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# Extracellular poly(ADP-ribose) is a pro-inflammatory signal for macrophages

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## Summary

Poly(ADP-ribose) polymerase 1 (PARP1) synthesizes poly(ADP-ribose) (PAR), an essential post-translational modification whose function is important in many cellular processes including DNA damage signalling, cell death, and inflammation. All known PAR biology is intracellular, but we suspected it might also play a role in cell-to-cell communication during inflammation. We found that PAR activated cytokine release in human and mouse macrophages, a hallmark of innate immune activation, and determined structure-activity relationships. PAR was rapidly internalized by murine macrophages, while the monomer, ADP-ribose, was not. Inhibitors of TLR2 and TLR4 signaling blocked macrophage responses to PAR, and PAR induced TLR2 and TLR4 signaling in reporter cell lines suggesting it was recognized by these TLRs, much like bacterial pathogens. We propose that PAR acts as an extracellular "Damage Associated Molecular Pattern" (DAMP) that drives inflammatory signaling.

## Introduction

Poly (ADP-ribose) (Figure 1A), a unique post-translational modification, is synthesized by poly(ADP-ribose) polymerases (PARPs). ADP-ribose subunits are attached to acceptor proteins using $NAD^+$ as a substrate (Diefenbach and Burkle, 2005). Additional ADP-ribose subunits are added to elongate the chain (Altmeyer et al., 2009). Chain length is variable and up to 200 units in length *in vitro* (D'Amours et al., 1999). Inside cells, PAR chains are rapidly cleaved by poly(ADP-ribose) glycohydrolase (PARG), TARG1, and other hydrolases such as phosphodiesterases (PDE) (Figure 1A) (Diefenbach and Burkle, 2005;



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The authors have no conflicts of interest to report.

**Author Contributions**

K.A.K., S.K., and T.J.M designed the study. K.A.K and S.K. performed the experiments and analyzed the data. E.T. prepared the purified and fractionated PAR and labeled ADPr and PAR. Z.M. prepared the human cells. K.A.K., S.K., and T.J.M wrote the manuscript.

Hassa and Hottiger, 2008; Perina et al., 2014). However, PAR is chemically quite stable. It is stable in 1 M NaOH (Tan et al., 2012) and might persist longer in the extracellular space. Notably, all proposed functions of PAR are inside the cell. Possible extracellular biology has not been investigated, to our knowledge.

PAR and PARPs have been most studied in the DNA damage response. PARP1, the most abundant family member, is activated by direct binding to strand breaks (Langelier et al., 2012; Tallis et al., 2014), increasing PARP1 activity 10–500 fold (D'Amours et al., 1999). Activation leads to modification of PARP1 itself and other proteins in the DNA repair pathway (Chapman et al., 2013; Daniels et al., 2014; Jungmichel et al., 2013; Zhang et al., 2013). It has been hypothesized that excessive DNA damage leads to PARP1 dependent cell death via necrosis (Ha and Snyder, 1999), whereas, PARP1 is cleaved and inactivated early in apoptosis (Kaufmann et al., 1993).

We hypothesized that PAR acts as a signaling molecule alerting the innate immune system to necrotic cells. PAR shares some structural motifs with DNA and ATP, both of which, when exposed to the extracellular space, stimulate phagocytosis via monocyte-derived dendritic cells (Cohen and Mosser, 2013; Haag et al., 2007; Kroemer et al., 2013). Could PAR be a substrate for extracellular receptors and act as an additional signal? To test this hypothesis we treated mouse macrophages and primary human macrophages with purified PAR and found that PAR activated both mouse and human macrophages. This study suggested that extracellular PAR could promote phagocytosis of PAR-modified cell debris and inflammatory cytokine production by immune cells.

## Results

### Extracellular PAR activates a mouse macrophage cell line

Macrophages respond to infection or cellular damage by engulfing foreign cells, dead cells or debris. They detect molecular patterns and in response, secrete pro- or anti-inflammatory cytokines, orchestrating innate immune and inflammatory responses (Murray and Wynn, 2011). To determine if extracellular PAR could activate macrophages, we treated a standard mouse macrophage cell line (RAW264.7) with enzymatically synthesized and purified PAR (Tan et al., 2012) in the media and measured secretion of 23 cytokines (Figure 1B). PAR strongly stimulated secretion of TNFα, MCP-1, eotaxin, MIP-1α, and MIP-1β all pro-inflammatory cytokines that are stimulated by the pathogen-associated molecular patterns (PAMPs) lipopolysaccharide (LPS), CpG DNA, and poly(I-C) RNA, known activators of innate immune responses (Caskey et al., 2011; Häcker et al., 2002).

