Prostaglandin E2 stimulates adaptive IL-22 production and promotes allergic contact dermatitis

Title page

Original Article

Prostaglandin E_2 stimulates adaptive IL-22 production and promotes allergic contact dermatitis

Calum T. Robb, PhD,[a] Henry J. McSorley, PhD,[a] Jinju Lee, MSc[b,c] Tomohiro Aoki, MD, PhD[b,c] Cunjing Yu, PhD,[a] Siobhan Crittenden, MSc,[a] Anne Astier, PhD,[a] Jennifer Felton, PhD[a] Nicholas Parkinson, MA, MS, VetMB[a] Adane Ayele, MD,[d] Richard M. Breyer, PhD,[e,f] Stephen M. Anderton, PhD,[a] Shuh Narumiya, MD, PhD,[b,c] Adriano G. Rossi, PhD, DSc[a] Sarah E. Howie, PhD,[a] Emma Guttman-Yassky, MD, PhD,[g,h] Richard B. Weller, MD, FRCP,[a] Chengcan Yao, PhD,[a]

[a] Medical Research Council (MRC) Centre for Inflammation Research, Queen's Medical Research Institute, The University of Edinburgh, Edinburgh EH16 4TJ, UK.

[b] Center for Innovation in Immunoregulative Technology and Therapeutics (AK Project), Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan.

[c] Core Research for Evolutional Science and Technology, Medical Innovation Center, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan.

[d] Department of Dermatovenereology, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.

[e] Department of Veterans Affairs, Tennessee Valley Health Authority, Nashville, TN 37212, USA.

[f] Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA.

[g] Department of Dermatology and the Laboratory for Inflammatory Skin Diseases, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[h] Laboratory for Investigative Dermatology, The Rockefeller University, NY 10065, USA

Corresponding author

Chengcan Yao, PhD

Medical Research Council (MRC) Centre for Inflammation Research,

Queen's Medical Research Institute,

The University of Edinburgh,

47 Little France Crescent

Edinburgh EH16 4TJ, UK.

Telephone: +4413-1242-6685

Fax: +4413-1242-6578

Email: Chengcan.Yao@ed.ac.uk

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Abstract

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease with a central role of Th22-derived IL-22 in its pathogenesis. Although prostaglandin E_2 (PGE_2) is known to promote inflammation, little is known about its role in processes related to AD development, including IL-22 up-regulation.

Objectives: To investigate whether PGE_2 has a role in IL-22 induction and development of allergic contact dermatitis (ACD), a disease related to AD.

Methods: T-cell cultures and *in vivo* sensitization of mice with hapten were used to assess the role of PGE_2 in production of IL-22. The involvement of PGE_2 receptors and their downstream signals were also examined. The effects of PGE_2 were evaluated using the oxazolone (OXA)-induced ACD mouse model. The relationship of PGE_2 and IL-22 signaling pathways were also investigated using genomic profiling in human lesional AD skin.

Results: PGE_2 induces IL-22 from T cells through its receptors EP2 and EP4 and involves cyclic adenosine monophosphate (cAMP) signaling. Selective deletion of EP4 in T-cells prevents hapten-induced IL-22 production *in vivo*, and inhibition of PGE_2 synthesis limits atopic-like skin inflammation in the OXA-induced ACD model. Moreover, both PGE_2 and IL-22 pathway genes were coordinately up-regulated in human AD lesional skin, but were below significant detection levels after corticosteroid or ultraviolet band B (UVB) treatments.

Conclusions: Our results define a crucial role for PGE_2 in promoting ACD by facilitating IL-22 production from T-cells.

Clinical Implications: (<30 words)

Atopic dermatitis is a common disabling disease characterized by elevated IL-22. The identification of a tightly regulated PGE_2 driven pathway controlling IL-22 dysfunction offers a novel target for therapeutic intervention.

Capsule Summary: (<35 words)

Prostaglandin E_2 promotes IL-22 production from T cells that mediates IL-22-driven development of atopic dermatitis.

Key words

Atopic dermatitis, Prostaglandin E_2, CD4+ T cells, Th22 cells, Th17 cells, Interleukin 22,

Abbreviations used

ACD: Allergic contact dermatitis

AD: Atopic dermatitis

AHR: Aryl hydrocarbon receptor

ANOVA: Analysis of variance

cAMP: Cyclic adenosine monophosphate

CCR: Chemokine receptor

COX: Cyclooxygenases

Db-cAMP: Dibutyryl cAMP

DC: Dendritic cell

DNFB: Dinitrofluorobenzene

IL-22: Interleukin 22

ILC3: Group 3 innate lymphoid cells

LN: Lymph node

110 mPGES: Microsomal prostaglandin E synthases

111 OXA: Oxazolone

112 PGE_2: Prostaglandin E_2

113 PKA: Protein kinase A

114 Th: Helper T cells

115 UVB: Ultraviolet band B

Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease, with a prevalence of up to 3-7% in adults and up to 25% among children[1,2]. Histologic features of affected eczematous skin include epidermal hyperplasia and spongiosis, and infiltration of immune cells (T-cells, dendritic cells/DCs, eosinophils, etc) in the dermis[1,3,4]. Barrier dysfunction is also recognized as important for development of AD[5-7]. Environmental allergens can penetrate through the skin in AD due to a dysfunctional epidermal barrier, where they are taken up by antigen presenting cells (such as dendritic cells) which in turn activate and polarize T-cells, resulting in AD initiation[1]. Dysregulation of the balance of type 1 and 2 helper T (Th1 and Th2) cells, characterized by production of IFN-γ or IL-4/IL-13, respectively, has traditionally been thought to contribute to AD pathogenesis[8]. However, recent studies have shown that IL-17 and IL-22, produced by Th17 and other activated T cells including Th22 cells, are also critical in mediating the initiation and progression of AD[9-13]. IL-22 levels in serum from patients with AD are higher than those from healthy individuals[14,15], and IL-22$^+$ T cells infiltrate into lesional AD skin[16] where IL-22 induces epidermal hyperplasia and inhibits epidermal differentiation[17,18]. Importantly, a neutralizing anti-IL-22 antibody (NCT01941537) or targeting components of the IL-23/Th17 pathway, are currently being tested in clinical trials[3,19]. To refine future therapies, there is a need to better understand the mechanisms that drive IL-22 production in response to cutaneous antigen stimulation.

Prostanoids, including prostaglandin D2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2, are bioactive lipid mediators that are generated from arachidonic acid by cyclooxygenases (COX) and then respective PG synthases. PGs have various roles in inflammatory skin diseases through regulating functions of immune cells, including Th1/Th17 T-cells, T regulatory cells, mast cells and DCs[20]. PGE_2 is synthesized by

141 microsomal prostaglandin E synthases (mPGES1 and mPGES2) and has essential roles in
142 modulating various inflammatory responses by binding to PG receptors (EP1-4) on cell
143 surfaces. Many cutaneous cells, including keratinocytes, mast cells, eosinophils, fibroblasts,
144 DCs and lymphocytes, express PGs and PG receptors[21]. Increased PGE_2 expression in
145 biologically active amounts has been reported in both lesional and non-lesional skin from AD
146 patients[22]. Blockade of PG production by a COX2 inhibitor was reported to enhance
147 eosinophil infiltration and elevate IL-4 expression in lesions of an OVA-sensitized mouse
148 model[23]. In contrast, PGE_2 was also suggested to induce AD by favoring a Th2 immune
149 milieu and directly enhancing B-cell production of IgE[24,25]. Furthermore, we and others have
150 recently reported that PGE_2 through EP2 and EP4 receptors augmented IL-17 and IL-22
151 productions, and blockade of PGE_2 signaling during T-cell differentiation limited acute
152 contact hypersensitivity[26-28]. These findings suggest that PGE_2 may have both suppressive
153 and provocative roles in the development of AD[21]. However, it is unclear how PGE_2
154 regulates IL-22 production and chronic, atopic skin inflammation.

155 Here we report that PGE_2 promotes IL-22 production from Th17 and Th22 T-cells
156 through its receptors EP2 and EP4. This effect is mediated by cAMP-protein kinase A (PKA)
157 signaling and induction of aryl hydrocarbon receptor (AHR), a transcription factor critical for
158 both adaptive and innate IL-22 expression[29]. T-cell specific EP4 deficiency diminishes
159 hapten-induced IL-22-expressing T cells in skin-draining lymph nodes (LNs). Accordingly,
160 inhibition of PGE_2 production limits skin inflammation in an animal model for ACD induced
161 by repeated OXA challenges in mice. Furthermore, genes related to PGE_2 signaling are over-
162 expressed in human atopic lesional skin, and positively correlate with expression of IL-22
163 pathway genes. This relationship between expression of PGE_2 and IL-22-related genes is no
164 longer evident after successful corticosteroid or UVB treatments. These findings suggest that
165 PGE_2 facilitates ACD through promoting adaptive IL-22 signaling.

