In vitro and *in vivo* investigation of the regulatory mechanisms controlling innate immunity

Thesis of the Ph.D. dissertation

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INTRODUCTION AND AIMS OF THE STUDY

The skin represents the first line of defence against invading microorganisms. Epithelial cells, considered as semi-professional immune cells, express a series of receptors known as Pattern Recognition Receptors (PRRs), which recognize distinct pathogen-associated molecular patterns (PAMPs). Activation of PRRs – for example Toll-like receptors (TLRs) – induces the secretion of different antimicrobial peptides (AMPs) and proinflammatory mediators resulting in local inflammation. Furthermore, the migration of different professional immune cells (e.g. dendritic cells, macrophages, T-cells) at the site of infection initiates, among other, adaptive immune response. Importantly, innate immunity must be properly controlled, as its continuous activation leads to the development of chronic inflammatory diseases. Although our knowledge regarding the direct role of different regulatory mechanisms controlling innate immune response is expanding, further investigations are needed in order to develop effective therapeutic procedures against chronic inflammatory diseases.

With respect to this, aims of this study were as follows:

1. Establishment of an *in vitro* experimental model for the examination of gene-specific regulatory mechanisms of innate immune processes that can be used for monitoring both acute and persistent inflammation.
2. To study the gene expression profile of different proinflammatory cytokines/chemokines and AMPs in keratinocytes treated with *Staphylococcus aureus*-derived peptidoglycan (PGN) using the above established model.
3. To investigate the global level of histone acetylation, a modification associated with transcriptional activation in keratinocytes after PGN stimulation/restimulation.
4., To study the expression profiles of microRNAs (miRNAs) – especially miR-203 and its targets (p63, SOCS3) – involved in the posttranscriptional regulatory mechanisms in keratinocytes, by utilizing to power of our experimental model.
5. To investigate the gene- and protein expression profile of TAM receptors and their ligand (GAS6) – which are negative regulators of inflammation – in both professional and semi-professional immune cells, after their immune activation achieved by different microbial agents.

6. To study the expression profile of TAM receptors and GAS6 in psoriasis, a common chronic skin disease.

METHODS

Cell cultures

In our experiments immortalized HaCaT human keratinocytes, PK E6/E7 cells (vaginal epithelial cells) and primary keratinocytes (pKC) were used as semi-professional immune cells.

Studying different professional immune cells, on the one hand peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat, which cells were treated for 5 days with granulocyte-macrophage colony stimulating factor (GM-CSF; Sigma) and recombinant human interferon-α (IFN-α; Sigma) for differentiation to immature dendritic cells (DCs). To create mature DCs, immature DCs were treated with recombinant human TNF-α (R&D Systems) for additional day.

On the other hand, THP-1 cells (human acute leukemia monocyte) were investigated, where activation of THP-1 cells was carried out with addition of phorbol myristyl acetate (PMA; Sigma) for 48 hours.

In each case, cells were cultured at 37 ^{o}C and 5% CO_2 under standard cell culture conditions; the cells were used at 60-70% of confluency.

Treatment of cell cultures

In our experiments stimulations of different cell types were made with the following microbial agents:

- PGN: *Staphylococcus aureus*-derived peptidoglycan (TLR2 ligand; Fluka)
- LPS: *Escherichia coli*-derived lipopolysaccharide (TLR4 ligand; Sigma)
- Poly I:C: polyinosine-polycytidylic acid as viral double strand RNA synthetic analog (TLR3 ligand; Enzo Life Sciences)

Studying the PGN-induced chromatin modification a part of HaCaT cells was treated with Trichostatin A (TSA, Sigma), a histone deacetylase (HDAC) inhibitor 2 hours prior to PGN stimulation, as well as 24 hours after PGN infection.

Transfection of cells

HaCaT cells were transfected with specific anti-miR-203 miRNA inhibitor or anti-miR negative control constructs using siPORT^{TM} Polyamine-Based Transfection Agent (Life Technologies) according to the manufacturer's „pre-plated" protocol instructions. Transfected cells were cultured at 37 ^{o}C under standard cell culture conditions. 24 hours after the first treatment, the transfection was repeated in order to ensure the constant presence of the anti-miR inhibitor.

RNA extraction

After the appropriate treatments total RNA was isolated with the RNeasy Mini Plus Kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of total RNAs were measured by NanoDrop, Qubit and Bioanalyzer.

Reverse transcription and quantitative real-time PCR

After quantification and qualification of RNA sample cDNA was synthesized with High Capacity RNA to cDNA Kit (Life Technologies) by using Applied Biosystems Veriti Thermal Cycler. The expression level of selected genes was determined with ABI StepOne Plus Real-Time PCR instrument using TaqMan Gene Expression Assays along with TaqMan Gene Expression Master Mix (Life Technologies). The ratio of each mRNA relative to the 18S rRNA was calculated using the $2^{\Delta\Delta C}{}_{T}$ method.

The expression patterns of miRNAs were determined by TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assays (LifeTechnologies) according to the manufacturer's instructions. The ratio of each miRNA relative to RNU48 (Life Technologies) was calculated using the $2^{\Delta\Delta C}{}_{T}$ method.

All experiments were carried out in at least three independent replicates with two technical parallels.

Histone extraction

To study the effect of different microbial agents on the global histone acetylation level, we used the Abcam histone acid extraction protocol. After acid extraction at 4 ^{o}C overnight, concentrations of histone proteins were quantified from supernatants by NanoDrop.

