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Genome-wide transcriptome analysis of dendritic cells in a model of acute and persistent infection

[Summary of PhD Thesis]

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Introduction

Immune response: the forfeit for surviving

Plants and animals are constitutively exposed to various microbes present in the environment, including commensal and pathogenic microorganisms. Despite their perpetual presence in the environment, infections still develop infrequently, because all multicellular organisms have evolved defense mechanisms to combat against harmful microbes.

The highly organized mammalian immune response can be divided into innate and adaptive (acquired) immunity. The innate immune system denotes an evolutionary more conserved, however less specialized defense mechanism. The non-specific effector cells (i.e. epithelial cells, such as keratinocytes) can eliminate invading pathogens and attract specific effector cells (i.e. macrophages, dendritic cells (DCs)) to the site of the infection by the secretion of pro-inflammatory mediators, such as cytokines and chemokines. The cellular elements of the innate immunity recognize and respond to pathogens in a generic way although it does not confer long-lasting protective immunity to the host. On the other hand, the adaptive immune response exhibits a more sophisticated and complex mechanism than the innate immunity with the ability to recognize and remember specific pathogens/antigens which finally results in a more pronounced immune response after the re-infection with the respective pathogen/antigen. Hence, for the development of immunological memory, antigen presentation is essential. For this professional antigen presenting cells (APCs, such as dendritic cells) take up invading pathogens at the periphery and after migrating to lymphoid organs they present antigens primarily to T lymphocytes. Antigen presentation to naïve T lymphocytes is called as lymphocyte activation. This activation process leads to the clonal expansion to the effector T lymphocytes which makes cells capable for fighting against pathogenic microbes in a microbe-specific way. Taken together, the successful activation of both the innate and the adaptive immune system finally results in the elimination of invading pathogens which is essential for the host's survival.

Beside cytokine and chemokine secretion, the epithelial cells form tight junctions which serve effective mechanical barrier between the host and the environment. This is particularly important, as the members of the commensal microflora are present on epithelial surfaces, such as gut, or skin. On epithelial surfaces, commensal microflora occupies niches which are suitable for bacterial grow. Epithelial cells are also capable for producing cationic antimicrobial peptides which all have direct antimicrobial activity thereby controlling commensal microflora.

The control of *Staphylococcus aureus* (*S. aureus*) infection

In order to induce the appropriate immune response the host must detect invading pathogens through their pattern recognition receptors (PRRs). The best characterized PRRs are the Toll-like receptors (TLRs) which recognize a wide variety of pathogen-associated molecular patterns (PAMPs, such as peptidoglycan; PGN), expressed by microorganisms including viruses, bacteria and protozoa. In addition to PAMPs, endogenous host-derived danger signals (danger-associated molecular patterns, DAMPs), like heat-shock proteins, and degradation products from necrotic tissues can also activate the TLRs.

Although *Staphylococcus aureus* (*S. aureus*) is the member of the normal microflora of the skin and the nasal passage, and more than 20% of the human population is long-term carriers of the bacteria, *S. aureus* is responsible for the majority of skin infections.

As the *Staphylococcal* cell wall is mostly composed by PGN, PGN is the most potent PAMP of *S. aureus*, inducing the host's immune response by cross-linking the TLR2. In brief, the activation of the TLR2 with PGN induces a signaling cascade which finally results in the

up regulation of pro-inflammatory effector molecules and up regulates the phenotypic maturation of dendritic cells.

Tolerance to LPS is essential for the control of inflammation

As inflammation causes dramatic changes in tissue physiology, inflammatory responses must be strictly regulated because uncontrolled inflammation could lead to serious pathologic conditions. On the other hand, there are a wide variety of regulatory mechanisms built in the TLR4 –mediated signaling pathway, such as the production of anti-inflammatory cytokines, and the induction of the negative regulators of the TLRs, like SOCS1, preventing the host from the uncontrolled side effects of the extended inflammation.

These molecules have pivotal role in the long-lasting hyporesponsiveness of the cells and organisms after prolonged/repeated LPS stimulation, which phenomenon is called as LPS tolerance. For a long time, LPS tolerance was thought to be a consequence of receptor desensitization, however recent experiments demonstrated that the transient silencing of pro-inflammatory genes at chromatin level also have essential role in the maintenance of LPS tolerance. In that experiments, primary mouse macrophages were treated once or repeatedly with LPS and the expression level of pro-inflammatory effector molecules were examined. Briefly, the relative gene expression of pro-inflammatory cytokines were robustly up regulated after the first LPS challenge, however they were not re-induced or induced in a much lesser degree after the second LPS treatment (defined as tolerizeable). In contrary to cytokines, antimicrobial peptides seemed to be re-inducible after the second LPS treatment (defined as non-tolerizeable) in order to protect the host from impending pathogens and the tissue damage caused by uncontrolled inflammation. The more accurate analysis demonstrated that covalent histone modifications have crucial role in the maintenance of LPS tolerance.

Dendritic cells: gatekeepers between innate and adaptive immune response

As it was mentioned earlier, professional APCs have central role in the induction of the adaptive immune response. In the periphery, DCs function as immune sentinels, because they are able to sense pathogens by their PRRs, which followed by antigen uptake. After taking up antigens, DCs migrate to lymphoid organs where they complete their maturation process and attract T lymphocytes by enhanced cytokine and chemokine secretion. Mature dendritic cells (mDCs) are able to present foreign antigens to T lymphocytes which finally lead to the induction of the adaptive immune response.

The majority of DCs function as immune sentinels at the periphery, i.e. in the skin, where they are called as Langerhans cells. Because DCs only constitute the minority of the peripherial blood where their separation is almost impossible, the investigation of human DCs are based on DC differentiation strategies from DC precursors (especially from $CD14^+$ monocytes) in the presence of appropriate cytokines. This differentiation procedure results in immature DCs (iDCs) with conserved phagocytic capacity and a moderate expression level of costimulatory molecules.

It is also possible to generate mDCs from iDCs by treating them with recombinant cytokines or challenging them with antimicrobial agents. mDCs are characterized with elevated pro-inflammatory cytokine production, increased antimicrobial response and enhanced antigen presenting capacity.

