Cellular/Molecular

Inflammasome-Induced IL-1β Secretion in Microglia Is Characterized by Delayed Kinetics and Is Only Partially Dependent on Inflammatory Caspases

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Inflammasomes are multiprotein complexes that link pathogen recognition and cellular stress to the processing of the proinflammatory cytokine interleukin-1β (IL-1β). Whereas inflammasome-mediated activation is heavily studied in hematopoietic macrophages and dendritic cells, much less is known about microglia, resident tissue macrophages of the brain that originate from a distinct progenitor. To directly compare inflammasome-mediated activation in different types of macrophages, we isolated primary microglia and hematopoietic macrophages from adult, healthy rhesus macaques. We analyzed the expression profile of NOD (nucleotide-binding oligomerization domain)-like receptors, adaptor proteins, and caspases and characterized inflammasome activation and regulation in detail. We here demonstrate that primary microglia can respond to the same innate stimuli as hematopoietic macrophages. However, microglial responses are more persistent due to lack of negative regulation on pro-IL-1β expression. In addition, we show that while caspase 1, 4, and 5 activation is pivotal for inflammasome-induced IL-1β secretion by hematopoietic macrophages, microglial secretion of IL-1β is only partially dependent on these inflammatory caspases. These results identify key cell type-specific differences that may aid the development of strategies to modulate innate immune responses in the brain.

Key words: **IL-1β; inflammasome; inflammatory caspases; macrophages; microglia**

Introduction

Microglia are the resident tissue macrophages of the CNS. Like other macrophages, they express multiple receptors of the innate immune system, including Toll-like receptors (TLRs), NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs), C-type lectin receptors, and retinoic acid-inducible gene-I-like receptors (Olson and Miller, 2004; Zuiderwijk-Sick et al., 2007; Furr et al., 2008; Shah et al., 2008; Shi et al., 2012). Recent studies have implicated NLR-mediated activation of microglia in several neurodegenerative (Salminen et al., 2008; Heneka et al., 2013) and infectious brain diseases (Hafner-Bratkovič et al., 2012; Jamilloux et al., 2013), providing an impetus for more detailed studies of this receptor family.

NLRs can sense disturbances in cellular homeostasis caused by amongst others pathogens, large protein aggregates, and neighboring cell death. To date, 23 NLRs have been described for humans (Martinon et al., 2009). Ligand recognition by NOD1 and NOD2 can directly induce transcription of proinflammatory cytokines and chemokines via NFκB and IRF3 signaling (Ting et al., 2010). Other NLRs, such as NALP1 (NACHT-, LRR-, and PYD-containing protein 1), NALP3, NALP7, AIM2 (absent in melanoma 2), and IPAF (ICE-protease activating factor) can form multiprotein complexes called inflammasomes. These receptors can either directly or indirectly via the adaptor proteins ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) or CARDINAL (CARD inhibitor of NF-κB-activating ligands) interact with inflammatory caspases and activate them. In turn, activated caspases can process precursor proteins, such as pro-IL-1β, to their bioactive and secreted forms. Thereby NLRs link perturbances in cellular homeostasis to the production of proinflammatory cytokines (Martinon et al., 2009; Latz, 2010; Khare et al., 2012).

Inflammasome-mediated activation of microglia is involved in both infectious (Chang et al., 2012; Kaushik et al., 2012; Lee et al., 2013) and noninfectious (Halle et al., 2008; Abulafia et al., 2009; Meissner et al., 2010) neurological diseases. Microglia express various components of the inflammasome, including NALP1, NALP3, ASC, and caspase 1 (Abulafia et al., 2009; Jamilloux et al., 2013; Shi et al., 2013). In addition, microglia can be induced to express pro-IL-1β, which can be processed to bioactive and secreted IL-1β in response to pathogens, protein aggregates, and more general cellular stressors, such as ATP and reactive oxygen species (Halle et al., 2008; Abulafia et al., 2009;

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Terada et al., 2010; Hanamsagar et al., 2011; Wu et al., 2013; Walsh et al., 2014).

Although microglia resemble hematopoietic macrophages both in phenotype and function, it has recently been uncovered that they originate from a different progenitor (Ginhoux et al., 2010). Furthermore, there are indications that regulation of signaling by innate immune receptors is different in microglia (Xiao et al., 2013), and microglia have been reported to specifically employ caspases 3/7 and 8 during inflammatory conditions (Burguillos et al., 2011). To directly compare inflammasome-mediated activation in different types of macrophages in an outbred system with close resemblance to humans, we isolated primary microglia and hematopoietic macrophages from adult, healthy rhesus macaques. We analyzed the expression profile of NLRs, adaptor proteins, and caspases and characterized inflammasome activation and regulation in detail. Our data reveal important cell type-specific differences pertaining to the negative regulation of pro-IL-1β expression as well as to the inflammasome-induced enzymatic processing of pro-IL-1β.

Materials and Methods

Animals and cell culture. Brain, bone marrow, and blood were obtained from adult rhesus monkeys (*Macaca mulatta*) of either sex without neurological disease that became available from the outbred breeding colony. No animals were sacrificed for the exclusive purpose of the initiation of primary cell cultures. Better use of experimental animals contributes to the priority 3Rs program of the Biomedical Primate Research Centre. Individual identification data of the animals are listed in Table 1. Primary microglia and bone marrow-derived macrophages were isolated and cultured as described previously (Zuiderwijk-Sick et al., 2007; van der Putten et al., 2009). In short, microglia isolations were initiated from cubes of ~3 g of prefrontal subcortical white matter tissue that were manually depleted of blood vessels and meninges. These were chopped into cubes of <2 mm^3 by using gentleMACS C tubes (Miltenyi Biotec) and incubated for 20 min at 37°C in PBS (Thermo Fisher Scientific) containing 0.25% (w/v) trypsin (Thermo Fisher Scientific) and 0.2 mg/ml bovine pancreatic DNase I (Roche Diagnostics). The pellet (no centrifugation) was washed, passed over a 100 μm nylon cell strainer (Corning), and spun for 7 min at 524 $\times$ *g*. After resuspending, this was followed by Percoll (GE Healthcare Bio-Sciences) gradient centrifugation for 30 min (1561 $\times$ *g*; slow brake). The pellet was washed and residual erythrocytes were depleted by hypotonic shock for 7 min on ice in milli-Q supplemented with 155 mM NH_4Cl (Calbiochem), 1 mM $KHCO_3$ (Merck), and 0.2% (w/v) bovine serum albumin (Sigma-Aldrich). After a final wash, cells were plated in tissue culture treated 6-well or 24-well plates (Corning Costar) in 1:1 v/v DMEM (high glucose)/HAM F10 Nutrient Mixture supplemented with 10% v/v heat-inactivated FCS, 0.5 mM glutamax, 50 U/ml penicillin, and 50 μg/ml streptomycin (all Thermo Fisher Scientific). After overnight incubation, unattached cells and myelin debris were removed by washing with PBS and attached cells were cultured in fresh medium supplemented with 20 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech).