RAW264.7 cells are sensitive to low levels of bacterial endotoxins such as LPS. To exclude the possibility that our purified PAR had endotoxin contamination, we digested purified PAR with snake venom phosphodiesterase (PDE) or bovine PARG and treated RAW264.7 cells with the digested polymer. Neither PDE nor PARG alone induced TNFα secretion when added to cells, and PAR digestion led to reduced TNFα and MCP-1 secretion upon treatment (Figure 1B,C, Figure S1). We concluded that macrophage activation was not caused by endotoxin contamination.

TNFα showed the most robust and PAR-specific response in a panel of 23 mouse cytokines (Figure 1B). It is also an extremely important pro-inflammatory cytokine in humans. We focused on experiments using TNFα secretion as a read-out. First, we treated RAW264.7 cells with increasing concentrations of PAR for 2 and 4 hours and measured the secretion of TNFα. TNFα secretion showed a robust time and dose-dependence for PAR (Figure 1D). PAR concentrations in all plots are expressed in units of the monomer, ADP-ribose.

We next explored the structure-activity relationship between PAR and immune cell activation. We observed no TNFα secretion with single ADP-ribose subunits, nicotinamide, or $NAD^+$. We also tested poly-deoxyriboadenosine (poly-dA) and poly-riboadenosine (poly-rA) that were 20 units in length and cyclic ADP-ribose (cADPr), a known secondary messenger. These molecules have similar structural motifs to PAR but did not induce TNFα secretion (Figure 2A,B). These experiments suggested that RAW264.7 macrophages recognized a specific structural moiety found in PAR.

To better define the stimulatory motif for RAW264.7 cells, we explored size requirements for PAR-dependent activation of these cells. By treating cells with size fractionated PAR (Tan et al., 2012), we found that small (2–17mer), medium (18–40mer), and large (>40mer) polymers induced TNFα secretion (Figure 2C). Because PAR concentration is expressed in units of the monomer, the concentration of polymer decreases as size increases. This may account for the decrease in TNFα secretion seen with longer polymers. We also tested purified PAR dimers and trimers and both induced TNFα secretion as well as bulk polymer (Figure 2D).

We concluded that PAR activated RAW264.7 macrophages in a manner similar to known PAMPs. We also concluded that RAW264.7 cells recognized a structural motif found in a dimer of PAR, i.e. the shortest polymer that has the unique 2’ –1” ribose-ribose linkage characteristic of PAR (Figure 2A). Iso-ADP-ribose, the digestion product of PDE (Figure 1A), contains the 2’ –1” ribose-ribose linkage. The loss of activity of PDE digested PAR (Figure 1C, Figure S1) indicated that RAW264.7 cells recognized more than just the ribose-ribose linkage.

## PAR acts through toll-like receptors to activate macrophages

We next explored the mechanism of PAR-dependent TNFα secretion. TNFα secretion is often activated in response to signaling via toll-like receptors (TLRs), a major family of pattern recognition receptors (Akira and Takeda, 2004). TLR signaling often occurs during or after endocytosis of the PAMP bound to its cognate TLR (Takeda and Akira, 2005). To test if PAR is internalized by cells, we incubated RAW264.7 cells with PAR labeled with the TAMRA fluorophore and measured uptake. HPLC characterization of TAMRA-PAR is shown in Figure S2A. Cells incubated with TAMRA labeled ADP-ribose and then washed briefly exhibited no fluorescent signal, whereas cells incubated with TAMRA-PAR exhibited fluorescent signal within 15 minutes of incubation (Fig 3A,B). Similar results were seen with unlabeled PAR visualized by immunofluorescence (Figure S2B). Immunofluorescence of the PAR treated cells with the anti-PAR 10H antibody indicated that the internalized PAR remained intact (Figure S2B,D). To confirm that TAMRA-PAR behaved like unlabeled PAR, we measured TNFα secretion after TAMRA-PAR treatment.

TAMRA-PAR induced TNFα secretion and secretion was blocked by digestion of TAMRA-PAR with PARG (Figure S2E). Uptake of TAMRA-PAR was also blocked by pre-digestion of the polymer with PDE (Figure 3C,D).

The internalized PAR signal localized to punctate structures throughout the cytoplasm that concentrated near the nucleus. This is the morphology expected for endosome compartments. Staining with endosomal markers showed partial co-localization with the early endosomal marker EEA1 and no co-localization with the late endosomal marker LAMP1 (Figure 3E,F). We also presented ADP-ribose or PAR attached to latex beads and performed time-lapse imaging of the cells for 3 hours after addition of the beads to the media. RAW264.7 cells preferentially phagocytosed PAR labeled beads (Figure 3C, S3, Movies S1 and S2).

