**PH.D. THESIS**

# CHARACTERIZATION OF THE CD14 MOLECULE AND CERTAIN TOLL LIKE RECEPTORS IN ATOPIC AND AUTOIMMUNE DISORDERS

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# 1. INTRODUCTION

## 1.1. Pattern recognition of innate immunity

The human body is constantly attacked by invading pathogenic microorganisms. The evolutionary ancient form of the host defense against these pathogens is the innate immune system. For a long time it was widely accepted that the non-specific response of innate immunity – mainly aiming the phagocytosis and degradation of microbes and foreign materials – serves only to provide a quick but less effective defense for the host until the development of the slower but more effective and specific adaptive immune response. Now it is already obvious that the innate immune system plays essential role in initiating the antigen-specific immune response (antigen presentation, costimulation) and in the regulation of the effector mechanisms. Since the last few years it is also clear that the innate immune response is not totally non-specific, it can make difference between the own molecules and the components of the pathogenic microorganisms, though the mechanisms of recognition is essentially different compared to the adaptive immune system. Pattern recognition receptors identifying the conserved structural elements of the pathogens ("pathogen-associated molecular patterns") have a crucial role in the initiation and regulation of the innate immune response. These receptors can be soluble and cell-associated and can be localized on the cell surface or intracellularly. Cell-associated receptors upon binding to their specific ligands activate specific signalization pathways and via these routes stimulate the production of effector molecules capable for the elimination of the pathogens. Depending on the receptors and the activated intracellular signalization pathways more-or-less antigen specific immune responses can be induced.

## 1.2. Toll-like receptors

Toll-like receptors (TLR) and their associated partners like the CD14 molecule have a central role among the cell-associated receptors. Until now 10 members of the human TLR

family have been identified. They belong to the type I transmembrane protein family and consist of an extracellular ligand binding LRR ("leucine rich repeat") domain, a transmembrane domain and an intracellular signaling TIR (Toll/IL-1R homology) domain. The TLRs differ in ligand-specificity, in cellular distribution and in signalization and as cellular components of the immune system express unique patterns of TLRs several relatively specific immune responses can be generated.

TLR4 recognizes LPS from the cell-wall of Gram-negative bacteria and certain host molecules (heat-shock proteins). TLR2 binds components of Gram-positive bacteria like peptidoglycan, lipoteichoic acid, lipopeptides, lipoarabinomannan of mycobacteria and zymosan, an important cell-wall component of yeasts. In contrast to the other TLRs TLR2 forms heterodimers with TLR1 and TLR6 that play a role in the discrimination between triacyl- and diacyl-lipopeptides. TLR5 identifies flagellin, the major molecular component of flagellum, responsible for bacterial movements. TLR3, TLR7 and TLR8 play an important role in recognizing viruses, TLR3 binds viral double-stranded RNA (dsRNA) while TLR7 and TLR8 single stranded RNA (ssRNA). On the other hand TLR7 and TLR8 can identify certain antiviral drugs (imiquimod). TLR9 is responsible for the recognition of viral and bacterial non-methylated CpG DNA. The LPS-binding receptor RP105 (radioprotective 105, CD180) shows similarity to TLR4 and can be found mainly on B lymphocytes and a lesser extent on macrophages and dendritic cells. It induces the proliferation of B cells, the upregulation of CD86 and enhances the resistance against the apoptosis inducing effect of irradiation.

Concerning cellular localization CD180, TLR2, TLR4 and the TLR2-associated TLR1 and TLR6 are expressed on the cell-surface, in the cytoplasm-membrane. The localization of TLR5 is similar, but characteristically they can be found on the basolateral surface of epithelial cells. In this way they initiate cellular response only if pathogens invade the intestinal mucosa. TLR3, TLR7, TLR8 and TLR9 are located intracellularly in the

endosomes/phagosomes, therefore they initiate cellular activation only if their ligands are internalized.

Specific co-receptor molecules help the ligand recognition and signalization of certain TLRs. MD2 is required for the proper activity of TLR4 and MD1 binds to CD180 showing similarity to TLR4. The initialization of the TRAM/TRIF signalization pathway of TLR4 is CD14-dependent. CD36 is essential for the diacyl-glycerol recognition of the TLR2/TLR6 complex. Finally, the Unc93b1 molecule localized in the endoplasmatic reticulum is required for the identification of viral/bacterial nucleic acids by intracellular TLR3, TLR7 and TLR9.

Certain members of the TLR family are able to recognize ligands originating from the human body and in this way during antigen presentation can help the activation and antigen production of latent autoreactive B cells. In this way these molecules can have a role in the development of autoimmune disorders.

### 1.3. The CD14 molecule

CD14 is an important, central pattern recognition receptor of the innate immune system and one of the most important LPS-binding proteins. It is not a transmambrane molecule, but inserted to the plasma membrane by a glycosyl-phosphatidyl-inositol (GPI) tail, that enables large lateral mobility.

