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Priming and activation of inflammasome by canarypox virus vector ALVAC via the cGAS/IFI16-STING-Type I IFN pathway and AIM2 sensor

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Abstract

Viral vectors derived from different virus families, including poxvirus (canarypox virus vector ALVAC) and adenovirus (human Ad5 vector), have been widely used in vaccine development for a range of human diseases including HIV/AIDS. Less is known about mechanisms underlying host innate response to these vectors. Increasing evidence from clinical vaccine trials testing different viral vectors has suggested the importance of understanding basic elements of host-viral vector interactions. In this study, we investigated the innate interactions of antigen presenting cells (APCs) with two commonly used HIV vaccine vectors, ALVAC and Ad5, and herein reported identification of AIM2 as an innate sensor for ALVAC triggering strong inflammasome activation in both human and mouse APCs. Microarray and comprehensive gene knockout analyses (CRISPR/Cas9) identified that ALVAC stimulated the cGAS/IFI16-STING-Type I IFN pathway to prime AIM2, which was functionally required for ALVAC-induced inflammasome activation. We also provided evidence that different from ALVAC, Ad5 vector itself was unable to induce inflammasome activation, which was related to its inability to stimulate STING-Type I IFN pathway and to provide inflammasome priming signals. In pre-conditioned APCs, Ad5 vector could stimulate inflammasome activation through AIM2-independent mechanism. Therefore, our study identifies AIM2 inflammasome and cGAS/IFI16-STING-Type I IFN pathway as novel mechanism for host innate immunity to ALVAC vaccine vector.

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Introduction

Viral vectors serve as an important antigen delivery platform and are widely used in vaccine development (1). Major advantages of using viral vectors as vaccine platform include their ability to infect broad range of host, to induce high levels of transgene expression and to stimulate both humoral and cellular immune responses (2, 3). Like their parental viruses, recombinant viral vectors contain pathogen-associated molecular patterns (PAMPs) to inherently stimulate innate immune responses, and therefore can confer intrinsic adjuvant effects to enhance vaccine-induced immunity (4). Due to these attractive properties, viral vectors have been widely employed in vaccine development for a range of human diseases, including HIV/AIDS and several types of cancers (1).

To date, a number of viral vectors for HIV vaccines have been developed, including those derived from adenovirus (5, 6), poxvirus (7, 8) and herpesvirus (9), among which the canarypox virus vector ALVAC and the human Ad5 vector were tested in late-stage human trials (10–12). Indeed, over the past decade or so, efficacy studies testing HIV vaccine regimens involving these different viral vectors have reported some unanticipated and distinct outcomes (10–12), which have underscored the importance to understand basic mechanisms for host innate immune response to these viral vectors. However, our current knowledge in host innate recognition of ALVAC as compared to Ad5 vector is limited. In response to pathogenic infections, mammalian hosts can detect a range of microbial components as danger signals (PAMPs) by a group of innate receptors, called pattern recognition receptors (PRRs), to trigger host defense responses (13). In the context of virally vectored vaccination, such innate recognition of vector-associated PAMPs by host APCs occurs as well, but has not been well explored. Prior studies have demonstrated that ALVAC could efficiently infect human dendritic cells (DCs) (14) and induced strong antiviral response (15, 16). Similarly, human Ad5 vectors were also shown to be able to infect a range of cell types including APCs (17). However, innate receptors or immune pathways that may differentially regulate the interactions of host APCs with ALVAC as compared to Ad5 vector remain less clear.

During viral vector immunization, various components of the vectors, including genomic DNA, can serve as potential PAMPs that could be sensed by host PRRs to trigger strong innate responses. A broad set of PPRs that recognize foreign DNAs have been identified, among which inflammasomes, a cytosolic protein complex, can induce strong pro-inflammatory responses upon activation. Inflammasome activation can trigger rapid conversion of pro-caspase zymogens into active proteases, resulting in a process of inflammatory cell death, called pyroptosis (18, 19). Importantly, activation of inflammasome pathway also leads to production of pro-inflammatory cytokines (IL-1β & IL-18) and has been shown to modulate host adaptive immunity (18, 19). Several canonical inflammasome sensors have been identified, including the NLR (nucleotide-binding domain and leucine-rich repeat-containing) proteins and the AIM2-like receptors (18).

In this study, we investigated interactions of host APCs with ALVAC and Ad5 vector, attempting to identify the innate receptors or immune pathways that differentially regulate

host recognition of these two important vectors. Using a combination of transcriptomics, cellular and comprehensive gene-editing (CRISPR/Cas9) approaches, we identified AIM2 as an innate sensor for ALVAC, triggering strong inflammasome activation and pyroptosis in both human and mouse DCs. In addition to inflammasome activation, we found that ALVAC could stimulate the cGAS/IFI16-STING-type I IFN pathway to prime AIM2, which was functionally required for the subsequent inflamasome activation and cell pytoptosis. Different from ALVAC, Ad5 vector itself was unable to trigger inflammasome activation. Our study provided evidence that failure of Ad5 vector alone to induce inflammasome activation was related to its inability to prime inflammasome. In LPS or MPLA-primed APCs, Ad5 vector could induce inflammasome activation through AIM2-independent mechanisms.

Materials and Methods

Ethics Statement

The study involves use of PBMC samples from healthy human donors (5 females and 3 males). PBMCs were obtained from the University of Texas Medical Branch (UTMB) blood bank. All samples were analyzed anonymously. The study was determined as non-human subject research and approved by UTMB's IRBs. Written informed consents were obtained from study participants. The study also involves use of mouse bone marrow cells for generation of mouse dendritic cells and macrophages. The Institutional Animal Care and Use Committee (IACUC) at UTMB approved the animal work. Mice were maintained under specific-pathogen-free (SPF) conditions in the animal facility of UTMB and were used when 6–8 weeks old. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cells and viral vectors

Mouse bone marrow from male AIM2$^{-/-}$ on the C57BL/6 background (20) (obtained from Dr. Thirumala-Devi Kanneganti) and WT (Stock No. 000664) C57BL/6 mice were used for generation of mouse BMDCs and BMDMs. Mouse bone marrow from female AIM2$^{-/-}$ mice on the C57BL/6J background (B6.129P2-Aim2$^{Gt(CSG445)Byg}$/J; Stock No. 013144) were used for generation of mouse BMDCs. AIM2$^{-/-}$ mice on the C57BL/6J background and WT mice were purchased from the Jackson Laboratory. THP-1 cells were maintained in PRMI 1640 medium (Gibco) containing 10% FBS. 293T cells were maintained in DMEM medium containing 10%FBS. Recombinant human Ad5 empty vector (Ad5) and Ad5 encoding GFP (Ad5GFP) (ΔE1/E2/E3) were obtained Vaccine Research Center of NIH. ALVAC vector was obtained from Sanofi Pasteur. Heat-inactivated viral vectors were generated by incubating vectors at 95°C for 2 hours.

