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Exopolysaccharides from *Cyanobacterium aponinum* induce a regulatory dendritic cell phenotype and inhibit SYK and CLEC7A expression in dendritic cells, T cells and keratinocytes

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ABSTRACT

Regular bathing in the Blue Lagoon has beneficial effects on psoriasis. Previously, we showed that exopolysaccharides (EPS-Ca) secreted by *Cyanobacterium aponinum*, a dominating organism in the Blue Lagoon, increased IL-10 secretion by human dendritic cells (DCs). In addition, co-culturing allogeneic $CD4^+$ T cells with DCs matured in the presence of EPS-Ca increased differentiation of T cells into T regulatory cells at the cost of the disease inducing Th17 cells. In the present study, EPS-Ca increased the proportion of DCs expressing CD141, a surface molecule linked to regulatory DCs, and the $CD141^+$ cells secreted more IL-10 than the $CD141^-$ cells. EPS-Ca decreased T cell secretion of IL-17, IL-13 and IL-10 and the proportion of T cells expressing the activation marker CD69 that has also been linked to lymphocyte retention. In addition, EPS-Ca reduced keratinocyte secretion of CCL20 and CXCL10, chemokines implicated in recruitment of inflammatory cells. EPS-Ca decreased DC expression of Dectin-1/CLEC7A and SYK, keratinocyte expression of CLEC7A, SYK and CAMP (the gene for LL37), and T cell expression of phosphorylated Zap70. These results indicate that EPS-Ca may induce a regulatory phenotype of DCs, T cells that are less active/inflammatory and less prone to being retained in the skin, and keratinocytes that induce less recruitment of inflammatory cells to the skin and that these effects may be mediated by the effects of EPS-Ca on CLEC7A and SYK. Overall the results indicate that EPS-Ca may be involved in the beneficial effects psoriasis patients experience when bathing in the Blue Lagoon.

1. Introduction

Psoriasis is a chronic inflammatory skin disease that results from a complex interplay between dendritic cells (DCs), T cells and keratinocytes [1]. It is characterized as a Th1/Th17 mediated autoimmune disease [2,3] in which the antimicrobial peptide LL37 has been determined to be an autoantigen [2,4]. That bathing in the Blue Lagoon in Iceland, in conjunction with UVB treatment, gives better and longer lasting results than UVB treatment alone has been confirmed in prospective, randomized clinical trials [5,6]. However, how bathing in the Blue Lagoon has beneficial effects is not fully understood, although many factors, including the 35 taxa that inhabit the Blue Lagoon [7], unique inorganic chemicals, heat, salinity, relaxation and sun exposure may be contributors. Extracts from silica mud and the two main microalgae from the Blue Lagoon improved skin barrier function and prevented premature skin ageing in humans [8], indicating that these may provide beneficial effects on skin. In addition, we have previously demonstrated that exopolysaccharides (EPS-Ca) secreted by *Cyanobacterium aponinum*, a dominating organism in the Blue Lagoon, has anti-inflammatory effects on DCs [9], leading us to hypothesize that EPS-Ca may contribute to the beneficial effects of regular bathing in the Blue Lagoon [9].

DCs that were matured in the presence of EPS-Ca in our previous study secreted more IL-10 than DCs matured in the absence of EPS-Ca

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and also differentiated co-cultured allogeneic $CD4^+$ T cells into T cells with a more T regulatory (Treg) and less Th17 phenotype than T cells co-cultured with DCs not treated with EPS-Ca [9]. These results indicate that EPS-Ca may induce a regulatory DC phenotype. DCs have previously been shown to be able to attain a regulatory phenotype [10,11] and recently a $CD141^+$ DC phenotype and a murine functional homologue, the $CD103^+$ DC phenotype, have been described and both these phenotypes have been linked to immune regulation and to mediate protection in several inflammatory diseases [12–17]. Whether the DC phenotype induced by EPS-Ca in the present study belongs to the $CD141^+$ phenotype remains to be seen.

Induction of regulatory DCs in mice was shown by Hang et al. to be mediated by a decrease in the Dectin-1 receptor (encoded by the CLEC7A gene) and the downstream Spleen tyrosine kinase (Syk) signaling pathway [13]. Overexpression of SYK induces inflammation, autoimmunity and allergy and has an imperative role in the pathogenesis of several autoimmune diseases, including psoriasis, multiple sclerosis and lupus erythematosus [18,19]. ZAP70 is one of two members of the Syk family and plays an important role in T cell activation [20], whereas SYK is broadly expressed in hematopoietic [21] and non-hematopoietic cells and plays a broader role in the immune system, e.g. in CCL20 secretion by keratinocytes [22] and IL-10 secretion by DCs [10].

Here we report that a higher proportion of DCs expressed CD141 and the DCs expressed higher levels of CD141 when they were treated with EPS-Ca than when they were not treated with EPS-Ca. In addition, EPS-Ca decreased DC expression of the Dectin-1 receptor and its mRNA levels (CLEC7A) as well as the down-stream signaling molecule SYK. Furthermore, EPS-Ca decreased cytokine secretion by stimulated T cells and their expression of the activation marker CD69, possibly by reducing their phosphorylation of the Syk family kinase ZAP70. EPS-Ca also inhibited CLEC7A and SYK mRNA expression in keratinocytes, decreased their expression of CAMP (the encoding gene for LL37) and their CCL20 and CXCL10 secretion upon either Th17-like or Th1-like stimulation.

2. Materials and methods

2.1. Cultivation of Cyanobacterium aponinum and preparation of EPS-Ca

C. aponinum obtained from the Blue Lagoon was cultured in Blue Lagoon geothermal seawater under controlled conditions in a closed tubular photobioreactors (160 μE/m^2/s; 40 °C; pH 7.5). The culture was collected and the biomass separated from the supernatant by centrifugation. The supernatant was lyophilized, dissolved in distilled water, dialysed for 4 days (Spectra/Por dialysis membrane with 3500 kDa cut-off, Spectrum Laboratories, CA), filtrated and lyophilized again. The exopolysaccharides obtained were named EPS-Ca. The monosaccharide content of EPS-Ca has been determined [9].

2.2. Dendritic cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized buffy coat obtained from healthy donors by density gradient centrifugation over Ficoll–Histopaque (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min. Monocytes were then isolated from the PBMCs using CD14 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The $CD14^+$ monocytes were differentiated into immature DCs (imDCs) by culturing them for 7 days with 12.5 ng/ml IL-4 and 25 ng/ml GM-CSF (both from R&D Systems, Bio-Techne, Abington, UK) in RPMI media, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco, Thermo Fisher Scientific, Paisley, UK) in 48-well flat bottom plates (Nunc, Roskilde, Denmark). The DCs were then cultured for 24 h in the presence or absence of EPS-Ca (100 μg/ml). DC viability was assessed by trypan blue staining.

