Closing report of the "Molecular determinants of NLRP3 inflammasome function in pro-inflammatory and anti-inflammatory human macrophages" grant.

BACKGROUND OF THE PROJECTS

Nlrp3 inflammasome is probably the most studied member of the NLR intracellular pattern recognition family. Due to the accumulating results it became obvious that the actual outcome of the activation of this multiprotein complex and subsequent IL-1b production strongly depends on the cell type and on the presence or absence of various intracellular or extracellular modulators. Although human monocytic leukemia cell line THP-1 is commonly used to investigate inflammasome activation human model, it is inappropriate to extrapolate results obtained on them to primary cells, as IL-1b release from THP-1 cells require different conditions (like priming with PMA but no ATP additions) from the primary cells. Furthermore, many of the inflammasome studies are carried out on murine macrophages (MFs), however, it is important to note that mouse MFs substantially differ from those of humans. In laboratory conditions, bone marrow (BM)-derived MFs are the most commonly used mouse MFs, nevertheless they are differentiated in the presence of M-CSF, and cultivation with GM-CSF or GM-CSF plus IL-4 leads to dendritic cell (DC) differentiation in the mouse.

While Nlrp3 inflammasome function has been studied in primary MFs, even in the classically (M1) and alternatively (M2) activated ones, there were no published comparative reports addressing expression of NLR proteins, as well as the molecular differences in Nlrp3 function and activation in GM-MFs and M-MFs. Our main aim was to delineate the molecular mechanism of the dramatically different Nlrp3 inflammasome-mediated IL-1b production of MCSF and GM-CSF macrophages (as well as DCs).

RESULTS

Project 1.

We have recently published a thorough study summarizing some basic points about the main aim of the project (Different dynamics of NLRP3 inflammasome-mediated IL‑1β production in GM-CSF- and M-CSF-differentiated human macrophages. Budai MM, Tőzsér J, Benkő S. J Leukoc Biol. 2017).

The main findings:

(1) LPS treatment of M-MFs and GM-MFs results in a different pattern of cytokine expression, as M-MFs produced very small amount of IL-6 and TNF, but comparable amount of IL-8 to GM-MFs. Nevertheless, the production of the anti-inflammatory IL-10 is hardly detectable from the supernatant of GM-MFs, while it is produced in a huge amount by the M-MFs.

We also found that in the presence of ATP the amount of IL-1beta production is comparable in the two MF subpopulations, however the time kinetics is very different, as it is very quick and robust in the M-MFs, while it is slow and gradually increasing in the GM-MFs. We also found that M-MFs require ATP supplementation for IL-1beta release, while GM-MFs, though in smaller amount, are able to release IL-beta even without ATP supplementation.

(2) Using caspase-1 specific inhibitor and also silencing NLRP3 by siRNA in both MF subpopulation, we proved that LPS-induced IL-1b production is mediated through the caspase-1–dependent NLRP3 inflammasome pathway in both MF subtypes.

(3) Upon LPS stimulation, monocytes have been reported to release endogenous ATP, which triggers NLRP3 inflammasome activation and IL-1b release. When we measured the extracellular ATP content of the medium, we found a 4-fold higher ATP concentration in the medium of untreated GM-MFs compared with that of the M-MFs. Nevertheless, using ATP hydrolyzing apyrase and specific P2X7R blocker we showed that extracellular ATP released by GM-MFs does not directly affect NLRP3 inflammasome activation.

(4) As ATP is quickly hydrolyzed to ADP, AMP and adenosine we sought to study the effect of adenosine in IL-1beta release. We found that CD39 and CD73 ectonucleotidases contribute to IL-1b secretion in GM-MFs, also that adenosine enhances IL-1b secretion in LPS-activated GM-MFs.

(5) We could also demonstrate differences in expression of NLRP3 and pro–IL-1b, activation of signal transduction pathways as well as in caspase-1 enzyme activity as underlying factors in diversity of IL-1b processing by GM- and M-MFs. We showed that while in M-MFs caspase-1 activation requires ATP supplementation, we detected constitutive activity of caspase-1 even in the absence of ATP in the case of GM-MFs, which could be further enhanced by ATP supplementation.

(6) We also showed that the rapid attenuation of IL-1b secretion by M-MFs is the result of robust secretion of IL-10 which reduces Akt signaling.

As a following step of this project, currently we are optimizing a co-culture technique to study the effect of NLRP3 inflammasome on T lymphocyte polarization by the two MF

subpopulations. Also, we try to determine possibly different post-translational modifications of NLRP3 sensor and degradation of caspase-1 in the two MF subpopulations.

Project 2.

As part of a collaboration aiming to delineate the molecular determinants of MF polarization at transcriptional level, we studied the NLRP3 inflammasome- mediated IL-1beta secretion in IL-4 treated LPS-activated murine MFs. The Transcription Factor STAT6 Mediates Direct Repression of Inflammatory Enhancers and Limits Activation of Alternatively Polarized Macrophages. Czimmerer Z, Daniel B, Horvath A, Rückerl D, Nagy G, Kiss M, Peloquin M, Budai MM, Cuaranta-Monroy I, Simandi Z, Steiner L, Nagy B Jr., Poliska S, Banko C, Bacso Z, Schulman IG, Sauer S, Deleuze JF, Allen JE, Benko S, Nagy L. Immunity, 2018).

(1) We could show that IL-4-STAT6 axis was able to reduce the basal and LPS-induced expression of a key inflammasome component, Nlrp3.

(2) The IL-4-dependent repression of basal and LPS-induced IL-1b mRNA expression was completely abolished in the absence of STAT6. LPS-induced NLRP3 and pro-IL-1b expression were also attenuated at the protein level by IL-4-STAT6 signaling, while the expression of other inflammasome components including caspase-1 and ASC was not altered following IL-4 and LPS stimulation of BMDMs.

