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This information is current as of March 11, 2012

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J Immunol; Prepublished online 5 March 2012;

doi:10.4049/jimmunol.1004227

<http://www.jimmunol.org/content/early/2012/03/05/jimmunol.1004227>

Supplementary Data

<http://www.jimmunol.org/content/suppl/2012/03/05/jimmunol.1004227.DC1.html>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



A Dissociated Glucocorticoid Receptor Modulator Reduces Airway Hyperresponsiveness and Inflammation in a Mouse Model of Asthma

Laurent L. Reber,^{*,1} François Daubeuf,^{*,2} Maud Plantinga,^{†,‡,2} Lode De Cauwer,^{§,¶} Sarah Gerlo,^{§,¶} Wim Waelput,^{†,||} Serge Van Calenbergh,[#] Jan Tavernier,^{§,¶} Guy Haegeman,^{**} Bart N. Lambrecht,^{†,‡,††} Nelly Frossard,^{*,3} and Karolien De Bosscher^{§,¶,3}

The glucocorticoid receptor (GR) is a transcription factor able to support either target gene activation via direct binding to DNA or gene repression via interfering with the activity of various proinflammatory transcription factors. An improved therapeutic profile for combating chronic inflammatory diseases has been reported through selectively modulating the GR by only triggering its transrepression function. We have studied in this paper the activity of Compound A (CpdA), a dissociated GR modulator favoring GR monomer formation, in a predominantly Th2-driven asthma model. CpdA acted similarly to the glucocorticoid dexamethasone (DEX) in counteracting OVA-induced airway hyperresponsiveness, recruitment of eosinophils, dendritic cells, neutrophils, B and T cells, and macrophages in bronchoalveolar lavage fluid, lung Th2, Tc2, Th17, Tc17, and mast cell infiltration, collagen deposition, and goblet cell metaplasia. Both CpdA and DEX inhibited Th2 cytokine production in bronchoalveolar lavage as well as nuclear translocation of NF- κ B and its subsequent recruitment onto the I κ B α promoter in the lung. By contrast, DEX but not CpdA induces expression of the GR-dependent model gene MAPK phosphatase 1 in the lung, confirming the dissociative action of CpdA. Mechanistically, we demonstrate that CpdA inhibited IL-4-induced STAT6 translocation and that GR is essential for CpdA to mediate chemokine repression. In conclusion, we clearly show in this study the anti-inflammatory effect of CpdA in a Th2-driven asthma model in the absence of transactivation, suggesting a potential therapeutic benefit of this strategy. *The Journal of Immunology*, 2012, 188: 000–000.

Glucocorticoids (GCs) exert most of their biological functions through activation of the glucocorticoid receptor (GR), a member of the nuclear receptor family. Upon ligand binding, the GR dimerizes and translocates into the nucleus, where it can both directly and indirectly regulate gene transcription. Once in the nucleus, the activated GR can act as a transcription factor and occupy specific genomic GC response elements (GREs) and as such transactivate nearby genes (1). Alternatively, the activated GR can also transrepress the activity of other transcription factors, as in particular reported for NF- κ B (2–4, reviewed in Ref. 5). The anti-inflammatory effects of steroids are believed to involve transrepression and the side effects to be predominantly linked to transactivation (6).

Compound A (CpdA), or 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, is an analog of the hydroxyphenylaziridine precursor found in the Namibian shrub *Salsola tuberculatifomis* Botschantzev (7). CpdA interferes with NF- κ B–driven expression of proinflammatory cytokine genes in a GR-dependent manner (8, 9). However, although CpdA is capable of activating GR, the chemical structure of CpdA has no relationship with that of a GC, like dexamethasone (DEX). The gene-repressive activity results from CpdA favoring GR monomer formation over dimer formation and involves the second zinc finger of the DNA-binding domain as well as the ligand-binding domain of GR (8). CpdA-activated GR does, however, not stimulate GRE-driven transactivation in various cell lines and tissues. As a consequence, treatment of animals with

*Laboratoire d'Innovation Thérapeutique, Unité Mixte de Recherche 7200, Centre National de la Recherche Scientifique-Université de Strasbourg, Faculté de Pharmacie, F-67400 Illkirch, France; [†]Laboratory of Immunoregulation, Department of Respiratory Medicine, University Hospital of Ghent, Ghent B-9000, Belgium; [‡]Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, B-9052 Ghent, Belgium; [§]Department of Medical Protein Research, Flanders Interuniversity Institute for Biotechnology, B-9000 Ghent, Belgium; [¶]Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, B-9000 Ghent, Belgium; ^{||}Department of Pathology, Brussels University Hospital, B-1090 Brussels, Belgium; [#]Laboratory for Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Ghent University, B-9000 Ghent, Belgium; ^{**}Laboratory for Eukaryotic Gene Expression and Signal Transduction, Department of Physiology, Ghent University, B-9000 Ghent, Belgium; and ^{††}Department of Pulmonary Medicine, Erasmus University Medical Center Rotterdam, 3050GE Rotterdam, The Netherlands

¹Current address: Department of Pathology, Stanford University School of Medicine, Stanford, CA.

²F.D. and M.P. contributed equally to this work.

³N.F. and K.D.B. contributed equally to this work.

Received for publication December 30, 2010. Accepted for publication January 25, 2012.

S.G. and K.D.B. are postdoctoral fellows at Fonds voor Wetenschappelijk Onderzoek-Vlaanderen, Brussels, Belgium. S.G., J.T., and B.N.L. are supported by a Multidisciplinary Research Partnerships grant from Ghent University (Group-ID consortium).

Address correspondence and reprint requests to Dr. Nelly Frossard, Unité Mixte de Recherche 7200, Centre National de la Recherche Scientifique/Université de Strasbourg, Faculté de Pharmacie, 74 Route du Rhin, 67400 Illkirch, France. E-mail address: nelly.frossard@unistra.fr

The online version of this article contains supplemental material.

Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; C_{dyn}, dynamic compliance; ChIP, chromatin immunoprecipitation; CpdA, Compound A; DEX, dexamethasone; GC, glucocorticoid; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MCh, methacholine; MHC-II, MHC class II; MKP1, MAPK phosphatase 1; MLN, mediastinal lymph node; MUC5, mucin 5; PAS, periodic acid-Schiff; PenH, enhanced pause; qPCR, quantitative PCR; R_L, lung resistance; siGR, small interfering RNA against glucocorticoid receptor; siRNA, small interfering RNA; Treg, regulatory T cell.

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CpdA is, in contrast to DEX, not accompanied with the development of particular GC-induced side effects. This was demonstrated by the lack of CpdA-induced hyperglycemia/hyperinsulinemia (8, 10) and by the lack of hypothalamus–pituitary–adrenal axis suppression (11). Additionally, CpdA treatment is not associated with homologous downregulation of GR levels either in vivo in mice or ex vivo in primary fibroblast-like rheumatoid arthritis synoviocytes (12). There, in sharp contrast to DEX, a prolonged treatment of synovial fibroblasts with CpdA fully retains anti-inflammatory effects in terms of cytokine gene repression. The anti-inflammatory activity of CpdA in vivo in arthritis models shows prevention of paw swelling or joint inflammation, as well as reduction of inflammation and neuronal damage in a model of experimental autoimmune encephalomyelitis (8, 10, 13). This anti-inflammatory activity of CpdA in vivo is evoked in disease models involving mainly Th1/Th17-driven mechanisms. We have in this study investigated the activity of the GR-monomer-inducing CpdA in the lung in a Th2-dependent asthma model. Asthma is a chronic inflammatory disease in which resistance to GC therapy occurs in parallel with the severity of the disease (14). Therefore, the effect of the dissociated GR modulator CpdA and of its steroid comparator DEX was studied in the lung in a murine model of asthma by assessing inflammatory cell infiltration, cytokine production, goblet cell metaplasia, mucus and Ig production, and airway hyper-responsiveness (AHR). At the molecular level, we studied MAPK phosphatase 1 (MKP1) expression as a model gene to investigate gene transactivation, the role of GR in CpdA- and DEX-induced chemokine repression, and NF- κ B nuclear translocation and DNA binding.

Materials and Methods

Mice

Nine-week-old BALB/c mice were purchased from Charles River and Harlan Laboratories. Animals were maintained under controlled environmental conditions with a 12-h/12-h light/dark cycle according to the European Union guide for use of laboratory animals. Food and tap water were available ad libitum. Animal experimentation was conducted with the approval of the government body that regulates animal research in France and the animal ethical committees of the Universities of Ghent and Strasbourg.

