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+RORγt+/IL-10+FoxP3+ ratio in CD4+ T cells

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ARTICLE INFO

ABSTRACT

Regular bathing in the Blue Lagoon in Iceland has beneficial effects on psoriasis. *Cyanobacterium aponinum* is a dominating member of the Blue Lagoon's microbial ecosystem. The aim of the study was to determine whether exopolysaccharides (EPSs) secreted by *C. aponinum* (EPS-Ca) had immunomodulatory effects *in vitro*. Human monocyte-derived dendritic cells (DCs) were matured in the absence or presence of EPS-Ca and the effects were determined by measuring the secretion of cytokines by ELISA and the expression of surface molecules by flow cytometry. DCs matured with EPS-Ca at 100 μg/ml secreted higher levels of IL-10 than untreated DCs. Subsequently, DCs matured in the presence or absence of EPS-Ca were co-cultured with allogeneic CD4+ T cells and their effects on T cell activation analysed by measuring expression of intracellular and surface molecules and cytokine secretion. Supernatant from allogeneic T cells co-cultured with EPS-Ca-exposed DCs had raised levels of IL-10 compared with control. A reduced frequency of IL-17+RORγt+ T cells was observed when co-cultured with EPS-Ca-exposed DCs and a tendency towards increased frequency of FoxP3+IL-10+ T cells, resulting in a lower IL-17+RORγt+/FoxP3+IL-10+ ratio. The study shows that EPSs secreted by *C. aponinum* stimulate DCs to produce vast amounts of the immunosuppressive cytokine IL-10. These DCs induce differentiation of allogeneic CD4+ T cells with an increased Treg but decreased Th17 phenotype. These data suggest that EPSs from *C. aponinum* may play a role in the beneficial clinical effect on psoriasis following bathing in the Blue Lagoon.

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1. Introduction

A clinical study has shown that bathing in the Blue Lagoon in conjunction with UVB light treatment is a useful alternative treatment for psoriasis and gives better results than UVB treatment alone [1]. This has been confirmed in a recent pilot clinical study [2], which has been repeated on a larger cohort [3].

The Blue Lagoon is located on the geothermally active Reykjanes peninsula in Iceland, consisting of porous lava, which allows water to slowly seep deep into its aquifers. The fluid in the geothermal aquifers, containing high silica concentration, is pumped from over 2000 m deep boreholes to the lagoon, where the majority of the silica precipitates [4]. The Blue Lagoon fluid consists of a mixture of seawater and fresh water (65:35), with 2.7% salinity, pH at 7.5 and its average temperature is approximately 37 °C [4]. The Blue Lagoon hosts an unusual ecosystem and the microbial diversity is low, probably due to the salinity and high silica concentration [4]. Two microorganisms; *Cyanobacterium aponinum* and *Silicabacter lacuscaerulensis* dominate the microbial community [4]. Cyanobacteria, sometimes referred to as blue-green algae, resemble bacteria

due to prokaryotic cellular organization but resemble algae and other higher plants by being photoautotrophic [5,6]. Polysaccharides produced by cyanobacteria are usually divided into three groups; storage polysaccharides, cell envelope polysaccharides and exopolysaccharides (EPSs) [7,8]. The EPSs can be soluble in the surrounding fluid or form a gelatinous layer around the organisms or both. They are usually complex heteropolysaccharides and generally consist of at least ten different monosaccharides [8].

Although bathing in the Blue Lagoon has increased over the years, still very little is known about the immunological activities present in the lagoon. However, extracts from silica mud and the two microorganisms found in the Blue Lagoon were capable of inducing expression of involucrin, loricrin, transglutaminase-1 and filaggrin in primary human epidermal keratinocytes, indicating an improvement in skin barrier function, which may explain some of the beneficial effects experienced by psoriasis patients [9]. The observed beneficial effects are most likely due to several factors, probably including bioactive components with specific biological/therapeutic effects.