(3) IL-1b secretion was induced dramatically in bone-marrow-derived MFs of WT and Stat6-KO mice following LPS treatment. However, LPS-dependent induction of IL-1b secretion was partially inhibited by IL-4 pretreatment in a STAT6-dependent manner.

Project 3.

We also aimed to study the effect of immunomodulatory compounds on the NLRP3 inflammasome mediated IL-1beta secretion. As we found in our previous study (Budai et al, JLB, 2017) that adenosine influences IL-1beta secretion, we decided to study the effect of caffeine on the LPS-activated two MF subpopulation. Caffeine is a methylxanthine molecule that inhibits adenosine receptors and has many reported effects on several physiological processes. Also, it was shown that it attenuates inflammatory responses. Reviewing the literature, we noticed that the immunomodulatory effect of caffeine is attributed to the observations that caffeine significantly reduces TNFa secretion. However, interestingly when we treated M- and GM-MFs with LPS we found that the cytokine production of the two subpopulations was significantly different. We found that LPS-induced TNFa secretion was

decreased by caffeine in both MF types, as well as, interestingly, that of the IL-10 anti-inflammatory cytokine.

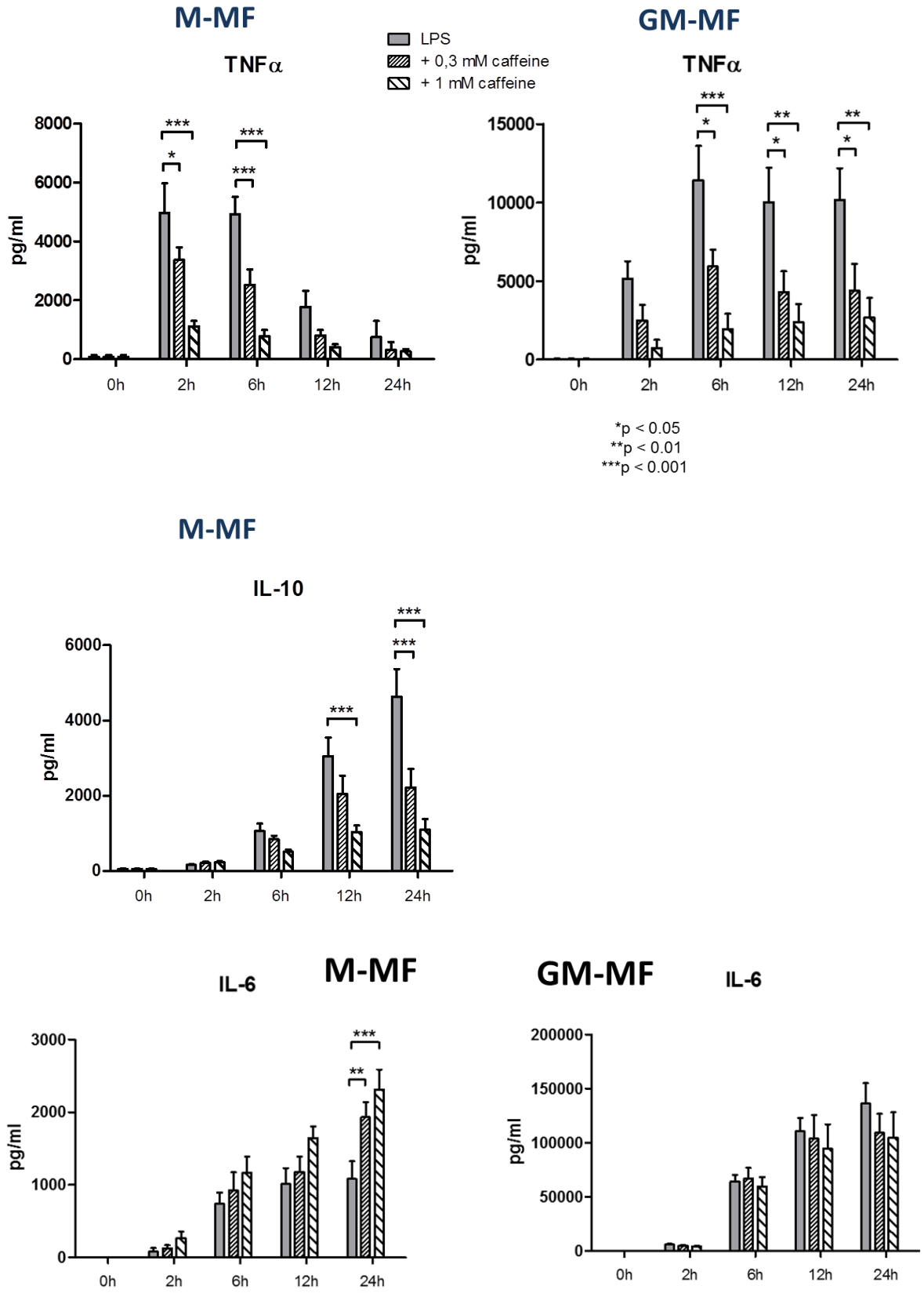

Figure 1. Cytokine production by M-MFs and GM-MFs (ELISA).