Allergen sensitization and challenge

Mice were sensitized (i.p.) on days 0 and 7 with 50 μ g OVA adsorbed on 2 mg aluminum hydroxide in saline (Sigma-Aldrich). Control animals received i.p. injections of aluminum hydroxide in saline only. Mice were challenged (intranasally) on days 19, 20, and 21 with 10 μ g OVA in saline or with saline alone for controls. Intranasal administrations (12.5 μ l/nostril) were performed under anesthesia (i.p.) with 50 mg/kg ketamine (Imalgene; Merial) and 3.33 mg/kg xylazine (Rompun; Bayer).

Treatment with CpdA or DEX

CpdA synthesized according to Louw and coworkers (7) or DEX (water-soluble; Sigma-Aldrich) was administered i.p. (200 μ l) 24 h before the first OVA challenge and 2 h before each OVA challenge at doses indicated in the figure legends. To avoid stability issues, compounds were freshly dissolved immediately before administration. Control animals received 200 μ l saline solution i.p.

Measurement of airway responsiveness

Airway responsiveness was measured on day 22 by two complementary techniques. In one set of experiments, airway responsiveness to increasing concentrations of methacholine (MCh; Sigma Chemicals) was measured by barometric plethysmography in unrestrained animals (Emka Technologies) (15). Mice were stabilized in the plethysmograph chamber for 30 min until stable baseline and then exposed to aerosolized saline (for 30 s) as a control. Mice were then challenged every 20 min with aerosolized MCh (0.05, 0.1, 0.2, and 0.3 M) for 30 s, and the enhanced pause (PenH) was recorded during 5 min and used as an index of airway obstruction.

In another set of experiments, airway responsiveness was measured invasively using Flexivent (Scireq). Mice were anesthetized (i.p.) with

xylazine (15 mg/kg), followed 15 min later by an i.p. injection of pentobarbital sodium (54 mg/kg). An 18-gauge metal needle was inserted into the trachea. Mice were connected to a computer-controlled small animal ventilator and quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to spontaneous breathing. After baseline measurement, mice were challenged for 10 s with saline aerosol and, at 4.5-min intervals, with MCh at increasing concentrations (0.05, 0.1, and 0.2 M). For each MCh dose, the peak response was calculated as the mean of the three maximal values and used for calculation of airway lung resistance (R_L) and dynamic compliance (C_{dyn}). Airway resistance was expressed as cm H₂O.s.ml⁻¹ and airway compliance as ml.cm H₂O⁻¹.

Determination of total and differential cell counts in bronchoalveolar lavage fluids

Bronchoalveolar lavage (BAL; 24 h after the last OVA challenge) was performed as described previously (16, 17). Cells were stained with CD3, CD19, CD11c, MHC class II (MHC-II), CD8 (all from eBioscience), Siglec-F, Gr-1 (both from BD Biosciences), and CD4 (Invitrogen) Abs. Acquisition of eight-color samples was done on an LSRII cytometer (BD Biosciences). Numbers of eosinophils (Siglec-F⁺CD11c⁻), macrophages (autofluorescent cells), neutrophils (Gr-1⁺), dendritic cells (CD11c⁺MHC-II⁺), B cells (CD19⁺), total T cells (CD3⁺MHC-II⁻), Th cells (CD3⁺MHC-II⁻CD4⁺), and cytotoxic T cells (CD3⁺MHC-II⁻CD8⁺) were analyzed using FlowJo software (Tree Star).

Determination of lung T cell populations

Lungs were collected 24 h after the last challenge for analysis of T cell subpopulations, and individual cell suspensions were prepared as previously described (18). Suspensions were stimulated with PMA/ionomycin for 4 h and stained with CD3, CD8, CD25 (all from eBioscience), and CD4 (Invitrogen) Abs (30 min, 4°C). Cells were then fixed and permeabilized (with buffers from both BD Biosciences and eBioscience) and stained with IL-17, IFN- γ , Foxp3 (all from eBioscience), and IL-4 (BD Biosciences) Abs (30 min, 4°C). Acquisition of eight-color samples was done on an LSRII cytometer (BD Biosciences). Final analysis and graphical output were performed using FlowJo software (Tree Star).

ELISA

IL-4, IL-5, IL-13, IL-10, IFN- γ , and TNF- α levels in BAL fluids were determined by ELISA (BD Pharmingen or Invitrogen). Serum OVA-specific IgG1, IgG2a, and IgE were determined by ELISA, as previously described (16). For the OVA recall assay, mediastinal lymph nodes (MLNs) were homogenized, and cells were plated at a density of 200,000 cells/well. Cells were restimulated for 4 d with 20 μ g/ml OVA. Mucin 5 (MUC5) AC and MUC5B ELISAs (Cusabio; Abs-online, Aachen, Germany) were performed according to the manufacturer's instructions.

Histological analysis

Lung tissues were fixed (4% formaldehyde) and paraffin-embedded. Six-micrometer sections were cut, mounted on Superfrost glass slides (Fischer Scientific), and stained with H&E, periodic acid-Schiff (PAS), or toluidine blue (all from Sigma-Aldrich) or immunolabeled with anti-MUC5AC Ab (Abcam). To determine the severity of the inflammatory cell infiltration, peribronchial cell counts were performed based on a five-point scoring system described by Myou et al. (19). The extent of mucus production was quantified using a five-point grading system described by Tanaka et al. (20).

Quantitative RT-PCR

Total RNA was prepared from lung tissue (right lobes) sampled 2 h after the last OVA-challenge using Tri-Reagent (Molecular Research Center). Reverse transcription was performed on 1 μ g total RNA (with all reagents from Promega). Quantitative PCR (qPCR) was performed using ABsolute SYBR Capillary Mix (Thermo Scientific) on a LightCycler (Roche Diagnostics) using the following primers: mouse MKP1 5'-GAGCTGTGCAGCAAA-CAGTC-3' (forward) and 5'-CTTCCGAGAAGCGTGATAGG-3' (reverse); and mouse β -actin 5'-AGCCATGTACGTAGCCATCC-3' (forward) and 5'-CTCTCAGCTGTGGTGGTGA-3' (reverse). It was verified that none of the inducing agents modulated the regulation of the housekeeping gene.

Preparation of nuclear extracts and Western blotting

Lungs (right lobes) were sampled 2 h after the last OVA challenge, sliced, and lysed in a solution containing 0.6% Nonidet P-40, 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA (all from Sigma Aldrich), and Complete Mini-

EDTA-free protease inhibitor mixture (Roche Diagnostics). Nuclear extracts were prepared as described previously (21). Immunoblotting was performed using anti-p65 (sc-109) and anti-CBP Abs (Santa Cruz Biotechnology).

Chromatin immunoprecipitation

Lungs (left lobe) were collected 2 h after the last OVA challenge, sliced, and washed with ice-cold PBS and the cross-linking buffer (10 mM NaCl, 0.5 mM EGTA, 1 mM EDTA, and 50 mM HEPES [pH 9]). Chromatin immunoprecipitation (ChIP) assays were performed as previously described (21) using rabbit anti-p65 polyclonal Ab (1/200, sc-109; Santa Cruz Biotechnology). The mouse *IkB α* promoter was amplified with the PCR primer pairs 5'-GGACCCCAACCAAAATCG-3' (forward) and 5'-TC-AGGCGCGGGGAATTTCC-3' (reverse) (22).

GR small interfering RNA knockdown analysis

At day -1, 25,000 A549 cells/well were seeded in 24-well plates. At day 0, cells were transfected with 25 pmol small interfering RNA (siRNA) against GR (siGR; siGENOMESMARTpool human NR3C1) or nontargeting control siRNA (siControl; siRenilla Luc) (both purchased from Dharmacon RNA Technologies) using Dharmafect (0.25 μ l/well). Twenty-four hours later, cells were pretreated for 1 h with solvent (EtOH, 10 μ M), DEX (1 μ M), or CpdA (1 and 10 μ M) followed by a treatment with TNF- α (2000 IU/ml) for 24 h. Total RNA was analyzed and further processed toward qPCR analysis as stated above, using the following primer sequences: for human GR, 5'-TGATGAAGCTTCAGGATGTCA-3' (forward) and 5'-TTCGAGCTTCC-AGGTTTCATTC-3' (reverse); for CCL2, 5'-CAGCCAGATGCAATCAATG-3' (forward) and 5'-TGGAATCCTGAACCCACTTCT-3' (reverse), for CCL5, 5'-TGCCACATCAAGGAGTATTT-3' (forward) and 5'-TTT-CGGGTGACAAAGACGA-3' (reverse); and for eotaxin, 5'-GTGGCATT-CAAGGAGTACCTC-3' (forward) and 5'-TGATGGCCTTCGATTCTGGA-TT-3' (reverse). The average of three verified stable housekeeping genes was used: human TBP, 5'-CACGAACACGGCACTGATT-3' (forward) and 5'-TTTTCTTGCTGCCAGTCTGGAC-3' (reverse); human GAPDH, 5'-TG-CACCACCAACTGCTTAGC-3' (forward) and 5'-GGCATGGACTGTGG-TCATAG-3' (reverse); and human cyclophilin, 5'-GCGTCTCCTTTGAG-CTGTTTGCA-3' (forward) and 5'-CCACCCTGACACATAAACCTGG-AA-3' (reverse).

