Effects of exopolysaccharides from *Cyanobacterium aponinum* from the Blue Lagoon in Iceland on immune responses *in vitro*

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Áhrif utanfrumufjölsykra *Cyanobacterium aponinum* úr Bláa Lóninu á ónæmissvör *in vitro*

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Ágrip

Lækningamáttur Bláa Lónsins var uppgötvaður af sórasjúklingum skömmu eftir myndun þess og hafa jákvæð áhrif böðunar í lóninu verið staðfest í klínískum rannsóknum. Enn fremur hefur verið sýnt að böðun í lóninu samhliða UVB meðferð sé árangursríkari en UVB meðferð ein og sér. Þrátt fyrir vinsældir lónsins er lítið vitað um hvernig það hefur áhrif á sóra. Blágrænþörungurinn *Cyanobacterium aponinum* er ríkjandi lífvera í jarðsjó Bláa Lónsins og framleiðir hann utanfrumufjölsykru (EPS-Ca) sem hann seytir í umhverfi sitt. Tilgáta okkar er að EPS-Ca hafi áhrif á ónæmiskerfið sem geti tekið þátt í að miðla þeim bata sem sórasjúklingar fá við böðun í lóninu. Tilgangur verkefnisins var að kanna verkun og verkunarmáta EPS-Ca í frumum sem taka þátt í meingerð sóra.

Utanfrumufjölsykran EPS-Ca var einangruð úr floti *C.aponium* ræktar frá Bláa Lóninu. Angafrumur, sérhæfðar út frá einkjörnungum, voru ræstar með boðefnunum IL-1β og TNF-α og inneitri (LPS) með eða án EPS-Ca í 24 tíma. $CD4^+$ T frumur úr mönnum voru örvaðar með mótefnum gegn CD3 og CD28 í 72 tíma með eða án EPS-Ca síðasta sólarhringinn. Hyrnisfrumur, fengnar frá ATCC, voru örvaðar með TNF-α og annaðhvort IFN-γ eða IL-17A og meðhöndlaðar með eða án EPS-Ca í 24 tíma. Áhrif EPS-Ca voru metin með því að mæla styrk boðefna í floti frumna með ELISA aðferð, tjáningu innanfrumu- og yfirborðssameinda með frumuflæðisjá og lagsjármyndum, og mRNA tjáningu með rauntíma PCR.

Angafrumur þroskaðar í návist EPS-Ca juku seytun sína á ónæmisbælandi boðefninu IL-10. Angafrumur þroskaðar í návist EPS-Ca ræstu einnig og sérhæfðu ósamgena $CD4^+$ T frumur í samrækt í T bælifrumur (Treg) á kostnað sérhæfingar þeirra í sjúkdómshvetjandi Th17 frumna. Ennfremur tjáðu angafrumur meðhöndlaðar með EPS-Ca meira af yfirborðssameindinni CD141 en angafrumur þroskaðar án EPS-Ca. CD141 er yfirborðssameind sem hefur verið tengd bæli-angafrumum og niðurstöðurnar sýndu að CD141 jákvæðar angafrumur seyttu meira af IL-10 en angafrumur sem tjáðu ekki CD141. Hins vegar dró EPS-Ca meðhöndlun úr tjáningu angafrumna á Dectin-1 viðtakanum, bæði á mRNA (CLEC7A) og próteinformi. EPS-Ca dró einnig úr umritun á SYK sem er lykilpróteinið í boðleiðinni sem liggur frá Dectin-1 viðtakanum. $CD4^+$ T frumur sem voru örvarðar í návist EPS-Ca seyttu minna af IL-17, IL-13 og IL-10 samanborið

við $CD4^+$ T frumur sem voru örvarðar án EPS-Ca. EPS-Ca minnkaði einnig hlutfall T frumna sem tjáðu CD69 á yfirborði sínu. EPS-Ca dró einnig úr fosfæringu á ZAP70 sem er lykilpróteinið í miðlun ræsingar inn í T frumur. Hyrnisfrumur meðhöndlaðar með EPS-Ca seyttu minna af flakkboðunum CXCL10 og CCL20 en hyrnisfrumur örvaðar án EPS-Ca. Enn fremur dró EPS-Ca meðhöndlun úr umritun SYK, CLEC7A og CAMP en CAMP er genið sem tjáir fyrir bakteríudrepandi peptíðinu LL37 sem er tjáð í yfirmagni í húð sórasjúklinga og talið eiga þátt í meingerð sjúkdómsins.

Niðurstöður rannsóknarinnar sýna að EPS-Ca breytir svipgerð angafrumna í bæli-angafrumur sem stuðla að sérhæfingu T frumna yfir í T bælifrumur. Einnig dregur EPS-Ca úr boðefnaseytun og ræsingu örvaðra T frumna. EPS-Ca gæti því dregið úr ræsingu og fjölda T frumna sem halda til í húðinni. EPS-Ca minnkaði einnig seytingu hyrnisfrumna á flakkboðum sem kalla T frumur til húðarinnar og minnkaði umritun gena sem tjá fyrir próteinum sem talin eru tengjast meingerð sóra. EPS-Ca virðist því hafa áhrif á allar lykilfrumurnar sem taka þátt í meingerð sóra og virðist áhrifunum miðlað með hindrun á Dectin-1 viðtakanum og Syk boðleiðinni. Niðurstöðurnar benda því til þess að EPS-Ca geti átt þátt í þeim bata sem sórasjúklingar fá við böðun í lóninu og leiða í ljós með hvaða leiðum þeim áhrifum er mögulega miðlað.

Lykilorð:

Angafrumur, T frumur, hyrnisfrumur, *Cyanobacterium aponinum,* sóri.

Abstract

Regular bathing in the Blue Lagoon in Iceland has been shown to have beneficial effects on the manifestations of psoriasis in clinical studies. Moreover, bathing in the Blue Lagoon in conjunction with UVB treatment gives better results than UVB treatment alone. The beneficial effects may be attributed to the unique chemical and ecological composition of the Lagoon. Our hypothesis is that an exopolysaccharide secreted by *Cyanobacterium aponinum* (EPS-Ca), one of the two main organisms in the Lagoon, may have specific immunomodulating effects that contribute to the therapeutic benefits obtained by regular bathing in the Lagoon. The objective of the study was to determine the effects of EPS-Ca on immune responses of cells involved in the pathogenesis of psoriasis and to elucidate how EPS-Ca mediates its effects.

EPS-Ca was isolated from supernatants of cultures of *C. aponinum* obtained from the Blue Lagoon. Human monocyte-derived dendritic cells (DCs) were matured with interleukin (IL)-1β, tumor necrosis factor (TNF)-α and lipopolysaccharides (LPS) with or without EPS-Ca for 24 h. $CD4^+$ T cells isolated from human blood were stimulated with antibodies against CD3 and CD28 for 72 h with or without EPS-Ca for the last 24 h of the stimulation. Finally, adult primary keratinocytes, obtained from ATCC, were stimulated with TNF-α and either interferon (IFN)-γ or IL-17, with or without EPS-Ca for 24 h. Cytokine concentration in the supernatant was measured by ELISA, expression of mRNA by RT-qPCR, expression of intra- and extracellular molecules by flow cytometry and confocal imaging.

DCs matured in the presence of EPS-Ca secreted more of the immunosuppressive cytokine IL-10 than DCs matured in the absence of EPS-Ca. EPS-Ca treatment also increased the capacity of the DCs to induce differentiation of T cells into T regulatory cells (Tregs) and diminished induction of the disease-inducing T helper (Th)17 cells. EPS-Ca-treated DCs expressed more CD141, a surface molecule that has been linked to regulatory DCs, than DCs stimulated without EPS-Ca. Interestingly, $CD141^+$ DCs secreted more IL-10 than $CD141^-$ DCs. EPS-Ca treatment decreased DC expression of Dectin-1 and also decreased transcription of key genes in the Syk signaling pathway (CLEC7A and SYK). T cells stimulated in the presence of EPS-Ca secreted less IL-10, IL-13 and IL-17 and expressed less

CD69 than T cells stimulated without EPS-Ca. Furthermore, EPS-Ca decreased the proportion of T cells expressing the active form of the T cell receptor signaling protein, ZAP70. EPS-Ca decreased keratinocyte secretion of CCL20 and CXCL10 and their transcription of CLEC7A, SYK and CAMP, the gene encoding for the antimicrobial peptide, LL37, that has been identified as an autoantigen in psoriasis.

These results show that following an in vitro treatment with EPS-Ca, DCs switch their phenotype towards a regulatory one, which has the potential to induce differentiation of T cells into Tregs at the cost of their differentiation into the disease inducing Th17 cells. In addition, EPS-Ca reduced activation of stimulated T cells and keratinocyte production of inflammatory chemokines. Furthermore, EPS-Ca also reduced keratinocyte expression of the gene encoding for LL37, one of the autoantigens in psoriasis. Interestingly, the effects of EPS-Ca seemed to be mediated by its effects on Dectin-1 and the Syk signaling pathway in all the cell types studied. These data indicate that EPS-Ca may contribute to the beneficial effects obtained by psoriasis patients by bathing in the Blue Lagoon and suggests a possible mechanism by which EPS-Ca has its effects.

Keywords:

Dendritic cells, T cells, Keratinocytes, *Cyanobacterium aponinum,* psoriasis

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List of abbreviations

APCs	Antigen-presenting cells
AMP	Antimicrobial peptide
BSA	Bovine serum albumin
CAMP	Cathelicidin antimicrobial peptides
CLA	Cutaneous leucocyte-associated antigen receptor
CLRs	C-type lectin receptors
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CLEC	C-type lectin
CLEC7A	C-type lectin domain family 7 member A
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	CXC-chemokine ligand
DCs	Dendritic cells
DAMP	Danger-associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPS-Ca	Exopolysaccharide from *Cyanobacterium aponinum*
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GM-CSF	Granulocyte colony-stimulating factor
HEV	High endothelial cells
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecule

ILC	Innate lymphoid cell
imDCs	Immature dendritic cells
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NK	Natural killer cell
NLR	NOD-like receptor
NMS	Normal mouse serum
NHS	Normal human serum
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pDCs	Plasmacytoid dendritic cells
PDL1	Programmed death-ligand 1
mDC	Myeloid dendritic cell
PRRs	Pattern recognition receptors
RPMI	Roswell Park Memorial Institute
RT	Room temperature
S1P	Sphingosine 1-phosphate
S1PR1	Sphingosine 1-phosphate receptor 1
SYK	Spleen tyrosine kinase
SEM	Standard error of the mean
Tc	Cytotoxic T cells
TCR	T cell receptor

Tfh	T follicular helper cells
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	T regulatory cells
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
VLA	Very late antigen

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List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Gudmundsdottir, A. B., Omarsdottir, S., Brynjolfsdottir, A., Paulsen, B. S., Olafsdottir, E. S., and Freysdottir, J. 2015. 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. Immunology Letters 163:157-162.

II. Gudmundsdottir, A. B., Brynjolfsdottir, A., Olafsdottir, E. S., Hardardottir, I., and Freysdottir, J. 2019. Exopolysaccharides from *Cyanobacterium aponinum* induce a regulatory dendritic cell phenotype and inhibit SYK and CLEC7A expression in dendritic cells, T cells and keratinocytes. International Immunopharmacology 69:328-336.

The thesis is also based on some unpublished data.

Both papers are reprinted by the kind permission of the publishers.

Declaration of contribution

Paper#1 Ása Bryndís Guðmundsdóttir planned and performed the experiments, analyzed and interpreted the data, performed statistical analysis and wrote the paper. Jóna Freysdóttir planned and discussed the experiments, participated in data interpretation and in writing the paper. Ása Brynjólfsdóttir provided the culture supernatants from the *C. aponinum* culture. Elín Soffía Ólafsdóttir and Sesselja Ómarsdottir provided expertise on isolation of the EPS-Ca. Monosaccharide analysis was performed at Berit S. Paulsen laboratory at the University of Oslo. All authors read and approved the final manuscript.

Paper#2 Ása Bryndís Guðmundsdóttir planned and performed the experiments, analyzed and interpreted the data, performed statistical analysis and wrote the paper. Ingibjörg Harðardóttir and Jóna Freysdóttir planned and discussed the experiments, participated in data interpretation and in writing the paper. Ása Brynjólfsdóttir provided the culture supernatants from the *C. aponinum* culture. Elín Soffía Ólafsdóttir provided expertise on isolation of the EPS-Ca. Sævar Ingþórsson assisted and advised on confocal imaging of the cells. Vala Jónsdóttir assisted in some of the keratinocyte experiments. All authors read and approved the final manuscript.

1 Introduction

1.1 The immune system

Every day the human body is exposed to numerous infectious agents and other potential threats. Preceding the immune system, we have physical and chemical barriers that hamper potential threats invading our bodies. Those barriers are generally not considered to be a part of the immune system for it is not until these barriers are breached that the immune system comes into action. Important frontline barriers include: The epithelial cells of the skin and mucosal surfaces that hinder pathogens entering the body. Movement in the respiratory and gastrointestinal tracts due to peristalsis and cilia on epithelial surfaces remove pathogens with the help of mucus. Tears, nasal secretions and saliva all contain lysozymes and phospholipases that destabilize bacterial membranes and break down their cell walls and low pH of gastric secretions and sweat prevent growth of bacteria (Murphy & Weaver, 2017).

The immune system is comprised of a widespread network of specific cells and molecules that work together by keeping infectious agents and harmful substances at bay and rid us of dead cells and tumor cells. A key function of the immune system is to distinguish between self and non-self molecules. It is commonly divided into the innate immune system and the adaptive immune system. The innate immune system is our first line of defense and delivers a swift response against invading pathogens or tissue damage. It is, however, rather limited in flexibility when fighting ever evolving pathogens that attempt evading its actions as it is only capable of responding to conserved molecular pathogenic motifs and limited to a fixed assortment of receptors. A range of specialized cell populations belong to the innate immune system including dendritic cells (DCs), monocytes (MCs), macrophages, natural killer (NK) cells and granulocytes. The adaptive immune system acts as a second line of defense, if the first one fails to clear the invading threat, through the means of their specialized immune cells, the T and B lymphocytes. The adaptive immune system uses antigen receptors to recognize practically any antigen and thus, responds in a pathogen specific manner. The adaptive immune system also insures protection against re-exposure to the same pathogen (Murphy & Weaver, 2017). Although DCs belong to the innate immune system they have a very special role in linking the innate and the adaptive immune systems as they are antigen presenting cells (APCs) that specialize in uptake and processing of antigens and presenting them to naïve T cells.

1.1.1 Dendritic cells and T cells

DCs were first described by Prof. Ralph Steinman in the 1970s as being a distinct hematopoietic lineage with unusual morphology (Steinman & Cohn, 1973). Prof. Steinman was awarded the Nobel Prize in Physiology or Medicine in 2011 for his contribution in discovering DCs and defining the role of DCs in adaptive immunity (Banchereau & Steinman, 1998). The main role of these specialized immune cells is to sample and present both endogenous and foreign antigens to the cells of the adaptive immune system, i.e. T cells and B cells, conducting their differentiation and thus the fate of the immune response (Banchereau & Steinman, 1998; Murphy & Weaver, 2017).

DCs play a fundamental role in bridging the innate and the adaptive immune systems. DCs are functionally classified into two heterogeneous subsets; the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs), where the mDCs are the main player in the bridging role and will, hence, from now on be referred to as DCs. DCs can be a challenging group of cells to study as investigators use different markers to identify subclasses of DCs and these are completely different between mice and humans, making interpretation and comparisons between studies difficult (Haniffa et al., 2013). Plasticity also seems to be a general feature of DCs as they can alter their role according to their surroundings (Lowes et al., 2014). However, DCs are often classified according to their surface marker expression, function or location in the body. For instance, several populations reside in skin; Langerhans cells that reside in the epidermis whereas a few subsets reside in the dermis. They are usually defined by their differential expression of CD141, CD1c and CD14 (Chu et al., 2012; Haniffa et al., 2012; Martini et al., 2017).

Collectively the DCs have their main role in common: They are distributed throughout nearly all the lymphoid and nonlymphoid organs where they reside in resting state, sampling their microenvironment for „danger signals“ using a selection of phagocytic receptors, such as C-type lectin receptors (CLRs), Fcγ receptors and complement receptors. DCs can sense a wide range of danger signals through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) and scavenger receptors, to name a few. Those PRRs recognize pathogen-associated molecular patterns (PAMPs) from invading pathogens and danger-associated molecular patterns (DAMPs), such as protein or nuclear components, released from injured host cells or tissues (Piccioli et al., 2007; Qian et al., 2014; Takeuchi & Akira, 2010). Upon recognition of a microbial product or tissue damage by PRRs, the DCs mature, which involves their activation

where they become able to migrate to lymphoid organs by expressing the lymphoid homing CC chemokine receptor (CCR)7, reduce their capacity of phagocytosis by downregulating their phagocytic receptors, while becoming powerful in presenting antigens to naïve T cells. Maturated DCs upregulate their expression of MHC molecules and co-stimulatory molecules and cytokine production, which is crucial for effective interaction with naïve T cells in the lymphoid organs (Geginat et al., 2013). Due to their exceptional antigen-presenting capacity they are the main APCs and play a crucial role in activating and controlling the adaptive immune response under inflammatory conditions but also the induction of tolerance to maintain homeostasis under steady state conditions (Morelli & Thomson, 2007; Sato & Fujita, 2007).

T cell progenitors originate in the bone marrow and migrate to the thymus where they complete their development. There they go through T cell receptor (TCR) gene rearrangement and selection followed by rigorous testing of their receptors by the means of positive selection (MHC restriction) and negative selection where thymocytes that have receptors that are strongly self-reactive are eliminated to prevent autoimmune reactions. After completion of the maturation process, $CD4^+$ or $CD8^+$ thymocytes are then released from the thymus to the peripheral tissue and become mature but naïve T cells (Murphy & Weaver, 2017).

When the T cells leave the thymus they circulate between blood and secondary lymphoid organs in search of a foreign peptide recognized by their TCR. Naïve T cells express the homing receptor L-selectin (CD62L) that directs them to lymphoid tissues where they migrate from blood into the lymphoid tissues through the high endothelial venules (HEV). The naïve T cells also express the chemokine receptor CCR7 which directs them towards the lymphoid tissues. In the lymphoid tissues, the naïve T cells test their TCR on peptide-MHC complexes expressed on the surface on various APCs, including DCs. If there is not recognition, the naïve T cells exit the lymphoid tissue via lymphatics and then re-enter the blood circulation. Those T cells that manage to bind their TCR to peptide-MHC complexes in the lymphoid organ get activated and start expressing CD69, an early activation marker, on their surface. Upregulation of CD69 leads to a downregulation in the expression of sphingosine 1-phosphate receptor 1 (S1PR1). This makes the T cells unable to respond to sphingosine 1-phosphate (S1P) gradient that is present in the lymphatics and are thus unable to leave the lymphoid tissue. Instead they remain in the lymphoid tissue where they proliferate and differentiate for several days, after which the CD69 expression decreases and they regain expression of S1PR1 on their surface. Now they can leave

the lymphoid tissue as fully activated effector T cells (Murphy & Weaver, 2017).

Following activation, the $CD8^+$ T cells (Tc) acquire cytotoxic functions, which are important in elimination of virus infected or tumor cells. The $CD4^+$ T cells play a central role in the elimination of pathogens and in self-tolerance. They become either T helper (Th) cells or regulatory T cells (Tregs), which either provide help to or regulate the function of other immune cells, respectively (Kuwabara et al., 2017; Murphy & Weaver, 2017). Multiple phenotypes of both $CD4^+$ and $CD8^+$ T cells have been identified, which have differing functions. These phenotypes are largely determined when they interact with DCs in the lymphoid organs where cytokines play the greatest role in regulating their differentiation pathway (Schmitt & Ueno, 2015).

For naïve T cells to become activated and differentiated, they need to obtain three signals, which are mainly delivered by DCs. The first signal is the main activation signal. It involves binding of the MHC molecule containing the peptide on the DC to the TCR and the CD4 co-receptor on the T cell. The second signal occurs through binding of the co-stimulatory molecules CD80 and/or CD86 on the DC to CD28 on the T cell, which provides a survival signal for the T cell. These two signals are sufficient to induce massive proliferation of the T cell, called clonal expansion. The third signal comes in the form of cytokines that trigger the differentiation of the T cells and steers their polarization into certain types of effector cells. The type of effector cell is dependent on which combination of cytokines bind to the activating T cell. The majority of these cytokines are secreted by the DC but some by other neighboring cells (Clark & Kupper, 2005; Gordon et al., 2014; Murphy & Weaver, 2017).

The main $CD4^+$ effector T cells are functionally divided into five major subsets, Th1, Th2, Th17, follicular T helper cells (Tfh) and Tregs. Each effector T cell type is induced by a combination of certain cytokines secreted by the DCs and neighboring cells during the activation stage (Clark & Kupper, 2005; Murphy & Weaver, 2017; Walsh & Mills, 2013). The $CD8^+$ T cells have corresponding effector cells (Tc cells) that share similar cytokine profiles as the Th cells, such as Tc1, Tc2, Tc17 and $CD8^+$ Tregs (Mittrücker et al., 2014). However, in the current study the focus will be on $CD4^+$ T cells. Figure 1 provides an overview of how cytokines from DCs and other neighboring cells polarize naïve T cells into different effector T cells producing different effector molecules. The Th1 cells are induced by interleukin (IL)-12, secreted by DCs, and interferon (IFN)-γ, most likely provided by NK cells, other Th1

cells and innate lymphoid cells (ILC)1 cells and express the transcription factor T-bet in response to bacterial, viral or protozoan infections. These cells produce type 1 cytokines such as IFN-γ that recruit and activate macrophages to terminate infected cells (Clark & Kupper, 2005; Geginat et al., 2013; Murphy & Weaver, 2017). Dysregulation of Th1 responses has been shown to have a causative role in the pathogenesis of a number of autoimmune diseases, such as type 1 diabetes and rheumatoid arthritis, as well as experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis, (Damsker et al., 2010; Jager & Kuchroo, 2010). Th2 cells are induced by IL-4 secretion and express the transcription factor GATA-3. IL-4 is not secreted by DCs; however, its origin has still not been determined but mast cells, ILC2 cells and other Th2 cells have been suggested (Murphy & Weaver, 2017). Th2 cells are central players in the protective role against helminths with their production of IL-4, IL-5 and IL-13 but are also participants in allergic reactions (Geginat et al., 2013; Jager & Kuchroo, 2010). Th17 cells are induced by IL-6 and IL-1β (in humans), and supported by IL-23, all cytokines secreted by DCs, and express the transcription factor RORγt (Acosta-Rodriguez et al., 2007; Jager & Kuchroo, 2010). Th17 cells promote inflammation and neutrophil recruitment to infected tissues by secreting IL-17A, IL-17F, IL-22, GM-CSF and tumor necrosis factor (TNF)-α. Th17 cells are now recognized to be the key inflammatory T cells that play a crucial role in the pathogenesis of many autoimmune diseases including psoriasis (Damsker et al., 2010; Geginat et al., 2013). Tfh cells are the only effector T cells that do not leave the lymphoid organs after activation, but migrate to the B cell area where they provide help for activating B cells. They secrete IL-21 but are also capable of secreting cytokines that resemble the cytokines secreted by Th1, Th2 and Th17 cells, which are responsible for the antibody class switch (Murphy & Weaver, 2017; Weinstein et al., 2016).

