A dual and conflicting role for imiquimod in inflammation: A TLR7 agonist and a cAMP phosphodiesterase inhibitor

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ABSTRACT

The Toll-like receptor 7 (TLR7) agonist imiquimod is an antitumor and antiviral drug used for the treatment of skin indications such as basal cell carcinoma, squamous cell carcinoma, and genital warts caused by the human papilloma virus. We show that imiquimod has TLR7-independent activity in which it directly inhibits phosphodiesterase (PDE), leading to cAMP increase, PKA-mediated CREB phosphorylation and subsequent CRE-dependent reporter transcription. The activation of the cAMP pathway by imiquimod is synergistically amplified by the β-adrenergic receptor agonist, isoproterenol. PDE inhibition is implied from cAMP measurements and CRE-reporter assays in intact RAW264.7 macrophages and HEK293T cells, and also directly demonstrated *in-vitro* using macrophages lysate. Moreover, molecular docking simulated the binding of imiquimod in the active site of PDE4B, enabled by the high molecular similarity between imiquimod and the adenine moiety of cAMP. As expected from the known anti-inflammatory role of cAMP inducers in stimulated macrophages, PDE inhibition by imiquimod results in reduced expression of the key pro-inflammatory cytokine TNFα, and enhanced expression of the key anti-inflammatory cytokine IL-10, compared to a different TLR7 agonist, loxoribine, as well as to the TLR4 agonist LPS. To conclude, our results indicate that the widely used inflammatory drug, imiquimod, is not only a TLR7 agonist, but also harbors a novel anti-inflammatory function as a PDE inhibitor. This off-target affects the desired therapeutic inflammatory activity of imiquimod and may be accountable for adverse side effects.

1. Introduction

Cyclic-AMP is a well-studied second messenger, known to modulate key inflammatory processes in macrophages, such as cytokine expression [1,2]. The rapid inflammatory response to infection initiates with the secretion of the key pro-inflammatory cytokine, TNFα, and is later followed by the highly essential anti-inflammatory process, in which IL-10 is a key mediator, destined to restore tissue homeostasis, as disproportional response of the immune system may cause excessive damage to the host. Co-stimulation of macrophages with an inflammatory trigger, such as the Toll-like receptor 4 (TLR4) ligand LPS, and a cAMP inducing agent, results in a prompt synergistic production of IL-10 [3–5] in parallel to acute suppression of TNFα production [6–8]. Multiple endogenous cAMP inducers were described to affect macrophages in such an anti-inflammatory direction, including adrenaline and noradrenaline [9,10], prostaglandins E_2 and I_2 (PGE_2 and PGI_2) [1,11] adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH) [12], α-melanocyte stimulating hormone (α-MSH) [13], and vasoactive Intestinal Peptide (VIP) and pituitary adenylate cyclase-

activating polypeptide (PACAP) [14,15]. The canonical cascade of cAMP involves its production by adenylyl cyclase whose enzymatic activity is turned on by a stimulated GPCR and the coupled G_s protein, PKA activation through cAMP binding to the regulatory unit and the release of the catalytic unit, which in turn phosphorylates CREB on serine residue 133, an event which is prerequisite to CREB-induced gene transcription via a CRE site [16,17]. We and others have shown that while several stimuli can lead to phosphorylation of CREB on Ser133 via different kinases, only phosphorylation by PKA induces CREB-mediated transcription activation [8,18–20]. These reports have clarified that CREB phosphorylation on Ser133 is required but not sufficient for its activation. For example, in macrophages, LPS stimulates CREB phosphorylation on Ser133, however via the kinase MSK1 rather than via the canonical cAMP-activated kinase PKA [21,22], and thus LPS is unable to stimulate CREB-dependent transcription [8]. This finding unraveled a common misconception regarding LPS signaling in macrophages [8]. Mechanistically, CREB phosphorylation on Ser133 in macrophages must be accompanied by cAMP- and PKA-mediated phosphorylation of the coactivator CRTC3 in order to enable

transcription via CRE sites [23]. Thus, cAMP inducers act via PKA to phosphorylate both CREB and CRTC3, thereby stimulating transcription of CREB-regulated genes, whereas LPS stimulates only phosphorylation of CREB which remains inactive due to the lack of concurrent CRTC3 phosphorylation. This signaling discrimination at the level of CREB explains how cAMP inducers can suppress TNFα and induce IL-10 in LPS-stimulated macrophages in a CREB-dependent manner although CREB phosphorylation on Ser133 is achieved also by LPS alone [8].

The inability of LPS to activate CREB was demonstrated in a short exposure (3 h) of RAW264.7 macrophages [8]. In contrast, a long (36 h) exposure of RAW264.7 macrophages to either of the two pathogen-associated molecular pattern (PAMP) molecules, LPS or peptidoglycan, results in phosphorylation as well as transcriptional activation of CREB [24]. However, the activation of CREB in such a long incubation is likely to be indirect. Therefore, we sought to determine whether various inflammatory stimuli can directly activate rapid CREB-dependent transcription. To that end, we exposed RAW264.7 macrophages for a short period of time (3 h) to inflammatory stimuli that activate a variety of pattern recognition receptors (PRRs): LPS (Gram-negative bacteria component; TLR4 agonist [25]); peptidoglycan (Gram-positive bacteria component; agonist of Nod2, an intracellular receptor that acts in synergism with stimulated TLR2 [26–29]); zymosan (yeast component; agonist of TLR2 and dectin-1 [30]); and imiquimod (a synthetic TLR7 agonist; [31]). The only stimulus that activated CREB-dependent transcription, both alone and in synergism with a cAMP inducer, was the TLR7 agonist imiquimod. This novel activity was specific to imiquimod and was not observed for a different TLR7 agonist, suggesting a TLR7-independent mechanism. Eventually, we found that imiquimod is a direct inhibitor of the cAMP hydrolyzing enzyme PDE, and that this activity partially counterbalances the TLR7-mediated inflammatory response to imiquimod in macrophages. These findings have a potential clinical implication due to the usage of imiquimod as a skin chemotherapy drug.

2. Materials and methods

2.1. Reagents

Imiquimod, lipopolysaccharide (LPS; Escherichia coli serotype 055:B5), loxoribine and isoproterenol were purchased from Sigma-Aldrich (St. Louis, MO). L-glutamine and penicillin–streptomycin-nystatin were purchased from Biological Industries (Beit Haemek, Israel). DMEM, OptiMEM and FBS were purchased from Gibco. Rolipram was purchased from Spectrum Chemical (New Brunswick, NJ). ELISA reagents sets for IL-10 and TNFα were purchased from R&D Systems (Minneapolis, MN). The antibody against general CREB (#7540) was purchased from Abcam (Cambridge, UK). The antibodies against phospho-Ser133 CREB (#9198) and PKA-Cα (#4782) were purchased from Cell signaling (Danvers, MA). Antibodies against PKA-Cβ (#904) and c-Jun (#45X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A CRE-containing EVX-1 promoter luciferase reporter construct [32] and a dominant negative Ser133Ala CREB construct [17] were a kind gift from Dr. M.R. Montminy. The CREx4 reporter construct, containing four copies of the consensus CRE sequence upstream to a luciferase reporter gene, was previously reported [5]. Vectors were amplified using DH10B bacteria (Invitrogen, Carlsbad, CA) and purified using Endofree Plasmid Maxi Kit (Qiagen, Hamburg, Germany). HD-fugene and TransIT2020 transfection reagents were purchased from Roche (Mannheim, Germany) and Mirus Bio (Madison, WI), respectively. Dual-luciferase reporter assay kit was from Promega (Fitchburg, WI). The HTRF-cAMP assay kit was from Cisbio (Codolet, France).

2.2. Cell culture

Mouse RAW264.7 macrophage cells and HEK293T cells were obtained from American Type Culture Collection (ATCC, Rockville, MD).

The cells were grown to 80–90% confluence in DMEM medium supplemented with 8 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1250 U/ml nystatin (hereafter culture medium), and with 10% FBS, at 37 °C in a humidified incubator with 5% CO2. The passage number was kept below 20.

2.3. Cytokine expression

RAW264.7 macrophages were maintained for 48 hr prior to the experiment in 96-well plates, at $1.0 \cdot 10^5$ cells per well, in culture medium supplemented with 5% FBS, up to a confluence of 90%. The cells were stimulated with the indicated compound at 37 °C for 3–4 h. IL-10 and TNFα secretion to the medium was measured by ELISA, according to the manufacturer's instructions, using a microplate reader (Bio-Tek, Winooski, Vermont). The samples were stored at −80 °C until used.

2.4. Transfection of RAW264.7 cells

RAW264.7 macrophages were grown for 24 h in 12-well plates, at $3.0 \cdot 10^5$ cells per well, in culture medium supplemented with 10% FBS. The cells were then transfected for 24 h with 0.6 μg of CRE-reporter plasmid, 0.2 μg of TK-renilla luciferase (for normalization), and where indicated – with an expression plasmid for the dominant negative (DN) CREB mutant Ser133Ala or with an empty vector. The plasmids were initially incubated with TransIT2020 transfection reagent in OptiMEM for 15 min at room temperature.

2.5. Transfection of HEK293T cells

HEK293T cells were grown for 24 h in 48-well plates, at $0.7 \cdot 10^5$ cells per well, in culture medium supplemented with 10% FBS. The cells were then transfected for 24 h with 25 ng of CRE-reporter plasmid and with 10 ng of TK-renilla luciferase (for normalization). The plasmids were initially incubated with HD-Fugene transfection reagent in OptiMEM for 15 min at room temperature.

2.6. Reporter gene assay

Following transfection, the cells were washed and stimulated for 3 h at 37 °C, after which luciferase activity in cell extracts was determined following the manufacturer's instructions. Data were expressed as a ratio of CRE-reporter luciferase activity divided by the renilla luciferase activity and control cells.

2.7. Whole cell cAMP measurements

RAW264.7 macrophages or HEK293 cells were seeded in 48-well plates 48 h prior to the experiment. The cells were incubated with or without 1 mM IBMX for 15 min and then stimulated for 5 min. Lysis was accomplished by addition of 1% Triton X-100 in PBS containing 1 mM IBMX. Intracellular cAMP was measured using the HTRF cAMP kit, according to the manufacturer's instructions, in a white 384-well plate, using a Synergy 2 multi-mode microplate reader (BioTek, Winooski, Vermont).

2.8. PDE activity assay

RAW264.7 cells lysate (500 ng protein) was allowed to hydrolyze cAMP (10 nM) for 0–60 min in the presence or absence of 0–250 μM imiquimod, 100 μM rolipram or 1 mM IBMX. The assay was stopped by addition of 1 mM IBMX, and the remaining cAMP level was measured using the HTRF-cAMP kit as described above.

2.9. CREB phosphorylation assay

RAW264.7 macrophages were maintained for 24 h prior to the experiment in 12-well plates, at $5 \cdot 10^5$ cells per well, in culture medium supplemented with 0.1% FBS. The cells were stimulated with 50 μM imiquimod and/or 1 μM isoproterenol at 37 °C for 0–60 min. The cells were then washed twice with cold PBS and lysed for 15 min at 4 °C with Tris buffer pH 8.0 (50 mM) containing Triton X-100 (1%), NaCl (100 mM), β-glycerophosphate (50 mM), sodium orthovanadate (1 mM), EDTA (1 mM), EGTA (1 mM), glycerol (30%), PMSF (1 mM) and a complete protease inhibitor mixture diluted according to the manufacturer instructions. Cell extracts were centrifuged (14,000 × g, 15 min at 4 °C) and the supernatants were stored at −80 °C.

