaline phosphatase-streptavidin (Sigma-Aldrich, Oakville, Canada) was used as a second-step reagent for detection of the biotinylated Abs. For total IgE measurement, 96 MaxiSorp plates were coated with 100 μ l of a 5 μ g/ml solution of LO-ME-3-purified capture antibody (Southern Biotech, Birmingham, AL, USA) in borate buffer pH 8.4 overnight. Coated wells were blocked with 1 % BSA solution for 2 h at room temperature, and ELISA was performed using purified mouse IgE (Pharmigen, Mississauga, Canada). Total IgE was detected using biotinylated rat-anti-mouse IgE (Southern Biotech, Birmingham, AL, USA) and alkaline phosphatase-streptavidin (Sigma-Aldrich, Oakville, Canada) as a second-step reagent.

Histology and immunohistochemistry for α -smooth muscle actin

The lungs were inflated with 10 % formalin at a standard pressure of 20 cm H₂O. Tissues were then paraffin embedded and cut at a thickness of 3 μ m, and sections were stained with hematoxylin and eosin (H&E) and Picro Sirius Red; we examined two sections from each sample for evidence of inflammation, injury, and number of mucus-containing cells using

periodic acid-Schiff (PAS) staining kit (Sigma Chemical Co., Milan Italy).

The presence of smooth muscle was detected with immunohistochemistry. Briefly, paraffin sections of the lungs were deparaffinized placed in freshly prepared methanol H₂O₂ solution to block endogenous peroxidase. Protein block was performed by incubating sections in 1 % v/v normal swine serum (NSS) for 15 min. Sections were incubated with α -smooth muscle actin primary antibody (1:150 in 1 % v/v NSS; Clone 1A4, Dako, Glostrup, Denmark) for 1 h; negative diluent control sections were run in parallel with the primary incubation. All sections were then incubated with biotinylated rabbit anti-mouse secondary antibody (1:300 in 1 % v/v NSS; Dako, Glostrup, Denmark) for 1 h at room temperature (RT), followed by streptavidin-peroxidase conjugate (1:600 in 1 % v/v NSS; Dako, Glostrup, Denmark) for 45 min at RT. Sections were placed in acetate buffer (pH 5.0) for 5 min, then incubated in freshly prepared chromogen substrate solution for 15 min. Tissues were counterstained in Mayer's hematoxylin for 1 min, washed, and mounted in glycerin gelatin. Washes using 0.05 M Tris-buffered saline were performed between steps.

Morphometry

Images for morphometric analysis were captured with OpenLab version 3.0.3 (Improvision, Guelph, ON, Canada), via a Leica camera and microscope attached to a Macintosh computer (Mac OS X operating system). Analysis of sections stained with α -smooth muscle actin (α -SMA) and Picro Sirius Red was performed on a custom computerized image analysis system (Northern Eclipse software, version 5 Empix Imaging, Mississauga, ON, Canada) as previously described (Johnson et al. 2004).

Isolation of mRNA from tissues and cDNA generation

Total RNA was isolated from frozen lung tissues by the cesium chloride method according to Chomczynski and Sacchi (modified method) as described previously (Chomczynski and Sacchi 1987). Briefly, 100 mg of the lung tissue was homogenized in 4 M guanidine thiocyanate/0.1 M sodium acetate pH 5.2/5 mM EDTA pH 8 (Sigma-Aldrich, Milan, Italy), put in ultracentrifuge tube with 2 ml of CsCl 5.7 M (Sigma-Aldrich, Milan, Italy) sterile solution. Pellets of total RNA were obtained by centrifugation for 17 h at 36,000 rpm at 20 °C and dissolved in 200 μ l of sterile water. Concentrations were determined spectrophotometrically at 260 nm. One microgram of total RNA was converted to complementary DNA (cDNA) with random primers with a first-strand cDNA synthesis kit (AMV; Roche Diagnostic, Mannheim, Germany).

Real-time quantitative PCR

Real-time quantitative RT-PCR analysis was performed on an ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA) with the SYBR Green I dye contained in the SYBR Green Master Mix (Applied Biosystems, Foster City, CA) as a probe. All PCR reactions contained 1× Master Mix, each specific primer at 200 nM and 5 ng of cDNA (total RNA equivalent) in a 25 µl total volume. Thermal cycling included an initial incubation at 95 °C for 10 min and then 45 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for extension. After each run, PCR amplification products were checked by melting curves and electrophoresis. Sequences of oligonucleotide primers for *CCL11* were forward 5'-CCCTCCACCATGCA GAGCT-3' and reverse 5'-TGGGATGGAGCCTGGGT-3'. All samples were amplified in triplicate, and the mean of the threshold cycle was used for further calculations. The probe signal was normalized to an internal reference, and the expression level of genes was calculated by relative quantification using *GAPDH* transcript level as endogenous reference. Expression data were analyzed following the comparative method as indicated in Applied Biosystems User Bulletin n.2.

Electrophoretic mobility shift assay (EMSA)

The lung samples were collected in liquid nitrogen and stored at −80 °C until use. Nuclear extracts were prepared according to Menegazzi et al. (2008), in the presence of 10 µg/ml leupeptin, 5 µg/ml antipain and pepstatin, and phenylmethylsulfonyl fluoride (PMSF) 1 mM (Sigma-Aldrich, Milan, Italy). The protein concentration in the nuclear extracts was determined using the method of Bradford (Bradford 1976). Ten micrograms of nuclear extract was incubated at room temperature for 20 min with $2\text{--}5 \times 10^4$ cpm of ^{32}P -labeled double-stranded oligonucleotide, containing the NF-κB binding site from IL-6 promoter (5'-GATCCAGAGGGGACTTTCCGAGT-3'), the STAT-1α binding site (sis-inducible factor-binding recognition element (SIE)/m67) from the c-fos promoter (5'-GTCCG ACATTTCCCGTAAATCG-3'), and the STAT-6 binding site (5'-GTATTTCCCGAGAAAAGGAAC-3') in 15 µl of binding reaction buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 10 % glycerol, 0.5 mM DTT, 0.1 mM EDTA, 2 µg poly(dIdC), 1 µg salmon sperm DNA). Products were fractionated on a nondenaturing 5 % polyacrylamide gel in Tris-borate-EDTA (TBE buffer, 0.5×). The intensity of the retarded bands was measured with a Phosphorimager (Molecular Dynamic, Milan, Italy). Oligonucleotides were purchased from Invitrogen, Milan, Italy.