Tregs are induced by IL-10 and transforming growth factor (TGF)-β, which can be secreted by DCs, and play a critical role in self-tolerance and regulation of immune responses. They mediate their regulation through regulatory molecules, such as cytotoxic T lymphocyte-associated protein (CTLA)-4 and programmed death-ligand 1 (PD-L)1 and their production of suppressive cytokines, primarily IL-10 and TGF-β (Geginat et al., 2013; Murphy & Weaver, 2017; Walker, 2013). The expression of their transcription factor FoxP3 is crucial in maintaining their suppressive activity (Jager & Kuchroo, 2010). Any abnormalities or mutations in FoxP3 result in loss of functional Tregs and, thus, have been studied extensively in many

autoimmune diseases, such as psoriasis, diabetes and systemic lupus erythematosus where Tregs have been discovered to be dysfunctional (Walsh & Mills, 2013).

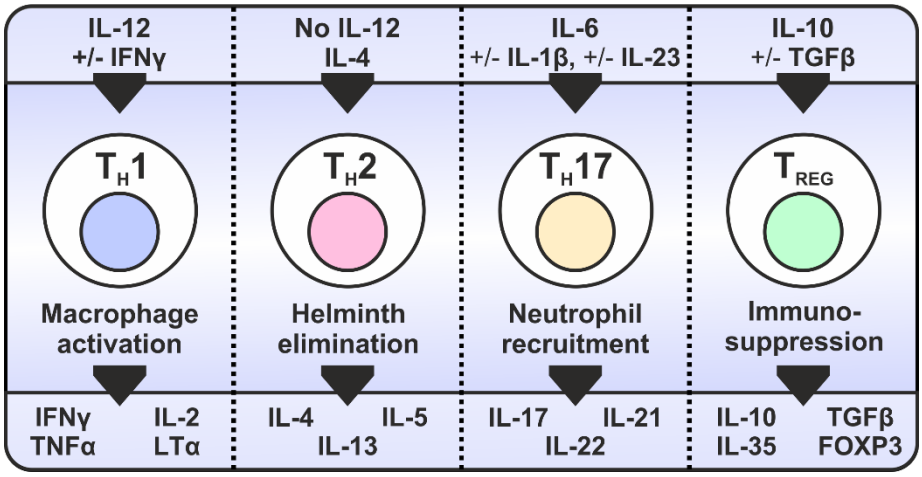

Figure 1. T cell polarization.

APCs secrete cytokines in signal 3 inducing naïve T cells to differentiate into different phenotypes which produce different effector molecules.

1.1.1.1 Tolerogenic DCs and regulatory DCs

In addition to the classical DCs that process antigens and present to naïve T cells, conducting their differentiation into different T effector cells, there are DCs with other functions, namely, tolerogenic DCs and regulatory DCs.

A definition of a tolerogenic DC phenotype has not been established. However, they are considered to express lower levels of MHC and costimulatory molecules than imDC and to be quite resistant to activation/maturation (Sato et al., 2017). The characteristic functions of tolerogenic DCs are reduction of T cell activation and increased induction of T cell apoptosis and generation of anergic T cells and Tregs resulting from their low expression of MHC and co-stimulatory molecules (signals 1 and 2 respectively required for naïve T cells for activation,). They mainly secrete anti-inflammatory cytokines, such as IL-10 and TGF-β, rather than pro inflammatory cytokines such as IL-12 (signal 3 required for naïve T cells for differentiation) (Sato et al., 2017). The tolerogenic capacity of these DCs greatly depends on their immature state and can be potentiated by

immunosuppressive mediators and various signals from immune cells, apoptotic cells, tissues and tumor microenvironment. A substantial effort has been made over the years to manipulate imDCs to retain their immature state to be subsequently able to induce tolerance. These efforts include, for example, immunosuppressive drugs, such as dexamethasone (corticosteroid), cyclosporine and rapamycin (calcineurin inhibitors), aspirin and anti-inflammatory factors, such as IL-10, TGF-β1, vitamin D3, and prostaglandin E2. (Gordon et al., 2014; Morelli & Thomson, 2007; Osorio et al., 2015; Rutella et al., 2006). Another way to maintain their immature state would be genetic manipulation, such as by overexpressing immunosuppressive molecules. However, the functional stability of human tolerogenic DCs has raised concern for application in the clinic as the possibility of them regaining immunogenicity when encountering inflammatory signals *in vivo* is a potential threat (Sato et al., 2017).

Regulatory DCs express moderately high levels of MHC molecules but low levels of costimulatory molecules, such as CD80 and CD86 compared with classical DCs (Sato et al., 2003a). Thus, these cells have a greater ability than tolerogenic DCs to interact with naïve T cells without delivering signal 2 and therefore inhibit the activation and induce hypo-responsiveness in T cells. Furthermore, even under inflammatory conditions, they retained their effective immunoregulatory properties (Sato et al., 2003a; Sato et al., 2003b). In light of these features of regulatory DCs dampening down immune responses, their use and further development may lead to promising therapeutic strategies for the attenuation of undesirable immune responses such as those that characterize autoimmune diseases, inflammatory diseases, graft rejections and allergic diseases (Sato et al., 2017).

Murine DCs expressing CD103 and their human functional homologue $CD141^{hi}$ DCs have been linked to tolerance/regulation and to protection against several inflammation-mediated diseases (Chu et al., 2012; Hang et al., 2016; Haniffa et al., 2013; Haniffa et al., 2012; Heier et al., 2017; Patel et al., 2018).

One of the signaling molecules linked to IL-10 secretion by DCs is the spleen tyrosine kinase (SYK) (Yin et al., 2016). The Syk family consists of two tyrosine kinases, SYK and ZAP70. SYK is a non-receptor tyrosine kinase that regulates diverse biological functions; it has an indispensable role in immune cell activation, and development of lymphocytes (Patel et al., 2018). C-type lectins (CLECs) are a group of CLRs which are mostly transmembrane receptors that engage their ligands to induce intracellular

signaling that alter cellular function. Many of which activate DCs via phosphorylation of the Syk signaling pathway (Hang et al., 2016; Plato et al., 2013). Several studies have shown that overexpression of SYK induces inflammation, autoimmunity and allergy and has an imperative role in the pathogenesis of several autoimmune diseases, including multiple sclerosis, psoriasis and systemic lupus erythematosus (Mócsai et al., 2010; Turner et al., 2000). ZAP70 plays an important role in T cell activation (Wang et al., 2010), whereas SYK is broadly expressed in hematopoetic (Au-Yeung et al., 2018) and non-hematopoietic cells and plays a broader role in the immune system, including. in CC chemokine ligand (CCL)20 expression of keratinocytes (Wu et al., 2015) and IL-10 secretion by DCs (Yin et al., 2016).

Using microarray analysis, Hang *et al.* found that DCs from mice, infected with the helminth *Heligmosomoides polygyrus bakeri* (Hpb), expressed decreased levels of mRNA for SYK and several CLECs which appeared to protect the mice from colitis. Similar inhibition of SYK and CLEC7A was found when DCs were incubated with the secretory product from Hpb. A decrease in Dectin-1 receptor (encoded by the CLEC7A gene) and the downstream Syk signaling pathway proved to be an important mechanism to promote regulatory DCs in mice (Hang et al., 2016). Another recent mouse study showed that SYK is upregulated in $CD11c^+$ DCs during imiquimod-induced psoriatic inflammation. Following SYK activation the DCs produced IL-6 and IL-23 and thus, induced differentiation of Th17 cells. Furthermore, a SYK-inhibitor R406 attenuated the psoriatic inflammation in the mice by suppressing the Th17 cells, via reduction of the IL-6 and IL-23 production by the DCs, and upregulating Tregs (Alzahrani et al., 2019).

1.2 The Blue Lagoon

The Blue Lagoon is one of Iceland's greatest tourist attractions. It is located on the Reykjanes peninsula in South-West Iceland which lies right on the Mid-Atlantic Ridge. The peninsula is mainly made up of very porous lava allowing seawater to seep deep into the aquifers. A geothermal power plant was built in Svartsengi in 1976. Wells were drilled through the lava to the depth of up to 2000 m obtaining geothermal fluid at 240°C from the geothermal reservoir. The steam phase is utilized to produce electricity and the liquid phase to warm up freshwater that is used to heat the houses in the neighboring communities. The Blue Lagoon was formed by the effluents of the power plant that pumps the effluent geothermal liquid at approximately 70°C in to the lava field. The liquid is 65% seawater and 35% freshwater. It is supersaturated with silica (SiO) resulting from its interaction with the

surrounding lava at the high temperatures in the geothermal reservoir. As the water cools down, the silica precipitates as white mud and fills in some of the cracks of the lava causing the Lagoon to slowly increase in size although most of the water seeps through the lava back into the aquifers or evaporates. The temperature of the Lagoon is approximately 37°C but can vary from 30-45°C due to local and seasonal fluctuations. The salinity is 2.5% and the average pH is 7.5 (Grether-Beck et al., 2008; Olafsson, 1996; Petursdottir et al., 2009).

Considering the great number of visitors, the Lagoon would seem like a perfect environment for many organisms including enteric bacteria. However, enteric bacteria are not to be found in the Lagoon. In fact, no plants, fungi, human coliform bacteria or environmental bacteria have been isolated from the Lagoon nor do they grow in water from the Lagoon. This has been confirmed by health authorities (Olafsson, 1996; Petursdottir et al., 2009; Pétursdóttir & Kristjánsson, 1996). The explanation for the low microbial diversity in the Lagoon is most likely found in the salinity and the unique chemical composition of the geothermal fluid with the high silica concentration. The high silica content is also thought to increase the sensitivity of bacteria to UV light (Olafsson, 1996; Petursdottir et al., 2009). Very few organisms have been able to adapt to this extreme environment. Although 35 taxa have been identified to inhabit the Blue Lagoon, two main organisms characterize the Blue Lagoon; *Cyanobacterium aponinum* and *Silicibacter lacuscaerulensis* (Petursdottir et al., 2009).

1.2.1 Cyanobacteria - Cyanobacterium aponinum

Cyanobacteria are simple microorganisms that have characteristics of both bacteria and algae. They resemble bacteria because of their prokaryotic cellular organization but are photoautotrophic similar to algae and other higher plants (Jaiswal et al., 2008). Cyanobacteria are larger than other bacteria, are mostly aquatic and are always unicellular although they frequently grow in colonies or filaments often surrounded by gelatinous or mucilaginous sheath depending on environmental conditions (Jaiswal et al., 2008; Singh et al., 2005).

Many cyanobacteria synthesize exopolysaccharides. The exopolysaccharides may form capsules, which are structures immediately associated with the cell surface; or be found as slime, a mucilaginous mass that is loosely attached to cells; or be released as colloids into the surroundings. The main function attributed to the exopolysaccharides is to act as a protective layer or boundary from the surroundings, protecting the

organism from predators or harsh environment (Kehr & Dittmann, 2015; Otero & Vincenzini, 2003). As the released polysaccharides can be relatively easily recovered from the culture medium, they have attracted much interest for their potential applications in various industries, such as the food, cosmetic and pharmaceutical industry (Philippis & Vincenzini, 1998). Cyanobacteria produce complex exopolysaccharides composed of at least 10 different monosaccharides. They are characterized by the presence of pentoses, which is unusual for polysaccharides of prokaryotic origin, and by their anionic nature due to acidic sugars, like glucuronic and/or galacturonic acids, as well as other anionic substituents, both organic (acetyl, pyruvil) and inorganic (phosphate, sulphate) (Otero & Vincenzini, 2003; Philippis & Vincenzini, 1998).

As carbohydrate structures such as exopolysaccharides are known for their safety and tolerability record they have been of interest as promising vaccine adjuvant candidates. In the field of vaccinology, the requirement to look for adjuvants capable of boosting a Th1-type immune responses without unacceptable toxicity is quite urgent (Petrovsky & Cooper, 2011). One such mouse study showed an exopolysaccharide derived from the cyanobacteria, *Aphanothece halophytica,* which showed promising effects as an adjuvant for a vaccine, promoting Th1 polarization (Zhu et al., 2016). Another study showed the effects of an exopolysaccharide derived from the cyanobacteria *Nostoc commune* on THP-1 cells that had been primed with IFN-γ and stimulated with LPS. Results showed anti-inflammatory effects with increased IL-10 secretion and reduced IL-6 secretion; however, some proinflammatory effects were also reported such as increased TNF-α and IL-8 secretion (Olafsdottir et al., 2014).

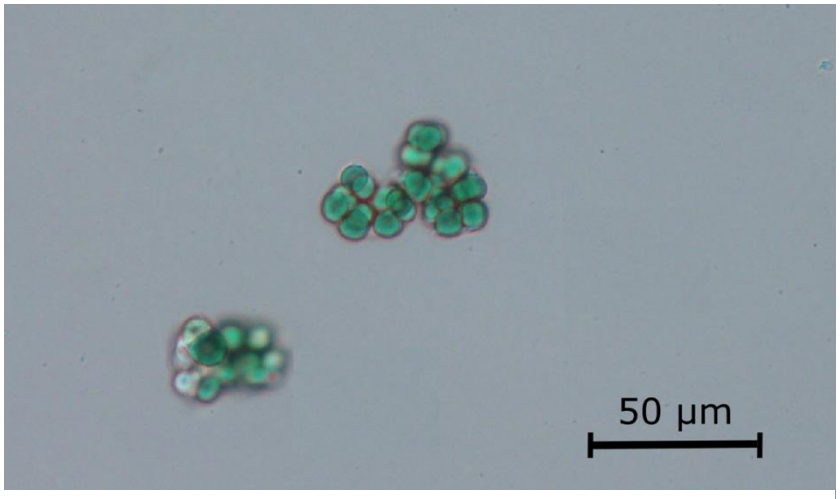

Figure 2. *Cyanobacteria aponinum.*

Microscopic image of *Cyanobacterium aponinum*, courtesy of the R&D team at the Blue Lagoon.

C. aponinum was first described when isolated from the Euganean thermal springs in Padua, Italy. It was found to be able to grow both in fresh water and seawater at temperatures up to 45°C (Moro et al., 2007). *C. aponinum* (figure 2) is a coccoid cyanobacteria and is the primary producer of the Blue Lagoon ecosystem. It uses sunlight as its energy source and, thus, is the dominating organisms in the Lagoon in the summertime. During fall and winter *Silicibacter lacuscaerulensis,* a heterothrophic alphaproteobacterium, is dominant in the Lagoon (Olafsson, 1996; Petursdottir et al., 2009; Pétursdóttir & Kristjánsson, 1996).

As cyanobacteria have been attracting increased interest over the past years, effort has been made to determine conditions for their cultivation (Ugwu et al., 2008). There are three ways to cultivate microalgae; open pond system that has obvious contamination problems, closed photobioreactor placed outside for natural illumination, and the final and the only method that is suitable for application in pharmaceutical and cosmetic industries, a closed photobioreactor with artificial illumination (Pulz, 2001; Ugwu et al., 2008). The last option is used by the Blue Lagoon R&D laboratory to cultivate *C. aponinum*.

1.2.2 Beneficial effects of bathing in the Blue Lagoon on psoriasis

The benefits of bathing in the Blue Lagoon on psoriasis was first discovered by an employee of the geothermal power plant suffering from psoriasis. His plaques gradually improved when he rubbed the white mud on his skin while bathing in the Lagoon. Word got out, and in the following years several people tried to cure their disease by bathing in the Lagoon and many of them claimed beneficial effects from the bathing. Since then several studies have been conducted and it has been reported that bathing in the Blue Lagoon has beneficial effects on psoriasis, atopic dermatitis, and healthy skin. Research has also shown that bathing in the Blue Lagoon in conjunction with ultraviolet B (UVB) phototherapy is a useful alternative treatment and gives better results than UVB treatment alone (Olafsson, 1996). Those results have been confirmed in two recent clinical studies, a pilot study and a study comprising a larger cohort, both conducted by Eysteinsdottir *et al.* (Eysteinsdottir et al., 2014; Eysteinsdottir et al., 2013). The results from the pilot study also suggested that the mechanism behind the beneficial effects of bathing in the Blue Lagoon may be linked to downregulation of Th17/Tc17 cells in peripheral blood (Eysteinsdottir et al., 2013). Grether-Beck *et al.* also demonstrated that extracts prepared from the silica mud and the two different microalgae found in the Lagoon, induce gene expression of involucrin, loricrin, transglutaminase-1 and filaggrin in primary human keratinocytes and that topical treatment of healthy human skin using the same extracts induced the same gene regulatory effects (Grether-Beck et al., 2008). These results indicate that extracts containing silica mud and microalgae from the Blue Lagoon may improve skin barrier function and that these effects might contribute to the beneficial effects psoriasis patients experience after bathing in the Blue Lagoon.

While bathing in the Blue Lagoon has increased immensely in popularity over the recent years, little is still known about the biological activities present in the Lagoon.

1.3 The skin

The skin is the largest organ of the body and the first line of defense against microbial pathogens and physical and chemical insult. It possesses many functions which together make the skin act as a protective barrier. It maintains body temperature, is a sensory organ and as such gathers information from the environment. Last but not least, it has essential immunological functions, both in homeostasis and in pathological conditions (Wickett & Visscher, 2006).

The commensal flora that populates the skin is in its own a very important nonspecific immune barrier. *Staphylococcus* and *Propionibacteria*, for example, colonize the skin and thereby compete for space and nutrients and thus, limiting those resources for pathogens. Shedding of cells also helps ridding the skin of microorganisms that are colonizing or infecting cells of the epidermis and prevents infection in the deeper layers of the skin. Intact skin can be considered a physical barrier that is resistant to most microorganisms and it is generally only when it is breached that they can invade through the skin, into underlying tissues, thereby causing infections (Baroni et al., 2012). The human skin is divided into two compartments; the epidermis and the dermis (figure 3). The epidermis contains four layers (strata); the stratum basale, the stratum spinosum, the stratum granulosum and the outermost layer that is responsible for the vital barrier function of the skin, the stratum corneum (Nestle et al., 2009a). The basal layer (stratum basale) of the epidermis, sits on the basement membrane that separates the epidermis from the dermis, and is responsible for the continuous renewal of the epidermis which consists predominantly of keratinocytes. The keratinocytes mature and move through the layers of the epidermis resulting in a fully differentiated keratinocytes of the corneum, often called corneocytes. These

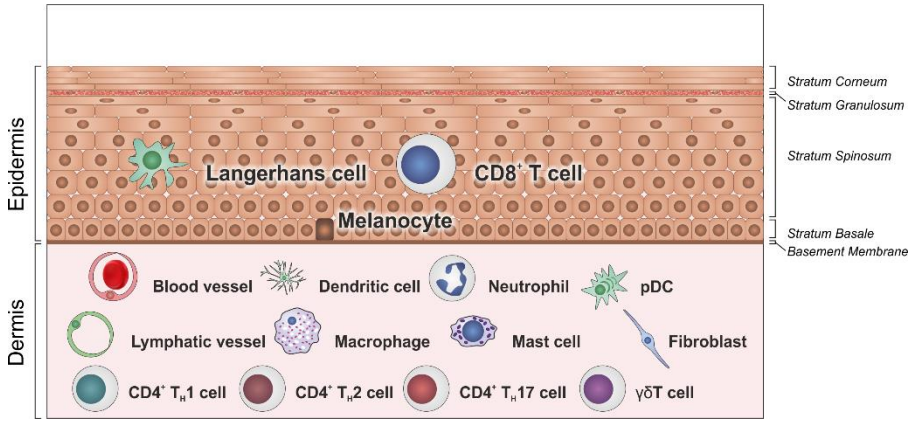

Figure 3. Skin anatomy.

The skin is divided in to two compartments that are separated by the basal membrane, the epidermis and the dermis. The epidermis contains the stratum basale, stratum, spinosum, stratum granulosum and stratum corneum. It contains many specialized cells including Langerhans cells, melanocytes and lymphocytes (mainly $CD8^+$ T cells). The dermis is a layer of connective tissue rich of collagen and elastic fibers. It contains a good network of blood vessles and lymphatic vessels and contains many specialized cells, including dendritic cells, plasmacytoid DCs (pDC), macrophages, mast cells, fibroblasts, melanocytes and $CD4^+$ T cells. Based on Nestle et al (Nestle et al., 2009a).

cells are dead, flat and have lost their nuclei and most of their organelles during the maturation process (Baroni et al., 2012; Nestle et al., 2009a) and are shed in the form of desquamation. In healthy epidermis, there is a balance between the proliferation process of the keratinocytes and the desquamation that results in a total renewal every 28 days (Baroni et al., 2012).

Keratinocytes also have many immunological functions and through the means of their PRRs can sense pathogens and discriminate between harmless microorganisms and harmful pathogens (Nestle et al., 2009a). Keratinocytes can secrete cytokines and chemokines and produce antimicrobial peptides (AMPs) (further discussed in chapter 1.3.1.4) (Nestle et al., 2009a; Nickoloff & Turka, 1994). They also have receptors for various cytokines and chemokines and, therefore, are able to receive signals from their inflammatory surroundings (Murphy & Weaver, 2017; Nestle et al., 2009b; Tuzun et al., 2007). Interestingly, when keratinocytes are stimulated with IFN-γ, they express MHC class II making them able to act as a non-professional APCs for T cells. They are also able to provide essential signals for T cell proliferation (Nestle et al., 2009a; Nickoloff & Turka, 1994).

Other cells that can be found in the epidermis include Langerhans cells (a type of DCs) that are crucial for the immune defense of the epidermis, melanocytes that lie in the basal layer of the epidermis and produce the pigment melanin that gives the skin its color and finally lymphocytes (Baroni et al., 2012; Wickett & Visscher, 2006).

The dermis is a layer of connective tissue that includes collagen and elastic fibers. It has a good network of lymphatic vessels, is highly vascularized and hosts hair follicles, nerve endings, sweat and sebaceous glands. It also contains numerous cells, including macrophages, mast cells, fibroblasts and specialized immune cells, such as DCs, $CD4^+$ T cells, γδ T cells and natural killer T (NKT) cells (figure 3) (Nestle et al., 2009a).

1.3.1 Psoriasis

Psoriasis is a chronic, autoimmune inflammatory disease that mainly affects the skin and joints. It has a complex etiology involving genetic risk factors and environmental triggers.

Psoriasis affects over 125 million people all over the world but the prevalence varies depending on geographic regions and ranges from 0.5% to 11.4% in adults (Michalek et al., 2017; Parisi et al., 2013). The disease is less common in children (prevalence 0% to 2.1%) than in adults and it is equally

common in women and men. Psoriasis can onset at any age, although it usually occurs between 18-39 or 50-69 years of age (Parisi et al., 2013).