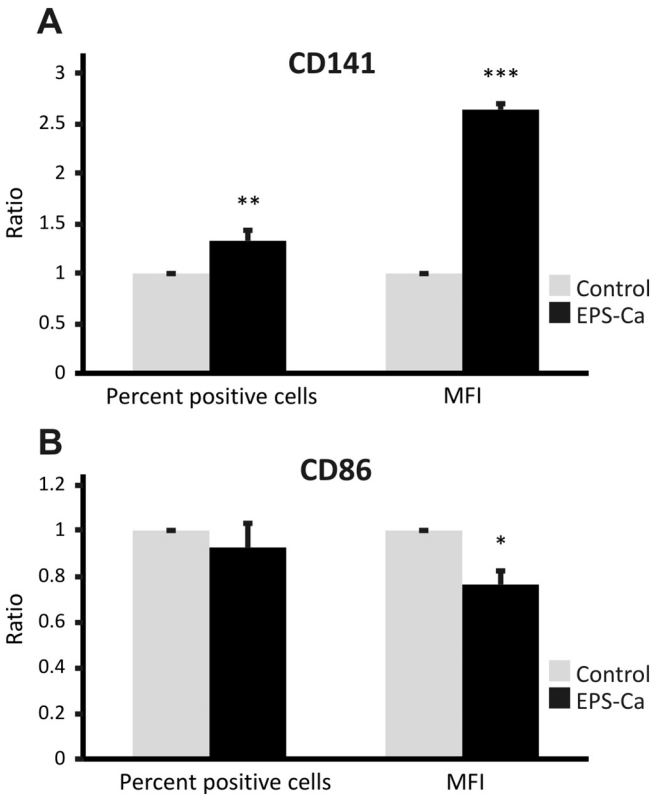

Fig. 1. EPS-Ca induces CD141 expression on DCs.
DCs, stimulated with cytokines and LPS, were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (control) for 24 h and expression of (A) CD141 and (B) CD86 assessed by flow cytometry with results expressed as ratio to control (percentage positive cells or mean fluorescence intensity (MFI)), n = 7. The absolute values for DCs cultured without EPS-Ca are 61% for CD141 and 97% for CD86; and MFI 28 for CD141 and 260 for CD86. Results are shown as mean + SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, different from control. Representative histograms of flow cytometric analysis are shown in Supplementary Fig. 1A and B.

There was no difference in viability between DCs treated with EPS-Ca and DCs not treated with EPS-Ca. When examining DCs for expression of surface and intracellular molecules, they were stimulated with 10 ng/ml IL-1β, 50 ng/ml TNF-α (both from R&D Systems) and 500 ng/ml LPS (Sigma-Aldrich), in addition to EPS-Ca, for the indicated times.

2.3. T cells

$CD4^+$ and $CD8^+$ T cells were isolated from PBMCs using CD4 and CD8 Microbeads (Miltenyi Biotec), respectively, according to the manufacturer's instructions. Isolated T cells were stimulated with 4 μg/ml plate-bound anti-CD3ε antibody and 1 μg/ml soluble anti-CD28 antibody (both from R&D Systems) for 72 h, in the presence or absence of EPS-Ca (100 μg/ml) for the last 24 h. The cells were cultured in RPMI media (supplemented with 10% FBS and 1% penicillin/streptomycin) in 96-well U bottom plates (Nunc). The viability of the T cells was assessed by trypan blue staining. There was no difference in viability between T cells treated with EPS-Ca and T cells not treated with EPS-Ca.

2.4. Keratinocytes

Normal adult human primary epidermal keratinocytes were acquired from ATCC (LGC Standards, Wesel, Germany) and cultured in Dermal Cell Basal Medium supplemented with Keratinocyte Growth Kit (LGC Standards). The cells were cultured in 48-well flat bottom plates

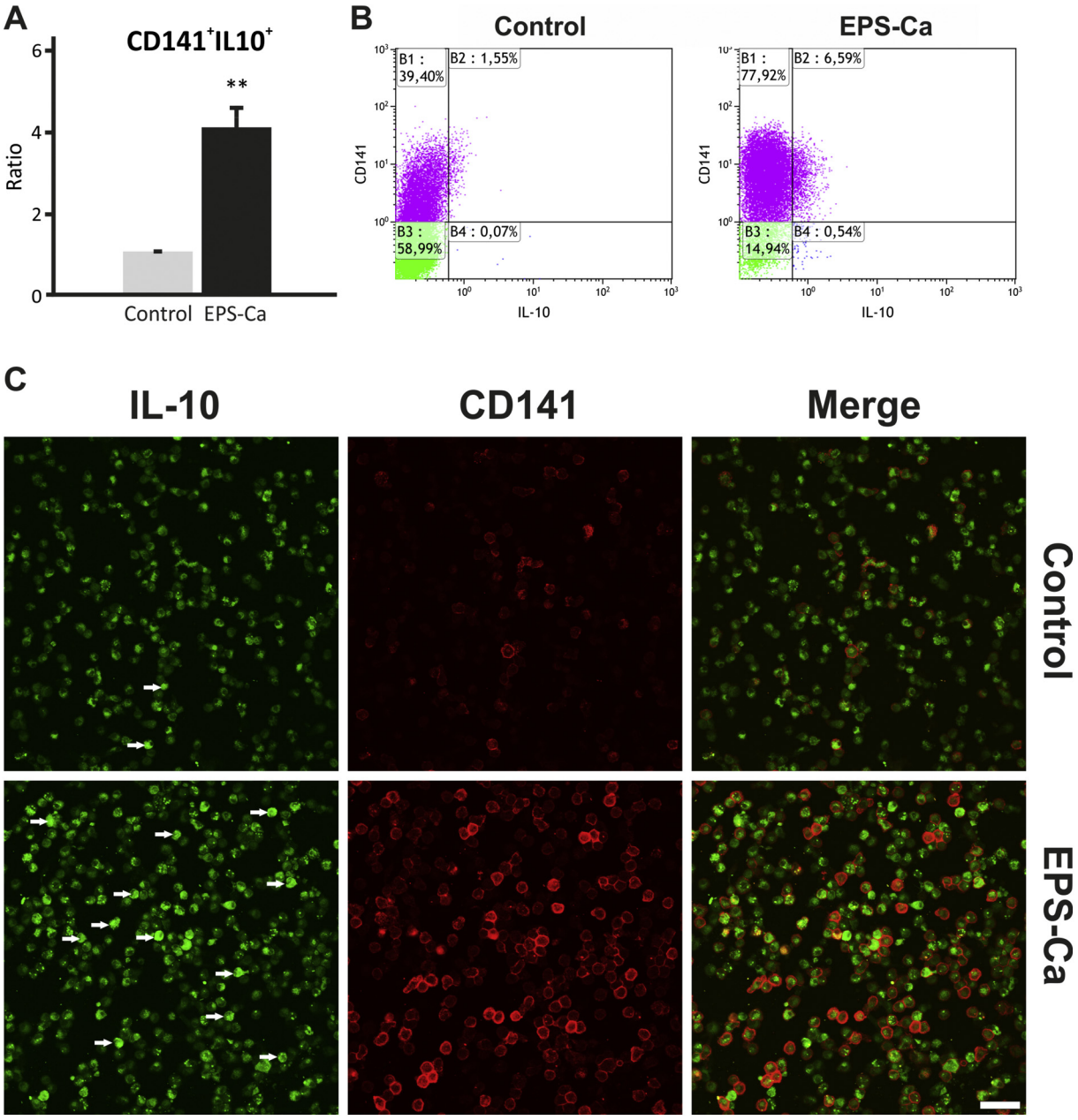

Fig. 2. EPS-Ca increases the proportion of CD141$^+$IL-10$^+$ DCs.
DCs were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (Control) for 24 h and stained with antibodies against CD141 and IL-10. (A) The ratio of CD141$^+$IL-10$^+$ DCs to control (percent positive cells), n = 3. 1.63% of control DCs were positive for CD141$^+$IL-10$^+$. Results are shown as mean + SEM, **p < 0.01, different from control. (B) Representative FACS dot plots of DCs. (C) Confocal microscopy images of DCs stained with antibodies against intracellular IL-10 (green) and CD141 (red). Arrows point to a few examples of IL-10$^+$ cells. Quantification of the fluorescence intensity was performed using CellProfiler and was for IL-10 8.2 ± 0.2 for cells cultured without EPS-Ca and 10.3 ± 0.3 for cells cultured with EPS-Ca. For CD141, the fluorescence intensity was 4.7 ± 0.2 for cells cultured without EPS-Ca and 8.2 ± 0.3 for cells cultured with EPS-Ca. Images of DCs were taken under 30× magnification. Scale bar, 50 μm.

and incubated for 24 h followed by Th17 or Th1 mimicking stimulation for 24 h, in the presence or absence of EPS-Ca (100 μg/ml). For Th17 mimicking stimulation the cells were cultured with 40 ng/ml TNF-α and 50 ng/ml IL-17A, whereas for Th1 mimicking stimulation they were cultured with 20 ng/ml TNF-α and 100 ng/ml IFN-γ (all from R&D Systems). The viability of the keratinocytes was assessed by trypan blue staining. There was no difference in viability between keratinocytes treated with EPS-Ca and those not treated with EPS-Ca.