DCs in health and disease

In some pathologic conditions, the function of the DCs disturbed and they induce immune response against self molecules. This process is called autoimmunity, which often results in serious, multi-organ autoimmune diseases such as psoriasis.

Psoriasis is one of the most common multifactorial autoimmune-like disease worldwide. The disease mainly affects the skin, however severe joint destructions (psoriatic arthritis) and nail alterations could be detected beside skin discrepancies.

As it is a multifactorial disease, environmental factors initiate psoriasis development in genetically susceptible individuals. Environmental stress leads to the release of self RNA and DNA from stressed keratinocytes which form complexes with antimicrobial peptides. These complexes than activates DCs through their TLRs and leads to the release of pro-inflammatory mediators such as IFN-α. The enhanced local level of IFN-α together with GM-CSF stimulates monocytes to differentiate to DCs. IFN-primed DCs than transform T limphocytes into Th1 and Th17 cells which migrate to the skin where they have central role in the initiation of psoriatic plaque formation, partly by elevated pro-inflammatory cytokine secretion.

The malfunction of the skin's immune system in psoriasis induces massive mononuclear leukocyte infiltration (T lymphocytes, plasmacytoid DCs and $CD11c^+$ DCs) compared with healthy skin. $CD11c^+$ DCs from psoriatic lesions express high level of TNF-α and inducible nitric oxide synthase (iNOS), both playing central role in pathogen elimination. In addition to TNF-α production, $CD11c^+$ DCs secrete T cell and keratinocyte activating cytokines, such as IL-23 and IL-20, respectively.

Psoriasis treatment strategies

Investigations of psoriasis at a molecular level demonstrated the up regulation of a wide variety of pro-inflammatory mediators. Thus, both conventional and biologic therapeutics approaches target the immune system. Conventionally, immunosuppressive therapies, including classical systemic or topical treatment and/or phototherapy are applied. As these conventional treatment strategies are based on the global suppression of the immune system often serious, sometimes life-threatening side effects are detected.

On the other hand, the inhibition of the respective pro-inflammatory mediators with antibodies or soluble receptors became the mainstream of the biologic therapy. As TNF-α is the main cytokine involved in the pathogenesis of psoriasis, most part of the biologic therapies target this cytokine, although the prevalent usage of this cytokine can cause gastrointestinal symptoms or liver damage. Yet, TNF-α blockers became the world's top selling medicine in 2012.

Based on the fact that Th17 cells are also involved in the pathogenesis of psoriasis, IL-17 blocking antibodies are also under extensive study, and they are successfully used to treat moderate to severe plaque type psoriasis. Some other cytokines, such as IL-23 and IL-22 can also be hampered by blocking antibodies in order to repress the elevated cytokine production of Th17 cells.

Transcriptome analysis

In microarray experiments, cDNA is hybridized on an array of complementary oligonucleotide probes corresponding to the gene of interest, and the relative abundance of mRNA is estimated from the hybridization capacity to the relevant probe. However one drawback of this approach is the limited number of probe sets included on the chip.

Recently launched technology, called next generation sequencing (NGS) has rapidly substituted microarray due to, among others, it has improved accuracy and throughput and less cost-per-experiment ratio. Beside *de novo* sequencing of genomes, NGS technology is also very powerful when it comes to genome resequencing and single nucleotide polymorphism (SNP) identification. Furthermore, NGS offers and unprecedented opportunity to jointly analyze cellular trascripts without any information of the nature of the transcript.

In order to analyze alterations at mRNA level, serial analysis of gene expression followed by sequencing (SAGE-Seq, also called as digital gene expression tag profiling; DGE) can be used. This method is based on the capture of polyadenylated mRNAs by oligo(dT) containing magnetic beads and only the captures mRNAs are further analyzed.

Aims

The aims of our investigations were

- To get a global view on the relative gene expression changes of primary human immature dendritic cells (iDCs) upon challenging them once or repeatedly with *S. aureus* derived PGN, a widely used TLR2 ligand
- To carry out pathway analysis based on the results of the SAGE-Seq experiments in order to investigate the impact of PGN on gene stes and signaling pathways
- To compare the relative gene expression pattern of the selected genes between immature and mature dendritic cells (iDCs and mDCs, respectively)
- To examine the relative gene expression pattern of selected genes on psoriatic skin biopsy samples
- To investigate the impact of soluble TNF-α blocking antibody on the relative gene expression of selected genes

Materials and methods

In vitro generation of primary human dendrtic cells

Human peripherial blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy individuals by Ficoll Paque Plus density gradient centrifugation. In brief, PBMCs were allowed to adhere for 2 hours on each wells of a 6-well plate. Adherent monocytes were washed extensively with pre-warmed phosphate buffered saline (PBS) in order to remove non-adherent cells. iDCs were obtained from monocytes by cultivating them in RPMI-1640 medium which was supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1% penicillin/streptomycin solution in the presence of 1000 unit/ml recombinant human GM-CSF and 1000 unit/ml recombinant human interferon-α (IFN-α) for 5 days. mDCs were obtained from iDCs by 10ng/ml recombinant human TNF-α stimulation for 24 hours. Cells were maintained in 37^{o} C in an atmosphere of 5% (v/v) CO_2 in air.

Stimulation of DCs and sample collection

Both iDCs and mDCs were stimulated with *S. aureus* derived PGN according to the model described at Figure 1. In brief, iDCs and mDCs were left untreated (hereafter defined as naïve, N) or stimulated with PGN once (hereafter defined as N+PGN) or twice (hereafter defined as T+PGN), in order to mimic acute and persistent infection, respectively.