Primary bone marrow-derived macrophages were isolated by flushing the bone marrow from the femur (~4 cm) with PBS, followed by passing the suspension over a 100 μm nylon cell strainer (Corning) and gradient centrifugation using Lymphoprep (Axis-Shield PoC) according to manufacturer's protocol. Cells were plated in tissue culture-treated 6-well or 24-well plates (Corning Costar) in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% v/v heat-inactivated FCS, 2 mM glutamax, 50 U/ml penicillin, 50 μg/ml streptomycin (all Thermo Fisher Scientific), and 20 ng/ml M-CSF (PeproTech).

Primary blood CD14$^+$ (cluster of differentiation antigen 14-positive) macrophages were isolated from heparinized blood using Lymphoprep (Axis-Shield PoC) and leucosep separation tubes. Interphases were collected and CD14$^+$ monocytes were isolated using CD14$^+$ microbeads (Miltenyi Biotec) and a magnetic activated cell sorting separation system. CD14$^+$ cells were plated in tissue culture-treated 6-well or 24-well plates (Corning Costar) in RPMI 1640 medium supplemented with 10% v/v heat-inactivated FCS, 2 mM glutamax, 50 U/ml penicillin, 50 μg/ml streptomycin (all Thermo Fisher Scientific), and 20 ng/ml M-CSF (PeproTech).

Table 1. Individual identification data of rhesus macaques

Monkey ID number	Age (years)	Gender	Weight (kg)	Origin
1XR	28	Female	4.3	India
2CL	24	Female	9.0	India
2CP	21	Female	5.2	India
9017	23	Female	7.7	India
94045	6	Female	5.0	Myanmar
94056	16	Female	9.0	India
96024	17	Male	13.1	India
9606117	9	Male	12.8	China
R00049	11	Male	9.6	India
R00063	11	Male	8.4	Myanmar
R011141	11	Male	10.1	Unknown
R02032	10	Male	8.9	Myanmar
R02052	11	Female	7.3	India
R02093	11	Female	9.8	Mix
R03042	9	Female	5.1	Myanmar
R04027	9	Female	4.3	Myanmar
R04053	10	Female	6.6	Mix
R04055	7	Male	9.2	Mix
R04058	7	Male	8.6	Mix
R04067	9	Female	10.6	Mix
R04080	6	Female	5.0	India
R04108	9	Female	8.1	Mix
R05074	9	Female	5.2	Myanmar
R05098	6	Male	13.3	Mix
R06026	8	Male	11.8	India
R06043	8	Male	11.0	India
R06084	4	Male	4.5	Myanmar
R06106	6	Male	10.4	Mix
R07097	6	Male	5.2	Myanmar
R07108	7	Male	6.7	India
R08045	4	Female	3.6	Myanmar
R11088	2	Female	2.7	India
R99007	14	Female	5.7	Mix
Ri0511002	5	Female	3.7	China
Ri201108	9	Female	5.4	China
Ri202062	4	Female	5.8	China
Ri202224	10	Female	6.0	China
Ri204252	10	Female	5.1	China
Ri303103	9	Male	8.9	China
Ri306029	6	Male	10.7	China

After isolation, cell populations were kept in culture for 7 or 8 d (without passaging) and received 1:1 fresh medium every 3–4 d supplemented with 20 ng/ml M-CSF (PeproTech). Cell cultures were synchronized for medium changes as well as for exposure to experimental stimuli. Different cell populations were analyzed (data not shown) for purity (there were no significant differences in percentage CD11b+ cells), morphological criteria indicative of activation (absent), and proliferation rate by Ki-67 immunostainings (there were no significant differences in percentage proliferating cells at time of stimulation). All cell culture media were analyzed for possible LPS contamination using a TLR4 bioassay: none of the media contained >10 fg of LPS/ml (data not shown).

Antibodies and reagents. Monoclonal antibodies against human caspase 1 (IMG5028, Novus Biologicals) and polyclonal antibodies against human caspase 4 and 5 (4450S and 4429S, Cell Signaling Technology) and human IL-1β (H-153, Santa Cruz Biotechnology) were used. Secondary mouse anti-rabbit-IgG-HRP and goat anti-mouse-IgG-HRP were obtained from Jackson ImmunoResearch Laboratories.

TLR ligands used were TLR1/2 agonist Pam_3CSK_4 (500 ng/ml), TLR3 agonist polyiosinic-polycytidylic acid (20 μg/ml), TLR8 agonist CL075 (1 μg/ml; all Invivogen) and TLR4 agonist LPS (100 ng/ml, unless indi-

cated otherwise; from *Escherichia coli* serotype O26:B6; Sigma-Aldrich). Inflammasome-activating agents used were monosodium urate (MSU) crystals (150 μg/ml; Invivogen), ATP (5 mM), and silica (500 μg/ml; Sigma-Aldrich).

Fluorochrome inhibitor of caspase assays (FLICA; AbD Serotec) to determine specific caspase 1 activity were performed according to manufacturer's instructions. Caspase inhibitors specific for caspase 1 (Z-YVAD-FMK; 10–40 μM), caspase 4 (Z-LEVD-FMK; 10–40 μM), and caspase 5 (Z-WEHD-FMK; 10–40 μM) were from BioVision. Concentrations of inflammasome-activating agents and caspase inhibitors were based on earlier studies (Zuiderwijk-Sick et al., 2007; Halle et al., 2008; Zhou et al., 2011).

RNA isolation, cDNA synthesis, and real-time PCRs. Total cellular RNA was isolated using TriReagent (Sigma-Aldrich) or the RNeasy minikit (Qiagen) according to manufacturer's protocol. Subsequently, 1 μg of template mRNA was reverse transcribed into cDNA with the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) according to manufacturer's protocol. Probes for real-time PCRs were designed using the Universal Probe Library design center (Roche Applied Science). Real-time quantitative PCRs were performed on the CFX96 real-time PCR detection system (Bio-rad Laboratories) using primer (Thermo Fisher Scientific) and probe (Exiqon ProbeLibrary, Roche) combinations listed in Table 2, and iTaq Universal Probes Supermix (Biorad). Relative gene expression was standardized to β-actin using the Pfaffl method (Pfaffl, 2001).

Cytokine analysis. Sandwich ELISA kits for human IL-1β (R&D Systems) were used for quantification of IL-1β in cell culture supernatants according to manufacturer's instructions.

Western blot analysis. Culture supernatants were collected and residual crystals or cellular debris was removed by short centrifugation at 12,000 $\times$ *g*. To remove large proteins, 1:1 v/v acentonitrile (ICN Biomedicals) was added, followed by 30 min incubation at room temperature and short centrifugation at 12,000 $\times$ *g*. Thereafter, supernatants were concentrated using 10 kDa microcentrifuge tubes (Merck). Concentrates were collected and standardized to volume.

Cell lysates were prepared in mammalian protein extraction reagent supplemented with Halt protease and phosphatase inhibitor mixture (Thermo Fisher Scientific) according to manufacturer's instructions. Cell lysates were standardized to protein concentrations that were determined using Bradford assays (Thermo Fisher Scientific).