2.5. Expression of intra- and extracellular molecules

DCs, T cells and keratinocytes were incubated with 2% normal human serum (NHS)/normal mouse serum (NMS) for 10 min for blocking of nonspecific binding sites. They were then incubated with fluorochrome-labeled monoclonal antibodies (mabs) against HLA-DR (clone L243), CD4 (clone RPA-T4), CD8 (clone SK1), CD69 (clone FN50), Dectin-1 (CD369, clone 15E2) (all from eBioscience, Thermo Fisher Scientific), ICAM-1 (CD54, clone 15.2), CD86 (clone BU63) (both from BioRad, Kidlington, UK), VLA-4 (CD49d, clone 9F10), CD141 (clone M80) (both from BioLegend, Nordic Biosite, Sweden), or appropriate isotype control antibodies on ice for 20 min. The cells were then washed with staining buffer (phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% sodium azide) and resuspended in 1% paraformaldehyde in PBS. The cells were collected on

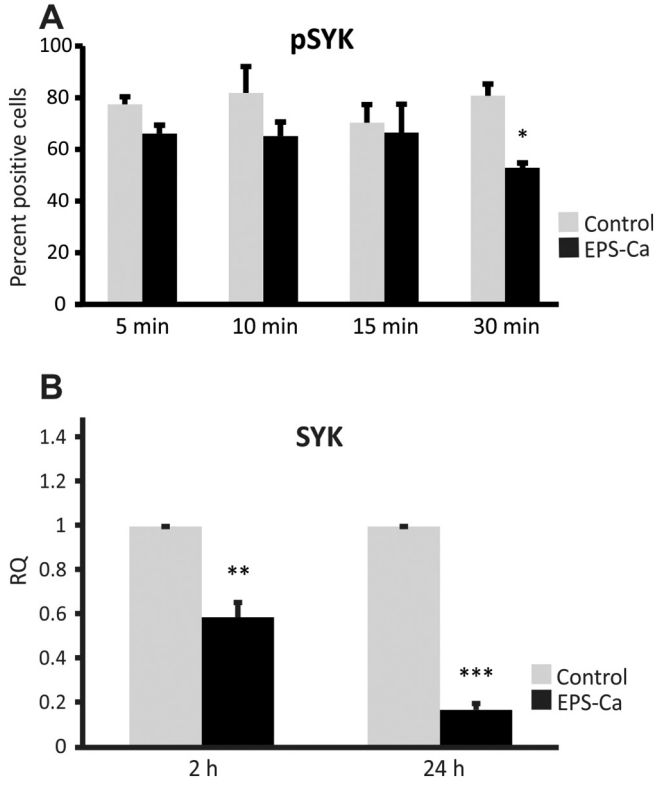

Fig. 3. EPS-Ca decreases phosphorylated SYK upon stimulation and decreases mRNA for SYK in DCs.
DCs were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (Control) for the times indicated. (A) The cells were additionally stimulated with IL-1β, TNF-α and LPS and then stained for pSYK and analyzed by flow cytometry, with results expressed as percentage of positive cells, n = 3. (B) Levels of mRNA for SYK were estimated using quantitative RT-PCR and normalized to the housekeeping gene glyceraldehyde 3-phosphate (GAPDH), n = 3. Results are shown as mean + SEM. **p < 0.01, ***p < 0.001, different from control. Representative histogram of flow cytometric analysis is shown in Supplementary Fig. 1C.

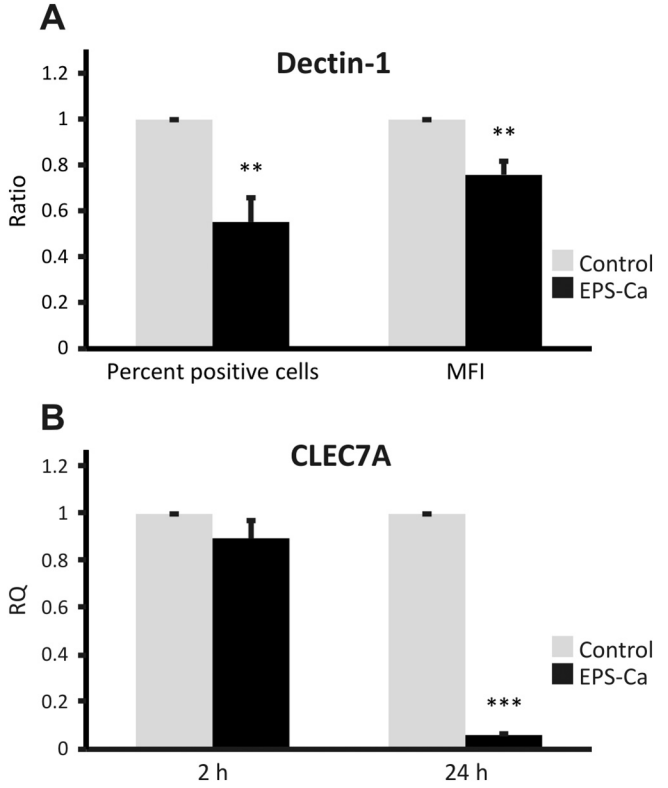

Fig. 4. EPS-Ca decreases Dectin-1 expression and mRNA levels for CLEC7A in DCs.
DCs, stimulated with cytokines and LPS (A) or not (B), were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (Control) for indicated times. (A) Expression of the Dectin-1 receptor was assessed after 24 h by flow cytometry with results expressed as ratio to control (percentage positive cells or mean fluorescence intensity (MFI)), n = 7. 49% of control DCs were positive for Dectin-1 and their MFI was 97. (B) Levels of CLEC7A (the encoding gene for the Dectin-1 receptor) mRNA was estimated using quantitative RT-PCR and normalized to the housekeeping gene glyceraldehyde 3-phosphate (GAPDH); n = 3. Results are shown as mean + SEM, **p < 0.01, ***p < 0.001, different from control. Representative histogram of flow cytometric analysis is shown in Supplementary Fig. 1D.

a FACSCalibur (BD Bioscience, San Jose, CA, USA) or Navios (Beckman Coulter, Atlanta, GA, USA) and analyzed with Kaluza analysis software (Beckman Coulter). The results are expressed as the ratio of the percentage of positive cells or mean fluorescence intensity (MFI) of cells treated with EPS-Ca to the percentage of positive cells or MFI of cells not treated with EPS-Ca.

