Synergism of NOD2 and NLRP3 activators promotes a unique transcriptional profile in murine dendritic cells

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ABSTRACT

NLRs are cytoplasmic proteins that sense cellular stress and intracellular damage resulting from pathogen uptake. To date, the role of NLRs has been studied using combinations of NLR and TLR agonists, but the interplay between two different NLRs remains uncharacterized. In this study, we employed microarrays to investigate in DCs the regulation of gene transcription mediated by activation of NOD2 and NLRP3 pathways using MDP and MSU. MDP and MSU co-stimulation of murine BMDCs up-regulated the expression of genes encoding molecules for antigen presentation and co-stimulation (MHC class II, CD80, CD86), integrins (ITGβ3, ITGAV), cytokines (IL-1α, IL-1β, IL-6, IL-2, IL-23p19, IL-12p40), and chemokines (CXCL1, CXCL2). Transcription of the cytokine genes induced by MDP and MSU partially depended on NOD2 but was independent of NLRP3. Finally, we showed that ERK1 and c-JUN activation increased upon MDP and MSU co-stimulation. As a whole, the results indicate that two different NLR activators synergize at the transcriptional level, leading to unique differential expression of genes involved in the innate immune response. J. Leukoc. Biol. 88: 000–000; 2010.

Introduction

DCs and macrophages sense danger using a range of receptor families, including the TLR and the more recently described NLRs [1, 2]. Upon stimulation, some NLRs form cytoplasmic complexes called inflammasomes [2]. One such complex is formed of NLRP3, the adaptor protein ASC, and caspase-1. The assembly of the inflammasome complex activates caspase-1, which then enables cleavage and secretion of the proinflammatory cytokines IL-1β and IL-18, thus promoting the early innate response to whatever stimulus initiated inflammasome activation [3]. The NLRP3 inflammasome is triggered by many different pathogen-associated molecules, including bacterial-derived peptidoglycan or pore-forming toxins, such as α-toxin from Staphylococcus aureus and listeriolysin O from Listeria monocytogenes [4, 5]. Moreover, large particulates, including uric acid (MSU), CPPD, silica, and asbestos, are also able to activate the NLRP3 inflammasome [6, 7]. Interestingly, the NLRP3 inflammasome is the only NLR complex able to sense endogenous danger molecules released by dying cells, including ATP, MSU, and CPPD, raising the question of how NLRP3 is able to be activated by such heterogeneous stimuli [5, 6].

NOD2 is an intracellular receptor belonging to the NLR family, which recognizes the peptidoglycan-derived moiety MDP and is associated with autoimmune disorders such as Crohn’s disease and Blau syndrome [8]. Through CARD–CARD interaction, NOD2 recruits receptor-interacting protein-2 kinase and activates NF-kB and MAPK pathways, leading to transcription of proinflammatory genes, including pro-IL-1β [9].

Inflammasome-mediated IL-1β secretion requires a two-step activation process in vitro. First, pro-IL-1β transcription is stimulated by TLR or NOD agonists, such as LPS or MDP, which is followed by inflammasome activation, enabling mature IL-1β protein to be produced and secreted [3, 10, 11]. However, recent findings highlighted other mechanisms contributing to
the two-step model. First, the classical inflammasome activator ATP cannot promote inflammasome formation in the absence of an additional proinflammatory stimulus [10–12]. Indeed, the activation of NF-κB by TLR or NLR ligands induces pro-IL-1β transcription, as well as NLRP3 expression, which is necessary for proper inflammasome activation [13]. Second, ATP may be responsible for cell membrane permeabilization, allowing cytotoxic access to microbial products [12]. In this model, LPS and MDP might be the genuine inflammasome activators [14].

It is important to understand how the NLRP3 inflammasome is activated and regulated in DCs, as there is increasing evidence for its involvement in pathological immunity. Crystals of MSU, which activate NLRP3 inflammasome, play a crucial role in the etiology of gout, a condition characterized by IL-1β release and neutrophil recruitment to the joints. Moreover, NLRP3 mutations resulting in inflammasome hyperactivation have been associated with autoinflammatory diseases, including Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and chronic infantile neurological, cutaneous, and articular syndrome [15, 16].

