Comprehensive Supplemental Information

Dual engagement of the NLRP3 and AIM2 inflammasomes by plasmodial-derived hemozoin and DNA during malaria

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Fig. S1

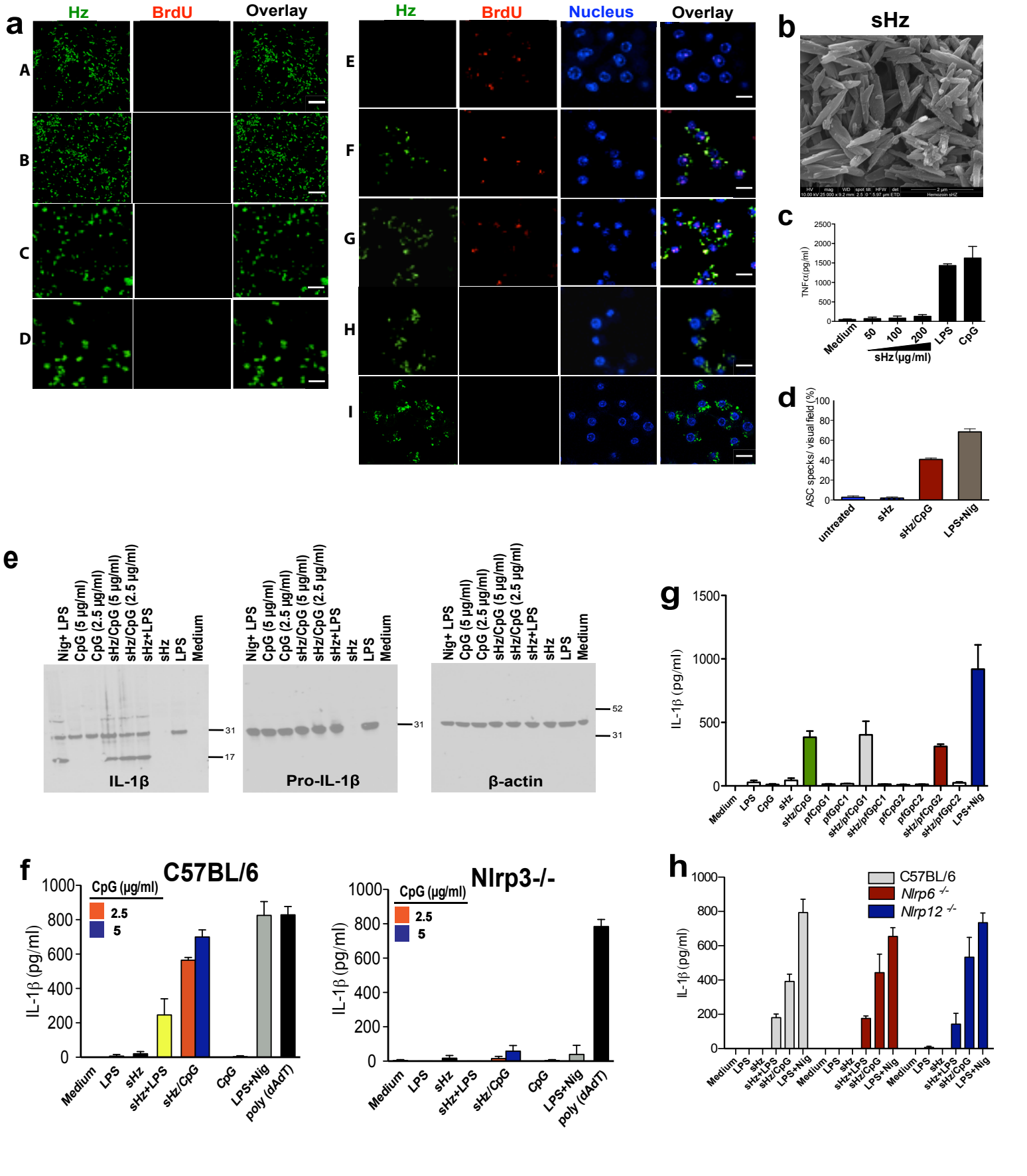
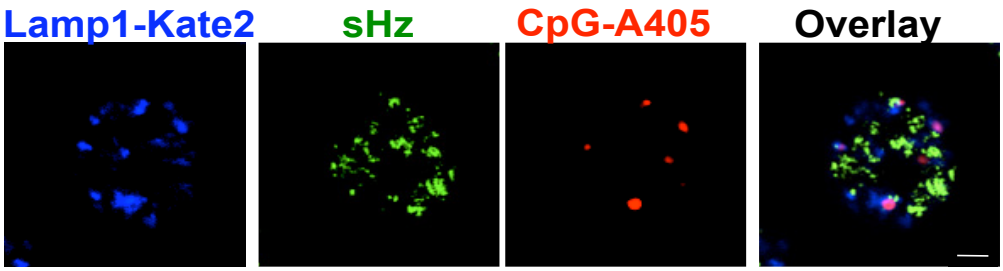

