Dendritic cells manipulating immune responses: Understanding the role of Flt3L and Flt3-dependent DCs in rheumatic diseases
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Reduced CLEC9A expression in synovial tissue after adalimumab therapy in psoriatic arthritis patients

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Submitted for publication
Abstract

Objectives: We investigated the early changes in CLEC9A expression, a C-type lectin expressed by a specific subset of dendritic cells (DCs), in three different compartments (skin, synovial tissue [ST] and serum) after short term adalimumab treatment in psoriatic arthritis (PsA) patients compared to placebo.

Methods: A total of 24 patients with active PsA and psoriasis were randomized to receive adalimumab (n=12) or placebo (n=12) for 4 weeks. Synovial and skin biopsies were obtained before and after 4 weeks of treatment and serum samples after 4 and 12 weeks, and one year after treatment. Skin (n=13) and serum (n=10) from healthy donors (HDs) were used as control. CLEC9A expression was assessed by immunohistochemistry (skin and ST) and by qPCR (ST). To detect CLEC9A associated with apoptotic cells, double immunofluorescence using TUNEL assay was performed. CLEC9A serum and synovial fluid (SF, n=10) levels were determined by ELISA.

Results: CLEC9A expression was significantly higher in psoriatic skin compared to HD. Importantly, in psoriatic skin and PsA ST CLEC9A+ cells were detected in close proximity with TUNEL+ cells. In SF, CLEC9A levels were significantly lower compared to paired PsA serum. Adalimumab treatment did not affect CLEC9A serum levels and skin expression. However, CLEC9A protein expression in ST was significantly lower after adalimumab treatment than in the placebo group while CLEC9A gene expression remained unchanged. Notably there was a positive correlation between T cell numbers and CLEC9A protein expression in ST.

Conclusion: Synovial CLEC9A expression was lower after adalimumab treatment than placebo while other compartments, namely serum and skin remained unaffected. Targeting CLEC9A might be valuable in reducing synovial inflammation in PsA patients by modulating T cell presentation and cross-presentation.
Introduction

Psoriatic arthritis (PsA) is an inflammatory disease in patients with psoriasis affecting the joints and presenting distinct clinical, genetic and immunohistochemical characteristics. Major advances in the study of PsA have occurred over the past several decades. The composition of the inflammatory infiltrate in the skin and synovial tissue has been the subject of multiple detailed investigations. In both tissues there is a prominent lymphocytic infiltrate, localized in the dermal papillae in skin and in the synovial sublining in the joint (1). Previous evidence suggests that a similar infiltrate is found in the inflamed enthesis (2).

There is growing evidence that CD8+ T cells colonize the developing skin lesion first, and that lymphocyte specific therapy results in a reduction of CD8+ T cells in the epidermis, which correlates with clinical signs of improvement (3-5). Recent work showed an increased presence of CD8+ IL-17- and IL-22-producing T cells in lesional psoriatic skin implying that these cells play a significant role in the pathogenesis of psoriasis (6). A dominant CD8+ T cell population was also found in PsA synovial fluid (SF) suggesting that these cells may be driving the immune response in the joint (7). This is further corroborated by historical data showing an association of PsA with human leucocyte antigen (HLA) class I (8).

Dendritic cells (DCs) are a crucial link between the adaptive and innate immune systems. DCs are vital for the initiation of primary immune responses and the mouse CD8α-type DC is particularly efficient at cross-presenting exogenous antigens for the activation of CD8+ T lymphocytes (9). A human DC subset expressing high CD141 (BDCA3) was recently reported to be the equivalent to the mouse CD8α-type DCs (10;11). This finding was confirmed phenotypically and functionally by identifying overlapping markers specific for these cell types and conserved across species, in particular the endocytic C-type lectin receptor CLEC9A (12;13) and the chemokine receptor XCR1 (11;14). We have recently observed that CD141+ DCs are increased in the synovial compartment in PsA patients (unpublished observations). CLEC9A is a group V C-type lectin-like receptor that functions as an activation receptor and is expressed on a specific subset of DCs, CD141+DCs, and small subset of CD14+CD16- monocytes (15). CLEC9A has been found to bind to dead cells and also regulates the cross-presentation of dead cell-associated antigens in a Syk-dependent manner (16). The recognition of damaged cells by CLEC9A in CD141+ DCs has the potential to play an important role in exacerbating chronic inflammatory processes.