Generation of human MDDCs, and mouse BMDCs and BMDMs

Human immature MDDCs (iMDDCs) were generated as in previous study (21). In brief, monocytes were positively selected from PBMCs using the Easy-Sep™ Human CD14 Positive Selection Kit II (Stemcell). Monocytes were seeded at 10^6 cells/ml in complete RPMI 1640 medium (Gibco) containing 20 ng/ml recombinant human IL-4 (Life Technologies) and 20 ng/ml recombinant human GM-CSF (Life Technologies) for 5 days in

culture plates. Fresh medium was added at day 3 of culture and iMDDCs were generally used at day 5. Mouse bone marrow-derived dendritic cells (BMDCs) were generated as previously described (22). Mouse bone marrow was isolated from femurs and tibia, and then cultured in 6-well plates at 10^6 cells/ml in complete RPMI 1640 medium containing 20 ng/ml recombinant mouse IL-4 (Life Technologies) and 20 ng/ml recombinant mouse GM-CSF (Life Technologies). Mouse bone marrow-derived macrophages (BMDMs) were generated as previously described (23). Bone marrow cultured in 6-well plates at 10^6 cells/ml in complete RPMI 1640 medium containing 20 ng/ml recombinant mouse M-CSF (Life Technologies). New medium was added at day 3 and BMDCs and BMDMs were used on day 5.

Viral vector infection of MDDC or THP-1 cells

MDDCs or BMDCs (1×10^5) were infected in with live or heated-inactivated Ad5 or ALVAC vector at various concentrations depending different experiments (ALVAC MOI: 1–3/cell; Ad5 viral particles: 3–200/cell). For THP-1, 5×10^4 cells were infected with vectors. 24 hours after infection, efficiency of vector infections in MDDC or THP-1 cells was validated by PCR measurement of vector DNA in cells. Vector-specific primers for PCR amplification were listed in Table 1. 48 hours after infection, cells were also measured for viability by immune staining and flow cytometer. Cells were first stained with LIVE/DEAD® Fixable Aqua (Life Technologies) and then stained for DC maturation markers, including CD83-PE-Dazzle 594 (Biolegend), CD86-PE-Cy5 (eBioscience) and HLA-DR-FITC (BD Biosciences). Cells were acquired on an LSRII Fortessa.

Caspase-1 and caspase-3 activity analysis

Caspase activation assay was performed using FAM-FLICA™ Caspase Assay Kits following the manufacturer's instructions. Briefly, two days after viral vector infection, culture medium was replaced with fresh medium containing FAM-FLICA™ Caspase-1 (FAM-FLICA™ Caspase-1 Assay Kit, ImmunoChemistry Technologies) or FAM-FLICA™ Caspase-3 (FAM-FLICA™ Caspase-3 Assay Kit, ImmunoChemistry Technologies) at 37 °C for one hour. Cells were washed twice with Apoptosis Wash Buffer provided with the kit and then acquired on an LSR-II flow cytometer.

Microarray

MDDCs (1×10^6) generated from 3 healthy donor PBMCs were infected with Ad5 or ALVAC vector (MOI=3) for 24 hours, followed by cellular RNA extraction and microarray analysis. Microarray was conducted using the HumanHT-12 v4 Expression Bead-Chip (Illumina) at the UT MD Anderson Sequencing and Microarray Core Facility. Microarray data analysis was conducted using the R Bio-conductor software package as previously described. The data have been deposited in NCBI's Gene Expression Omnibus (24) and are accessible through GEO Series accession number GSE103204 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103204). To analyze the role of interferon in gene regulation induced by viral infection, Interferome v2.01 (http://www.interferome.org/interferome/home.jspx) (25), an online database of IFN-inducible genes, was used to identify IFN-related genes and evaluate the weight of different type of IFN (Type I, II and III) on the gene expression induced by viral infection in this study.

Real-time PCR for gene expression

Total RNA was extracted from viral vector-infected BMDCs or THP-1 cells collected (24 hours post-infection) using Quick-RNA MicroPrep kit (Zymo) according to the manufacturer's protocol. Gene expression was quantified using iTaq Universal SYBR Green Supermix (Bio-rad) and the CFX Connect Real-Time PCR Detection System (Bio-rad) after reverse transcription from RNA into cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-rad). Primer sequences for quantification of gene expression were shown in Table 2. The relative quantity of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Gene editing by CRISPR/Cas9

Gene editing/knockout (KO) in THP-1 cells by CRISPR/Cas9 was conducted as previously described with modifications (26). In brief, gene-specific guide sequences (Table 3) were designed to target exons using the online tool (http://crispr.mit.edu/) and were cloned into Lenti-CRISPR v2 vector (a gift from Dr. Feng Zhang; Addgene plasmid # 52961) (27). The constructed plasmids or empty Lenti-CRISPR V2 plasmid, together with the Lentivirus packaging plasmids, psPAX2 (a gift from Didier Trono; Addgene plasmid # 12260) and pCMV-VSV-G (a gift from Bob Weinberg; Addgene plasmid # 8454) (28), were used to co-transfect 293T cells for producing Lentivirus. Packaged lentivirus were then used to transduce THP-1 cells. Gene knockout and the knockout control THP-1 cells were selected and maintained in complete RPMI 1640 medium supplemented with 2 μg/ml puromycin. To validate the efficacy of CRISPR/Cas9-mediated gene knockout, genomic DNA was extracted from selected stable THP-1 and targeted cleavage was measured though T7 enzyme-1 assay as previously described (29), after PCR amplification spanning the targeted cleavage sites. Primer sequences for PCR amplification are listed in Table 4. Furthermore, the PCR product was also clone into pGEM®-T vector (Promega) for sequencing to confirm gene editing in THP-1 cells.