Besides its membrane expressed form (mCD14) CD14 exists as a soluble molecule (sCD14), too. In human serum two different forms of sCD14 can be identified: a 48 and a 56 kDa molecule, which have different origin. The 48 kDa form is cleaved from the surface-expressed mCD14 upon different stimuli, and the role of serine-proteases was proved in this process. The 56 kDa form keeps its C-terminal part, escapes GPI-attachment and secreted directly. This form is probably stored intracellularly and serine-proteases do not play a role in its release.

mCD14 is a myeloid differentiation marker that is expressed mainly on mature myeloid cells, but at a lesser extent can be detected on B lymphocytes, dendritic cells, basophile granulocytes, trophoblasts and gingival fibroblasts. Hepatocytes also participate in sCD14 production.

CD14, as an LRR containing pattern recognition receptor has an important role in the host defense against different pathogens, because it can recognize not only LPS but other conserved structural molecules originating from Gram-negative and Gram-positive bacteria and yeasts. Such molecules are peptidoglycan, lipoteichoic acid, mycobacterial lipoarabinomannan or polyuronic acid from *Pseudomonas*. As CD14 has no intracellular domain it cannot induce signal transduction on its own, but the interaction with certain coreceptors is required for this. CD14 associated with the TLR4/MD2 complex is already able to induce cellular activation, and via the activation of NF-κB it can induce the production of proinflammatory cytokines.

LPS associated with sCD14 can bind to the surface of mCD14-negative (like endothel, epithel and smooth muscle) cells and in this way can activate them. On the other hand sCD14 competes with mCD14 for LPS-binding and large amounts of sCD14 can neutralize the biological effects of LPS. sCD14 with LBP forms an effective phospholipid-transfer protein pair, enhances the transport of LPS to HDL and helps the clearance and detoxification of endotoxin. CD14 is able to bind and mediate the internalization of circulating apoptotic bodies without the initiation of an inflammatory reaction. CD14 can have a role in binding and phagocytosis of Gram-negative bacteria.

In the proximal promoter region of the CD14 gene a functionally important polymorphism – C-159T – has been described. The T allele showed enhanced transcriptional activity in those cells where the inhibitory transcription factor Sp3 had lower concentration (like monocytes), while in Sp3-rich hepatocytes the transcriptional activity of the T and C allele showed no

difference. It is also known that the serum sCD14 concentration is higher in individuals homozygous for the T/T than in the two other genotypes (C/C and C/T).

CD14-expression, serum sCD14 concentration, polymorphisms of the CD14 gene and alterations in the expression and function of TLRs showed association with certain autoimmune and immune mediated disorders. Furthermore, specific therapeutic approaches could modulate components of this system.

### 1.4. The studied atopic and autoimmune disorders

#### 1.4.1. Atopic dermatitis

Atopic dermatitis (AD) is a frequent, multifactorial, relapsing or chronic inflammatory skin disorder with severe itching, that is frequently associated with or preceding other atopic disorders (like allergic rhinitis or asthma). AD patients are very much susceptible for skin infections mediated by viruses, bacteria and fungi. Two types of AD have been described. 70-80% of the patients belong to the extrinsic group associated with enhanced specific IgE production against environmental and/or nutritive allergens and with positive prick and intracutan test. In the intrinsic (non-allergic) group (20-30% of the patients) the IgE-mediated sensitization is not present and the serum IgE concentration is not elevated. In the extrinsic form a type I hypersensitivity reaction is going on induced by environmental and/or nutritive allergens that becomes more severe in the chronic phase due to a Th1 response initiated by microbial infections. Cutan infections are also frequent in intrinsic AD, because of the diminished host defense against pathogenic microbes. Certain microbial toxins acting as superantigens can induce the activation of T cells, furthermore, they can stimulate the production of specific IgE as classical allergens. In this way, hyperreactivity against components of pathogens might induce the clinical symptoms of intrinsic AD.

In the pathogenesis of AD the major factor is the polarization of the Th1/Th2 immune system to a Th2 direction that can result in the downmodulation of the cutan defense mechanisms.

The exact cause of the Th2 dominance observed in the initial phase of the disease is not known. According to the hygiene hypothesis the lack of Th1 responses induced by microbial infections in early childhood can explain the dominance of Th2 response, but the altered functions of regulatory (Treg) T cells essential in keeping mucosal tolerance might also result in the observed changes.

### 1.4.2. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an idiopathic, multifactorial and multiorgan systemic autoimmune disorder with variable symptoms and disease courses. A complex abnormality of immune regulation develops in SLE. The major elements of the pathogenesis are the breaking of immune tolerance against self antigens, increase in the autoantigen repertoire, diminished clearance of cell debri that results in the development of autoreactive T cells and then in the T-dependent and -independent (via TLRs) polyclonal activation and autoantibody production of B cells. The presence of nucleus-specific autoantibodies – like antinuclear antibody (ANA) or anti-DNA antibody – is very specific for SLE. Autoantibodies can directly cause cell and tissue damage but concerning the pathogenesis the formation and deposition of antigen-antibody complexes – that cause an inflammatory reaction in the involved tissues – seems to be the most important. Due to the decreased elimination of the immune-complexes the inflammatory reaction becomes chronic, resulting in tissue destruction (type III hypersensitivity reaction, immune-complex disease).

