Antimicrobial expression in keratinocytes

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University of Iceland
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Tjáning varnarpeptíða í hyrnisfrumum

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Ágrip

Sóri er algengur bólgusjúkdómur með alvarlega fylgikvilla og minnkuð lífsgæði. Meðal þeirra þátta er einkenna meingerð sóra er aukin tjáning á varnarpeptíðum ónæmiskerfisins. Varnarpeptíðið LL-37 er mikilvæg vörn gegn sýkingum jafnt sem ræsingu ónæmiskerfisins og er stór partur af ósérhæfðu ónæmissvari húðar. Í sóra er tjáning þess stórlega aukin og er tengd viðhaldi bólgusvörunar í húðinni. Tjáningarmynstur LL-37 í húð sórasjúklinga hefur ekki verið sýnt áður. Þekking á hlutverki og dreifingu tjáningar LL-37 í húð sórasjúklinga ásamt mögulegu sambandi við alvarleika sjúkdóms eða tjáningu annarra bólguboðefna gæti því leitt til aukins skilnings á sjúkdómnum.

Húðsýni voru fengin frá sórasjúklingum sem tóku þátt í rannsókn um áhrif Bláa Lóns meðferðar á skellusóra. Sjúklingum var skipt í þrjá hópa sem hver fékk mismunandi meðferð. Einn hópur fékk innlagnarmeðferð í Bláa Lóninu ásamt NB-UVB meðferð, annar hópurinn fékk göngudeildarmeðferð í Bláa Lóninu ásamt NB-UVB meðferð og sá þriðji fékk einungis NB-UVB meðferð sem viðmið við hina tvo. Húðsýnum var safnað með húðsýnapenna fyrir og eftir 6 vikna meðferð. Psoriasis Area Severity Index (PASI) gildi var metið fyrir og eftir meðferð ásamt Trozak gildi hvers sýnis, sem segir til um sjúkdómseinkenni í vef. Sýnin voru síðan fryst í OCT, skorin í örþunnar sneiðar og merkt með flúrljómandi mótefni gegn LL-37 sem og IL-10. Einnig var fjöldi $CD3^+$, $CD4^+$ og $CD8^+$ T-frumna í húð fyrir og eftir innlagnar meðferð annars vegar og NV-UVB meðferð hinsvegar ákvarðaður með vefjalitun.

Fjöldi $CD3^+$, $CD4^+$ og $CD8^+$ T-frumna lækkaði marktækt í hópnum sem lagðist inn í Bláa Lónið ($p=0.0255$, 0.0282 and 0.0234) og sýnir fylgni við Trozak gildi sýnanna ($p=0.0016$, 0.0077 and 0.0004). Ekki fékkst tölfræðilega marktæk fylgni milli fjölda T-frumna og PASI gildis. Fjöldi $CD4^+$ T-frumna lækkaði einnig marktækt í NB-UVB hópnum ($p=0.0089$) en tilhneiging var til fækkunar á $CD3^+$ og $CD8^+$ T frumum án þess að ná tölfræðilegu marktæki ($p=0.0600$ og 0.0727).

Engin breyting sást á tjáningu IL-10 fyrir og eftir meðferð, hvorki með einkunnakerfinu ($p=0.8422$) né með notkun ImageJ til magngreiningar ($p=0.3394$).

Staðsetning LL-37 litunar í húð sýnir tölfræðilega marktæka fylgni með Trozak gildi sjúklinga ($p<0.0001$) en fylgni við PASI gildi nær ekki tölfræðilegu marktæki ($p=0.1248$) með notkun einkunnakerfisins. Við upphaf meðferðar sýndu einstaklingar samfellda/dreifða LL-37 tjáningu í húð frá yfirborði hornlags alla leið niður í neðri frumulög yfirhúðar (stratum spinosum). Í þeim tilfellum þar sem sjúkdómseinkenni minnkuðu eftir meðferð var tjáningarmynstrið orðið allt annað og takmarkaðist við neðsta lag epidermis, þ.e stratum basale. Þetta tjáningarmynstur sést einnig í heilbrigðu sýni sem fengið var til viðmiðs.

Samkvæmt okkar niðurstöðum er það greinilegt að fjöldi T-frumna í húð sórasjúklinga lækkar með meðferð sem er í samræmi við gögn sem hópurinn okkar hefur gefið út sem sýnir lækkun í T-frumum í blóði sjúklinga eftir meðferð. Sú niðurstaða að IL-10 tjáningin hafi ekkert breyst við meðferð kemur á óvart þar sem við hefðum búist við að sjá aukna tjáningu eftir meðferð. Gögnin sem fengust í þessari rannsókn sýna að greinileg breyting er á LL-37 tjáningarmynstri þegar sjúkdómseinkenni minnka. Þessi staðsetning LL-37 í neðsta frumulaginu hefur ekki verið sýnd áður.

Abstract

Psoriasis is a common chronic skin disease accompanied by many comorbidities and a reduced quality of life. One of the factors defining the pathogenesis of psoriasis is the increased expression of antimicrobial peptides of the innate immune system. The antimicrobial peptide LL-37 is an important defender against infection while also playing a part in activating the immune system in the skin. In psoriasis, expression of this peptide is greatly increased in the skin and is connected to the maintenance of the inflammatory cycle. Expression patterns of LL-37 in psoriatic lesions have not been determined before. Knowledge of the location and distribution of the expression of LL-37 in the skin of psoriasis patients along with potential correlation with clinical scores or expression of other inflammatory markers might lead to increased understanding of the pathophysiology of the disease.

Skin samples were obtained from psoriasis patients participating in a study on the effects of the Blue Lagoon psoriasis treatment. Patients were randomly divided into three different treatment groups. One group received an in–patient treatment at the Blue Lagoon spa along with NB-UVB treatment, one group received out-patient treatment at the Blue Lagoon spa clinic along with NB-UVB treatment and the third group received NB-UVB treatment alone as a control. Punch biopses were collected from patients before and after 6 weeks of treatment. Psoriasis Area Severity Index (PASI) was determined for the samples at each time point along with the histological Trozak score. The samples were then frozen and cryosectioned before immunohistochemistry was performed. Samples were stained with a fluorescent antibody against LL-37 and IL-10. The number of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes was also determined before and after in-patient treatment and NB-UVB treatment alone using immunohistochemistry.

The number of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells was significantly reduced in patients receiving the in-patient Blue Lagoon treatment ($p=0.0255$, 0.0282 and 0.0234 respectively) and showed correlation with the Trozak score of the patients ($p=0.0016$, 0.0077 and 0.0004 respectively) while no statistically significant correlation was seen between T-cell numbers and PASI score. The NB-UVB group showed a significant reduction in $CD4^+$ T-cells ($p=0.0089$) and a trend for reduction in $CD3^+$ and $CD8^+$ T-cells without reaching statistical significance ($p=0.0600$ and 0.0727 respectively).

No change was seen in the expression of IL-10 before and after treatment using a grading system ($p=0.8422$) or the ImageJ software ($p=0.3394$) for quantification.

The expression pattern of LL-37 shows significant correlation with the Trozak scores of patients ($p<0.0001$) while a correlation between expression pattern and PASI score did not reach statistical significance ($p=0.1248$) using the grading system for LL-37 expression. At the start of treatment the expression of LL-37 is diffused throughout the epidermis, from the horny layer (stratum corneum) down to the lower cell layers. The expression pattern in patients showing progress in disease severity after treatment changed to basal layer (stratum basale) only. This basal layer expression was also seen in the one healthy control sample we had.

Thus, according to this study it is clear that the number of T-cells in the skin of psoriasis is reduced following treatment. This is in accordance with previously published data including work done by our

group showing a reduction in circulating T-cell numbers. The lack of change in IL-10 expression before and after treatment came as a surprise as we would have expected an increase in expression. The data obtained in this study also makes it clear that LL-37 expression changes as histological severity goes down. The localization of LL-37 in the basal layer of the epidermis after treatment has not been demonstrated before.

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Abbreviations

AMP – Antimicrobial peptide

APC – Antigen presenting cell

cDC – Conventional Dendritic cell

CLA - Cutaneous Lymphocyte-associated Antigen

hCAP-18 – Human Cathelicidin Antimicrobial Protein-18

IFN-γ – Interferon gamma

IL – Interleukin

NK cell – Natural killer cell

PAMP – Pathogen-associated molecular pattern

PASI – Psoriasis Area Severity Index

pDC – Plasmacytoid dendritic cell

PRR – Pattern recognition receptor

qPCR – Quantitative Real-Time polymerase chain reaction

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor alpha

1 Introduction

1.1 The skin

The skin is the largest organ of the body, covering around $2m^2$ of surface area; it is also our first line of defense against chemical and microbiological agents, protecting the body from the plethora of bacteria, fungi, viruses and parasites that we are constantly in contact with (2).

The skin is divided into two structurally different components: the epidermis, consisting of keratinocytes connected by tight junctions, who make up the waterproof outer barrier of the skin and produce a steady stream of antimicrobial peptides (AMPs) to help fight against invading pathogens, and the collagen-rich dermis which forms the connective layer of the skin (Fig. 1) (2, 3).

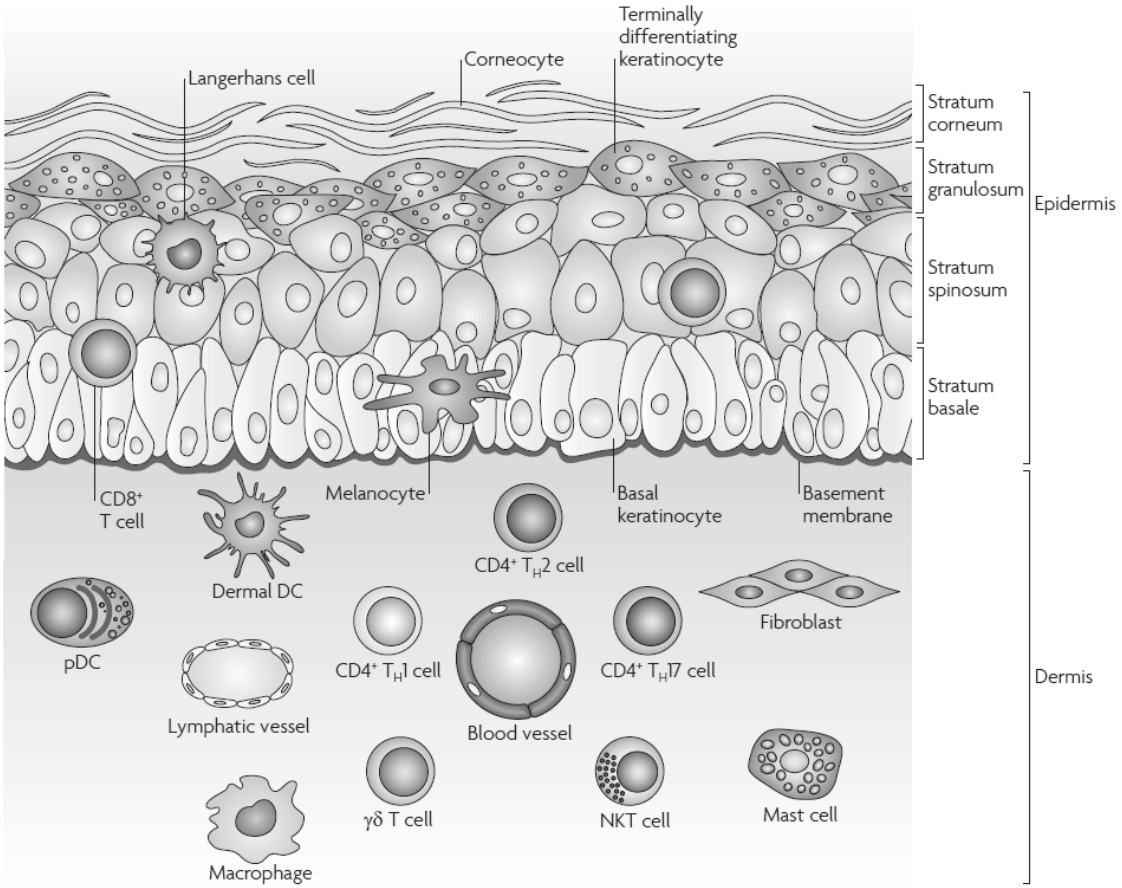

Figure 1. Cells of the skin. The upper layer of the skin, the epidermis consists mostly of stratified layers of keratinocytes along with melanocytes and the resident immune cells; $CD8^+$ T-cells and Langerhans cells. The dermis has a more diverse cell population. The largest portion of the cells of the dermis are fibroblasts and macrophages but other cells of the immune system, such as dendritic cells, mast cells and T-cells are common (4).

1.1.1 Epidermis

The epidermis is a continually renewing stratified epithelium largely composed of keratinocytes. These cells change their appearance as they move through the different layers of the epidermis starting just above dermis in the basal layer (stratum basale). The keratinocytes in the basal layer are the only ones that divide and are responsible for the continuous renewal of the epidermis. Though most of these cells maintain a resting or quiescent state, they are ready to start proliferating if required, for example during wound healing (2). The next layer is the spinous layer (stratum spinosum) and as the cells move from the spinous to the granular layer (stratum ganulosum) the cells flatten considerably and increase in size, forming tight junctions. It is the granular cell layer that forms the waterproof barrier of the skin. This is also the last layer in which the cells are alive. The outer most layer of the epidermis is called the horny layer (stratum corneum) which consists of completely flattened keratinocytes whose intracellular organelles have disappeared completely (3). This is the layer that plays the biggest part in shielding the host from potentially hazardous external agents, though the lower layers of the epidermis provide important components as well (2).

The keratinocyte is the predominant cell type found in the epidermis. These cells synthesize and express various structural proteins and lipids during their maturation. In the healthy epidermis there's a balance between the processes of proliferation and desquamation that result in a complete renewal approximately every 28 days. This delicate balance must be maintained as some skin disorders arise from alterations in these processes. Increased proliferation rates for example are seen in inflammatory skin disease, such as psoriasis. This leads to further disturbance in cell differentiation and the formation of parakeratotic scales, in which the keratinocytes in the uppermost layers of the epidermis retain their nuclei (2).

The epidermis consists of more cell types than just keratinocytes. Melanocytes are found in the epidermis along with the antigen presenting Langerhans cells and the occasional T cell residing in the basal and spinous layers. Residential epidermal T-cells are often found in close proximity to Langerhans cells. While T-cells are normally found in small numbers in the epidermis their numbers are increased during inflammation or infection when they infiltrate the skin as a response to invaders. This increased infiltration is also seen in auto inflammatory diseases (4, 5). The T-lymphocytes normally found in human skin have already been presented with an antigen and are mostly found in hair follicles and thus are more frequent in skin where hair follicles are more abundant (6).

1.1.2 Dermis

The dermis is located beneath the epidermis and is rich in collagen and elastin fibers. The main cells of the dermis are fibroblasts and macrophages (2), however the cell diversity in the dermis is quite a bit greater than in the epidermis, it is also much more dynamic due to easy acces to the bloodstream. Along with the fibroblasts and macrophages, the dermis contains immune cells such as dendritic cells and various types of T cells (4).

1.2 Immunity of the skin

Immunity of the body is divided into innate immunity and adaptive immunity. Both are essential for identifying and killing invading pathogens but the way in which they do so differs drastically. The adaptive immunity reacts in a targeted manner after induction by a specific immune stimulus to which the organism has previously been exposed while innate immunity is an immediate, nonspecific, and diverse response that protects against a wide range of invading microorganisms. The innate immune system also serves a role in summoning agents of the adaptive immune system to help fight off the invaders. Even though the adaptive immune system reacts more specifically and efficiently it takes days to activate. Meanwhile the innate immune system fights off the invading pathogens (7, 8). The two immune systems also differ in another key feature, in that the innate immune system does not have a memory while the adaptive immune system forms memory T and B cells specific to invaders already defeated, resulting in the adaptive response getting stronger with repeated exposure. This means that if the body encounters these pathogens again the adaptive immune system knows how to fight them and the induction period of the adaptive immunity is shortened (9). The immune response is highly dependent on cytokine secretion. Cytokines are secreted by the cells of the immune system along with other cells and can be divided roughly into two major groups based on their function; the anti-inflammatory cytokines such as interleukin 10 (IL-10), and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (7). However, cytokines are pleiotropic by nature and can have different effects depending on the situation and location of their expression so there is some fluidity in the classification of cytokines as either pro- or anti-inflammatory. IFN-γ for example is classified as a pro-inflammatory cytokine based on the effects it has on TNF-α but also has antiviral activity and serves a role in activating the pathway leading to cytotoxic T-cells (10).