Western blot

Efficacy for CRISPR/Cas9-mediated ablation of AIM2, caspase-1, STING, cGAS and IFI16 proteins in human THP-1 cells was evaluated by western blot. For AIM2 detection, control or AIM2 KO THP-1 cells were treated with human IFN-γ (75ng/ml, R&D System) or infected with Ad5 or ALVAC vector for 24 hours, followed by cell lysis in RIPA buffer (Cell Signaling). For detection of caspase-1, STING, cGAS and IFI16, control and KO THP-1 cells are directly lysed in RIPA buffer (Cell Signaling). Cell lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-rad). After blocking in 5% non-fat milk (TBS+0.1% Tween 20), membranes were incubated with anti-AIM2 antibody (1:500, Cell Signaling), anti-caspase-1 antibody (1:1000, Thermo Scientific), anti-STING antibody (1:1000, Cell Signaling), anti-cGAS (1:1000, Cell signaling), or anti-IFI16 (1:1000, Thermo Scientific) at 4°C overnight and developed with Rabbit IgG Horseradish Peroxidase-conjugated Antibody (1:1000, Cell Signaling) or mouse IgG Horseradish Peroxidase-conjugated Antibody (1:1000, Cell Signaling) and SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific). β-actin was also detected as control, using anti-β-actin antibody (1: 50,000, R&D System) for incubation at room temperature for 1

hour and then mouse IgG Horseradish Peroxidase-conjugated Antibody (1:3000, Cell Signaling) after striping.

Quantification of cytokine production in culture supernatants of vector-infected cells

Production of human IL-1β and IL-18 by vector-infected MDDCs or THP-1 cells was quantified using the LEGENDplex assay (Biolegend) following the manufacturer's instruction. Briefly, cell-free supernatant was collected 48 hours post-infection and then incubated with Legendplex beads for 2 hours at room temperature on a plate shaker, followed by incubation with detection antibodies for 1 hour. After incubating with SA-PE for 30 min, beads were acquired on an LSR-II flow cytometer. Raw data were analyzed using the LEGENDplex™ software (Biolegend). Mouse IL-1β and IL-18 in supernatants of vector-infected mouse BMDCs were measured using the Mouse IL-1β ELISA MAX™ Deluxe (Biolegend) and Mouse IL-18 Platinum ELISA (eBioscience) according to the manufacturer's instruction.

Measurement of intracellular IFN-β by flow cytometry

Twenty-four hours after viral vector infection, MDDCs and THP-1 were stained with FITC conjugated anti-human IFN-β antibody (PBL Assay Science) after incubating with protein transport inhibitor (BD Biosciences) at 37°C for 5 hours as well as fixation/ permeabilization. Cells were acquired on an LSRII Fortessa flow.

Type I IFN blockade

To study the role of type I IFN in ALVAC-induced gene up-regulation, MDDCs, THP-1 and BMDCs were infected with ALVAC in the presence or absence of anti-human IFNAR2 (Thermo Scientific) or anti-mouse IFNAR1 (EMD Millipore) antibodies, to block type I IFN signaling. Cells were collected 24 hours post infection for real-time PCR quantification for target gene expression.

Ad5-induced inflammasome activation assay

WT (Ctrl) or KO THP-1 cells were first treated with LPS (100 ng/ml, Sigma-Aldrich) or MPLA Synthetic VacciGrade™ (2.5 μg/ml, Invivogen) for 6 hours or human IFN-γ (75ng/ml, R&D System) for 24 hours, followed by Ad5 infection (1200 viral particle/cell). Two days after infection, cells were collected for caspase-1 analysis.

Statistical analysis

Statistical analysis was performed using Prism 6.0 (Graph-Pad). Statistical comparison between groups was performed using paired or non-paired t test. Two-tailed p values were denoted, and p values < 0.05 were considered significant. Quantitative data displayed in all figures are expressed as means ± SD (represented as error bars)

Results

ALVAC induces maturation and significant cell death of human MDDCs as compared to Ad5 vector

To model the interactions between viral vectors and host APCs, human monocyte-derived dendritic cells (MDDCs) were generated from PBMC of healthy donors and infected with empty ALVAC or Ad5 vector. Effects of vectors on DC maturation was assessed by flow cytometry based on expression of CD83, CD86 and HLA-DR. We found that unlike Ad5 vector, which exhibited negligible effect on DCs, ALVAC induced substantial DC maturation as indicated by enhanced expression of maturation markers (n=8; ALVAC vs. Ad5: $p < 0.001$) (Fig. 1A). To preclude the possibility that minimum stimulatory effect of Ad5 on DCs was due to limited amounts of Ad5 vectors used, we infected MDDCs with Ad5 of high concentrations for longer time (>72 hours) and still found no DC maturation (Fig. S1A). The inability of Ad5 vector to stimulate DC maturation was consistent with some previous reports (30). Both ALVAC and Ad5 vector DNAs were detected in cells, indicating that both vectors could efficiently transduce MDDCs (Fig. S1B). Efficient transduction by Ad5 vector was also confirmed by transgene GFP expression in MDDCs after Ad5-GFP infection (Fig. S1C). These results suggest that ALVAC selectively induces strong DC maturation.

In addition to DC maturation, we noted that different from Ad5 vector, ALVAC induced substantial MDDC cell death (viability: ALVAC vs. Ad5 vs. mock: 53.3%, 84.6%, 87.9%) ($p < 0.0001$) (Fig. 1B). We also found that only live, but not heat-inactivated, ALVAC significantly induced DC maturation (Fig. S2A) and cell death (Fig. S2C), suggesting that active vector infection was required in this process. As controls, no significant difference was observed in induction of DC maturation or cell death by live or heat-inactivated Ad5 vectors (Fig. S2B–S2D).

ALVAC induces inflammasome activation and pyroptosis in human and mouse DCs

To understand mechanisms underlying differential effects of ALVAC and Ad5 vector on DCs, we conducted microarray analysis for vector-infected MDDCs and identified that ALVAC and Ad5 induced distinct gene expression profiles in MDDCs (Fig. 2A and 2B), with substantially larger number of genes that were up-regulated in ALVAC-infected DCs as compared to Ad5-infected DCs (Fig. 2A). Of interest, further analysis indicated that ALVAC selectively induced pro-inflammatory pyroptosis, but not apoptosis, of MDDCs, as evidenced by up-regulation of caspase-1 gene (CASP1) ($p<0.001$), but not other caspase members that are associated apoptosis (Fig. 2C). In addition to gene expression, we evaluated caspase activities in vector-infected MDDCs using FLICA probes (31) and identified that only active caspase-1 (Fig. 2D), but not caspase-3 (Fig. 2E), was detected in ALVAC-infected MDDCs. In contrast, little caspase activity was detected in Ad5-infected DCs (Fig. 2D and 2E). Altogether these data suggest that ALVAC, but not Ad5 vector, induces pro-inflammatory cell pyroptosis, but not apoptosis, in human MDDCs.