To test the role of TLRs, we treated cells with small molecules that block TLR signaling. Pre-treatment of cells with the oxidized phospholipid OxPAPC, which inhibits TLR2 and TLR4 by binding to specific adaptor proteins and preventing ligand binding (Erridge et al., 2008), dramatically reduced both TNFα secretion and labeling of cells with fluorescent PAR (Figure 4A-C). Chloroquine, an inhibitor of endosomal acidification that also disrupts TLR signaling by binding nucleic acid ligands (Kuznik et al., 2011), also reduced PAR-induced TNFα secretion but to a lesser extent than OxPAPC. Chloroquine also inhibited labeling of cells with TAMRA-PAR.

To test the requirement for TLR2 versus TLR4 we treated NfkB-luciferase reporter cell lines expressing either TLR2 or TLR4 with PAR. Both cell lines responded to PAR while a cell line expressing only the NFkB-luciferase reporter did not (Figure 4D) indicating that PAR can signal through both TLR2 and TLR4.

### Extracellular PAR activates specific human macrophage types

To test if PAR activates human primary immune cells, we isolated monocytes, precursors of macrophages, from human blood, differentiated them into macrophages (M0) with M-CSF and into M1 and M2 polarized macrophages using LPS/interferon-γ and IL-4 respectively. We treated the resulting four monocyte/macrophage types with PAR, and found that M0 and M2 macrophages responded to PAR by secreting TNFα, while monocytes and M1 macrophages did not (Figure 4E, F, S4). Secretion of TNFα from M0 and M2 macrophages was blocked by OxPAPC, suggesting a similar TLR2 or TLR4 dependent mechanism as seen in the mouse macrophage line (Figure 4E, S4B). We concluded that PAR is also a strong pro-inflammatory signal for certain primary human monocyte-derived lineages.

## Discussion

In this study, we found that highly purified PAR added to the media activated both mouse and human macrophages to secrete pro-inflammatory cytokines. The activation was dose-dependent and was strongly decreased by enzymatic pre-hydrolysis of PAR. It required a minimum of two subunits, suggesting that a cell surface receptor is recognizing, at least in part, the ribose-ribose linkage unique to PAR among standard biomolecules. TLRs are a main class of receptors for innate immune response activation in macrophages (Cohen and

Mosser, 2013; Takeda and Akira, 2005). The PAR-dependent cytokine profile closely resembled the profiles for known binders and activators of TLRs, LPS, CpG DNA, and poly(I-C) RNA (Wang et al., 2014). PAR triggered uptake of labelled beads or internalization of fluorescently labelled PAR, and an inhibitor of TLR2 and TLR4, OxPAPC, blocked PAR-dependent TNFα secretion and the uptake of fluorescently labelled PAR. PAR induced NFkB signalling in both TLR2 and TLR4 reporter cell lines indicating that PAR can signal through both receptors. OxPAPC almost completely blocked TNFα secretion suggesting that PAR mainly signalled through either TLR2 or TLR4.

An interesting question is whether, and if so how, PAR makes its way to the extracellular space. PAR antibodies have been found in the serum of Alzheimers and systemic lupus erythematosus patients (Kanai and Sugimura, 1981; Kanai et al., 2007) suggesting that PAR can become extracellular in humans. One model is that excessive activation of PARP1 through oxidative stress or DNA damage leads to cell death by necrosis. Necrotic death would expose PAR-modified acceptor proteins, or free PAR that has been cleaved off acceptors, to the extracellular space. PAR could also be made extracellularly. ART2, an enzyme found on the surface of T-cells, was recently shown to synthesize PAR (Morrison et al., 2006).

The recognition of PAR by macrophages may serve multiple biological purposes. One possibility is that this represents an alternative mechanism for activation of the innate immune response by the DNA damage response (DDR). DDR signalling is known to be involved in the production of pro-inflammatory signals from damaged cells (Chatzinikolaou et al., 2014). Unrepaired DNA damage can also trigger apoptosis generating many new DNA strand breaks through activation of the CAD nuclease (Samejima and Earnshaw, 2005). PARP1 itself is cleaved early during apoptosis (Kaufmann et al., 1993) for unknown reasons. An interesting possibility is that high PARP1 activity due to CAD-induced strand breaks would otherwise generate a lot of PAR, and if pro-inflammatory, this PAR would be problematic for the physiology of apoptosis. Apoptosis is thought to be a form of cell death that is not pro-inflammatory, unlike necrosis, which is highly pro-inflammatory.

