Immunobiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

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

Glucocorticoid dexamethasone down-regulates basal and vitamin D3 induced cathelicidin expression in human monocytes and bronchial epithelial cell line

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ARTICLE INFO

Article history:
Received 30 June 2015
Accepted 1 September 2015
Available online xxx

Keywords:
Antimicrobial polypeptides
CAMP gene
LL-37
Glucocorticoid Receptor
Anti-inflammatory

ABSTRACT

Glucocorticoids (GCs) have been extensively used as the mainstream treatment for chronic inflammatory disorders. The persistent use of steroids in the past decades and the association with secondary infections warrants for detailed investigation into their effects on the innate immune system and the therapeutic outcome. In this study, we analyse the effect of GCs on antimicrobial polypeptide (AMP) expression. We hypothesize that GC related side effects, including secondary infections are a result of compromised innate immune responses. Here, we show that treatment with dexamethasone (Dex) inhibits basal mRNA expression of the following AMPs; human cathelicidin, human beta defensin 1, lysozyme and secretory leukocyte peptidase 1 in the THP-1 monocytic cell-line (THP-1 monocytes). Furthermore, pre-treatment with Dex inhibits vitamin D3 induced cathelicidin expression in THP-1 monocytes, primary monocytes and in the human bronchial epithelial cell line BCi NS 1.1. We also demonstrate that treatment with the glucocorticoid receptor (GR) inhibitor RU486 counteracts Dex mediated down-regulation of basal and vitamin D3 induced cathelicidin expression in THP-1 monocytes. Moreover, we confirmed the anti-inflammatory effect of Dex. Pre-treatment with Dex inhibits dsRNA mimic poly IC induction of the inflammatory chemokine IP10 (*CXCL10*) and cytokine *IL1B* mRNA expression in THP-1 monocytes. These results suggest that GCs inhibit innate immune responses, in addition to exerting beneficial anti-inflammatory effects.

1. Introduction

Glucocorticoids or corticosteroids (GCs) are currently the most preferred mainstream treatment of inflammatory disorders including asthma, chronic obstructive pulmonary disease and cystic fibrosis (Barnes, 2011). GCs act via binding to the glucocorticoid receptor (GR), leading to receptor dimerization (GR-GR). This ligand-homo-dimer complex (GC-GR-GR) binds to glucocorticoid response elements (GRE) in the promoter regions of GC responsive genes with subsequent activation or repression of target genes (Barnes, 2011). GCs inhibit airway inflammation via suppression of genes encoding pro-inflammatory cytokines together with up-regulation of anti-inflammatory cytokines (Barnes, 2011). GC treatment has been associated with secondary infections such as oral candidiasis and long term side effects include osteoporosis, diabetes, cataracts and pneumonia (Barnes, 2011). Thus, investigations into side effects of GC treatment in general and on health status of patients on long term treatment are warranted. We hypothesize that these secondary infections may be associated with negative effects on the innate immune system.

The innate immune system comprises early immediate defense against invading pathogens. Monocytes, macrophages, neutrophils and epithelial cells constitute an important part of the innate

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http://dx.doi.org/10.1016/j.imbio.2015.09.001
0171-2985/

immune system, providing early defense by secretion of multiple antimicrobial and inflammatory polypeptides. Antimicrobial polypeptides (AMPs) include cationic antimicrobial peptides such as cathelicidins and defensins and larger polypeptides such as lactoferrin, lysozyme and secretory leukocyte peptidase inhibitor (SLPI) (Laube et al., 2006). LL-37 is the main cathelicidin in humans, encoded by the *CAMP* gene. LL-37 is stored as a pro-form (pro-LL-37) in cells and is activated upon secretion to the mature form LL-37 by specific proteases. In neutrophils the processing enzymes has been shown to be proteinase 3 (Sørensen et al., 2001). LL-37 has direct antimicrobial activity against multiple pathogens and has been demonstrated to exhibit both anti-inflammatory and pro-inflammatory activities, wound healing and angiogenic properties (Cederlund et al., 2011). Pathogen colonization and invasion have been associated with decreased *CAMP* gene expression. The physiological relevance of cathelicidin is underlined by the fact that the cathelicidin deficient mice (*cnlp* −/−) are susceptible to infections (Nizet et al., 2001). The active form of vitamin D3 1α, 25-dihydroxy vitamin D3 (1,25D3) is a direct inducer of *CAMP* gene expression via the binding and activation of the vitamin D receptor (VDR). VDR binding leads to formation of VDR-RXR (retinoid X receptor) heterodimer. This complex activates target genes including the *CAMP* gene by binding to vitamin D response elements (VDREs) within the gene promoter (Wang et al., 2004). Recent reports have shown positive effects of co-treatment of vitamin D3 and GCs as compared to treatment with GCs alone. Co-treatment with vitamin D3 and GCs was shown to reverse the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients with a positive outcome on the anti-inflammatory treatment (Xystrakis et al., 2006).

In this study, we have analysed the effect of GCs on AMP expression and the effect of co-treatment with vitamin D3. We demonstrate that treatment with Dexamethasone (Dex) down-regulates gene expression of the following AMPs: cathelicidin, human beta defensin 1, lysozyme, lactoferrin and SLPI in the human monocytic cell line THP-1. Furthermore, we show that pre-treatment with Dex suppresses vitamin D3 enhanced cathelicidin expression in both THP-1 and primary monocytes. Dex mediated suppression of *CAMP* gene expression is counteracted by pre-treatment with the GR inhibitor RU486 in THP-1 monocytes. Pre-treatment with RU486 also reverses Dex mediated inhibition of vitamin D3 enhanced cathelicidin expression. Similarly, we observed that Dex treatment inhibited basal and vitamin D3 induced *CAMP* gene expression in the human bronchial epithelial cell line BCi NS 1.1. Finally, we have confirmed the anti-inflammatory effects of Dex in THP-1 monocytes. Pre-treatment with Dex inhibits poly IC induction of genes encoding inflammatory cytokines. In conclusion, we show that treatment with GCs have negative effects on AMP expression *in vitro,* which may have possible therapeutic implications in patients on GC therapy.