Although psoriasis was formerly categorized as a Th1-mediated skin disorder, it also appears to be mediated by Th17 cells, according to the cytokine profile in peripheral blood and skin lesions of psoriasis patients [2,10–12]. Furthermore, T regulatory cells (Tregs) are dysfunctional in patients with psoriasis, both in peripheral blood and in psoriatic skin [13].

In a recent clinical study, bathing in the Blue Lagoon in conjunction with UVB light treatment resulted in a reduction of the Th17-mediated response in psoriasis patients [2], raising the question whether the Blue Lagoon contained compounds with immunomodulating effects. Since polysaccharides from other organisms, e.g. plants, lichen and fungi, have been shown to have immunomodulating effects [14,15], the main objective of this study was to explore whether EPSs from *C. aponinum*, the characteristic organism of the Blue Lagoon, affects DCs, and moreover which effects DCs matured in the presence of the EPSs have on T cell stimulation.

2. Materials and methods

2.1. Cultivation of C. aponinum and preparation of EPS fraction

C. aponinum obtained from the Blue Lagoon was cultured in the Blue Lagoon geothermal seawater under controlled conditions in a closed tubular photobioreactor ($160\,\mu E/m^2/s$; $40\,^{\circ}C$; pH 7.5; no nutrition added), thus mimicking the culture conditions for *C. aponinum* in the Blue Lagoon. The culture was collected and the biomass separated from the supernatant by centrifugation. The supernatant (600 ml) 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 and named EPS-Ca.

2.2. Determination of monosaccharide composition and mean M_r of EPS-Ca

The monosaccharide composition of EPS-Ca was determined by gas chromatography (GC) as described earlier [16,17]. The mean M_r of EPS-Ca was determined by HP-GPC on a Superose 6 HR 10/30 column (Amersham, GE Healthcare) eluted with 0.05 M sodium phosphate buffer pH 6.0, containing 0.15 M NaCl, with a flow rate of 0.1 ml/min, using refractive index detection (Hewlett Packard 1047A RI detector). The samples were applied in 1% solutions in the mobile phase, and the injected volume was $20\,\mu l$. For the M_r-estimation, calibration was performed using dextrans of known M_r (T10, T40, T70, T500 and T2000, Amersham, GE Healthcare).

2.3. Isolation of CD14^{+} monocytes and CD4^{+} T cells

CD14^{+} monocytes and CD4^{+} T cells were purified from mononuclear cells obtained from peripheral blood from healthy volunteers using CD14 and CD4 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according the manufacturer's instructions. The purity of both the CD14^{+} monocytes and CD4^{+} T cells was > 95%, confirmed by flow cytometry.

2.4. Dendritic cells matured in the presence of EPSs

Immature dendritic cells (imDCs) were differentiated from peripheral blood CD14^{+} monocytes and subsequently matured into mature DCs (mDCs), as previously described [18]. In short, CD14^{+} monocytes were cultured for seven days in RPMI medium (Gibco®, Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum (Gibco® and 1% penicillin/streptomycin (Gibco®) and their differentiation into imDCs induced with IL-4 at 12.5 ng/ml and GM-CSF at 25 ng/ml (both from R&D Systems, Abingdon, UK). To induce maturation of the imDCs into mDCs, the imDCs were cultured with RPMI medium supplemented with FCS and antibiotics in the presence of IL-1β at 10 ng/ml, TNF-α at 50 ng/ml (both from R&D Systems) and lipopolysaccharide (LPS) from *E. coli*, serotype 055:B5 at $0.5\,\mu g/ml$ (Sigma–Aldrich, St. Louis, MO) for 48 h, without (control) or with EPS-Ca at concentrations of 1, 10 and $100\,\mu g/ml$. The effect of EPS-Ca on DC maturation was evaluated by measuring expression of surface molecules by flow cytometry and cytokine secretion by ELISA.