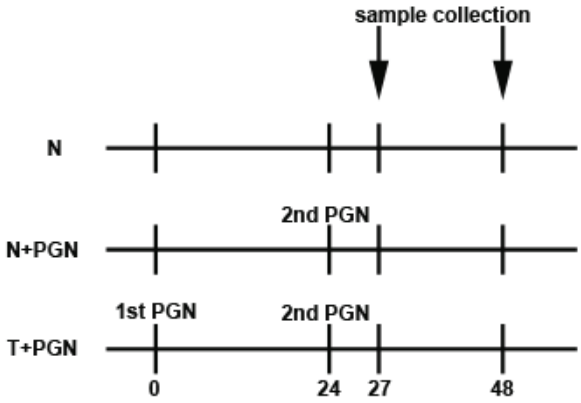

Figure 1. The model of acute (N+PGN) and persistent (T+PGN) *Staphylococcal* infection

In some experiments soluble chimeric monoclonal anti-TNF-α antibody (infliximab, Remicade) was added to primary monocytes in parallel with GM-CSF and IFN-α. Infiximab has a long half life of approximately 10 days, which ensured the neutralization of all secreted TNF-α during iDC generation. Moreover, freshly prepared infiximab was added to culture supernatants along with PGN stimulation to neutralize *de novo* secreted TNF-α.

Total RNA and cell culture supernatants were collected at 27 and 48 hours, that is 3 and 24 hours post-second PGN treatment.

Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR)

Total RNA was extracted from iDCs and mDCs using RNeasy Plus Mini Kits according to the manufacturer's instructions.

cDNA was synthesized from 100ng of total RNA. The relative abundance of selected mRNAs was determined by QRT-PCR by using commercially available TaqMan probes or SybrGreen based QRT-PCR assays. The ratio of each mRNA is relative to the 18S rRNA calculated by using the $\Delta\Delta C_T$ method.

RNA extraction from skin biopsy samples

Skin biopsy samples from psoriatic lesional and non-lesional areas and from healthy controls were collected. After the removal of the subcutaneous tissues, skin biopsies were incubated overnight at 4^o C in Dispase solution. On the following day, the epidermis was separated from the dermis and samples were mounted in 1ml TRIzol Reagent to which 400µl chloroform was added and vortexed vigorously. Samples were centrifuged at 13000rpm for 10 minutes and the upper phase was loaded onto a gDNA eliminator spin columns of the RNeasy Plus Mini Kits. Subsequent RNA extraction as well as cDNA synthesis and QRT-PCR were performed as described above.

All tissue samples were taken with the patient's informed consent and the approval of the local ethics committee. The study was conducted according to the declaration of the Helsinki Priciples.

SAGE-Seq

SAGE-Seq was performed using SOLiD SAGE Kit according to the manufacturer's instructions. Briefly, purified total RNA was bind to Dynabeads Oligo (dT) EcoP15 magnetic beads in order to capture polyadenylated RNAs. cDNA was synthesized on the magnetic beads by using SuperScript III Reverse Transcriptase and *E. coli* DNA polymerase. Next, NlaIII restriction was performed in order to generate GTAC containing sticky end at the complementer DNA strand. Barcode adaptor A (P2 Seq-IA-EcoP15I) was ligated which

contains an EcoP15 restriction enzyme recognition site and truncated internal adaptor sequence. An EcoP15I digestion was carried out in order to cleave the construct off from the magnetic beads. EcoP15I restriction also resulted in a 2 base-pair long overhanging tag. Adaptor B was ligated to 5' end of each tag which contains the P1 primer binding site. Each library was amplified in an emulsion PCR, sequencing beads deposited onto sequencing slide and sequenced on SOLiD V4 System.

Measurement of secreted cytokine levels

Harvested cell culture supernatants were centrifuged and the concentrations of secreted TNF-α and CCL1 were measured by Quantikine Human Immunoassay Kits following the manufacturer's instructions. Serial dilutions of the respective recombinant human proteins were used to generate standard curves. The optical density of each wells were determined by using microplate reader set to 450nm with a wavelength correction set to 540nm.

Data representation and statistical analysis

Data show average ± standard error of the mean. The significance of differences between sets of data was determined by Student's paired t-test or one-way ANOVA following Neuman-Keuls post-hoc test using Graph Pad Prism for Windows. A probability value (p) of less than 0,05 was considered significant.

Results

SAGE-Seq studies demonstrate that PGN stimulation and re-stimulation has appreciable impact on the gene expression profile of primary human DCs

In order to get a global view on the gene expression profile of primary human iDCs after single or prolonged PGN stimulation SAGE-Seq experiments were performed. Bioinformatics analysis of SAGE-Seq data evidenced that single or prolonged PGN stimulation resulted in significant alterations of the relative gene expression of altogether 5411 genes ($p<0,05$). In this current study we mainly focused on those 566 genes which were uniquely changed between N+PGN and T+PGN samples because they represent the differences between acute and persistent *Staphylococca*l infections at a relative gene expression level.

To characterize the potential relevance of the alterations caused by single or prolonged PGN stimulation pathway analysis was performed by BioProc and Kegg pathway analysis softwares. We used both pathway analysis tools to reduce the possibility of false results. After single or prolonged PGN stimulation compared with naïve cells both BioProc and Kegg analysis resulted in massive enrichment of pathways from the function group "Immune response" "Apoptosis" and "Motion". Importantly, comparison between N+PGN and T+PGN samples resulted in more down regulated pathways than up regulated from these function groups, which strongly suggest the induction of tolerogenic mechanisms after prolonged PGN stimulation. In contrary with "Immune response" "Apoptosis" and "Motion", the pathway analysis of the function group "Cell cycle" and "Energy production" demonstrates that single or prolonged PGN stimulation significantly down regulates the expression of the members of these function groups. It is important to note here that BioProc pathway analysis also demonstrated the induction of several pathways from "Cell cycle". Single or prolonged PGN stimulation also results in the induction of a number of other pathways compared with naïve cells, for example "Systemic lupus erythematosus" or "Autoimmune thyreoid diseases".