### 1.4.3. Polymyositis, dermatomyositis

Polymyositis (PM) and dermatomyositis (DM) are idiopathic, multifactorial systemic autoimmune disorders that belong to the group of inflammatory myopathies. Common feature of the two disorders is the immune-mediated chronic inflammation of the proximal muscles, that leads to symmetric proximal muscle weakness. In the case of patients with DM typical inflammatory skin alterations can be also observed.

Though the pathogenesis of the two disorders is different, in both cases the altered inflammatory reactions result in the destruction of the muscle fibers and in the development of chronic inflammation and fibrosis of the muscles. The presence of myositis-specific and myositis-associated autoantibodies is a typical autoimmune phenomenon in PM/DM.

In PM cellular immunity plays the major role in maintenance of inflammation and the two dominant cell types in the endomyseal infiltrate within the fascicules are macrophages and CD8+ cytotoxic T-cells (Tc). Macrophages produce proinflammatory cytokines. Probably this takes also a part in the enhanced expression of MHC-I and other costimulatory molecules on the infiltrated and even on some further muscle fibers, too. In this way muscle fibers, acting as antigen presenting cells, react and activate autoinvasive T cells that clonally proliferate and destruct muscle cells by production of perforin. The muscle autoantigen presented by MHC-I is not know, yet. The Th1/Th2 balance in the peripheral blood of PM patients is polarized in a Th1 direction.

In dermatomyositis the pathological, dominantly humoral immune processes are targeting the endothelial cells of the microvasculature. The major cellular elements in the perivascular and perifascicular inflammatory infiltrate are Th cells, B lymphocytes, macrophages, pDC cells and neutrophil granulocytes. Pathogenic autoantibodies activate the complement system and the lytic C5b-9 MACs deponate into the wall of the capillaries that results in the destruction of the vasculature and development of muscle ischemia and perifascicular atrophy. The autoantibody of the endothelial cells inducing the pathological processes is not known, yet. The Th1/Th2 balance in the peripheral blood of DM patients is polarized in a Th2 direction.

## 2. AIMS OF THE THESIS

CD14 expression, serum sCD14 concentration, polymorphism of the CD14 gene, and alterations in the expression and function of TLRs showed association with certain autoimmune and immune mediated disorders. Furthermore, specific therapeutic approaches could modulate components of this system. In our work we studied the expression and function of CD14 and certain TLRs in systemic autoimmune disorders with different pathogenesis (systemic lupus erythematosus, poly- and dermatomyositis). We analyzed these parameters in samples of patients with atopic dermatitis known to be associated with frequent skin infections, furthermore, we tested the effect of glucocorticoids – used widely in the management of autoimmune disorders – on these parameters.

The major questions asked at the beginning of the study:

- How does the expression of CD14 and certain TLRs on peripheral leukocytes, the ligand binding of CD14 and the serum concentration of sCD14 change in samples of patients with intrinsic and extrinsic atopic dermatitis?
- Are there any associations between the CD14-expression of monocytes, the serum sCD14 concentration, the isotype distribution of serum sCD14, the C(-159)T polymorphism of the CD14 gene and the clinical parameters of myositis?
- What is the effect of *in vivo* systemic glucocorticoid therapy on CD14-expression, CD14-mediated LPS binding and LPS-induced cellular activation of peripheral monocytes in SLE?

## 3. PATIENTS

### 3.1. Atopic dermatitis

Thirty patients with AD (18 men, 12 women, aged: 19.7±9.3 years) and 56 – age and sex matched – healthy control subjects were enrolled into this study. All patients with AD

fulfilled the diagnostic criteria of Hanifin and Rajka. 10 patients with negative skin prick test results, with negative serum allergen-specific IgE test and with serum total IgE below 120 kU/L were categorized as intrinsic AD, while 20 other patients formed the extrinsic AD group. None of the healthy controls had a personal history of allergic disease and their serum IgE levels were within the normal range.

### 3.2. Systemic lupus erythematosus

Altogether 18 patients (16 women, 2 men; mean age: 38.8±11.7 years) suffering from systemic lupus erythematosus (SLE) were selected from the individuals followed by the Outpatient Unit of the 3rd Department of Internal Medicine, University of Debrecen, Hungary. During the time of the study ten patients (10 women; mean age: 40.6±14.3 years) were in the inactive stage of the disease and were free of any treatment for more than 3 months ("No steroid" group). The other group consisted of eight patients (6 women, 2 men; mean age: 36.5±7.5 years) who received regular low dose corticosteroid (4-16 mg methylprednisolon per day) as maintenance therapy. During the time of the study, due to the observed severe disease flare all of these patients underwent a short pulse steroid treatment (1g/day for 3 days). Samples were taken before the pulse steroid treatment (when the patients still received the low dose maintenance therapy; "low dose steroid" values of the group) and 24 hours after the injection of the last pulse steroid dose ("pulse steroid" values of the group). The control group was age and sex matched and consisted of 11 healthy laboratory persons.

