SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

INVESTIGATION OF THE ELEMENTS OF INNATE IMMUNE SYSTEM IN ATOPIC DERMATITIS

by Georgina Nagy

Supervisor: Andrea Szegedi MD, PhD, DSc

UNIVERSITY OF DEBRECEN

GYULA PETRÁNYI DOCTORAL SCHOOL OF CLINICAL IMMUNOLOGY AND ALLERGOLOGY

DEBRECEN, 2017

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By **GEORGINA NAGY**
biologist

Supervisor: Andrea Szegedi MD, PhD, DSc

Gyula Petrányi Doctoral School of Clinical Immunology and Allergology
University of Debrecen

Head of the Examination Committee:	Edit Bodolay MD, PhD, DSc
Members of the Examination Committee:	Norbert Wikonkál MD, PhD, DSc
	Szilvia Benkő PhD

The Examination take place at the Conference Room of Building C, Department of Internal Medicine, Faculty of Medicine, University of Debrecen 10:00 a.m., June 26, 2017.

Head of the Defense Committee:	Edit Bodolay MD, PhD, DSc
Reviewers:	Attila Bácsi PhD
	Péter Holló MD, PhD
Members of the Defense Committee:	Norbert Wikonkál MD, PhD, DSc
	Szilvia Benkő PhD
	Attila Bácsi PhD
	Péter Holló MD, PhD

The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 1:00 p.m., June 26, 2017.

INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing, non-contagious inflammatory skin disease. The prevalence of the symptoms of AD is higher in children with positive family history. Besides genetic backgrounds environmental factors also play an important role in the development of the disease.

The pathogenesis of AD is complex; it is intensively studied topic, although there are many unclarified and controversial data in the literature in connection with the disease. The three main elements of the pathogenesis of AD are skin barrier disruption and the altered innate and adaptive immune responses. Two main hypotheses have been proposed to explain the development of AD, an immunological (inside-out) and a skin barrier (outside-in) hypothesis. According to immunological theory the primary reason of the disease is an immunological dysregulation results form an imbalance of T cells which leads to skin inflammation and consequential skin barrier distraction. According to outside-in theory first there is an impaired skin barrier which results in immune-mediated inflammation. Data from the literature show that these two hypotheses are connected, one does not exist without the other. It is also known, that the elements of innate immune system, mostly keratinocytes can make bridge between the two theories, since they are not just the building blocks of the physicochemical barrier of the skin, but also member of the skin immune system. When its role in physicochemical barrier is impaired because of genetical or acquired reasons, it results in alterations of their immunological role too. Other members of the innate immune system [dendritic cells (DC), invariant natural killer T cells (iNKT), etc.] are also disrupted in AD, which alteration are partly reasons, partly consequences of the damage of the physicochemical barrier of the skin or of the alteration of skin immune system, and these three system by strengthening each other develop a vicious circle, which maintains a severe skin inflammation characteristic in AD.

In our study we investigated primarily the role of cells (kertinocytes, DCs, iNKT cells) from innate immune system in AD patients. The first part of my thesis concludes our results from the investigation of the role of interleukin (IL)-16 as a possible biomarker, second part shows the results of peripheral blood DCs experiments, and the third part displays the results of iNKT studies.

Pathogenesis of atopic dermatitis

The research of recent years has significantly increased our knowledge of the pathomechanism of the disease. Basically there are two hypotheses about the development of AD, an immunological (inside-out) and a skin barrier (outside-in) hypothesis. According to immunological theory the primary reason of the disease is such an immunological dysregulation which results from an imbalance of T cells, particularly T helper cell types 1, 2, 17 and 22, and also regulatory T cells. In acute AD mainly Th2 and Th22 cells predominate in the skin, which causes an increased production of IL-4, IL-5, and IL-13, which then leads to skin inflammation, resulting in consequent skin barrier damage, additional inflammatory cell infiltration (eosinophil, macrophage, mast cell), inhibited Th1 cell differentiation and enhanced IgE production. According to this theory dysregulated immune system comes first, and skin barrier damage is the consequence.

In skin barrier hypothesis the damage of the barrier is the first step, which can be genetic or acquired destruction. Filaggrin (FLG) mutation is a good example for this theory, which can be detected in 30-40% of all patients. With gene defects, less filaggrin is produced, leading to skin barrier dysfunction and transepidermal water loss increase. The impaired skin barrier leads to increased penetration of allergens, and keratinocytes start to produce inflammatory cytokines. The most important cytokine is thymic stromal lymphopoetin (TSLP), which activates DCs to polarize Th2 type cells. The infiltration of Th2 cells results in the recruitment of other inflammatory cell types. So according to outside-in theory the primary barrier destruction leads to an immune mediated inflammation. Presumably both hypotheses are true, in one of patients dominate the first theory, and in another patient outweigh the other hypothesis, so these two theories complement each other, don't exist one without the other.

The importance of the cells of immunological skin barrier (innate immune response) in AD

The most important elements of the skin physicochemical barrier are keratinocytes, the lipid-protein matrix between them and the desmosomes and tight junctions which connecting them. However, keratinocytes are not only the building blocks of skin physicochemical barrier, but also of the immunological barrier of the skin, primarily of innate immune response. If the role of keratinocytes in the physicochemical barrier is impaired because of genetic or acquired reasons, it also results in divergences in their immune function. Other members of the innate immune system (DCs, iNKT cells) are also impaired in AD, and these alterations are partly reasons, partly consequences of the destructed skin physicochemical barrier or the

dysregulated adaptive immune response. The close correlation between the 3 systems also points to the close connection between the physicochemical barrier and immunological barrier of the skin.
In my own studies I investigated the role of innate immune cells (keratinocytes, DCs, iNKT cells) in AD.

Keratinocytes, IL-16

In the last decades the results of research have proved that the role of keratinocytes is not limited to the formation of skin physicochemical barrier, but these cells play an active role in the induction of innate immune response, as well in the operation of the skin immunological barrier. With their Toll-like receptors and other pathogen associated molecular pattern (PAMP) and endogenous damage associated molecular pattern (DAMP) recognition receptors (for example NLR gene family encoded proteins) they are able to differentiate between pathogen and non-pathogen signs. In keratinocytes TLR activation induces a predominant Th1 type immune response, which associated with type I interferon (IFN) production. As a result of NLR induction, other types of proinflammatory signaling pathways are activated. Keratinocytes also play an important role in AMP production (β-defensins, cathelicidins). During skin infection, T cell cytokines increase the production of AMPs form keratinocytes, especially IL-17A and IL-22. Keratinocytes are also able to express cytokines and chemokines, so that they can modulate the immune system by activation and recruitment of immune cells. It is known that keratinocytes express MHC class II molecules suggesting that they might act as non-professional antigen-presenting cells, it seems that although keratinocytes cannot prime naive T cells, they can potentially induce a recall immune response in antigen-experienced T cells.
The role of keratinocytes is highly important in the development of AD, both in the initiation of the disease, and also in the maintenance of the symptoms. Best known for their role in the production of TSLP, a proinflammatory cytokine, which cannot be detected in normal skin, although increased levels of TSLP can be found both in the acute and chronic lesions in AD, and this increased production mostly connected to the fully differentiated keratinocytes. Following exposure to TSLP immature CD11c+ DCs become mature and induce Th2 type polarisation in naïve CD4+ T cells, and at the same time they produce chemokine (CC motif) ligand (CCL)17 and CCL22, which when connected to C-C chemokine receptor type 4 (CCR4) are capable of attracting Th2-type CD4+ T-cells to the skin. TSLP can also co-stimulate the activation of both mast cells and NKT cells. The iNKT cells express TSLP

receptor and respond to the binding of the ligand by producing IL-4 and IL-13. Besides TSLP, keratinocytes are able to produce many other cytokines, namely IL-1, IL-6, IL-10, IL-18, GM-CSF and tumor necrosis factor α (TNFα), and also IL-16, on which we have less information in AD.

Interleukin-16 (IL-16) protein and bioactivity were initially identified in 1982 as a lymphocyte chemoattractant factor (LCF). The human IL-16 gene consists of seven exons and six introns which encodes the full-length precursor IL-16. The IL-16 gene has been sequenced from a large number of species and found to be highly conserved within all species investigated and this high evolutionary conservancy not only for IL-16 protein but for the binding relationship with its receptor CD4. This suggests a basic role for an IL-16/CD4 connection in regulating cellular responses.