### Significance

Overall, this study elucidated a novel extracellular function for PAR and provides an exciting connection between PAR, PARP activity, and the innate immune system. The link between PAR and the innate immune systems warrants further study and presents exciting possibilities for the role of PAR in inflammation and cancer.

## Materials & Methods

### Cell culture and reagents

RAW264.7 cells (ATCC), 293-NFkB-Luc, 293-TLR4HA-MD2-CD14-NFkB-Luc, 293-TLR2-FLAG-NFkB-Luc cell lines were grown in DMEM (Mediatech/Corning) with 10% FBS (Gibco) and 1% penicillin/streptomycin (Mediatech/Corning). Isolation and manipulation of primary human immune cells were adapted from Martinez, et. al. (Martinez et al., 2006). For more detail see supplemental.

PAR used in this study was purified and fractionated as previously described by Tan et al. (Tan et al., 2012). TAMRA (Life Technologies) labeled PAR was synthesized by coupling the carboxyl group on TAMRA with the free amine on PAR using EDC or 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Oakwood Products) as coupling reagents. For more details see supplemental. For PAR digestions, PAR was incubated with either 13 ng/ml bovine PARG (Enzo Life Sciences) or 2 ug/ml PDE (Affymetrix) for 2 hours at 37°C in 100mM Tris buffer pH 8.0.

### Multiplex cytokine analysis

RAW264.7 cells were seeded in 96-well plates and allowed to adhere overnight. Media was then exchanged for media containing either PAR, LPS (Sigma), CpG DNA (Invivogen) or poly(I-C) RNA (Invivogen). At each timepoint, media was collected and frozen at −80°C until further analysis. Secreted cytokines were measured using the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (BioRad) and the FlexMap3D (Luminex) following the manufacturer's instructions.

### TNFα ELISA assay

Cells were seeded in 96-well plates a day before treatment at a density of $1x10^5$ cells per ml. Media was exchanged for media containing PAR and other stimulants. After treatment, media was collected and stored at −80°C until analysis. Levels of secreted TNFα were measured using the BioLegend TNFα ELISA kit according to the manufacturer's instructions.

### NFkB reporter assay

Cells were seeded in 96-well plates a day before treatment at a density of $1x10^5$ cells per ml. Cells were treated with 30 μM PAR, 100 ng/ml LTA-BS (Invivogen), 1 ng/ml LPS-B5 ultrapure (Invivogen), or 30 μM ADPr for 24 hours. Luciferase activity was determined using the Promega Luciferase Assay system according to manufacturer's instructions.

### Imaging

For confocal imaging, Raw264.7 cells were seeded onto coverslips. After a day, cells were treated with TAMRA-labelled PAR for 30 min in a 37°C incubator and washed twice with PBS to remove residual PAR. Cells were fixed with 4% formaldehyde for 15–20 minutes at room temperature and kept in PBS during imaging or mounted with Prolong Gold Mounting Media (Invitrogen). Images at up to five random positions per condition were obtained. For quantification of PAR uptake, cell edges were outlined and fluorescent intensity was analysed in single cells using Image J.

For live cell imaging ADP-ribose (Sigma) and purified PAR were conjugated to 1 μm carboxylated latex beads (Sigma) using DMTMM as a coupling reagent (see supplemental). $2.5x10^5$ beads/ml were added to cells growing in MatTek glass bottom tissue culture plates and imaged.

See supplemental for more information on the microscope systems used.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- Poly(ADP-ribose) stimulates cytokine secretion in human and mouse macrophages
- Poly(ADP-ribose) activates phagocytosis in mouse macrophages
- A dimer of poly(ADP-ribose) is sufficient for macrophage activation
- TLR2 and TLR4 mediate poly(ADP-ribose) induced cytokine secretion