In this study, we addressed the question of whether the integration of multiple stimulatory signals via NLRs would lead to a unique transcriptional signature. Little is known about the mechanism of MSU-mediated activation of DCs during inflammation. We therefore investigated the transcriptional responses of murine DCs stimulated with the NOD2 agonist MDP and the endogenous NLRP3 activator MSU. We identified unique transcriptional signatures of DCs stimulated in vitro with MDP or MSU, and a distinctive differential expression of genes associated with simultaneous stimulation by MSU and MDP. The interaction of these two NLR activators regulated the expression of genes related to DC maturation and the inflammatory response, including cytokines, chemokines, costimulatory molecules, and integrins. The synergistic signaling of MDP and MSU was partially dependent on NOD2, whereas the NLRP3/IL-1β/IL-18 axis seems to be dispensable. Our data suggest that the mechanisms regulating inflammasome activation by MSU and MDP could also involve a fine-tuning mechanism at transcriptional level, promoting a specific reprogramming of DCs toward an inflammatory phenotype.

**MATERIALS AND METHODS**

**BMDC preparation and stimulation**

BMDCs from C57BL/6 (Biological Resource Center, Agency for Science, Technology and Research, Singapore), NOD2-deficient mice (The Jackson Laboratory, Bar Harbor, ME, USA; stock #005763), and NLRP3-deficient mice (from Jürg Tschopp, Department of Biochemistry, University of Lausanne, Epalinges, Switzerland [6]) were prepared as described [17]. After 8 days of culture, BMDCs were stimulated with MDP (10 μg/ml, InvivoGen, San Diego, CA, USA), or MDP and MSU to-gether for the indicated times. MSU crystals were prepared as described [6] and endotoxin tested by Limulus amoebocyte lysate assay.

**Caspase activation and cell viability**

BMDCs (2×10⁶) were stimulated in black, 96-well plates (NUNC, Rochester, NY, USA) with MDP, MSU, or MDP and MSU together, as above, or camptothecin (1 nM, Sigma Chemical Co., St. Louis, MO, USA). At defined time-points, activated polycaspases and caspase-1 were detected using FLICA™ red or green (Immunochrometry Technologies, Bloomington, MN, USA), respectively, during the last hour of culture. After washing, fluorescence was measured using a Tecan M200 infinite plate reader. Percentage of caspase activation was calculated using the formula ([stimulated/unstimulated]×100) – 100.

**Total RNA extraction**

Total RNA was isolated by double extraction using Trizol® (Invitrogen, Carlsbad, CA, USA), followed by the RNeasy clean-up procedure (Qiagen, Valencia, CA, USA). RNA purity and integrity were assessed by spectrophotometer and Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Only high-quality RNA samples with a 260/280 ratio >1.9 and RNA Integrity Number >8.5 were used for microarray analysis and quantitative real-time RT-PCR analyses.

**Quantitative real-time RT-PCR**

Genomic DNA was removed from total RNA using the Turbo DNA-free kit (Ambion, Austin, TX, USA) before RNA was copied into cDNA by high-capacity cDNA RT kits (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed using primer sets shown in Table 1 Amplification was performed using Applied Biosystems 7500 real-time PCR. The relative expression level for each gene was evaluated using the ΔΔCt method. Briefly, the difference between Ct of the target gene and Ct of the housekeeping gene (GAPDH or HPRT) was normalized to the difference between the Ct of the same genes in the untreated condition.

**Flow cyt fluorimetry**

BMDCs stimulated for 24 h with MDP, MSU, or MDP and MSU were labeled with allophycocyanin-conjugated anti-mouse CD11c (clone N418, Bio-...