Based on the results of the pathway analysis and considering that NlaIII has recognition site of approximately every 200-300 nucleotides in the human genome we investigated the expression patterns of individual tags (tag represents the short sub-sequence of a gene which is used to identify gene transcripts in SAGE-Seq experiments; a gene may contain more than

one tag). We found that 132 tags out of 427 tested were re-inducible, as in N+PGN samples significant gene expression down regulation was detected compared with naïve cells, in contrast, we detected gene expression up regulation in T+PGN samples compared with N+PGN. The comparison of these results with the pathway analysis demonstrated that "Antigen presentation" and pathways from autoimmune diseases such as "Systemic lupus erythematosus" and "Autoimmune thyreoid disease" fell into this category. Based on the results of Foster et. al we were mostly interested in tolerizeable tags. Tolerizeability means that single PGN stimulation results in a robust induction in the relative gene expression compared with naïve cells however in T+PGN samples significant relative gene expression down regulation was detected compared with N+PGN samples. The importance of the decreased relative gene expression level after prolonged PGN stimulation plays role in is the prevention of the host against the development of serious side effects resulting from uncontrolled inflammation. In this study 181 tags out of 427 fell into this pattern category; most of them from the "Toll-like receptor signaling pathway". In contrast, the expression of 40 tags out of 427 monitored were down regulated in N+PGN samples compared with naïve cells and a more robust down regulation was detected after prolonged PGN stimulation: the members of "Cell cycle" and "Apoptosis" function group fell into this category. Finally, 74 tags out of 427 tested were robustly up regulated in N+PGN samples as compared to naïve cells and a more significant relative gene expression up regulation was detected after prolonged PGN stimulation. Comparing these results with the pathway analysis the members of the "Cytokine and chemokine signaling pathway" (the member of the function group "Immune response") fell into this category.

Taken these findings together, our data strongly suggest that our experimental model together with SAGE-Seq is a valuable approach to monitor the impact of acute and persistent *Staphylococcal* infection. Nevertheless, we also aimed to validate the SAGE-Seq results on a set of genes. Based on the results of the pathway analysis and enrichment studies we have chose members of the "Immune response", "Apoptosis" and "Cell cycle" function group which were more accurately analyzed at relative gene expression and secreted protein level by QRT-PCR and ELISA. The validation procedure was carried out on mature dendritic cells (mDCs) as well, because the presence of foreign antigens and the inflammatory milieu induces DC maturation process. During maturation, the antigen processing capacity of DCs decreases while their antigen presentation and expression of co-stimulatory molecules increase. We hypothesized that these features may result in significant difference in their relative gene expression pattern as compared to iDCs after single or prolonged PGN stimulation according to the model described at Figure 1.

PGN stimulation has significant impact on the relative gene expression of inflammation-related effector molecules and their respective receptors

In iDCs single PGN stimulation resulted in significant relative gene expression up regulation in case of CXCL8, IL-6, CCL1 and IL-17A as compared with naïve cells. Furthermore, a more robust relative gene expression up regulation was detected in T+PGN samples compared with N+PGN samples. Surprisingly, although IL-10 has anti-inflammatory role, single or prolonged PGN stimulation caused identical alterations in the relative gene expression pattern with the previously mentioned effector molecules. In mDCs we detected the same relative gene expression patterns as for iDCs, however the values of the relative gene expression were less prominent than iDCs.

Interestingly, the relative gene expression of the neutrophil-attractant chemokine, CXCL7 was significantly down regulated in N+PGN samples in both iDCs and mDCs compared with naïve cells. Uniquely from the function group "Cytokines and cytokine receptors" prolonged

PGN treatment had no effect on the relative gene expression of CXCL7 in neither iDCs, nor mDCs compared with naïve cells.

In contrary to the previously mentioned effector molecules, the IFN-γ inducible CXCL9 and CXCL10 genes showed different expression pattern after single or prolonged PGN stimulation. We demonstrated that CXCL9 in mDCs and CXCL10 in both iDCs and mDCs are tolerizeable, as single PGN stimulation resulted in gene expression up regulation, however prolonged PGN stimulation caused robust gene expression down regulation compared with both naïve cells and N+PGN samples. Furthermore, in iDCs the relative gene expression of CXCL9 was significantly down regulated after both single and prolonged PGN stimulation compared with naïve cells.

Because the cytokines show altered gene expression, we aimed to determine the impact of PGN stimulation on the relative gene expression pattern of their respective receptors. Our data demonstrate that single or prolonged PGN stimulation significantly down regulated the relative gene expression of IL-10RA (the receptor of IL-10) and IL-17RA (the receptor of IL-17A) in both iDCs and mDCs. In addition, the same gene expression alterations were detected in the case of gp130 (IL-6 signal transducer, the receptor of IL-6) in iDCs, although in mDCs we demonstrated the tolerizeability of gp130. Moreover, we detected the tolerizeable capacity of CCR8 (the receptor of CCL1) in both iDCs and mDCs.

PGN stimulation has distinct effect on the relative gene expression of TNF-α and the members of the TNF-α family

Although TNF-α is an acute-phase protein which has pivotal role in the early immune response against invading pathogens, QRT-PCR data demonstrated that in both iDCs and mDCs prolonged PGN stimulation resulted in significant relative gene expression down regulation of TNF-α as compared with N+PGN samples. In order to validate this result at a translational level, secreted TNF-α measurement was carried out from cell culture supernatants by ELISA 24 hours post-second PGN treatment. ELISA measurements supported the QRT-PCR data: in iDCs PGN stimulation resulted in significantly elevated secreted TNF-α level as compared with naïve cells. Because recombinant TNF-α was used to generate mDCs, it is not surprising that in mDCs enhanced secreted TNF-α level was measured in cell culture supernatants from naïve cells. It is important to note here that neither single nor prolonged PGN stimulation had impact on secreted TNF-α level in mDCs.

The relative gene expression of TNFSF15 (also known as TL1A) was significantly induced after PGN stimulation in iDCs. Although it is not fully understood to which pathogens TNFSF15 have evolved to, it is well known that in psoriatic skin samples the relative gene expression of TNFSF15 was significantly up regulated than healthy controls.

In case of TNFAIP6 the identical relative gene expression pattern was detected as compared with TNFSF15; PGN stimulation resulted in significantly up regulated relative gene expression. In mDCs we also demonstrated PGN derived relative gene expression up regulation, however the degree of the up regulation was less prominent than in iDCs. It is important to note that TNFAIP6 is a multifactorial cytokine which has pivotal role in extracellular matrix remodeling thus it might be involved in the pathogenesis of autoimmune diseases.