Tumor necrosis factor (TNF) plays a key pathogenic role in both psoriasis and PsA. Increased levels of TNF have been reported in serum, SF, skin and synovial tissue (ST) of affected patients (17;18) and blocking this molecule has a beneficial effect in this condition (19;20). Adalimumab is a fully human, anti-TNF monoclonal antibody that binds both to soluble and cell membrane-bound TNF with high affinity, preventing the interaction between TNF and its receptor (21). Clinical improvement
Reduced expression of CLEC9A in synovial tissue in PsA patients after adalimumab treatment is associated with a reduction of synovial inflammation in the joint and skin. Accordingly, there is a decrease in leukocyte numbers (including T cells and macrophages), vascularity and expression of proinflammatory cytokines and matrix metalloproteinases (MMPs) in the synovium of patients with PsA after treatment with the chimeric anti-TNF antibody infliximab (22). The T cell reduction observed in PsA patients after adalimumab therapy (23) might be due in part to a direct effect on T cell trafficking but also by modulating DCs numbers/function resulting in reduced T cell activation/expansion.

Taking into account that CD8+ T cells play an important role in PsA pathology and that CLEC9A is expressed in cross-presenting CD141+ DCs, we aimed to assess whether CLEC9A expression might be modulated after successful response to adalimumab therapy, studying three different compartments (skin, ST and peripheral blood).

PATIENTS AND METHODS

Patients and controls

Patients with PsA fulfilling the CASSification of Psoriatic ARthritis (CASPAR) criteria for PsA (24) and healthy donors (HD) were included in this study. PsA patients were enrolled in a randomized, double-blind, placebo-controlled, single centre study performed at the Academic Medical Center of the University of Amsterdam (Current Controlled Trials ISRCTN23328456) (23). All patients and controls were included into the study after written informed consent was obtained. Demographics and clinical features for PsA patients and HDs can be found in Supplementary Table 1, and in Supplementary Table 2 for the PsA patients enrolled in the adalimumab study. In Supplementary Table 2, the parameter values for serum (samples from all 12 patients available), skin and ST differ because skin and/or ST specimens were not available anymore from some patients. The 28-joint Disease Activity Score (DAS28) was chosen to monitor changes in clinical disease activity after therapy (25).

Treatment

Patients were randomized to receive subcutaneous injections with either adalimumab 40 mg or matching placebo at baseline (day 0) and day 15. After the second arthroscopy all patients received adalimumab 40 mg every other week.

Skin Biopsies

4-mm punch biopsies were taken from lesional (L) and non-lesional (NL) skin of PsA patients, preferentially from non-sun-exposed areas, at baseline and at week 4. The first and second biopsies were collected from the same psoriatic plaque, separated by at least 1 cm. Normal adult skin was obtained from healthy individuals undergoing plastic surgery of the breast or abdomen after informed consent. The skin biopsies were randomly coded, snap-frozen in Tissue-Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, the Netherlands) by immersion in liquid nitrogen and stored...
at -80°C until processing. Five-micrometer cryostat sections were cut and mounted on glass slides before being stored at -80°C until immunohistochemical staining.

**Arthroscopic synovial biopsy sampling**

Controls and PsA patients, under local anesthesia, underwent miniarthroscopy of a knee joint, wrist, or ankle, as described previously in detail (26;27). The samples were snap frozen en bloc in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN). The frozen blocks were stored in liquid nitrogen. Cryostat sections (5 μm) were mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Braunschweig, Germany). The glass slides were sealed and stored at -80°C until analysis.

**ELISA**

Levels of soluble CLEC9A were determined by ELISA (Cusabio Life Sciences, Wuhan, China) following the manufacturer’s instructions. Paired serum and synovial fluid (SF) from PsA patients not treated with biologicals (n=10) (see supplementary Table 1) and healthy donor serum (n=10); and from placebo (n=13) and adalimumab (n=11) treated PsA patients were included (see supplementary Table 2).