a
Hz
BrdU
Overlay
Nucleus
A
B
C
D
E
F
G
H
I
b
sHz
c
TNFα(pg/ml)
Medium
50
100
200
LPS
CpG
sHz (μg/ml)
d
ASC specks/ visual field (%)
untreated
sHz
sHz/CpG
LPS+Nig
e
Nig+ LPS
CpG (5 μg/ml)
CpG (2.5 μg/ml)
sHz/CpG (5 μg/ml)
sHz/CpG (2.5 μg/ml)
sHz+LPS
sHz
LPS
Medium
IL-1β
Pro-IL-1β
β-actin
31
17
52
g
IL-1β (pg/ml)
CpG
sHz
sHz/CpG
pfCpG1
pfGpC1
sHz/pfCpG1
sHz/pfGpC1
pfCpG2
pfGpC2
sHz/pfCpG2
sHz/pfGpC2
LPS+Nig
f
C57BL/6
Nlrp3-/-
CpG (μg/ml)
2.5
5
poly (dAdT)
h
C57BL/6
Nlrp6 -/-
Nlrp12 -/-

Figure S1 (Related to Figure 1): (a) **BrdU does not bind to synthetic and natural Hz.** Synthetic Hz was incubated with BrdU (A) and PBS (B) and nHz was incubated with BrdU (C) and PBS (D) for 2h at 37^{o}C. Synthetic Hz and nHz in A, B, C and D were washed with PBS X 3. Immortalized BMDMs were incubated with BrdU for 2h at 37^{o}C (E). Immortalized BMDMs were incubated with sHz for 6h and then treated with BrdU for 2h at 37^{o}C (F) Immortalized BMDMs were incubated with nHz for 6h and then treated with BrdU for 2h at 37^{o}C (G) Natural Hz was incubated with BrdU for 2h at 37^{o}C, washed 3 times with PBS and then incubated immortalized BMDMs with nHz for 6h (H) Synthetic Hz was incubated with BrdU for 2h at 37^{o}C, washed 3 times with PBS and then incubated immortalized BMDMs with nHz for 6h (I). BrdU concentration used in A-I was 10mM. DAPI and anti-BrdU mAb were used in Figures A-I as described in Materials and Methods and Hz was visualized using *reflection microscopy*. (b) **Scanning electron micrographs of sHz**. Sample was coated with Au/Pd of about 4 A^{o} in thickness and SEM pictures were taken using a FEI Quanta 200 MK II FESEM **(**c) **sHz is devoid of *Pf* gDNA and fails to elicit TNFα production.** Immortalized murine BMDMs were stimulated with different concentrations of sHz, CpG B (2.5μM), or 100ng/ml LPS for 12 h, and the production of TNFα was assessed by ELISA**.** (d) **sHz/CpG induces ASC pyroptososme formation in the absence of alternative priming signals.** Immortalized murine BMDMs stably expressing ASC-CFP were treated with sHz/CpG (5μg/ml), sHz only (100μg/ml) or LPS (100ng/ml) primed for 2h and then treated with nigericin (10μM). The formation of ASC pyroptosomes were quantified using confocal microscopy. Fields are representative of at least 10 fields of view and three independent experiments. (e) Original scans related to the composite blot shown in Fig. 1f **(**f**) sHz/CpG activates the NLRP3 inflammasome in primary macrophages without the**