Methods

Mice

Wild-type C57BL/6 mice were purchased from Harlan UK. Lck-Cre mice were crossed to lox-flanked *Ptger4* (EP4-floxed) mice[30] to generate mice with selective EP4 deficiency in T cells (EP4cKO) as previously reported[27,28]. All mice were bred and maintained under specific pathogen-free conditions in accredited animal facilities in the University of Edinburgh and Kyoto University. Mice were aged >7 weeks old at the beginning of use and sex-matched. All experiments were conducted in accordance with the UK Scientific Procedures Act of 1986 and had local institutional ethical approval.

Reagents

Antibodies to mouse CD3 (clone 145-2C11), CD28 (clone 37.51), CD45 (clone 30-F11), CD4 (clone L3T4), CD8 (clone 53-6.7), IL-17A (clone eBio17B7), IL-22 (clone IL22J0P) and to human CD3 (clone OKT3) and IL-22 (clone 142928) were from eBioscience or Biolegend. Anti-human IL-22 was from R&D Systems. Mouse CD4 microbeads were from Miltenyi Biotec. Recombinant human TGF-β1 and recombinant mouse IL-6 and IL-23 were purchased from Biolegend. PGE_2, 17-phenyl trinor PGE_2 (EP1/3 agonist), Butaprost (EP2 agonist), CAY10598 or L-902,688 (EP4 agonist), PF-04418948 (EP2 antagonist), and L-161,982 (EP4 antagonist) were from Cayman. Db-cAMP, PKA Inhibitor 14-22 (PKI), CH-223191, oxalozone, indomethacin, phorbol myristate acetate (PMA), Ionomycin were from Sigma or Calbiochem.

Oxazolone-induced ACD model

The OXA-induced mouse ACD model was induced as reported[11]. Briefly, mice were sensitized with 3% OXA in EtOH on shaved abdominal skin and after 5 days were repeatedly

challenged with 0.6% OXA in EtOH on one ear once every two days for a total of 5 challenges. The opposing ear was challenged with vehicle (pure EtOH) to serve as a control. Mice were culled at 6 h after the last challenge and the ears and ear-draining LNs collected for further analysis. Ear samples were fixed with 10% neutral buffered formalin solution (Sigma), embedded in paraffin wax, and 5μm sections used for staining with hematoxylin and eosin (H&E). In some experiments, EP4cKO or control mice were sensitized with 0.5% dinitrofluorobenzene (DNFB), and 5 days later skin-draining LNs were collected for further analysis.

T-cell isolation and culture

Mouse $CD4^+$ T-cells were isolated from spleens using autoMACS (Miltenyi). Cells were cultured in complete RPMI1640 medium containing 10% FBS and stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) antibodies plus various cytokines and other compounds as indicated in figures. For certain experiments, Th17 cells were differentiated from $CD4^+$ T-cells by TGF-β1 and IL-6 for 3-4 d. IL-22 levels in supernatants were measured using mouse IL-22 enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go!® kits (eBioscience).

Surface and intracellular staining

For surface staining, cells were stained on ice for 30 min with anti-CD45, anti-CD3e, anti-CD4 and anti-CD8 Abs. For intracellular staining of IL-22, cells were stimulated with PMA and ionomycin for 4-5h in the presence of GolgiPlug (BD Bioscience). Cells were then harvested and fixed by BD Cytofix/Cytoperm Fixation Buffer (BD Bioscience) for 30min and then stained with anti-human/mouse IL-22 (clone IL22JOP, eBioscience) or anti-human

IL-22 in BD Perm/Wash Buffer for 1h. Flow cytometry was performed on a BD LSRFortessa (BD Bioscience) and analyzed by FlowJo software (Tree Star).

Real-time PCR

RNA purification from T-cells was performed using the Rneasy Mini Kit (Qiagen). cDNA was obtained by reverse transcription using High-capacity cDNA Reverse Transcription Kits (ABI). Samples were analyzed by real-time PCR with SYBR Premix Ex Taq II (Tli RNase H Plus) kit (Takara) or GoTaq qPCR Master Mix (Promega) on the Applied Biosystem 7900HT Fast machine. The following primers were used. *Gapdh* forward, 5'-TGAACGGGAAGCTCACTGG-3'; *Gapdh* reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. *Il22* forward, 5'-CATGCAGGAGGTGGTACCTT-3'; *Il22* reverse, 5'-CAGACGCAAGCATTTCTCAG-3'. *Ahr* forward, 5'-TGCACAAGGAGTGGACGA-3'; *Ahr* reverse, 5'-AGGAAGCTGGTCTGGGGTAT-3'. *Ccr4* forward, 5'-TGTCCTCAGGATCACTTTCAGA-3'; *Ccr4* reverse, 5'-GGCATTCATCTTTGGAATCG-3'. Expression was normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and presented as relative expression to control group by the $2^{-\Delta\Delta Ct}$ method.

Gene expression of human skin biopsies from microarray datasets

Microarray gene expression data of human skin biopsies were retrieved from Gene Expression Omnibus datasets (GSE16161, GSE32924, GSE36842, GSE32473 and GSE27887)[31-35]. Patients information and skin sample have been described previously[31-33,35]. In brief, skin biopsy specimens were collected from patients with moderate-to-severe AD (Scoring of Atopic Dermatitis 20-97) and healthy volunteers under institutional review board–approved protocols (written consent obtained). Patients (age 16-81, mean age 40 from three cohorts) with an acute exacerbation of chronic AD and without any therapy for more

than 4 weeks were included. Biopsy specimens were obtained from acute lesional skin which was actively involved, and erythematous lesions with atopic dermatitis and were frozen in liquid nitrogen for RNA extraction[31-33,35]. To standardize data across a wide range of experiments and to allow for the comparison of microarray data independent of the original hybridization intensities, gene expression levels were transformed to z-score values[36]. *P* values were calculated by nonparametric Wilcoxon-Mann-Whitney test, paired nonparametric tests with post-hoc Dunn's multiple comparisons or paired 2-way analysis of variance (ANOVA) test with post-hoc Bonferroni's multiple comparisons test. Correlations between expression levels of two genes were calculated by nonparametric Spearman correlation test.

Statistical analyses

All data were expressed as mean ± SEM or scatter dot-plots in which each dot represents one mouse, one AD patient or healthy individual. Statistical significance between two groups was examined by the Student's *t*-test or Mann-Whitney test, while the one-way and two-way ANOVA with post-hoc Bonferroni's multiple comparisons test were used to evaluate multiple groups unless otherwise indicated in figure legends. Statistical analyses were performed using Prism 6 software (GraphPad) and a $P<0.05$ was considered as statistically significant.

Results

PGE_2 promotes IL-22 production *in vitro* through its receptors EP2 and EP4

We have recently reported that PGE_2 promotes IL-22 production from group 3 innate lymphoid cells (ILC3s)[28]. We thus evaluated whether PGE_2 also promoted IL-22 production from T-cells. To address this question, we isolated $CD4^+$ T-cells from mouse spleens, activated with anti-CD3 and anti-CD28 antibodies (Abs), and co-cultured with various cytokines. Addition of exogenous PGE_2 enhanced *Il22* gene expression with IL-6 or IL-23 alone, IL-6 + IL-23 (Th22 priming condition) or IL-6 + IL-23 + TGF-β (Th17 priming condition) (**Fig 1A**). IL-22 protein production in supernatants of primary cell cultures was also elevated by PGE_2 in the presence of IL-23 or IL-23 + IL-6 (**Fig 1B**). It is important to note that these data are in agreement with previous findings[37] that TGF-β inhibits IL-22 production even in the presence of PGE_2 (Fig 1A,B). Moreover, when same numbers of PGE_2-stimulated T-cells and control T-cells were washed and then re-stimulated with T-cell receptors (i.e. anti-CD3 and anti-CD28), PGE_2-stimulated T-cells produced more IL-22 than control T-cells even in the presence of TGF-β (**Fig 1C**), suggesting that PGE_2-treated cells have higher capability to produce IL-22 at the single cell level.