STAT6 immunofluorescence assay

A549 cells, seeded on coverslips and incubated in serum- and phenol red-free medium for 24 h, were pretreated for 1 h with solvent (EtOH, 10 μ M), DEX (1 μ M), or CpdA (10 μ M) before incubation with 50 ng/ml recombinant human IL-4 for 30 min. Fixation, permeabilization, and staining protocol was performed as described previously (8). Endogenous STAT6 was visualized using a 1:200 dilution of the anti-STAT6 Ab (E-10, sc 271213; Santa Cruz Biotechnology) followed by Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) secondary Ab. DAPI staining (0.4 mg/ml) allowed visualization of nuclei upon UV illumination. Images were recorded using an Olympus FV1000 confocal microscope (Olympus).

Statistical analysis

Data are presented as means $\pm$ SEM. Differences in airway responses between different groups were statistically analyzed using a two-way ANOVA followed by a Bonferroni posttest. For all other experiments, statistical differences were analyzed using Student *t* test. Data were considered significantly different when *p* < 0.05.

Results

CpdA counteracts OVA-induced AHR

OVA sensitization and subsequent challenge is known to lead to the development of AHR. We therefore assessed the effect of CpdA, as compared with the GC DEX, on airway responses to aerosolized MCh by noninvasive (measuring the Penh) and invasive (measuring R_L and C_{dyn}) methods 24 h after the last challenge. CpdA and DEX exhibit a totally different molecular structure. Consequently, effective dose ranges for CpdA use in vivo have previously been shown to be between 3- and 10-fold higher as compared with DEX (8, 10, 11). For this reason, we chose a dose range for CpdA between 30 and 300 μ g as compared with the known effective dose for DEX in AHR, which can be as low as 5 μ g.

As expected, OVA-sensitized/challenged mice exhibited increased Penh responses to MCh as compared with saline-treated

mice. CpdA and DEX had no effect on airway responsiveness in control saline-challenged mice (Fig. 1A). By contrast, OVA-induced AHR was totally inhibited with 100 and 300 μ g CpdA, with similar responses to MCh than those in saline-treated animals (control) (Fig. 1B). Moreover, CpdA (100 and 300 μ g) was more efficient than DEX (5 μ g) in the prevention of AHR to inhaled MCh.

We further analyzed the inhibitory effect of CpdA (100 μ g) and DEX (5 μ g) on airway responses to MCh by invasive measurements of R_L and C_{dyn} . OVA-sensitized/challenged mice exhibited increased R_L and decreased C_{dyn} as compared with control groups (Fig. 1C, 1D). CpdA (100 μ g) and DEX (5 μ g) reduced AHR to a similar extent, although neither of them totally prevented AHR.

CpdA decreases OVA-induced inflammatory cell infiltration in BAL fluid

Induction of bronchial hyperreactivity often results from inflammatory cell influx in the lungs. Although eosinophils are the most abundant cells to infiltrate the allergic lung, other cell types, like T and B lymphocytes, macrophages, neutrophils, and Ag-presenting dendritic cells, can also infiltrate the lung (23). As expected, OVA-sensitized/challenged mice displayed a significantly increased number of inflammatory cells in BAL fluid, consisting mainly of eosinophils (62.5% of the total cells) as well as 19% macrophages, 9% neutrophils, 1.1% dendritic cells, 0.6% B cells, and 7.3% T cells (mainly CD4⁺ Th cells) (Fig. 2). CpdA inhibited the recruitment of all inflammatory cell types, with an almost complete inhibition observed at the highest dose (300 μ g). As expected, DEX also inhibited the recruitment of all inflammatory cell types (Fig. 2). In contrast, CpdA and DEX had no effect on BAL fluid cell counts in control mice.

CpdA decreases OVA-induced T cell infiltration in the lung

OVA-sensitized/challenged mice had increased numbers of CD3⁺ T cells in the lung consisting mainly of CD4⁺ Th cells but also of

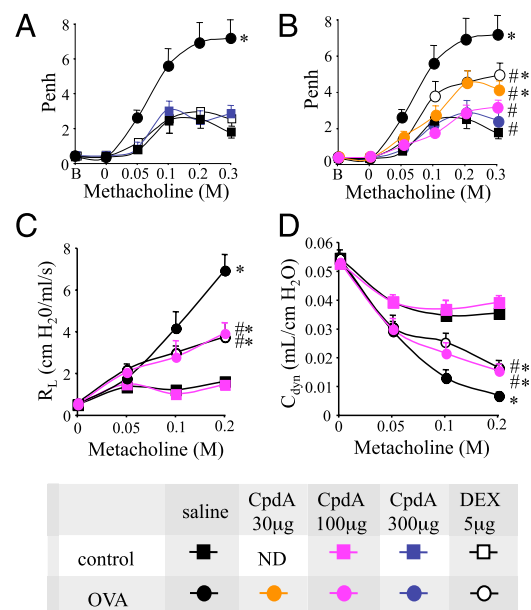


FIGURE 1. CpdA attenuates AHR to MCh in OVA-sensitized and -challenged mice. (A and B) Penh responses to increasing doses of aerosolized MCh 24 h after the last challenge. Data in (A) and (B) represent mean values $\pm$ SEM from *n* = 6 mice/group (control) and *n* = 9–12 mice/group (OVA). Effects of CpdA and DEX on R_L (Flexivent) (C) and lung C_{dyn} (Flexivent) (D) values in response to increasing doses of aerosolized MCh 24 h after the last challenge. Data in (C) and (D) represent mean values $\pm$ SEM from *n* = 5 to 6 mice per group. **p* < 0.05 versus control group, #*p* < 0.05 versus OVA-sensitized/challenged mice.

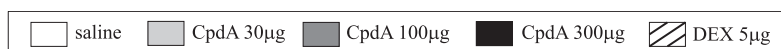
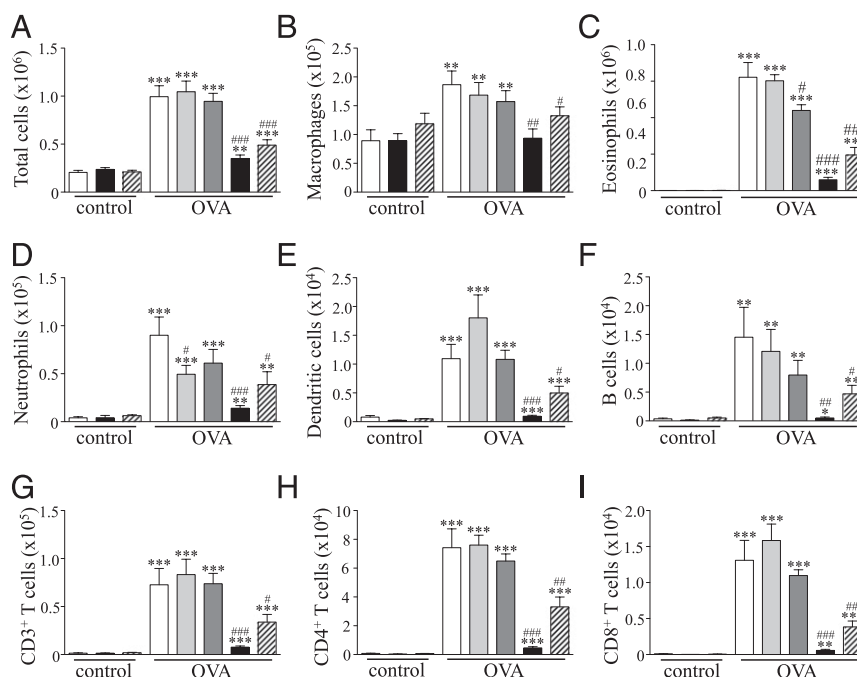


FIGURE 2. CpdA inhibits OVA-induced leukocyte recruitment in BAL fluid. Numbers of total leukocytes (A), macrophages (B), eosinophils (C), neutrophils (D), dendritic cells (E), B cells (F), CD3⁺ T cells (G), CD4⁺ Th cells (H), and CD8⁺ cytotoxic T cells (Tc) (I) in BAL fluid 24 h after the last challenge. Data represent mean values $\pm$ SEM from $n = 9$ –26 mice/group from two to five independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus corresponding controls, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus OVA-treated mice.