Psoriasis can manifest as various phenotypes including psoriasis vulgaris, guttate psoriasis (scaly droplet shaped spots), inverse psoriasis (usually found in folds of skin), pustular psoriasis, palmoplantar psoriasis (pustular psoriasis of the palms and soles) and erythrodermic psoriasis. The same individual can have one or multiple phenotypes. Symptoms that are shared by all phenotypes are burning, itching and soreness and most of them have a cyclic process, i.e. flaring up for a few weeks and then subsiding for a period of time or even going into period of remission (Griffiths & Barker, 2007; Naldi & Gambini, 2007).

Individuals with psoriasis are at high risk of developing other chronic health diseases. A number of comorbidities have been associated with psoriasis including psoriatic arthritis, cardiovascular diseases, metabolic syndrome and psychiatric complications, such as depression and anxiety and the disease has well described negative effects on quality of life (de Korte et al., 2004; Gelfand et al., 2004; Griffiths & Barker, 2007; Nestle et al., 2009b).

A complex interplay between genetic predisposing factors and environmental factors leads to the onset or exacerbation of psoriasis. The environmental factors include physical and psychological stressors (stress, major life events and trauma) (Mazzetti et al., 1994; Pacan et al., 2003), lifestyle (alcohol and smoking) (Armstrong et al., 2014; Poikolainen et al., 1999), hormonal changes (menopause, puberty and postpartum period) (Ceovic et al., 2013), infections (e.g. *Streptococcus pharyngitis*, *Staphylococcus aureus* and HIV infections) (Fry & Baker, 2007), weather changes (cold and humidity) (Raychaudhuri & Farber, 2001) and drugs (e.g. beta-blockers, lithium, anti-malarial drugs, interferons, imiquimod and terbinafine) (Balak & Hajdarbegovic, 2017; Fry & Baker, 2007). It is unclear how these environmental factors trigger psoriasis. However, it is likely that psoriasis patients inherit a predisposition to the disease that requires an exogenous stimulus to express its phenotype (Ortonne, 1999).

1.3.1.1 Clinical manifestation and histopathology of psoriasis vulgaris

Psoriasis vulgaris (or chronic plaque psoriasis) is the most common form of the disease. It accounts for about 90% of cases and will be referred to as psoriasis here after. The disease usually manifests as red, raised, well-demarcated, erythematous plaques with adherent silvery scales surrounded

by normal skin. Psoriasis can affect any skin site; however, commonly affected sites include the scalp, elbows, knees, and the lumbar region (Griffiths & Barker, 2007). Psoriasis of the scalp develops in 75-90% of patients with psoriasis (Ortonne et al., 2009). The psoriatic scale is formed by hyperproliferative epidermis with premature maturation of keratinocytes. The mitotic rate of the keratinocytes in the basal layer is increased up to 10 fold compared with normal proliferation, which results in incomplete cornification with retention of the nuclei in cells of the stratum corneum (parakeratosis) (Lowes et al., 2007). The redness of the plaque is the result of increased angiogenesis that leads to vascularization and further influx of leucocytes into the inflamed skin (Griffiths & Barker, 2007; Nestle et al., 2009a).

Histopathological features of a typical psoriatic plaque are depicted in figure 4. They include epidermal thickening, retention of nuclei in the upper layers of the skin, elongated rete ridges, increase in number and size of dermal blood vessels and an increase in inflammatory cell infiltrate, consisting mostly of neutrophils, in the stratum corneum (Munro's microabsesses) and epidermis, and T cells and DCs in the dermis (Boehncke & Schön, 2015; Griffiths & Barker, 2007).

1.3.1.2 Immunopathogenesis of psoriasis

Psoriasis is a disease involving both the innate and the adaptive immune system, with immune cells interacting with other cells of the skin tissues, where the T cells, DCs and keratinocytes have central roles (Lowes et al., 2007). The importance of unraveling its pathogenesis is undisputed since new and deeper understanding may unravel novel therapeutic targets.

The development of a psoriatic lesion is a result of interaction between hyperproliferating keratinocytes, inflammatory DCs, T cells, neutrophils and mast cells (Chiricozzi et al., 2018). The model for pathogenesis of psoriasis has been under constant revision over the last thirty years or so and still the pathogenesis is a long way from being fully understood. Early on, psoriasis was thought to be a disease of the hyperproliferating keratinocytes, where a defect in the keratinocytes would trigger an inflammatory response. However, it was soon postulated that hyperproliferation of the keratinocytes was secondary to the inflammation/immune response (Lowes et al., 2014). A breakthrough was made when the imperial role of the immune system, particularly the T cells, in the pathogenesis of the disease was established by Gottlieb and coworkers (Gottlieb et al., 1995). Their study showed a

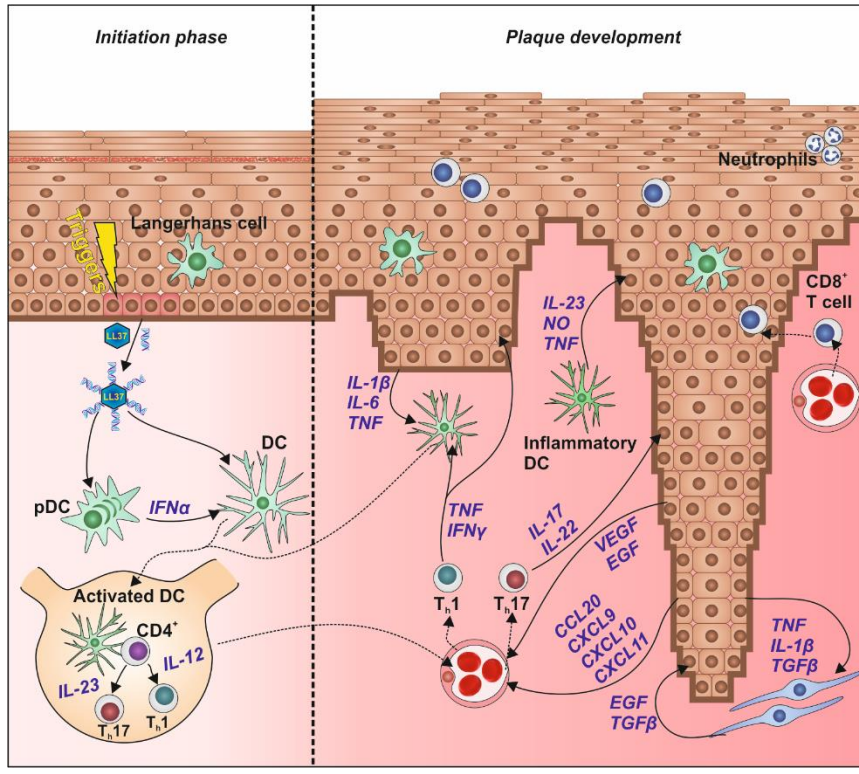

Figure 4. Immunopathogensis of psoriasis from initiation phase to plaque development.

As depicted on the left panel on the figure (initiation phase) the antimicrobial peptide LL37 released from keratinoctes can bind DNA or RNA and form complexes that activate pDCs to release IFN-α which in turn activates resident DCs. The mature DCs migrate to the nearby lymphoid organs, secrete IL-12 and IL-23 which induces polarization of naive T cells into Th1 and Th17 cells. Those T cells circulate back to the skin where they contribute to the cytokine millieu that acts on keratinocytes (right panel, plaque development). Furthermore, keratinocytes can produce chemokines and antimicrobial peptides that amplify the immune response. Based on Nestle et al 2009 (Nestle et al., 2009a).

resolution of psoriatic plaques upon selective apoptosis of activated T cells, without any effect on keratinocyte survival or activation. They hypothesized that psoriasis is a disease of activated T cells. Soon they found confirmation in other studies, further supported by the availability of a number of effective immune therapies, including, Abatacept that was used to block CD80/CD86 mediated co-stimulation to T cells (Abrams et al., 1999; Lowes et al., 2014; Valdimarsson et al., 1986). Initially, the pathogenic mechanism was thought to involve IL-12/IFN-γ signaling where the $CD4^+$ Th1 cells and $CD8^+$ Tc1 cells

played the biggest roles (Lew et al., 2004; Schlaak et al., 1994). Recent revisions of the pathogenesis model for psoriasis favors the IL-23/IL-17 axis where cells producing IL-17 play the greatest role, in particular the Th17 and Tc17 cells (Krueger et al., 2012; Martin et al., 2013).

1.3.1.3 Initiation of psoriasis and key contributing cells

Psoriasis can be triggered by various factors such as injury, trauma, infection or medication causing damage to cells as previously discussed. pDCs produce large amounts of type 1 IFNs when activated and are considered to be the primary source of IFN-α in the skin. Their activation is by many considered to be the first step in the initiation phase of psoriasis (Nestle et al., 2005; Theofilopoulos et al., 2005; Zaba et al., 2009b). They can be activated by TLR agonists, such as DNA or RNA from damaged cells complexed with LL37, β-defenins, lysozyme or IL-26 (Ganguly et al., 2009; Lande et al., 2015; Meller et al., 2015). pDC activation was shown to be crucial in the pathogenesis of psoriasis in a mouse study by Nestle *et al.,* as development of skin lesions was inhibited by suppressing pDC activity (using anti-BDCA-2 antibody) and thereby IFN-α production (Nestle et al., 2005). Keratinocytes are a rich source of AMPs, including LL37, β-defensins and S100A7 (psoriasin). LL37 combines with DNA or RNA to form complexes that activate pDCs through TLR9 or TLR7, respectively. Activated pDCs produce IFN-α/β that activates DCs (Ganguly et al., 2009; Gilliet & Lande, 2008; Lande et al., 2007).

DCs are key immune sentinels that drive the adaptive immune response in psoriasis and their numbers are greatly increased in psoriatic plaques. As APCs secreting IL-12 and IL-23, the DCs drive the differentiation of T cells into Th1 and Th17 cells, respectively (Johnson-Huang et al., 2009; Zaba et al., 2009a). Activated Th17 cells produce cytokines, including IL-17A, IL-17F and IL-22. IL-17 may also be released by Tc17 cells, γδ T cells, ILCs, mast cells and neutrophils (Chiricozzi, 2014). Keratinocytes are activated both by the Th17-associated cytokines (IL-17A, IL-17F and IL-22) and the Th1-associated cytokines (TNF-α and IFN-γ), which leads to their proliferation and production of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and chemokines (CCL20 and CXC chemokine ligand (CXCL)10). This forms a positive feedback loop that further drives this cytokine storm (Lowes et al., 2013; Nickoloff & Nestle, 2004).

DCs expressing CD11c are abundant in lesions of psoriatic skin. They are believed to be derived from circulating precursors that migrate to the skin in response to inflammatory and chemotactic signals and thus differentiate in

psoriatic inflammatory environment (Zaba et al., 2009a). The DCs are usually divided into two subpopulations. The DC phenotype that is more prevalent in psoriatic skin is $CD11c^{+}CD1c^{-}$ DC and often called inflammatory DCs (Zaba et al., 2009b). Their number is estimated to be 30 fold greater in lesional psoriatic skin than in normal skin and they are considered to be the key players in the pathogenesis of psoriasis (Zaba et al., 2009a). They secrete TNF-α, IL-6, IL-20, IL-23 and IL-12 and produce nitric oxide (NO). Due to these products they are able to cause inflammation (TNF-α, NO), epidermal hyperplasia (IL-20) and induce T cell differentiation (IL-6, IL-23 and IL-12). DCs can secrete both IL-12 and IL-23 (which are members of the same cytokine family and share the IL-12p40 chain) that drives T cell differentiation towards Th1/Tc1 and Th17/Tc17 phenotypes, respectively. However, they mainly release IL-23 and thus amplify and maintain the IL-17 mediated response (Johnson-Huang et al., 2009; Zaba et al., 2009a). The other DC phenotype is $CD11c^{+}CD1c^{+}$ DC and phenotypically more similar to those of normal skin. They act as resident, mature APCs and the number of these cells does not increase in lesional skin compared with uninvolved skin (Zaba et al., 2009b).

In human lesional skin and in the blood of psoriatic patients the number of $CD4^{+}$ and $CD8^{+}$ T cells is increased (Kagami et al., 2010; Lowes et al., 2008; Ortega et al., 2009). These cells express the skin homing molecule cutaneous leucocyte-associated antigen receptor (CLA) and chemokine receptors and interact with endothelial cells expressing adhesion molecules e.g. P-selectin and E-selectin (Lowes et al., 2014). The $CD8^{+}$ T cells infiltrate the epidermis while the $CD4^{+}$ T cells infiltrate the dermis. Th1, Th17, Th22, Th9 and Tfh cells have been identified within the cellular infiltrates as have their $CD8^{+}$ T cell counterparts (Tc cells) (Hijnen et al., 2013; Ortega et al., 2009). γδ T cells also contribute greatly to the IL-17 production (Cai et al., 2011).

ILCs are a heterogenous group of cells that do not express specific antigen receptors. One of the ILC subsets is the ILC3 that express NKp44 and the RORγt transcription factor and have been suggested to take part in the pathogenesis of psoriasis as they are able to produce IL-17 and IL-22 upon stimulation with IL-23 and IL-1β (Teunissen et al., 2014; Villanova et al., 2014). ILC3 have even been suggested as a biomarker for psoriasis as their number is consistently higher in the circulation of psoriatic patients compared with healthy individuals or atopic dermatitis patients; however, their role in the pathogenesis of psoriasis needs to be clarified (Teunissen et al., 2014).

Keratinocytes have receptors for all the major cytokines observed in lesional skin making them the main responding cells in the psoriatic milieu. They react to the psoriatic cytokines by proliferating and releasing a number of pro-inflammatory products such as cytokines (e.g. IL-19, TNF-α, IL-17C and thymic stromal lymphopoietin (TSLP)), proliferation stimulating factors (e.g. epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF)), chemokines (e.g. CCL20, CXCL1 and CXCL10) and AMPs (e.g. LL37) that are important in the recruitment of T cells, neutrophils, and inflammatory mDCs (Chiricozzi et al., 2011; Chiricozzi et al., 2014; Guilloteau et al., 2010; Harper et al., 2009). The keratinocytes, thus, further amplify the inflammation and take part in the psoriatic positive feedback loop.

1.3.1.4 Antimicrobial peptides

AMPs are host defense molecules that are produced by all living species. They are small, amphipathic peptides or proteins that are highly effective in killing pathogenic microorganisms, including Gram-negative and Gram-positive bacteria, enveloped viruses, fungi and protozoa. They even have potential to act as cytotoxic agents against certain types of cancers (Lai & Gallo, 2009; Morizane & Gallo, 2012). AMPs are also capable of modifying host inflammatory responses in vertebrates via assortment of mechanisms, including acting as chemotactic agents, angiogenic factors and regulators of cell proliferation (Lai & Gallo, 2009). One such peptide is the only human cathelicidin, hCAP18, which is believed to take a substantial part in the pathogenesis of psoriasis.

hCAP18 is encoded by the CAMP gene and is an inactive precursor protein with the approximate mass of 18 kDa. Several cell types in humans produce hCAP18, including neutrophils, NK cells and mast cells, as well as epithelial cells in skin, lung, gut, mammary gland and epididymis. All AMPs need to be cleaved by proteases from their precursor proteins to become active and efficient peptides. hCAP18 is processed to release the active antimicrobial peptide of 37 amino acids beginning with two leucines named LL37. LL37 has a rapid, potent and broad spectrum of antimicrobial activity when released from the C-terminus of hCAP18 (Lai & Gallo, 2009).

The presence of LL37 in normal non-inflamed skin is negligible. It has been shown to be dependent on inflammatory conditions, upregulated by keratinocytes during inflammatory disorders (Kim et al., 2005), and it is overexpressed in psoriatic plaques but not in atopic dermatitis. Both diseases are common inflammatory diseases with defective skin barriers, but have different disease characteristics (Fuentes-Duculan et al., 2017; Ong et al.,

2002). Serum levels of LL37 are higher in psoriasis patients than in healthy individuals. Furthermore, the interesting observation was made by Kanda *et al.* that treatment with cyclosporin A, a treatment that inhibits T cell activation and has been shown to revoke T cell influx into skin, also reduces serum levels of LL37 (Kanda et al., 2010). Only recently, LL37 was identified as one of the autoantigens in psoriasis (Fuentes-Duculan et al., 2017; Lande et al., 2014). Lande *et al.* reported that two thirds of patients with moderate to severe plaque psoriasis have $CD4^+$ and/or $CD8^+$ T cells specific for LL37 and that those LL37 specific T cells produce pathogenic cytokines, including IL-17 (Lande et al., 2014).

1.3.1.5 Available treatment options

No cure has yet been found for psoriasis and therefore only symptomatic treatment is available. Topical therapy, UVB phototherapy and systemic therapy are widely used to treat psoriasis and often a combination of all three is used.

With increasing understanding of the immunopathogenesis of psoriasis over the last years, new biological medications that target key players in the pathogenesis of the disease have been developed. These include T cell modulating agents (alfacept and efalizumab), TNF-α inhibitors (adalimumab, etanercept and infliximab), IL-12 and IL-23 inhibitors (ustekinumab) and the newest and most promising, i.e. IL-17 antagonists (secukinumab, ixekizumab and brodalumab) (Weger, 2010; Wu et al., 2017). These treatments are administered to patients with the most severe form of psoriasis, however, further discussion about these clinical resources are beyond the scope of this thesis.

2 Aims

Psoriasis is a disease which seriously affects patients' quality of life. Clinical studies, discussed in previous chapters, have shown beneficial effects of bathing in the Blue Lagoon on psoriasis. Many contributing factors are likely to be at play, e.g. salinity, heat, relaxation, silica and more. However, it remains to be determined whether the organisms living in the Blue Lagoon secrete a compound or compounds that may contribute to the beneficial effects of bathing in the Blue Lagoon. Therefore, the general aim of the study was to determine whether the exopolysaccharide secreted by *C. aponinum*, the dominating member of the microbial environment of the Blue Lagoon, modulates *in vitro* the immune response of cells known to participate in the pathogenesis of psoriasis, and may, therefore, contribute to the clinical betterment observed in psoriasis patients after bathing in the Lagoon.

2.1 Specific aims

The specific aims were to determine the effects of the exopolysaccharide from the *C. aponinum* (EPS-Ca) from the Blue Lagoon in Iceland on:

1. Maturation of human dendritic cells and their ability to activate allogeneic $CD4^+$ T cells (Paper I, Paper II and unpublished data)
2. Activation/function of polyclonally stimulated T cells (Paper II and unpublished data)
3. Function of keratinocytes stimulated with Th1 and Th17 mimicking environment (Paper II and unpublished data)
4. Intracellular signaling in DCs, T cells, and keratinocytes (Paper II and unpublished data)

3 Materials and methods

3.1 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 a small amount of distilled water, dialyzed for 4 days (Spectra/Por dialysis membrane with 3500 kDa cut-off, Spectrum Laboratories, CA), filtrated and lyophilized again. The exopolysaccharide obtained was named EPS-Ca with reference to the species they originate from.

3.2 Monosaccharide analysis and molecular weight of EPS-Ca

An EPS-Ca sample was analyzed with regard to monosaccharide composition and molecular weight at Prof. Berit Smestad Paulsen's laboratory at the University of Oslo, Norway. The monosaccharide composition of the sample was analyzed by capillary gas chromatography on a Carla Erba 600 Vega Series 2 chromatograph with an ICU 600 programmer as previously described (Barsett & Smestad Paulsen, 1992; Reinhold, 1972). The mean Mr of EPS-Ca was determined by high pressure-gel permeation chromatography (HP-GPC) on a Superose 6 HR 10/30 column (GE Healthcare, Amersham, UK) 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, San Jose, USA). The sample was injected on the column in a 1% solution in the mobile phase. For the Mr-estimation, calibration was performed using dextrans of known Mr (T10, T40, T70, T500 and T2000, GE Healthcare).

3.3 Cell isolation

Heparinized buffy coat from healthy donors was obtained from the Blood bank at Landspitali. All donors signed an informed consent and the study approved by the National Bioethics Committee (06-068). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll–Histopaque (Sigma-Aldrich, St. Louis, MO, USA) at room

temperature (RT) for 30 min.

Monocytes, $CD4^+$ and $CD8^+$ T cells were isolated from the PBMCs using CD14, CD4 and CD8 Microbeads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

3.4 Maturation of dendritic cells

Freshly isolated $CD14^+$ monocytes were cultured at 0.5×10^6 cells/ml for 7 days 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 bottomed tissue culture plates (Nunc, Roskilde, Denmark). The monocytes were differentiated into immature DCs by culturing them with 12.5 ng/ml IL-4 and 25 ng/ml GM-CSF (both from R&D Systems, Bio-Techne, Abington, UK). At day 7, the immature DCs were cultured at 1.25×10^6 cells/ml in 48-well flat bottomed tissue culture plates for 24 h and maturation induced by stimulating them with 10 ng/ml IL-1β, 50 ng/ml TNF-α (both from R&D Systems) and 500 ng/ml LPS (Sigma-Aldrich), in the presence or absence of EPS-Ca (100 µg/ml). The effect of EPS-Ca on maturation of the DCs was analyzed by measuring expression of surface and intracellular molecules by flow cytometry and cytokine secretion by enzyme-linked immunosorbent assay (ELISA).

In some experiments the DCs were left unstimulated and cultured without stimulation in the absence or presence of EPS-Ca at 100 µg/ml. In other experiments, several concentrations of EPS-Ca were used, i.e. 1, 10 and 100 µg/ml. For data presented in paper I DCs were maturated for 48 h. However, later it was determined that 24 h gave similar results and 24 h was used for maturation of DCs for data presented in paper II. For measurements of active intracellular signaling molecules, the DCs were maturated for 5, 10, 15 and 30 min.

DC viability was assessed by trypan blue staining. There was no difference in viability between DCs treated with EPS-Ca and DCs not treated with EPS-Ca.

3.5 Co-culture of mature DCs and allogeneic $CD4^+$ T cells

In order to analyze the effect of DCs matured in the presence of EPS-Ca on stimulation of allogeneic $CD4^+$ T cells, the DCs were matured in the presence or absence of EPS-Ca at 100 µg/ml for 48 h and subsequently cultured for 6 days in the presence of allogeneic $CD4^+$ T cells. Prior to co-culturing them with T cells, matured DCs were harvested and washed to remove any EPS-

Ca and cytokines and then transferred into a 96-well round-bottomed tissue culture plates at 2.5 × 10^5 cells/ml. Freshly isolated $CD4^+$T cells at 2 × 10^6 cells/ml 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 10% FBS and 1% penicillin/streptomycin. For comparison, $CD4^+$ T cells and DCs were cultured alone. The effect of the co-culture on T cell and DC activation was analyzed by measuring proliferation by ^{3}H thymidine uptake, expression of surface and intracellular molecules by flow cytometry and cytokine secretion by ELISA. The DC model and the following co-culture is described in figure 5.

3.5.1 Proliferation of allogeneic $CD4^+$ T cells co-cultured with mature DCs

The number of $CD4^+$ T cells was kept constant at 2 x 10^5 cells/well but the number of DCs was 0.42 x 10^4 cells/well (DC:T cell ratio of 1:48), 0.14 x 10^4 cells/well (DC:T cell ratio of 1:143), 0.046 x 10^4 cells/well (DC:T cell ratio of 1:428) and 0.015 x 10^4 cells/well (DC:T cell ratio of 1:1286). T cells alone were cultured at 2 x 10^5 cells/well and DCs alone at 2.5 x 10^4 cells/well in RMPI culture medium. The plates were incubated at 37°C in 5% CO_2 and 95% humidity for 6 days (culture days 9-15).