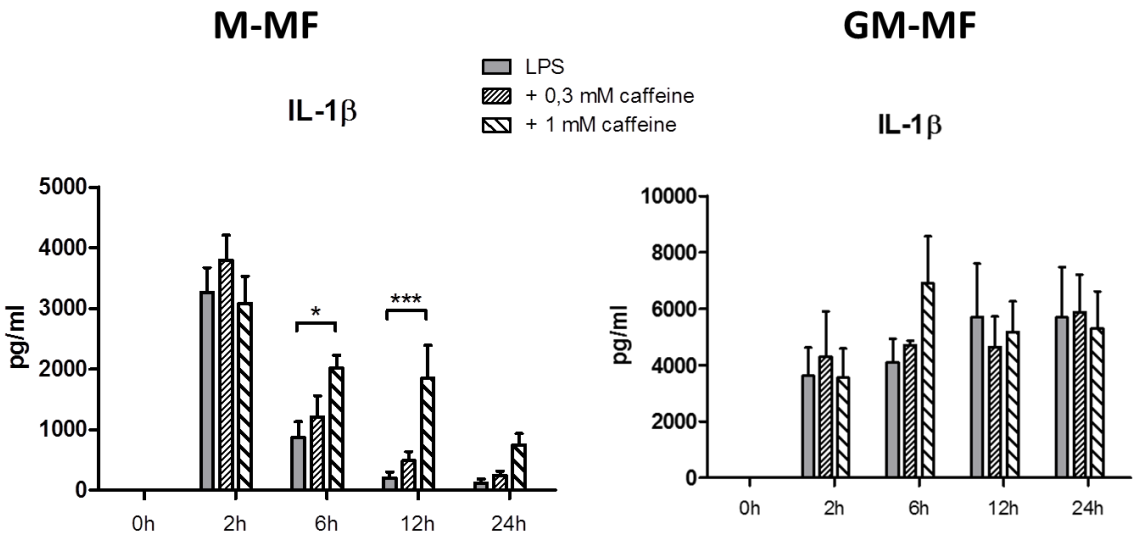

Figure 1. Cytokine production by M-MFs and GM-MFs (ELISA) (continued)

However, while caffeine did not affect the secretion of IL-6, IL-8 and IL-1beta by GM-MFs, it significantly induced the production of these cytokines by M-MFs (Figure 1.)

To determine the molecular mechanism behind the diverse IL-1beta secretion, we aimed to study the expression of inflammasome components.

We found that caffeine treatment significantly enhanced the LPS-induced expression of NLRP3, pro-IL-1beta and the cleaved form of caspase-1 (Figure 2).

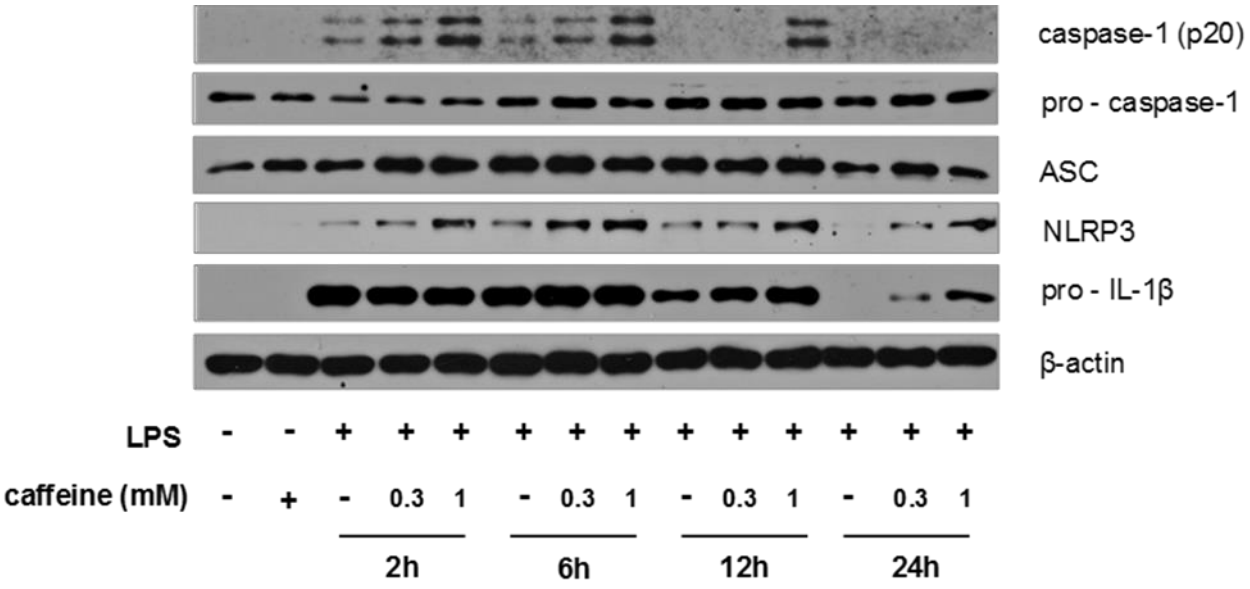

Figure 2. Expression of inflammasome components in M-MFs (Western blot).

We found that these expressions were effected at transcription level, as using Q-RT-PCR technique we detected the induced expression of NLRP3 and pro-IL-1beta at mRNA level (Figure 3).

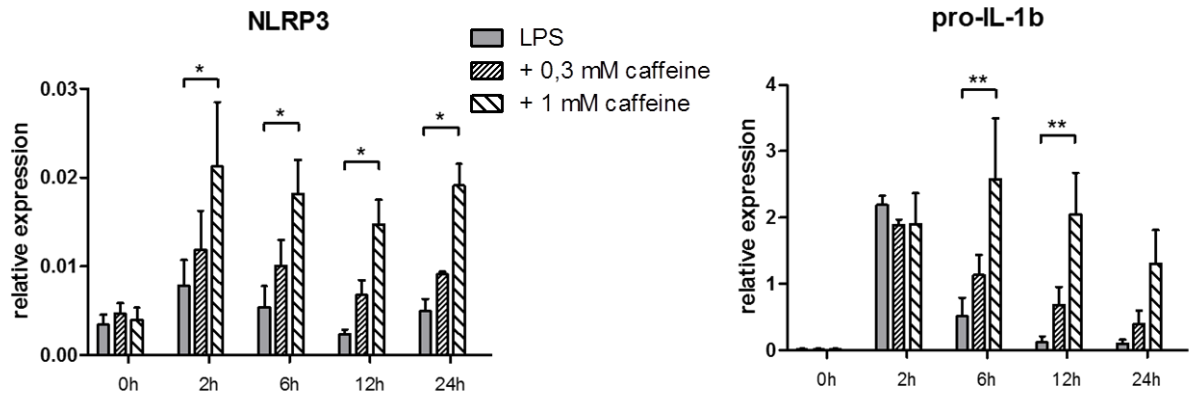

Figure 3. Expression of NLRP and pro-IL-beta in M-MFs (Q-RT-PCR).