To define which PGE_2 receptor mediates IL-22 production, we cultured T-cells under Th22-priming conditions and selectively activated EP2 and EP4. Both EP2 and EP4 agonists individually mimicked augmentation of IL-22 production (**Fig 1D**). To further confirm the roles of PGE_2-EP2/EP4 signaling in inducing IL-22 production of T-cells, we also cultured differentiated Th17 T-cells and found that PGE_2 still promotes IL-23-driven IL-22 gene and protein expression (**Fig 1E and 1F**). Moreover, enhancement of IL-22 production by EP2 or EP4 agonists was prevented by co-treatment of antagonists against EP2 or EP4, respectively (**Fig 1F**); elevated IL-22 production by PGE_2 was also diminished by co-administration of

EP2 and EP4 antagonists (**Fig 1F**). In addition, PGE_2 also increased IL-22 production in Th17 cells in the absence of other cytokines (**Fig 1E and 1F**), suggesting a potentially direct action of PGE_2 to promote *Il22* gene expression. These data indicate the involvement of EP2 and EP4 in PGE_2 facilitation of IL-22 production from T cells *in vitro*.

Cyclic AMP promotes IL-22 production from T cells through the transcription factor aryl hydrocarbon receptor (AHR).

As both EP2 and EP4 receptors activate cAMP-PKA pathway in T cells[26], we next examined whether cAMP mediates PGE_2 facilitation of adaptive IL-22 production. Like PGE_2, db-cAMP promoted IL-22 production in a concentration-dependent manner in either presence or absence of IL-23 (**Fig 2A**), confirming a direct action of cAMP on IL-22 induction. The increased IL-22 production by cAMP was prevented by PKI, a PKA inhibitor, in a concentration-dependent manner (**Fig 2B**). Cyclic AMP also up-regulated *Il22* gene expression in IL-23-stimulated T-cells, which was prevented by cycloheximide, an inhibitor for eukaryote protein synthesis (**Fig 2C and 2D**), suggesting that the PGE_2-cAMP pathway promoted adaptive IL-22 production through synthesis of new protein(s).

The transcription factor AHR has been reported to regulate IL-22 production in both T-cells and ILC3s[29]. We thus investigated whether AHR contributes to PGE_2-cAMP signaling-dependent increase in adaptive IL-22 production. We first checked that both PGE_2 and cAMP up-regulated *AHR* gene expression in T-cells independently of cytokine stimuli (**Fig 2E and 2F**). Importantly, a small-molecule AHR inhibitor CH-223191 effectively suppressed cAMP-dependent IL-22 gene expression and protein production from T cells (**Fig 2G and 2H**) although it had no effect on AHR expression itself (**Fig 2F**). These results suggest that PGE_2-cAMP signaling promotes IL-22 production from T-cells through induction of AHR.

309

310 **PGE_2-EP4 signaling promotes adaptive IL-22 production *in vivo***

311 To investigate whether PGE_2 promotes adaptive IL-22 production *in vivo*, we topically
312 sensitized the abdominal skin of control mice and mice with specific EP4 deletion in T cells
313 (EP4cKO mice[27]) with DNFB and measured IL-22-producing T-cells in skin-draining LNs 5
314 days after sensitization using flow cytometry. Both percentages and absolute numbers of IL-
315 22-producing T-cells were markedly reduced in EP4cKO compared to control mice (**Fig 3A**
316 **and 3B**). Furthermore, EP4 deficiency reduced *Il22* gene expression in skin-draining LN
317 $CD4^+$ T cells (**Fig 3C**). These data indicate that PGE_2-EP4 signaling promotes adaptive IL-22
318 production *in vivo*.

319

320 **Endogenous PGE_2-EP4 signaling in T cells promotes allergic contact dermatitis**

321 We next investigated whether PGE_2 has a pathogenic role in development of allergic skin
322 inflammation. To address this question, we used an animal model mimicking human ACD.
323 We sensitized wild-type C57BL/6 mice with 3% oxalozone (OXA) on abdominal skin and
324 after 5 days we repeatedly challenged mice with 0.6% OXA every two days for a total of 5
325 challenges. Indomethacin was administrated to mice in drinking water to inhibit endogenous
326 PGE_2 production. Once DCs in the skin capture the antigen OXA, they migrate to skin dLNs
327 and present antigen to T cells for their activation. Because the migration of antigen-capturing
328 DCs to skin dLNs peaks between 18-24h requires PGE_2-EP4 signaling[38], we only treated
329 mice with indomethacin 24 h post the OXA sensitization (**Fig 4A**). Consistent with previous
330 reports[11], repeated challenge with OXA induced allergic skin inflammation (**Fig 4B and 4C**).
331 Ears of OXA-challenged, indomethacin-treated mice showed decreased swelling compared to
332 vehicle-treated mice (**Fig 4B**). Indomethacin had no effect on the thickness of vehicle
333 (EtOH)-challenged ears. Compared to EtOH-challenged ears, histology of OXA-challenged

ear skin showed features of eczema, namely parakeratosis, acanthosis and focal spongiosis with a dense and diffuse dermal infiltrate containing eosinophils (**Fig 4C**). However, in skin from the indomethacin-treated mice the epidermal changes are less pronounced, and the dermal infiltrates are markedly reduced (**Fig 4C**). Furthermore, whilst repeated OXA challenge recruited $CD4^+$ and $CD8^+$ T cells to ear-draining LNs (**Fig 4D**), indomethacin treatment reduced both this recruitment and the T-cell capacity to produce IL-22 (**Fig 4E**). To further investigated whether this pro-inflammatory effect of PGE2 on OXA-induced ACD development was mediated by EP4 and T cells, we performed this model on EP4cKO and control ($EP4^{fl/fl}$) mice. OXA induced significantly less ear skin inflammation in EP4cKO mice compared to control mice (**Fig. 4F**), suggesting that PGE_2-EP4 signaling in T cells promotes ACD development.

PGE_2-dependent IL-22 production and signaling in human atopic lesional skin

We next investigated whether PGE_2-dependent adaptive IL-22 production and signaling can be found in human atopic skin. We analyzed mRNA expression of genes related to PGE_2 metabolism (**Fig 5A**) and IL-22 signaling pathways in human lesional AD and normal skin biopsies. As reported previously[32], mRNA expression levels for Th17/Th22 related genes (*IL17A, IL22*, *IL23R, AHR*), IL-22-induced products (e.g. *S100A7, S100A8, S100A9*), and skin-homing chemokines (*CCR4*) were over-expressed in lesional AD compared to normal skin (**Fig 5B**). Strikingly, mRNA expression levels for PGE_2 synthases (*PTGS2, PTGES, PTGES2*) were also up-regulated in human atopic lesional skin, and expression levels for the PGE_2 degradation enzyme *HPGD* (hydroxyprostaglandin dehydrogenase 15-(NAD)) was in contrast down-regulated in lesional AD skin (**Fig 5B**). These gene expression data are consistent with previous reports showing increased PGE_2 levels in lesional AD skin[21]. There was a trend that expression of *PTGER4* gene (encoding PGE_2 receptor EP4) was up-regulated

in lesional AD skin, but expression of *PTGER2* (encoding PGE_2 receptor EP2) was significantly down-regulated in lesional AD skin (Fig. 5B). Expression of Th2 cytokines (e.g. IL4, IL5, IL13) were not significantly up-regulated in lesional AD skin, however Th2 chemokines such as *CCL26, CCL18, CCL22, CCL17, CCL11* and *CCL5* were up-regulated in lesional AD skin compared to normal skin (**Fig 5B**). Interestingly, expression of IL-22 pathway genes showed strongly positive correlation with those of *PTGES* in biopsy samples from lesional AD but not normal skin (**Fig 5C**). There were no correlations between *PTGES* expression and Th2 cytokines (data not shown). However, weak correlations between *PTGES* gene expression and Th2 chemokines in lesional AD skin were observed (**Fig 5D**). These results suggest that PGE_2 signaling is activated and positively correlates with the IL-22 signaling pathway and, probably, the Th2 pathway in human atopic skin.