During the elimination of invading pathogens/antigens immune pathogenesis is a common side effect of extended inflammation. To maintain the transient nature, immune response must be strictly regulated. Recent studies demonstrated that there are two classes of molecules which are involved in this process. The first class of these molecules limits the strength of the immune activation, these include anti-inflammatory cytokines, negative transcription factors and the negative regulators of the TLR signaling. The second class of the regulatory

molecules is involved in programmed cell death. In order to gain inside the negative regulators we examined the expression pattern of TNFAIP3 and TNFAIP8, respectively.

The relative gene expression of TNFAIP3 (also known as A20) was similar to TNF-α: as prolonged PGN stimulation resulted in significant relative gene expression down regulation in both iDCs and mDCs. Beside their role in the down regulation of the immune response, gene targeting experiments demonstrated that the absence of TNFAIP3 is associated with chronic inflammation and autoimmunity.

The relative gene expression of the negative regulator, TNFAIP8 (also known as TIPE2) was significantly down regulated in iDCs and mDCs after PGN stimulation. Moreover, in T+PGN samples more robust relative gene expression decline was detected that N+PGN samples. The absence of TNFAIP8 expression results in hyper-responsiveness of TCR and TLR activation and enhanced pro-inflammatory cytokine production. This is in line with our findings that prolonged PGN stimulation resulted in significant relative gene expression up regulation in case of CXCL8, CCL1, IL-6 and IL-17A as compared with naïve cells.

SOCS proteins have function in the regulation of PGN induced inflammation

Beside the previously mentioned negative regulators of the innate immunity from the TNF-α super family, suppressor of cytokine signaling (SOCS) proteins have been identified as an inducible inhibitors of cytokine signaling and these molecules have pivotal role in the limitation of inflammatory responses. Beside the down regulation of the immune response SOCS proteins have also been shown to interfere with DC physiology; the absence of SOCS1 in DCs showed aberrant activation of the adaptive immune system which finally resulted in the induction of autoimmunity.

In accordance with SAGE-Seq, we demonstrated the tolerizeability of SOCS1 in iDCs. Our findings strongly suggest that the down regulation of SOCS1 in iDCs after prolonged PGN stimulation results in the massive induction of cytokine production which may lead to the development of autoimmune diseases.

Similar with SOCS1 the tolerizeable nature of SOCS2 was demonstrated in iDCs after prolonged PGN stimulation. Uniquely from SOCS proteins the relative gene expression of SOCS2 was down regulated in mDCs after single PGN treatment compared with naïve cells, however in T+PGN samples more pronounced relative gene expression down regulation was detected that N+PGN samples. A previous report demonstrated that the decreased level of SOCS2 results in enhanced cytokine production, especially in case of IL-10 and IL-1β.

We also examined the impact of single or prolonged PGN stimulation on the relative gene expression pattern of SOCS3 which is one of the most potent suppressor of cytokine signaling beside SOCS1. In both iDCs and mDCs PGN stimulation resulted in enhanced relative gene expression level of SOCS3 compared with naïve cells.

Tryptophan deprivation has possible role in the control for *Staphylococcal* infection

Beyond the elevated cytokine expression and the induction of the adaptive immune response, tryptophan deprivation is another possible mechanism to control **1)** pathogenic infections and **2)** the maintenance of self tolerance. For these, a potential mechanism is the over expression of the tryptophan catabolizing enzyme, indolamine 2,3-dioxigenase (IDO). The enhanced IDO activity suppresses effector T cell responses, promotes regulatory T cell differentiation and activation and inhibits IL-6 production by DCs.

The relative gene expression of IDO was significantly up regulated in both iDCs and mDCs in N+PGN samples, and a more pronounced gene expression up regulation was detected in both cell types after prolonged PGN stimulation.

Single PGN stimulation resulted in significant relative gene expression up regulation in iDCs and mDCs in case of kynureninase (Kynu) compared with naïve cells. After prolonged

PGN stimulation a more robust relative gene expression up regulation was detected as compared with naïve and N+PGN samples in iDCs. In contrary with iDCs the second PGN stimulation had no impact on the relative gene expression of Kynu in mDCs.

These data strongly suggest that the over expression of tryptophan catabolizing enzymes has role in the control for acute and more interestingly in persistent *Staphylococcal* infection in DCs.

PGN stimulation induces cell cycle arrest and resistance to apoptotic processes

The strict regulation of cell division cycle has pivotal role in the maintenance of tissue homeostasis and the protection against cancer. As cyclins and cyclin-dependent kinases have pivotal role in the transition of different phases of cell cycle we examined the impact of PGN treatment on the gene expression of cyclin B2 (CycB2) which is involved in G2-M transition and cyclin D1 (CycD1) which has central role in G1-S transition.

SAGE-Seq and QRT-PCR demonstrated that single or prolonged PGN stimulation resulted in relative gene expression down regulation in case of CycB2 in iDCs and in case of CycD1 in both iDCs and mDCs. In mDCs we demonstrated the tolerizeable capacity of CycB2.

As mentioned previously the induction of apoptotic processes has important role in the maintenance of the transient nature of the immune response. We examined the impact of PGN stimulation on the relative gene expression of two genes involved in the regulation of cell fate and apoptosis. Fas-associated protein with death domain (FADD) has essential role in the activation of the caspase cascade by cleaving pro-caspase 8 during extrinsic apoptotic pathway. In both iDCs and mDCs single and prolonged PGN stimulation resulted in significant gene expression down regulation compared with naïve cells although we did not find any further difference in the gene expression of FADD between N+PGN and T+PGN samples.

We also examined the impact of single and prolonged PGN stimulation on the relative gene expression of transforming growth factor β (TGF-β). In both iDCs and mDCs PGN stimulation resulted in significant relative gene expression down regulation as compared to naïve cells, although the relative gene expression of TGF-β was up regulated in T+PGN cells than N+PGN samples in iDCs.