For intracellular IL-10 staining, the cells were incubated with Brefeldin A (BioLegend) for the last 10 h of the culture. The cells were then fixed using 4% paraformaldehyde for 20 min at room temperature (RT), permeabilized with saponin buffer (0.5% saponin, 0.5% BSA in PBS) and incubated with Alexa-488-labeled mab against IL-10 (clone JES3-19F1) (BD Bioscience) for 20 min on ice and finally washed and resuspended in staining buffer.

For phospho-flow staining, freshly isolated T cells or imDCs were allowed to rest for 1 h and then stimulated for the indicated times. The cells were then fixed with 3.7% paraformaldehyde for 10 min at RT and permeabilized with ice cold 95% MeOH for 30 min in a − 20 °C freezer. The cells were then washed and resuspended in staining buffer. Next, the cells were incubated for 10 min at RT with NHS/NMS and then with APC-labeled mabs against pSYK (clone moch1ct) or pSYK/ZAP70 (clone n3kobu5) (both from eBioscience, Thermo Fisher Scientific) for 30 min at RT. Cells were then washed and resuspended in staining buffer.

2.6. Immunofluorescence staining and confocal imaging

DCs previously stained with mabs against CD141 and IL-10 for flow cytometry were mounted on glass slides using Cytospin (Thermo Fisher Scientific). Keratinocytes were fixed in 3.7% formaldehyde for 15 min and permeabilized with 0.1% Triton-X (Sigma-Aldrich) for 30 min. Samples were incubated with 10% FBS and then with antibody against LL37 (Innovagen AB, Lund, Sweden) overnight at 4 °C. The slides were then stained with Alexa-488-labeled goat anti-rabbit IgG (Thermo Fisher Scientific), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI), which stains nuclei (Sigma-Aldrich) and Alexa-546-labeled phalloidin, which stains actin (Thermo Fisher Scientific). The slides were embedded in fluoromount (Sigma-Aldrich) and viewed in an Olympus Fluoview FV1200 confocal microscope (Olympus, Tokyo, Japan).

2.7. Secretion of cytokines

Cytokine concentration in cell culture supernatants was determined by DuoSet® ELISA (R&D Systems) according to the manufacturer's protocol. The cytokines IL-10, IL-13, IL-17 and IFN-γ were measured in supernatants from T cells and CCL20 and CXCL10 in supernatants from keratinocytes. The results are expressed as the ratio of the cytokine concentration in supernatants from cells treated with EPS-Ca to the cytokine concentration in supernatants from cells not treated with EPS-Ca.

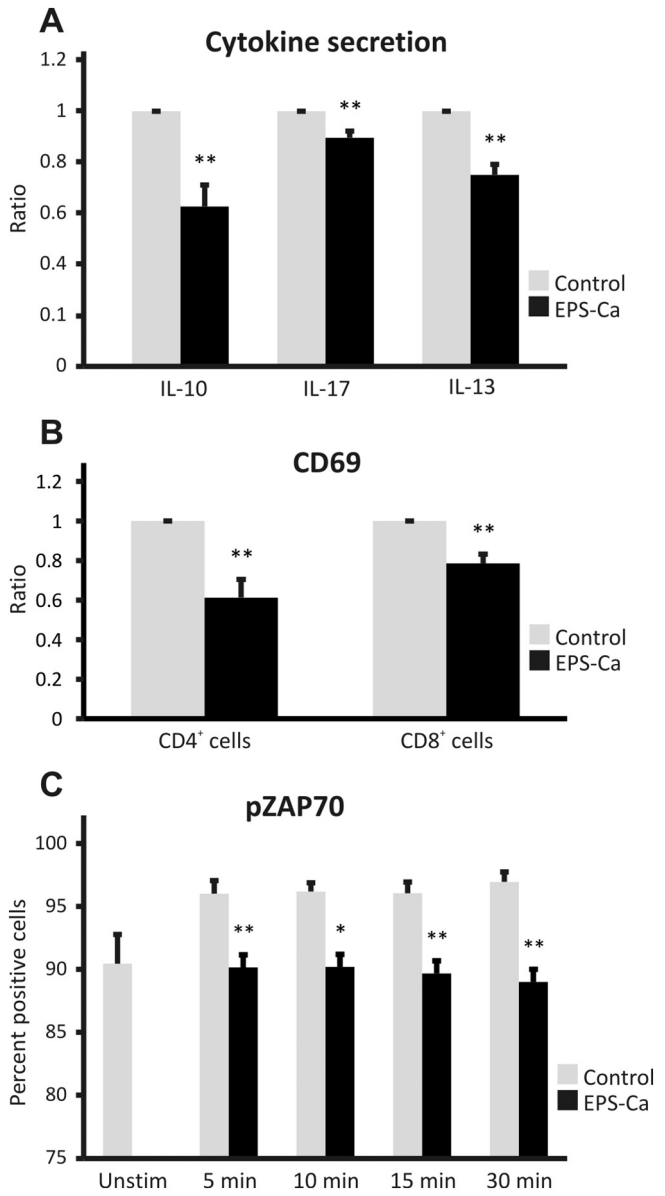

Fig. 5. EPS-Ca decreases cytokine secretion and expression of CD69 and pZAP70 by stimulated T cells.
T cells were stimulated with 4 μg/ml plate-bound anti-CD3ε antibody and 1 μg/ml soluble anti-CD28 antibody for 72 h, with 100 μg/ml EPS-Ca present (EPS-Ca) or absent (Control) for the last 24 h (A and B) or for the times indicated (C). (A) The concentrations of IL-10, IL-17 and IL-13 in the supernatants of $CD4^+$ T cells were measured by ELISA and are expressed as ratio of control, n = 6. The absolute values for T cells cultured without EPS-Ca are 19.3 ng/ml for IL-10, 5338 pg/ml for IL-17 and 952 pg/ml for IL-13. (B) Expression of CD69 by $CD4^+$ or $CD8^+$ T cells was determined by flow cytometry and the results expressed as ratio of control (percent positive cells), n = 6 for $CD4^+$ T cells and n = 3 for $CD8^+$ T cells. The absolute values for T cells cultured without EPS-Ca are 16% for the $CD4^+$ T cells and 49% for the $CD8^+$ T cells. (C) $CD4^+$ T cells were stained with antibody against pZAP70 and analyzed by flow cytometry with results shown as percent positive cells, n = 3. Results are presented as mean + SEM. *p < 0.05, **p < 0.01, different from control. Representative histograms of flow cytometric analysis are shown in Supplementary Fig. 2A and B.

2.8. mRNA expression

Total RNA was isolated from DCs, T cells and keratinocytes using Tri Reagent® solution (Sigma-Aldrich) and reverse transcribed using random hexamer primers and Superscript IV (Thermo Fisher Scientific). The resulting cDNA was used as a template for quantitative RT-PCR, using primers and a probe (TaqMan) acquired from Integrated DNA Technologies (Skokie, IL, USA). The levels of CLEC7A, SYK, ZAP70 and CAMP were estimated and normalized to the housekeeping gene glyceraldehyde 3-phosphate (GAPDH), where each experiment was performed in triplicate.

2.9. Statistical analysis

Results are presented as mean + standard error of the mean (SEM) unless stated otherwise. Differences between groups were assessed with Student two tailed *t*-test. Differences were considered statistically significant if p-values were lower than 0.05.

