Short thesis for the degree of Doctor of Philosophy (PhD)

INVESTIGATION OF IMPAIRED IMMUNOREGULATION AND TISSUE REMODELING IN ATOPIC DERMATITIS

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The Examination takes place at the Library of the Dept. of Pediatrics, Faculty of Medicine, University of Debrecen, on 14 April 2015 at 11 am.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on 14 April 2015 at 1 pm.

1. INTRODUCTION

Atopic dermatitis (AD) is a common, chronic inflammatory skin disease showing remissions and relapses, and characterized by skin dryness, intense pruritus, as well as tissue remodeling in the most severe cases.

Previous studies have helped to understand the pathophysiology of the disease, however details must be elucidated. In the background of the impaired immune response the changes in immune tolerance may play a possible role. Regulatory T-cells are important contributors to peripheral immune tolerance. Three main subgroups of these cells have been identified, one of them is the group of $CD4^{+}CD25^{bright}FOXP3^{+}$ T regulatory (Treg) cells. The natural Treg (nTreg) cells are in focus of current studies in AD, however the results are controversial.

Another important process in chronic recalcitrant AD is tissue remodelling that is characterized by skin structural changes, however the underlying mechanisms are unknown. The proinflammatory cytokines and chemokines participate not only in the initiation, maintenance and regulation of the inflammation in AD, but also may play role in tissue remodelling, however there are no data about it.

The first part of the thesis summarizes the results of our investigations about the role of immunregulatory changes in AD, while in the second part the results of the investigations of a possible communication pathway in atopic skin tissue remodelling is revealed.

2. ATOPIC DERMATITIS

2.1. AD – ethiology, genetics, epidemiology, effects of environmental factors

Genetic and environmental factors (including gene-gene and gene-environment interactions) play role in the development of AD. The skin physic-chemical barrier function as well as the innate and adaptive immune responses are genetically well-defined processes that are affected by specific and aspecific environmental factors. The compound and sophisticated interactions among these factors determine the development of the disease.

The prevalence of the disease has benn increasing sice the 70’s among the children population, however the geographical diversities show broad differencies. The prevalence in the adult population is lower (1-3%).

Genomic studies have identified many AD-related locuses. Beside the genes responsible for correct immune responses, the skin barrier related genes are also important in AD development. Among the latter ones filaggrin (*FLG*) gene plays important part.

Upon genetic predisposition after the affection of environmental factors the disease may be manifested. Environmental factors are divided into two groups; the specific (allergens, microbes) and the aspecific (mechanical irriations, climate, stress, drugs, and pregnancy) factors.

In homeostatic conditions many microorganisms colonize the skin, however the toxin-producing *S. aureus* is not part of the normal skin microbiom. In the majority of AD patients (about 90 %) the *S. aureus* colonization is obvious on lesional and non-lesional skin (vs healthy people where 5-10 % is appearent). The degree of colonization correlates the severity of the disease and the clinical signs are proved to improve after antibiotic treatment. The AD skin-colonized bacteria and their toxins (enterotoxin A-D) attribute broad biological activity spectra and participate in the worsening of atopic skin inflammation.

2.2. AD pathogenesis

In AD, behind the well characterized clinical symptoms complex pathomechanism is found. In the background of the disease the epidermal barrier dysfunction and the abnormalities of immunmechanisms are described in extent that the individuals prone to develop the disease are unable to resist outer afflictions and hypersensitivity reactions may develop upon otherwise harmless environmental elements.

Barrier impairment in AD

The human skin barrier is a metabolically active, physiologically important layer that serves as a physical and chemical protection against environmetal factors, and also helps to prevent transepidermal waterloss (TEWL). The structural elements of the barrier are the keratinocytes, proteins, cell-cell connections, and the intercellular lipids. The cornified envelope is in connection with the epidermal structural proteins among which FLG plays an important role. FLG provides a mechanical scaffold and its end-products form the natural moisturizing factor, playing crucial task in skin hydration, acidic pH, as well as regulates protease activity, barrier permability, and anti-microbial protection. The impairment of the barrier may be genetic or acquired. Among genetic factors the *FLG* gene mutation is the most common variant, while microorganisms appering on the skin surface as well as irritant chemicals may cause acquired changes in the barrier.

Tissue remodelling in AD

As the result of the structural and functional changes in the skin barrier as well as the perpetuate inflammation present in the skin there may be other changes in atopic skin. In AD patients proinflammatory cytokines are responsible for the initiation and maintenance of skin inflammation, and may also participate in the pathogenesis of remodeling. The underlying mechanisms (participating cellular and humoral elements) of tissue remodeling in chronic, reractory AD are not well understood.

Changes in immunological mechanisms in AD

The disturbances in the regulation of immune responses (e.g. accelerated Th2 responses and allergic senzitizations, impaired immune tolerance and innate immunity) and in the elements of barrier functions play very important and integrated role in the pathomechanism of AD.

To understand the immunology of AD it is inevitable to know the components of adaptive immune responses. The role of T cells are crucial in the development and progression of the disease. In the acute phase of AD IL-5 and IL-13 producing memory cells and Th1/Th2 inbalance in favour of Th2 cells may be appearent. There is a dual connection between the physic-chemical barrier and the overweight Th2 inbalance: inflammation promotes barrier dysfunction, while allergens penetrating through the impaired barrier cause Th2 production. Because of the increased Th2 inflammation the amount of antimicrobial peptides (AMP) decreases, and the Th2 cytokines (IL-4, IL-13) accelerate the expression of adhesion molecules on endothelial cells, as well as induce IgE production in B cells, while CCL11 and CCL26 chemokines enhance the recruitment of eosinophils into the inflammation. In the chronic phase of AD the cutaneous Th2 dominance is ceased and the Th1 and Th22 cells and their products (interferon (IFN)-γ, IL-12, IL-5, granulocyta-makrofág-CSF, illetve IL-22) play crucial role.