Culture supernatants and cell lysates were separated on 12% Bis/Tris gel in NuPage MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer (Thermo Fisher Scientific). Proteins were transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences) by semidry blotting. Membranes were probed with indicated primary and secondary antibodies, and developed with chemiluminescence (Thermo Fisher Scientific).

Statistics. All data are depicted as means ± SD. Graphpad Prism 6 (Graphpad Software, 2014, version 6.0e) for Macintosh was used for graphical representations. Statistical analyses were performed using Microsoft Excel for Mac (Microsoft, 2011, version 14.4.1) and the R statistical package (R Development Core Team, 2009, version 3.02).

Table 2. Overview of primer/probe combinations used for real-time quantitative PCR

Gene name	Universal probe number	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon length (nt)
AIM2	3	agcctgagcagaaacagagg	ccataactggcaaacagtctttc	78
ASC	65	gaactggacctgcaaggact	tcctccaccaggtaggactg	66
β-Actin	63	gcccagcacgatgaagat	cgccgatccacacagagta	65
CARDINAL	81	tgacgattgggtttggttc	agccactgttcatggtgct	67
CASP1	4	ccaggacattaaaataaggaa attgt	ccaaaaacctttacagaaggatctc	77
CASP3	68	tggaattgatgcgtaatgtt	tggctcagaagcacacaaac	73
CASP4	26	ttccgggcaattgaaaatgg	tgcaagctgtactaatgaaggtg	85
CASP5	80	ttgctttctgttcttcaacacc	tgaagatggagccctttg	66
CASP7	25	gctgacttcctcttcgccta	caaaccaggagcctcttcct	76
CASP8	55	catggaccacagtaacaagga	gccatagatgatgcccttgt	73
CD14	74	gcatcctgcttgttgctg	tcgtccagctcacaaggtt	77
EBP1	62	gacgaggcagctgagttga	ttccgaagtagaaatccctctct	88
IL-1α	6	aataacctggaggccatcg	gctaaaaggtgccgacctg	69
IL-1Rα	16	tgcctgtcctgtgtcaagtc	cgcttgtcctgttttctgttc	95
IL-6	40	acaaaagtcctgatccagttcc	gtcatgtcctgcagccact	131
IL-8	4	tctgtgtaaacatgacttccaagc	cactccttggcaaaactgc	96
IPAF	18	aagtgaaccctgtgaccttga	accaaattgtgaagattctgagc	96
MALT	4	ccagactcagttcactgcaaaa	gcaatgagaggtttcccaac	130
MYD88	80	gcaaggaatgtgacttccaga	gatggggatcagtcgcttc	77
NAIP	6	gacagcgtggtggaaattg	gttgtccagtgctcgaaagaaa	129
NALP1	45	catcctgcctgccaactca	cctcagctcctgcctcatct	75
NALP2	11	caccctccagacactccg	cagtatcaataatcagttgtgggttg	104
NALP3	74	cacctgttgtgcaatctgaag	gcaagatcctgacaacacgc	74
NALP4	87	cggtcctggtatacctgatgct	tcagagatgtattcacagcac	67
NALP5	14	gcctctcagtgatgccttg	tgatgccacagtcctcca	71
NALP6	75	tctcgaggcaccacaaaaca	gactttgcagtgggacagc	111
NALP7	30	gctggactggacagactgc	tccttgcagctgaggtagaac	66
NALP8	1	ccctgaagaaccctgactgt	agcagatagaggtgaacagg	69
NALP9	7	ctggacgaaggctcaggaag	cagggacgggaagacaggtt	110
NALP10	40	gaggggtttgagtcccaag	cgtggggagtgtatgtctcc	64
NALP11	1	ccttaatgatatttcggaaaggattc	gcagtcgagatataggacaactt	93
NALP12	83	gcctaggggaatgtgtcaac	gggtttgagtgctccttcac	70
NALP13	80	tcagcttgtaacctcaagtatc	caaggccaggtcctgacagc	75
NALP14	82	ttgagatatccaaactgtaacattca	cattttatcagtctttggttgcag	117
NOD1	24	acaacaatctcaacgactacgg	cagtgatctggtttacgctgag	90
NOD2	74	gactacaactctgtgggtgacatt	tgagatattgttatcgcgcaaat	93
NOD3	34	aggtcggcaaggacttctc	acacagcttctcgtgggtgt	71
NOD4	38	catcagagctgtgggtcctc	caggtacttcttggccaactcta	75
PKR	13	aaaacacagaattgacggaaaga	tcaagttttgccaatgctttt	96
Pro-IL-1β	10	aaagcttggtgatgtctggtc	ggacatggagaacaccacttg	89
Pro-IL-18	87	gccaactctggctgctaaa	cagcagccatctttattcctg	135
TGFβ	31	actactacgccaaggaggtcac	tgcttgaacttgtcatagatttcg	73
TLR2	1	cggcctgtggtacatgaaa	atgtccctgttgggagctt	78
TLR4	69	aatcccctgaggcatttagg	tcaattgtctggatttcacacc	92
TNFα	79	aagcctgtagcccatgttgt	gctggttatctgtcagctcca	112

Overview of primer/probe combinations used for real-time quantitative PCR, including sequences (5′ → 3′) of forward and reverse primer, human Universal Probe Library number of corresponding probe, and amplicon length (in nucleotides (nt)). AIM2, Absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; CARDINAL (CARD8), CARD inhibitor of NF-κB-activating ligands; CASP, caspase; CD14, cluster of differentiation antigen 14; EBP, end binding protein; IPAF, ICE-protease activating factor; MALT, mucosa-associated lymphoid tissue lymphoma-translocation gene; MyD88, myeloid differentiation primary response 88; NAIP, neuronal apoptosis inhibitor protein; NALP, NACHT-, LRR-, and PYD-containing protein; NOD, nucleotide-binding oligomerization domain; PKR , double stranded RNA-activated protein kinase; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumor-necrosis factor.

Results

We first assessed mRNA expression levels of different inflammasome components in primary microglia, bone marrow-derived macrophages (BMDMs), and blood CD14$^+$-derived macrophages (CD14Ms; Fig. 1*A*). mRNA transcripts for NLR family members NALP1–NALP3, AIM2, IPAF, NAIP (neuronal aptosis inhibitory protein), MALT (mucosa-associated lymphoid tissue lymphoma-translocation gene), and NOD1–NOD4 were detectable in all cell types, while transcripts for NALP4–NALP14 were below detection levels. In addition, all cell types expressed transcripts for the adaptor proteins ASC, CARDINAL, EBP1 (end binding protein 1), and PKR (double-stranded RNA-activated protein kinase) as well as transcripts for caspase 1, 3–5, 7, and 8. Expression levels of adaptor protein- and caspase-encoding transcripts were relatively abundant when compared with levels of NLR-encoding transcripts.