Immunoistochemistry for poly(ADP-ribose) polymers

This assay was carried out on histological sections, 5 µm thick, of Mota-fixed, paraffin-embedded lung tissue

fragments. Sections were treated with 0.3 % (v/v) H_2O_2 in 60 % (v/v) methanol to quench endogenous peroxidase, permeabilized with 0.1 % (w/v) Triton X-100 in PBS for 20 min, and incubated overnight with goat polyclonal anti-poly(ADP-ribose) polymerase antibodies (1:500 in PBS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Immune reaction was revealed by indirect immunoperoxidase method (Vectastain Elite kit; Vector Laboratories, Burlingame, CA), using 3,3'-diaminobenzidine as chromogen. As negative controls, sections incubated with only the primary or the secondary antisera were used.

Data analysis

Data were analyzed with Sigma Stat version 2.03 (SPSS Inc., Chicago, IL) and are expressed as means ± SEM. Results were interpreted by analysis of variance followed by the Tukey post hoc test, when applicable. Differences were considered to be statistically significant when $p < 0.05$.

Results

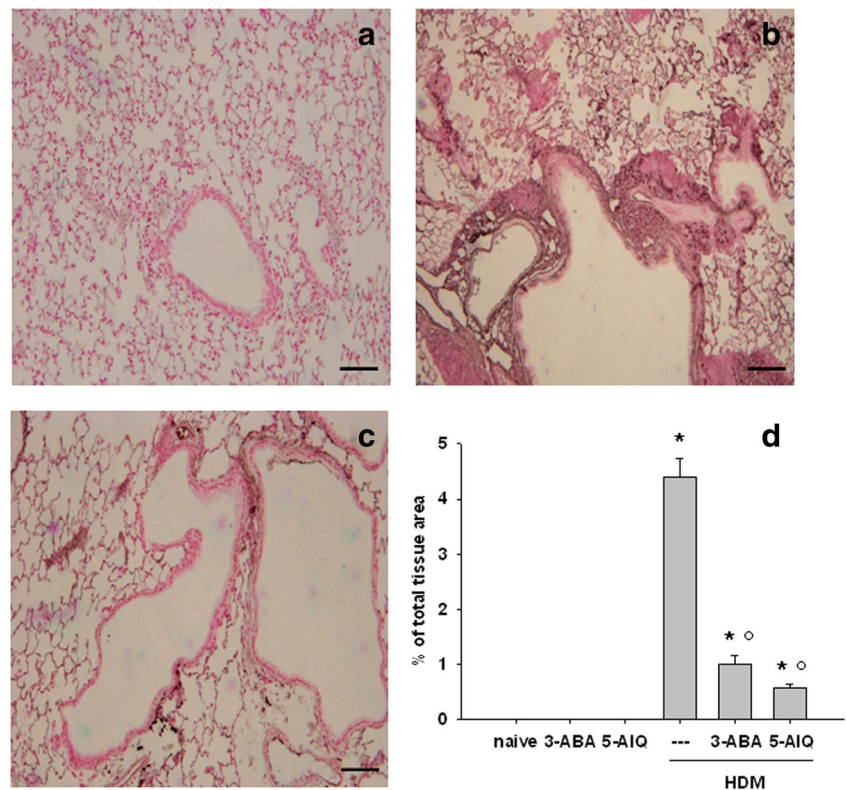
Immunohistochemical localization of poly(ADP-ribose)

Detection of PAR binding to target proteins, a marker for PARP activity, in the lung tissue cells showed no apparent staining in the naïve (Fig. 1a) and control groups (not shown). Immunostained cells increased markedly in HDM-exposed mice. In particular, positive staining for PAR was mostly located in the airway epithelium and at the inflammatory infiltrate within the stromal septa (Fig. 1b). In HDM-exposed mice treated with 3-ABA or 5-AIQ, immunostained cells decreased dramatically and only a few, scattered positive cells could be observed (Fig. 1c and data not shown). Morphometric analysis of lung immunohistochemistry of poly(ADP)-ribosylated proteins is reported in the histogram (Fig. 1d).

Effect of PARP inhibition treatment in the BALF

Mice exposed to HDM for up to 5 weeks mounted a robust inflammatory response that was clearly evident in the BALF (Fig. 2). At this time, we observed an increase in total cells from $\sim 1 \times 10^6$ to $\sim 5.5 \times 10^6$ in naïve mice (Fig. 1a). The cellular composition consisted of 72 % mononuclear cells, 25 % eosinophils (Fig. 2b, c), and 3 % neutrophils (data not shown). To investigate the effects of PARP-1 inhibition on established inflammation, mice were treated daily during the last 2 weeks of HDM exposure with the PARP-1 inhibitors 3-ABA or 5-AIQ. After the treatment, we observed a significant decrease in the overall extent of airway inflammation (45 % decrease in total cell number, Fig. 2a). This decrease was characterized by a conspicuous reduction of eosinophils (70 % with 3-ABA and