**TUNEL assay and immunofluorescence staining**

To detect cell apoptosis, the terminal deoxynucleotidyl-transferase dUTP nick-end labeling (TUNEL) assay was performed using a commercial kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL) according to the manufacturer’s protocol. Positive and negative controls were set up each time. Apoptotic cells were identified by positive nuclear staining. After the TUNEL assay was performed immunofluorescence was performed using a mouse monoclonal purified antibody against CLEC9A (Clone: 8F9 from Biolegend Europe b.v., Uithoorn, the Netherlands) on the same sections. After washing with PBS/BSA1% sections were incubated with Alexa 594-labeled goat anti-mouse antibody (Invitrogen, Bleiswijk, the Netherlands) for 30 min. After washing with PBS/BSA1% the slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA,) and analyzed on a fluorescence microscope (Leica DMRA, Wetzlar, Germany) coupled to a charge-coupled device (CCD) camera.

**Immunohistochemistry**

Briefly, endogenous peroxidase activity was inhibited in the acetone-fixed ST and skin sections by 0.1% sodium azide and 0.3% hydrogen peroxide in PBS. Sections were stained by using mouse monoclonal antibodies against CLEC9A (Clone: 8F9, Biolegend). Sections were sequentially incubated with HRP-labelled anti-mouse antibody, followed by HRP detection using an AEC kit (Brunschwig, Amsterdam, the Netherlands), and haematoxylin (Klinipath, Duiven, the Netherlands) as counter stain. Parallel sections were incubated with isotype- and concentration-matched monoclonal antibodies as negative controls. All samples were coded and stained in a single run to minimize technical biases. Due to the “filamentous” staining...
Reduced CLEC9A expression in synovial tissue... pattern, coded sections stained for CLEC9A were analyzed in a random order using a 4-point semiquantitative scale, as described previously (28). CD8+ T cell staining was performed as previously described (29). For the analysis of the CD8+ T cell count, the images of the high-power fields (18 high-power fields from different parts of the synovial tissue section were analyzed, e.g. the mean of the 18 high-power fields was calculated) were analyzed using the Qwin analysis system (Leica, Cambridge, UK) (30). Positive staining was expressed as cell count per mm². Scoring was carried out by two independent observers who were blinded to each patient’s diagnosis and clinical data.

Quantitative measurement of mRNA expression
Total RNA was isolated from ST biopsy samples using RNA Stat-60 (Tel-Test, Friendswood, TX), then treated with DNase I (Invitrogen), and reverse transcribed using RevertAid H Minus First-Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). 500 ng of total RNA was reverse-transcribed using SuperScript™ II RT (Invitrogen). The RNA concentration was determined with a NanoDrop spectrophotometer. Duplicate PCR reactions were performed using SYBR green (Applied Biosystems, Foster City, CA) with an ABI Prism® 7000 sequence detection system (Applied Biosystems). cDNA was amplified using specific primers: CLEC9A we used primer mix from Qiagen PPH24284A; XCR1 primer mix from Qiagen PPH01042B and GAPDH forward, TTCACCACCATGGAGAAG, GAPDH reverse, GGCATGGACTGTGGTCAT.

All PCR data were normalized to the expression of GAPDH, used as an internal control. PCR data were obtained as Ct values and the mean of the duplicate Ct values of each sample was calculated. Relative levels of gene expression were normalised to GAPDH housekeeping gene (HK) using the comparative Ct method.

Statistical evaluation
The expression of CLEC9A in HD serum and PsA paired serum and SF was compared using GraphPad Prism Software (V.5, GraphPad Software, La Jolla, CA). Differences between groups were analyzed using Kruskall-Wallis test with post-Dunn’s multiple comparison tests or non-parametric Mann Whitney U-test where appropriate. P values less than 0.05 were considered statistically significant.