2. Materials and methods

2.1. Cell culture and reagents

The THP-1 cell line (THP-1 monocytes) is a human monocytic leukemia cell line commonly used as a model to study monocyte-macrophage differentiation (Auwerx, 1991). This cell line is available from ATCC (Cat. No. TIB-202) and was a kind gift from Dr. Petur Henry Petursen, University of Iceland. The cells were cultured in complete RPMI 1640 medium (Gibco, Cat. No. 52400) supplemented with 10% Fetal Bovine Serum (Gibco, Cat. No. 10270-106), 0.05 mM 2-merceptoethanol (Sigma–Aldrich, USA, Cat. No. M7522) with 20 U/ml penicillin and 20 μg/ml streptomycin as antibiotics (Gibco, Cat.No. 15140-122). For monocyte to macrophage differentiation THP-1 monocytes (1×10^6) were treated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma, Cat. No. P8139) for 24 h. After 24 h treatment the cells were washed thrice with 1x phosphate buffered saline (PBS). Adherent cells were then rested and cultured in PMA free media for 24 h followed by different treatments. Primary monocytes were isolated by adherence method as described previously (Elkord et al., 2005). Blood was isolated from donors with informed consent and approval of the internal ethical committee at Blood Bank, University of Iceland. Briefly, 10 ml buffy coat and 30 ml 1x PBS were mixed in a 50 ml centrifuge tube. This diluted buffy coat was overlaid on 10 ml ficoll-paque (Cat. No. 17-1440-02, Sigma–Aldrich, USA). After 20 min centrifugation at 2000 rpm, the middle mononuclear cell layer was collected and washed twice with 1x PBS at 1200 rpm for 10 minutes. The resulting live mononuclear cells were counted with trypan blue dye (Sigma–Aldrich, USA, Cat.No.T8154) exclusion assay (more than 95% live cells were recovered). A total of 10×10^6 isolated mononuclear cells were then seeded in each well in a 6 well cell culture plate. After that the cells were incubated at 37 °C, 5% CO_2 for 2–3 h and washed three times with 1x PBS. The adherent monocytic cells were then rested in complete RPMI 1640 medium for another 3 h before desired treatments. The purity of adherent primary monocytes was analysed with with anti-CD14 antibody (BD Pharmingen, La Jolla, California, USA, Cat. No. 555398) on FACS Calibur flow cytometer, equipped with a 488 nm argon laser and standard band pass filters, and Cell Quest Pro software version 6.0 (Becton Dickinson, La Jolla, California, USA) and was more than 80% pure. BCi. NS 1.1 is a human bronchial epithelial cell line was a kind gift from Dr. Matthew S Walters, Weill Cornell Medical College, New York NY, USA (Walters et al., 2013) and was established by immortalization with retrovirus expressing human telomerase (hTERT), The BCi. NS 1.1 cells were cultured in Bronchial/Tracheal Epithelial Growth medium (Cell Applications, USA, Cat. No. 511A-RA) supplemented with antibiotics as described above. 2×10^5 cells were seeded in each well in a 6 well cell culture plate and grown to 70–80% confluence before desired treatments. The active form of vitamin D3 1α, 25-Dihydroxy vitamin D3 (1,25D3) (Cat. No. 2551), Dexamethasone (Dex) (Cat. No.1126), Fluticasone Propionate (FP) (Cat. No. 2007), Budesonide (BD) (Cat.No. 2671) and glucocorticoid receptor inhibitor Milfepristone (RU 486) (Cat. No. 1479) were all purchased from Tocirs (R and D Biosystems, USA). The inactive form of vitamin D3 25-Dihydroxy vitamin D3 (25D3) was purchased form Sigma–Aldrich, USA (Cat. No. C9756). The GCs were reconstituted in Dimethyl sulfoxide (DMSO) and vitamin D3 (1,25D3 and 25D3) in 100% ethanol according to manufactures instructions. The final concentration of solvents was kept at 0.1% v/v or less and had no significant effects on expression of genes of interest. Appropriate solvent controls were included in all the experiments.

2.2. RNA isolation and quantitative real time PCR

Total RNA was isolated with NucleoSpin RNA kit (Macherey-Nagel, Germany, Cat. No. 740955) and quantified on a spectrophotometer (Nanodrop, Thermo Scientific, USA). Isolated RNA was reverse transcribed into first strand cDNA according to manufacturer's instructions (High capacity cDNA reverse transcription kit, Life Technologies, USA, Cat. No. 4368814). The cDNA was quantified with Power SYBR green Universal PCR master mix (Applied Biosystems, USA, Cat. No. 4367659) on a 7500 Real time PCR machine (Applied Biosystems, USA). Ubiquitin C (UBC) and Hypoxanthine phosphoribosyltransferase 1 (HPRT1) were utilized as reference genes in all the quantitative real time PCR (q-RT PCR) experiments. An arithmetic average of the Ct values of both reference genes were used. A non-template control was included in all the experiments. Primers for human beta

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Table 1
Primers from Integrated DNA Technologies used in q-RT PCR.

Gene Name	RefSeq ID	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
CAMP	NM_004345	GCACACTGTCTCCTTCACTG	CTAACCTCTACCGCCTCCT
MKP-1 (*DUSP1*)	NM_004417	CCTTCCTCCAGCATTCTTGA	CAGTACAAGAGCATCCCTGTG
IP-10 (*CXCL10*)	NM_001565	CAGTTCTAGAGAGAGGTACTCCT	GACATATTCTGAGCCTACAGCA
IL1B	NM_000576	GAACAAGTCATCCTCATTGCC	CAGCCAATCTTCATTGCTCAAG
UBC	NM_021009	CCTTATCTTGGATCTTTGCCTTG	GATTTGGGTCGCAGTTCTTG
HPRT1	NM_000194	GCGATGTCAATAGGACTCCAG	TTGTTGTAGGATATGCCCTTGA
LZY	NM_000239	CTCCACAACCTTGAACATACTGA	AGATAACATCGCTGATGCTGTAG
LTF	NM_001199149	AATAGTGAGTTCGTGGCTGTC	TGTATCCAGGCCATTGCG
SLPI	NM_003064	CATAAGTCACTGGGCACTTCC	GATACAAGAAACCTGAGTGCCA

