

Poxvirus dsDNA genomes differentially activate AIM2 or NLRP3 inflammasomes in human primary cells

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Abstract

The innate immune system is known for its ability to recognize cytosolic DNA as evidence of infection, but detailed studies of this process have been mostly limited to mice and cell lines. To investigate inflammasome responses in human primary cells, we used engineered viruses encoding the inflammasome reporter caspase-1^{CARD}-EGFP. We show that released genomes of vaccinia virus and monkeypox virus trigger robust inflammasome assembly in human primary cells. To determine the involved inflammasome sensors, we generated nanobodies against AIM2. Three of them inhibit AIM2 inflammasome assembly by blocking the polymerization of the AIM2 Pyrin domain, most potently as bivalent nanobodies. Utilizing an engineered vaccinia virus expressing bivalent AIM2 nanobodies, we demonstrate that inflammasomes in primary human macrophages and keratinocytes are nucleated by AIM2, while CD14⁺ monocytes assemble NLRP3 inflammasomes. This finding resolves the discrepancy between the previously reported activation of AIM2 inflammasomes in mice and NLRP3 inflammasomes in humans, and provides the first evidence for cell-type-specific regulation of DNA-triggered inflammasome activation. The newly developed AIM2-specific nanobodies offer a precise tool to dissect and potentially target AIM2 inflammasome assembly in other disease contexts.

Keywords AIM2; Inflammasome; Nanobody; NLRP3; Poxvirus

Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

In a healthy functional cell, double-stranded DNA (dsDNA) is strictly confined to the nucleus or mitochondria. dsDNA outside these cellular compartments is a sign of cellular damage or pathogen infection. Several germline-encoded pattern recognition receptors recognize cytosolic dsDNA and initiate a cascade of events that either lead to an antiviral state, inflammation, or cell death (Briard et al, 2020). Of these, absent in melanoma 2 (AIM2) nucleates inflammasome assembly, while cyclic GMP-AMP synthase (cGAS) produces the secondary messenger 2'-5'/3'-5' cyclic GMP-AMP (2'3' cGAMP) to activate STING, resulting in the transcriptional activation of type I interferons and NF-κB-driven genes, lysosomal cell death, and autophagy (Sun et al, 2013; Hornung et al, 2009; Gaidt et al, 2017).

AIM2 consists of the N-terminal Pyrin domain (PYD) and a C-terminal DNA-binding HIN200 domain. The positively charged HIN200 domain binds to the negatively charged sugar-phosphate backbone of dsDNA in a sequence-independent manner (Jin et al, 2012). An initial study suggested that the interaction of AIM2^{HIN200} with AIM2^{PYD} yields an autoinhibited conformation in the absence of dsDNA, which is released upon the engagement of dsDNA by the HIN200 domain (Jin et al, 2012). Yet, a subsequent study concluded that binding of the AIM2^{HIN200} to dsDNA requires oligomerization of AIM2^{PYD}. This implies a cooperative binding model that ensures AIM2 is locally concentrated and thus able to nucleate inflammasomes even in the presence of excess dsDNA (Morrone et al, 2015). Short AIM2^{PYD} filaments then recruit the inflammasome adapter protein ASC (apoptosis-associated speck-like protein containing CARD) and initiate polymerization of ASC^{PYD} filaments (Lu et al, 2014b). The latter are cross-linked by the ASC^{CARD} to form ASC specks, which recruit and activate the effector protein caspase-1 through the caspase-1^{CARD}, forming the AIM2 inflammasome (Schmidt et al, 2016b). Active caspase-1 cleaves GSDMD and the pro-inflammatory cytokines IL-1β and

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IL-18 into their mature forms. The N-terminal domain of GSDMD inserts into the plasma membrane, oligomerizes to assemble pores with more than 30 subunits, and facilitates the release of cytokines and a highly inflammatory form of cell death described as pyroptosis (Kayagaki et al, 2015; Xia et al, 2021; Shi et al, 2015; Schiffelers et al, 2024).

Similarly, cGAS binds to dsDNA through its positively charged C-terminus in a sequence-independent manner. dsDNA-bound cGAS undergoes a conformational change into its active state and catalyzes the production of 2'3' cGAMP. cGAMP activates the cyclic-dinucleotide sensor STING, which recruits and activates TANK-binding kinase 1 (TBK1) to consecutively phosphorylate STING and the interferon regulatory factor 3 (IRF3) transcription factor. Phosphorylated IRF3 dimerizes and translocates into the nucleus, driving the expression and secretion of type I interferons and the ensuing expression of interferon-stimulated genes (ISGs), establishing an antiviral state of the cell (Sun et al, 2013). STING-induced lysosomal rupture and subsequent potassium efflux has also been reported to activate another inflammasome sensor, NLRP3 (Blevins et al, 2022; Gaidt et al, 2017). The cGAS-STING-NLRP3 axis was proposed to mediate the inflammasome response to dsDNA in human myeloid cells, while the role of AIM2 as the relevant inflammasome sensor to dsDNA in mice and human THP-1 cells is well established (Hornung et al, 2009). In vivo, both Aim2 and cGAS have been shown to play a protective role against bacteria and DNA virus infections in mice (Rathinam et al, 2010).

Poxviruses are brick/oval-shaped, large dsDNA viruses with a genome size of about 200 kbp. Unlike most DNA viruses, they replicate in the host cell cytosol (Lefkowitz et al, 2006). Poxviruses have co-evolved with most vertebrates and include many pathogens with narrow or broad host range. The most detrimental human pathogen of the poxvirus family, variola virus, caused smallpox and has been eradicated by a successful vaccination campaign with live vaccines based on the related vaccinia virus (VACV) (Henderson, 2011). Molluscum contagiosum virus causes a relatively benign disease in immunocompetent humans, whereas other vertebrate poxviruses, such as cowpox virus, regularly cause zoonotic infections (Oliveira et al, 2017). Monkeypox virus (MPXV) was historically endemic in Western and Central parts of Africa, largely caused by zoonotic infections. A recent global outbreak of clade IIB strains fueled by human-to-human transmissions led to about 100,000 Mpox cases worldwide since 2022. A more virulent strain of clade I is currently emerging in the Democratic Republic of the Congo (Kaler et al, 2022; Kibungu et al, 2024).

Poxviruses undergo their full viral life cycle in the cytoplasm of the infected host cells. The two infectious forms of poxviruses, mature virions (MVs) and extracellular virions (EVs), enter cells by macropinocytosis, followed by fusion of the single (MVs) or innermost (EVs) membrane with the limiting membrane of endosomes (Schmidt et al, 2011; Mercer and Helenius, 2008; Schmidt et al, 2012). This deposits viral cores into the cytosol, which start to transcribe and export early mRNAs. Uncoating of viral genomes relies on early viral as well as host proteins (Kilcher et al, 2014). VACV, and in particular the attenuated and host-restricted strain modified vaccinia virus Ankara (MVA), has been widely used to study the immune response to poxviruses. VACV infection of mouse bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) have been shown to activate Aim2 (Hornung et al, 2009; Rathinam et al, 2010). MVA infection of

human monocytes, BMDMs, and the monocyte-like cell line BlaER1 was shown to activate the NLRP3 inflammasome (Gaidt et al, 2017). The lack of specific inhibitors for inflammasome sensors other than NLRP3 has limited inflammasome research to the use of genetically modified cell lines or mouse models. Consequently, the inflammasome response to poxvirus infections in human primary cells has not been adequately addressed. Nevertheless, the evolution of viral host-modulatory proteins to inhibit caspase-1 and other caspases by VACV B13 (OPG199), as well as viral soluble IL-1 β and IL-18 receptors VACV B15 (OPG201) and C12 (OPG022), clearly demonstrates the relevance of inflammasome responses to contain poxvirus infections (Smith et al, 2013; Hernaez and Alcamí, 2024).

In this study, we investigated the inflammasome response to VACV infection in human primary monocytes, macrophages, and keratinocytes. We engineered recombinant VACV strains to quantitatively measure inflammasome activation in primary cells and developed antagonistic nanobodies as the first AIM2-specific inhibitors. We found that the release of poxvirus genomes prior to any DNA replication is sufficient to initiate inflammasome responses. Importantly, VACV activated AIM2 in human macrophages and keratinocytes, while infection of human monocytes triggered the activation of NLRP3 inflammasomes, indicating cell-type-specific regulation of inflammasome responses to VACV in human primary cells. Our findings highlight the intricate regulation of inflammasomes and imply the involvement of additional factors that positively or negatively regulate inflammasome activation in distinct cell types.

Results

VACV infection activates inflammasomes in IFN- γ -treated macrophage-like cell lines

To obtain first insights into the inflammasome responses to poxviruses in human myeloid cells, we infected PMA-differentiated human macrophage-like THP-1 cells with VACV strain Western Reserve (WR). In unprimed THP-1 cells, no substantial secretion of IL-1 β was observed. Previous studies have shown that poxvirus lesions in murine skin are associated with strong IFN- γ release (Liu et al, 2010), while other inflammasomes rely on TLR-mediated priming (Bauernfeind et al, 2011). We therefore pretreated PMA-differentiated THP-1 cells with LPS, IFN- γ , or IFN- α prior to infection with VACV (Fig. 1A). VACV infection induced strong IL-1 β secretion when cells were pretreated with IFN- γ , and a weak response in LPS-pretreated cells. No IL-1 β was detected in cells treated with IFN- α . We next measured LDH release and uptake of the DNA dye DRAQ7 as readouts for membrane rupture and increased plasma membrane permeability, respectively (Fig. 1B–D). Cell death, as well as pyroptotic morphology, were only observed in IFN- γ pretreated cells, but not in any of the other conditions.

To quantify inflammasome activation at a single-cell level, we generated a THP-1 reporter cell line expressing caspase-1^{CARD}-EGFP (C1C-EGFP). C1C-EGFP recapitulates the recruitment of caspase-1 and thus is efficiently recruited to nascent ASC specks (Jenster et al, 2023). The reporter is extensively characterized in a separate manuscript (Gohr et al, 2025). When cells are stimulated in the presence of the caspase-1 inhibitor VX-765 (VX), cell death

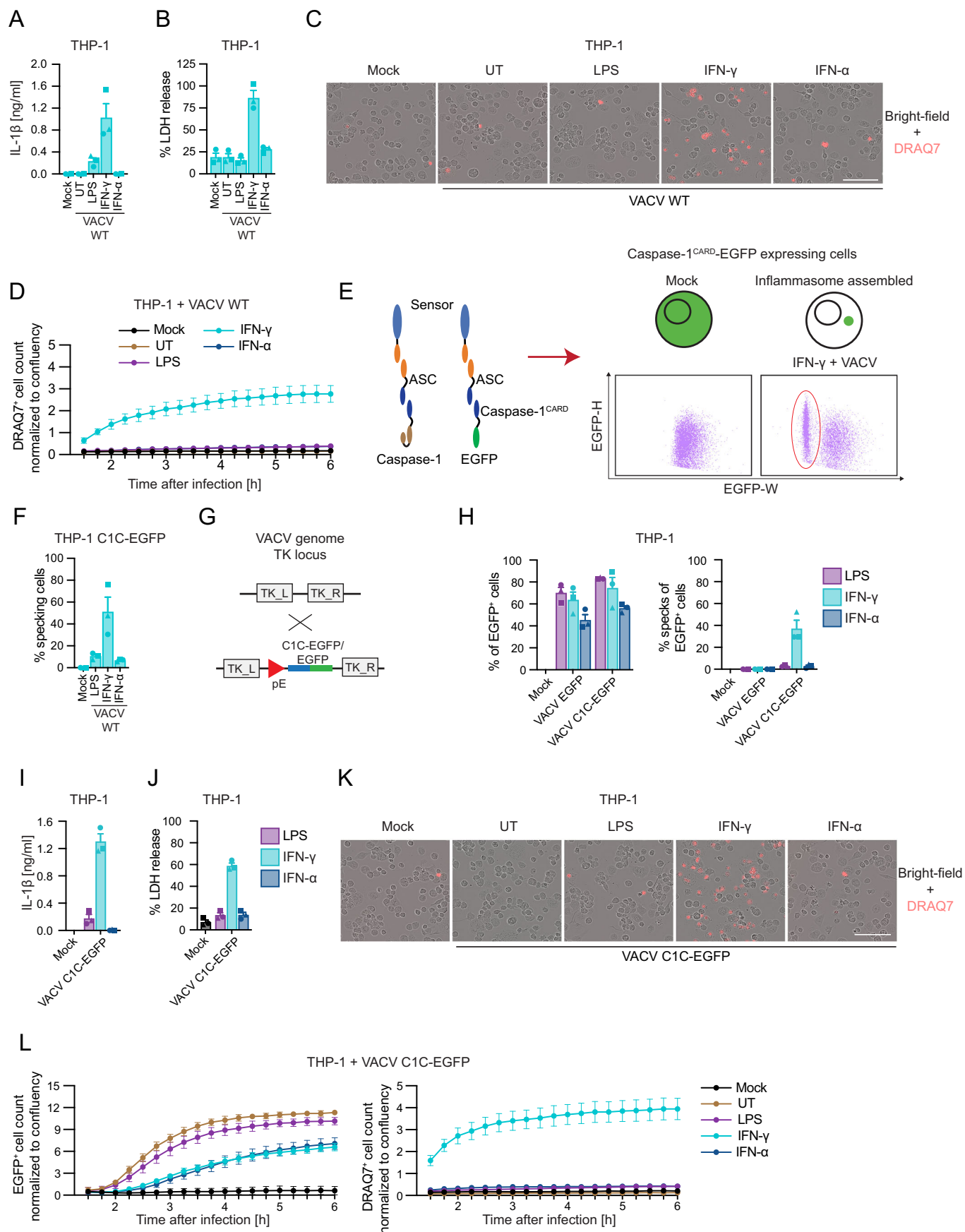


Figure 1. VACV infection induces inflammasome activation in IFN- γ -treated macrophage-like cell lines.

(A–D) PMA-differentiated THP-1 macrophages were either pretreated for 3 h with LPS (200 ng/mL), or overnight with 500 U/mL IFN- γ or IFN- α and infected with VACV WR WT at a multiplicity of infection (MOI) of 5 for 6 h. IL-1 β in the supernatant was measured by homogeneous time-resolved fluorescence (HTRF) (A). Cell death was measured by LDH release, normalized to cells lysed in 1% Triton X-100 (B). Membrane permeabilization was quantified by the uptake of DNA dye DRAQ7 over 6 h in an Incucyte Live-Cell Imaging system. Representative images after 6 h (C) and graphs of normalized DRAQ7 uptake over 6 h are displayed (D). (E) Scheme of inflammasome reporter caspase-1^{CARD}-EGFP (C1C-EGFP), and detection of C1C-EGFP specks with flow cytometry, by plotting height (EGFP-H) against width (EGFP-W) of the EGFP signal. Data were from a representative sample of PMA-differentiated THP-1 C1C-EGFP cells pretreated with IFN- γ and infected with VACV WR WT. (F) PMA-differentiated THP-1 macrophages expressing doxycycline (dox)-inducible C1C-EGFP were treated with LPS, IFN- γ , or IFN- α as mentioned before and infected with VACV at an MOI 5 for 6 h in the presence of 40 μ M VX-765 (VX). Infected cells were harvested, fixed in 4% PFA, and speck assembly was quantified by flow cytometry. (G) Genome structure of recombinant VACV strains expressing C1C-EGFP from the J2R early promoter (pE). The transgene was inserted into the VACV TK locus by homologous recombination. (H–L) PMA-differentiated THP-1 macrophages were pretreated as mentioned before and infected with the indicated recombinant VACV at an MOI of 5 in the presence (H) or absence (I–L) of VX for 6 h. Infection (EGFP⁺ cells, left) and C1C-EGFP speck assembly in infected or mock-treated cells was quantified by flow cytometry (H). IL-1 β in the supernatant was measured by HTRF (I), and cell death by LDH release (J) as described before. Representative images of cells infected in the presence of DRAQ7 for 6 h (K), and graphs of normalized infection (EGFP⁺, left) and DRAQ7 uptake (right) over 6 h (L) are displayed. Scale bar = 100 μ m. Data represents average values (with individual data points) from $N = 3$ independent experiments $\pm$ SEM. Source data are available online for this figure.

by pyroptosis is prevented, and intact cells can be analyzed by flow cytometry. The characteristic redistribution of C1C-EGFP into a single speck per cell allows us to specifically quantify cells with assembled inflammasomes (Sester et al, 2015): In cells with inflammasomes, the height of the EGFP signal is increased, while the width of the signal is decreased in the flow cytometer (Fig. 1E). We observed robust inflammasome assembly in the IFN- γ pretreated cells, while LPS pretreated cells responded much weaker (Fig. 1F). As we are interested to study the inflammasome response to VACV in primary cells, we next engineered recombinant VACV strains that either express the inflammasome reporter C1C-EGFP or the control protein EGFP under the early J2R promoter (Fig. 1G). Recombinant VACV C1C-EGFP allows us to specifically quantify inflammasome assembly in infected, i.e., EGFP-positive cells. We infected PMA-differentiated THP-1 cells and found that infection was not affected by the different cytokine pre-treatments at the applied MOI of 5. Importantly, we observed a strong C1C-EGFP specking response in IFN- γ pretreated cells infected with VACV C1C-EGFP, but no recruitment of fluorescence to ASC specks in cells infected with control VACV EGFP (Fig. 1H). Similar to wild-type VACV, infection with recombinant VACV C1C-EGFP induced IL-1 β secretion, LDH release, and uptake of DNA dye DRAQ7 in IFN- γ pretreated cells (Fig. 1I–L). Expression of C1C-EGFP from an early promoter did not affect VACV-mediated IL-1 β release or cell death (Fig. EV1A). This indicates that the expression of the reporter does not affect the viral life cycle or inflammasome responses, allowing us to robustly quantify inflammasome assembly at the single-cell level and specifically in infected cells.

Poxvirus infection triggers inflammasome activation in diverse human primary cells

After establishing that VACV infection induces an inflammasome response in IFN- γ pretreated THP-1 cells, we next tested whether VACV also induces inflammasome assembly in human primary cells. We infected IFN- γ pretreated or untreated CD14⁺ monocytes, GM-CSF differentiated monocyte-derived macrophages (hMDMs), and normal human epidermal keratinocytes (NHEK) with VACV C1C-EGFP/EGFP. To narrow down the potential inflammasome sensors involved in the sensing of VACV, we included CRID3 (also known as MCC950), a specific inhibitor of NLRP3 (Coll et al, 2015). Unfortunately, no specific inhibitors for other inflammasome sensors are available. VACV infected both GM-CSF-

differentiated macrophages and NHEKs. IFN- γ pretreated cells assembled inflammasomes (Fig. 2A,C,D,F), although the fraction of responding primary cells was lower than in THP-1 cells. Accordingly, we observed cell death as measured by DRAQ7 uptake in IFN- γ -pretreated cells (Figs. 2B,E and EV2A,B). Inflammasome assembly was not inhibited by CRID3, indicating that the responses were independent of NLRP3. Infection of CD14⁺ monocytes also elicited a robust inflammasome response and cell death (Figs. 2G–I and EV2C). While IFN- γ pretreatment boosted VACV infection, inflammasome responses did not require IFN- γ pretreatment, and IFN- γ even seemed to reduce inflammasome activation. Interestingly, inflammasome assembly was completely blocked by CRID3, indicating that responses in monocytes were mediated by NLRP3. This confirms the NLRP3 activation in monocytes after dsDNA delivery or MVA infection that was observed before (Gaidt et al, 2017). We were not able to detect IL-1 β release or infection-induced cell death in any of the primary cell types investigated (Fig. EV2D–I). Cell death by LDH release is generally difficult to detect if only a fraction of cells assemble inflammasomes. The absence of inflammasome-related cytokines indicates that infection may counteract later stages of the inflammasome response. It also highlights the high sensitivity of the specking-based readout, which reports on the first stages of inflammasome assembly, and which is therefore less sensitive to any perturbations that affect cytokine levels and e.g., the upregulation of pro-IL-1 β .