2.5. Co-culture of mature DCs and allogeneic CD4^{+} T cells

In order to analyse the effect DCs matured in the presence of EPS-Ca on stimulation of allogeneic CD4^{+} T cells, the DCs were matured in the presence of EPS-Ca (DC-EPS-Ca) and without EPS-Ca (DC-C) (see Section 2.4) and subsequently cultured for 6 days in the presence of allogeneic CD4^{+} T cells, as described before [18]. In short, the DCs were harvested and washed to remove any EPS-Ca and then transferred into a 96-well round-bottomed tissue culture plates. Freshly isolated CD4^{+} T cells were added to the wells and the cells co-cultured at a DC:T cell ratio of 1:8 (2.5×10^4 DCs/well:2×10^5 CD4^{+} T cells/well) in RPMI medium supplemented with FCS and antibiotics. For comparison, CD4^{+} T cells and DCs were cultured alone. The effect of the co-culture on T cell and DC activation was analysed by measuring expression of surface and intracellular molecules by flow cytometry and cytokine secretion by ELISA.

2.6. Measurement of cytokines by ELISA

Cytokine concentration in supernatants was determined by ELISA (DuoSet®, R&D Systems) according to the manufacturer's protocol. The cytokines IL-10 and IL-12p40 were measured in supernatants from DCs matured with or without EPS-Ca and IL-10, IL-12p40, IL-17, IL-22 and IFN-γ in supernatants from co-cultures of allogeneic CD4^{+} T cells and DCs matured in the absence or presence EPS-Ca. The results are shown as secretion index (SI), which was calculated by dividing the cytokine concentration (pg/ml) in supernatants from DCs cultured in the presence of EPS-Ca by the cytokine concentration (pg/ml) in supernatants from DCs cultured in the absence of EPS-Ca. In the co-cultures, SI was calculated by dividing the cytokine concentration in supernatant of allogeneic CD4^{+} T cells and DCs that had been cultured in the presence of EPS-Ca by the cytokine concentration in supernatants of allogeneic CD4^{+} T cells and DCs that had been cultured in the absence of EPS-Ca.

2.7. Measurement of surface and intracellular molecules by flow cytometry

The purity of $CD14^+$ monocyte and $CD4^+$ T cell isolation was confirmed by staining the cells with fluorochrome-labelled antibodies against CD14 and CD4, respectively. In order to determine the effects of EPSs on the maturation of DCs, the cells were stained with fluorochrome-labelled monoclonal antibodies against CD14, CD40, CD86 and HLA-DR. For analysing the effects of co-culturing DCs and allogeneic $CD4^+$ T cells, the DCs were stained with fluorochrome-labelled antibodies against CD1c, CD40, CD86, CCR7, PD-L1, HLA-DR and IL-10 and the $CD4^+$ T cells were stained with fluorochrome-labelled antibodies against CD4 (to identify T cells), CD25, CD40L, CD54, CD69 and CTLA-4 (to identify activated cells), FoxP3 and IL-10 (to identify Tregs), and RORγt and IL-17 (to identify Th17 cells). Cells stained with fluorochrome-labelled isotype-matched antibodies were used as controls. Antibodies were obtained from AbD Serotec, Kidlington, England, BD Bioscience, San Jose, CA, eBioscience, San Diego, CA, Miltenyi Biotec, and R&D Systems (see further in Suppl. Table 1). Ten thousand cells were acquired using FACScalibur (BD Bioscience) and analysed using CellQuest (BD Bioscience). Dot plots of forward and side scatter were formed, gates drawn around the DCs and the T cells and the cells analysed further using histograms. Results are expressed as percentage positive cells compared with cells stained with isotype control and expression levels (mean fluorescence intensity, MFI).

2.8. Statistical analysis

Means and standard error of means were calculated and the difference between groups was evaluated using one-way ANOVA followed by Tukey's post hoc test when the data were normally distributed. When the data were not normally distributed one-way ANOVA of ranks followed by Dunn's post hoc test was used to determine whether group medians differed, but means ± SEM listed in the corresponding data tables for clarity. Statistical analysis was performed in SigmaStat 3.1. p-Value <0.05 was considered statistically significant.