Caspase-1-dependent pyroptosis is usually triggered by inflammasome activation, leading to production of mature IL-1β and IL-18. We quantified human IL-1β and IL-18 in supernatants of vector-infected MDDCs and found that compared to Ad5- or mock-treated

DCs, ALVAC-infected DCs produced markedly larger amounts of IL-1β (Fig. 2E, $p = 0.006$) and IL-18 (Fig. 2F, $p = 0.02$). These data strongly suggest that ALVAC induced inflammasome activation in human MDDCs.

To explore if ALVAC also induces inflammasome activation in mouse, bone-barrow derived dendritic cells (BMDCs) from wild-type (WT) mice were infected with vectors. Highly consistent with human MDDCs, ALVAC, but not Ad5, also stimulated strong inflammasome activation and pryoptosis in mouse BMDCs, as evidenced by enhanced mouse caspase-1 activity (Fig. 2G), increased expression of inflammasome genes (*mCASP1, mIL-1β* and *mIL-18*) (Fig. 2H) and production of mature IL-1β and IL-18 (Fig. 2I). Accordingly, these data suggest that ALVAC stimulates inflammasome activation and cell pryoptosis in both human and mouse DCs.

To identify the innate sensor that mediates ALVAC-induced inflammasome activation, microarray analysis identified that among all the known inflammasome sensors, only AIM2 was significantly up-regulated for expression in ALVAC-infected DCs as compared to Ad5-infected DCs (fold change >8; $p = 0.000008$); no significant changes in expression for other inflammasome sensors, including NLRC4, NLRP1 and NLPR3, were observed (Fig. 2J). Importantly, we confirmed AIM2 protein expression in ALVAC-infected cells by western blot, while no AIM2 protein was detected in mock- or Ad5-infected cells (Fig. 2K). These data suggest that ALVAC could selectively stimulate priming of AIM2 inflammasome in DCs.

Generation of AIM2 KO THP-1 cells using the CRISPR/Cas9 approach

We next aimed to determine the functional role of AIM2 in ALVAC-induced inflammasome activation. THP-1 cells, a human monocyte-like cell line that is widely used in dissecting innate immune pathways (32), were used for AIM2 genetic manipulation using the CRISPR/Cas9 system. First, we evaluated the feasibility and relevance of THP-1 cells for this purpose by measuring induction of inflammasome activation in these cells following vector infection. We showed that similar to human and mouse DCs, infection of THP-1 cells with ALVAC, but not Ad5 vector, led to significant cell death (Fig. 3A). Importantly, ALVAC also induced remarkable inflammasome activation and cell pyroptosis in THP-1, as evidenced by enhanced caspase-1 activity (Fig. 3B), higher levels of inflammasome gene expression (*AIM2, CASP1 and IL-1β*) (Fig. 3C) and production of mature IL-1β and IL-18 (Fig. 3D). Of note, ALVAC infection did not enhance IL-18 mRNA levels in THP-1 cells (Fig. 3C), which is different from that in ALVAC-infected mouse BMDCs (Fig. 2H); however, ALVAC was able to enhance mature IL-18 secretion (Fig. 3D), suggesting that in THP-1 cells, ALVAC infection does not significantly affect pro-IL-18 priming but promote its cleavage. This data is consistent with the observation using STING-KO THP-1 cells, which will be presented later (Fig. 5O). Collectively, these data suggest that THP-1 cells can serve as a feasible model to study vector-induced inflammasome activation.

Genetic editing of AIM2 by the CRISPR/Cas9 system was conducted as previously described (29) (Fig. 3E). We validated the editing efficacy and showed that on-target AIM2 mutation were successfully introduced by the CRISPR/Cas9 based on the T7 endonuclease I (T7E1) cleavage assay (Fig. 3F). In addition, sequencing analysis confirmed efficient AIM2

editing with deletion of two nucleotides, resulting in formation of an immature stop codon (TAA) shortly downstream of mutant (Fig. 3G). Lastly, we verified efficient ablation of AIM2 protein expression in ALVAC-infected, AIM2-KO THP-1 cells by western blot (Fig. 3H).

AIM2 is functionally required for ALVAC-induced inflammasome activation and pyroptosis

We next aimed to determine the functional role of AIM2 in ALVAC-induced inflammasome activation and cell pyroptosis using the AIM2 KO or CRISPR/Cas9 internal control (Ctrl) THP-1 cells generated above. We found that while ALVAC induced significant cell death in the Ctrl THP-1 cells as anticipated (viability: 24.6%), such effect by ALVAC on cell death was substantially inhibited in the AIM2 KO cells (viability: 69.1%) ($p = 0.02$) (Fig. 4A). As controls, no significant change in cell viability was observed between AIM2 KO and Ctrl THP-1 cells when infected with Ad5 vector (Fig. 4A).

We then evaluated the impact of AIM2 KO on ALVAC-induced activation of inflammasome pathway in THP-1 cells. Consistent with the observation for cell death, AIM2 KO almost completely abrogated caspase-1 activity in ALVAC-infected cells (Fig. 4B). This finding, together with our microarray data showing that AIM2 was the only inflammasome sensor up-regulated by ALVAC (Fig. 2J), indicates that AIM2 is the predominant inflammasome sensor for ALVAC. Lastly, we quantified human mature IL-1β and IL-18 cytokines produced by AIM2 KO or Ctrl THP-1 cells following infection and found that AIM2 KO also led to substantial reduction in IL-1β ($p = 0.0067$) and IL-18 ($p = 0.0242$) compared to the Ctrl THP-1 cells (Fig. 4C). Of importance, AIM2 KO did not significantly change the transcription of IL-1β and IL-18 induced by ALVAC (Fig. S3A), suggesting that AIM2 KO does not affect priming of inflammasome pathway by ALVAC.