CD8⁺ cytotoxic T cells 24 h after the last challenge (Fig. 3A–C). These mice also had significant numbers of CD25⁺Foxp3⁺ lung regulatory T cells (Tregs) (Fig. 3D). Infiltration of all of these T cell subtypes was inhibited by both DEX and CpdA in a largely dose-dependent manner (Fig. 3A–D). Intracellular staining with IL-17 and IL-4 revealed a small number of Th17 (IL-17⁺CD4⁺), Tc17 (IL-17⁺CD8⁺), Th2 (IL4⁺CD4⁺), and Tc2 (IL4⁺CD8⁺) cells in the lung of control mice, and the numbers of these cells were all significantly increased in OVA-sensitized/challenged mice (Fig. 3E–H). Infiltration of these T cell subtypes was also inhibited by both DEX and CpdA in a dose-dependent manner (Fig. 3E–H). By contrast, no significant change was observed in numbers of Th1

(IFN- γ ⁺CD4⁺) and Tc1 (IFN- γ ⁺CD8⁺) cells between naive and OVA-sensitized/challenged mice (data not shown).

CpdA effectively reduces OVA-induced Th2 cytokine production

We next assessed the levels of cytokines in BAL fluid collected 24 h after the last challenge. OVA-treated mice showed significantly increased levels of the proinflammatory cytokine TNF- α and the Th2 cytokines IL-4, IL-5, and IL-13. These cytokine levels were inhibited by CpdA in a dose-dependent manner, without any effect on baseline levels of control mice (Fig. 4). DEX also significantly reduced levels of TNF- α , IL-4, and IL-5 but not IL-13 in BAL. By

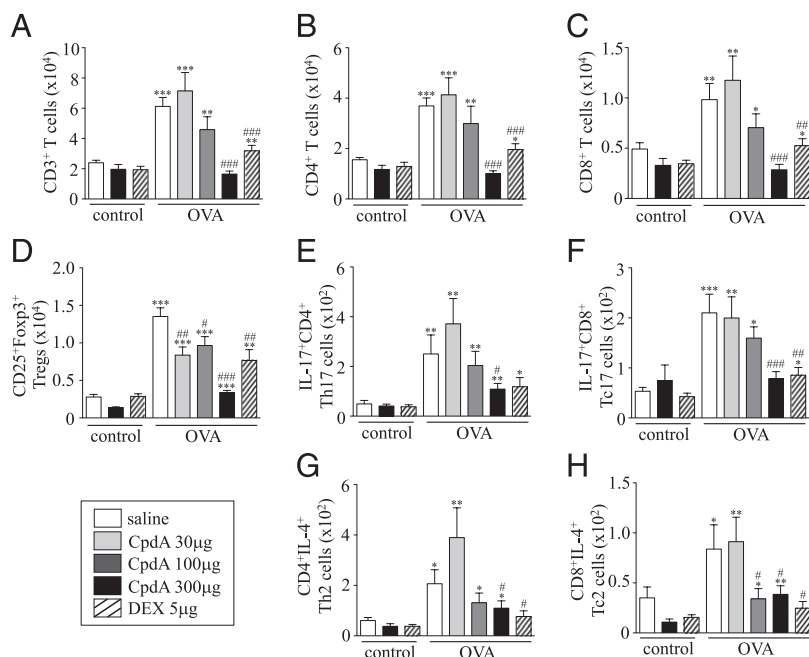


FIGURE 3. CpdA inhibits OVA-induced recruitment of different T cell populations in the lung. Numbers of total CD3⁺ T cells (A), CD4⁺ Th cells (B), CD8⁺ cytotoxic T cells (Tc) (C), CD25⁺Foxp3⁺ Tregs (D), IL-17⁺CD4⁺ Th17 cells (E), IL-17⁺CD8⁺ Tc17 cells (F), CD4⁺IL-4⁺ Th2 cells (G), and CD8⁺IL-4⁺ Tc2 cells (H) in BAL fluid 24 h after the last challenge. Data represent mean values $\pm$ SEM from $n = 9$ –13 mice/group from two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus corresponding controls, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus OVA-treated mice.

contrast, OVA treatment led to reduced levels of the Th1 cytokine IFN- γ , which was counteracted either by CpdA or DEX (Fig. 4E).

We also analyzed the levels of IL-13, IL-5, and IL-10 in cells from the MLNs following an OVA recall assay (Supplemental Fig. 1). CpdA inhibited OVA-induced IL-13 and IL-5 production in a dose-dependent manner. In agreement with our data in BAL fluids, DEX significantly reduced IL-5 but not IL-13 levels in OVA-restimulated MLN cells (Supplemental Fig. 1A, 1B). OVA-induced IL-10 production was completely inhibited by all doses of CpdA but not DEX (Supplemental Fig. 1C).

CpdA inhibits OVA-induced inflammation, mast cell infiltration of the lung, and goblet cell metaplasia

Lung tissues collected 24 h after the last challenge showed marked peribronchiolar inflammation and increased mast cell numbers (Fig. 5A–C). These features were inhibited by DEX and CpdA in a dose-dependent manner (Fig. 5B, 5C). We also observed a strong increase of mucus-producing goblet cells in OVA-sensitized/challenged mice as revealed by staining with PAS and immunostaining with anti-MUC5AC Abs (Fig. 5A). Goblet cell metaplasia, MUC5B, and MUC5AC protein levels in BAL fluids were all inhibited by both DEX and CpdA (Fig. 5A, 5D–F).

CpdA efficiently inhibits OVA-induced serum Ig levels

Serum was collected 24 h after the last OVA challenge. OVA treatment led to a significant increase in Th2 cytokine-dependent OVA-specific IgE and IgG1 serum levels as compared with control mice (Fig. 6A, 6B). Th1 cytokine-dependent OVA-specific IgG2a levels were also slightly increased in OVA-treated mice, although the difference did not reach statistical significance (Fig. 6C). These increases were inhibited by DEX and CpdA in a dose-dependent manner (Fig. 6B, 6C). By contrast, the increase of OVA-specific IgE was only inhibited by 60% at the highest dose of CpdA, which was nevertheless to an extent similar to that obtained with DEX (Fig. 6A).

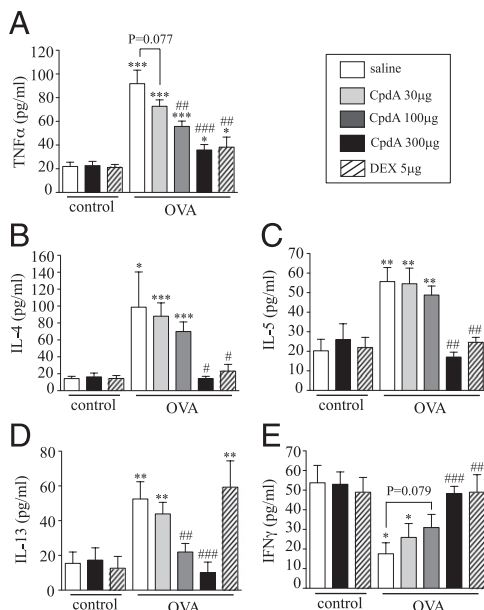


FIGURE 4. CpdA inhibits OVA-induced TNF- α and Th2 cytokine production in BAL. Effects of CpdA and DEX on TNF- α (A), IL-4 (B), IL-5 (C), IL-13 (D), and IFN- γ (E) levels in BAL fluid 24 h after the last challenge. Data represent $n = 4$ –6 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control (saline) group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus OVA-treated mice.

CpdA induces selective transrepression versus transactivation in the lung

One of the key mechanisms of inflammatory gene downregulation by GCs is the transrepression of proinflammatory transcription factors. We therefore assessed the effect of CpdA (300 μ g) and DEX (5 μ g) on lung NF- κ B activation in OVA-sensitized/challenged mice. We measured the protein levels of the p65 NF- κ B subunit by Western blot in the nuclear fraction of lungs sampled 2 h after the last challenge. OVA challenge-induced nuclear translocation of NF- κ B was prevented by CpdA or DEX (Fig. 7A). ChIP experiments assessing binding of p65 to a prototypical NF- κ B-regulated promoter, the I κ B α promoter, was performed on lung tissues isolated 2 h after the last challenge. OVA-induced binding of p65 to the I κ B α promoter was prevented by CpdA or DEX treatment (Fig. 7B). The transrepressive action of both CpdA and DEX is therefore similar in lung tissue.