**TABLE 1. Quantitative RT-PCR Primer Sets**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Set</th>
<th>For TaqMan RT-PCR (Applied Biosystems)</th>
<th>For iQ™ SYBR® Green real-time PCR (BioRad Laboratories)</th>
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<td>reverse 5'-CGGCTGAGGCTAACTGATGCC-3'</td>
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<td>IL-6</td>
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<tr>
<td>GAPDH</td>
<td>(RefSeq: NM_008084.2)</td>
<td>forwa...</td>
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**For TaqMan RT-PCR (Applied Biosystems)**

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<th>Gene</th>
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<td>reverse 5'-GGTTGCCGTTTGGCCGACC-3'</td>
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legend, San Diego, CA, USA) combined with PE-conjugated antibodies specific for CD80 (clone 16-10A1), CD86 (clone GL1), CD40 (clone 3/23), or I-A<sup>b</sup> MHC class II (clone AF6-120.1; BD Pharmingen, San Diego, CA, USA). Data were collected using a FACSCalibur (BD Biosciences, San Jose, CA, USA) and Flowjo software for analysis (Tree Star, Ashland, OR, USA).  

Western blot  
Total cell lysates of BMDCs were prepared according to standard methods. Membranes were immunoblotted with antibodies specific for IL-1β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-c-Jun (Ser<sup>73</sup>; Cell Signaling, Danvers, MA, USA), p44/p42 ERK1/2 (clone MAPK-YT, Sigma Chemical Co.), p38 (clone D37, Cell Signaling), and GAPDH (clone 6C5, Millipore, Billerica, MA, USA).  

Phosphoprotein assay  
Unstimulated BMDCs or cells stimulated with MDP, MSU, or MDP and MSU were lysed using the Bio-plex cell lysis kit. The Bio-plex phospho-c-Jun assay (BioRad Laboratories, Hercules, CA, USA) was used, according to the manufacturer’s instructions.  

Array hybridization and analysis  
Total RNA (8 μg) was used for cRNA target preparation, according to the Affymetrix GeneChip expression analysis technical manual (Affymetrix, Santa Clara, CA, USA), using the one-cycle target-labeling kit, according to the manufacturer’s instructions. Biotinylated cRNA (15 μg) was hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 arrays through the service provided by Genopolis Consortium (Milan, Italy). Microarray analysis was performed using R language and bioconductor packages [18]. Probe set signals were generated using a GC robust multiarray average background adjustment and normalized with a quantile method [19]. The data were filtered, discarding the probe sets showing an absent call in all of the conditions and signal intensity lower than the 95th percentile of the overall absent call distribution. The differentially expressed genes were selected over a threshold of Log2 (ratios) of 1.5-fold among the treatments at 4 h versus the control at 0 h. To address a biological framework involved in the stimulation processes, we focused on different gene families extracted from DAVID [20]. The changes in expression levels for each gene in each family were represented using MeV software, Version 4.4, generating hierarchical clustering of Log2 (ratios) signals and setting a Pearson correlation as a metric measure and an average linkage as a linkage method. All microarray data are available from the ArrayExpress database under Accession Code E-MEXP-2905.  

RESULTS AND DISCUSSION  
Stimulation by MDP, MSU, or MDP and MSU induces distinctive transcriptional signatures in BMDCs  
We investigated the effect of the NLRP3 activator MSU on modulation of gene transcription in DCs. As inflammasome activation is two-step in vitro, we used the NOD2 agonist MDP to prime the BMDCs. Although a direct structural interaction between MDP and NOD2 has not yet been proven, there is substantial evidence that NOD2 is required for MDP-mediated activation of NF-κB and MAPK pathways in macrophages [21]. MDP has the advantage of being a weak activator of innate cells compared with TLR agonists (i.e., LPS and CpG) [9], enabling us to detect its interaction with MSU at the transcriptional level.  

BMDCs were stimulated with MDP, MSU, or MDP and MSU for 4 h. Cellular transcriptomes were generated using high-density mouse oligonucleotide Affymetrix gene arrays, interrogating 45,101 transcripts. DEG for each treatment were selected over a threshold of Log2 (ratios) of 1.5-fold compared with the untreated control. Using an unsupervised hierarchical cluster analysis, we found that gene expression profiles were significantly different in MDP-, MSU-, or MDP/MSU-treated BMDCs (Fig. 1A). The transcriptional signature induced by MDP/MSU in BMDCs was more similar to that induced by MSU than the one induced by MDP. This suggests that MDP enhanced MSU-mediated gene expression, but MSU was the stimulus dictating the molecular signature (Fig. 1A). MDP alone mostly induced down-regulation of gene transcription, whereas MSU and MDP/MSU triggered a stronger up-regulation of several genes (Fig. 1B and C).  