The well-operating immune system recognizes self and foreign antigen structures, and differentiates between harmful and harmless impulses. To maintain the normal immune homeostasis the immune system must protect the host from foreign structures and must tolerate self structures. This is defined as immunetolerance that is configured by positive and negative selection processes. The T lymphocytes reaching the periphery are controlled by the mechanisms of peripheral tolerance. There are 3 subgroups of regulative T cells at the periphery: $TGF\beta^{+}FOXP3^{-}$ Th3 sejtek; Type-1 $IL\text{-}10^{+}FOXP3^{-}$ regulatory T (Tr1) cells, and $CD4^{+}CD25^{bright}FOXP3^{+}$ natural regulative (nTreg) cells.

The nTregs control the allergen specific T cell responses. Only the FOXP3 expressing $CD4^{+}CD25^{bright}$ regulative cells possess suppressing qualities. They inhibit T cell

differentiation, activation, proliferation, cytokine secretion, migration, as well as they prevent the development of lymphoproliferative disorders, maintain normal immune homeostasis, and modulate immune responses.

There is little known about the role and functions of nTregs in atopic skin inflammation. Some studies described the protective effect of increased nTreg cell numbers in early age where allergen specific tolerance developed in AD children due to elevated nTreg cell number. In adult AD population the elevated numbers of circulating $CD4^{+}CD25^{bright}FOXP3^{+}$ nTregs are described; however functional characterizations are carried out only by a few study groups and the results are controversial.

Beside the accelerated and impaired adaptive immune responses AD may be characterized by the dysregulative innate immunity as well. One of the tasks of innate immunity is to recognize microbial structures by pathogen-associated molecular patterns and pattern recognition receptors (PRR). After PRR binding the microbial molecules are eliminated. In AD, due to TLR2, CD14, NOD1 polymorphisms, the recognition of microorganisms is impaired and it leads to disturbed innate immunity. These changes along with the AMP deficiencies and barrier dysfunctions may promote bacterial skin colonization, protective mechanism deficiencies, and possible epidermal infections.

Chemokines and their roles in AD

Chemokines (46 ligands and 19 receptors) are small cytokine-like proteins that regulate leukocyte trafficking under homeostatic and inflammatory conditions. The main sources of chemokines are the inflammatory cells and the skin structural resident cells. Recently, a defined set of chemokines was identified to initiate and perpetuate atopic skin inflammation. It has also been postulated that chemokines may have a role in the pathogenesis of cutaneous tissue remodeling in atopic skin; however, no report demonstrates their contribution yet.

2.3. AD – clinical signs, diagnostics, therapy

In the clinical pattern of the disease we can distinguish 3 stages (acute, subacute, chronic) that are characterized by unique clinical features. The leading symptoms are skin dryness and pruritus. The most severe complications of AD are the secondary bacterial and viral superinfections. The management of the disease may require complex treatment. The primary aim is to improve the skin barrier functions, and furthermore to have the inflammation ceased and to eliminate the provoking environmental factors. Our final goal may be to reach symptom-free state and to improve quality of life.

2.4. OBJECTIVES

In our study our aim was to investigate 2 important mechanisms of AD pathogenesis in details.

2.4.1. Investigation of nTreg cells in AD

Because of the controversial results found in literature we investigated the role of peripheral nTrges in AD. Our aims were the followings:

1. what changes may be seen in the functional and quantitative characters of circulating $CD4^{+}CD25^{bright}FOXP3^{+}CD127^{-}$ nTreg cells and skin homing nTregs in AD peripheral blood compared with healthy controls,

2. investigation of functional changes in the suppressor activity of nTreg cells in healthy controls and AD patients; effect of SEB on the cells,

3. possible correlations between nTreg numbers and clinical data.

2.4.2. Investigation of tissue remodelling in AD

Chemokines play key role in atopic skin inflammation, thus we were wondering if they have a direct effect on normal human dermal fibroblasts (NHDF) and if they take part in tissue remodeling. Our aim was to determine chemokine-mediated pathways in the tissue remodelling during atopic skin inlammation:

1. the chemokine receptor repertoire of human dermal fibroblasts, and the determination of cell surface chemokine receptor expressed the most,

2. distribution of CCR3 ligands' (CCL5, CCL8, CCL11, CCL24 és CCL26) mRNA expression in chronic skin inflammations and *in vivo* in atopic skin,

3. CCL26 chemokine expression and its regulation,

4. effect of CCL26 on cell migration and repair activity of fibroblasts by functional tests.

3. PATIENTS AND METODS

3.1. Patients

To investigate nTreg cells, peripheral blood of 27 moderate-to-severe AD patients with high level IgE (>1000 U/ml) and 11 age and sex matched healthy volunteers were recruited. To investigate tissue remodelling six-millimeter punch biopsies were taken from healthy

individuals (n = 29), non-lesional (n = 9) and lesional (n = 37) psoriasis, non-lesional (n = 29) and lesional (n = 65) chronic AD, and prurigo nodularis (n = 46) patients. Atopy patch tests (APT) were performed with mixed house dust mite preparations (Stallergen SA, Antony, France) on 17 patients with atopic dermatitis and positive house dust mite prick tests. Of those tested, seven patients reacted positively and were chosen for the study. AD was identified according to the criteria defined by Hanifin and Rajka, and AD severity was assessed by SCORing AD (SCORAD) index. Patients were given no systemic corticosteroid or other immunomodulant therapy 4 weeks, or local treatments 2 weeks prior to sample taking. The studies were approved by the local ethics committee, and patients gave written informed consent. The experiments were performed according to the Helsinki Declaration.