SPSS version 15.0 (SPSS, Chicago, IL) was also used for statistical analysis. Correlations of clinical parameters and CLEC9A serum levels, immunohistochemical CLEC9A expression and qPCR gene expression at week 4 after adalimumab therapy were analyzed with Spearman rank correlation. Additionally, an analysis of covariance model (ANCOVA) after rank transformation was used to correct for baseline differences (31). The model included expression level at week 4 as dependent variable, treatment group as fixed effect and expression at week 0 as covariate.
Results

CLEC9A expression is increased in the skin of psoriasis patients compared with healthy donors

To investigate CLEC9A expression in skin and potential differences between HD skin and psoriasis non-lesional and lesional skin we performed immunohistochemical analysis. We found that CLEC9A expression was confined to the dermis and was significantly higher in both psoriasis lesional and non-lesional skin compared to HD skin (Figure 1A, p= 0.0021; p= 0.0246; respectively). In addition, the CLEC9A staining pattern (cellular indicated by the punctuated arrows and “filamentous” structures indicated by the straight arrows) and distribution was similar in psoriasis lesional skin and non-lesional skin CLEC9A (Figure 1A, right panel).

CLEC9A binds to dead cells present in the skin

CLEC9A expressed by CD141+ DCs was recently reported to couple the recognition of necrotic cells to the subsequent cross-presentation of dead-cell-associated antigens to CD8+ T cells (16). We performed TUNEL assays in combination with CLEC9A staining to study whether CLEC9A expression is present in close proximity with dead cells in inflamed tissues of psoriasis patients. The TUNEL assay has been designed to detect cells that undergo extensive DNA degradation during the late stages of apoptosis. We observed that CLEC9A co-localizes with late-stage apoptotic cells (Figure 1B), but not with live cells in the skin and ST of psoriasis and PsA patients, respectively, suggesting that recognition of dead cells by CLEC9A+ cells might take place in the inflamed skin and ST. Using this staining it is not possible to discriminate between soluble and membrane bound CLEC9A expression.

Lower CLEC9A protein expression after adalimumab treatment than placebo in PsA synovial tissue but not in psoriatic skin

Next we investigated whether CLEC9A expression in psoriatic skin might be modulated by TNF blockade. ANCOVA was applied to correct for baseline imbalances for the placebo and adalimumab treated groups. There was no difference in CLEC9A expression in the skin between placebo and adalimumab treated group after 4 weeks (Figure 2A; Table 1). Skin biopsies from later time points were not available to study whether any changes in CLEC9A expression might occur in a later phase after treatment. We observed that CLEC9A expression in ST was much lower than in the skin (Figure 2A and 2B). CLEC9A expression in PsA ST was significantly lower in the adalimumab treated group compared to placebo after 4 weeks (Figure 2B; Table 1), although the change from baseline per se did not reach statistical significance (p=0.187), possibly due to the relatively small size of the study. We performed qPCR analysis to understand whether differences in CLEC9A protein levels could be due to changes in CLEC9A gene expression upon treatment. In contrast to reduced CLEC9A protein expression, we found that CLEC9A gene expression was not altered after adalimumab treatment (Table 1).
Figure 1. CLEC9A expression in healthy donor (HD) skin and serum (S) and psoriatic skin, synovial tissue (ST) and paired serum (S) and synovial fluid (SF). (A) Representative immunohistochemical staining of CLEC9A expression in HD and psoriatic skin (right). Quantification of CLEC9A expression in HD and PsA skin (lesional and non-lesional). Each dot represents one donor (left). Cellular staining is indicated by the punctuated arrows and “filamentous” structures are indicated by the straight arrows. Original magnification 100x. (B) Representative CLEC9A/TUNEL double immunofluorescence staining in HD and PsA ST and psoriatic skin. Original magnification 250x. (C) CLEC9A quantification in HD serum and paired S and SF of PsA patients.
Soluble CLEC9A levels in PsA patients are similar to those in HD and are not affected by adalimumab therapy

CLEC9A is present as a cell surface receptor, but can also be found in a soluble form. We found that soluble CLEC9A is detectable in serum of HD and PsA at similar levels (approximately 1 ng/ml). Remarkably, CLEC9A levels were significantly lower in PsA SF compared to paired serum samples (Figure 1C, p=0.0037). To study whether adalimumab treatment would affect CLEC9A serum levels we collected serum samples at baseline (week 0), week 4, 12 and after 1 year of treatment with adalimumab or placebo. In PsA patients, CLEC9A serum levels were not altered after adalimumab treatment compared to placebo at any time point studied (Table 1). The effect of adalimumab on CLEC9A levels in PsA SF could not be determined as these specimens were only available at baseline; synovial fluid cannot be obtained after successful treatment.