To characterize the ability of MDP, MSU, and MDP/MSU to induce specific reprogramming of gene expression in BMDCs, we next compared the global gene expression variations induced by the three stimulation conditions. DEG (116) were shared among MDP-, MSU-, and MDP/MSU-treated BMDCs (Fig. 1D). DEG (315) were modulated exclusively in MDP-treated BMDCs, whereas only 37 genes were exclusive to MSU as a result of the major overlap with the MDP/MSU condition (272 genes), which emphasizes the similarity in responses induced by MSU alone and MSU in combination with MDP (Fig. 1D). Interestingly, 468 DEG were expressed exclusively after MDP and MSU co-stimulation (Fig. 1B and D). Taken together, these results indicated that MSU can be considered a strong modulator of gene transcription in BMDCs. By combining MSU with MDP, we observed an increase in the proinflammatory activity of MDP but also the induction of a unique gene expression profile co-modulated by both stimuli.  

MDP and MSU induce exclusive up-regulation of genes related to inflammation and intracellular transport in BMDCs  
To identify the specific transcriptional signature derived from the interaction between MDP and MSU in BMDCs, we ranked the top 30 up-regulated genes within the 468 DEG expressed exclusively following stimulation by MDP/MSU (Supplemental Table 1). The ranking was based on the fold-change expression of every gene compared with the untreated condition. We observed that genes encoding the cytokines IL-2, IL-23p19, and IL-1F9, as well as other genes related to inflammation, such as the transcription factor FOSoB, ITGβ8, TNFRSF13C, and semaphorin 7A, ranked in the highest positions (Supplemental Table 1). We also identified genes encoding plasma membrane-mediated processes, such as endocytosis (caveolin 1, RAB11 GTPase FIP1) and ion exchange (Gap junction 43 kDa heart protein, SLC22A4; solute carrier family 15 H<sup>+</sup>/pep-
tide transporter-member 2), and molecules involved in signaling pathways (GPCR, family C, group 5, member A, Supplemental Table 1). Finally, we undertook functional clustering analysis using gene-annotation enrichment by DAVID Bioinformatics Researches. The MDP/MSU-specific genes identified were related to inflammation, the immune response, cell motility, and matrix remodeling (data not shown). Endocytosis and potassium cation transport are cellular processes studied as putative mechanisms involved in NLRP3 activation [12, 22, 23]. We hypothesized that activation of DCs by MDP and MSU may lead to plasma membrane modifications, promoting uptake and intracellular trafficking of crystals and simultaneously promoting the inflammatory response, although a direct involvement of these mechanisms has not been shown. Interestingly, polymorphisms in the gene encoding SLC22A4 seem to be associated with Crohn’s disease, a genetic disorder caused
by NOD2 variants, leading to intestinal chronic inflammation [8]. A functional association between SLC22A4 and NOD2 has not been reported; however, it seems possible that commensal flora might trigger expression of the SLC22A4 gene through MDP, ultimately contributing to the pathogenesis of Crohn’s disease.

**BMDCs activated by MDP and MSU exhibit an inflammatory phenotype characterized by upregulation of integrins and costimulatory molecules**

Upon activation, BMDCs initiate genetic reprogramming, leading to maturation, which is associated with upregulation of costimulatory molecules required for T cell help, antigen presentation, and adhesion. Thus, we studied the transcription of integrins and maturation markers induced by MDP, MSU, and MDP/MSU stimulation in BMDCs. The genes encoding ITGA5 and CD47 were strongly upregulated in response to MSU alone and MSU/MDP, whereas the ITGB3-encoding gene was induced exclusively by MDP/MSU (Fig. 2A). Moreover, flow cytometric analysis of BMDCs stimulated for 24 h with MDP/MSU revealed increased expression of costimulatory and antigen-presenting molecules, including CD80 (B7.1), CD86 (B7.2), and MHC class II (Fig. 2A and B). In agreement with the transcriptome data, CD40 expression was upregulated predominantly after stimulation with MDP alone.