3.2. Cell cultures

Human primary epidermal keratinocytes, and normal human dermal fibroblasts (NHDF) were cultured in keratinocyte (KGM-2), or fibroblast (FGM-2) growth medium (all Clonetics, San Diego, CA, USA).

3.3. Measurement of serum IgE level

Serum total IgE levels were measured by using an enzyme-linked immunosorbent assay (Radim SpA, Pomezia (Rome), Italy) according to the manufacturer's instructions.

3.4. Flow cytometry analysis

Investigation of nTreg cells by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Sigma-Aldrich, Munich, Germany) gradient centrifugation from heparinised blood. CD4+CD25+ Treg cells were separated by positive selection with a regulatory T-cell isolation kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Flow cytometry was utilised to determine cutaneous lymphocyte-associated antigen positive (CLA^+) $CD4^+CD25^{bright}FOXP3^+$ Tregs in the blood samples of AD patients and healthy controls. Because of the limitation of employable antibodies (regarding colours), first we proved that the gated $CD4^+CD25^{bright}$ cells were almost exclusively FOXP3 positive and CD127 negative/low. These proportions were calculated as the percentage of gated $CD4^+CD25^{bright}$ cells. Next, the anti-CD127 antibody was replaced by anti-CLA antibody. CLA positivity was investigated on the gated $CD4^+CD25^{bright}FOXP3^+$ T cells. Cell surface and intracellular staining was carried out according to the manufacturer's instructions. Briefly,

after PBMC preparation, cells were washed in PBS and cell surface staining was performed using CD4-APC or CD4-FITC, CD127-APC, CLA-FITC (all Becton Dickinson, BD Pharmingen, Heidelberg, Germany), and CD25-PC5 (Immunotech, Marseille, France) antibodies. After cell surface staining, cells were washed in cold Flow Cytometry Staining Buffer and re-suspended in Fixation/Permeabilisation working solution (eBioscience, San Diego, CA, USA), and washed with Permeabilisation Buffer (eBioscience, San Diego, CA, USA) anti-human FOXP3-PE (clone PCH101; eBioscience, San Diego, CA, USA) antibody was added to the cell suspension. Samples were analysed on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Data were analysed by CellsQuest software (Becton Dickinson, BD Pharmingen, Heidelberg, Germany).

Chemokine receptors on fibroblasts by flow cytometry

Systematic flow cytometric analyses of the chemokine receptor repertoire of cultured NHDFs were performed by PE-conjugated antibodies [CCR1-10, CXCR1-6 and isotype control (all R&D Systems Inc., Minneapolis, MN, USA)]. To investigate CCR3 expression cells were stained with phycoerythrin-conjugated rat anti-human anti-CCR3 (IgG2a) monoclonal antibody (BD Pharmingen, San Diego, CA, USA), or appropriate isotype control. Fluorescence was quantified by FACScan and CellQuest software (BD Pharmingen, Heidelberg, Germany).

3.5. Cell suppression tests

The $CD4^+CD25^+$ Regulatory T-Cell Isolation Kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) was used to obtain Tregs from peripheral blood (PBMC), according to the manufacturer's advice. To detect the suppressor activity of $CD4^+CD25^+$ Tregs, $1x10^5$ $CD4^+CD25^+$ T-cells were co-cultured with $1x10^5$ $CD4^+CD25^-$ effector T-cells in a 96-well microtitre plate. For T-cell stimulation, anti-CD3/CD28 microbeads (Invitrogen Dynal AS, Oslo, Norway) were applied alone or together with *Staphylococcus* enterotoxin B (SEB) (Sigma-Aldrich, Munich, Germany). For the basic experiment, one anti-CD3/CD28 microbead per cell and a concentration of 1 pg/ml SEB were used. To prove a selective response of Tregs for suppressing SEB-stimulated effector T-cells, dose-response experiments were utilised. Cell proliferation was measured by EZ4U colorimetric cell proliferation assay (Biomedica, Vienna, Austria). All of the measurements were performed in triplicate and averages were calculated. Suppressor activity was determined by an index calculated from the optical density (OD) values. The OD of mixed $CD4^+CD25^-$/$CD4^+CD25^+$ lymphocyte culture

was corrected with the OD of $CD4^+CD25^+$ T-cells as the background. The suppressor activity index was equal to $OD_{CD25-}/(OD_{MLR}-OD_{CD25+})$, where MLR stands for mixed lymphocyte reaction.

3.6. Quantitative real-time RT- polymerase chain reaction (PCR) (TaqMan) analysis

Human primary epidermal keratinocytes, and normal human dermal fibroblasts were either left untreated or stimulated for different period of time with either TNF-α plus IL-1β, or IFN-γ, or IL- 13, or IL-4, or hGM-CSF (Schering- Plough Research Institute, Kenilworth, NJ, USA). All other reagents were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Quantitative real-time RT-PCR analyses were performed from skin biopsies. Specimens were homogenized in liquid nitrogen using Mikro-DismembratorU (Braun Biotech, San Diego, CA, USA) and RNA was extracted using TRIzol (Invitrogen, Karlsruhe, Germany). Total RNA of 4 μg was treated with DNase I (Boehringer Mannheim, Germany) and reverse transcribed. The cDNA was subjected to qPCR analyses (ABI PRISM 7000 Sequence Detection Systems (Applied Biosystems) continu-ously during 40 cycles) of chemokine and receptor expression. Target gene expression was normalized to 18S RNA expression. Primers and probes specific for chemokines and their receptors were obtained from Applied Biosystems (Darmstadt, Germany).

