SUPPLEMENTAL FIGURE AND VIDEO LEGENDS

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Supplemental Figure 1 – Supplemental Digital Content 2. ATP release through pannexin-1 (panx1) channels and autocrine P2 receptor signaling are required for LPS-induced p38 MAPK activation. ***A***, Primary human monocytes freshly isolated from the peripheral blood of healthy volunteers were pretreated with the panx1 inhibitor CBX (100 µM) or not for 5 min and stimulated with different TLR ligands (TLR1/2: Pam3CysSerLys4, 1 µg/ml; TLR2: heat-killed *Listeria monocytogenes*, 10^8 cells/ml; TLR3: Poly(I:C), 10 µg/ml; TLR4: LPS, 10 ng/ml; TLR5: flagellin, 10 µg/ml; TLR6/2: FSL-1, 1 µg/ml; TLR7: Imiquimod, 10 µg/ml; TLR8: ssRNA40, 10 µg/ml; TLR9: ODN2006, 5 µM) . After stimulation for 5 s, reactions were stopped by chilling in an ice bath. Cells were removed and ATP concentrations in the supernatants determined with a luciferase assay kit. ***B***, Monocytes were stimulated with LPS (10 ng/ml) and ATP concentrations in the cell culture supernatants were measured at the indicated time points. ***C***, Differentiated U937 macrophages were pretreated with the indicated concentrations of suramin for 5 min, stimulated with LPS (1 µg/ml) for 30 s, and p38 MAPK activation was assessed by immunoblotting and comparing the ratio between the phosphorylated and the total forms of p38 MAPK. Data shown are mean values ± SD of n=3 independent experiments; $^{*}P<0.05$ vs. unstimulated controls; $^{\#}P<0.05$ vs. stimulated cells in the absence of inhibitors.

Supplemental Figure 2 – Supplemental Digital Content 3. LPS triggers rapid ATP release and mitochondrial ATP production. ***A-B***, Human monocytes plated onto glass coverslips were stained with the cell membrane-targeting ATP probe 2-2Zn, stimulated with LPS (10 ng/ml), and changes in ATP levels at the cell surface and in the supernatant were imaged by fluorescence

microscopy. Representative images and fluorescence traces corresponding to the indicated regions of interest (ROI) are shown in **A**. Relative changes in mean fluorescence (average + SD) of n=4 separate experiments are shown in ***B***. 100x objective; scale bar: 10 µm; pseudocoloring as indicated by the calibration bar was used to monitor changes in fluorescence intensity. See also **Suppl. Video 1**, Supplemental Digital Content 7). ***C***, U937 macrophages plated onto glass coverslips were loaded with the mitochondrial Ca^{2+} probe Rhod-2-AM. Cells were stimulated with LPS (10 ng/ml) and changes in mitochondrial Ca^{2+} levels were monitored by measuring the Rhod-2 signal using live-cell fluorescence microscopy (objective: 100x oil; scale bar: 10 µm). ***D***, The Rhod-2 signals of different cells were averaged (n=15 cells) and plotted over time (mean ± SEM). Results are representative of n=3 separate experiments (see also **Suppl. Video 2**, Supplemental Digital Content 8). ***E***, U937 macrophages were stimulated with LPS (10 ng/ml) for the indicated times. Then samples were chilled in an ice bath, cells were centrifuged, supernatants removed, and intracellular ATP concentrations in cell lysates assessed. Data shown represent mean values ± SD of n=3 independent experiments; **P*<0.05 vs. unstimulated control.

Supplemental Figure 3 – Supplemental Digital Content 4. Mitochondrial inhibitors do not affect viability of monocytes. ***A***, Human PBMCs were treated with CCCP (10 µM), rotenone (10 µM) or oligomycin (10 µM) for 2 h and viability of monocytes was assessed by propidium iodide staining and flow cytometry. The gating strategy to identify live, propidium iodide negative CD14 positive monocytes (left) and averaged results (mean ± SD) of n=3 experiments (right) are shown. ***B***, Monocytes were isolated from PBMCs by adherence and stimulated with LPS (10 ng/ml) in the presence or absence of CCCP (1 µM) for 24 h. Viability was assessed by

propidium iodide staining and fluorescence microscopy. Data are means ± SD of n=3 experiments each comprising at least 100 cells (40x objective, NA 0.75).

Supplemental Figure 4 – Supplemental Digital Content 5. Flow cytometric analysis of cell activation and polarization to inflammatory phenotype of peripheral mouse monocytes in sepsis. C57BL/6 mice were subjected to cecal ligation and puncture (CLP) and CD11b and Ly6C expression by peripheral blood monocytes was measured using the gating strategy shown. Representative histograms demonstrate monocyte activation (CD11b) and polarization to inflammatory monocytes ($Ly6C^{++}$) before (0 h, grey) and at different times (6 h, blue; 24 h, red) after CLP.

Supplemental Figure 5 – Supplemental Digital Content 6. Proposed model of TLR sensing and inflammasome activation: TLR stimulation triggers rapid ATP release that fuels a panx1/ATP/P2Y2 receptor driven feed-forward signaling loop needed to amplify TLR danger signals. This purinergic sensing mechanism alerts cells to the presence of dangers, ignites mitochondrial ATP production, and promotes downstream signaling events that are needed for the initial host response to bacterial invasion. This initial TLR-induced signaling mechanism contributes to inflammasome priming by generating pro-IL-1β, pro-caspase1, and other building blocks that are needed for the assembly of the NLRP3 inflammasome complex. TLR signaling thereby sets the stage for inflammasome activation and for a full-blown inflammatory response to combat bacterial invasions, if needed.

SUPPLEMENTAL VIDEO LEGENDS

Supplemental Video 1 – Supplemental Digital Content 7. LPS stimulation rapidly increases ATP release from monocytes (related to **Suppl. Fig. 2**, ***A*** and ***B***, Supplemental Digital Content 3)**.** Human monocytes were seeded onto fibronectin-coated chambered glass slides, incubated with the cell membrane targeting fluorescent ATP probe 2-2Zn, and ATP release at the cell surface and into the supernatant was monitored by time-lapse fluorescence microscopy. LPS (10 ng/ml) was added after 20 s and ATP (1 mM) was added as a positive control after 2.5 min as indicated. Fluorescence images were captured at a frame rate of 60 frames per minute; objective: 100x oil (NA 1.39). Pseudocoloring was applied to show changes in extracellular ATP levels.

Supplemental Video 2 – Supplemental Digital Content 8. LPS stimulation rapidly increases mitochondrial Ca^{2+} influx (related to **Suppl. Fig. 2**, ***C*** and ***D***, Supplemental Digital Content 3). U937 cells were seeded onto fibronectin-coated chambered glass slides, differentiated to macrophages, loaded with the mitochondrial Ca^{2+} indicator Rhod-2, and mitochondrial Ca^{2+} uptake was recorded with time-lapse fluorescence microscopy. LPS (10 ng/ml) was added after 15 s. Fluorescence images were captured at a frame rate of 60 frames per minute; objective: 100x oil (NA 1.3).

Supplemental Video 3 – Supplemental Digital Content 9. Autocrine purinergic signaling upregulates mitochondrial activity following LPS stimulation (related to **Fig. 2** ***C***). U937 cells were prepared for monitoring of mitochondrial Ca^{2+} uptake as described for **Suppl. Video 2**. LPS (10 ng/ml) alone or in combination with CCCP (10 µM), CBX (100 µM), or suramin (100

μM) was added and fluorescence images were captured at a frame rate of 60 frames per minute; objective: 100x oil (NA 1.3).