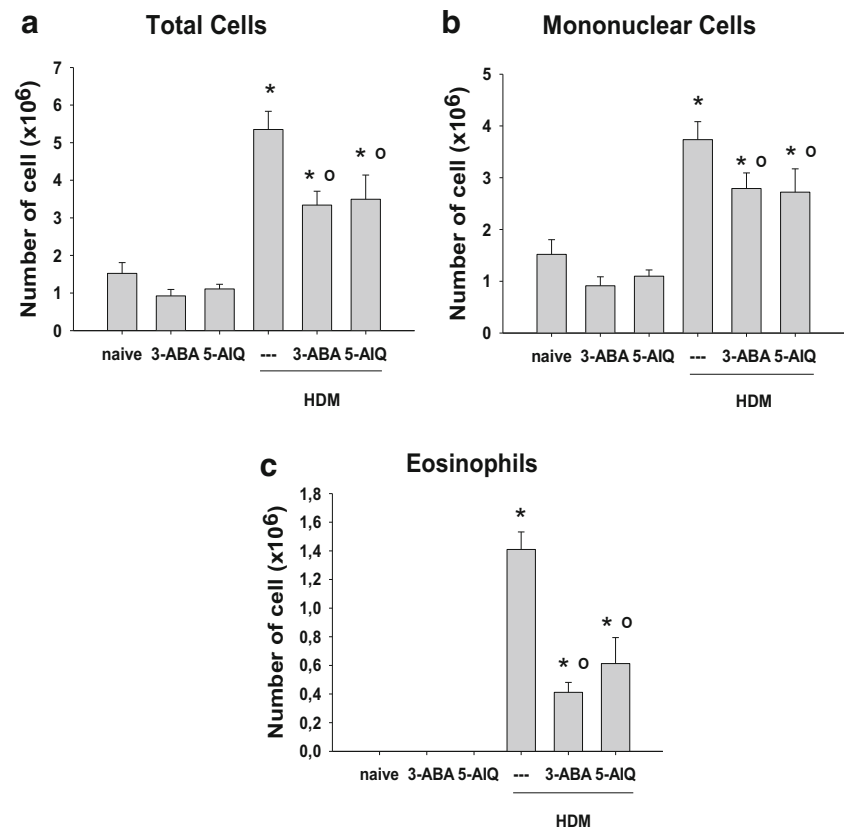
Fig. 1 Immunohistochemical detection of poly-ADP ribose (PAR) in the lung proteins. **a** Naïve mice. **b** HDM-exposed mice for 5 days/week for up to five consecutive weeks. **c** HDM-exposed mice plus 5-AIQ treatment in the last 2 weeks for 7 days/week. Poly-ADP ribose immunoreactivity is intense in the sensitized, HDM-challenged mice, especially in the bronchiolar and alveolar epithelium. On the contrary, immunostaining was dramatically decreased in the group treated with PARP-1 inhibitors (**c** shows group treated with 5-AIQ). **d** Morphometric analysis of lung immunohistochemistry of poly(ADP)-ribosylated proteins. Data are expressed as a percentage of the area of interest stained with hematoxylin. Data represent means $\pm$ SEM ($n = 6-8$); $*p < 0.05$ compared with naïve mice and $^{\circ}p < 0.05$ compared with HDM-exposed mice



60 % with 5-AIQ, Fig. 2c), a lower but significant decrease of mononuclear cells (Fig. 2b), and a negligible lowering of

neutrophils (data not shown). Administration of 3-ABA or 5-AIQ in naïve mice did not elicit any noticeable effect (Fig. 2).

Fig. 2 Airway inflammation in the BALF fluid of Balb/c mice exposed to HDM or HDM and PARP-1 inhibitors. Total cells (**a**), mononuclear cells (**b**), and eosinophils (**c**) in the BALF: diminution of inflammation after treatment with 3-ABA and 5-AIQ. Mice were administered with antigen intranasal, 5 days/week, for five consecutive weeks. The last 2 weeks, PARP inhibitors were administered i.p. daily. Data represent means $\pm$ SEM ($n = 6-8$); $*p < 0.001$ compared with naïve animals and $^{\circ}p < 0.001$ compared with HDM-treated animals. Statistical analysis was performed by one-way ANOVA with the Fisher post hoc test



Histological analysis of the lung

Histological evaluation of the lung tissue from mice subjected to continuous HDM exposure corroborated the findings detected in the BALF. The lungs of HDM-exposed mice displayed extensive peribronchial and perivascular inflammation (Fig. 3b, e) as well as goblet cell hyperplasia (Fig. 3h) compared with naïve (Fig. 3a, d, g) and control mice (3-ABA or 5-AIQ alone). Histological analysis of control mice did not show any inflammatory infiltration or abnormality (data not shown). After treatment with 3-ABA and 5-AIQ, we observed

a considerable decrease of cellular infiltration (Fig. 3c, f) and goblet cell hyperplasia (Fig. 3i). Higher magnification revealed extensive eosinophilia among the infiltrate after 5 weeks of HDM exposure (Fig. 3e) that decreased in mice treated with 3-ABA (data not shown) or 5-AIQ (Fig. 3f).

Impact of PARP inhibition on structural changes in the airway

After 5 weeks of HDM exposure, lung histology demonstrated marked morphological changes: We observed significant