### 3.3. Polymyositis/dermatomyositis

Seventy-six patients with PM (60 women, 16 men, 23 active, 53 inactive, aged 54.0±12.1 years) and 34 patients with DM (27 women, 7 men, 14 active, 20 inactive, aged 53.1±13.9 years) were enrolled in this study. The disease course was evaluated by the number of active phases during the follow-up (at least two years). We categorized 53 patients into the monophasic, 40 patients into the polycyclic and 17 patients into chronic disease course group.

Thirty-five healthy individuals served as controls. In the case of the C(-159)T genotyping the control group was increased to 110 healthy persons in order to enhance the statistical power of the analysis. Based on literature data a virtual, larger control population was also created. Among the publications containing Caucasian control data about the C(-159)T polymorphism we found only 8 studies, where the gender distribution showed equal or female dominance similarly to our myositis patient group. The number of controls with C/C, C/T and T/T genotypes was summarized from these publications.

## 4. METHODS

### 4.1. Quantitation of cell-surface receptors

To perform the quantitation of cell surface receptors we used anticoagulated whole blood, directly and indirectly labeled monoclonal antibodies and isotype controls. After labeling, the erythrocytes were lysed and the leukocytes were fixed with paraformaldehyde. The fluorescence intensity of the samples were measured by a Coulter EPICS XL, or a FACSCalibur flow cytometer. Monocytes, lymphocytes and granulocytes were separated on the basis of their size and scatter properties. As background, MFI values, measured using the appropriate isotype controls were subtracted from the fluorescence intensity of the samples contained receptor specific antibodies. In certain cases the absolute count of the cell surface receptors was determined with the help of a calibration curve, which was made using standard beads coated with known amounts of a monoclonal antibody (Qifikit; DAKO).

### 4.2. CD14-mediated LPS binding

Anticoagulated, washed whole blood or isolated mononuclear cells of healthy controls and patients were used to study the CD14-dependent LPS binding of monocytes and granulocytes. Samples were preincubated in the presence or absence of a blocking anti-CD14 mAb (60bca) and then incubated with FITC-LPS (FITC-labeled *S.minnesota* Re595), or bodipy-LPS

(bodipy-labeled LPS from *S.minnesota* Re595) in the presence of 4% normal human serum (NHS). After that in the case of whole blood samples erythrocytes were lysed, and finally leukocytes were fixed with paraformaldehyde. The samples were measured by a Coulter EPICS XL, or a FACSCalibur flow cytometer. Monocytes and granulocytes were gated on the basis of their scatter properties. The fluorescence intensity of the unlabeled cells – as background – was subtracted from the MFI values of all samples. To define the amount of CD14-dependently bound LPS, the fluorescence intensity of the cells in the presence of the blocking anti-CD14 mAb (CD14-independent LPS binding) was subtracted from the fluorescence intensity of the cells incubated without the mAb 60bca (total LPS binding).

**4.3. CD14-mediated phagocytosis of monocytes and granulocytes**

Washed and diluted whole blood samples of healthy controls and patients – anticoagulated by EDTA – were incubated with bodipy-labeled *Escherichia coli* (strain K-12, Molecular Probes, Eugene, OR; cell to bacterium ratio = 1:8) in the presence of EDTA plasma. EDTA plasma promote the CD14-mediated, but inhibit the Fc and complement receptor mediated phagocytosis. After the lysis of erythrocytes, leukocytes were fixed with paraformaldehyde. The fluorescence intensity and the percentage of phagocytosing cells in the samples were measured by a FACSCalibur flow cytometer. We also measured the fluorescence intensity of the bacteria and calculated the average number of bacteria taken up by one cell ("phagocytosis index"):

$$\% \text{ of phagocytosing cells} \times ((MFI^{ph} - MFI^{nph}) / MFI^{b}) / 100$$

where $MFI^{ph}$, $MFI^{nph}$ and $MFI^{b}$ are the mean FL1 fluorescence intensity of phagocytosing and non-phagocytosing cells and bacteria, respectively.

