Summary

Monocytes and macrophages, as professional phagocytes, are important part of the innate immune system, and they are able to produce diverse cytokines, reactive oxygen and nitrogen species and participate in activation of the adaptive immune system. The aim of this study was to compare activation status of NF-κB system and to examine response to oxidative stress in human monocyte and macrophage models and to investigate how it is modulated during pathogen recognition.

Despite unquestionable advantage of *ex vivo* cell models and their natural origin, work with primary blood cells generates many problems including: high cost and labour-intensive procedure, low available cell number, contamination with other blood components, high variability from donor to donor. The use of a simpler model system, human cell lines originating from cancer cells, circumvents this problems and provides an important tool to study cellular functions, mechanisms and responses.

In this study, two types of model cell lines have been used: THP-1 and Mono Mac 6. They represent monocyte-like cells at the different stage of maturation. Both can be further differentiated into macrophage-like cell types with stimuli such as phorbol-12-myristate-13-acetate or vitamin D3. For THP-1 cells, treatment with PMA was shown to be most potent agent to induce macrophage-like phenotype in terms of induction of adherence, loss of proliferation, phagocytic activity, expression of markers for monocyte differentiation: CD14, CD11a, CD11b, at the mRNA and protein level, production of cytokines: TNF-α, IL-1β, IL-6, IL-8, IL-10. THP-1 was shown to be a good model system for studying gene expression profile after bacterial ligand challenge. Mono Mac 6 appears to have phenotypic and functional characteristic of mature blood monocytes in terms of CD14 expression and cytokines production: IL-1, TNF-α, IL-6, in response to bacterial ligands. Mono Mac 6 was shown to be effectively differentiated to the more mature, macrophage-like phenotype using mixture of vitamin D3 and TGF-β1. Differentiation resulted in cells clustering, expression of 5-lipooxygenase, CD69 and was accompanied by the upregulation of *CD14*, *CD11a*, *CD11b*, *CD11c*, *MMP9* at mRNA level.

Modulation of cell function: phagocytosis, production of reactive oxygen species (ROS) and cytokines, was examined during pathogen recognition. Pam3CSK4 was shown to play a role in the phagocytosis of pathogens by monocytes and macrophages similar to other bacterial ligands: FSL-1 and peptidoglycan. TLR2 stimulation of undifferentiated THP-1 cells

and differentiated MM6 cells resulted in enhanced phagocytosis of *E. coli*. Phagocytic activity of monocytes was higher than that of monocyte-like cells and was enhanced in macrophages. We did not observe changes in amount of produced superoxide anion during TLR2 activation in THP-1 and MM6 cells. It was probably due to high activity of antioxidant system in this cells, in contrast to monocytes where larger amounts of ROS were detected after stimulation with bacterial ligand. The stimulation of analysed cell lines, both undifferentiated and differentiated, with Pam3CK4 resulted in higher amounts of secreted cytokines, while differentiation primed cells for the faster and enhanced ability to respond to stimulating factor.

During normal life cycle of aerobic organism, reactive oxygen species are constantly generated as a byproducts of the activity of enzymes participating in oxygen metabolism. Even more ROS are generated during recognition and phagocytosis of pathogens, as a main product of the activity of NADPH oxidase enzyme. This is of great importance for specialized phagocytic cells, like monocytes and macrophages, that use reactive oxygen species as a part of defense mechanism against invading pathogens. Despite their positive role as signaling molecules and a weapon against pathogens, ROS are also known to be involved in many pathological conditions, leading to DNA, protein and lipid damage and dysfunction. To avoid deleterious effects of reactive oxygen species, cells have evolved diverse antioxidant mechanisms. Enzymes and molecules involved in antioxidant defense can be classified into three main systems: the superoxide dismutase/catalase system, the glutathione system and the thioredoxin system.