The expression profiles are consistent with the notion that all these cell types can form functional inflammasomes. Yet, comparison of relative mRNA expression levels of microglia, BMDMs, and CD14Ms (Fig. 1*B*) revealed interesting differences. Where mRNA expression levels in BMDMs and CD14Ms were

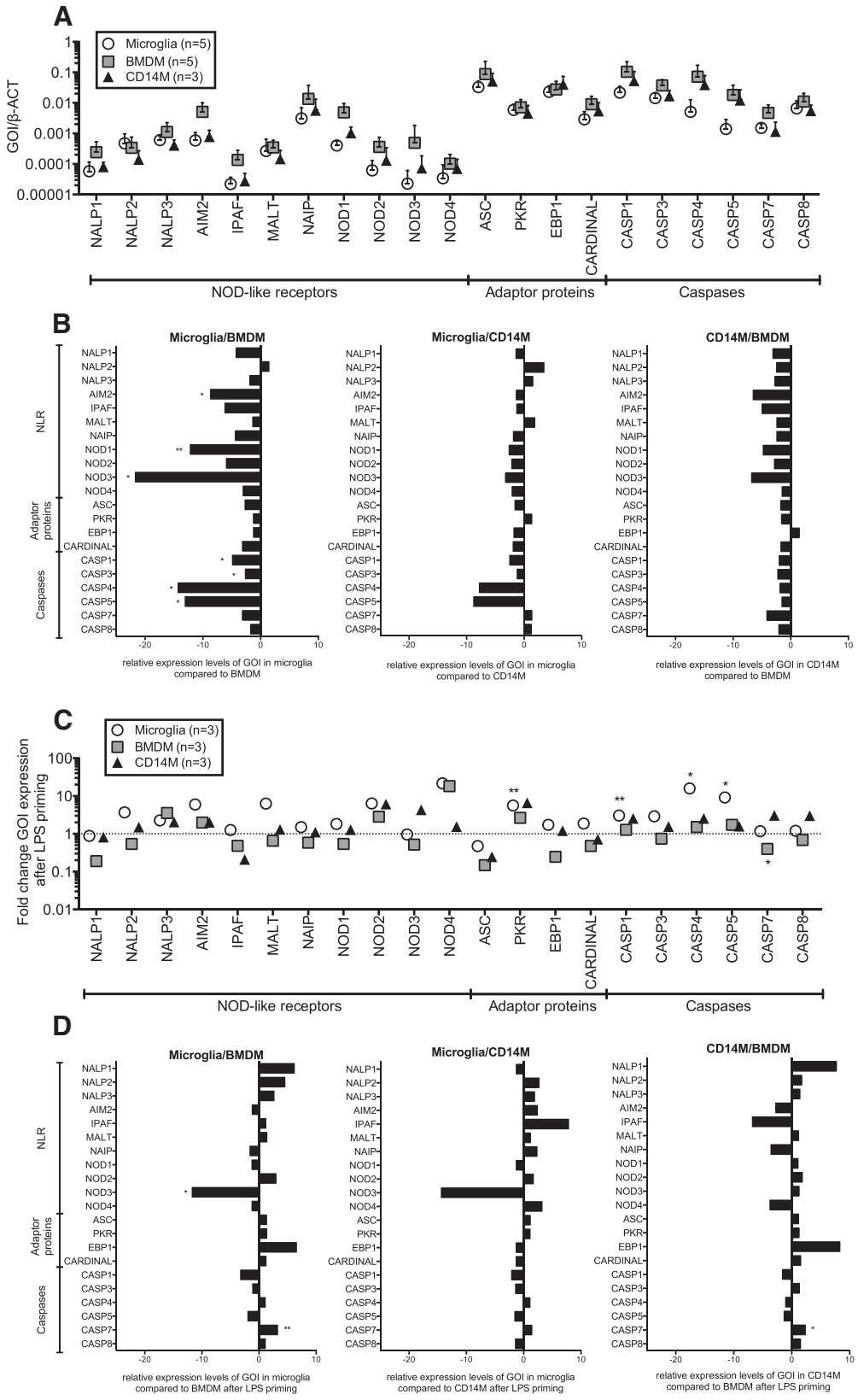

Figure 1. mRNA expression profile of inflammasome components in primary rhesus microglia, BMDMs, and CD14Ms. ***A***, mRNA expression levels of NLRs, adaptor proteins, and caspases in primary rhesus microglia, BMDMs, and CD14Ms. mRNA expression levels of genes of interest (GOI) are expressed relative to reference gene [β-actin (β-ACT)] mRNA expression levels. Data are represented as means ± SD. NALP4-NALP11, NALP13, and NALP14 mRNA expression levels were below detection limits and low expression levels of NALP12 prohibited reliable quantification of all samples. ***B***, Relative mRNA expression levels compared between microglia and BMDMs, between microglia and CD14Ms, and between CD14Ms and BMDMs. ***C***, Fold increases in relative mRNA expression levels of NLRs, adaptor proteins, and caspases after 16 h exposure to LPS (100 ng/ml) in microglia, BMDMs, and CD14Ms. Data are represented as mean values. ***D***, Relative mRNA expression levels after 16 h exposure to LPS (100 ng/ml) compared between microglia and BMDMs, between microglia and CD14Ms, and between CD14Ms and BMDMs. *$p < 0.05$, **$p < 0.01$; ANOVA with Tukey's HSD correction.

comparable, microglia expressed significantly lower levels of AIM2, NOD1, NOD3, caspase 1, 3, 4, and 5-encoding mRNA transcripts compared to BMDMs. In addition, levels of caspase 4 and 5-encoding mRNA transcripts were lower in microglia when compared with CD14Ms, although this was not significant.

As *in vitro* inflammasome activation is most often monitored in cells that have been primed with a TLR agonist (Martinon et al., 2002; Netea et al., 2009; Zhou et al., 2011), we also analyzed mRNA expression levels after overnight exposure to the TLR4 agonist LPS. Comparison to nonstimulated cells revealed LPS-induced changes in mRNA expression levels of many inflammasome components in all cell types (Fig. 1*C*). Priming of microglia significantly enhanced expression levels of PKR and caspase 1, 4, and 5 transcripts. Comparison of relative expression levels between cell types after LPS priming revealed that the mRNA expression profile of microglia now more closely resembled that of hematopoietic macrophages (Fig. 1*D*). The expression levels of transcripts encoding caspase 1, 3, 4, and 5 were now similar in all macrophages, but LPS-primed microglia still expressed markedly lower levels of NOD3-encoding transcripts compared with BMDMs and CD14Ms.

For functional analysis of inflammasome-mediated activation in microglia and BMDMs, cells were primed with different TLR agonists before triggering the inflammasome with silica, MSU crystals, or ATP (Martinon et al., 2006; Zhou et al., 2011). Priming of microglia and BMDMs by exposure to TLR1/2, TLR4, or TLR8 agonists strongly induced pro-IL-1β-encoding mRNA expression levels, whereas exposure to a TLR3 agonist was much less effective (Fig. 2*A*). Consistent with literature (Halle et al., 2008; Netea et al., 2009), secretion of IL-1β protein in response to TLR priming alone was $<$500 pg/ml or below detection levels both in microglia as well as in BMDMs (Fig. 2*B*). Exposure of microglia and BMDMs that were primed with the TLR4 agonist LPS to inflammasome activators strongly induced processing and secretion of IL-1β (Fig. 2*C*,*D*). Whereas the physiological relevance of MSU and ATP for microglia is clear (Shi et al., 2003; Rock and Kono, 2008; Euser et al., 2009; Hanamsagar et al., 2011; Maetzler et al., 2011; McFarland et al., 2013; Martins et al., 2014), it is less likely that they will encounter silica. However, as silica and MSU were more potent in triggering inflammasome-mediated activation in microglia than ATP, we chose to continue our studies with these stimuli.