need for alternative priming signals. ELISA of IL-1β production by primary C57BL/6 BMDMs and primary *Nlrp3*$^{-/-}$ BMDMs primed for with LPS (100ng/ml x 2h) and then left unstimulated or stimulated with sHz crystals (100μg/ml) or stimulated with sHz/CpG (5μg/ml), CpG (5μg/ml), nigericin (10μM), or poly(dAdT) (1.5. μg/ml). Supernatants were analyzed for IL-1β 12h after stimulation. (g) **sHz/*Pf* CpG complex mediates the release of mature IL-1β.** Immortalized murine BMDMs were stimulated with sHz coated with CpG 1826, *Pf*CpG1, *Pf*GpC1, *Pf*CpG2 or *Pf*GpC2 (sHz: 100μg/ml and CpG or GpC: 5μg/ml) and assayed by ELISA for released IL-1β. Sequences of the *Pf*CpG1, *Pf*GpC1, *Pf*CpG2 and *Pf*GpC2 are described in Ref (Parroche et al., 2007). (h) **sHz/CpG mediated release of IL-1β is NLRP6 and NLRP12 independent**. *Nlrp6*$^{-/-}$ or *Nlrp12*$^{-/-}$ primary macrophages were primed with LPS, or left untreated, and then stimulated with sHz crystals (100μg/ml) or sHz/CpG (5μg/ml) or nigericin (10μM). Supernatants were assessed for released IL-1β 12 hours later. Data are presented as mean of triplicate determinations ± SD and are representative of 3 independent experiments.

Figure S2

a


Lamp1-Kate2
sHz
CpG-A405
Overlay

b

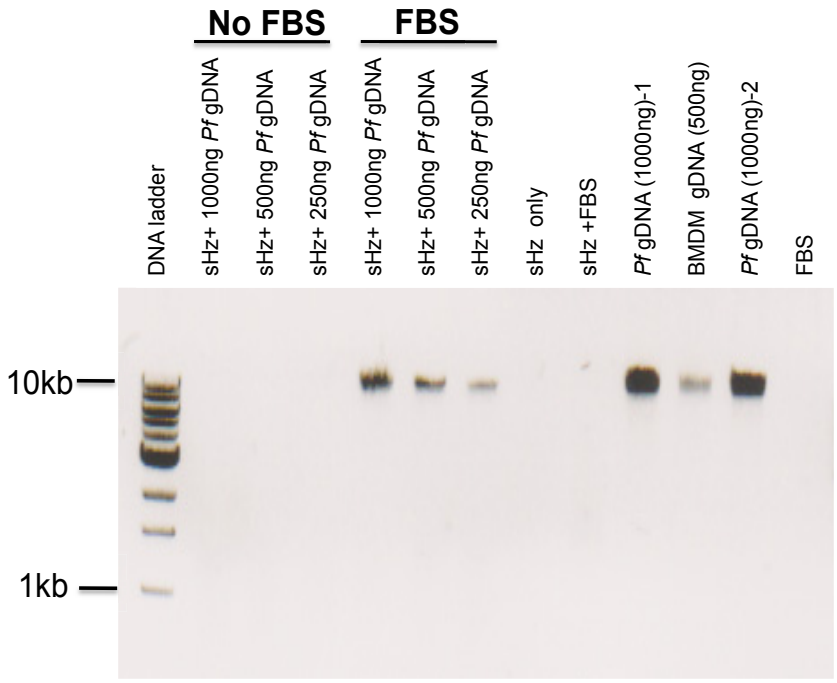

No FBS
FBS
DNA ladder
sHz+ 1000ng Pf gDNA
sHz+ 500ng Pf gDNA
sHz+ 250ng Pf gDNA
sHz+ 1000ng Pf gDNA
sHz+ 500ng Pf gDNA
sHz+ 250ng Pf gDNA
sHz only
sHz +FBS
Pf gDNA (1000ng)-1
BMDM gDNA (500ng)
Pf gDNA (1000ng)-2
FBS
10kb
1kb

Figure S2 (Related to Figure 2): (a) **sHz is released into cytosol while CpG remains bound to the phagosomal remnants of wt macrophages. Related to Figure 2.** (a) Confocal microscopy of immortalized murine BMDMs stably transduced with Lamp1-kate2, incubated for 6h with sHz/CpG (5μg/ml). Most sHz is released into cytosol, while CpG DNA stays in lysosomes. sHz was visualized using reflection microscopy (green). Scale bar: 5μm. Fields are representative of at least 10 fields of view and three independent experiments. (b) **Synthetic Hz coated with FBS binds to *Pf* gDNA.** 100 μg of sHz was left untreated or was coated with FBS overnight and then incubated with different amounts of *Pf* gDNA for 2h and washed 3x with PBS or incubated with gDNA (without coating with FBS) and washed 3x with PBS. The samples were run in a 1% agarose gel. As positive controls, 1000ng of *Pf* gDNA (1 and 2 are *Pf* gDNA extracted from malaria cultures at different times) and 500ng of immortalized murine BMDMs gDNA was used.