Integrins are plasma membrane proteins involved in cell–cell contact, cell-matrix adhesion, cytoskeleton contraction, migration, and signal transduction [24]. CD47 is a receptor for the extracellular matrix protein thrombospondin and is expressed in association with integrins. Moreover, CD47 interacts with transmembrane signal regulatory protein α (SIRPalpha) on macrophages and DCs, resulting in inhibition of phagocytosis [25]. CD47 expression on DCs is essential for their transmigration across high endothelial venules and trafficking to spleen and LNs [26]. Taken together, the upregulation of ITGA5 and ITGB3 with CD47 in BMDCs in response to MSU may support an involvement of these molecules in the interaction and phagocytosis of MSU crystals by DCs and macrophages, as well as induction of DC migration. Furthermore, it has been shown that a multireceptor complex composed by the scavenger receptor CD36, integrin ITGA6B1, and CD47 mediates the proinflammatory properties of fibrillar β-amyloid, which induces ROS, NLRP3 inflammasome activation, and IL-1β secretion, with some similarities to MSU [27].

**Stimulation of BMDCs by MDP and MSU induces modulation of cytokine- and chemokine-encoding genes related to inflammation**

DCs are a major source of cytokines and chemokines important for eliciting and regulating the immune response. Thus, we investigated the cytokine expression pattern induced in BMDCs in response to MDP, MSU, and MDP/MSU. To identify cytokine- and chemokine-encoding genes, the microarray data generated in our study were interrogated using the anno-

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**Figure 2. Functional clustering of costimulatory molecule- and integrin-encoding genes expressed in response to MDP, MSU, and MDP/MSU stimulation of BMDCs.** (A) Differential gene expression of integrin and surface molecule genes modulated during 4 h stimulation with MDP, MSU, or MDP/MSU in BMDCs. (B) Surface expression of BMDC maturation markers 24 h after stimulation with MDP, MSU, or MDP/MSU. Each plot shows superimposed histograms representing the surface expression of CD80, CD86, CD40, and MHC class II, as assessed by flow cytometry. Different line styles represent distinct BMDC treatments with the isotype and untreated controls, according to the legend.
tation resources provided by Gene Ontology. To confirm gene-expression changes, BMDCs were stimulated with MDP, MSU, or MDP/MSU for 2, 4, 8, 18, and 24 h, and cDNA from three independent experiments was used as a template for quantitative real-time RT-PCR targeting selected cytokine and chemokine genes. Transcription of IL-6, TNF-α, and IL-1β-encoding genes was induced by MDP, and MSU stimulation elicited significant up-regulation of IL-1α and TNF-α genes (Fig. 3A and B). Simultaneous stimulation of BMDCs with MDP/MSU induced robust up-regulation of genes encoding IL-1α, IL-1β, IL-6, and TNF-α, suggesting a synergistic relationship of these two stimuli in triggering a proinflammatory immune response. The expression of pro-IL-1β p35 protein in response to MDP, MSU, or MDP/MSU stimulation was validated further by Western blot (Fig. 3C). IL-23p19- and IL-12p40-encoding genes were up-regulated significantly by MDP and MSU co-stimulation, whereas the IL-2 gene was induced exclusively by MDP/MSU synergism (Fig. 3A and B). With the exception of IL-6, all cytokines tested were induced primarily at early time-points (2 h), suggesting that they were transcribed as a result of direct activation of NOD2- and NLRP3-mediated pathways by MDP and MSU. In addition, expression of the proinflammatory chemokines CXCL1 and CXCL2 was enhanced by the