The relative gene expression of the members of the TNF-α super family form psoriatic samples show strong correlations with our *in vitro* model

Pro and anti-inflammatory members of the TNF-α superfamily maintain critical balance during acute and persistent *Staphylococcal* infection to successfully eliminate invading pathogens and to prevent the host against the harmful side effects of uncontrolled inflammation. Although the pathogenesis of psoriasis has not been fully understood yet, the immunological basis of this disease has been demonstrated. Although it is well known that the enhanced level of TNF-α has pivotal role in the pathogenesis of psoriasis, little is known about the expression pattern of the other members of the TNF-α super family in this autoimmune disease. In order to determine the relative gene expression pattern of TNFAIP3, TNFAIP6 and TNFAIP8 in plaque-type psoriasis we performed QRT-PCR experiments on psoriatic non-leisonal and lesional dermal and epidermal samples. As controls, healthy skin biopsy samples were used.

We found that the relative gene expression level of TNF-α was up regulated in psoriatic non-leisonal epidermal and dermal samples compared with healthy biopsies. Furthermore, in lesional samples a more robust up regulation was detected than in non-lesional samples. As these data corroborate earlier findings we used these samples for further experiments.

In accordance with PGN stimulated DCs the relative gene expression of TNFAIP6 was significantly up regulated in psoriatic non-lesional and lesional epidermal samples compared with healthy skin samples. In non-leisonal dermal samples significant gene expression down regulation was detected as compared to healthy skin samples, however in lesional samples robust gene expression up regulation was detected.

In contrary with the pro-inflammatory mediators the anti-inflammatory effector molecules, TNFAIP3 and TNFAIP8 were both down regulated in psoriatic non-lesional and lesional epidermal and dermal samples as compared to healthy controls. It is important to note that these results show strong correlations with the results from PGN stimulated DCs.

TNF-α blockade down regulates pro-inflammatory cytokines at relative gene expression and secreted protein level in T+PGN samples

Beside conventional treatment strategies, TNF-α blocking antibodies became the most common therapeutic approach to cure psoriasis. Thus, and based on the results which demonstrate that iDCs after prolonged PGN stimulation show strong correlations with psoriatic DCS we aimed to test the impact of TNF-α deprivation at cellular level. For this we added soluble chimeric monoclonal anti-TNF-α antibody (Remicade, infliximab) in parallel with the differentiation procedure as it was described earlier.

First we monitored the impact of Remicade treatment on the relative gene expression pattern of TNF-α. Single PGN stimulation in combination with Remicade treatment resulted in significant relative gene expression up regulation compared with naïve cells. Similar with only PGN stimulated iDCs, prolonged PGN stimulation with Remicade treatment resulted in significant relative gene expression down regulation as compared with N+PGN samples. In N+PGN samples Remicade treatment resulted in more robust relative gene expression up regulation than only single PGN stimulated cells. In T+PGN samples TNF-α blockade had no impact on the average fold change as compared with only PGN stimulated samples.

Single or prolonged PGN stimulation resulted in significant relative gene expression up regulation in case of IL-6 compared with naïve cells. Interestingly, in T+PGN samples Remicade treatment resulted in robust relative gene expression down regulation of IL-6 compared with prolonged PGN stimulated iDCs.

As mentioned earlier, PGN stimulation results in the up regulation of the relative gene expression of CCL1 and a more robust relative gene expression up regulation was detected after prolonged PGN stimulation. Importantly, Remicade treatment resulted in relative gene expression down regulation in N+PGN samples. Although Remicade treatment in T+PGN samples moderately up regulated the relative gene expression of CCL1, the average fold change was significantly less than only PGN treated iDCs. To analyze this result at secreted protein level, ELISA measurement from cell culture supernatants was performed. ELISA measurements strongly supported our QRT-PCR results: the blockade of secreted TNF-α significantly down regulated secreted CCL1 level in T+PGN samples compared with iDCs after prolonged PGN stimulation.

Discussion

In mammals DCs have pivotal role in orchestrating innate and adaptive immune response against impending pathogens. For this, they have sentinel function at the periphery by sensing and taking up pathogens/antigens. Pathogen/antigen uptake initiates maturation processes which transform immature DCs into mDCs that are professional antigen presenting cells (APCs). Professional APCs present pathogen-derived antigens through their MHC II molecules to naïve T lymphocytes at the proximate lymph nodes and initiate adaptive immune response.

PGN stimulation and re-stimulation has appreciable impact on the gene expression profile of primary human iDCs

Pathway analysis strongly suggest that iDCs maintain critical balance between inflammation and tolerance in order to maintain the transient nature of the immune response and to protect the host from immune pathologic side effects caused by extended inflammation. In contrary, PGN stimulation resulted in the down regulation of numerous members of the cell cycle and energy production pathways, which supports the fact that antigen challenge resulted in DC maturation characterized by functional changes rather than the numeral expansion of iDCs. It is also important to note that PGN stimulation results in the induction of autoimmune inflammatory disease pathways, such as systemic lupus erythematosus or autoimmune thyreoid disease.

The relative gene expression profile of pro-inflammatory mediators after single or prolonged PGN stimulation show strong correlations with autoimmune diseases

Chemokines are small secreted proteins which have pivotal role in the migration and the organ-specific homing of distinct leukocyte subsets *in vivo*. In accordance with SAGE-Seq, QRT-PCR validation demonstrated that single or prolonged PGN stimulation significantly up regulates the relative gene expression level of CXCL8, CCL1, IL-6 and IL-17A. Beside their pivotal role in pathogen clearance, the robust induction of these cytokines is also characteristic for autoimmune diseases. As IL-17A is involved in the defense against extracellular pathogens by enhancing T cell dependent immune response, it is not surprising that enhanced IL-17A level is characteristic for subsequent T cell dependent inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, etc. As this cytokine has crucial role in the pathogenesis of psoriasis, it is not surprising that ixekizumab (humanized IgG4 monoclonal antibody against IL-17A) is successfully used to treat moderate to severe plaque type psoriasis.