In PsA synovial tissue CLEC9A expression correlates positively with T cell numbers and CLEC9A+ cells are in proximity to CD8+ T cells

Recently, CLEC9A was found to be required for efficient cross-presentation of dead cell associated materials (16). To study the link between CLEC9A-expressing cells and T cells in PsA ST we investigated whether the expression of these markers after 4 weeks of adalimumab treatment are associated with each other. Indeed, we observed that CLEC9A expression correlates positively with the number of CD8+ T cells and CD4+ T cells (Figure 2C; CD8+ T cells p=0.01, r=0.43; CD4+T cells p=0.003, r=0.8) in ST of PsA patients. Importantly, there was no correlation between CD68+ macrophages (in the synovial sublining (SL) and intimal lining layer (L)) and CD163+ macrophages with CLEC9A expression (Supplementary Figure 1A)). We performed immunofluorescence to understand whether CLEC9A and CD8 T cells interact in the synovial compartment. We observed that in ST from PsA patients not treated with biologicals, CLEC9A+ cells are in close proximity to CD8+ T cells (Figure 2D). In addition, in the same PsA ST samples, most of the CD141+ DCs co-expressed CLEC9A (Supplementary Figure 1B).

Discussion

We found that CLEC9A expression is significantly upregulated in psoriatic skin and that CLEC9A binds to TUNEL+ dead cells in inflamed psoriatic skin and PsA ST. After adalimumab treatment CLEC9A serum levels and expression of CLEC9A in skin were not changed compared to placebo. However, we observed that CLEC9A protein expression in ST was significantly decreased after adalimumab therapy, although CLEC9A gene expression remained unchanged. This reduction in CLEC9A protein levels might be the result of posttranslational modifications. Posttranslational modifications are important to diversify protein functions and dynamically
coordinate their signaling networks affecting enzymatic activity of kinases (32) and protein degradation (33). Importantly, in PsA ST CLEC9A expression was positively correlated with the numbers of CD8+ and CD4+ T cells after 4 weeks of adalimumab treatment. Moreover, we showed that in ST from PsA patients not treated with biologics CLEC9A+ cells are in close contact with CD8+ T cells suggesting a possible interaction. CD141+ DCs excel in antigen cross-presentation to CD8+ T cells, but can concomitantly present antigen to both CD4+ and CD8+ T lymphocytes, allowing optimal delivery of CD4+ T-cell help for CD8+ T priming (34). In PsA ST the numbers of CD8+ T cells and CD4+ T cells were significantly correlated with the expression of CLEC9A suggesting that cross-presentation and CD4+ T cell help might occur at the site of inflammation. In recent years it has become clear that DCs can in addition to being superior antigen-presenting cells for T cell priming also activate innate lymphocytes such as natural killer cells (NK) (35). NK cells are activated by IFN and TNF released by DCs upon stimulation of TLRs, usually endogenous TLR3, 7 or 9 which are activated by viral nucleic acids (36).

Expression of human CLEC9A in peripheral blood is highly restricted to CD141+ DCs and on a small subset of CD14+CD16– monocytes (15). We found that in PsA ST

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**Table 1.** ANCOVA analysis for CLEC9A expression levels after 4 weeks of adalimumab treatment comparing placebo versus adalimumab treated and corrected for week 0 after ranking of the data.