In this study, oxidative stress was mimicked directly by addition of hydrogen peroxide or indirectly by exposure of cells to oxygen-radical generating systems: paraquat or iron-ascorbate mixture.

Cell viability and ROS-induced protein modification analysis showed that MM6 cells, representing a more mature monocytic phenotype than THP-1, were more resistant to H_2O_2. This effect was accompanied by lower level of oxidative protein damage. Increased resistance to H_2O_2 was observed after THP-1 differentiation into macrophage-like phenotype and after TLR2 activation and caused a decrease in the production of protein carbonyl groups. MM6 cells were also more resistant to paraquat, while there was no difference in resistance to iron/ascorbate (Fe/Asc), a system generating reactive oxygen species highly toxic to cell compounds, between less mature THP-1 and more mature MM6. In MM6 cells, more resistant to milder prooxidant systems (H_2O_2, paraquat), TLR2 activation resulted in increased resistance to highly toxic ROS generating system (Fe/Asc).

The NF-κB transcription factor was shown to regulate expression of several antioxidant factors. For that reason the activity of this transcription factor was analysed in monocyte- and macrophage-like cells stimulated with bacterial ligand.

Following ligand stimulation, TLR2 initiates MyD88-IRAK1/IRAK2-TRAF6 or Rac1-PI3K-Akt signaling pathways, resulting in NF-κB activation. Stimulation with TLR2 ligand triggers rapid degradation of inhibitory protein IkBα and slower degradation of IkBβ and IkBε. Activated NF-κB dimers comprise all members of the NF-κB family. NF-κB activity is tightly controlled by numerous feedback loops, whereas positive feedback loops, i.e. paracrine and autocrine regulation via TNF-α, IL-1β and other cytokines, has a crucial role in amplification and prolongation of innate immune response. TLR2 activation resulted, in both analysed cell lines, in increased expression of NF-κB family members and NF-κB activity. Differentiation also plays a significant role in NF-κB activation. Particularly in PMA-differentiated THP-1 cells, for most of NF-κB family members expression level was increased and differentiation was responsible for more rapid and enhanced ability of cells to respond to stimulating factor, as determined e.g. by TNF-α secretion. Stimulation with pam3CSK4 induced the translocation of NF-κB p65 from the cytoplasm into the nucleus, whereas this process was more rapid in differentiated than in undifferentiated cells. A synergistic effect of differentiation and stimulation with pam3CSK4 was observed on NF-κB transcriptional activity. Expression levels of *RELB* and *NFKB2* were increased in undifferentiated and differentiated THP-1 and MM6 cells after bacterial ligand stimulation, so that cells were able to activate the alternative signaling pathway which can result in prolonged immune response. Differentiation of THP-1 cells and TLR2 activation in undifferentiated THP-1 cells led to a significant rise in the gene expression of several pattern recognition receptors (PRR) and increased expression of *TLR2* that was mediated by NF-κB.

We examined the expression and the activity of the main components of the antioxidant system to test their involvement in increased cellular resistance to oxidative stress during differentiation and TLR2 activation.

We observed that the expression and the activity of the antioxidants was dependent on cell maturation and ligand recognition. An increased expression of superoxide dismutase in line with increased resistance to oxidative stress and inhibition of SOD activity resulting in raised cell susceptibility to oxidative stress, suggested that SOD may have important protective capacities towards oxidative stress. On the other hand SOD could facilitate formation of H_2O_2, another ROS capable of inducing cell injury at high concentrations. It is of great importance to tighly control the concentration of H_2O_2 to protect cells against formation