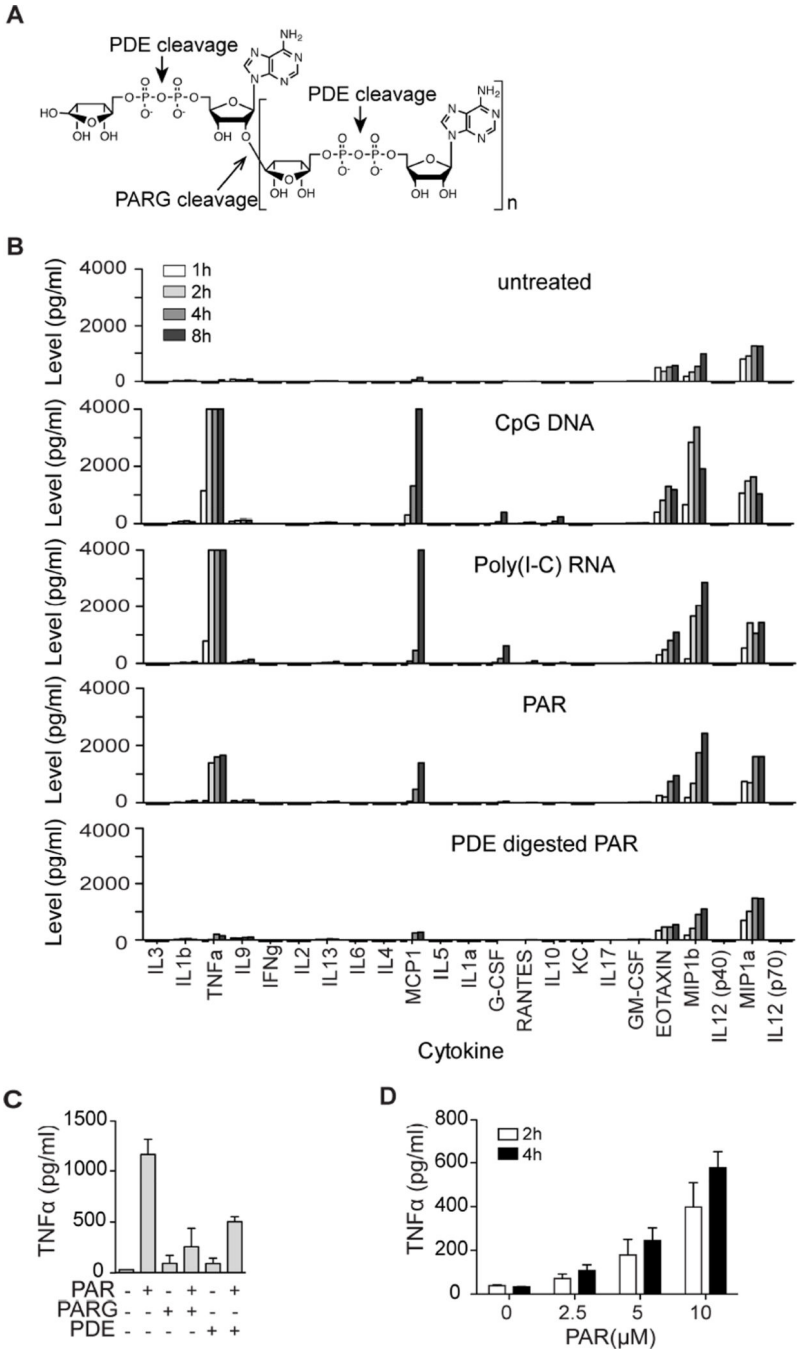


**Figure 1. PAR induces cytokine secretion in RAW264.7 cells**

A) Structure of PAR and cleavage sites for PARG and PDE. B) Multiplexed profiling of secreted mouse cytokines from RAW264.7 cells was performed in control cells or after treatment with 30 μM CpG DNA, 30 μM poly(I-C) RNA, 30 μM PAR, or 30 μM PAR digested with PDE. C) Cells were treated for 4 hours with PAR, PDE digested PAR, PDE alone, PARG digested PAR or PARG alone. Secreted TNFα is represented as the mean ± SD and n=4. D) RAW 264.7 cells were treated with PAR for given times. Secreted TNFα is represented as the mean ±SD and n=3.