Finally, we investigated whether current therapies for atopic dermatitis modulate PGE_2 signaling in human AD. We analyzed changes in expression levels of IL-22 and PGE_2 signaling genes in lesional AD skin before and after treatments such as betamethasone (a corticosteroid) by re-analyzing a public microarray dataset[34]. Compared to baseline levels, treatments with betamethasone reduced *IL22* gene expression in atopic lesional skin. Betamethasone also significantly reduced expression of IL-22-related genes such as *AHR, S100A8, S100A9* (**Fig 6A**). Expression of PGE_2 synthases (e.g. *PTGES, PTGES2) and EP4 receptor (PTGER4*) was significantly downregulated by betamethasone treatment, while EP2 receptor (*PTGER2*) expression was not changed (**Fig 6A**). This observation is consistent with findings that betamethasone treatment reduces PGE_2 production[39,40]. Interestingly, expression of *PTGES, PTGES2* and *PTGER4*, but not *PTGER2*, positively correlated with *IL22* expression in atopic biopsies at baseline, while expression of *HPGD*, which mediates PGE_2 degradation, negatively correlated with *IL22* expression (**Fig 6B**). Furthermore, we have also

384 reanalyzed gene expression of skin biopsies before and after treated with UVB from our
385 previous dataset[35]. Expression of PGE_2 synthases *PTGS1* and *PTGES* in lesional AD skin
386 were down-regulated after treatment with UVB (**Fig 6C**). Interestingly, although IL-22 gene
387 expression was not changed by UVB treatment, most likely due to the small number of
388 samples, changes in expression of IL-22 activated genes (e.g. *S100A8* and *S100A9*) positively
389 correlated with changes in expression of PGE_2 synthases (i.e. *PTGS1, PTGES* and *PTGES2*)
390 in atopic lesional, but not in non-lesional biopsies (**Fig 6D and data not shown**). Together,
391 these data indicate that PGE_2, most likely through the EP4 receptor, plays a role in IL-22
392 regulation in human skin.

Discussion

The cytokine IL-22, notably produced by Th22 and Th17 T-cells, is emerging as a key player in AD[3,12,16], leading to a new therapy targeting IL-22 signaling in AD clinical trials (NCT01941537). Genome-wide association studies have identified that gene polymorphisms of the PGE_2 receptor EP4 were associated with T cell-mediated human autoimmune and allergic inflammatory diseases[41,42]. However, the role of PGE_2 in promoting IL-22 production and development of AD remain to be determined. Therefore, in this study, we examined this using *in vitro* cell cultures and *in vivo* ACD mouse model as well as interrogating gene expression in human AD lesional skin before and after common treatments for AD.

We show that PGE_2 markedly stimulates IL-22 production from T-cells, which complements our previous findings that PGE_2 promotes innate IL-22 production from ILC3s[28]. It is worth noting that PGE_2 promotes IL-22 production from freshly activated $CD4^+$ T-cells under various conditions including Th17 and Th22-priming conditions. The effect of PGE_2 was mimicked by activation of EP2 and EP4 receptors and was prevented by antagonists against these receptors, confirming the involvement of these two receptors in adaptive IL-22 production *in vitro*. Further studies showed that the cAMP-PKA signaling pathway, activated by engagement of EP2 and EP4, mediates PGE_2 enhancement of IL-22 production through induction of newly expressed AHR. Our results thus uncover a new, targetable molecular mechanism for regulation of adaptive IL-22 by the PGE_2-cAMP-AHR axis.

The role of PGE_2 in promoting adaptive IL-22 production was also confirmed *in vivo*. T-cell specific EP4 deficiency led to large reduction of $IL\text{-}22^+$ T-cells in skin-draining LNs in response to hapten sensitization and attenuated repeated OXA challenge-induced allergic skin inflammation in the mouse model of ACD, indicating a critical role of endogenous EP4

signaling in T-cells for IL-22 expression and function *in vivo*. This is similar to innate IL-22 expression which also requires PGE_2-EP4 signaling[28], suggesting a shared mechanism for regulating both the adaptive and innate IL-22 responses. Inhibition of endogenous PGE_2 production by a COX inhibitor successfully prevented accumulation of T-cells in ear-draining LNs, reduced T-cell production of IL-22 and attenuated allergic skin inflammation induced by repeated OXA challenges. Given that PGE_2 also promotes IL-17 production from both mouse and human Th17 T-cells[26,43] and that IL-17 may participate in creating the ACD phenotype[9-12], IL-17 may also be involved in the PGE_2-facilitated ACD pathogenesis.

Facilitation of IL-22 production by PGE_2 may contribute to several human inflammatory diseases such as AD and psoriasis[44]. Indeed, parallel up-regulations of IL-22 pathway genes, PGE_2 synthases were observed in human atopic lesional skin, whereas the PGE_2 degrading enzyme 15-PGDH (encoded by *HPGD*) was down-regulated in AD skin. This is consistent with previous observations showing increased PGE_2 levels in lesional AD skin[22]. Moreover, our data indicate that IL-22 signaling positively correlated with PGE_2 signaling in atopic lesional skin, and these correlations were absent in both normal skin or in lesional AD skin following successful steroid or UVB treatments. These findings potentially suggest a PGE_2-dependent IL-22 production and signaling in human atopic skin.

Besides IL-22^+ T-cells, Th2 cells (especially cutaneous lymphocyte antigen-positive population with skin-homing capacity) are expanded in severe AD[45]. Th2 cells suppress major terminal differentiation proteins (i.e. filaggrin and loricrin) and predominates in the acute phase of AD through production of cytokines (e.g. *IL4* and *IL13*) and chemokines (e.g. *CCL17, CCL18, CCL26*, etc)[33,46]. The Th2 response is thus critical for AD pathogenesis. Inhibition of COX2 has been reported to enhance Th2 cytokine production and Th2 response

443 to ovalbumin-induced epicutaneous sensitization[23], suggesting a potential inhibitory role of
444 PGs in Th2 cell-driven allergic inflammation. In human lesional AD skin, however, there was
445 no correlations between the PGE_2 pathway and Th2 cytokines and, in contrast, weak positive
446 correlations between the PGE_2 pathway and Th2 chemokines, suggesting a possible
447 promoting role of PGE_2 in Th2 response in AD. This may be because PGE_2 promotes IL-17
448 and IL-22 production[26-28,42] and subsequently IL-17 exacerbates Th2 type inflammation
449 during the initiation of AD[11,47].
450
451 We acknowledge this report has several limitations. For example, our analysis of gene
452 expression profiles in human skin biopsies were retrieved from public GEO datasets and
453 confirmation of these findings was not performed by methods with higher sensitivity such as
454 real-time PCR using fresh biopsies. Moreover, cytokine expression in protein levels in
455 different T-cell subsets were not measured by flow cytometry in skin of patients and animals.
456 Furthermore, although in mice, the OXA-repeated challenge-induced chronic allergic skin
457 inflammation represents a robust model, additional animal models are required in attempt to
458 fully mimic most pathogenic and physiologic processes during the development of human
459 AD. In addition, due to limitation of resources, the effects of PGE_2 signaling in T cells,
460 especially the involvement of both EP2 and EP4 in T cells, on initiation and progression of
461 ACD could not be interrogated, e.g. using mice with T cell-specific deficiency of both EP2
462 and EP4 receptors. Future prospective studies are therefore required to further understand not
463 only the role of PGE_2 during perpetuating AD skin inflammation but also during its onset.
464
465 In conclusion, our data ascertain that PGE_2 acts as a potent promoter of both adaptive and
466 innate IL-22 production. We have previously found that PGE_2 signaling promotes DC
467 production of IL-23, a cytokine essential for IL-22 expression, as well as for proliferation and

468 maturation of Th17 T-cells[26]. Taken together, our results highlight the PGE_2 signaling
469 pathway as an important stimulus of T-cell responses and AD development. Thus targeting
470 PGE_2 synthesis or its receptors may represent a possible therapeutic strategy for the treatment
471 of AD, and other inflammatory skin diseases such as psoriasis with an active role for IL-22,
472 such as psoriasis.
473

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Figure Legends

Figure 1. PGE_2 promotes T cell production of IL-22 through its receptors EP2 and EP4. **A,** *Il22* mRNA expression in naïve $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 (anti-CD3/CD28) antibodies (Abs) with indicated cytokines in the absence or presence of PGE_2 (100 nM) for 3 days. **B,** IL-22 levels in supernatants of cultures in A. **C.** IL-22 levels in supernatants of naïve $CD4^+$ T cells were cultured as in A and then the same numbers of T cells were re-stimulated with anti-CD3/CD28 Abs for another 3 days. **D,** IL-22 levels in supernatants of naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 Abs with IL-6+IL-23 with or without an EP2 agonist (butaprost, 1 μM) or EP4 agonist (L-902,688, 1 μM) for 3 days and then the same numbers of T cells were re-stimulated with anti-CD3/CD28 Abs for another 3 days. **E,** IL-22 levels in supernatants from Th17 cells re-stimulated with anti-CD3/CD28 Abs without or with IL-23 and various small molecule compounds activating or inhibiting PGE_2 receptors for 3 days. **F,** *Il22* mRNA of re-stimulated Th17 cells with PGE_2 and/or IL-23 for 3 days. EP1/3 agonist 17-phenyl trinor PGE_2 was used at 1 μM while EP2 antagonist PF-04418948 and EP4 antagonist L-161,982 were used at 10 μM. All experiments were performed in duplicates or triplicates and repeated for 2-3 times independently. **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001.