IL-16 is generated as a precursor molecule, which is cleaved into a pro-molecule and a secreted, mature component. Unlike other precursor molecules both forms of IL-16 are biologically active. In lymphocytes pro-IL-16, the N-terminal domain of the precursor molecule, functions as a transcriptional repressor with regulatory effects on cell cycle progression, whereas mature IL-16, the C-terminal domain, is secreted from the cell as a ligand for CD4 with chemoattractant, growth factor, and differentiation factor capabilities on a variety of hematopoietic cell types involved in an inflammatory response.

IL-16 produced by keratinocytes, epithelial cells, lymphocytes, macrophages, synovial fibroblasts, eosinophil cells. It was also showed that $CD4^+$ and $CD8^+$ are able to produce IL-16 as well after antigen or mitogen stimulation. IL-16 expression primarily linked to such inflammatory diseases as asthma, rheumatoid arthritis, systemic lupus erythematosus, colitis, atopic dermatitis, and multiple sclerosis. IL-16 was originally described as chemoattractant factor for $CD4^+$ T cells, later it was proved, that IL-16 is also chemoattractant for all of $CD4^+$ peripheral immune cells, like monocytes, eosinophils and DCs. From $CD4^+$ T cells, IL-16 promotes mainly Th1 and Treg cell migration; moreover it can directly induce forkhead box P3 (FOXP3) expression, thus expanding the inducible Treg cell population. Also play a role in upregulation of the IL-2 receptor (CD25 and CD122), leading to proliferation of certain T cell subsets (for example Tregs, malignant transformed T cells) in the presence of IL-2 or IL-15. Furthermore, IL-16 upregulates HLA-DR, thereby facilitating antigen recognition by T cells.

Several studies showed a clear correlation between the elevated levels of serum IL-16 and the activity and severity of AD. However not all of the studies can confirm this, and

we did not found data about the connection between allergic sensitisation and the serum levels of IL-16.

Role of dendritic cells in AD

DCs are important members of the innate immune system; they are a vital link between innate and adaptive immune responses. DCs by antigen presentation are able to initiate adaptive immunity, and they can determine its orientation too. The quality of the antigen binding PRR, characteristic to the given DC, the expressed costimulatory molecules and the cytokines produced during maturation determine the type and the quality of the resulting immunogenic Th response (Th1/Th2), or the tolerance in certain circumstances. In the peripheral blood two types of myeloid DCs (mDCs) were identified. One of them is $CD1c^{+}$blood dendritic cell antigen (BDCA)1^{+} mDC, which coexpress CD11c and CD11b markers, the other type is $CD141^{+}BDCA3^{+}$ mDC, which is CD11b negative and express lower levels of CD11c and C-type lectin domain family 9 member A (CLEC9A) on its surface. Both types of mDCs lack CD14 and CD16, so they can be separated from monocyte populations.

In the skin we distinguish steady state and inflammatory DC subtypes. During steady-state conditions Langerhans cells reside in the epidermis, while resident dermal mDCs and plasmocytoid DCs can be detected in the dermis. However, during inflammation besides LCs and resident DCs also appear the inflammatory dendritic epidermal cells (IDEC) and myeloid dermal inflammatory DCs. Dermal DCs (dDCs) can be further subdivided into three subsets: $CD1a^{+}/CD1a^{-}CD1c^{+}CD14^{-}$ DCs, $CD14^{+}$ DCs and $CD141^{hi}CD14^{-}$ DCs. $CD1a^{+}CD1c^{+}CD14^{-}$ dermal DCs further coexpress CD11c, HLA-DR and CD45. In the presence of decent stimuli the express high levels of CD80 and CD86, strongly induce $CD4^{+}$ and $CD8^{+}$ T cells which resulting in a polarization toward Th2 and Th22 cells.

Besides monocytes, blood DCs are thought to be the precursors of DCs located in the skin and other tissues, and are often described as precursor DCs (pre-DCs). The precursor-progeny connection between $CD1c^{+}$ blood DCs and tissue $CD1c^{+}$ DCs is also supported by a recent in vitro differentiation and gene expression analysis

In AD skin both T cell and DC numbers are increased, especially of those, which express CD11c and CD1a. In lesional skin besides LCs, infiltrating IDEC are present; since the majority of these cells are found in the dermis of AD, they are also referred to as myeloid inflammatory DCs. Phenotypical analysis shows that IDEC are: $CD1a^{+}$,$CD1b^{dim}$,$CD1c^{dim}$,$CD11b^{bright}$,$CD11c^{+}$,$CD23^{dim}$,$CD32^{dim}$,$CD36^{bright}$,$CD206^{bright}$,$CD209^{+}$,$FcεRI^{bright}$,IgE^{+},$HLA\text{-}DR^{bright}$. Both LC and IDEC express high levels of FcεRI. The

expression of this FcεRI, in combination with a low expression of CD32/FCγRII, strongly distinguishes AD patients from patients with other lesional skin conditions.

During the acute phase of AD, LC may capture antigens via FcεRI-bound IgE leading to secretion of CCL2 (monocyte chemoattractant protein 1 (MCP1)), IL-16 and probably CXCL8. As a result, monocytes, eosinophils, precursors of IDECs, and T cells recruit to the site of inflammation. Through impaired barrier, re-entering antigens bind to surface IgE equipped LCs, and after activation, even locally or in the skin DLNs, they prime antigen specific T cells generating Th2 polarization.

LCs express high levels of TSLP receptor, its ligand is secreted in high amounts by keratinocytes. After binding TSLP, LCs become mature and secrete the following cytokines: CXCL8, CCL17, CCL22, CCL24 és IL-15. These agents contribute to the recruitment of Th2 cells in the site of inflamed skin, where they are activated by TSLP induced LCs.

In the subacute and chronic phase of AD, besides dominant Th2/Th22 presence, lesions are more characterized by a Th1 signature. IDEC cells are responsible for this phenomenon; they have a key role in the maintenance of chronic lesions. After antigen activation, IDEC cells are able to produce CCL3, IL-1, IL-16 and IL12p70; these latter two cytokines induce Th1 polarization.

In AD besides the well characterized epidermal mDCs, the role of dermal DCs seems to be especially important. In AD is even more typical that pre-DCs are not only precursors of skin dermal DCs, but they may also serve as the precursors of skin inflammatory DCs. In the dermis during inflammation inflammatory dDCs are also present, which can be identify according to their cell surface $FcεRI^{+}FcεRII^{+}CD207^{-}CD206^{+}CD1b^{+}CD1a^{+}$ expression. These cells also express CCR7, so they are able to migrate to the draining lymph nodes, and at the same time by their CCL17 and CCL18 production they contribute to the development of Th2 dominance characteristic to AD skin. Their cytokines (IL-5, IL-13, IL-6, TNFα) create such a tissue microenvironment which also participate in the maintenance of Th2/Th22 imbalance.

It is important to realize that the function of DCs is highly influenced by the amount and quality of tissue stimuli.

Although skin-infiltrating Th cell subpopulations and skin DCs are well characterised in the context of AD, it is not known exactly when or where mDCs acquire their ability to polarise Th cells towards the characteristic atopic subtypes. There is only a few data about the maturation of these cells and about the possible precursors in the blood.

Role of iNKT cells in AD

NKT cells are a subset of T cells. Common feature with T cells, that these cells express the semi-invariant T cell receptor (TCR) consisting of the Vα24/Jα18 α chain paired preferentially with the Vβ11 β chain. This receptor separate them from NK cells, although they are also able to express CD161 and like NK cells, NKT cells express cytotoxic granules such as perforin and granzyme.

At the same time they differ from T cells and Tregs because the TCR receptor of NKT cells are not able to bind peptid antigen presented by MHCI and II, they only recognize glycolipid antigens presented by CD1d, a non-polymorphic non-classical MHC class I molecule.

According to recent data activating agents can be divided two groups: ceramide based glycolipids (glycosphingolipids) and glycerol based lipids (membrane phospholipids).

Invariant natural killer T (iNKT) cells develop in the thymus from the same precursor pool as MHC-restricted T cells. NKT cells differentiate from $CD4^+CD8^+$ T cells, they can be divided into single positive, $CD4^+$ or $CD8^+$ populations and double negative cells. Moreover, two types of NKT cells have been described: the invariant $V\alpha14^+$ NKT, also called type I NKT cells. Within the type I NKT cells, there are four defined subpopulations: a $CD4^+$ and CD^+ single positive and $CD4^-CD8^-$ (double negative; DN) population. In addition to type I NKT cells, there is a population of $V\alpha14^-$ cells, also called type II NKT cells, which does not recognize Gal-Cer-CD1d complex.