In contrary with the previously mentioned pro-inflammatory cytokines, in some cases, such as CCL18 or TNF-α prolonged PGN stimulation resulted in dramatic relative gene expression down regulation than N+PGN samples. As TNF-α is essential in the early defense against invading pathogens its rapid up regulation is not surprising, although it has essential role in the induction of apoptosis, and enhanced TNF-α level is characteristic for inflammation-related diseases, such as psoriasis.

Because the diverse functional role of TNF-α and its tolerizeable relative gene expression pattern after prolonged PGN stimulation we determined the relative gene expression pattern of the other members of the TNF-α super family. Single or prolonged PGN stimulation resulted in significant relative gene expression up regulation in case of TNFSF15. This cytokine shows the most conserved architecture compared to TNF-α based on the presence of conserved subdomains. As TNFSF15 is a potent inducer of the effector mechanisms of T lymphocytes it is not surprising that it has pivotal role in the pathogenesis of psoriasis.

The tolerizeability of CCL18 together with CXCL9, CXCL10 and TNF-α strongly suggest the induction of tolerogenic mechanisms rather than inflammation in order to prevent the host against the side effects of extended inflammation. Furthermore, the enhanced expression levels of those genes which are involved in the pathogenesis of psoriasis strongly support our hypothesis that prolonged PGN stimulation not only models persistent infection but it is a useful model to investigate psoriatic dendritic cells. This is particularly important since it is almost impossible to separate Langerhans cells from psoriatic skin samples, thus the characterization of the gene expression pattern of these cells was only possible using *ex vivo in situ* hybridization.

Tryptophan deprivation has central role in the control of *Staphylococcal* infection and the induction of autoimmune inflammatory diseases

In this study we demonstrated that single or prolonged PGN stimulation resulted in significant relative gene expression up regulation in case of IDO and Kynu which are involved in tryptophan catabolism. More than ten years ago it was demonstrated that the up regulation of tryptophan breakdown confers antiparasitic and antimicrobial effector functions in semi-professional and professional immune cells.

The induction of tryptophan catabolism is not limited to the elimination of invading pathogens, it also has central role in the inhibition of T cell proliferation. Upon stimulation with Th1 cytokines such as IFN-γ and TNF-α, the expression of IDO can be induced in PBMCs. Accordingly, it is not surprising that enhanced IDO activity can be detected in a wide variety of Th1 mediated inflammatory diseases such as inflammatory bowel disease or psoriasis.

Based on these facts, the significant up regulation of the tryptophan catabolic pathway after single and prolonged PGN stimulation not only suggests the induction of the adequate immune response against invading pathogens, but it strongly supports our hypothesis that prolonged PGN stimulation models inflammatory diseases such as psoriasis.

PGN stimulation has distinct effect on the relative gene expression of the negative regulators of inflammatory processes

As mentioned earlier, immune response must be strictly regulated in order to protect the host against the serious side effects of extended inflammation. The mediators which are involved in this phenomenon can be divided into two groups based on their regulating capacity.

The first class of these regulatory molecules includes inhibitory cytokines and the negative regulators of the TLR signaling pathway, which limits the strength of immune activation. In our experimental model PGN stimulation resulted in robust relative gene expression up regulation in case of IL-10. This cytokine has pleiotropic effects in the regulation of the immune response and inflammatory processes, moreover it has been associated with subsequent inflammation-related diseases. Moreover, enhanced IL-10 expression was detected in metastatic melanoma samples where it has role in the down regulation of the immune response against tumor cells.

Identically with the inflammation-related TNFSF15, enhanced TNFAIP6 relative gene expression level was demonstrated after single or prolonged PGN stimulation. Our data show strong correlations with those previous reports where LPS stimulation resulted in a dose-dependent up regulation of TNFAIP6 in primary macrophages. Although enhanced TNFAIP6 level was demonstrated in numerous inflammation-related diseases, no correlations between the relative gene expression level of TNFAIP6 and psoriasis has been reported so far.

In case of TNFAIP3 we demonstrated tolerizeability after prolonged PGN stimulation. Our findings show strong correlations with a previous report where flagellin-mediated TLR5 induction resulted in abundant TNFAIP3 expression in intestinal epithelial cells which was also demonstrated in an *in vivo* acute colitis model in mice. This report also showed that the enhanced TNFAIP3 expression does not influence tolerance induced with flagellin restimulation.

In case of TNFAIP8 PGN stimulation resulted in relative gene expression down regulation in both iDCs and mDCs. In thymocytes, the down regulation of TNFAIP8 effectively protected the cells against glucocorticoid induced apoptosis. Furthermore, the decreased expression level of TNFAIP8 establishes the significantly enhanced level of pro-inflammatory effector molecules such as CXCL8, IL-6, CCL1 and IL-17A, which is in good

agreement with our observations, showing a reversible expression of TNFAIP8 with the previously mentioned pro-inflammatory molecules.

Beside the previously mentioned negative regulators, SOCS proteins also have essential role in the inhibition of extended inflammation. Moreover, the absence of SOCS proteins in DCs results in the aberrant activation of the adaptive immune response which finally leads to the development of autoimmunity. The tolerizeability of SOCS1 and SOCS2 strongly support their central role in the up regulation of pro-inflammatory cytokines after single or prolonged PGN stimulation. In case of SOSC3 enhanced relative gene expression was detected after single or prolonged PGN stimulation. A previous report demonstrated that enhanced expression level of SOCS3 form psoriatic keratinocytes leads to the resistance to apoptosis.

The second class of the regulatory molecules is involved in the induction of programmed cell death [60]. Apoptosis is essential in the maintenance of the transient nature of the immune response and to prevent the host against cancer which can be induced by environmental triggering factors. Our data demonstrated significant relative gene expression down regulation after PGN stimulation in case of Cyclin B2 and Cyclin D1. These findings strongly supported by the fact that cross-linking of TLR with its respective ligand induces maturation process which is characterized by functional differentiation of the antigen presenting cells without numeral expansion. PGN stimulation significantly reduced the relative gene expression of FADD and TGF-β in both iDCs and mDCs. Importantly, the role of programmed cell death is not limited to the maintenance of tissue homeostasis, it has pivotal role in preventing the organism against the development of autoimmune diseases. However, the role of FADD is not limited to the initiation of TNF-dependent apoptosis it is a key factor in cell proliferation, differentiation, autophagy, genome surveillance and innate immunity. Our data show strong correlations with earlier findings showing that the down regulation of FADD enhances the relative gene expression of IL-1β, IL-6, IL-10 and TNF-α in mouse epidermis.