Figure S3

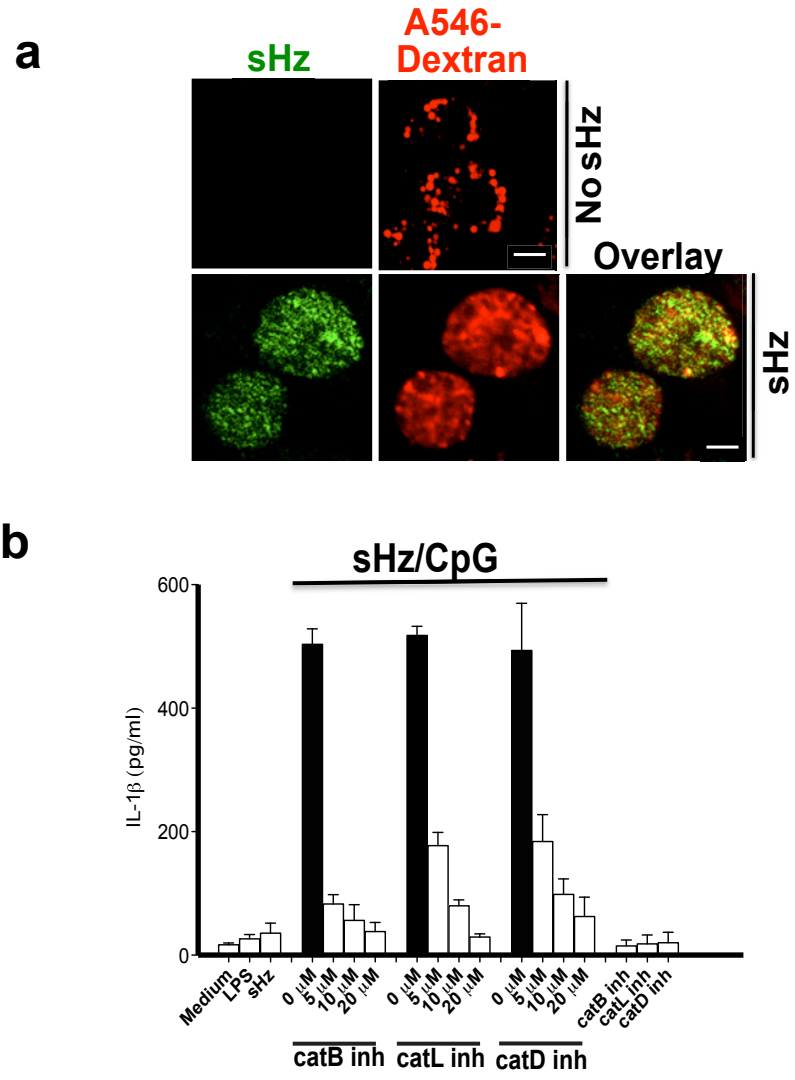

a
sHz
A546-Dextran
No sHz
Overlay
sHz
b
sHz/CpG
600
400
200
0
IL-1β (pg/ml)
Medium
LPS
sHz
0 μM
5 μM
10 μM
20 μM
0 μM
5 μM
10 μM
20 μM
0 μM
5 μM
10 μM
20 μM
catB inh
catL inh
catD inh
catB inh
catL inh
catD inh

Figure S3 (Related to Figure 3): (a) **Synthetic Hz induces phagolysosomal destabilization.** Confocal microscopy of immortalized murine BMDMs incubated with A546-Dextran 10kDa for 45 min and then either left untreated or stimulated with 100μg/ml sHz for 4 h. Reflection microscopy was used to visualize sHz (green). Scale bar (top and bottom panels: 10μm). The fields are representative of at least 10 fields of view and three independent experiments. (b) **Cathepsins are involved in sHz-mediated release of IL-1β.** Effect of various concentrations (0μM, 5μM, 10μM and 20 μM) of inhibitors of cathepsin B (catB inh), cathepsin L (catL inh) and cathepsin D (catD inh) on the sHz/CpG (5μg/ml) mediated release of IL-1β from wt murine immortalized BMDMs. Data are presented as mean ± SD of triplicates and are representative of 3 independent experiments.