3. Results

3.1. Monosaccharide composition analysis and mean molecular weight of EPS-Ca

The cultured *C. aponinum* originating from the Blue Lagoon was shown to release complex heteroglycan named EPS-Ca. The monosaccharide analysis revealed that the polysaccharide was composed of GalA/Fuc/3-OMe-GalA/Glc/Ara/Gal/Man/Rha in a molar ratio of 24:24:17:16:10:4:3:2 and traces of 4-OMe-GluA. The mean M_r was determined to be 1060 kDa by comparison to dextran standards.

3.2. The effects of EPS-Ca on maturation of DCs

DCs were matured in the absence or presence of the EPS-Ca in various concentrations and the effect on cytokine secretion and expression of surface molecules determined.

DCs matured in the presence of EPS-Ca at 100 μg/ml secreted higher levels of IL-10 than DC matured without EPS-Ca ($p = 0.004$) (Fig. 1 and Suppl. Table 2), demonstrating immunomodulating effects of the EPS-Ca. However, EPS-Ca did not affect the level of IL-12p40 secretion (Fig. 1 and Suppl. Table 2). Maturation of DCs in the presence of the EPS-Ca did not affect the expression of CD86, HLA-DR, CD14 and CD40 (Suppl. Table 3).
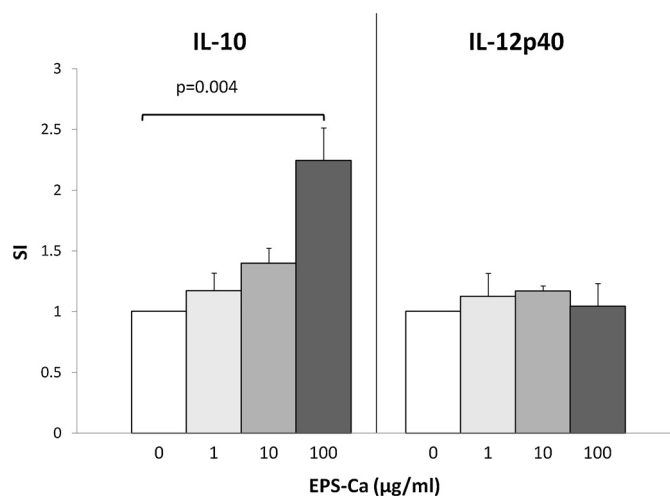

Fig. 1. Cytokine secretion by DCs matured in the absence or presence of exopolysaccharides from *C. aponinum*. DCs were matured in the absence or presence of exopolysaccharides from *C. aponinum* (EPS-Ca) at the concentrations 1, 10 and 100 μg/ml and the levels of IL-10 and IL-12p40 measured by ELISA. The results are shown as the mean + standard error of the mean of three experiments expressed as secretion index (SI), calculated by dividing the concentration of cytokine secreted by DCs matured with the EPS-Ca by the concentration of cytokine secretion by DCs matured without EPS-Ca. Statistical difference between mDCs and DCs matured with EPSs was calculated by one-way ANOVA with significant p-values indicated.

3.3. The effects of DCs matured in the presence of EPS-Ca on the stimulation of allogeneic $CD4^+$ T cells

As the DCs matured in the presence of EPS-Ca (DC-EPS-Ca) secreted significantly higher levels of IL-10 than DCs matured without EPS-Ca (DC-C), the effects of these DCs on the activation of allogeneic $CD4^+$ T cells was analysed by measuring cytokine secretion by ELISA and expression of surface and intracellular molecules by flow cytometry.

Co-culturing $CD4^+$ T cells with DC-EPS-Ca did not affect the percentage of T cells expressing CD4, CD40L, CD54, CD69 or CTLA-4 (Suppl. Table 4).

Supernatant from allogeneic $CD4^+$ T cells co-cultured with DC-EPS-Ca contained higher levels of IL-10 than supernatant from allogeneic $CD4^+$ T cells co-cultured with DC-C ($p = 0.005$) (Fig. 2a and Suppl. Table 5). The IL-10 in the co-culture supernatant was, at least partly, derived from the $CD4^+$ T cells, as the frequency of IL-10 secreting $CD4^+$ T cells expressing CD25 was higher when co-cultured with DC-EPS-Ca than when co-cultured with DC-C, although it did not reach a significant level ($p = 0.095$) (Fig. 2b). There was no difference in the concentration of IFN-γ, IL-17 and IL-22 in the supernatant from allogeneic $CD4^+$ T cells co-cultured with DC-EPS-Ca or DC-C (Fig. 2a and Suppl. Table 5).