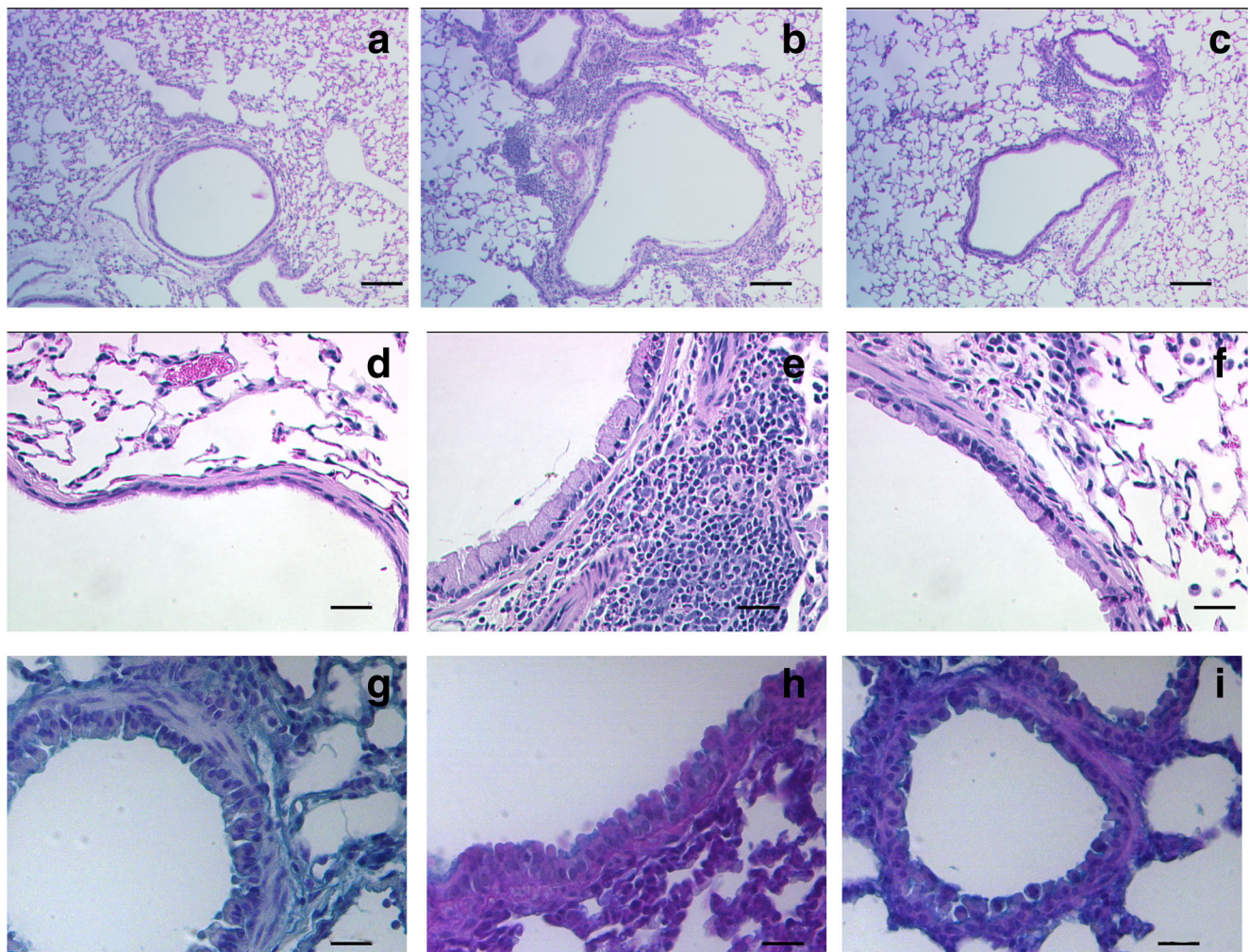


Fig. 3 Histological analysis of lung inflammation in mice exposed to HDM alone or HDM and PARP-1 inhibitors. Naïve mice were not manipulated (**a**, **d**). Low-magnification analysis of the lung tissue stained with H&E shows a robust infiltration in the perivascular and peribronchial area in the mice exposed to HDM for 5 weeks (**b**). After treatment with 3-ABA for the last 2 weeks, we obtained a considerable decrease of the inflammatory infiltration (**c**). Higher magnification allows to see the particular of the inflammatory infiltration in the lung parenchyma and shows the eosinophilic component in the group of mice treated only with HDM (**e**) and the partly resolution of the infiltration in the group treated with HDM and 5-AIQ (**f**). HDM-exposed animals were

administered for five consecutive weeks, 5 days/week, whereas HDM-PARP-1 inhibitor-exposed groups were administered for five consecutive weeks, 5 days/week with HDM, and the last 2 weeks, they were treated with 3-ABA or 5-AIQ. Control groups were treated only with 3-ABA or 5-AIQ for two consecutive weeks and show a histology similar to naïve mice group (data not shown). Scale bars 40 μ m (**a**, **b**, **c**), 10 μ m (**d**, **e**, **f**). Moreover, paraffin sections of the lung were stained with periodic acid-Schiff (PAS) to detect the presence of goblet cells. HDM treatment for 5 weeks induced PAS-positive purple staining (**h**). On the contrary, 5-AIQ treatment reduced PAS-positive purple staining (**i**). No positive staining was observed in naïve group (**g**). Scale bar 20 μ m (**g**, **h**, **i**)

increases of subepithelial collagen deposition (Fig. 4a (B)) and peribronchial contractile elements (Fig. 4a (E)) compared to naïve mice (Fig. 4a (A and D), respectively). The increases

of contractile elements and collagen deposition were confirmed by morphometric analysis (Fig. 4b). Interestingly, mice treated with 5-AIQ during the last 2 weeks of HDM exposure