IL-10 is one of the most potent cytokines of its kind and is produced by cells of both the adaptive and the innate immune system. Its potent immunomodulatory effects stretch throughout the entirety of the immune system as it can prevent production of pro-inflammatory cytokines, antigen presentation and cell proliferation (11, 12). The importance of this cytokine has been demonstrated in the fact that IL-10 deficient mice spontaneously develop a fatal inflammatory bowel disease as a result of the imbalance between the two groups of cytokines (10). In healthy skin, IL-10 expression is low and is confined to the keratinocytes in the basal layer of the epidermis (13). However in the event of injury such as tape stripping or poison ivy, keratinocytes in the epidermis begin secreting IL-10 as a response to the accompanying inflammation (14).

1.2.1 Innate immunity of the skin

The human skin represents the first line of defense against potentially hazardous environmental threats, such as infection by microbes, and is also the largest organ of the body, functioning as an interface between the human host and the environment. The skin has several key elements working for the innate immune system. These include AMPs and the recruitment and activation of leukocytes, whose role it is to kill or phagocytize invading microorganisms (2). The balance of the immune response is critical to keep the host safe, since an overreactive immune system can potentially lead to

autoimmune and inflammatory diseases while a lacking immune response can lead to recurring infections and tumor growth (4).

The innate immune system has evolved to launch an immediate response when sensing invading pathogens and to destroy them before they infect cells, to kill already infected cells or, if needed, keep the pathogen population in check until the adaptive immunity has been activated. In order to eliminate pathogens before they infect cells the innate immunity has AMPs whose role is to destroy pathogens (15). A characteristic of the innate immune system is the recognition of pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). PAMPs are highly conserved and are essential for the survival or function of the pathogens, these include the lipopolysaccharide from gram-negative bacteria and peptidoglycans from gram-positive bacteria (16). Another integral part of the innate immune system are the phagocytic cells that consume the pathogens. There are three main types of phagocytic cells in the body, macrophages/monocytes, dendritic cells and granulocytes. The granulocytes include basophils, eosinophils and the neutrophils which are the most common cells of the innate immune system (8). These defenses aren't infallible and should they fail to neutralize the pathogens; the innate immune system has natural killer cells (NK cells) whose role is to identify and kill already infected cells. These cells play a crucial role in maintaining control over pathogens until the cytotoxic T cells of the adaptive immune system can take over (9). This immediate response is then followed by an induced phase of the innate immune system. In the induced phase invading pathogens are met with an inflammatory response which prevents the pathogens from spreading through the bloodstream. This is achieved by inducing clotting in small blood vessels close to the site of infection while simultaneously recruiting effector cells and molecules out of the bloodstream and into the tissues through chemokines (7).

1.2.1.1 Antimicrobial peptides

One of the oldest lines of defense against invading pathogens such as bacteria, fungi and viruses are the antimicrobial peptides (17), a diverse group consisting of over 1700 known members throughout the biosphere (18). AMPs are ever present in the skin but expression by keratinocytes is up-regulated as a response to damage in epithelial surfaces where they strive to limit microbial invasions. This increased production can be attributed to recruited neutrophils and other leukocytes. In healthy skin keratinocytes are the main source of AMPs, where they are stored within lamellar bodies in the cells and secreted once the keratinocytes reach the stratum granulosum of the epidermis, along with other proteins and lipids important for barrier function (18). Antimicrobial peptides have built-in mechanisms which allow them to target pathogens. This includes a cationic and amphipathic structure giving them the ability to disrupt microbial membranes (17), as well as their net positive charge which allows them to interact with the anionic components of viruses and fungi and the negatively charged phospholipid bilayer of bacteria. The AMPs insert themselves into this bilayer and form trans membrane pores, which leads to bacterial lysis of both Gram negative and Gram positive bacteria (18). There are three major classes of antimicrobial peptides found in mammals; defensins, histatins and cathelicidins (17).

Cathelicidins were the first AMPs found in mammalian skin. In humans, cathelicidin is mostly found in granules within keratinocytes of the epidermis as well as in the extracellular spaces of the stratum

corneum (19). Other sources of cathelicidin are neutrophils and mast cells in the dermis (20) and the sweat glands in the skin (21). The human cathelicidin gene is responsible for encoding the 18 kDa α-helical precursor protein, human cathelicidin antimicrobial protein (hCAP-18). The protein is comprised of two different parts; the N-terminal cathelin like prodomain region and a structurally variable cationic C terminal antimicrobial peptide, which needs to be cleaved from the pro-peptide before activation (fig. 2) (18).

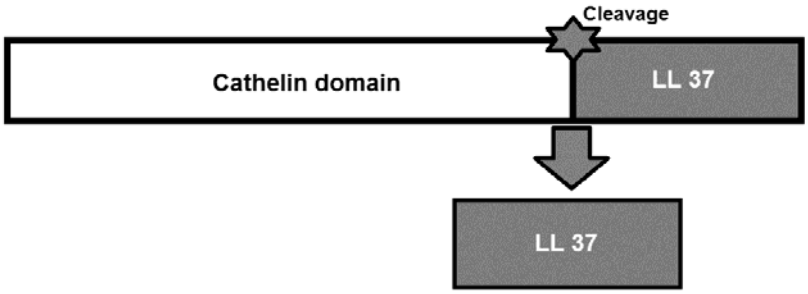

Figure 2. hCAP-18 precursor protein cleavage into LL-37 by proteases. The full length protein is considered inactive and has to be cleaved by proteases such as serine proteases or neutrophil proteases in order to release the active peptide LL-37.

The only cathelicidin expressed in humans is a 37 amino-acid peptide beginning with two leucine recidues, giving the name LL-37. hCAP-18 is thought to be inactive in its uncleaved form and is reliant on proteases to release the mature peptide LL-37. The proteases responsible are the serine proteases kallikrein 5 and 7 found in keratinocytes and the neutrophil proteases such as serine protease 3 among others (fig. 2). Unlike uncleaved hCAP-18, processed LL-37 demonstrates fast, potent, broad-spectrum antimicrobial activity as well as possessing the ability to kill Gram-negative and Gram-positive bacteria, fungi and viruses (18). Additionally, it has been shown in vitro that LL-37 can act as a chemoattractant for neutrophils, monocytes and T-cells (17).

The fact that cathelicidin is expressed as a precursor protein which requires cleaving in order to function as an antimicrobial peptide allows for a very stable and specific control of its activity as a response to certain stimuli (18).

1.2.1.2 Toll-like receptors

Toll-like receptors (TLRs) are one of the PRRs responsible for recognizing PAMPs, leading to the activation of host cell signalling pathways and responses by the innate and adaptive immune systems (4). They are type I transmembrane proteins evolutionarily conserved among insects and vertebrates. At least 10 members of the TLR family have been identified in humans (22). Different subfamilies of TLRs sense different PAMPs; TLRs 3, 7, 8, and 9, belong to the same subfamily and are found within endosomal compartments in the cell where they recognize pathogen-derived nucleic acid components (17).

TLRs play an important role in the immunity of the skin and are responsible for most of the immune functions of keratinocytes. Keratinocytes have the ability to distinguish between invading pathogens and the harmless organisms living in and on the body and mount a response against the former

through the use of TLRs located either inside endosomes, such asTLR3 and TLR9 or on the cell surface like TLR1, 2, 4, 5 and 6 (4).

1.2.1.3 Dendritic cells

Dendritic cells are the bridge between the innate and adaptive immune system. They are derived from the bone marrow and are found in every part of the body. They are the most common antigen presenting cell (APC) in the body and serve to prime and activate T-cells to mount a response against invading pathogens (23). In healthy skin there are two major types of dendritic cells, the dermal myeloid dendritic cells, also known as conventional dendritic cells (cDCs) and the epidermal Langerhans cells who are vital for antigen-specific skin immunity (8). A third type of dendritic cell which is rarely found in healthy skin is the plasmacytoid dendritic cell (pDC).

cDCs account for most of the dermal dendritic cells. These cells can be further subcategorized by their surface markers with each subset having a unique specialization. They are short-lived and are replaced by new cells on a regular basis (24).

Langerhans cells play a role in Th2 cell differentiation and are the first type of dendritic cell that microbial antigens come into contact with. They also have the ability to prime and cross-prime naïve CD8+ T-lymphocytes (4).

pDCs comprise a dendritic cell population highly specialized for sensing viral infection. In contrast to cDCs, pDCs uniquely express TLR7 and TLR9 which recognize viral DNA and RNA within endosomal compartments. In response to TLR7 and 9 activation, pDCs are capable of producing very large amounts of type I interferons which play a central role in the initiation of antiviral defense through replication restriction of the virus and initiation of the adaptive immune response (17). These cells are normally absent from the skin but enter from the blood stream when inflammation occurs. They are also found in the skin in autoinflammatory diseases and skin tumors (1).

1.2.2 Adaptive immunity of the skin

The response of the adaptive immune system is reliant on proper function of the innate immune system. APCs are responsible for encouraging the initial adaptive response. The APCs present antigens to appropriate lymphocytes primed to recognize the antigens (8). They also serve a role in immunomodulation, secreting anti-inflammatory cytokines such as IL-10 which inhibitis antigen presentation to T-cells (11).

T-cells comprise one group of lymphocytes in the body. They, along with the B-cells belong to the adaptive immune system. A third type of lymphocyte, NKs, belong to the innate immune system (8) though recent studies suggest that these cells have both important innate and adaptive abilities (25).

T-cells can be divided into cytotoxic $CD8^+$ T cells whose role it is to kill infected cells, helper $CD4^+$ T cells who send out signals to influence the behaviour of other cells such as B cells, and regulatory T cells who serve to control other cells and dampen immune responses (9). These three cell groups can then be further subcategorized, such as the T helper cells being divided into for example the Th1, Th2 and Th17 subsets (26) and the Tc17 subset of $CD8^+$ T-cells (27). Each subset has its own cytokine expression pattern related to their specific cell fates (26).

The Th17 subset of T-cells is relatively newly recognized, and its name stems from the cytokine IL-17 which is generated by this subset of cells along with IL-22 (26). Th17 cells take part in host defense where they help protect against extracellular bacteria and fungi (26). In concurrance with its active role in the immune system, Th17 has been shown to participate in numerous autoimmune diseases such as MS and psoriasis (28).

Another new addition to the T-cell family are the dermal γδ T-cells found in the skin. These cells, like the Th17 subset are a great producer of IL-17. However, this subset of T-cells, unlike most others, belongs to the innate immune system (29).

1.3 Psoriasis

Psoriasis is a common autoimmune disease of the skin affecting 2-4% of the population of western countries. Higher prevalence of the disease is seen at higher latitudes with Caucasians being more prone than other ethnic groups (30). There are several different forms of psoriasis ranging in features and severity with plaque psoriasis being the most common form, accounting for approximately 90% of affected individuals (31). This form is characterized by well-defined round or oval plaques that differ in size. Plaque psoriasis lesions are generally found on the arms, legs, scalp, buttocks and trunk. Inverse psoriasis is less scaly than plaque and occurs in skin folds such as the ones found under the breasts, joints, perineum, armpit and other areas (30). Erythodermic psoriasis is characterized by wide-spread generalized erythema, involving over 90% of the skin, and is often associated with systematic symptoms (31). The localized form of pustular psoriasis consists of pustules on the palms and soles, without plaque formation. The von Zumbusch variant, a severe and acute form can cause life-threatening complications. Guttate psoriasis is more common in patients younger than 30 years, and lesions are usually located on the trunk (30).

The symptoms of psoriasis can be mild, such as a few scaly red plaques, or so severe that plaques cover nearly the entire surface of the body (32). New lesions may appear on patients as a direct result of injury to the skin; this effect is known as the Koebner phenomenon (33). There are several different ways to assess the severity of psoriasis. Psoriasis area and severity index (PASI) uses the features of psoriatic plaques such as redness and thickness along with the body surface area covered by plaques to quantify disease severity (34) and is the most used method to determine the effectiveness of clinical trials (35). PASI is scored on a scale of 0-72 with 72 being the most severe form. The usual way in which clinical trials apply PASI is by measuring the percentage of improvement before and after treatment. The most common endpoint of clinical trials evaluating treatment methods is the percentage of patients who achieve PASI75 which is a 75% or greater reduction in the PASI score (35). Histological features are also sometimes graded using Trozak's histological grading system on skin biopsies. It grades the severity of several predetermined features of psoriatic skin such as elongation of rete ridges and parakeratosis with each feature scored from 1-3 and the cumulative score describes the severity of histological features (36).

1.3.1 Pathogenesis and histological features

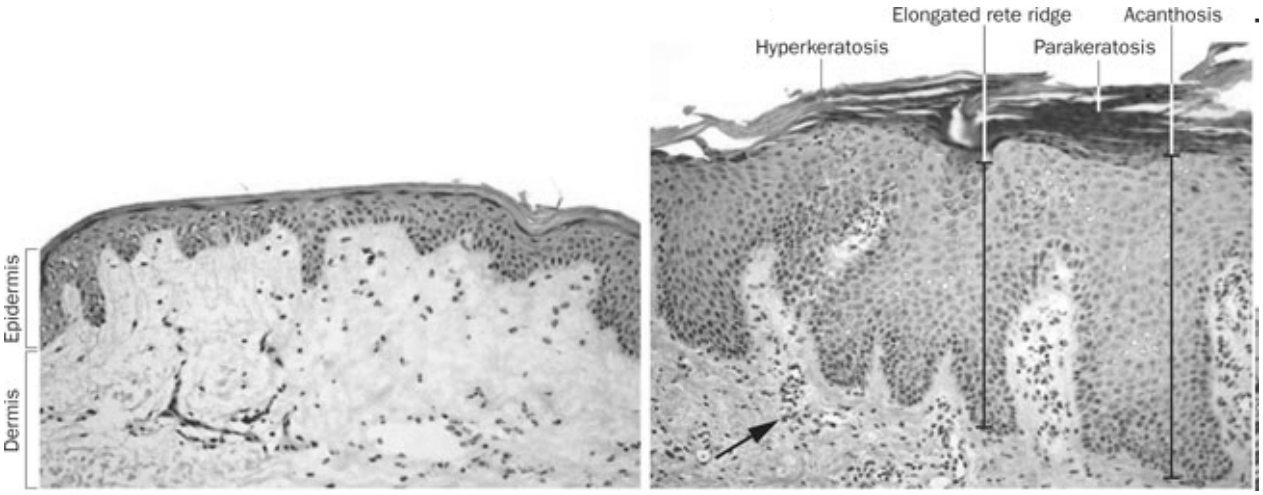

Figure 3. Histological changes in psoriasis lesions (right) compared to healthy skin (left).

Changes in skin accompanied by psoriasis are hyperkeratosis, elongation in rete ridges, parakeratosis, acanthosis and infiltration of immune cells into the dermis and epidermis (marked by the arrow) (37).

Histological features of psoriasis include increased thickness of the epidermis (acanthosis) with elongated epithelial extensions projecting into the underlying connective tissue, also known as rete ridges (fig 3. right). This increase is caused by the abnormal behaviour of keratinocytes in psoriatic plaques. They hyperproliferate and don't undergo normal differentiation as the granular layer, where differentiation normally starts, is greatly reduced or absent in psoriasis while the horny layer is greatly increased (hyperkeratosis). They also retain their nucleus (parakeratosis) which is degraded along with all other organelles in healthy skin (37, 38). These keratinocytes then produce a substantial amount of antimicrobial peptides and proteins which contribute to the autoinflammatory cycle (29).

Another key feature of psoriasis is the infiltration of cells of the immune system such as T-cells, dendritic cells, macrophages and neutrophils into both the dermis and the epidermis of the skin (37, 39). This leads to an altered cytokine profile in both the sera of patients and lesional skin. In skin this is defined by an over expression of pro-inflammatory cytokines such as TNF-α and IFN-γ while the expression of anti-inflammatory cytokines such as IL-10 is low and can't counteract the inflammation (11). In the serum however there is a marked increase in IFN-γ while TNF-α levels remain the same as in healthy controls and IL-10 is virtually undectable compared to controls (40).