2.10. Western blotting

Cell extracts (30 μg protein) were boiled for 5 min in SDS-PAGE buffer, subjected to 10% SDS-PAGE, and proteins were transferred to PVDF membrane. An antibody raised against phospho-Ser133 CREB was used together with an antibody against α-tubulin. Two-color imaging and quantitative analysis of western blots was performed using the Odyssey infrared imaging system (Li-Cor Biosciences), according to the manufacturer's instructions.

2.11. Protein determination

Protein was determined by a modification of the Bradford procedure, which yields linear and thus more accurate results, increased sensitivity, and reduced detergent interference, as previously described by us [33,34]. BSA served as standard.

2.12. Molecular modeling

In order to understand the chemical nature of the interaction between imiquimod and PDE4B, we performed a docking simulation, using PatchDock [35] for geometrical fitting, followed by side chain refinement with Firedock [36]. The structure of imiquimod was taken from a co-crystal with human quinone reductase 2 (PDB ID: 5LBT). Polar hydrogens were added to imiquimod using PyMol. Imiquimod was docked into a ligand-free PDE4B, derived from an AMP-bound structure (PDB ID: 1TB5) from which the AMP molecule was removed using PyMol. The model with the highest score was chosen for analysis.

2.13. Statistical analysis

Data were analyzed using one- or two-ways ANOVA with the appropriate multiple comparison test wherever applicable, or Student's t-test, as indicated in the figure legend. In all cases, differences of $p < 0.05$ were considered to be significant. All experiments were repeated as least twice with similar results.

3. Results

3.1. Imiquimod induces TLR7-independent CRE transcriptional activity

We have previously unraveled a common misconception regarding LPS signaling in macrophages, by demonstrating that phosphorylation of CREB following a short (3 hr) exposure is not an indication for its activation [8]. Now, we sought to test whether additional inflammatory stimuli, some of which were reported to stimulate CREB phosphorylation, can activate CREB-dependent transcription. To that end, RAW264.7 macrophages were transiently transfected with a construct coding for the firefly luciferase reporter gene downstream to the EVX-1 promoter which is regulated by a strong cAMP responsive element (CRE) [32]. The macrophages were stimulated for a short period of time (3 hr) with various inflammatory stimuli: LPS, imiquimod,
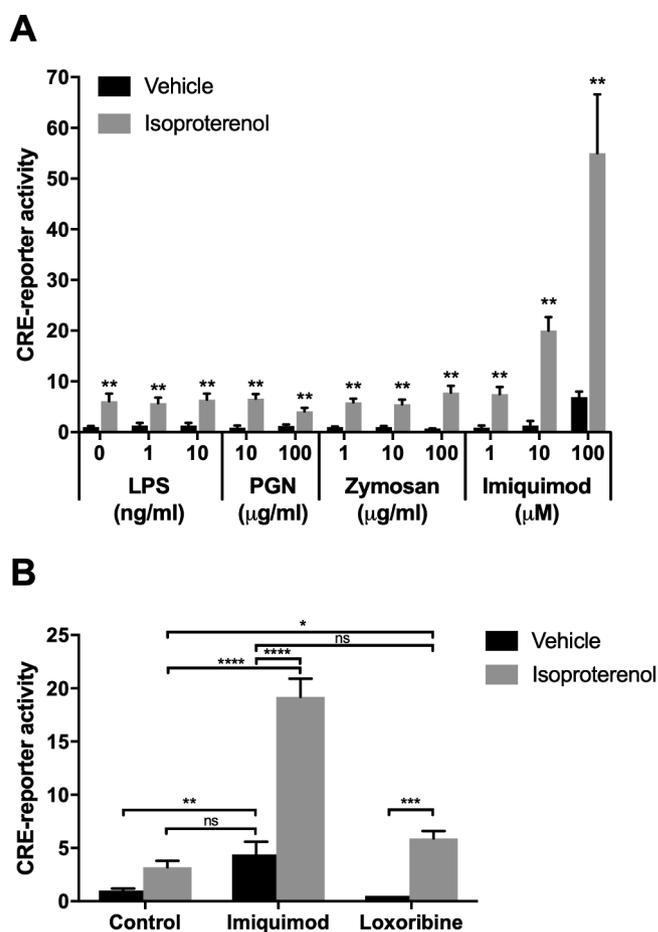
Fig. 1. Imiquimod stimulates CRE-reporter activity in a TLR-independent mechanism. RAW264.7 macrophages (A) and HEK293T cells (B) were transfected with a CRE-reporter construct which codes for firefly luciferase downstream to the EVX-1 promoter regulated by a strong CRE sequence (A), or to four copies of the consensus CRE sequence (B). The cells were treated for 3 hr with various inflammatory stimuli at the indicated concentration (A) or with either of two alternative TLR7 agonists – imiquimod (50 μM) or loxoribine (500 μM) (B), with or without isoproterenol (1 μM). PGN = peptidoglycan. Luciferase reporter data represent two (A) or three (B) independent experiments and are expressed as mean ± SD of values normalized against renilla luciferase activity, divided by unstimulated control cells. (A) **p < 0.01 for cells transfected with isoproterenol compared to vehicle (Student's t-test). (B) *p = 0.0366, **p = 0.008, ***p = 0.0001, ****p < 0.0001 (two-way ANOVA followed by Tukey's multiple comparison test).

peptidoglycan, and zymosan, in the presence or absence of the cAMP inducer isoproterenol (β-adrenergic receptor (β-AR) agonist). Fig. 1A shows that imiquimod was the only PRR agonist that induced CRE-dependent luciferase activity. At 100 μM imiquimod alone induces a 7-fold increase in CRE-reporter activity, similarly to the induction by the β-adrenergic receptor agonist, isoproterenol, alone. Strikingly, imiquimod and isoproterenol synergistically stimulated CRE-reporter activity 55-fold compared to resting cells. In contrast, while LPS, zymosan and peptidoglycan rapidly stimulate phosphorylation of CREB at a relevant peak time point of 0.5 hr [8,24,37], they all failed to induce CRE-dependent luciferase activity or to intensify the effect of isoproterenol. These results suggest that imiquimod is unique among the inflammatory stimuli in its ability to activate CREB, and that the activation mechanism is different that isoproterenol that acts via a GPCR to induce cAMP formation.

We continued by testing the effect of imiquimod on CRE-reporter activity in HEK293T cells, which do not express TLR7 [38], the established receptor for imiquimod. In this experiment we used a construct
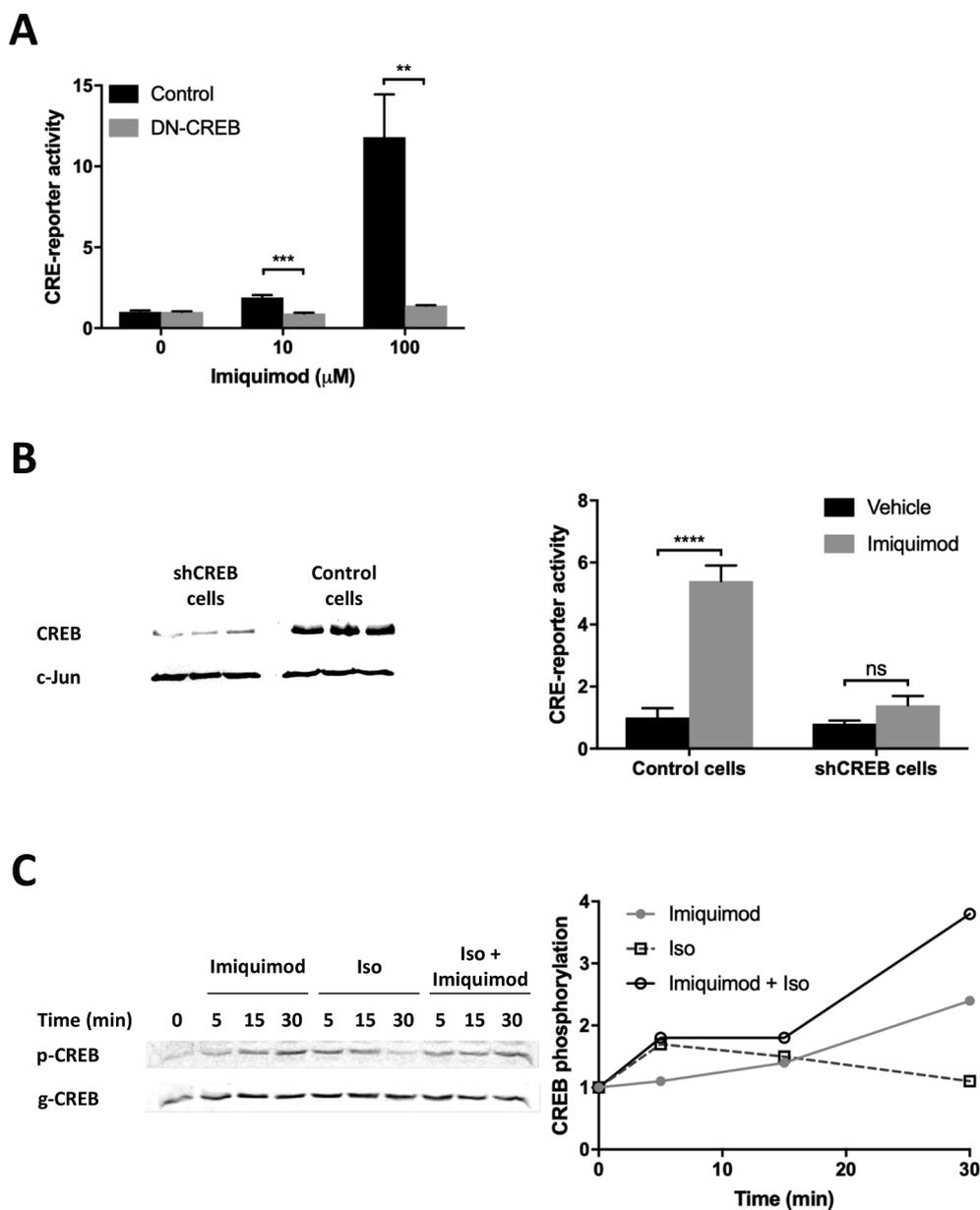
Fig. 2. Imiquimod stimulates CRE-reporter via CREB. (A) RAW264.7 macrophages were transiently transfected with a CRE-reporter construct which codes for firefly luciferase under regulation of the CRE-containing EVX-1 promoter, and with either a dominant negative construct expressing Ser133Ala CREB (DN-CREB) or a control vector. The cells were treated with the indicated concentration of imiquimod for 3 hr. (B) RAW264.7 macrophages stably expressing shCREB or shControl were transiently transfected with the consensus CREx4 reporter (see legend of Fig. 1B) and then treated with imiquimod (50 µM) for 3 hr. The expression of CREB (and of c-Jun – for normalization) in shCREB and shControl cells is shown. (A-B) Luciferase reporter data represent two (A) or three (B) independent experiments and are expressed as mean ± SD of values normalized against renilla luciferase activity, divided by unstimulated control cells. (A) **p = 0.002, **p = 0.0004 (Student's t-test). (B) ****p < 0.0001 (two-way ANOVA followed by Sidak's post-test). (C) RAW264.7 macrophages were treated with imiquimod (50 µM) and/or isoproterenol (Iso; 1 µM) for the indicated time. Cells lysates (30 µg) were subjected to SDS-PAGE and blotted with antibodies raised against phospho-Ser133 CREB and the general CREB. Quantitative western blot analysis is shown as the ratio of intensities of the phosphorylated and total CREB, relative to unstimulated cells. The results are representative of two experiments.

coding for luciferase under the regulation of four copies of the consensus CRE sequence. As can be seen in Fig. 1B, imiquimod induced CRE activity by nearly 5-fold, higher than the effect observed for isoproterenol. Together, imiquimod and isoproterenol synergized to induce a 20-fold increase in activity. For comparison, we have used another TLR7 agonist, loxoribine, which was unable alone to stimulate CRE-reporter activity, and demonstrated only low activity in combination with isoproterenol. These data strongly suggest that imiquimod regulates CRE transcriptional activity in a TLR7-independent manner.