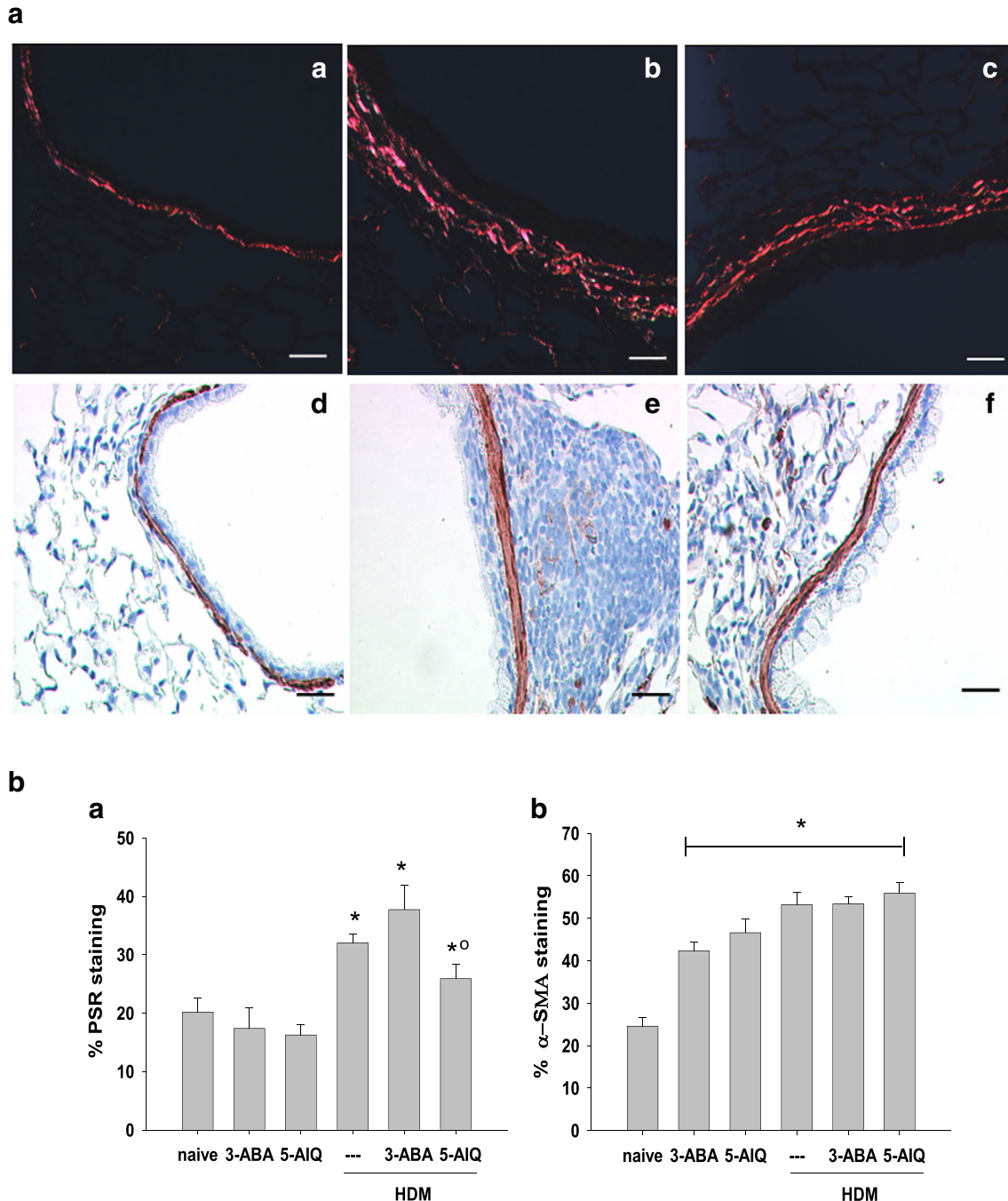


Fig. 4 **a** Histological analysis of lung structure in mice after treatments. Picro Sirius Red staining visualized under polarized light showing subepithelial accumulation of collagen in HDM-exposed mice (B) and in HDM-5-AIQ-exposed animals (C) compared to naïve mice (A). Immunohistochemistry for anti- α smooth muscle actin indicates increased contractile elements in the airway wall in mice exposed to HDM alone (E) and in mice exposed to HDM-3-ABA (F) compared to naïve mice (D). Naïve animals were not manipulated; HDM-exposed mice were administered with HDM 5 days/week for five consecutive weeks. HDM-PARP inhibitor animals were administered with HDM

5 days/week for five consecutive weeks, and the last 2 weeks were administered with PARP inhibitors 7 days/week. Scale bars 10 μ m. **b** Morphometric analysis of lung histology. Structural changes were assessed in naïve mice, in control groups of animals (treated only with 3-ABA or 5-AIQ), in HDM-exposed mice only 5 days/week for five consecutive weeks, and in animals HDM-exposed plus 3-ABA or 5-AIQ 5 days/week in the last 2 weeks. Data are expressed as a percentage of the area of interest stained with Picro Sirius Red. Data are means $\pm$ SEM ($n = 6-8$); * $p < 0.05$ compared with naïve mice and $^{\circ}p < 0.05$ compared with HDM-exposed mice

showed a modest but significant reduction in collagen deposition (Fig. 4a (C), b) with no effect on α -smooth muscle actin (Fig. 4b). On the other hand, HDM-exposed 3-ABA-treated mice showed no changes in collagen deposition and α -smooth muscle actin (Fig. 4a (F), b).

Impact of PARP inhibition on transcription factor activation (NF-kB, STAT-1, STAT-6)

To study the effect of PARP-1 inhibition on the DNA binding activity of transcription factors nuclear factor-kB (NF-kB), signal transducer and activator of transcription-1 (STAT-1), and signal transducer and activator of transcription-6 (STAT-6), we performed an electrophoretic mobility shift assay. As shown in Fig. 5a, b, c, HDM exposure increases the baseline DNA binding activity of STAT-1, STAT-6, and NF-kB in the lung tissue. Conversely, HDM-challenged mice treated with 3-ABA or 5-AIQ showed a marked decrease in STAT-6 DNA binding activity (Fig. 5d) compared to HDM-exposed mice. In addition, a negligible decrease in NF-kB and STAT-1 DNA binding activity was observed following treatment with either the PARP inhibitors (Fig. 5a, b and data not shown).

Effect on expression of CCL11

Real-time PCR (Syber Green) was performed to examine the expression of CCL11 in the lung tissue, a small chemokine that is a chemoattractant for eosinophils. We observed an up-regulation in the relative messenger RNA (mRNA) levels of CCL11 in the lungs of mice exposed to HDM (Fig. 6). Following the treatment with PARP inhibitors, we observed a significant decrease in the expression of CCL11 gene (Fig. 6).

Impact of PARP inhibition on in vitro cytokine recall responses

Splenocytes isolated from HDM-exposed mice and stimulated in vitro with HDM produced considerable levels of the Th2-associated cytokines IL-5, IL-4, and IL-13 (Fig. 7) and of some Th1-associated IFN- γ compared to control groups. Treatment with 3-ABA or 5-AIQ during the last 2 weeks of HDM exposure caused no reduction of splenocyte Th2-associated cytokine ex vivo production (Fig. 7a, b, c). On the contrary, treatment with 5-AIQ increased the levels of all