of highly reactive ROS. We therefore examined the expression and the activity of H_2O_2 detoxifying enzymes. Significantly higher basal level of catalase and glutathione peroxidase activity in MM6 cells can be potentially responsible for their higher resistance towards H_2O_2. The importance of catalase in THP-1 cells was demonstrated using its inhibitor, sodium azide. Our experiments revealed increased susceptibility to H_2O_2 in undifferentiated cells, in differentiated cells and differentiated cells stimulated with pam3CSK4. Two forms of glutathione peroxidase were expressed predominantly in THP-1 and MM6 cells, glutathione peroxidase 1 and 4. Basal glutathione peroxidase activity was higher in MM6 cells. It was further increased, in both analysed cell lines, during differentiation, as well as in undifferentiated THP-1 cells during TLR2 stimulation. Recent findings have highlighted the importance of GPx-4 in protection against oxidative stress. Its unique ability to directly reduce phospholipid hydroperoxides makes it an important tool to inhibit lipid peroxidation and to protect against oxidative damage of cell membrane caused by e.g. H_2O_2 or Fe/Asc. GPx-1 was shown, in other reports, to be important in protection against oxidative stress generated by high concentration of paraquat.

Action of glutathione peroxidase requires a regenerating system composed of glutathione and glutathione reductase. MM6 cells had significantly higher levels of glutathione than THP-1 cells. As we observed, it was accompanied by higher expression level of γ-glutamylcysteine synthase (γGLCL) subunits: *GCLM* and *GCLC*, resulting in elevated γGLCL activity; this enzyme acts as the rate-limiting step in GSH biosynthesis. An increased glutathione level was further demonstrated in differentiated THP-1 cells. Inhibition of γ-glutamylcysteine synthase resulted in decreased basal resistance of THP-1 cells against H_2O_2 and decreased viability of (differentiated and pam3CSK4 activated) THP-1 cells exposed to H_2O_2. Higher basal activity of glutathione peroxidase was accompanied by higher activity of glutathione reductase in MM6 cells. Increased GR activity was observed, like for other components of glutathione system, in undifferentiated, pam3CSK4-stimulated THP-1 cells and in differentiated THP-1 cells. Glutathione is also used by peroxiredoxin 6 and glutaredoxin to reduce thiol groups in catalytic center. We observed increased *PRDX6* expression in pam3CSK4 stimulated and in differentiated THP-1 cells. Peroxiredoxin 6 was shown, like GPx-4, to play a unique role in antioxidant defense, as it is able to reduce phospholipid hydroperoxides. Taken together, the glutathione system is an important component of protection against oxidative stress.

We subsequently examined the expression and the activity of thioredoxin system components, containing: thioredoxin, thioredoxin reductase and other enzymes that use

thioredoxin as electron donor, e.g peroxiredoxins. Their role in protection against oxidative stress was shown previously and particularly peroxiredoxin can eliminate H_2O_2 more effectively than catalase, because of its high affinity for H_2O_2. Expression level of cytoplasmic isoforms of thioredoxin, thioredoxin reductase and peroxiredoxin was increased in bacterial ligand-stimulated and differentiated cells. However, a decrease in the activity of thioredoxin reductase (inhibited by auranofin) was not able to change cell susceptibility to H_2O_2; thus, this system seems to be less important in protection against oxidative stress. As the amount of thioredoxin in cells is lower in comparison with other antioxidants, its protective mechanism can be related to regulation of signal transduction pathways rather than to direct engagement in oxidation-reduction reactions.

In MM6 cells, a decrease in the activity of one component of the antioxidant system was not able to change cell susceptibility to H_2O_2. Probably all antioxidant systems participate in cellular protection against oxidative stress and active systems are able to compensate for inactive components. Depletion of more than one system is needed to change cell sensitivity to oxidative stress.

Expression level of most of examined antioxidant system genes was much higher for primary human monocytes than that observed for monocytic cell lines. Increased gene expression was observed for M1 macrophages, where it was activated to a higher extent than for M2 macrophages. After bacterial ligand challenge, gene expression profile of antioxidant system components was comparable for monocytes and macrophages. Expression of *GCLM*, *TXN*, *TXNRD1*, *PRDX1* and *SOD2* was increased, and these components can be of particular importance for regulation of redox homeostasis.