Although there was no difference in the proportion of allogeneic $CD4^+CD25^+$ T cells expressing FoxP3 when co-cultured with DC-EPS-Ca or DC-C, there was a higher, although not significant, proportion of allogeneic $CD4^+$ T cells expressing FoxP3 and IL-10 ($p = 0.053$) (Fig. 3a), suggesting that EPS-Ca enhances Treg differentiation. In addition, the proportion of allogeneic $CD4^+$ T cells expressing RORγt and IL-17 was lower when co-cultured with DC-EPS-Ca than when co-cultured with DC-C ($p = 0.031$) (Fig. 3a), indicating that EPS-Ca hampers the differentiation of Th17 cells. This resulted in a decreased ratio of allogeneic $CD4^+$ T cells expressing RORγt and IL-17 to those expressing FoxP3 and IL-10 when co-cultured with DC-EPS-Ca compared with the ratio when the allogeneic $CD4^+$ T cells were co-cultured with DC-C ($p = 0.005$) (Fig. 3b).
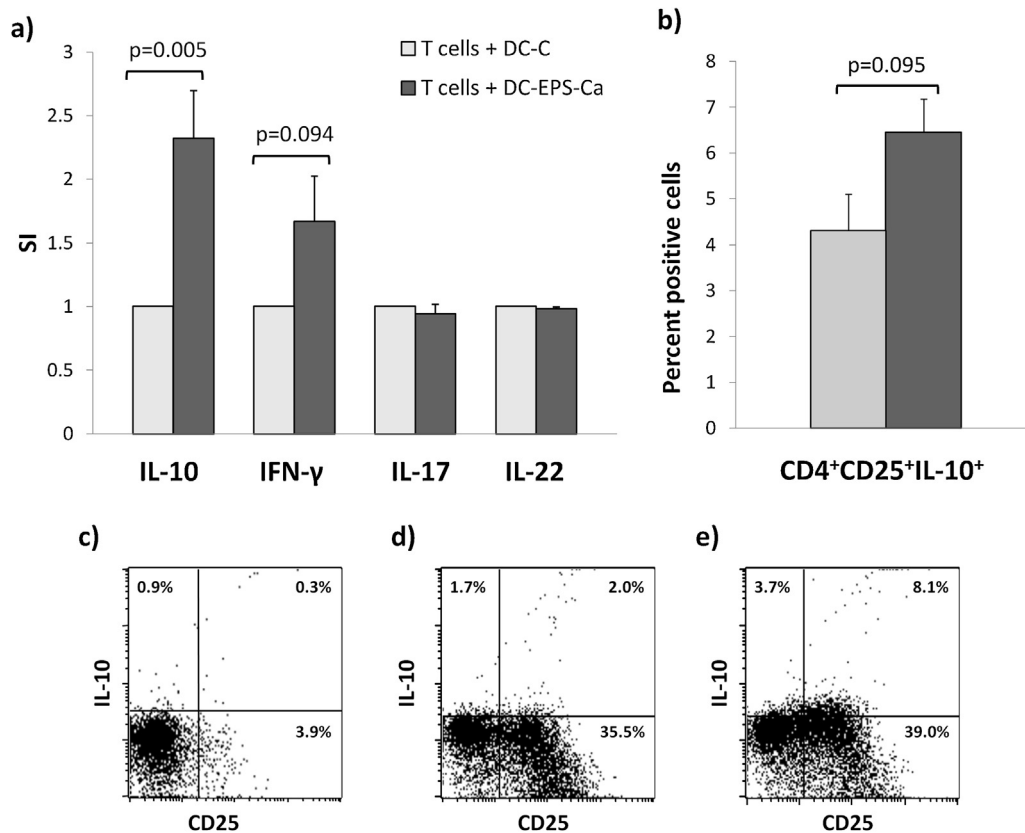
Fig. 2. Cytokine production following co-culture of allogeneic CD4+ T cells and DCs matured in the absence (DC-C) or presence (DC-EPS-Ca) of EPS-Ca at 100 μg/ml. (a) Levels of IL-10, IFN-γ, IL-17 and IL-22 secretion in co-culture supernatants were measured by ELISA and the results expressed as secretion index (SI), calculated by dividing the concentration of cytokines secreted by T cells co-cultured with DC-EPS-Ca by concentration of T cells co-cultured with DC-C. The results are shown as mean + standard error of the mean of 3–6 experiments. (b) The percentage of IL-10+ cells within the CD4+ T cell population expressing high levels of CD25 (CD4+CD25++IL-10+) was measured by flow cytometry. The results are shown as mean + standard error of the mean of 6 experiments. Statistical difference between T cells + DC-C and T cells + DC-EPS-Ca was calculated by one-way ANOVA with significant (and near significant) *p*-values indicated. (c–e) Representative dot plots showing the percentage of CD4+ T cells stained with antibodies against CD25 and IL-10, when (c) cultured alone, (d) with DC-C or (e) DC-ESP-Ca.