**4.4. LPS-induced cell activation**

The degree of cell activation was determined by measurement of TNFα production of isolated mononuclear cells. Mononuclear cells attached to the wells of a microtiter plate were

preincubated in the presence or absence of anti-CD14 mAb 60bca. Then LPS with NHS was added to the wells. The samples were incubated for 16 hours and the TNFα levels of the supernatants were measured with a commercial cytokine ELISA (OptEIA$^{TM}$system, Pharmingen, San Diego, CA) according to the manufacturers' instructions. The cells incubated without LPS and NHS served as background and their TNFα production was subtracted from each sample. The CD14-dependent stimulation of the cells was calculated by subtracting the TNFα production in the presence of the blocking anti-CD14 mAb (CD14-independent activation) from the TNFα amount measured in the absence of the mAb (total activation).

**4.5. Developing a novel method to quantify soluble CD14**

We developed a novel, easy and rapid flow cytometric assay to measure sCD14 levels in human serum and plasma. The assay is based on the competition between membrane-expressed CD14 of isolated monocytes from healthy volunteers and sCD14 present in the sample sera for binding to an anti-CD14 monoclonal antibody. The amount of cell-associated mAb is determined with an FITC-labeled anti-mouse conjugate and flow cytometry, and the fluorescence signal is inversely proportional with the amount of serum sCD14. Using dilutions of a standard serum the concentration of sCD14 in the samples was calculated and compared to results obtained by a commercial sCD14 ELISA (Quantikine, R&D systems). The comparison by Pearson regression revealed a strong correlation between the two methods (r=0.92)

**4.6. Quantitation of sCD14 isoforms by Western blotting**

Quantitation of the 56 and 48 kDa isoforms of serum sCD14 was performed with the help of Western blotting and densitometry. Serum proteins were separated by polyacrylamide gel electrophoresis and then blotted to a nitrocellulose membrane. sCD14 isoforms were detected with the help of biotin-labeled polyclonal anti–human CD14 antibody (R&D Systems, Minneapolis, MN, USA), alkaline phosphatase conjugated streptavidine (DAKO) and

NBC/BCIP substrate (Promega, Madison, WI, USA). Dried nitrocellulose membranes were analyzed with a GelDoc1000 (BioRad) gel documentation system and the density of the lanes for 48 kDa and 56 kDa sCD14 were measured. Knowing the ratio of the two isoforms and the total sCD14 concentration in a given sample, the concentration of both isoforms could be calculated.

**4.7. Genotyping of the C(-159)T polymorphism of the CD14 gene**

The C(-159)T polymorphism of the CD14 gene was determined by the use of PCR followed by RFLP. Genomic DNA of patients and controls was isolated with the help of QIAamp DNA Blood Mini Kit (QIAGEN). A part of the genomic DNA surrounding the polymorphic site was amplified and the PCR product was digested with *Ava*II restriction endonuclease (New England Biolabs, Beverly, MA, USA). The digested products were fractionated on agarose gel and visualized with the help of ethidium-bromide. As the PCR product contained a recognition site of the *Ava*II restriction enzyme only in the case of the T allele, the genotype of the tested persons could be determined on the basis of the length of the digestion fragments.

**4.8. Other applied (routine) laboratory methods**

The serum total IgE concentration was measured by a Modular E170 automated analyzer (Roche, Basel, Switzerland), while the allergen-specific IgE concentration by a MAST CLA1 analyzer (Mountain View, CA). The serum lactate dehydrogenase (LDH) activity was measured by an Integra 700 automated analyzer (Roche). The absolute count of the monocytes was determined using a Sysmex SF3000 hematology analyzer (Sysmex Corporation, Kobe, Japan).

**4.9. Statistical analysis**

Normal distribution of the tested parameters was evaluated by the Kolmogorov-Smirnov test. In the case of results showing a normal distribution the data of control and patients

groups were compared by the Student's unpaired t-test, while in the case of non-normal distributions a Mann-Whitney U-test was used. The differences between parameters measured on the same SLE patients before and after pulse steroid treatment were analyzed by the Wilcoxon paired test. During the development of the flow cytometric sCD14 assay, the sCD14 results measured by the two tests in samples of the same controls and patients were compared by the Student's paired t-test and the correlation between the two tests was evaluated by the Pearson regression analysis. In the case of PM/DM patients the associations between CD14-expression, serum sCD14 concentrations and LDH values were determined by a Spearman rank regression analysis. The genotype distribution of the C(-159)T polymorphism in the control and different patient groups was compared by the Chi-square test. The statistical analysis was performed with the help of the "Statistica" software, significant differences were defined at $p<0.05$.

## 5. RESULTS

### 5.1. Alterations of CD14/TLR expression and CD14-dependent functions in atopic dermatitis

#### *5.1.1. Quantitation of numbers of TLR2, TLR4, CD14 and CD180 molecules on monocytes, granulocytes and lymphocytes of patients with atopic dermatitis*

Based on the serum total and allergen specific IgE concentrations patients were divided into an intrinsic and an extrinsic AD group. The number of TLR2 showed significantly higher values on the surface of monocytes if data of all AD patients were compared to that of the controls. Similarly to all AD patients the TLR2 expression on monocytes of intrinsic AD individuals was significantly higher compared to the healthy controls. Furthermore, there was a significant increase in the number of TLR4 on the intrinsic AD monocytes compared to that of the controls. The TLR2 and TLR4 expression on monocytes of extrinsic AD patients

showed lower values compared to the intrinsic AD group, but these differences were not significant. There was also non-significant difference in the amount of the monocytes' CD14 and CD180 expression between the controls and the patient groups.