3. Results

3.1. EPS-Ca induces CD141 expression on DCs

Following our previous findings that EPS-Ca induces IL-10 production by DCs and their ability to differentiate T cells into Tregs [9], we examined the effects of EPS-Ca on DC expression of CD141, a surface marker expressed on regulatory DCs [12,15]. A higher proportion of DCs expressed CD141 when the DCs were treated with EPS-Ca than when they were not treated with EPS-Ca and EPS-Ca-treated DCs also expressed higher levels (higher MFI) of CD141 (Fig. 1A). EPS-Ca did not affect the proportion of DCs expressing the co-stimulatory molecule CD86 but decreased slightly the level (MFI) of CD86 expression on the DCs (Fig. 1B). These data show that EPS-Ca has the potential to increase the proportion of DCs with a regulatory phenotype. This was also confirmed using DCs that were unstimulated and cultured with or without EPS-Ca (data not shown).

3.2. EPS-Ca treatment increases the proportion of $CD141^+IL\text{-}10^+$ DCs

Next, we examined whether the increase in the proportion of $CD141^+$ DCs obtained when treating the cells with EPS-Ca was linked to the increased IL-10 secretion by EPS-Ca-treated DCs demonstrated previously. Treatment with EPS-Ca increased the proportion of $CD141^+IL\text{-}10^+$ DCs by four-fold (Fig. 2A) and most (> 90%) of the EPS-Ca treated DCs expressing IL-10 intracellularly also expressed CD141 (Fig. 2B). Confocal imaging demonstrated the presence of IL-10 in the $CD141^+$ EPS-Ca-treated DCs (Fig. 2C).

3.3. EPS-Ca decreases SYK expression by DCs

Downregulation of the Syk signaling pathway has recently been shown to promote induction of regulatory DCs in mice [13]. We, therefore, determined the effects of EPS-Ca on phosphorylated SYK (pSYK). A lower proportion of DCs expressed pSYK when the DCs had been treated with EPS-Ca as compared with that when the DCs had not been treated with EPS-Ca (Fig. 3A). To determine whether the reduction in the proportion of DCs expressing pSYK was an actual reduction in phosphorylation of the SYK protein or whether EPS-Ca was affecting production of the protein on a gene level, the effects of EPS-Ca on mRNA levels for SYK were determined using quantitative RT-PCR. As shown in Fig. 3B, mRNA levels for SYK were lower in DCs treated with EPS-Ca than DCs not treated with EPS-Ca. These results indicate that the decrease in pSYK in DCs treated with EPS-Ca was the result of a decrease in mRNA levels for SYK.

3.4. EPS-Ca decreases Dectin-1 expression and inhibits CLEC7A gene expression in DCs

Next, we determined the effects of EPS-Ca on expression of Dectin-1, one of the receptors that induces the Syk signaling pathway in DCs. When DCs were treated with EPS-Ca a lower proportion of the cells

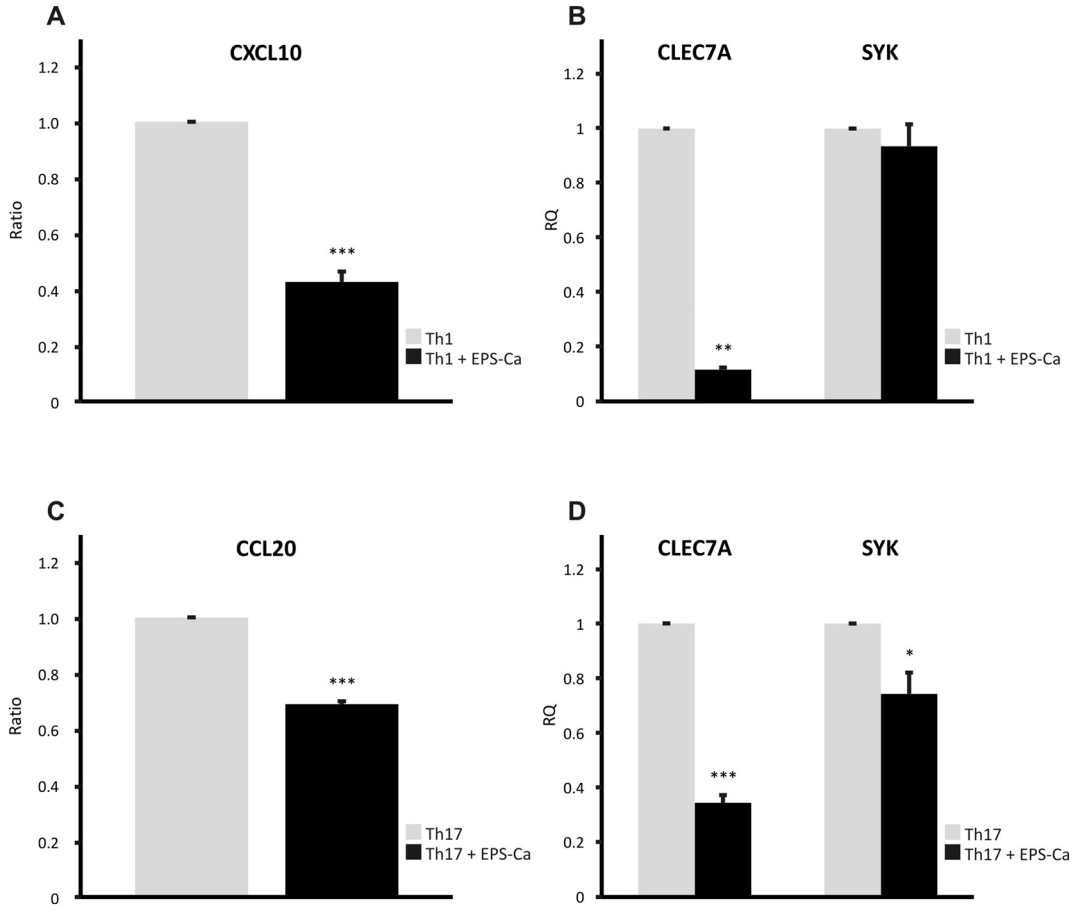

Fig. 6. EPS-Ca decreases CXCL10 and CCL20 secretion and mRNA levels for CLEC7A and SYK in keratinocytes.
Primary keratinocytes were stimulated for 24 h with Th1 (A and B) or Th17 (C and D) mimicking environments in the presence of EPS-Ca at 100 μg/ml (Th1 + EPS-Ca or Th17 + EPS-Ca, respectively) or not (Th1 or Th17, respectively). The concentration of (A) CXCL10 and (C) CCL20 in the supernatants were measured by ELISA, with results expressed as ratio of control, n = 3. The absolute values for keratinocytes cultured without EPS-Ca are 1349 ng/ml for CXCL10 and 1329 pg/ml for CCL20. (B and D) Levels of mRNA for CLEC7A (the encoding gene for the Dectin-1 receptor) and SYK was estimated using quantitative RT-PCR and normalized to the housekeeping gene glyceraldehyde 3-phosphate (GAPDH), n = 3. Results are shown as mean + SEM, *p < 0.05, **p < 0.01, ***p < 0.001, different from control.

expressed Dectin-1 on their surface compared with that on DCs not treated with EPS-Ca (Fig. 4A). EPS-Ca-treated DCs also expressed lower levels (lower MFI) of Dectin-1 than DCs not treated with EPS-Ca (Fig. 4A). This was also confirmed using DCs that were unstimulated and cultured with or without EPS-Ca (data not shown). In order to determine whether EPS-Ca affected the expression of CLEC7A, the gene encoding for Dectin-1, rather than down-regulating the receptor by internalization, mRNA levels for CLEC7A were determined in DCs treated with or without EPS-Ca. As shown in Fig. 4B, EPS-Ca decreased mRNA levels for CLEC7A when the DCs were treated with EPS-Ca for 24 h. These results show that EPS-Ca inhibited mRNA levels for CLEC7A, which in turn resulted in down-regulation of DC expression of the Dectin-1 receptor.