A number of adverse effects of the classical GCs are claimed to result from the transactivation of genes containing a GRE element in their promoter region (24). In contrast, a number of GC-driven genes may also contribute to the anti-inflammatory effect (25). CpdA was previously shown not to support transactivation of GRE-dependent promoters (8). To further explore this assumption for CpdA in the lung, we measured mRNA levels of the GC-dependent MKP1 gene normalized to mRNA of the domestic gene β -actin 2 h after the last OVA challenge. mRNA levels of MKP1 were similar in control and OVA-treated mice (Fig. 7C). As expected, DEX treatment increased MKP1 mRNA levels. By contrast, CpdA had no effect on MKP1 mRNA levels (Fig. 7C), confirming its dissociative nature.

The anti-inflammatory effect of CpdA in lung cells depends on the presence of sufficient levels of GR

To address the question to what extent the anti-inflammatory effects of CpdA in lung cells are GR mediated, we performed a GR siRNA-knockdown analysis using A549 lung epithelial cells and subsequently measured mRNA levels of GR, the GC-dependent genes MKP1 and GR-induced leucine zipper (GILZ), and the TNF-induced chemokines CCL5, CCL2, and eotaxin (Fig. 8). We confirm that DEX, but not CpdA, induced the expression of MKP1 and GILZ in a GR-dependent manner (Fig. 8B, 8C). The results also clearly demonstrate that the NF- κ B-regulated chemokines CCL5, CCL2, and eotaxin were only suppressed by DEX and CpdA when sufficiently high levels of endogenous GR were present (Fig. 8D–F).

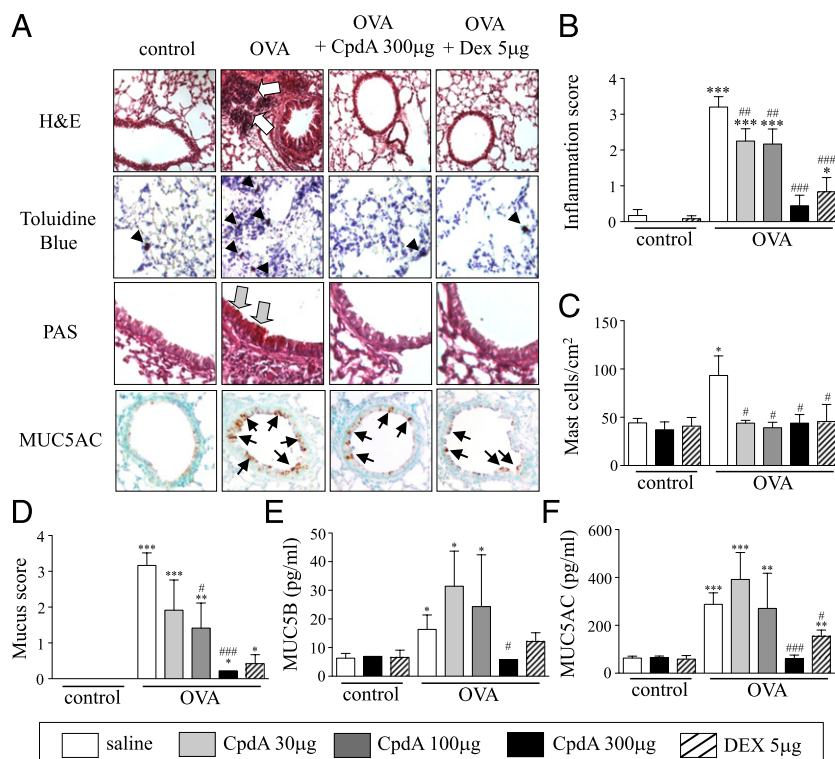
CpdA is able to inhibit the nuclear translocation of STAT6

IL-4-mediated activation of the JAK/STAT6 pathway is essential for Th2 cell development and cell expansion. Moreover, this signaling pathway also contributes to an enhanced IgE production in B cells and increased production of chemokines, such as eotaxin, by bronchial epithelial cells (26). Because the transcription factor STAT6 accumulates in the nucleus upon IL-4 signaling, we studied whether CpdA could affect its nuclear translocation. Because of the short time window in which transcription factor translocation events generally occur, to be able to address this question, we reverted to an indirect immunofluorescence analysis of the human lung epithelial cell line A549. As expected, stimulation of cells with IL-4 (50 ng/ml) for 30 min promoted the nuclear accumulation of STAT6 (Fig. 9). Both DEX and CpdA blocked the IL-4-induced nuclear translocation of STAT6 (Fig. 9).

CpdA induces adverse events on tissue weights

OVA-sensitized/challenged mice exhibited a 6% decrease in body weight 24 h after the last OVA challenge on D22 as compared with

FIGURE 5. CpdA inhibits OVA-induced lung inflammation, mast cell recruitment, and mucus production. **(A)** Representative H&E, toluidine blue, PAS staining, and MUC5AC immunostaining of lung sections 24 h after the last challenge. White arrows show peribronchial and perivascular infiltrates of inflammatory cells, black arrowheads indicate toluidine blue-positive mast cells, gray arrows show goblet cell metaplasia, and black arrows show MUC5AC⁺ goblet cells. Quantification of the effect of CpdA and DEX on lung inflammation **(B)**, toluidine blue-positive lung mast cell numbers **(C)**, lung goblet cell metaplasia **(D)**, and MUC5B **(E)** and MUC5AC **(F)** protein levels in BAL fluid. Data represent mean values $\pm$ SEM from $n = 4-6$ mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus OVA-sensitized/challenged mice.



D0 (not shown). Treatment with DEX (5 μ g) had no effect on body weight. By contrast, the highest dose of CpdA (300 μ g), although acting as a fully dissociated GC agonist (Fig. 8), induced a significant loss in body weight in the OVA-sensitized/challenged mice (-13%) (Fig. 10A). A similar weight loss was observed in CpdA-treated control mice (data not shown). This was accompanied with a dose-dependent loss in liver weight (-27% with 300 μ g CpdA), spleen weight (-34% with 300 μ g CpdA), and kidney weight (-18% with 300 μ g CpdA) (Fig. 10B–D) in both control and OVA-sensitized/challenged mice (not shown). DEX treatment had no significant effect on liver and kidney weight, but induced a 22% loss in spleen weight as compared with control or OVA-sensitized/challenged mice.

Discussion

GCs remain the most effective anti-inflammatory therapy for asthma (27), but their use is limited by side effects and by GC resistance (reviewed in Refs. 24, 28). GCs can control the expression of genes either by transactivation (i.e., by acting as a transcription factor) or transrepression (i.e., by repressing the activity of other transcription factors, such as NF- κ B). The anti-inflammatory effects of GCs are believed to involve mainly transrepression, whereas particular side effects have been linked to transactivation (6, 29, 30). This concept has led to the development of so-called dissociated GR ligands, which lack trans-

activating properties. CpdA is a fully dissociated nonsteroidal GR agonist that has demonstrated promising anti-inflammatory activity in Th1-driven arthritis and experimental autoimmune encephalomyelitis models (8, 10, 11, 31). To date, there is no report on the effect of CpdA in Th2-dependent models of inflammatory diseases. The present study demonstrates that CpdA also exerts strong anti-inflammatory properties in a Th2-dependent mouse model of asthma.

We show that in this murine asthma model, CpdA is a potent inhibitor of AHR, lung inflammation, mucus production, infiltration of eosinophils, neutrophils, dendritic cells, B cells, T cells, macrophages, and mast cells in the lung. Infiltration of all T cell subtypes, not only CD4⁺ Th2 and CD8⁺ cytotoxic Tc2 cells, but also CD25⁺Foxp3⁺ Tregs and Th17 and Tc17 cells, was inhibited both by DEX and CpdA in a largely dose-dependent manner. This concurs with the CpdA-induced suppression of Th2 cytokine production as demonstrated in this study for IL-4, IL-13, and IL-5 in BAL fluid. In parallel, both DEX and CpdA are able to restore OVA-suppressed Th1 cytokine IFN- γ levels, which is in agreement with previous demonstrations for DEX (32). Of note, and in contrast to this model, in a rat model for experimental autoimmune neuritis, which is a Th1 cell-mediated inflammatory demyelinating disease of the peripheral nervous system, CpdA significantly reduced mRNA levels of IFN- γ , but increased those of IL-4, suggesting that Th2 cell polarization is favored by CpdA

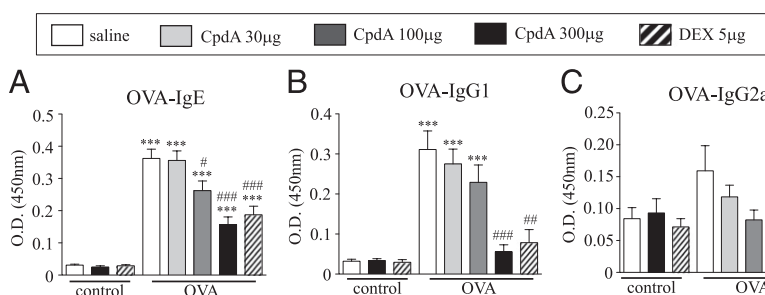


FIGURE 6. CpdA inhibits OVA-specific Ig levels. Effects of CpdA and DEX on OVA-specific IgE **(A)**, IgG1 **(B)**, and IgG2a **(C)** levels in the serum 24 h after the last challenge. Data represent $n = 6$ mice/group. *** $p < 0.001$ versus control group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus OVA-treated mice.