3.2. Imiquimod stimulates CREB phosphorylation and transcriptional activity

We set to determine whether the transcription factor CREB mediates imiquimod-stimulated CRE activity by over-expression of a dominant negative CREB as well as by shRNA-mediated CREB silencing. Both approaches resulted in elimination of the transcriptional activity, indicating that CREB mediates the activation of CRE in response to imiquimod (Fig. 2A-B). Next we examined CREB phosphorylation on Ser133 in response to imiquimod, in the presence and absence of

isoproterenol (Fig. 2C). Interestingly, imiquimod induced CREB phosphorylation comparably to isoproterenol, but with significantly slower kinetics, and in a prolonged and accumulative, rather than transient, manner. Together, the two stimuli induced a synergistic response at the later time point. These observations are consistent with a mechanism of phosphodiesterase (PDE) inhibition for imiquimod vs. the established GPCR-mediated adenylyl cyclase activation for isoproterenol, as also inferred from the synergistic CRE-reporter activation (Fig. 1A-B).

3.3. Imiquimod and isoproterenol synergistically induce cAMP formation

Following our discovery that imiquimod stimulates CRE-reporter activity via CREB, we sought to determine whether imiquimod can stimulate the production of cAMP in macrophages, under two settings – in the absence or presence of the pan PDE inhibitor IBMX, commonly used to block cAMP degradation and thus to enable detection of low transient cAMP elevation. As shown in Fig. 3A, isoproterenol elevated cAMP level in macrophages 2.5-fold, whereas neither imiquimod, nor the PDE4 inhibitor rolipram or the pan PDE inhibitor IBMX, affected cAMP on their own. However, imiquimod amplified isoproterenol-
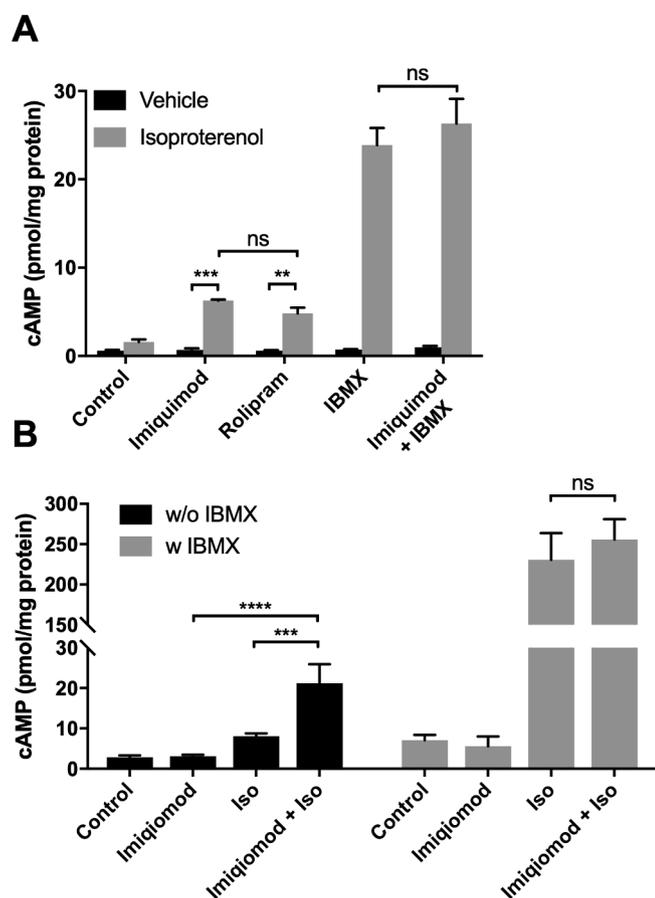
Fig. 3. Imiquimod synergizes with isoproterenol to induce cAMP accumulation. (A) RAW264.7 cells (A) and HEK293T cells (B) were pre-incubated for 10 min with imiquimod (50 μM), the specific PDE4 inhibitor rolipram (20 μM), or the pan PDE inhibitor IBMX (1 mM), prior to the addition of isoproterenol (Iso, 1 mM) for 5 min. Cells were lysed using 1% Triton X-100 in the presence of 1 mM IBMX and cAMP levels were measured using an HTRF-cAMP assay. Data expressed as mean ± SD (n = 3). (A) **p = 0.0057, ***p = 0.0002 (two-way ANOVA followed by Sidak's post-test); (B) ***p = 0.0007, ****p < 0.0001 (one-way ANOVA followed by Tukey's post-test).

induced cAMP 4-fold, and thus cAMP level was increased 11-fold by the combination of imiquimod and isoproterenol. For comparison, rolipram presented a quantitatively-similar effect to imiquimod, whereas IBMX dramatically amplified isoproterenol-induced cAMP 15-fold, and thus cAMP level was increased 45-fold by the combination of IBMX and isoproterenol. Importantly, in the presence of IBMX, imiquimod cannot further amplify isoproterenol-induced cAMP level (Fig. 3A). A similar trend was observed in HEK293T cells, where the effect of imiquimod was slightly lower compared to RAW264.7 cells, while the effect of IBMX was even stronger than in RAW264.7 cells (Fig. 3B). Taken together, these data suggest that imiquimod acts as a selective PDE inhibitor.

3.4. Imiquimod inhibits PDE activity in-vitro

In order to directly test the hypothesis that imiquimod inhibits PDE, we measured its effect on degradation of exogenous cAMP by PDE activity present in RAW264.7 macrophage cells extract. Fig. 4A shows that while cAMP was rapidly degraded in untreated cells extract, the pan PDE inhibitor IBMX completely blocked cAMP degradation, and imiquimod partially prevented cAMP hydrolysis in a time-dependent manner, similarly to the selective PDE4 inhibitor rolipram. Fig. 4B presents a dose response for imiquimod in the cell-free assay for cAMP
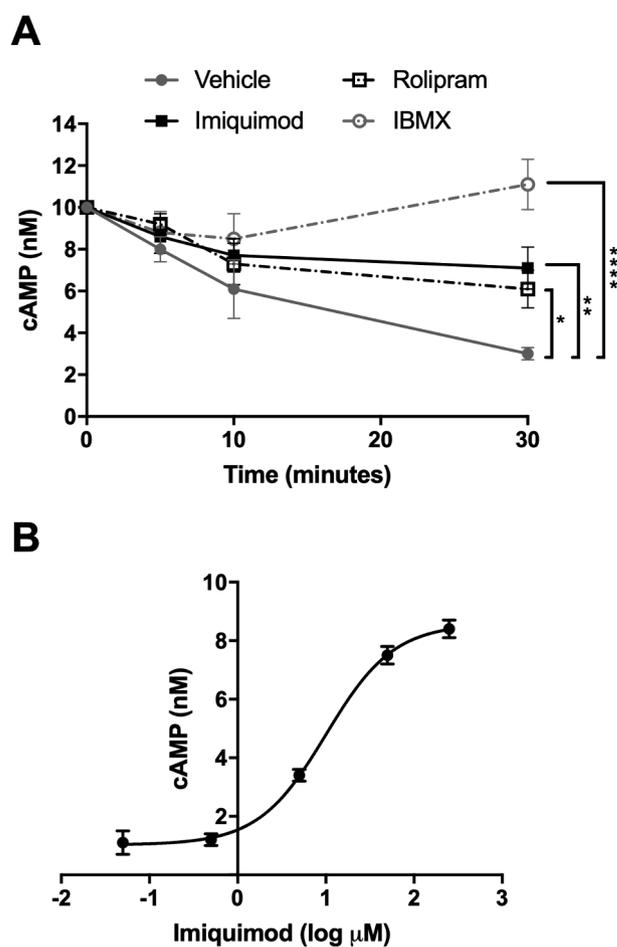

Fig. 4. Imiquimod inhibits macrophages PDE activity in a cell-free system. RAW264.7 cells were lysed and cAMP (10 nM) was added for the indicated time (A) or for 60 min (B) in the presence of rolipram (100 μM), IBMX (1 mM), or imiquimod – at 250 μM (A) or as indicated (B). The reaction was terminated with the addition of IBMX (1 mM) and cAMP was measured using an HTRF-cAMP assay. Data expressed as mean ± SD (n = 6). (A) *p = 0.013, **p = 0.0026, ****p < 0.0001 (one-way ANOVA followed by Tukey's post-test).

hydrolysis inhibition, in which the IC$_{50}$ was determined to be 11 μM. These experiments demonstrate that imiquimod is indeed a selective PDE inhibitor.