Figure S4

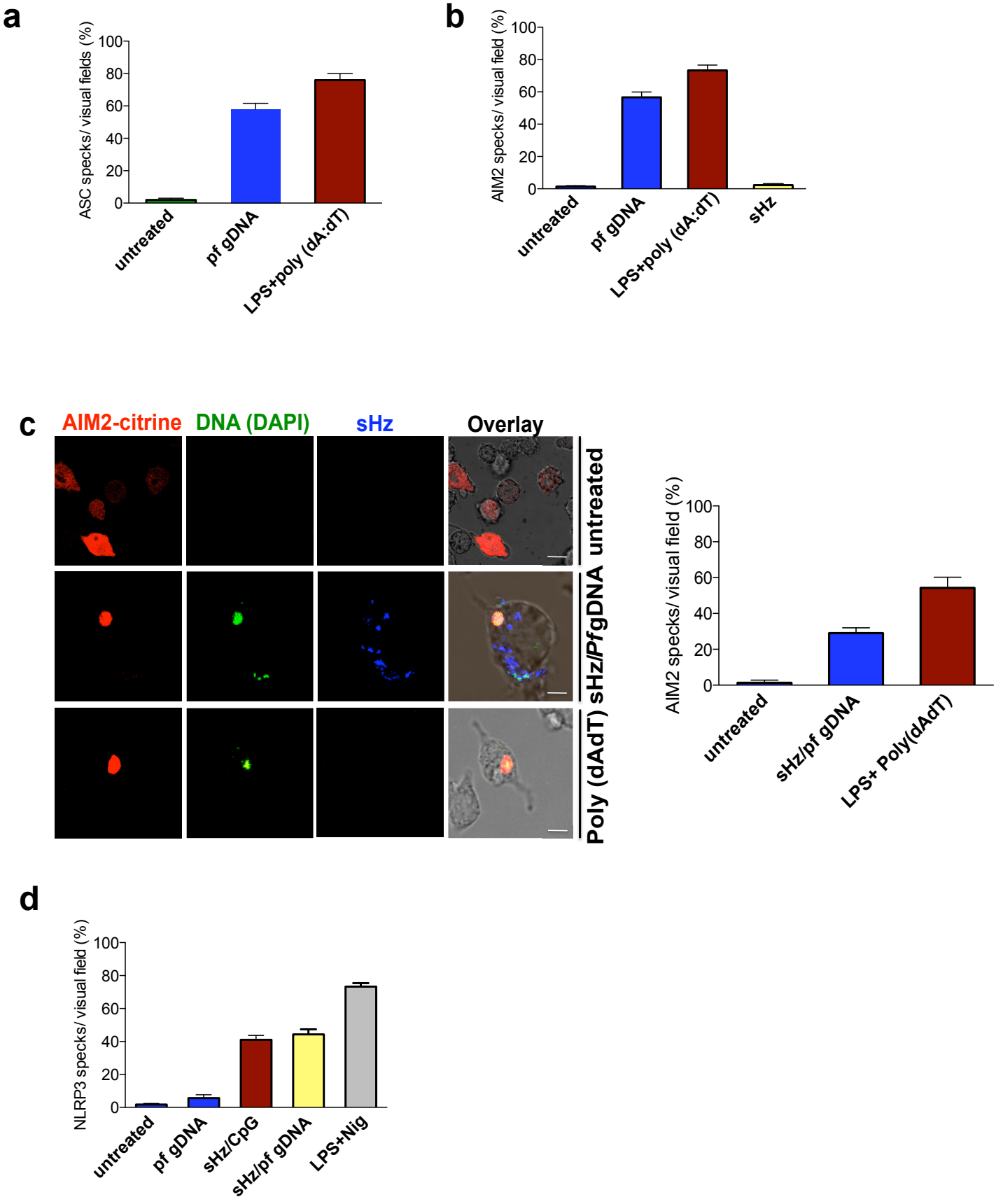

a
ASC specks/ visual fields (%)
100
80
60
40
20
0
untreated
pf gDNA
LPS+poly (dA:dT)
b
AIM2 specks/ visual field (%)
100
80
60
40
20
0
untreated
pf gDNA
LPS+poly (dA:dT)
sHz
c
AIM2-citrine
DNA (DAPI)
sHz
Overlay
untreated
sHz/PfgDNA
Poly (dAdT)
AIM2 specks/ visual field (%)
100
80
60
40
20
0
untreated
sHz/pf gDNA
LPS+ Poly(dAdT)
d
NLRP3 specks/ visual field (%)
100
80
60
40
20
0
untreated
pf gDNA
sHz/CpG
sHz/pf gDNA
LPS+Nig