3.5. EPS-Ca decreases activation and cytokine secretion by T cells

As activated T cells play an important role in the pathogenesis of psoriasis, the effects of EPS-Ca on stimulated T cells were analyzed. $CD4^+$ T cells, stimulated with antibodies against CD3 and CD28 and then treated with EPS-Ca, secreted lower levels of IL-10, IL-17 and IL-13 than those not treated with EPS-Ca (Fig. 5A). EPS-Ca did not affect secretion of IFN-γ (data now shown). Little or negligible cytokine concentration was detected in the supernatants of $CD8^+$ T cells (data not shown). Lower proportion of $CD4^+$ and $CD8^+$ T cells expressed CD69 after EPS-Ca treatment as compared with T cells not treated with EPS-Ca (Fig. 5B). EPS-Ca did not affect expression of the adhesion molecules ICAM-1 and VLA-4 on activated T cells (data not shown). The Syk family kinase ZAP70 is essential for signaling through the T cell receptor and, therefore, the effects of EPS-Ca on phosphorylated ZAP70 (pZAP70) were determined. EPS-Ca reduced the proportion of activated T cells expressing pZAP70 as compared with T cells not treated with EPS-Ca (Fig. 5C). EPS-Ca did, however, not affect mRNA levels for ZAP70 in activated T cells (data not shown). These data suggest that activation of T cells was down-regulated following treatment with EPS-Ca and that it was linked to down-regulation of ZAP70 phosphorylation.

3.6. EPS-Ca decreases CXCL10 and CCL20 secretion and mRNA levels for CLEC7A and SYK by primary keratinocytes

Keratinocytes are important in the pathogenesis of psoriasis and, therefore, the effects of EPS-Ca on keratinocytes were determined. Primary keratinocytes were stimulated with TNF-α + IFN-γ or TNF-α + IL-17A, mimicking the proposed Th1 and Th17 cytokine environment in psoriasis [3]. EPS-Ca decreased CXCL10 secretion by keratinocytes stimulated with Th1 mimicking stimulation (Fig. 6A) and CCL20 secretion by keratinocytes stimulated with Th17 mimicking stimulation (Fig. 6C). As EPS-Ca decreased mRNA levels for CLEC7A and SYK in DCs, its effects on mRNA levels for these genes in keratinocytes were also determined. EPS-Ca decreased mRNA levels for CLEC7A regardless of whether the keratinocytes were stimulated with Th1 or Th17 mimicking stimulation (Fig. 6B and D) but only decreased mRNA levels for SYK upon Th17 mimicking stimulation (Fig. 6D).

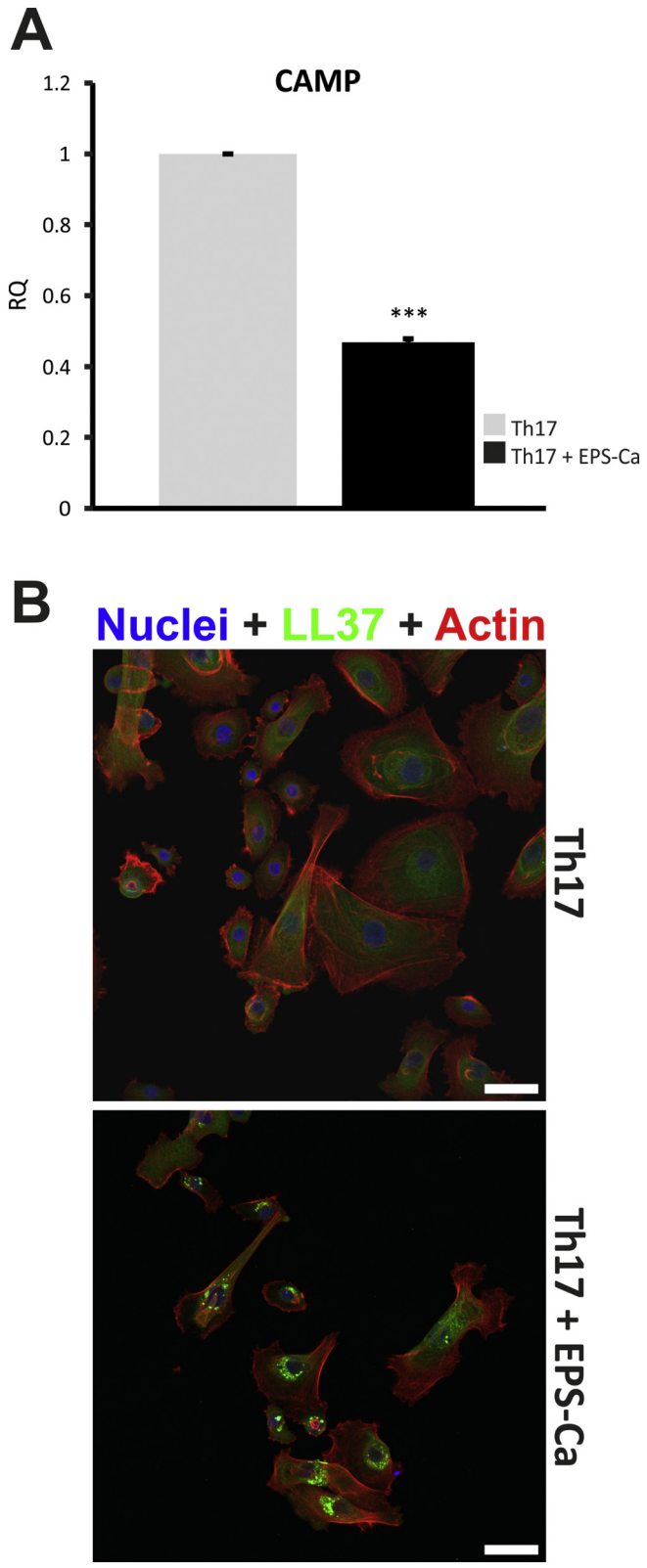

Fig. 7. EPS-Ca decreases mRNA levels of CAMP and alters LL37 expression in keratinocytes.
Keratinocytes were stimulated with Th17 mimicking environment (TNF-α and IL-17A) for 24 h with 100 μg/ml EPS-Ca present (Th17 + EPS-Ca) or absent (Th17). (A) Levels of mRNA for CAMP were estimated using quantitative RT-PCR and normalized to the housekeeping gene glyceraldehyde 3-phosphate (GAPDH), n = 3. Results are shown as mean + SEM, ***$p < 0.001$, different from control. (B) Confocal microscopy imaging of Th17 stimulated keratinocytes stained for intracellular LL37 (green), actin (red) and nuclei (blue). Data show that LL37 is dispersed throughout the cytosol when the keratinocytes are stimulated with Th17 mimicking cytokines; however, LL37 seems to accumulate into vesicles when treated with EPS-Ca. Images of keratinocytes were taken under 30× magnification. Scale bars, 50 μm.

3.7. EPS-Ca decreases mRNA levels for CAMP in primary keratinocytes

The antimicrobial peptide LL37, encoded by the CAMP gene, has been reported to be one of the autoantigens in psoriasis [4]. The Th17 mimicking stimulation increased mRNA levels for CAMP in primary keratinocytes and this increase was attenuated by treatment with EPS-Ca (Fig. 7A). To explore the fate of the existing LL37 peptide, keratinocytes were stained with antibody against LL37 and viewed by confocal microscopy. As shown in the upper panel of Fig. 7B, LL37 is dispersed fairly evenly throughout the cytoplasm. After treatment with EPS-Ca, LL37 accumulated into small vesicles (Fig. 7B, lower panel). These results show that EPS-Ca had an effect on LL37 expression in keratinocytes both on a gene level and on distribution of the protein.