3.5. Imiquimod binds to PDE4B in-silico

The ability of imiquimod to inhibit cAMP degradation by PDE is not surprising, considering the very high chemical similarity between imiquimod and the adenine moiety of cAMP (Fig. 5A). To further rationalize our experimental data in structural terms, a model of the complex between imiquimod and PDE4B was derived, based on the crystal structure of PDE4B bound to its product, AMP [39]. In-silico, the AMP was removed from the active site and PDE4B-imiquimod binding was initially simulated using PatchDock, a molecular docking algorithm based on shape complementarity principles [35]. Subsequently, the models were refined and rescored using FireDock, a molecular docking algorithm based on energy minimization considerations [36]. As expected, the highest score model derived from PatchDock demonstrates docking of imiquimod into the active site of PDE4B (Fig. 5B). The planar adenine-like moiety of imiquimod is rotated in the model by 60° relative to the adenine moiety of AMP in the crystal structure [39] (Fig. 5B&C, respectively). This rotation is likely due to the presence of
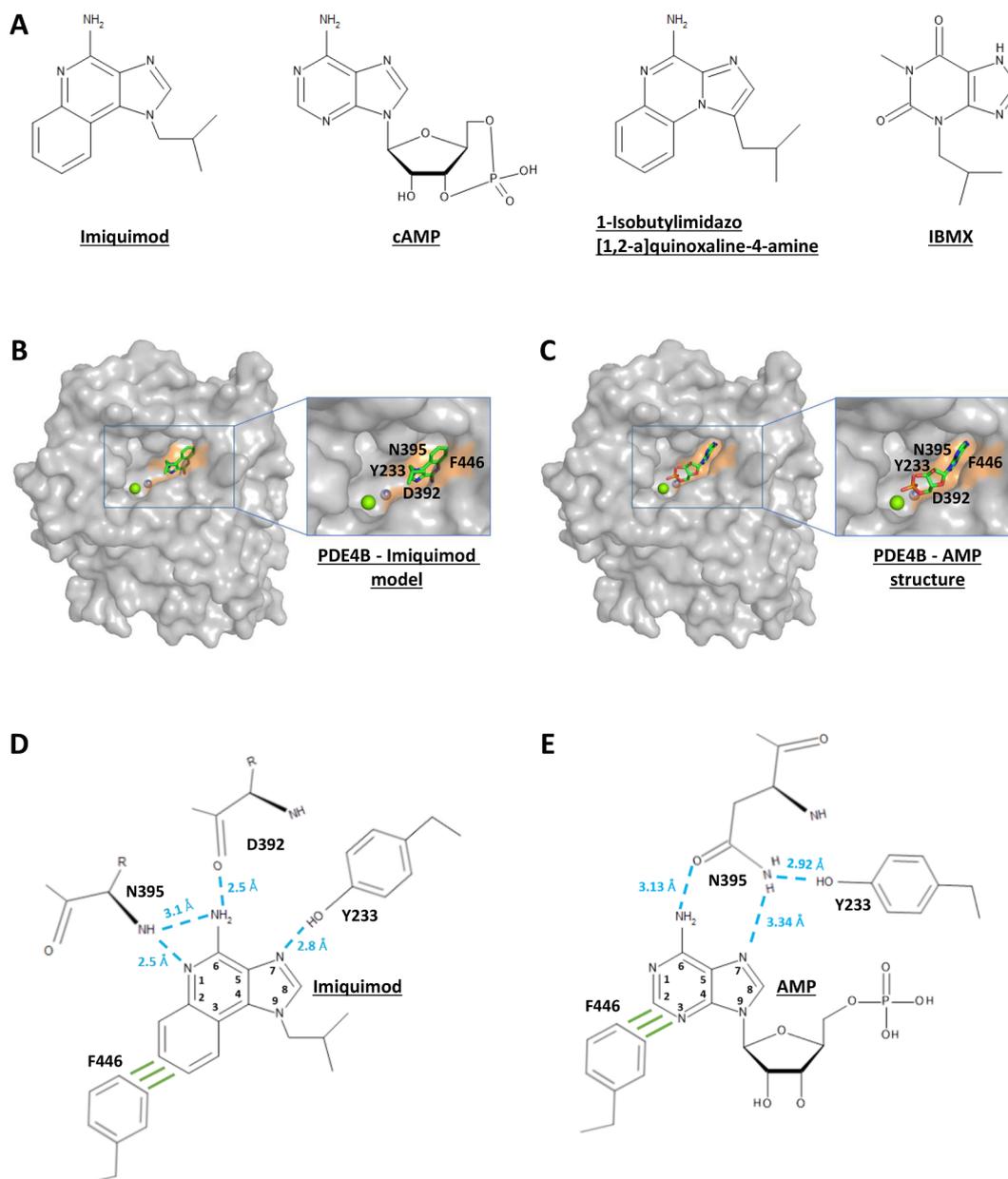
Fig. 5. Imiquimod docks into the active site of PDE4B. Molecular docking was performed by the sequential algorithms of PatchDock and FireDock, using the AMP-bound conformation of PDE4B [39]. (A) Chemical structures of imiquimod, cAMP, IBMX and the PDE4 inhibitor 1-Isobutylimidazo[1,2-a]quinoxaline-4-amine [79]. (B–E) Comparison of PDE4B binding by imiquimod (B&D, model) and by the AMP product (C&E, crystal structure). Key interacting amino acids are depicted by orange color on the gray enzyme surface; AMP and imiquimod are presented as *sticks*. The Mg^{2+} and Zn^{2+} ions are shown as green and purple spheres, respectively. Hydrogen bonds and stacking interactions are shown by dashed cyan lines and green lines, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the additional aromatic hydrocarbon ring of imiquimod and the optimal stacking interaction observed in the model between Phe446 and this particular ring, which is distinct from the interaction between Phe446 and the adenine-like ring observed in the AMP-bound structure. The most important predicted interactions calculated by FireDock include those made by Asn395, Phe446, Tyr233 (Fig. 5D), which also make key interactions in the AMP-bound structure [39,40] (Fig. 5E). In addition, the backbone carbonyl of Asp392 is predicted to hydrogen bond to the amine group of imiquimod (Fig. 5D). The *in-silico* molecular modeling thus strongly supports the conclusion derived from the *in-vitro* enzymatic experiments together with the cell-based assays, namely that imiquimod directly binds PDE and inhibits cAMP degradation.

3.6. cAMP induction by imiquimod synergizes with TLR-mediated IL-10 expression in macrophages

We have recently reported that the TLR4 agonist LPS and the cAMP inducer isoproterenol synergistically induce early secretion of the anti-inflammatory cytokine IL-10 in macrophages, whereas LPS alone demonstrates only a modest effect in a short (3–4 hr) assay [4,5]. As imiquimod can activate the cAMP pathway by preventing cAMP degradation, we hypothesized that it can induce IL-10 secretion to a higher extent than a TLR7 agonist which does not target PDE. Indeed, as seen in Fig. 6A, treatment of RAW264.7 macrophages with imiquimod for 4 hr stimulated secretion of IL-10, in contrast to loxoribine which
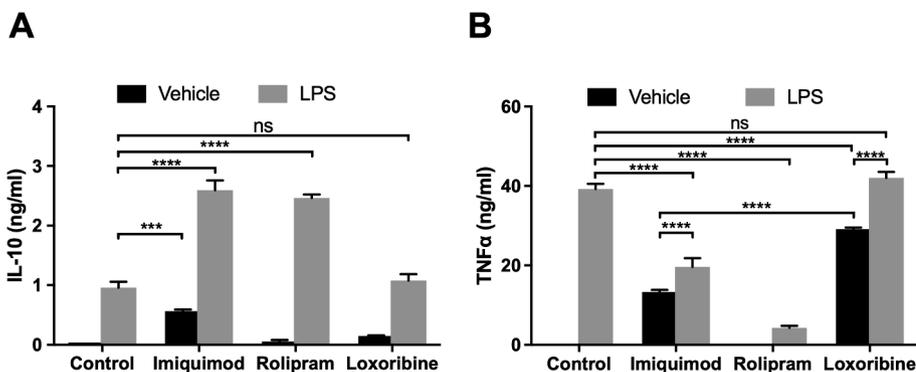
Fig. 6. The TLR7-mediated pro-inflammatory response to imiquimod is diverted by an anti-inflammatory pathway. (A) RAW264.7 cells were treated for 4 hr with imiquimod (50 μM), rolipram (20 μM) or loxoribine (500 μM), in the presence or absence of LPS (100 ng/ml). IL-10 (A) and TNFα (B) were measured by ELISA. Data expressed as mean ± SD (n = 6). ***p = 0.0004, ****p < 0.0001 (two-way ANOVA followed by Tukey's post-test).

induces TLR7 signaling but does not inhibit PDE, as well as to rolipram which inhibits PDE but does not induce TLR signaling (Fig. 6A). This finding suggests that imiquimod can induce IL-10 only due to the combination of TLR7 activation and PDE inhibition. The minimal effect of loxoribine, compared to imiquimod and to LPS, suggests that TLR7 signaling alone is significantly less effective than TLR4 signaling regarding IL-10 induction in a short assay. As expected, rolipram synergistically amplified LPS effect, due to CRE-dependent transcription at the IL-10 promoter [5]. Interestingly, imiquimod, but not loxoribine, also synergized with LPS (Fig. 6A), reinforcing our conclusion regarding a similar role for imiquimod and rolipram as PDE inhibitors.

3.7. cAMP induction by imiquimod partially counterbalances TLR7-mediated TNFα expression in macrophages

We [8,41] and others [2,6,7] have reported that cAMP inducers block transcription of the pro-inflammatory cytokine TNFα via PKA. We therefore hypothesized that imiquimod can suppress TNFα secretion to a larger extent than a TLR7 agonist which does not target PDE. Indeed, as shown in Fig. 6B, imiquimod can only induce 33% out of the LPS-induced TNFα level, whereas the other TLR7 agonist used in this study, loxoribine, induced considerably higher levels of TNFα, reaching 75% of the response to LPS. Interestingly, although both LPS and imiquimod independently induce TNFα via their respective TLRs, in co-stimulated macrophages imiquimod suppressed 50% of the TNFα induced by LPS alone (Fig. 6B). For comparison, the specific PDE4 inhibitor rolipram suppressed 89% of LPS-induced TNFα production, whereas the TLR7 agonist loxoribine was unable to affect the induction of TNFα by LPS (Fig. 6B). These results suggest that both imiquimod and loxoribine effectively induce TNFα via TLR7, while in parallel, imiquimod (but not loxoribine) abrogates TNFα expression via PDE inhibition and the cAMP pathway, similarly to the established PDE4 inhibitor rolipram. Thus, our results suggest that imiquimod acts via PDE inhibition to divert TLR7 signaling to a reduced inflammatory state, by reciprocal modulation of TNFα and IL-10. This conclusion can be quantitatively demonstrated by the TNFα/IL-10 ratio, determined to be 200 for loxoribine but only 25 for imiquimod – an 8-fold anti-inflammatory switch.

3.8. Synergistic IL-10 induction by imiquimod is mediated by PKA

To further demonstrate that activation of the cAMP pathway modulates the TLR7-mediated inflammatory response to imiquimod, we determined IL-10 secretion in response to treatment with imiquimod and/or LPS for 3 hr in RAW264.7 macrophages in which both the α and β catalytic subunits of PKA were stably silenced by shRNA. As shown in Fig. 7, PKA down-regulation reduced imiquimod-induced IL-10 expression by 70%, almost to the minimal level obtained in response to
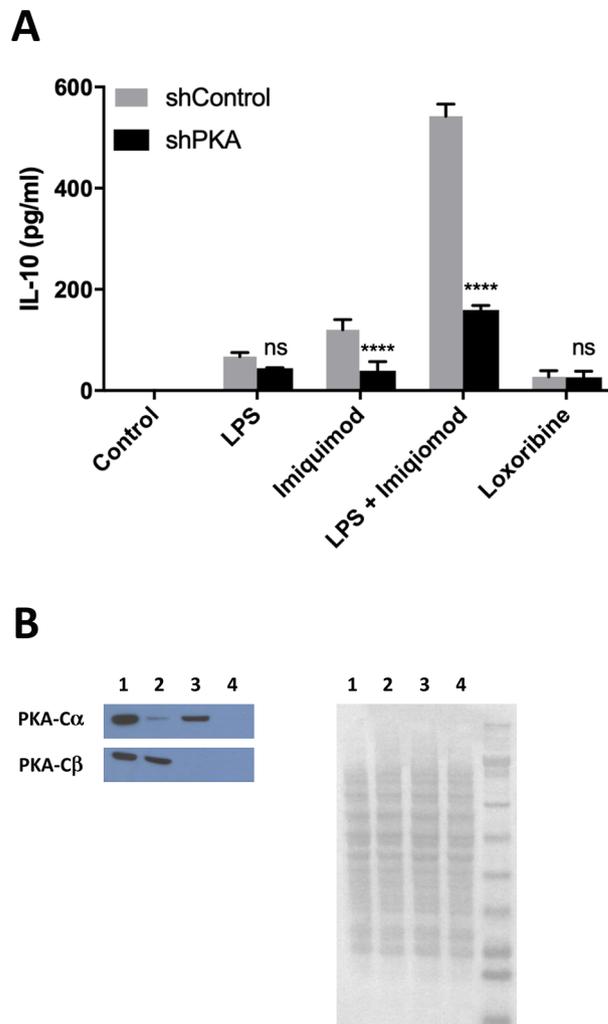