We first characterized the influence of priming on inflammasome-mediated activation by varying the length of the priming period before inflammasome activation. In microglia, silica-induced IL-1β secretion was detectable from 2 h after LPS priming onwards and increased gradually for $\leq$12 h after LPS priming (Fig. 3*A*). Priming for $>$16 h led to lower levels of secreted IL-1β. In BMDMs, silica-induced IL-1β secretion was detected as early as 1 h after LPS priming. Much higher levels of secreted IL-1β were obtained if BMDMs were primed with LPS for 6–8 h, whereas priming for $>$8 h led to lower levels of secreted IL-1β (Fig. 3*A*). Similar differences in kinetics were found between microglia and CD14Ms and also applied to MSU-induced inflammasome activation. Analysis of the ratio of inflammasome-induced secretion of IL-1β after 16 h of LPS priming to inflammasome-induced secretion of IL-1β after 4 h of LPS priming confirmed that these kinetics differ significantly between microglia and hematopoietic macrophages (Fig. 3*B*).

We examined whether the different kinetics of IL-1β secretion in microglia could be attributed to differences in sensitivity to LPS-induced signaling. However, basal expression levels of TLR2-, TLR4-, CD14-, and MyD88 (myeloid differentiation primary response 88)-encoding transcripts were similar for microglia and other macrophages (Fig. 4*A*). In addition, priming of microglia and BMDMs with different concentrations of LPS resulted in similar differences in IL-1β secretion profiles between microglia and BMDMs (Fig. 4*B*). Finally, we assessed pro-IL-1β-encoding mRNA levels in more detail after LPS priming (Fig. 4*C*). Pro-IL-1β mRNA transcripts were strongly induced already after 1 h of LPS priming in both microglia and BMDMs. However, while pro-IL-1β mRNA transcript levels were downregulated already 4 h after LPS priming in BMDMs, they remained high in microglia. Analysis of other cytokine-encoding mRNA transcripts after priming with LPS demonstrates that the lack of negative regulation on pro-IL-1β transcripts also applied to IL-1α and IL-8 (Fig. 4*C*). Other LPS-inducible transcripts like those of IL-1Rα (IL-1 receptor antagonist), IL-6, and TNF-α (tumor-necrosis factor α) were subject to regulation in both microglia as well as in BMDMs.

To examine the processing and secretion of IL-1β in more detail, we first monitored the activation of caspase 1, the principal inflammasome-activated caspase described (Martinon and Tschopp, 2004). To our surprise, silica-induced caspase 1 activation could not be detected in microglia that were primed with LPS for 4 h. Only after 16 h of LPS priming some caspase 1 activation could be observed (Fig. 5*A*). This was in marked contrast to BMDMs, where robust silica-induced caspase 1 activation was detectable 4 h after LPS priming, while longer priming resulted in decreased caspase 1 activation. Western blot analysis of caspase 1 expression revealed that although both microglia and BMDMs expressed caspase 1, BMDMs expressed an additional isoform at 30 kDa and an additional high molecular-weight band at 55 kDa, indicative of a protein complex (Fig. 5*B*). In addition, Western blot analysis revealed that both microglia and BMDM expressed approximately similar amounts of the inflammatory caspases 4 and 5 under basal conditions and after LPS priming. Similar to caspase 1, BMDMs expressed an additional isoform of caspase 4 at 40 kDa. To assess their functional involvement in the secretion of IL-1β, we specifically inhibited caspase 1, 4, and 5 before activating the inflammasome (Fig. 5*C*). In LPS-primed microglia and BMDMs, inhibition of caspase 1 inhibited silica-induced IL-1β secretion by $\leq$46 and 59% respectively. Inhibition of caspase 4 and 5 inhibited silica-induced IL-1β secretion in microglia by $\leq$41 and 53% respectively, compared with $\leq$84 and 86% inhibition in BMDMs respectively. Whereas inhibition of caspase 4 and 5 in BMDMs was clearly dose dependent, this was not the case for microglia, suggesting that exposure to even higher concentrations of caspase inhibitors would not lead to a further reduction in silica-induced IL-1β secretion in microglia. This could not be directly tested, as the necessary dissolvent controls affected cellular homeostasis (data not shown). However, simultaneous inhibition of caspase 1, 4, and 5 inhibited silica-induced IL-1β secretion in microglia by $\leq$38%, whereas this inhibited silica-induced IL-1β secretion in BMDMs by $\leq$84%. Together these data indicate that microglia are less dependent on inflammatory caspases than BMDMs for the secretion of silica-induced IL-1β.

Discussion

Although microglia have long been considered similar to other myeloid macrophages, it is becoming more and more apparent that they differ in many respects, as illustrated by the distinct roles that resident microglia and peripheral macrophages play in CNS injury (Raivich and Banati, 2004; Greenhalgh and David, 2014). Differences in regulation of innate immune responses (Xiao et al., 2013) and alternative use of caspases have been described for