To determine proliferation of the T cells, 0.5 µCi ^{3}H-thymidine (PerkinElmer, Boston MA, USA) was added to each well on day 14 and the cells further incubated for 16 hours. On day 15 the cells were harvested on filter paper using a cell harvester (FilterMate Universal Harvester, PerkinElmer, Shelton, CT, USA). The filter paper was placed in a counting plate and 30 µl of MicroScint-O cocktail (PerkinElmer) was added to each well. The radioactivity bound to the filters was determined using a scintillation counter (TopCount NXMT^{TM}, Packard, PerkinElmer) and data presented as mean counts per minute (cpm).

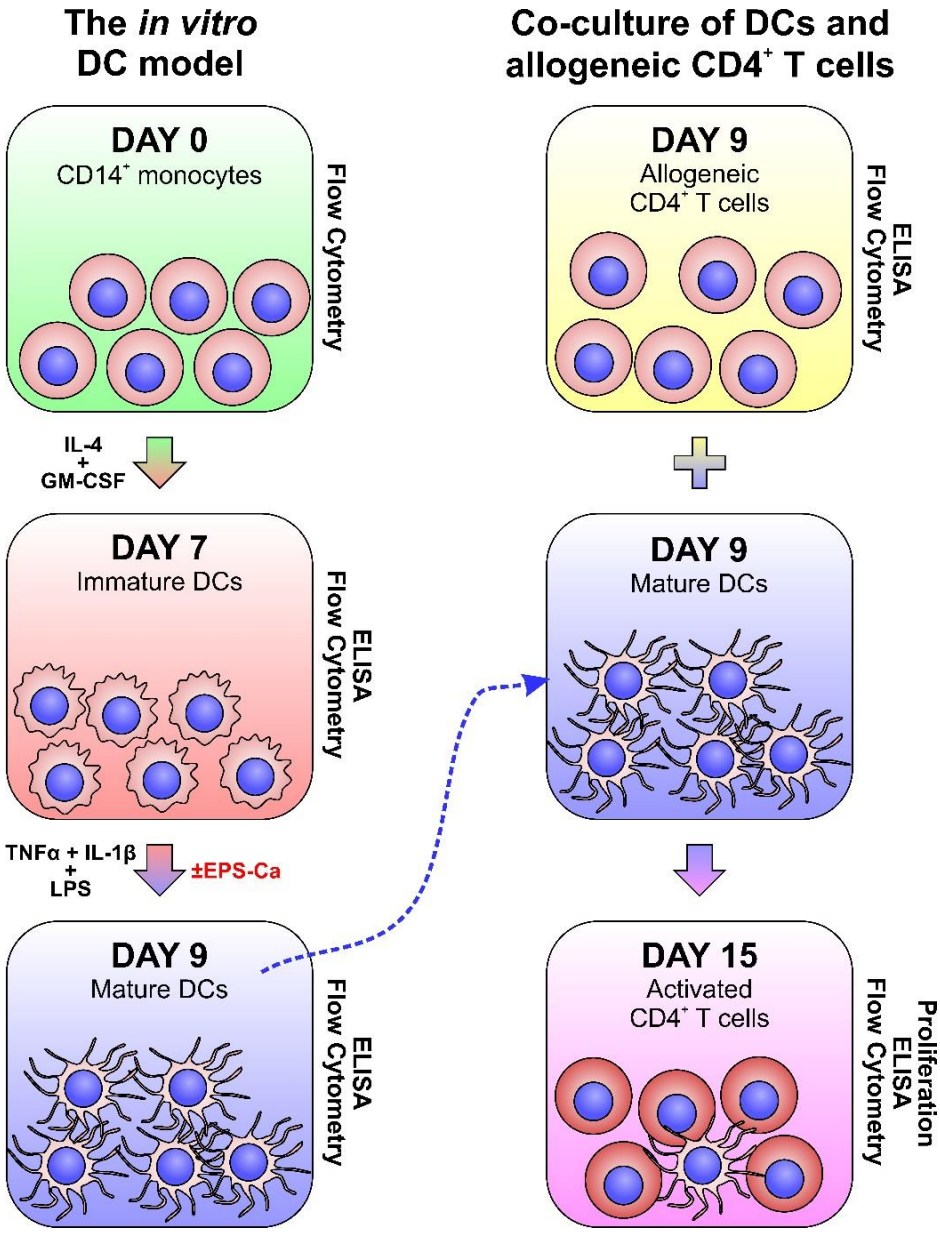

Figure 5. The DC model.

A schematic overview of the DC model and the following co-culture of DCs and allogeneic naïve T cells.

3.6 Stimulation of T cells

Freshly isolated T cells at 1 × 10^6 cells/ml 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-bottomed tissue culture plates (Nunc). The effect of EPS-Ca on T cell activation was analyzed by measuring expression of surface and intracellular molecules by flow cytometry and cytokine secretion by ELISA.

For measurements of active intracellular signaling molecules, the cells were only stimulated in the presence or absence of EPS-Ca for 5, 10, 15 and 30 min.

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 those not treated with EPS-Ca.

3.7 Stimulation of 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 bottomed tissue culture plates 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). Unstimulated keratinocytes were used as a control to confirm the upregulation of measured molecules by the stimulations before the effects of EPS-Ca treatment was determined.

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.

3.8 Expression of intra- and extracellular molecules

For staining of surface 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 (see detailed list in

appendix, table 3) 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 a FACSCalibur (BD Bioscience, San Jose, CA, USA) or Navios (Beckman Coulter, Atlanta, GA, USA) and analyzed using 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 divided by the percentage of positive cells or mean fluorescence intensity (MFI) of cells not treated with EPS-Ca.

For intracellular staining for IL-10, the cells were incubated with Brefeldin A (BioLegend, San Diego, CA, USA) for the last 10 h of the culture in order to stop golgi-mediated exocytosis. The cells were then fixed using 4% paraformaldehyde for 20 min at RT, permeabilized with saponin buffer (0.5% saponin, 0.5% BSA in PBS) and incubated with anti-IL-10-Alexa-488 (eBioscience, Thermo Fisher Scientific) antibody for 20 min on ice and finally washed and resuspended in staining buffer.

For staining of phosphorylated intracellular signaling molecules (phospho-flow staining), freshly isolated T cells or imDCs were rested for 1 h and then stimulated for the indicated times (5, 10, 15, 30 min). The cells were subsequently fixed with 3.7% paraformaldehyde for 10 min at RT and then permeabilized with ice cold 95% methanol for 30 min in a -20°C freezer. The cells were washed and resuspended in staining buffer. Next, nonspecific binding of antibodies to the cells was blocked for 10 min at RT with 2% NHS/NMS. Cells were then incubated with APC-labeled antibodies against pSYK or pSYK/ZAP70 (both from eBioscience, Thermo Fisher Scientific) for 30 min at RT. Finally, cells were washed and resuspended in staining buffer.

3.9 Immunofluorescence staining and confocal imaging

Surplus DCs stained for 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. Unstained binding sites were blocked using 10% FBS and the cells incubated with monoclonal antibody against LL37 (Innovagen AB, Lund, Sweden) overnight at 4°C. The slides were then stained with isotype specific goat anti-mouse antibodies labeled with Alexa Fluor 488 (Life Technologies, Thermo Fisher Scientific) and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and phalloidin

(Thermo Fisher Scientific). The slides were embedded in fluoromount (Sigma-Aldrich) and viewed in an Olympus Fluoview FV1200 confocal microscope (Olympus, Tokyo, Japan).

3.10 Concentration of cytokines

Cytokine concentration in cell culture supernatants was determined by ELISA using DuoSet® (R&D Systems) according to the manufacturer's protocol. The cytokines IL-10, IL-12p40, IL-17, IL-22 and IFN-γ were measured in supernatants from DCs and the co-culture of DCs and allogeneic T cells, 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 (secretion index, SI) of the cytokine concentration (pg/ml) in the supernatants from cells treated with EPS-Ca to the cytokine concentration (pg/ml) in the supernatants from cells not treated with EPS-Ca.

3.11 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) (see detailed list in appendix, table 4) acquired from Integrated DNA Technologies (Skokie, IL, USA) and each sample run in triplicate. The level of CLEC7A, SYK, ZAP70 and CAMP were estimated and normalized to the levels of the housekeeping gene glyceraldehyde 3-phosphate (GAPDH).

3.12 Statistical analysis

Results are presented as mean + standard error of the mean (SEM) unless stated otherwise. Differences between groups were assessed with Student's two tailed t-test if two groups were compared or with one-way ANOVA if more than two groups were compared. Differences were considered statistically significant if P-values were lower than 0.05.

4 Results

4.1 Molecular weight and monosaccharide composition analysis of EPS-Ca

An EPS-Ca sample was analyzed with regard to monosaccharide composition and molecular weight at Berit Smestad Paulsen's laboratory at the University of Oslo, Norway. The monosaccharide composition of the exopolysaccharide is presented in Table 1.

Table 1. Monosaccharide composition of EPS-Ca presented as mol% of total carbohydrate content.

	(mol%)
Galacturonic acid	24.0
Fucose	23.8
3-*O*-Methyl-Galactose	16.7
Glucose	15.8
Arabinose	10.3
Galactose	3.8
Mannose	2.6
Rhamnose	2.3
4-*O*-Methyl-Glucuronic acid	0.6

The mean molecular weight (Mr) of EPS-Ca was estimated, by comparison to dextran standards, to be 1060 kDa.

4.2 The DC model

Paper I, paper II and unpublished data

4.2.1 The effects of EPS-Ca on cytokine secretion by DCs

Once EPS-Ca had been isolated and characterized our first step was to screen for immunomodulatory activity using the DC model. The DCs were matured in the absence or presence of EPS-Ca at 1, 10 or 100 µg/ml. DCs matured in the presence of the highest concentration of EPS-Ca secreted more IL-10 than DCs matured without EPS-Ca (figure 6). However, maturing DCs in the presence of EPS-Ca had no effect on IL-12p40 secretion (figure 6).

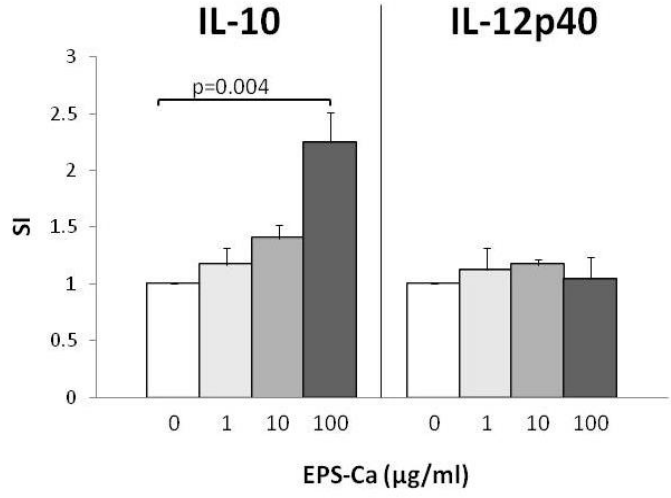

Figure 6. The effects of EPS-Ca on cytokine secretion by DCs.

DCs were matured with IL-1β, TNF-α and LPS for 48 h in the presence of 0, 1, 10 or 100 µg/ml of EPS-Ca. The concentration of IL-10 and IL-12p40 in the supernatant was measured by ELISA. The results are expressed as secretion index (SI), which is the ratio between the concentration of cytokines (ng/ml) in the supernatant of DCs matured in the presence of EPS-Ca and the concentration of cytokines (ng/ml) in the supernatant of DCs matured in the absence of EPS-Ca. The absolute values for DC matured in the absence of EPS-Ca are 1.2 ng/ml for IL-10 and 7.6 ng/ml for IL-12p40. Results are shown as the mean + SEM, n=3. The p-value shows statistically significant difference from control as measured by one-way ANOVA.

4.2.2 The effects of EPS-Ca treated DCs on activation and differentiation of allogeneic $CD4^+$ T cells

The effects EPS-Ca had on cytokine secretion by DCs indicated that DCs matured in the presence of EPS-Ca might affect T cell activation and differentiation. Therefore, the next step was to analyze the effects of DCs matured in the presence of EPS-Ca on activation and differentiation of allogeneic $CD4^+$ T cells. This was done by measuring by flow cytometry, the expression of RORγt, the key transcription factor of Th17 cells, and their signature cytokine IL-17 as well as FoxP3, the transcription factor of Tregs, and their key cytokine IL-10. As shown in figure 7a, the proportion of $CD4^+$ T cells expressing RORγt and IL-17 was lower when the T cells had been co-cultured with DCs that had been matured in the presence of EPS-Ca (DC-EPS-Ca) compared with when the T cells had been co-cultured with DCs that had been matured in the absence of EPS-Ca (DC-C). Conversely, there was a tendency towards a higher percentage of $FoxP3^+IL\text{-}10^+$ $CD4^+$ T cells when the DCs had been matured in the presence of EPS-Ca compared with when the DCs had been matured in the absence of EPS-Ca. The ratio between the

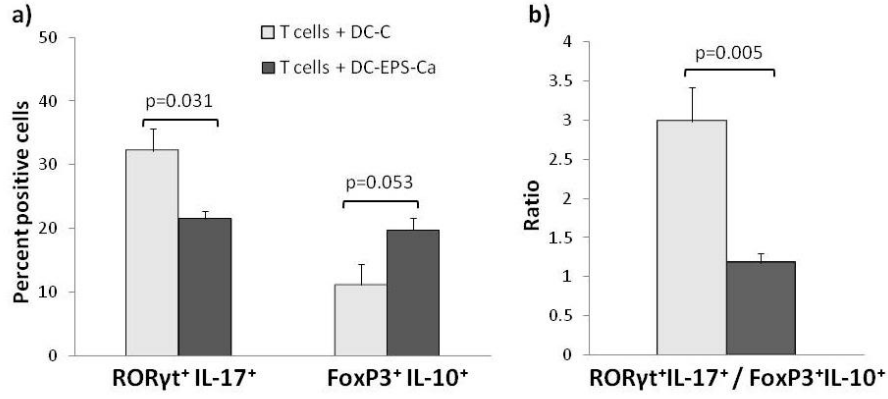

Figure 7. The effects of EPS-Ca-treated DCs on Th17 and Treg phenotypes of allogeneic $CD4^+$ T cells.

DCs were matured with IL-1β, TNF-α and LPS for 48 h in the absence (DC-C) or presence of 100 µg/ml EPS-Ca (DC-EPS-Ca) and then co-cultured with allogeneic $CD4^+$ T cells for 6 days. The effects of the co-culture on the percentage of Th17 ($ROR\gamma t^+IL\text{-}17^+$) and Treg ($FoxP3^+IL\text{-}10^+$) phenotypes was measured by flow cytometry and expressed as (a) percent positive cells or (b) the ratio between the two phenotypes $ROR\gamma t^+IL\text{-}17^+$ and $FoxP3^+IL\text{-}10^+$. The results are shown as the mean + SEM, n=6. The p-values show statistically significant and near significant differences as measured by Student's t-test.

two phenotypes (RORγt$^+$IL-17$^+$: FoxP3$^+$IL-10$^+$) was 3:1 when the T cells were co-cultured with DCs that were matured in the absence of EPS-Ca (DC-C) compared with 1:1 when the T cells were co-cultured with DCs matured in the presence of EPS-Ca (DC-EPS-Ca) (figure 7b).

When CD4$^+$ T cells were co-cultured with DCs that had been matured in the presence of EPS-Ca (DC-EPS-Ca), IL-10 concentration in the culture supernatant was higher than it was when CD4$^+$ T cells were co-cultured with DCs matured in the absence of EPS-Ca (DC-C) (figure 8a). There was no difference in the concentration of IL-17 or IL-22 in supernatants from CD4$^+$ T cells co-cultured with DCs matured in the presence or absence of EPS-Ca. To determine whether the IL-10 in the culture supernatant was derived from the DCs only, or whether the T cells were also contributing by producing IL-10, an intracellular staining for IL-10 was combined with staining for extracellular markers to differentiate between DCs and T cells.

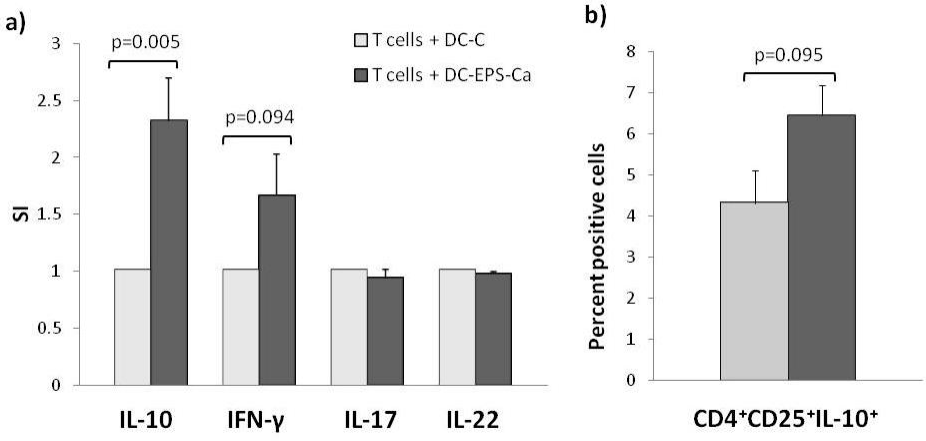

Figure 8. The effects of EPS-Ca treated DCs on cytokine secretion and expression of CD4$^+$CD25$^+$IL-10$^+$ T cells

DCs were matured with IL-1β, TNF-α and LPS for 48 h in the absence (DC-C) or presence of 100 µg/ml of EPS-Ca (DC-EPS-Ca) and then co-cultured with allogeneic CD4$^+$ T cells for 6 days. The effect of the DCs matured in the presence or absence of EPS-Ca on T cells was determined by measuring (a) concentration of IL-10, IFN-γ, IL-17 and IL-22 in the supernatants by ELISA. The results are expressed as secretion index (SI), which is the ratio between the concentration of cytokines (pg/ml) in the supernatant from the T cell and DC-C co-culture and the concentration of cytokines (pg/ml) from the supernatant from the T cell and DC-EPS-Ca co-culture, n=3-6. (b) The proportion of IL-10$^+$ cells within the CD4$^+$CD25$^+$ T cells was determined by flow cytometry with results expressed as percent positive cells, n=6. The results are shown as the mean + SEM. Statistically significant differences and near significant differences are indicated in the figure by the p-values.

As shown in figure 8b, there was a tendency towards a higher proportion of $CD4^+CD25^+IL\text{-}10^+$ T cells when the T cells had been co-cultured with DCs matured in the presence of EPS-Ca (DC-EPS-Ca) compared with that when the T cells had been co-cultured with DCs matured in the absence of EPS-Ca (DC-C). These results demonstrate that the T cells in the co-culture produce IL-10 and, therefore, contribute to the total IL-10 detected in the co-culture supernatant.

The effects of DCs matured in the presence of EPS-Ca on proliferation of the T cells were also determined. As shown in figure 9, the proliferation rate of the allogeneic $CD4^+$ T cells was not affected by co-culturing them with DCs matured in the presence of EPS-Ca (DC-EPS-Ca) as compared with co-culturing them with DCs matured in the absence of EPS-Ca (DC-C).

Next, the effect of DCs matured in the presence of EPS-Ca on the percentage of allogeneic $CD4^+$ T cells expressing the activation molecules CD40L, CD54, CD69 and CTLA-4 was determined. Expression of the activation molecules was not different between T cells co-cultured with DCs that had been matured in the presence or absence of EPS-Ca (figure 10).

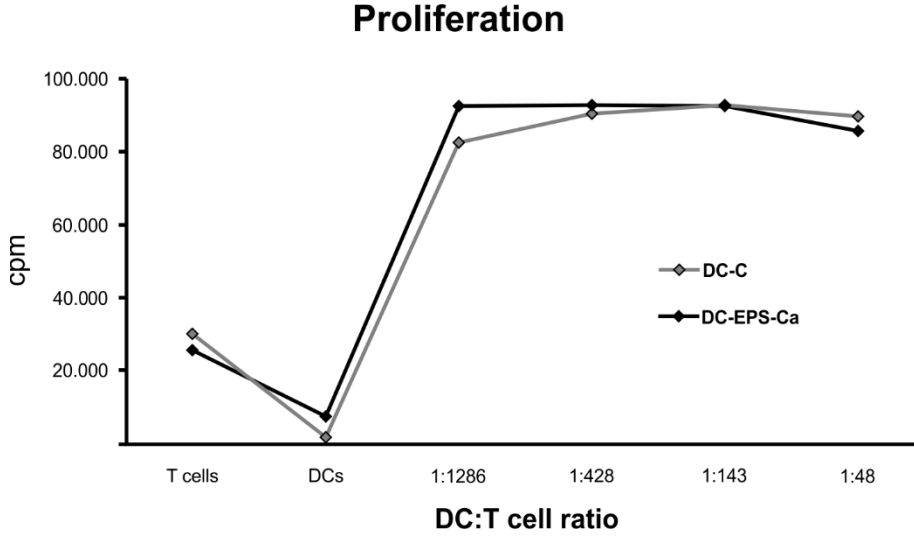

Figure 9. The effects of EPS-Ca-treated DCs on proliferation of $CD4^+$ T cells.

DCs were matured with IL-1β, TNF-α and LPS for 48 h in the absence (DC-C) or presence of 100 µg/ml EPS-Ca (DC-EPS-Ca) and then co-cultured with allogeneic CD4+ T cells for 6 days at various DC-T cell ratios, with 0.5 µCi present for the last 16 h. DCs and T cells were also cultured alone. The proliferation rate of the T cells was measured by determining their uptake of ^3H thymidine. Results are shown as counts per minute (cpm), n=3.

4.2.3 The effects of EPS-Ca on DC activation and cytokine secretion following their co-culture with allogeneic $CD4^+$ T cells

The effects of maturing DCs in the presence of EPS-Ca on their expression of surface molecules linked with antigen presentation and activation, following their co-culture with allogeneic $CD4^+$ T cells, was determined. No difference was observed in the percentage of DCs expressing HLA-DR, CD86, CD40 and PD-1L between co-cultures in which the DCs had either been matured in the presence (DC-EPS-Ca) or in the absence of EPS-Ca (DC-C) (figure 11a). However, a higher proportion of DCs expressing CD1c was observed in the co-culture where the DCs had been matured in the presence of EPS-Ca (DC-EPS-Ca) compared with the control co-culture where DCs had been matured in the absence of EPS-Ca (DC-C). Conversely, a lower percentage of DCs expressing the lymph node homing receptor, CCR7, was observed in the co-culture of DCs matured in the presence of EPS-Ca (DC-EPS-Ca) than in co-culture of DCs matured in the absence of EPS-Ca (DC-C) (figure 11a).