To further verify the functional role of AIM2, we assessed ALVAC infection in BMDCs that were generated from *AIM2*$^{-/-}$ mice on the C57BL/6J background as compared to those from the matched WT mice. Consistent with the findings for THP-1 cells, AIM2 KO in mouse BMDCs almost completely abrogated ALVAC-induced cell death (viability for *AIM2*$^{-/-}$ and WT: 36.2% vs. 75.4%) (Fig. 4D) as well as the caspase-1 activity (Fig. 4E). Quantification of cytokines showed that production of mature mouse IL-1β and IL-18 were also almost completely abrogated in *AIM2*$^{-/-}$ BMDCs as compared to the WT BMDCs (Fig. 4F). Also, AIM2 KO in mouse BMDCs did not significantly change the transcription of mouse IL-1β and IL-18 induced by ALVAC in these cells (Fig. S3B), suggesting that AIM2 KO did not affect inflammasome priming by ALVAC in mouse BMDCs. In addition, recent studies reported that targeted mutagenesis in mice is prone to introduce potential passenger mutations in genetically modified mice (33). To further confirm the above observations, we used AIM2−/− mice generated on the C57BL/6 genetic background (20). Highly consistent with the results from AIM2−/− on mixed background, AIM2 deficiency also abrogated IL-1β production in ALVAC-infected BMDCs and BMDMs (bone marrow-derived macrophages) from AIM2−/− C57BL/6 mice (Fig. 4G). Taken together, these data provide strong evidence that AIM2 is functionally required for inflammasome activation and pyroptosis induced by ALVAC in both human and mouse cells.

ALVAC stimulates priming of AIM2 via the cGAS/IFI16-STING-Type I IFN pathway

Inflammasome activation usually requires two signals, including the priming signal to up-regulate inflammasome-associated genes and a second signal that directly activates inflammasomes through the up-regulated sensor molecules (34). Our data have shown that AIM2 is a sensor for ALVAC triggering inflammasome activation, but also importantly, ALVAC infection can provide priming signal to up-regulate AIM2 (Fig. 2J and 2K). It remains unclear as to how ALVAC primes AIM2. AIM2 is an interferon (IFN)-inducible protein and can be induced by type I and type II IFNs (35, 36). To evaluate which type of IFN response was induced by ALVAC, we used the Interferome database (25) to analyze the category of genes in the microarray data. Among all the up-regulated genes (n = 622; fold changes > 2), the majority (579 genes) belongs to those induced by Type I IFNs (Fig. 5A). To confirm the bioinformatics analysis result, we assessed type I IFN production in vector-infected cells and found that ALVAC infection caused substantial increase in expression and production of IFN-β in human DCs (Fig. 5B), mouse DCs (Fig. 5C) as well as in THP-1 cells (Fig. 5D). In contrast, Ad5 vector infection did not stimulate IFN-β production (Fig. 5B–D). This result, together with the Interferome database analysis, showed that unlike Ad5 vector, ALVAC selectively induced strong type I IFN response in APCs.

To determine if Type I IFN signaling is required for ALVAC-induced AIM2 up-regulation, we used antibodies specific to human and mouse Type I IFN receptor (IFNAR), respectively, to block the signaling. We found that that IFNAR blockade significantly reduced AIM2 expression in all the tested cell types, including human MDDCs, mouse BMDCs and THP-1 cells (Fig. 5E–G), suggesting that ALVAC-induced type I IFN production mediates AIM2 expression. Moreover, IFNAR blockade in ALVAC-infected THP-1 also decreased caspase-1 activity (Fig. 5H), suggesting that type-I IFN blockade also inhibits inflammasome activation.

STING is an important adaptor molecule in type I IFN response to cytosolic DNA (37, 38). Given that ALVAC is a DNA virus, we next determined if ALVAC-induced type I IFN response relied on STING signaling. We generated two STING KO THP-1 cell lines (Fig. 5I) using the CRISPR/Cas9 system, as described for the generation of AIM2 KO THP-1 cell lines. Indeed, we found that STING was functionally required for ALVAC-stimulated IFN-β transcription and production (Fig. 5J–K) as well as for AIM2 expression (Fig. 5L). Together with the IFNAR blocking analysis, these data suggest that ALVAC primes AIM2 expression through the STING-Type I IFN pathway.

Next we explored the role of STING-Type I IFN pathway in ALVAC-induced AIM2 inflammasome activation and cell pyroptosis. Importantly, we found that STING KO almost completely abrogated ALVAC-induced cell death (Fig. 5M), caspase-1 activation (Fig. 5N), pro-IL1β mRNA expression (Fig. 5O), and mature IL-1β secretion (Fig. 5P). Of note, we found that STING KO did not significantly affect pro-IL-18 mRNA level in ALVAC-infected THP-1 cells (Fig. 5O), which is consistent with the data that ALVAC infection itself had little effect on pro-IL-18 transcription in WT THP-1 cells, as shown earlier (Fig. 3C). Therefore, these data suggest that ALVAC primes AIM2 inflammasome via the STING-Type I IFN pathway, leading to AIM2 inflammasome activation and cell pyroptosis.

We next sought to identify upstream DNA sensors responsible for recognition of ALVAC. cGAS, IFI16 and DDX41 are known as STING-dependent sensors recognizing cytosolic DNA (39). We therefore used similar CRISPR/Cas9 methods and generated additional KO THP-1 cells deficient for cGAS KO, IFI16 KO (Fig. 5Q) and DDX41 KO (data not shown). Infection of these cells with ALVAC showed that both cGAS and IFI16 deficiency could significantly reduce the expression of IFN-β and AIM2 in infected THP-1 cells (Fig. 5R), suggesting that both sensors are functionally involved in recognition of ALVAC. This result is consistent with recent studies showing that cGAS and IFI16 cooperate to sense intracellular viral DNAs together (40, 41). Collectively, using a combination of antibody blockade and gene-editing approaches, we demonstrate that ALVAC stimulates cGAS/IFI16-STING-Type I IFN pathway, leading to priming of AIM2 for subsequent inflammasome activation by ALVAC.

Ad5 vector itself fails to induce inflammasome activation but can stimulate AIM2-independent inflammasome activation in primed cells

In an attempt to explore potential mechanisms why Ad5 vector itself failed to stimulate inflammasome activation in our system, we first found that Ad5 vector alone could not induce IFN-β production as shown above in Fig. 5B–D. Next, we tested Ad5 vector infection at high dose (1200 viral particle/cell) and showed still no induction of inflammasome activation, precluding the possibility that failure to induce inflammasome activation by Ad5 vector alone was simply due to limited vector doses (Fig. 6A). We then hypothesized that there might be lack of inflammasome priming signals stimulated by Ad5 vector infection. To test this, we pre-conditioned the THP-1 cells with LPS or IFN-γ, followed by Ad5 vector infection. Interestingly, priming THP-1 cells with LPS and IFN-γ both led to efficient induction of inflammasome activation by Ad5 vector, as evidenced by enhanced caspase-1 activity (Fig. 6B–C).