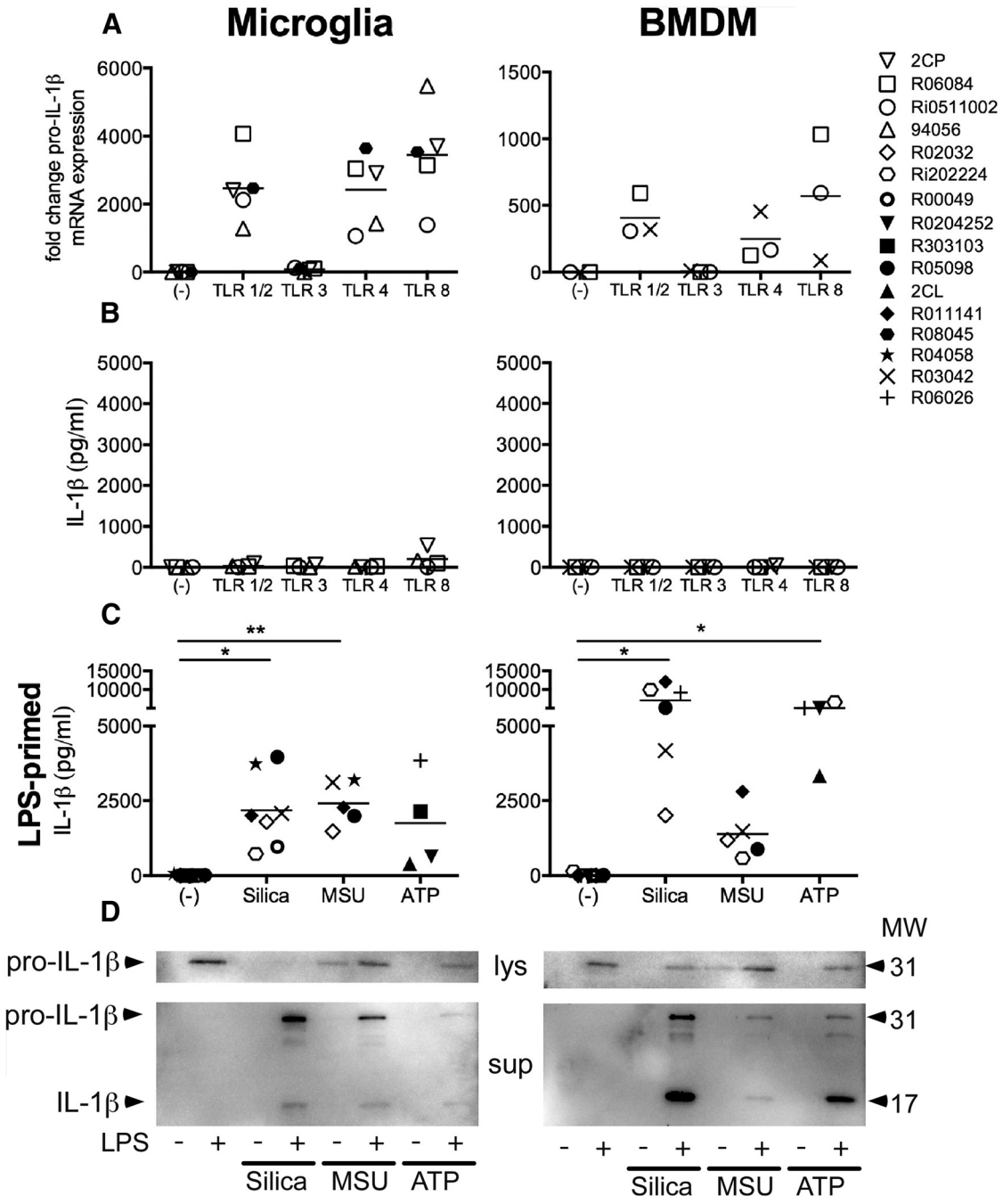

Figure 2. TLR-mediated priming of microglia and BMDMs induces the expression of pro-IL-1β-encoding mRNA, but processing and secretion of IL-1β is measured only after inflammasome activation. ***A***, Relative pro-IL-1β-encoding mRNA expression levels in primary rhesus microglia and BMDMs. Cells were exposed for 16 h to TLR1/2 (PAM_3CSK_4, 500 ng/ml), TLR3 (polyiosinic-polycytidylic acid, 20 μg/ml), TLR4 (LPS, 100 ng/ml), and TLR8 (CL075, 1 μg/ml) agonists. ***B***, Levels of secreted IL-1β by TLR-primed microglia and BMDMs. ***C***, Levels of secreted IL-1β by LPS-primed (100 ng/ml, 4 h) microglia and BMDMs exposed to inflammasome inducers silica (500 μg/ml, 6 h), MSU (150 μg/ml, 6 h), or ATP (5 mM, 6 h). *$p < 0.05$, **$p < 0.01$; paired *t* tests with Bonferroni's correction compared with unstimulated cells (-). ***D***, Western blot analysis of pro-IL-1β and cleaved IL-1β protein in cell lysates (lys) and supernatant (sup) of LPS-primed (100 ng/ml, 4 h) microglia and BMDMs that were exposed to silica (500 μg/ml, 6 h), MSU (150 μg/ml, 6 h), or ATP (5 mM, 6 h). Arrows indicate molecular weight (MW) in kilodaltons.

microglia in earlier studies (Burguillos et al., 2011). Our data now identify new and important cell type-specific differences in inflammasome-mediated responses.

Results from this study demonstrate that microglia and hematopoietic macrophages are in principle endowed with similar inflammasome machinery. Published or online datasets on gene expression profiles of rodent microglia and macrophages (Chiu et al., 2013; Hickman et al., 2013; Butovsky et al., 2014) did not reveal differences in expression levels of inflammasome components between microglia and hematopoietic macrophages and are thus in line with most of our results. However, we report here that there are relatively large differences for two transcripts in particular. Resting microglia express less caspase 4 and 5 than resting BMDMs and CD14Ms (Fig. 1*A*). As caspase 4 and 5 are not encoded for in rodents, which express the orthologue caspase 11 (Martinon and Tschopp, 2004), this is a new finding that underlines the added value of studies using primate material.

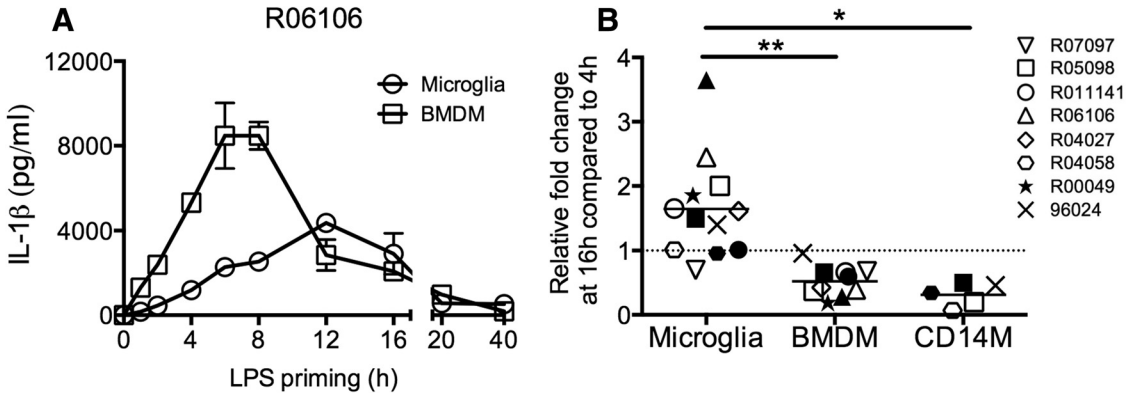

Figure 3. Kinetics of IL-1β secretion by LPS-primed microglia differ from that by LPS-primed hematopoietic macrophages. ***A***, Silica-induced (500 μg/ml, 6 h) IL-1β secretion in LPS-primed (100 ng/ml, indicated time points) microglia and BMDMs. Data of one representative donor of $\geq$3 independent experiments are shown and are represented as mean $\pm$ SD. ***B***, Analysis of the ratio of inflammasome-induced IL-1β secretion after 16 h to inflammasome-induced IL-1β secretion after 4 h. Open symbols, Silica-induced IL-1β secretion; closed symbols, MSU-induced IL-1β secretion; $*p < 0.05$, $**p < 0.01$; paired *t* tests with Bonferroni's correction compared with microglia.