Fig. 7. Imiquimod-induced IL-10 expression is PKA-dependent. (A) RAW264.7 cells stably expressing shRNA against the α and β catalytic subunits of PKA, or shControl, were treated for 3 hr with imiquimod (50 μM) and/or LPS (100 ng/ml), or with loxoribine (500 μM). IL-10 was measured by ELISA. Data expressed as mean ± SD (n = 6). ****p < 0.0001 for cells transfected with shPKA compared to cells transfected with shControl (two-way ANOVA followed by Sidak's post-test). (B) The expression of PKA-Cα and PKA-Cβ is shown in cells transfected with shControl (lane 1), shPKA-Cα (lane 2), shPKA-Cβ (lane 3), shPKA-Cα and shPKA-Cβ (lane 4). Ponceau staining of the blot probed with the anti-PKA-Cα antibody is shown to confirm equal loading (right panel).

the TLR7 agonist loxoribine. Moreover, imiquimod amplified LPS-stimulated IL-10 expression by 8-fold in shControl cells, while PKA downregulation reduced the synergistic IL-10 production in response to imiquimod and LPS by 70% (Fig. 7). These observations suggest that PKA is essential for induction of IL-10 by imiquimod, both in the absence and in the presence of LPS.

4. Discussion

Phosphorylation of CREB on Ser133 is essential for its interaction with the transcriptional coactivator CBP [42,43] and therefore for its activation [16,17], but it is not sufficient [8,18–20]. Although several kinases can carry out this phosphorylation event, only PKA can mediate CREB activation as it also phosphorylates an essential coactivator, CRTC3 [23]. In macrophages, we [5,8] and others [23] have previously exemplified this misconception for LPS which stimulates phosphorylation, but not activation of CREB and its essential involvement in IL-10 induction. In this study we turned to test whether other PRR agonists can activate CREB. Similarly to LPS, we found that peptidoglycan and zymosan failed to induce transcriptional activation of CREB in macrophages at 3 hr, although they were both reported to rapidly stimulate CREB phosphorylation, and to induce CREB-dependent transcription [24,37]. Our results suggest that like LPS, neither peptidoglycan nor zymosan can directly induce CREB activation in macrophages. The reported activation of CREB by these PAMPs [24,37] is therefore likely to be indirect and mediated by an autocrine cAMP inducer, such as PGE$_2$ [44,45], in particular with regards to peptidoglycan which activated CREB only at a late time point of 36 hr [24].

We found that in contrast to peptidoglycan and zymosan, the TLR7 agonist imiquimod does activate CREB-dependent transcription, and we deciphered the mechanism to be PDE inhibition leading to cAMP elevation. Consistently, a study performed with airway smooth muscle cells stimulated with agonists of two TLRs that sense viral PAMPs, showed that imiquimod, but not the TLR3 agonist poly I:C, stimulated cAMP increase by 3-fold, yet the mechanism was assumed to be TLR7 activation and was not further explored [46]. As expected, we also found that activation of the cAMP pathway by imiquimod is synergistically amplified by the β-AR agonist isoproterenol. Importantly, PDE inhibition markedly changes the outcome of TLR7 activation by imiquimod. Simulation of a TLR in macrophages initiates inflammation, propagated by a burst of pro-inflammatory cytokines, among which TNFα is prominent [47,48]. Timely resolution is ensured by the delayed kinetics of expression and secretion of the key anti-inflammatory cytokine IL-10 – low at the early phase and gradually increasing at the late phase due to autocrine/paracrine type I IFN activity [49–54]. Co-stimulation of macrophages by a cAMP inducer promotes vast IL-10 expression already at the early phase due to synergism of the cAMP pathway via CREB with the TLR-MyD88 pathway [4,5]. In addition, the cAMP pathway suppresses TNFα expression [4], and thus by reciprocal regulation of TNFα and IL-10 expression, the cAMP pathway restricts inflammation at its onset. Accordingly, in the present study we show that by inhibiting PDE and elevating cAMP level, imiquimod reciprocally regulates the expression of TNFα and IL-10 which is governed by its own activation of TLR7. For comparison, loxoribine, an unrelated TLR7 agonist that does not induce cAMP, is considerably more effective than imiquimod at TNFα expression, and in contrast it hardly stimulates IL-10 expression at the early phase. We calculated the TNFα/IL-10 ratio to be 8-fold higher for loxoribine relative to imiquimod. The extremely low IL-10 expression by loxoribine-activated TLR7, compared to LPS-activated TLR4 (Fig. 6A), which is also unable to stimulate cAMP signaling, may be because both MyD88 and TRIF contribute to IL-10 expression downstream to TLR4, whereas TLR7 is able to activate only MyD88 and not TRIF [47,48]. The ability of imiquimod to activate both the TLR7-MyD88 pathway and the cAMP-CREB pathway is therefore crucial for IL-10 expression at the early phase, as neither of these pathways alone is sufficient (Fig. 6A). Overall, the

inflammatory capacity of imiquimod as a TLR7 agonist is considerably curbed by being also a PDE inhibitor. The two contrasting effects occur as a similar concentrations range: CREB signaling is affected by imiquimod at the 10–100 μM range, and TNFα is induced by imiquimod with an EC$_{50}$ of 30 μM, as we previously reported [4].

Imiquimod is an immunoregulatory molecule that mimics single stranded RNA, a viral PAMP, by binding to TLR7 on antigen-presenting cells, such as tissue macrophages, monocytes and dendritic cells. These imiquimod-stimulated cells secrete pro-inflammatory cytokines that promote Th1 immunity [55]. In this capacity imiquimod is widely used as an antitumor and antiviral topical medication in skin cancers such as basal cell carcinoma and squamous cell carcinoma, infectious skin diseases such as molluscum contagiosum, and genital and anal warts caused by the human papilloma virus [55,56]. In light of the high efficacy, additional indications for topical imiquimod, such as melanoma *in situ* and also cutaneous melanoma metastases are often tested [55].

In this study we show that imiquimod has an additional unappreciated function, i.e. cAMP induction via PDE inhibition, which may have positive and negative implications on the effectiveness of treatment. First, PDE inhibition and activation of the cAMP pathway in tissue macrophages and other antigen-presenting cells, is expected to negatively impact the magnitude of the inflammatory response. As we have shown here, due to the suppressive role cAMP plays in macrophages, imiquimod acts simultaneously as a pro-inflammatory agent via TLR7, and as an anti-inflammatory agent via PDE inhibition. To fully appreciate the obstacle presented for imiquimod therapy by PDE inhibition, these imiquimod-treated skin pathologies should be compared to psoriasis and atopic dermatitis, which are skin pathologies treated by PDE4 inhibitor drugs. UV light induces immunosuppression which promotes the pathology of basal cell carcinoma and squamous cell carcinoma, and therefore stimulation of inflammation by the TLR7 agonist imiquimod is an approved clinical strategy [55]. Indeed, the local inflammatory response to imiquimod is well correlated with therapy success [55,57]. In contrast, psoriasis is a chronic inflammatory autoimmune skin disease, that can be initiated by excessive activation of endosomal TLRs, including TLR7 [58], and therefore the anti-inflammatory activity of the selective PDE4 inhibitor apremilast is an approved clinical strategy [59,60]. Similarly, atopic dermatitis is another inflammatory skin pathology treated by a selective PDE4 inhibitor, crisaborole [60]. Therefore, if considering the effect of PDE4 inhibition only on the inflammatory response, this off-target of imiquimod may reduce therapy success compared to alternative TLR7 agonists which do not inhibit PDE4.

In addition to the central role of TNFα in inflammation, type I IFN are of particular importance in the imiquimod-stimulated immune response during treatment of skin tumors [55]. We have recently shown that cAMP negatively regulates LPS-stimulated secretion of type I IFN from macrophages [4], and therefore it is likely that PDE inhibition by imiquimod interferes with the expression of type I IFN, as it does with TNFα expression. IL-10 which is synergistically elevated by TLR7 activation and PDE inhibition, down-regulates inflammation, for example by inhibiting TNFα expression [61], and in addition it aids cancer cells to escape immune surveillance and is also anti-angiogenic [55]. Therefore, the reciprocal effect of PDE inhibition in the macrophages on secretion of pro- and anti-inflammatory cytokines seems to have a negative impact on the effectiveness of treatment with imiquimod.

Apart from its effect on immune cells and inflammation, clinical application of imiquimod in skin disorders is expected to directly affect via PDE inhibition also non-immune cell types, such as epidermal cells that express various PDE4 isoforms [62]. This may actually represent an advantage for the usage of imiquimod in skin cancers, as cAMP signaling was reported to have multiple antitumor effects in a variety of cancer cell types, including inhibition of cell growth and stimulation of apoptosis, inhibition of migration, induction of mesenchymal-to-epithelial transition, and sensitization to chemotherapy [63]. Most relevantly, PDE4 is up-regulated in the highly malignant squamous cell

carcinoma cell line CarB, and a selective PDE4 inhibitor stimulated their apoptosis [64]. Furthermore, imiquimod stimulates p53-dependent apoptosis in a human basal cell carcinoma cell line via a TLR7-independent mechanism [65]. Finally, imiquimod inhibits hedgehog signaling, which drives basal cell carcinoma malignancy, in a TLR7-independent manner via PKA. The mechanism of PKA activation by imiquimod has been only partially attributed to adenosine receptors, and remained partially unknown [66,67]. Considering these reports regarding the antitumor effects of cAMP signaling, in particular in the relevant skin cancers [64–67], together with our discovery of PDE as an imiquimod target, it is conceivable that imiquimod is effective in treating skin carcinomas due to both TLR7-mediated inflammation which elicits anti-tumor Th1 immunity, and PDE inhibition in the cancer cells which inhibits growth and promotes apoptosis. It is thus plausible that the net result of PDE inhibition on top of TLR7 activation is positive for the antitumor indications of imiquimod, but negative for its antiviral indications.