3.4. The effects of co-culturing DCs matured in the presence or absence of EPS-Ca with allogeneic CD4+ T cells

Following co-culture with allogeneic CD4+ T cells the percentage of DCs expressing HLA-DR, CD86, CD40 and PD-L1 was the same, regardless of whether the DCs were matured in the absence or presence of EPS-Ca (Fig. 4a). The percentage of CD1c+ DCs co-cultured with allogeneic CD4+ T cells was higher and the percentage of CCR7+ DCs was lower for DC-EPS-Ca compared with DC-C (*p* < 0.001 and *p* = 0.050, respectively) (Fig. 4a), indicating that these DCs are less capable of migrating to draining lymph nodes.

After co-culture with CD4+ T cells the percentage of DCs expressing intracellular IL-10 was similar in DC-EPS-Ca and DC-C (Fig. 4b). However, the difference in MFI of the IL-10+ DCs was increased in the DC-EPS-Ca compared with the DC-C (*p* = 0.034) (Fig. 4b), suggesting that EPS-Ca enhances the tolerogenic DC phenotype.

4. Discussion

Human DCs cultured in the presence of EPSs, isolated from cultures of *C. aponinum* obtained from the Blue Lagoon (EPS-Ca), secreted higher levels of the anti-inflammatory cytokine IL-10 in comparison to DCs cultured without EPS-Ca, demonstrating their immunomodulating effects, which may contribute to the beneficial effects experienced by psoriasis patients.

IL-10 secretion by DCs is known to contribute to the differentiation of naive CD4+ T cells into Tregs [19,20]. Thus, to elucidate the immunomodulating effects of EPS-Ca further, we studied the

effect of co-culturing allogeneic CD4+ T cells with DCs matured in the presence of EPS-Ca and showed an increase in the IL-10 levels in the supernatant from co-cultures of allogeneic CD4+ T cells and DCs matured in the presence of EPS-Ca compared with those matured without EPS-Ca. Since IL-10 can be secreted by both the T cells and the DCs intracellular expression of IL-10 was analysed and both DCs and CD4+ T cells were found to produce IL-10. There was a non-significantly higher frequency of IL-10 positive CD4+ CD25++ T cells in the co-cultures with DCs matured in the presence of EPS-Ca than in the co-cultures with DCs matured in the absence of EPS-Ca. Although the frequency of DCs secreting IL-10 was the same regardless of whether they were matured in the absence or presence of EPS-Ca, the DCs matured in the presence of EPS-Ca secreted higher levels as indicated by higher MFI.