Inflammasome responses in VACV-infected THP-1 cells were insensitive to the NLRP3 inhibitor CRID3 (Figs. 2J and EV2J). To define the involved inflammasome sensor, we next infected various monoclonal THP-1 knockout (KO) cell lines with VACV C1C-EGFP. We observed that infection activated inflammasomes in NLRP3, STING, and cGAS KO cells, while inflammasome responses were blunted in AIM2 and ASC KO cells (Figs. 2K and EV2K), confirming earlier findings in THP-1 cells (Gaidt et al, 2017). Taken together, IFN- γ pretreatment is necessary for VACV-triggered inflammasome assembly in THP-1 cells, NHEKs, and GM-CSF macrophages. The inflammasome response in THP-1 cells is AIM2- and ASC-dependent, whereas CD14⁺ monocytes assemble NLRP3 inflammasomes, independent of IFN- γ pretreatment. Due to the absence of inhibitory drugs or knockouts, the involved sensor for inflammasome assembly in primary human GM-CSF macrophages and NHEKs could not be verified.

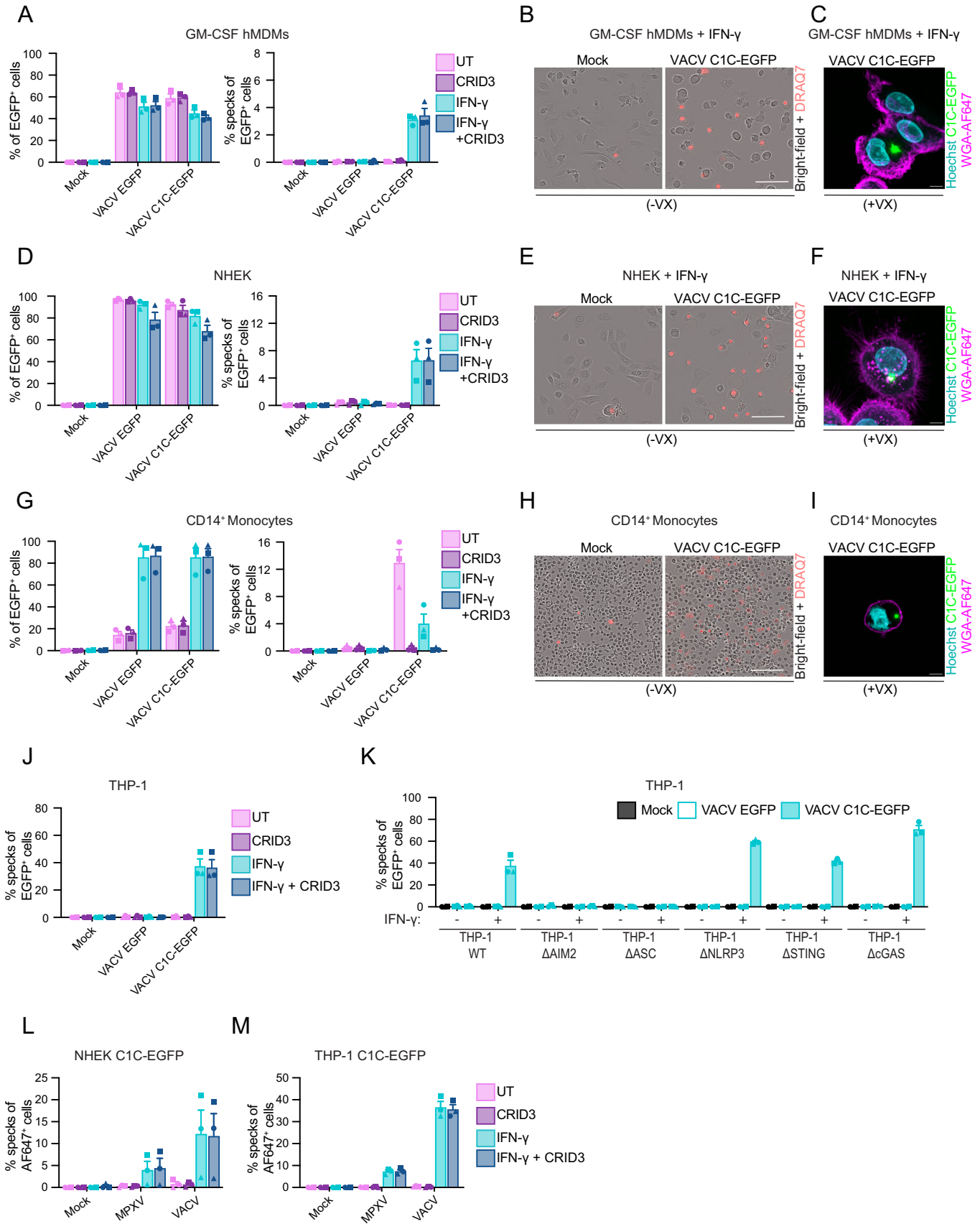


Figure 2. Poxvirus infection induces inflammasome activation in primary human cell types.

(A–C) Human monocyte-derived macrophages (hMDMs) differentiated in GM-CSF, (D–F) normal human epidermal keratinocytes (NHEK), (G–I) CD14⁺ monocytes, and (J) PMA-differentiated THP-1 macrophages were left untreated or pretreated with IFN- γ overnight. Cells were infected with indicated recombinant VACV strains at an MOI of 5 (A–F, J) or MOI 10 (G–I), in the presence (A, C, D, F, G, I, J) or absence (B, E, H) of VX, and where indicated, in the presence of 2.5 μ M CRID3. Cells were harvested and fixed 6 h post infection. Infection (EGFP⁺ cells) and C1C-EGFP speck assembly in infected or mock-treated cells was quantified by flow cytometry (A, D, G, J); infection data (EGFP⁺ cells) corresponding to the experiment shown in panel (J) is displayed in Fig. EV2J. Membrane permeabilization was quantified by the uptake of DNA dye DRAQ7 over 6 h in an Incucyte live-cell imaging system (B, E, H). Scale bar = 100 μ m. Samples for confocal microscopy were fixed and stained with 5 μ g/mL wheat germ agglutinin (WGA)-Alexa Fluor (AF) 647 and 4 μ M Hoechst 33342 (C, F, I). Scale bar = 5 μ m. (K) PMA-differentiated WT or indicated knockout THP-1 cells were left untreated or pretreated with IFN- γ overnight and infected with the indicated reporter VACV strains at an MOI of 5 in the presence of VX. C1C-EGFP speck assembly was quantified as before. The corresponding infection data (EGFP⁺ cells) is shown in Fig. EV2K. (L, M) NHEK (L) or PMA-differentiated THP-1 cells (M) constitutively expressing C1C-EGFP were left untreated or pretreated with IFN- γ overnight and infected with VACV WT or monkeypox virus (MPXV) at an MOI of 5, and where indicated, in the presence of CRID3. Cells were harvested and fixed 6 h post infection. VACV- or MPXV-infected cells were stained with rabbit polyclonal anti-H5 serum and AF647-labeled goat anti-rabbit IgG antibodies, and measured by flow cytometry. Corresponding infection data (α H5-AF647⁺ cells) is shown in Fig. EV2L. M. C1C-EGFP speck assembly was analyzed in infected or mock-treated cells. Data represent average values (with individual data points) from $N = 3$ independent donors (primary cells) $\pm$ SEM, or from $N = 3$ independent experiments $\pm$ SEM (THP-1 cells). Source data are available online for this figure.

The currently emerging MPXV clade IIB shares 96% similarity with VACV (Ahmed et al, 2022). Clinical symptoms of Mpox disease manifest mainly in the skin as rashes and skin/mucosal lesions, in addition to other systemic symptoms (Harapan et al, 2022). We therefore wondered if MPXV infection would also activate inflammasomes in NHEK. We plaque-purified a clinical MPXV isolate and purified virus stocks comparable to our VACV stocks. NHEKs were transduced with lentivirus to express the inflammasome reporter C1C-EGFP and infected with MPXV and VACV. Comparable to VACV, NHEKs were robustly infected by MPXV (Fig. EV2L). MPXV infection induced inflammasome activation in IFN- γ pretreated cells, and the response was not blocked by CRID3 (Fig. 2L). Yet, the fraction of cells assembling inflammasomes was lower than after VACV infection. We next tested the MPXV infection with PMA-differentiated THP-1 cells and again observed that inflammasome responses depend on IFN- γ pretreatment and were lower than the response after VACV infection (Figs. 2M and EV2M). Our findings highlight that, similar to VACV, MPXV activates inflammasomes other than NLRP3 in IFN- γ pretreated NHEKs and PMA-differentiated THP-1 macrophages. As inflammasome activation was reduced, MPXV may be better equipped to counteract inflammasome responses or may expose less of the critical ligands necessary for inflammasome activation.

Identification and characterization of AIM2 nanobodies

If available, small compound inhibitors can rapidly impair protein function in human primary cells. They thus help to define the contribution of individual proteins to a biological phenotype, even if the cells are not amenable to genetic modification. Accordingly, we used the NLRP3 inhibitor CRID3 to show that the inflammasome response in primary CD14⁺ monocytes entirely depends on NLRP3. We next wondered whether the CRID3-insensitive inflammasome response to VACV infection in primary GM-CSF differentiated macrophages and NHEKs relies on AIM2. As no specific AIM2 inhibitors exist, we decided to generate AIM2-specific nanobodies to perturb AIM2 function. We raised nanobodies against the AIM2^{PYD} protein by immunizing an alpaca (*Vicugna pacos*) with MBP-AIM2^{PYD} L10A L11A, a mutant of the PYD that cannot polymerize into filaments (Lu et al, 2014a). We cloned the coding sequences for the variable domains of heavy chain-only antibodies (VHHs) into a phagemid vector as described before (Ingram et al, 2018; Koenig et al, 2021). We employed two

strategies to identify AIM2^{PYD}-specific nanobodies by phage display. In the first approach, we expressed full-length human AIM2-Strep2-HA (AIM2-SH) in HEK293 Flp-In T-REx cells, lysed the cells, and immobilized the SH-tagged protein on Strep-Tactin beads. The beads were used to enrich for phages displaying AIM2-specific nanobodies in two consecutive selection rounds. In a second approach, we prepared phages from the library, depleted phages displaying nanobodies binding to MBP, and subsequently used recombinant His-MBP-AIM2^{PYD} L10A L11A immobilized on magnetic amylose beads to enrich for phages displaying AIM2^{PYD}-specific nanobodies. Supernatants from 95 nanobody candidates of each phage display campaign were tested by ELISA with immobilized His-MBP-AIM2^{PYD} L10A L11A. Nanobodies corresponding to positive wells were sequenced, and a representative candidate of each cluster was selected for further characterization (Figs. 3A and EV3A). Altogether, six hits were identified using phage display against full-length AIM2 expressed in HEK293 cells, and 12 hits using phage display against the recombinantly expressed His-MBP-AIM2^{PYD} L10A L11A. We confirmed nanobody binding and specificity by ELISA (Figs. 3B,C and EV3B) and found that all nanobodies bound AIM2^{PYD} but not NLRP3^{PYD} (Fig. EV3B). Some of the nanobodies identified with His-MBP-AIM2^{PYD} L10A L11A exhibited background binding to the fusion protein MBP. We next performed LUMIER assays to test the interaction of cytosolically expressed HA-tagged nanobodies with AIM2 expressed as a fusion to Renilla luciferase in the cytosol of living cells. We selected three nanobodies, VHH_{AIM2-1}, VHH_{AIM2-2} and VHH_{AIM2-3}, which are functional in the reducing environment of the cytosol and bind AIM2, but not the control protein NLRP1^{CARD} (Fig. 3D). As VHH_{AIM2-1} and VHH_{AIM2-3} share the same CDR2, and their CDR3 only differs by one amino acid, it is likely that they employ the same binding mode. All three nanobodies were specific to human AIM2 and did not bind mouse Aim2 (Fig. 3D). LUMIER assays with AIM2^{HIN200} or AIM2^{PYD} domains showed that nanobodies bound the full-length AIM2 and AIM2^{PYD}, but not AIM2^{HIN200}, as expected based on the antigen used for immunization (Fig. 3E).

We conducted competition ELISA experiments and used an excess of VHH-LPETG to test interference with the binding of HA-tagged nanobodies, which was detected with HRP-coupled HA antibodies. As expected, binding of VHH_{AIM2-1}-HA to MBP-AIM2^{PYD} was blocked by an excess of VHH_{AIM2-1}-LPETG (Fig. 3F). Similarly, binding of VHH_{AIM2-2}-HA to MBP-AIM2^{PYD} was

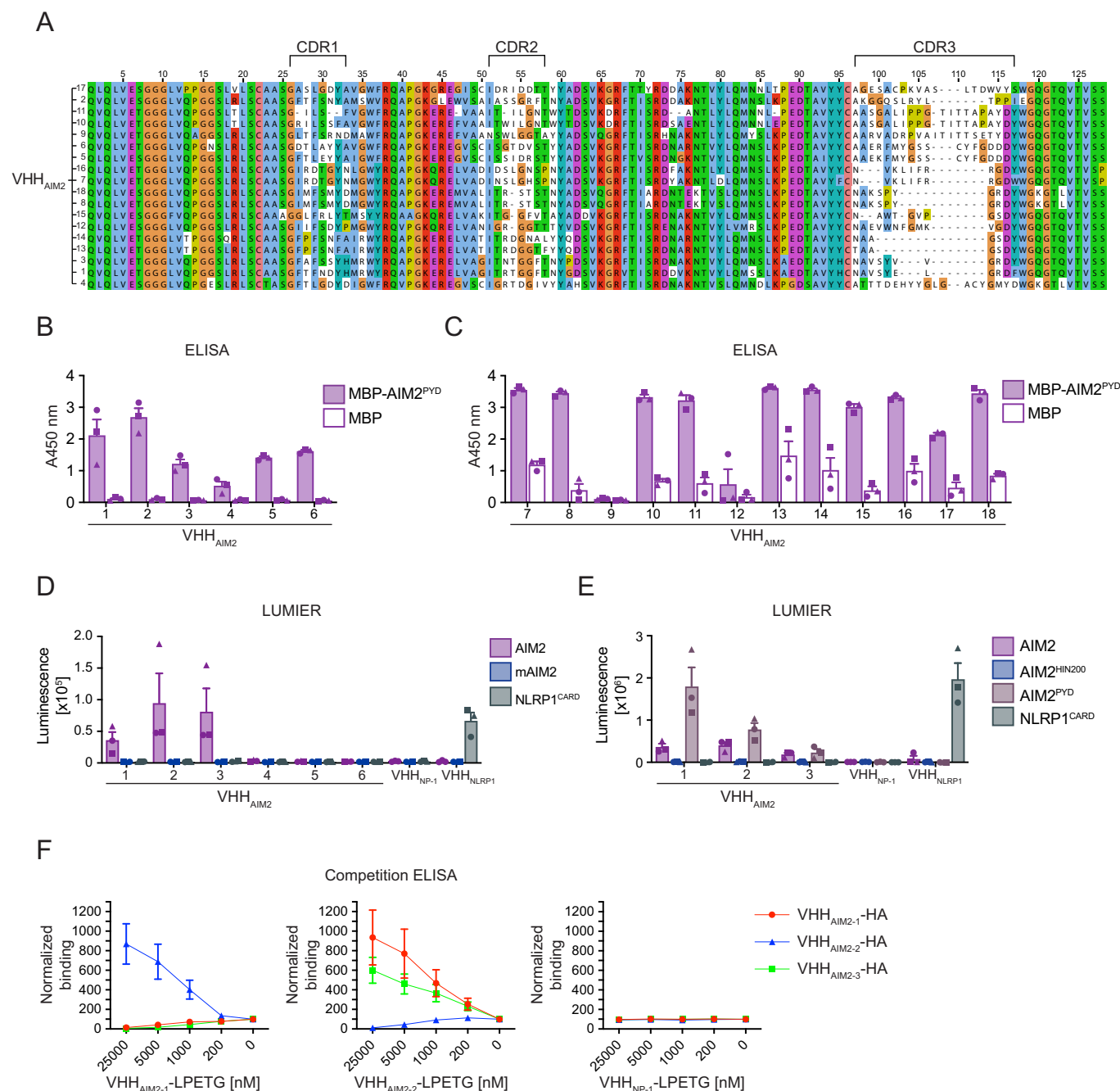


Figure 3. Identification and characterization of AIM2 nanobodies.

(A) Sequence alignment of the 18 AIM2 nanobody candidates. Complementarity-determining regions (CDRs) are indicated. (B, C) Binding of the indicated recombinant HA-tagged nanobody candidates (1 μ M VHH_{AIM2}(1-6)-HA-His) and (0.1 μ M VHH_{AIM2}(7-18)-HA-His) to immobilized MBP-AIM2^{PYD} or control protein MBP was quantified by ELISA with anti-HA HRP antibodies and the chromogenic substrate TMB. Nanobodies identified by phage display with full-length eukaryotically expressed AIM2-SH (B) or bacterially expressed MBP-AIM2^{PYD} (C) were analyzed, and absorption at 450 nm (A450) is displayed. (D, E) Binding of indicated nanobodies in the cytosol was quantified by LUMIER assay. HEK293T cells were co-transfected with expression vectors for the specified HA-tagged nanobodies and the indicated proteins fused to Renilla luciferase (mAIM2 indicates murine AIM2). 24 h post transfection, cells were lysed and VHH-HA was immunoprecipitated with immobilized anti-HA antibody. Coelenterazine-h was added and luminescence of co-purified Renilla luciferase was measured. Binding to full-length mouse/human AIM2 (D), or to different AIM2 domains (E) was analyzed. (F) Competition ELISA: MBP-AIM2^{PYD} was immobilized to ELISA plates, and binding of 1 μ M of the indicated HA-tagged nanobodies in the presence of an increasing concentration of VHH_{AIM2}(1-6)-LPETG-His, VHH_{AIM2}(7-18)-LPETG-His or control nanobody VHH_{NP-1}-LPETG-His was quantified by ELISA with anti-HA HRP antibodies and TMB as in (A). Values were normalized to binding in the absence of VHH-LPETG-His. Data represent average values from $N = 3$ independent experiments $\pm$ SEM. Individual data points are shown for (B-E). Source data are available online for this figure.