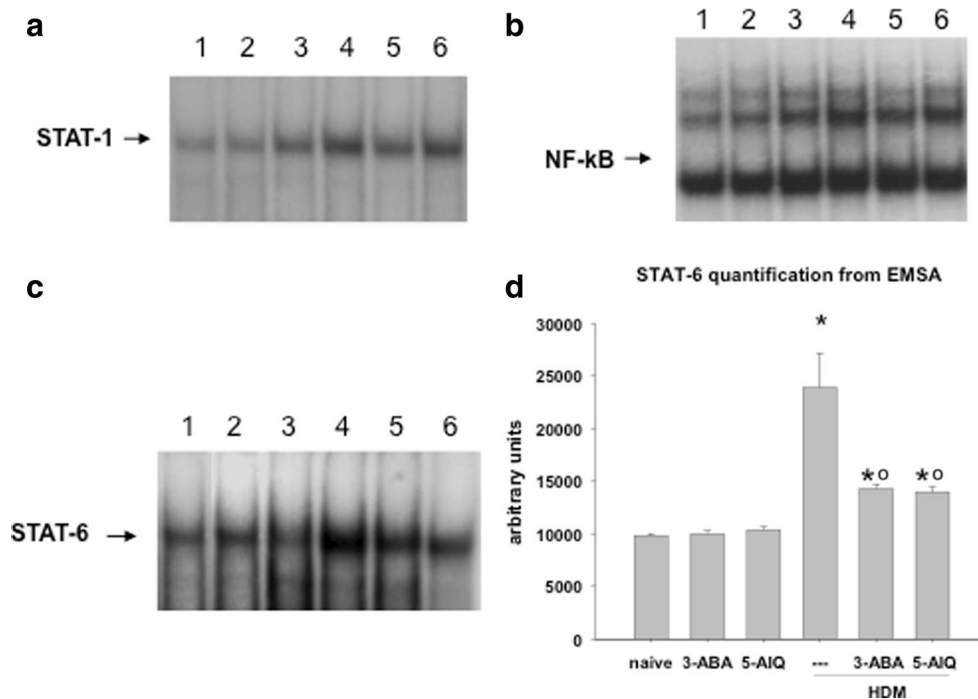


Fig. 5 Effect of HDM exposure and PARP-1 inhibition in mice on STAT-1, NF-kB, and STAT-6 activation. **a, b, c** Naïve (lane 1), control mice treated with 3-ABA 7 days/week for two consecutive weeks (lane 2), control mice treated with 5-AIQ 7 days/week for two consecutive weeks (lane 3), HDM-exposed mice 5 days/week for five consecutive weeks (lane 4), HDM-exposed mice 5 days/week for five consecutive weeks plus 3-ABA 7 days/week for the last two consecutive weeks (lane 5), HDM-exposed mice 5 days/week for five consecutive weeks plus 5-AIQ 7 days/week for the last two consecutive weeks (lane 6). Gel shift

experiments using a radiolabeled oligonucleotide containing a consensus STAT-1 α (**a**) or NF-kB (**b**) or STAT-6 (**c**) binding site and nuclear extract from the lung tissue of mice. The positions of DNA protein complexes are indicated. The gels are representative of eight gels, for each different transcription factor, showing similar results. **d** Data expressed as arbitrary units of the area of DNA protein complexes and representing means $\pm$ SEM ($n = 8$); * $p < 0.05$ compared with naïve animals and ° $p < 0.05$ compared with HDM-treated animals

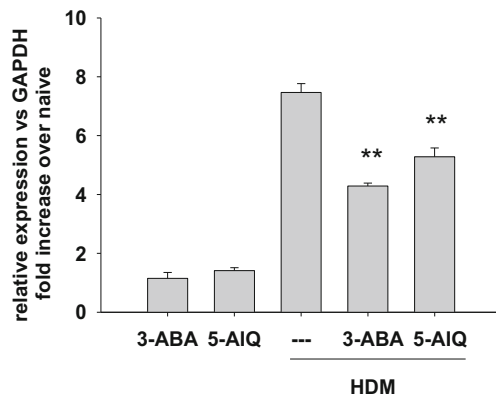


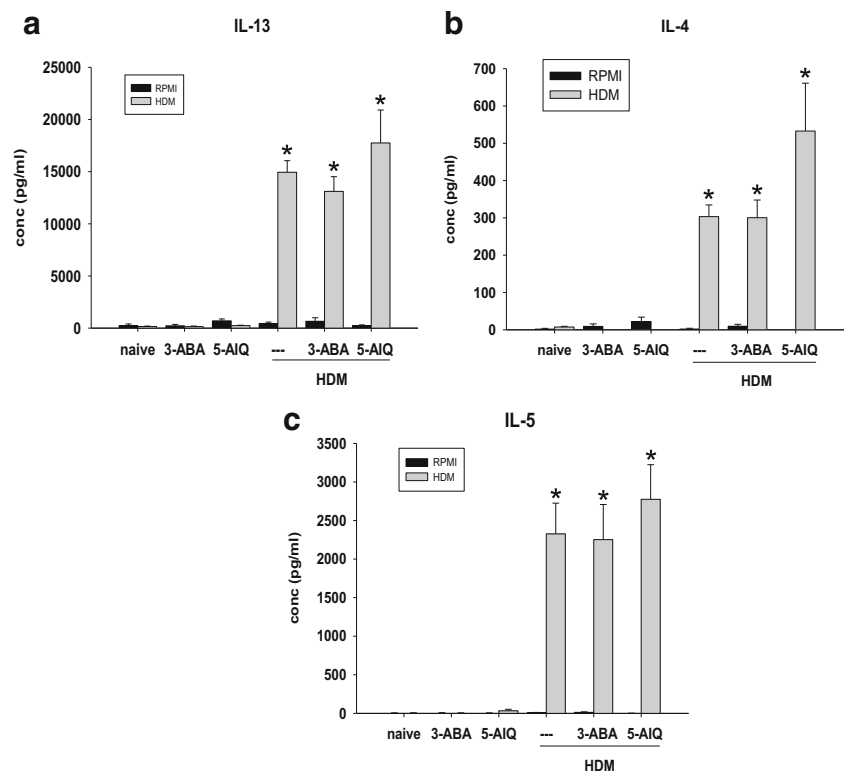
Fig. 6 Effect of HDM exposure and PARP-1 inhibitor treatment on CCL11 expression. Mice were intranasally exposed for five consecutive weeks 5 days/week with HDM and in the last 2 weeks were treated daily with 3-ABA and 5-AIQ. RNA was isolated from the lung tissue after its removal from thoracic site, and CCL11 relative expression was measured by SYBER Green ($n = 8$; statistical analysis was performed on triplicate measurements, $**p < 0.01$ compared with HDM-exposed mice)

cytokine production. Moreover, we did not observe any significant differences in IFN- γ production (457 ± 72 , 369 ± 46 , and 345 ± 67 pg/ml for HDM, HDM + 3-ABA, and HDM + 5-AIQ groups, respectively).

Impact of PARP inhibition on serum immunoglobulin levels

Serum levels of total IgE were significantly increased in mice exposed to HDM for 5 weeks if compared with naïve animals

Fig. 7 Effects of HDM exposure and PARP inhibition in in vitro splenocyte cytokine production. Splenocytes were isolated after 72 h after the last treatment and were cultured for 120 h in medium alone (black bars) or medium plus HDM (gray bars). In vitro expression of Th2-associated cytokines interleukin (IL)-13 (a), IL-4 (b), and IL-5 (c) was elevated in splenocytes of sensitized animals continuously exposed to HDM, and these levels did not change after treatment with 3-ABA and 5-AIQ. Data represent means $\pm$ SEM ($n = 6-8$); $*p < 0.05$ compared with naïve and control animals but not with HDM group



(Table 1). In addition, serum levels of HDM-specific IgG1 and IgG2a were also increased. Treatment with either 3-ABA or 5-AIQ appeared to augment total IgE and HDM-specific IgG1 levels, although these increases were not statistically significant in comparison with the ones of HDM-exposed mice (Table 1).