1.3.2 IL-23/Th17 pathogenic axis in psoriasis

T-cells play a central role in the pathogenesis of psoriasis as they contribute to inflammation by producing a variety of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-12, IL-17A, IL-17C, IL-17F, IL-22 and IL-23 (39). T-cells infiltrate the skin in psoriasis and research has shown a marked increase in Th17 cells in the dermis of psoriasis patients (41) as well as in the circulation along with Th1 and Th22 cells (42). The importance of T-cells in psoriasis is demonstrated by the fact that T-cells from psoriasis patients are capable of transmitting the disease to animal models (26).

Originally, psoriasis was classified as a Th1 mediated disease as IL-12 was thought to be an integral part in the pathogenesis of psoriasis. It was later discovered that IL-23, a heterodimer with the same p40 chain as IL-12 is an important element in psoriasis as IL-23 is up regulated in psoriasis and IL-12 is not. IL-23 is highly expressed by keratinocytes and dendritic cells in psoriatic lesions and is responsible for inducing differentiation of naïve $CD4^+$ T-cells into pathogenic Th17 cells (26). After the discovery of IL-17 and IL-23 the disease was reclassified as Th17 mediated with a focus on the IL-23/Th17 axis (43). While most research considering IL-17 focuses on Th17 cells there is a subset of $CD8^+$ T-cells who also express IL-17, this subset is called Tc17 (27). Psoriasis also shows a marked increase in γδ T cells when compared to healthy skin and these cells are also capable of producing great amounts of IL-17 (43). IL-17 is expressed by non T-cells such as neutrophils and mast cells in the skin of psoriasis patients but IL-17 positive mast cells have also been found in healthy skin (27). The cytokine IL-17 has a major effect on the cells involved in psoriasis, inducing production of many cytokines, chemokines and antimicrobial peptides (43). In psoriasis, IL-17A for example is responsible for keratinocyte chemokine expression (44) and decreases adhesion in the skin causing a disruption in the barrier function of the skin (45). The most common member of the IL-17 family at the protein level in psoriatic lesions is IL-17C (46). IL-17C is produced by epithelial cells and it serves an important role in inflammation through expression of proinflammatory cytokines, chemokines and antimicrobial peptides (47). It has also been shown to promote the development of Th17 cells which respond to IL-17C by upregulating the expression of IL-17A and IL-17F (48). IL-17F is abundantly expressed in psoriatic lesions and mRNA levels have been shown to correlate with disease activity. IL-17F serves to attract neutrophils in psoriatic lesions (26).

1.3.3 Proposed mechanism of LL-37/nucleic acid interaction in psoriasis

Under normal circumstances expression of cathelicidin in skin is low and not in the form of the cleaved peptide LL-37 (39). However, during injury or infection the expression of activated LL-37 is greatly increased. This happens through secretion by both resident keratinocytes and infiltrating neutrophils. Keratinocyte production of hCAP18/LL-37 is dependent on vitamin D_3, TLR2 and TLR6 while neutrophils constantly express hCAP18/LL-37 in their granules and release it by degranulation. The enzymes responsible for cleaving and activating the peptide are expressed by cells attracted by inflammatory signals. This dramatic increase in LL-37 production and activation happens very fast and lasts only a short while (17). In psoriasis however the chronic expression and proteolytic cleavage of cathelicidin into LL-37 is proposed to maintain inflammation through its interaction with self-DNA/RNA which is continuously being released by dead or dying cells (39). This interaction forms a complex between LL-37 and self-DNA which seems immune to extracellular degradation. pDCs along with monocytes in the skin sense this complex through TLR9 after it has been internalized by these cells (17, 49), activating them to produce large quantities of type I IFN, thus leading to the activation of cDCs. This event further activates the autoinflammatory cascade by inducing Th1/Th17 differentiation and keratinocyte activation (fig. 4) (39). It has been demonstrated that keratinocytes in psoriatic lesions show an increased TLR9 protein expression compared to healthy skin and that this expression is increased in the presence of LL-37. It has also been shown that keratinocytes respond to DNA by releasing type I IFNs in a LL-37/DNA dependant manner similar to pDCs (50). LL-37 has been

reported to interact with self-RNA in a similar manner. The LL-37/self-RNA complex formed is resistant to degradation by RNase and is internalized by both pDCs and cDCs leading to pDC activation through TLR7 and direct cDC activation through TLR8 leading to the secretion of TNF-α and IL-6 along with the differentiation into mature dendritic cells (51).

The interaction between LL-37 and self-DNA is not unique to psoriasis, it has also been demonstrated that keratinocytes in cholesteatoma respond to self-DNA in a LL-37 dependant manner leading to increased IFN response (52). The same kind of interaction has been demonstrated in atherosclerotic lesions (53). It has also recently been demonstrated that LL-37/DNA complexes affect NK cells *in vitro* through the type I IFN response by monocytes leading to an increased production of IFN-γ and cytotoxicity of NK cells (54).

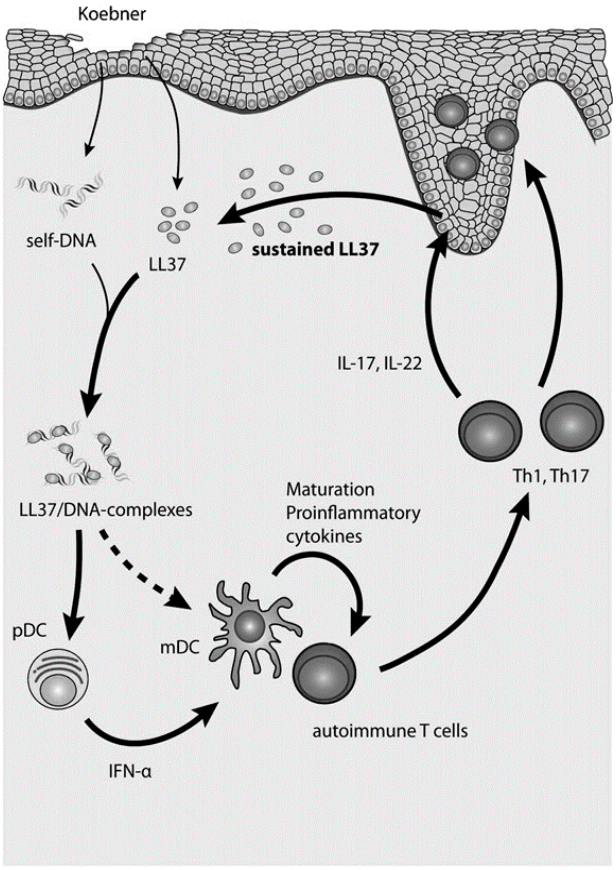

Figure 4. Proposed mechanism of interaction between LL-37/DNA complexes and pDCs in psoriasis (1).

1.3.4 LL-37 as a T-cell autoantigen

It has long been known that psoriasis is mediated by T cells but their specific autoantigens have remained elusive. Studies have shown that keratins, proteins produced by keratinocytes, show autoantigen activity in some psoriasis patients who suffer from frequent streptococcal throat infections. This activity is likely caused by molecular mimicry as keratins and streptococcal protein M share some sequence homologies (55). Several other keratinocyte proteins such as ezrin, maspin and heat shock protein 27 have been linked to increased T-cell activation in psoriasis patients. These proteins also share some sequence homologies with proteins associated with streptococcal infections (56).

A recent study has identified LL-37 as an auto-antigen in psoriasis. LL-37 specific T-cells were found in up to 75% of patients with severe cases of psoriasis. These T-cells are found in both CD4+ and CD8+ populations. The LL-37 specific T-cells produce IFN-γ and Th17 cytokines which are well known as driving factors in the pathogenesis of psoriasis. The auto-antigen function of LL-37 is probably not caused by molecular mimicry but rather by the break of T-cell tolerance caused by overexpression and subsequent effects of LL-37 in psoriasis (57).

1.3.5 Causes

Psoriasis has as genetic component and approximately one-third of patients with psoriasis have a first degree relative with the condition (30). Research suggests a multifactorial mode of inheritance.

Many stressful physiological and psychological events are associated with the onset and worsening of the condition. Direct skin trauma can trigger the formation of psoriatic lesions and streptococcal throat infection can also trigger the condition or exacerbate existing disease (32). The connection of streptococcus infections and psoriasis lead to several studies assessing whether tonsillectomy could prove beneficial to psoriasis patients and found that it may reduce symptoms in patients who show disease exacerbation at the time of streptococcal throat infection (55, 58). Smoking increases the risk of psoriasis and disease severity, as do obesity and excessive alcohol use and abuse (32).

1.3.6 Comorbidities

In addition to the symptoms of psoriasis, patients can also suffer from a number of comorbidities. The most common one, affecting 20-30% of psoriasis patients, is psoriatic arthritis of the joints. The symptoms of psoriatic arthritis, like those for psoriasis, vary in severity from mild symptoms such as stiff or tender joints to crippling arthritis essentially rendering the joints useless (35).

Patients with psoriasis are at increased risk of a variety of medical conditions such as depression, Crohn's disease or ulcerative colitis, lymphoma and squamous cell carcinoma, obesity and myocardial infarction (30). Social isolation may also contribute to increased risk of certain medical conditions that are mediated by exercise and lifestyle factors (32).

Not only do psoriasis patients have to live with the potentially crippling physical comorbidities, but their psychological wellbeing and overall quality of life are affected by the disease as well. Studies have shown that nearly 60% of the psoriasis patients consider their quality of life affected by the disease in a major way, regardless of disease severity. Large studies focused on the psychological state and work productivity impairment of patients have shown that over 80% of participants found psoriasis to affect their overall enjoyment of life with feelings of anger and helplessness being common. These studies also showed that of the patients who were employed nearly half regularly missed work because of the disease and of those who were unemployed, over 90% claimed that psoriasis or psoriatic arthritis were the reasons behind their unemployment. So even with recent advances, psoriasis still has major economic consequences to patients (59).

1.4 Treatment options

A number of therapeutic options to treat psoriasis are available with the most common line of treatment for patients with mild symptoms being topical agents such as corticosteroids and vitamin D analogues as well as oral immunosuppressants such as methotrexate and cyclosporin (43). The topical agents mentioned are ineffective for patients with more severe symptoms however and treatments targeted for that group usually involve phototherapy and the use of biological agents against different targets (60).

Current gold standard biologic therapies targeting anti-tumor necrosis factor alpha (TNF-α) and anti-IL-12/23p40, which inhibits both IL-12 and IL-23, have dramatically improved clinical outcomes in patients with psoriasis with improved short term safety profiles compared to widely used systemic agents. However, current biological agents still have the potential to cause significant side effects in a subset of patients and their long-term safety have yet to be fully evaluated (60, 61).

1.4.1 Biological agents

Biological agents are widely used to treat psoriasis. These are agents designed specifically to block certain pathways in the autoinflammatory cascade (61).

The TNF-α pathway is an established target for psoriasis treatment with current licensed anti-TNF medications including infliximab, adalimumab, and etanercept. Anti-TNF agents have been used to treat over 2 million patients with immune-mediated diseases for extended durations (32). This method was the first anti-cytokine treatment used to treat psoriasis but for a long time before that it was used to treat rheumatoid arthritis. What this treatment seems to do is target the regulation of antigen presenting cells as dendritic cells seem less capable of stimulating T cell proliferation when generated *in vitro* in the presence of etanercept. IL-23 produced by dendritic cells is a key factor in T-cell stimulation, so it seems that TNF-α is an activator of IL-23 production and could mean that the anti-TNF-α treatment is suppressing the IL-23/Th17 axis in some way (43). There are agents designed to target IL-23 directly. Ustekinumab is a blocker directed at IL-12 and IL-23 which has shown superiority to the TNF-α blocker etanercept in patients with moderate to severe psoriasis (61).

Blocking IL-17 seems to be the most promising anti-psoriasis strategy as patients in studies using these agents have rapidly improved with significant changes seen after 1-2 weeks of treatment, with a vast majority of high dose patients achieving PASI75 by 12 weeks and 75% and 62% achieving PASI90 and PASI100 respectively (43). Secukinumab, an IL-17 blocker that has recently been approved to treat adult patients has been shown to have a more dramatic effect than any of the TNF-α blockers as well as the IL-12 and IL-23 blocker Ustekinumab (62).

1.4.2 The Blue Lagoon Treatment and its effects on psoriasis

Water based therapies, i.e. balneotherapies; have long been used to treat psoriasis. This includes bathing in the Dead Sea in Israel, Palestine and Jordan, the Black Sea in Bulgaria, the Blue Lagoon in Iceland and bathing with the Kangal “doctor fish” in Turkey (63).

The Blue Lagoon was formed in 1976 when water from the newly built geothermal power plant Svartsengi pooled together. An employee of the power plant noticed an improved condition of his psoriatic plaques when he rubbed the white mud from lagoon on them while bathing there. More and more psoriasis patients started bathing in the lagoon in an attempt to cure their condition (64) which eventually led to the opening of the Blue Lagoon Clinic. It has been shown that bathing in the Blue Lagoon has a beneficial effect on psoriasis but in some cases it might not be sufficient as the sole treatment (64). Other studies have shown that the combination of bathing in the Blue Lagoon along with NB-UVB treatment is significantly more beneficial to patients than NB-UVB treatment alone (36, 65). The combined therapy shows faster reduction in Trozak histological score and PASI score, the PASI score showing significant results as early as in week one of treatment. The accelerated improvement of the condition means that fewer exposures and less time is needed to reach at least PASI75 than with NB-UVB treatment alone and patients were less likely to relapse (start another therapy) than those receiving only NB-UVB treatment (36).

The water in the lagoon is composed of 65% seawater and 35% freshwater with a salt content of 2.5% and high silica content. The pH of the water is 7.5 and the average temperature 37°C (36, 66).

The composition of the water in the lagoon causes the ecosystem to be very homogeneous with two organisms accounting for the majority of the microbial community with several other species in low numbers (66). These two major organisms are *Cyanobacterium aponium* and *Silicabacter lacuscaeulensis* and they may, along with the silica mud from the lagoon account for some of the benefits psoriasis patients see from receiving treatment there through an increase in IL-10 secretion by dendritic cells as well as a reduction in IL-17 expression (67). The silica and microalgae from the Blue Lagoon have previously been shown to improve the barrier function of the skin and prevent ageing through increased keratinocyte differentiation (68).

2 Aims

The aim of this project was to study the expression and role of the antimicrobial peptide LL-37 in psoriasis and the effects of Blue Lagoon therapy on inflammation in psoriasis.

2.1 Specific aims

I. The effect of the Blue Lagoon treatment upon immunohistological inflammation in psoriasis.

To determine the effects the treatment has on psoriasis lesions:

- **a.** T cell numbers (CD3, CD8, CD4)
- **b.** Anti-inflammatory markers (IL-10)

II. The role of antimicrobial peptides in psoriasis

a. The effects of Blue Lagoon treatment on LL-37 expression in the skin of patients

3 Materials and Methods

3.1 Blue Lagoon patient samples

Patients in this study were either referred by a dermatologist or answered an advert in the paper. The inclusion criteria was threefold, 1) patients had to have been diagnosed with chronic plaque psoriasis; 2) patients had to have a Psoriasis Area Severity Index (PASI) score (20) of 7 or higher; and 3) patients had to be unresponsive to topical treatment and be candidates for phototherapy or systemic treatment.

Patients were randomized to receive either 1) outpatient treatment for six weeks in the Blue Lagoon which included bathing in the Blue Lagoon for one hour and NB-UVB therapy afterwards three times a week, 2) in-patient treatment for two weeks in the Blue Lagoon which included bathing in the Blue Lagoon for one hour twice a day and NB-UVB therapy daily followed by maintenance NB-UVB therapy three times a week for four weeks or 3) outpatient NB-UVB therapy treatment for six weeks. This group received a regular, monitored NB-UVB therapy three times a week for four weeks.

Disease severity was recorded and skin punch biopsies obtained from lesions on enrolment and after two and six weeks of therapy. Histological changes were evaluated with Trozak's histological score at enrollment and after two and six weeks of treatment.

This study was performed with the approval of the Icelandic National Bioethics Committee and the Icelandic Data Protection Authority.