In humans the peripheral blood iNKT cell levels ranging from 0.01% up to nearly 1% and it varies considerably between subjects. Recent data suggest that there are correlations between the frequency of iNKT cells and sex-based distribution. Indeed higher numbers are more commonly observed in women, probably because of the effects of sex hormones.

The general ligand of iNKT cells TCR is a synthetic glycolipid, namely α-galactosylceramide (α-Gal-Cer). The newest data from literature suggest to use α-GalCer-CD1d complex instead of CD161 markers to identify iNKT cells. Besides ligand binding, for the complete maturation of these cells, they also need cytokine stimulation. The best characterized cytokine mediator for these cells is IL-12, but TSLP, IL-18, IL-23 and IL-25 receptor were also identified on the surface of iNKTs. After activation, iNKT cells respond with fast cytokine production, like IFNγ, tumor necrosis factor (TNF), IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, IL-21 and GM-CSF.

According to current data it can be stated that iNKT cells have a key role in the coordination of innate and adaptive immune responses in infections, autoimmune diseases, allergies and tumor diseases. Overall it is known that the main function of these cells besides cytotoxic ability is to produce inflammatory and tolerogenic response inducing cytokines.

At the same time little is known about the role of iNKT cells in AD. Indeed, few and conflicting results have been published on iNKT cell frequencies and numbers in the peripheral blood and in lesional skin in AD to date; the functional properties of iNKT cells have not been determined in details. Since it is well known that iNKT cells are able to bridge innate and adaptive immune responses, they can be good therapeutic target in the early phase of the development of the disease. For this more experiments are needed to understand more precisely the role of these cells in the pathogenesis of AD.

AIMS

Our aim was to study the importance and possible role of the three important elements of innate immune system in the pathogenesis of AD. The first part of our study we investigated the role of IL-16 as a possible biomarker, in the second part we made the phenotypical and functional analysis of peripheral blood mDCs and in the third part we display the results of iNKT studies.

Role of IL-16 in AD

Although it was already known, that the levels of serum IL-16 correlate to the severity of AD. However not all of the studies can confirm this, and we did not found data about the connection between IL-16 and the allergic sensitisation. Our aims were defined as follows:

1. to assess serum levels of IL-16 in patients with AD and psoriatic patients and non-atopic healthy controls,

2. to analyse the possible correlation between IL-16 and laboratory markers, reflecting to the severity of AD (Scoring Atopic Dermatitis (SCORAD), total IgE, eosinophil cell number),

3. to analyse the connection of serum IL-16 levels and the degree of allergic sensitisation,

4. to compare the usefulness of IL-16 as biomarker with total IgE in the evaluation of the severity of AD, and the degree of allergic sensitisation.

Role of peripheral blood mDCs in AD

Skin DCs play a key role in the initiation and maintenance of atopic skin inflammation, and it is also known that blood CD1c+ DCs are “precursors” of tissue mDCs. We wanted to

investigate how mDCs of AD patients differ phenotypically and functionally from healthy controls. Our aims were defined as follows:

1. to determine the differences of nuclear morphology and activation state of mDCs between AD patients and healthy individuals,
2. to investigate whether isolated peripheral blood mDCs from AD patients and healthy controls differ in their cytokine expression profiles de novo without stimuli and after stimulation in an AD skin-specific tissue microenvironment (Staphylococcus enterotoxin B (SEB) and TSLP),
3. to find out when or where mDCs acquire their ability to polarise Th cells towards the characteristic atopic subtypes.

Role of iNKT cells in AD

The unique ability of iNKT cells to produce high amounts of both Th1-type and Th2-type cytokines suggested the possibility that they may have a pathogenic role in AD. However only few and conflicting results have been published on iNKT cell frequencies and function in AD to date, so we defined our goals as follows:

1. to elucidate whether the frequency and/or the absolute number of peripheral iNKT cells are altered in AD patients compared to controls,
2. to determine whether the frequency and/or the absolute number of peripheral iNKT cells are altered in AD patients compared to controls, when we divided the cells into subsets according to their cell surface markers (CD4 and CD8),
3. to investigate their effector functions by examining intracellular IFNγ and IL-4 cytokine production in the different subsets of AD patients and controls.

PATIENTS AND METHODS

In all three parts of the thesis AD patients fulfilled the diagnostic criteria established by Hanifin and Rajka. All participants gave written informed consent according to the Declaration of Helsinki principles, and the study was approved by the local Ethics Committee of the University of Debrecen, Hungary. Patients had not received any oral glucocorticosteroids or other systemic immunomodulatory agents for at least 4 weeks and had not been treated with antihistamines or topical corticosteroids for at least 5 days before the collection of blood samples.

Role of IL-16 in AD

Patients

Overall, 85 patients with AD were studied (46 males, 39 females, mean age 18.6 years, range 7–49 years). The severity of the disease was determined by the SCORAD index: the mean SCORAD score was 32 (range 8–56). In all AD patients, serum IL-16 levels, total IgE levels and eosinophil cell counts were determined, as described. In 48 AD patients, allergen-specific serum IgE was measured and prick tests were performed. Based on the specific IgE levels, we divided AD patients into the following two subgroups: group 1 (nonsensitized, n = 13), which consisted of patients with no detectable specific IgE, and group 2 (sensitized, n = 35), which consisted of patients in which 1 or more IgEs were detected. The prick test was performed according to a standardized procedure. Thirty-two different aeroallergens, including perennial, seasonal [house dust mite (Dermatophagoides pteronyssinus, Dermatophagoides farinae), birch pollen, cat dander, grass pollen (Phleum pratense)] and food allergens were applied. Based on prick test results, patients were also divided into a nonsensitized, prick test-negative (group A, n = 24), and a sensitized, prick test-positive (group B, n = 24), subgroup. Blood samples from 36 healthy age-matched volunteers and 20 psoriasis patients that were suffering from chronic plaque type psoriasis (mean age 28.2 years; mean Psoriasis Area Severity Index 15.2) but were not being treated with local or systemic immunomodulatory agents served as controls.

Eosinophil Cell Count

The rate and absolute count of eosinophils in whole blood were determined by Advia 120 haematology analyser (Siemens) as part of the routine clinical investigation of the patients. The eosinophils in the analyser were identified based on their scatter properties and myeloperoxidase positivity.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of IL-16 were determined by solid phase sandwich enzyme-linked immunosorbent assay (ELISA; Biosource International Inc.) according to the manufacturer's instructions. Blood samples were also evaluated for total IgE and specific IgE for common food and aeroallergens. Serum levels of total IgE were determined by solid-phase sandwich ELISA (Adaltis Italia S.p.A.) according to the manufacturer's instructions. All assays were

performed in duplicates. Serum levels of specific IgE were determined by the ALLERgen Systems kit, which is based on the immunoenzyme capture system (Adaltis Italia S.p.A.).

Statistical Analysis

Statistical analyses were performed using SPSS 15.0 software. Nonparametric Mann-Whitney and Kruskal-Wallis tests were used to compare the data of AD patients to healthy and psoriasis controls in the subgroup analysis. The nonparametric Spearman rank correlation test was performed between serum total IgE and serum IL-16 levels, between serum total IgE and eosinophils and also between SCORAD score and the levels of serum IL-16 or total IgE. $p<0.05$ was considered statistically significant. Data are presented as means ± standard deviation.

Role of mDCs in AD

Patients

Eleven patients with AD and 8 age-matched healthy controls were enrolled in this study. The activity of the disease was determined using the subjective SCORAD index, and the sensitivity of the patients was assessed based on the total serum IgE level. In all patients, Hyper IgE Syndrome (HIES) was excluded according to the HIES clinical scoring system.