The down regulation of the anti-inflammatory members of the TNF-α super family has essential role in the pathogenesis of psoriasis

Beside keratinocyte hyperproliferation, extended inflammation and immune cell infiltration are also hallmarks of psoriasis. The abnormalities leading to psoriasis development remain controversial; however the cross-talk between keratinocytes and T cells represents a necessary step during the course of the disease. According to the brand new model of the pathogenesis of psoriasis, dermal DCs from psoriatic lesions drive the cytokine production towards Th1 and Th17 cytokines. In accordance with the literature, significantly up regulated TNF-α relative gene expression level was detected in psoriatic non-lesional and lesional dermal and epidermal samples.

In psoriatic skin samples slightly up regulated TNFAIP6 relative gene expression level is characteristic. TNFAIP6 was first detected in fibroblasts under the stimulation of TNF-α. This glycosaminoglycan-binding protein has role in experimental model of arthritis, acute myocardial infarction and chemically burned rat cornea. These facts together with our results strongly suggest that first, TNFAIP6 is one of the key molecules in the pathogenesis of psoriasis, and, second, our model is a useful tool in mimicking the expression pattern of psoriatic DCs.

In psoriatic skin samples, the relative gene expression of TNFAIP3 was significantly down regulated as compared to healthy skin. Originally, TNFAIP3 was identified as a TNF-α inducible gene which functions as negative feedback inhibitor of TNF-α signaling, by inhibiting NF-κB mediated immune response. Furthermore, genome-wide association studies have revealed associations between TNFAIP3 and psoriasis. Based on these facts our data strongly suggest that the significantly decreased level of TNFAIP3 has crucial role in the

maintenance of enhanced level of pro-inflammatory cytokines in psoriatic lesions which serve as a homing signal of infiltrating leukocytes.

TNFAIP8 is also an essential negative regulator of inflammation and immune homeostasis by down regulating the T cell receptor and TLR signaling and, as it was mentioned earlier, it is involved in the protection against apoptosis. To the best of our knowledge we were the first who demonstrated the significant down regulation of TNFAIP8 in psoriatic dermis and epidermis at a relative gene expression level which in accordance with the literature explains the enhanced cytokine production and the decreased sensitivity to pro-apoptotic processes which is characteristic for psoriasis.

The blockade of soluble TNF-α significantly reduces the relative gene expression and secreted protein levels of pro-inflammatory cytokines

Based on the more particular understanding of the pathogenesis of psoriasis at a molecular level, the inhibition of pro-inflammatory mediators with antibodies or soluble receptors became the mainstream therapy to treat psoriasis. As TNF-α plays central role in the pathogenesis of this disease, it represents an active target for biologic therapies.

We showed that Remicade treatment markedly reduced the relative gene expression of IL-6 after PGN stimulation. This result show strong correlations with those earlier findings where the blockade of TNF-α by Remicade significantly reduced the relative gene expression of acute-phase proteins such as IL-1β and IL-6. Soluble TNF-α blockade also significantly down regulated CCL1 at relative gene expression and secreted protein level after prolonged PGN stimulation. To the best of our knowledge, we were the first who demonstrated the negative impact of TNF-α blockade on the expression of CCL1.

Taken these findings together, our data strongly suggest that in inflammatory conditions, enhanced TNF-α level robustly up regulates other cytokines and chemokines which have pivotal role in the maintenance of chronic inflammation. When enhanced TNF-α level is blocked by Remicade treatment, the expression level of the other effector mechanisms are down regulated, that is why Remicade successfully used to treat a wide variety of autoimmune inflammatory diseases.

The most important highlight of our study

- Our experimental model together with SAGE-Seq is a valuable approach to monitor the impact of single or prolonged PGN stimulation on primary human iDCs
- Pathway analysis demonstrated that single or prolonged PGN stimulation causes alterations on different pathways; although these alterations were mostly characteristic for “Immune response’, “Cell cycle” and “Apoptosis”
- In accordance with SAGE-Seq experiments, QRT-PCR and ELISA-based validation demonstrated that:
    - after single or prolonged PGN stimulation the host cell maintain critical balance between inflammation and tolerance to eliminate impending pathogens and to protect the host against the harmful side effects resulting from uncontrolled inflammation
    - the decreased pro-inflammatory cytokine producing capacity of mDCs compared with iDCs
    - PGN stimulation results in the induction of the pro-inflammatory mediators from the TNF-α super family, yet we demonstrated the tolerizeable nature of TNF-α and TNFAIP3, however PGN treatment significantly down regulated the relative gene expression of TNFAIP8 (which has anti-inflammatory role) compared with naïve cells

- the over expression of the tryptophan catabolizing enzymes has role in the control for acute and more interestingly in persistent *Staphylococcal* infection
- the enhanced relative gene expression level of SOCS1 and SOCS3 may be responsible for the down regulation of pro-inflammatory effector molecules in mDCs
- the decreased level of SOCS1 and SOCS2 in iDCs after prolonged PGN stimulation strongly explains the enhanced production of inflammation-related effector molecules
- acute and persistent *Staphylococcal* infections decreases cell division cycle by down regulating the relative gene expression levels of cyclins
- the reduced relative gene expression level of pro-apoptotic genes suggest the induction of survival mechanisms which may lead to the elimination of invading pathogens

- TNFAIP6 has important role in the maintenance of chronic inflammation, which is characteristic for psoriasis
- The down regulation of TNFAIP3 and TNFAIP8 strongly suggest that braking mechanisms of the inflammation is disturbed, which is also characteristic for psoriasis
- It has not been published yet that Remicade treatment significantly decreased the relative gene expression and secreted protein level of CCL1 as compared with only PGN stimulated iDCs.
- *Ex vivo* PGN stimulated DCs are identical with psoriatic IFN-primed DCs

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