3.2 Immunohistochemistry

Punch biopsies were cut to 5um slices in the cryotome and put on Starfrost slides. The slides were kept at -80°C until they were used for immunohistochemistry. The patient samples were blinded at this stage with each patient getting a random 4-digit number as their ID.

Sections stained for $CD3^+$, $CD4^+$ and $CD8^+$ T-cells were stained by the LSH Department of Pathology where IHC was performed by clinical standards.

Sections intended for IHC for LL-37, IL-10 and neutrophils were air dried for 10 minutes at room temperature before being fixed in ice cold methanol for 10 minutes. Slides were tapped on paper and then washed in 1x PBS for 5 minutes, three times. Blocking was done in PBS with 5% serum (goat, donkey) in PBS for 30 minutes before incubating with primary antibodies against LL-37 (Polyclonal rabbit anti-LL-37, Innovagen, Pa-LL37100), IL-10 (Anti-hIL-10, RD systems, AF-217-NA), and neutrophils (Monoclonal mouse a-Neutrophil elastase, Dako, M 0752) in PBS with 5% serum (goat, donkey), 0,1% Triton-X solution overnight in a refrigerator at 4°C. Slides were tapped on paper and then washed with 1x PBS for 5 minutes, three times before incubation with secondary fluorescence labeled antibodies for LL-37 (AF goat anti rabbit 488 (H+L), Invitrogen, A11008), IL-10 (Donkey anti-goat IgG (H+L) Dylight 650, Thermo Scientific, SA5-10089), and neutrophils (Alexa Fluor 546 goat anti-mouse IgG1, Life Technologies, A21123) in 5% serum (goat, donkey) in PBS for an hour at room temperature. Slides were then tapped on paper and washed with 1x PBS for 5 minutes, three times. A nuclear stain was performed using DAPI (Invitrogen, D3571) diluted 1/10.000 in PBS for one minute

before washing again three times for 5 minutes in 1x PBS. Slides were mounted with Fluoromount (Sigma, F4680) and left to dry in darkness until they were analyzed in a confocal microscope (Olympus FV1200) at 20x magnification with laser settings always the same. Three fields per sample were used for quantification.

3.3 Analysis and Statistical methods

3.3.1 T-cell counts using Axiovision

Positive CD3, CD4 and CD8 T-cells were counted using AxioVision 4.6.3 in 20x magnification. The samples were divided into three fields and the average number of cells were used for statistical analysis. Cells were counted in both the dermis and the epidermis.

3.3.2 IL-10 expression analysis using ImageJ

Pictures were analyzed using Fiji (ImageJ) 1.50d. The factors used to determine expressions were intensity of the stain and percentage of area stained positive for IL-10. Three fields were analyzed for each sample and the mean of the three counted for the sample. The data was then normalized to week 0.

3.3.3 IL-10 expression analysis using the grading system

IL-10 expression was analyzed using a grading system based on the intensity of the stain with the grades ranging from 1-3. The grade for each sample was the mean of the grade given by the graders for three fields for each sample.

3.3.4 LL-37 expression analysis using ImageJ

Pictures were analyzed using Fiji (ImageJ) 1.50d. For each LL-37 staining there was an isotype control which served as a threshold to measure positive fluorescence in the samples. This threshold was then used to assess the percentage of epidermis stained positive for LL-37. This method was used to evaluate whether there was a change in expression patterns after treatments and to compare expression patterns between treatments.

3.3.5 LL-37 expression analysis using the grading system

LL-37 expression was analyzed using a grading system where expression contained in the basal layer only got the grade *1* or *basal* and expression covering the entire epidermis or undefined expression got the grade *3* or *diffused*. An intermediate grade of 2 was given to samples where expression was mostly located in the basal layer while stretching up further into the epidermis and the average grade given independently by the graders for three fields of each sample was then rounded to either basal or diffused.

3.3.6 Statistical methods

Graphpad Prism 5 version 5.04 for Windows was used for statistical analysis. Statistical methods used were binomial tests, correlation tests, linear regression tests and t-tests where the significance is

set on p=0.05 and p values p≤0.05, p≤0.01, p≤0.001 and p≤0.0001 are symbolized with *, **, *** and **** respectively.

4 Results

4.1 Changes in disease severity and histological scoring

Our research group has previously demonstrated that the number of patients achieving PASI75 (fig. 5A) and PASI90 (fig. 5B) was higher in the groups that received Blue Lagoon treatment (73,1% and 42,3% respectively for the in-patient group (n=27) and 68,1% and 18,2% respectively for the out-patient group (n=23)) than in the control group receiving NB-UVB treatment only (16,7% and 0% (n=24)) at week 6 (fig 5A and 5B). In the case of drop outs the last observation was carried over to week 6 (36).

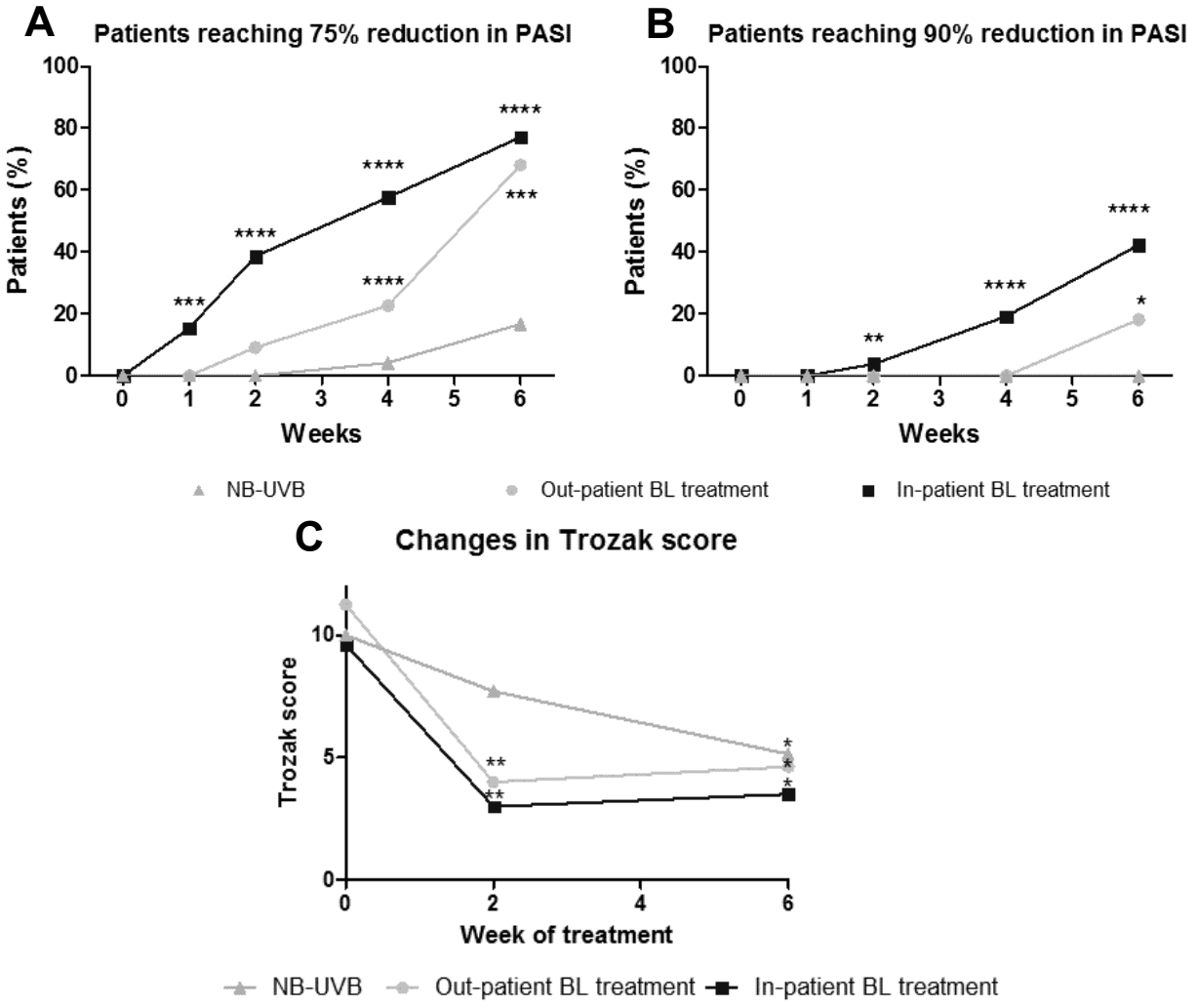

Figure 5. Reduction in disease severity and histological scoring during treatments. PASI score was determined at regular intervals during treatment and Trozak score was evaluated at the times of acquiring punch biopsies. A t-test was performed in order to determine significance. (A) Percentage of patients attaining 75% reduction from baseline PASI score. (B) Percentage of patients attaining 90% reduction from baseline PASI score. (C) Reduction in the histological Trozak score. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

We also demonstrated that the histological score of patients in the groups receiving Blue Lagoon treatments show a faster and more dramatic reduction, with a t-test showing a statistically significant reduction by week two (p=0.0088 for in-patient (n=8) and p=0.0032 for out-patient treatment (n=8)), compared to the NB-UVB group (ns by week two (n=7)). All treatment groups reach statistical significance by week six when compared to week 0 (p=0.0252 for in-patient, p=0.0192 for out-patient and p=0.0074 for NB-UVB treatment) (fig 5C).

4.2 Immunohistochemistry

4.2.1 T-cell count ($CD3^+/CD4^+/CD8^+$)

In order to determine the effects treatments have on the number of T-cells in the skin of psoriasis patients immunohistochemistry was performed and $CD3^+$, $CD4^+$ and $CD8^+$ T-cells counted in the dermis and epidermis of patients undergoing NB-UVB treatment (n=6) and patients receiving in-patient Blue Lagoon treatment (n=5). A t-test showed that the patients receiving in-patient Blue Lagoon treatment showed a statistically significant reduction in $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes in the skin (fig. 6B) while the group receiving NB-UVB treatment showed a statistically significant reduction in CD4+ T-lymphocytes only and a tendency for reduction in $CD3^+$ and $CD8^+$ T-lymphocytes (fig. 6A).

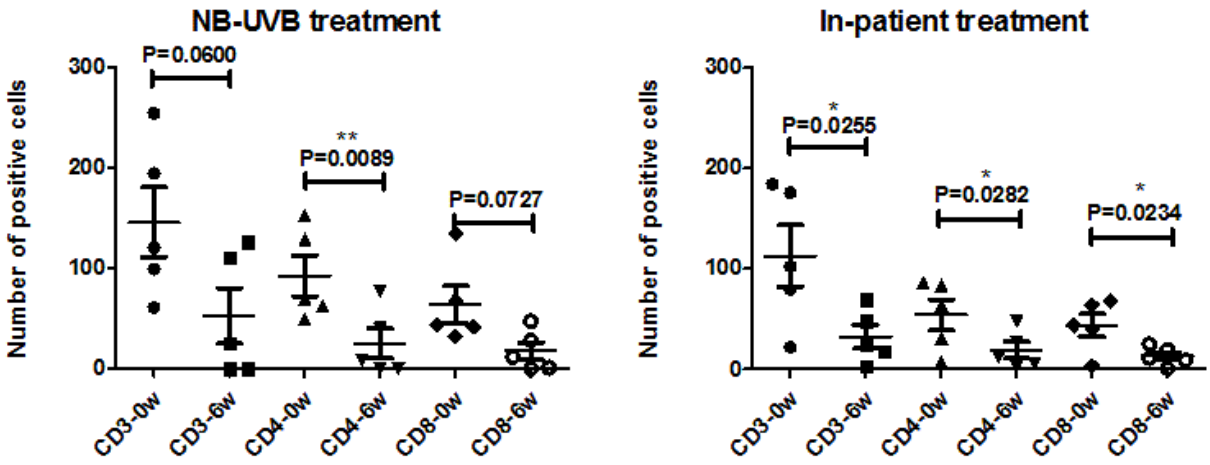

Figure 6. T-cell count per field. Skin samples before and after treatment were stained for $CD3^+$, $CD4^+$ and $CD8^+$ T-cells using IHC and the cells counted in the dermis and epidermis of the skin of patients receiving in-patient Blue Lagoon treatment and patients receiving NB-UVB treatment alone. A t-test was performed for statistical analysis. (A) Number of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells before and after NB-UVB treatment. (B) Number of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells before and after in-patient Blue Lagoon treatment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2.1.1 T-cell count correlation with PASI score

A correlation test was performed to determine whether the amount of T-cells counted in three fields in the skin correlated with the PASI score of the patients. Both treatment groups were combined for this analysis. No statistically significant correlation was detected in any cell type (fig. 7A-C).

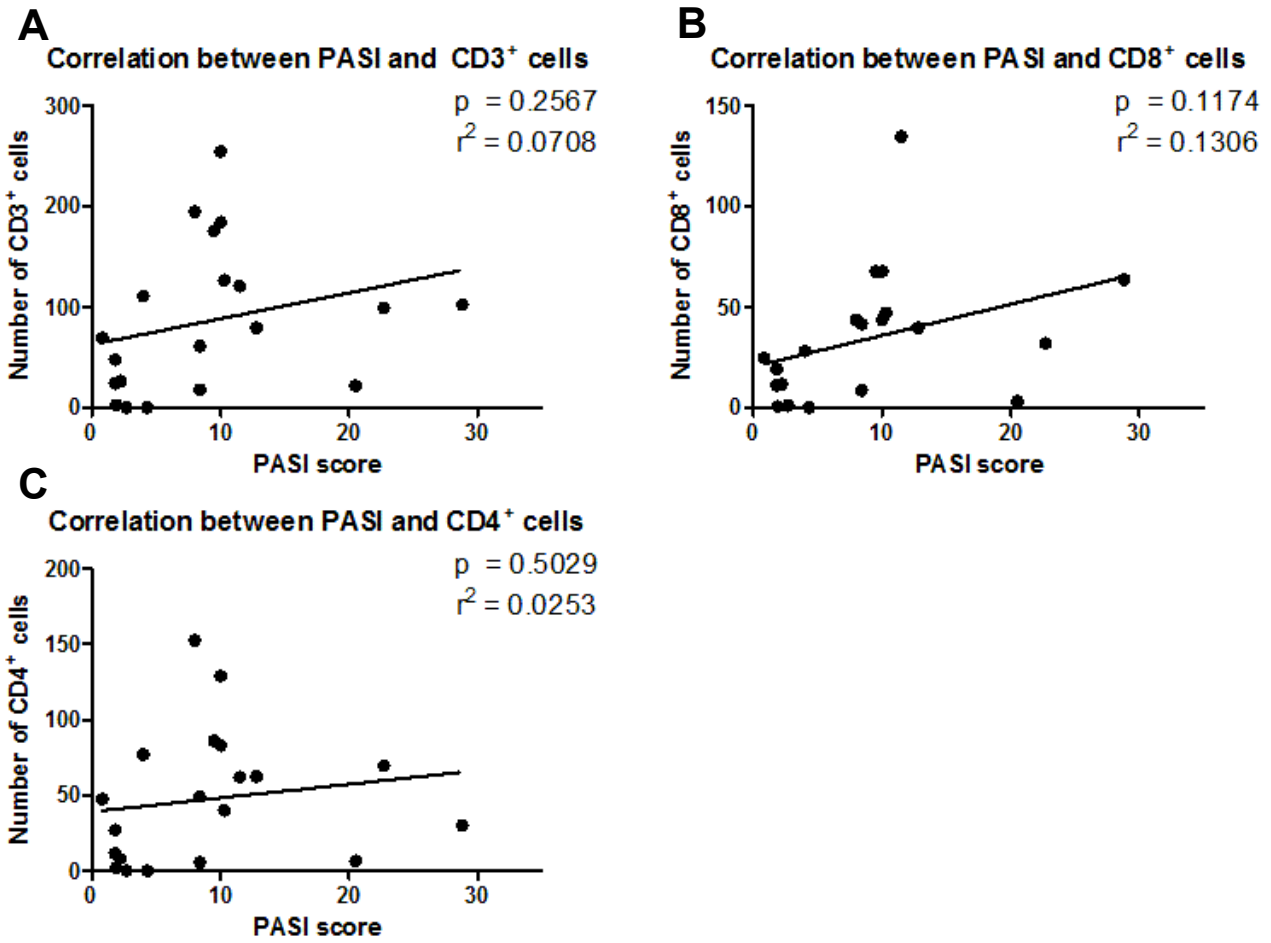

Figure 7. Correlation between PASI score and T-cell count, all treatments combined. A correlation test was performed for statistical analysis. (A) Correlation between PASI score and number of $CD3^+$ T-cells. (B) Correlation between PASI score and number of $CD8^+$ T-cells. (C) Correlation between PASI score and number of $CD4^+$ T-cells.