<table>
<thead>
<tr>
<th></th>
<th>adalimumab</th>
<th>Change upon treatment</th>
<th>placebo</th>
<th>Change upon treatment</th>
<th>Ancova (P value)</th>
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<tr>
<td><strong>CLEC9A expression</strong></td>
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<td><strong>Serum (ng/ml)</strong></td>
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<tr>
<td>At baseline</td>
<td>1.052</td>
<td>4 weeks -0.07</td>
<td>1.126</td>
<td>4 weeks -0.03</td>
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<td>12 weeks -0</td>
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<tr>
<td>1 year -0.10</td>
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<td><strong>Skin (week 4)</strong></td>
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<tr>
<td>(semiquantative score 0-4)</td>
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<tr>
<td>Lesional</td>
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<td>-0.1</td>
<td>1.6</td>
<td>-0.1</td>
<td>0.544</td>
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<tr>
<td>Non-lesional</td>
<td>1.4</td>
<td>0.3</td>
<td>1.3</td>
<td>-0.3</td>
<td>0.957</td>
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<td><strong>Synovial tissue</strong></td>
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<td>(week 4)</td>
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<tr>
<td>IHC (semiquantative score 0-4)</td>
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<tr>
<td>1.7</td>
<td>-0.5</td>
<td></td>
<td>2.1</td>
<td>-0.1</td>
<td>0.045</td>
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<tr>
<td>qPCR (RQ2–DDCT)</td>
<td>1.229</td>
<td>-0.30</td>
<td>0.929</td>
<td>0.22</td>
<td>0.943</td>
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A positive or negative value for change represents an increase or decrease in CLEC9A expression respectively. After ANCOVA was applied to correct for baseline imbalances, the effect of adalimumab versus placebo treatment after 4 weeks was significant only for the reduction in CLEC9A expression in ST by IHC (p = 0.045).
most of the CD141+ DCs express CLEC9A. CLEC9A has been shown to be expressed only on immature CD141+ DCs and surface expression is rapidly lost after TLR-mediated maturation (37). This differential expression of CLEC9A by CD141+ DCs in PsA ST might reflect different maturation/activation status as previously reported for other DC subsets (38). CLEC9A+CD141- cells in PsA ST might be infiltrating monocytes.

As mentioned above, CLEC9A was increased in psoriatic skin. As dermal DCs are increased in psoriatic lesions (39) it is tempting to speculate that the observed increase in CLEC9A expression in the skin of psoriasis patients might be related to an increase in DC numbers. CLEC9A has been shown to bind actin filaments that are exposed upon loss of membrane integrity, when cells undergo primary or secondary necrosis (16;40). In the skin and ST of PsA we observed that CLEC9A+ cells were in close proximity with TUNEL+ cells supporting previous observations that CLEC9A is a damaged cell-recognition molecule. In addition to the above mentioned function, CLEC9A was suggested to induce pro-inflammatory cytokine production and thus function as an activation receptor (15) contributing to the inflammatory process. We found that in SF of PsA patients soluble CLEC9A is reduced compared to paired serum. This reduction of soluble CLEC9A in the synovial compartment might be due to binding to dead cells, hijacking this receptor from solution. Indeed here we have demonstrated that CLEC9A+ cells recognize dead cells in ST and skin of PsA patients, the same phenomena might occur with soluble CLEC9A in PsA SF.

CLEC9A might be a receptor that allows CD141+ DCs to respond to tissue damage signals generated during inflammation in PsA patients. As previously mentioned, CLEC9A surface expression is downregulated after TLR-mediated maturation (37). As SF DCs have a semi-mature phenotype (41), this might also account for the lower CLEC9A expression in PsA SFMCs compared to PBMCs.

We observed that CLEC9A expression in blood is stable after adalimumab treatment. This might perhaps be explained by the very low frequency of CLEC9A expressing cells (CD141+ DCs and CD14+CD16- monocytes) in peripheral blood. Similarly, we did not observe differences in CLEC9A expression levels in the skin after adalimumab therapy compared to placebo. Despite studies in the skin of psoriasis patients have shown that TNF blockers can selectively induce apoptosis of dermal DC in plaques of responding patients (42;43), this remains controversial(22). Indeed our results suggest that adalimumab treatment does not target CD141+ DCs in the skin since no change was observed in CLEC9A expression after 4 weeks of adalimumab treatment between placebo and treated group. It should be noted however that we cannot completely exclude the possibility that the results for the skin were influenced by patient selection. The patients were primarily included in the study based on arthritis activity rather than activity of the skin lesions, and this may have contributed to a relatively low PASI (44) and possibly lower inflammatory infiltrate in the skin. Previous work has shown that the severity of arthritis and skin disease often do not correlate with each other (45). Further studies on larger groups of patients
Figure 2. CLEC9A expression in psoriatic skin (lesional and non-lesional) and ST before and after adalimumab treatment. (A) Representative immunohistochemical staining of CLEC9A expression in skin of PsA patients before (baseline) and after treatment with adalimumab. Original magnification 100x. (B) Representative immunohistochemical staining of CLEC9A expression in ST of PsA patients before (baseline) and after treatment with adalimumab. Original magnification 100x. (C) Correlation between CLEC9A expression and T cells (CD8+ and CD4+ T cells). (D) Representative CLEC9A/CD8 double immunofluorescence staining in PsA ST. Original magnification 250x.
with higher PASI are required to confirm our data on the effect of adalimumab on CLEC9A expression in the skin.