ELISA method

The amounts of secreted cytokines (IL-6 and CXCL8) were quantified from culture supernatants using the Quantikine Single Analyte ELISArray Kit (R&D Systems). Serial dilutions of recombinant human IL-6 and CXCL8 were used to generate standard curves. The optical density of the wells was determined using a Microtitre Plate Reader (FluoStar Optima, BMG Labtech) set to a wavelength of 450 nm, with a wavelength correction set to 570 nm.

Total protein extraction

Harvested cells were homogenized in PBS containing proteinase inhibitor (PIC) and phenylmethanesulfonyl fluoride (PMSF). Samples were frozen quickly in liquid nitrogen and were thawed slowly on ice by gentle vortexing. This procedure was repeated 3-4 times. Samples were than centrifuged, pellets were discarded and total protein concentrations of supernatants were determined by NanoDrop.

Western blot

For quantitative protein analysis equivalent amounts of total- or histone proteins were electrophoresed by Tris-SDS-PAGE and transferred to Pure Trans-Blot Transfer Medium Nitrocellulose Membrane (Bio-Rad). The successful blotting was confirmed with Ponceau staining. The expression profiles of selected proteins were determined by using specific primary antibodies. Chemiluminescent detection was performed using appropriate IgG-HRP (Bio-Rad) and Immobilon Western Chemiluminescent HRP Substrates (Millipore).

Immunofluorescent labelling

For immunofluorescent detection of investigated proteins cells were grown on flexiPERM (Sarstedt) subdividing slides using silicone inserts. After fixation with

paraformaldehyde cells were incubated with primary antibodies at 4 ^{o}C overnight. On the next day samples were incubated with the appropriate secondary fluorescein isothiocyanate (FITC)/ tetramethyl rhodamine isothiocyanate (TRITC) -conjugated antibodies for 1 hour at room temperature. The nuclei of the cells were labeled with 4',6-diamidino-2-phenylindole (DAPI). Samples were mounted with Citifluor (Citifluor Ltd.) to prevent quenching and the images were captured with a Zeiss Axio Observer Z1 fluorescent microscope.

RNA extraction from psoriatic and healthy skin biopsies

Shave biopsies from uninvolved and involved skin of psoriatic patients and control skin biopsies were collected,epidermis was separated from the dermis following an overnight incubation at 4 °C in Dispase solution (Roche Diagnostics). On the next day samples were vortexed vigorously in TRIzol Reagent (Life Technologies) containing chloroform (Sigma). After removing the genomic DNA from supernatant (RNeasy Plus Mini Kit, Qiagen), further steps (e.g. purification and quantification of total RNA, reverse transcription and quantitative real-time PCR) were performed as described above.

All tissue samples were taken with the patient's written informed consent and the approval of the local ethics committee; the study was conducted according to the Declaration of Helsinki Principles.

RESULTS

First, we found in our *in vitro* experimental model, that the gene expression profile of different proinflammatory cytokines/chemokines and AMPs is differently altered when keratinocytes are stimulated or restimulated with *Staphylococcus aureus*-derived peptidoglycan (PGN). The expression of cytokines/chemokines (e.g. IL-6, CXCL8) remains unchanged upon PGN restimulation, these genes are therefore considered non-tolerizable; in contrast, PGN restimulation significantly decreases AMP expression (e.g. DEFB4, CAMP) - these genes are considered tolerizable. However, this non-tolerizable/tolerizable phenotype is altered when TSA, a HDAC inhibitor pretreatment is applied: the expression of proinflammatory mediators is silenced and AMPs are reactivated.

These observations prompted us to investigate the role of epigenetic factors (e.g. histone acetylation), miRNAs and negative regulatory molecules in the regulation of innate

immunity by using our *in vitro* model. In order to identify mechanisms underlying non-tolerizable/tolerizable phenotype, first we examined the global level of histone acetylation, a modification associated with transcriptional activation. We found that PGN stimulation/restimulation differentially influences the acetylation levels of H3 and H4 histone proteins. These global epigenetic changes - which can specifically modify the gene expression are, partly, responsible for the tolerization of innate immune system.

By investigating the posttranscriptional effect of PGN stimulation/restimulation in keratinocytes we found that miR-146a and miR-155 miRNAs show non-tolerizable phenotype; in contrast, the keratinocyte-specific miR-203 is tolerizable. Furthermore, the expression profiles of miR-203 and its target proteins p63 and SOCS3) change in opposite directions, showing the immediate role of miR-203 in the posttranscriptional regulation of its targets.

By studying the expression profile of the TAM receptor family members, which is a negative feedback regulators of innate immune processes, we found that the expression of these receptors is altered in several cell types (e.g. dendritic cells, monocytes, vaginal epithelial cells, keratinocytes) as a response to treatment with different microbial agents. Our data are showing that the expression pattern of TAMs upon challenge with different microbial agents negatively correlates with thatof pro- inflammatory mediators: in parallel with the downregulation of TAMs the expression of proinflammatory mediators (e.g. TNF-α, IL-6) is upregulated.

Finally, in order to test our observations made in the *in vitro* experimental model, we studied the expression profile of TAM receptor family *in vivo* in samples from psoriatic patients. We found that the expression of TAM receptors remarkably decreases in the epidermis of diseased patients. Importantly, this down-regulated gene expression is present both in lesional and non-lesional epidermis of psoriatic patients. Furthermore, down-regulated expression of TAMs and their ligand (GAS6) is associated with significant up-regulation of proinflammatory mediators (e.g. TNF-α, CXCL8), pointing toward a possible regulatory role of TAM receptors in skin immune processes.

Summarizing the results we can conclude, that our *in vitro* experimental model is suitable to study different gene-specific regulatory mechanisms responsible for controlling acute and persistent infection.

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