Drug off-targets are considered a potential cause for side effects. Imiquimod is only administrated topically at the skin lesion, and is currently considered the safest drug for the given indications [55]. The most common adverse side effects associated with imiquimod in two phase III clinical trials of superficial basal cell carcinoma treatment included application site itching, burning and pain [68,57]. Other local skin reactions, e.g. erythema, edema and crusting, were highly correlated with therapy success (as assessed by histological clearance rate) and are apparently TLR7-derived [57]. Imiquimod-elicited itching was suggested to be a TLR7-independet side effect, possibly mediated by an IP_3 receptor-dependent Ca^{2+} release in DRG sensory neurons [38]. Yet, we note that itching may be mediated by histamine [69], and interestingly, non-selective PDE inhibitors (but not the selective PDE4 inhibitor rolipram) blocked histamine secretion from human skin mast cells [69,70]. Further research is warranted to identify the mechanisms of imiquimod's side effects and to determine whether these are TLR7-dependent, or perhaps mediated by PDE inhibition. Interestingly, appearance of psoriatic lesions was reported to be a side effect of imiquimod during therapy of a skin cancer [71], and subsequently, an imiquimod-induced mouse psoriasis model has been established [72]. It can therefore be assumed that a TLR7 agonist that does not inhibit PDE, and hence causes a stronger inflammatory response, bears a higher risk of inducing psoriasis as an adverse effect.

We show here that imiquimod and the selective PDE4 inhibitor rolipram similarly amplify isoproterenol-induced cAMP level in RAW264.7 macrophages, while the pan PDE inhibitor IBMX is roughly 5-fold more effective. This finding therefore implies that imiquimod displays selectivity towards PDE isoforms. Furthermore, it insinuates that imiquimod, like rolipram, is a PDE4 inhibitor, and the molecular modeling of imiquimod in the active site of PDE4B supports this suggestion. Notably, PDE4B is specifically induced by LPS in primary macrophages, and it is solely accountable for the modulation of LPS-stimulated TNFα expression in primary monocytes and macrophages and *in-vivo*, even though macrophages express additional isoforms of PDE in general, and PDE4 in particular [73,74]. It was therefore suggested that TNFα expression can be suppressed in LPS-stimulated macrophages strictly by physical sequestration of PDE4B and formation of a minor pool of cAMP, rather than by a global change in cellular cAMP level [73,74]. Yet, it should be noted that this central and nearly exclusive role of PDE4B may be relevant for TNFα induction by TLR4, and not necessarily by TLR7. Moreover, RAW264.7 macrophages may express and/or use different PDE isoforms than the primary macrophages. For example, it was suggested that PDE3 inhibition partially inhibits LPS-stimulated TNFα expression in J774.1 macrophages [75]. Finally, the potential inhibition of other PDE isoforms by imiquimod can affect processes other than TNFα suppression (e.g. IL-10 induction) in the same macrophage cell, as well as in other cell types. The selectivity of imiquimod for PDE isoforms should be explored in the future using purified recombinant PDE enzymes.

Imiquimod shares a strikingly similar dinuclear heteroaromatic structure with the adenine moiety of cAMP (Fig. 5A). In fact, the single difference in the shared part between the two molecules is the substitution of 3-N in adenine to a carbon in imiquimod. Additionally, imiquimod contains a third aromatic hydrocarbon ring fused to the adenine-like moiety, and atom 9-N has a bond to a phospho-ribose vs. an isobutyl in cAMP and imiquimod, respectively (Fig. 5A). We intentionally chose to dock imiquimod into PDE4B, in light of the central and unique role this isoform plays in cAMP-regulated TNFα expression in macrophages [73,74]. The molecular docking illustrates how the highly similar core structure enables imiquimod to mimic cAMP by binding to the active site of PDE, thereby inhibiting cAMP degradation. Imiquimod appears to interact with the side chains of Asn395, Phe446, Tyr233 and the backbone carbonyl of Asp392 (Fig. 5D). Asn395 is conserved in all cAMP-selective PDEs; its side chain forms bidentate hydrogen bonds with the $6-NH_2$ and 7-N of the adenine, and is therefore considered to be a key switch residue in dictating nucleotide selectivity between cAMP and cGMP [39,40]. In the PDE4B-imiquimod model we observed hydrogen bonds between the backbone NH of Asn395 and both $6-NH_2$ and 1-N of imiquimod. Phe446 and Tyr233, highly conserved in all PDEs, are located at opposite sides of the hydrophobic pocket. Phe446 makes a pi stacking interaction with the adenine base of AMP which accommodates the pocket [39,40], while in the model, we rather observed a strong pi stacking interaction of Phe446 with the third aromatic hydrocarbon ring of imiquimod. The different ring with which Phe446 interacts also dictates its orientation: Phe446 is parallel to the adenine of AMP whereas it appears to be in a 60° angle to the aromatic hydrocarbon ring of imiquimod. These different orientations are consistent with the stacking interaction modes considered optimal for pairing an aromatic hydrocarbon ring (as in phenylalanine) with an aromatic heterocyclic structure (containing two nitrogen atoms, as in AMP) vs. another aromatic hydrocarbon structure (as in imiquimod) [76–78]. In the PDE4B-AMP structure, Tyr233 hydrogen bonds to Asn395, thereby stabilizing it in the conformation that interacts with both $6-NH_2$ and 7-N of the adenine [39,40]. The hydroxyl group of Tyr233 appears to hydrogen bond with 7-N of imiquimod, suggesting a more direct role for this residue in the modeled interaction of the enzyme with imiquimod compared to its structure with AMP. An additional hydrogen bond is observed in the model between the backbone carbonyl of Asp392 and the $6-NH_2$ group of imiquimod. Together, these modeled interactions are suggested to enable the specific binding of imiquimod to the PDE active site.

Interestingly, the tri-aromatic structure present in imiquimod also exists in a series of imidazo[1,2–a]quinoxalines, previously developed as PDE4 inhibitors [79]. In fact, compound 7a (1-Isobutylimidazo[1,2-a]quinoxaline-4-amine) in that report [79] is identical to imiquimod, with the exception of a single shift in the location of one of the two nitrogen atoms of the imidazole ring (Fig. 5A). These high chemical similarities between imiquimod and a previously reported PDE4 inhibitor, as well as the PDE substrate cAMP, together with the molecule modeling simulation, strongly support our suggestion that imiquimod directly binds to and inhibits PDE4. The other TLR7 agonist used in this study, loxoribine, is a guanosine analog so it is less suited to bind a cAMP-selective PDE. Yet, it may bind weakly due to additional interactions made by the ribose which is present in cAMP and loxoribine, but not in imiquimod. This may explain the slightly higher CRE reporter activity in response to a co-stimulus of loxoribine and isoproterenol, compared to isoproterenol alone (Fig. 1B).

The identification of PDE as an imiquimod target paves the way to revisit previous reports where imiquimod effects were considered to be TLR7-independent. For example, intravenous administration of imiquimod causes rapid bronchodilation via an unidentified TLR7-independent mechanism [80]. It can now be understood that the mechanism in question is most likely PDE4 inhibition, as PDE4 inhibitors are strong bronchodilators [81], and accordingly the selective PDE4 inhibitor roflumilast is an approved drug for the treatment of chronic

obstructive pulmonary disease (COPD) [60,82]. Consistently, imiquimod elevates cAMP level in airway smooth muscle cells [46]. In another respiration-related example, imiquimod reduced chronic inflammation, airway hypersensitivity and airway deformation in a mouse model of chronic asthma [83]. We speculate that these outcomes are due to the anti-inflammatory effect of PDE inhibition on the highly inflamed airway tissue in the ovalbumin (OVA)-sensitized mice.

Obviously not all TLR7-independent effects of imiquimod should necessarily be attributed to PDE inhibition. It was previously demonstrated that imiquimod can also bind and antagonize the activity of the human A_1, A_{2A} and A_3 (but not significantly A_{2B}) adenosine receptors [84]. The A_1 and A_3 receptors preferentially couple to G_s and therefore stimulate cAMP production, whereas the A_{2A} and A_{2B} receptors preferentially couple to $G_{i/o}$ and therefore inhibit cAMP production [85]. The potential effect of imiquimod as an adenosine receptor antagonist on cAMP level is therefore complex and depends on the expression pattern of the receptors. RAW264.7 macrophages express the A_1 and A_3 receptors, through which adenosine was reported to inhibit LPS-induced TNFα expression [86,87], as well as the A_{2B} receptor, through which adenosine was reported to augment LPS-stimulated IL-10 expression [88]. In contrast, RAW264.7 cells do not significantly express the A_{2A} receptor [86,88] which was however shown to mediate IL-10 elevation by adenosine in LPS-stimulated peritoneal macrophages [89]. Taken together, the induction of cAMP by imiquimod via PDE inhibition may be offset in RAW264.7 macrophages by inhibition of cAMP formation via antagonism of A_1 and A_3 receptors that are activated by autocrine/paracrine adenosine. Yet, in primary macrophages imiquimod may elevate cAMP via both PDE inhibition and A_{2A} receptor antagonism. The binding of imiquimod to adenosine receptors is not surprising considering the finding presented here that imiquimod inhibits PDE due to its high chemical similarity to the adenine base present in both cAMP and adenosine. Moreover, the pan PDE inhibitor IBMX is also an antagonist of adenosine receptors [90,91], giving further support to our suggestion that the core adenine-like moiety present in both imiquimod and IBMX enable these molecules to bind PDE as well as adenosine receptors.

To conclude, we found that the TLR7 agonist imiquimod functions also as a PDE inhibitor and therefore TLR7-mediated inflammatory responses in macrophages are strongly skewed. Molecular modeling suggests that due to a strong chemical similarity, imiquimod mimics the adenine portion of cAMP in the active site of PDE4B, thereby curbing the inflammatory response. These findings may have therapeutic considerations regarding the usage of imiquimod as an antitumor and antiviral drug in certain pathological skin conditions. The present identification of a new off-target for imiquimod may facilitate development of novel anti-tumor and anti-viral skin drugs with improved efficacy and reduced side effects.

CRediT authorship contribution statement

Orna Ernst: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Hila Failayev:** Investigation, Visualization. **Muhammad Athamna:** Investigation. **Haoming He:** Investigation. **Yossi Tsfadia:** Supervision. **Tsaffrir Zor:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing - original draft. **:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] M. Takano, H. Nishimura, Y. Kimura, J. Washizu, Y. Mokuno, Y. Nimura, Y. Yoshikai, Prostaglandin E2 protects against liver injury after Escherichia coli infection but hampers the resolution of the infection in mice, J. Immunol. 161 (1998) 3019–3025.

[2] [2] R.E. Kast, Tumor necrosis factor has positive and negative self regulatory feed back cycles centered around cAMP, Int. J. Immunopharmacol. 22 (2000) 1001–1006.

[3] M. Goldsmith, D. Avni, O. Ernst, Y. Glucksam, G. Levy-Rimler, M.M. Meijler, T. Zor, Synergistic IL-10 induction by LPS and the ceramide-1-phosphate analog PCERA-1 is mediated by the cAMP and p38 MAP kinase pathways, Mol. Immunol. 46 (2009) 1979–1987.

[4] O. Ernst, Y. Glucksam-Galnoy, M. Athamna, I. Ben-Dror, H. Ben-Arosh, G. Levy-Rimler, I.D.C. Fraser, T. Zor, The cAMP pathway amplifies early MyD88-dependent and type I interferon-independent LPS-induced interleukin-10 expression in mouse macrophages, Mediators Inflamm. 2019 (2019) 3451461.

[5] O. Ernst, Y. Glucksam-Galnoy, B. Bhatta, M. Athamna, I. Ben-Dror, Y. Glick, D. Gerber, T. Zor, Exclusive temporal stimulation of IL-10 expression in LPS-stimulated mouse macrophages by cAMP inducers and type I interferons, Front. Immunol. 10 (2019) 1788.