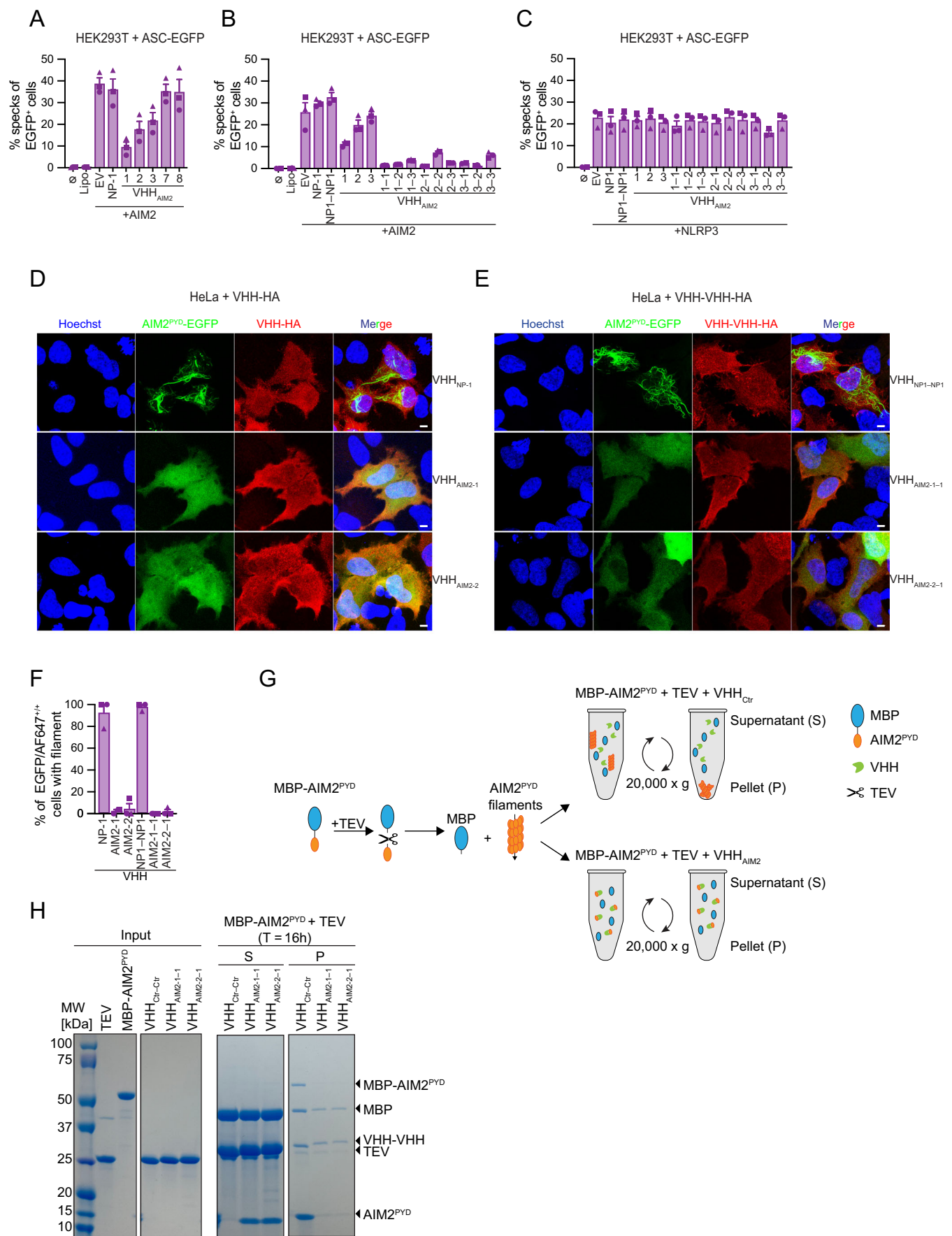


Figure 4. AIM2 nanobodies inhibit inflammasome assembly by preventing oligomerization of AIM2^{PYD} filaments.

(A–C) HEK293T cells expressing ASC-EGFP were co-transfected with empty vector (EV), AIM2 (A, B), or NLRP3 (C) expression vectors as well as expression vectors for the indicated monovalent VHH-HA (A) or bivalent VHH-(G₄S)₃-VHH-HA (B, C). ASC-EGFP speck assembly was quantified by flow cytometry 24 h post transfection. (D–F) HeLa cells were co-transfected with expression vectors for AIM2^{PYD}-EGFP and the indicated monovalent VHH-HA (D) and bivalent VHH-(G₄S)₃-VHH-HA (E). 24 h post transfection, cells were fixed and stained with Hoechst 33342 and anti-HA AF647. Cells were recorded by confocal microscopy, and images representative of at least 3 independent experiments are displayed (D, E). Scale bar = 5 μm. The fraction of EGFP-positive cells containing AIM2^{PYD}-EGFP filaments was quantified by manual counting (at least 50 EGFP-positive cells per condition and repeat were analyzed). (G) Schematic representation of the AIM2^{PYD} polymerization assay. Soluble MBP-AIM2^{PYD} is cleaved by TEV protease. The released AIM2^{PYD} polymerizes into insoluble filaments that can be sedimented by high-speed centrifugation. (H) MBP-AIM2^{PYD} and a 1.5-fold molar excess of control or AIM2-inhibitory bivalent nanobodies were incubated with TEV protease at RT for 16 h. AIM2^{PYD} filaments were sedimented at 20,000 × g, and supernatants and pellets were analyzed by SDS-PAGE and colloidal Coomassie staining. Input as well as supernatants and pellets from samples with bivalent nanobodies are shown in this panel. The uncropped gels with samples of the reaction at *t* = 0 and 16 h as well as samples with monovalent nanobodies are shown in Fig. EV4A. Average values with individual data points from *N* = 3 independent experiments ± SEM are displayed (A, B, C, F). Source data are available online for this figure.

inhibited by an excess of VHH_{AIM2-2}-LPETG. Binding of VHH_{AIM2-3}-HA to MBP-AIM2^{PYD} was also blocked by an excess of VHH_{AIM2-1}-LPETG, indicating that VHH_{AIM2-1} and VHH_{AIM2-3} share an overlapping epitope. VHH_{AIM2-1}-HA and VHH_{AIM2-3}-HA still bound their target in the presence of VHH_{AIM2-2}-LPETG, as did VHH_{AIM2-2}-HA in the presence of VHH_{AIM2-1}-LPETG, indicating that they bind to two different epitopes. Surprisingly, VHH binding was substantially enhanced by increasing amounts of nanobodies specific for the respective other epitope on AIM2^{PYD}, but not by control nanobody VHH_{NP-1}-LPETG. We speculate that an excess of nanobodies binding to an independent epitope may render more of AIM2^{PYD} accessible for binding, e.g., by counteracting the oligomerization of AIM2^{PYD}.

AIM2 nanobodies inhibit inflammasome assembly by preventing oligomerization of AIM2^{PYD} filaments

We next evaluated whether cytosolic expression of AIM2^{PYD} nanobodies interfered with AIM2 inflammasome assembly in living cells. We transfected ASC-EGFP expressing HEK293T cells with expression vectors for AIM2-FLAG and VHH-HA. This enables the transcription and translation of AIM2, while the plasmid dsDNA itself serves as an AIM2 ligand. AIM2 binding to dsDNA induces AIM2 oligomerization and the nucleation of ASC-EGFP specks. While co-expression of a control nanobody against influenza A virus NP (VHH_{NP-1}) (Ashour et al, 2015) did not alter AIM2 inflammasome assembly, co-expression of the three cytosolically functional AIM2 nanobodies reduced inflammasome assembly to varying degrees (Fig. 4A). Given that two of the nanobodies have non-overlapping epitopes, we next wanted to test if bivalent nanobodies would interfere with AIM2 inflammasome assembly even more efficiently. We constructed expression vectors of bivalent nanobodies with a 15 aa linker (GGGGS)₃ containing all possible combinations of VHH_{AIM2-1}, VHH_{AIM2-2}, and VHH_{AIM2-3}, as well as bivalent control nanobodies composed of two concatenated copies of VHH_{NP-1}. All bivalent combinations of AIM2 nanobodies substantially improved inhibition, and most combinations completely inhibited AIM2 inflammasome assembly (Fig. 4B). In control experiments, we activated NLRP3 inflammasomes using a similar overexpression system in HEK293T^{ASC-EGFP} cells (Fig. 4C). Of note, NLRP3 also recruits and nucleates ASC filaments and specks through its PYD. However, none of the AIM2^{PYD} nanobodies interfered with NLRP3 inflammasome assembly, indicating specificity for AIM2^{PYD}.

We next wanted to address the mechanism of action of the inhibitory AIM2 nanobodies. Nucleation of inflammasomes by AIM2 involves the formation of small oligomers of the AIM2^{PYD}, which in turn nucleate the polymerization of ASC^{PYD} (Lu et al, 2014b). As a macroscopic readout for AIM2^{PYD} oligomerization, AIM2^{PYD}-EGFP can be overexpressed in HeLa cells, where it polymerizes into microscopically detectable filaments (Fig. 4D,E). While control nanobodies did not affect AIM2^{PYD}-EGFP polymerization, overexpression of monovalent VHH_{AIM2-1}, VHH_{AIM2-2}, or bivalent VHH_{AIM2-1}-VHH_{AIM2-1}, VHH_{AIM2-2}-VHH_{AIM2-1}, completely abrogated polymerization (Fig. 4D–F). To independently confirm inhibition of AIM2^{PYD} oligomerization by AIM2 nanobodies, we employed a biochemical assay based on the sedimentation of AIM2^{PYD} filaments. While MBP-AIM2^{PYD} is a soluble protein, removal of the MBP fusion protein by cleavage with Tobacco Etch Virus (TEV) protease initiates the polymerization of AIM2^{PYD} filaments, which are no longer soluble (Fig. 4G). Insoluble filaments can be separated from soluble monomers and small oligomers by high-speed centrifugation, followed by analysis of pellet and supernatant fractions by SDS-PAGE and Coomassie staining. In the presence of bivalent control nanobody, the entire pool of AIM2^{PYD} was sedimented upon MBP removal, indicating that all monomers polymerize into insoluble filaments (Figs. 4H and EV4A). In the presence of bivalent inhibitory AIM2 nanobodies, AIM2^{PYD} remained in the supernatant, indicating that nanobody binding blocked oligomerization and filament formation (Figs. 4H and EV4A). Similar trends were observed with monovalent nanobodies, although the interpretation was impaired by the similar molecular weight of monovalent nanobodies and AIM2^{PYD}, which prevented a clear distinction by SDS-PAGE (Fig. EV4A). Taken together, our findings imply that the inhibitory AIM2 nanobodies bind to monomeric AIM2 and prevent the oligomerization of AIM2^{PYD}, which consequently prevents the recruitment of ASC and the nucleation of inflammasomes.

VACV-encoded antagonistic AIM2 nanobodies reveal cell-type-specific inflammasome activation in human primary cells

While the contribution of individual proteins to a biological process in cell lines (or transgenic mice) can be evaluated with CRISPR/Cas9-mediated knockouts, this is not possible in many human primary cell types. We therefore intended to employ the antagonistic AIM2 nanobodies to answer the outstanding question

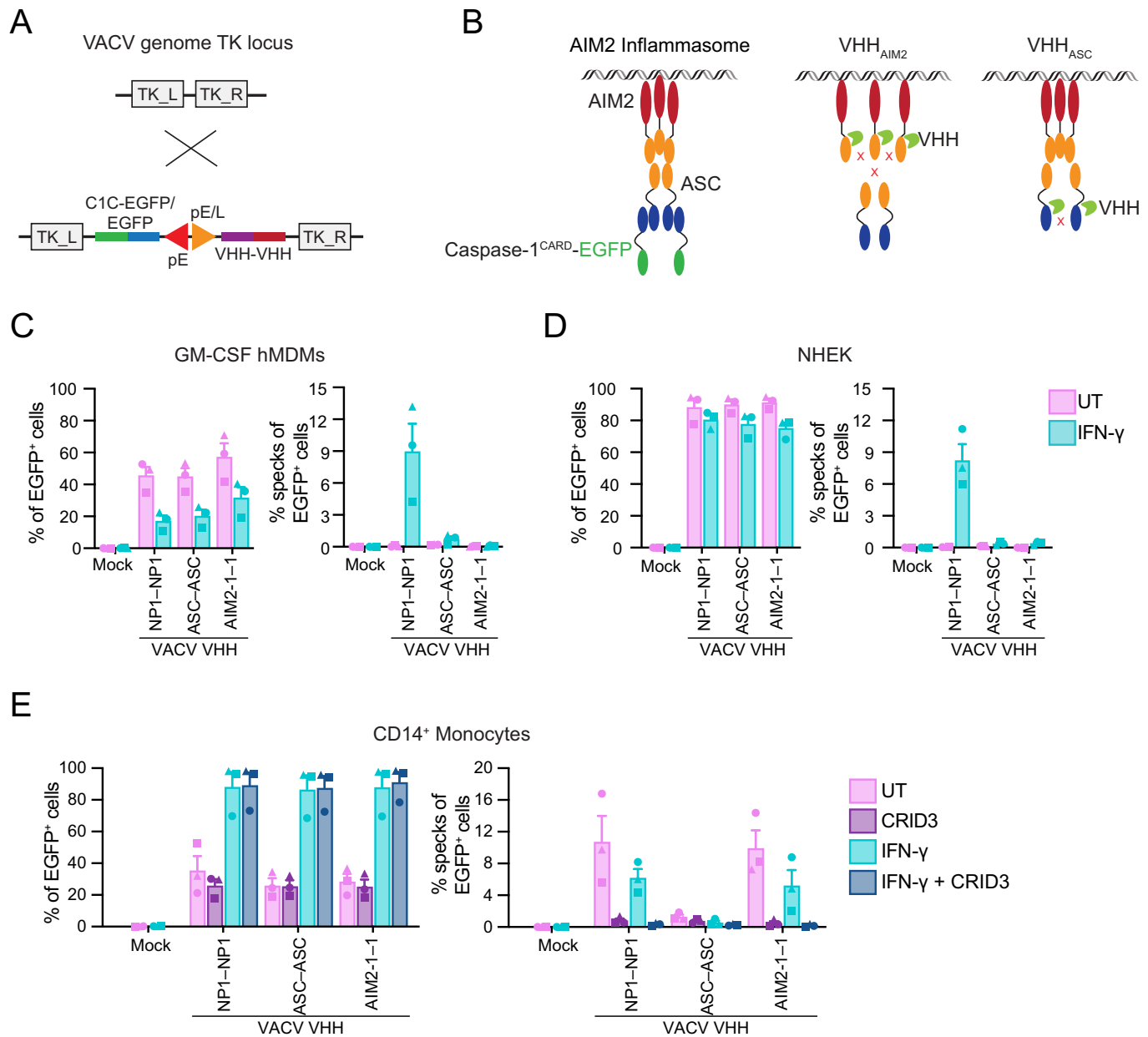


Figure 5. VACV encoded antagonistic AIM2 nanobodies reveal cell-type-specific inflammasome activation in human primary cells.

(A) Genome structure of recombinant VACV strains expressing C1C-EGFP from the J2R early promoter (pE) and bivalent nanobodies from a synthetic early/late promoter (pE/L). Transgenes were inserted into the VACV TK locus by homologous recombination. (B) Scheme of AIM2 inflammasome components indicating the VACV-encoded nanobodies used to perturb inflammasome activation. (C–E) Human MDMs differentiated with GM-CSF (C), NHEKs (D), and CD14⁺ monocytes (E) were left untreated or treated with IFN-γ overnight. Cells were infected with VACV C1C-EGFP expressing the indicated nanobodies at an MOI of 5 (C, D) or 10 (E), in the presence of VX and, where indicated, CRID3. Six hours post infection, cells were harvested, fixed, and infection and C1C-EGFP speck assembly was analyzed by flow cytometry. Average values (with individual data points) from $N = 3$ independent donors $\pm$ SEM are displayed. Source data are available online for this figure.

of which inflammasome is activated by poxviruses in primary human cell types. To monitor inflammasome assembly in any cell type or tissue susceptible to infection, we genetically engineered recombinant VACV to encode (1) the inflammasome reporter C1C-EGFP/EGFP under the control of J2R early promoter and (2) different bivalent nanobodies under the control of a strong synthetic early/late promoter (Fig. 5A). We generated recombinant VACV encoding the bivalent VHH_{NP-1}-VHH_{NP-1} as a negative

control, VHH_{ASC}-VHH_{ASC} as a positive control for inflammasome inhibition (Schmidt et al, 2016b), and VHH_{AIM2-1}-VHH_{AIM2-1} or VHH_{AIM2-2}-VHH_{AIM2-1} to inhibit AIM2 (Fig. 5B).

To test whether the virally expressed nanobodies were indeed able to prevent inflammasome assembly, we first infected IFN-γ-treated PMA-differentiated THP-1 cells, which assemble AIM2 inflammasomes upon infection (Fig. 2K). Infection with vaccinia virus strains expressing control nanobodies triggered inflammasome assembly in IFN-γ

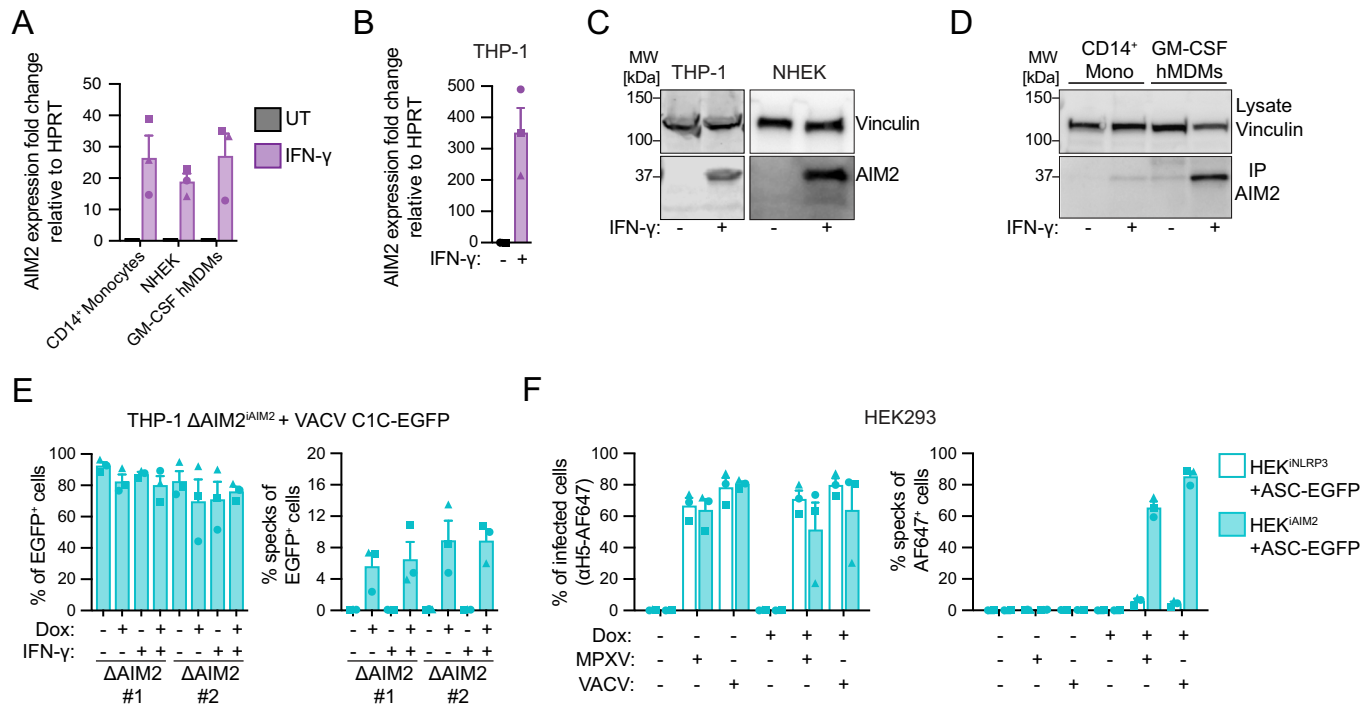


Figure 6. AIM2 upregulation by IFN- γ allows detection of VACV infection by AIM2.

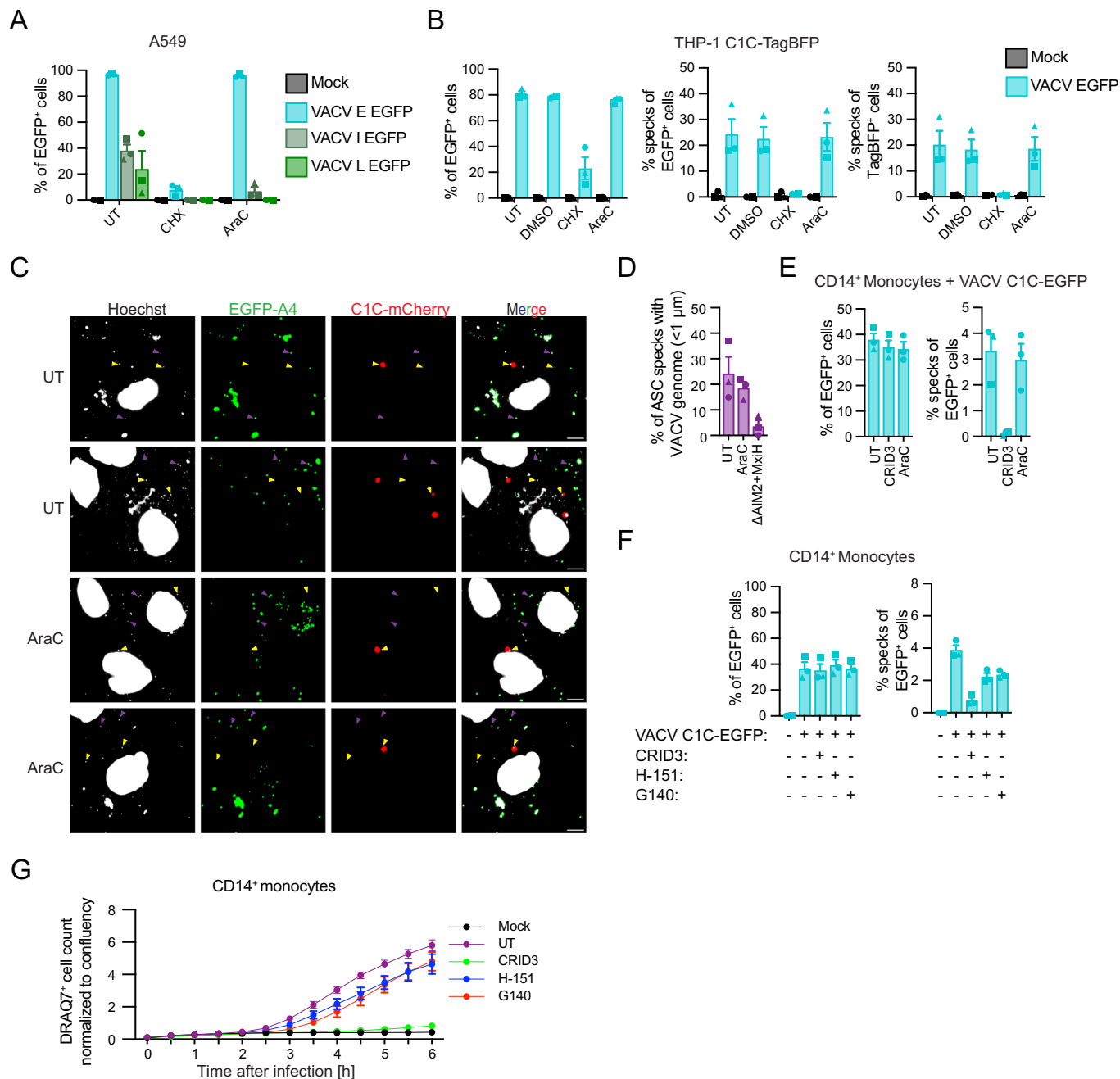
(A–D) The indicated cell types were treated with IFN- γ as before. (A, B) mRNA transcript levels of AIM2 were quantified by qPCR. The fold change of AIM2 expression upon IFN- γ treatment was normalized to HPRT transcript levels. (C) PMA-differentiated THP-1 cells or NHEKs were lysed and AIM2 protein levels were analyzed by immunoblot. (D) CD14⁺ monocytes and GM-CSF hMDMs were lysed and AIM2 was immunoprecipitated using VHH_{AIM2-2} covalently coupled to sepharose beads. AIM2 protein levels were analyzed by immunoblot. (E) Two monoclonal THP-1 Δ AIM2 cell lines were lentivirally transduced to dox-inducibly express AIM2. Cells were PMA-differentiated, treated with dox and IFN- γ as indicated, and infected with VACV C1C-EGFP at an MOI of 5 in the presence of VX, followed by analysis of infection and C1C-EGFP speck formation as before. (F) HEK293 cells expressing ASC-EGFP constitutively and either NLRP3 or AIM2 upon dox induction were either left untreated or treated with dox for 4 h. Cells were infected with VACV or MPXV (MOI 5) and harvested and fixed after 6 h. Infected cells were stained with anti-H5 serum as in Fig. 2L/M. Infection and ASC-EGFP speck assembly was analyzed by flow cytometry. Data represent average values (with individual data points) from $N = 3$ independent donors (primary cells) $\pm$ SEM, or from $N = 3$ independent experiments $\pm$ SEM (Cell lines). Source data are available online for this figure.

pretreated THP-1 cells (Fig. EV5A). Inflammasome assembly was completely blunted by viruses expressing ASC-specific nanobodies, as well as by the recombinant viruses expressing VHH_{AIM2-1}-VHH_{AIM2-1} or VHH_{AIM2-2}-VHH_{AIM2-1} (Fig. EV5A). Similarly, virus-triggered IL-1 β secretion was substantially reduced after infection with viruses expressing VHH_{ASC}-VHH_{ASC}, VHH_{AIM2-1}-VHH_{AIM2-1} or VHH_{AIM2-2}-VHH_{AIM2-1} (Fig. EV5B). Cell death as measured by LDH release and uptake of DNA dye DRAQ7, was likewise reduced to background levels after infection with the same viruses (Fig. EV5C–F). This demonstrates that the designed poxvirus reporter strains are functional, as they allow the quantification of inflammasome assembly and confirm the requirement of functional AIM2 and ASC for VACV-induced inflammasome assembly in THP-1 cells.

Taking advantage of the newly generated poxvirus reporter strains, we next set out to investigate inflammasome assembly in human primary cells. Human GM-CSF-differentiated macrophages were robustly infected with the reporter viruses. Although infection was reduced upon IFN- γ pretreatment, inflammasome assembly was only observed in IFN- γ pretreated macrophages infected with viruses expressing control nanobodies. Expression of bivalent ASC and AIM2 nanobodies completely blocked inflammasome assembly (Fig. 5C). This indicates that inflammasome assembly in VACV-infected primary macrophages relied on AIM2, although it was

previously postulated that dsDNA activates NLRP3 in all human primary myeloid cells (Gaidt et al, 2017). Similarly, NHEKs derived from human skin were robustly infected and only assembled inflammasomes after IFN- γ pretreatment and in the presence of control nanobodies (Fig. 5D). ASC and AIM2 nanobodies completely abrogated inflammasome assembly, proving that this primary cell type also employs AIM2 to detect the dsDNA of VACV. Lastly, we infected human CD14⁺ monocytes with the reporter viruses. Infection was robust and -as before- was boosted by IFN- γ pretreatment (Fig. 5E). Inflammasome assembly was detected in both untreated and IFN- γ -treated monocytes. Viruses expressing ASC nanobodies inhibited inflammasome assembly as expected, but inflammasome activation was not altered when viruses expressed AIM2 nanobodies, indicating that inflammasome assembly did not rely on the AIM2 sensor. Instead, inflammasome assembly was sensitive to the NLRP3-specific inhibitor CRID3, confirming that VACV infection is sensed by NLRP3 in human monocytes.

Taken together, our set of inhibitory AIM2 nanobodies (in combination with a small molecule inhibitor of NLRP3) proves that poxvirus infection (and by extension dsDNA) is sensed by different mechanisms in inflammasome-competent cell types. While IFN- γ -treated keratinocytes and macrophages employ AIM2 to nucleate



inflammasomes, infection of monocytes triggers NLRP3 inflammasome assembly.

As inflammasome assembly in THP-1 cells, GM-CSF macrophages, and NHEKs was only observed after IFN- γ pretreatment, we tested if AIM2 itself is upregulated by IFN- γ treatment in primary and THP-1 cells. AIM2 mRNA was upregulated by IFN- γ pretreatment in all cell types (Fig. 6A,B). In THP-1 cells and NHEKs, protein levels in cell lysates were sufficient to readily detect upregulation of AIM2 by immunoblot (Fig. 6C). Given the lower expression of AIM2 in primary monocytes and macrophages, we immunoprecipitated endogenous AIM2 with VHH_{AIM2-2} covalently coupled to sepharose beads and observed

robust upregulation by IFN- γ (Fig. 6D). We also analyzed the proteins immunoprecipitated from CD14⁺ monocytes by mass spectrometry and found that AIM2 was the most enriched protein, indicating both the presence of AIM2 in monocytes and the specificity of the nanobody (Fig. EV5G). As IFN- γ alters the expression of numerous genes, we wondered if other proteins upregulated by IFN- γ were necessary for AIM2 inflammasome activation. To decouple AIM2 expression from IFN- γ pretreatment, we reconstituted AIM2-deficient THP-1 cells with doxycycline (dox)-inducible AIM2 and infected them with VACV C1C-EGFP. Dox-induced AIM2 expression was sufficient for AIM2 activation upon VACV infection, although the inflammasome

Figure 7. VACV incoming genomes are sufficient for the activation of inflammasomes, independent of viral DNA replication.

(A) A549 cells were infected with an MOI 5 of recombinant VACV expressing EGFP under the control of the J2R early (VACV E EGFP), the G8R intermediate (VACV I EGFP) or the F17R late (VACV L EGFP) promoter, where indicated, in the presence of 50 μ M CHX or 50 μ M AraC. EGFP expression was quantified by flow cytometry. (B) PMA-differentiated THP-1 macrophages constitutively expressing C1C-TagBFP were pretreated with IFN- γ overnight and infected with VACV E EGFP in the presence of VX and the indicated inhibitors for 6 h. Infection (EGFP⁺) and inflammasome assembly (C1C-TagBFP specks) in TagBFP⁺ cells, or in infected (EGFP⁺) cells, were quantified by flow cytometry as before. (C, D) PMA-differentiated THP-1 cells were infected with VACV expressing core protein A4 fused to EGFP (EGFP-A4), and C1C-mCherry at an MOI of 2 for 6 h, where indicated, in the presence of AraC. Where indicated, PMA-differentiated THP-1 Δ AIM2 were infected with VACV EGFP-A4 E C1C-mCherry to express C1C-mCherry and treated with 1 μ g/mL PA and 0.1 μ g/mL LFn-MxiH to activate NLRC4 as a control. Cells were fixed and stained for cellular and viral DNA with Hoechst 33342. Cells were recorded by confocal microscopy, and representative images of at least three independent experiments are displayed (C). Purple arrowheads indicate MVs or cores with genomes (positive for Hoechst and EGFP), while yellow arrowheads highlight released DNA genomes (positive for Hoechst, no EGFP). Scale bar = 5 μ m. Hoechst-positive VACV genomes and C1C-mCherry specks were detected, and the fraction of C1C-mCherry specks in close proximity (<1 μ m) to released viral genomes was quantified. A total of at least 100 specks per condition were analyzed in $N = 3$ independent repeats. (E–G) Primary CD14⁺ monocytes were infected with VACV C1C-EGFP at an MOI of 10 in the presence (E, F) or absence (G) of VX, as well as the indicated inhibitors. Six hours post infection, cells were harvested, fixed, and infection (EGFP⁺) and C1C-EGFP speck assembly were analyzed by flow cytometry (E, F). Membrane permeabilization was quantified by the uptake of DNA dye DRAQ7 over 6 h (G). Data represent values with individual data points from $N = 3$ independent donors (primary cells), or from $N = 3$ independent experiments (cell lines) $\pm$ SEM. Source data are available online for this figure.

response was reduced (Fig. 6E). Additional IFN- γ pretreatment did not boost the specking response.

VACV infection is directly sensed by reconstituted AIM2 inflammasomes

MVA infection has been reported to trigger NLRP3 inflammasome responses indirectly through activation of cGAS-STING by dsDNA and the ensuing lysosomal rupture, while AIM2 is assumed to directly bind to viral dsDNA (Gaidt et al, 2017). To test whether VACV infection activated AIM2 or NLRP3 directly, we reconstituted AIM2 and NLRP3 inflammasomes in HEK293 Flp-In T-REx cells. We constructed cell lines, which constitutively express ASC-EGFP and either AIM2 or NLRP3 after dox induction. Upon VACV and MPXV infection, only AIM2- but not NLRP3-expressing HEK293 cells assembled ASC-EGFP specks (Fig. 6F), highlighting the direct activation of AIM2 by VACV and MPXV infection and the requirement of other upstream processes for NLRP3 activation. Of note, the HEK293 cell lines do not express functional amounts of cGAS and therefore cannot undergo cGAS-STING-mediated lysosomal rupture (Sun et al, 2013). Interestingly, MPXV infection of the AIM2-expressing HEK293 cells induced a robust ASC specking response comparable to that induced by VACV, while a substantially weaker response had been observed in the NHEK or PMA-differentiated THP-1 cells (Fig. 2L,M). This indicates that the inflammasome response to MPXV infection likely relies on similar molecular features, i.e., detection of poxvirus genomes by IFN- γ -induced AIM2.

Incoming VACV genomes are sufficient for the activation of inflammasomes, independent of viral DNA replication

We next investigated which phases of VACV replication trigger inflammasome assembly and whether differences in the VACV viral life cycle could explain the differential activation of AIM2 or NLRP3 in different cell types. VACV gene expression occurs in three consecutive phases: Early, intermediate and late gene expression yield viral proteins crucial for uncoating and immune evasion (early), viral DNA replication (intermediate), and virus assembly (late). Hereby, each phase of gene expression provides the transcription factors for the next. While early genes are transcribed from viral genomes in the core using transcription factors packaged in the virion, intermediate and late gene expression rely on

successful uncoating and replication of viral genomes (Assarsson et al, 2008). We used the translation inhibitor cycloheximide (CHX) to inhibit all viral gene expression, and the DNA replication inhibitor cytosine arabinoside (AraC) to inhibit viral DNA replication and late gene expression. We first verified inhibition using recombinant VACV strains that express EGFP under early (E EGFP), intermediate (I EGFP), or late (L EGFP) VACV promoters in A549 cells. CHX inhibited all phases of viral gene expression, whereas AraC inhibited the intermediate and late gene expression and had no effect on early gene expression (Fig. 7A). We next analyzed inflammasome responses in THP-1 cells constitutively expressing C1C-TagBFP to quantify inflammasome assembly independent of viral gene expression. Cells were pretreated with IFN- γ and infected with VACV E EGFP in the presence of CHX or AraC (Fig. 7B). CHX treatment inhibited early gene expression and completely abrogated inflammasome assembly, whereas AraC treatment did not affect either early gene expression or inflammasome assembly. Hence, VACV DNA replication was not required for AIM2 inflammasome activation in THP-1 cells, which implies that incoming viral genomes are sufficient for AIM2 activation. To test whether incoming genomes are associated with AIM2-mediated inflammasomes, we infected IFN- γ -treated THP-1 cells with VACV strains expressing a fluorescent fusion of the core protein A4 (EGFP-A4) as well as C1C-mCherry (Fig. 7C). As viral genomes can be stained with the DNA dye Hoechst 33342, we can distinguish intact mature virions (MVs) and cores released by fusion (positive for EGFP-A4 and DNA, purple arrowheads), from released genomes (only positive for DNA, yellow arrowheads). We indeed observed that around 20% of C1C-mCherry specks co-localized with released viral genomes in both untreated and AraC-treated cells (Fig. 7D). To test whether such a co-localization may also occur by chance, we infected AIM2 knockout cells with VACV C1C-mCherry (to provide the reporter but avoid inflammasome assembly) and initiated NLRC4 inflammasome assembly by delivering *Shigella flexneri* MxiH into the cytosol using the anthrax toxin delivery system (Yang et al, 2013). Hardly any NLRC4 inflammasomes were found near released viral genomes, supporting that the observed co-localization was indicative of genuine viral DNA binding to AIM2 (Fig. 7D).

We next tested which viral structures triggered inflammasome assembly in CD14⁺ monocytes. As for THP-1 cells, we found that inflammasome assembly in monocytes was not affected by AraC,

highlighting that VACV-induced NLRP3 activation in primary monocytes was independent of viral DNA replication and likely triggered by incoming genomes as well (Fig. 7E). Since cGAS-STING signaling has been implicated in promoting lysosomal rupture and subsequent NLRP3 inflammasome activation, we next tested whether pharmacological inhibition of STING or cGAS would affect VACV-induced NLRP3 activation in primary monocytes. Both the STING inhibitor H-151 and the cGAS inhibitor G140 only partially inhibited NLRP3 activation and DRAQ7 uptake in VACV-infected primary monocytes (Fig. 7F,G), suggesting that VACV-induced NLRP3 activation is not solely dependent on the cGAS-STING pathway and may involve additional mechanisms that warrant further investigation. Taken together, our data shows that incoming poxvirus genomes are sufficient to induce inflammasome responses, although virus detection is achieved by different sensors, depending on the cell type, and potentially on cell-type-specific inflammasome regulatory factors.

Discussion

Cytosolic dsDNA is one of the strongest indications of pathogen infection and triggers inflammasome assembly to release pro-inflammatory cytokines acting on the entire tissue, as well as transcriptional responses that coordinate cell-autonomous responses. Yet, despite the central role of dsDNA sensing, major questions remain, as accurate and comparable experiments are difficult to achieve due to the lack of suitable readouts and tools. This is exemplified by the reported differences between the human and murine responses to cytosolic dsDNA. Poxviruses are a medically relevant family of pathogens that cause frequent zoonotic infections and include variola virus, which has afflicted the world for centuries, and MPXV, which has caused two recent outbreaks. More importantly, they are a well-controlled physiological model for cytosolic dsDNA delivery, as dsDNA deposition of single molecules from uncoated viral cores does not rely on transfection or electroporation of excess amounts of (complexed) DNA. In the context of poxvirus infections, dsDNA is typically not exposed in endosomes, and early gene expression allows an accurate quantification of the release of viral cores into the cytosol, which precedes exposure of DNA due to uncoating. The combination of a virus-encoded inflammasome reporter, virus-encoded antagonistic AIM2 nanobodies, as well as NLRP3 inhibitors, now allows us to analyze dsDNA-mediated inflammasome assembly in different human primary cell types under comparable conditions. These experiments yielded a clear picture: We found that uncoating of viral genomes is sufficient to nucleate AIM2 inflammasomes in primary human macrophages and keratinocytes when AIM2 is upregulated by IFN- γ . In human primary CD14⁺ monocytes, however, the inflammasome response to released viral genomes is completely dependent on NLRP3.

VACV is equipped with soluble IL-1 β , IL-18, and IFN- γ decoy receptors, and a host-modulatory protein that targets a broad range of caspases, including caspase-1, as well as the cGAS-STING axis, type I interferon, NF- κ B, and IFN- γ signaling (Hernaiz and Alcamí, 2024). This proves that the inflammasome pathway and other dsDNA-sensing pathways are evolutionarily relevant to poxviruses. They also highlight the importance of readouts for inflammasome assembly that are as upstream as possible, such as ASC speck formation. This avoids cytokine-based readouts that are affected by several host-modulatory proteins. Recombinant VACV strains expressing the inflammasome

reporter C1C-EGFP thus allowed us to quantify inflammasome responses at a single-cell resolution in any susceptible cell type or tissue. As a case in point, we detected robust IL-1 β secretion in VACV-infected THP-1 macrophages, but we were not able to detect IL-1 β from infected primary cells, where the fraction of cells assembling inflammasomes was substantially lower, and host cell modulation may be more prominent (Fig. EV2D,F,H). Genetically encoding the antagonistic AIM2 nanobodies in the poxvirus genome elegantly avoids the need to genetically manipulate primary cells. This is particularly critical, as many primary cell types, including monocytes, cannot be cultivated long enough or differentiate during extended cultivation, ruling out CRISPR-mediated loss of function after natural protein turnover.

To the best of our knowledge, this study presents the first evidence that inflammasome responses to VACV are cell-type-specific. The AIM2-dependent inflammasome response in human primary macrophages and keratinocytes relies on the upregulation of AIM2, e.g., by IFN- γ , as well as on the uncoating of viral genomes, as inflammasome assembly could also be reconstituted in HEK293 cells or THP-1 cells that expressed AIM2 after dox induction. Interestingly, higher steady state levels of AIM2 seem to be difficult to tolerate, as no THP-1 or HEK293T cell lines constitutively expressing AIM2 could be generated. It may thus impose an additional safety measure to only express sufficient AIM2 after IFN- γ signaling, a cytokine that is known to be released in VACV skin lesions (Liu et al, 2010). It is likely that AIM2 is not only regulated at the transcriptional level, as human monocytes express detectable amounts of AIM2, but still do not assemble AIM2 inflammasomes after VACV infection. In contrast, HEK293 cells with reconstituted NLRP3 inflammasomes, as well as human macrophages, do not assemble NLRP3 inflammasomes after poxvirus infection despite release of viral genomes, but respond well to canonical NLRP3 triggers, such as the potassium ionophore nigericin. Loss or absence of AIM2 did not restore NLRP3 activation, indicating that it is unlikely that AIM2 and NLRP3 are activated in a mutually exclusive manner by some inhibitory mechanism. It rather seems like some factor or response unique to human monocytes is required to allow cytosolic dsDNA to activate NLRP3. Of note, human macrophages and THP-1 cells are responsive to cGAS-STING activation and secrete type I interferons (Wiser et al, 2020), ruling out that the mere functionality of this pathway is decisive. We had speculated that the differential activation of NLRP3 was explained by different outcomes of cGAS-STING activation, e.g., the lysosomal cell death postulated by (Gaidt et al, 2017). Yet, we were not able to detect lysosomal rupture in primary monocytes with virus-encoded Galectin-3-mCherry reporters, perhaps due to limitations in the sensitivity. In addition, chemical inhibition of cGAS and STING only somewhat reduced NLRP3 inflammasome activation in monocytes (Fig. 7F,G). A well-described difference between the VACV strain MVA used by Gaidt et al, and strain WR is the loss of many immunomodulatory genes in MVA, resulting, e.g., in the release of IFN- β in MVA-, but not WR-infected mice or cells (Waibler et al, 2008). Loss of the cGAS inhibitor E5 led to IFN- β induction in VACV WR-infected cells and boosted IFN- β release in MVA-infected cells (Yang et al, 2023). While cGAS activation may thus be different in VACV WR and MVA, we observed robust NLRP3 activation in monocytes infected with VACV WR despite the expression of functional E5. Taken together, this suggests additional, yet unidentified pathways beyond the cGAS-STING

axis may be involved in NLRP3 activation. These may be functional in monocytes, but not in other NLRP3-competent cell types.

Lastly, the newly developed antagonistic nanobodies close an important gap in our toolbox to study AIM2 inflammasomes, as no inhibitor specific to AIM2 had been described to date. A commercially available suppressive 24-mer oligodeoxynucleotide (ODN) TTAGGG, also known as A151, has been reported to inhibit dsDNA sensing by AIM2, cGAS and TLR9, but is not specific for AIM2 (Kaminski et al, 2013; Steinhagen et al, 2018; Araie et al, 2018). Our genetically encoded inhibitory AIM2 nanobodies allow precise perturbation of AIM2 inflammasome assembly by preventing the oligomerization of AIM2^{PYD} filaments. While its application requires the transfer of genetic information, it is expected to act immediately on the target protein and is thus substantially faster than any knockout or knockdown could be. The proposed model of cooperative binding of AIM2 to dsDNA implies that oligomerization of AIM2^{PYD} filaments is necessary to achieve sufficient affinity of AIM2^{HIN200} for dsDNA (Morrone et al, 2015). We thus expect that nanobody-bound (i.e., inhibited) AIM2 does not bind viral genomic DNA as PYD filaments cannot form.

Antagonistic AIM2 nanobodies may be used to study the inflammasome-dependent and—independent roles of AIM2 in microglia, regulatory T cells, and B cells (Yang et al, 2021; Ma et al, 2021; Chou et al, 2021). Aberrant AIM2 activation is critically involved in a growing list of autoinflammatory diseases, including systemic lupus erythematosus (SLE), neuroinflammatory disease, psoriasis, inflammatory bowel diseases (IBD), and in cancer, as well as in pathologies triggered by infection-induced AIM2 activation (Man et al, 2016; Dombrowski et al, 2011; Yang et al, 2015; Vanhove et al, 2015; Denes et al, 2015). Antagonistic AIM2 nanobodies could be therapeutically expressed in the relevant cell types by transduction with viral vectors, or mRNA delivery as a more suitable alternative. Therapeutic applications may also benefit from any of the protein delivery methods that are currently in development (Chan and Tsourkas, 2024). Altogether, the AIM2-specific nanobodies are valuable tools to study the role of AIM2 in different disease settings and to disentangle the inflammasome-dependent and -independent roles of AIM2 in different cell types.

Methods

Reagents and tools table

Reagent/resource	Reference or source	Identifier or catalog number
Experimental models		
Cell lines		
A549	ATCC	CCL-185; RRID:CVCL_0023
BSC-40	ATCC	CRL-2761; RRID:CVCL_3656
HEK293T	ATCC	CRL-3216; RRID:CVCL_0063
HEK293T ASC-EGFP	Jenster et al, (2023)	Lab-internal number: H1
HEK Flp-In 293 T-REx	Thermo Fisher Scientific	R78007 RRID:CVCL_U427

Reagent/resource	Reference or source	Identifier or catalog number
HEK293 Flp-In T-REx AIM2-SH	This study (Flp-In HEK293 T-REx + plasmid pFS1917; Flp recombination)	Lab-internal number: HFT23
HEK293 Flp-In T-REx AIM2-SH + ASC-EGFP	This study (HFT23 + lentivirus generated with plasmid pFS833)	Lab-internal number: HFT105
HEK293 Flp-In T-REx NLRP3-SH	This study (Flp-In HEK293 T-REx + plasmid pFS353; Flp recombination)	Lab-internal number: HFT3
HEK293 Flp-In T-REx NLRP3-SH + ASC-EGFP	This study (HFT3 + lentivirus generated with plasmid pFS833)	Lab-internal number: HFT104
Hela	ATCC	CCL-2; RRID:CVCL_0030
THP-1	ATCC	TIB-202; RRID:CVCL_0006
THP-1 ΔASC	Schiffelers et al, (2024)	Clone 5a1; lab-internal number: T137
THP-1 ΔAIM2 #1	This study	Clone 1; Dr. Thomas Zillinger, University of Bonn, Germany; lab-internal number: T146
THP-1 ΔAIM2 #2	This study	Clone 15; Dr. Thomas Zillinger, University of Bonn, Germany; lab-internal number: T147
THP-1 ΔAIM2 + AIM2-HA(i)	This study (T146 + lentivirus generated with plasmid pFS1512)	Lab-internal number: T185
THP-1 ΔAIM2 + AIM2-HA(i)	This study (T147 + lentivirus generated with plasmid pFS1512)	Lab-internal number: T186
THP-1 ΔcGAS	Christensen et al, (2016)	Søren Riis Paludan Aarhus University, Denmark; lab-internal number: T128
THP-1 ΔNLRP3	Gritsenko et al, (2020)	Prof. Veit Hornung Ludwig-Maximilians-University Munich, Germany; lab-internal number: T151
THP-1 ΔSTING	This study	Clone 3A6; Dr. Thomas Zillinger, University Hospital Bonn, Germany; lab-internal number: T122
THP-1 C1C-EGFP	Gohr et al, (2025) (THP-1 + lentivirus generated with plasmid pFS846)	Lab-internal number: T76s
THP-1 C1C-EGFP(i)	Schiffelers et al, (2024) (THP-1 + lentivirus generated with plasmid pFS720)	Lab-internal number: T55
THP-1 C1C-TagBFP	This study (THP-1 + lentivirus generated with plasmid pFS2993)	Lab-internal number: T276

Reagent/resource	Reference or source	Identifier or catalog number
VACV strains		
VACV Western Reserve	N/A	Ari Helenius, ETH Zurich, Switzerland (originally from Paula Traktman, then Medical College of Wisconsin, WI, USA)
VACV WR E EGFP	Schmidt et al, (2013)	Lab-internal number: WR027
VACV WR I EGFP	Kilcher et al, (2014)	Lab-internal number: WR029
VACV WR L EGFP	Schmidt et al, (2013)	Lab-internal number: WR031
VACV WR EGFP-A4	Schmidt et al, (2011)	Lab-internal number: WR039
VACV WR E C1C-EGFP EL NeoR	This study (VACV WR + plasmid pFS1923)	Lab-internal number: WR157
VACV WR E EGFP EL NeoR	This study (VACV WR + plasmid pFS1924)	Lab-internal number: WR158
VACV WR EGFP-A4 E C1C-mCherry EL NeoR	This study (VACV WR EGFP-A4 [WR039] + plasmid pFS1925)	Lab-internal number: WR163
VACV WR E C1C-EGFP EL VHH _{NP1} -(G ₄ S) ₃ -VHH _{NP1} -HA	This study (VACV WR + plasmid pFS4279)	Lab-internal number: WR178
VACV WR E C1C-EGFP EL VHH _{ASC} -(G ₄ S) ₃ -VHH _{ASC} -HA	This study (VACV WR + plasmid pFS4281)	Lab-internal number: WR179
VACV WR E C1C-EGFP EL VHH _{AIM2-1} -(G ₄ S) ₃ -VHH _{AIM2-1} -HA	This study (VACV WR + plasmid pFS4277)	Lab-internal number: WR180
VACV WR E C1C-EGFP EL VHH _{AIM2-2} -(G ₄ S) ₃ -VHH _{AIM2-1} -HA	This study (VACV WR + plasmid pFS4278)	Lab-internal number: WR181
MPXV/Germany/2022/BN001	This study	N/A
Recombinant DNA		
Bacterial expression vectors		
pEXPR His-MBP-AIM2 PYD L10A L11A	This study	Lab-internal number: pFS1516
pHEN6 VHH _{Enhancer} -LPETG-His	Ashour et al, (2015)	Lab-internal number: pFS216
pHEN6 VHH _{NP-1} -LPETG-His	Ashour et al, (2015)	Lab-internal number: pFS2801
pHEN6 VHH SN-32-A12-HA-His (VHH _{AIM2-1})	This study	Lab-internal number: pFS3609
pHEN6 VHH SN-32-C07-HA-His (VHH _{AIM2-2})	This study	Lab-internal number: pFS3610
pHEN6 VHH SN-32-H12-HA-His (VHH _{AIM2-3})	This study	Lab-internal number: pFS3611

Reagent/resource	Reference or source	Identifier or catalog number
pHEN6 VHH SN-32-F11-HA-His (VHH _{AIM2-4})	This study	Lab-internal number: pFS3777
pHEN6 VHH SN-32-G04-HA-His (VHH _{AIM2-5})	This study	Lab-internal number: pFS3778
pHEN6 VHH SN-32-G07-HA-His (VHH _{AIM2-6})	This study	Lab-internal number: pFS3779
pHEN6 VHH YM-01-H06-HA-His (VHH _{AIM2-7})	This study	Lab-internal number: pFS3612
pHEN6 VHH YM-01-D12-HA-His (VHH _{AIM2-8})	This study	Lab-internal number: pFS3782
pHEN6 VHH YM-01-H02-HA-His (VHH _{AIM2-9})	This study	Lab-internal number: pFS3780
pHEN6 VHH YM-01-H03-HA-His (VHH _{AIM2-10})	This study	Lab-internal number: pFS3781
pHEN6 VHH YM-01-H04-HA-His (VHH _{AIM2-11})	This study	Lab-internal number: pFS3791
pHEN6 VHH YM-02-E9-HA-His (VHH _{AIM2-12})	This study	Lab-internal number: pFS3784
pHEN6 VHH YM-01-D11-HA-His (VHH _{AIM2-13})	This study	Lab-internal number: pFS3785
pHEN6 VHH YM-01-H05-HA-His (VHH _{AIM2-14})	This study	Lab-internal number: pFS3786
pHEN6 VHH YM-01-F05-HA-His (VHH _{AIM2-15})	This study	Lab-internal number: pFS3787
pHEN6 VHH YM-01-C10-HA-His (VHH _{AIM2-16})	This study	Lab-internal number: pFS3789
pHEN6 VHH YM-01-E09-HA-His (VHH _{AIM2-17})	This study	Lab-internal number: pFS3792
pHEN6 VHH YM-02-E6-HA-His (VHH _{AIM2-18})	This study	Lab-internal number: pFS3783
pSB_Init VHH SN-32-A12-(G ₄ S) ₃ -VHH SN-32-A12-LPETG-His (VHH _{AIM2-1} -VHH _{AIM2-1})	This study	Lab-internal number: pFS5388
pSB_Init VHH SN-32-C07-(G ₄ S) ₃ -VHH SN-32-A12-LPETG-His (VHH _{AIM2-2} -VHH _{AIM2-1})	This study	Lab-internal number: pFS5389
Eukaryotic expression vectors		
pCAGGS VHH SN-32-A12-HA (VHH _{AIM2-1})	This study	Lab-internal number: pFS2589
pCAGGS VHH SN-32-C07-HA (VHH _{AIM2-2})	This study	Lab-internal number: pFS2590
pCAGGS VHH SN-32-H12-HA (VHH _{AIM2-3})	This study	Lab-internal number: pFS2595
pCAGGS VHH YM-01-H06-HA (VHH _{AIM2-7})	This study	Lab-internal number: pFS2962
pCAGGS VHH YM-01-D12-HA (VHH _{AIM2-8})	This study	Lab-internal number: pFS2955
pCAGGS VHH _{NP-1} -HA	Schmidt et al, (2016a)	Lab-internal number: pFS390
pCAGGS VHH SN-32-A12-(G ₄ S) ₃ -VHH SN-32-A12-HA (VHH _{AIM2-1} -VHH _{AIM2-1})	This study	Lab-internal number: pFS4119

Reagent/resource	Reference or source	Identifier or catalog number
pCAGGS VHH SN-32-A12-(G ₄ S) ₃ -VHH SN-32-C07-HA (VHH _{AIM2-1} -VHH _{AIM2-2})	This study	Lab-internal number: pFS4120
pCAGGS VHH SN-32-A12-(G ₄ S) ₃ -VHH SN-32-H12-HA (VHH _{AIM2-1} -VHH _{AIM2-3})	This study	Lab-internal number: pFS4121
pCAGGS VHH SN-32-C07-(G ₄ S) ₃ -VHH SN-32-A12-HA (VHH _{AIM2-2} -VHH _{AIM2-1})	This study	Lab-internal number: pFS4122
pCAGGS VHH SN-32-C07-(G ₄ S) ₃ -VHH SN-32-C07-HA (VHH _{AIM2-2} -VHH _{AIM2-2})	This study	Lab-internal number: pFS4123
pCAGGS VHH SN-32-C07-(G ₄ S) ₃ -VHH SN-32-H12-HA (VHH _{AIM2-2} -VHH _{AIM2-3})	This study	Lab-internal number: pFS4124
pCAGGS VHH SN-32-H12-(G ₄ S) ₃ -VHH SN-32-A12-HA (VHH _{AIM2-3} -VHH _{AIM2-1})	This study	Lab-internal number: pFS4125
pCAGGS VHH SN-32-H12-(G ₄ S) ₃ -VHH SN-32-C07-HA (VHH _{AIM2-3} -VHH _{AIM2-2})	This study	Lab-internal number: pFS4126
pCAGGS VHH SN-32-H12-(G ₄ S) ₃ -VHH SN-32-H12-HA (VHH _{AIM2-3} -VHH _{AIM2-3})	This study	Lab-internal number: pFS4127
pCAGGS VHH _{NP-1} -(G ₄ S) ₃ -VHH _{NP-1} -HA	This study	Lab-internal number: pFS4214
pcDNA3.1 MCS-FLAG	This study	Lab-internal number: pFS54
pEXPR AIM2-FLAG	This study	Lab-internal number: pFS3195
pEXPR NLRP3-FLAG	This study	Lab-internal number: pFS2149
pEXPR AIM2 PYD-EGFP-FLAG	This study	Lab-internal number: pFS5075
pEXPR AIM2-Renilla	This study	Lab-internal number: pFS2510
pEXPR AIM2 PYD-Renilla	This study	Lab-internal number: pFS2511
pEXPR AIM2 HIN200-Renilla	This study	Lab-internal number: pFS2512
pEXPR TO FRT AIM2-SH	This study	Lab-internal number: pFS1917
pEXPR TO FRT NLRP3-SH	This study	Lab-internal number: pFS353
Lentiviral vectors		
pInducer20	Meerbrey et al, (2011)	Stephen Elledge, Harvard Medical School, Boston, MA, USA
pInducer20 AIM2-HA	This study	Lab-internal number: pFS1512

Reagent/resource	Reference or source	Identifier or catalog number
pInducer20-NA	Schmidt et al, (2016b)	Lab-internal number: pFS251
pInducer20-NA caspase-1 CARD-EGFP	Schiffelers et al, (2024)	Lab-internal number: pFS720
pRRL pUbC C1C-EGFP Hygro	Gohr et al, (2025)	Lab-internal number: pFS846
pRRL pUbC C1C-TagBFP Puro	This study	Lab-internal number: pFS2993
pRRL pUbC ASC-GS-EGFP Puro	Jenster et al, (2023)	Lab-internal number: pFS833
pMD2.G	N/A	Didier Trono, École polytechnique fédérale de Lausanne, Switzerland
psPax2	N/A	Didier Trono, École polytechnique fédérale de Lausanne, Switzerland
Vectors for recombinant VACV generation		
pJS4	Chakrabarti et al, (1997)	Bernard Moss, NIH, Bethesda, MD, USA
pJS4 EL NeoR E caspase-1 CARD-EGFP (C1C-EGFP)	This study	Lab-internal number: pFS1923
pJS4 EL NeoR E EGFP	This study	Lab-internal number: pFS1924
pJS4 EL NeoR E caspase-1 CARD-mCherry (C1C-mCherry)	This study	Lab-internal number: pFS1925
pJS4 E C1C-EGFP EL VHH _{NP1} -(G ₄ S) ₃ -VHH _{NP1} -HA	This study	Lab-internal number: pFS4279
pJS4 E C1C-EGFP EL VHH _{ASC} -(G ₄ S) ₃ -VHH _{ASC} -HA	This study	Lab-internal number: pFS4281
pJS4 E C1C-EGFP EL VHH _{AIM2-1} -(G ₄ S) ₃ -VHH _{AIM2-1} -HA	This study	Lab-internal number: pFS4277
pJS4 E C1C-EGFP EL VHH _{AIM2-2} -(G ₄ S) ₃ -VHH _{AIM2-1} -HA	This study	Lab-internal number: pFS4278
Antibodies		
Goat polyclonal anti-mouse IgG (H + L)-HRP	Thermo Fisher Scientific	31430; RRID:AB_228307
Goat polyclonal anti-rabbit IgG (H + L)-HRP	Thermo Fisher Scientific	31460; RRID:AB_228341
Highly cross-adsorbed goat polyclonal anti-rabbit IgG (H + L)-Alexa Fluor™ Plus 647	Thermo Fisher Scientific	A32733; RRID:AB_2633282
Rabbit anti-AIM2 monoclonal D5X7K	Cell Signaling Technology	12948; RRID:AB_2798067
Rabbit polyclonal anti-E-tag-HRP	Bethyl	A190-133P; RRID:AB_345222
Mouse anti-HA.11 Epitope tag clone 16B12	BioLegend	901503; RRID:AB_2565005

Reagent/resource	Reference or source	Identifier or catalog number
Rabbit monoclonal anti-HA-Tag clone C29F4	Cell Signaling Technology	3724; RRID: AB_1549585
Mouse anti-HA-HRP clone 6E2	Cell Signaling Technology	2999S; RRID:AB_1264166
Rabbit polyclonal anti-VACV H5 serum	DeMasi et al, (2000)	Paula Traktman, Medical University of South Carolina; USA
Mouse anti-vinculin clone hVIN-1	Sigma-Aldrich	V9131; RRID:AB_477629
Oligonucleotides and other sequence-based reagents		
qPCR Primers and guide RNAs	This study	Appendix Table S1
Chemicals, enzymes and other reagents		
Amylose magnetic beads	New England Biolabs	E8035S
Buffy coats	University Hospital Bonn	N/A
CD14 (human) MicroBeads	Miltenyi Biotec	130-050-201
CNBr-activated Sepharose 4B	Sigma-Aldrich	GE17-0430-01
cOmplete™ Mini protease Inhibitor Cocktail	Roche	11836153001
CRID3 (MCC950)	Tocris	5479
Criterion™ TGX™ pre-cast protein gel	Bio-Rad Laboratories	5671094
Cycloheximide	Sigma-Aldrich	C7698-1G
Cytarabine	Abcam	ab141924
DermaCult™ Keratinocyte Expansion Medium	STEMCELL Technologies	100-0500
DMEM GlutaMax	Thermo Fisher Scientific	61965059
Doxycycline	Biomol	Cay14422-1
DRAQ7	Biologend	424001
Ficoll-Paque™ PLUS	VWR	17-1440-02
G140	Invivogen	inh-g140
H-151	Biozol	FBM-10-4132
Halt phosphatase and protease inhibitor cocktail	Thermo Fisher Scientific	78420
HiLoad 16/600 Superdex 75 pg column	Cytiva	28989333
Hoechst 33342	Thermo Fisher Scientific	62249
Human IFN-α	PBL Assay Science	11100-1
Human IFN-γ	Immunotools	11343536
Human IL-1β HTRF kit	Cisbio	62HIL1BPEH
LDH Cytotoxicity Detection Kit	Roche	11644793001
Lipofectamine™ LTX transfection reagent	Thermo Fisher Scientific	11100-1

Reagent/resource	Reference or source	Identifier or catalog number
MagStrep type 3 Strep-Tactin beads	IBA Lifesciences	2-4090-002
Maxima SYBR Green/ROX qPCR Master Mix	Steinbrenner Laborsysteme GmbH	SL-9903RB
Ni-NTA agarose	Qiagen	30230
PD MiniTrap G-25 columns	Cytiva	28918007
PEI Max transfection reagent	Polysciences	24765-1
PMA	Sigma-Aldrich	P8139
Polyvinylidene difluoride membranes 0.45 μm	Merck	IPFL(IPVH)00010
Recombinant human GM-CSF	Immunotools	11343125
RNeasy Kit	QIAGEN	74136
RPMI 1640 GlutaMax medium	Thermo Fisher Scientific	61870044
SuperScript III Reverse Transcriptase	Thermo Fisher Scientific	18080044
TMB	Thermo Fisher Scientific	002023
VX-765/ belnacasan	Selleckchem	S2228
Western Lightning Ultra/Plus-ECL	Revvity	NEL112001EA/ NEL103001EA
Software		
EvolutionCapt SL6 software	Vilber	N/A
FACS DIVA Software	BD	N/A
FIJI version 2.14 (ImageJ)	NIH	N/A
FlowJo version 10.8.1	FlowJo	N/A
GraphPad Prism version 9.5.0	GraphPad Software Inc.	N/A
Imaris software version 9.9	Bitplane	N/A
Incucyte 2021 C software	Sartorius	N/A
SoftMax Pro 6.3 Software	Molecular Devices	N/A
Other		

Ethics statement

Experiments with cells derived from human blood were approved by the Ethics Committee of the Medical Faculty of the University of Bonn (300/17). Informed consent was obtained from all subjects, and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Alpaca immunizations were approved by the Landesuntersuchungsamt Rheinland-Pfalz (23 177-07/A 17-20-005 HP).

Cell lines

Human THP-1 cells (ATCC TIB-202, RRID: [CVCL_0006](#)) were cultured in RPMI 1640 GlutaMax medium (Thermo Fisher Scientific) containing 10% FBS, 50 μ M 2-mercaptoethanol, and 100 U/mL penicillin-streptomycin. Human A549 (ATCC CCL-185, RRID: [CVCL_0023](#)), HEK293T (ATCC CRL-3216, RRID: [CVCL_0063](#)), HeLa (ATCC CCL-2, RRID: [CVCL_0030](#)), and Vervet monkey BSC-40 (CRL-2761, RRID: [CVCL_3656](#)) cells were cultivated in DMEM GlutaMax containing 10% FBS, and 100 U/mL penicillin-streptomycin; BSC-40 media was supplemented with non-essential amino acids and 1 mM sodium pyruvate. Flp-In 293 T-REx cells (Thermo Fisher Scientific, [R78007](#), RRID: [CVCL_U427](#)), dox-inducibly expressing AIM2-SH or NLRP3-SH were generated according to manufacturer's recommendation, and cultured in DMEM containing 10% FBS, GlutaMax, 4 μ g/ml blasticidin S, and 50 μ g/ml hygromycin B. Lentivirus produced with packaging vectors psPax2 and pMD2.G (kind gifts provided by Didier Trono, École polytechnique fédérale de Lausanne, Switzerland), were used to generate genetically modified cell lines (see reagents and tools table for details on all cell lines used). THP-1 cell lines expressing caspase-1^{CARD}-EGFP (C1C-EGFP) or C1C-TagBFP inflammasome reporter under a dox-inducible promoter were generated using lentiviral vectors derived from pInducer20 (Meerbrey et al, 2011) (a kind gift provided by Stephen Elledge, Harvard Medical School), and selected with 500 μ g/mL geneticin (Thermo Fisher Scientific). Cell lines constitutively expressing transgenes under the control of the human elongation factor-1 α promoter (pEF1 α) or human ubiquitin C promoter (pUbC) were generated using lentiviral vectors constructed by Gateway cloning (Thermo Fisher Scientific) using vectors modified from pRLL (a kind gift of Susan Lindquist, Whitehead Institute of Biomedical Research), followed by antibiotic selection. THP-1 knockouts were generated by lentiviral transductions using vectors modified based on pLenti CRISPR v2 (Sanjana et al, 2014) (a kind gift of Feng Zhang, Broad Institute, Cambridge, MA, USA) and antibiotic selection, or by electroporation with 50 μ g/ml of an EF1 α -Cas9-2A-EGFP, U6-sgRNA expression plasmid (Neon system, Thermo Fisher Scientific, 1250 V, 50 ms, 1 pulse) and sorting for EGFP-positive cells 18 h post transfection (see Appendix Table S1 for sgRNA target sequences used). Monoclonal knockout cell lines were generated by limiting dilution; the best clones were selected based on analysis with Sanger sequencing, functional testing, and immunoblot. THP-1 Δ NLRP3 and THP-1 Δ cGAS were kindly provided by Veit Hornung (Ludwig-Maximilians-University Munich, Germany) and Søren Riis Paludan (Aarhus University, Denmark), respectively (Gritsenko et al, 2020; Christensen et al, 2016).

Primary cells

For the isolation of primary human cells, whole blood buffy coats were obtained from the blood bank of the University Hospital Bonn, with consent of healthy donors and according to protocols accepted by the institutional review board of the University of Bonn. PBMCs were isolated using Ficoll-Paque™ PLUS (VWR) according to the manufacturer's instructions and CD14⁺ were isolated by positive selection using paramagnetic CD14 (human) MicroBeads (Miltenyi Biotec). 10⁷ CD14⁺ monocytes were then

differentiated into macrophages using 500 U/mL recombinant human GM-CSF (Immunotools) for 4 days. Primary cells were cultured in RPMI 1640 GlutaMax medium supplemented with 10% FBS, 100 U/mL penicillin-streptomycin, and 1 mM sodium pyruvate.

Normal human skin was obtained from excessive tissue removed during plastic surgery (e.g., abdominoplasty or reduction mammaplasty) at the University Hospital Bonn with the consent of patients and according to protocols accepted by the institutional review board of the University of Bonn. Primary keratinocytes were isolated from the skin utilizing a previously published protocol (Johansen, 2017) and cultured in DermaCult™ Keratinocyte Expansion Medium (STEMCELL Technologies), supplemented with hydrocortisone (96 ng/ml, STEMCELL Technologies), 100 U/mL penicillin-streptomycin, 5 μ g/mL gentamicin, and 2.5 μ g/mL amphotericin B (all Thermo Fisher Scientific). The epidermal layer was scraped off with a foot planer from cleaned skin. Skin pieces were incubated in sterile-filtered 0.25% trypsin-EDTA solution with 0.1% glucose for 30 min at 37 °C. Trypsin was inactivated by adding DMEM with 2% FBS, and the suspension was vortexed to release cells from the tissue. The cell suspension was filtered through a 70 μ m cell strainer, sedimented by centrifugation at 450 $\times$ g for 10 min, and 8 $\times$ 10⁶ cells were seeded in T175 flasks in DermaCult™ media and cultivated until near confluency. NHEK cells expressing caspase-1^{CARD}-EGFP (C1C-EGFP) under dox-inducible or constitutive UbC promoter were generated by lentiviral transduction and antibiotic selection as described above.

Generation and production of recombinant VACV and a clinical MPXV isolate

Recombinant VACV strains were generated based on the VACV Western Reserve (WR) strain by transfecting infected cells with derivatives of plasmid pJS4 containing the flanking regions of the tk locus, as well as sequences of genes of interest with viral promoters as described in (Chakrabarti et al, 1997) (see reagents and tools table for full list of recombinant VACV strains). Recombinant VACV strains expressing C1C-EGFP (WR E C1C-EGFP) or EGFP (WR E EGFP) were generated with pJS4 derivatives encoding C1C-EGFP/EGFP under the J2R early promoter and neomycin phosphotransferase under a synthetic early/late promoter as described before (Schmidt et al, 2013). Recombinant VACV expressing inflammasome reporter and bivalently nanobodies were generated similarly using pJS4 derivatives encoding C1C-EGFP or EGFP under the control of the J2R early promoter and the bivalent nanobodies under the control of a synthetic early/late promoter. VACV WR mCherry-A4 was described previously (Schmidt et al, 2011) and was modified to encode C1C-EGFP under the control of the J2R early promoter as described above. Recombinant VACV strains were isolated by at least three rounds of fluorescent plaque purification. The MPXV strain MPXV/Germany/2022/BN001 was isolated on Vero E6 cells from a skin lesion of a patient who presented at the University Hospital Bonn in June 2022 and amplified from a single plaque on BSC-40 cells. PCRs for orthopoxvirus and MPXV were positive and identified the strain as a clade II virus (formerly West African clade). VACV and MPXV mature virions (MVs) were produced using BSC-40 cells and purified from cytoplasmic extracts through a sucrose cushion as described in (Schmidt et al, 2013). Virus titers were determined by

plaque assay on BSC-40 cells. All work involving VACV and MPXV was performed according to BSL2 and BSL3 safety standards, respectively.

Plasmids

Expression vectors, lentiviral vectors, and plasmids for homologous recombination of VACV described in the individual experiments were generated by Gateway and Gibson cloning (see reagents and tools table of used plasmids). Plasmid maps and oligonucleotide sequences are shared on request.

Protein expression and purification

Expression of His-MBP-AIM2^{PYD} L10A L11A for immunization

To produce a soluble AIM2^{PYD} mutant L10A L11A (Lu et al, 2014a), we generated a bacterial expression vector pEXPR His-MBP-AIM2 PYD L10A L11A (encoding AA 1–107 of AIM2) with a gateway-compatible derivative of modified pDB-His-MBP. *Escherichia* (*E.*) *coli* LOBSTR (Andersen et al, 2013) was transformed with pEXPR His-MBP-AIM2 PYD L10A L11A, and bacteria were grown in Terrific Broth, induced with 0.2 mM IPTG at an OD₆₀₀ of 0.6, and cultivated for another 24 h at 18 °C. Cells were lysed with a Bandelin Sonopuls HD2070 sonicator with a TT13 tip, followed by Ni-NTA affinity purification with Ni-NTA agarose (Qiagen) and size exclusion chromatography with a HiLoad 16/600 Superdex 75 pg column (Cytiva) in reducing HEPES gel filtration buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 2 mM DTT, and 10% glycerol).

Expression and purification of nanobodies

AIM2 nanobody sequences were cloned into bacterial expression vectors based on pHEN6 for periplasmic expression with C-terminal LPETG-His for large scale, or C-terminal HA-His for small-scale protein expression. Nanobodies were expressed and purified as described before (Koenig et al, 2021). *E. coli* WK6 were transformed with nanobody expression vectors and grown in Terrific Broth, induced with 1 mM IPTG at an OD₆₀₀ of 0.6, and cultured at 30 °C for 16 h. Expression vectors for bivalent nanobodies were generated by Gibson cloning using a modified version of pSBInit (Zimmermann et al, 2018). For expression of bivalent nanobodies, *E. coli* MC1061 was transformed with pSBInit-based expression vectors and grown in Terrific Broth, induced with 0.02% arabinose at an OD₆₀₀ of 0.6, and cultivated at 22 °C for 16 h. Bacteria were sedimented and resuspended in TES buffer (200 mM Tris-HCl, pH 8.0, 0.65 mM EDTA, 0.5 M sucrose), and incubated for 1 h at 4 °C with shaking. Periplasmic extracts were obtained by osmotic shock in 0.25x TES overnight at 4 °C. Nanobodies were purified with Ni-NTA agarose beads, followed by desalting with PD MiniTrap G-25 columns (Cytiva) for small-scale expression, or by gel filtration with a HiLoad 16/600 Superdex 75 pg column in HEPES gel filtration buffer (20 mM HEPES pH 7.4, 150 mM NaCl, and 10% glycerol) for large-scale expression.

Production of nanobody-coupled beads for immunoprecipitation

To immunoprecipitate AIM2, recombinant AIM2 or control nanobodies were covalently coupled to cyanogen bromide (CNBr)-activated Sepharose 4B beads (Sigma-Aldrich) according to the manufacturer's recommendations.

Antibodies

The following antibodies were used: rabbit monoclonal anti-AIM2 (Cell Signaling Technology Cat# 12948, RRID:AB_2798067), rabbit polyclonal anti-VACV H5 serum (DeMasi and Traktman, 2000) (kindly provided by Paula Traktman, Medical University of South Carolina), mouse anti-vinculin clone VIN-1 (Sigma-Aldrich Cat# V9131, RRID:AB_477629), rabbit monoclonal anti-HA-Tag (Cell Signaling Technology Cat# 3724, RRID: AB_1549585), mouse anti-HA.11 Epitope tag clone 16B12 (BioLegend Cat# 901503, RRID:AB_2565005), mouse anti-HA-HRP clone 6E2 (Cell Signaling Technology Cat# 2999S, RRID:AB_1264166), rabbit polyclonal anti-E-tag-HRP (Bethyl Cat# A190-133P, RRID:AB_345222), goat polyclonal anti-mouse IgG (H + L)-HRP (Thermo Fisher Scientific Cat# 31430, RRID:AB_228307), goat polyclonal anti-rabbit IgG (H + L)-HRP (Thermo Fisher Scientific Cat# 31460, RRID:AB_228341), and highly cross-adsorbed goat polyclonal anti-rabbit IgG (H + L)-Alexa FluorTM Plus 647 (Thermo Fisher Scientific Cat# A32733, RRID:AB_2633282).

Small compound inhibitors and reagents

The following small compound inhibitors and reagents were used: CRID3 (MCC950) (Tocris), cycloheximide (Sigma-Aldrich), Cytarabine (AraC) (Abcam), doxycycline (Biomol), G140 (Invivogen), H-151 (Biozol), human IFN- α (PBL Assay Science), human IFN- γ (Immunotools), LPS-EK Ultrapure (Invivogen), PMA (phorbol 12-myristate 13-acetate) (Sigma-Aldrich), and VX-765/belnacasan (Selleckchem).

Nanobody library generation

To raise AIM2-specific VHHs, a male alpaca from local husbandry was immunized six times with 200 μ g MBP-AIM2^{PYD} L10A L11A using GERBU Adjuvant Fama (GERBU Biotechnik GmbH), according to locally authorized protocols (Landesuntersuchungsamt Rheinland-Pfalz, 23 177-07/A 17-20-005 HP). The M13 phagemid vector pD (pJSC) was used to generate the VHH plasmid library as described before in (Koenig et al, 2021). In short, RNA from peripheral blood lymphocytes was isolated and used as a template to generate cDNA using three sets of primers (random hexamers, oligo(dT), and alpaca heavy chain gene-specific primers). VHH coding sequences were amplified by PCR using VHH-specific primers, cut with *Asc*I and *Not*I, and ligated into a linearized M13 phagemid vector (pJSC). *E. coli* TG1 cells (Agilent) were electroporated with ligation reactions, and the obtained ampicillin-resistant colonies were harvested, pooled, and stored as glycerol stocks.

Nanobody screening by phage display

AIM2^{PYD}-specific VHHs were obtained by phage display and panning as described in (Koenig et al, 2021). *E. coli* TG1 cells containing the VHH library were infected with helper phage VCSM13 to produce phages displaying encoded VHHs as pIII fusion proteins. Phages collected from the supernatant were purified and concentrated by precipitation. Phages displaying AIM2^{PYD}-specific VHHs were enriched using two approaches: (1) phages were incubated with chemically biotinylated MBP-AIM2^{PYD}

immobilized on amylose magnetic beads (New England Biolabs) and (2) phages were incubated with full-length AIM2-SH expressed in HEK293 Flp-In T-REx cells and immobilized on MagStrep type 3 Strep-Tactin beads (IBA Lifesciences). Bound phages were eluted by low pH elution or biotin elution buffers, respectively, and used to infect *E. coli* ER2738, followed by a second round of panning. *E. coli* ER2837 colonies from the second round of panning were picked and grown in 96-well plates. VHH expression was induced with IPTG, and expressed VHHs that leaked into the supernatant were tested for specificity using ELISA plates coated with control protein MBP or MBP-AIM2^{PYD} L10A L11A. VHH binding was detected with HRP-coupled rabbit anti-E-Tag antibodies (1:10,000), and the chromogenic substrate tetramethylbenzidine (TMB) (Life Technologies). About 1 M HCl was used to stop the reaction prior to recording absorption at 450 nm using a SpectraMax i3 instrument, and the SoftMax Pro 6.3 Software (Molecular Devices). Positive candidates were sequenced and representative VHHs were cloned into bacterial and mammalian expression vectors for further analysis.

ELISA

To test AIM2-specific VHHs, MBP or MBP-AIM2^{PYD} L10A L11A in PBS were immobilized on ELISA plates at a concentration of 1 µg/mL overnight. On the following day, the immobilized antigens were incubated with the HA-tagged VHHs in 10% FBS/PBS in a tenfold dilution series ranging from 1 µM to 100 pM. Bound VHHs were detected using the mouse anti-HA HRP antibody (1:5000) and developed with the chromogenic substrate TMB. 1 M HCl was used to stop the reaction prior to measuring absorption at 450 nm using a SpectraMax i3 instrument and the SoftMax Pro 6.3 Software (Molecular Devices).