defensin 1 (DEFB1, Ref Seq ID: NM 5,218) were designed with Perl Primer (Marshall, 2004) (forward 5′-CCAGTCGCCATGAGAACTTCC-3′ and reverse 5′-GTGAGAAAGTTACCACCTGAGGC-3′) and used at a final concentration of 300 nM. All additional primers were purchased from Integrated DNA technologies (PrimeTime™ predesigned qPCR Assays) and were utilized at a final concentration of 500 nM according to manufacturer's instructions. All the primers gave one single PCR product, which was evaluated with the aid of a melting curve generated on the Real Time PCR machine. Default cycling conditions were as followed: (1) hold stage; 95 °C for 10 min followed by 40 cycles of (2) denatured stage; 95 °C for 15 s (3) annealed/extended stage: 60 °C for 1 min. The $2^{(-\Delta\Delta CT)}$ Livak method was utilized for calculating fold difference over untreated control (Livak and Schmittgen, 2001). The complete list of primers from Integrated DNA Technologies used in the q-RT PCR assay are shown in Table 1.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Total cell lysate was prepared by addition of complete RIPA buffer (Sigma–Aldrich, USA, Cat.No. R0278) containing 1x protease inhibitory cocktail (Halt Protease Inhibitor Cocktail, Life Technologies, USA, Cat.No. 87786) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Life Technologies, USA, Cat.No. 78420). Cells were then washed three times with cold 1x PBS, incubated for 30 min on ice with complete RIPA buffer and spun down at 12.000 rpm for 10 min at 4 °C. Supernatants thus obtained were used for Western Blot analysis. The protein content of supernatants was analyzed by utilizing a protein assay reagent (Bio-Rad, USA, Cat. No. 500-0006). SDS-PAGE (4–12% gradient Bis–Tris SDS gels, Life Technologies, USA, Cat.No. NP0322BOX) and subsequent Western blot analysis was performed using the NuPage blotting kit (Life Technologies, USA, Cat.No. EI0002). Equal amount of proteins was loaded onto the SDS gel and run at 200 V for 35 min. The proteins in the gel were transferred onto a PVDF membrane (Millipore, USA, Cat.No. ISEQ00010) and blocked with 5% non-fat skimmed milk (Blotto, Santacruz Biotechnologies, USA, Cat.No. sc-2325) in 1x PBS with 0.05% Tween 20 (Sigma–Aldrich, USA, Cat.No. P2287). Antibody against LL-37 (polyclonal Rabbit, Cat No. PA-LL37-100) was purchased from Innovagen (Sweden). Interleukin 1-beta (IL1B) (polyclonal Rabbit, Cat. No.sc-7884), VDR (Monoclonal Mouse, Cat No. sc-13133) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (polyclonal Rabbit, Cat No. sc-25778) antibodies were all from Santa Cruz Biotechnologies (USA). The primary antibodies were diluted at a concentration of 1:200–1:1000 in 1x PBS with 0.5% non-fat skimmed milk powder and 0.05% Tween according to manufacturer instructions, and the membrane was incubated in this solution overnight at 4 °C. The following day, the membrane was incubated with 1:10000Horseradish Peroxidase (HRP)-linked secondary anti-Rabbit IgG antibody (Sigma–Aldrich, USA, Cat.No. A0545) or anti-Mouse IgG HRP (Santacruz Biotechnologies, USA, Cat. No. sc-2005), diluted in PBS with 0.5% non-fat skimmed milk powder and 0.05% Tween. Protein bands were visualized by Pierce ECL plus chemiluminescence substrate (Thermo Scientific, USA, Cat.No. 32132) on Image Quant LAS 4000 station (GE Healthcare, USA).

2.4. Statistical analysis

Normally distributed results for q-RT PCR experiments are represented as means and standard error of the means from at least three independent experiments. For comparison of differences between two groups the unpaired Student's *t*-test was used. P value of less than 0.05 was considered statistically significant. All the statistical analysis was performed with the Prism 6 software (Graph Pad, USA). The western blots are representative of at least three independent experiments, showing similar results.

3. Results

3.1. Dexamethasone treatment down-regulates vitamin D3 induced human cathelicidin expression in THP-1 monocytes

First, we analyzed the effect of dexamethasone (Dex) treatment on *CAMP* gene expression encoding the human cathelicidin LL-37 in THP-1 monocytes (Fig 1A). THP-1 monocytes were treated with 10 μM Dex at different time points (3–48 h). We analyzed the mRNA expression of mitogen activated protein kinase phosphatase-1 (MKP-1) and the *CAMP* gene with q-RT PCR. MKP-1 is known to be activated on Dex treatment and was used as a positive control (Zhang et al., 2013a). The expression of MKP-1 was detected at 3 h and continued to increase up to 48 h. Dex treatment inhibited basal *CAMP* gene expression at 3 h (0.24 fold, $p = 0.0457$), 6 h (0.37 fold, $p < 0.0001$), 24 h (0.45 fold, $p < 0.0001$) and 48 h (0.61 fold, $p < 0.0001$) post-treatment. We further analysed the effect of Dex treatment on mRNA level of several other antimicrobial polypeptides (AMPs) such as human beta defensin 1 (*DEFB1*), lysozyme (*LZY*), seceretory leukocyte protease inhibitor 1 (*SLPI*) and lactoferrin (*LTF*). Treatment with 10 μM Dex for 48 h down-regulated *DEFB1* (0.49 fold, $p < 0.0001$), *LZY* (0.69 fold, $p = 0.0005$) and *SLPI* (0.21 fold, $p = 0.0011$) gene expression (Fig 1B). Next, we investigated the effect of the glucocorticoids Dex, Fluticasone Propionate (FP) and Budesonide (BD) on vitamin D3 (1,25D3) induced cathelicidin expression (Fig 1C). THP-1 monocytes were pre-treated with either 100 nM Dex, 10 μM Dex, 100 nM FP or 100 nM BD for 24 h, followed by treatment with 1,25D3 for another 24 h. After 1,25D3 treatment, cells were processed for Western blot analysis. The blot was probed for antibodies against the human cathelicidin LL-37 and vitamin D receptor (VDR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Pre-treatment with Dex suppressed 1,25D3 enhanced pro-LL-37 expression and the reduction was more pronounced in cells treated with 10 μM Dex as compared to 100 nM Dex (Fig 1C). Similarly, pre-treatment with both FP and BD also inhibited 1,25D3 induced pro-LL-37 protein expression (Fig 1C). The active form of