Figure 2. Cyclic AMP promotes IL-22 production from T cells through induction of aryl hydrocarbon receptor (Ahr). A, IL-22 levels in supernatants from Th17 cells re-stimulated with anti-CD3/CD28 Abs without or with IL-23 and indicated concentrations of db-cAMP for 3 days. **B,** IL-22 levels in supernatants from Th17 cells re-stimulated with anti-CD3/CD28 Abs with IL-23, db-cAMP (100 μM) and indicated concentrations of PKI for 3 days. **C,D,** *Il22* mRNA expression levels in Th17 cells re-stimulated with anti-CD3/CD28 Abs with IL-23 and db-cAMP in the absence (C) or presence (D) of cycloheximide (CHX, 1

μM) for 3 days. **E,** *Ahr* mRNA expression in naïve $CD4^+$ T cells cultured with anti-CD3/CD28 Abs and indicated cytokines in the absence or presence of PGE_2 for 3 days. **F-H,** *Ahr* (F) and *Il22* (G) mRNA expression and IL-22 levels (H) in supernatants of Th17 cells cultured with anti-CD3/CD28 Abs, IL-23, db-cAMP and CH-223191 (10 μM) for 3 days. All experiments were performed in duplicates or triplicates and repeated for 2-3 times independently. **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001. ns, non significant.

Figure 3. PGE_2-EP4 signaling in T cells facilitates IL-22 production *in vivo*. A, Representative flow cytometry dot-plot of IL-22 and IL-17 expression in skin-draining LN $CD4^+$ T cells from T cell-specific EP4 deficient (EP4cKO, n=9) mice or control mice (n=10) 5 days post sensitization with 0.5% DNFB. **B,** Percentages and numbers of IL-22^+ $CD4^+$ T cells. Each dot represents one mouse. **C,** *Il22* mRNA expression levels in $CD4^+$ T cells isolated from skin-draining LNs of EP4cKO (n=6) or control (n=6) mice. Each dot represents one mouse. **P*<0.05.

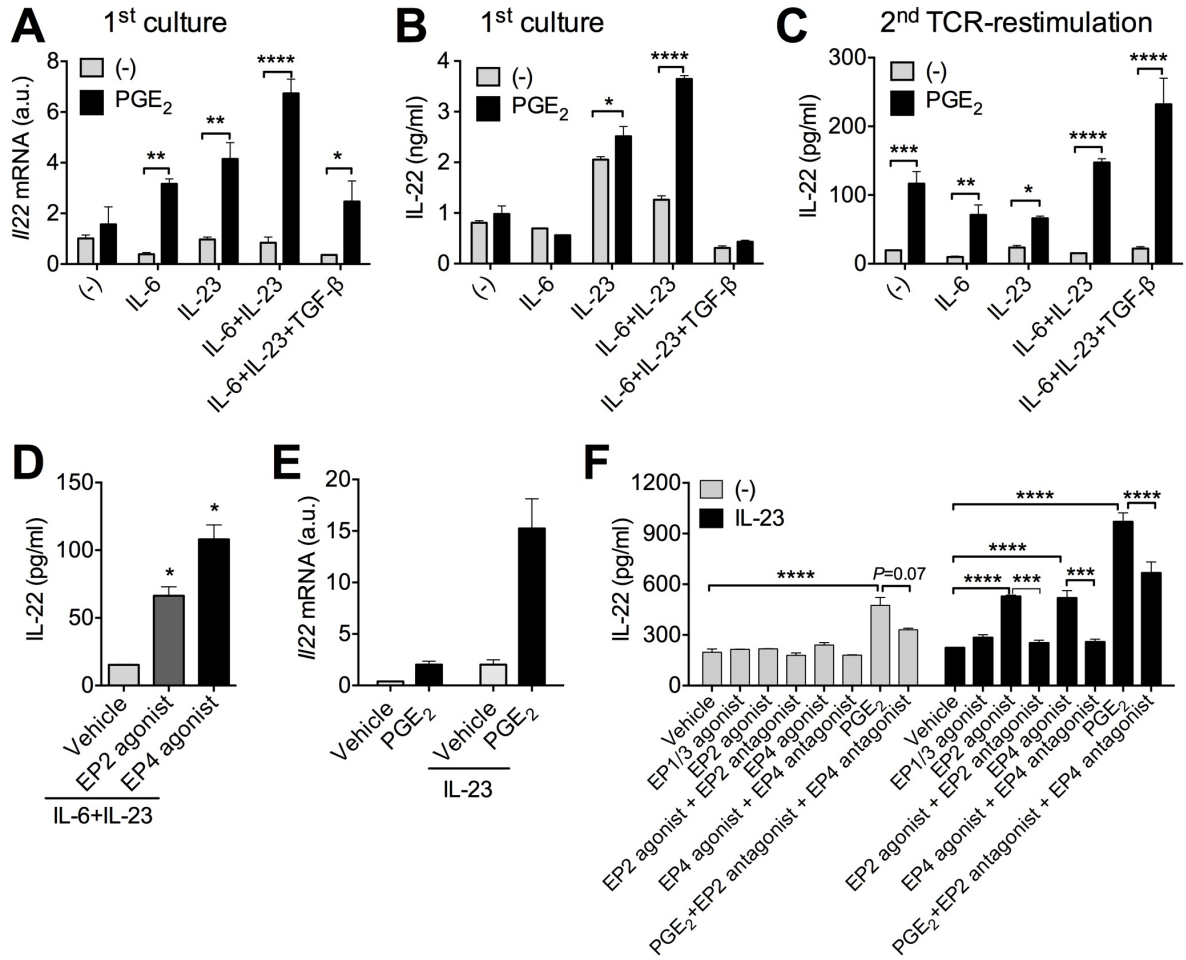
Figure 4. PGE_2 exacerbates atopic skin inflammation in the repeated oxalozone (OXA) challenge model. A-E, WT C57BL/6 mice were sensitized with 3% OXA on abdominal skin on (day 0) and then challenged with 0.6% OXA or EtOH on ears 5 days later. Challenge with OXA was repeated once every two days for a total of 5 challenges. Ears and ear-draining LNs were collected 6 h after the last OXA challenge (n=4-5). **A,** Schematic representation of the experimental protocol. **B,** Ear thickness. **C,** Ear histology. Scale bar, 50 μM. **D,** $CD4^+$ and $CD8^+$ T cells in ear-draining LNs. **E,** IL-22 production by ear-draining LN cells cultured with soluble anti-CD3 or medium only *in vitro* for 3 days. **F,** Swelling of ears from control and EP4cKO mice (n=9-13) sensitized and repeatedly challenged with OXA as in **A** but without indomethacin treatment. **P*<0.05; ****P*<0.001 and *****P*<0.0001.

Figure 5. Over-expression of PGE_2 signaling genes in human lesional AD skin which positively correlate with expression of IL-22 signaling genes. A, Schematic depicting synthesis and metabolism of PGE_2 mediated by COX2 (encoding by *PTGS2*), PGE synthases (encoding by *PTGES* or *PTGES2*) and 15-PGDH (encoding by *HPGD*), respectively. A PGE_2 receptor EP4 (encoding by *PTGER4*) is also shown. **B,** Heat map of expression profiles of Th2 cytokine and chemokine genes and genes related to IL-22 and PGE_2 pathways in human lesional AD skin (n=38) and normal skin (n=32). A color scale bar indicates the Z-score transformed values of microarray gene expression data[31-33]. Z-ratios represent the changes in gene expression levels between the normal and AD lesional groups[36]. *P* values were calculated by nonparametric Wilcoxon-Mann-Whitney tests. Probes with the largest Z-ratios were chosen when several probes represented single genes. **C,D,** Correlations of *PTGES* gene expression versus expression of IL-22 pathway genes (**C**) or Th2 chemokines (**D**) in atopic lesional and normal skins. *P* value was calculated by nonparametric Spearman correlation test.