When we analyzed the effect of caffeine on the LPS-induced signal transduction pathways we did not found changes in the p38, JNK and ERK signaling in any of the two MF subpopulations (*GM-MF is not shown*) (Figure 4). Although, we found that caffeine inhibited the phosphorylation of Akt, this effect was detected in both cell types. However, we detected attenuation in p-IκBα upon caffeine treatment in M-MFs, while it did not affect GM-MFs (*GM-MF is not shown*).

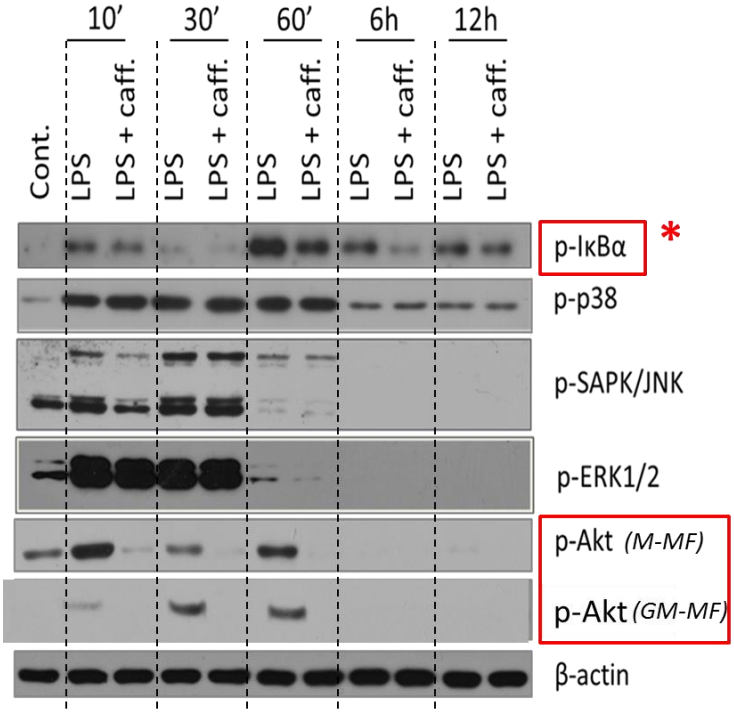

Figure 4. Activation of signal transduction pathways (Western blot).

Caffeine binds to adenosine receptors that mediate adenylate cyclase activity and this way may regulate cAMP level. As NLRP3 inflammasome activation may be regulated by cAMP, we sought to see whether CREB signaling is affected. We did not found changes in the CREB phosphorylation (Figure 5. *GM-MF is not shown*).

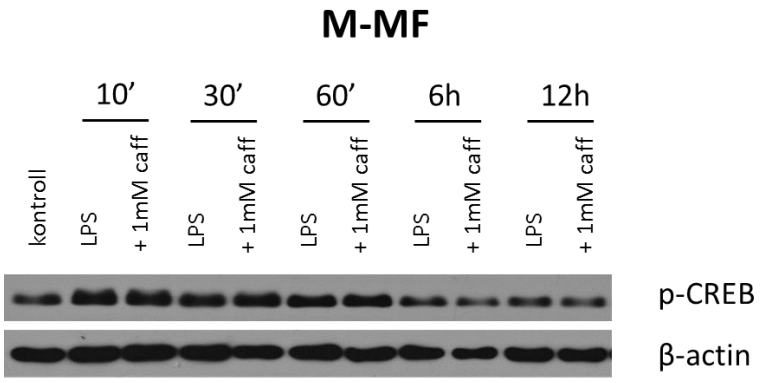

Figure 5. CREB sigmaling (Western blot).

As caffeine was reported to inhibit phosphodiesterase (PDE) activity, which is responsible for the conversion of cAMP to AMP, then AMP may activate AMPK signaling pathway. Activation of AMPK signaling may lead to increased ATP production and activation of autophagy machinery, and both of these may be related to NLRP3 inflammasome activation. Interestingly, we observed that caffeine attenuated AMPK phosphorylation in GM-MFs, but in M-MFs it was not changed (Figue 6).

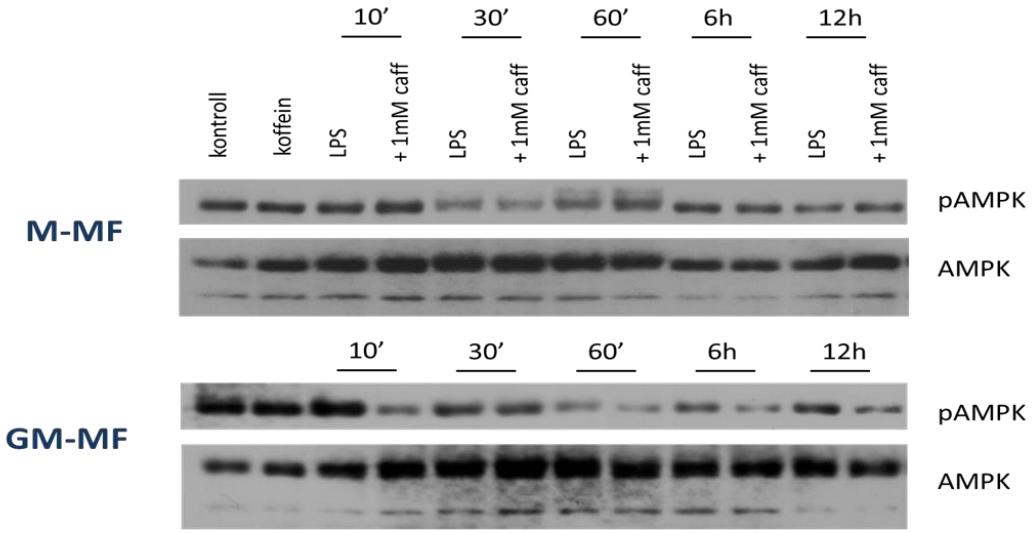

Figure 6. AMPK sigmaling (Western blot).