Cell isolation and cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained using Ficoll Paque Plus (GE Healthcare Bio-Sciences AB) gradient centrifugation from the peripheral blood of AD patients and healthy controls. mDCs were isolated from PBMCs using the CD1c (BDCA-1)+ Dendritic Cell Isolation Kit (Miltenyi Biotec GmbH) according to the manufacturer's instructions. Briefly, after the depletion of CD19+ B cells, CD1c (BDCA-1)+ cells were positively selected. Purified mDCs were then cultured in RPMI-1640 medium (Miltenyi Biotec GmbH) supplemented with 10% FBS (Lonza Group Ltd) and antibiotics (PAA Laboratories GmbH) for 48 hours in the presence (stimulated) or absence (unstimulated) of 30 ng/ml TSLP (eBioscience Inc.) and 100 ng/ml SEB (Sigma-Aldrich)**.**

Cell surface marker and intracytoplasmic cytokine staining

We analysed mDC-derived cytokines/chemokines that have well-characterised roles in the lineage commitment of the different Th cell subtypes. Cytokine production by unstimulated and stimulated cells was analysed after 6 hours of incubation with 3 μg/ml brefeldin A

solution to inhibit cytokine secretion. After incubation, the cells were harvested, washed with FACS buffer (phosphate-buffered saline containing 1% bovine serum albumin) and aliquoted into tubes. The cells were first stained with the cell surface marker-specific antibodies anti-CD1c-allophycocyanin (APC) (L161, BioLegend), anti-CD83-fluorescein isothiocyanate (FITC) (HB15e, BD) and anti-CD86-phycoerythrin (PE) (IT2.2, BioLegend) for 30 minutes in the dark at 4°C. The stained cells were then washed with FACS buffer and fixed by adding 100 µl of IC Fixation Buffer (eBioscience) for 20 minutes in the dark at room temperature (RT). Then, the cells were washed with 1x Permeabilisation Buffer (eBioscience), and after centrifugation, the pellet was resuspended in 100 µl of 1x Permeabilisation Buffer and stained with various combinations of the following intracellular cytokine-specific antibodies: anti-IL-10-PE (JES3-19F1), anti-IL-2-PerCP-Cy5.5 (MQ1-17H12), anti-IL-4-PerCP-Cy5.5 (MP4-25D2), anti-IL-6-FITC (MAb11), anti-TNFα-PerCP-Cy5.5 (MQ2-13A5) (BioLegend), anti-IL-23p19-PE (23dcdp, eBioscience), anti-IL-12(p40/p70)-FITC (C11.5, BD), anti-TGFβ1-CFS (9016) and anti-CCL17/TARC-PE (54015) (R&D Systems). Then, the cells were incubated in the dark at RT for 20 minutes and washed first with 1x Permeabilisation Buffer and subsequently with FACS buffer. After centrifugation, the pellet was resuspended in 30 µl of FACS buffer and loaded into the channels of an ibidi µ-Slide VI (ibidi GmbH). Before storage, the channels were filled with 150 µl of FACS buffer, and, prior to measurement, 3 µg/ml DAPI (Sigma-Aldrich) was added for 20 minutes at RT. Because we did not observe any significant changes in the fluorescence intensities of the stained cells for any of the antibodies in the presence or absence of an Fc receptor blocker (FcR Blocking Reagent, Miltenyi Biotec GmbH), unstained cells served as a negative control.

Laser scanning cytometry

To acquire and analyse multiparametric cytometry data from a limited number of cells, an iCys Research Imaging Cytometer and the accompanying software were utilised (iNovator Application Development Toolkit and iBrowser Data Integration Software, Compucyte Corporation). The following lasers were used in multi-track mode to separately excite the fluorophore-conjugated antibodies: a 405-nm violet diode laser for DAPI; a 488-nm argon-ion laser for FITC, CFS, PE and PerCP-Cy5.5; and a 633-nm HeNe gas laser for APC dyes. Emission from an area of 6x 12x 1024x 768x 0.06125 µm2 (6 x 12 scanning area, 1024 x 768 pixel/ scanning area, sampling area of one pixel of 0.25 µm x 0.245 µm size) of the sample was collected through an Olympus 40x objective (0.75 NA) using photomultiplier tubes (PMT). With the appropriate emission filters, each PMT monitored a specific fluorescence

channel; for example, DAPI was detected at 463±20 nm, FITC and CFS were detected at 530±15 nm, PE was detected at 580±15 nm, and PerCP-Cy5.5 and APC were detected at 675±25 nm. Cellular events were segmented independent of the cytokine fluorescence when the sum of the scatter signal from the membranes and the DAPI signal from the nuclei was higher than a pre-set threshold value. The fluorescence intensities of all fluorochromes and the other cellular properties inside the contour line were measured and presented as imaging and cytometric parameters. The following parameters were analysed: Integral, which is the sum of the pixel intensities for a given event that provides information about the cellular level of the labelled protein; Max pixel, which is the highest pixel value for an event that provides information about the highest concentration of the label in a cell; Area, which is the area bounded by the contour line in square micrometres; and cell location. From these parameters, data regarding cytokine production, cell and nuclear size alterations, cell cycle phase (according to the DNA content) and DNA condensation could be assessed. Data on the cytokine expression and membrane markers of at least 1000 gated cells were collected and analysed on a cell-by-cell and a cell-population basis using statistical software (GraphPad Prism and Microsoft Excel). A compensation matrix was generated according to the iCys manual and BD's recommendations using polystyrene microparticles from the BD CompBeads Set Anti-Mouse Ig, κ (BD Biosciences).

Statistics

The data were normally distributed, significant differences were identified using an unpaired Student's t-test with GraphPad Prism version 5 (GraphPad), and the data are presented as the means ± SEM. P values less than 0.05 (*) or 0.025 (**) were considered statistically significant.

Role of iNKT cells in AD

Patients

Forty-one patients with AD (16 female and 25 male, mean age 18.59±8.9 years) and 16 healthy non-atopic controls (HCs) (11 female and 5 male, mean age 18.9±5.9 years) were selected. The severity of the disease was determined by the SCORAD index. The mean objective SCORAD was 26.7±13.1. When the subjective scores were also added, the mean SCORAD was 41.2±14.6. The median serum total IgE was 584.3 kU/L with a range of 251.3

– 245.0 kU/L, and the median serum lactate dehydrogenase (LDH) was 475 U/L with a range of 268.1–732.4 U/L, reference: 230–460 U/L.

Isolation of Peripheral Blood Mononuclear Cells

Heparin anti-coagulated blood samples were layered over Histopaque-1077 (Sigma-Aldrich Chemie GmbH) and centrifuged at 700×g for 30 min, and the mononuclear cells were isolated from the plasma-Ficoll interface.

Determination of Frequency and Number of iNKT Cells and CD4/CD8 iNKT Cell Subsets by Flow Cytometry

The number and frequency of iNKT cells were determined by flow cytometry on peripheral blood mononuclear cells (PBMCs) from 41 patients with AD using anti-CD3 and 6B11 (specific for the CDR3 loop on the Vα24 chain of the TCR) monoclonal antibodies (mAbs). Cells (1×106) were preincubated with 100 μg/mL purified human IgG (Sigma) for 20 min on ice to prevent nonspecific binding to Fc receptors. After two washes in staining buffer (phosphate buffered saline, 5% fetal calf serum, and 1% bovine serum albumin, all from Sigma), cells were then incubated with anti-CD3-FITC, 6B11-PE (BD Pharmingen), anti-CD4-PE-Texas Red (ECD) and anti- CD8-PE-Cyanine7 (PC7) (Immunotech) mAbs for 45 min on ice. Cells were then washed and analyzed by four-color flow cytometry on a Beckman Coulter FC500 flow cytometer and CXP software. For all staining experiments, appropriate isotype-matched controls were included. The absolute cell numbers were calculated by applying the two platform method using lymphocyte counts.

Determination of Functional Properties of iNKT Cells and Their CD4/CD8 Subgroups by Intracellular Cytokine Analysis

PBMCs of ten AD patients and ten HCs were investigated. Intracellular cytokine levels of iNKT cells and their CD4/ CD8 subsets were determined by five-color flow cytometry as described. Briefly, after stimulating PBMCs with 25 ng/mL phorbol-12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin (Io) in the presence of 10 μg/mL Golgistop Brefeldin A (all from Sigma), the cells were washed, and surface staining of the cells was performed with appropriate mAbs to CD3 (FITC-labeled for IL-4-APC staining and APC-labeled for IFNγ-FITC staining, both from BD Pharmingen), 6B11, CD4, and CD8. The cells were then fixed and permeabilized with IntraprepTM permeabilization reagent (Immunotech) according to the

manufacturer's instructions, and intracellular cytokines were stained with IFNγ-FITC or IL-4-APC mAbs (both from BD Pharmingen) for 30 min. After two washes with staining buffer, the cells were analyzed on a Beckman Coulter FC500 flow cytometer and CXP software. At least 300,000 cells were counted from each sample. Appropriate isotypic controls were included in all experiments. The specific response of the cells to PMA/ionomycin is calculated by substracting the percentage of positive events in the unstimulated sample from the percentage of positive events in the activated sample. Results were expressed as net percentage of cytokine positive cells.

Statistical Analysis

Results are presented as median, interquartile ranges (IQR 25th–75th percentiles), and ranges (5th–95th percentiles). Statistical analysis was performed with SigmaPot v.11.0 software (SyStat Software Inc, USA). A non-parametric Mann–Whitney rank-sum test or Student's t test was used. Correlation analysis was performed using a Spearman's rank test. A p value less than 0.05 was considered statistically significant.