4.2.1.2 T-cell count correlation with Trozak score

A correlation test was performed to determine if a relationship exists between the average number of lymphocytes in skin counted in three fields in IHC and the histological Trozak score of patients from both groups before and after treatment. There is clear significant correlation between the Trozak score and the number of all three cell types found in the skin of the patients (fig. 8A-C).

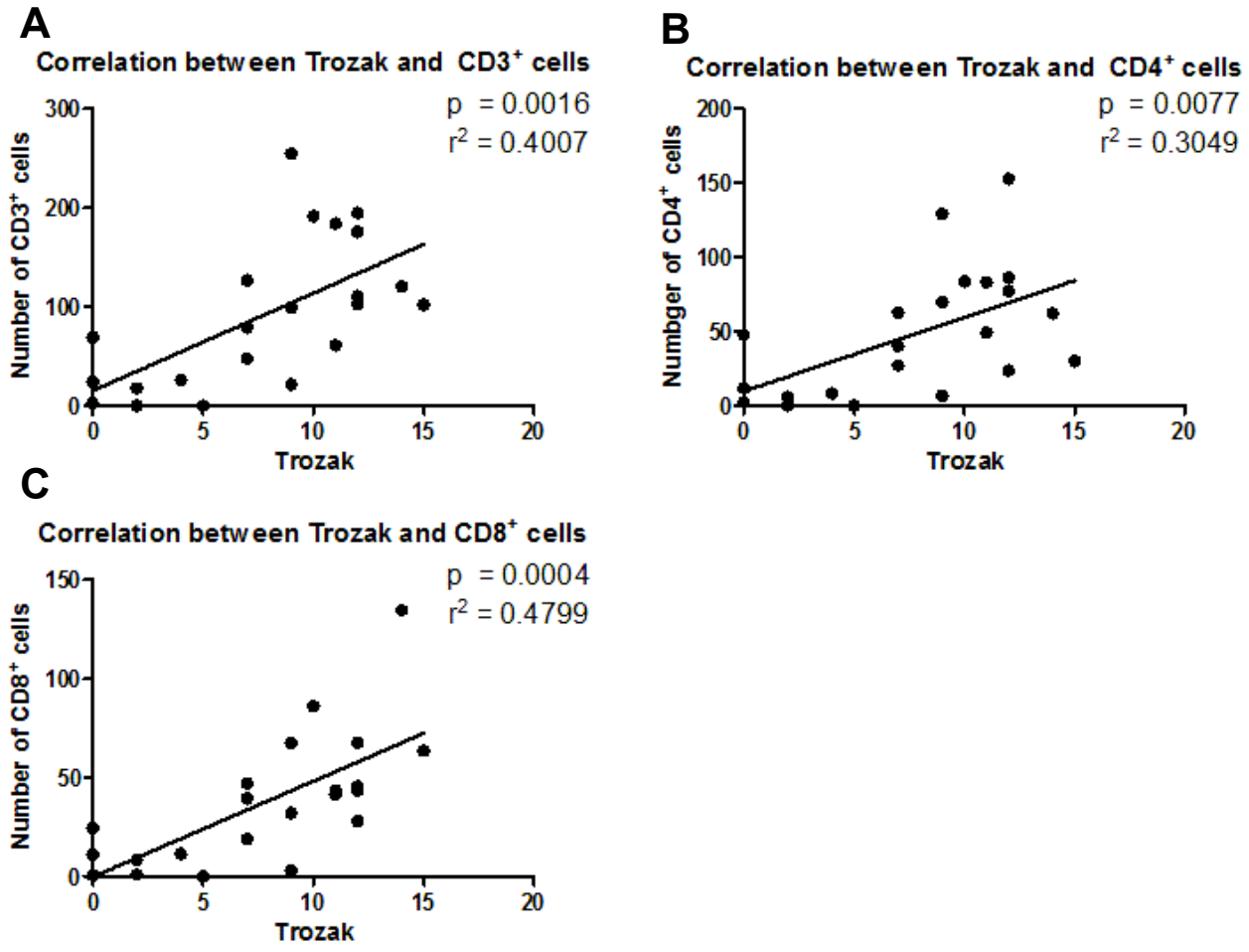

Figure 8. Correlation between Trozak score and T-cell count, all treatments combined. A correlation test was performed for statistical analysis. (A) Correlation between the histological Trozak score and number of $CD3^+$ T-cells. (B) Correlation between the histological Trozak score and number of $CD4^+$ T-cells. (C) Correlation between the histological Trozak score and number of $CD8^+$ T-cells.

4.2.2 IL-10 expression

Two different methods were used to analyze IL-10 expression, as determined by IHC, before and after treatment (n=7). One was a grading method where the expression pattern was determined as explained on page 25 (fig. 9A) and for the other method we used ImageJ to determine the percentage of the epidermis area that stained positive for IL-10. The percentage given by ImageJ was then normalized to week 0 (fig. 9B). A t-test was performed in order to determine statistical significance. Neither method showed any statistically significant difference in expression before and after treatment, regardless of the treatment given. The grading method showed a mean grade of 2.125 before treatment and 2.05 after treatment (p=0.8422) (fig. 9A) while the mean percentage of fluorescence increased from 100% (normalized) before treatment to 111% after treatment (p=0.3394) (fig. 9B).

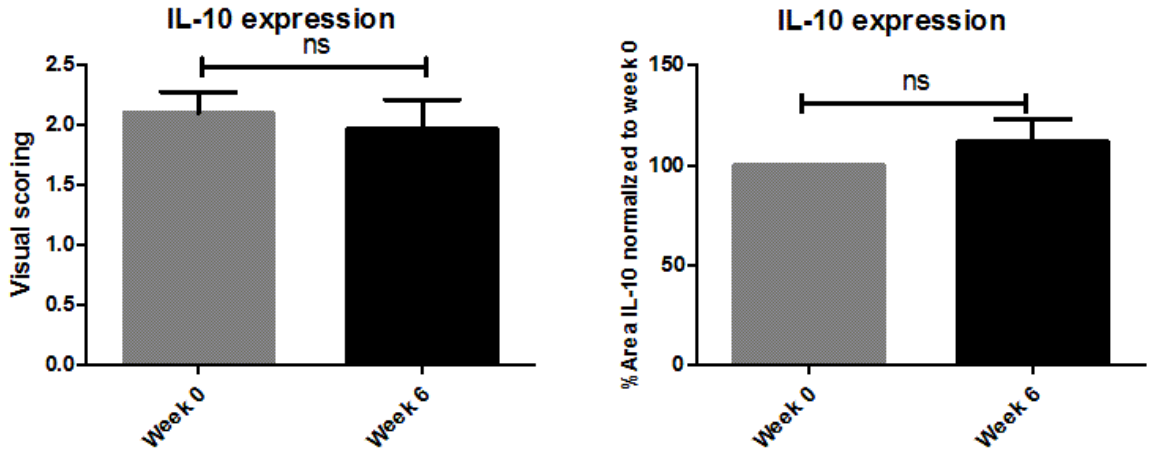

Figure 9. IL-10 expression before and after treatment, all treatments combined. (A) IL-10 expression using the grading system, before and after treatment (n=7). (B) IL-10 expression using ImageJ for fluorescent quantification before and after treatment (n=7).

The expression pattern seen in IL-10 before and after treatment was localized to the basal layer of the epidermis (fig. 10A and 10B). The expression pattern seen in the healthy control was also localized in the basal layer of the epidermis (fig. 11) and no detectable difference was seen in expression between the treatment groups and healthy control.

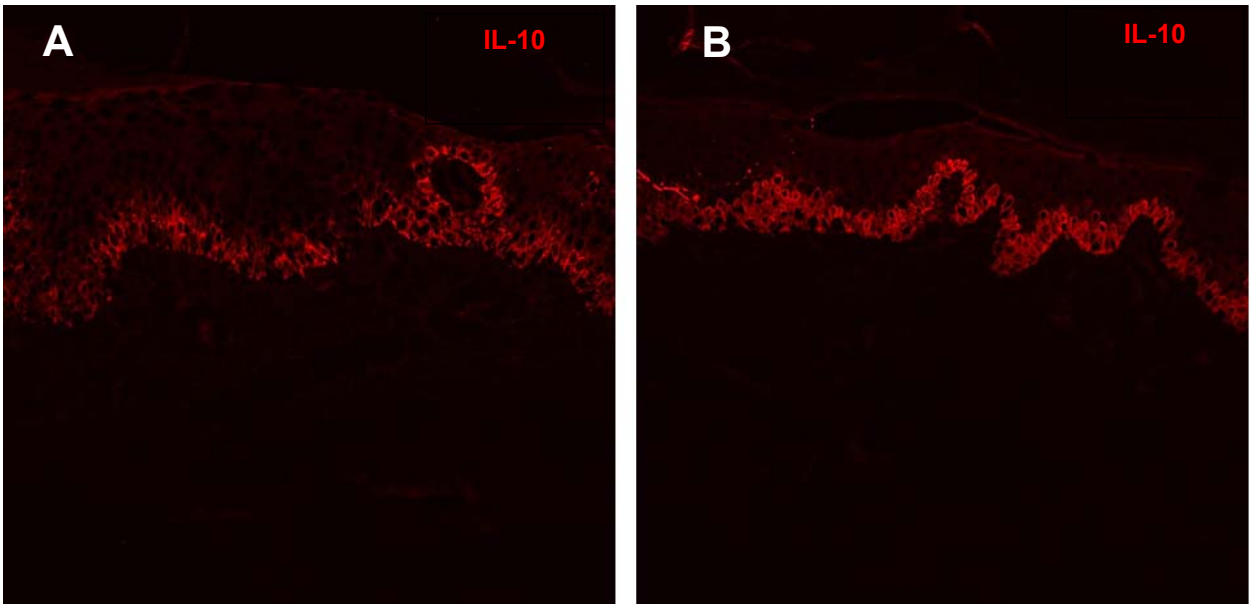

Figure 10. Example of IL-10 expression before and after treatment. (A) IL-10 expression before treatment. The patient had a histological Trozak score of 9 and a PASI score of 20.5. (B) IL-10 expression after 6 weeks of in-patient Blue Lagoon treatment. The patient had a histological Trozak score of 0 and a PASI score of 1.9. The pictures are taken at 20x magnification.

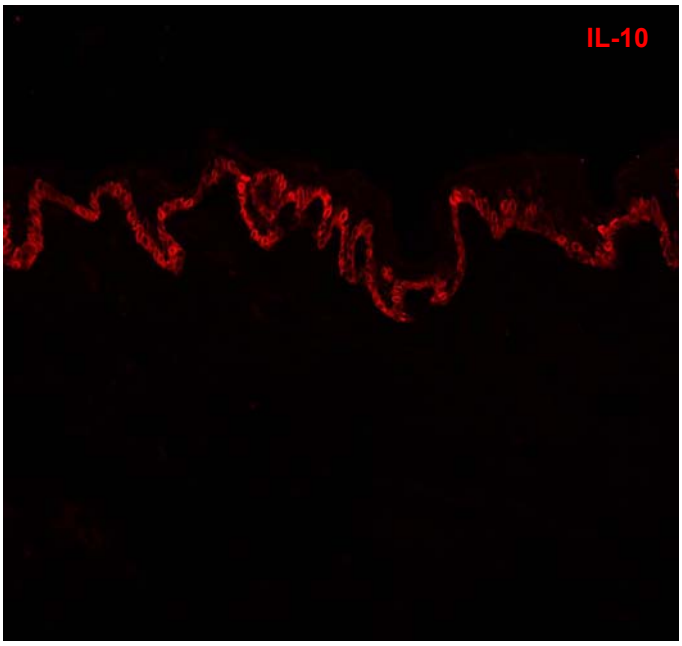

Figure 11. Example of IL-10 expression in the healthy control. Healthy control obtained from a patient undergoing elective surgery. The picture is taken at 20x magnification.

4.2.3 LL-37 expression pattern

Two different methods were used to quantify LL-37 expression before and after treatment. A grading method where the expression pattern on IHC skin samples from 10 patients was determined as explained on page 25 (fig. 12A). The other method involved using ImageJ to determine the percentage of the epidermis area stained positive for LL-37, each patient sample normalized for expression at week 0 (fig. 12B).

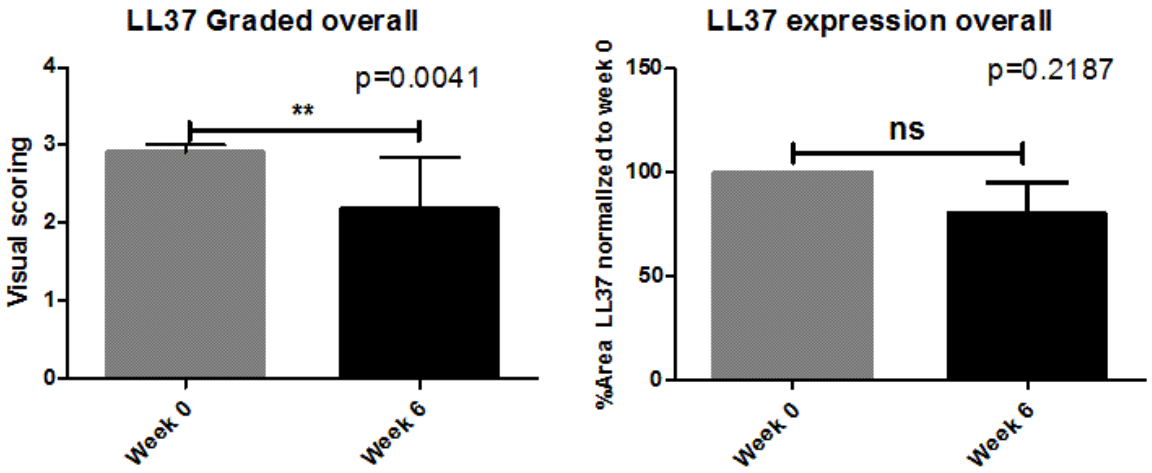

Figure 12. LL-37 expression in psoriasis patients before and after treatment.
Immunohistochemistry was performed on skin samples in order to determine expression levels of LL-37 in the epidermis before and after treatment. All treatment groups are combined. A t-test was performed for statistical analysis. (A) LL-37 expression quantified with the grading system before assigning them to either basal or diffused expression. (B) LL-37 expression quantified with ImageJ. Percentage area of expression was normalized to week 0. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

A t-test of the grading system showed a clear change in expression pattern before and after treatment (p=0.0041) (fig 12A). A t-test of the ImageJ analysis was inconclusive and showed no statistically significant difference (p=0.2187) (fig. 12B). The grades shown in this figure are the raw grades prior to assigning them to either diffused expression or basal layer expression.

The samples were assigned either diffused expression or basal layer expression based on the average grade given for three fields by the graders. Before treatment all patients showed a diffused expression throughout the epidermis. After treatment 60% of patients showed a basal layer expression and 40% continued to show a diffused expression. A binomial test showed a statistically significant increase in basal layer expression after treatment regardless of treatment given (p<0.0001) (fig. 13).