Next, we would like to know whether caffeine effects ic cAMP concentration, for this reason currently we measuring cAMP with a specific ELISA kit. Also we plan to detect the

expression of PDEs in the two cell types, as PDE family has several members and we hypothesize that there are differences between the two cell types in PDE expression that may affect related signaling pathways.

We have also found that LPS-induced IFNb expression is significantly downregulated by caffeine in M-MFs (Figure 7).

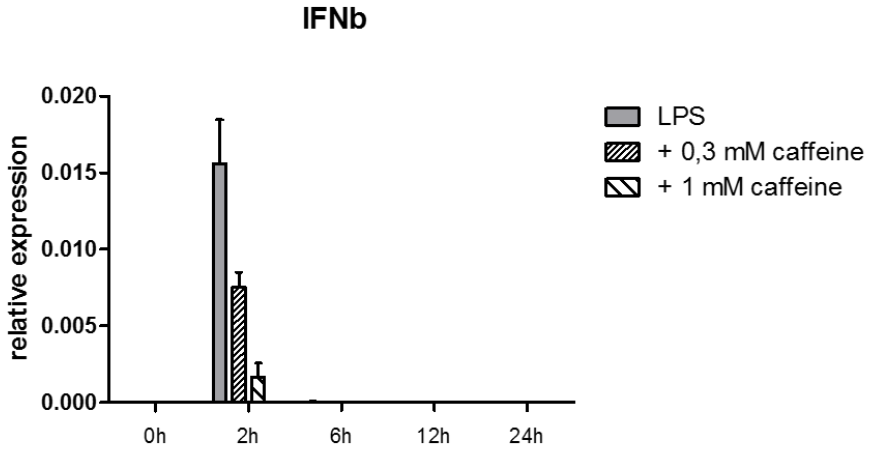

Figure 7. IFNb expression (Q-RT-PCR).

IFNb signaling leads to the activation of STAT1 that will induce the expression of IL-10 which activates STAT3 signaling. We detected abolished LPS-induced phosphorylation of STAT1 in the presence of caffeine (Figure 8).

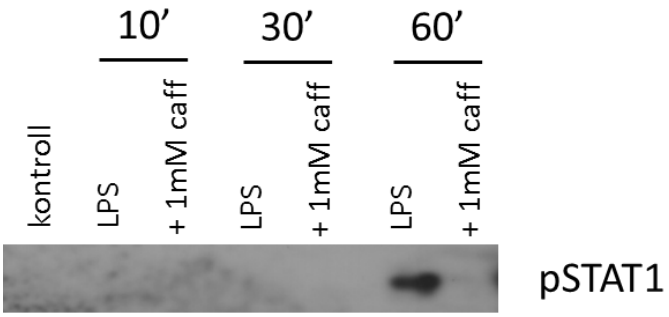

Figure 8. STAT1 signaling (Western blot).

As we also detected reduced IL-10 secretion by M-MFs following caffeine treatment, in the next step we aim to study STAT3 signaling. As STAT3 was reported to play a central role in NLRP3 inflammasome-mediated IL-1beta production, this result may give an important aspect to the multifactorial and very complex mechanism of inflammasome activation.

Furthermore, we also aim to study ROS production in the cell, as caffeine is involved in ROS production, and ROS is an important inducer of NLRP inflammasome activation.

We hope to finalize our work within a few months and we will send our results to publish.

Project 4.

It is known that the different pattern recognition receptors are in a "cross-talk" via the regulation of each other's expression through signal transduction pathways. Furthermore, it is also known that the expression of NLRs is very plastic as it shows big differences among cell types and highly depends on the microenvironment and the activating stimulus of the cell.

We know that although myeloid cells are indispensable for the proper innate immune responses, they have very different morphological and functional characteristics. We aimed to study the NLR expression profile of the human blood monocyte-derived M-MF, GM-MFs and dendritic cells (DCs). When we observed the general pro-inflammatory cytokine production by the LPS-activated cells, we measured very different cytokine production profile of these cells (Figure 9).