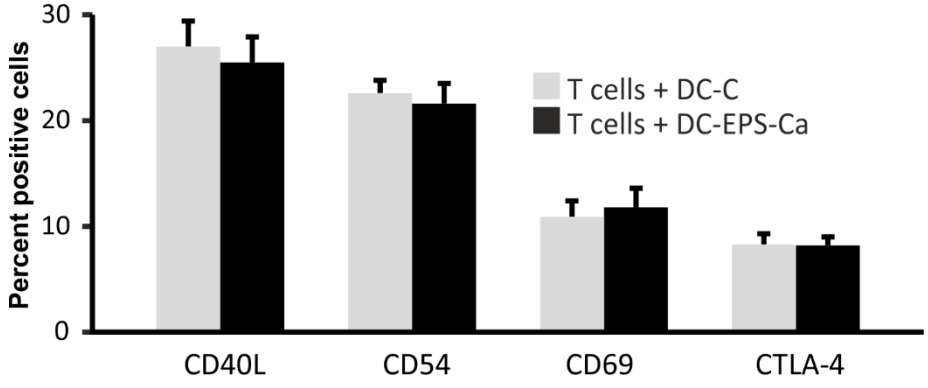

Figure 10. The effects of EPS-Ca-treated DCs on surface molecule expresson by $CD4^+$ T cells.

DCs were matured with IL-1β, TNF-α and LPS for 48 h in the absence (DC-C) or presence of 100 µg/ml of EPS-Ca (DC-EPS-Ca) and then co-cultured with allogeneic $CD4^+$ T cells for 6 days. Surface expression of CD40L, CD54, CD69 and CTLA-4 by the T cells was measured by flow cytometry and expressed as percent positive cells. The results are shown as the mean + SEM, n=6.

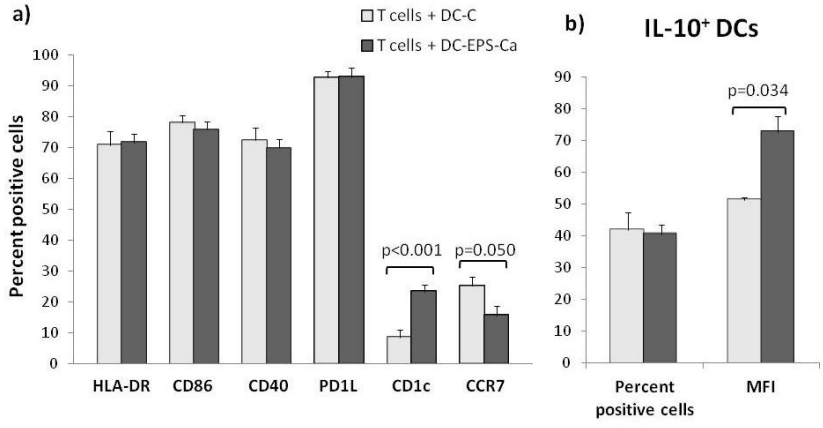

Figure 11. The effects of EPS-Ca on surface molecule expresson and IL10 expression by DCs following co-culture of allogeneic $CD4^+$ T cells and DCs.

DCs were matured with IL-1β, TNF-α and LPS for 48 h in the absence (DC-C) or presence of 100 µg/ml of EPS-Ca (DC-EPS-Ca) and then co-cultured with allogeneic $CD4^+$ T cells for 6 days. The effects of the co-culture on (a) Surface expression of HLA-DR, CD86, CD40, PD1L, CD1c and CCR7 by DCs as measured by flow cytometry and expressed as percent positive cells, n=3-6. (b) Proportion of $IL\text{-}10^+$ DCs and mean fluorecence intensity (MFI) of the $IL\text{-}10^+$ positive DCs, n=6. The results are shown as the mean + SEM. Statistically significant differences and near significant differences are indicated in the figure by the p-values.

Intracellular staining of IL-10 in DCs following co-culture with allogeneic $CD4^+$ T cells showed no difference in the proportion of DCs producing IL-10 between DCs matured in the presence (DC-EPS-Ca) or in the absence of EPS-Ca (DC-C) (figure 11b). In contrast, DCs matured in the presence of EPS-Ca (DC-EPS-Ca) had higher expression levels (MFI) of IL-10 than DCs matured in the absence of EPS-Ca (DC-C). Thus, although the same percentage of DCs from the two co-cultures produced IL-10, the DCs that had been matured in the presence of EPS-Ca (DC-EPS-Ca) produce higher levels of IL-10 than DCs that had been matured in the absence of EPS-Ca (DC-C).

4.2.4 The effects of EPS-Ca on CD141 expression by DCs

The findings that maturing DCs in the presence of EPS-Ca a) increased their production of IL-10 and b) enhanced their ability to induce differentiation of Tregs, led to our interest in examining the effects of EPS-Ca on DC expression of CD141, a surface marker that has been linked to regulatory DCs. In line with the previous results, a higher percentage of DCs expressed CD141 when the DCs had been matured in the presence of EPS-Ca compared with that when the DCs had been matured in the absence of EPS-

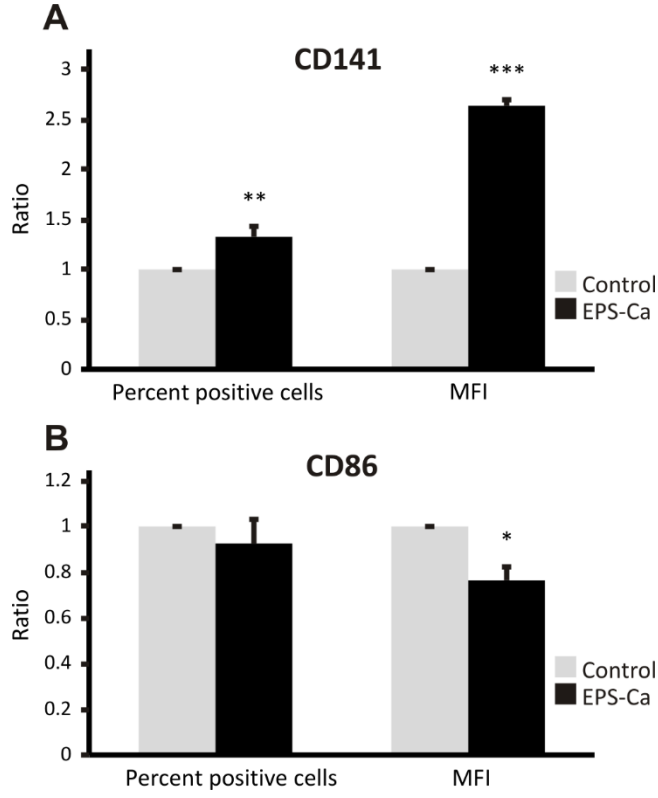

Figure 12. The effects of EPS-Ca on CD141 and CD86 expression by DCs.

DCs were matured with IL-1β, TNF-α and LPS for 24 h in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca). The effects of EPS-Ca on expression of the surface molecules (A) CD141 and (B) CD86 was measured by flow cytometry. Results are expressed as ratio to the control (percent positve cells or mean fluorescence intensity (MFI)). The absolute values for controls are 61% for CD141 and 97% for CD86; and MFI 28 for CD141 and 260 for CD86. The results are shown as mean + SEM, n=7. The stars show statistically significant differences from control as measured by Student's t-test. *p<0.05, **p<0.01, ***p<0.001.

Ca (figure 12A). In addition, DCs matured in the presence of EPS-Ca expressed higher levels of CD141 as indicated by the higher MFI (figure 12A) than DCs matured in the absence of EPS-Ca. Interestingly, maturation of DCs in the presence of EPS-Ca did not affect the proportion of them expressing the co-stimulatory molecule CD86 but slightly decreased the expression levels (MFI) of CD86 on DCs, compared with DCs matured in the absence of EPS-Ca (figure 12B). These results indicated that treatment with EPS-Ca leads to a higher percentage of DCs having a regulatory phenotype with little effect on the expression of co-stimulatory molecules like CD86.

In order to better understand the nature of the DCs following EPS-Ca treatment, the connection between CD141 expression and IL-10 secretion was examined. The majority of the IL-10 secreting cells also expressed CD141, regardless of whether they were matured in the presence or absence of EPS-Ca (figure 13B), indicating that IL-10 production was linked to the regulatory DC phenotype. The proportion of $CD141^{+}IL\text{-}10^{+}$ DCs was higher in DCs that had been matured in the presence of EPS-Ca than in DCs matured in the absence of EPS-Ca (figure 13A).

Confocal imaging of the cells stained from flow cytometry further confirmed the increase in IL-10 production and CD141 expression, as seen in figure 14.

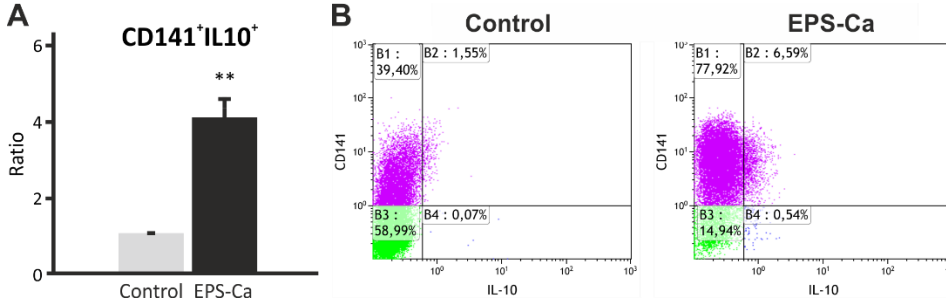

Figure 13. The effects of EPS-Ca on CD141 and IL-10 co-expression by DCs.

DCs were matured with IL-1β, TNF-α and LPS for 24 h in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca) and stained for CD141 and intracellular IL-10. DC expression of CD141 and IL-10 was measured by flow cytometry and expressed as (A) a ratio to the control (percent positive cells). 1.63% of control DCs were $CD141^{+}IL\text{-}10^{+}$. (B) Shows representative flow cytometry dot plots. The results are shown as mean + SEM, n=3. The stars show statistically significant difference from control as measured by Student's t-test. **p<0.01.

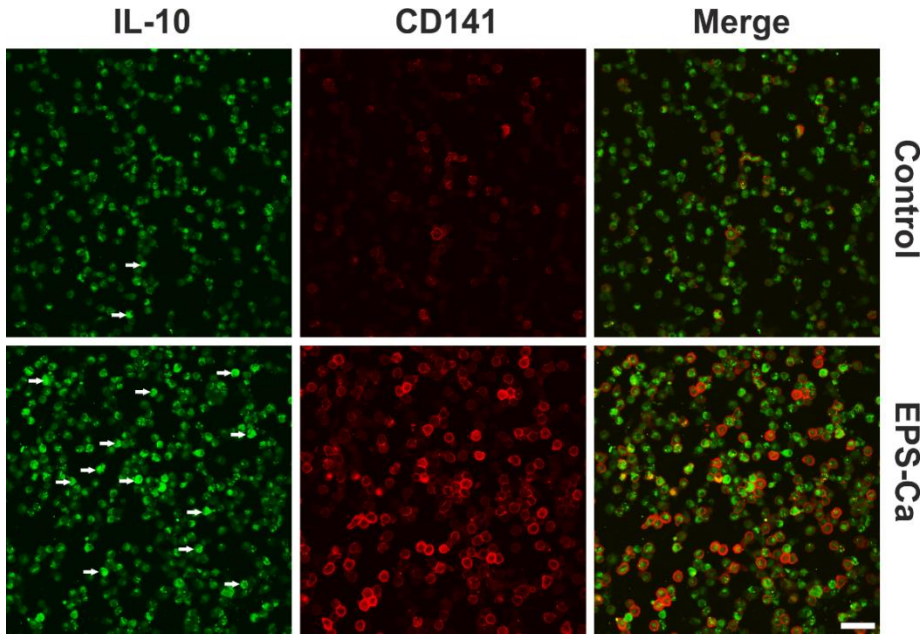

Figure 14. Confocal microscopy images of the effects of EPS-Ca on IL-10 and CD141 expression by DCs.

DCs were matured with IL-1β, TNF-α and LPS for 24 h in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca) and stained for intracellular IL-10 (green) and CD141 (red). Arrows point at several IL-10^{+} cells. Images were taken under 30 x magnification. Scale bar, 50 µm.

4.2.5 EPS-Ca inhibits SYK expression in DCs

A recent study indicated that downregulation of the Syk signaling pathway is an important mechanism to promote regulatory DCs in mice (Hang et al., 2016). Therefore, we determined the levels of phosphorylated SYK (pSYK) in DCs matured in the presence or absence of EPS-Ca. A lower proportion of DCs expressed pSYK, at the 30 min time point, when the DCs had been matured in the presence of EPS-Ca compared with DCs that had been matured in the absence of EPS-Ca (figure 15A). 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 the protein production on mRNA level, imDCs were treated with or without EPS-Ca for 2 or 24 h and the mRNA expression for SYK was analyzed using quantitative RT-PCR. As figure 15B reveals, SYK mRNA expression was substantially lower in EPS-Ca treated cells after 2 h compared with untreated

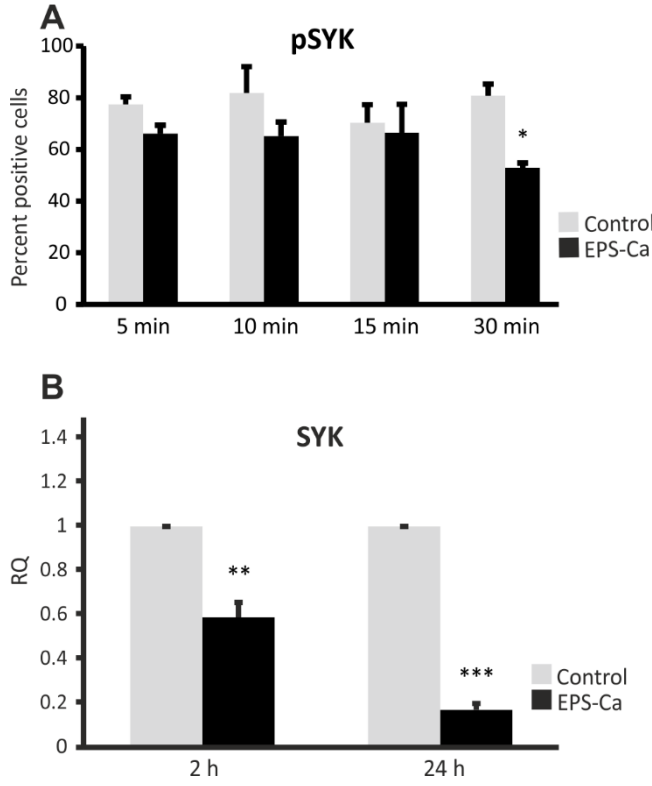

Figure 15. The effects of EPS-Ca on the phosphorylation of SYK by DCs and the mRNA expression of its encoding gene, SYK.

(A) DCs were matured with IL-1β, TNF-α and LPS for 5, 10, 15 or 30 min in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca). Expression of phosphorylated SYK was measured by flow cytometry and expressed as percent positive cells, n=3. (B) mRNA expression of SYK by DCs after 2 h or 24 h incubation in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca), assesed by RT-PCR and normalized to the houskeeping gene glyceraldehyde 3-phosphate (GAPDH), n=3. The results are shown as the mean + SEM. The stars show statistically significant differences from control as measured by Student's t-test. *p<0.05, **p<0.01, ***p<0.001.

DCs. The difference in SYK mRNA expression was even greater after 24 h (figure 15B). These results demonstrate that EPS-Ca not only decreased expression of phosphorylated SYK but also mRNA expression of SYK.

4.2.6 The effects of EPS-Ca on surface expression of Dectin-1 by DCs and mRNA expression of the encoding gene CLEC7A

In order to further investigate the effects of EPS-Ca on the Syk signaling pathway, its effects on expression of Dectin-1, one of the surface receptors that trigger SYK expression, was investigated. A lower proportion of DCs matured in the presence of EPS-Ca expressed Dectin-1 on their surface than of DCs matured in the absence of EPS-Ca (figure 16A). Furthermore, the EPS-Ca-treated DCs also expressed lower levels of Dectin-1 than DCs not treated with EPS-Ca (indicated by lower MFI) (figure 16A).

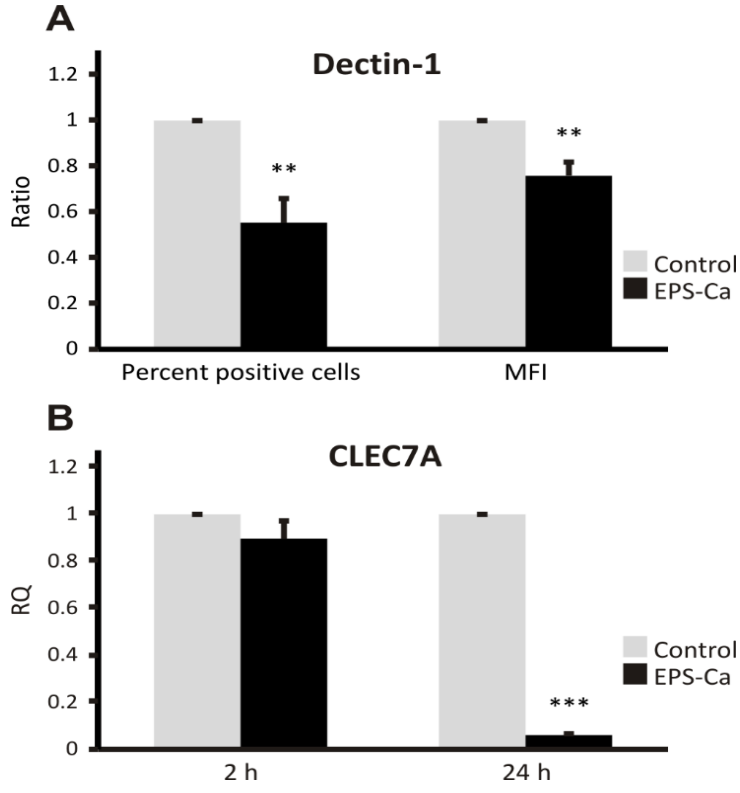

Figure 16. The effects of EPS-Ca on surface expression of Dectin-1 by DCs and mRNA expression of the encoding gene CLEC7A.

(A) DCs were matured with IL-1β, TNF-α and LPS for 24 h in the absence (control) or presence of 100 µg/ml EPS-Ca (EPS-Ca). Dectin-1 expression was measured by flow cytometry and expressed as ratios to the control (percent positve cells or mean fluorescence intensity (MFI)), n=7. 49% of control DCs were positive for Dectin-1 and their MFI was 97. (B) mRNA expression of CLEC7A by DCs after 2 h or 24 h incubation in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca), assessed by RT-PCR and normalized to the houskeeping gene glyceraldehyde 3-phosphate (GAPDH), n=3. The results are shown as the mean + SEM. The stars show statistically significant differences from control as measured by Student‘s t-test. **p<0.01, ***p<0.001.

To determine whether downregulated surface expression of the Dectin-1 receptor by EPS-Ca could be caused by a decrease in its production, the effects of EPS-Ca on mRNA expression of CLEC7A, the encoding gene for Dectin-1, were determined. DCs were treated with or without EPS-Ca for 2 h or 24 h and the CLEC7A mRNA expression measured using quantitative RT-PCR. CLEC7A mRNA expression was minimally affected in EPS-Ca-treated DCs compared with non-treated DCs after 2 h (figure 16B). However, following 24 h treatment with EPS-Ca the CLEC7A mRNA expression was almost completely inhibited (figure 16B). These results demonstrate that EPS-Ca inhibits the mRNA expression of CLEC7A, which in turn results in downregulation of the surface expression of Dectin-1 on DCs.

When the effects of maturing DCs in the presence of EPS-Ca on their expression of CD141 and Dectin-1 became apparent, we wanted to analyze the effects of treating imDCs with EPS-Ca without maturing them simultaneously, i.e. in the absence of LPS, IL-1β and TNF-α, on their CD141 and Dectin-1 expression. Table 2 shows the effects of EPS-Ca on expression of CD141, Dectin-1 and CD86 in imDCs that were left immature (unmaturated) but treated with or without EPS-Ca and in imDCs that are matured in the absence or presence of EPS-Ca. The results shown in table 2 originate from DCs from one donor, DCs from other donors followed the same pattern (data not shown). Interestingly, EPS-Ca decreased the percentage of cells expressing Dectin-1 and increased the percentage of cells expressing CD141 regardless of whether the imDCs were treated with maturation factors (LPS, IL-1β and TNF-α) or not. Surprisingly, EPS-Ca seemed to be fully capable of maturating the DCs without the maturation factors being present. The maturation factors seem to have a greater effect on MFI levels than percent positive cells although the level of the effect is different for each surface molecule tested. Therefore, when determining the effects of EPS-Ca on the expressions of mRNA for CLEC7A and SYK no maturation factors were added to the cultures in order to minimize the variables at play (figures 15B and 16B).

Table 2. Comparison of the effects of EPS-Ca (100 µg/ml) on surface molecule expression on matured and unmatured DCs. Data from one donor.

% positive cells	**CD86**	**Dectin-1**	**CD141**
Unmatured DCs	41.7	62.0	52.0
Unmatured DCs + EPS-Ca	96.8	29.4	71.6
Matured DCs	99.7	60.9	47.5
Matured DCs + EPS-Ca	98.9	51.7	66.2

MFI	**CD86**	**Dectin-1**	**CD141**
Unmatured DCs	48.0	92.4	14.4
Unmatured DCs + EPS-Ca	96.8	93.8	54.6
Matured DCs	374.0	131.3	18.6
Matured DCs + EPS-Ca	411.4	116.2	36.5

4.3 T cells

Paper II and unpublished data

The experiments where the EPS-Ca-treated DCs were co-cultured with allogeneic $CD4^+$ T cells had shown that EPS-Ca-treated DCs were capable to affect the differentiation of T cells, without interfering in their activation status (see chapter 4.2.2). This lead to the question of whether EPS-Ca could have direct effects on activation and differentiation of T cells. In an attempt to resemble the situation of T cells in skin of psoriatic patients with an active disease, the T cells were stimulated prior to treating them with EPS-Ca and the effects of EPS-Ca on expression of surface markers and the cytokine secretion by already activated T cells were investigated. First, a time curve for cytokine secretion of T cells stimulated for 24, 48, 72 and 96 h was established. The cytokine levels were highest in the supernatants when the T cells had been stimulated for 48 and 72 h and had diminished slightly at 96 h (data not shown). Therefore, in the following experiments the T cells were stimulated for 72 h with EPS-Ca being absent or present during the last 24 h.

4.3.1 The effects of EPS-Ca on cytokine secretion and surface expression of CD69 by stimulated T cells

In order to investigate the hallmark cytokines for Th1, Th2, Th17 and Treg cells, the concentration of IFN-γ, IL-13, IL-17 and IL-10, respectively, was

measured in the supernatants by ELISA. As can be seen in figure 17, a marked reduction was observed in the concentration of IL-10, IL-17 and IL-13 in the supernatants of EPS-Ca-treated $CD4^+$ T cells compared with untreated $CD4^+$ cells. No difference was observed in the concentration of IFN-γ (data not shown). When $CD8^+$ T cells were stimulated in the absence of $CD4^+$ T cells cytokine concentration in the supernatants was very low. When both $CD4^+$ T cells and $CD8^+$ T cells were stimulated together, similar results were obtained as obtained for $CD4^+$ T cells alone (data not shown).