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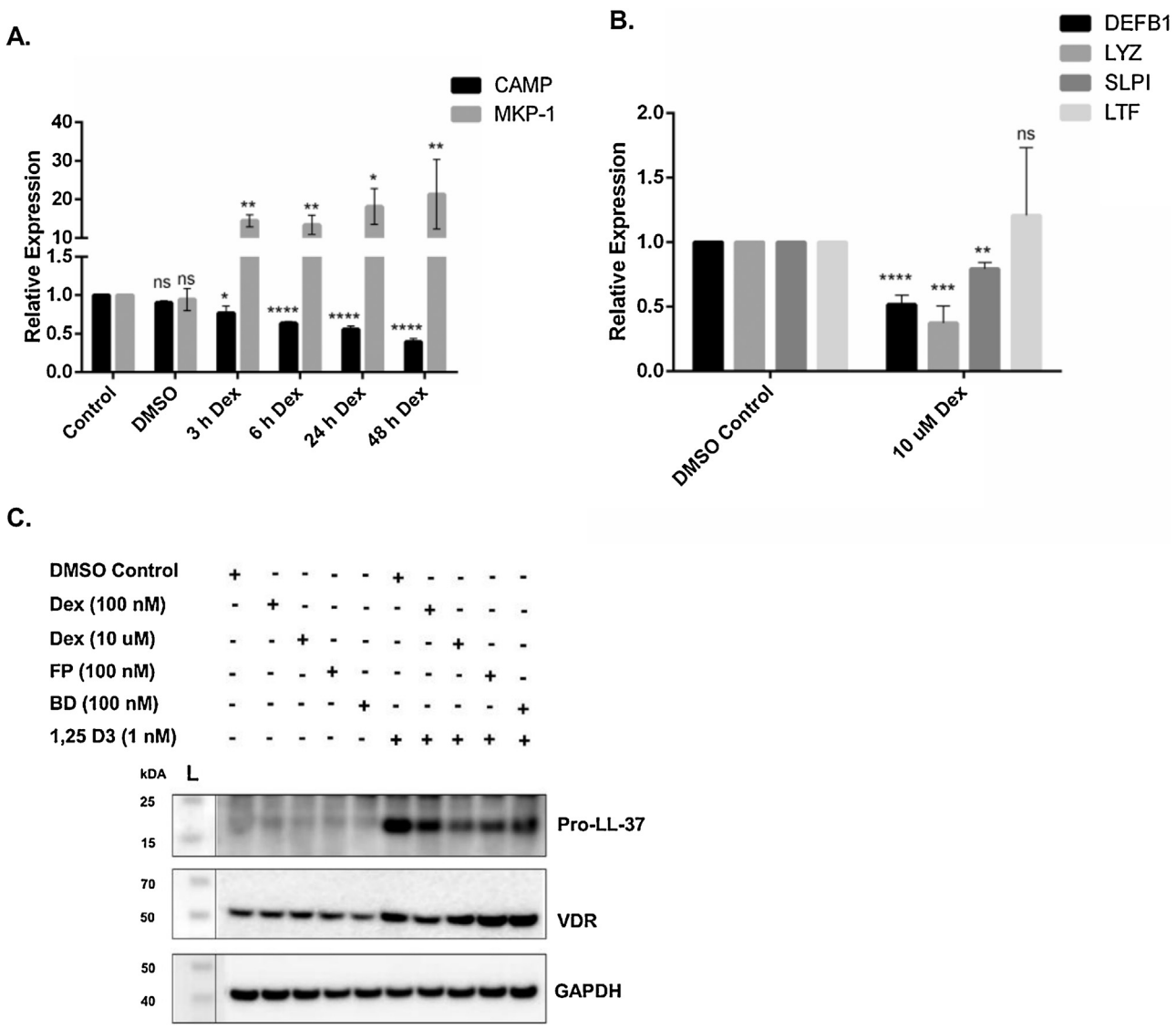

Fig. 1. Dexamethasone down-regulates basal and vitamin D3 induced human cathelicidin expression in THP-1 monocytes. THP-1 monocytes were treated with 10 μM Dex at different time points (3 h-48 h). DMSO was used as a solvent control (A). The cells were then harvested and relative gene expression of the human cathelicidin *CAMP* and MAP kinase Phosphatase-1 (MKP-1) was assessed with q-RT PCR ($n = 3$). In B similarly THP-1 monocytes were treated with 10 μM Dexamethasone (Dex) for 48 h and screened for effects on gene expression of human beta defensin 1 (*DEFB1*), lysozyme (*LZY*), secretory leukocyte peptidase inhibitor-1 (*SLPI*) and lactoferrin (*LTF*) with q-RT PCR. Poly Ubiquitin C (*UBC*) and hypoxanthine-guanine phosphoribosyltransferase -1 (*HPRT1*) were used as reference genes ($n = 3$). In C, THP-1 monocytes were pre-treated with either 100 nM Dex, 10 μM Dex, 100 nM Fluticasone Propionate (FP) or 100 nM Budesonide (BD) for 24 h, followed by treatment with 1 nM 1,25D3 for another 24 h. Protein expression of the human cathelicidin (Pro-LL-37) and vitamin D receptor (VDR) was analyzed with Western blot analysis. GAPDH was used as a loading control. (L = Molecular weight ladder; ns indicates non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$)

vitamin D3 (1,25D3) up-regulates LL-37 expression via activation of VDR (Gombart et al., 2005). Pre-treatment with Dex did not affect 1,25D3 mediated increase in VDR protein expression. FP and BD pre-treatment enhanced 1,25D3 mediated increase in VDR expression (Fig 1C). Similar effects were observed in PMA differentiated THP-1 macrophages (Fig. S1). In contrast to above results Dex treatment enhanced basal *CAMP* gene expression in THP-1 macrophages (Fig. S1).