Psoriasis is a T cell-mediated skin disorder accepted to be mainly mediated by Th17 cells [10,12]. Furthermore, down-regulation of the Th17 cells was proposed to explain the beneficial effects from bathing regularly in the Blue Lagoon [2]. As psoriasis has also been associated with dysfunctional Tregs [13], the effect of IL-10 secreting DCs obtained following maturation with EPS-Ca on the development of Tregs and Th17 cells was analysed. The DCs matured in the presence of EPS-Ca clearly affected the development of both Th17 and Tregs as the percentage of T cells expressing IL-10 and the Treg-associated transcription factor FoxP3 increased at the same time as the percentage of T cells expressing IL-17 and the Th17-associated transcription factor RORγt decreased. This resulted in a lower IL-17+RORγt+/IL-10+FoxP3+ ratio for the CD4+ T cells co-cultured with DC-ESP-Ca as compared with CD4+ T cells
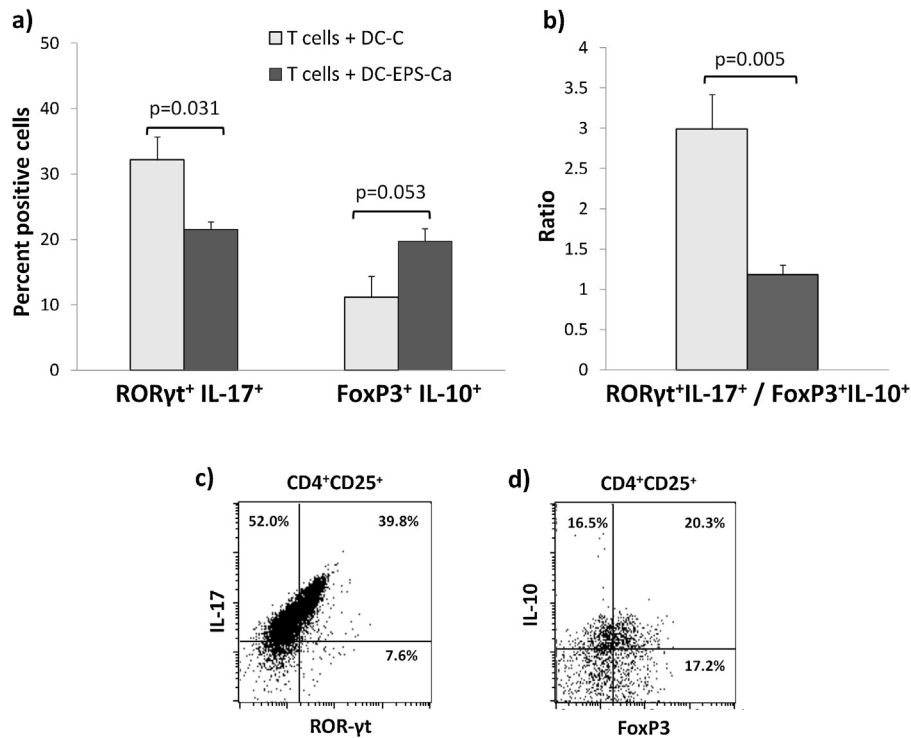
Fig. 3. The expression of transcription factors and cytokines by allogeneic CD4+ T cells co-cultured with DCs matured in the absence (DC-C) or presence (DC-EPS-Ca) of EPS-Ca at 100 μg/ml. Following the co-culture (a) the percentage of positive allogeneic CD4+ T cells expressing RORγt+IL-17+ or FoxP3+IL-10+ was measured by flow cytometry and (b) the ratio of RORγt+IL-17+/FoxP3+IL-10+ allogeneic CD4+ T cells calculated. The results are shown as mean + standard error of the mean of 6 experiments. Statistical difference between T cells + DC-C and T cells + DC-EPS-Ca was calculated by one-way ANOVA with significant (and near significant) p-values indicated. (c) A representative dot plot of CD4+ T cells stained with antibodies against RORγt and IL-17. (d) A representative dot plot of CD4+CD25+ T cells stained with antibodies against FoxP3 and IL-10.

co-cultured with DC-C, indicating a decrease in Th17 cells and a non-significant increase in Tregs. This effect of the EPSs may play a role in the clinical improvement observed for psoriasis patients following bathing in the Blue Lagoon [1–3].