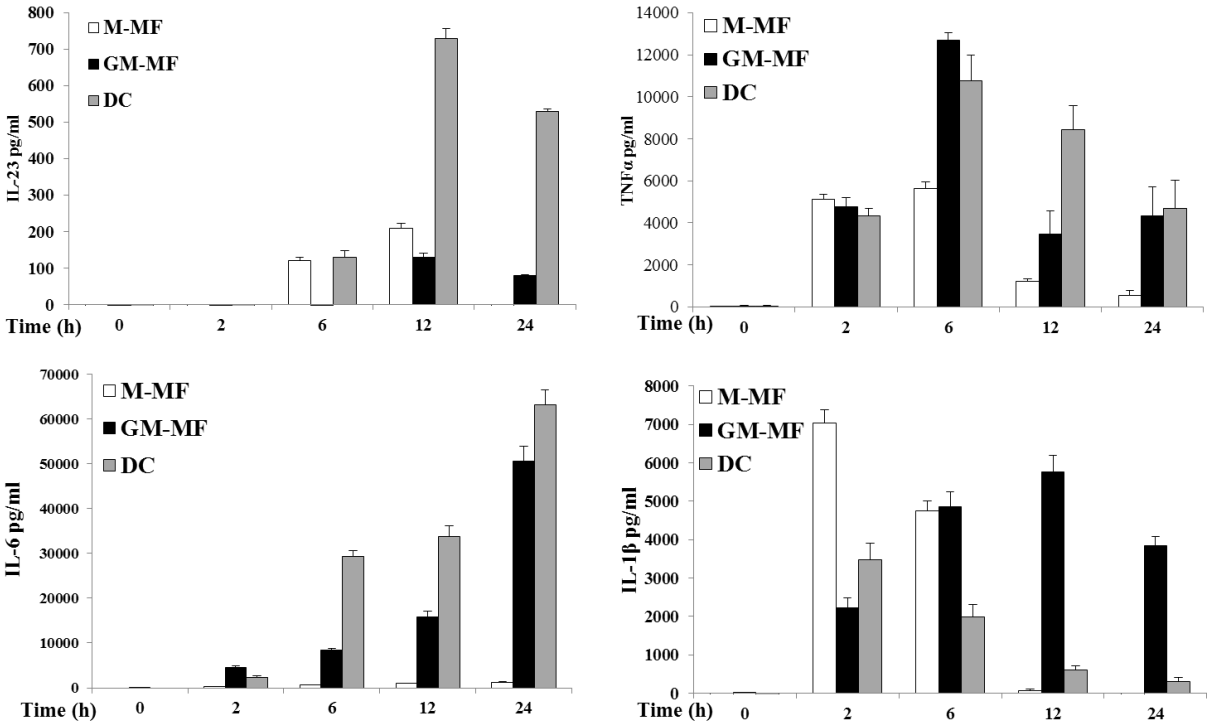

Figure 9. IL-23, TNFa, IL-6 and IL-1beta secretion by M-MF, GM-MFs and DCs (ELISA).

We also studied the NLR expression changes during the differentiation from monocytes to M-MFs, GM-MFs and DCs. We found that during the differentiation process the expression of the NLRs dramatically changes. Some of them strongly increased (like NOD1) or some of them are dramatically decreases (like NOD2). We have also found some where the change of

NLR expression affected only one type of cell population but not the others (like NLRP2) (Figure 10.). We screened for the expression of each member of the NLR family (22 members), also some of the enzymes of inflammasome complexes (caspase-1 as canonical infalmmasome, caspase-4 and caspase-5 as non-canonical inflammasome). Only results from some selected members are represented.

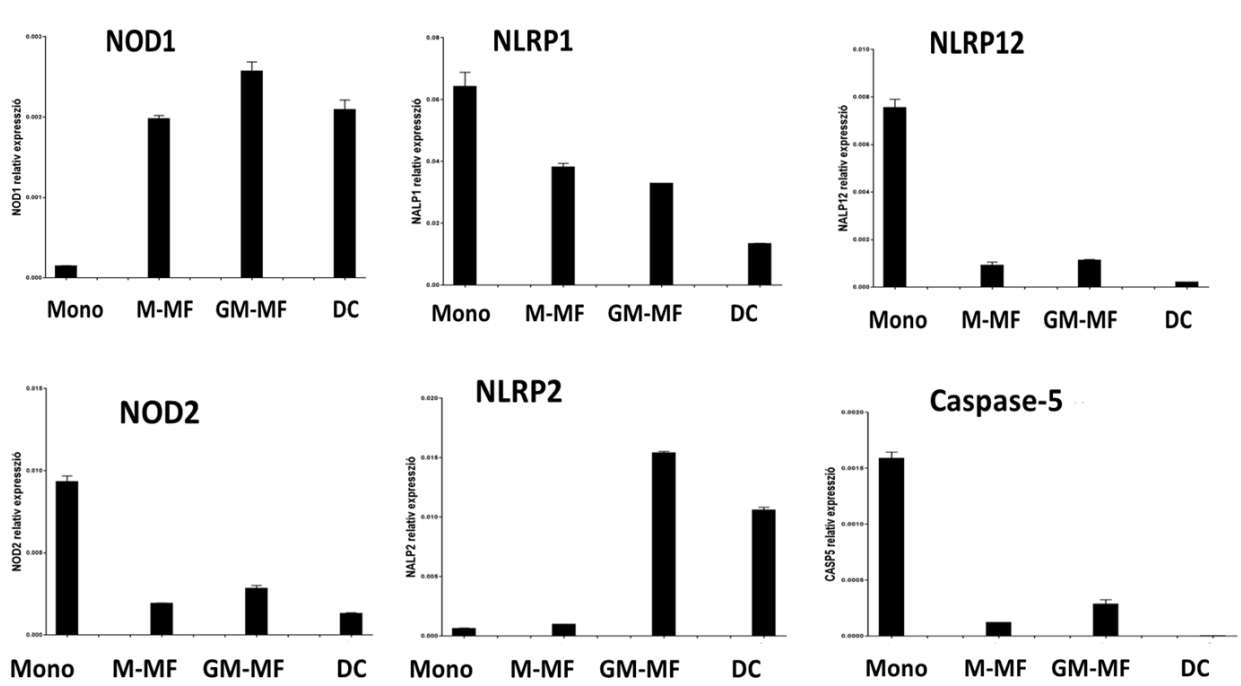

Figure 10. Changes of NLR expression in myeloid cells during differentiation (Q-RT-PCR).

We have also studied the LPS-induced changes in expression of each NLR family members. Our results show very versatile changes in the expression profiles (Figure 11, only some representative results shown). These results indicate that the expression of NLRs in myeloid cells is highly changeable and variable. In many cases the expression profile of DC follows GM-MFs profile, nevertheless we observed that in almost each cases the basal expression of an NLR is lower in DC compared to GM-MFs (which is probably due to the IL-4 which presents in the differentiation medium od DCs) (Figure 10). Upon LPS activation, the expression profile detected in DCs also follows or very similar to that of observed in the GM-MFs. However, many times M-MFs are characterized by a very different profile from the two other cell populations. Currently, we are studying double activations (like LPS plus NOD1 ligand as both derives from bacteria, or LPS plus NOD2 ligand as both from bacteria) to see how the synchronic and asynchronic activation of these TLR-NLR pathways contribute to cellular functions like cytokine productions.