[6] K. Koga, G. Takaesu, R. Yoshida, M. Nakaya, T. Kobayashi, I. Kinjyo, A. Yoshimura, Cyclic adenosine monophosphate suppresses the transcription of proinflammatory cytokines via the phosphorylated c-Fos protein, Immunity 30 (2009) 372–383.

[7] E.A. Wall, J.R. Zavzavadjian, M.S. Chang, B. Randhawa, X. Zhu, R.C. Hsueh, J. Liu, A. Driver, X.R. Bao, P.C. Sternweis, M.I. Simon, I.D.C. Fraser, Suppression of LPS-induced TNF-alpha production in macrophages by cAMP is mediated by PKA-AKAP95-p105, Sci. Signal. 2 (2009) ra28.

[8] D. Avni, O. Ernst, A. Philosoph, T. Zor, Role of CREB in modulation of TNFalpha and IL-10 expression in LPS-stimulated RAW264.7 macrophages, Mol. Immunol. 47 (2010) 1396–1403.

[9] A. Severn, N.T. Rapson, C.A. Hunter, F.Y. Liew, Regulation of tumor necrosis factor production by adrenaline and beta-adrenergic agonists, J. Immunol. 148 (1992) 3441–3445.

[10] J.J. Grailer, M.D. Haggadone, J.V. Sarma, F.S. Zetoune, P.A. Ward, Induction of M2 regulatory macrophages through the β2-adrenergic receptor with protection during endotoxemia and acute lung injury, J. Innate Immun. 6 (2014) 607–618.

[11] S. Shinomiya, H. Naraba, A. Ueno, I. Utsunomiya, T. Maruyama, S. Ohuchida, F. Ushikubi, K. Yuki, S. Narumiya, Y. Sugimoto, A. Ichikawa, S. Oh-ishi, Regulation of TNFα and interleukin-10 production by prostaglandins I2 and E2: studies with prostaglandin receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists, Biochem. Pharmacol. 61 (2001) 1153–1160.

[12] G. Fantuzzi, E. Di Santo, S. Sacco, F. Benigni, P. Ghezzi, Role of the hypothalamus-pituitary-adrenal axis in the regulation of TNF production in mice. Effect of stress and inhibition of endogenous glucocorticoids, J. Immunol. 155 (1995) 3552–3555.

[13] D.M. Ignar, J.L. Andrews, M. Jansen, M.M. Eilert, H.M. Pink, P. Lin, R.G. Sherrill, J.R. Szewczyk, J.G. Conway, Regulation of TNF-alpha secretion by a specific melanocortin-1 receptor peptide agonist, Peptides 24 (2003) 709–716.

[14] M. Delgado, D. Pozo, C. Martinez, J. Leceta, J.R. Calvo, D. Ganea, R.P. Gomariz, Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-induced TNF-alpha production by macrophages: in vitro and in vivo studies, J. Immunol. 162 (1999) 2358–2367.

[15] J.R. Temerozo, S.S.D. de Azevedo, D.B.R. Insuela, R.C. Vieira, P.L.C. Ferreira, V.F. Carvalho, G. Bello, D.C. Bou-Habib, The neuropeptides vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide control HIV-1 infection in macrophages through activation of protein kinases A and C, Front. Immunol. 9 (2018) 1336.

[16] M.R. Montminy, K.A. Sevarino, J.A. Wagner, G. Mandel, R.H. Goodman, Identification of a cyclic-AMP-responsive element within the rat somatostatin gene, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 6682–6686.

[17] G.A. Gonzalez, M.R. Montminy, Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133, Cell 59 (1989) 675–680.

[18] B.L. Wagner, A. Bauer, G. Schütz, M. Montminy, Stimulus-specific interaction between activator-coactivator cognates revealed with a novel complex-specific antiserum, J. Biol. Chem. 275 (2000) 8263–8266.

[19] B. Mayr, M. Montminy, Transcriptional regulation by the phosphorylation-dependent factor CREB, Nat. Rev. Mol. Cell Biol. 2 (2001) 599–609.

[20] B.M. Mayr, G. Canettieri, M.R. Montminy, Distinct effects of cAMP and mitogenic signals on CREB-binding protein recruitment impart specificity to target gene activation via CREB, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 10936–10941.

[21] M. Caivano, P. Cohen, Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages, J. Immunol. 164 (2000) 3018–3025.

[22] A.G. Eliopoulos, C.D. Dumitru, C.-C. Wang, J. Cho, P.N. Tsichlis, Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals, EMBO J. 21 (2002) 4831–4840.

[23] K.F. MacKenzie, K. Clark, S. Naqvi, V.A. McGuire, G. Nöehren, Y. Kristariyanto, M. van den Bosch, M. Mudaliar, P.C. McCarthy, M.J. Pattison, P.G.A. Pedrioli, G.J. Barton, R. Toth, A. Prescott, J.S.C. Arthur, PGE(2) induces macrophage IL-10 production and a regulatory-like phenotype via a protein kinase A-SIK-CRTC3 pathway, J. Immunol. 190 (2013) 565–577.

[24] D. Gupta, Q. Wang, C. Vinson, R. Dziarski, Bacterial peptidoglycan induces CD14-dependent activation of transcription factors CREB/ATF and AP-1, J. Biol. Chem. 274 (1999) 14012–14020.

[25] A. Poltorak, X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, B. Beutler, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282 (1998) 2085–2088.

[26] U. Zähringer, B. Lindner, S. Inamura, H. Heine, C. Alexander, TLR2 – promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity, Immunobiology 213 (2008) 205–224.

[27] M.A. Müller-Anstett, P. Müller, T. Albrecht, M. Nega, J. Wagener, Q. Gao, S. Kaesler, M. Schaller, T. Biedermann, F. Götz, Staphylococcal peptidoglycan colocalizes with Nod2 and TLR2 and activates innate immune response via both receptors in primary murine keratinocytes, PLoS ONE 5 (2010) e13153.

[28] T. Volz, M. Nega, J. Buschmann, S. Kaesler, E. Guenova, A. Peschel, M. Röcken, F. Götz, T. Biedermann, Natural Staphylococcus aureus-derived peptidoglycan fragments activate NOD2 and act as potent costimulators of the innate immune system exclusively in the presence of TLR signals, FASEB J. 24 (2010) 4089–4102.

[29] H. Schäffler, D.D. Demircioglu, D. Kühner, S. Menz, A. Bender, I.B. Autenrieth, P. Bodammer, G. Lamprecht, F. Götz, J.-S. Frick, NOD2 stimulation by Staphylococcus aureus-derived peptidoglycan is boosted by Toll-like receptor 2 costimulation with lipoproteins in dendritic cells, Infect. Immun. 82 (2014) 4681–4688.

[30] S. Suram, G.D. Brown, M. Ghosh, S. Gordon, R. Loper, P.R. Taylor, S. Akira, S. Uematsu, D.L. Williams, C.C. Leslie, Regulation of cytosolic phospholipase A2 activation and cyclooxygenase 2 expression in macrophages by the beta-glucan receptor, J. Biol. Chem. 281 (2006) 5506–5514.

[31] H. Hemmi, T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, S. Akira, Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway, Nat. Immunol. 3 (2002) 196–200.

[32] M.D. Conkright, E. Guzmán, L. Flechner, A.I. Su, J.B. Hogenesch, M. Montminy, Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness, Mol. Cell 11 (2003) 1101–1108.

[33] T. Zor, Z. Selinger, Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies, Anal. Biochem. 236 (1996) 302–308.

[34] O. Ernst, T. Zor, Linearization of the bradford protein assay, J. Vis. Exp. (2010), https://doi.org/10.3791/1918.

[35] D. Schneidman-Duhovny, Y. Inbar, R. Nussinov, H.J. Wolfson, PatchDock and SymmDock: servers for rigid and symmetric docking, Nucleic Acids Res. 33 (2005) W363–W367.

[36] E. Mashiach, D. Schneidman-Duhovny, N. Andrusier, R. Nussinov, H.J. Wolfson, FireDock: a web server for fast interaction refinement in molecular docking, Nucleic Acids Res. 36 (2008) W229–W232.

[37] S.E. Elcombe, S. Naqvi, M.W.M. Van Den Bosch, K.F. MacKenzie, F. Cianfanelli, G.D. Brown, J.S.C. Arthur, Dectin-1 regulates IL-10 production via a MSK1/2 and CREB dependent pathway and promotes the induction of regulatory macrophage markers, PLoS ONE 8 (2013) e60086.

[38] H. Hwang, H. Min, D. Kim, S.-W. Yu, S.J. Jung, S.-Y. Choi, S.J. Lee, Imiquimod induces a Toll-like receptor 7-independent increase in intracellular calcium via IP (3) receptor activation, Biochem. Biophys. Res. Commun. 450 (2014) 875–879.

[39] K.Y.J. Zhang, G.L. Card, Y. Suzuki, D.R. Artis, D. Fong, S. Gillette, D. Hsieh, J. Neiman, B.L. West, C. Zhang, M.V. Milburn, S.-H. Kim, J. Schlessinger, G. Bollag, A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases, Mol. Cell 15 (2004) 279–286.

[40] R.X. Xu, W.J. Rocque, M.H. Lambert, D.E. Vanderwall, M.A. Luther, R.T. Nolte, Crystal structures of the catalytic domain of phosphodiesterase 4B complexed with AMP, 8-Br-AMP, and rolipram, J. Mol. Biol. 337 (2004) 355–365.

[41] D. Avni, A. Philosoph, M.M. Meijler, T. Zor, The ceramide-1-phosphate analogue PCERA-1 modulates tumour necrosis factor-alpha and interleukin-10 production in macrophages via the cAMP-PKA-CREB pathway in a GTP-dependent manner, Immunology 129 (2010) 375–385.

[42] I. Radhakrishnan, G.C. Pérez-Alvarado, D. Parker, H.J. Dyson, M.R. Montminy, P.E. Wright, Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions, Cell 91 (1997) 741–752.

[43] T. Zor, B.M. Mayr, H.J. Dyson, M.R. Montminy, P.E. Wright, Roles of phosphorylation and helix propensity in the binding of the KIX domain of CREB-binding protein by constitutive (c-Myb) and inducible (CREB) activators, J. Biol. Chem. 277 (2002) 42241–42248.

[44] B.-C. Chen, C.-C. Liao, M.-J. Hsu, Y.-T. Liao, C.-C. Lin, J.-R. Sheu, C-H. Lin, Peptidoglycan-induced IL-6 production in RAW 264.7 macrophages is mediated by cyclooxygenase-2, PGE2/PGE4 receptors, protein kinase A, I kappa B kinase, and NF-kappa B, J. Immunol. 177 (2006) 681–693.

[45] Y. Alvarez, S. Municio, S. Alonso, M. Sánchez Crespo, N. Fernández, The induction of IL-10 by zymosan in dendritic cells depends on CREB activation by the coactivators CREB-binding protein and TORC2 and autocrine PGE2, J. Immunol. 183 (2009) 1471–1479.