RESULTS

Role of IL-16 in AD

Serum IL-16 Levels in AD Patients, in Healthy Individuals and in Patients with Psoriasis

Serum IL-16 levels in patients with AD were compared to those of healthy individuals and patients with psoriasis. We found that IL-16 levels in the sera of patients with AD were significantly elevated compared to those in healthy individuals and those of patients with psoriasis

Association among Serum Levels of IL-16 and laboratory parameters reflecting to the severity of AD (SCORAD, Total IgE and Eosinophil Counts)

In the total patient group, we examined the connection of serum IL-16 to the laboratory parameters reflecting to the severity of the disease (SCORAD score, serum levels of total IgE and absolute eosinophil cell count). In the case of the SCORAD index, no association was found between SCORAD score and serum IL-16 even though the value of the R coefficient was 0.27, which is very close to the statistical limit of correlation. At the same time significant correlations were found between serum levels of IL-16 and total IgE, but there was no correlation between IL-16 and eosinophils. On the other hand we detected a positive

correlation between the serum levels of total IgE and SCORAD score and there was positive association between total IgE and eosinophil counts.
Our results show that IL-16 exhibits only a small correlation with the severity parameters, the serum levels of total IgE indicates better the severity of the disease than IL-16.

Association between IL-16 and the sensitization of patients with AD

According to specific IgE levels and prick test results, we divided AD patients into sensitized (group 2 and group B) and nonsensitized (group 1 and group A) subgroups. Serum IL-16 and total IgE levels were further assessed within these groups. Serum IL-16 levels were significantly elevated in group 2 compared to those in group 1. No significant differences between groups A and B were detected. Serum total IgE levels were significantly increased in both in sensitized subgroups compared to nonsensitized groups.

According to our results we can demonstrate that although serum IL-16 levels showed some correlation with the severity of the disease and the degree of sensitization in patients with AD, serum total IgE levels were found to be more accurate markers in predicting the condition of the disease.

Role of mDCs in AD

Az AD-s betegek mDC-inek morfológiai és fenotípusos jellemzői

Cellular and cytoplasmic cytokine staining in mDCs isolated from the peripheral blood of AD patients were performed with nuclear morphological assay using DAPI staining. In AD patients, both in unstimulated and in stimulated samples, a greater population of larger, more active cells with decondensed nuclei was generally observed than in healthy individuals. These cells were identified by their low DAPI intensities. In general, the observed changes in the cytokine profiles could be assigned only to these larger cells with decondensed chromatin. Regarding CD83 and CD86 expression, there were no significant differences between the unstimulated control cells and the patient-derived mDCs. However after stimulation, a significant up-regulation of CD83 and CD86 was found, which was accompanied by further chromatin decondensation in stimulated cells from AD patients relative to unstimulated atopic mDCs. Between healthy individuals these markers were not elevated significantly. Despite the fact that these nuclear changes most likely reflect increased transcription, mDCs derived from patients and controls all remained in the G0/G1 phase of the cell cycle.

Cytokine profile of unstimulated mDCs from AD patients and healthy controls

The cytokines are grouped based on their known T-cell-polarising features and on the three different patterns of changes in their intracellular expression levels. Box 1: IL-2, CCL7, IL-6 and TNFα, which induce Th2 and Th22 polarisation; Box 2: IL-12p40/p70, which induces Th1 polarisation; and Box 3: IL-10, TGFb1 and IL-23p19, which induce Th17 and Treg polarisation. Under unstimulated conditions, circulating mDCs from AD patients seem to have the ability to produce all Th-cell-polarising cytokines, similar to control cells, except IL-4. Atopic mDCs produced higher levels of IL-2, CCL17, IL-6 and TNFa, and the alterations in CCL17 and TNFa expression were statistically significant. In the cases of IL-12p40/p70, IL-10, TGFb1 and IL-23p19, no differences between the two examined unstimulated groups were observed. Our results proved that the utilized three box separation was appropriate in terms of expression pattern as well.

The effect of atopic skin like microenvironment on the T-cell-polarising cytokine production of mDCs

After stimulation with SEB and TSLP, we observed three different patterns of cytokine production by mDCs from AD patients compared with controls. First, the levels of previously elevated Th2/Th22 polarising cytokines (IL-2, CCL17, IL-6 and TNFα) further increased, and the differences became statistically significant, except that for CCL17, when atopic mDCs were compared with control cells. Second, the IL-12p40/p70-producing capacity (Th1 polarisation) of mDCs from atopic donors significantly decreased relative to that of control cells. Third, the expression levels of IL-10, TGFb1 and IL-23p19 by stimulated mDCs did not differ between healthy individuals and AD patients. IL-4 was not expressed by either of the mDC populations under any culture conditions.

Role of iNKT cells in AD

Percentage and Number of Total iNKT Cells in AD Patients

Circulating iNKT cells were identified as $CD3^+6B11^+$ cells among the lymphocytes of PBMCs obtained from peripheral blood of AD patients and HCs. Notably, the frequency of $CD3^+6B11^+$ iNKT cells was significantly reduced in AD patients. In addition, the median circulating number of iNKT cells was also significantly diminished in AD patients compared with the controls. Because alteration in the T cell numbers may affect the iNKTcell count, we determined the percentage of the $CD3^+$ T cell, $CD4^+$, $CD8^+$ T cell subsets and the CD4/CD8 ratio in PBMC of both AD patients and HCs, and in this sense, the AD population did not

differ significantly from the investigated controls. No correlations were found between the frequency or number of iNKT cells and the age or sex of the patients, disease severity (measured by the SCORAD index), or the serum levels of total IgE and LDH.

Subgroup Analysis of iNKT Cells in AD Patients

Among the gated CD3/6B11 double-positive total iNKT cells, the four iNKT subsets were identified on the basis of their CD4 and CD8 expression. Analysis of the CD4/CD8 phenotype of iNKT cells revealed a significant decrease in the percentage of $CD4^-$ subpopulations (DN and $CD4^-CD8^+$) in AD patients compared with HCs. When the absolute numbers were counted, the results showed an even more pronounced decrease in the CD4/CD8 iNKT subpopulations of AD patients compared with HCs.

Positive correlations were found between the frequency of total iNKT cells and the frequencies of their subsets in AD patients except for DP iNKT cells, and these correlations were more pronounced when the absolute cell counts were analysed. For both the frequencies and the absolute numbers, the strongest correlation was found between the values of the total iNKT group and the DN iNKT subgroup.

Functional Analysis of iNKT Cells in AD Patients

To assess the effector functions of iNKT cells in AD patients, the intracellular IFNγ and IL-4 cytokine content of total iNKT cells, and subgroups was determined. After comparing cells from ten AD patients and ten HCs, the intracellular IFNγ expression was found to be decreased, whereas that of IL-4 was increased in the total iNKT cells from AD patients compared with those from HCs; however, these alterations did not reach the limit of statistical significance. Next, the functional properties of the different subsets of iNKT cells were analysed. When the intracellular cytokine levels of iNKT subgroups from AD patients and HCs were compared, the DN iNKT subgroup from AD patients exhibited significantly lower IFNγ levels and significantly increased intracellular IL-4 levels. With respect to the other three iNKT subgroups, no significant differences were detected in the intracellular IFNγ and IL-4 cytokine levels between AD patients and controls.

DISCUSSION

AD is one of the most common chronic inflammatory skin diseases; in the complex pathogenesis of AD besides damaged skin barrier and innate immune response, overactivated acquired immune response has a critical role. During the development of the disease the

above mentioned systems have a significant influence on each other and as a vicious circle they worsening the discrepancies and functional disorders, which results in an increasingly severe, clinically visible inflammation.

Our current studies focus on the alterations of innate immune system. Our aim was to analyse three members of this system. First we investigated the role of IL-16, which produced by many innate immune cells, than we determined the alterations of the numbers and functional properties of mDCs and iNKT cells in the peripheral blood of AD patients.