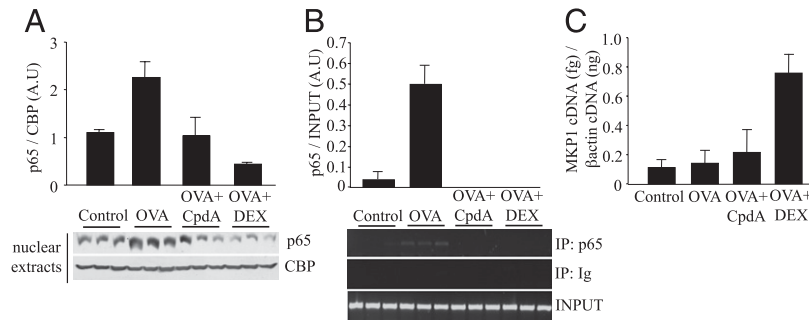


FIGURE 7. CpdA induces selective transrepression versus transactivation in the lung. OVA-sensitized mice were treated with CpdA (300 μ g) or DEX (5 μ g), and lungs were harvested 2 h after the last challenge. **(A)** Western blot analysis was performed on nuclear extracts by using an anti-p65 Ab. Probing with an anti-CBP Ab served as a control. **(B)** ChIP analysis was performed using p65 Ab on cross-linked and sonicated lung lysates. Recruitment of p65 at the IkB α gene promoter was assessed by PCR. Input reflects the relative amounts of sonicated DNA fragments before immunoprecipitation. Results in (A) and (B) show three individual mice per group. **(C)** Total lung RNA was extracted and reverse transcribed, and cDNA was subjected to qPCR with primers to detect MKP1 and β -actin. Data represent mean values $\pm$ SEM from $n = 3$ mice/group. A.U., arbitrary units.

in this context (31). Therefore, even if all T cell subtypes were found to be affected by CpdA in the OVA model, we speculate that CpdA-activated GR may exert a regulatory function on different Th cell phenotypes in other disease models and target tissues, hence fine-tuning equilibrium in the immune response.

Our study further shows that CpdA displays a dissociative effect on GR signaling in the lung, because it fails to upregulate mRNA levels of the GC-dependent MKP1 gene, which is in contrast to DEX, whereas CpdA also interferes with the nuclear translocation and promoter recruitment of NF- κ B observed upon OVA stimulation. This is in agreement with recent findings using this non-steroidal GR phyto modulator in L929sA murine fibroblasts and A549 human alveolar cells, demonstrating that a GR-dependent transrepression of NF- κ B-driven genes is achieved without supporting the transactivation properties of GR through GRE-driven gene expression (8). We have further extended these data by demonstrating that the presence of sufficient levels of GR is not only crucial to support the DEX-mediated transactivation of

MKP1 and GILZ, but also proves essential for the direct anti-inflammatory effects of both CpdA and DEX in A549 cells. The proposed mechanism implies active GR monomer formation for the dissociated, transrepression-favoring effects of CpdA (10, 33). We show in this study that CpdA also displays this effect *in vivo* in the lung. The results therefore suggest that GR-dependent transrepression without transactivation is sufficient for counteracting a Th2-dependent inflammatory response in the lung. Another example of a marked discrepancy in the molecular mechanism of CpdA as compared with DEX became apparent upon measuring IL-10 in an OVA recall assay (Supplemental Fig. 1C). Only CpdA, but not DEX, was able to inhibit OVA-induced IL-10 production in cells derived from the MLNs.

The transcription factor GATA-3, which plays a major role in allergic diseases by regulating the expression of Th2 cytokines, is expressed in bronchial epithelial cells in humans (34). One of the mechanisms of GC action in allergic diseases is referred to as blockade of GATA-3 nuclear translocation, as demonstrated by the

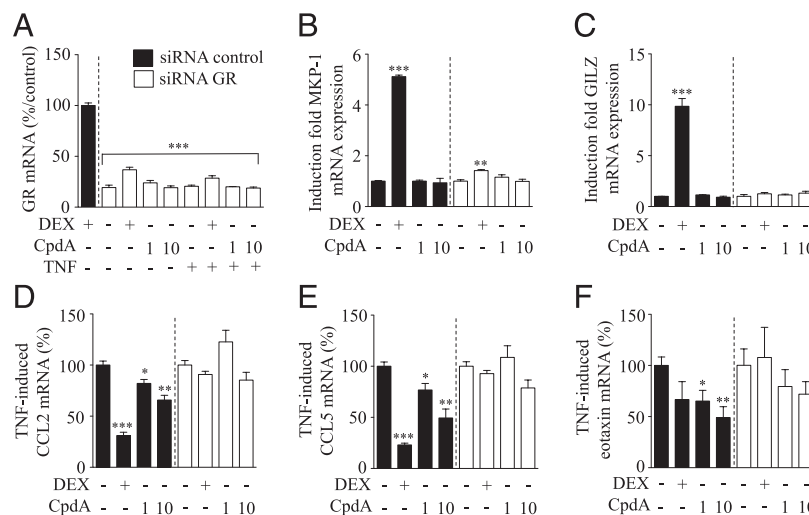


FIGURE 8. GR is essential to mediate chemokine repression by DEX and CpdA. A549 cells were transfected with control siRNA (siRNA control) or siRNA against GR (siRNA GR). At 24 h thereafter, cells were treated with EtOH control solvent, DEX (1 μ M), or CpdA (10 μ M) for 1 h, then TNF- α (2000 IU/ml) was added and incubated for 24 h. Total RNA was isolated and subjected to RT-PCR. **(A)** Silencing of GR was monitored by qPCR analysis. The GR expression levels in control siRNA-transfected cells were set at 100%, and values in GR-specific siRNA-transfected cells were determined as the percent silencing relative to siControl-transfected samples. To monitor GC inducibility, the amount of cDNA for the model genes MKP1 **(B)** and GILZ **(C)** after siControl or siGR transfection was measured by qPCR. The relative induction fold to solvent control set at 1 is represented. The amount of cDNA for the chemokines CCL2 **(D)**, CCL5 **(E)**, and eotaxin **(F)** after siControl or siGR transfection was measured by qPCR. Expression levels in each treatment group were normalized to the values in the TNF-stimulated samples. Bars show the mean and SD results from quadruplicates. Of note, the TNF inducibility ranged from 15-fold for CCL5 to several 100-folds for CCL2 and eotaxin (data not apparent from the graphic representation). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus corresponding controls.

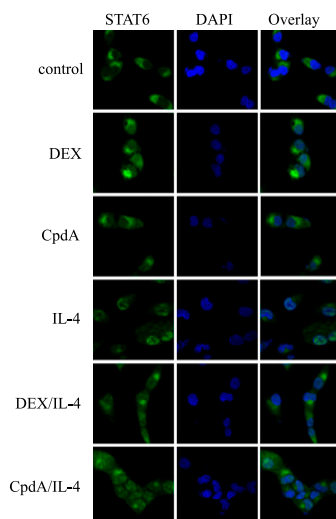


FIGURE 9. STAT6 nuclear accumulation is inhibited both by CpdA and DEX. A549 cells were treated with solvent, DEX (1 μ M), or CpdA (10 μ M) for 1 h, followed by treatment with IL-4 for 30 min. Through indirect immunofluorescence using an anti-STAT6 Ab, endogenous STAT6 was visualized (green), and DAPI staining (blue) indicates the nuclei of the cells. In the *right panels*, an overlay of both signals is presented.

corticosteroid fluticasone in human T lymphocytes (35). However, we did not observe any effect of either CpdA or DEX on OVA-induced GATA-3 expression and subcellular localization in our model (Supplemental Fig. 2). STAT6 is a transcription factor required for many biologic functions of Th2 cytokines and contributing to IgE production in B cells (26). As a novel piece of the molecular mechanism in A549 human lung epithelial cells, we found that CpdA, as well as DEX, interfered with the IL-4-induced nuclear accumulation of STAT6. These results are in contrast to a previous report that GCs do not target STAT6 nuclear translocation in BEAS-2B cells (36), but are in line with another report that STAT6 activity, in particular the binding to its response element, can be targeted as part of the anti-inflammatory mechanism of activated protein C (37). As such, the result we obtained may