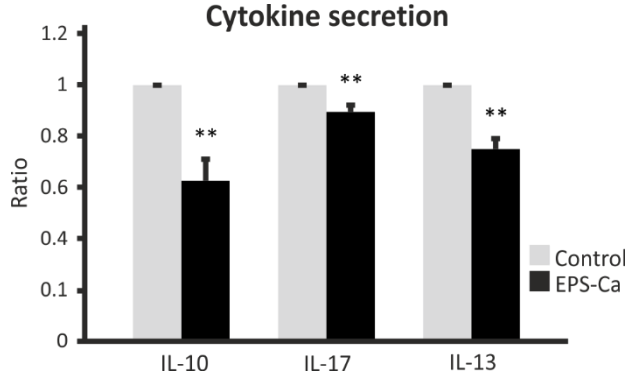

Figure 17. The effects of EPS-Ca on cytokine secretion by anti-CD3/CD28 stimulated $CD4^+$ T cells.

$CD4^+$ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 72 h in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca) for the last 24 h of the culture. The concentration of IL-10, IL-17 and IL-13 in the supernatant was measured by ELISA and results expressed as ratio to the control. The absolute values for the controls are 19.3 ng/ml for IL-10, 5338 pg/ml for IL-17 and 952 pg/ml for IL-13.The results are shown as the mean + SEM, n=6. The stars show statistically significant differences from control as measured by Student's t-test. **p<0.01.

A significant reduction in the percentage of both $CD4^+$ and $CD8^+$ T cells expressing CD69 on their surface was observed when they were treated with EPS-Ca as compared with that by untreated T cells (figure 18), indicating a lower activation status of the EPS-Ca-treated T cells. EPS-Ca did not affect expression of the adhesion molecules ICAM-1 and VLA4 on activated T cells (data not shown).

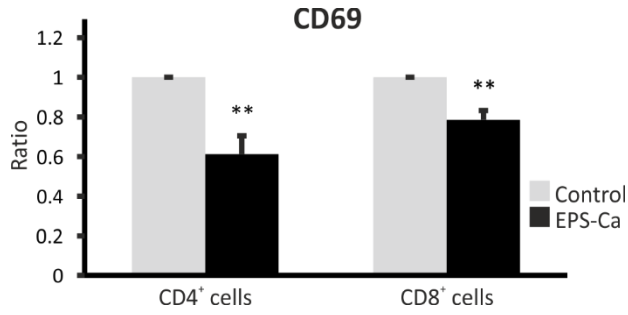

Figure 18. The effects of EPS-Ca on surface expression of CD69 by antiCD3/CD28 stimulated $CD4^+$ and $CD8^+$ T cells.

$CD4^+$T cells (n= 6) and $CD8^+$ T cells (n=3) were stimulated with plate bound anti-CD3 and anti-CD28 antibodies for 72 h in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca) for the last 24 h of the culture. CD69 expression was measured by flow cytometry and expressed as ratios to the control (percent positive cells). The absolute values for the controls are 16% for the $CD4^+$ T cells and 49% for the $CD8^+$ T cells. The results are shown as the mean + SEM. The stars show statistically significant differences from control as measured by Student‘s t-test. **p<0.01.

4.3.2 The effects of EPS-Ca on expression of phosphorylated ZAP70 and ZAP70 mRNA expression by $CD4^+$ T cells

To investigate the potential mechanism for the lowered activation status and cytokine secretion observed for both $CD4^+$ T cells and $CD8^+$ T cells exposed to EPS-Ca, phosphorylation of ZAP70, a Syk family kinase which is crucial for signaling through the TCR, was determined. EPS-Ca reduced the proportion of activated $CD4^+$ T cells expressing pZAP70 compared with that for $CD4^+$ T cells not treated with EPS-Ca (figure 19). EPS-Ca did, however, not affect mRNA levels for ZAP70 in activated T cells (data not shown). These results are consistent with activation of T cells being downregulated following treatment with EPS-Ca and that it may be linked to downregulation of ZAP70 phosphorylation.

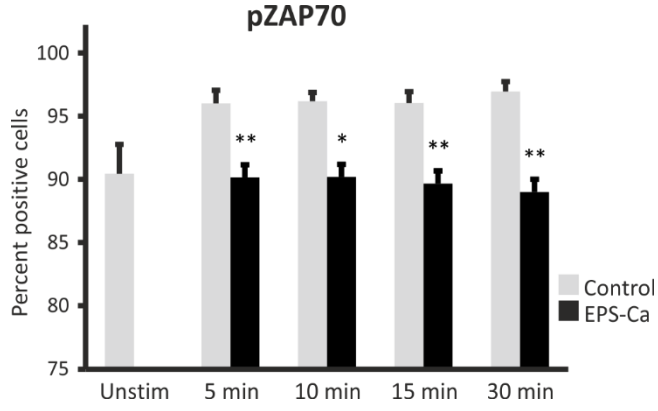

Figure 19. The effects of EPS-Ca on expression of phosphorylated ZAP70 by $CD4^+$ T cells.

$CD4^+$T cells were stimulated with plate bound anti-CD3 and anti-CD28 antibodies for 5, 10, 15 or 30 min in the absence (control) or presence of 100 µg/ml of EPS-Ca (EPS-Ca). Phosphorylated ZAP70 was measured by phospho-flow cytometry and expressed as percent positve cells. The results are shown as the mean + SEM, n=3. The stars show statistically significant differences from control as measured by Student's t-test. *p<0.05, **p<0.01.

4.4 Keratinocytes

Paper II and unpublished data

As keratinocytes are an important cell type in the immunopathogenesis of psoriasis, the effect of EPS-Ca on the stimulation of keratinocytes was investigated. The keratinocytes were stimulated with different cytokines mimicking either a Th1- or a Th17-like environment, which are the phenotypes present in psoriatic skin lesions.

4.4.1 The effects of EPS-Ca on primary keratinocytes in a Th1 mimicking environment

CXCL10 has been strongly linked to the Th1 cytokine environment and contributes greatly to chemotaxis of immune cells (Ferrari et al., 2015; Smit et al., 2003). EPS-Ca treated primary keratinocytes stimulated with the Th1 mimicking stimulation secreted less CXCL10 than keratinocytes stimulated in the absence of EPS-Ca (figure 20A). As EPS-Ca had a great effect on mRNA expression of CLEC7A in DCs and the fact that Dectin-1 is highly expressed in psoriatic epidermis (de Koning et al., 2010) it was of interest to assess the effect of EPS-Ca on CLEC7A and SYK mRNA expression in keratinocytes.

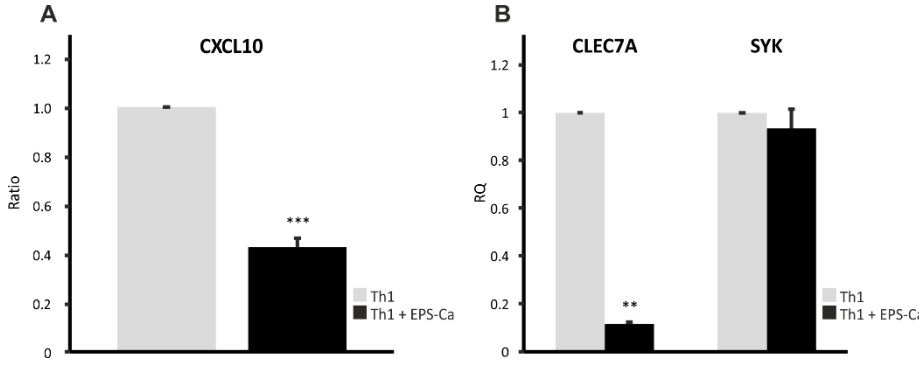

Figure 20. The effects of EPS-Ca on primary keratinocytes stimulated with Th1 mimicking environment.

Primary keratinocytes were stimulated for 24 h with IFN-γ and TNF-α in the absence (Th1) or presence of 100 µg/ml of EPS-Ca (Th1 + EPS-Ca). (A) The concentration of CXCL10 in the supernatants was measured by ELISA with results expressed as ratios to the control, n=3. The absolute value for keratinocytes cultured in the absence of EPS-Ca is 139.7 ng/ml for CXCL10 (B) mRNA expression of CLEC7A and SYK was assessed by RT-PCR and normalized to the houskeeping gene glyceraldehyde 3-phosphate (GAPDH), n=3. The results are shown as the mean + SEM. The stars show statistically significant differences from control as measured by Student's t-test. **p<0.01, ***p<0.001.

mRNA levels for CLEC7A were lower in keratinocytes treated with EPS-Ca than in keratinocytes not treated with EPS-Ca (figure 20B). In contrast, mRNA levels for SYK were not affected by EPS-Ca treatment of keratinocytes stimulated with Th1 mimicking stimulation (figure 20B).

4.4.2 The effects of EPS-Ca on primary keratinocytes in a Th17 mimicking environment.

CCL20 is important for chemotaxis of IL-17 producing cells such as lymphocytes and secretion of the chemokine has been shown to be linked to Th17 mimicking environment (Wu et al., 2015). When the primary keratinocytes were stimulated in a Th17 mimicking environment in the presence of EPS-Ca they secreted less than keratinocytes stimulated without EPS-Ca (figure 21A). Figure 21B depicts the effects of EPS-Ca treatment on mRNA expression of CLEC7A and SYK in the Th17 stimulated primary keratinocytes. mRNA levels for CLEC7A and SYK were lower when the keratinocytes had been stimulated in the presence of EPS-Ca compared with keratinocytes stimulated in the absence of EPS-Ca (figure 21B).

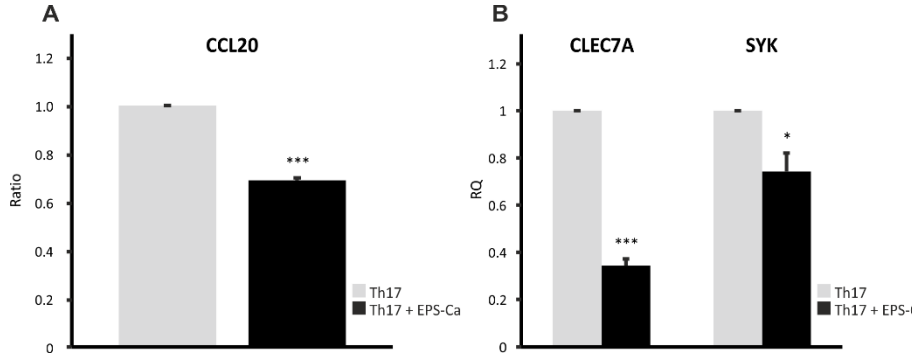

Figure 21. The effects of EPS-Ca on primary keratinocytes stimulated with Th17 mimicking environment.
Primary keratinocytes were stimulated for 24 h with IL-17A and TNF-α in the absence (Th17) or presence of 100 µg/ml of EPS-Ca (Th17 + EPS-Ca). (A) The concentration of CCL20 in the supernatants was measured by ELISA with results expressed as ratios to the control, n=3. The absolute value for keratinocytes cultured in the absence of EPS-Ca is 1329 pg/ml for CCL20 (B) mRNA expression of CLEC7A and SYK was assessed by RT-PCR and normalized to the houskeeping gene glyceraldehyde 3-phosphate (GAPDH), n=3. The results are shown as the mean + SEM. The stars show statistically significant differences from control as measured by Student‘s t-test. *p<0.05, ***p<0.001.

4.4.3 The effects of EPS-Ca on CAMP mRNA expression and LL37 distribution in primary keratinocytes

The AMP LL37, encoded by the CAMP gene, has been reported to be overexpressed in psoriatic skin and to be one of the autoantigens in psoriasis (Fuentes-Duculan et al., 2017). It was, therefore, of interest to investigate the effects of EPS-Ca on mRNA levels of CAMP in primary keratinocytes. Unstimulated primary keratinocytes expressed very low levels of CAMP but CAMP expression was upregulated following stimulation with Th17 mimicking stimulation (figure 22). Stimulating primary keratinocytes in the presence of EPS-Ca led to lower mRNA levels of CAMP compared with keratinocytes stimulated in the absence of EPS-Ca.

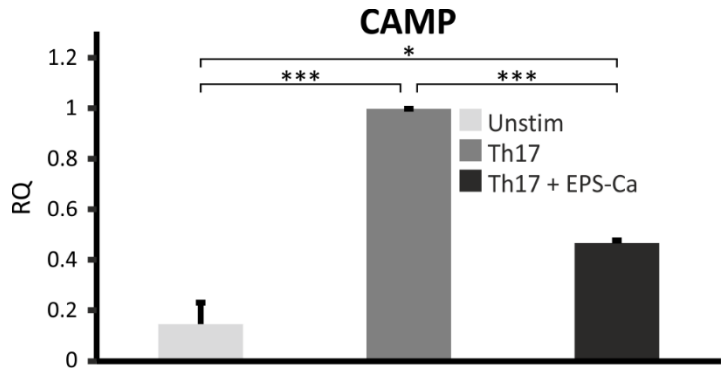

Figure 22. The effects of EPS-Ca on expression of CAMP in primary keratinocytes stimulated with Th17 mimicking environment.

mRNA expression of CAMP (encodes for LL37) in unstimulated primary keratinocytes (unstim) and following a 24 h Th17 mimicking stimulation (IL-17A and TNF-α) in the absence (Th17) or presence of 100 µg/ml of EPS-Ca (Th17 + EPS-Ca) was assessed by RT-PCR and normalized to the housekeeping gene glyceraldehyde 3-phosphate (GAPDH). The results are shown as the mean + SEM, n=3. The stars show statistically significant differences from control as measured by Student‘s t-test. *p<0.05, ***p<0.001.

To explore the expression pattern of existing LL37 within keratinocytes, the keratinocytes were stained for intracellular LL37 (green) and viewed by confocal microscopy. Interestingly, LL37 was dispersed fairly evenly throughout the cytoplasm when the keratinocytes had been stimulated with Th17 mimicking cytokines (figure 23, left panel) but accumulated into small perinuclear vesicles following treatment with EPS-Ca (figure 23, right panel). These vesicles have not yet been identified. However, these results demonstrate that EPS-Ca affects LL37 expression by keratinocytes both on transcription and protein levels.

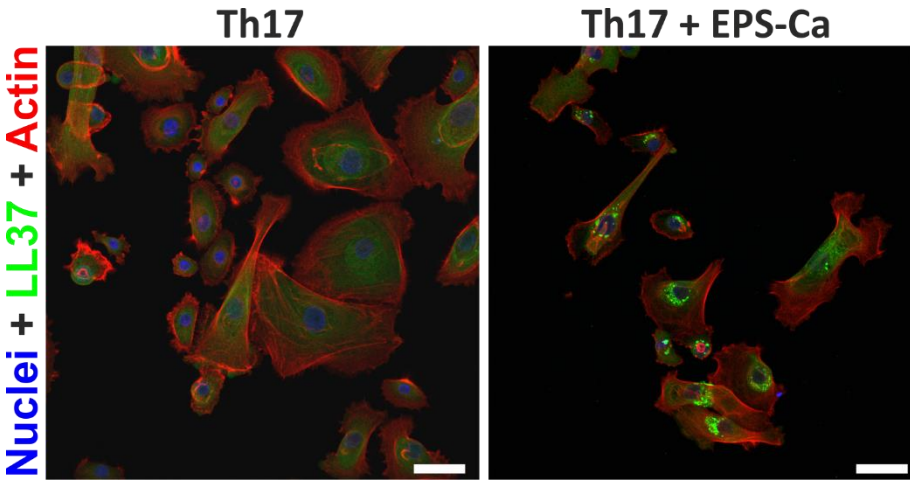

Figure 23. The effects of EPS-Ca on LL37 distribution by primary keratinocytes stimulated with Th17 mimicking environment.

Primary keratinocytes were stimulated with Th17 mimicking cytokines (IL-17A and TNF-α) in the absence (Th17) or presence of 100 µg/ml of EPS-Ca (Th17 + EPS-Ca) for 24 h and stained for intracellular LL37 (green), actin (red) and the nuclei (blue). Analysis was performed by confocal microscopy. Images were taken under 30 x magnification. Scale bars, 50 µm.

5 Discussion

5.1 Summary of findings

The main objective of the study presented in this thesis was to seek an answer to the research question whether an exopolysaccharide isolated from the cultured *Cyanobacterium aponinum* from the Blue Lagoon had immunomodulating effects *in vitro.* The research question is based on the fact that regular bathing in the Blue Lagoon offers therapeutic benefit to psoriasis patients (Eysteinsdottir et al., 2014; Olafsson, 1996), hence the exopolysaccharide secreted by the main organism in the Blue Lagoon, *C. aponinum*, could contribute to these beneficial effects. Here we show for the first time that the exopolysaccharide EPS-Ca induces IL-10 secretion by DCs and that this immunosuppressive effect of EPS-Ca coincides with a phenotypic change of the DCs towards a regulatory phenotype. After treatment with EPS-Ca, DCs expressed CD141 at high levels and the IL-10 secreting cells were all within the $CD141^{hi}$ population. In addition, EPS-Ca downregulated the Syk signaling pathway in the DCs along with its receptor, Dectin-1. A link between downregulation of the Syk signaling pathway and induction of a regulatory phenotype of DCs has not been shown before in humans, although it has been demonstrated in a murine model (Hang et al., 2016). In line with the phenotypic changes, these regulatory DCs were capable of activating allogeneic $CD4^{+}$ T cells and increased their differentiation into a Treg phenotype whilst reducing their differentiation into an inflammatory Th17 phenotype. In addition, EPS-Ca decreased cytokine secretion and expression of the activation molecule CD69 by polyclonally stimulated T cells resulting in less active T cells. Furthermore, EPS-Ca attenuated induction of mRNA levels for SYK, CLEC7A (the gene that encodes for the Dectin-1 receptor) and CAMP (the gene that encodes for LL37) by primary keratinocytes stimulated with cytokines to mimic a psoriatic environment. EPS-Ca also downregulated keratinocyte secretion of the chemokines CXCL10 and CCL20 that are important in recruitment of immune cells, including T cells. Thus, in the three *in vitro* cell models studied, EPS-Ca had inhibitory effects on the activation and/or inflammatory responses of the cells, in such a way that might explain, in part, the therapeutic benefit obtained by psoriatic patients bathing in the Blue Lagoon.

5.2 Technical considerations

The cell models used in this study; the DC model, the polyclonally stimulated T cells and the Th1/Th17 stimulated keratinocytes, all have advantages and disadvantages that are classical for *in vitro* cell culture systems. The advantages include simplified, definable experimental conditions that offer possibilities of dissecting complicated biological phenomena and of manipulating single variables. The disadvantages, or limitations, obviously include that the *in vitro* conditions are not representing the complex biological system found in the body, which raises the question whether the experimental findings obtained *in vitro* have much relevance for the *in vivo* situation. Clearly, extreme care is needed when attempting to extrapolate information obtained from *in vitro* tissue culture experiments to the properties and functions of cells and tissues of the living animal.

The DC model has proven to be an excellent model to screen for anti-inflammatory effects of compounds that possibly can become drug leads and has successfully been utilized for many years in screening a number of extracts and compounds, including polysaccharides (Di et al., 2017; Di et al., 2018; Freysdottir et al., 2008; Jonsdottir et al., 2011; Omarsdottir et al., 2006). The model allows for determination of the maturation profile of the DCs (by measuring expression of surface molecules linked with antigen presentation) and their potential to direct T cell differentiation towards different T cell phenotypes (by measuring both inflammatory and anti-inflammatory cytokines). Using this information, the direction of the immune response in which these DCs participate can be predicted. To put that prediction to the test, a co-culture of DCs and T cells is carried out to determine the polarization of naïve T cells, where characteristic cytokines and transcription factors are used to identify the T cell phenotypes.

The keratinocyte model was set up using primary keratinocytes that were stimulated with cytokines to mimic a Th1 or a Th17 associated microenvironment found in psoriatic skin. In healthy skin, the keratinocytes grow in continuous three-dimensional layers, whereas cultured keratinocytes grow in a monolayer and often in a subconfluent manner, which is an obvious shortcoming and an artificial form of keratinocyte growth. An air-liquid interface (ALI) system, culturing the keratinocytes on a filter with media below and atmosphere above, would probably mimic more their growth in the skin and create a more realistic setting. In the ALI culture system, the keratinocytes could be allowed to reach confluence and take the next natural step in stratification and differentiation. The ALI culture system would also

allow for a merger of the cell culture approaches presented in the thesis, as co-cultures of keratinocytes and immune cells might provide information regarding how the interactions between the two cells are affected by EPS-Ca.

One limitation of the present study is the limited number of, and the selection of, cell surface and secreted molecules, that were analyzed. Although the selection of cell surface molecules and cytokines/chemokines studied was based on experience and literature, there is a strong possibility that molecules important in mediating the effects of EPS-Ca on cellular functions may have been overlooked. To overcome this limitation, a total RNA sequencing would be optimal to study the effects of EPS-Ca on the transcription profile and proteomic analysis by mass spectrometry to measure proteins and protein modifications which are not represented in RNA analysis, such as phosphorylation. Results from these analyses could then be confirmed with other methods, such as quantitative PCR, phospho-flow, flow cytometry, ELISA, Luminex and/or Western blotting.

In this study all the cells were obtained from healthy individuals. It would have been of interest to compare the effect of EPS-Ca treatment on cells obtained from psoriatic patients with that observed in cells obtained from healthy donors.

Apart from several possible improvements of the *in vitro* cell culture models used in this thesis, an *in vivo* investigation is of interest. The imiquimod mouse model entails a topical application of imiquimod which is a TLR7/8 ligand and a potent immune activator that induces and exacerbates psoriasis via the IL-17/IL23 axis (van der Fits et al., 2009). A model, such as the imiquimod mouse model, could be an ideal starting point for *in vivo* investigations of a topical treatment containing EPS-Ca.

The exopolysaccharide studied in this thesis is derived from *C. aponinum* that are cultured in bioreactors at the Blue Lagoon‘s R&D laboratory for cosmetic production purposes. It would have been interesting to investigate the concentration of the exopolysaccharide in the Lagoon itself and compare it to the concentration of EPS-Ca used in the present study. It would also have been interesting to be able to successfully label the polysaccharide to be able to determine a possible binding/blocking site(s) on the cells used. The approximate size and monosaccharide composition of the exopolysaccharide was determined (Table 1), but the complete structure of the exopolysaccharide has not been determined which would have been interesting, especially in comparing it with other structurally similar molecules. However, the unfractionated product of *C. aponinum*, rather than a purified

compound, may better mimic what the skin of psoriatic patients come in contact with when bathing in the Blue Lagoon.