Different cell types, including T and B lymphocytes and a variety of innate immune cells, eosinophils, mast cells, airway epithelial cells, dendritic cells and keratinocytes, are able to synthesize IL-16. IL-16 was recognized as a chemoattractant for all peripheral immune cells expressing CD4, such as CD4+ T lymphocytes, monocytes and dendritic cells. In allergic diseases, IL-16 has been shown to promote secretion of pro-inflammatory cytokines, which suggests a pathophysiological role for IL-16 as a mediator of inflammation. The secretion of IL-16 from allergen activated epidermal Langerhans cells may contribute to the recruitment and activation of dendritic cells, T cells and eosinophils in AD, providing a possible link between IgE-mediated and cellular inflammatory responses. In AD it has been shown that one mechanism for the exacerbation of the disease may be the allergen-induced IL-16 production by eosinophils, which recruits additional eosinophils producing IL-4. Increased expression of IL-16 mRNA has been detected in epidermal keratinocytes and in dermal T lymphocytes both in acute and chronic AD lesions. The number of IL-16 mRNA+ cells was significantly higher in acute lesions compared to that in chronic lesions and correlated with the number of CD4+ cells in both the epidermis and dermis, underlying the important role of IL-16 in the initiation and perpetuation of skin inflammation in AD. The connection between IL-16 and AD has also been confirmed by clinical studies, so that IL-16 could be a proper marker to measure the activation state of the disease. On the other hand, the association between serum IL-16 levels and the degree of sensitization in AD has not been described previously.

In this study, we demonstrated significantly higher serum IL-16 levels in AD patients compared to those in healthy controls. Moreover, a significant difference was detected between AD and psoriatic patients. Our results are in accordance with the findings of Masuda et al. because they also demonstrated significantly increased serum IL-16 levels in patients suffering from AD compared to those in patients with psoriasis. They concluded that the more pronounced monocyte/macrophage infiltration in the dermis of AD patients could be one of the reasons for the high IL-16 serum levels. Also, the difference between psoriasis and AD in

a Th1/Th2 balance may contribute to the dissimilarity in IL-16 levels in the two inflammatory disorders.

In AD, the association between high serum IL-16 levels and different clinical and laboratory parameters of the disease has been investigated extensively. Although serum IL-16 seems to correlate with SCORAD score, the relationship between IL-16 and laboratory parameters is controversial in the literature. Frezzolini et al. did not detect an association between total IgE and serum IL-16 levels, while Masuda et al. found a positive correlation between IgE and IL-16 and also between IL-16 and the eruption score of AD patients.

In our study, we found a significant correlation between serum IL-16 and serum total IgE levels, and also between total IgE and eosinophil cell counts. In addition, a positive correlation was found between the SCORAD index and the total IgE level, although this association was not significant. On the other hand, no association was found between SCORAD score and serum IL-16, yet the value of the R coefficient was 0.27, which is very close to the statistical limit of correlation. This shows a modest correlation between SCORAD score and serum IL-16, and this correlation could probably have been increased by more patients. Significant correlation was detected between serum IL-16 and serum levels of total IgE, however there was no association between serum IL-16 and eosinophil cell count. The severity of AD can be well determined by SCORAD score; serum total IgE and eosinophil counts are less proper parameters reflecting on severity. Results from our experiments with total IgE show, that there is positive, but not significant correlation between serum levels of total IgE and SCORAD. Significant correlation was found between serum total IgE and eosinophil cell count.

Since it turns out, that IL-16 cannot indicate properly the severity of the disease, we investigated the connection between serum IL-16 and the degree of allergic sensitization. Based on the degree of sensitization detected by specific IgE measurements and prick tests, AD patients were divided into sensitized and nonsensitized subgroups, and serum total IgE and IL-16 levels were further assessed within these subgroups. According to our results, serum IL-16 levels showed significant elevation in the sensitized subgroup compared to those of the nonsensitized subset only when sensitization was determined by specific IgE. Serum total IgE levels were significantly increased in both sensitized subgroups compared to those of nonsensitized subjects. Our data suggest that although serum IL-16 levels show a correlation with the degree of allergic sensitization, this association is not as strong as the relationship between serum total IgE and allergic sensitization.

Previous studies found, that in the peripheral blood of AD patients the Th1 type cytokine production of both $CD4^+$ and $CD8^+$ T cells is impaired and Th2/Th22 type cytokines are dominant. In acute skin lesions in addition to the previously described Th2 dominance, the presence of Th22 cells was also defined, and this distribution remains in the chronic lesions too. At the same time, in the skin infiltration of Th1 cells was also observed. DCs responsible for this Th cell imbalance. DCs themselves contribute to the initiation of AD skin inflammation not only with their T cell polarizing capacity but also with their remarkable proinflammatory cytokine production. The differentiation and lineage commitment of the above mentioned Th cell subpopulations basically depend on their interaction with DC and the tissue microenvironment. Skin colonization with superantigen-producing *Staphylococcus aureus* is a characteristic feature of AD in 80-100% of patients and it correlates with disease severity. The SEB acts as a superantigen, it can penetrate the epidermis and the dermis, leading to polyclonal T cell activation and inducing professional or non-professional antigen presenting cells. SEB conditioned human DCs promote Th2 polarization and SEB induces IL-22 secretion by $CD4^+$ T cells. Another characteristic feature of both acute and chronic AD skin is that keratinocytes overproduce TSLP, due to the dysfunctional barrier and the presence of proinflammatory cytokines. TSLP stimulation induces Th2 immune response and contributes to the survival and maturation of antigen-presenting cells. Recent data indicate that besides monocytes, blood $CD1c^+$ DCs are considered precursors of tissue mDCs. In AD DC sin the skin are well characterized, but there is only few data in connection with circulating precursors of these cells.

According to our results we have three conclusions: First, the unstimulated, circulating mDCs from AD patients seem to have the ability to produce all Th-cell-polarising cytokines, similar to control cells, although atopic mDCs showed biased elevation of cytokines for Th2 (IL-2, CCL17) and Th22 (IL-6, TNFa) polarisation relative to non-atopic control cells. These alterations were significant in case of CCL17 and TNFα. We also found that in AD patients, a population of larger, more active cells with decondensed nuclei was generally seen in a bigger proportion compared to healthy persons. In general, our observed changes of cytokine profiles could only be assigned to these larger cells with decondesed chromatin. Based on this observation we think that these cells are in a "preactivated" state.

Second, our present study indicates that mDCs from AD patients and healthy controls respond differently to the specific, AD skin-like tissue micromilieu. When mDCs were cultured in the presence of SEB and TSLP, which we used to create a model of an AD tissue environment, the biased alterations in the levels of Th2- and Th22-polarising cytokines became statistically

significant in cells from atopic patients relative to those from healthy controls. Fujita et al. also confirmed the pluripotent Th-cell (Th1, Th2, Th17, Th22)-polarising capacity of freshly isolated skin-derived resident and inflammatory DCs, and they emphasised that the recruitment of disease-specific Th subsets is determined primarily by the microenvironment associated with chronic inflammation in AD. In an analogous experimental system, Reefer et al. demonstrated that in DC-T cell co-cultures after TSLP, allergen or SEB stimulation, Th cells from AD patients produce Th2-type cytokines, although that study did not investigate Th22 cytokine expression. In our experiments, we also observed that unstimulated mDCs had a similar capacity to polarise Th1 cells in atopic and non-atopic individuals; however, this ability of atopic mDCs became impaired after stimulation. Consistent with the results of our study, Lebre et al. found that CD1c+ DCs exhibit aberrant functions in AD patients because these cells could not induce a Th1 immune response, even in the presence of a strong Th1 stimulus.

Because of the low number of mDCs in the peripheral blood and the limited blood sample volume that is available, especially in childhood, it is challenging to directly investigate the function of these cells from patients; however the results of these kinds of studies reflect more to the physiological conditions. Because of this in our experiments we investigated freshly isolated peripheral blood CD1c (BDCA-1)$^{+}$ mDCs using slide-based citometry (LSC). Consequently, we want to emphasise a third point: that LSC is a useful technique to monitor mDC functions in vitro. To our knowledge, we are the first to directly determine the intracellular cytokine profile and activation of circulating mDCs in AD patients using multiparametric laser scanning cytometry (LSC). Measurements utilising this slide-based technique allow for the analysis of specimens with low cell numbers (10^5 or less) but provide results with statistical relevance.

Although considerable evidence suggests a role for iNKT cells in autoimmune and infectious disorders, there are scarce data on their distribution and function in atopic diseases. Data on the role of iNKT cells in human asthma are conflicting, since some authors found iNKT cells in low numbers in the airways of subjects with asthma, while others reported opposite results. iNKT cells are able to produce large amounts of Th1 and Th2 type cytokines upon activation, which suggests that they may play a role in the pathogenesis of another atopic disease, such as AD. So far, there have been few and conflicting data published on iNKT cells in AD; the contrary findings can be explained by the different sensitivities and specificities of the distinct methods that were used for the detection of these cells. Currently, CD1d multimers or

invariant TCRα chain-specific 6B11 monoclonal antibodies are regarded as the most accurate markers for the identification of iNKT cells by flow cytometry.