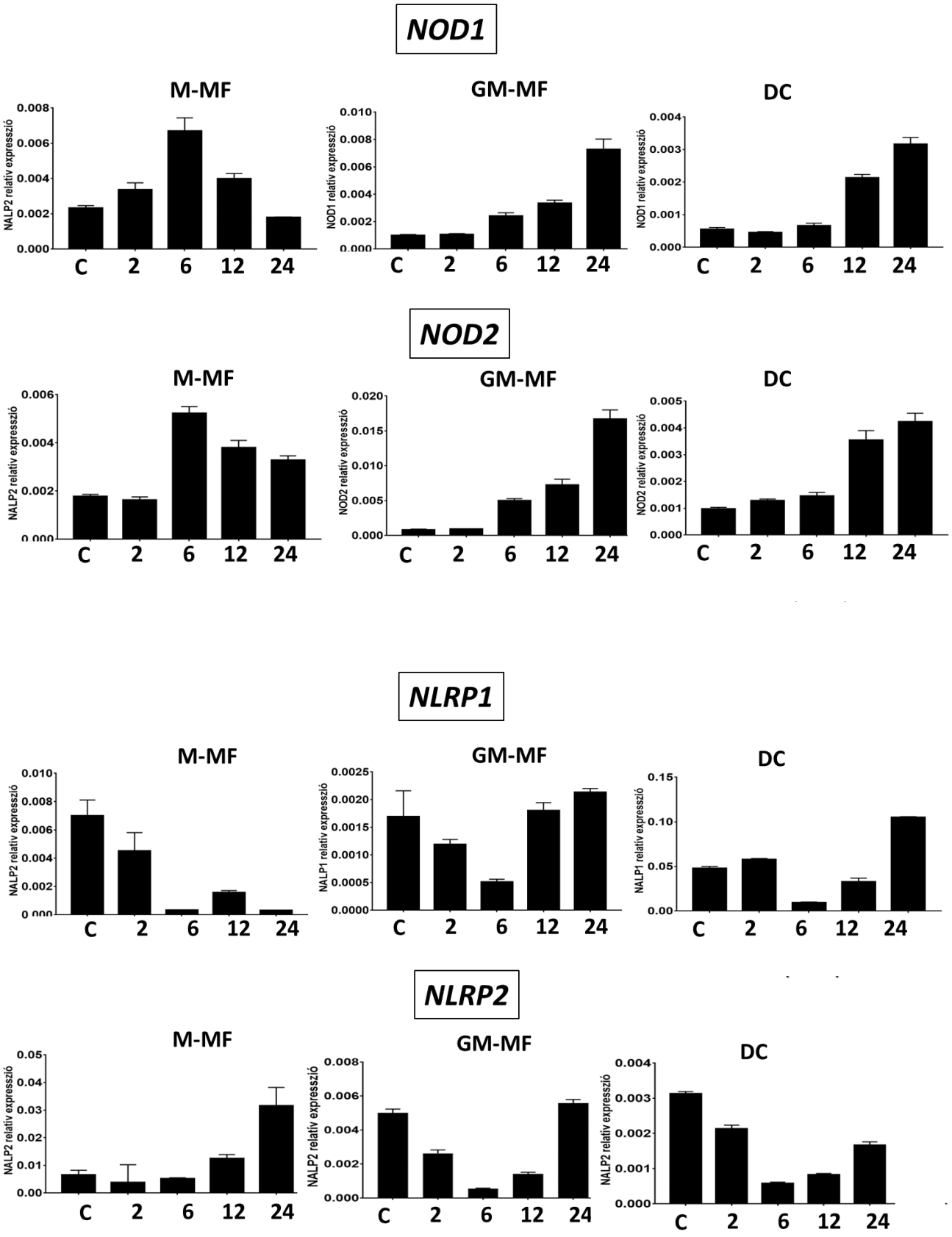

Figure 11. Changes of NLR expression in myeloid cells upon LPS treatment (Q-RT-PCR).

Furthermore, as GM-MFs and DCs show different pattern of IL-1beta secretion, we currently to delineate some molecular aspects of the NLRP3 inflammasome activation in these two cell types, similarly as we did it with GM-MFs and M-MFs (Budai et al., JLB, 2017). We plan to send a manuscript summarizing these observations for publication within a few months.

Project 5.

NLRP3 inflammasome-mediated IL-1beta secretion is the underlining problem of many chronic inflammatory diseases. Recently, NLRP3 inflammasome was recognized by pharmaceutical companies (like Novartis) to study small molecular compounds that as potential regulators (mainly inhibitors) of the protein complex (Novartis trial validates inflammasome as chronic disease driver, *Nature Biotechnology*, 2017; Ridker PM, MacFadyen JG, Thuren T, Everett BM, Libby P, Glynn RJ; CANTOS Trial Group. Effect of interleukin-1β inhibition with canakinumab on incident lung cancer in patients with atherosclerosis: exploratory results from a randomized, double-blind, placebo-controlled trial. *Lancet*. 2017).

Currently we study the effect of two Bimoclomol compounds on human, LPS-activated MF functions. BGP-15, a hydroxylamine derivative, prevents insulin resistance in humans and protects against several oxidative stress-related diseases in animal models. It also has a cytoprotective effect with potential HSP72 co-inducing and PARP inhibiting properties. BRLP42 is another bimoclomol derivative compound which is not used in health treatment currently. However, these compounds have not been studied on immune responses and macrophage functions.