LUMIER assay

To test the intracellular target binding of VHHs in the reducing environment of the cytosol, LUMIER assays were performed as previously described (Schmidt et al, 2016a). In short, Renilla luciferase fusions of target proteins were co-expressed with HA-tagged VHHs in HEK293T cells. VHHs from cell lysates were immunoprecipitated with anti-HA antibodies, and the co-purified luciferase activity was determined as a readout for the cytosolic interaction of VHHs and target proteins. About 2.5×10^5 HEK293T cells per well were seeded into 24-well plates, and the following day co-transfected with 0.25 µg pCAGGS VHH-HA expression vectors and 0.25 µg of pcDNA3.1-based expression vectors for the Renilla-fused target proteins human full-length AIM2, mouse full-length AIM2, human AIM2^{HIN200} (aa 144–343), human AIM2^{PYD} (aa 1–107), or the control human NLRP1^{CARD} using transfection reagent PEI Max (Polysciences). High-binding Lumitrac 600 white 96-well plates (Greiner) were coated with 20 µg/mL of the mouse anti-HA.11 Epitope tag clone 16B12 antibody in PBS overnight. The next day, HEK293T cells were lysed in LUMIER lysis buffer (50 mM HEPES-KOH pH 7.9, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 5% glycerol and Roche cOmplete™ Mini protease Inhibitor Cocktail), and lysates were incubated in the anti-HA-coated Lumitrac 600 plates for one hour to immunoprecipitate HA-tagged VHHs. After repeated washing, Renilla luciferase substrate coelenterazine-h was added, and

luminescence was measured using a SpectraMax i3 instrument, and the SoftMax Pro 6.3 Software (Molecular Devices).

Immunoblot

To confirm the expression of AIM2, cells were either treated with 500 U/mL IFN-γ (Immunotools) or left untreated overnight. The following day, cells were washed with PBS and lysed in 100 µL SDS-PAGE buffer (50 mM Tris, pH 6.8; 0.01% bromophenol blue, 10% glycerol, 2% SDS, 100 mM dithiothreitol). Proteins were separated by SDS-PAGE using a 12% polyacrylamide gel. Separated proteins were transferred to polyvinylidene difluoride membranes (0.45 µm; Merck) by wet transfer. Immunoblots were blocked in 3% BSA/TBS and probed with anti-AIM2 (1:1000), or anti-vinculin (1:1000), in 1% BSA/TBST overnight at 4 °C. The following day, immunoblots were washed and probed with HRP-conjugated secondary antibodies (1:5000) for 2 h at room temperature. A chemiluminescent signal was induced by Western Lightning Ultra-ECL or Western Lightning Plus-ECL (Revvity). Signal was detected using a Fusion Advancer imaging system (Vilber), and images were recorded using the EvolutionCapt SL6 software (Vilber).

To confirm the expression of AIM2 in primary cells by immunoprecipitation with AIM2 nanobodies and subsequent detection by immunoblot, cells were treated with IFN-γ overnight, washed with PBS, and lysed on ice in 500 µL lysis buffer (50 mM HEPES-KOH, 150 mM NaCl, 2 mM EDTA, 0.5% triton X-100, 5% glycerol, pH 7.9) supplemented with cOmplete™ Mini protease Inhibitor Cocktail (Roche). Lysates were cleared of debris and nuclei by sedimentation, and lysates were supplemented with NaCl to reach a final concentration of 500 mM. About 150 µL of VHH_{AIM2-2} coupled to CNBr-activated Sepharose 4B beads was subsequently incubated with lysates to immunoprecipitate AIM2. Beads were washed five times with high salt lysis buffer (50 mM HEPES-KOH, 500 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 5% glycerol, and pH 7.9). Beads were resuspended in 50 µL SDS-PAGE buffer and boiled at 95 °C for 5 min and sedimented. SDS-PAGE for protein separation and immunoblot were performed as described above.

Gene expression analysis by quantitative PCR (qPCR)

To quantify gene expression, up to 10^6 cells were lysed in RTL buffer (QIAGEN), and RNA was isolated using the RNeasy Kit (QIAGEN). Equal amounts of RNA from each sample were reversely transcribed into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), and oligo dT₍₁₈₎ primers. Quantitative real-time PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (Steinbrenner Laborsysteme GmbH) on a QuantStudio6 cycler. Hypoxanthine-Guanine Phosphoribosyltransferase (HPRT) was used to normalize the expression of target genes. The primers used for human AIM2 (Ran et al, 2017) and HPRT (Lauterbach et al, 2019) are listed in Appendix Table S1.

Virus infections

For virus infections in THP-1 cells, 3×10^5 cells were differentiated into macrophage-like cells using 50 ng/mL PMA (Sigma-Aldrich) overnight (16 h) in a 24-well plate. The following morning, medium was changed to PMA-free medium, where indicated, containing 1 µg/mL dox to induce gene expression. For infection experiments in

primary cells, we seeded 1.5×10^5 primary macrophages, 5×10^4 keratinocytes (both in 24-well plates), or 3×10^5 CD14⁺ monocytes (in 96-well plates). Twenty-four hours post seeding, adherent cells (differentiated THP-1, primary macrophages and keratinocytes) were treated with 500 U/mL IFN- γ overnight, where indicated. CD14⁺ monocytes were treated with IFN- γ at the time of seeding. On the following day, cells were infected with 300 μ L (for 24-well plate) and 100 μ L (for 96-well plate) of virus inoculum containing the indicated MOI of freshly sonicated virus in serum-free medium. Cells were incubated for 1 h with gentle rocking every 15 min. About 1 h post infection, the virus inoculum was replaced with 500 μ L full medium, and cells were cultivated for 6 h. CD14⁺ monocytes were cultivated for 6 h (in serum supplemented media) without media change to enhance infection efficiency. Cells were analyzed by flow cytometry or microscopy, while supernatants were analyzed by HTRF (see below).

Quantification of inflammasome assembly by flow cytometry

To quantify the assembly of inflammasomes, the assembly of ASC-EGFP specks or recruitment of caspase-1^{CARD}-EGFP (C1C-EGFP) or C1C-TagBFP to endogenous ASC specks was quantified by flow cytometry. Reporter cell lines were either infected with VACV WT or transfected with the indicated expression vectors, or WT cells were infected with recombinant VACV expressing C1C-EGFP. All infections were done in the presence of 40 μ M VX to prevent cell death by pyroptosis. Infected cells were trypsinized, fixed in 4% formaldehyde for 20 min, resuspended in FACS buffer (PBS, 2% FBS, 5 mM EDTA, 0.02% NaN₃), and analyzed using a BD FACS Canto flow cytometer. To determine the frequency of cells with ASC or C1C-EGFP/TagBFP specks, we first gated a homogenous population from the FSC-A vs. SSC-A plot, followed by selection of single cells using SSC-A vs. SSC-W and FSC-A vs. FSC-W plots. If not mentioned otherwise, cells expressing fluorescent transgenes or stained with fluorescent antibodies were gated using the area of the respective fluorescent channel. Only EGFP⁺ or TagBFP⁺ cells (except for mock-infected samples) were included for the analysis of inflammasome speck formation. We plotted the width against the height of EGFP/TagBFP and gated cells with C1C specks as described in (Jenster et al, 2023). Flow cytometry data were analyzed using FlowJo 10.8.1 software.

Cell death quantification by LDH release (plasma membrane rupture)

To quantify the release of cytosolic LDH as a consequence of cell death upon VACV infection, 3×10^5 THP-1 cells were differentiated and infected in 24-well plates as described before. Cells were infected for 6 h in Opti-MEMTM. Supernatants were collected, and LDH release was quantified using the LDH Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions. Absorption at 492 nm was measured using a SpectraMax i3 instrument and the SoftMax Pro 6.3 Software (Molecular Devices). Cell death was normalized to control well, in which cells were lysed in 1% Triton X-100, and background signal from medium was subtracted from all samples.

Cell death quantification by DRAQ7 uptake (membrane integrity)

To quantify VACV infection-induced loss of plasma membrane integrity over time, 3×10^5 THP-1 cells were differentiated and

infected in 24-well plates as described before. Cells were infected with virus inoculum for 1 h, the inoculum was removed, and cells were covered with full medium containing 100 nM DRAQ7 (Biolegend). DRAQ7 uptake and infection (EGFP⁺ cells) was recorded by taking four images per well every 15 min, for a total of 6 h using the Incucyte Live-Cell Imaging system (Sartorius). The number of DRAQ7-uptake, infection and cell confluency were analyzed using the Incucyte 2021C software. For every single image, the cell death count was determined. The cell death was first corrected by subtracting the cell death count at $T=0$ and was further normalized to the cell confluency, and average values from all four images were calculated and plotted over time.

Cytokine quantification by homogenous time-resolved fluorescence (HTRF)

To quantify IL-1 β secretion post VACV infection, 3×10^5 cells were seeded in 24-well plates as described before. For IL-1 β secretion quantification experiments, cells were infected for 6 h in Opti-MEMTM in the absence of VX. Supernatants were then collected, and IL-1 β levels were quantified using the Human IL-1 β HTRF kit (Cisbio) according to the manufacturer's instructions. Emissions at 620 and 665 nm were measured using a SpectraMax i3 instrument, and IL-1 β levels were calculated by the SoftMax Pro 6.3 Software (Molecular Devices) based on a standard curve.

Confocal microscopy

To quantify ASC^{PYD}-EGFP filament formation by confocal microscopy, HeLa cells were seeded on 12 mm cover slips in 24-well plates and co-transfected the next day with 0.25 μ g of monovalent or bivalent VHH-HA, and AIM2^{PYD}-EGFP expression vectors using LipofectamineTM LTX transfection reagent (Thermo Fisher Scientific). 24 h later, transfected cells were fixed in 4% formaldehyde in PBS for 20 min, followed by permeabilization with permeabilization buffer (PBS with 0.5% Triton X-100, 10% goat serum) for 20 min. Cells were consecutively stained with primary rabbit anti-HA antibody (1:1000) and secondary goat anti-rabbit IgG AF647 (1:1000) in permeabilization buffer for 1 h each. DNA was stained with Hoechst 33342 (Thermo Fisher Scientific) (1:5000), and images were recorded with the HC PL APO CS2 63x/1.20NA oil objective on a Leica SP8 Lightning confocal microscope. Images were processed and quantified using ImageJ 2.3.0 software.

To record confocal microscopy images of VACV-infected THP-1 cells, cells were infected as described above, fixed in 4% formaldehyde in PBS, and stained for DNA as before. Confocal images were recorded with the HC PL APO CS2 63x/1.20NA water objective on a Leica Stellaris 8 microscope.

To quantify localization of viral genomes with inflammasomes, cells were infected with VACV WR EGFP-A4 E C1C-mCherry. Fixed cells were stained with Hoechst 33342, and Z stacks with 0.5 μ m intervals were recorded by confocal microscopy as described above. We used the Imaris (Bitplane) spot detection tool to detect both C1C-mCherry specks (XY-diameter = 2 μ m, Z-diameter = 2 μ m, quality >1.40) and viral genomes (XY-diameter = 0.4 μ m, Z-diameter = 1.5 μ m, quality >2) in the respective channels. Parameters for viral genome detection in the Hoechst channel were optimized to detect viral genomes in intact cores, colocalizing with core protein EGFP-A4. The intensity sum for the Hoechst channel

was set to a maximum of 2000 to filter out signals from stained nuclei. For each C1C-mCherry speck, we calculated the distance to the next released genome and quantified the fraction of C1C-mCherry with a released viral genome less than 1 μm away.

AIM2 immunoprecipitation from primary CD14⁺ monocytes

To confirm the expression of AIM2 in CD14⁺ monocytes by mass spectrometry, 4.5×10^6 cells were treated with IFN- γ overnight, washed with PBS, and lysed on ice in 500 μL lysis buffer (0.2% NP-40, 150 mM NaCl, 20 mM HEPES, 1 mM MgCl_2 , 50 U/mL Benzonase) supplemented with Halt phosphatase and protease inhibitor cocktail (Thermo Fisher Scientific). Lysates were cleared of debris and nuclei by sedimentation, and subsequently supplemented with NaCl to reach a final concentration of 500 mM. 100 μL of VHH_{AIM2-2} or control VHH_{Enhancer} coupled to CNBr-activated Sepharose 4B beads were subsequently incubated with lysates to immunoprecipitate AIM2. Beads were washed five times with high salt lysis buffer (0.2% NP-40, 500 mM NaCl, 20 mM HEPES, 1 mM MgCl_2 , 50 U/mL Benzonase) and once with 40 mM HEPES.

Proteins were digested off the beads by sequentially adding 1 μg LysC (Wako Chemicals) in 6 M urea (Sigma-Aldrich), and 40 mM HEPES for 1 h. Urea concentrations were then diluted with water to reach concentrations below 2 M, followed by the addition of 1 μg Trypsin (Sigma-Aldrich) for overnight digestion at room temperature (RT), and shaking at 800 rpm. Cysteines were reduced by adding 10 mM dithiothreitol (Sigma-Aldrich) for 20 min at RT, followed by alkylation with 55 mM iodoacetamide (Sigma-Aldrich) for 20 min at RT and in the dark. Excess iodoacetamide was quenched with 150 mM thiourea (Sigma-Aldrich) at RT for 15 min prior to acidification for peptide desalting with trifluoroacetic acid (TFA) (0.6% TFA final conc. v/v, 2% acetonitrile) (Thermo Fisher Scientific, Riedel-de-Haen). Peptides were desalted on in-house-produced double-layer C18 StageTips (Affinisep # SPE-Disks-Bio-C18-100.47.20) (Rappsilber et al, 2007). StageTips were activated with methanol and equilibrated with acetonitrile buffer (80% acetonitrile, 0.1% formic acid) (VWR), followed by 0.1% formic acid in water (VWR). Acidified samples were loaded onto StageTips and washed twice with 0.1% formic acid in water, before elution with acetonitrile buffer. After each step, centrifugation was carried out at $500 \times g$ for two to four minutes or until all liquid passed through the double layer. After drying in a SpeedVac (Eppendorf, Concentrator plus), peptides were resuspended in 0.1% formic acid in water and the peptide concentration was determined using a nanodrop (Thermo Fisher Scientific).

Liquid chromatography coupled to mass spectrometry

For each sample, 300 ng of tryptic peptide was separated using a Vanquish Neo UHPLC System (Thermo Fisher Scientific) in trap-and-elute mode with a trap column (Thermo Fisher Scientific) and a 25 cm analytical column (25 cm, 75 μm inner diameter, C18 filling material (Ionopticks). Peptides were separated using a non-linear 90 min gradient of 2–25% acetonitrile buffer in the first 70 min, followed by an increase to 55% over 8 min and a wash out with 95% for 10 min. Eluted peptides were transferred online to a quadrupole orbitrap tandem mass spectrometer (MS, Orbitrap Exploris 480, Thermo Fisher Scientific) and peptide ions were generated by a

nano electrospray ionization source (Thermo Fisher Scientific). The mass spectrometer was operated in data-independent acquisition (DIA) mode. Full spectra were recorded with a scan range from 380 to 1020 m/z with a resolution of 60,000. Automatic gain control was set to 300% and maximum injection time was limited to 55 ms. MS2 scans were acquired at a resolution of 30,000, an automatic gain control target of 1000% and a maximum injection time of 55 ms. Twenty-four staggered DIA isolation windows of 25 m/z were acquired in a range from 400–1,000 m/z and were fragmented with a higher-energy collisional dissociation energy of 27%.

MS data analysis

MSConvert was used for pre-processing of raw mass spectra and to deconvolute the staggered DIA windows (Peak Picking, -Zero Samples, -demultiplex). Peptide identification and protein quantification was computed with DIA-NN version 1.8.1 (Demichev et al, 2019). Mass spectra were matched against an in silico digested FASTA file of the UniProt SWISSPROT human proteome (version from 18-11-2021 with additional sequences for VHH_{AIM2-2} and VHH_{Enhancer}), and filtered on the peptide level for a false discovery rate of 1%. Trypsin was selected as the digestion enzyme, the maximum missed cleavages was set to 1 and carbamidomethylation was selected as a fixed modification. The scan window radius was set to 10, and mass accuracies were fixed to 1.2×10^{-5} (MS2) and 1.0×10^{-5} (MS1). Precursor masses were fixed between 300 and 1800 m/z with peptide sequence length from 7 to 30 amino acids. The DIA-NN main output table was used as input for label-free quantification (LFQ) of protein intensities based on the maxLFQ algorithm implemented in the DIA-NN R package (Cox et al, 2014) after a median normalization and filtering for proteotypic/unique peptides. Persus (version 2.0.10.0) was used for further statistical analysis (Tyanova et al, 2016). Protein group intensities were log2 transformed, and replicates of treatment conditions were annotated. The dataset was filtered for at least three valid values in at least one treatment group, followed by an imputation of missing values from a normal distribution (width 0.3 and downshift 1.8). A Student's *T*-test with an FDR of 0.5% and an *S0* of 0.1 was performed to compare the treatments and create a volcano plot.

AIM2^{PYD} filament assay

About 100 μg MBP-AIM2^{PYD} (34.4 μM) was incubated with 5 μg TEV protease (4 μM) at RT for 16 h in the presence of 77.5 μg of either control monovalent nanobody (VHH_{NP-1}), monovalent AIM2 nanobodies (VHH_{AIM2-1}, VHH_{AIM2-2}) (103.2 μM each), bivalent control nanobody (VHH_{Ctrl-Ctrl}), or bivalent AIM2 nanobodies (VHH_{AIM2-1}-VHH_{AIM2-1}, VHH_{AIM2-2}-VHH_{AIM2-1}) (51.6 μM each) in a total volume of 50 μL Hepes buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, and 1 mM DTT). Samples were sedimented at $20,000 \times g$, 4°C for 10 min. Supernatants and pellets were analyzed by SDS-PAGE using a 4–20% Criterion™ TGX™ pre-cast protein gel (Bio-Rad Laboratories).

Data availability

The amino acid sequences of the described nanobodies VHH_{AIM2-1} (SD-5FPK; <https://nanosaurus.org/entry/SD-5FPK>; lab-internal

name: VHH SN-32-A12-HA), VHH_{AIM2-2} (SD-U91X, <https://nanosaurus.org/entry/SD-U91X>; lab-internal name: VHH SN-32-C07), and VHH_{AIM2-3} (SD-9SRJ, <https://nanosaurus.org/entry/SD-9SRJ>; lab-internal name: VHH SN-32-H12) are deposited in the Nanosaurus nanobody database (<https://nanosaurus.org/>) under the indicated Nanosaurus IDs/links. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al, 2025) partner repository with the dataset identifier PXD069678.

The source data of this paper are collected in the following database record: [biostudies:S-SCDT-10_1038-S44318-025-00690-z](https://biostudies.org/study/S-SCDT-10_1038-S44318-025-00690-z).

Expanded view data, supplementary information, appendices are available for this paper at <https://doi.org/10.1038/s44318-025-00690-z>.