In this study, by using an anti-CD3/6B11 mAb combination to characterize iNKT cells, we were able to show that both the percentage and the number of iNKT cells were significantly decreased in the peripheral blood of patients with AD compared with HCs. In our study, the number of iNKT cells in the HCs was higher than usually observed by other investigators. The mean age was about 18 years for HCs, and we believe that the higher number of iNKT cells in this population can partly be explained by the younger age. On the other hand, a high interindividual variation in the frequency of iNKT cells can also been considered. There was no alteration found in the frequency of the total T cells and the CD4/CD8 subsets in AD patients, presuming that the reduction in the prevalence of iNKT cells was not resulted from differences in T cell populations. In agreement with our results, four previous publications demonstrated diminished numbers of iNKT cells in AD patients; conversely, two groups found increased numbers of these cells. These contradictory results suggest that further investigations are needed, as it is possible that not only differences in the applied methods but also differences in the patient selection (extrinsic–intrinsic AD, mild–moderate–severe AD) may alter the iNKT cell number. It is important to emphasize that among the abovementioned studies only one employed a method similar to ours.

Using phenotypic analysis of the CD4/CD8 subsets of iNKT cells, we investigated which subpopulation was mainly responsible for the decreased iNKT number in AD patients. In HCs, we found that the CD4+CD8− and DN subpopulations of iNKT cells were the most frequent subpopulations, and both of them represented a similar percentage of iNKT cells, the CD8+CD4− iNKT cells had an intermediate frequency, and the DP iNKT cells had the lowest frequency. Montoya et al. demonstrated similar prevalence of iNKT subsets in healthy individuals, while various frequencies of these subsets have also been reported. In the peripheral blood of patients with AD, the most pronounced alteration was observed in the DN iNKT subset. Although the numbers of all iNKT subsets were found to be decreased, only CD4− (DN and CD4−CD8+) iNKT subsets showed significantly diminished percentages in AD patients. The strongest positive correlation was found between the DN iNKT subset and total iNKT cells. These findings are consistent with the results of previous studies. Takahashi et al. detected a decreased tendency of CD4−CD8+ and DN iNKT subsets, while Oishi et al. found a greatly diminished DN iNKT cell number in patients with asthma and with AD. In contrast to these results, some authors reported significantly reduced CD4+ iNKT subset in the peripheral blood of patients with AD. The cause for the varied results of the different

reports on iNKT subsets are unknown, but may arise from a distinct patient population selection or a difference in gating on CD4+ and CD8+ iNKT subsets, namely, whether dimly positive cells were involved in these studies.

Differentiating between iNKT subsets is crucially important because distinct subsets may have different functions; furthermore, a crossregulation between the various iNKT subsets markedly influences the immune response to self and foreign antigens. In healthy individuals, the circulating CD4+ iNKT subset can produce both Th1 and Th2 cytokines (IFNγ, IL-4, and IL-13), whereas the DN iNKT subset secretes predominantly Th1 cytokines (IFNγ and TNFα). Elimination of pathogens and tumor rejection are generally correlated with Th1 responses, whereas tolerogenic mechanisms, such as suppression of graft rejection and inhibitory effects, are usually associated with Th2 responses by iNKT cells. Because data on the number of iNKT cells or even iNKT subgroups are limited if functional properties are not analyzed, intracellular IFNγ and IL-4 cytokine production were also measured. We have found that the overall IFNγ production by iNKT cells was decreased, whereas the IL-4 production was increased in AD patients; however, these alterations were not significant. On the other hand, these functional alterations were significant when the DN subgroup was investigated. The DN iNKT cells from AD patients produced significantly less IFNγ and significantly more IL-4 compared with HCs. According to our best knowledge, this is the first demonstration of the altered intracellular cytokine pattern in the DN iNKT subgroup of AD patients. The significantly decreased number and altered function of iNKT cells demonstrated in our results are in good agreement with previous studies, which found that increased plasma IL-18 level in AD patients correlated with the decreased number and dysfunction of iNKT cell in these patients. Lind et al. found that elevated plasma levels of IL-18 exerted an inhibitory effect on iNKT cells and that IL-18-mediated dysregulation of iNKT cells may play a role in the pathogenesis of AD.

It is a yet unsolved question whether changes in the number and cytokine expression of total iNKT cells or iNKT subgroups may act as primary pathogenic factors in the development of AD, or whether the disease is a consequence of elevated Th2-type cytokine levels in the peripheral blood of these patients. The decreased number and altered cytokine production of iNKT cells may lead to an impaired immunoregulatory capacity of these cells, which could contribute to the development of the characteristic cytokine milieu and chronic inflammation observed in AD patients.

In conclusion, the significantly altered number and cytokine production of iNKT cells of AD patients suggest that these cells may play an important role in the pathogenesis of AD.

SUMMARY

The first part of our work we investigated the role of IL-16 – one of the key molecule of the innate immune system, as a possible biomarker in AD. We demonstrated significantly higher serum IL-16 levels in AD patients compared to those in healthy controls and in psoriatic patients. When we studied the association between high serum IL-16 levels and different severity parameters of the disease we found that IL-16 is not suitable for characterize the activity of AD, therefore we investigated whether it can indicate the allergic sensitisation of the patients. We demonstrated that although serum IL-16 levels showed some correlation with the degree of sensitization in patients with AD, it is less accurate marker of disease activity and sensitivity.

Based on the results from DC experiments we made three conclusions: first, unstimulated, circulating mDCs from AD patients seem to have the ability to produce all the investigated Th cell-polarising cytokines, similar to control cells, but the Th2/Th22 polarizing capacity seems to be over activated even in the blood. We suggest that these cells are in a preactivated state. Second, when mDCs were cultured in a model of an AD tissue microenvironment, Th2- and Th22-polarising cytokines became statistically significant in cells from atopic patients relative to those from healthy controls. Third, we would like to emphasise that LSC is a useful technique to monitor mDC functions in vitro.

According to our results with iNKT cells we were able to show that both the percentage and the number of iNKT cells were significantly decreased in the peripheral blood of patients with AD compared with HCs. Using phenotypic analysis of the CD4/CD8 subsets of iNKT cells, we found CD4-CD8+ and DN subpopulations of iNKTcells were the most frequent subpopulations. After the functional analysis of iNKT cells we have found that the overall IFNγ production by iNKT cells was decreased, whereas the IL-4 production was increased in AD patients; however, these alterations were not significant. On the other hand, these functional alterations were significant when the DN subgroup was investigated.

The present work emphasizes the possible role of innate immune system in AD which was considered as a disease primarily influenced by the element of the adaptive immune system.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor, Professor Andrea Szegedi, who helped my work in the past few years with her outstanding professional knowledge, ideas, attention and enduring patience. Encouraged me both professionally and humanly, and provided indispensable support for my scientific achievements.

I would also like to thank the directors of the Department of Dermatology, Éva Remenyik and János Hunyadi, and OMNINVEST Ltd. to make it possible to carry out my experiments in their laboratory.

I would like to express my appreciation to my colleagues in the work group of Andrea Szegedi and in the laboratory of the Department of Dermatology (Krisztián Gáspár, Gábor Mócsai, Anikó Kapitány, Zsolt Dajnoki, Gábor Boross, Eszter Emri and Edit Miko) for giving me their valuable time, advices and support.

Special thanks go to our collaborating partners for their work, scientific knowligde and constructive suggestions: Sándor Baráth and Edit Gyimesi in the III. Department of Internal Medicine, Professor Éva Rajnavölgyi and Erzsébet Nagyné Kovács in the Department of Immunology, Professor Tamás Bíró, Nóra Dobrosi and Balázs Tóth in The Department of Physiology and Zsolt Bacsó and Minh Doan in the Department of Biophysics and Cell Biology.

I would like to thanks to my family for their love and support.

My work was supported by the Hungarian Scientific Research Grant OTKA K108421 and OTKA-PD 112077, the projects of TÁMOP-4.2.2-08/1-2008-0019, TÁMOP-4.2.2.A-11/1/KONV-2012- 0023-”DEFENSE-NET” and TÁMOP-4.2.2/B-10/1-2010-0024. (implemented by the New Hungary Development Plan co-financed by European Social Fund and European Regional Development Fund). The work was also supported by the GINOP-2.3.2- 15-2016- 00050 project. The project is co-financed by the European Union and the European Regional Development Fund.