Discussion

Asthma is the most common airway disease characterized by recurrent exacerbations and the environmental stimuli, such as aeroallergen, virus, and occupational exposure, cause its inception and evolution (Holgate 2002). More than 300 million persons are affected by asthma all over the world, with approximately 250,000 annual deaths, and the number of the asthmatic patients is expected to increase by more than 100 million by 2025 (Bousquet et al. 2007; Masoli et al. 2004).

Characteristic feature of the disease is a persistent airway inflammation that includes infiltration in the airway of inflammatory cells and is accompanied with hypertrophy of the smooth muscle, angiogenesis, thickening of the lamina reticularis, and, often, disruption of the epithelium. Loss of epithelial cells leads to goblet cell hyperplasia and hypertrophy that contribute, with the concurrent hypertrophy of smooth muscle, to the narrowing of airway caliber. All these characteristics are known as airway remodeling that contribute to clinical symptoms (Singh and Busse 2006; Fahy 2001).

Table 1 Effects of continuous HDM exposure and PARP inhibitor treatment on serum immunoglobulin production

	IgE (ng/ml)	HDM-IgG1 (U/ml)	HDM-IgG2a (U/ml)
Naïve	59.7 ± 13.3	0.00	0.00
3-ABA	65.9 ± 18.9	0.00	0.00
5-AIQ	29.7 ± 8.3	0.00	0.00
HDM	630.9 ± 123.3	92,225.0 ± 30,826.0	1097.5 ± 357.4
HDM + 3-ABA	1214.8 ± 349.9	155,750.0 ± 11,168.3	2255.2 ± 517.7
HDM + 5-AIQ	946.3 ± 251.3	151,150.0 ± 14,236.8	1099.5 ± 270.8

In HDM-exposed animals, the levels of serum immunoglobulins were significantly increased ($p < 0.05$) compared with naïve and control animals. Treatment with 3-ABA or 5-AIQ does not affect the Ig's levels. Mice were sacrificed 72 h after the last treatment, and Ig content in the serum was measured by ELISA. Data represent means ± SEM ($n = 6-8$)

At present, treatment with corticosteroid and long-acting β_2 agonists in combination is the most effective therapy, and it is very difficult to develop new classes of therapeutic agents. Although the existing drugs are effective and safe for controlling symptoms, a significant number of patients still show poor responses to available therapies because of the severity of the pathology (Barnes 2004; Robinson et al. 2012).

The difficult development of new asthma therapy is almost due to the absence of natural disease like asthma in the animal kingdom, so that the experimental animal models of asthma used are often inadequate to simulate human asthma (Kurucz and Szeleny 2006). Conventional protocols that utilize OVA as allergen are useful to study the molecular mechanisms of the acute inflammation, whereas they seem inadequate to establish chronic airway eosinophilic inflammation, that is a characteristic feature of asthma and airway remodeling. In the end, the biochemical and presumably immunogenic differences between OVA and HDM may impact not only the outcome of allergen exposure but also the mechanisms through which these outcomes are brought about (Cates et al. 2007; Fattouh et al. 2011).

In this study, we used a nonconventional murine model of asthma utilizing HDM as allergen that allows to obtain a Th2 immune response accompanied with a robust inflammation and remodeling (Johnson et al. 2004). Indeed, it is well known that 80 % of asthmatic people are sensitized to environmental allergens leading to eosinophilic inflammation (Singh and Busse 2006). House dust mites are the predominant aeroallergens; in this study, we used an extract of *Dermatophagoides pteronyssinus* that is the predominant mite species in Europe (Zock et al. 2006) and two different PARP inhibitors: 3-ABA and 5-AIQ. These early PARP inhibitors have been chosen on the bases of the different PARP selectivity and potency. In fact, small, basic PARP inhibitors that target only the nicotinamide site (such as 3-ABA) are very unselective across PARP enzymes. Conversely, 5-AIQ has been designed to bind the nicotinamide site and adjacent sites to gain potency and selectivity for PARP-1/2 (Steffen et al. 2013).

In a preliminary experiment, we have administered these PARP inhibitors in mice previously HDM exposed,

intranasally (Cates et al. 2004), following a 10-day protocol and obtaining a decrease of cell number in the BALF, especially of eosinophils (80 % less than HDM-treated only group; data not shown). These results encouraged us to investigate the potentiality of this class of drugs on this asthma model. The experiments described here show the effects of 3-ABA and 5-AIQ in the airway compartment and in the periphery (spleen and serum), taking into account the Th1 and Th2 components of the disease.

We intranasally treated animals for the first 3 weeks with the HDM allergen leading to a robust eosinophilic inflammation and airway remodeling (Johnson et al. 2004). Thus, we administered mice for the last 2 weeks of the experiments with 3-ABA and 5-AIQ to test their therapeutic potential in this asthma model framework. PARP inhibitor doses were chosen on the basis of dose-response curves (data not shown), and we checked by immunohistochemistry the PAR polymers, the final product of this enzyme (Bürkle 2005), in the lung tissue, in order to verify the occurred inhibition of PARPs. As shown in Fig. 1, we obtained a decrease of 80 % with 3-ABA and 90 % with 5-AIQ of PAR polymers, confirming that PARP inhibition occurred.

Continuous exposure of animals to HDM leads to a remarkable airway inflammation with an eosinophilic component that was quantified in the BALF and histopathologically pointed out (Figs. 2 and 3). After treatment with 3-ABA and 5-AIQ, we observed a decrease in the number of cells in the BALF, but the greater decrease was in the eosinophilic fraction. This result is in line with the previous finding of Oumouna et al. (2006) that shows a role of PARPs in the eosinophil recruitment process during allergen-induced lung inflammation.