In contrast to the effects on the skin, we observed a decrease in CLEC9A expression in the ST of PsA patients after 4 weeks of treatment with adalimumab compared to placebo. This might be due to a reduction of CD141+ DCs or infiltrating monocytes. Blocking TNF can have different effects. DC trafficking to draining lymph nodes is orchestrated through a complex interplay of pro-inflammatory cytokines (TNF, IL-1β, IL-18), chemokine and chemokine receptors (CCL19, CCR2, CCR7), leukotrienes, and cell adhesion molecules (e.g. E-cadherin down-regulation) (46). The lower TNF availability might lead to a reduction in DC migration to the ST after adalimumab which might account for the reported reduction of the CLEC9A expression after therapy. It has been suggested in mice that adalimumab binds to and has an apoptosis-inducing effect on monocytes in vivo (47). However, our previous work suggested that TNF blockade in PsA results in reduced celularity which could not be explained by apoptosis induction (19) supporting our hypothesis that adalimumab might affect trafficking of CLEC9A+ cells to the inflamed synovium.

CD141+ DCs expressing CLEC9A are particularly efficient at cross-presenting necrotic antigens on MHC class I inducing CD8+ T cell responses (48). Therefore CLEC9A might be involved in cross-presentation at the site of inflammation. In agreement with this we observed a positive correlation between CLEC9A expression and CD8+ T cell numbers in PsA ST. Consistent with this observation CLEC9A+ cells were localized in the vicinity of CD8+ T cells in inflammatory infiltrates in PsA ST suggesting a possible interaction between these two cell types. Therefore we suggest that targeting CLEC9A (blockade of its function) might modulate CD8+ T cell responses in PsA ST by reducing cross-presentation.

Since T cells play such a central role in PsA, it is important to define the DC role as these cells may influence activation/expansion of T cell responses. Investigating the impact of effective therapies that modulate particular DCs subsets with specific pattern recognition receptors, such as CLEC9A might provide a valuable insight into how T cell responses are maintained during inflammation and how these responses can be dampened.

Conclusion

We observed that adalimumab therapy in PsA patients leads to a specific significant decrease in CLEC9A expression in the ST compared to placebo. Targeting this molecule known to be expressed by a specific subset of DCs (CD141+ DCs) and monocytes (CD14+CD16-) might be promising in reducing inflammation in PsA patients by modulating (cross)presentation and T cell responses.
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Supplementary Figure 1. CLEC9A expression in PsA ST. (A) Correlation between CLEC9A expression and macrophages numbers (defined by the expression of CD68 in the lining (L) and sublining (SL) and CD163) in PsA ST. No correlation was observed. (B) Representative CLEC9A/CD141 double immunofluorescence staining in PsA ST. Original magnification 250x.
## Supplementary Tables