Figure 3. Analysis of cytokine and chemokine gene expression profiles in BMDCs stimulated with MDP, MSU, or MDP/MSU. (A) Heat-map representation of cytokine (upper panel) and chemokine (lower panel) genes differentially expressed upon 4 h stimulation with MDP, MSU, or MDP/MSU in BMDCs. Each column represents each treatment, and each row represents the expression profile of a single gene listed according to their similarities, as depicted by the dendrogram on the left. (B) Quantitative real-time RT-PCR analysis of the expression of cytokine (IL-1α, IL-1β, IL-1RN, IL-6, TNF-α, IL-2, IL-12p40, IL-23p19) and chemokine (CXCL1, CXCL2, CCL3) genes in BMDCs stimulated for 2, 4, 8, 18, and 24 h with MDP, MSU, or MDP/MSU. The relative expression level for each gene was calculated by using the ΔΔCt method. (C) Western blot analysis of IL-1β p35 expression in total cell extracts from untreated BMDCs or BMDCs treated with MDP, MSU, or MDP/MSU for 4 h.
combination of MDP and MSU, and CCL3 expression was also up-regulated by MSU alone (Fig. 3B). These data suggest that the NOD2 and NLRP3 signaling pathways induced by MDP and MSU synergize, leading to a unique transcriptional regulation of cytokine and chemokine genes.

In support of our data, MSU-mediated up-regulation of specific proinflammatory transcripts (i.e., IL-6, TNF-α, and IL-1β) with a similar kinetic profile was also observed in a murine air-pouch model resembling the human joint synovium [28]. Moreover, increased expression of chemokine genes, such as CXCL1 and CXCL2, is associated with the neutrophil recruitment typical of some inflammatory diseases, including gout [29].

In this study, we have also observed for the first time that transcription of genes encoding IL-2 and IL-23p19 is associated with MSU activation of DCs. It has been shown that DCs can secrete IL-2 upon bacterial and fungal encounter, which is crucial for DC-mediated NK cell activation, T cell homeostasis, and T regulatory cell maintenance [17, 30]. IL-23p19, together with IL-12p40, forms the active complex of the IL-23 cytokine, which is secreted primarily by DCs and macrophages upon activation [31]. IL-23 is important during the immune response against pathogens but also promotes pathological intestinal and systemic inflammation by targeting a subset of memory T cells that secretes the proinflammatory cytokines IL-17 and IL-6 [32]. Further studies will elucidate the functional role of IL-2 and IL-23 during MSU-induced inflammation in the presence of a priming signal.

Role of NOD2 and NLRP3 signaling in induction of cytokine expression by MDP and MSU stimulation of BMDCs

The contribution of NOD2 and NLRP3 pathways to the activation of genes encoding proinflammatory cytokines was also studied. BMDCs were generated from NOD2-deficient, NLRP3-deficient, and C57BL/6 mice, and were stimulated with MDP, MSU, or MDP/MSU for 2, 4, 8, and 24 h. The transcription of IL-1α, IL-1β, IL-6, IL-12p40, and TNF-α genes was assessed by quantitative RT-PCR. This revealed that the induction of cytokine genes by MDP/MSU co-stimulation was partially dependent on NOD2 and independent of NLRP3 (Fig. 4). MSU stimulation elicited cytokine gene transcription independently of NOD2 and NLRP3 (data not shown). Interestingly, transcription of the IL-23p19 gene was fully dependent on NOD2 signaling, and IL-2 transcription was abrogated in NOD2- and NLRP3-deficient BMDCs (Fig. 4).

It is known that MSU induces NLRP3-dependent release of IL-1β and IL-18 ([6] and data not shown), which through the autocrine-feedback loop, leads to transcription of other inflammatory cytokines (i.e., IL-6 and TNF-α). Our data showed that the gene-expression profile of these cytokines was independent of NLRP3. Therefore, this further indicates that the transcription of these cytokines is also independent of the production of IL-1β and IL-18 activated by NLRP3. Regardless, the cooperative transcription of genes encoding cytokines was detected in BMDCs following co-stimulation by MDP with MSU only. To explain the synergism observed in our study, we suggest that other possible mechanisms induced by MSU bypassing NLRP3 activation contribute to transcriptional regulation. Indeed, it has been shown that MSU stimulation of innate cells can also lead to production of ROS, activation of SYK tyrosine kinase pathway, as well as cell death [33].