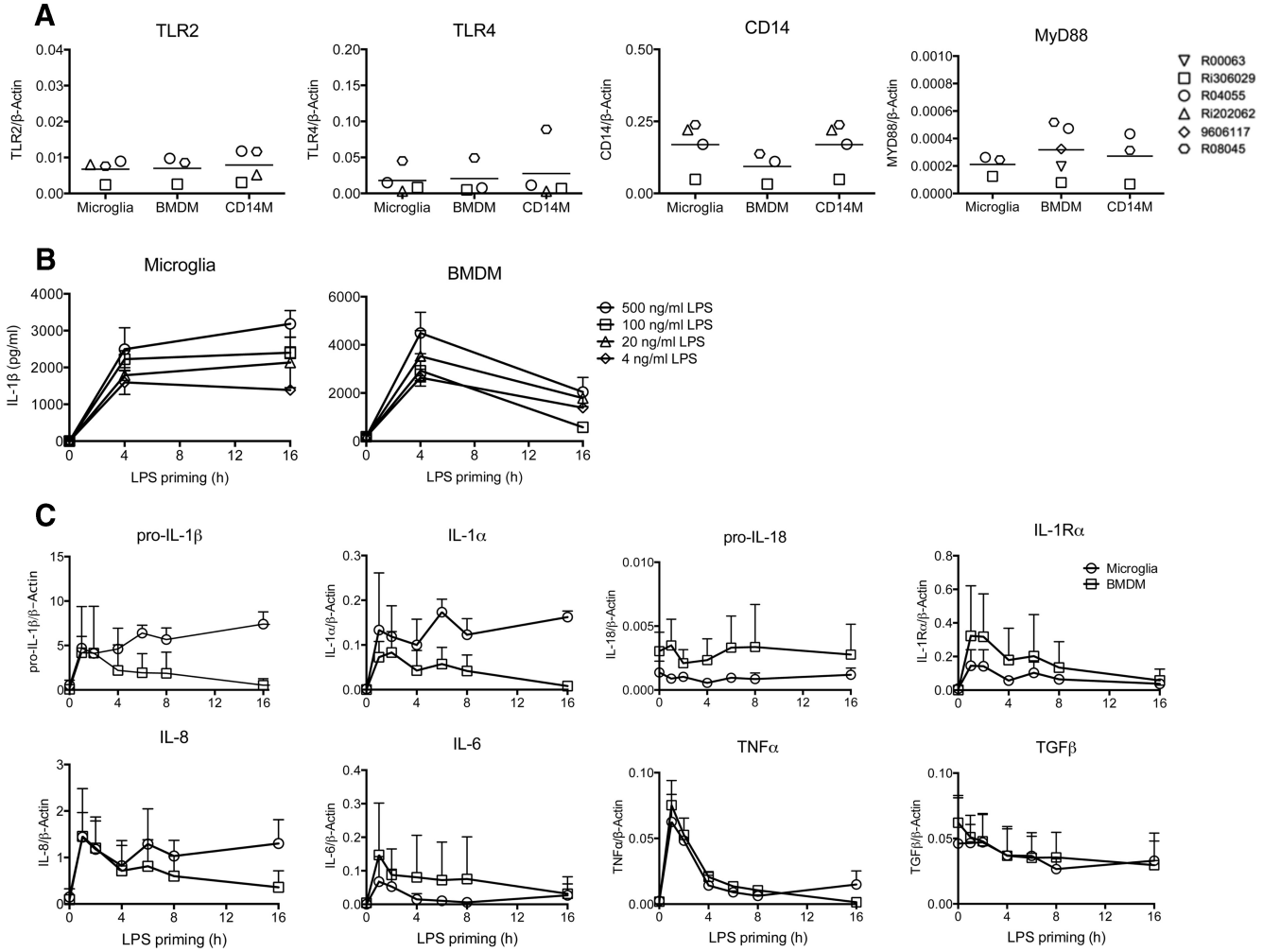

Figure 4. Negative regulation of pro-IL-1β transcription is impaired in microglia. ***A***, Relative mRNA expression levels of TLR2, TLR4, CD14, and MyD88 in unprimed microglia, BMDMs, and CD14Ms. ***B***, Silica-induced (500 μg/ml, 6 h) IL-1β secretion of microglia and BMDMs primed with different concentrations of LPS (4 –500 ng/ml, 4 or 16 h). ***C***, Relative pro-IL-1β, IL-1α, pro-IL-18, IL-1Rα, IL-8, IL-6, TNFα, and TGFβ (transforming growth factor β) mRNA expression levels in LPS-primed (100 ng/ml, indicated time points) microglia ($n = 3$) and BMDMs ($n = 3$). Data are represented as mean $\pm$ SD.

Furthermore, we show that although microglia and hematopoietic macrophages respond to the same innate stimuli, microglial IL-1β secretion is optimal when longer priming periods are used and is more persistent when compared with hematopoietic macrophages. Together, our data demonstrate that the latter is due to a lack of negative regulation at the transcriptional level. This not only applies to LPS-induced pro-IL-1β mRNA expression levels, but also to IL-1α and IL-8 mRNA expression levels.