**Supplementary Table 1.** Demographic and clinical characteristics of the patients with psoriatic arthritis

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<tr>
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<th>Serum/ Synovial Fluid</th>
<th>Skin</th>
<th>Synovial tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, female/male (n)</td>
<td>5/5 (10)</td>
<td>4/9 (13)</td>
<td>2/3 (5)</td>
</tr>
<tr>
<td>Age in years, median (range)</td>
<td>56 (45-80)</td>
<td>46.6 (21-56.8)</td>
<td>43 (37-54)</td>
</tr>
<tr>
<td>Currently receiving MTX, n (%)</td>
<td>4 (40)</td>
<td>8 (62)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Duration of PsA in years, median (range)</td>
<td>5.12 (0.4-23.2)</td>
<td>6.6 (0.4-18.2)</td>
<td>10.2 (4.5-19)</td>
</tr>
<tr>
<td>DAS28 score, median (range)</td>
<td>4 (2.09-4.82)</td>
<td>5.26 (3-5.97)</td>
<td>4.86 (4.20-5.76)</td>
</tr>
<tr>
<td>VAS disease activity, median (range)</td>
<td>n.a.*</td>
<td>71 (34-92)</td>
<td>72.50 (50-76)</td>
</tr>
<tr>
<td>VAS pain, median (range)</td>
<td>n.a.*</td>
<td>76 (41-88)</td>
<td>n.a*</td>
</tr>
<tr>
<td>CRP (mg/liter), median (range)</td>
<td>17.15 (0.7-174.4)</td>
<td>5.65 (1.7-26.7)</td>
<td>25.6 (1.3-51)</td>
</tr>
<tr>
<td>ESR mm/h, median (range)</td>
<td>14 (2-64)</td>
<td>14 (4-66)</td>
<td>45 (8-48)</td>
</tr>
<tr>
<td>PASI, median (range)</td>
<td>n.a.*</td>
<td>6 (1.5-13.8)</td>
<td>6.2 (0-6.2)</td>
</tr>
</tbody>
</table>

*n.a.: not available; MTX, methotrexate; DAS28, disease activity score of 28 joint; VAS, visual analogue scale (100 mm); CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PASI, psoriasis area severity index.
Supplementary Table 2. Demographic and clinical characteristics of the patients with psoriatic arthritis enrolled in the trial

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Skin</th>
<th>Synovial tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adalimumab (n=12)</td>
<td>Placebo (n=12)</td>
<td>Adalimumab (n=9)</td>
</tr>
<tr>
<td>Sex, female/male (n)</td>
<td>9/3 (12)</td>
<td>6/6 (12)</td>
<td>3/6 (9)</td>
</tr>
<tr>
<td>Age in years, median(range)</td>
<td>42.8 (21–61)</td>
<td>47.2 (25–78)</td>
<td>39.4 (21-56.8)</td>
</tr>
<tr>
<td>Current receiving MTX, number (%)</td>
<td>7 (58)</td>
<td>5 (42)</td>
<td>6 (6.6)</td>
</tr>
<tr>
<td>MTX dose, mg/week</td>
<td>18.2 (10–25)</td>
<td>19.0 (15–25)</td>
<td>17.5</td>
</tr>
<tr>
<td>Duration of psoriasis in years, median (range)</td>
<td>6.8 (0.1-26.5)</td>
<td>15.3 (1,9-53,2)</td>
<td>6.2 (0.1-22.1)</td>
</tr>
<tr>
<td>Duration of PsA in years, median (range)</td>
<td>5.5 (0.4–14.1)</td>
<td>8.4 (1.9-18.2)</td>
<td>4.1 (0.4-11.2)</td>
</tr>
<tr>
<td>DAS28 score (range)</td>
<td>4.67 (3.0–5.78)</td>
<td>5.07 (2.21–6.83)</td>
<td>4.79 (3-5.78)</td>
</tr>
<tr>
<td>VAS disease activity, median (range)</td>
<td>73 (45–94)</td>
<td>62.8 (18–92)</td>
<td>71 (45-85)</td>
</tr>
<tr>
<td>VAS pain, mean (range)</td>
<td>72.8 (55–91)</td>
<td>67.4 (11–89)</td>
<td>73 (55-85)</td>
</tr>
<tr>
<td>CRP (mg/liter), median (range)</td>
<td>19.9 (2.3–81.6)</td>
<td>9.9 (1.3–26.7)</td>
<td>5.70 (2.3-81.6)</td>
</tr>
<tr>
<td>ESR mm/h, median (range)</td>
<td>24.2 (4–66)</td>
<td>22.4 (3–66)</td>
<td>16 (4-66)</td>
</tr>
<tr>
<td>PASI (range)</td>
<td>5.89 (0-13.8)</td>
<td>4.72 (0-7.1)</td>
<td>3.7 (1.5-13.8)</td>
</tr>
</tbody>
</table>

MTX, methotrexate; DAS28, disease activity score; VAS, visual analogue scale (100 mm); CRP, C reactive protein; ESR, erythrocyte sedimentation rate, PASI, psoriasis area severity index.