Figure S4 (Related to Figure 4): (a) **Quantification of ASC and AIM2 speck formation in response to transfection of *Pf* gDNA.** Confocal microscopy of ASC-CFP stably expressing macrophages left untransfected or transfected with 100ng/ml Syto60-labeled *Pf* gDNA or Syto60-labeled poly (dAdT) using lipofectamine. The formation of pyroptosomes was quantified in 10-15 fields of cells by confocal microscopy. (b) AIM2-citrine stably expressing macrophages were left untransfected or LPS primed (2h, 100ng/ml) and then transfected with 100ng/ml DAPI-labeled *Pf* gDNA and DAPI-labeled poly (dAdT) using lipofectamine or sHz only (100μg/ml). The formation of AIM2 pyroptosomes was quantified using confocal microscopy (c) **The complex sHz/*Pf* gDNA induce AIM2 pyroptosome formation**. left panel: AIM2-citrine stably expressing macrophages were left untransfected or LPS primed (2h, 100ng/ml) and then transfected with DAPI-labeled poly (dAdT) using lipofectamine or incubated with DAPI-labeled sHz/*Pf*gDNA (4μg/ml). Scale bar: 20μm (top panel), 15μm (middle panel) and 25μm (bottom panel). Right panel: The formation of AIM2 pyroptosomes in figure shown in left panel was quantified using confocal microscopy (d) NLRP3-citrine stably expressing macrophages were left untreated or treated with sHz/CpG (5μg/ml), sHz/*Pf* gDNA (4μg/ml), nigericin or transfected with 100ng/ml *Pf* gDNA using lipofectamine. The formation of NLRP3 pyroptosomes was quantified using confocal microscopy. The fields are representative of at least 10 fields of view and three independent experiments.