3.7. Histology investigations

To determine tissue remodeling and changes within the dermal compartment, skin specimens from healthy volunteers and AD patients with chronic lesions were stained by elastic van Giesson, and Masson's trichrome.

3.8. Immunfluorescent investigations

Immunfluorescent investigations in skin

Immunofluorescence analyses of CCL26 protein expression in normal skin of healthy volunteers and lesional skin of AD patients was performed using acetone fixed cryo-sections. Sections were stained overnight with a goat anti-human CCL26 or isotype controls antibodies, respectively (goat IgG; Santa Cruz Biotechnology, Heidelberg, Germany; Jackson Immunoresearch, West Grove, PA). Subsequently, sections were stained with an AlexaFluor 555-labeled donkey anti-goat antibody (R&D Systems, Mineapolis, MN, USA) and DAPI (Invitrogen, Karlsruhe, Germany). Images were captured using a Zeiss Cell Observer System (Zeiss, Oberko- chen, Germany).

Immunofluorescent investigations on NHDF cells

NHDFs were cultured in chamber slides and fixed. Staining was performed with primary antibodies against human CCR3 (goat IgG; Santa Cruz Biotechnology, Heidelberg, Germany), and goat IgG (Jackson Immunoresearch, West Grove, PA) as isotype control. Binding was detected by rabbit anti-goat fluorescent secondary antibodies (1:200; Molecular Probes, Eugene, OR, USA). For double immunofluorescent staining in healthy (n = 10), and lesional AD (n = 10) skin, frozen sections were fixed and fibroblast surface protein (FSP) was stained by anti-FSP (mouse IgG; Sigma, Taufkirchen, Germany), and then with fluorescent secondary anti-mouse IgG antibody (R&D Systems, Mineapolis, MN, USA). Afterwards CCR3 was detected by anti-CCR3 (goat IgG; Santa Cruz Biotechnology, Heidelberg, Germany), and fluorescent secondary anti-goat IgG antibody (R&D Systems, Mineapolis, MN, USA). DAPI (Invitrogen, Karlsruhe, Germany) was used for nuclear staining. Cells and samples were examined with Zeiss Axiovert2 MOT microscope (Zeiss, Oberkochen, Germany).

3.9. Functional analysis on NHDF cells

***In vitro* reparation**

NHDFs were cultured to 80% confluence, and a defined single path scratch was made with a sterile pipette tip through the intact cell layer. Cells were washed, and either medium or 100 ng/ml CCL26 was added to each well. For neutralizing experiments cells were treated for 30 min with mouse anti-human CCR3 (2 μg/ml) before addition of CCL26 (both R&D Systems Inc., Minneapolis, MN). The experiment was performed in triplicates. The status of the single path wound was monitored for 15 h by time-lapse video microscopy (Inverse Leitz Microscope with Incubator, Zeiss AxioCam HRc, Zeiss AxioVision Software, Carl Zeiss, Oberkochen, Germany).

Fibroblast migration on 3D collagen matrix

To gain spheroids, NHDFs were plated into agarose gel-coated wells (Agarose TypVII Low Gelling, Sigma, Taufkirchen, Germany), and cultured for 24 h. Fibroblast spheroids (1 spheroid/well) were embedded in Matrigel (BD Bioscience, Erembodegem, Belgium) in 24-well plate, into which 100 ng/ml CCL26 was added, or was left untreated. The experiment was performed in quadruplicates. To gain migratory activity of cells, the area of spheroids was determined at day 0 and day 6. Motility of individual cells was also tracked. In the

measurement and analyses digital camera system (Olympus, Hamburg, Germany), and Zeiss AxioVision Software (Carl Zeiss, Oberkochen, Germany) were used.

Calcium-imaging by confocal microscopy

Changes in intracellular calcium ([Ca2+]i) upon drug applications were detected by fluorimetric Ca2+ imaging. Cells were seeded on glass coverslips at pre-confluent density one day before measurements. Cells were incubated in Hank's solution (Sigma, Taufkirchen, Germany) containing 1% bovine serum albumin and 2.5 mM Probenecid (both from Sigma, Taufkirchen, Germany) with 2 mM of the cytoplasmic calcium indicator Fluo-4 AM (Invitrogen, Karlsruhe, Germany) at 37 ^{o}C for 40 min. The cells were washed and incubated then coverslips were placed in a custom-made mount, and imaged using an LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Cells were treated with 100 ng/ml CCL26 (R&D Systems Inc., Minneapolis, MN, USA). For neutralization mouse anti-human CCR3 (2 μg/ml) was used. A total of 200 images were taken during one treatment (Zeiss LSM Image Browser version 4.2.0.121, Zeiss, Oberkochen, Germany). The relative fluorescence intensity of responding cells was determined with the ImageJ program (NIH, Bethesda MD, USA) in each frame.

BrdU cell proliferation assay

NHDFs were cultured in standard 96-well microtiter plates in the presence, or absence of either 10 ng/ ml, or 100 ng/ml, or 1000 ng/ml CCL26. 24 h after seeding and incubating, 20 μl BrdU was added to each well. After incubation DNA synthesis was assayed with Cell Proliferation ELISA, BrdU (Roche Molecular Biochemicals, Indianapolis, IN, USA) using colorimetric detection according to the manufacturer's instructions. Newly synthesized BrdU-DNA was determined by ELISA-reader (BioRad, 450).