It is well known that asthma elicits an increasing expression of cytokines and inflammatory proteins in the respiratory tract, and most of them are regulated at the transcription level. Transcription factors are proteins that regulate gene expression through DNA binding and play a key role in the etiopathogenesis of asthma. In order to verify the molecular mechanism leading to the prevalent eosinophilic inhibition in the BALF, we checked the activation of the main transcription

factors in asthma: STAT-1, NF- κ B, and STAT-6. One of the prominent transcription factors found activated in asthma is NF- κ B (Barnes 2006). On the one hand, it is well known that asthma is characterized from a predominant Th2 immune response and cytokines, enzymes, and inflammatory proteins are STAT-6 dependent. On the other hand, it was also found a constitutive activation of STAT-1 that is a typical feature of a Th1 immune response. These evidences support the hypothesis that a counter-regulation between Th1 and Th2 immune responses in asthma exists, when the former is found to be activated in this disease, but the latter remains the main component (Sampath et al. 1999; Ngoc et al. 2005; Fulkerson et al. 2004). As shown in Fig. 5, HDM treatment induced STAT-1, NF- κ B, and STAT-6 DNA binding activity in the lung tissue; however, only STAT-6 DNA binding was reduced by 3-ABA and 5-AIQ administration. We obtained a reduction of almost 50 % STAT-6 DNA binding activity, whereas activation of STAT-1 and NF- κ B DNA binding remained unchanged. Besides its role in Th2 cell development, STAT-6 mediates a multitude of actions that are relevant in the pathogenesis of asthma: For instance, a large part of chemokine expression, playing a pivotal role in the infiltration of Th2 cells and eosinophils, are STAT-6 dependent (Hebenstreit et al. 2006). CCL11 is a small chemokine produced in the lungs of asthmatic patients and is a potent chemoattractant for eosinophils (Pease and Williams 2001). This chemokine is regulated in a STAT-6-dependent manner (Fulkerson et al. 2004; Hoeck and Woisetschlager 2001), and as a probable consequence of STAT-6 DNA-binding downregulation, we found that its mRNA expression is inhibited. Our data are consistent with the results of Kuperman et al. (1998) showing a lack of eosinophil influx into the lung in STAT6-deficient mice affected by lung allergic inflammation. The authors attribute this effect, at least partially, to defective CCL11 production in these animals.

As shown in Fig. 6, we obtained a 50 % decrease of CCL11 gene expression after 3-ABA treatment and 40 % after 5-AIQ treatment. On the other hand, eosinophils have a central role in the etiopathogenesis of asthma, and they are a peculiar characteristic of this disease. In this respect, several studies have addressed to eosinophils a critical role in displaying some asthma features, such as mucus accumulation and airway remodeling (Kay 2005). The previous results suggested us to investigate the impact of this eosinophil reduction on the HDM-induced airway remodeling. After 5 weeks of HDM exposure, we observed goblet cell hyperplasia, collagen deposition in the subepithelial layer, and increased expansion of peribronchial contractile elements (Figs. 3 and 4). These airway structure changes were evident through histology and immunohistochemistry and were quantified by morphometric analysis. As shown in Fig. 4a, b, after 5-AIQ treatment, we obtained a partial decrease in the subepithelial collagen deposition, but peribronchial changes were not affected after 2-

week PARP inhibitor treatment. It is worth noting that even if we obtain a decrease of 60–70 % eosinophils in the BALF, this decrease is not sufficient to affect airway remodeling.

As eosinophil accumulation and the resulting airway remodeling are the terminal events of Th2 response in asthma, an interaction between antigen-presenting cells and T cells is representative of the initiation of the process named sensitization (Hammad and Lambrecht 2006). Considering that allergic sensitization is a systemic process, we investigated two different markers of systemic Th2 immune activity: in vitro splenocyte expression of Th2 cytokines and serum levels of total IgE and HDM-specific IgG1. Our data show elevated production of Th2-affiliated cytokines such as IL-13, IL-4, and IL-5 in the group treated with HDM for 5 weeks, but these productions do not change after 3-ABA or 5-AIQ treatments (Fig. 7). Elevated serum levels of Th2-affiliated immunoglobulins, total IgE and HDM-specific IgG1, provide evidence of a humoral response in HDM-exposed mice. Then, after exposure to 3-ABA and 5-AIQ, we did not detect any significant decrease in the immunoglobulin production as well (Table 1). The lack of effects of PARP inhibitors on the allergenic-induced systemic response could be not surprising as it is well known that different immunologic processes mediate different facets of asthma, and various types of inflammatory reactions differentially contribute to the multiple features of the disease in a coordinated effort. During an immune response, the ability to recruit and activate leukocytes is dependent upon the local production of a set of chemokines able to influence the outcome of the disease by altering the profile of cytokines locally produced and by activating multiple effector cell populations (Lukacs et al. 1999). In our experiments, we obtained the suppression of eosinophil recruitment in the airway compartment by the administration of PARP inhibitors. This effect is probably due to the inhibition of STAT6 activation/CCL11 expression. This, in turn, allows to locally improve the lung function without affecting the degree of systemic response (Fig. 7 and Table 1). Nevertheless, the efficacy of these drugs remains confident because it is well known that the major problems of the asthmatic pathology concern the lung tissue.

Conclusions

We can conclude that PARP inhibitors used in this mouse model of HDM-induced airway disease are partly effective on airway remodeling with a decrease of the subepithelial collagen deposition. This effect is specific for 5-AIQ, the most potent and selective inhibitor used in this study.

PARP inhibition treatments did not affect sensitization when this process is already in progress but significantly reduced allergic airway inflammation with a decrease of inflammatory cell migration. Both PARP inhibitors used have

demonstrated this ability, but 3-ABA is more effective on eosinophil recruitment reduction. These findings also confirm the different interactions and action profile of the two different PARP inhibitors used: In fact, the unselective 3-ABA appears more effective than 5-AIQ against airway inflammation, although the latter is effective on collagen deposition. This study shows that this effect may be selective, mainly on the migration of eosinophils, in this model of asthma. This conclusion is in contrast with a previous report (Virag et al. 2004) but is in line with another study (Oumouna et al. 2006), anyway both utilizing OVA-based model of airway disease. In this model, reduced eosinophil recruitment is probably carried out through a mechanism that involves, at least in part, a STAT-6 DNA binding activity inhibition and a consecutive decrease of CCL11 mRNA levels. In conclusion, these results are in line with the suggestion that this class of therapeutic agents can have a potential role in the treatment of severe asthma, especially in a multimechanistic approach.

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This paper is dedicated to the memory of Hisanori Suzuki, who passed away on 19 March 2012 due to a rapidly overwhelming disease. We all remember our friend as a master in life and science, and we hope that he will continue to help us from the place where he is now.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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