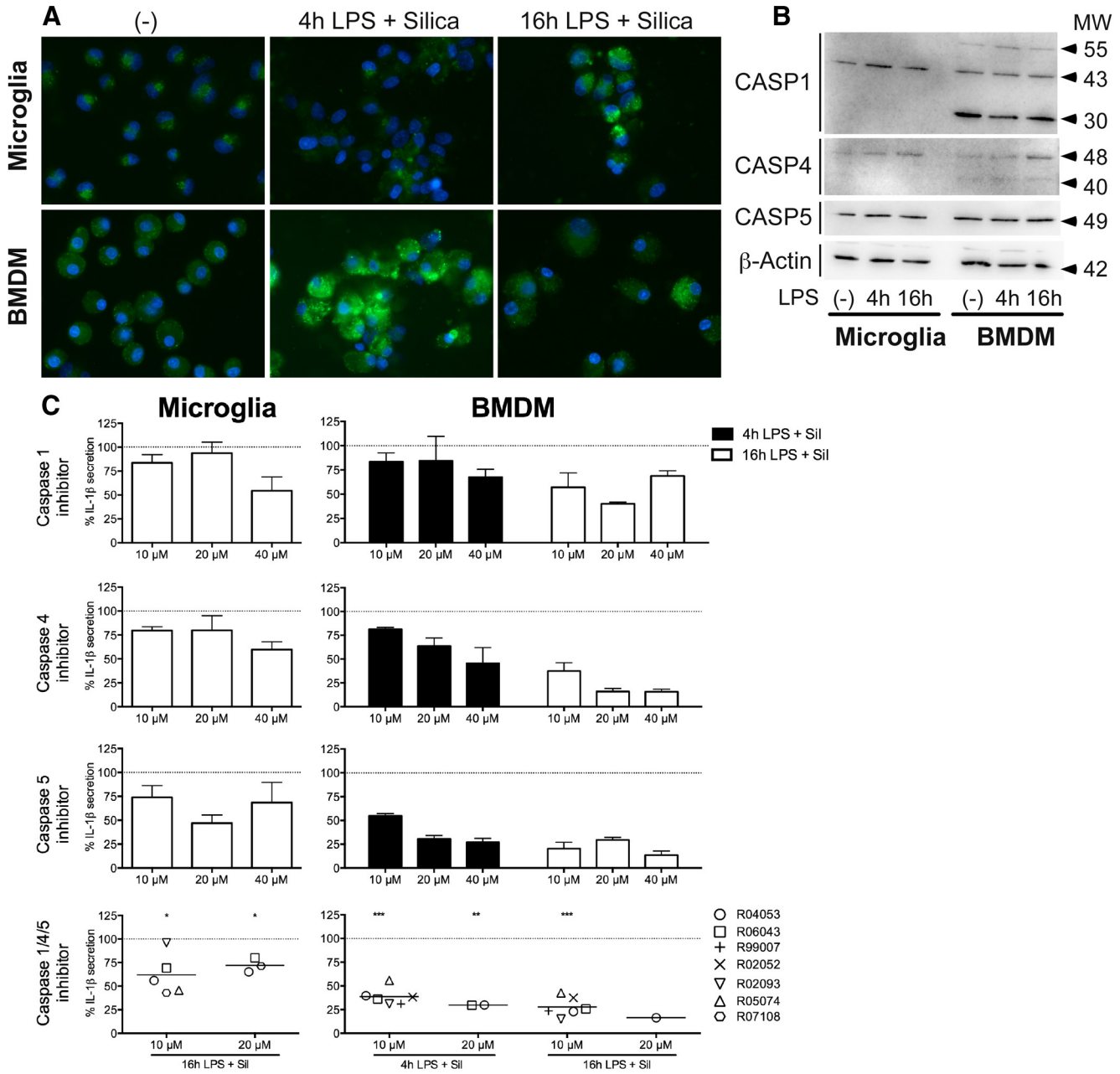

Figure 5. Silica-induced IL-1β secretion in microglia is only partially dependent of caspase 1, 4, and 5. ***A***, Silica-induced (500 μg/ml, 30 min) caspase 1 activation in LPS-primed (100 ng/ml, indicated time points) microglia and BMDMs. Caspase 1 activation was assessed using fluorochrome inhibitor of caspase (FLICA) assays and results in a green fluorescent signal. Nuclei are counterstained with DAPI. Original magnifications 400×. ***B***, Western blot analysis of caspase 1, 4, and 5 expression in cell lysates of unprimed and LPS-primed (100 ng/ml, indicated time points) microglia and BMDMs. Arrows indicate molecular weight (MW) in kilodaltons. ***C***, Analysis of silica-induced (500 μg/ml, 6 h) IL-1β secretion by LPS-primed (100 ng/ml, indicated time points) microglia and BMDMs in the presence of 10 – 40 μM specific caspase inhibitors or a combination of caspase 1, 4, and 5 inhibitors (10 –20 μM each, all applied 15 min before silica activation). Data of one representative donor of $\geq$3 independent experiments are shown for the separate caspase inhibitors, while data of multiple donors are shown for the combination of the caspase inhibitors. IL-1β secretion is represented relative to DMSO controls (dotted line, 100%). DMSO or caspase inhibitors alone did not induce IL-1β secretion in unprimed or LPS-primed microglia and BMDMs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; paired *t* tests with Bonferroni's correction compared with DMSO controls.

Transcription factor analysis (Champion ChiP Transcription Factor Search Portal, Qiagen; Lee et al., 1987; Cogswell et al., 1994; De Bosscher et al., 2003) revealed that the promoter regions of these cytokines share binding sites for the transcription factors NFκB, AP-1, C/EBPβ (CCAAT/enhancer binding protein β), and glucocorticoid receptor. Many proteins have been described that regulate the activation of these transcription factors, including regulatory proteins A20, SOCS1, TRIAD3A, SHP1, TOLLIP, IRAK-M (IL-1 receptor-associated kinase M), PELI1 (pellino E3 ubiquitin protein ligase 1), FLN29, and MAPK phosphatases (Bajramovic, 2011). In microglia specifically, PELI1 can positively regulate NFκB signaling (Xiao et al., 2013). However, mRNA expression levels of PELI1 in microglia and hematopoietic macrophages were similar (data not shown), rendering it unlikely that this can directly explain the observed lack of negative regulation in microglia. Interestingly, NOD3 (also called NLRC3) has been found to negatively regulate NFκB activation (Schneider et al., 2012). As our mRNA expression analysis revealed that microglia constitutively express much lower levels of NOD3-encoding transcripts than hematopoi-

etic macrophages, NOD3 is an interesting candidate for future studies.

Various studies using primary microglia describe inflammasome-induced IL-1β secretion in response to protein aggregates and pathogens as caspase 1 dependent (Halle et al., 2008; Meissner et al., 2010; Terada et al., 2010; Hanamsagar et al., 2011; Hafner-Bratkovič et al., 2012; Jamilloux et al., 2013; Lee et al., 2013; Walsh et al., 2014). Our results confirm that microglial secretion of IL-1β is dependent on caspase 1, but also demonstrate that this dependence is only partial. In addition, we demonstrate that although caspase 4 and 5 are involved in both microglia and hematopoietic macrophages, their relative contribution to inflammasome-induced IL-1β secretion appears to be less important in microglia. Our data on the simultaneous inhibition of caspase 1, 4, and 5 are in line with this notion. It has been reported that activation of caspase 4 and 5 can potentiate the activity of caspase 1 (Martinon et al., 2002; Sollberger et al., 2012) and this process might well be less efficient in microglia.

Overall our data show that microglia are less dependent than BMDMs on inflammatory caspases for the processing and secretion of IL-1β and suggest that they also employ other mechanisms. Alternative mechanisms that have been described for IL-1β processing and secretion include activation of a noncanonical caspase 8 inflammasome (Gringhuis et al., 2012; Antonopoulos et al., 2013) and inflammasome-independent mechanisms, such as matrix metalloproteinases (Schönbeck et al., 1998), cathepsins (Edye et al., 2013), and serine proteases (Joosten et al., 2009; Stehlik, 2009; Karmakar et al., 2012; Cassel et al., 2014). Preliminary results from our laboratory indicate that inhibition of caspase 8 does not affect silica-induced IL-1β secretion in either microglia or BMDMs (data not shown). The relative contribution of other pathways in microglial IL-1β secretion remains to be investigated.

This study, describing cell type-specific differences in the negative regulation of pro-IL-1β expression and in the enzymatic processing of pro-IL-1β, concurs with recently described fundamental differences in inflammasome-mediated activation of monocytes and macrophages (Netea et al., 2009). Whether such differences are cell inherent (Ginhoux et al., 2010) or induced by prolonged exposure to the neuronal microenvironment is currently unknown. It also remains to be demonstrated how the differences described here translate to tissue-specific responses to chronic or acute cellular stress *in vivo*. Currently, inhibition of caspase 1 activation is considered a therapeutic strategy to reduce inflammation in neuroinflammatory diseases (Rabuffetti et al., 2000). Our results suggest that this strategy might only be partially effective on microglia. To develop therapeutic strategies that target IL-1β processing and secretion in microglia specifically, it is important to delineate the additional mechanisms used by microglia for the secretion of IL-1β.

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