3.10. Statistical analysis

Statistical analysis was performed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). The normal distribution of data was tested using the Kolmogorov-Smirnov test. Student's t-test or the nonparametric Mann–Whitney test and Kruskal-Wallis test were used to compare the data of patients with AD to those from healthy controls. The correlation between the percentage of $CD4^{+}CD25^{bright}FOXP3^{+}$ Tregs and $CLA^{+}CD4^{+}CD25^{bright}FOXP3^{+}$ cells as well as IgE levels and the SCORAD index was assessed by Pearson's correlation. Data are

presented as mean ± SD, and $p < 0.05$ was considered statistically significant (*$p<0.05$; **$p<0.01$; ***$p<0.005$).

4. RESULTS

4.1. Investigation of nTreg cells

Determination of nTreg population in the peripheral blood of AD patients by $CD4^+CD25^{bright}CD127^{-/low}FOXP3^+$ staining (4 colour combination)

The presence or absence of surface markers may help to identify the $CD4^+CD25^+$ cells with regulative features; however there is no unified method available. First we used a 4-colour-method and we determined $CD4^+CD25^{bright}CD127^{-/low}FOXP3^+$ Tregs by flow cytometry in a small number of AD patients (n=3). We proved that the gated $CD4^+CD25^{bright}$ cells were almost exclusively FOXP3 positive (95.3% + 3.4%), or CD127 negative/low (93.4% + 4.1%). The proportion was calculated as the percentage of gated $CD4^+CD25^{bright}$ cells (97.3% + 2.0% of the $CD4^+CD25^{bright}$ cells were $FOXP3^+$ and $CD127^{-/low}$). The cells with $CD4^+CD25^{bright}FOXP3^+$ phenotype were examined in further analysis.

Determination of nTreg numbers in the peripheral blood of AD patients by $CD4^+CD25^{bright}FOXP3^+$ staining (3 colour combination)

Flow cytometric analyses were performed to evaluate the frequencies of $CD4^+CD25^{bright}FOXP3^+$ and $CLA^+CD4^+CD25^{bright}FOXP3^+$ Treg cells among $CD4^+$ T-cells in the peripheral blood of AD patients (n=27) with high IgE levels and age- and sex-matched healthy controls (n=11). The percentage of $CD4^+CD25^{bright}FOXP3^+$ Tregs in total $CD4^+$ T-cells was significantly elevated in patients with AD compared to healthy controls (AD: 3.62% ± 1.55; control: 2.19% ± 0.84; $p=0.014$). To address whether the phenotype of Tregs could reflect the ability of cells to migrate to the skin, we determined the proportion of CLA expressing $CD4^+CD25^{bright}FOXP3^+$ Tregs in the $CD4^+$ T-cells as well as among the peripheral Treg population. The frequency of $CLA^+CD4^+CD25^{bright}FOXP3^+$ Tregs was significantly higher in patients with AD compared to healthy controls (AD: 0.78% ± 0.46; control: 0.43% ± 0.17; $p=0.048$).

4.2. Correlations between the laboratory parameters of nTregs and the clinical signs

A statistically significant correlation was found between the percentage of $CD4^+CD25^{bright}FOXP3^+$ Tregs and serum levels of IgE ($r= 0.514$, $p= 0.041$) or SCORAD

index (r= 0.584, p= 0.018). We also detected a significant correlation between $CLA^+CD4^+CD25^{bright}FOXP3^+$ Tregs and IgE level (r= 0.561, p= 0.024) as well as SCORAD (r= 0.789, p= 0.0003).

4.3. The *in vitro* functional examination of nTregs

Changes in Treg suppressor activity after SEB stimulation

We compared the functional activity of Tregs obtained from randomly selected AD patients (n=11) and healthy controls (n=11). Since $CD4^+CD25^+$ cells can be either effector or regulatory cells, to investigate the suppressor activity of $CD4^+CD25^+$ T-cells, magnetically isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells were cultured alone and together in the presence of anti-CD3/CD28 microbeads, and the effect of exotoxin SEB was also tested. In the culture of mixed lymphocyte reaction (MLR) of $CD4^+CD25^-/CD4^+CD25^+$ cells, the presence of $CD4^+CD25^+$ cells caused a significant decrease in the proliferation of $CD4^+CD25^-$ effector T-cells. The suppressor function of these cells was characterised using a suppressor activity index. The suppressor activity of $CD4^+CD25^+$ T-cells that were not subjected to SEB stimulation was higher in patients with AD than in healthy controls (AD: 4.30 +/- 1.79; control: 2.71 +/- 0.72), although the difference was not significant. However, in the presence of SEB, the suppressor activity of $CD4^+CD25^+$ T-cells was significantly decreased in both AD patients and healthy controls (AD: 1.79 +/- 0.09; control: 1.49 +/- 0.10).

Dose-dependent changes of nTreg cell suppressor activity after SEB effect

The selective, dose-response reaction of Tregs, suppressing effector T-cells in the presence of SEB, was proved first when an increasing Treg : Teffector cell ratio (1:2, 1:1, 3:1) was utilised with constant SEB concentration (1 pg/ml). The higher the Treg : Teffector ratio was increased, the more the Tregs suppressed effector T-cells (20-60% decrease in the proliferation of effector T-cells). When the effect of increasing concentrations of SEB (1 pg/ml, 500 pg/ml, 5ng/ml) was analysed together with an equal ratio of Treg : Teffector cells (1:1 ratio), a high concentration of SEB (>500 pg/ml) diminished the suppressor activity of Tregs, causing increased proliferation of effector T-cells.

4.4. Investigation of tissue remodelling in atopic skin inflammation

To determine changes in matrix deposition during chronic atopic skin inflammation histochemical analyses were performed in specimens of lesional skin of chronic AD patients (n = 37) and that of healthy volunteers (n = 10). Elastica van Giesson staining showed marked

decrease of normal elastic fibers in AD patients compared to healthy skin, while Masson's trichrome staining showed that the amount of collagen bundles in the papillary dermis is markedly increased in chronic atopic skin inflammation compared to normal skin.