In the case of the granulocytes higher numbers of TLR2 were observed in all AD patients and in the intrinsic AD group than in the controls, but the differences were significant only in the case of the individuals suffering from intrinsic AD. Furthermore the TLR2 expression of intrinsic AD granulocytes was significantly elevated compared to the extrinsic AD cells. Concerning the CD14 expression granulocytes of all AD and the intrinsic AD patients showed significantly higher numbers of surface CD14 compared to the controls. The number of surface CD180 on the CD180-positive lymphocytes of all AD patients and patients of the intrinsic and extrinsic subgroups was significantly elevated compared to the healthy individuals.

The number of TLR4 and CD180 on granulocytes and the number of TLR2, TLR4 and CD14 on lymphocytes were below the detection level.

### *5.1.2. The CD14-mediated LPS- and bacterium-uptake of monocytes and granulocytes in patients with atopic dermatitis*

Our data showed no difference in the CD14-mediated LPS-binding of atopic monocytes and granulocytes compared to healthy controls. The CD14-mediated bacterium phagocytosis of monocytes and granulocytes showed insignificant difference between the controls and the patient groups.

### *5.1.3. Determination of serum sCD14 levels in patients with atopic dermatitis*

The sCD14 concentration of all and the extrinsic AD patients was significantly lower than that of the healthy controls.

## 5.2. Alterations of CD14 expression and CD14-dependent functions in SLE upon glucocorticosteroid therapy

### *5.2.1. Effect of glucocorticosteroid therapy on CD14-expression of monocytes of SLE patients*

Our results showed that the amount of CD14 on the surface of these cells decreased with the dose of the applied steroid, though the differences were significant only between the data of pulse steroid treated patients and the data of other patients and control groups. A similar significant difference was observed when the paired values (before and after the therapy) of pulse steroid treated individuals were compared with the Wilcoxon matched pairs test.

### *5.2.2. Effect of glucocorticosteroid therapy on CD14-dependent binding of FITC-LPS to monocytes*

The CD14-dependent LPS binding capacity of monocytes isolated from SLE patients showed a decrease as a function of the increasing *in vivo* steroid doses. The differences were significant between the controls and the patients with low dose and pulse steroid treatment but not between the controls and the untreated SLE patients. The pulse steroid treated patients' monocytes showed significantly lower LPS-binding than that of the untreated and the low dose steroid patients' cells. Similar significant differences in CD14-mediated LPS-binding were observed when the paired values (before and after the therapy) of pulse steroid treated individuals were compared with the Wilcoxon matched pairs test.

### *5.2.3. Effect of glucocorticosteroid therapy on CD14-dependent activation of monocytes by LPS*

The LPS-induced TNFα production was significantly lower in the case of pulse steroid treated SLE monocytes compared to the controls. The data of the low dose steroid SLE group was non-significantly lower compared to controls similar to the untreated patients, while the pulse steroid values were significantly lower than the data of the untreated patients. In

contrast to the calculation based on the grouping of data before and after pulse steroid treatment the comparison of the paired values (before and after the therapy) of the CD14-dependent TNFα production of pulse steroid treated individuals, using the Wilcoxon matched pairs test, revealed significant differences. The rather high individual variation of TNFα production might explain the difference between the results of the two statistical analyses.

## 5.3. Association between sCD14 concentration, sCD14 isoforms, CD14 expression, the C(-159)T genotype of the CD14 gene and disease activity and course in PM/DM

### *5.3.1. The serum concentration of the total and the 48/56 kDa sCD14, the peripheral monocytes' CD14-expression and the distribution of the C(-159)T polymorphism of the CD14 gene in PM/DM*

First we compared the phenotypic/genotypic properties of CD14/sCD14 in the samples of PM and DM patients. We could not find significant differences in these parameters between the two patient groups, and similarly, no significant difference was found in the CD14-expression of monocytes. We also determined the distribution of the C(-159)T polymorphism of the CD14 gene and found similar data in the two patient groups. We also compared these parameters between the patient groups (PM, DM, PDM) and the healthy controls. No significant differences were found except the concentration of the 56 kDa form of sCD14 that was significantly elevated in the patients compared to controls. Based on these results we could not detect any significant difference in the listed parameters between the PM and DM patient groups, therefore we handled these patients hereafter as one homogenous group named PDM.