[46] D. Van Ly, M. De Pedro, P. James, L. Morgan, J.L. Black, J.K. Burgess, B.G.G. Oliver, Inhibition of phosphodiesterase 4 modulates cytokine induction from toll like receptor activated, but not rhinovirus infected, primary human airway smooth muscle, Respir. Res. 14 (2013) 127.

[47] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783–801.

[48] B. Beutler, Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du, K. Hoebe, Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large, Annu. Rev. Immunol. 24 (2006) 353–389.

[49] E.Y. Chang, B. Guo, S.E. Doyle, G. Cheng, Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production, J. Immunol. 178 (2007) 6705–6709.

[50] S.S. Iyer, A.A. Ghaffari, G. Cheng, Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages, J. Immunol. 185 (2010) 6599–6607.

[51] J.G. Bode, C. Ehlting, D. Häussinger, The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis, Cell. Signal. 24 (2012) 1185–1194.

[52] M.J. Pattison, K.F. Mackenzie, J.S.C. Arthur, Inhibition of JAKs in macrophages increases lipopolysaccharide-induced cytokine production by blocking IL-10-mediated feedback, J. Immunol. 189 (2012) 2784–2792.

[53] M. Baliu-Piqué, G. Jusek, B. Holzmann, Neuroimmunological communication via CGRP promotes the development of a regulatory phenotype in TLR4-stimulated macrophages, Eur. J. Immunol. 44 (2014) 3708–3716.

[54] A. Howes, C. Taubert, S. Blankley, N. Spink, X. Wu, C.M. Graham, J. Zhao, M. Saraiva, P. Ricciardi-Castagnoli, G.J. Bancroft, A. O'Garra, Differential production of type I IFN determines the reciprocal levels of IL-10 and proinflammatory cytokines produced by C57BL/6 and BALB/c macrophages, J. Immunol. 197 (2016) 2838–2853.

[55] V.M. Voiculescu, C.V. Lisievici, M. Lupu, C. Vajaitu, C.C. Draghici, A.V. Popa, I. Solomon, T.I. Sebe, M.M. Constantin, C. Caruntu, Mediators of inflammation in topical therapy of skin cancers, Mediators Inflamm. 2019 (2019) 8369690.

[56] S.V. Bozrova, V.A. Levitsky, S.A. Nedospasov, M.S. Drutskaya, Imiquimod: the biochemical mechanisms of immunomodulatory and anti-inflammatory activity, Biochem. Moscow Suppl. Ser. B 7 (2013) 136–145.

[57] J. Geisse, I. Caro, J. Lindholm, L. Golitz, P. Stampone, M. Owens, Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: results from two phase III, randomized, vehicle-controlled studies, J. Am. Acad. Dermatol. 50 (2004) 722–733.

[58] C.-Y. Lai, Y.-W. Su, K.-I. Lin, L.-C. Hsu, T.-H. Chuang, Natural modulators of endosomal Toll-like receptor-mediated psoriatic skin inflammation, J. Immunol. Res. 2017 (2017) 7807313.

[59] G.M. Keating, Apremilast: a review in psoriasis and psoriatic arthritis, Drugs 77 (2017) 459–472.

[60] H. Li, J. Zuo, W. Tang, Phosphodiesterase-4 inhibitors for the treatment of inflammatory diseases, Front. Pharmacol. 9 (2018) 1048.

[61] E. Di Santo, M. Adami, R. Bertorelli, P. Ghezzi, Systemic interleukin 10 administration inhibits brain tumor necrosis factor production in mice, Eur. J. Pharmacol. 336 (1997) 197–202.

[62] C.S. Chujor, F. Hammerschmid, C. Lam, Cyclic nucleotide phosphodiesterase 4 subtypes are differentially expressed by primary keratinocytes and human epidermoid cell lines, J. Invest. Dermatol. 110 (1998) 287–291.

[63] L. Sapio, M. Gallo, M. Illiano, E. Chiosi, D. Naviglio, A. Spina, S. Naviglio, The natural cAMP elevating compound forskolin in cancer therapy: is it time? J. Cell. Physiol. 232 (2017) 922–927.

[64] D. Marko, K. Romanakis, H. Zankl, G. Fürstenberger, B. Steinbauer, G. Eisenbrand, Induction of apoptosis by an inhibitor of cAMP-specific PDE in malignant murine carcinoma cells overexpressing PDE activity in comparison to their nonmalignant counterparts, Cell Biochem. Biophys. 28 (1998) 75–101.

[65] S.-W. Huang, S.-H. Chang, S.-W. Mu, H.-Y. Jiang, S.-T. Wang, J.-K. Kao, J.-L. Huang, C.-Y. Wu, Y.-J. Chen, J.-J. Shieh, Imiquimod activates p53-dependent apoptosis in a human basal cell carcinoma cell line, J. Dermatol. Sci. 81 (2016) 182–191.

[66] F. Wolff, A. Loipetzberger, W. Gruber, H. Esterbauer, F. Aberger, A.M. Frischauf, Imiquimod directly inhibits Hedgehog signalling by stimulating adenosine receptor/protein kinase A-mediated GLI phosphorylation, Oncogene 32 (2013) 5574–5581.

[67] W. Gruber, A.-M. Frischauf, F. Aberger, An old friend with new skills: imiquimod as novel inhibitor of Hedgehog signaling in basal cell carcinoma, Oncoscience 1 (2014) 567–573.

[68] H. Gollnick, R. Barasso, U. Jappe, K. Ward, A. Eul, M. Carey-Yard, K. Milde, Safety and efficacy of imiquimod 5% cream in the treatment of penile genital warts in uncircumcised men when applied three times weekly or once per day, Int. J. STD AIDS 12 (2001) 22–28.

[69] D. Green, X. Dong, The cell biology of acute itch, J. Cell Biol. 213 (2016) 155–161.

[70] N. Eskandari, R. Bastan, P.T. Peachell, Regulation of human skin mast cell histamine release by PDE inhibitors, Allergol. Immunopathol. (Madr.) 43 (2015) 37–41.

[71] U. Patel, N.M. Mark, B.C. Machler, V.J. Levine, Imiquimod 5% cream induced psoriasis: a case report, summary of the literature and mechanism, Br. J. Dermatol. 164 (2011) 670–672.

[72] K. Bocheńska, E. Smolińska, M. Moskot, J. Jakóbkiewicz-Banecka, M. Gabig-Cimińska, Models in the research process of psoriasis, Int. J. Mol. Sci. 18 (2017).

[73] S.-L.-C. Jin, M. Conti, Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-alpha responses, PNAS 99 (2002) 7628–7633.

[74] S.-L.-C. Jin, L. Lan, M. Zoudilova, M. Conti, Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages, J. Immunol. 175 (2005) 1523–1531.

[75] Z.H. Németh, C. Szabó, G. Haskó, A.L. Salzman, E.S. Vizi, Effect of the

phosphodiesterase III inhibitor amrinone on cytokine and nitric oxide production in immunostimulated J774.1 macrophages, Eur. J. Pharmacol. 339 (1997) 215–221.

[76] C.A. Hunter, J.K.M. Sanders, The nature of.pi.-.pi. interactions, J. Am. Chem. Soc. 112 (1990) 5525–5534.

[77] D.D. Boehr, A.R. Farley, G.D. Wright, J.R. Cox, Analysis of the π-π Stacking Interactions between the aminoglycoside antibiotic kinase APH(3′)-IIIa and its nucleotide ligands, Chem. Biol. 9 (2002) 1209–1217.

[78] Y. Wu, H. Chen, D. Liu, J. Xiao, Y. Qian, H. Xi, Effective ligand functionalization of zirconium-based metal-organic frameworks for the adsorption and separation of benzene and toluene: a multiscale computational study, ACS Appl. Mater. Interfaces 7 (2015) 5775–5787.

[79] C. Deleuze-Masquéfa, G. Gerebtzoff, G. Subra, J.-R. Fabreguettes, A. Ovens, M. Carraz, M.-P. Strub, J. Bompart, P. George, P.-A. Bonnet, Design and synthesis of novel imidazo[1,2-a]quinoxalines as PDE4 inhibitors, Bioorg. Med. Chem. 12 (2004) 1129–1139.

[80] E.H. Kaufman, A.D. Fryer, D.B. Jacoby, Toll-like receptor 7 agonists are potent and rapid bronchodilators in guinea pigs, J. Allergy Clin. Immunol. 127 (2011) 462–469.

[81] D. Spina, PDE4 inhibitors: current status, Br. J. Pharmacol. 155 (2008) 308–315.

[82] M.A. Giembycz, S.K. Field, Roflumilast: first phosphodiesterase 4 inhibitor approved for treatment of COPD, Drug Des. Dev. Ther. 4 (2010) 147–158.

[83] Q. Du, L.-F. Zhou, Z. Chen, X.-Y. Gu, M. Huang, K.-S. Yin, Imiquimod, a toll-like receptor 7 ligand, inhibits airway remodelling in a murine model of chronic asthma, Clin. Exp. Pharmacol. Physiol. 36 (2009) 43–48.

[84] M.P. Schön, M. Schön, K.-N. Klotz, The small antitumoral immune response modifier imiquimod interacts with adenosine receptor signaling in a TLR7- and TLR8-independent fashion, J. Invest. Dermatol. 126 (2006) 1338–1347.

[85] R.O. Godinho, T. Duarte, E.S.A. Pacini, New perspectives in signaling mediated by receptors coupled to stimulatory G protein: the emerging significance of cAMP efflux and extracellular cAMP-adenosine pathway, Front. Pharmacol. 6 (2015) 58.

[86] Z.H. Németh, S.J. Leibovich, E.A. Deitch, E.S. Vizi, C. Szabó, G. Hasko, cDNA microarray analysis reveals a nuclear factor-kappaB-independent regulation of macrophage function by adenosine, J. Pharmacol. Exp. Ther. 306 (2003) 1042–1049.

[87] L. Martin, S.C. Pingle, D.M. Hallam, L.P. Rybak, V. Ramkumar, Activation of the adenosine A3 receptor in RAW 264.7 cells inhibits lipopolysaccharide-stimulated tumor necrosis factor-alpha release by reducing calcium-dependent activation of nuclear factor-kappaB and extracellular signal-regulated kinase 1/2, J. Pharmacol. Exp. Ther. 316 (2006) 71–78.

[88] Z.H. Németh, C.S. Lutz, B. Csóka, E.A. Deitch, S.J. Leibovich, W.C. Gause, M. Tone, P. Pacher, E.S. Vizi, G. Haskó, Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism, J. Immunol. 175 (2005) 8260–8270.

[89] G. Haskó, D.G. Kuhel, J.F. Chen, M.A. Schwarzschild, E.A. Deitch, J.G. Mabley, A. Marton, C. Szabó, Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor-dependent and independent mechanisms, FASEB J. 14 (2000) 2065–2074.

[90] J.W. Daly, K.A. Jacobson, D. Ukena, Adenosine receptors: development of selective agonists and antagonists, Prog. Clin. Biol. Res. 230 (1987) 41–63.

[91] A.J. Morgan, K.J. Murray, R.A. Challiss, Comparison of the effect of isobutylmethylxanthine and phosphodiesterase-selective inhibitors on cAMP levels in SH-SY5Y neuroblastoma cells, Biochem. Pharmacol. 45 (1993) 2373–2380.