4.5. Chemokine receptor expressions *in vitro* és *in vivo* expressions in human fibroblasts

Recently, chemokines have been shown to play a crucial role in the initiation and amplification of atopic skin inflammation. Next we sought to investigate whether chemokines directly interact with human dermal fibroblasts and contribute to tissue remodeling. Systematic flow cytometric analyses of the chemokine receptor repertoire of cultured NHDFs showed that fibroblasts abundantly express CCR3 on their cell surface. Immunofluorescence analyses confirmed the expression of CCR3 on NHDFs *in vitro*, also on FSP-positive dermal fibroblasts *in vivo*.

4.6. CCR3 chemokine receptor and ligand expression in AD

In vitro mRNA expressions

Although some information on the chemokine signature of AD was recently reported, no systematic analysis of the expression of CCR3 and its ligands in chronic inflammatory and autoimmune skin diseases in the context of tissue remodeling exists, to date. Hence, we aimed to compare the expression profiles of CCR3, CCL5, CCL8, CCL11, CCL24 and CCL26 in lesional (n = 65) and non-lesional (n = 29) skin of AD patients with lesional (n = 37) or non-lesional skin (n = 37) of psoriatic patients. Moreover, we investigated expressions in prurigo nodularis (n = 46) and normal skin of healthy individuals (n = 29). The qPCR analyses showed that CCL26 represents the most AD-specific CCR3 ligand. On average CCL26 was expressed 150-fold higher in lesional AD skin compared with skin samples from non-lesional AD and healthy skin, and more than 15 times higher when compared with lesional psoriatic skin (**$p < 0.01$). Interestingly, CCL11, the most exten-sively studied CCR3 ligand, did not show a dominant regulation during atopic versus psoriatic skin inflammation. Although CCL11 was 4-fold higher expressed in lesional AD compared to healthy skin and to non-lesional atopic skin and 3-fold higher than in lesional psoriatic skin the results did not meet statistical significance. Overall, CCL24 transcript levels were low but showed significant up-regulation in inflamed AD skin compared with non-lesional AD or lesional psoriatic skin (***$p < 0.005$). In contrast, CCL5 and CCL8 were predominantly expressed in psoriatic skin and down-regulated in lesional AD skin. Their corresponding receptor CCR3 showed highest expression in lesional AD followed by prurigo nodularis. CCR3 transcripts were significantly

induced in lesional AD compared with non-lesional atopic, lesional psoriatic or healthy skin. Taken together, our findings identify CCL26 among the ligands of CCR3 as the chemokine showing the strongest regulation and association with AD.

***In vivo* tissue expressions**

To obtain insights into the cellular origin of CCL26 in healthy skin of normal volunteers and lesional skin of atopic dermatitis patients, we performed immunofluorescence analyses using specific antibodies directed against CCL26 or isotype controls. CCL26 displayed abundant expression in the epidermis of lesional AD skin. Computer-assisted image analyses of skin specimens of healthy volunteers (n = 9) and lesional skin of atopic dermatitis patients (n = 10) revealed that CCL26 protein is significantly overexpressed within the epidermal compartment during atopic skin inflammation (***$p < 0.005$; Student's t-test). During atopic skin inflammation, keratinocytes of basal and suprabasal layers of the epidermis represented the dominant source of CCL26 protein. Furthermore, endothelial cells of the superficial dermal plexus displayed immunoreactivity for CCL26 protein.

4.7. CCR3 and CCL26 regulations *in vivo* and *in vitro*

Expression kinetics of CCR3 and CCL26 in atopic skin

To further investigate the regulation of CCL26 and its receptor during atopic skin inflammation *in vivo*, we determined their expression kinetics during atopy patch test reactions. In patients with a history of house dust mite allergy and positive atopy patch tests (n = 7), CCL26 and CCR3 mRNA expression were determined 2, 6, and 48 h post relevant allergen exposure and compared with untreated skin. Six out of seven patients showed a more than 2-fold upregulation of CCL26 expression during the course of the atopy patch test reaction. Overall, highest CCL26 mRNA expression was observed at early time points after allergen exposure (2–6 h; three patients). Patients showing peak expression at 48 h post allergen challenge showed on average lower mRNA expression levels. With regard to CCR3 mRNA, in four out of six patients peak CCR3 expression followed CCL26 transcription indicating an orchestrated program of transcription in sensitized patients post allergen exposure.

Regulations of CCL26 expressions in primary keratinocytes and NHDF cells

To gain insights into the regulatory pathways of CCL26 and other eotaxin family members (CCL11 and CCL24), we treated human primary epidermal keratinocytes and NHDFs with

proinflammatory cytokines (TNF-a/IL-1b), and Th cell-derived effector cytokines (IFN-γ, IL-4 and IL-13) that are known to play a crucial role in the initiation or amplification of atopic skin inflammation. The qPCR analyses showed that keratinocytes constitutively express low levels of CCL26 that can be significantly induced by IL-4 and IL-13 following stimulation for 6 or 24 h. Direct comparison with other eotaxin family members indicated that CCL26 was expressed at more than 100-fold higher levels in keratinocytes compared to CCL11 or CCL24 indicating its dominant role as CCR3 ligand expressed in the epithelial compartment. Although CCL26 expression was very low in NHDFs, 24-h stimulation with IL-4 and IL-13 markedly induced CCL26 expres-sion. Similar to CCL26, resting NHDFs expressed low levels of CCL11. Direct comparison of the expression of eotaxin family members in NHDF indicated that CCL11 is homeostatically expressed and both CCL11 and CCL26 are markedly inducible by TH2 cytokines. Overall CCL24 was expressed at negligible levels in keratinocytes and fibroblast and some minor induction of CCL24 was observed in NHDFs after IL-4 and IL-13 stimulation. Hence, a picture emerges indicating that TH2 cytokines drive CCL11 and CCL26 expression in structural cells of the skin and that CCL26 represent the major CCR3 ligand present within the epidermal compartment.

4.8. *In vitro* functional analyses regarding CCR3

Effect of CCL26 on intracellular Ca^{2+} mobilization

To investigate whether the chemokine receptor CCR3 is functionally active on NHDFs, we performed Ca2+ mobilization assays and observed a steady rise in cytosolic Ca2+ after the addition of CCL26 (100 ng/ml), confirming that calcium flux occurs following chemokine stimulation of primary cells. The pretreatment with a neutralizing anti-CCR3 antibody inhibited the stimulatory effect of CCL26 in fibroblasts, indicating that CCR3 is functionally active on human dermal fibroblasts.

CCL26 biologic effects on dermal fibroblasts

After that we aimed to study the role of CCR3 in the modulation of fibroblast proliferation and migration. Interestingly, increasing concentration of CCL26 stimulation did not result in differences of fibroblast proliferation; however, signifi-cant effects were observed in migratory responses of NHDFs. In vitro-wound repair assays using NHDF layers and inducing a linear injury indicated that CCL26 markedly increased the repair capacity of fibroblasts from 40.37 % $\pm$ 1.84% (unstimulated controls) to 70.06 % $\pm$ 12.86% (CCL26, *$p < 0.05$) in a 15-h time period. Pretreatment of NHDFs with a neutralizing anti-CCR3 antibody

completely inhibited CCL26-induced in vitro wound repair indicating that this process critically depends on CCR3 signaling. To further support these findings, we performed migration assays of NHDF spheroids in 3D collagen matrix in the presence or absence of CCL26. The chemokine enhanced the migration of fibroblasts from the spheroids. The total area of fibroblast spheroids (36.5% increase; $^{**}p < 0.01$) and the mean motility of the cells from the focal point (95.7% increase; $^{***}p < 0.005$) were significantly augmented when stimulated with 100 ng/ml CCL26 compared to untreated spheroids. Our results are in line with the notion that CCL26 directly affects fibroblast migration.

5. DISCUSSION

5.1. Investigations of nTreg cells int he peripheral blood of AD patients

Regulatory T-cells have an important control function in immunologic processes; however, their proper functions in AD are not yet fully elucidated. Because of the heterogeneity of these cells and the lack of specific cell markers, the results of different studies regarding the characterisation of Tregs are difficult to compare. There is evidence indicating that the percentage of Tregs is either normal or increased in the peripheral blood in patients suffering from AD; however, there have only been a few studies investigating both the number and function of Tregs simultaneously.

We have depicted the complex quantitative and functional characterisation of Tregs as well as specific skin-homing Tregs in AD patients with moderate or severe clinical signs and high IgE levels. Furthermore, for the first time we also clarified the correlation between CLA+ skin homing Tregs with disease severity, the connection between which might reveal a more complex pathogenic role of Tregs in AD development.

Previously we have identified high numbers of $CD4^{+}CD25^{bright}FOXP3^{+}$ Tregs in close connection to dendritic cells in lesional AD skin and in atopy patch test positive biopsies. However, elevated numbers of $CD4^{+}CD25^{bright}FOXP3^{+}$ cells in AD patients does not necessarily mean that these cells have regulatory functions only, since FOXP3 alone is insufficient to determine the $CD4^{+}CD25^{bright}$ T-cell population with regulatory properties in humans. In AD, a proportion of $CD4^{+}CD25^{bright}FOXP3^{+}$ and $CLA^{+}CD4^{+}CD25^{bright}FOXP3^{+}$ cells exhibit Th2-like effector function after activation by specific triggers.

To overcome the difficulty of the inappropriate characterisation of Tregs, we first verified the $CD4^{+}CD25^{bright}CD127^{-/low}FOXP3^{+}$ T-cell population. As a calculated proportion, almost the entire percentage of gated $CD4^{+}CD25^{bright}$ cells were FOXP3 positive and CD127

negative/low. Henceforth, we identified the Treg population as $CD4^{+}CD25^{bright}FOXP3^{+}$ cells and detected significantly increased percentages of these cells in the peripheral blood of AD patients compared with healthy controls. In the next phase, we quantified the specific subset of $CLA^{+}CD4^{+}CD25^{bright}FOXP3^{+}$ skin-homing Tregs, and described them as also significantly increased in the peripheral blood in comparison with healthy controls.

A significant correlation of $CD4^{+}CD25^{bright}FOXP3^{+}$ Tregs and SCORAD was previously shown, yet serum IgE levels and skin-homing Tregs in association with disease severity have never been assessed. Our current report is the first to demonstrate significant correlations between the percentage of both Tregs and skin-homing Tregs and disease severity and sensitization, as assessed in a relatively larger AD population by SCORAD and serum IgE levels consequtively.

Previously, conflicting results have been published on Treg responses to antigenic stimuli in AD. Insufficient nTreg functions were reported after allergen or skin-colonizing microorganism effects. In the majority of the patients *S. aureus* colonization is found, and in the presence of SEB the nTregs lost their suppressor activity on effector Tcells. Demonstrated with a modified method, including multiple dilutions of SEB, we characterised the impact of SEB on AD pathogenesis and progression, by showing the SEB-mediated suppression of Treg function. Our functional tests revealed that Tregs, stimulated with anti-CD3/CD28, maintained their suppressor activity in the peripheral blood of AD patients. However, when stimulated with additional SEB, Tregs lost their suppressive ability in both AD patients and normal controls, indicating that the primary functional characteristics of Tregs in AD are not different from those in healthy controls. Thus, we conclude that the main distinctive factor in AD is the surrounding environment in the skin due to the nearly continuous presence of SEB that leads to secondary impairment of the nTreg cell functions.