3.2. Treatment with glucocorticoid receptor inhibitor RU486 counteracts Dexamethasone mediated decrease in human cathelicidin expression

Next, we analysed the role of Glucocorticoid Receptor (GR) in Dex mediated decrease in cathelicidin expression in THP-1 monocytes.THP-1 monocytes were pre-treated with 10 μM of the GR inhibitor RU 486 for 3 h, followed by treatment with 10 μM Dex for 48 h and the mRNA level of *CAMP* transcript was analysed with q-RT PCR. As shown in Fig. 2A, Dex treatment reduced basal *CAMP* gene expression by 0.60 fold ($p < 0.001$). Pre-treatment with RU486 prevented Dex mediated decrease in *CAMP* mRNA level ($p = 0.0010$). Further, THP-1 monocytes were pre-treated with 10 μM RU 486 for 3 h, followed by treatment with 10 μM Dex for 24 h. The cells were further treated with 1 nM 1, 25D3 for 24 h and pro-LL37 expression was analysed with Western blot (Fig. 2B). Pre-treatment with RU486 counteracted Dex mediated decrease in 1,25D3 induced cathelicidin (pro-LL-37) protein expression (Fig. 2B). We also confirmed Dex mediated decrease in *CAMP* gene expression in primary monocytes isolated from fresh buffy coats of two healthy human volunteers (Donor1 and Donor 2, Fig. 2C). Primary monocytes were pre-treated with 10 μM Dex for 24 h, followed by treatment with 10 nM 1,25D3 for another 24 h and *CAMP* mRNA expression was analysed with q-RT PCR. As shown in Fig. 2C, Dex treatment reduced basal *CAMP* mRNA levels in both Donor 1 (0.62 fold) and Donor 2

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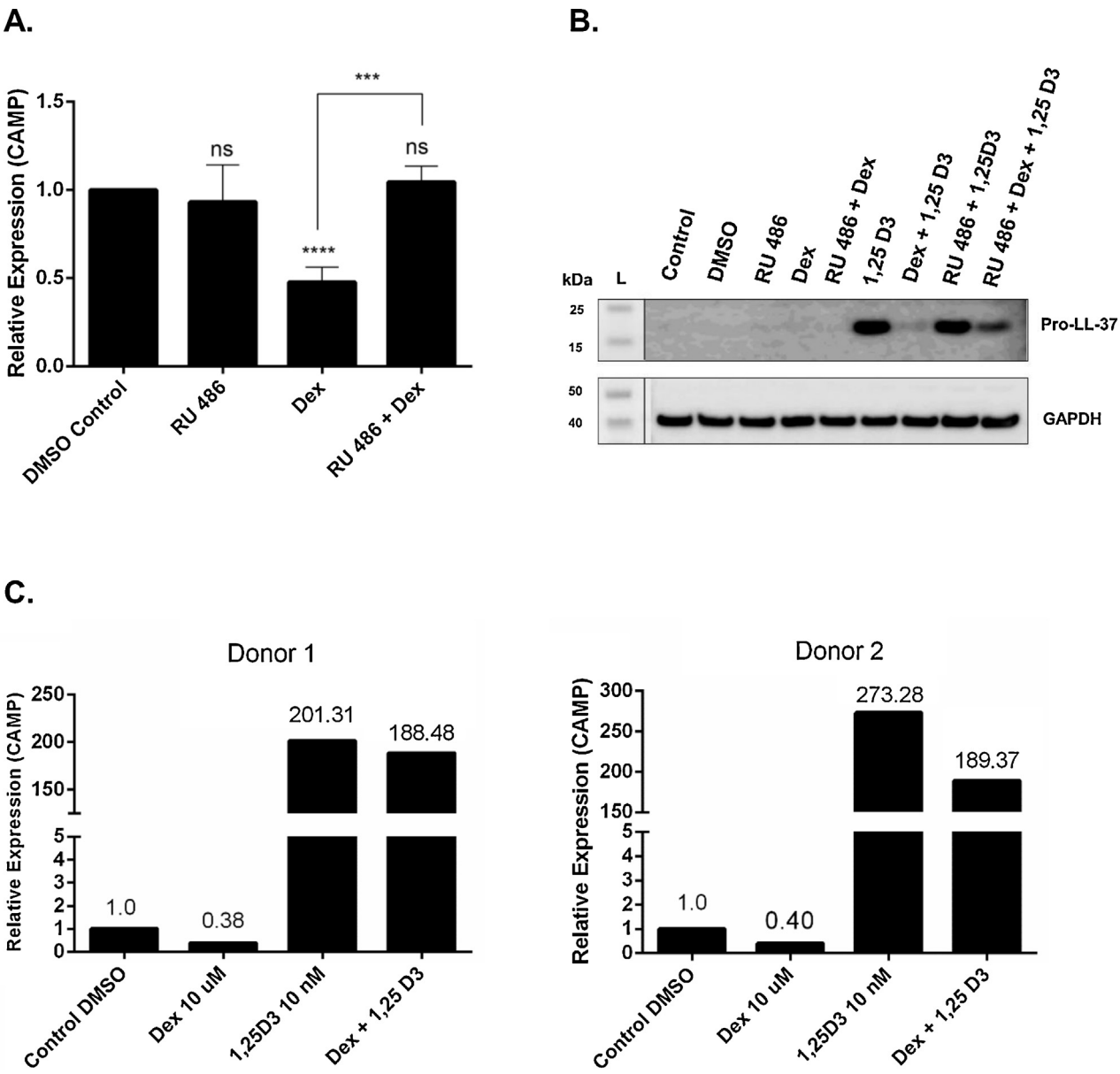

Fig. 2. Glucocorticoid receptor (GR) inhibition counteracts Dexamethasone mediated cathelicidin down-regulation in THP-1 monocytes. THP-1 monocytes were pre-treated with 10 μM of the GR inhibitor RU486 for 3 h, followed by treatment with 10 μM Dex for 48 h (A). The cells were harvested and gene expression of *CAMP* was analyzed with q-RT PCR. *UBC* and *HPRT1* were used as reference genes (n=3). Further, the effect of GR inhibition on Dex mediated decrease in pro-LL-37 expression was analyzed with Western blot (B). THP-1 monocytes were pre-treated with 10 μM RU486 for 3 h, followed by treatment with 10 μM Dex for 24 h. The cells were further treated with 1 nM 1,25D3 for 24 h. The blot was probed with antibody against LL-37 (human cathelicidin). GAPDH was used as a loading control. Furthermore, we confirmed the effects of Dex treatment on *CAMP* gene expression in primary monocytes isolated from fresh buffy coats of two healthy human donors (Donor 1 and Donor 2, C). Primary monocytes (1×10^6) were pre-treated with 10 μM Dex for 24 h, followed by treatment with 10 nM 1,25D3 for 24 h. The level of mRNA of *CAMP* transcript was analyzed with q-RT PCR. *UBC* and *HPRT1* were used as reference genes.The relative gene expression values are shown on top of the different bar. (L = Molecular weight ladder; ns indicates non-significant; **, $p < 0.01$; ***, $p < 0.001$).