To determine whether certain adjuvants can act similarly to LPS or IFN-γ to provide appropriate priming signals and therefore might be able to modulate the Ad5 vector-stimulated innate inflammasome pathway, we used the adjuvant MPLA, a nontoxic derivative of the lipid A region of LPS (42). Interestingly, pretreatment of THP-1 cells with MPLA adjuvant also led to strong induction of inflammasome activation by Ad5 vector (Fig. 6D). These data suggest that the lack of early priming signals after Ad5 vector infection is an important mechanism explaining the failure of Ad5 vector alone to trigger inflammasome activation.

Lastly, we explored whether Ad5 vector-induced inflammasome activation in pre-conditioned THP-1 cells is mediated by AIM2. AIM2-KO THP-1 cells were used and compared with CRISPR/Cas9 empty vector control (Ctrl) THP-1 cells. We found that even in the absence of AIM2, Ad5 vector could still induce significant inflammasome activation (Fig. 6E), indicating that AIM2 may not be critically required and other inflammasome pathway may be involved. Furthermore, by using STING-KO THP-1 cells, we found that Ad5 vector could also stimulate inflammasome activation in LPS-primed cells in the absence of STING (Fig. 6E), which is not surprising given that priming signals required for inflammasome activation were provided by LPS conditioning. These data altogether suggest

that Ad5 vector can trigger inflammasome activation in LPS-primed THP-1 cells independent of AIM2.

Discussion

Recombinant viral vectors are an important platform for antigen delivery and have been widely used for vaccine development. Increasing evidence from vaccine studies has suggested the importance to understand molecular mechanisms that regulate host innate immunity to different viral vectors. The current study reports identification of novel innate receptors and pathways involved in recognition of canarypox virus vector ALVAC as compared to human Ad5 vector, two vectors widely used in vaccine development, by host APCs. Major discoveries are that different from Ad5 vector, which are less immune stimulatory on DCs, ALVAC induces priming of inflammasome pathway in DCs via cGAS/IFI16-STING-Type I IFN signaling, and that AIM2 is a predominant inflammasome sensor for ALVAC, triggering strong inflammasome activation and pro-inflammatory pyroptosis in both human and mouse DCs. Data of our study also suggest that failure of Ad5 vector alone to induce inflammasome activation is related to its inability to stimulate the STING/Type I IFN pathway, thereby failing to provide appropriate inflammasome priming signals in infected APCs. A schematic model for priming and activation of AIM2 inflammasome by ALVAC is summarized in Fig. 7.

Similar to pathogenic infections, the innate interactions between host and viral vectors are thought to occur following virally vectored vaccination. We here performed a comparative analysis for the effects of ALVAC and Ad5 vector on human and mouse DCs and our data showed that these two vectors manifested distinct innate immune stimulatory properties, based on the maturation status and cytokine production in DCs after vector transduction. ALVAC vector induced strong maturation of both human and mouse DCs, while Ad5 vector appeared to exert minimal effects (Fig. 1). This phenotype is also supported by our microarray analysis showing that significantly larger number of genes were up-regulated in DCs infected by ALVAC as compared to Ad5 vector (Fig. 2A). This finding is consistent with several previous studies reporting that human Ad5 virus or recombinant Ad5 vector alone is generally less innate immune stimulatory and induces very little activation of DCs (30); in contrast, ALVAC vector can more potently stimulate activation of APCs, leading to expression of antiviral gene expression (15) and production of inflammatory cytokines (16). Mechanisms for limited stimulatory activity of Ad5 vector on DCs are not fully known but may be related to the fact that Ad5 virus is a human pathogen and may evolve immune escape mechanisms from innate recognition by host defense responses. Indeed, we showed that different from ALVAC, Ad5 vector alone did not stimulate the STING/Type I IFN pathway in both human and mouse DCs, providing an explanation why Ad5 vector is less innate immune stimulatory and induces little activation of DCs.

A new class of PRRs that recognize foreign DNAs as danger signals to trigger inflammasome activation as a distinct host defense response from the conventional TLR-mediated pathways have been more recently identified (18). Compared to pathogenic infections, little has been explored for potential induction of inflammasome pathway in virally vectored vaccination. We identify that ALVAC vector selectively induces priming and

activation of inflammasome pathway, leading to pro-inflammatory cell pyroptosis and production of cytokines IL-1β and IL-18. Importantly, we show that AIM2 is the predominant sensor that mediates inflammasome activation pathway by ALVAC. We have provided the following evidence to support this: 1) ALVAC selectively induces AIM2 priming at both gene and protein levels (Fig. 2J–K); 2) no induction or priming of other inflammasome sensors (NLRC4, NLRP1 and NLRP3) by ALVAC; 3) ablation of AIM2 by CRISPR/Cas9 or use of cells from AIM2$^{-/-}$ mice (on both C57BL/6J and C57BL/6 background) abrogates activation of inflammasome pathway (Fig. 3–5). In our study, caspase-1 activity was measured as an indication of inflammasome activation, by using the FLICA staining method as reported in previous study (31). To validate this method, we further measured caspase-1 activation by immunoblotting and showed that ALVAC could induce active caspase-1 in the infected cells in a dose-dependent manner (Fig. S4A), supporting the specificity of FLICA staining to detect active caspase-1 in our system.

We noted that only ALVAC, but not Ad5, could stimulate transcriptional activation of inflammasome component AIM2 gene (priming), which is intriguing given that both ALVAC and Ad5 are DNA viruses with vector DNA being readily detectable in cells after transduction for both vectors. As part of our efforts in understanding why ALVAC selectively stimulates AIM2 inflammasome, we found that different from Ad5 vector, ALVAC could induce Type I IFN response in DCs. Further studies based on Type-I IFN blockade and gene-knockout analyses (STING, cGAS and IFI16) discovered that cGAS/IFI16-STING-Type I IFN pathway is functionally required for priming AIM2 inflammasome by ALVAC. Therefore, we speculate that the inability of Ad5 vector to trigger inflammasome activation was possibly due to the lack of priming signals, which is consistent with a recent study by Eichholz *et al* showing that Ad5 virus alone could not stimulate inflammasome activation in human MDDCs (43). This could be related to the intracellular trafficking and viral replication of Ad5 virus in infected cells. Ad5 usually enters target cells by endocytosis after binding to its cell surface receptor (coxsackievirus adenovirus receptor: CAR). After entry into cells, Ad5 virus can be transported to nuclear pore complex and release viral DNA to nucleus, thereby preventing efficient exposure of viral DNA to cytosol for innate stimulation. However, interestingly, we further identified that Ad5 vector alone could stimulate inflammasome activation in THP-1 cells when pre-conditioned with IFN-γ, or LPS or its adjuvant derivative MPLA (Fig. 6A–D), which appeared not to require AIM2 (Fig. 6E). We speculate that induction of inflammasome activation by Ad5 vector in primed THP-1 cells might be mediated by non-AIM2 inflammasome pathway. Indeed, other than AIM2, NLPR3 has also been shown to recognize adenovirus and trigger inflammasome activation (44, 45). It is possible that pre-conditioning of THP-1 cells can prime NLPR3 or other sensors, which mediate Ad5 vector-induced inflammasome activation in THP-1 cells. Nonetheless, much remains unknown about mechanisms underlying stimulation of inflammasome activation by Ad5 vector in the absence or presence of priming signals. Further investigation is warranted in future studies.