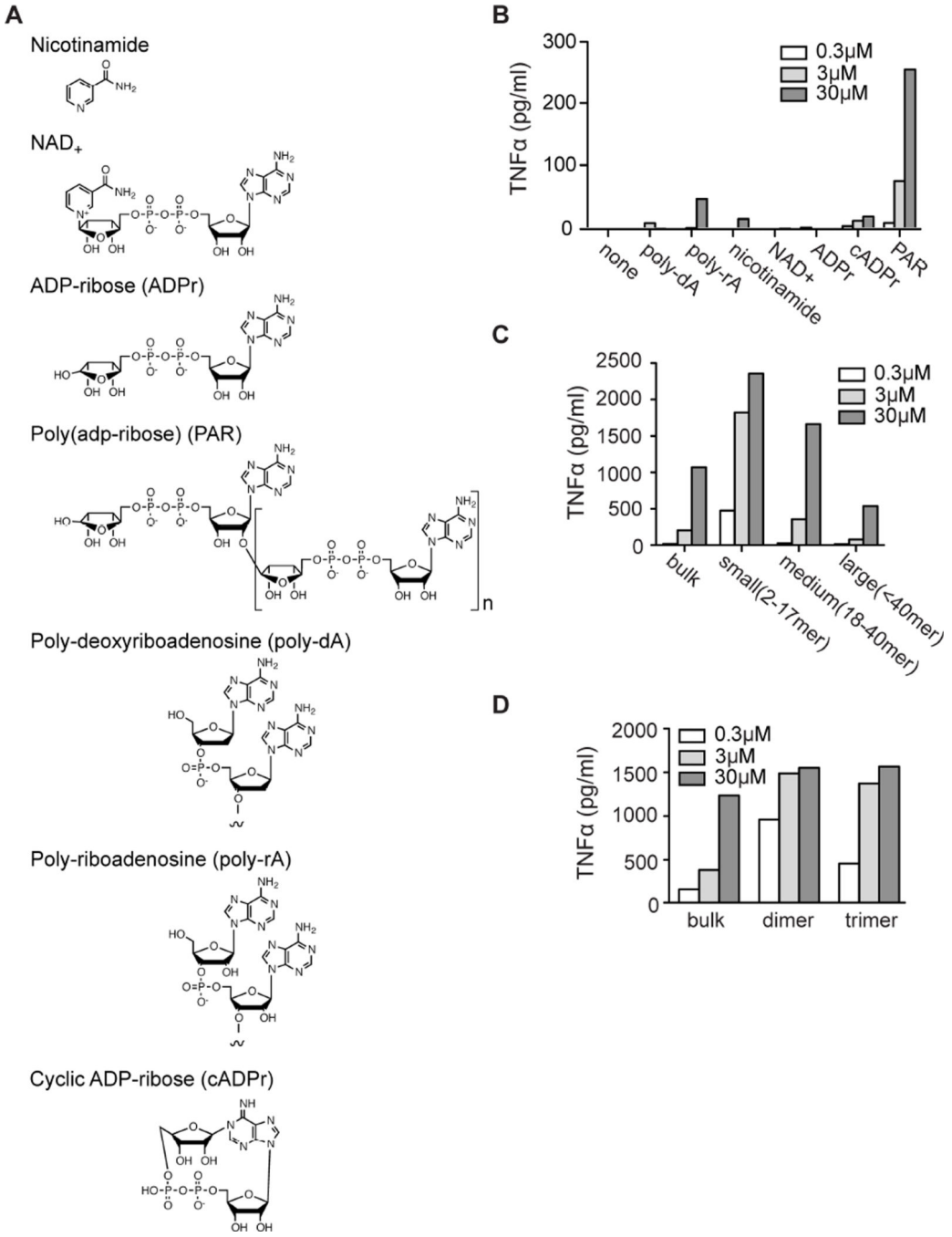


**Figure 2. A dimer of PAR is sufficient to induce TNFα secretion**

A) Structurally similar compounds to PAR were B) added to RAW264.7 cells and secreted TNFα was measured. Cells were treated for 4 hours. Data represent one of three independent experiments. C) Size fractionated PAR was added to cells for 4 hours before TNFα secretion was measured. Data represent one of two independent experiments. D) Bulk PAR, a dimer or a trimer of ADP-ribose was incubated with RAW cells for 4 hours before secreted TNFα was measured.