(0.60 fold). Dex also reduced 1,25D3 mediated increase in *CAMP* gene expression in both the donors 1 and 2 (12.83 fold and 83.91 fold, respectively) (Fig. 2C).

3.2. Dexamethasone down-regulates poly IC induced genes encoding pro-inflammatory cytokines in THP-1 monocytes

THP-1 monocytes were pre-treated with 10 μM Dex for 24 h, followed by treatment with 1 nM 1,25D3 and/or 10 μg/ml poly IC for 6 h. The mRNA level of *CAMP* transcript, the inflammatory chemokine interferon gamma induced protein 10 (IP10/ *CXCL10*) and the inflammatory cytokine interleukin beta 1 (IL1B) was analysed with q-RT PCR (Fig. 3 A–C). Poly IC treatment significantly inhibited 1,25D3 induced *CAMP* expression ($p = 0.0426$) (Fig. 3A). This reduction was further enhanced by Dex ($p = 0.0033$) (Fig. 3A). Pre-treatment with Dex inhibited poly IC increased mRNA level encoding IP10 ($p = 0.0016$) (Fig. 3B). Treatment with 1,25D3 enhanced mRNA level of the genes encoding the inflammatory cytokines IP10 (*CXCL10*) ($p = 0.0240$) (Fig. 3B) and *IL1B* ($p = 0.0298$) (Fig. 3C) in cells treated with poly IC. This enhanced expression of mRNA encoding IP10 (*CXCL10*) and *IL1B* was inhibited on pre-treatment with Dex ($p = 0.0235$ and $p = 0.0093$, respectively) (Fig. 3B and C). Dex mediated inhibition of poly IC induced IL1B expression was confirmed with Western blot (Fig. 3D). THP-1 monocytes were pre-treated with 10 μM Dex for 24 h, followed by treatment with 1 nM 1,25D3 and/or 10 μg/ml poly IC for 18 h. Protein expression

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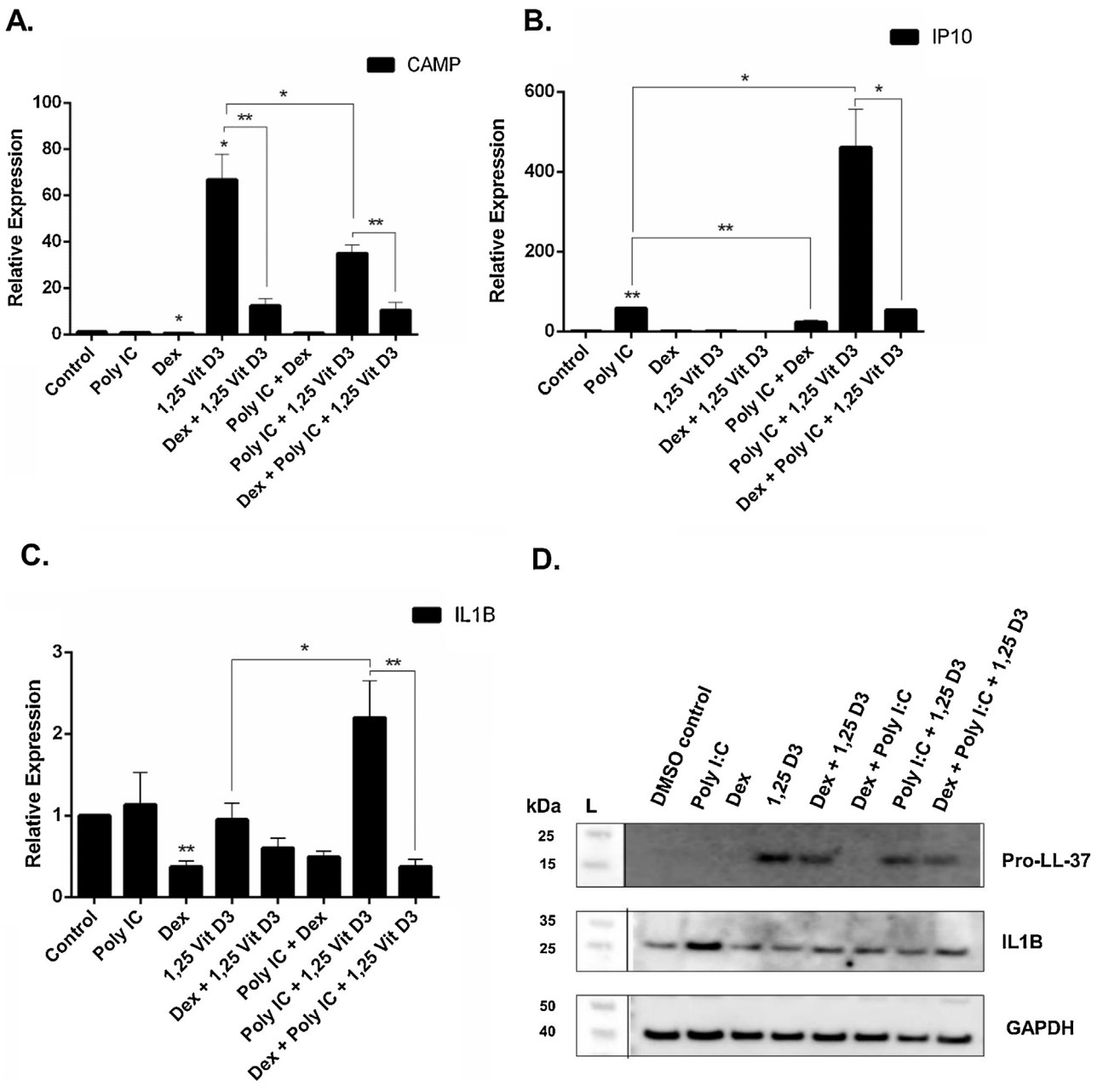

Fig. 3. Dexamethasone treatment inhibited poly IC induced gene expression of pro-inflammatory cytokines. THP-1 monocytes (1×10^6) were pre-treated with 10 μM Dex for 24 h, followed by treatment with 1 nM 1,25D3 and/or 10 ug/ml poly IC for 6 h (A–C). The cells were then harvested and mRNA level of *CAMP* transcript (A) and the genes encoding the pro-inflammatory cytokines IP10 (*CXCL10*) (B) and *IL1B* (C) was analyzed with q-RT PCR. *UBC* and *HPRT1* were used as reference genes ($n = 3$). In D, cells were pre-treated with 10 μM Dex for 24 h, followed by treatment with 1 nM 1,25D3 and/or 10 μg /ml poly IC for 18 h. Protein expression of pro-LL-37 and IL1B was analyzed with Western blot analysis. GAPDH was used as a loading control. (L= Molecular weight ladder; *, $p < 0.05$; **, $p < 0.01$)

of pro-LL-37 and IL1B was analysed by Western blot. Interestingly both poly IC and Dex treatment reduced 1,25D3 mediated increase in pro-LL37 expression (Fig. 3D). Further, we show both Dex and 1,25D3 treatment inhibited poly IC mediated increase in IL1B protein expression (Fig. 3D).

3.3. *Dexamethasone treatment down-regulates vitamin D3 induced human cathelicidin expression in the human bronchial epithelial cell line BCi NS1.1*

Finally, we investigated whether Dex treatment also has similar effects on *CAMP* gene expression in other cell types. BCi NS 1.1 is a basal human bronchial epithelial cell line established by immortalization with hTERT (Walters et al., 2013). BCi NS1.1 cells were grown to 70–80% confluence. The cells were then pre-treated with either 100 nM Dex or 10 μM Dex for 24 h, followed by treatment with 20 nM 1,25D3 for 24 h. mRNA level of *CAMP* transcript was analysed with q-RT PCR. Pre-treatment with 100 nM Dex and 10 μM Dex reduced basal expression of *CAMP* gene by 0.34 fold ($p = 0.001$) and 0.63 fold ($p < 0.001$), respectively (Fig. 4A). Further, pre-treatment with 100 nM Dex and 10 μM Dex reduced 1,25D3 induced *CAMP* gene expression by 1.14 fold ($p = 0.0012$) and 1.72 ($p < 0.001$) fold, respectively. We confirmed this inhibition at protein level with Western blot (Fig. 4B). The cells were pre-treated with either 100 nM Dex or 10 μM Dex, followed by treatment with either 20 nM 1,25D3 (active form of vitamin D3) or 20 nM 25D3 (inactive pro-form of vitamin D3) for 24 h. Expression of pro-LL37 was analysed with Western blot. GAPDH was used as a loading

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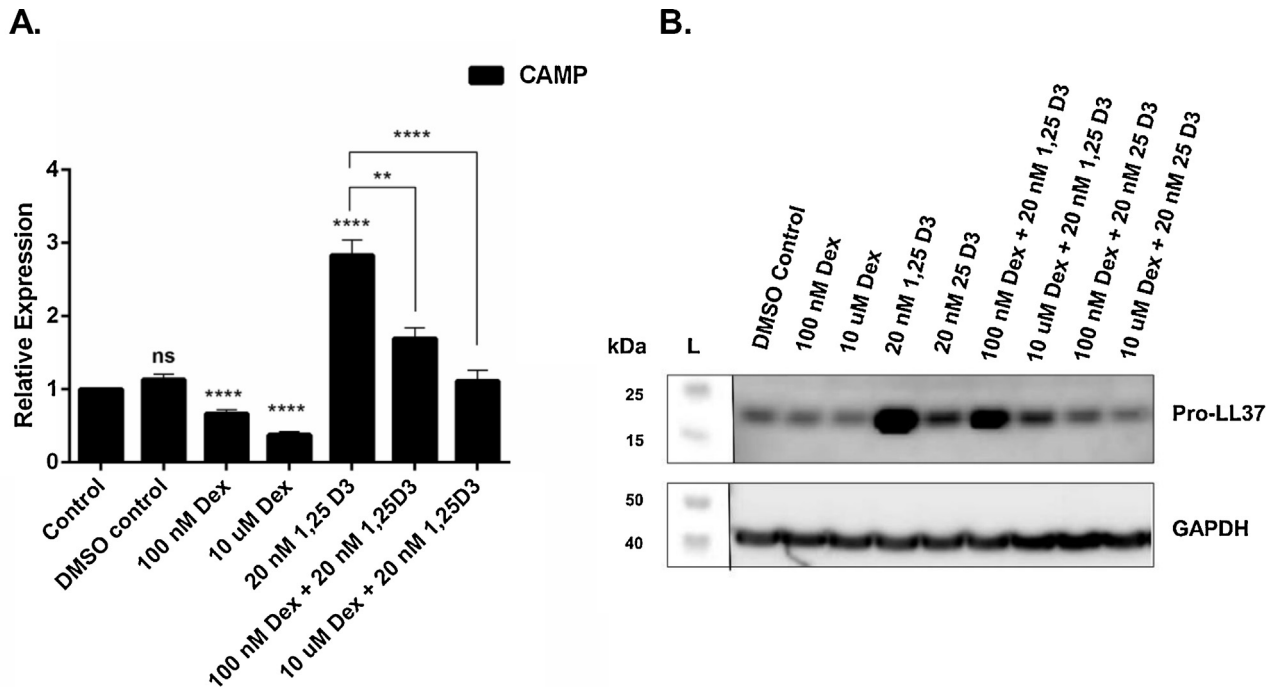

Fig. 4. Dexamethasone down-regulates vitamin D3 induced human cathelicidin expression in the human bronchial epithelial cell line BCi NS1.1. The human bronchial epithelial Bci NS 1.1 cells (2×10^5) were grown to 70–80% confluence. Then the cells were pre-treated with 100 nM or 10 μM Dexamethasone (Dex) for 24 h, followed by treatment with either 20 nM 1, 25D3 for another 24 h. DMSO was used as a solvent control (A). After that the cells were harvested and relative gene expression of *CAMP* gene was assessed with q-RT PCR. *UBC* and *HPRT1* were utilized as reference genes ($n = 3$) (A). In B, cells were pre-treated with 100 nM or 10 μM Dex for 24 h, followed by treatment with either 20 nM 1, 25D3 (active form of vitamin D3) or 25D3 (pro-form of vitamin D3) for another 24 h and harvested for Western blot analysis. The blot was probed with antibody against LL-37 (human cathelicidin). GAPDH was used as a loading control. (L = Molecular weight ladder; ns indicates non-significant; **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$).

control. Pre-treatment with Dex reduced both 1,25D3 and 25D3 increased pro-LL-37 protein expression (Fig. 4B).

4. Discussion

In this study, we demonstrate that glucocorticoids (GCs) suppress innate immunity *in vitro* by down-regulation of genes encoding antimicrobial peptides. Dexamethasone treatment down-regulated the basal mRNA expression of the antimicrobial peptides human cathelicidin (LL-37) and human beta defensin 1 in addition to the antimicrobial polypeptides lysozyme and secretory leukocyte peptidase inhibitor-1 in THP-1 monocytes (Fig. 1). GCs are the most effective treatment for chronic inflammatory disorders, however, the consistent use of these steroids in the past decades with associated side effects including secondary infections has made it imperative to study the long term effects of treated patients. In general, GCs have been shown to spare innate immunity, while down-regulating inflammatory responses (Schleimer, 2004). However, recent reports suggest that GC treatment can modulate innate immunity by differentially affecting antimicrobial peptide expression. Budesonide was shown to suppress mCRAMP (mouse cathelicidin) expression, leading to impaired anti-bacterial clearance in an allergic asthma mouse model (Wang et al., 2013). Treatment with the GC fluticasone propionate either enhanced or inhibited or spared the expression of antimicrobial proteins in human primary bronchial epithelial cells and in the BEAS-2B human bronchial epithelial cell line. These effects were mediated via activation of C/EBPβ transcription factor (Zhang et al., 2007). Further, tracheal antimicrobial peptide (TAP), a bovine defensin, expression was shown to be down-regulated in bronchial biopsies from dexamethasone treated calves (Mitchell et al., 2007).

Most patients with asthma respond to standard therapy with GCs, however about 15% patients fail to respond to treatment due to steroids insensitivity (Kerley et al., 2015). Vitamin D3 insufficiency has been associated with poor outcome in patients on GC therapy with impaired lung function, increased airway hyper responsiveness and reduced GC response (Sutherland et al., 2010). Interestingly, an inverse correlation was noted in asthmatic children between 25D3 levels and use of anti-inflammatory medication including GCs (Searing et al., 2010). Furthermore, it was also shown that treatment with vitamin D3 enhanced the GC action in PBMCs from asthmatic patients and anti-inflammatory functions of Dex *in vitro* (Searing et al., 2010; Zhang et al., 2013b). In this study, we analyzed the possible effects of GC pre-treatment on vitamin D3 induced cathelicidin expression. The active form of the secosteroid hormone vitamin D3 (1,25D3) induces human cathelicidin expression via VDR binding with subsequent formation of VDR-RXR heterodimer, leading to active *CAMP* gene transcription (Liu et al., 2007). Pre-treatment with Dex, fluticasone propionate and budesonide inhibited 1,25D3 induced cathelicidin expression in THP-1 monocytes (Fig. 1). Interestingly, basal *CAMP* mRNA transcription was enhanced in PMA differentiated THP-1 macrophages (Fig. S1). However, pre-treatment with Dex led to down-regulation of 1,25D3 induced *CAMP* gene expression in THP-1 macrophages (Fig. S1) in agreement with the results in THP-1 monocytes. Similarly, the basal expression of human beta defensin 1 and lysozyme was reduced in Dex treated THP-1 macrophages (Fig. S1). In primary monocytes isolated from buffy coats, Dex treatment reduced basal and 1,25D3 induced *CAMP* gene expression also in agreement with the results in THP-1 monocytes (Fig. 2). To our knowledge this is the first report, demonstrating GC mediated down-regulation of human cathelicidin expression. GC act via binding to the glucocorticoid receptor (GR), leading to trans-activation of genes encoding anti-inflammatory cytokines such as IL-10 and trans-repression of pro-inflammatory cytokines and chemokines (Barnes, 2011). We show that pre-treatment with the GR inhibitor RU486 counteracted

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the Dex mediated inhibition of 1,25D3 enhanced pro-LL-37 expression (Fig. 2). The down-stream pathways leading to GR mediated inhibition of *CAMP* gene expression will need further investigation. The most likely explanation for this effect would be GR mediated p38 mitogen activated protein kinase (MAPK) inhibition (Barnes, 2011). The p38 and JNK pathways have been shown to cooperatively trans-activate VDR via c-Jun/AP-1 in breast cancer cells (Qi et al., 2002). We further demonstrate that Dex treatment also down-regulated basal and vitamin D3 induced human cathelicidin expression in a human bronchial epithelial cell line BCi NS 1.1 (Fig. 4).

Finally, we confirmed the anti-inflammatory effects of Dex in THP-1 monocytes (Fig. 3). The double stranded RNA mimic poly IC is a Toll like Receptor 3 ligand known to induce inflammatory responses similar to viral infections (Marshall-Clarke et al., 2007). Viral infections in asthma patients are a frequent cause of exacerbations (Rosenthal et al., 2010). Dex pre-treatment inhibited dsRNA mimic the poly IC induced inflammatory chemokine IP10 gene expression (Fig. 3). Interestingly, treatment with 1,25D3 enhanced poly IC induced mRNA encoding IP10 (*CXCL10*) and *IL1B* gene expression was inhibited upon Dex pre-treatment (Fig. 3). In conclusion, we demonstrate that treatment with GC dexamethasone down-regulated human cathelicidin expression via the GR receptor. Thus, vitamin D3 treatment might act as a promising co-therapeutic intervention in patients on GC therapy, reversing GC mediated suppression of AMPs and promoting innate immunity. This treatment would have possible clinical therapeutic implications for GC treatment of asthma patients and additional inflammatory disorders.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

This work was supported by a grant to Gudmundur Hrafn Gudmundsson from Icelandic Centre for Research (RANNIS) (Project Number-130202-052) and the University of Iceland Research Fund. Nikhil N. Kulkarni was supported by University of Iceland Research fund for PhD students. This work was supported by grants from the Swedish Foundation for Strategic Research (SSF, Grant No. RBd08-0014), the Swedish Heart-Lung Foundation (Grant No. 2013-0366), the Swedish Research Council (Grant No. K2014-67X-11217-20-3). Gudmundur Hrafn Gudmundsson was supported by a Wenner-Gren Foundation as a visiting scientist at Karolinska Institutet.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2015.09.001.

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