Figure 6. Steroid and UVB therapies down-regulate PGE_2 and IL-22 signaling pathway genes in human atopic lesional skin. A, Expression profiles of genes related to PGE_2 and IL-22 pathways in human lesional AD skin (n=10) at baseline (AD Base) or after treatment with betamethasone for 3 weeks. The z-score transformed values of microarray gene expression data[34] were used. *P* value was calculated by paired nonparametric tests with post-hoc Dunn's multiple comparisons test. **B,** Correlations of *PTGES, PTGES2, HPGD, PTGER4* gene expression versus that of *IL22* gene in human atopic lesional skins at baseline. *P* value was calculated by nonparametric Spearman correlation test. **C,** Expression of genes related to PGE_2 and IL-22 signaling in human atopic lesional (AL, n=8) and non-lesional (ANL, n=7)

679 skin before (PRE) or 12 weeks after (POST) treatment with UVB. The z-score transformed
680 values of microarray gene expression data[35] were used. *P* value was calculated by paired 2-
681 way ANOVA test with post-hoc Bonferroni's multiple comparisons. **D,** Correlations of
682 changes in expression of IL-22-induced genes (*S100A8, S100A9*) and PGE_2 synthase genes
683 (*PTGS1, PTGES* and *PTGES2)* before (PRE) and after (POST) UVB therapy in atopic AL or
684 NL skins. *P* value was calculated by nonparametric Spearman correlation test.
685

Fig.1



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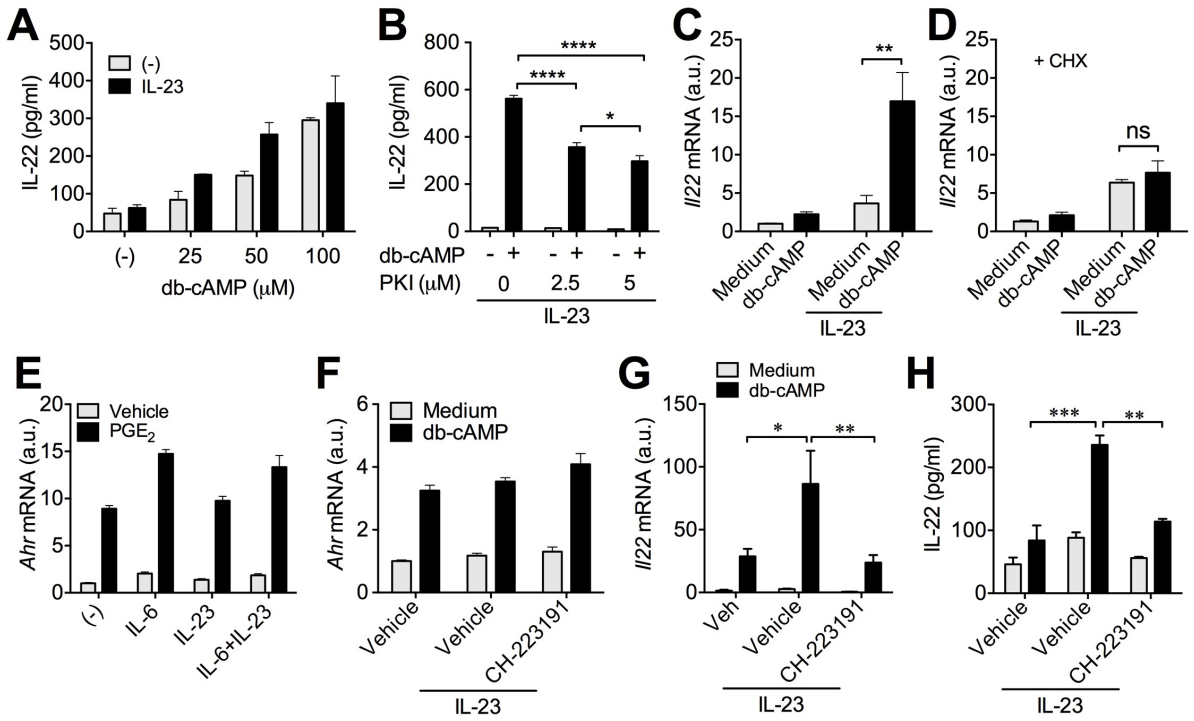
687

688

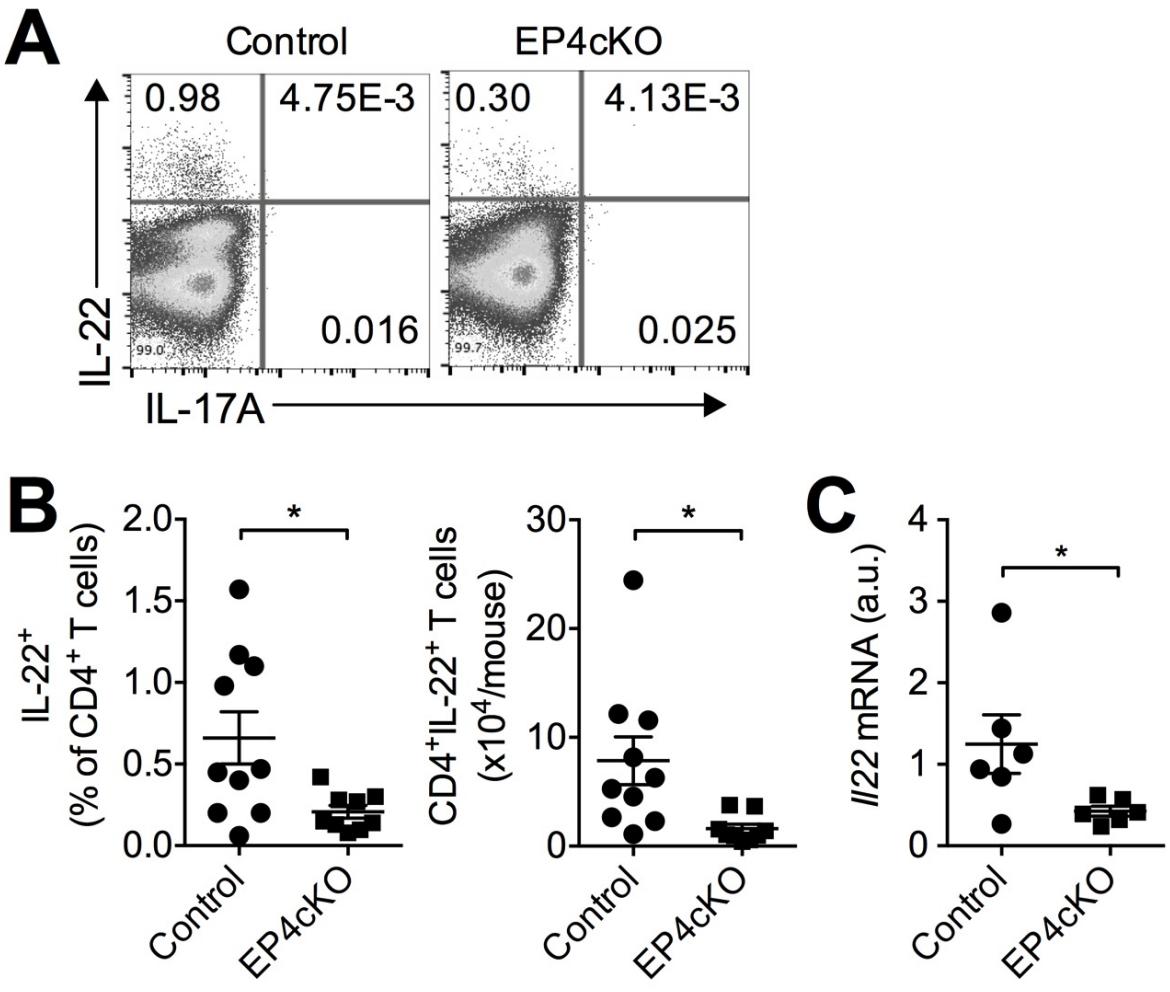
Fig. 2


A
IL-22 (pg/ml)
(-)
IL-23
(-) 25 50 100
db-cAMP (μM)
B
IL-22 (pg/ml)
****
****
*
db-cAMP - + - + - +
PKI (μM) 0 2.5 5
IL-23
C
Il22 mRNA (a.u.)
**
Medium db-cAMP Medium db-cAMP
IL-23
D
Il22 mRNA (a.u.)
+ CHX
ns
Medium db-cAMP Medium db-cAMP
IL-23
E
Ahr mRNA (a.u.)
Vehicle
PGE2
(-) IL-6 IL-23 IL-6+IL-23
F
Ahr mRNA (a.u.)
Medium
db-cAMP
Vehicle Vehicle CH-223191
IL-23
G
Il22 mRNA (a.u.)
Medium
db-cAMP
*
**
Veh Vehicle CH-223191
IL-23
H
IL-22 (pg/ml)
***
**
Vehicle Vehicle CH-223191
IL-23