Registry number: DEENK/38/2017.PL
Subject: PhD Publikációs Lista

Candidate: Georgina Nagy
Neptun ID: G8HUZY
Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology

List of publications related to the dissertation

1. **Nagy, G.**, Doan-Xuan, Q. M., Gáspár, K., Mócsai, G., Kapitány, A., Törőcsik, D., Bacsó, Z., Gyimesi, E., Remenyik, É., Bíró, T., Szegedi, A.: The atopic skin-like microenvironment modulates the T-cell-polarising cytokine production of myeloid dendritic cells, as determined by laser scanning cytometry.
   Exp. Dermatol. 23 (4), 276-278, 2014.
   DOI: http://dx.doi.org/10.1111/exd.12342
   IF: 3.762
2. Gyimesi, E., **Nagy, G.**, Remenyik, É., Sipka, S., Zeher, M., Bíró, T., Szegedi, A.: Altered peripheral invariant natural killer T cells in atopic dermatitis.
   J. Clin. Immunol. 31 (5), 864-872, 2011.
   DOI: http://dx.doi.org/10.1007/s10875-011-9551-5
   IF: 3.077
3. **Nagy, G.**, Gáspár, K., Irinyi, B., Gál, M., Tumpek, J., Gyimesi, E., Sipka, S., Remenyik, É., Szodoray, P., Szegedi, A.: Association between Serum IL-16 Levels and the Degree of Sensitization in Patients with Atopic Dermatitis.
   Int. Arch. Allergy Immunol. 156 (1), 69-74, 2011.
   DOI: http://dx.doi.org/10.1159/000321959
   IF: 2.403

Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

List of other publications

4. Kapitány, A., Béke, G., **Nagy, G.**, Doan-Xuan, Q. M., Bacsó, Z., Gáspár, K., Boros, G., Dajnoki, Z., Bíró, T., Rajnavölgyi, É., Szegedi, A.: CD1c+ Blood Dendritic Cells in Atopic Dermatitis are Premature and Can Produce Disease-specific Chemokines.
Acta Derm.-Venereol. [Epub ahead of print], 2016.
DOI: http://dx.doi.org/10.2340/00015555-2540
IF: 3.638 (2015)

5. Emri, E., Mikó, E., Bai, P., Boros, G., **Nagy, G.**, Rózsa, D., Juhász, T., Hegedűs, C., Horkay, I., Remenyik, É., Emri, G.: Effects of non-toxic zinc exposure on human epidermal keratinocytes.
Metallomics. 7 (3), 499-507, 2015.
DOI: http://dx.doi.org/10.1039/C4MT00287C
IF: 3.54

6. 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.
Acta Derm. Venereol. 95 (2), 151-155, 2015.
DOI: http://dx.doi.org/10.2340/00015555-1882.
IF: 3.638

7. Mócsai, G., Gáspár, K., **Nagy, G.**, Irinyi, B., Kapitány, A., Bíró, T., Gyimesi, E., Lajszné Tóth, B., Maródi, L., Szegedi, A.: Severe skin inflammation and filaggrin mutation similarly alter skin barrier in atopic dermatitis patients.
Br. J. Dermatol. 170 (3), 617-624, 2014.
DOI: http://dx.doi.org/10.1111/bjd.12743
IF: 4.275

8. Gáspár, K., Nagy, M., Herédi, E., **Nagy, G.**, Szegedi, A.: A psoriasisos betegek kezelésében megfigyelhető változások az elmúlt évtizedben a Debreceni Egyetem Orvos- és Egészségtudományi Centrum Bőrgyógyászati Klinikáján és a területi járóbeteg szakrendeléseken = Changes in the treatment of psoriatic patients in the last decade at the Department of Dermatology, University of Debrecen Medical and Health Science Center and at the outpatient clinics.
Bőrgyógyász. Venerol. Szle. 89 (4), 99-104, 2013.

9. Herédi, E., Csordás, A., Clemens, M., Ádám, B., Gáspár, K., Törőcsik, D., **Nagy, G.**, Ádány, R., Gaál, J., Remenyik, É., Szegedi, A.: The prevalence of obesity is increased in patients with late compared with early onset psoriasis.
Ann. Epidemiol. 23 (11), 688-692, 2013.
DOI: http://dx.doi.org/10.1016/j.annepidem.2013.08.006
IF: 2.145

10. Boros, G., Mikó, E., Muramatsu, H., Weissman, D., Emri, E., Rózsa, D., **Nagy, G.**, Juhász, A., Juhász, I., Horst, G. T. J. v. d., Horkay, I., Remenyik, É., Karikó, K., Emri, G.: Transfection of pseudouridine-modified mRNA encoding CPD-photolyase leads to repair of DNA damage in human keratinocytes: a new approach with future therapeutic potential.
J. Photochem. Photobiol. B. 129, 93-99, 2013.
DOI: http://dx.doi.org/10.1016/j.jphotobiol.2013.09.010
IF: 2.803

11. Törőcsik, D., **Nagy, G.**, Remenyik, É., Szegedi, A.: A psoriasis kezelésében alkalmazható jelenlegi és új szisztémás terápiás eljárások.
Immunol. Szle. 3 (5), 22-26, 2011.

12. Matta, C., Juhász, T., Szíjgyártó, Z., Kolozsvári, B., Somogyi, C., **Nagy, G.**, Gergely, P., Zákány, R.: PKCdelta is a positive regulator of chondrogenesis in chicken high density micromass cell cultures.
Biochimie. 93 (2), 149-159, 2011.
DOI: http://dx.doi.org/10.1016/j.biochi.2010.09.005
IF: 3.022

13. Juhász, T., Matta, C., Mészár, Z., **Nagy, G.**, Szíjgyártó, Z., Molnár, Z., Kolozsvári, B., Bakó, É., Zákány, R.: Optimalized transient transfection of chondrogenic primary cell cultures.
Cent. Eur. J. Biol. 5 (5), 572-584, 2010.
DOI: http://dx.doi.org/10.2478/s11535-010-0053-x
IF: 0.685

14. Gaál, J., Lakos, G., Szodoray, P., Kiss, J., Horváth, I., Horkay, E., **Nagy, G.**, Szegedi, A.: Immunological and clinical effects of alphacalcidol in patients with psoriatic arthropathy: results of an open, follow-up pilot study.
Acta Derm. Venereol. 89 (2), 140-144, 2009.
DOI: http://dx.doi.org/10.2340/00015555-0555.
IF: 3.007

15. Juhász, T., Matta, C., Veress, G., **Nagy, G.**, Szíjgyártó, Z., Molnár, Z., Fodor, J., Zákány, R., Gergely, P.: Inhibition of calcineurin by cyclosporine A exerts multiple effects on human melanoma cell lines HT168 and WM35.
Int. J. Oncol. 34 (4), 995-1003, 2009.
DOI: http://dx.doi.org/10.3892/ijo_00000225
IF: 2.447

16. Szegedi, A., Baráth, S., **Nagy, G.**, Szodoray, P., Gál, M., Sipka, S., Bagdi, E., Banham, A. H., Krenács, L.: Regulatory T cells in atopic dermatitis: epidermal dendritic cell clusters may contribute to their local expansion.
Br. J. Dermatol. 160 (5), 984-993, 2009.
DOI: http://dx.doi.org/10.1111/j.1365-2133.2009.09035.x
IF: 4.26

17. Dobrosi, N., Tóth, I. B., **Nagy, G.**, Dózsa, A., Géczy, T., Nagy, L., Zouboulis, C. C., Paus, R., Kovács, L., Bíró, T.: Endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling.
FASEB J. 22 (10), 3685-3695, 2008.
DOI: http://dx.doi.org/10.1096/fj.07-104877
IF: 7.049

18. Szegedi, A., **Nagy, G.**, Baráth, S., Gaál, J.: A D3 vitamin immunmoduláló hatása.
Magyar Immunol. 6 (1-2), 4-10, 2007.

19. Gaál, J., Lakos, G., Aleksza, M., Kiss, J., Horváth, I., Horkay, E., **Nagy, G.**, Szegedi, A.: Az alfakalcidol psoriasisos arthopathiában: klinikai és immunológiai hatások.
Magyar Immunol. 6 (5), 31-37, 2007.

20. Sárközi, S., Almássy, J., Lukács, B., Dobrosi, N., **Nagy, G.**, Jóna, I.: Effect of natural phenol derivatives on skeletal type sarcoplasmic reticulum Ca2+ -ATPase and ryanodine receptor.
J. Muscle Res. Cell. Motil. 28, 167-174, 2007.
DOI: http://dx.doi.org/10.1007/s10974-007-9113-x
IF: 1.731

Total IF of journals (all publications): 51,482
Total IF of journals (publications related to the dissertation): 9,242

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

01 March, 2017

Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu