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iGLuc: a luciferase-based inflammasome and protease activity reporter

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Abstract

Measurement of protease activity in living cells or organisms remains a challenging task. We here present a transgene-encoded biosensor that reports the proteolytic activity of caspase-1 in the course of inflammasome activation and that of other proteases in a highly sensitive and specific manner. This protease reporter is based on the biological activity of a pro-interleukin (IL)-1β-Gaussia luciferase (iGLuc) fusion construct, in which pro-IL-1β-dependent formation of protein aggregates renders GLuc enzyme inactive. Cleavage leads to monomerization of this biosensor protein, resulting in a strong gain in luciferase activity. Exchange of the canonical caspase-1 cleavage site in this reporter construct allows the generation of protease biosensors with additional specificities. The high sensitivity, signal-to-background ratio and specificity of the iGLuc system renders it a useful tool to study proteolytic events in mouse and human cells at high throughput and to monitor protease activity in mice in vivo.

Inflammasomes are large multiprotein signaling platforms that form in the cytoplasm upon sensing microbe or damage-associated molecular patterns[1]. Several NLR (NOD-like receptor) proteins (such as NLRP3) and the PYHIN (pyrin and HIN domain–containing) protein family member AIM2 (absent in melanoma 2) have been shown to function as initiators of inflammasome activation[2]. Via their shared adaptor protein—ASC, or apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)—they recruit the cysteine protease caspase-1, which is in turn activated by proximity-induced dimerization leading to its autocatalytic cleavage into two heterodimer-forming subunits. One of the main targets of active caspase-1 is IL-1β, a highly pro-

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inflammatory cytokine that is expressed mainly by cells of the myeloid lineage. Upon being expressed, IL-1β is initially present as a cytoplasmic precursor polypeptide (pro–IL-1β) that requires maturation by proteolytic cleavage[3]. Active caspase-1 processes human IL-1β at two cleavage sites to generate the active, mature C-terminal, 153-residue cytokine (152 amino acids for mouse IL-1β). Upon maturation, IL-1β is released via a still poorly defined mechanism into the extracellular space, where it can bind to its receptor to exert its pro-inflammatory activity. NLRP3 was initially identified in a series of heritable sterile fever syndromes, now commonly referred to as autoinflammatory diseases[4, 5]. Here gain-of-function mutations in the *NLRP3* gene are the basis for the spontaneous activation of this inflammasome cascade. At the same time, it has become clear that ligand-dependent NLRP3 activation is operational not only in infectious but also in sterile inflammatory conditions. In this latter context, it is noteworthy that in many lifestyle-related diseases, such as gout[6], atherosclerosis[7], type 2 diabetes[8] and Alzheimer's disease[9], engagement of the NLRP3–ASC–caspase-1–IL-1β axis has been shown to contribute to disease progression.

IL-1β release measured with ELISA-based techniques is commonly used as an indirect readout to study inflammasome activation. However, this technique is unable to distinguish mature IL-1β from unprocessed IL-1β, which can be released upon cell stress as well. In addition, a pro-inflammatory priming signal is required to induce IL-1β expression. The same priming signal, however, also strongly modulates the activity of the NLRP3 inflammasome itself, which makes it difficult to interpret IL-1β ELISA data when investigating NLRP3 inflammasome activation[10, 11, 12]. For these reasons, the gold-standard technique for the assessment of inflammasome activation has so far been the analysis of direct caspase-1 cleavage using western blotting, even though this method is inherently time consuming and suitable only for low-throughput analysis.

Here we report a novel tool to monitor proteolytic cleavage events on the basis of the unique functionality of a pro–IL-1β–GLuc fusion protein: iGLuc. The iGLuc reporter system allows the monitoring of cytosolic proteolytic cleavage events with not only high sensitivity and specificity but also an extraordinarily high signal-to-background ratio (SBR). Moreover, its ease of use and its applicability as a transgene in cell-based assays make it an attractive tool to study cytoplasmic cleavage events at high throughput. Although initially developed to study caspase-1 activation in the course of inflammasome activation, the iGLuc reporter system can also be applied to other proteases such as caspase-3 or the nuclear inclusion a (NIa) protease from tobacco etch virus (TEV). Its functionality as a gain-of-function reporter makes the iGLuc system particularly attractive to study proteolytic events that also affect cell viability, such as caspase activation.

RESULTS

Cleavage of iGLuc is required for luciferase activity

To study the activity of caspase-1, we generated a fusion construct of pro–IL-1β and GLuc (iGLuc). We expected that this fusion construct could be used to assess IL-1β secretion using luciferase activity in the supernatants as a readout. To achieve this, we fused the open reading frame of murine pro–IL-1β to GLuc lacking its N-terminal secretion signal (Fig. 1a and Supplementary Fig. 1a). Unexpectedly, upon transient expression in HEK 293T cells,

we could not detect a significant luciferase signal in the cell lysate or cell supernatant, despite the fact that the iGLuc fusion construct was strongly expressed (Fig. 1a) without displaying toxicity (Supplementary Fig. 1b). In contrast, an EGFP-GLuc fusion construct generated in a similar fashion led to a high luminescence signal, both in the cell lysate and the cell supernatant, and its expression level was comparable to the that of the iGLuc construct (Fig. 1a and Supplementary Fig. 1c). Notably, when we coexpressed caspase-1 with iGLuc, which led to the cleavage of the iGLuc construct, we observed a robust increase in luciferase activity in cell lysates (Fig. 1b). Further experiments revealed that luciferase activity closely correlated with the cleavage of the construct, with an SBR of 571-fold (Fig. 1c). We next reconstituted an inflammasome pathway in 293T cells by stepwise introduction of components of the AIM2 inflammasome cascade. First, we expressed limiting amounts of caspase-1 that did not trigger iGLuc cleavage by themselves in conjunction with increasing amounts of ASC (Fig. 1d). Here we observed a strong, dose-dependent increase in iGLuc cleavage and luciferase activity, which indicated that we could mimic an ASC-dependent activation of caspase-1 activation using this assay. Next we expressed caspase-1 and ASC at limiting conditions and titrated increasing amounts of the inflammasome receptor AIM2 (Fig. 1e). Again, a robust gain in luciferase signal closely correlating with cleavage of the iGLuc construct was seen. This technique could also be applied to other ASC-activating inflammasome sensors such as NLRP3, with a disease-associated mutant of NLRP3 (R262W)[13] showing higher luciferase activity, as expected (Supplementary Fig. 2). Together, these results indicated that GLuc activity was almost completely suppressed when GLuc was fused to pro–IL-1β and that caspase-1–mediated cleavage relieved this suppression, leading to a robust gain in luciferase signal with high SBR.

iGLuc reports inflammasome activation in macrophages

We next tested the applicability of the iGLuc reporter system for studying inflammasome activation in cells that are inherently functional for this pathway (Fig. 2). Therefore, we transduced immortalized mouse macrophages (MΦs) with lentivirus expressing the iGLuc reporter construct and generated various clones from these cells, in which iGLuc expression was in the same range as endogenous IL-1β (Supplementary Fig. 3). iGLuc MΦs challenged with increasing amounts of the NLRP3 inflammasome stimulus nigericin revealed a similarly high SBR as observed for 293T cells (Fig. 2b). iGLuc MΦs challenged with prototypical inflammasome stimuli showed a close correlation of luciferase activity with both the processing of the reporter construct and the activation of endogenous caspase-1 (Fig. 2c). In contrast to 293T cells, iGLuc-expressing MΦs secreted the processed, active reporter construct alongside cleaved endogenous IL-1β into the supernatant, and iGLuc activity could be conveniently monitored in the supernatant without lysing the cells (Supplementary Fig. 4). Overexpression of iGLuc did not affect the responsiveness of these MΦs toward other stimuli and did not perturb the activity of the inflammasome pathway in these cells (Supplementary Figs. 5 and 6 and Supplementary Note 1). To assess the specificity of this system, we next transduced NLRC4- and NLRP3-deficient MΦs using the iGLuc construct and tested the response elicited by these MΦs when exposed to various inflammasome stimuli. As expected, NLRC4-deficient MΦs showed no luciferase activity upon Salmonella enterica infection, whereas NLRP3-deficient MΦs were unresponsive to the specific NLRP3 activator nigericin (Fig. 2d,e). Listeria monocytogenes–infected

NLRP3-deficient iGLuc MΦs showed only a partial reduction in luciferase activity, which is in concordance with the fact that Listeria are sensed in a both AIM2- and NLRP3-dependent fashion[14]. Again, a close correlation between caspase-1 activation, luciferase signal and cleavage of the iGLuc construct was observed in MΦs. Furthermore, loss-of-function experiments in iGLuc reporter MΦs using siRNA or small-molecule inhibitors recapitulated known signaling cascades, indicating that this system is broadly applicable to perturbation assays aimed at studying inflammasome activation (Supplementary Fig. 7). Analogous results were obtained when we used mouse primary bone marrow–derived MΦs or dendritic cells, or when we used the human monocytic cell line THP1 (Supplementary Figs. 8 and 9). Together these results indicate that the iGLuc reporter system is well suited for studying caspase-1 activation in myeloid cells without affecting the cells' physiology.

The iGLuc scaffold is applicable to study other proteases

To further explore the applicability of this protease reporter to other proteases, we exchanged the second caspase-1 cleavage site in pro–IL-1β for DEVD ↓ G, a well-known caspase-3 cleavage site (Fig. 3a)[15, 16]. Transient expression of this reporter construct (herein named iGLuc(C3)) in conjunction with increasing amounts of caspase-3 led to a high increase in luciferase activity (Fig. 3b). Moreover, 293T cells stably expressing the iGLuc(C3) construct responded to apoptosis-inducing compounds such as staurosporine (Fig. 3b) or cycloheximide (Supplementary Fig. 10) with a high increase in luciferase signal. Notably, specificity of the individual caspase-1 or caspase-3 iGLuc reporter constructs toward their respective caspases was retained (Supplementary Fig. 11). The specificity of the iGLuc(C3) reporter was further addressed in MΦs lacking caspase-1. The NLRP3 stimulus nigericin did not activate caspase-3 in the absence of caspase-1, whereas staurosporine and cycloheximide induced activation of caspase-3 independently of caspase-1 (Fig. 3c). In line with these findings, wild-type MΦs stably expressing the iGLuc(C3) reporter showed increased luciferase activity when stimulated with either one of these compounds; yet, in the absence of caspase-1, nigericin was inactive (Fig. 3d). In comparison to established caspase-3 reporter systems (for example, luminometric or fluorometric tetrapeptide substrates), the iGLuc(C3) reporter construct showed higher sensitivity and also far superior SBRs when applied within a cell-based caspase-3 activation assay (Supplementary Fig. 12). Of note, the applicability of this system was not restricted to caspase-mediated cleavage events. Introducing the recognition site of the NIa protease from TEV (ENLYFQ ↓ G), we could observe a similar gain in luciferase signal upon cleavage of the reporter construct (iGLuc(TEV)) (Fig. 3e,f).

Structural requirements of the iGLuc reporter

Other C-terminal tags such as EGFP or firefly luciferase showed neither discernable difference in biological activity when fused to pro–IL-1β nor a gain in function when cotransfected with caspase-1 (Supplementary Fig. 13). However, when we used luciferase derived from Metridia longa, which is closely related to GLuc in sequence[17], the resulting reporter behaved similarly to iGLuc (Supplementary Note 2 and Supplementary Fig. 14). Pro–IL-1β has two well-confirmed caspase-1 cleavage sites (site I, Asp27 ↓ Gly28, and site II, Asp117 ↓ Val118, in the murine system) that are processed in a sequential manner. A truncated version of pro–IL-1β lacking the N-terminal part up to the first cleavage site

displayed 17-fold higher luciferase activity compared to the full-length protein (Fig. 4a). In addition, shortening of the N-terminal portion of pro–IL-1β even further led to an increase in baseline luciferase activity in the absence of caspase-1 activity. Indeed, a construct lacking the entire pro-domain (Δ1-117–iGLuc) showed maximal luciferase activity within the range of iGLuc processed by caspase-1 (Fig. 4a). Similar results were obtained when we truncated the C-terminal domain of the protein (Supplementary Fig. 15a), indicating that the presence of both the full pro-domain and the C-terminal 17-kDa part was critically required for the inhibition of GLuc activity in this protein configuration. Notably, an increase in the length of the peptide linker between pro–IL-1β and GLuc also resulted in a loss of the inhibitory activity (Supplementary Fig. 15b), suggesting that pro–IL-1β exerted a steric hindrance on its C-terminal GLuc tag that required a critical distance between the two proteins.

Functionality of the iGLuc reporter system ex vivo

To address whether the iGLuc construct required the presence of accessory proteins in the cytoplasm or certain subcellular compartments to unfold its activity upon cleavage, we next studied the activity of purified iGLuc, iGLuc(TEV) or Δ1–117–iGLuc in the presence or absence of caspase-1 or TEV protease ex vivo. Both purified iGLuc and iGLuc(TEV) showed no luciferase activity in the absence of protease-mediated cleavage, whereas the N-terminally truncated version showed high activity per se with no further enhancement upon caspase-1 or TEV protease addition (Fig. 4b–d). However, a strong gain of function in GLuc activity was seen for both iGLuc or iGLuc(TEV) when we added recombinant caspase-1 or TEV protease, respectively. This gain in activity was specific for the respective protease and paralleled by cleavage of the constructs (Fig. 4e). Together, these results indicated that cleavage-mediated activation of the iGLuc construct could be observed under cell-free conditions and that the gain of function in luciferase signal could be attributed to a special functionality of the fusion protein itself.

Monomerization is required for iGLuc activity

We observed that the pro-form of iGLuc showed a strong propensity to form aggregates under nondenaturing conditions (data not shown), so we subjected purified iGLuc constructs to size-exclusion chromatography. Here we observed that the unprocessed iGLuc construct did not emerge as a monomeric protein but rather eluted in aggregates with no specific size preference. As expected, no substantial luciferase activity could be detected in the fractions in which aggregates eluted (Fig. 5a). However, when we expressed iGLuc under conditions that led to its caspase-1–mediated cleavage, a clearly discernable protein peak could be observed at about 40 kDa, corresponding to the predicted size of the cleaved monomeric iGLuc construct (Fig. 5b). Moreover, a strong luciferase signal could be detected in the corresponding elution fractions. When we expressed a truncated version of iGLuc lacking the pro-domain, a similar picture was seen. The majority of the protein eluted as a putative monomer, and luciferase activity was confined to this fraction (Fig. 5c). Analogous results were obtained when we studied iGLuc and also endogenous IL-1β in mouse MΦ cell lines. As observed for 293T cells, in cell lysates of unstimulated MΦs, iGLuc and IL-1β emerged as broad peaks indicative of large protein aggregates having formed. However, upon NLRP3 stimulation, monomeric iGLuc or IL-1β peaks could be detected in the macrophage supernatants, with luciferase activity and the iGLuc protein peak displaying a complete

overlap (Supplementary Fig. 16). On the other hand, subjecting EGFP-GLuc to size-exclusion chromatography mainly revealed a monomeric protein peak, with GLuc activity strictly confined to the monomeric protein fraction (Supplementary Fig. 17a). Together, these data implied that aggregation of the iGLuc construct was responsible for the observed lack in luciferase activity and that its processing resulted in monomerization and, thus, activity. To corroborate this hypothesis, we generated fusion constructs in which GLuc was linked to protein domains that are known to induce aggregation upon overexpression[18]. We chose the pyrin domain of AIM2 (PYD(Aim2)) and the CARD domain of caspase-1 (CARD(Casp1)), and we used EGFP and the precleaved version of IL-1β (Δ1–117) as non-aggregating controls. Fluorescently tagged versions of these constructs showed a strong propensity to form large cytoplasmic aggregates when expressed at high concentrations (Fig. 5d and Supplementary Fig. 18). When we transfected increasing amounts of GLuc-tagged versions of these constructs, a dose-dependent and linear increase in protein expression could be seen for all constructs studied (Fig. 5e). However, although luciferase activity dose-dependently increased for all constructs at low expression levels (<25 ng per transfection), both CARD(Casp1)-GLuc and PYD(Aim2)-GLuc luciferase activity reached plateau levels at higher concentrations (Fig. 5f). EGFP-GLuc or Δ1–117–iGLuc, on the other hand, displayed a close linear correlation between luciferase activity and expression level (Fig. 5f). Comparing these constructs, we saw ~17-fold lower luciferase activity for the aggregation-prone GLuc constructs at high expression (Fig. 5g). Studies of purified PYD(Aim2)-GLuc using size-exclusion chromatography corroborated these findings, showing that only a minor fraction was indeed monomeric protein and that luciferase activity was largely confined to this protein fraction (Supplementary Fig. 17b). Overall, these results confirmed that protein aggregates hinder luciferase activity of the C-terminally tethered GLuc tag.

Studying caspase-1 activation using iGLuc in vivo

Finally, we wanted to assess the applicability of the iGLuc reporter system for studying inflammasome activation in vivo. We chose to study caspase-1 activation in response to intraperitoneal Listeria infection, which constitutes a potent trigger of the NLRP3 and AIM2 inflammasomes. Wild-type iGLuc-expressing macrophage cell lines were transferred intraperitoneally into mice and infected 18 h later with Listeria via the same route. Subsequently, luminescence was monitored ex vivo and assessed in serum samples of mock-treated and infected animals 3–24 h after treatment. In addition, IL-1β protein levels were assessed in serum samples using ELISA (Fig. 6a). Mice infected with Listeria displayed a strong luciferase signal in the peritoneal cavity, reaching an up to 21-fold increase in luminescence signal (Fig. 6b,c). In addition, in the serum of infected mice, we could observe an increase in luciferase activity that correlated with the production of IL-1β (Fig. 6d,e). Together, these results indicated that the iGLuc construct could be used as a biosensor to study caspase-1 activation in vivo.

DISCUSSION

Caspase-1 activity can be studied in vitro using purified enzymes and substrates; as such, various established protease activity-reporter systems are in principle suitable for studying

caspase-1 activation. Nonetheless, these assays are usually unable to reliably distinguish different caspase activities in complex milieus such as cell lysates or live cells. However, such milieus are required to study functional inflammasome cascades that operate in a ligand-dependent manner (Supplementary Note 3). In the course of the work reported here, we tested several approaches to develop a cell-based, transgenic reporter system that would be amenable to studying inflammasome activation. Initially, we adapted previously published caspase-3 biosensors using the consensus cleavage site of caspase-1 (for example, loss of a FRET signal[19] or complementation of a locked, cyclic luciferase[20]). However, none of these systems worked as a caspase-1 reporter, even though caspase-3 activity could be monitored (data not shown). We hypothesize that this was due to the fact that substrate requirements for caspase-1 cleavage are more difficult to mimic in vivo, a notion that is well in line with discrepancies of consensus cleavage sites found with peptide mimetics or cell-based experiments[21]. A possible explanation could be that interactions of caspase-1 with its substrate distant from the recognition site are important determinants for cleavage activity in an in vivo setting. At the same time, it is possible that the IL-1β scaffold provides a unique presentation and also conformation of the respective cleavage site that are particularly favorable for caspase accessibility[22].

On the basis of various independent experimental approaches, we suggest the following model of action for the iGLuc system: because of its pro-domain, full-length pro–IL-1β–GLuc has a strong propensity to form protein aggregates. In fact, gel-shift experiments suggest that only a negligible fraction of pro–IL-1β–GLuc protein is present in its monomeric state under native conditions. Aggregation, however, inhibits GLuc in its luciferase activity, and thus no signal is obtained even though the protein is expressed at high amounts. In the presence of caspase-1, the pro–IL-1β scaffold is cleaved, leading to the release of the C-terminal part of iGLuc. This event leads to the monomerization of the protein and thus a gain in functionality of the GLuc tag. This unique functionality is also seen with GLuc homologs of the copepod luciferase family, yet not with other distantly related luciferases. The exact nature of the pro–IL-1β–GLuc protein aggregates is currently unknown, but our data suggest that the iGLuc complex formation reconstitutes the physiological situation of pro–IL-1β and does not represent an overexpression artifact. In this context it is also important to note that transient or stable iGLuc reporter expression does not affect cell viability and does not alter the physiology of the cell. Immortalized or primary myeloid cells expressing the iGLuc construct still respond normally to innate immune stimuli, most importantly to inflammasome activators.

The iGLuc reporter system constitutes a versatile and simple tool to study proteolytic events in cell-based assays and in vivo. Its unique functionality as a specific and sensitive caspase-1 reporter and its ease of use will allow the study of inflammasome activation at high throughput, which has not been achieved by any other method so far. This could contribute invaluably to our understanding of inflammasome activation itself as well as benefiting clinical drug development for the treatment of inflammasome-related illnesses.

METHODS

Plasmids

Expression plasmids coding for caspase-1 and ASC-HA are based on modifications of the plasmid pCI as previously described [18]. The expression construct coding for TEV protease (pCMV-TEV) was kindly provided by Dr. Rossner. pCI empty or pBluescript were used as stuffer plasmids. All other constructs were cloned into pEFBOS via XhoI/NotI fusion. For lentiviral transductions, constructs were subcloned into a modified pFUGW vector via XhoI/NotI fusion [9]. PCR site-directed mutagenesis was carried out in pMini, a shuttle vector based on pCDNA. A detailed list of all constructs is provided in Supplementary Table 1 and primer sequences are provided in Supplementary Table 2.

Reagents

Unless otherwise specified, 293T cells were transiently transfected with GeneJuice transfection reagent (Novagen) and lysed with passive lysis buffer (Promega). Coelenterazine (CTZ-Native) was obtained from P•J•K. Poly(dA:dT), nigericin, staurosporine and cycloheximide (CHX) were from Sigma-Aldrich. Ultra-pure LPS from E. coli was from Invivogen. Macrophages were transfected with poly(dA:dT) using Lipofectamine 2000 (Invitrogen). Milk powder, calcium chloride dihydrate, sodium chloride, potassium chloride, Triton X, paraformaldehyde (PFA) and sodium phosphate dibasic were from Carl Roth. HRP-conjugated monoclonal Anti-Flag M2 was from Sigma-Aldrich. Anti-HA (sc-805), anti-GFP (sc-9996) and anti-caspase1 p10 (sc-514), anti-goat-HRP and anti-rabbit HRP were from SantaCruz, the anti-GLuc antibody from NEB, the anti-caspase1 p20 (Casper-1) from Adipogen, and the murine anti-IL1β antibody from R&D Systems.

Cell culture and stimulation experiments

293T cells and immortalized mouse macrophages were cultivated in DMEM supplemented with L-glutamine, sodium pyruvate and 10 % v/v FBS (Gibco). For transfection or stimulation experiments, cells were plated in 96-well at 1×10^5 (macrophages) or 3×10^4 (293T) cells per 96-well. Direct inflammasome stimulation in macrophages was carried with the indicated activators as previously described [14]. In brief, *Listeria monocytogenes* strain EGD and *Salmonella enterica* were used for bacterial infections. ATP and nigericin were used at a concentration of 5 mM or 5 μM if not otherwise indicated. Poly(dA:dT) was transfected at 200 ng per 96-well.

Derivation of bone-marrow macrophages (BMDM) and dendritic cells (BMDC)

Bone marrow was isolated from the femur and tibia of female, 7-8 week old C57BL/6 mice that were housed under SPF conditions. Bone marrow cells were filtered and subjected to Erylysis (BD PharmLyse). For the derivation of macrophages, the bone-marrow cells were incubated with 30 % L929 supernatant for a total of 11 days. To generate dendritic cells, bone-marrow cells were incubated for 11 days with 3 % GM-CSF supernatant.

Lentivirus generation and transduction of primary and immortalized cells

All lentiviruses were generated via standard calcium phosphate transfection of 293T cells[23]. THP-1 cells were transduced via spin-transduction for 45 min at 800 g and 32 °C with 8 μg/mL hexadimethrine bromide (Sigma). Immortalized macrophages and primary BMDMs and BMDCs were transduced via incubation with viral supernatants for approximately 4 days. After transduction of the immortalized macrophages and THP-1 cells, single-cell clones were plated and expanded. Clonal cell lines were chosen based on the fold-activity of the iGLuc reporter.

Transient transfections and luciferase experiments

293T cells were transiently transfected with 0.5 μL GeneJuice with a total amount of 200 ng DNA per 96-well. For titrations, an empty PCI vector served as a stuffer. After 20 hours the supernatants were removed, and the cells were lysed in 35μL passive lysis buffer, of which equal amounts were taken for the GLuc readout and immunoblotting. In 293T cells, the iGLuc signal was measured in the cell lysate. In macrophage experiments, iGLuc was secreted after caspase-1 cleavage, and the signal was measured directly from the supernatant. In all assays, supernatants or cell lysates were combined 1:1 with distilled water containing 4.4 μM coelenterazine with a final concentration of 2.2 μM. The luciferase signal was measured on a 2104 EnVision Multilabel reader from Perkin-Elmer.

Immunoblotting and ELISA

293T cell lysates in passive lysis buffer and cell-free assays in TEV protease buffer were combined with 2× Laemmli buffer. In macrophage experiments, the cells were lysed in 1× Laemmli buffer, and the supernatants were precipitated as previously described. All immunoblotting probes were denatured at 99 °C, separated on 10 %, 12 % or 15 % PAGE and transferred to a nitrocellulose membrane. The membranes were incubated in the respective antibody mixed with 0.5% milk powder, washed afterwards in 0.05 % Tween/PBS and developed using Thermo Scientific ECL Substrate. ELISA kits for murine and human IL-1β, IL-1α and IL-6 were from BD Biosciences. The murine/human HMGB1 ELISA is from IBL International, and the murine IP-10 ELISA is from R&D Systems. All ELISAs were performed according to the manufacturer's recommendations. EGFP-GLuc and iGLuc were quantified by ELISA using the anti-GLuc antibody as capture (1:5000 coated in PBS) and the anti-Flag antibody (1:10,000) as the detection antibody. Where pg/mL is shown, a standard was made using overexpressed iGLuc-Δ1-117, which was measured in parallel using the murine IL-1β ELISA kit from BD.

Protein Expression in 293T cells and Immunoprecipitation

Cell-free assays and size exclusion chromatography experiments used, when not otherwise noted, proteins overexpressed in HEK 293T cells. 3-4×10^6 cells were plated in polyornithine-coated 10 cm dishes and transfected with calcium phosphate using 30 μg DNA in total (6 μg per plasmid to be expressed and 24 μg pBluescript as a stuffer). After 40 hours, the cells were washed with PBS and lysed with 2 mL lysis buffer with protease inhibitor. The Flag-IP was then performed using Anti-Flag M2 Affinity Gel (Sigma) according to the manufacturer's recommendations and proteins were then eluted using 200

μg/mL 3× Flag Peptide. For the cell-free protease assays, the protein was eluted directly into 1 × TEV Protease Buffer (Promega), whereas, for size-exclusion experiments, the protein was eluted in PBS.

Recombinant proteolysis

After elution, the iGLuc, iGLuc(TEV) and iGLuc-Δ1-117 were serially diluted with a total probe volume of 100 μL in TEV Protease Buffer (Promega), combined with recombinant TEV protease (Promega) or recombinant Caspase 1 (Enzo) and then incubated for 1 hour at 37°C. From the 100 μL, 15 μL was taken for the GLuc readout, 15 μL for immunoblotting and 1 μL for Elisa.

Preparation of whole cell lysates for size exclusion chromatography

iGLuc and wildtype immortalized macrophages were harvested, washed once with cold PBS and lysed for 10 min on ice in CHAPS lysis buffer (20 mM Tris•HCl, pH 7.6; 150 mM $NaCl_2$; 1 % CHAPS; 1 mM EDTA; 2 mM DTT) with Protease Inhibitor Cocktail. Afterwards, lysates were spun for 2 min at 1000 g and 4 °C, and the resulting supernatant was further centrifuged at 45,000 g and 4 °C for 45 minutes. 200 μL of the cleared supernatant was subjected to the Superdex 200 10/300 GL.

Size exclusion chromatography

Size exclusion chromatography experiments were performed on a Superdex 200 10/300 GL (GE Healthcare) calibrated with the Gel Filtration Standard from BioRad. Of note, two different Superdex columns were used for the size exclusion chromatography experiments, explaning slight differences in resolution. The samples were run in PBS at 4 °C with a flow rate of 0.5 ml/min using the ÄKTA purifier UPC10 and a sample volume 200-250 μl in PBS or CHAPS lysis buffer, as specified. The fraction volume was 150 μl starting at the retention volume of 2.5 ml unless otherwise noted. 30μL was taken from each fraction for luciferase measurements. iGLuc and endogenous IL-1β levels were quantified using a murine IL-1β or GLuc-FLAG ELISA.

Microscopy

Confocal microscopy was performed on a Leica SP2 AOBS confocal laser-scanning microscope. Cell membranes were stained with choleratoxin subunit B (Alexa 555). For staining of FLAG-tagged proteins, the cells were fixed with 4 % PFA and then permeabilized with 0.3 % Triton × in 10% FBS before staining with a direct-conjugated anti-FLAG (Alexa 647) antibody.

In vivo imaging

Wild-type, female, 7-8 week old C57BL6/J mice, purchased from Jackson Laboratory, were injected intraperitoneally (i.p.) with 4×10^7 iGLuc transduced macrophages. 18 hours later mice were injected i.p. either with 200 μL PBS alone (vehicle) or 200 μL PBS containing 5×10^6 *Listeria monocytogenes*. After the time indicated, mice were injected with 1mL of PBS/coelenterazine solution (50 μg/mL) and subjected to IVIS measurement. For each measurement one individual animal was used. The GLuc signal was measured during 5

minutes' exposition with the acquisition mode set to luminescent and photography overlay, an object height of 1.5 cm and medium binning. During the measurements, mice were placed under anesthesia with isoflurane (Abbott). All animal procedures were approved and performed according to the guidelines of the local authorities.

siRNA Transfection

siRNA molecules (Ambion, silencer select) were reverse transfected into iGLuc macrophages (2×10^4 cells per 96-well) at a final concentration of 50 μM using 0.5 μl Hiperfect (Qiagen). 48h after transfection cells were washed and stimulated as indicated.

Quantitative real-time PCR analysis

RNA from macrophages was reverse transcribed and quantitative PCR analysis was performed on a Roche LC480. All gene expression data are presented as relative expression to HPRT1. Primer sequences are available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. Annu Rev Immunol. 2009; 27:229–265. [PubMed: 19302040]
2. Bauernfeind F, et al. Inflammasomes: current understanding and open questions. Cell Mol Life Sci. 2011; 68:765–783. [PubMed: 21072676]
3. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol. 2009; 27:519–550. [PubMed: 19302047]
4. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet. 2001; 29:301–305. [PubMed: 11687797]
5. Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (*). Annu Rev Immunol. 2009; 27:621–668. [PubMed: 19302049]
6. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 2006; 440:237–241. [PubMed: 16407889]
7. Duewell P, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 2010; 464:1357–1361. [PubMed: 20428172]
8. Masters SL, et al. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. Nat Immunol. 2010; 11:897–904. [PubMed: 20835230]
9. Halle A, et al. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol. 2008; 9:857–865. [PubMed: 18604209]

10. Bauernfeind F, et al. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. J Immunol. 2011; 187:613–617. [PubMed: 21677136]
11. Bauernfeind FG, et al. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol. 2009; 183:787–791. [PubMed: 19570822]
12. Franchi L, Eigenbrod T, Nunez G. Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. J Immunol. 2009; 183:792–796. [PubMed: 19542372]
13. Dode C, et al. New mutations of CIAS1 that are responsible for Muckle-Wells syndrome and familial cold urticaria: a novel mutation underlies both syndromes. Am J Hum Genet. 2002; 70:1498–1506. [PubMed: 11992256]
14. Kim S, et al. Listeria monocytogenes is sensed by the NLRP3 and AIM2 inflammasome. Eur J Immunol. 2010; 40:1545–1551. [PubMed: 20333626]
15. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature. 1994; 371:346–347. [PubMed: 8090205]
16. Garcia-Calvo M, et al. Purification and catalytic properties of human caspase family members. Cell Death Differ. 1999; 6:362–369. [PubMed: 10381624]
17. Takenaka Y, et al. Two forms of secreted and thermostable luciferases from the marine copepod crustacean, Metridia pacifica. Gene. 2008; 425:28–35. [PubMed: 18725274]
18. Hornung V, et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature. 2009; 458:514–518. [PubMed: 19158675]
19. Mahajan NP, Harrison-Shostak DC, Michaux J, Herman B. Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis. Chem Biol. 1999; 6:401–409. [PubMed: 10375546]
20. Fan F, et al. Novel genetically encoded biosensors using firefly luciferase. Acs Chem Biol. 2008; 3:346–351. [PubMed: 18570354]
21. Demon D, et al. Caspase substrates: easily caught in deep waters? Trends Biotechnol. 2009; 27:680–688. [PubMed: 19879007]
22. Timmer JC, Salvesen GS. Caspase substrates. Cell Death Differ. 2007; 14:66–72. [PubMed: 17082814]
23. Kutner RH, Zhang XY, Reiser J. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc. 2009; 4:495–505. [PubMed: 19300443]

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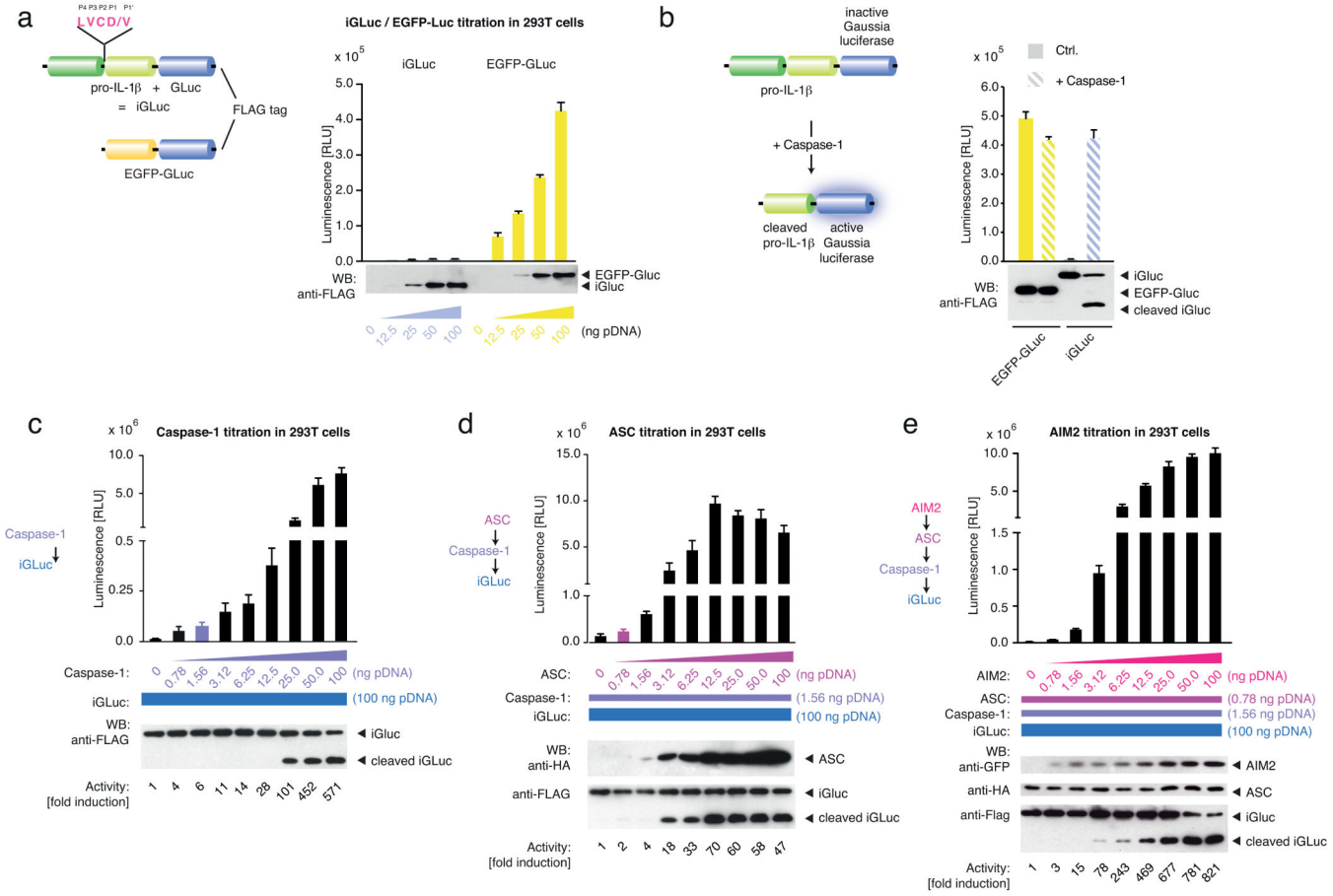

Figure 1. iGLuc is active upon caspase-1 mediated cleavage

(a) 293T cells were transiently transfected with iGLuc or EGFP-GLuc plasmid DNA (pDNA) at the amounts indicated. Cell lysates were assessed 20 h after transfection for luciferase activity, and iGLuc expression was assessed with western blotting (WB) using anti-Flag antibody. (b) 293T cells were transfected with 100 ng of the indicated reporter constructs in the presence or absence of 100 ng caspase-1, and luciferase activity and reporter construct expression were analyzed as in a. (c) 293T cells were transfected with increasing concentrations of caspase-1 (0.78–100 ng in twofold increases) in the presence of 50 ng iGLuc, with the final amount of DNA kept at 200 ng per well. Analysis was performed as described in a. (d,e) 293T cells were transfected and analyzed as in c, now with increasing concentrations of ASC in the presence of 1.56 ng caspase-1 (d) or with increasing concentrations of AIM2 (e) in the presence of 1.56 ng caspase-1 and 0.78 ng ASC. ASC was monitored by WB using an antibody against hemagglutinin (anti-HA), whereas AIM2 was detected using anti-GFP. Data are presented as mean + s.e.m. and are representative of three (c–e) or four (a,b) independent experiments each. r.l.u., relative light units.

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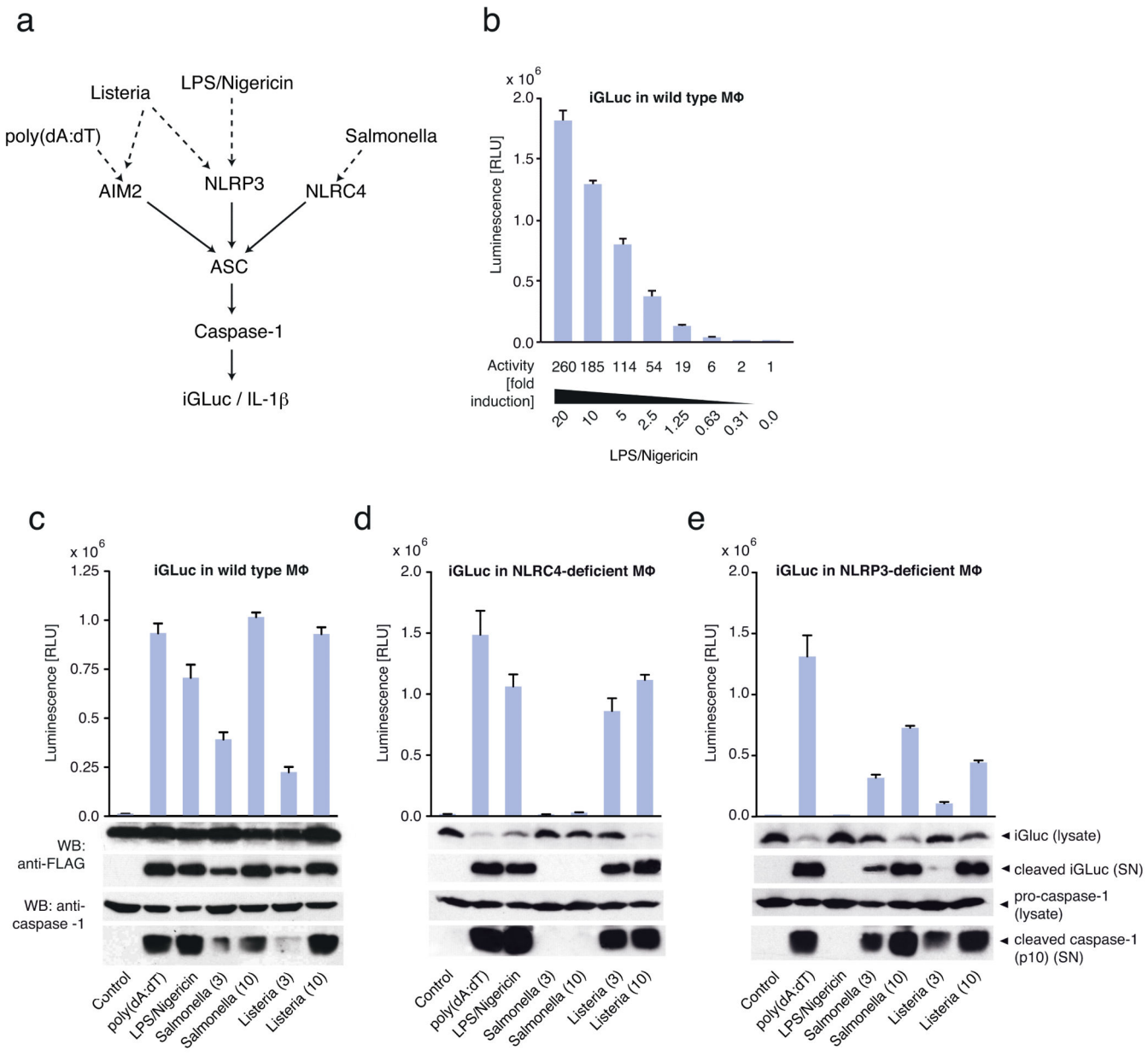

Figure 2. iGLuc reports inflammasome activation

(a) Overview of the AIM2, NLRP3 and NLRC4 inflammasome pathways and the pathogens or stimuli that trigger them. LPS, lipopolysaccharide. (b) Wild-type immortalized mouse macrophages (MΦs) stably expressing the iGLuc reporter construct were stimulated with increasing concentrations of nigericin in the presence of LPS (denoted as LPS/nigericin). Luciferase activity was assessed 2 h after stimulation. Fold-induction numbers are given in relation to measurements from unstimulated cells. (c–e) Wild-type (c), NLRC4-deficient (d) and NLRP3-deficient (e) immortalized MΦs, all stably expressing the iGLuc reporter, were treated with the indicated stimuli. Numbers in parenthesis indicate the multiplicity of infection of the respective bacteria used. Cell lysates and cell supernatants (SN) were analyzed 6 h after stimulation for IL-1β and caspase-1 expression, and luciferase activity

was assessed in the cellular supernatants. Data are presented as mean + s.e.m. and are representative of three (b) or five (c–e) independent experiments each. r.l.u., relative light units. WB, western blot.

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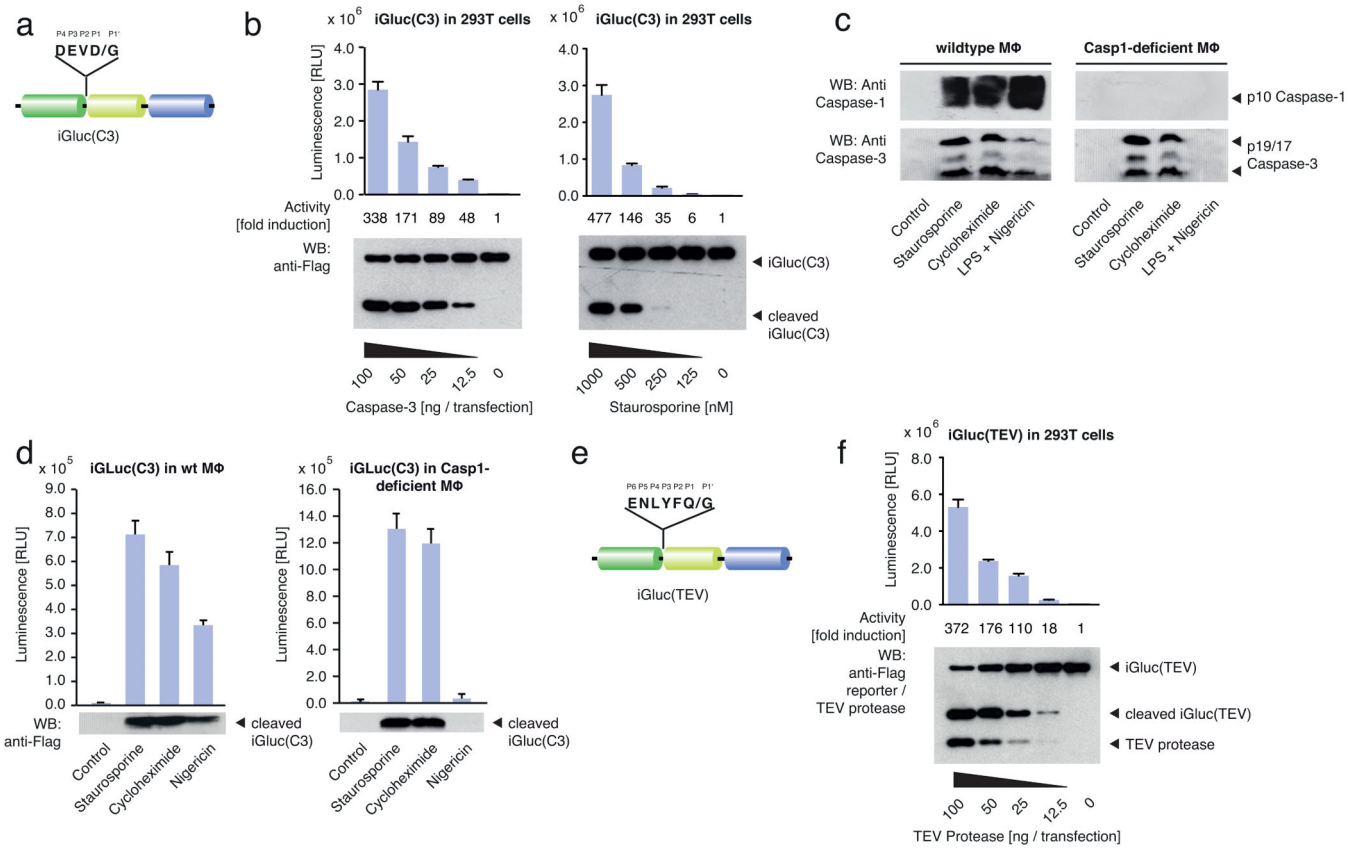

Figure 3. Building other protease biosensors based on iGLuc

(a) Schematic of the iGLuc(C3) reporter. (b) 293T cells stably expressing iGLuc(C3) were transfected with increasing concentrations of a caspase-3 expression construct or treated with increasing concentrations of staurosporine. Luciferase activity was analyzed 24 h after stimulation in cell lysates, and iGLuc expression was assessed using western blotting (WB). (c) Wild-type or caspase-1–deficient murine MΦ cell lines were stimulated with staurosporine (1 μM), cycloheximide (10 nM) or LPS and nigericin. Caspase-1 and caspase-3 activity were determined 6 h after stimulation using WB. (d) Wild-type or caspase-1–deficient murine MΦ cell lines transduced with iGLuc(C3) were stimulated as in c, and luciferase activity and cleavage of the reporter construct were assessed 6 h after stimulation in the supernatant. (e) Schematic of the iGLuc(TEV) reporter. (f) 293T cells expressing iGLuc(TEV) were transfected with increasing concentrations of a TEV expression construct and subsequently analyzed as in b. Data are presented as mean + s.e.m. and are representative of two (c,d) or four (b,f) independent experiments each. r.l.u., relative light units.

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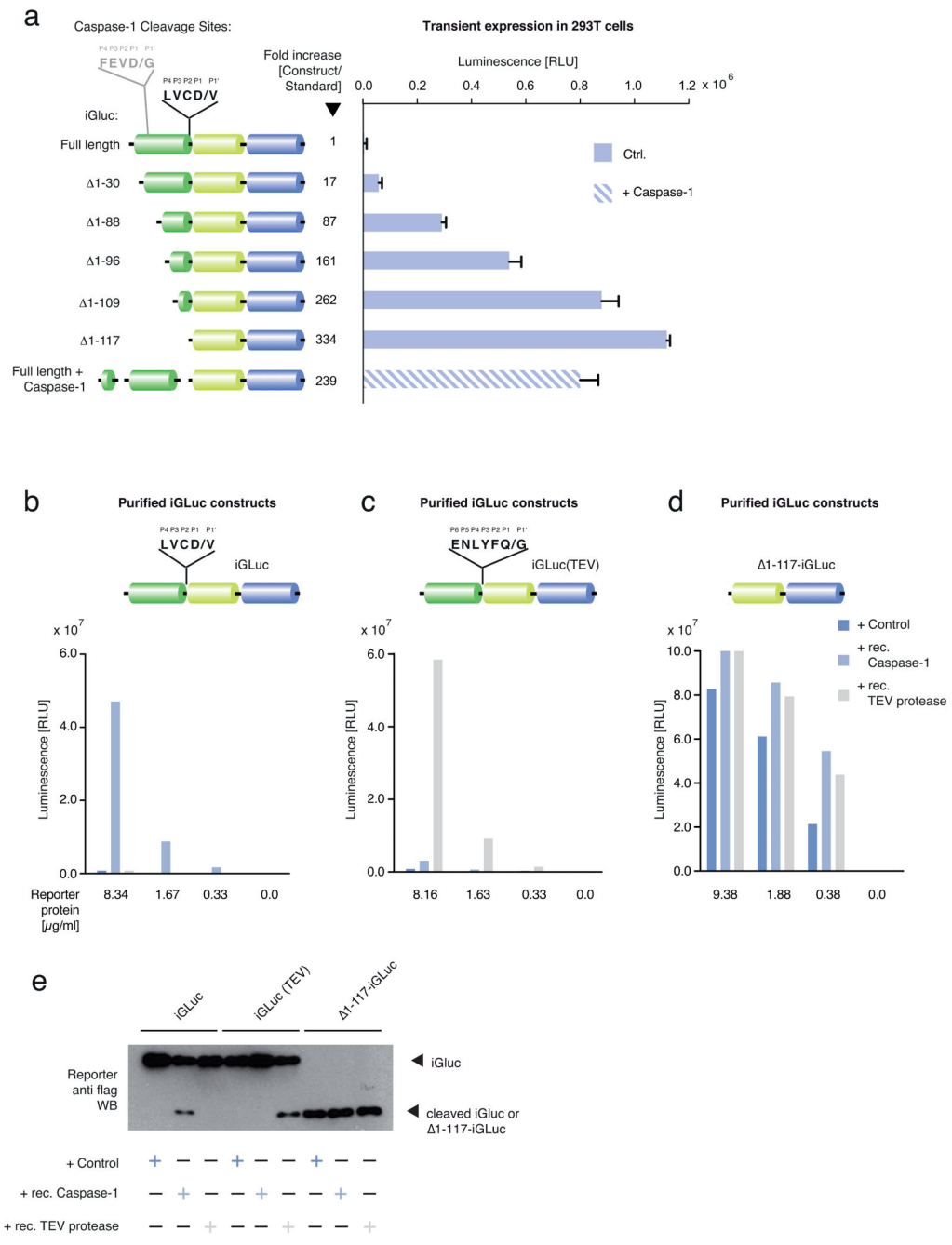

Figure 4. Functionality of purified iGLuc

(a) 293T cells were transiently transfected with 100 ng of the indicated N-terminally truncated variants, and luminescence of each construct was assessed in cell lysates. The shortest variant Δ1–117–iGLuc is the same length as mature IL-1β. Full-length iGLuc in the presence of caspase-1 was included as a positive control (100 ng, hatched bars.) (b–d) iGLuc (b), iGLuc(TEV) (c) and Δ1–117–iGLuc (d) were expressed in 293T cells and subsequently purified from cell lysates by affinity purification using anti-Flag. Purified iGLuc constructs were incubated at the indicated amounts in the presence of recombinant enzymes (rec.

caspase-1 or TEV protease) and then assessed for luciferase activity. (e) Western blot (WB) analysis was performed to study protein sizes under the different conditions. Data are presented as mean + s.e.m. and are representative of three independent experiments each. r.l.u., relative light units.

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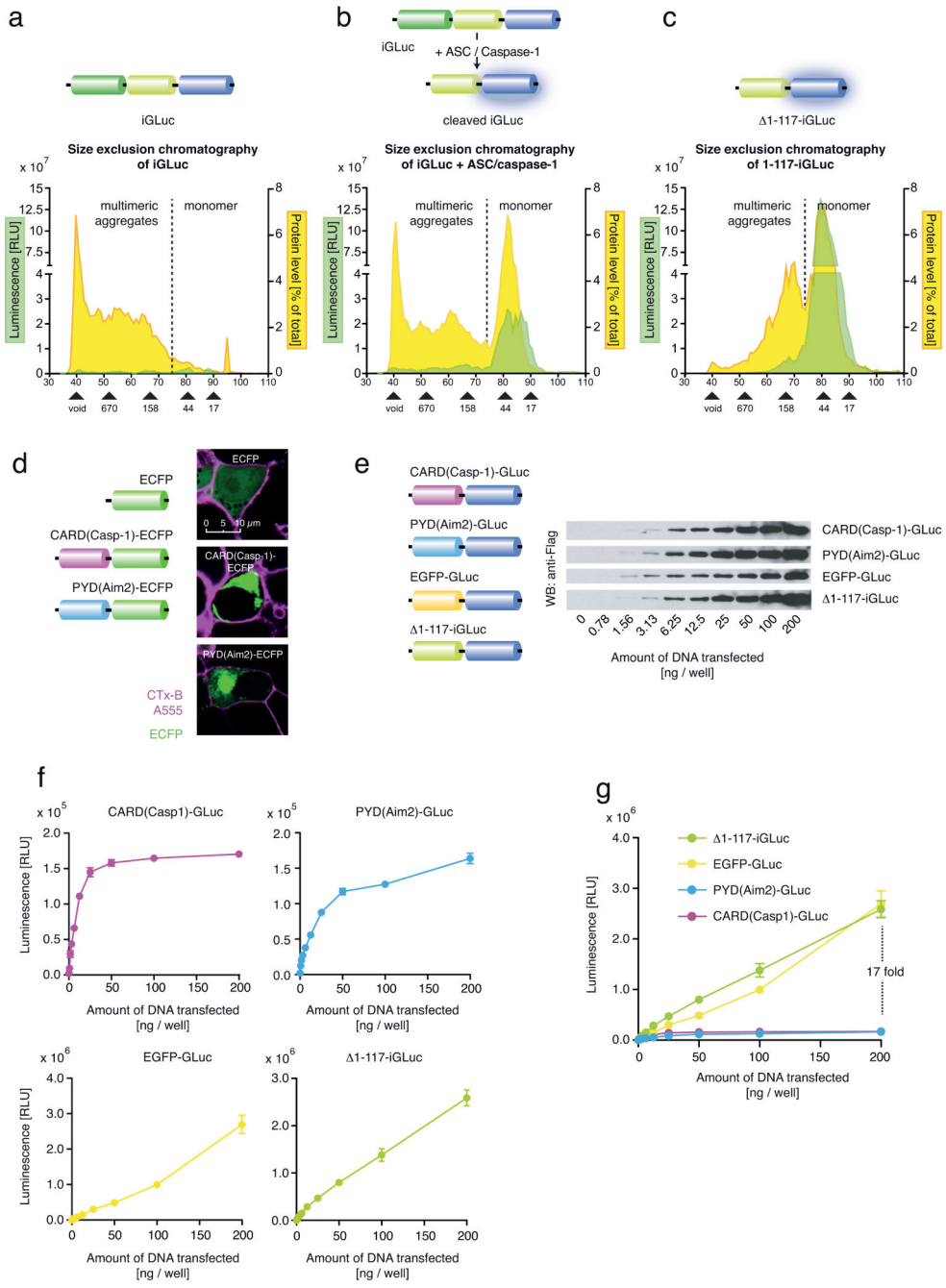

Figure 5. iGLuc is inactive in the context of protein aggregates

(a–c) Size-exclusion chromatography (SEC) analysis of iGLuc and its variants. iGLuc (a) and iGLuc in the presence of ASC and caspase-1 (b) or Δ1–117–iGLuc (c) were expressed in 293T cells, purified and subsequently subjected to SEC. Eluted fractions were then quantified for iGLuc levels using an IL-1β ELISA that detects the C-terminal part of IL-1β (yellow graphs); luciferase activity was assessed in parallel (green graphs). To allow for comparison, each data set was normalized to total protein amount (area under the curve). (d) ECFP-tagged versions of the CARD of caspase-1 (CARD(Casp1)-ECFP) or the PYD of

AIM2 (PYD(Aim2)-ECFP), and ECFP itself, were transiently expressed in 293T cells and subsequently analyzed by confocal microscopy. Cholera toxin subunit B conjugated to Alexa Fluor 555 (CTx-B A555) was used to stain the cell membrane. (e) GLuc fusion constructs of CARD(Casp1)-GLuc, PYD(Aim2)-GLuc, EGFP-GLuc and Δ1–117–iGLuc were expressed in increasing concentrations in 293T cells, and protein expression was assessed 24 h after transfection using western blotting (WB). (f) Luciferase activity in cells that were treated as in e was assessed. (g) For direct comparison, all data sets were plotted together in one graph. Data are presented as mean + s.e.m. and are representative of three (a–c,f,g) or two (d,e) independent experiments each. r.l.u., relative light units.

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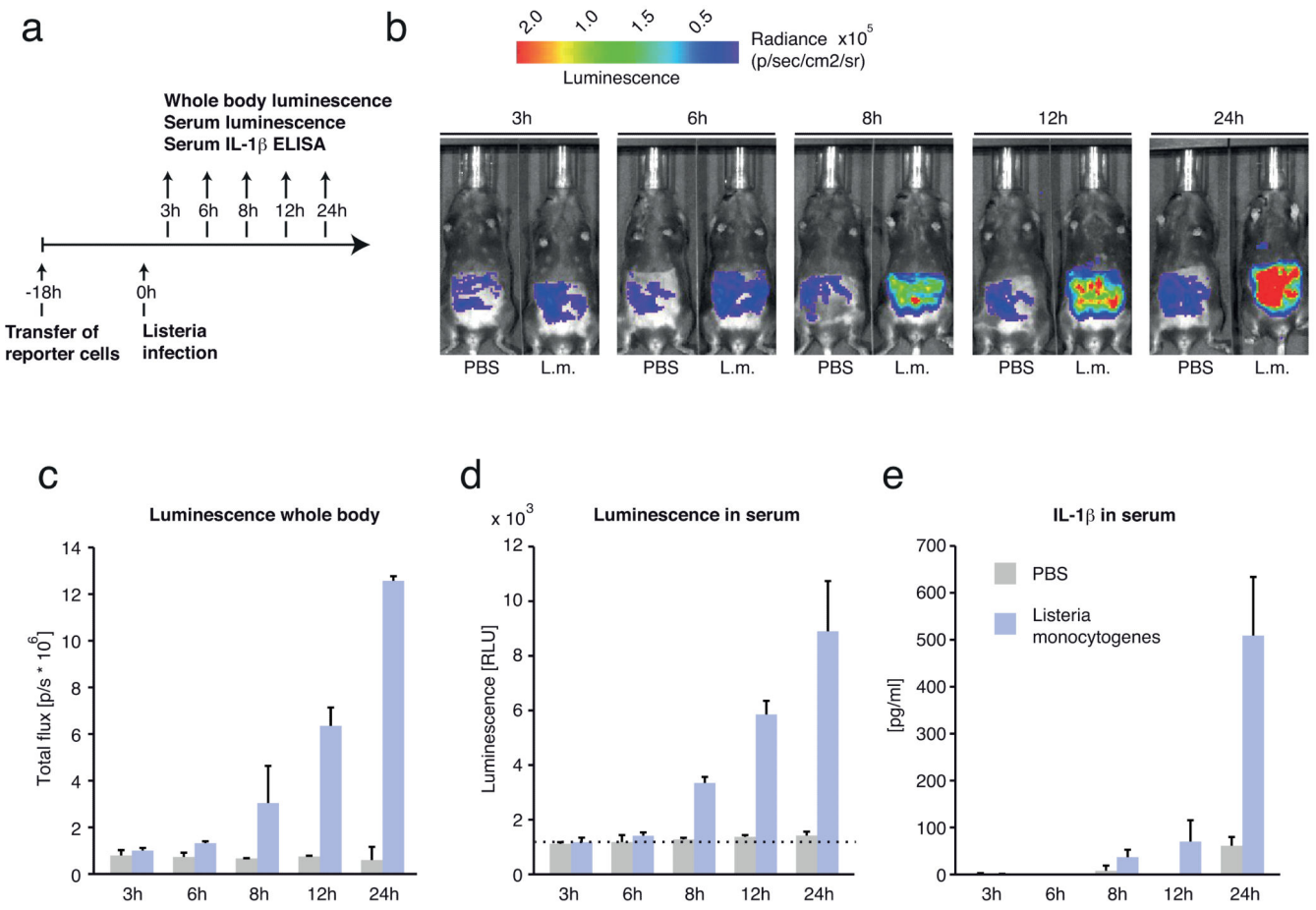

Figure 6. iGLuc as a tool to study in vivo inflammasome activation

(a) Schema of the experimental protocol. (b–e) Unmodified iGLuc reporter macrophages were transferred intraperitoneally into C57BL/6 mice. After 18 h, mice were challenged with Listeria monocytogenes (L.m.) or PBS intraperitoneally. At time points 3, 6, 8, 12 and 24 h after Listeria injection, luminescence was assessed using an IVIS in vivo imaging instrument (b) and represented quantitatively (c). In parallel, serum GLuc luminescence (d) and IL-1β protein levels (e) were measured in the serum of mice treated as in a at each respective time point. ELISA and luminescence data are mean + s.e.m. calculated for two mice treated with PBS and three mice infected with Listeria per time point. Representative data out of two independent test series are shown. r.l.u., relative light units.

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