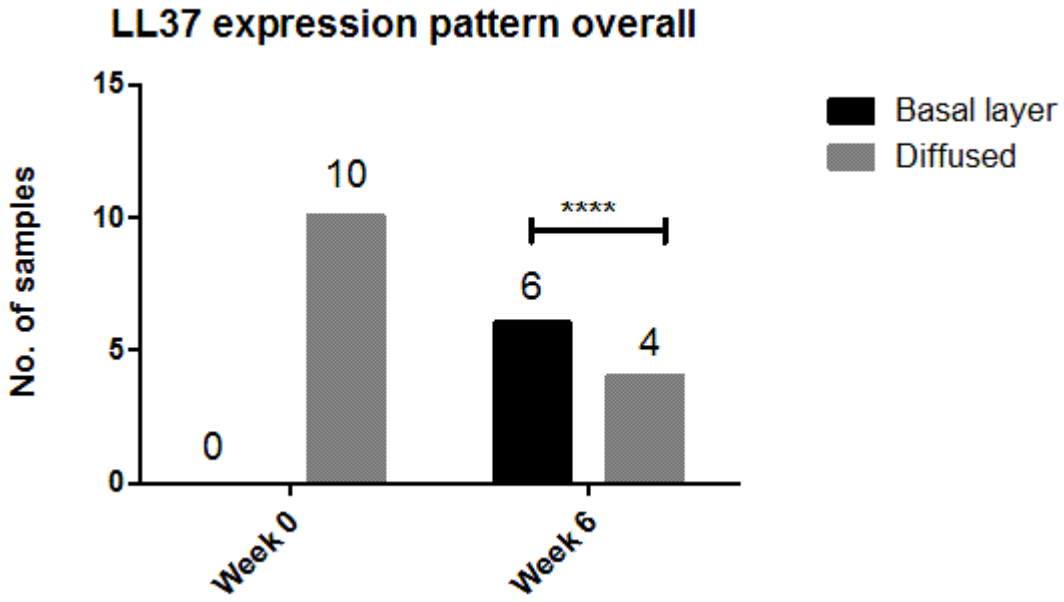

Figure 13. LL-37 expression patterns before and after treatment. Number of samples showing diffused and basal layer expression before and after treatment. A binomial test was performed in order to determine the difference in expression patterns before and after treatment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The diffused expression seen in skin samples of psoriasis patients before treatment covered every layer of the epidermis along with a few positive cells in the dermis (fig. 14A) while the basal layer expression seen in a large number of patients after treatment was localized solely in the basal layer of the epidermis along with a few scattered positive cells in the dermis (fig. 14B). A healthy control obtained from a patient undergoing elective surgery shows a bright basal layer expression with very few positive cells in the dermis (fig. 15A) similar to the expression seen in patients after treatment. A rabbit isotype was used as a control for LL-37 and shows no expression in the epidermis (fig. 15B).

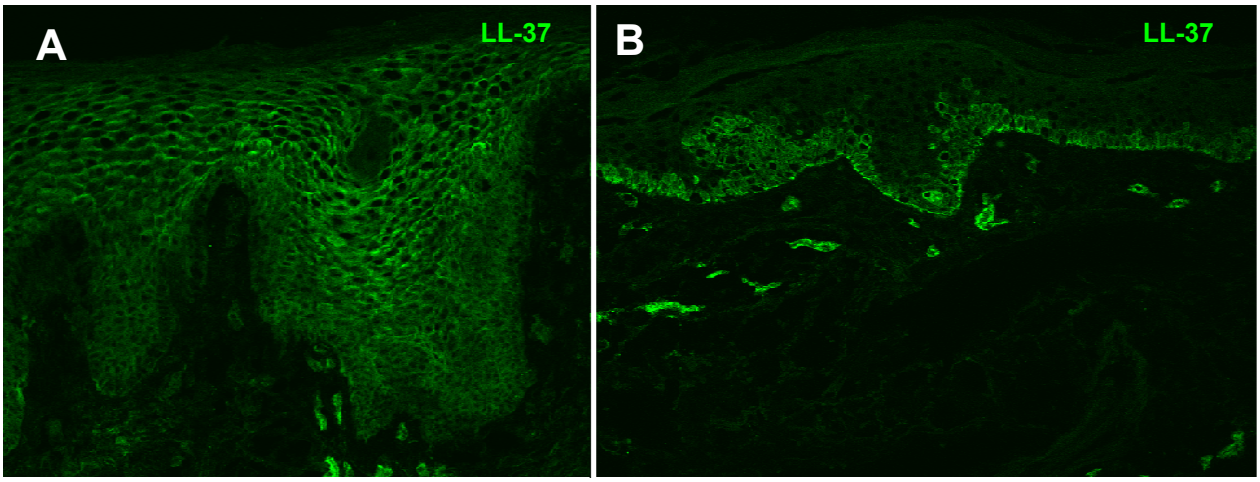

Figure 14. LL-37 expression in psoriatic skin before and after treatment. Immunohistochemical staining was performed on samples from 10 patients to visualize the expression of LL-37 before and after treatment. (A) Diffused expression in the epidermis of a psoriasis patient before treatment. The patient had a histological Trozak score of 15 and a PASI score of 7. (B) Basal layer expression in the epidermis of a patient after undergoing out-patient Blue Lagoon treatment. The patient had a Trozak score of 0 and a PASI score of 1.2 after treatment. The pictures are taken at 20x magnification.

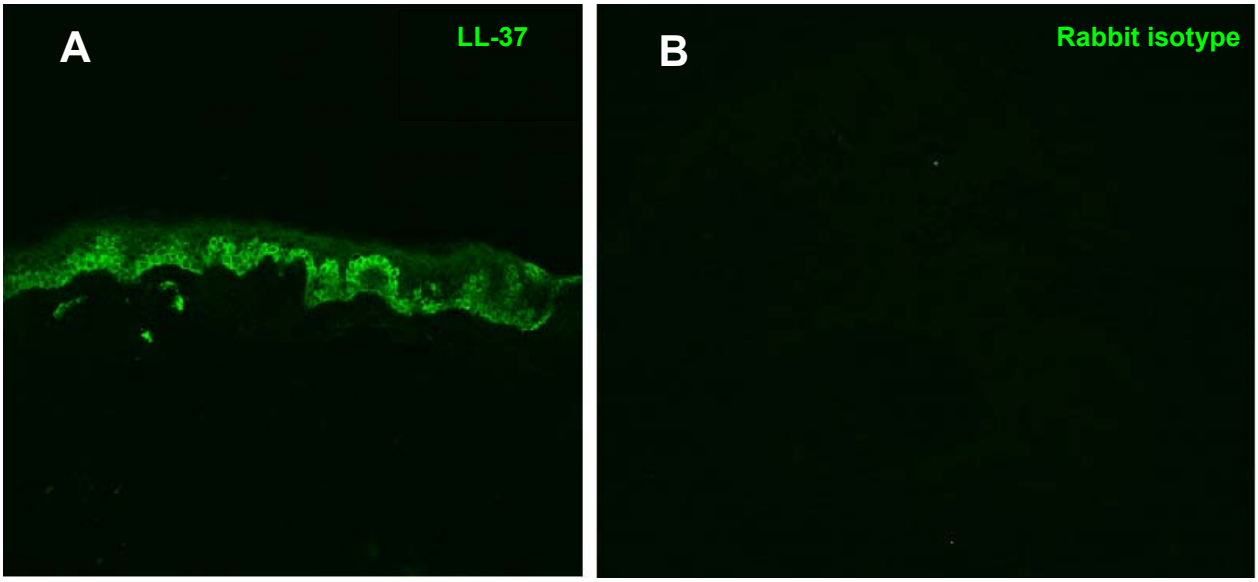

Figure 15. LL-37 expression and control. IHC was performed on controls to compare to expression seen in psoriasis patients. (A) LL-37 expression in healthy control obtained from a patient undergoing elective surgery. (B) Rabbit isotype used as a negative control for LL-37 and IFN-γ. The pictures are taken at 20x magnification.

4.2.3.1 LL-37 correlation with PASI score

A correlation test was performed to determine whether percentage area of LL-37 expression, as determined by IHC, quantified with ImageJ correlated with the PASI score of the patients. No statistically significant correlation was detected in any of the treatment groups (fig. 16A-D).

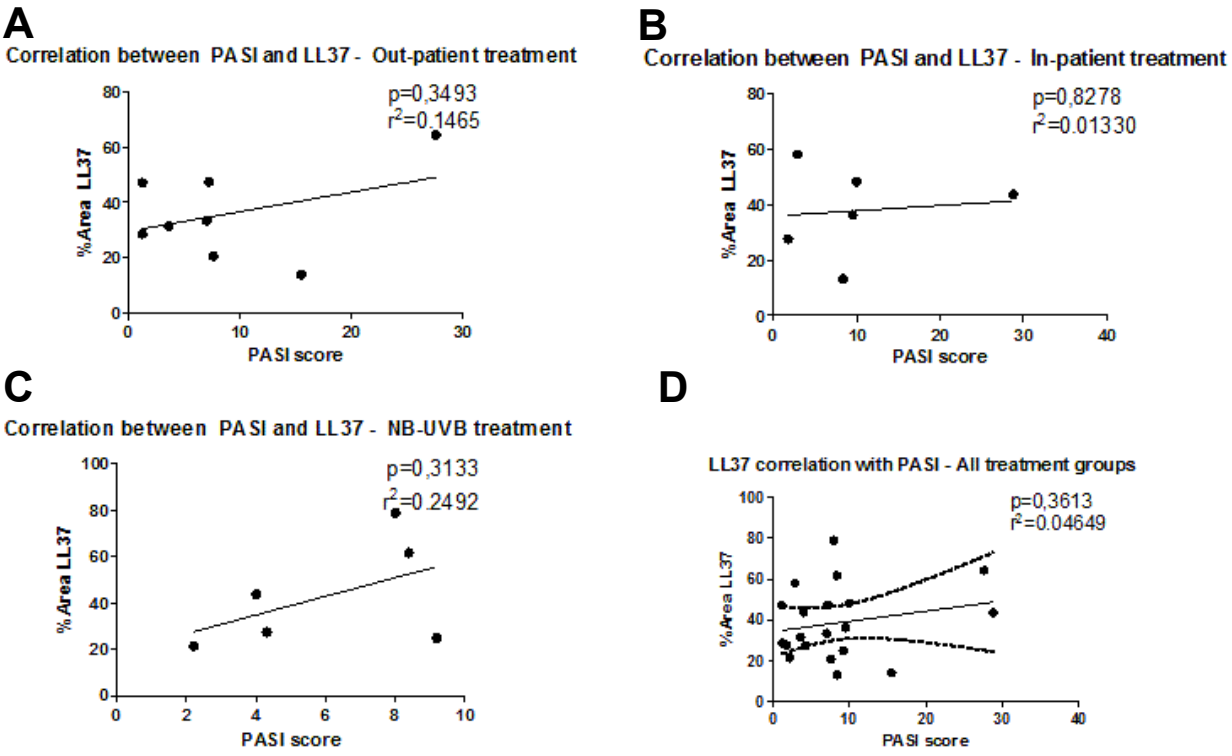

Figure 16. Correlation between PASI score and LL-37 expression analyzed with ImageJ. A correlation test was performed for statistical analysis. (A) Correlation between PASI score and LL-37 expression in patients receiving out-patient Blue Lagoon treatment. (B) Correlation between PASI score and LL-37 expression in patients receiving in-patient Blue Lagoon treatment. (C) Correlation between PASI score and LL-37 expression in patients receiving NB-UVB treatment. (D) Correlation between PASI score and LL-37 expression in all treatment groups combined.

A t-test was performed in order to determine whether there was a relationship between the PASI score of the patient and the expression pattern the patient was assigned through the grading system. The mean PASI score for patients showing diffused expression (n=14) was 10.16 ± 2.126 and 4.367 ± 2.275 for patients showing basal layer expression (n=6) (fig. 17). The difference in PASI score between the two groups did not reach statistical significance (p=0.1248).

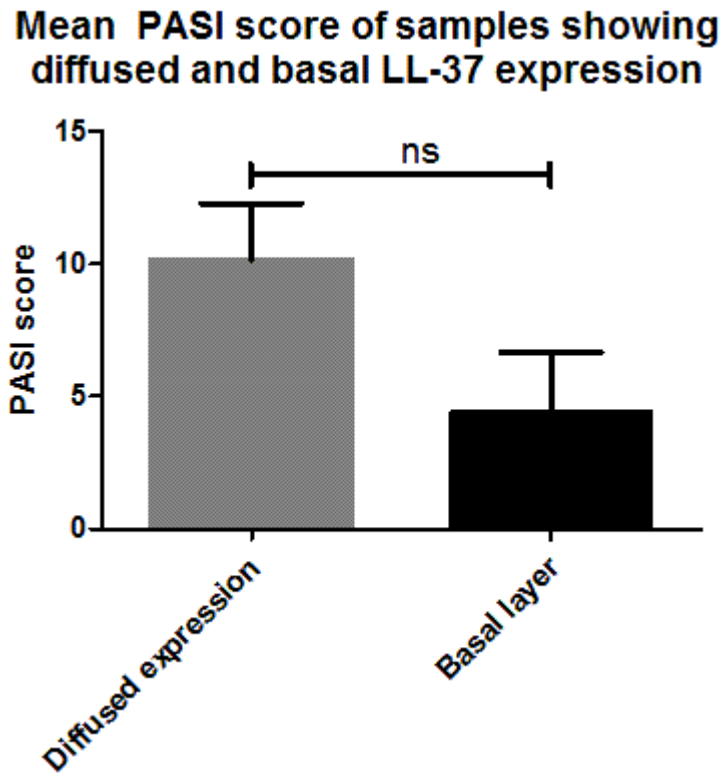

Figure 17. PASI score for the different expression patterns. The mean PASI score for patients whose skin samples showed either diffused expression or basal layer expression, as determined by IHC, regardless of week of treatment or treatment group. A t-test was performed for statistical analysis.

4.2.3.2 LL-37 correlation with Trozak score

A correlation test was done in order to determine whether there was a relationship between the percentage area of LL-37 expression in IHC stained skin samples quantified with ImageJ and the histological Trozak score the patients received. The only group showing statistically significant correlation was the group receiving NB-UVB treatment (p=0.0303) (fig. 18C) while the patient group receiving in-patient Blue Lagoon treatment showed a trend towards correlation as well as the data from all the groups combined (p=0.0800 and 0.0609 respectively) (fig. 18B and 18D). The patients receiving out-patient Blue Lagoon treatment did not reach statistical significance (p=0.6733) (fig. 18A).

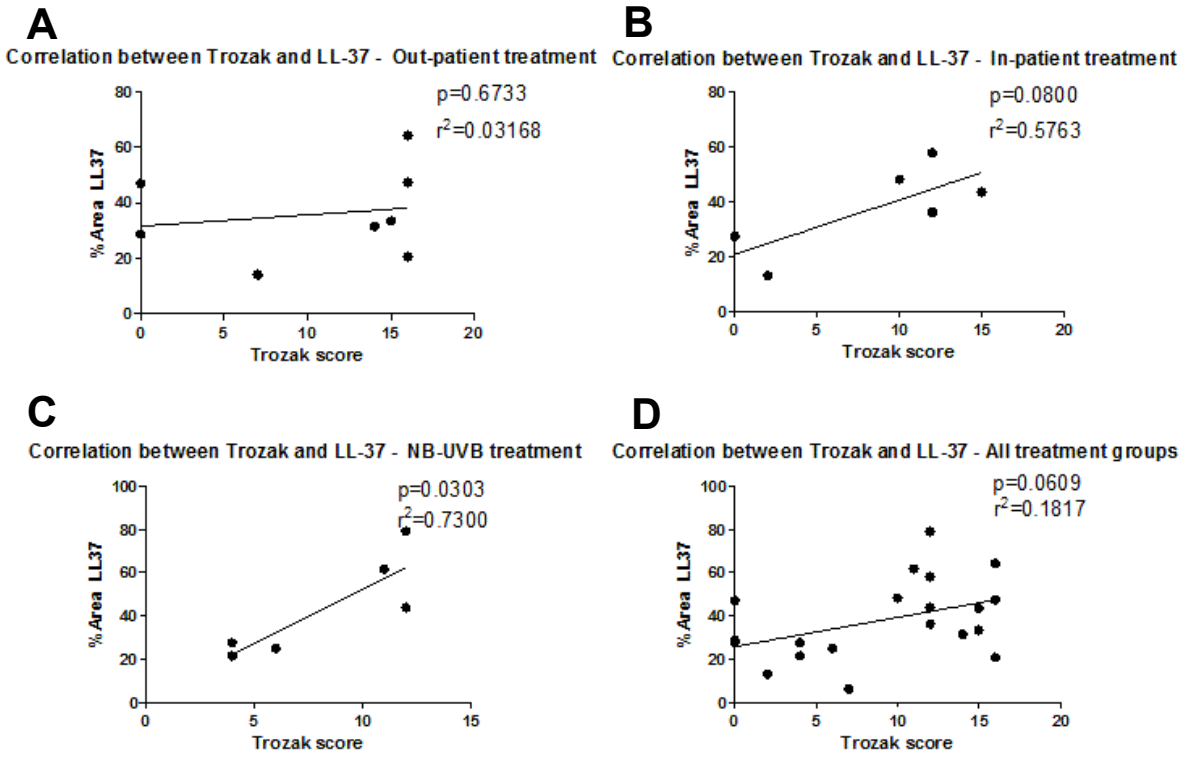

Figure 18. Correlation between the histological Trozak score and LL-37 expression quantified with ImageJ. A correlation test was performed for statistical analysis. (A) Correlation between Trozak score and LL-37 expression out-patient Blue Lagoon treatment group. (B) Correlation between Trozak score and LL-37 expression in in-patient Blue Lagoon treatment group. (C) Correlation between Trozak score and LL-37 expression in NB-UVB treatment group. (D) Correlation between Trozak score and LL-37 expression in all treatment groups combined. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

A t-test was performed in order to determine whether there was a relationship between the histological Trozak score of the patient and the expression pattern the patient was assigned through the grading system. The mean Trozak score for patients showing diffused expression (n=14) was 12.07 ± 1.077 and the mean for the patients showing basal layer expression (n=6) was 2.50 ± 1.204 (fig. 19). The difference in Trozak score between the two groups showed statistical significance (p<0.0001).