689

690

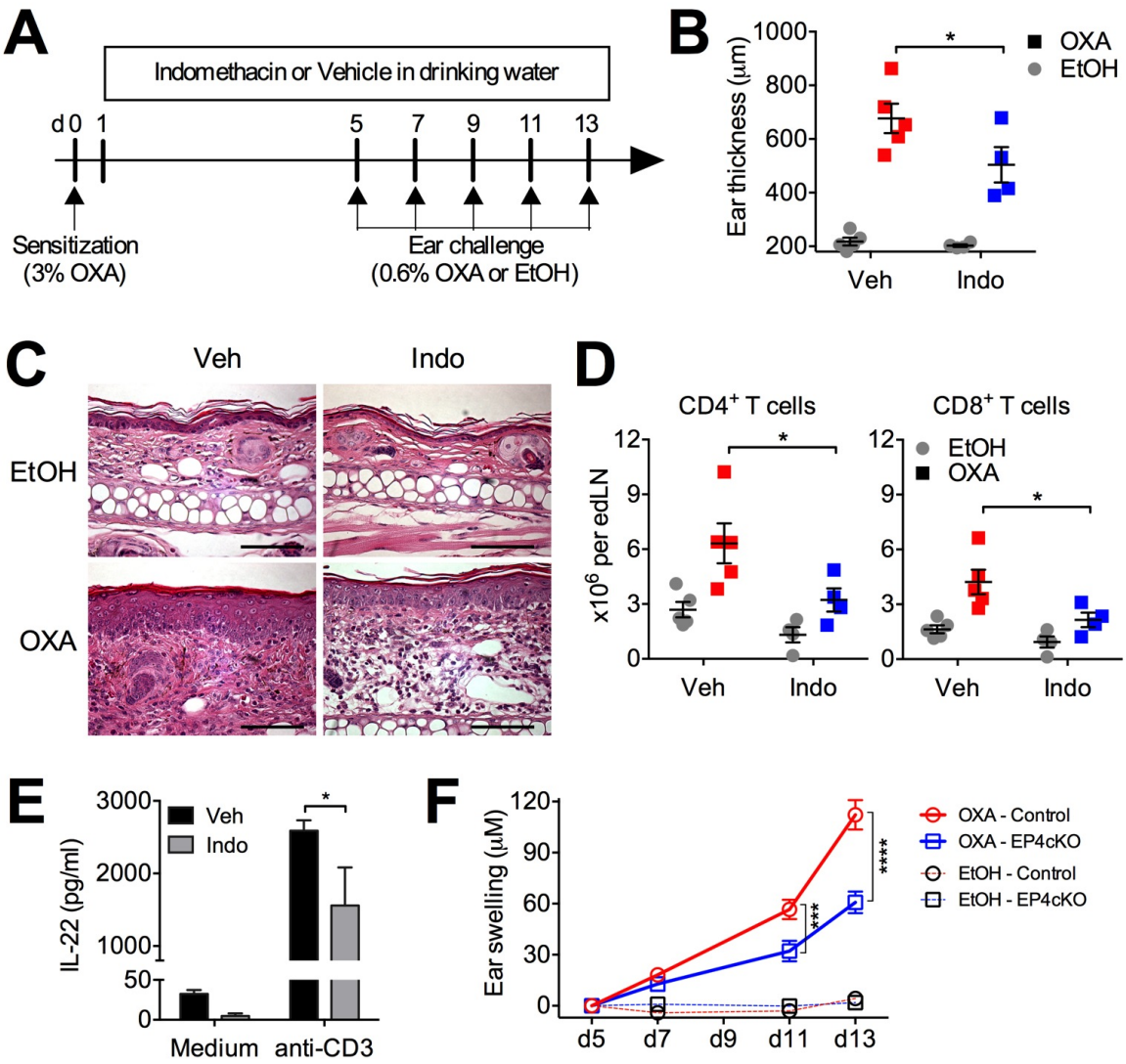
Fig. 3


A
Control
EP4cKO
0.98
4.75E-3
0.30
4.13E-3
0.016
0.025
99.0
99.7
IL-22
IL-17A
B
IL-22+ (% of CD4+ T cells)
CD4+IL-22+ T cells (x10^4/mouse)
C
Il22 mRNA (a.u.)
*
Control
EP4cKO

691

692

Fig. 4


A
Indomethacin or Vehicle in drinking water
d 0 1 5 7 9 11 13
Sensitization (3% OXA)
Ear challenge (0.6% OXA or EtOH)
B
Ear thickness (μm)
1000
800
600
400
200
*
OXA
EtOH
Veh
Indo
C
Veh
Indo
EtOH
OXA
D
CD4+ T cells
CD8+ T cells
x10^6 per edLN
12
9
6
3
0
*
EtOH
OXA
Veh
Indo
E
IL-22 (pg/ml)
3000
2000
1000
50
0
Veh
Indo
*
Medium
anti-CD3
F
Ear swelling (μM)
120
90
60
30
0
d5
d7
d9
d11
d13
***
****
OXA - Control
OXA - EP4cKO
EtOH - Control
EtOH - EP4cKO