Stimulation of STING-Type I IFN axis can activate multiple downstream pathways. In addition to AIM2, type-I IFN signaling can induce expression of POP3 (human) and p202 (mouse), both of which can inhibit AIM2 inflammasome (46, 47). In our study, we found that ALVAC infection also induced enhanced expression of POP3 in THP-1 cells (Fig. S4B)

as well as p202 in mouse BMDCs (Fig. S4C), which appeared to involve the cGAS-STING-Type I IFN pathway (Fig. S4D–E). These data suggest that following ALVAC infection, co-induction of p202 or POP3 may provide a regulatory mechanism to attenuate inflammasome activation, leading to reduced pyroptosis at later times. It is also worth noting that AIM2 can negatively regulate IFN-β production (48, 49). We speculate that in addition to attenuating inflammasome activation, co-induction of POP3 and p202 by ALVAC infection may also indirectly promote Type-I IFN response through inhibiting AIM2, leading to a well-controlled feedback loop for STING-Type I IFN, AIM2 inflammasome and p202/POP3 pathways. A better understanding of the interactions between these pathways following ALVAC infection should be further pursued in future studies. Additional research should also be warranted to investigate the differences in molecular signatures of ALVAC and Ad5 vectors that contribute to their differential ability to stimulate STING-Type I-IFN pathway, inflammasome activation and DC maturation. Knowledge from these studies will help understand mechanisms for differential adjuvant effects of these vectors and provide novel insights into future vector design.

Cleavage of pro-IL-1β/18 into mature cytokines is a characteristic event following inflammasome activation. In our study, we showed that AIM2 deficiency almost completely abrogated IL-1β/18 productions in ALVAC-infected mouse cells (Fig. 2I), but not in human THP-1 cells (a partial reduction; Fig. 4C), suggesting that IL-1β and IL-18 might be differentially processed in mouse and human cells. The incomplete reduction of IL-1β and IL-18 secretion in AIM2-KO THP-1 cells could be possibly explained by multiple mechanisms. First, it has been shown that cleavage and maturation of IL-1β and IL-18 are not entirely inflammasome-dependent (50, 51); therefore, ALVAC infection may also trigger non-inflammasome pathway to process pro-IL-1β/18 in THP-1 cells. Second, an interesting study by *Webster SJ et al.* demonstrated that in response to bacterial infection, activation of STING pathway could mediate priming of both canonical and non-canonical inflammasomes (52). We observed similar results that ALVAC induces strong stimulation of STING, and speculate that in THP-1 cells, ALVAC may also activate other inflammasome pathways, potentially contributing to IL-1β/18 cleavages independent of AIM2. Nevertheless, the mechanisms for this observation are less clear and a more detailed understanding of whether and how this may occur in ALVAC-infected THP-1 cells should be explored in future studies.

Emerging evidence has suggested that inflammasome pathway can influence or modulate host adaptive immune responses in infection and immunization (53–55). For examples, IL-1β and IL-18 are key cytokines that not only activate monocytes, macrophages and neutrophils but have also been shown to drive the development of CD4 T-cell response (54, 56) and antibody response (57, 58). A recent study testing DNA vaccination in mice reported that deletion of AIM2 inflammasome led to significant reduction in antigen-specific antibody response (58). While it remains to be determined in our future studies whether and how ALVAC-induced AIM2 inflammasome pathway may regulate vaccine-induced immune responses, we speculate that compared Ad5 vector, the ALVAC vector may potentially manifest a more effective adjuvanting effect that could modulate vaccine-induced antibody response and T-cell (CD4) response, due to strong DC maturation and the production of pro-inflammatory cytokines IL-1β and IL-18 induced by ALVAC. Nevertheless, future studies

involving wild-type and gene-deficient animals (e.g. AIM2$^{-/-}$ mice) will be needed to further define *in vivo* roles of inflammasome pathway in modulating vaccine-induced adaptive immune responses in virally vectored vaccination. In addition, based on our data that activation of inflammasome pathway can be regulated at the priming stage involving STING-Type I IFN pathway, discovery and development of potential new adjuvant approaches or modulators based on these identified innate pathways should also be explored in future studies to enhance vaccine immunity.

In summary, our study herein reports identification of AIM2 as a predominant inflammasome sensor for ALVAC, triggering inflammasome activation and pro-inflammatory pyroptosis in human and mouse DCs. We discover that ALVAC primes and activates AIM2 inflammasome via the cGAS/IFI16-STING-Type I IFN pathway, providing a novel mechanism for innate host APC-ALVAC interactions. In addition, failure of Ad5 vector alone to trigger inflammasome activation is due to their inability to stimulate STING-Type I IFN pathway for priming inflammasome signals. Taken together, our research highlights the importance of understanding the basic mechanisms underlying host-viral vector interactions and provides novel insights into design and development of vaccine vectors in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	antigen presenting cells
BMDCs	bone marrow derived dendritic cells
BMDMs	bone-marrow derived macrophages
DC	dendritic cells
iMDDCs	immature monocyte-derived dendritic cells
IFNAR	Type I IFN receptor
KO	knockout
MDDCs	monocyte-derived dendritic cells
MPLA	monophosphoryl lipid A