### *5.3.2. Associations between disease activity and the monocytes' CD14 expression and serum sCD14 concentrations in myositis patients*

We grouped the patients on the basis of disease activity which was determined based on clinical symptoms and laboratory parameters. Our results showed that the concentration of the total sCD14 and the 56 kDa isoform of the active patients (aPDM) were significantly increased compared to those of inactive patients (iPDM) and controls. On the contrary, the CD14 expression of monocytes was significantly lower in active than in inactive patients and controls.

### *5.3.3. Associations between disease course and the monocytes' CD14 expression and serum sCD14 concentrations in myositis patients*

PDM patients were divided into subgroups based on the disease course, namely: monophasic, polycyclic and chronic. We observed significantly elevated total sCD14 concentration in the chronic course group compared to the monophasic and polycyclic groups and the healthy controls. Patients with chronic disease course had also the highest value of the 56 kDa sCD14 but the differences between this and the other course groups were not significant. On the other hand, every course subgroup had a significantly higher 56 kDa sCD14 concentration compared to the healthy controls. Concerning the monocytes' CD14-expression patients with chronic course showed the lowest value and the difference was significant between this group and the monophasic group and also the controls.

As significant differences were found in total and 56 kDa sCD14 concentrations and monocytes' CD14-expression between patients in the active and inactive disease state, we analyzed the data of these groups subdivided by disease course. Within the inactive patients we could not find significant differences between the monophasic and polycyclic groups, neither between the different course groups and the controls in the monocytes' CD14 expression and serum sCD14 concentrations. In the case of active PDM patients there were no

differences between the course groups in these parameters, but the total sCD14 and 56kDa sCD14 concentrations were significantly elevated in each group compared to the controls. Concerning the monocytes' CD14 expression the active chronic patients showed significantly decreased expression compared to controls. The rate of patients in the active disease state was significantly higher in the chronic course group compared to the monophasic and polycyclic patients.

### *5.3.4. Association between serum sCD14 concentration, monocytes' CD14-expression and another biochemical disease activity marker*

As the concentration of the total and the 56 kDa sCD14 and the monocytes' CD14-expression showed association with disease activity, we examined the correlation of these parameters with a known myositis activity marker. The association between patients' total sCD14 and the lactate dehydrogenase concentration really proved to be significant. Between the monocytes' CD14 expression and the lactate dehydrogenase concentration a significant negative correlation could be observed.

### *5.3.5. Association between the T/T genotype of the C(-159)T polymorphism in the CD14 gene and disease course and activity*

We compared the genotype distribution of the active PDM patients with that of the inactive PDM group. Our results showed that the rate of the T/T genotype was significantly higher within the active patients than in the inactive patient group and our own or the literature controls. The rate of the T/T genotype was also increased in the group of patients with chronic disease course compared to controls and patients with monophasic or polycyclic disease, but the difference was significant only between the chronic and polycyclic patients and the chronic patients and the controls. Within the patients having a T/T genotype the rate of individuals with active disease state and chronic disease course was significantly higher than in the C/C+C/T group.

### *5.3.6. Association between the T/T genotype of the C(-159)T polymorphism in the CD14 gene and serum sCD14 concentrations*

We compared the sCD14 concentrations of patients having different genotype, concerning the C(-159)T polymorphism of the CD14 gene. In the case of the TT genotype the concentration of the total sCD14 and the 56 kDa isoform was significantly higher than that of the other genotypes

## 6. DISCUSSION

Toll-like receptors and their associated molecules like CD14 are the most important pattern-recognition receptors of the innate immune system. They support the elimination of invading microorganisms, furthermore have an important role in the initiation of the adaptive immune response and even can participate in the development of autoimmune disorders. In our studies we analyzed the expression and functions of the CD14 system and certain TLRs in autoimmune disorders (systemic lupus erythematosus – SLE, poly/dermatomyositis – PM/DM) and in atopic dermatitis (AD) characterized by frequent bacterial skin infections.

The association of infections with CD14 and TLRs might occur in two ways. Reduced expression or activity of these receptors can result in frequent infections, on the other hand severe local and systemic infections can stimulate the systemic immune response leading to the increased expression and activity of CD14 and certain TLRs. As the expression of the tested CD14 and TLR receptors did not show decrease in AD patients, a diminished expression of these molecules does not play a role in the development of bacterial infections associated with AD. On the other hand it is known, that different bacterial products are able to induce the upregulation of CD14, TLR2 and TLR4 on peripheral leukocytes, furthermore, the expression of these molecules on these cells are significantly elevated in different forms of bacterial sepsis. These data and our own results suggest that the frequent local bacterial

infections associated with altered barrier functions of the skin might lead to the leakage of bacterial molecules (superantigens, LPS, peptidoglycan) that stimulate and upregulate the leukocytes' CD14/TLR expression.

The observed alterations in CD14, TLR2 and TLR4 expression were even more expressed and significant in the case of the intrinsic AD patients, while the data of the extrinsic group did not differ significantly from that of the healthy controls. As the frequency of bacterial infections is not higher in the intrinsic form of the disease compared to extrinsic AD, our data suggest a kind of hyperreactivity to microbial components in the case of intrinsic AD that might be a trigger factor of this form of the disease. Recent data in the literature showed that allergen specific IgE against microbial components could be detected in 50% of intrinsic AD patients and this observation also raised the possibility that a hyperreactivity to microbial components might be a trigger factor of this subtype of AD.