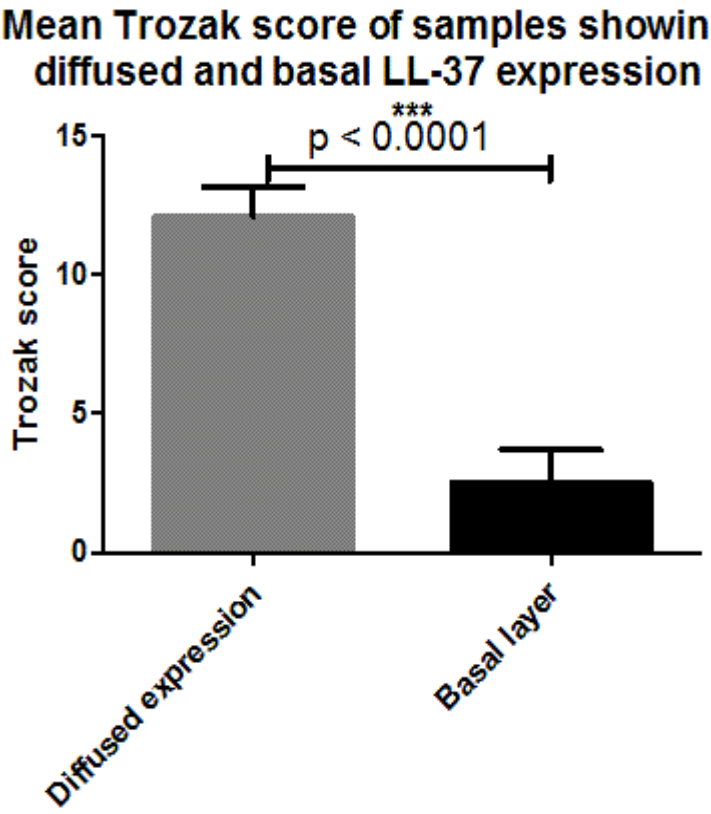

Figure 19. Trozak score for the different expression patterns. The mean histological Trozak score for patients showing diffused expression and patients showing basal layer expression for all treatment groups combined. A t-test was performed for statistical analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2.3.3 LL-37 and neutrophils costain

In order to determine which cells were responsible for the scattered LL-37 expression seen in the dermis of the patients an elastase IHC staining for neutrophils was performed. The antibody used shows cloudy stains in frozen IHC samples. All positive neutrophils seen in the dermis colocalized with LL-37 (fig. 20A-B)

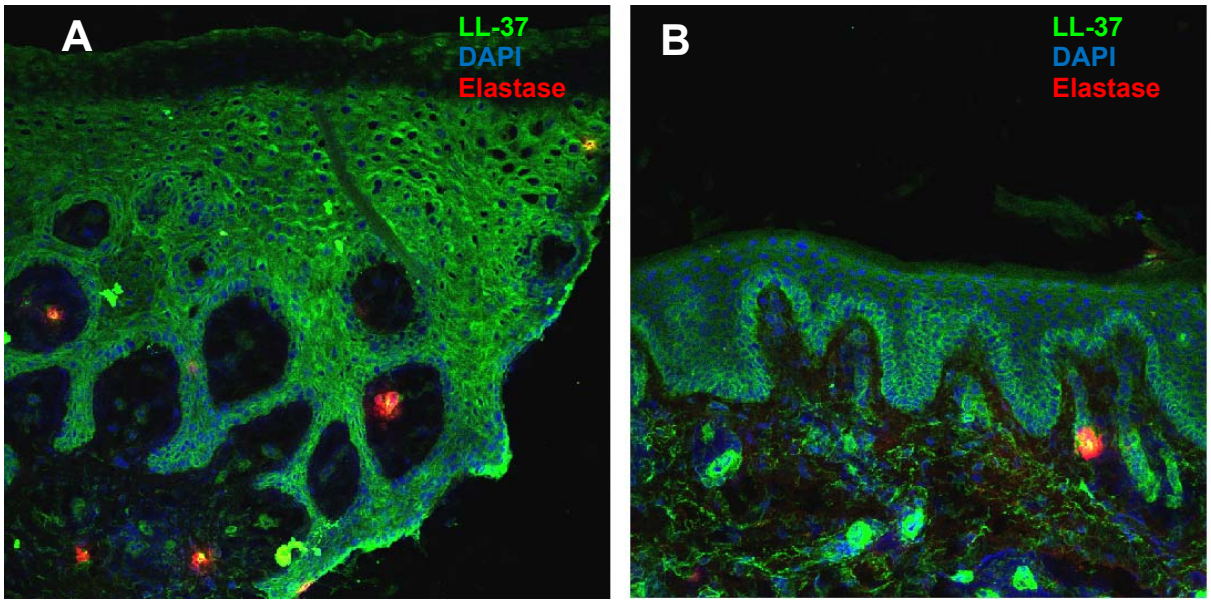

Figure 20. Neutrophil elastase (red) costains with LL-37 (green) in the dermis of psoriasis patients. (A) Neutrophil elastase staining before treatment. The patient has a Trozak score of 16 and a PASI score of 27.6. Elastin costains with LL-37 in the dermis. (B) Neutrophil elastase staining two weeks into out-patient Blue Lagoon treatment. Patient has a Trozak score of 7 and a PASI score of 23.5. The pictures are taken at 20x magnification.

4.2.4 Other immunohistochemistry

Other immunohistological stainings were performed but were discontinued because of lack of optimization, antibodies and/or time.

An IFN-γ staining was performed on patients before and after treatment (fig. 21A-B) as well as a healthy control (fig. 22) and an isotype control (fig. 15B). All samples showed the same kind of expression, covering the entire epidermis, seemingly unspecific regardless of treatment given or week of treatment. The same kind of expression was seen in the healthy control.

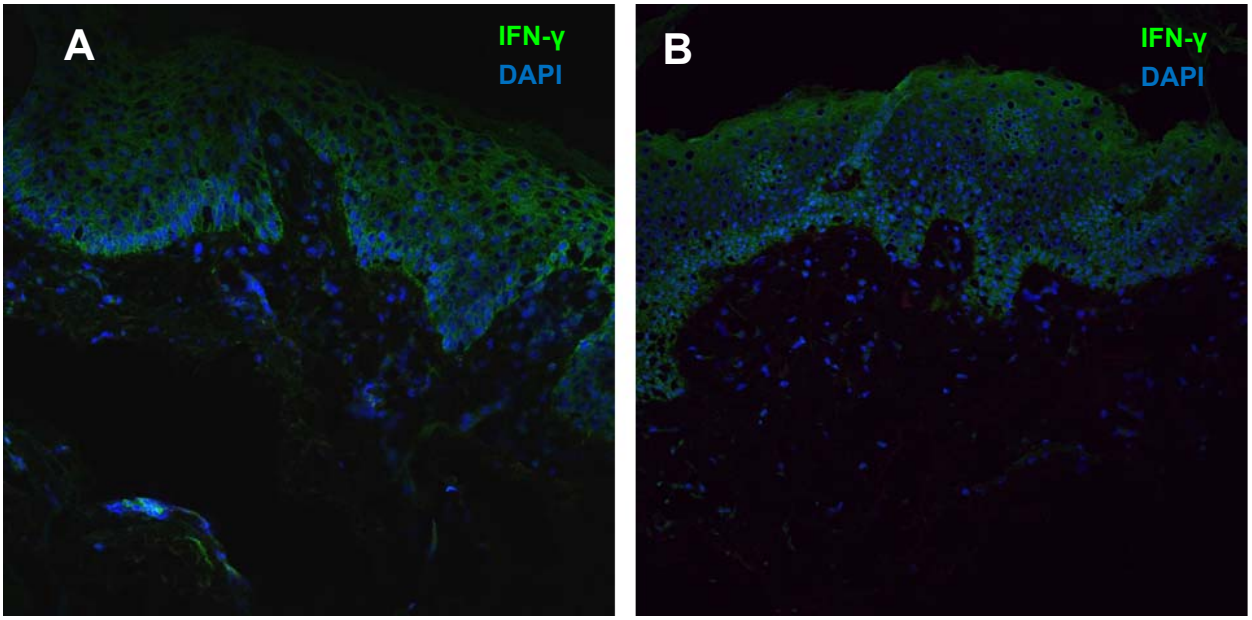

Figure 21. IFN-γ expression before and after treatment. (A) IFN-γ expression before treatment. The patient had a Trozak score of 11 and a PASI score of 10. (B) IFN-γ expression after 6 weeks of in-patient Blue Lagoon treatment. The patient had a Trozak score of 7 and a PASI score of 1.8. The pictures were taken at 20x magnification.

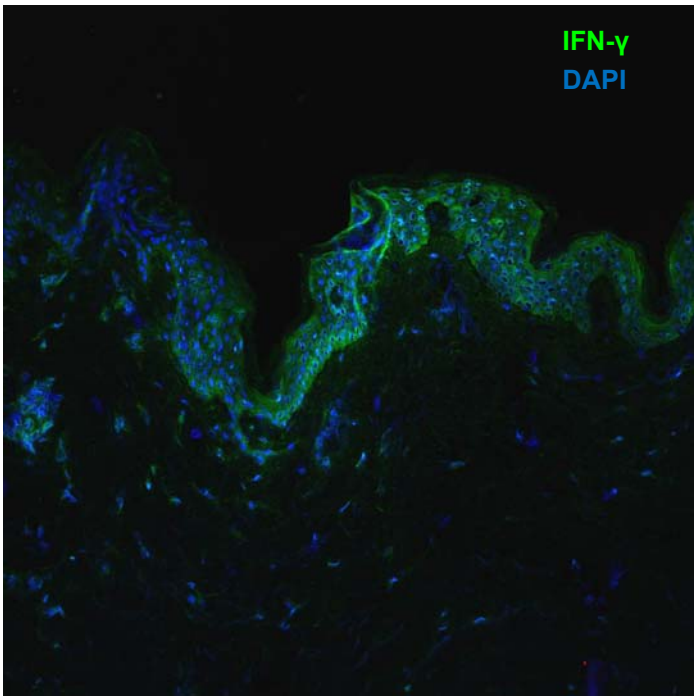

Figure 22. IFN-γ expression in healthy control. Expression in a healthy control sample obtained from a patient undergoing elective surgery. The picture is taken at 20x magnification.

A cutaneous lymphocyte-associated antigen (CLA) IHC staining was performed before and after treatment to determine the number and location of T-cells in the epidermis of the patients (Fig 23A-B). However this data was never processed and CLA staining was only performed on four samples.

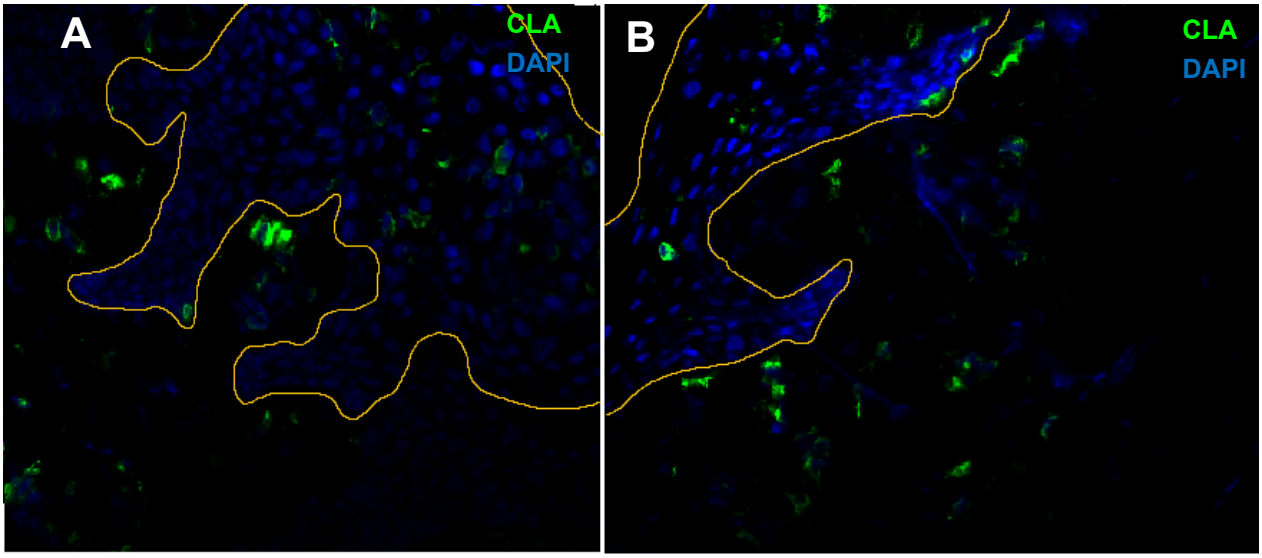

Figure 23. CLA expression before and after treatment. (A) CLA IHC staining in the epidermis (defined by the orange border) of a patient before treatment. The patient had a Trozak score of 9 and a PASI score of 20.5. (B) CLA staining in the epidermis of a patient after 6 weeks of in-patient Blue Lagoon treatment. The patient had a Trozak score of 0 and a PASI score of 1.9. The pictures are taken at 30x magnification.

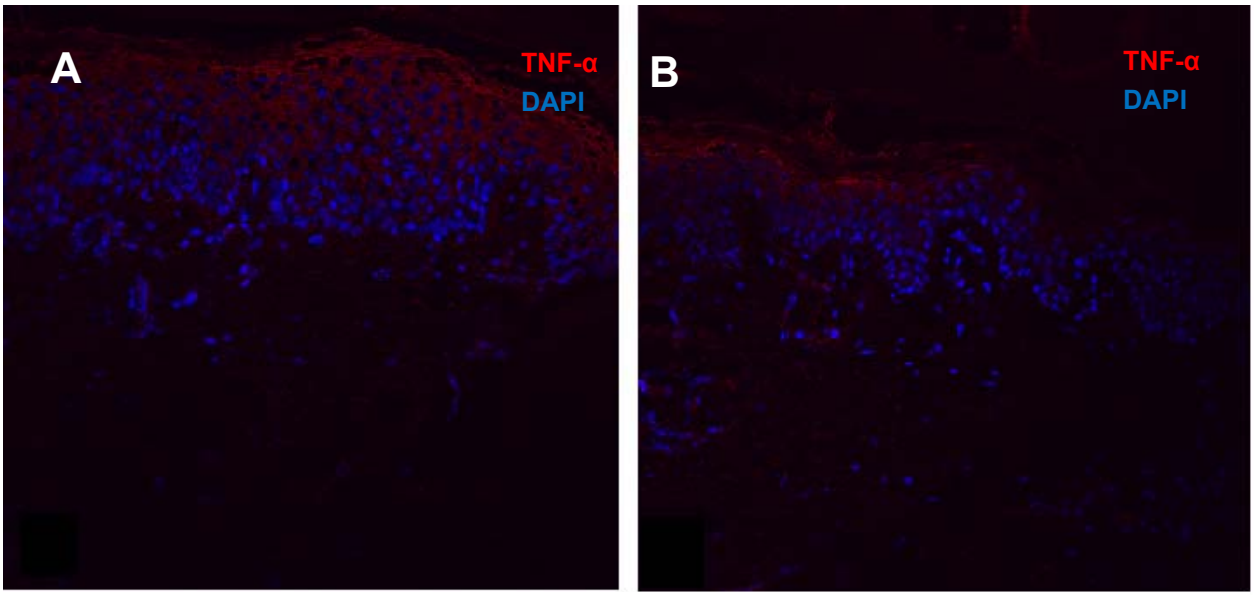

Figure 24. TNF-α expression before and after treatment. IHC staining for TNF-α was performed on samples before and after treatment. (A) TNF-α expression before treatment. The patient had a Trozak score of 11 and a PASI score of 10. (B) TNF-α expression after 6 weeks of in-patient Blue Lagoon treatment. The patient had a Trozak score of 7 and a PASI score of 1.8. The pictures are taken at 20x magnification.