693

694

Fig. 5

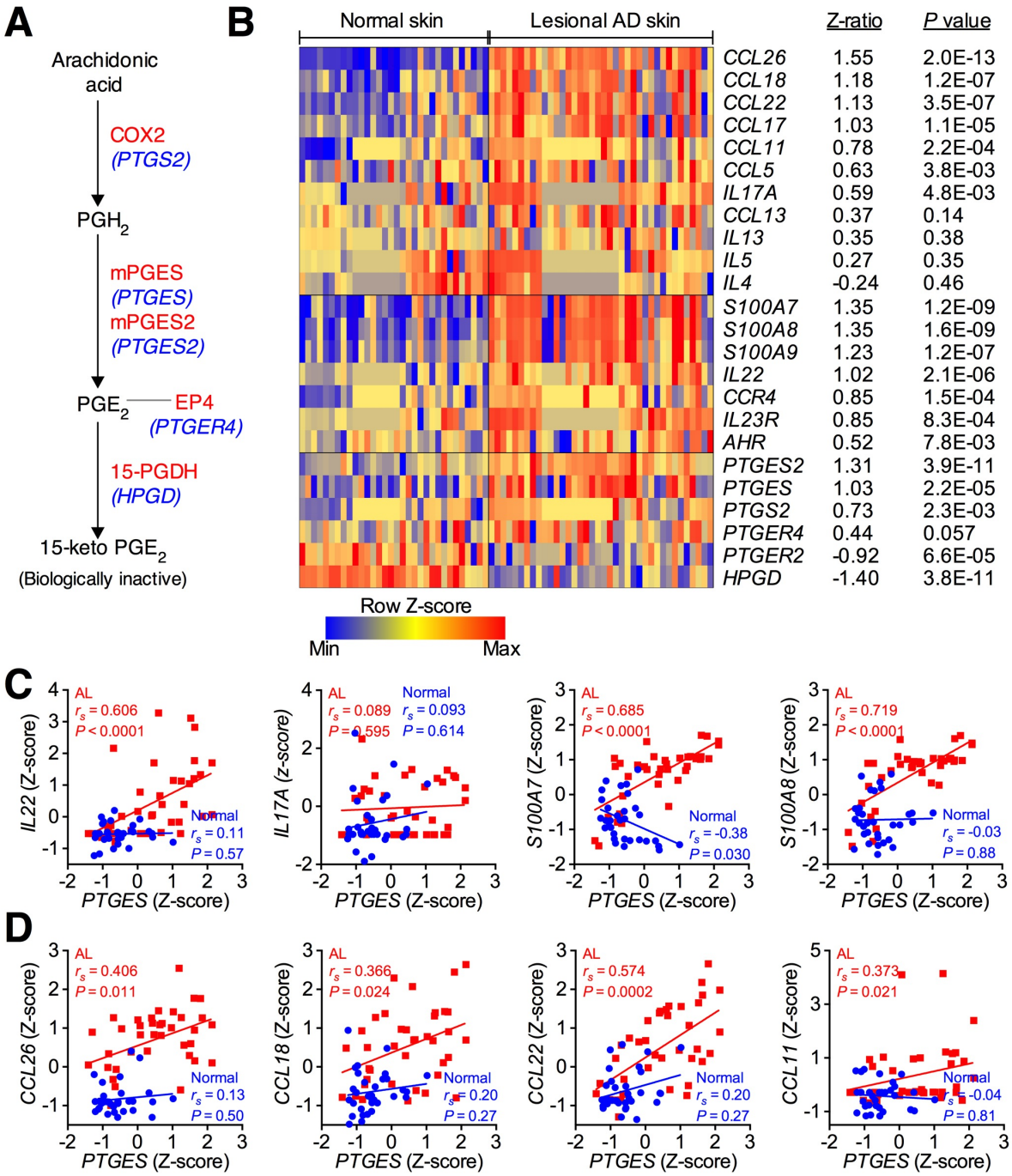
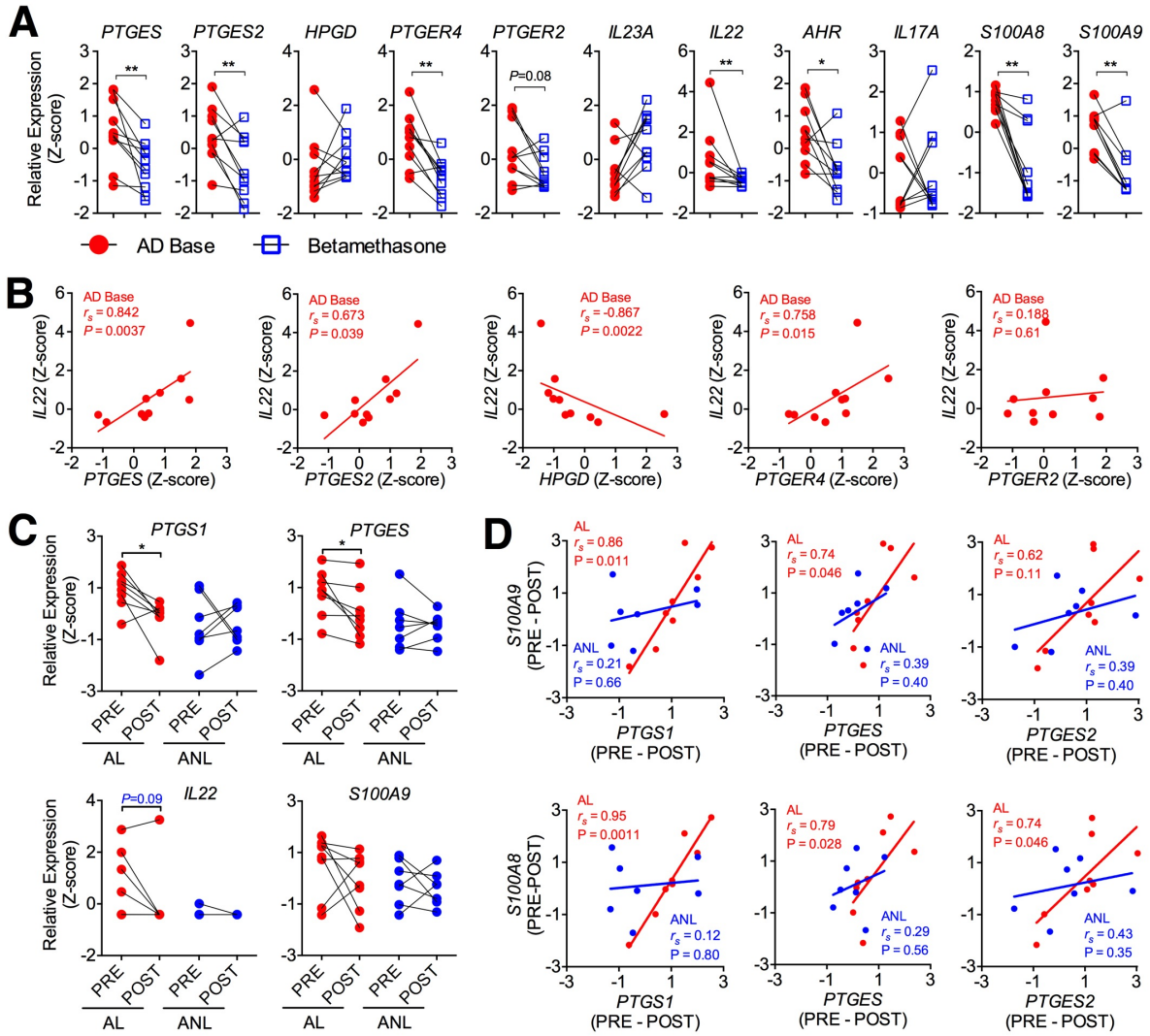

A
Arachidonic acid
COX2 (PTGS2)
PGH2
mPGES (PTGES)
mPGES2 (PTGES2)
PGE2
EP4 (PTGER4)
15-PGDH (HPGD)
15-keto PGE2
(Biologically inactive)
B
Normal skin
Lesional AD skin
Z-ratio
P value
CCL26 1.55 2.0E-13
CCL18 1.18 1.2E-07
CCL22 1.13 3.5E-07
CCL17 1.03 1.1E-05
CCL11 0.78 2.2E-04
CCL5 0.63 3.8E-03
IL17A 0.59 4.8E-03
CCL13 0.37 0.14
IL13 0.35 0.38
IL5 0.27 0.35
IL4 -0.24 0.46
S100A7 1.35 1.2E-09
S100A8 1.35 1.6E-09
S100A9 1.23 1.2E-07
IL22 1.02 2.1E-06
CCR4 0.85 1.5E-04
IL23R 0.85 8.3E-04
AHR 0.52 7.8E-03
PTGES2 1.31 3.9E-11
PTGES 1.03 2.2E-05
PTGS2 0.73 2.3E-03
PTGER4 0.44 0.057
PTGER2 -0.92 6.6E-05
HPGD -1.40 3.8E-11
Row Z-score
Min
Max
C
IL22 (Z-score)
AL rs = 0.606 P < 0.0001
Normal rs = 0.11 P = 0.57
IL17A (z-score)
AL rs = 0.089 P = 0.595
Normal rs = 0.093 P = 0.614
S100A7 (Z-score)
AL rs = 0.685 P < 0.0001
Normal rs = -0.38 P = 0.030
S100A8 (Z-score)
AL rs = 0.719 P < 0.0001
Normal rs = -0.03 P = 0.88
PTGES (Z-score)
D
CCL26 (Z-score)
AL rs = 0.406 P = 0.011
Normal rs = 0.13 P = 0.50
CCL18 (Z-score)
AL rs = 0.366 P = 0.024
Normal rs = 0.20 P = 0.27
CCL22 (Z-score)
AL rs = 0.574 P = 0.0002
Normal rs = 0.20 P = 0.27
CCL11 (Z-score)
AL rs = 0.373 P = 0.021
Normal rs = -0.04 P = 0.81
PTGES (Z-score)

Fig. 6


A
PTGES
PTGES2
HPGD
PTGER4
PTGER2
IL23A
IL22
AHR
IL17A
S100A8
S100A9
Relative Expression (Z-score)
P=0.08
AD Base
Betamethasone
B
AD Base
rs = 0.842
P = 0.0037
rs = 0.673
P = 0.039
rs = -0.867
P = 0.0022
rs = 0.758
P = 0.015
rs = 0.188
P = 0.61
IL22 (Z-score)
PTGES (Z-score)
PTGES2 (Z-score)
HPGD (Z-score)
PTGER4 (Z-score)
PTGER2 (Z-score)
C
PTGS1
PTGES
IL22
S100A9
P=0.09
PRE
POST
AL
ANL
D
AL
rs = 0.86
P = 0.011
ANL
rs = 0.21
P = 0.66
rs = 0.74
P = 0.046
rs = 0.39
P = 0.40
rs = 0.62
P = 0.11
rs = 0.39
P = 0.40
rs = 0.95
P = 0.0011
rs = 0.12
P = 0.80
rs = 0.79
P = 0.028
rs = 0.29
P = 0.56
rs = 0.74
P = 0.046
rs = 0.43
P = 0.35
S100A9 (PRE - POST)
S100A8 (PRE-POST)
PTGS1 (PRE - POST)
PTGES (PRE - POST)
PTGES2 (PRE - POST)