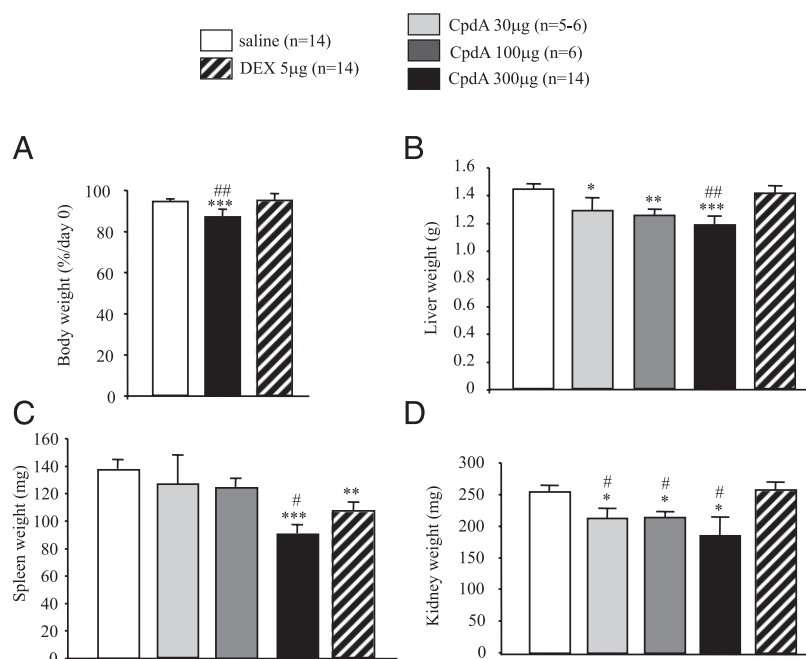
represent part of the underlying mechanism to explain the lower levels of IgE and general therapeutic antiasthmatic response of both CpdA and DEX in the animal model and emphasizes that cytokine-signaling pathways can be targeted by different GR modulators at the level of nuclear accumulation of transcription factors.

The NF- κ B-driven cytokine TNF- α , which is also induced upon OVA challenge, was efficiently suppressed both by CpdA and DEX in BAL. This is in accordance with the observed inhibition of p65 translocation by CpdA and DEX in the lung. Similarly, CpdA also prevented translocation of the p65 subunit of NF- κ B in prostate cells, whereas in the same cells, classic GCs induced transrepression without affecting nuclear translocation of NF- κ B (38). Another report demonstrates that CpdA, but not DEX, can inhibit NF- κ B nuclear translocation in primary murine microglia but not in astrocytes (8). Indeed, it has been recognized for quite some time that, depending on the target tissue, GR-dependent inhibition of NF- κ B can either occur through inhibition of NF- κ B translocation or NF- κ B transactivation in the nucleus, leaving its translocation unhampered (39). In concordance with a drop in nuclear NF- κ B levels in lung tissues, ChIP experiments unveiled a complete blockade of OVA-induced recruitment of p65 onto the NF- κ B-driven I κ B α promoter by either CpdA or DEX.

The MKP1 (DUSP1) protein is known to dephosphorylate MAPKs and thus to impact the inflammatory process (40). The MKP1 promoter is stimulated by GC-activated GR (41). The team of Clark (42) identified different GC-responsive regions containing GR-binding site consensus sequences, located at various positions upstream to the MKP-1 transcription start site. The *cis*-acting region at 29 kb upstream of the transcription start site was positively identified to confer the transcriptional response to DEX (42). We show in this study that only the combination of DEX with OVA, but not CpdA with OVA or OVA alone, is able to stimulate MKP1 mRNA expression in the lung. Our data further imply that induction of GR-driven anti-inflammatory genes like MKP1 is not a requirement to the improved phenotype of CpdA-treated mice and clearly show that CpdA acts as a dissociated GR modulator also in the lung in this murine asthma model.

Despite its clear dissociated activity, we also found that systemic treatment with CpdA induced side effects that included losses in

FIGURE 10. CpdA induces adverse events. (A) Whole-body weight loss was measured 24 h after the last challenge (day 22) and represented as a percentage from day 0 weight. Liver (B), spleen (C), and kidney (D) weight were measured 24 h after the last challenge. * p < 0.05, ** p < 0.01, *** p < 0.001 versus control (saline) group, # p < 0.05, ## p < 0.01 versus DEX-treated mice.



kidney, liver, and spleen weights. This was observed in a dose-dependent manner. By contrast, DEX induced a loss in spleen weight, only at a dose as low as 5 µg/mice/d. Such CpdA-induced adverse events, most probably induced by its metabolites, were also recently reported by Wüst and collaborators (13). These authors showed that the aziridine metabolites of CpdA can induce apoptosis of various cell types, including lymphocytes *ex vivo*, and demonstrated that this occurred via a Bcl-2- and caspase-dependent pathway and independently of the presence of GR. To further address potential cytotoxic effects on tissues or organs, liver, and kidney tissue samples were examined and showed normal histology in all groups (Supplementary Figs. 3, 4). These results are in line with earlier results using CpdA in the experimental autoimmune encephalomyelitis model, in which no elevation in levels of the liver enzyme aspartate aminotransferase and alanine aminotransferase were detected in the blood after CpdA (150 µg) treatment (11).

In conclusion, we demonstrate in this study that monomeric GR stimulation, as obtained by the use of the selective GR modulator CpdA, results in a profound inhibitory effect on airway inflammation and hyperresponsiveness in a murine asthma model at least in part via inhibiting Th2 cytokines. Furthermore, a contribution to the overall anti-inflammatory effect of CpdA in the lung tissue most likely occurs in a mainly GR-dependent manner via inhibiting NF-κB activity and possibly also STAT6 translocation at an early step of the activation pathway. Therefore, our study indicates that sharply dissociated GR modulators like CpdA are potentially active anti-inflammatory agents for asthma and thus justifies the use of CpdA as a scientific research tool, but that new compounds will have to be developed and compared with classical GCs before entering the clinic.

Acknowledgments

We thank Dr. Manuel Neves (Unité Mixte de Recherche 7200, Centre National de la Recherche Scientifique/Université de Strasbourg) for kind help with plethysmography analysis.

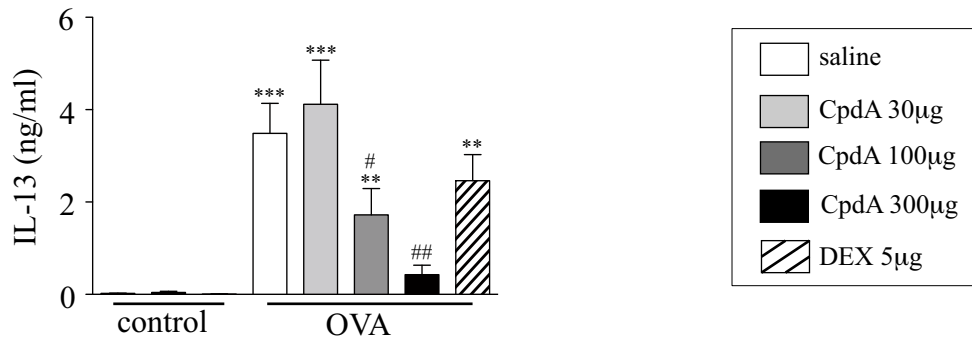
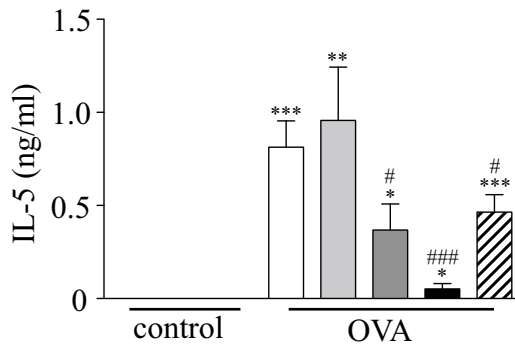
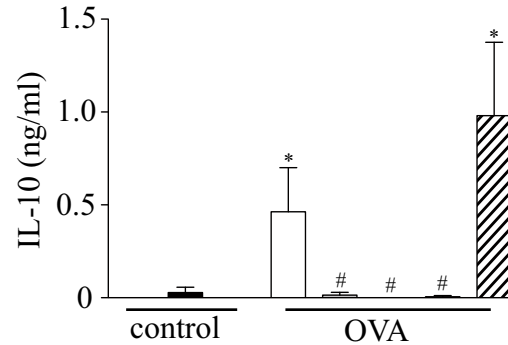
Disclosures

The authors have no financial conflicts of interest.