No difference in the IL-17 concentration in the supernatant of CD4+ T cells following co-culture with DCs regardless of their pre-treatment was found. A possible explanation is that the time point for collecting the supernatant may not have been optimal or the IL-17 may have started to break down.

A higher percentage of DCs matured in the presence of EPS-Ca expressed CD1c following the co-culture than DCs matured without the EPS-Ca. A recent study analysing the two human blood myeloid DCs expressing either CD1c or CD141 showed that CD1c+ DCs produced high levels of IL-10, which could suppress T cell proliferation in an IL-10-dependent manner [21]. Our findings suggest that the monocyte-derived DCs matured in the presence of EPS-Ca acquire a phenotype resembling these immunosuppressive CD1c+ blood DCs.
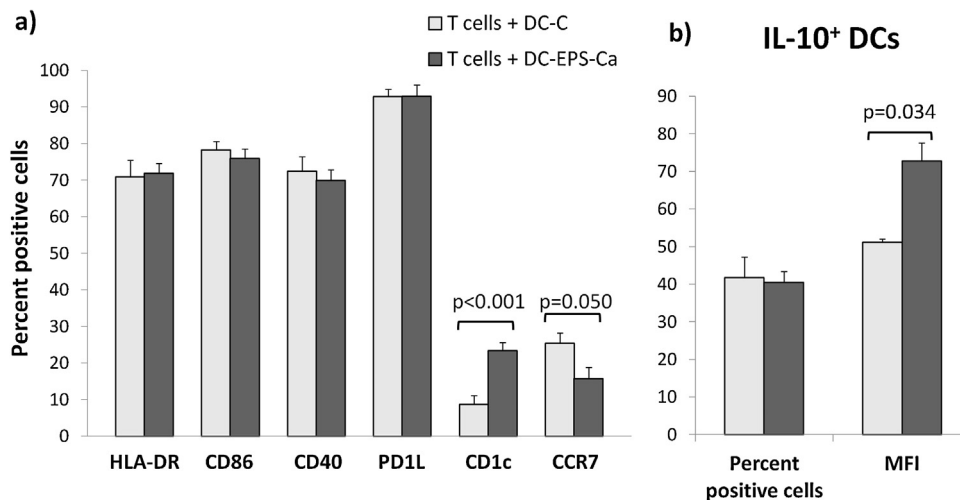

Fig. 4. The effects of co-culturing DCs matured in the absence (DC-C) or presence (DC-EPS-Ca) of EPS-Ca at 100 μg/ml with allogeneic CD4+ T cells on their expression of surface molecules and cytokines. (a) Percentage of DCs expressing the surface molecules HLA-DR, CD86, CD40, PD-L1, CD1c and CCR7 was measured by flow cytometry. The results are shown as mean + standard error of the mean of 3–6 experiments. (b) Percentage of IL-10+ DCs and the mean fluorescent intensity (MFI) of the IL-10 positive cells. The results are shown as mean + standard error of the mean of 6 experiments. Statistical difference between T cells + DC-C and T cells + DC-EPS-Ca was calculated by one-way ANOVA with significant (and near significant) p-values indicated.

A lower percentage of DCs exposed to EPS-Ca expressed CCR7 compared with unexposed DCs in the co-cultures. This indicates that DCs exposed to EPS-Ca are less able to migrate to lymph nodes and hence less likely to be able to interact with naive T cells, raising the question whether the effect of the Blue Lagoon treatment for psoriasis patients is partly due to reduced levels of DC–T cell interaction.

In summary, exopolysaccharides, EPS-Ca, produced by the defining cyanobacterium of the Blue Lagoon, *C. aponinum*, affect the maturation of DCs *in vitro*, which in turn induce differentiation of T cells with an increased Treg phenotype but decreased Th17 phenotype. These findings suggest that the exopolysaccharides may be involved in the therapeutic results observed in psoriasis patients following a treatment in the Blue Lagoon.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2014.11.008.

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