4. Discussion

Here we take important steps towards clarifying how exopolysaccharides produced by the *Cyanobacterium aponinum* in the Blue Lagoon induce IL-10 production by DCs and also elucidate the effects of the exopolysaccharides on T cells and keratinocytes. The main findings are that EPS-Ca increased the proportion of DCs with a regulatory phenotype ($CD141^+$ DCs), decreased DC expression of Dectin-1, on both protein and mRNA (CLEC7A) levels, and decreased mRNA levels for the downstream signaling protein SYK. EPS-Ca also decreased mRNA levels for CLEC7A in keratinocytes. Furthermore, EPS-Ca reduced activation of stimulated T cells and reduced the efficacy of the T cell receptor signaling by down-regulating phosphorylation of ZAP70. As Dectin-1 and SYK/ZAP70 have different roles within different cell types the effects of EPS-Ca may manifest differently, and in a cell specific manner, but overall seem to reduce inflammation. The results can explain, to some extent, the beneficial effect of bathing in the Blue Lagoon on psoriasis (Fig. 8).

In a previous study, we showed that EPS-Ca increased IL-10 secretion by DCs and increased the ability of the DCs to differentiate T cells into Tregs [9]. The present study further demonstrates that EPS-Ca increases the proportion of DCs expressing the regulatory DC marker CD141 and that these $CD141^+$ DCs are the major producers of IL-10. Thus, EPS-Ca may increase the proportion of regulatory DCs, which can direct the immune response away from a pro-inflammatory one towards a more regulatory one. EPS-Ca treatment of the DCs resulted in down-regulation of expression of the Dectin-1 receptor and of SYK, the major protein in the signaling pathway from Dectin-1. As down-regulation of Syk has previously been shown to induce a regulatory phenotype of DCs [13], EPS-Ca's down-regulation of SYK may be the key event in inducing an increase in regulatory DCs with increased ability for IL-10 production and increased potential to induce Tregs instead of Th17 cells.

Most of the psoriatic patients bathing in the Blue Lagoon are in an active stage of their psoriasis, with their psoriatic plaques heavily infiltrated by Th1 and Th17 effector cells [3]. Therefore, we examined the effects of EPS-Ca on stimulated T cells and demonstrated that EPS-Ca attenuated T cell activation, evidenced by a lowered proportion of the cells expressing CD69 and a decrease in their cytokine secretion. Since CD69 also acts as a retention signal [23], one can speculate that decreased CD69 expression on T cells following treatment with EPS-Ca might increase T cell migration from the skin; thereby decreasing inflammation in skin-inflammatory diseases, such as psoriasis. In addition to lowering their activation state, EPS-Ca decreased the proportion of T

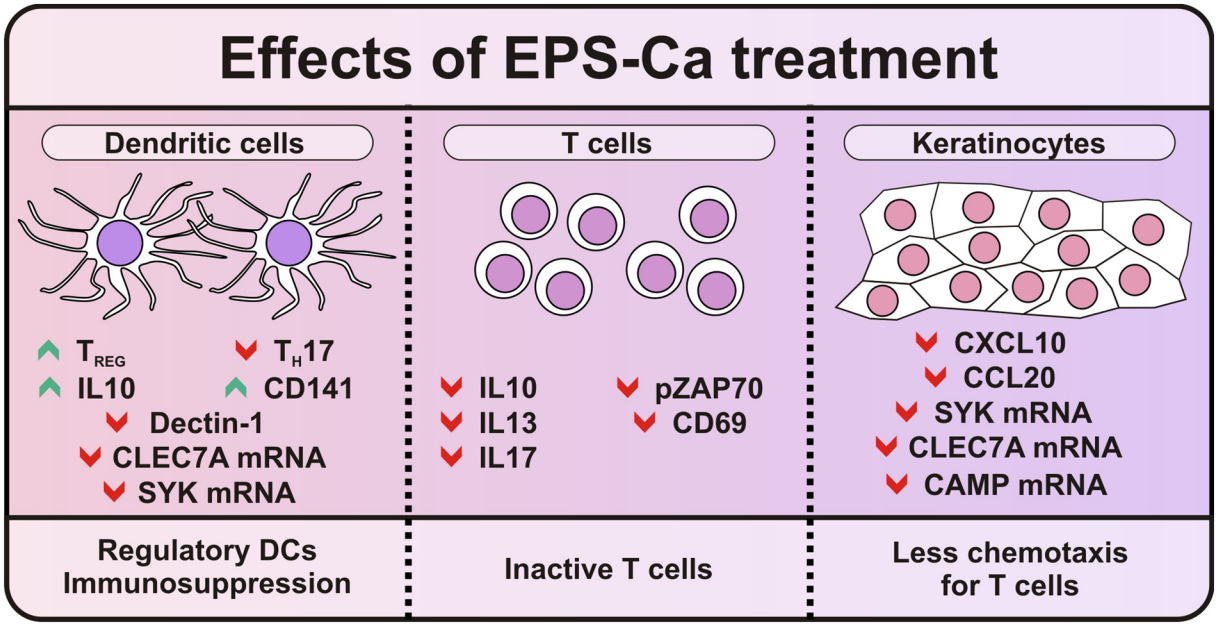

Fig. 8. The effects of EPS-Ca on dendritic cells, T cells and keratinocytes *in vitro*.

cells expressing pZAP70, indicating that EPS-Ca may dampen activation of the T cells via their T cell receptor signaling, as ZAP70 is known to play a central role in mediating activation signals from the T cell receptor [20].

EPS-Ca treatment of activated keratinocytes led to reduced secretion of the chemokines CXCL10 and CCL20, which both are strong chemotactic agents for lymphocytes and other inflammatory cells. Therefore, it may be concluded that EPS-Ca treatment can lead to reduced inflammatory cell recruitment to the skin. Accompanying the decrease in CCL20 secretion, following Th17 mimicking stimulation of the keratinocytes, was a decrease in mRNA levels for SYK. SYK is a signaling molecule in IL-17A stimulated keratinocytes and inhibition of SYK has been shown to attenuate CCL20 production [22]. Therefore, the decrease in SYK, following treatment with EPS-Ca, may have led to the decreased CCL20 secretion by the Th17 stimulated keratinocytes in the present study. This highlights SYK as a potential therapeutic target for inflammatory skin diseases, such as psoriasis, and may explain the beneficial effects detected in clinical studies after regular bathing by psoriasis patients in the Blue Lagoon.

In the present study, Th1 and Th17 mimicking stimulation markedly induced mRNA levels for CLEC7A (the gene for Dectin-1) in the keratinocytes and this induction was drastically reduced by EPS-Ca. Dectin-1 has previously been shown to be highly expressed in psoriatic epidermis, but not in normal skin, and to be induced by psoriasis-associated cytokines such as IFN-γ and IL-17 [24]. Therefore, the ability of EPS-Ca to reduce mRNA levels for this receptor may be beneficial in psoriasis, however, its role in the disease remains to be clarified.