In order to evaluate the role of alterations concerning CD14 molecule in the development and course of systemic autoimmune disorders, we studied certain parameters of the CD14 system in samples of patients suffering from PM and DM. No differences were found in membrane CD14 expression of monocytes, in the serum concentration of different forms of CD14 and in the genotype distribution of the C(-159)T polymorphisms between the two types of myositis. Based on these data, we can conclude that alterations in the CD14 system do not explain the differences in the pathogenesis of PM and DM.

The total serum sCD14 concentration of PDM patients showed no significant difference compared to controls and similarly the membrane CD14 expression of monocytes was not altered, either. Analyzing the data of patients with active and inactive disease revealed that sCD14 concentration was higher while membrane CD14 was lower in active patients compared to inactive patients or controls. Defining subgroups based on disease course disclosed that patients with chronic course have higher sCD14 concentrations and express

lower number of membrane CD14 in chronic disease course. The significant alterations of sCD14 and membrane CD14 in chronic course group can be explained by the significantly higher rate of active patients among them compared to the other groups and controls. Based on these data we can conclude that elevation of serum sCD14 and – a lesser extent – reduction of membrane CD14 are disease activity markers. Indeed, these parameters showed significant association with a known activity marker, LDH. The association of CD14 changes with a wild variety of inflammatory reactions might suggest that sCD14 act as a positive acute phase protein. Indeed in two different studies the association of sCD14 levels or CD14-expression with CRP was described, furthermore in a recent publication Bas and his colleagues proved this hypothesis.

Our results showed that the source of elevated serum sCD14 in the active myositis patients is the direct secretion of the 56 kDa isoform. The facilitation of this route and the probable consequent downmodulation of the GPI-anchor attachment explain the lower expression of membrane CD14 observed in active PDM patients.

Several lines of evidence indicate that a known promoter polymorphism – C(-159)T – in the gene of CD14 alters the serum sCD14 concentration. The T/T genotype shows association with elevated serum sCD14 levels in different disorders, and we found similar data in our patient cohort. Based on our data the rate of T/T genotype was significantly higher in patients with active PDM and with chronic disease course. As these are the subgroups characterized by elevated serum sCD14 levels this further proves the association of the T/T genotype and higher serum sCD14 concentrations. As sCD14 can activate CD14-negative endothelial, epithelial and smooth muscle cells, the presence of elevated number of serum sCD14 molecules can be important in the maintenance of a chronic activation state of these cells. In this way the T/T genotype might mean a genetic susceptibility for chronic disease course in myositis patients as its association with higher serum sCD14 might induce a slight but

constant cellular activation, production of proinflammatory cytokines and preservation of a chronic inflammatory reaction.

The CD14 system can be modulated by therapeutic agents. The immunosuppressive and anti-inflammatory glucocorticosteroids affect several different cells in different ways. According to our results pulse steroid treatment significantly reduced CD14-expression, CD14-mediated LPS-binding and LPS-induced cellular activation in monocytes of SLE patients that can mean a new biochemical pathway of steroid action.

**SUMMARY**

Toll-like receptors and their associated molecules like CD14 are the most important pattern recognition receptors of the innate immune system. They support the elimination of invading microorganisms, furthermore have an important role in the initiation of the adaptive immune response and even can participate in the development of autoimmune disorders. In our studies we analyzed the expression and functions of the CD14 system and certain TLRs in autoimmune disorders (systemic lupus erythematosus, poly/dermatomyositis) and in atopic dermatitis characterized by frequent bacterial skin infections.

**The most important results and new findings of the studies:**

- A novel competitive flow cytometric assay was developed to measure serum sCD14 concentrations.
- In the intrinsic form of AD we observed an upregulation of CD14, TLR2, TLR4, CD180, and the CD14 mediated LPS- and bacteria-binding was not altered. Based on these data, the presence of frequent infections in AD are probably not attributable to the diminished functions of these molecules. It is more likely that bacterial components are able to translocate through the altered barrier of the skin and can induce the systemic activation of the immune system and upregulation of certain receptors.
- The T/T genotype of the C(-159)T polymorphism showed association with high sCD14 concentrations and with the chronic disease course in myositis. In this way the T/T genotype might mean susceptibility for the development of chronic disease course by maintaining elevated sCD14 levels that can induce the mild but constant activation of CD14-negative endothelial and muscle cells.
- Pulse steroid treatment significantly reduced CD14-expression, CD14-mediated LPS-binding and LPS-induced cellular activation in monocytes of SLE patients that can mean a new biochemical pathway of steroid action.

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**Sum of the impact factors: 12,797**

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