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Expanded View Figures

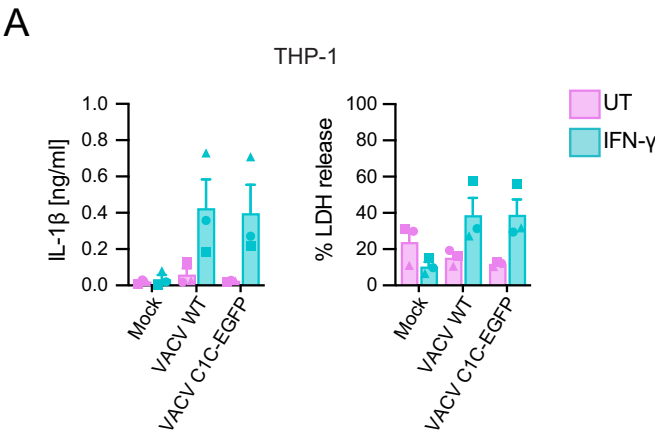
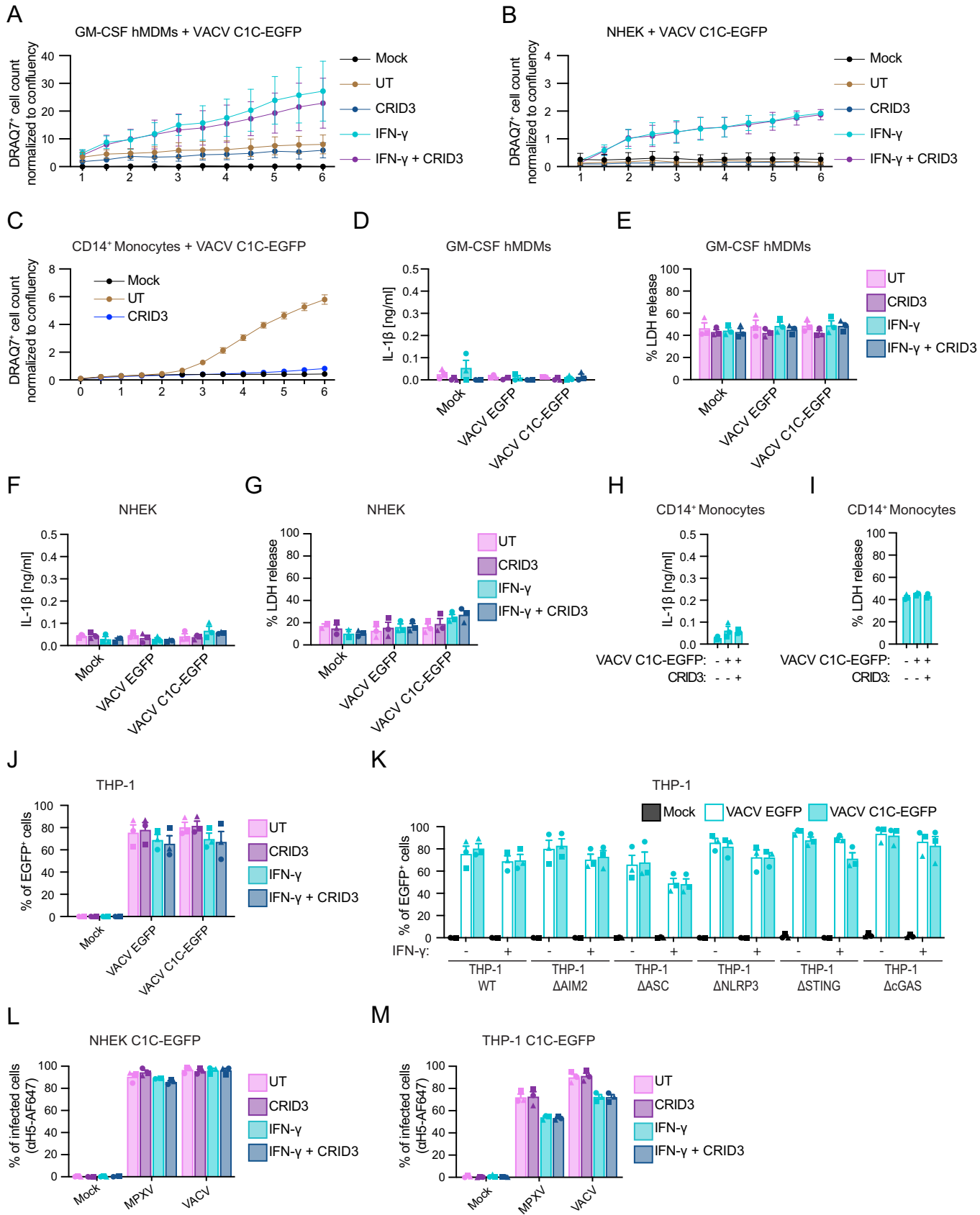


Figure EV1. VACV WT and VACV C1C-EGFP induce comparable cell death and IL-1 β secretion.

(A) PMA-differentiated THP-1 cells were either left untreated or pretreated overnight with 500 U/mL IFN- γ and infected with the indicated viruses at an MOI of 5. Six hours post infection, IL-1 β in the supernatant was measured by homogeneous time-resolved fluorescence (HTRF), and cell death was measured by LDH release, normalized to cells lysed in 1% Triton X-100. Data represent values with individual data points from $N = 3$ independent experiments $\pm$ SEM.



◀ **Figure EV2. Poxvirus infection induces inflammasome activation in primary human cell types.**

(A–I) Human monocyte-derived macrophages (hMDMs) differentiated in GM-CSF (A, D, E), normal human epidermal keratinocytes (NHEK) (B, F, G), or human primary CD14⁺ monocytes (C, H, I), were left untreated or pretreated with IFN- γ overnight. Cells were infected with VACV C1C-EGFP at an MOI of 5 (A, B) or MOI 10 (C), in the absence of VX, and where indicated in the presence of 2.5 μ M CRID3. Membrane permeabilization was quantified by the uptake of DNA dye DRAQ7 over 6 h in an Incucyte Live-Cell Imaging system (A–C). DRAQ7 uptake normalized to cell confluency are shown. IL-1 β in the supernatant was measured by homogeneous time resolved fluorescence (HTRF) (D, F, H). Cell death was measured by LDH release and normalized to cells lysed in 1% Triton X-100 (E, G, I). (J, K) PMA-differentiated WT (J) or indicated knockout THP-1 cells (K) were left untreated or pretreated with IFN- γ overnight and infected with indicated VACV strains in the presence of VX, and where indicated, CRID3. Infection levels are displayed here; specking data from the same experiments are shown in Fig. 2J, K. (L, M) NHEK (L) and PMA-differentiated THP-1 cells (M) constitutively expressing C1C-EGFP were left untreated or pretreated with IFN- γ overnight and infected with VACV or monkeypox virus (MPXV) at an MOI of 5 in the presence of VX, and where indicated, CRID3. Cells were fixed 6 h post infection and stained with rabbit polyclonal anti-H5 serum and AF647-labeled goat anti-rabbit IgG antibodies. Infected cells (EGFP⁺) were analyzed in infected or mock-treated cells by flow cytometry as described before. Infection levels are displayed here; specking data from the same experiments are displayed in Fig. 2L, M. Data represent average values (with individual data points) from $N = 3$ independent donors (primary cells) $\pm$ SEM, or from $N = 3$ independent experiments $\pm$ SEM (THP-1 cells).

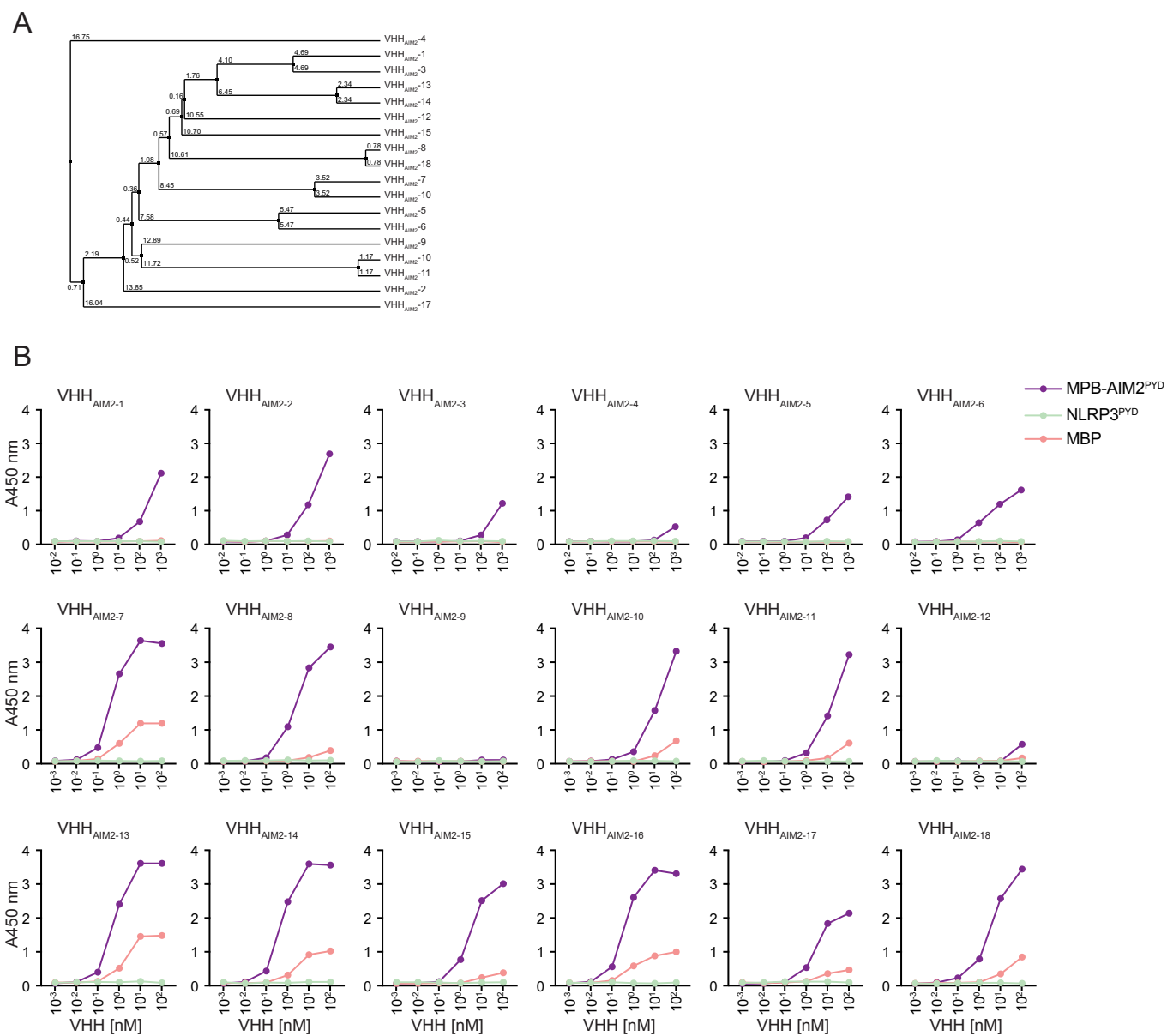


Figure EV3. Identification and characterization of AIM2 nanobodies.

(A) Average distance tree calculated based on the percentage identity between the indicated nanobody sequences displayed in Fig. 3A. (B) Binding of the indicated recombinant HA-tagged nanobodies to MBP-AIM2^{PYD}, NLRP3^{PYD}, or MBP was quantified by ELISA as described in Fig. 3B. The full data set with nanobody concentrations from 1000 nM to 1 pM (VHH_{AIM2-1} - VHH_{AIM2-6}) and 100 nM to 1 pM (VHH_{AIM2-7} - VHH_{AIM2-18}) in tenfold dilutions is shown. Data represent average values from N = 3 independent experiments.

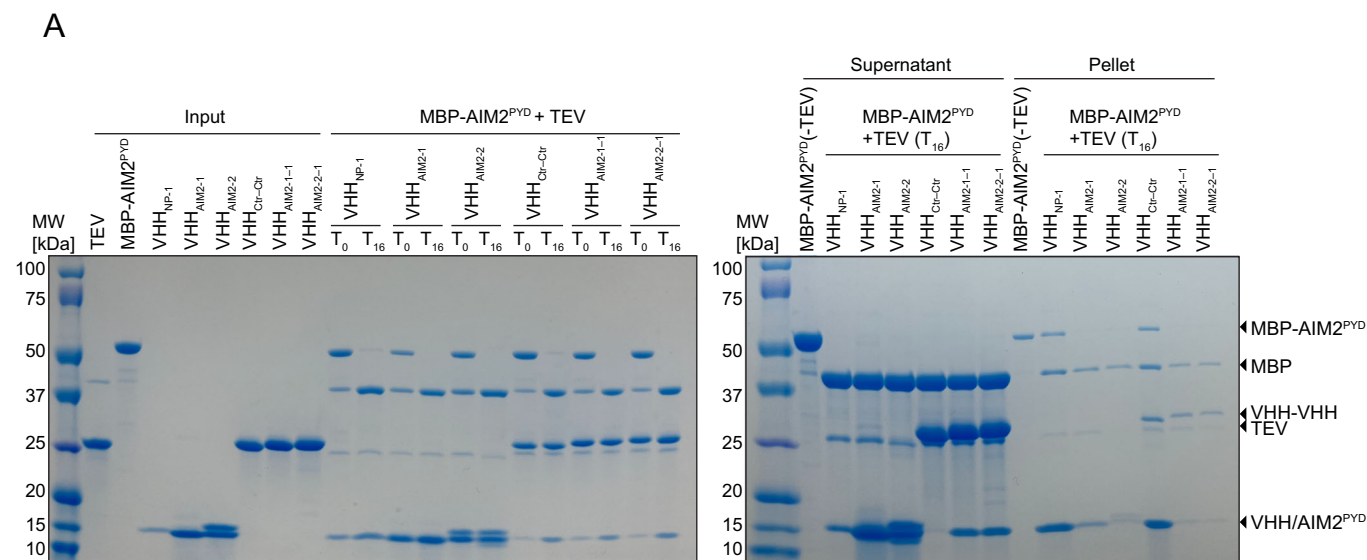


Figure EV4. AIM2 nanobodies inhibit AIM2^{PYD} oligomerization and filament formation.

(A) 100 μ g of MBP-AIM2^{PYD} (34.4 μ M) was incubated with TEV protease at RT for 16 h in the presence of 75 μ g of monovalent (103.2 μ M) or bivalent (51.6 μ M) control or AIM2-inhibitory nanobodies as indicated. 2 μ g of each input protein and samples before ($t = 0$ h, T₀) and after incubation ($t = 16$ h, T₁₆) were analyzed by SDS-PAGE and displayed on the left. AIM2^{PYD} filaments were sedimented at 20,000 $\times$ g, and samples of supernatants and pellets are shown on the right. Excerpts from the entire gels shown here are displayed in Fig. 4H. Control samples without TEV display that MBP-AIM2^{PYD} is almost completely found in the supernatant, with very little fusion protein in the pellet (and no processing in the absence of TEV is observed). Note that AIM2^{PYD} is exclusively found in the pellet fraction after release from MBP in the presence of bivalent control nanobodies. In the presence of bivalent AIM2 nanobodies, AIM2^{PYD} is mostly found in the supernatant, indicating that nanobody binding to AIM2^{PYD} prevents filament formation. Monovalent nanobodies cannot be distinguished from AIM2^{PYD} by SDS-PAGE (compare input nanobodies and pure AIM2^{PYD} released at T16 in the presence of bivalent nanobodies). In the presence of monovalent control nanobodies, the VHH/AIM2^{PYD} band in the pellet corresponds to the intensity of the AIM2^{PYD} band in the pellet in the presence of bivalent control nanobodies, indicating that the pellet mostly contains AIM2^{PYD}. In the presence of inhibitory AIM2 nanobodies, the band intensity of VHH/AIM2^{PYD} in the supernatant is drastically increased, while the VHH/AIM2^{PYD} band in the pellet is much weaker. Representative gels from at least three independent experiments are displayed.

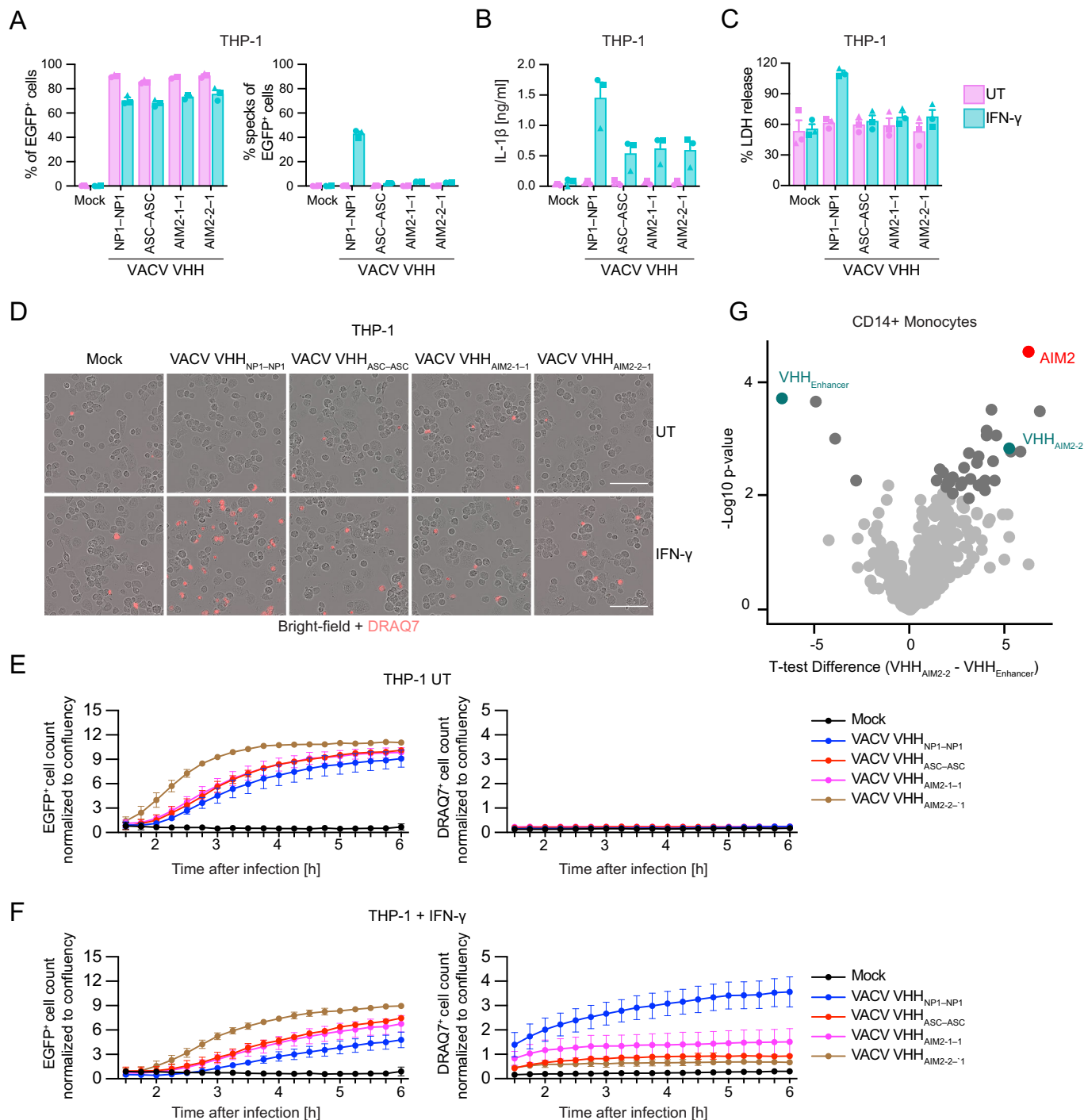


Figure EV5. VACV-encoded AIM2-inhibitory nanobodies abrogate inflammasome response in THP-1 macrophages.

(A–F) PMA-differentiated THP-1 cells were either left untreated or treated with IFN- γ overnight and infected with VACV (MOI 5) expressing C1C-EGFP and the indicated nanobodies for 6 h in the presence (A) or absence (B–F) of VX (see Fig. 5A, B for overview of virus strains). Cells were harvested, fixed, and infected, as well as ASC speck assembly were analyzed by flow cytometry as described before (A). IL-1 β in the supernatant was measured by HTRF (B) and cell death was measured by LDH release, normalized to cells lysed in 1% Triton X-100 (C). (D–F) DRAQ7 uptake was monitored over 6 h in an Incucyte live-cell imaging system. Representative images after 6 h (D), and graphs showing infection (EGFP⁺ cells) and DRAQ7 uptake normalized to confluency in untreated (E), and IFN- γ -treated (F) cells over 6 h are displayed. Scale bar = 100 μ m. Data represent average values (with individual data points) from $N = 3$ independent experiments $\pm$ SEM (A–C). Graphs represent average values from $N = 3$ independent experiments $\pm$ SEM (E, F). (G) CD14⁺ monocytes were treated with IFN- γ overnight and proteins were immunoprecipitated with Sepharose beads covalently coupled to VHH_{AIM2-2} or VHH_{Enhancer}, an EGFP-specific control nanobody. Enriched proteins are displayed in a volcano plot, demonstrating specific enrichment of AIM2 from IFN- γ -treated CD14⁺ monocytes with VHH_{AIM2-2}, but not VHH_{Enhancer}.