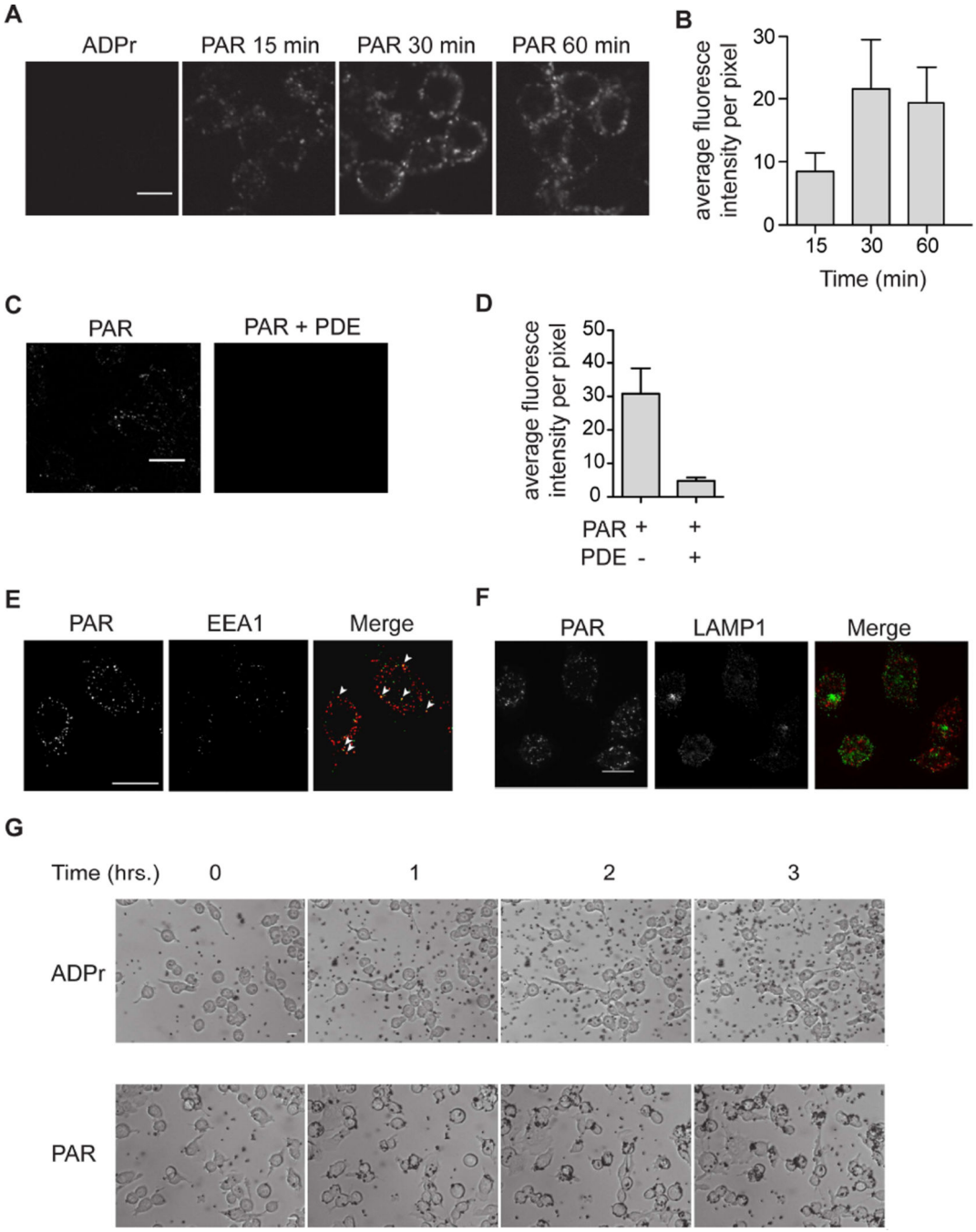


**Figure 3. PAR but not ADP-ribose is uptaken by RAW264.7 cells**

A) Raw264.7 cells were treated with 20 μg/ml TAMRA-PAR for indicated times. Representative images are shown with a scale bar of 10 μm. B) Quantification of uptake is shown as the mean intensity per cell ±SD with n≥20 cells. Data represent one of three independent experiments. C) Cells were treated with 20 μg/ml TAMRA-PAR or PDE digested TAMRA-PAR for 1 hour. Representative images are shown with a scale bar of 10 μm. D) Quantification of uptake is shown as mean intensity per cell ±SD with n≥25. E-F) Cells treated with 20 μg/ml TAMRA-PAR for 1 hour were subsequently stained for E)

EEA1 (BD Biosciences) or F) LAMP1 (Abcam). G) ADP-ribose (ADPr) or PAR conjugated latex beads were incubated with RAW264.7 cells and imaged for 3 hours at 1 minute intervals. Stills at given times are shown with a scale bar of 10 mm. See also Movies S1 and S2 and Figure S3 where bead uptake was quantified with confocal imaging.