Injured cells release a number of endogenous danger signals that activate innate immune cells and induce sterile inflammation [34]. During infection, sterile cell death can co-occur with the presence of bacterial or viral components. To rule out the possibility that the observed synergism between MDP and MSU was a result of MSU-induced cell death, we quantified the active polycaspases and caspase-1 in BMDCs treated by MDP, MSU, or MDP/MSU. Camptothecin was used as a positive control to induce apoptosis, and all cells were as-
Figure 5. Activation of poly-caspases and caspase-1 upon treatment of BMDCs with MDP, MSU, MDP and MSU, or camptothecin. BMDCs were stimulated with MDP, MSU, MDP/MSU, or camptothecin. Polycaspases (A) and caspase-1 activation (B) were measured at the indicated time-points by using FLICA™. Data are shown as differential percentage of activation related to unstimulated condition (UT).

Figure 6. Specific expression profile and activation of transcriptional modulators by MDP/MSU activation of BMDCs. (A) Microarray data representation of genes encoding transcription factors, which are induced by MDP, MSU, or MDP/MSU stimulation of BMDCs. (B) Western blot analysis showing phosphorylation of c-JUN (Ser73), p44/p42 ERK 1/2 (Thr183, Tyr185 on ERK2), and p38 MAPK (Thr180, Tyr182) in BMDCs left untreated or stimulated for 15, 30, 60, or 120 min by MDP, MSU, or MDP/MSU. GAPDH expression was used to assess the equal gel loading. (C) Phosphoprotein assay for detection of phosphorylated c-JUN (Ser73) from BMDCs stimulated with MDP, MSU, and MDP/MSU for 2 h.

sayed by FLICA™ staining. Activation of polycaspases was observed after 2 h in BMDCs treated with MSU alone but not by MDP, MDP/MSU, or camptothecin (Fig. 5A). At 18 h, camptothecin treatment was the most potent activator of polycaspases. MSU and MDP/MSU treatment also induced polycaspase activation but to a lower and similar level. Caspase-1 was most acti-
tant contribution of cell death to sterile inflammation in the absence of a priming signal.

**SynergiSM of MDP and MSU in BMDCs engages the MAPK signaling pathway**

The synergistic effects of MDP with MSU treatment of BMDCs may be the result of the activation of additional signaling pathways. Thus, we used Gene Ontology to select DEG from our microarray data that encode transcription factors. We found that MDP/MSU stimulation of BMDCs induced up-regulation of several transcription factors that are involved in DC activation, including STAT1, STAT2, STAT5A, IRF4, IRF7, Nfatc1, Nfatc2, Nfat5, the NF-κB family members IκBα, NF-κBp50/p105, and NF-κB1B, as well as c-REL, JUNb, and Fosb (Fig. 6A).

To determine the contribution of the MAPK signaling pathway to the activation of BMDCs in response to our stimuli, we carried out Western blots to assess the phosphorylation of c-JUN. To revist the paper; G.L. performed caspase activation experiments and contributed significantly to setting up Western blot analysis of phosphoproteins; H.L.Q. performed part of real-time quantitative RT-PCR; M.U. contributed to microarray experiments; F.V. contributed to microarray analysis; P.R-C. designed the research and revised the paper, A.M. designed and coordinated the research, analyzed the data, and wrote the paper.

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**DISCLOSURES**

The authors declare no financial or commercial conflict of interest.

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**AUTHORSHIP**

C.C-A. performed most research, analyzed the data, and wrote the paper; O.B. performed microarray analysis, clustering, functional analysis, prepared part of some pictures, and revised the paper; G.L. performed caspase activation experiments and contributed significantly to setting up Western blot analysis of phosphoproteins; H.L.Q. performed part of real-time quantitative RT-PCR; M.U. contributed to microarray experiments; F.V. contributed to microarray analysis; P.R-C. designed the research and revised the paper, A.M. designed and coordinated the research, analyzed the data, and wrote the paper.


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