Figure S5

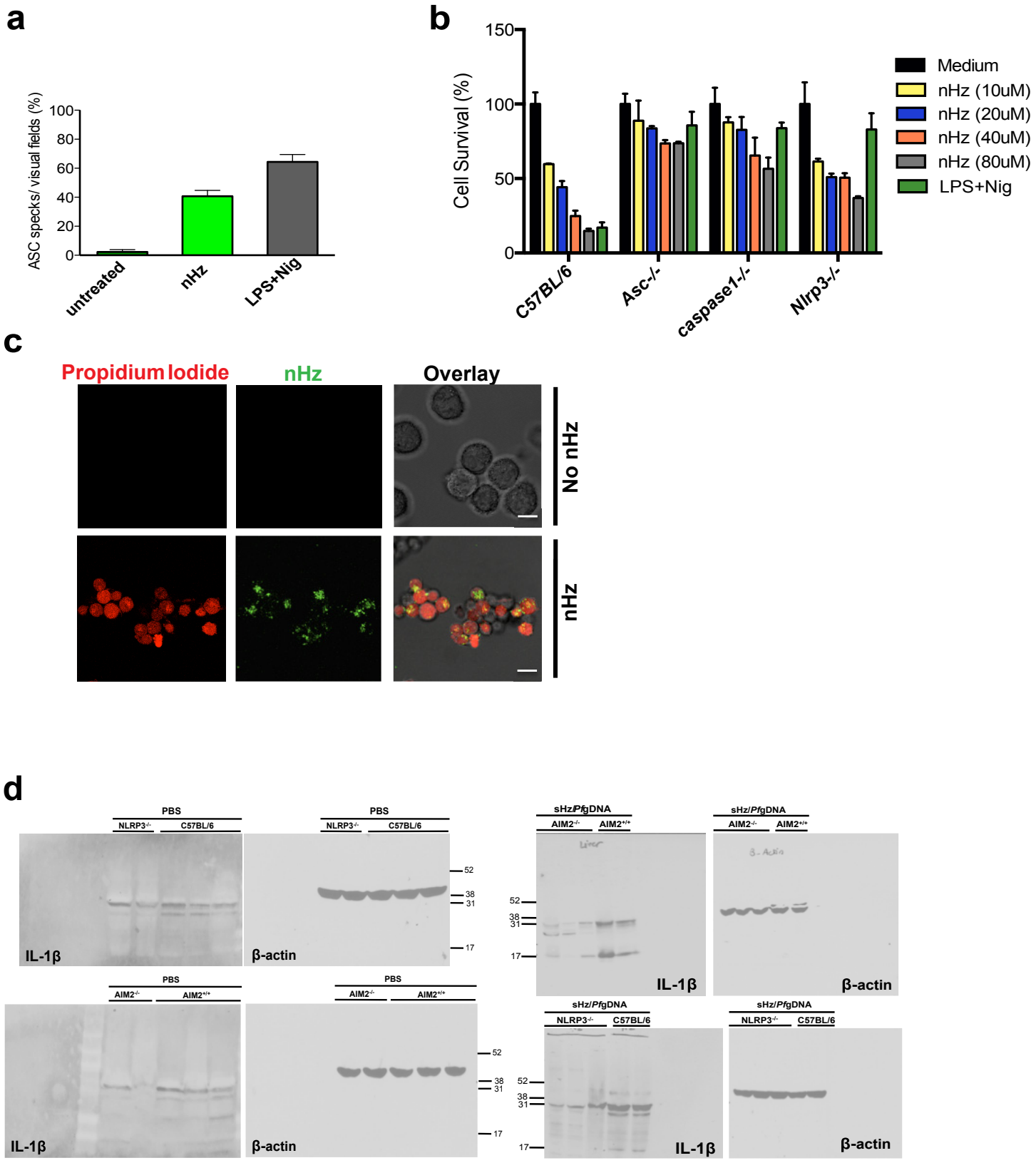

a
ASC specks/ visual fields (%)
100
80
60
40
20
0
untreated
nHz
LPS+Nig
b
Cell Survival (%)
150
100
50
0
C57BL/6
Asc-/-
caspase1-/-
Nlrp3-/-
Medium
nHz (10uM)
nHz (20uM)
nHz (40uM)
nHz (80uM)
LPS+Nig
c
Propidium Iodide
nHz
Overlay
No nHz
nHz
d
PBS
NLRP3-/-
C57BL/6
IL-1β
β-actin
52
38
31
17
AIM2-/-
AIM2+/+
sHz/PfgDNA

Figure S5 (Related to Figure 5): (a) **Quantification of ASC speck formation in response to nHz treatment.** ASC-CFP stably expressing macrophages were left untreated or treated with nHz (100μM). The formation of ASC pyroptosomes was quantified in 10-15 fields of cells by using confocal microscopy. (b) **nHz induced cell death is ASC, caspase-1 and NLRP3 dependent.** wt, *Asc*$^{-/-}$, *casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ immortalized murine BMDMs were incubated with 10 μM, 20 μM, 40μM and 80 μM of nHz for 12 h. Cell survival was measured using Calcein AM. Medium was set as 100%. Data are presented as mean ± SD of triplicates and are representative of 3 independent experiments. (c) wt BMDMs were incubated with 40μM nHz for 24h, then rinsed with PBS and stained with fluorescent Propidium Iodide. nHz was visualized using reflection microscopy (green). Scale bar: 30μm (top panel) and 20μm (bottom panel). Fields are representative of at least 10 fields of view and three independent experiments (d) Original scans related to the blot shown in Fig. 5d.