To affect psoriatic plaques the exopolysaccharide in the Blue Lagoon would have to penetrate the skin. As it has been shown previously that bathing in a salt water solution increases the permeability of minerals through psoriatic skin (Shani et al., 1985), bathing in the Blue Lagoon's seawater probably also increases the permeability of psoriatic skin. Furthermore, when psoriasis patients bathe in the Blue Lagoon, they are instructed to rub some of the silica mud on their skin. The silica mud acts as a coarse scrub, ridding the plaques of scales leaving the plaques exposed as wounds and thus providing greater access for the exopolysaccharide to infiltrate the deeper layers of the epidermis and dermis.

5.3 DC model

This study demonstrates that EPS-Ca-treated DCs have a regulatory phenotype and function, where IL-10 seems to play a central role. As a major immunosuppressive cytokine, IL-10 has a critical role in limiting the extent of the activation of both innate and adaptive immune cells to maintain homeostatic state and ultimately to limit and terminate inflammatory responses (Moore et al., 2001). Therefore, IL-10 is of critical importance in protecting the host from unwanted excessive inflammation, such as occurs in autoimmunity and allergy.

To investigate the possible mechanism(s) behind the effects of EPS-Ca on IL-10 secretion, the Syk signaling pathway was analyzed as previous studies have linked changes in SYK signaling with IL-10 regulation and expression (Kotthoff et al., 2017; Robinson et al., 2009; Rogers et al., 2005; Slack et al., 2007; Yang et al., 2017; Yin et al., 2016). For instance, increased SYK phosphorylation was associated with an increase in IL-10 secretion by Dectin-1 stimulated DCs (Kotthoff et al., 2017) and IL-10 secretion was dependent on SYK signaling in bone marrow derived DCs from mice upon activation of Dectin-1 or Dectin-2 (Robinson et al., 2009; Rogers et al., 2005; Slack et al., 2007). Intriguingly, in the present study the induction of IL-10 secretion by DCs treated with EPS-Ca was accompanied by hampered mRNA expression and activation of SYK, an observation not in agreement with the aforementioned research. However, other studies in mice have shown results more similar to the ones obtained in the present study. Yin *et.al.* showed that SYK negatively regulates IL-10 production in mice, as SYK deficiency in murine DCs resulted in induction of IL-10 secretion upon LPS

stimulation (Yin et al., 2016). Additionally, an extract of *Cordyceps bassiana* reduced expression of multiple inflammatory mediators but induced IL-10 expression by murine macrophages and simultaneously blocked activation of the SYK signaling cascade (Yang et al., 2017).

In the present study EPS-Ca also downregulated mRNA expression of CLEC7A (the gene encoding for Dectin-1), which is in line with results obtained by Hang and coworkers (Hang et al., 2016). They showed in a mouse model downregulation of both Dectin-1 and the Syk signaling pathway by a helminthic infection in the mice. This downregulation seemed to be important for conversion of the phenotype of the DCs into regulatory DCs. Furthermore, Hang *et al.* showed that SYK deficient DCs transferred into Rag mice reduced colonic inflammation. Cytokine analysis of OVA stimulated lamina propria mononuclear cells (LPMCs) isolated from the terminal ileum showed that LPMCs from the Rag mice, reconstituted with SYK deficient DCs, secreted lower levels of IL-17 and IFN-γ than the controls (Hang et al., 2016). However, they did not measure IL-10 levels in their model. Another study, using an imiquimod psoriasis mouse model, demonstrated that SYK activation plays an essential role in imiquimod-induced psoriatic inflammation and that a SYK-inhibitor reduced IL-6 and IL-23 levels in skin DCs which led to a downregulation of Th17 cells and upregulation of Tregs in the skin (Alzahrani et al., 2019). Collectively, these results suggest that SYK may be a potential therapeutic target in inflammatory diseases such as psoriasis and that inhibition of the Syk signaling pathway by EPS-Ca as observed in the present study may be a potential explanation for the benefits observed in clinical studies after regular bathing in the Blue Lagoon.

The two subtypes of myeloid DCs found in psoriatic skin are defined as being $CD1c^+$ and $CD1c^-$. The $CD1c^+$ DCs are not increased in numbers in psoriatic skin and are similar to those found in normal skin but the $CD1c^-$ are increased around 30 fold in psoriatic skin and are often termed inflammatory DCs (Zaba et al., 2009b). EPS-Ca treatment led to an increase in the proportion of DCs expressing CD1c, i.e. the DC phenotype less inclined to contribute to inflammation. Interestingly, DCs expressing CD1c have been shown to produce high levels of IL-10 and to suppress T cell proliferation in a T cell dependent manner (Kassianos et al., 2012). Although in the present study it was demonstrated that EPS-Ca led to a higher proportion of DCs expressing CD1c and to an increase in IL-10 production by DCs, we did not establish that the $CD1c^+$ DCs were the DCs producing IL-10. Furthermore, the data presented in this thesis shows that EPS-Ca-treated DCs did not

affect T cell proliferation and is therefore not in total agreement with the results by Kassianos and coworkers.

Interestingly, our data showed a lower proportion of DCs expressing CCR7 on their surface when treated with EPS-Ca. CCR7 is a receptor involved in homing of cells to secondary lymph nodes in response to CCL19 and CCL21 produced in the lymph nodes (Murphy & Weaver, 2017; Sallusto et al., 1998). Thus, our results indicate that the EPS-Ca treated DCs may be less able to migrate to draining lymph nodes and subsequently to interact with naïve T cells. This potential reduction of interaction between DCs and naïve T cells could lead to less activation of T cells and thus, dampening of the hyperactive immune response in psoriasis. These effects could play a role in the betterment obtained by psoriatic patients bathing in the Blue Lagoon.

The function of the EPS-Ca-treated DCs, i.e. their increased secretion of IL-10 and their increased ability to induce Treg cells, is consistent with a tolerance/regulation function. However, the phenotype of the EPS-Ca-treated DCs does not completely comply with current definition of tolerogenic or regulatory DCs. These two phenotypes have been shown to have low or moderate levels of MHC expression and to be defective in their expression of co-stimulatory molecules (Sato et al., 2003a; Sato et al., 2003b). However, as EPS-Ca-treated DCs have high expression levels of both MHC class II and co-stimulatory molecules concomitant with secretion of high levels of IL-10, the question arises whether these DCs constitute a third phenotype of tolerogenic/regulatory DCs. Supporting that is the finding that the EPS-Ca-treated DCs were just as capable as untreated DCs in activating allogeneic $CD4^+$ T cells and therefore were not inhibiting T cell activation. At the same time, the EPS-Ca-treated DCs were able to induce more T cells with a Treg phenotype and decreased the proportion of T cells with a Th17 phenotype. This suggests that the increased secretion of IL-10 observed for the EPS-Ca-treated DCs was an important factor in rendering the DCs capable of affecting the T cell phenotype towards regulation.

5.4 T cells

Many psoriatic patients go to the Blue Lagoon when their disease is in an active stage with psoriatic plaques present. Active plaques have been shown to be heavily infiltrated by Th1 and Th17 effector cells (Zaba et al., 2009a). Therefore, it was of interest to investigate the effects of EPS-Ca on polyclonally stimulated T cells and explore whether EPS-Ca treatment would

attenuate their activation. EPS-Ca treatment did attenuate activation of stimulated T cells, evidenced by a lowered proportion of cells expressing CD69 as well as a decrease in their of cytokine secretion.

CD69 is known as an early activation marker of T cells and reduced CD69 expression is, hence, a sign of reduced activation of the EPS-Ca-treated T cells. However, since CD69 also acts as a retention signal (Mackay et al., 2015) it follows that after EPS-Ca treatment, T cell emigration from the skin might be increased, resulting in reduced number of effector cells being present in the skin. CD69 expression by T cells in the lymphoid tissue entails a downregulation of S1PR1, making the T cells unable to leave the lymphoid tissue whilst they differentiate and proliferate. Subsequently, when they are ready to leave, they downregulate CD69 and upregulate S1PR1, enabling them to react to S1P gradient and leave the lymphoid tissue (Murphy & Weaver, 2017). Thus, it was of interest to determine if EPS-Ca, in addition to decreasing CD69 expression by the T cells, also upregulated their S1PR1 expression. Unfortunately, staining for S1PR1 expression was not successful, and remains a work in progress. Had the staining been successful and showed that EPS-Ca enhanced expression of S1PR1 by the T cells, that would have supported the hypothesis that EPS-Ca treatment could increase T cell mobility and enable them to leave the skin.

In addition to the lowered activation stage of the T cells, evidenced by lower CD69 expression following EPS-Ca treatment, EPS-Ca treatment also decreased the proportion of T cells expressing phosphorylated ZAP70, indicating that EPS-Ca may be dampening the activation of the T cells via their TCR signaling, as ZAP70 is known to have a central role in mediating activation signals from the TCR (Wang et al., 2010).

Although reduced cytokine secretion by the EPS-Ca-treated T cells is an indication of reduced activation, it is of interest that EPS-Ca treatment did not affect IFN-γ secretion by the T cells. This indicates that the T cells are still functioning and that the reduction in cytokine secretion is selective and not dampening secretion of all cytokines. The reduction in the IL-17 secretion by the polyclonally activated T cells following EPS-Ca treatment is in concordance with the decreased proportion of Th17 cells observed in co-cultures of T cells and EPS-Ca-treated DCs. This is an interesting finding as Th17 cells are the dominant inflammatory T cells found in psoriatic skin (Zaba et al., 2009a) and biologic therapies targeting IL-17, especially the IL-17 antagonists, have proven to be promising candidates in psoriasis treatment (Wu et al., 2017).

5.5 Keratinocytes

Keratinocytes are the main responding cells in psoriasis and active contributors to the cytokine storm present in the psoriatic plaques. Interestingly, Dectin-1 (CLEC7A) expression has been shown to be high in psoriatic skin compared to normal skin (de Koning et al., 2010) and thus, of interest to study its expression in our model. The induction of CLEC7A mRNA expression in the proinflammatory Th1 and Th17 mimicking environments was drastically inhibited by EPS-Ca. As Dectin-1 is minimally expressed in normal skin but is highly expressed in psoriatic epidermis, this observation suggests that the upregulation of CLEC7A in keratinocytes may be under control of psoriasis-associated cytokines and that the keratinocyte model may be mimicking psoriatic inflamed skin. However, the exact role of Dectin-1 expression in psoriasis has yet to be clarified.

Keratinocyte secretion of the chemokines CXCL10 and CCL20 was upregulated when the cells were stimulated with Th1 and Th17 mimicking cytokines, respectively. As TNF-α was used in both stimulations the different pattern of chemokine secretion must be induced by IFN-γ and IL-17, respectively. CXCL10 is also known as interferon gamma-induced protein 10 (IP-10) as it is secreted in response to IFN-γ by various cells, including keratinocytes (Lee et al., 2009). CXCL10 appears to take part in the pathogenesis of many autoimmune diseases, such as cutaneous lupus erythematosus, rheumatoid arthritis and type 1 diabetes (Antonelli et al., 2014; Braegelmann et al., 2016; Lee et al., 2009).

EPS-Ca treatment of Th1 or Th17 stimulated keratinocytes led to reduced secretion of CXCL10 and CCL20, respectively. As both chemokines are strong chemotactic agents for lymphocytes and other inflammatory cells (Ferrari et al., 2015; Schutyser et al., 2003), it may be concluded that EPS-Ca treatment can lead to reduced inflammatory cell recruitment to the skin. EPS-Ca also hampered SYK transcription in Th17 stimulated keratinocytes which can explain the decrease observed in CCL20 by EPS-Ca-treated keratinocytes, as it has been shown that SYK mediates IL-17A-induced CCL20 expression (Wu et al., 2015). Previous studies have shown a link between expression of SYK and CXCL10. SYK-mediated CXCL10 production was, for example, abrogated with lentivirus SYK silencing (Redhu et al., 2009). Braegalmann *et al.* also showed analogue expression of pSYK and CXCL10 in cutaneous lupus erythematosus, a Th1 mediated disease, and that pSYK inhibition attenuated CXCL10 secretion in an immortalized keratinocyte cell line (HaCat cells) as well as in primary keratinocytes

(Braegelmann et al., 2016). In the present study, the downregulation of CXCL10 in the Th1 stimulated keratinocytes following EPS-Ca treatment was not accompanied by a significant downregulation of SYK mRNA expression. It might, therefore, be important to investigate the level of phosphorylation of SYK in the Th1 stimulated keratinocytes following EPS-Ca treatment.

In the present study, the Th17 mimicking cytokine environment induced an upregulation of CAMP, the gene that encodes for LL37, in keratinocytes. In this environment, EPS-Ca treatment had a substantial effect, by both reducing the mRNA levels for CAMP and altering the localization of LL37 within the cells. LL37 is an antimicrobial peptide that is overexpressed in psoriatic skin and it has recently been identified as one of the autoantigens in psoriasis (Fuentes-Duculan et al., 2017; Lande et al., 2014). LL37-specific T cells produce IFN-γ and Th17 cytokines consistent with an important LL37 related contribution to the pathogenesis of the disease (Lande et al., 2014). In view of the proposed role of LL37 in the pathogenesis of psoriasis, an inhibition of CAMP mRNA expression, and thus the production of its product LL37, could clearly contribute to the beneficial effects seen in clinical studies after regular bathing in the Blue Lagoon.

The localization of the LL37 peptide within the keratinocytes before and after treatment with EPS-Ca was visualized by confocal microscopy. In untreated keratinocytes the peptide was diffusely dispersed whereas it had accumulated into what appeared to be small cytoplasmic vesicles in the EPS-Ca-treated keratinocytes. The importance of this finding is not clear but one can hypothesize that the keratinocytes could be relocating the peptide for its removal following downregulation of the gene expression. However, further work is needed for elucidation of the nature and function of these vesicles. In a previous, unpublished clinical study an interesting observation was made; a diffused expression of LL37 was observed in the epidermis before a 6 week outpatient Blue Lagoon treatment but after the treatment the LL37 had gathered to the basal layer of the epidermis (Bjornsdottir, 2016). It is intriguing that in that study the Blue Lagoon treatment affected histological distribution of LL37, whereas in the present study exopolysaccharide isolated from the main organism of the Lagoon (EPS-Ca) altered intracellular distribution of LL37. As LL37 is an important contributor in the pathogenesis of psoriasis it is of great interest to study whether the mechanisms mediating these effects on LL37 localization are of a similar nature and importance.

5.6 Future aspects

A substantial amount of data has been gathered on the effects of EPS-Ca on different cell types in several *in vitro* models. As always, with new information more questions arise. The next immediate steps would be to investigate the immunomodulatory effects of EPS-Ca in the DC model and polyclonally stimulated T cells, using blood from psoriatic patients and comparing with the data obtained using healthy donor cells. It would also be interesting to culture the keratinocytes in the ALI culture system as discussed in chapter 5.2, in order to determine the effects of EPS-Ca on keratinocytes cultured in a more organotypic culture environment. In that setting, the effects of EPS-Ca on the localization of LL37 could be determined in a more organotypic model and compared to the observed changes in localization of LL37 in skin samples from patients before and after treatment in the Blue Lagoon (Bjornsdottir, 2016). If successful, a co-culture of keratinocytes and other immune cells in the ALI culture system would be the next step to perform. An ALI culture could in this regard serve as an intermediate platform between the culture systems presented in this study, and *in vivo* studies. In order to obtain more detailed information about the predicted effects of EPS-Ca, along with any unanticipated effects, a non-selective screening of samples could be performed. By collecting cells and cell culture supernatants from all cell models following EPS-Ca treatment (using cells derived from blood of healthy individuals and psoriatic patients) and conducting full RNA sequencing and proteomic analysis a comprehensive overview would be obtained over EPS-Ca-induced effects. A topical imiquimod-induced psoriatic inflammation model in mice would be an optimal platform to start *in vivo* studies, to fully assess the effects of EPS-Ca on *in situ* skin. Finally, it would be of major interest to perform a pilot clinical study where the effect of a crème or a lotion containing the EPS-Ca would be investigated as treatment of psoriatic plaques.

6 Conclusions

Over the last few decades the views and understanding of the pathogenesis of psoriasis has changed quite drastically and evolved from a model of a primary disease of the keratinocytes to being viewed as an IL-12/Th1 mediated disease. The current pathogenic model has, however, centered on the IL-23/IL-17 axis but is being revised almost every day in response to new findings and acquisitions, such as the recently discovered autoantigens and autoreactive T cells. Although increased information is accumulating regarding the pathogenesis of psoriasis, much is still unknown.

The study presented in this thesis has shed new light on how the exopolysaccharide termed EPS-Ca, produced by the *Cyanobacterium aponinum* in the Blue Lagoon, affects *in vitro* the key cells participating in the pathogenesis of psoriasis, i.e. DCs, T cells and keratinocytes (figure 24). We

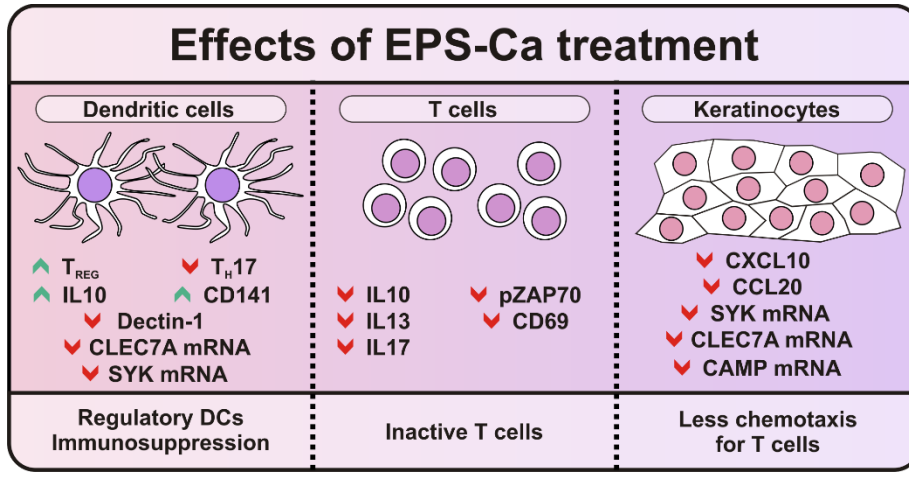

Figure 24. Schematic overview of the effects of EPS-Ca on DCs, T cells and keratinocytes.

have established that EPS-Ca induces IL-10 production by DCs and that EPS-Ca-treated DCs have a regulatory phenotype. EPS-Ca also increased the capacity of the DCs to induce differentiation of T cells into T regulatory cells, diminishing induction of the disease associated Th17 cells. Furthermore, we explored the effect of EPS-Ca on polyclonally stimulated T

cells where EPS-Ca decreased the proportion of T cells expressing CD69, an activation marker and a retention signal for T cells, indicating that the T cells cultured in the presence of EPS-Ca may be less active and better equipped to leave the skin than T cells cultured without EPS-Ca. EPS-Ca also decreased T cell secretion of several cytokines, and decreased the proportion of T cells with activated ZAP70, further supporting that EPS-Ca may lead to decreased activation of T cells. When cultured in the presence of EPS-Ca, Th17 or Th1 stimulated primary keratinocytes expressed less SYK and CLEC7A mRNA and secreted less of the chemokines CCL20 and CXCL10, respectively, indicating a reduction in their inflammatory state. Finally, our results demonstrate that EPS-Ca inhibited Th17 cytokine-induced upregulation of CAMP mRNA expression, an important finding as the product of CAMP - LL37, is one of the autoantigens in psoriasis and is upregulated in psoriatic skin.

EPS-Ca had an immunomodulatory effect on all the cell types tested in the present study and the effects appeared to be anti-inflammatory. Interestingly, in all the cell types tested, EPS-Ca affected SYK/ZAP70, indicating a common mechanism behind its effects in the different cell types. As CLEC7A and SYK/ZAP70 have diverse roles within different cell types, the inhibitory effects of EPS-Ca manifested differently in the cells analyzed. However, overall EPS-Ca seemed to have a beneficial effect with regard to reducing inflammation. This supports our hypothesis that EPS-Ca may be a contributing factor in the beneficial effects obtained by psoriatic patients when bathing in the Blue Lagoon.

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Paper I

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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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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.

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

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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 μE/m^2/s; 40 °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 μl. 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 μg/ml (Sigma–Aldrich, St. Louis, MO) for 48 h, without (control) or with EPS-Ca at concentrations of 1, 10 and 100 μg/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).

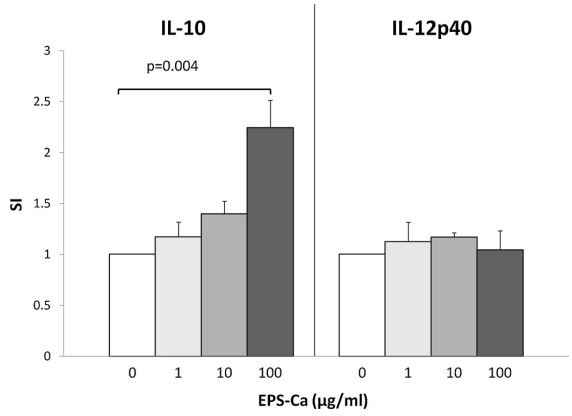

Fig. 1. Cytokine secretion by DCs matured in the absence or presence of exopolysaccharides from *C. aponinum*. DCs were matured in the absence or presence of exopolysaccharides from *C. aponinum* (EPS-Ca) at the concentrations 1, 10 and 100 μg/ml and the levels of IL-10 and IL-12p40 measured by ELISA. 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).