We believe that assessing $CD4^{+}CD25^{bright}FOXP3^{+}$ and $CLA^{+}CD4^{+}CD25^{bright}FOXP3^{+}$ Tregs in patients with AD can be important novel markers for disease severity.

5.2. Investigation of tissue remodeling in atopic dermatitis

Remodeling is an important pathophysiologic feature of chronic atopic skin inflammation; however, the underlying mechanisms remain largely elusive. Current concepts suggest the involvement of inflammatory cells, in particular eosinophils, the alteration of microvascular structures, as well as the recruitment and activation of resident mesenchymal cells (e.g. fibroblasts). Complex communication pathways within the skin may be important to regulate eosinophil–fibroblast interactions that finally lead to dermal remodeling.

In this study, we aimed to unravel the chemokine-driven communication pathways between epithelial, stromal and inflammatory cells within atopic skin. So far, no systematic analysis of the chemokine receptor repertoire of human dermal fibroblasts exists. Chemokine signaling in general leads to biological responses such as migration, invasion, proliferation and gene expression.

First we analyzed the NHDFs' chemokine receptor repertoire. Our investigations identified CCR3 as a receptor expressed on the dermal fibroblast surface the storngest. Further more our findings also showed that NHDFs express functionally active forms of CCR3 *in vitro* and *in vivo*. The present study demonstrates for the first time that CCR3-driven pathways may also play a role in fibroblast activation and tissue remodeling within the skin.

CCR3 binds a variety of different ligands. Here we demonstrate for the first time a comprehensive overview of the expression of CCR3 ligands in chronic inflammatory and autoimmune skin diseases and show that among these ligands, keratinocyte-derived CCL26 proves significant, strong, and selective expression in atopic when compared to psoriatic skin inflammation, or to healthy skin. We suggest that CCL26 is an inflammatory and atopy-associated chemokine induced during atopic skin inflammation playing an important role in the pathogenesis of AD. Moreover we demonstrate that CCR3 on resident dermal fibroblasts is directly activated by CCL26, results in intracellular calcium mobilization in NHDFs and in strong, and selective activation of cell migration, invasion, and repair activity. However, we did not detect any significant modulation of cell proliferation.

Taken together, within the complex puzzle of mediators regulating leukocyte trafficking and tissue remodeling during atopic skin inflammation, a concept emerges suggesting an orchestrated process of chemokine-driven communication that leads to the formation of a profibrogenic microenvironment or ‘‘niche’’ created by keratinocytes, eosinophils, Th2 cells, and fibroblasts. Atopy-associated, keratinocyte-derived CCL26 will support Th2 and eosinophil recruitment to sites of atopic skin inflammation. Moreover, CCL26 will support the recruitment and migratory activation of CCR3-expressing resident mesenchymal cells. In turn, Th2 effector cytokines will locally enhance the production of CCR3 ligands such as CCL26 perpetuating the inflammatory process that will lead to atopy-associated cutaneous remodeling.

6. SUMMARY

We investigated the immunreglatory alterations and the chemokine-mediated communication pathways in cutaneous remodelling during atopic dermatitis.

According to our results the $CD4^{+}CD25^{bright}CD127^{-/low}FOXP3^{+}$ and the skin homing $CLA^{+}CD4^{+}CD25^{bright}FOXP3^{+}$ T cell populations with regulative characteristics occur in a significantly higher percentage in the sera of AD patients when compared with healthy volunteers. We demonstrated the strong connection between the precentage of these cell populations and the severity of the disease that may indicate an important pathogenetic role of these nTregs in AD. Furthermore by functional tests we proved that the nTregs in the peripheral blood of AD patients primarily maintained their suppressor activity, however, when stimulated with SEB, they lost their suppressive ability similar to normal controls. We concluded from our experiments that unlike in healthy people the main distinctive factor in AD is the surrounding environment of the skin due to the nearly continuous presence of SEB.

Next we investigated the cutaneous remodelling during atopic skin inflammation. It was previously postulated that activated inflammatory and resident skin cells may play crucial role in remodelling, however the regulating pathways have not yet been investigated. In our work we characterized the most significantly expressed chemokine receptor on NHDF cells (CCR3) and then we analysed the chemokine-mediated communication routes via this receptor. Among its corresponding ligands CCL26 demonstrated a significant and specific up-regulation in atopic when compared to healthy skin or other skin inflammations. *In vivo*, epidermal keratinocytes showed most abundant CCL26 protein expression in lesional atopic skin. In structural cells of the skin, Th2-cytokines such as IL-4 and IL-13 were dominant inducers of CCL26 expression. We proved that in dermal fibroblasts, CCL26 induced CCR3 signaling resulting in intracellular Ca^{2+} mobilization, as well as enhanced fibroblast migration, invasion and repair capacity that are inevitable processes in the remodelling during atopic skin inflammation.

Our investigations in AD clarified that the nTregs are important indicators of the severity of the disease and proved that the participation of CCR3-mediated pathways in tissue remodelling during atopic skin inflammation is crucial.

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List of publications related to the dissertation

1. **Gáspár, K.**, Baráth, S., Nagy, G., Mócsai, G., Gyimesi, E., Szodoray, P., Irinyi, B., Zeher, M., Remenyik, É., Szegedi, A.: Regulatory T-cell Subsets in Atopic Dermatitis: Important Indicators of Disease Severity with Acquired Functional Impairment.
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