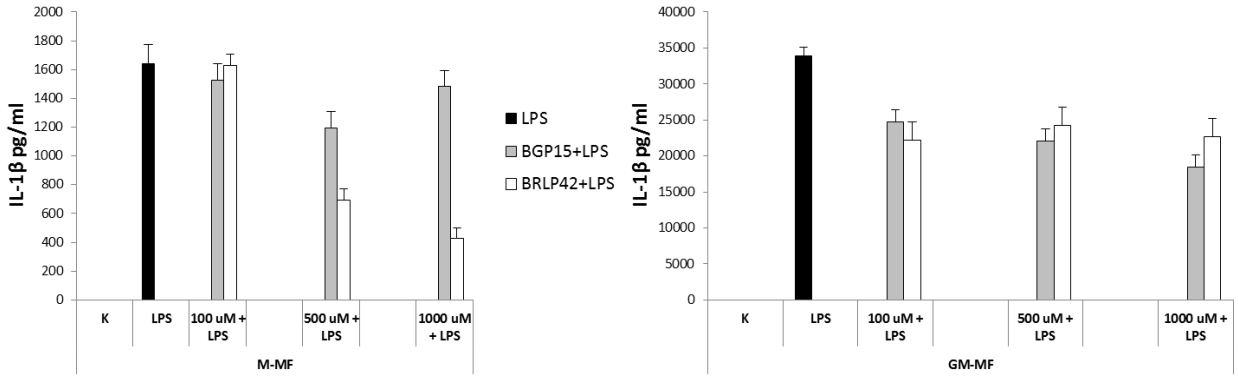

Figure 12. IL-1beta secretion(ELISA).

Our results show that LPS-induced IL-1beta secretion is significantly attenuated with BLRP42 in M-MFs, while this compound had a moderate effect on GM-MFs (Figure 12). In the case of BGP-15, we did not observe that pronounced effect for the cytokine secretion. Currently, we are studying inflammasome expression and signaling pathways to delineate the molecular mechanism of these effects. We see that BRLP42 changes the expression of inflammasome components as well as pro-IL-1beta.

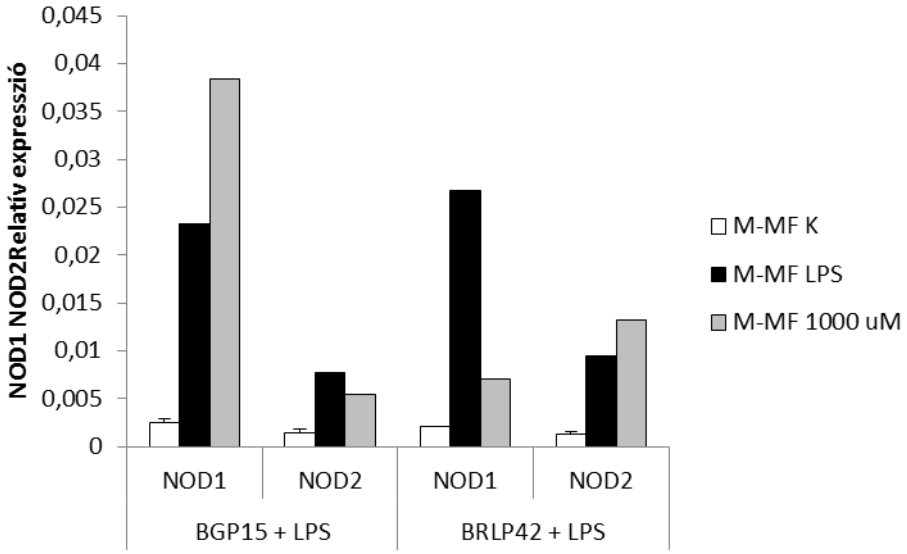

Figure 13. Expression of NOD1 and NOD2 (Q-RT-PCR).

Interestingly, we also see changes in the expression of many other members of the NLR family. Like, while BGP15 significantly enhances the expression of NOD1, it has no effect on NOD2 in M-MFs (Figure 13). On the other hand, while BRLP43 strongly inhibits NOD1 expression, it has no significant effect on NOD2 expression. As both receptors recognize some specific patterns of bacterial cell wall, these compounds could facilitate or attenuate immune responses mediated by these receptors.

We have also shown that these compounds induced HSP70 expression in M-MFs and they induced the activation of p38 signaling pathway (Figure 14), while they did not have effect on other common pathways, like NFkB, JNK, ERK (results not shown).

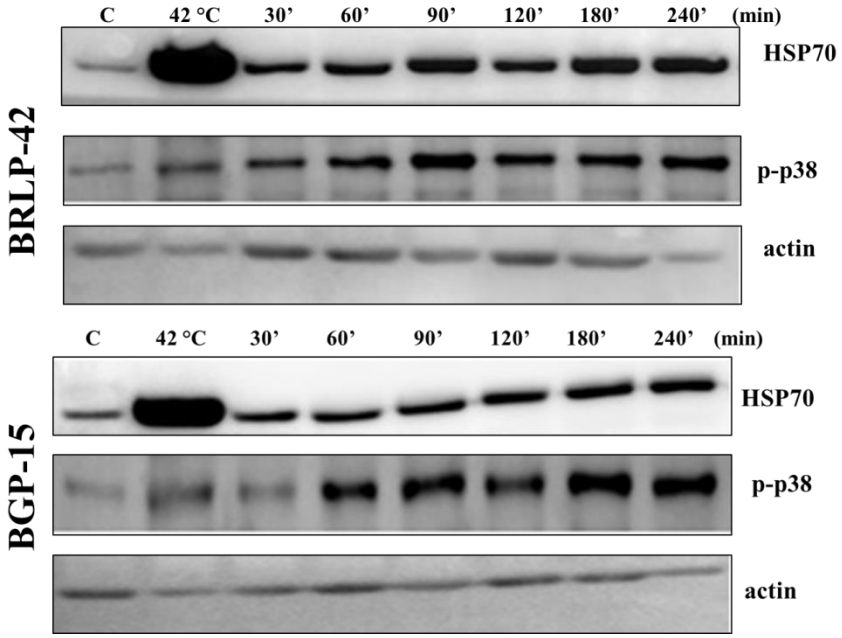

Figure 14. Expression of HSP70 and p-p38(Western blot)

Currently, we are studying cellular and molecular effects of the bimoclomol compounds. We plan to publish these results in this year.