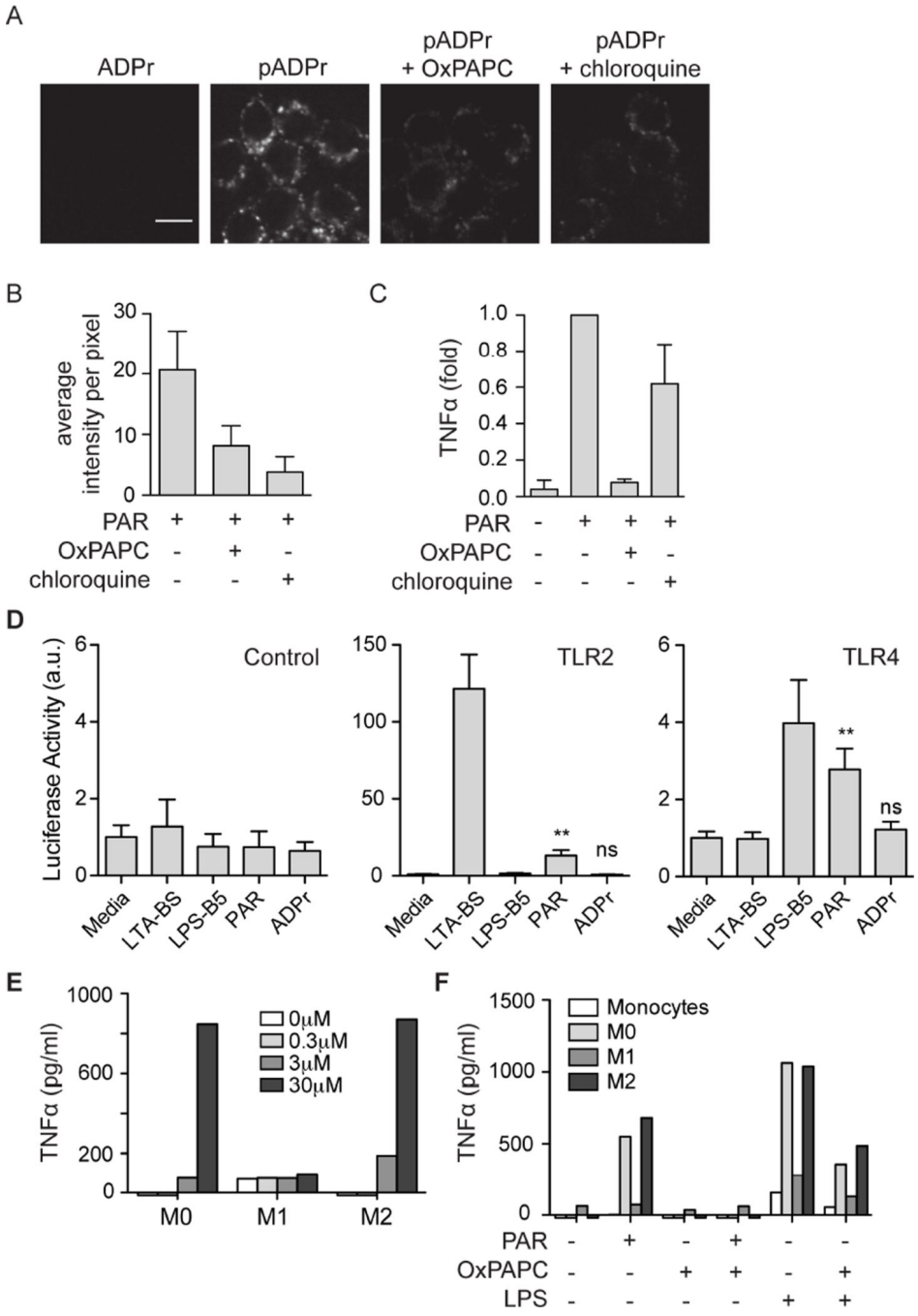


**Figure 4. TLR2/TLR4 are required for activation of both mouse and human macrophages**

A) Cells, pretreated with vehicle, 30 μg/ml OxPAPC, or 100 μM chloroquine for 1 hour, were incubated with TAMRA-PAR for 30 minutes. Scale bar represents 10 μm. B) Quantification from one of two independent experiments is shown as the mean intensity per cell ±SD with n≥20. C) Cells were pretreated with vehicle, 30 μg/ml OxPAPC, or 100 μM chloroquine for 1 hour and then incubated with PAR for 4 hours. Data represents mean TNFα secretion ±SD and n=4. D) 293 NFkB reporter cells expressing no receptor (control), TLR2, or TLR4 were treated with a TLR2 specific agonist, LTA-BS, a TLR4 specific

agonist, LPS-B5, PAR or ADPr. Luciferase activity was normalized by the mean of the media alone condition. Results are shown as the mean ±SD and n≥4. E) Primary human monocytes and macrophages were treated with PAR for 6 hours before media was collected and analyzed for TNFα. For the second of two independent experiments see Figure S4. F) Primary human monocytes and macrophages were pretreated with 30 μg/ml OxPAPC for 1 hour followed by 30 mM PAR or 1 μg/ml of LPS for 6 hours. For the second of two independent experiments see Figure S4.