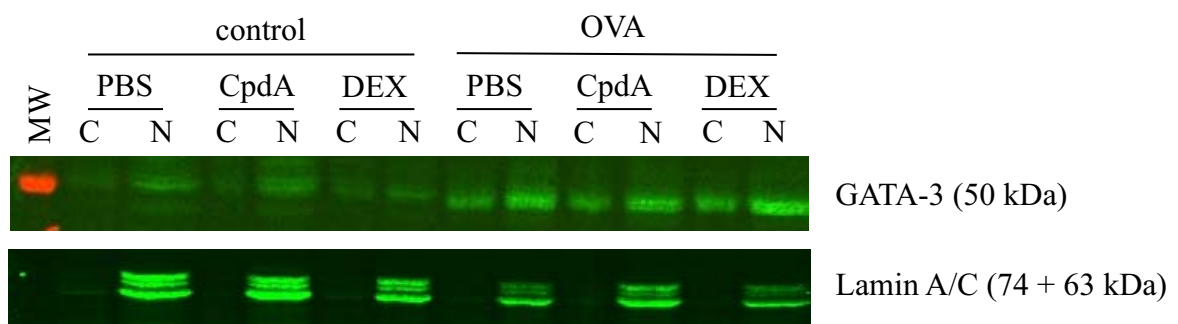
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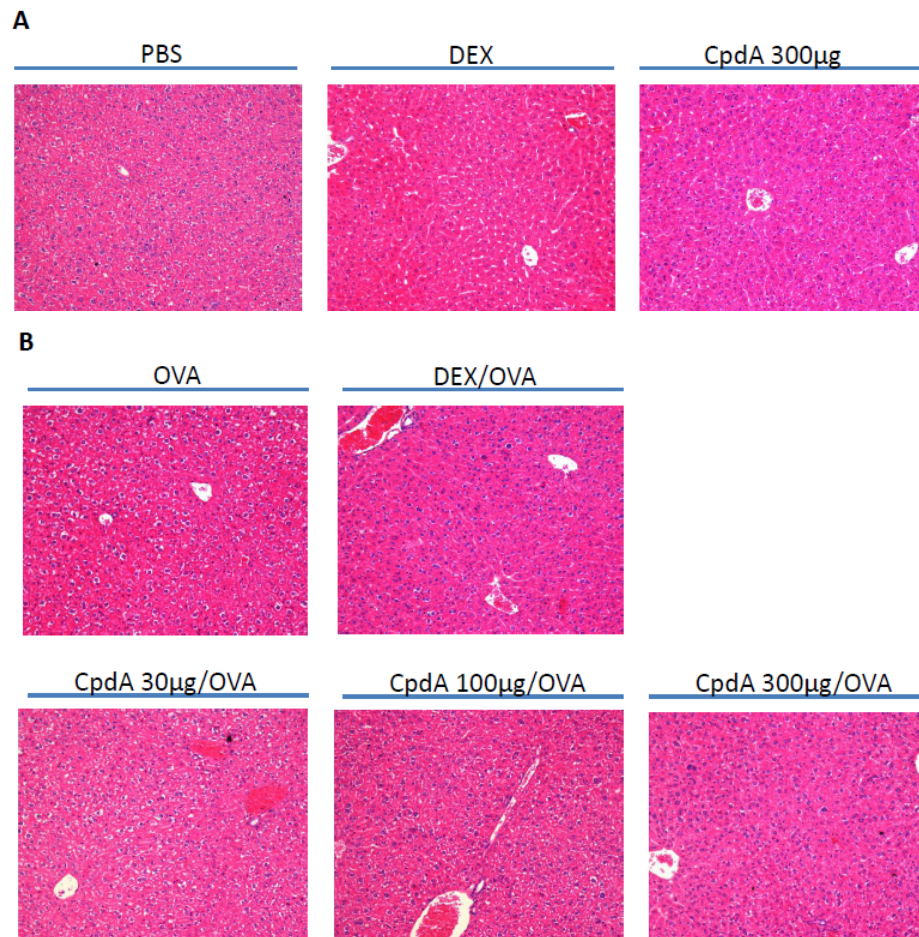
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A**B****C**

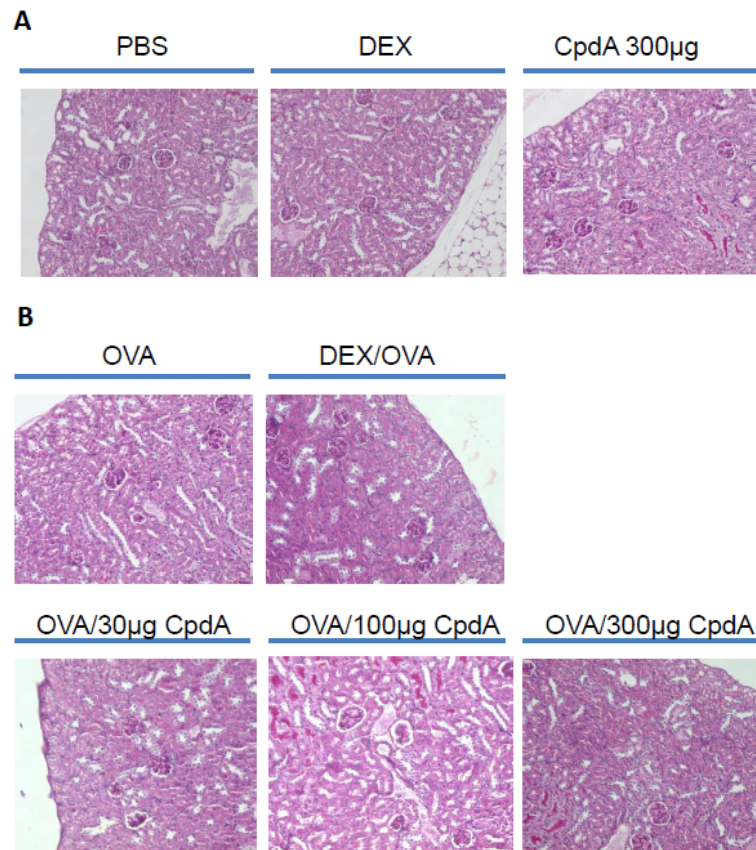
Supplementary figure 1. Effect of Cp dA OVA-specific responses in *ex vivo* cultured mediastinal lymph node (MLN) cells. Mediastinal lymph nodes (MLN) were collected and homogenized 24 h after the last challenge and cells were plated at a density of 200,000 cells/well. Cells were cultured with OVA (20 µg/ml) for 4 days, after which the supernatant was assayed for IL-13 (A), IL-5 (B) and IL-10 (C) cytokine production by ELISA. Each bar represents the mean $\pm$ SEM of $n=5$. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ versus corresponding controls; # $P < 0.05$, ## $P < 0.01$ or ### $P < 0.001$ versus OVA-treated mice.



Supplementary figure 2. CpdA does not affect GATA3 expression and nuclear translocation in the lung. Lungs (right lobes) were collected 24 h after the last OVA-challenge, sliced and subjected to a nuclear-cytoplasmic protein fractionation assay. Western blot analysis was performed on nuclear extracts by using an anti-GATA3 antibody (sc-268, Santa Cruz Biotechnologies). Probing with an anti-lamin A/C (sc-6215, Santa Cruz Biotechnologies) antibody served as a control. MW: molecular weight marker; C: cytoplasmic extract; N: nuclear extract.



Supplementary figure 3. CpdA does not induce liver toxicity. Liver tissues were fixed (4% formaldehyde) and paraffin-embedded. 6- μ m sections were cut, mounted on Superfrost glass slides (Fischer Scientific) and stained with haematoxylin & eosin. Sections were made for minimum 4 mice from each group of control (**A**) and OVA sensitized/challenged (**B**) mice. Slides were examined by a certified pathologist using a classical bright field microscope, Olympus BX53. No differences between the different groups were observed. Representative images are depicted.



Supplementary figure 4. CpdA does not induce kidney toxicity. Kidney tissues were fixed (4% formaldehyde) and paraffin-embedded. 6- μ m sections were cut, mounted on Superfrost glass slides (Fischer Scientific) and stained with periodic acid-Schiff (PAS). Sections were made for minimum 4 mice from each group of control (**A**) and OVA sensitized/challenged (**B**) mice. Slides were examined by a certified pathologist using a classical bright field microscope, Olympus BX53. No differences between the different groups were observed. Representative images are depicted.