IHC staining for TNF-α were performed before and after treatment (fig. 24A-B) as well as on a healthy control (fig. 25). No difference was seen in the expression before and after treatment. The expression was weak and covered the entire epidermis. The same expression was seen in the healthy control. However there is bright expression in the uppermost layer not seen in the samples from the patients.

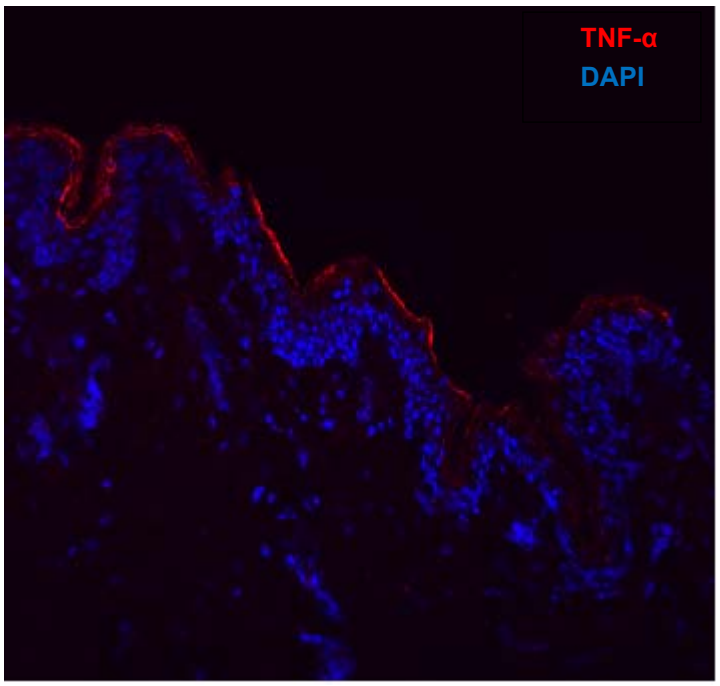

Figure 25. TNF-α expression in healthy control. IHC was performed to determine TNF-α expression in healthy control obtained from a patient undergoing elective surgery. Picture is taken at 20x magnification.

5 Discussion

The aim of this study was to determine the effect the Blue Lagoon treatment has on LL-37 and IL-10 expression in the skin of psoriasis patients compared to NB-UVB treatment alone. The effects the treatments had on the amount of T-lymphocytes in the skin were also assessed. It was shown that the Blue Lagoon treatment significantly reduces the amount of T-cells in the skin of patients. We have also demonstrated that the expression pattern of LL-37 changes in the epidermis of patients undergoing any of the treatments. We did not however see any changes in IL-10 expression in any of the treatments. The data acquired in this study demonstrates that treatment in the Blue Lagoon along with NB-UVB treatment is superior to NB-UVB treatment alone, both in the time it takes for the patients to go into remission and in the time the patients stay in remission.

5.1 Changes in disease severity and histological scoring.

As previously published by the group (36) the PASI score was reduced for all groups receiving treatment but the groups receiving Blue Lagoon treatments had a higher percentage of patients reaching PASI75 and PASI90 than did the NB-UVB group. The groups receiving a Blue Lagoon treatment also showed a faster response to treatment, already showing a dramatic reduction by week 2 (fig. 5A-B). The Blue Lagoon treatments also showed better responses in areas poorly accessed by NB-UVB treatment alone and a longer relapse time than the group receiving NB-UVB treatment alone.

The Trozak score was reduced in all treatments. However the Blue Lagoon treatments both showed a much more dramatic response at an earlier time point than did the NB-UVB treatment (fig. 5C). This is consistent with the data from the PASI score and confirms that the Blue Lagoon treatment combined with NB-UVB therapy is a more effective method of treating psoriasis than NB-UVB treatment alone.

5.2 Immunohistochemistry

5.2.1 T-cell count ($CD3^+/CD4^+/CD8^+$)

The T-cell count was performed on samples from patients receiving either the in-patient Blue Lagoon treatment or NB-UVB treatment alone. It has been demonstrated using FACS that there is an increase of the Th17 subset of $CD4^+$ T-cells in the dermis of psoriasis patients (41) as well as the Tc17 subset of $CD8^+$ T-cells (27) in the dermis compared to healthy skin and unaffected skin from psoriasis patients and an increase in primarily $CD8^+$ T-cells in the epidermis of psoriatic lesions while virtually none were found in healthy epidermis (69). As a result of this increase in T-cells we would expect the number of T-cells in the skin to go down following treatment. As expected there was a reduction in the numbers of T-cells in the skin of psoriasis patients after treatment. The in-patient treatment showed a statistically significant reduction in $CD3^+$, $CD4^+$ and $CD8^+$ T-cell count (fig. 6B) while the NB-UVB treatment alone showed only a statistically significant reduction in $CD4^+$ T-cells. We believe we have seen a trend for reduction in $CD3^+$ and $CD8^+$ T-cells as well in this group but it did not reach statistical significance (fig. 6A). This reduction in T-cells found in the skin is in accordance with data previously

published by our group showing a reduction in circulating $CD4^+$ and $CD8^+$ T-cells following treatment in the Blue Lagoon clinic (70).

5.2.1.1 T-cell count correlation with PASI score

The amount of T-cells in the skin does not correlate with the patient's PASI score (fig. 7A-C). This was not entirely unexpected as the PASI score measures overall disease severity while the T-cells are counted locally in the plaque, and while the patient as a whole may be in remission the specific site of the punch biopsy is possibly still quite affected by psoriasis. Our group has previously demonstrated a correlation between PASI score and circulating CLA^+ T-cells and $CD103^+$ T-cells (70) and other research has shown a correlation between $CD8^+$ Tc17 cells and PASI score (71). In those cases however the T-cells were circulating in the blood and not residing in the exact location of the plaque and therefore a better representation of the patient's disease severity as a whole.

5.2.1.2 T-cell count correlation with Trozak score

The amount of T-cells in the skin correlates with the patient's Trozak score (fig. 8A-C). This is not surprising as the Trozak score measures disease severity at the exact location of the punch biopsy where the T-cells are also counted leading it to be a more accurate representation than the PASI score for that particular site. It has been established that there is an abundance of T-cells in psoriasis plaques so it is to be expected that as the disease severity at the site goes down and inflammation recedes, so does the amount of T-cells in the area.

5.2.2 IL-10 expression

We did not see any change in IL-10 expression in the skin of patients before and after treatment (fig. 9A-B). This was surprising as IL-10 has been shown to increase on an mRNA level following conventional psoriasis treatments (72) and UVB irradiation (73) as well as in IHC in mice following UVB radiation, in which case the expression seen was diffused throughout the epidermis (74). Similar diffused expression was shown as a response to tape stripping and poison ivy injury (14) and an increased expression was shown in the epidermis following UVB treatment although not in a diffused pattern (75). The expression seen in this study was bright and localized in the basal layer only and did not change at all following treatment. The intensity of the expression and the area stained positive remained the same (fig. 10A-B). The same expression was seen in the healthy control (fig. 11) which is consistent with other published IHC data showing basal layer expression in healthy skin (13). It is not surprising that the expression seen in the healthy control mirrors that seen in the patients before treatment as IL-10 expression is generally low in the skin without injury (14) and expression is downregulated in psoriasis. An *in vitro* study performed at our lab has previously shown that dendritic cells co-cultured with expolysaccharides produced by bacteria in the Blue Lagoon affect dendritic cell maturation and IL-10 expression (76) so it could have been expected that we would see some IL-10 positive dendritic cells in the dermis of the patients after treatment, however we did not see this in our samples. The fact that no change was observed could be because the increase in expression is located in the basal layer but our setup made it so no change in intensity was seen. It is also possible that although previously published data shows an increase in IL-10 expression following UVB treatment the prolonged nature of the treatment the patients underwent has a different effect. It is

possible the patients showed increased resistance for NB-UVB radiation leading to a diminished IL-10 response as the damage caused to the skin by the radiation lessened with time. It could be interesting to see the expression in samples from an intermediate timepoint.

5.2.3 LL-37 expression

It has been demonstrated that mRNA levels of LL-37 are increased in both lesional and non-lesional skin of psoriasis patients while not found in healthy skin (77, 78). We had expected to see a decrease in expression intensity following treatment as studies using IHC have previously demonstrated (78, 79). Surprisingly, we instead saw a drastic change in the expression pattern of the peptide with a grading system showing statistically significant change in expression after treatment (fig. 12A and fig. 13). Before treatment LL-37 expression covered most of the epidermis (fig. 14A), other studies using IHC have shown similar diffused expression with the most intense expression shown in the granular and horny layers of the skin (78-80). After treatment this expression moved from covering the entirety of the epidermis to localizing almost exclusively in the basal layer along with some positive cells in the dermis (fig. 14B). This is the same expression pattern as we saw in the one healthy control that we had (fig. 15A). To our knowledge this expression pattern has not been shown in other studies.

It has been shown that LL-37 is kept in lamellar bodies in the granular layer in the healthy skin of mice (19) so we were expecting to see a localization of positive LL-37 staining in that area in the healthy control and possibly in patients showing improvement with treatment and it was surprising to see it in the basal layer only. However the basal layer is the most active layer as the cells differentiate and die as they move their way up through the layers. Hence, the cells in the basal layer might be more prone to express cytokines and AMPs than the ones in the layers above. It would be interesting to have qPCR data to compare to the expression seen in IHC. Further work will be done by our group researching LL-37 in the skin such as how incubation with LL-37 affects secretion by keratinocytes.

The fact that the antibodies available for LL-37 stainings aren't selective for the active peptide but also for the entire pro-protein made it so we could not determine whether the increased expression also included increased cleavage and activation of the peptide. Thus, it would be interesting to costain LL-37 with the appropriate protease to determine whether the expression increase is confined to hCAP-18 only or if there is an increased cleaving activity in the skin as well. The staining was also very variable in intensity between samples even though they were stained in batches and the exact same settings were used on the microscope every time. Therefore there was no reliable quantification possible based only on intensity of the stain. The fact that we were dealing with a complete change in the location of the expression also made it difficult to quantify using a computer program as it is not as equipped to determine qualitative expression like the eye is.

5.2.3.1 LL-37 correlation with PASI score

Using ImageJ to quantify the expression there was no statistically significant correlation between area LL-37 expression in the epidermis and PASI score (fig. 16A-D). As is the case with the T-cell count this is not surprising. High LL-37 expression is connected to sites of active inflammation so while the PASI score of the patient may have been greatly reduced, the punch biopsy most likely came from

an active plaque meaning LL-37 expression would be upregulated in the local cells. The lack of correlation could also be attributed to the method of quantification as using ImageJ did not prove to be an accurate way of measuring the changes in expression pattern (fig. 12B).

The grading system seemed to show some correlation with PASI. Patients showing basal layer expression had a low PASI score (<4.5) in all cases but one (14.5) while patients showing diffused expression ranged from 2.9 to 28.8 in PASI. This difference however did not reach statistical significance (fig. 17).

5.2.3.2 LL-37 correlation with Trozak score

The ImageJ method shows correlation between LL-37 expression and Trozak in the NB-UVB group and we believe we see a trend in the other groups (fig. 18A-D). However this method of quantification was not suited for detecting changes in expression patterns as it only measured the percentage of area showing positive staining and not the location of the expression itself.

The grading system resulted in a statistically significant difference in Trozak score between basal layer expression and diffused expression (fig. 19).

5.2.3.3 LL-37 and neutrophil costain

LL-37 expression is frequently found in the dermis of psoriasis patients in close proximity to pDCs (81). We wanted to find out which cells were responsible for the expression we saw in the dermis. Neutrophils are a known source of LL-37 and infiltrate the skin of psoriasis patients and LL-37 expression seems to be more abundant in neutrophil rich dermis than in neutrophil poor, though there seem to be other sources in the dermis as well (81). All positive neutrophils in the dermis colocalize with LL-37; however they do not account for all of the LL-37 positive cells seen in the dermis (fig. 20A-B). Other potential sources of either uncleaved hCAP-18 or LL-37 in the dermis of the skin are mast cells and the sweat glands of the skin as well as potential leaking from the keratinocytes in the epidermis.

5.2.4 Other immunohistochemistry

Other immunohistochemistry we did were IFN-γ, TNF-α and CLA but these were never completed since the antibodies we had didn't seem to work consistently despite many attempts at optimization as fixation, preservation and different antibodies can affect the way in which samples react to IHC.

It would have been interesting to compare IFN-γ and TNF-α expression in the skin before and after treatment as these cytokines are heavily upregulated in psoriasis and visualizing their expression in the epidermis of psoriasis patients as they progressed through treatment would have given valuable insight into the pathophysiology of the disease. However, these cytokines didn't show any difference in expression levels or patterns before and after treatment. Both showed what appears to be unspecific binding throughout the epidermis no matter which dilutions, secondary antibodies and fixation methods were used. The same sort of expression was seen in the healthy control (fig. 21A-B, 22, 24A-B and 25).

The number of circulating CLA^+ T-cells before and after treatment has previously been determined by our research group (70) and having the corresponding data from the skin of these patients to

compare to the circulation data would have been interesting. The staining initially showed promise (fig. 23A-B); however, IL-10 and LL-37 were prioritized over CLA as all T-cells located in the skin are expected to express this marker and IHC staining for T-cells had already been performed.

The CLA immunohistochemistry was done prior to obtaining the healthy control sample so no healthy control exists for CLA and no isotype controls exist for TNF-α and CLA.

One of the flaws of this experiment was the fact that there was only one healthy control sample; we had arranged to receive more but this was not possible due to a general strike at the hospital.

6 Conclusion

In this study the effects of the Blue Lagoon treatment and NB-UVB treatment on T-cell numbers, and two different Blue Lagoon treatments and NB-UVB treatment on LL-37 and IL-10 expression in the skin, were assessed.

Previous research demonstrates that T-cells infiltrate the skin of active psoriatic lesions (27, 41, 69) and data previously published by our group shows a significant reduction in circulating T-cells in psoriasis patients after treatment (70). The data acquired in this study shows a statistically significant reduction in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the skin of psoriasis patients following in-patient Blue Lagoon treatment and a statistically significant reduction in $CD4^+$ T-cells, but only a tendency towards reduction in $CD3^+$ and $CD8^+$ T-cells following NB-UVB treatment. This reduction correlated with a reduction in disease severity.

LL-37 expression, associated with inflammation, has been reported to be increased in psoriatic lesions while the expression of IL-10, an immune modulator, is downregulated. Conversely, in our setup no change was detected in the expression of IL-10 in seven patients before and after treatment or when compared to the healthy control. However, a significant change in LL-37 expression pattern was detected after treatment in all three treatment groups, changing from a diffused pattern covering most of the epidermal layers to a pattern confined mostly to the basal layer, correlating with the histological Trozak score of the patients. This basal layer expression is reflected in the expression seen in the healthy control and has, to our knowledge, not been demonstrated before. Further work should be done exploring this change in expression and what it means.

As expected samples also showed histological changes associated with treatment, including shorter rete ridges and fewer cell layers in the epidermis, consistent with the reduction in disease severity and histological scoring.

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