LL37 is an antimicrobial peptide that is overexpressed in psoriatic skin and it has been identified as one of the autoantigens in psoriasis [2,4]. LL37-specific T cells produce IFN-γ and Th17 cytokines, supporting the hypothesis that LL37 may contribute to the pathogenesis of the disease [2]. In the present study, Th17 mimicking stimulation of the keratinocytes induced upregulation of CAMP (the gene for LL37) and that induction was attenuated by EPS-Ca treatment. In view of the proposed role of LL37 in the pathogenesis of psoriasis, the ability of EPS-Ca to decrease CAMP expression in keratinocytes may indicate that EPS-Ca could have a beneficial effect on psoriasis. When visualizing LL37 within the cells, by confocal microscopy, EPS-Ca did not seem to affect the amount of the peptide but changed its localization, such that the diffusely dispersed peptide present in cells not treated with EPS-Ca had accumulated into small vesicles. The significance of this change in LL37's location within the keratinocytes remains to be elucidated.

In this paper, we demonstrate how activation of cells involved in the pathophysiology of psoriasis can be affected by *in vitro* treatment with EPS-Ca. We show that DCs treated with EPS-Ca switch to a regulatory DC phenotype and that T cells become less active. We also demonstrate that EPS-Ca reduces keratinocyte production of chemokines involved in chemotaxis of inflammatory cells. In addition, EPS-Ca reduces keratinocyte production of LL37, one of the autoantigens in psoriasis. The effects of EPS-Ca seem to be mediated by inactivation of the Dectin-1 receptor and its downstream signaling protein SYK. These data suggest that exopolysaccharides secreted by *Cyanobacterium aponinum* may contribute to the beneficial effect of bathing in the Blue Lagoon and indicate a possible mechanism by which they mediate their effects.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

[1] M.A. Lowes, A.M. Bowcock, J.G. Krueger, Pathogenesis and therapy of psoriasis, Nature 445 (2007) 866.

[2] R. Lande, E. Botti, C. Jandus, et al., The antimicrobial peptide LL37 is a T-cell autoantigen in psoriasis, Nat. Commun. 5 (2014) 5621.

[3] L.C. Zaba, J. Fuentes-Duculan, N.J. Eungdamrong, et al., Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells, J. Invest. Dermatol. 129 (2009) 79.

[4] J. Fuentes-Duculan, K.M. Bonifacio, J.E. Hawkes, et al., Autoantigens ADAMTSL5 and LL37 are significantly upregulated in active psoriasis and localized with keratinocytes, dendritic cells and other leukocytes, Exp. Dermatol. 26 (2017) 1075.

[5] J.H. Olafsson, The Blue Lagoon in Iceland and psoriasis, Clin. Dermatol. 14 (1996) 647.

[6] J.H. Eysteinsdottir, J.H. Olafsson, B.A. Agnarsson, B.R. Luethviksson, B. Sigurgeirsson, Psoriasis treatment: faster and long-standing results after bathing in geothermal seawater. A randomized trial of three UVB phototherapy regimens, Photodermatol. Photoimmunol. Photomed. 30 (2014) 25.

[7] S.K. Petursdottir, S.H. Bjornsdottir, G.O. Hreggvidsson, S. Hjorleifsdottir, J.K. Kristjansson, Analysis of the unique geothermal microbial ecosystem of the Blue Lagoon, FEMS Microbiol. Ecol. 70 (2009) 425.

[8] S. Grether-Beck, K. Muhlberg, H. Brenden, I. Felsner, A. Brynjolfsdottir, S. Einarsson, J. Krutmann, Bioactive molecules from the Blue Lagoon: *in vitro* and *in vivo* assessment of silica mud and microalgae extracts for their effects on skin barrier function and prevention of skin ageing, Exp. Dermatol. 17 (2008) 771.

[9] A.B. Gudmundsdottir, S. Omarsdottir, A. Brynjolfsdottir, B.S. Paulsen, E.S. Olafsdottir, J. Freysdottir, Exopolysaccharides from *Cyanobacterium aponinum* from the Blue Lagoon in Iceland increase IL-10 secretion by human dendritic cells and their ability to reduce the IL-17$^+$RORgammat$^+$/IL-10$^+$FoxP3$^+$ ratio in CD4$^+$ T cells, Immunol. Lett. 163 (2015) 157.

[10] H. Yin, H. Zhou, Y. Kang, et al., Syk negatively regulates TLR4-mediated IFNbeta and IL-10 production and promotes inflammatory responses in dendritic cells, Biochim. Biophys. Acta 1860 (2016) 588.

[11] J.R. Gordon, Y. Ma, L. Churchman, S.A. Gordon, W. Dawicki, Regulatory dendritic cells for immunotherapy in immunologic diseases, Front. Immunol. 5 (2014) 7.

[12] C.C. Chu, N. Ali, P. Karagiannis, et al., Resident CD141 (BDCA3)$^+$ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation, J. Exp. Med. 209 (2012) 935.

[13] L. Hang, A.M. Blum, S. Kumar, et al., Downregulation of the Syk signaling pathway in intestinal dendritic cells is sufficient to induce dendritic cells that inhibit colitis, J. Immunol. 197 (2016) 2948.

[14] M. Haniffa, M. Collin, F. Ginhoux, Ontogeny and functional specialization of dendritic cells in human and mouse, Adv. Immunol. 120 (2013) 1.

[15] M. Haniffa, A. Shin, V. Bigley, et al., Human tissues contain CD141^{hi} cross-presenting dendritic cells with functional homology to mouse CD103$^+$ nonlymphoid dendritic cells, Immunity 37 (2012) 60.

[16] E.-C. Heier, A. Meier, H. Julich-Haertel, et al., Murine CD103$^+$ dendritic cells protect against steatosis progression towards steatohepatitis, J. Hepatol. 66 (2017) 1241.

[17] D. Patel, S. Gaikwad, N. Challagundla, M. Nivsarkar, R. Agrawal-Rajput, Spleen tyrosine kinase inhibition ameliorates airway inflammation through modulation of NLRP3 inflammosome and Th17/Treg axis, Int. Immunopharmacol. 54 (2018) 375.

[18] A. Mócsai, J. Ruland, V.L.J. Tybulewicz, The SYK tyrosine kinase: a crucial player in diverse biological functions, Nat. Rev. Immunol. 10 (2010) 387.

[19] M. Turner, E. Schweighoffer, F. Colucci, J.P. Di Santo, V.L. Tybulewicz, Tyrosine kinase SYK: essential functions for immunoreceptor signalling, Immunol. Today 21 (2000) 148.

[20] H. Wang, T.A. Kadlecek, B.B. Au-Yeung, H.E.S. Goodfellow, L.-Y. Hsu, T.S. Freedman, A. Weiss, ZAP-70: an essential kinase in T-cell signaling, Cold Spring Harb. Perspect. Biol. 2 (2010) a002279.

[21] B.B. Au-Yeung, N.H. Shah, L. Shen, A. Weiss, ZAP-70 in signaling, biology, and disease, Annu. Rev. Immunol. 36 (2018) 127.

[22] N.L. Wu, D.Y. Huang, H.N. Tsou, Y.C. Lin, W.W. Lin, Syk mediates IL-17-induced CCL20 expression by targeting Act1-dependent K63-linked ubiquitination of TRAF6, J. Invest. Dermatol. 135 (2015) 490.

[23] L.K. Mackay, A. Braun, B.L. Macleod, et al., Cutting Edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cCell retention, J. Immunol. 194 (2015) 2059.

[24] H.D. de Koning, D. Rodijk-Olthuis, I.M.J.J. van Vlijmen-Willems, L.A.B. Joosten, M.G. Netea, J. Schalkwijk, P.L.J.M. Zeeuwen, A comprehensive analysis of pattern recognition receptors in normal and inflamed human epidermis: upregulation of Dectin-1 in psoriasis, J. Invest. Dermatol. 130 (2010) 2611.