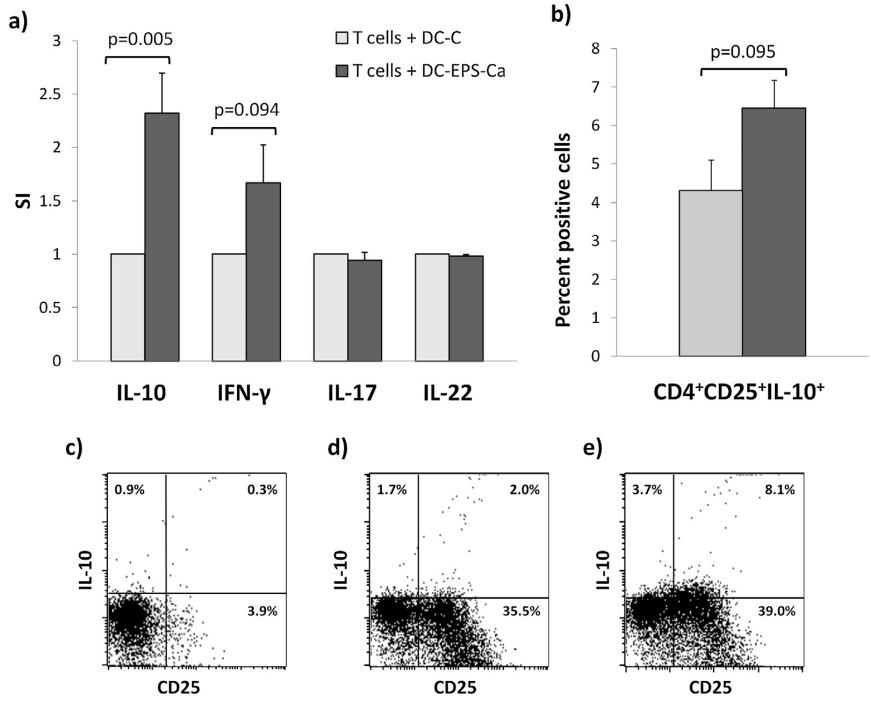

Fig. 2. Cytokine production following co-culture of allogeneic CD4$^+$ T cells and DCs matured in the absence (DC-C) or presence (DC-EPS-Ca) of EPS-Ca at 100 μg/ml. (a) Levels of IL-10, IFN-γ, IL-17 and IL-22 secretion in co-culture supernatants were measured by ELISA and the results expressed as secretion 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

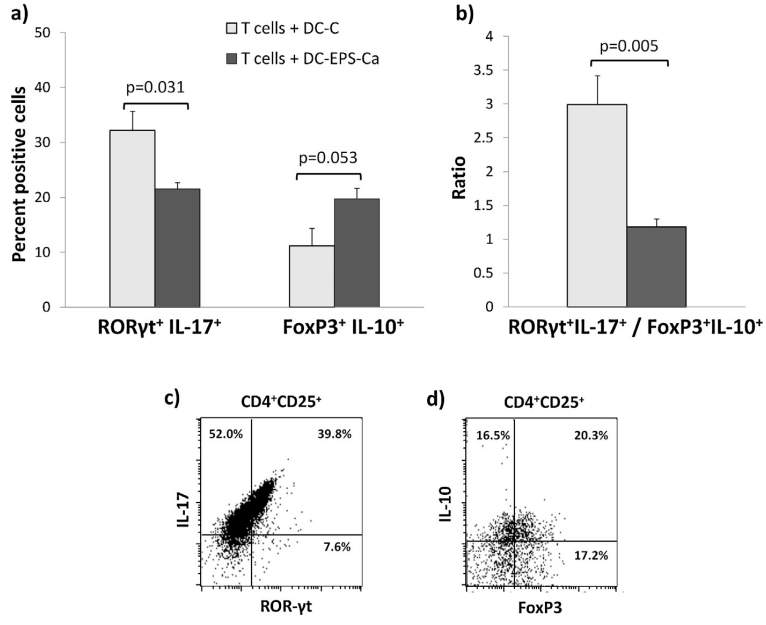

Fig. 3. The expression of transcription factors and cytokines by allogeneic CD4⁺ T cells co-cultured with DCs matured in the absence (DC-C) or presence (DC-EPS-Ca) of EPS-Ca at 100 μg/ml. Following the co-culture (a) the percentage of positive allogeneic CD4⁺ T cells expressing RORγt⁺IL-17⁺ or FoxP3⁺IL-10⁺ 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.

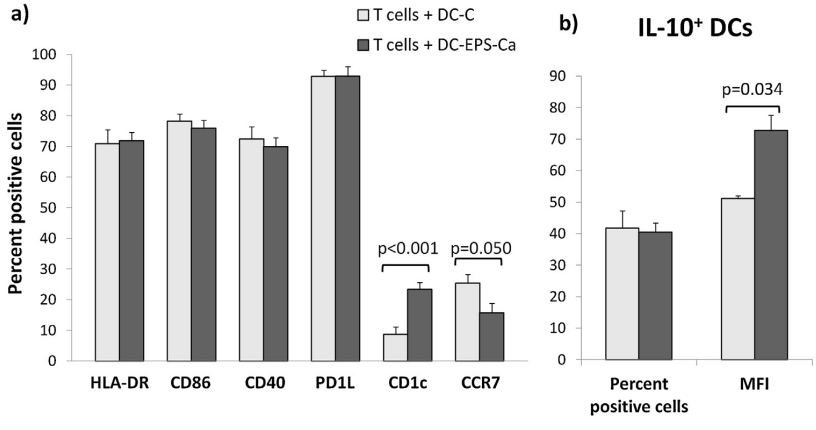

Fig. 4. The effects of co-culturing DCs matured in the absence (DC-C) or presence (DC-EPS-Ca) of EPS-Ca at 100 μg/ml with allogeneic CD4⁺ T cells on their expression of surface molecules and cytokines. (a) Percentage of DCs expressing the surface molecules HLA-DR, CD86, CD40, PD-L1, CD1c and CCR7 was 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.

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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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Paper II

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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.

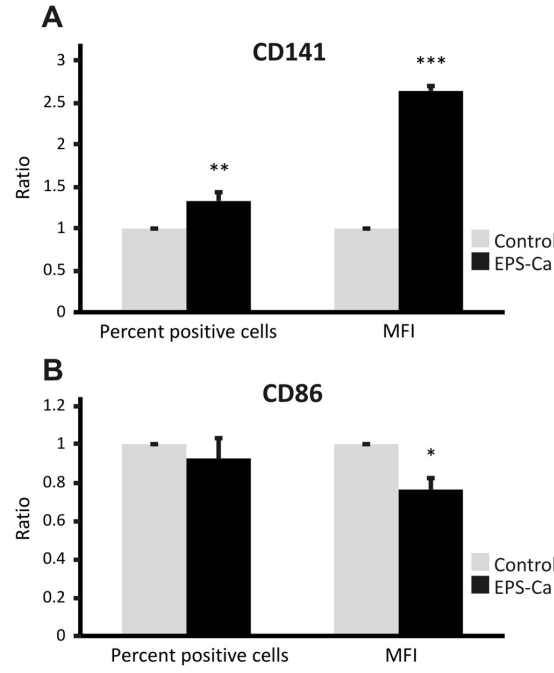

Fig. 1. EPS-Ca induces CD141 expression on DCs.
DCs, stimulated with cytokines and LPS, were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (control) for 24 h and expression of (A) CD141 and (B) CD86 assessed by flow cytometry with results expressed as ratio to control (percentage positive cells 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

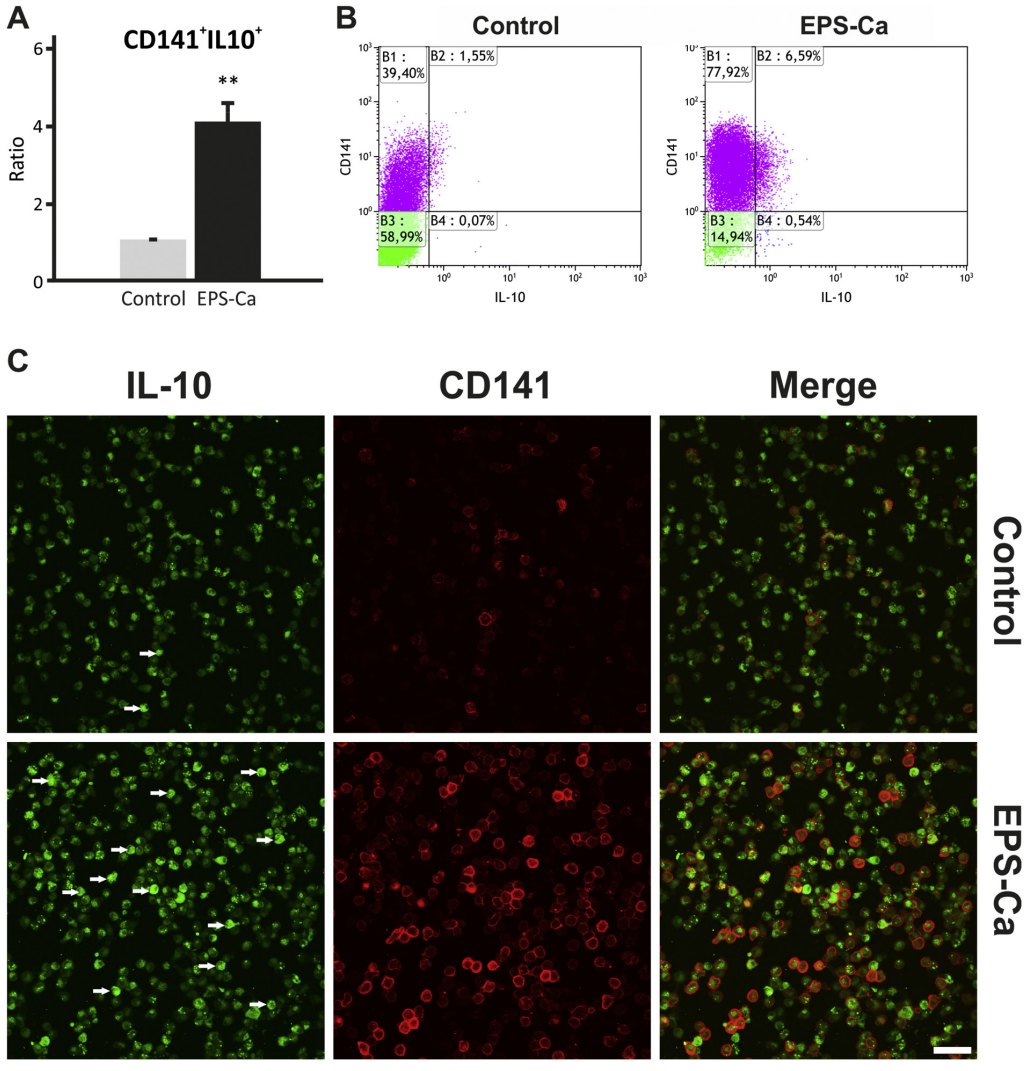

Fig. 2. EPS-Ca increases the proportion of CD141$^+$IL-10$^+$ DCs.
DCs were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (Control) for 24 h and stained with antibodies against CD141 and IL-10. (A) The ratio of CD141$^+$IL-10$^+$ DCs to control (percent positive cells), n = 3. 1.63% of control 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

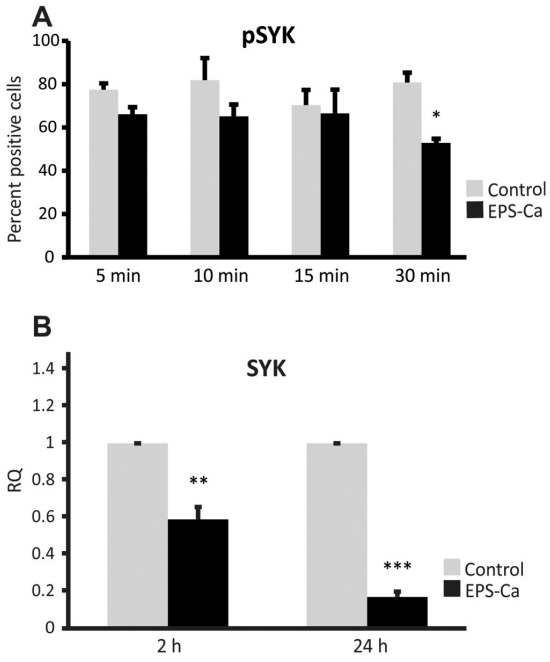

Fig. 3. EPS-Ca decreases phosphorylated SYK upon stimulation and decreases mRNA for SYK in DCs.
DCs were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (Control) for the times indicated. (A) The cells were additionally stimulated with IL-1β, TNF-α and LPS and then stained for pSYK and analyzed 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.

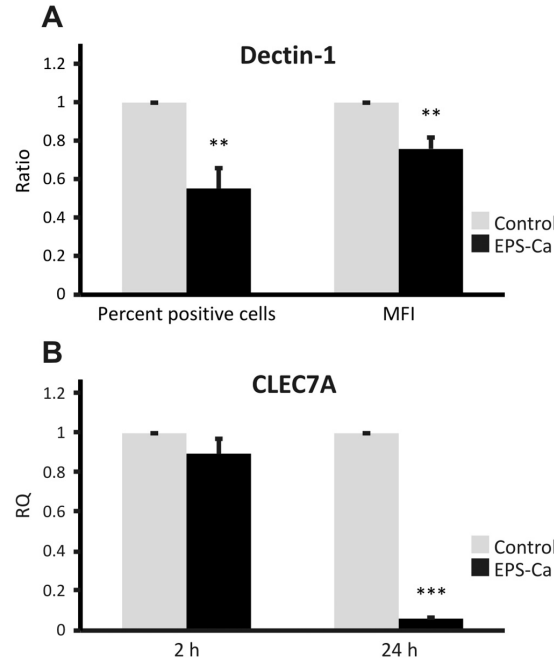

Fig. 4. EPS-Ca decreases Dectin-1 expression and mRNA levels for CLEC7A in DCs.
DCs, stimulated with cytokines and LPS (A) or not (B), were cultured with EPS-Ca at 100 μg/ml (EPS-Ca) or without (Control) for indicated times. (A) Expression of the Dectin-1 receptor was assessed after 24 h by flow cytometry 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.

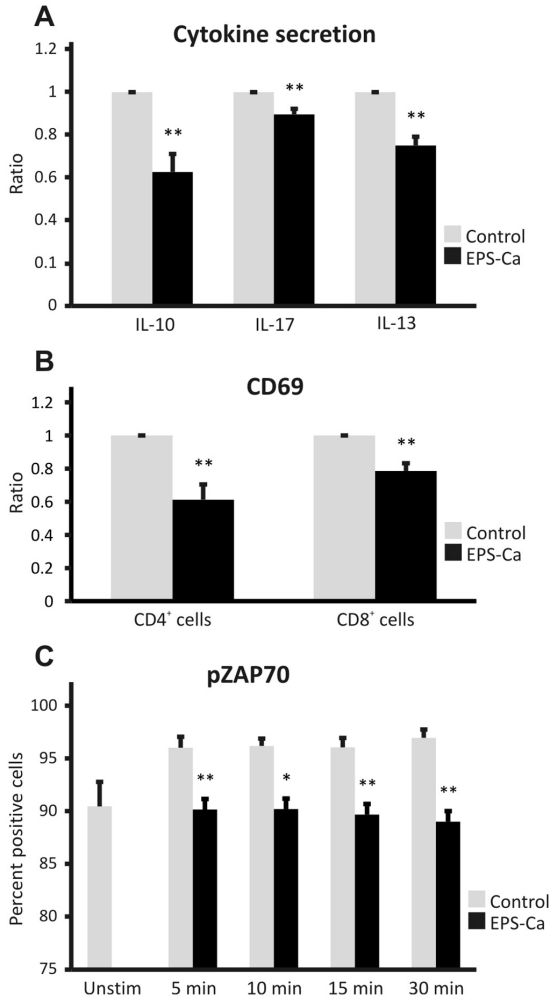

Fig. 5. EPS-Ca decreases cytokine secretion and expression of CD69 and pZAP70 by stimulated T cells.
T cells were stimulated with 4 μg/ml plate-bound anti-CD3ε antibody and 1 μg/ml soluble anti-CD28 antibody for 72 h, with 100 μg/ml EPS-Ca present (EPS-Ca) or absent (Control) for the last 24 h (A and 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

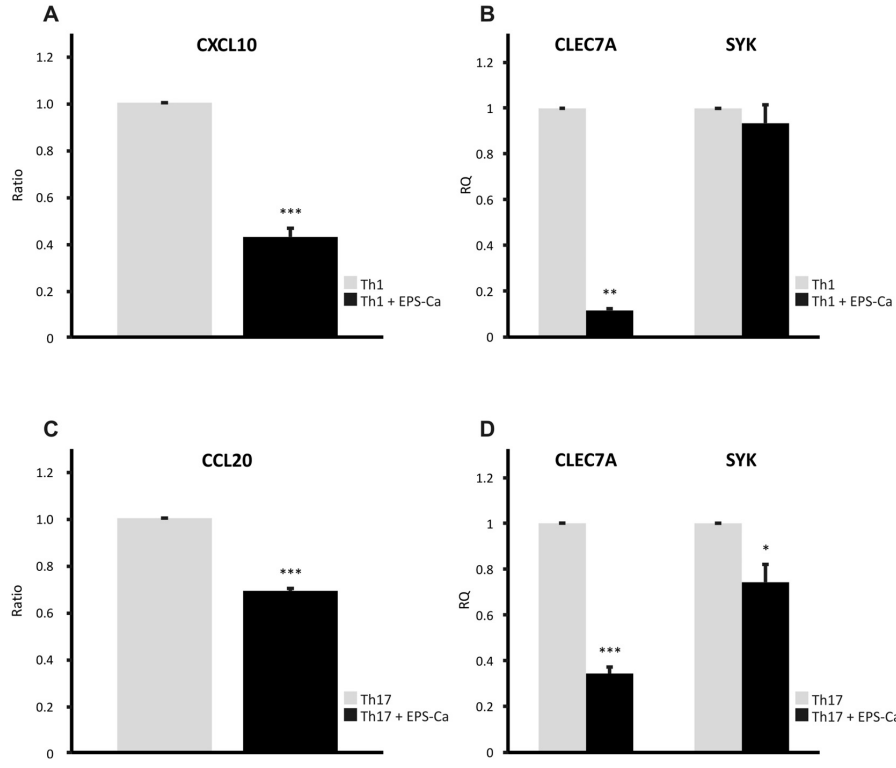

Fig. 6. EPS-Ca decreases CXCL10 and CCL20 secretion and mRNA levels for CLEC7A and SYK in keratinocytes.
Primary keratinocytes were stimulated for 24 h with Th1 (A and B) or Th17 (C and D) mimicking environments in the presence of EPS-Ca at 100 μg/ml (Th1 + EPS-Ca or Th17 + EPS-Ca, respectively) or not (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).

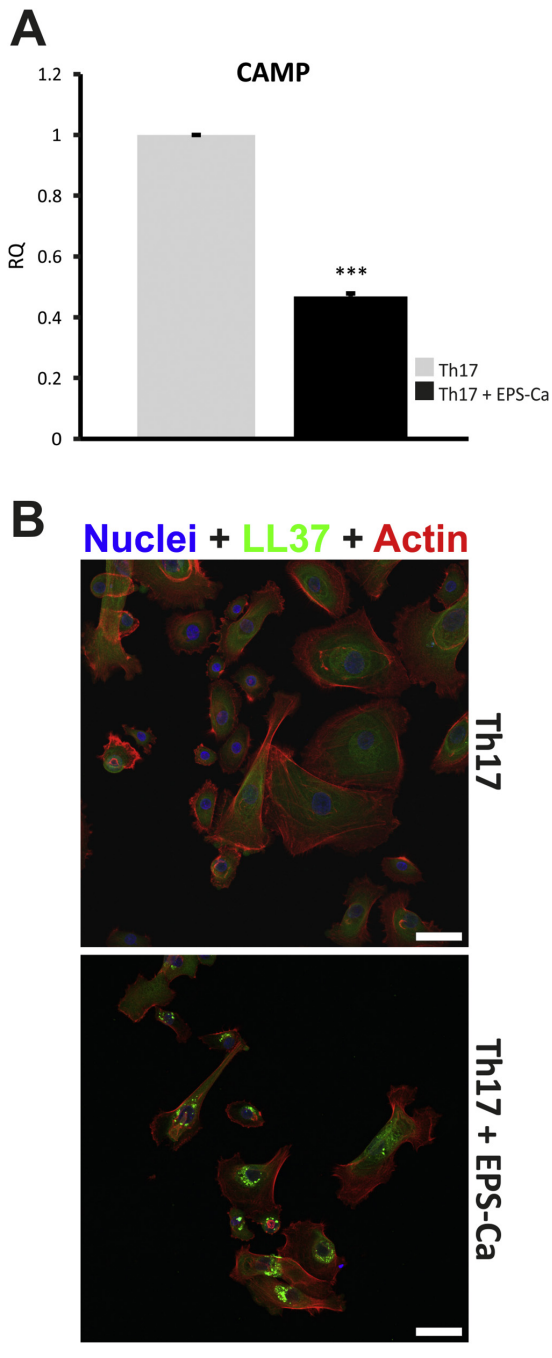

Fig. 7. EPS-Ca decreases mRNA levels of CAMP and alters LL37 expression in keratinocytes.
Keratinocytes were stimulated with Th17 mimicking environment (TNF-α and IL-17A) for 24 h with 100 μg/ml EPS-Ca present (Th17 + EPS-Ca) or absent (Th17). (A) Levels of mRNA for CAMP were estimated using quantitative 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

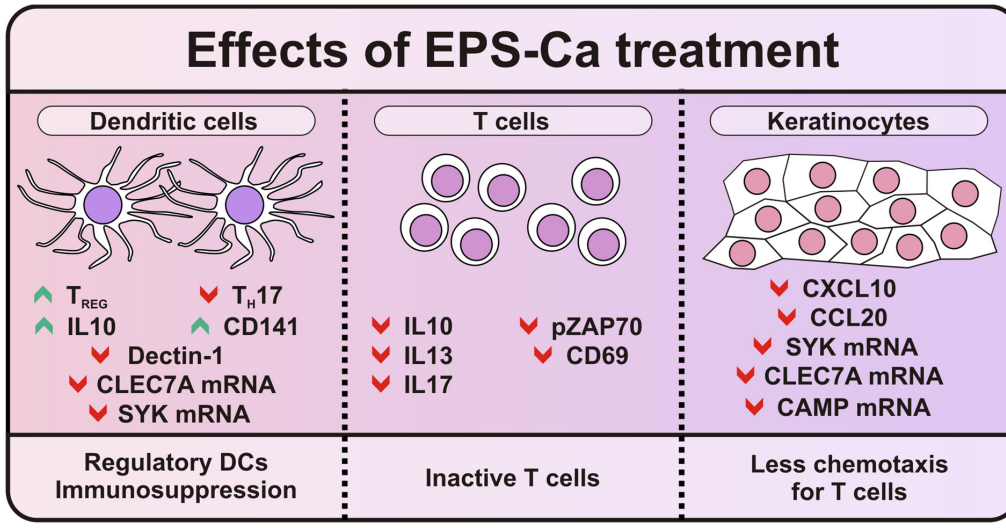

Fig. 8. The effects of EPS-Ca on dendritic cells, T cells and keratinocytes *in vitro*.

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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.044.

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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Appendix

Table 3. Antibodies used for flow cytometry analysis

Antigen	Clone	Supplier
CD1c	AD5-8E7	Miltenyi Biotec
CD4	SK3	BD Bioscience
CD4	RPA-T4	eBioscience
CD8	SK1	eBioscience
CD14	UCHM1	AbD Serotec
CD25	BC96	eBioscience
CD40	LOB7/6	AbD Serotec
CD49d (VLA-4)	9F10	BioLegend
CD54 (ICAM-1)	15.2	AbD Serotec
CD69	FN50	eBioscience
CD86	BU63	AbD Serotec
CD141	M80	BioLegend
CCR7 (CD197)	150503	R&D Systems

CTLA-4 (CD152)	14D3	eBioscience
CD40L (CD154)	24-31	eBioscience
Dectin-1 (CD369)	15E2	eBioscience
PD1L (CD274)	MIH1	eBioscience
HLA-DR	G46-6 (L243)	eBioscience
FoxP3	236A/E7	eBioscience
RORγt	AFKJS-9 (rat)	eBioscience
IL-10	JES3-19F1 (rat)	BD Bioscience
IL-17	eBio64DEC17	eBioscience

Table 4. Primers used for quantitative RT-PCR

Antigen	IDT identification number
SYK	Hs.PT.58.38446151.g
CLEC7A	Hs.PT.58.3686547.g
ZAP70	Hs.PT.58.3371269.g
CAMP	Hs.PT.56a.45325005.g
GAPDH	Hs.PT.39a.22214836