Figure S6

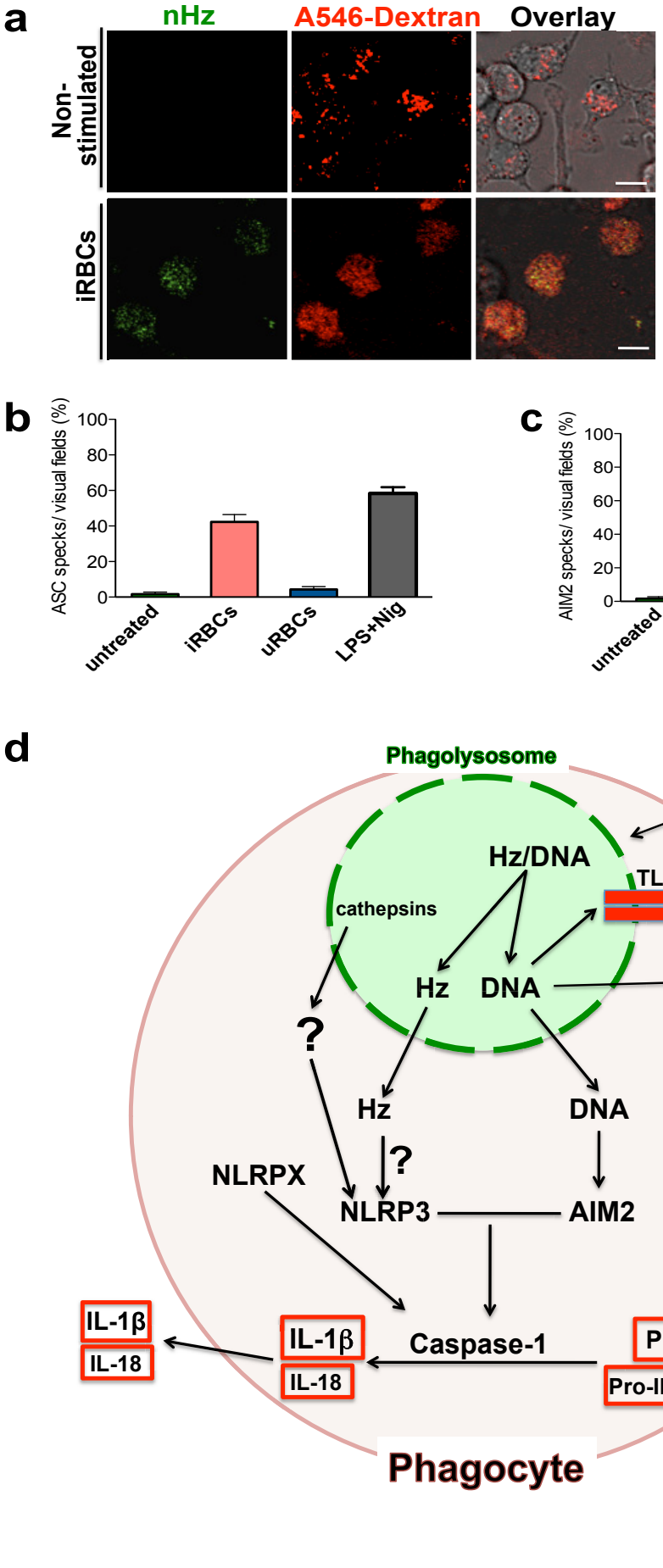

a
nHz
A546-Dextran
Overlay
Non-stimulated
iRBCs
b
ASC specks/ visual fields (%)
100
80
60
40
20
0
untreated
iRBCs
uRBCs
LPS+Nig
c
AIM2 specks/ visual fields (%)
100
80
60
40
20
0
untreated
iRBCs
uRBCs
LPS+poly (dA:dT)
d
Phagolysosome
Free Hz/DNA
Hz/DNA
TLR9
cathepsins
Hz
DNA
Type I IFNs
Ringed form
Schizont
?
Hz
DNA
?
NLRPX
NLRP3
AIM2
IL-1β
IL-18
IL-1β
IL-18
Caspase-1
Pro-IL-1β
Pro-IL-18
Phagocyte

Figure S6 (Related to Figure 6 and summary figure): (a) ***P. berghei*-RBCs induce phagolysosomal destablilization.** Confocal microscopy of immortalized BMDMs incubated with A546-Dextran 10kDa for 45 min and then either left untreated or stimulated with 8x 10^5 *P. berghei*-RBCs (iRBCs) for 18h. Reflection microscopy was used to visualize Hz. Scale bar (top and bottom panels: 15μm) (b) **Infected RBCs induce ASC and AIM2 speck formation.** ASC-CFP stably expressing macrophages were incubated with 8x 10^5 *P. berghei*-RBCs (iRBCs) or uRBCs for 12h and the formation of ASC pyroptosomes were quantified using confocal microscopy. (c) *P. berghei*-RBCs and uRBCs were incubated with DAPI for 1h and then co-cultured with AIM2-citrine macrophages for 12h. The formation of AIM2 pyroptosomes was quantified using confocal microscopy. Fields are representative of 10 fields of view and three independent experiments. (d) **Summary model of how Hz/DNA drives the innate immune response.** During the asexual phase of parasite life cycle, circulating schizonts release merozoites into the systemic circulation, as are fragments of parasites that are turning over within erythrocytes. Infected RBCs of all types (schizonts and ringed trophozoites are shown), as well as the liberated Hz that has been coated within the erythrocyte with DNA, encounter phagocytes. These microbial products, as well as iRBCs are internalized by phagocytosis. DNA on the surface of the Hz triggers TLR9 translocation and activation, which induces the transcriptional induction of NLRP3, pro-IL-1β and other NF-κB inducible genes, thus providing signal 1 for inflammasome activation. Once internalized, phagosomal enzymes digest the DNA off of the surface of the Hz. Hemozoin crystals subsequently destabilize the phagolysosome, providing access to the cytosol by the contents of this subcellular compartment. Hemozoin and *Plasmodium* gDNA are then released into the cytosol where they activate the NLRP3 and AIM2 inflammasomes. Cathepsins seem to be critical in Hz mediated inflammasome activation. It is not yet known if other inflammasomes ("NLRPX") play a role in IL-1β production, but this is likely. *Plasmodium* gDNA in the cytosol also triggers type I Interferon production. Activation of NLRP3 and AIM2 inflammasomes

(signal 2) leads to pro-IL-1β and pro-IL-18 cleavage and release of mature IL-1β and IL-18. The arrow shows the Hz crystal inside a *Pf* infected RBC (schizont).