PAMPs pathogen-associated molecular patterns

PRRs pattern recognition receptors

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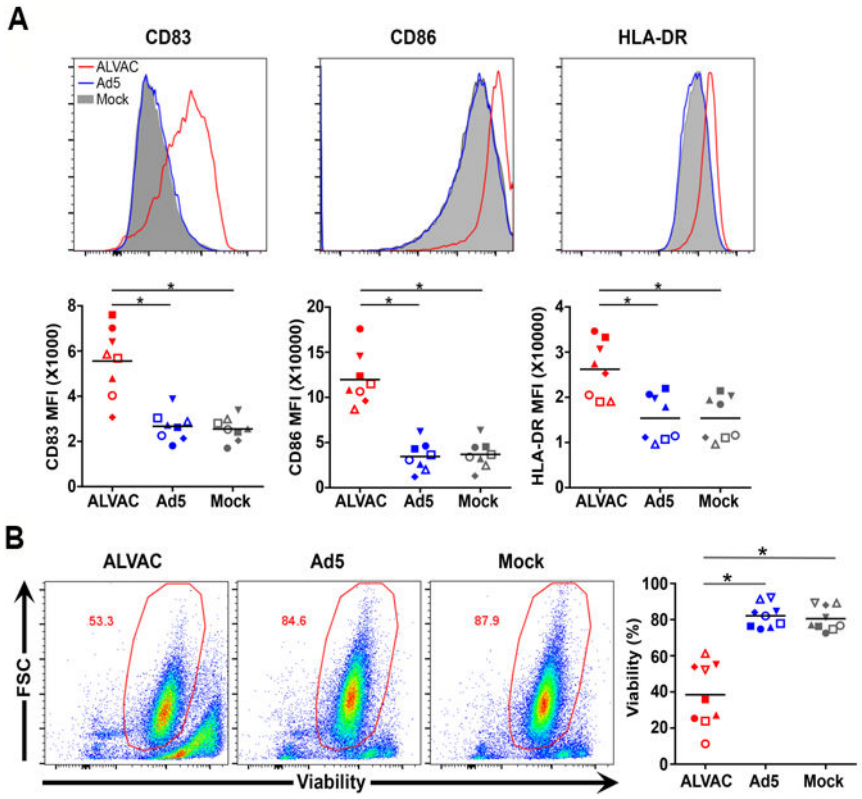

Figure 1. ALVAC induces maturation and cell death of human MDDCs

(A) Human iMDDC were infected with ALVAC or Ad5 vector (MOI=3, same MOI in all figures unless otherwise indicated) or un-infected (Mock) for 48 hours. DC maturation was measured based on expression of cell surface markers CD83, CD86 and HLA-DR. Both representative plot (top) and cumulative results from multiple subjects (n = 8, bottom) were shown. (B) Viability of human MDDCs was measured by flow cytometry 48 hours after infection with ALVAC or Ad5 vector. No infection (mock) was included as control. Cumulative results for viability of MDDCs from multiple subjects (n = 8) were also shown. *, $p < 0.05$.

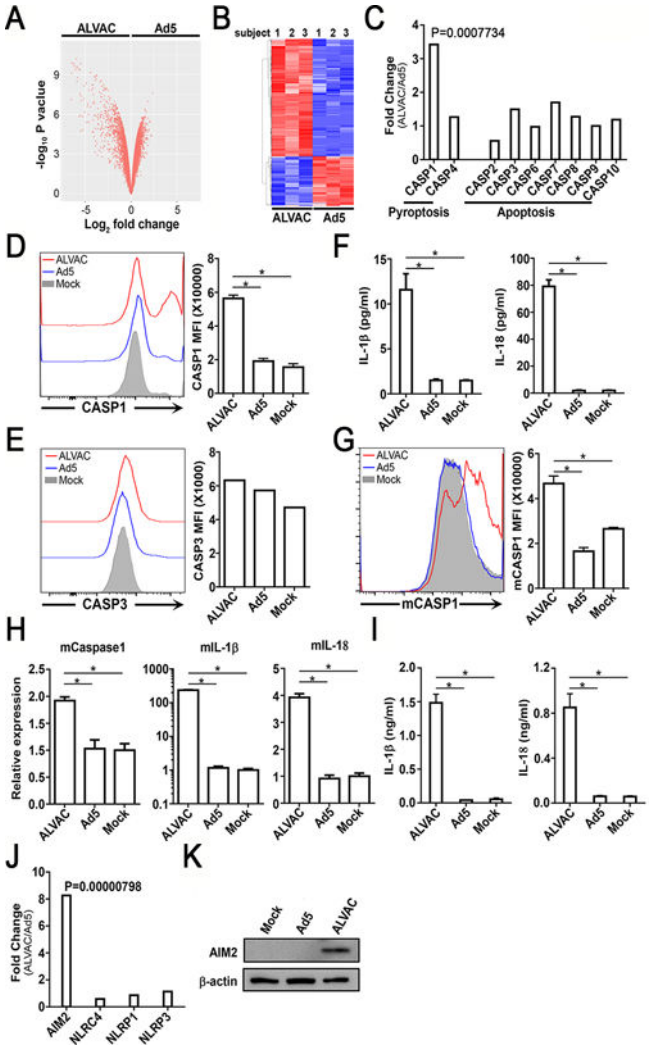

Figure 2. ALVAC induces inflammasome activation and pyroptosis in human and mouse DCs

(A–B) Human iMDDCs derived from 3 normal human subjects were infected with ALVAC or Ad5 vector for 24 hours, followed by RNA extraction and microarray analysis. Distinct gene-expression profiles between ALVAC- and Ad5-infected MDDCs were presented in Volcano plot (A) and heat-map analysis (B). (C) Differential gene expression for caspase family members (CASP1-10) between ALVAC- and Ad5-infected MDDCs from microarray analysis. (D–E) Flow cytometric measurement of caspase-1 (D) and caspase-3 (E) activity in ALVAC- (red) or Ad5- (blue) infected, or un-infected (grey) MDDCs at 48 hours post-infection. Activity was quantified as mean fluorescence intensity (MFI) and compared between ALVAC, Ad5 or mock infection. (F) Quantification of mature IL-1β and IL-18 in culture supernatants of human MDDCs infected with ALVAC, Ad5 or un-infected. Supernatants were collected at 48 hours post-infection and subject to LEGENDplex assay. (G–I) Measurement of inflammasome activation in mouse DCs. BMDCs from WT mice were infected with ALVAC, Ad5 or un-infected. Caspase-1 activity (G) and production of mature IL-1β and IL-18 (I) were measured at 48 hours after infection by flow cytometry and ELISA, respectively. Cells were also collected at 24 hours post-infection to measure gene-

expression of mouse caspase-1, IL-1β and IL-18 by real-time PCR (H). (J–K) Identification of AIM2 as an inflammasome sensor primed by ALVAC. Significantly increased expression of AIM2, but not NLRC4, NLPR1 and NLRP3, in ALVAC-infected MDDCs as compared to Ad5-infected MDDCs identified by microarray (J); Confirmation of enhanced AIM2 protein expression in ALVAC-infected MDDCs by western blot analysis (K). Cells were collected for western blot analysis at 24 hours post-infection. *, $p < 0.05$.

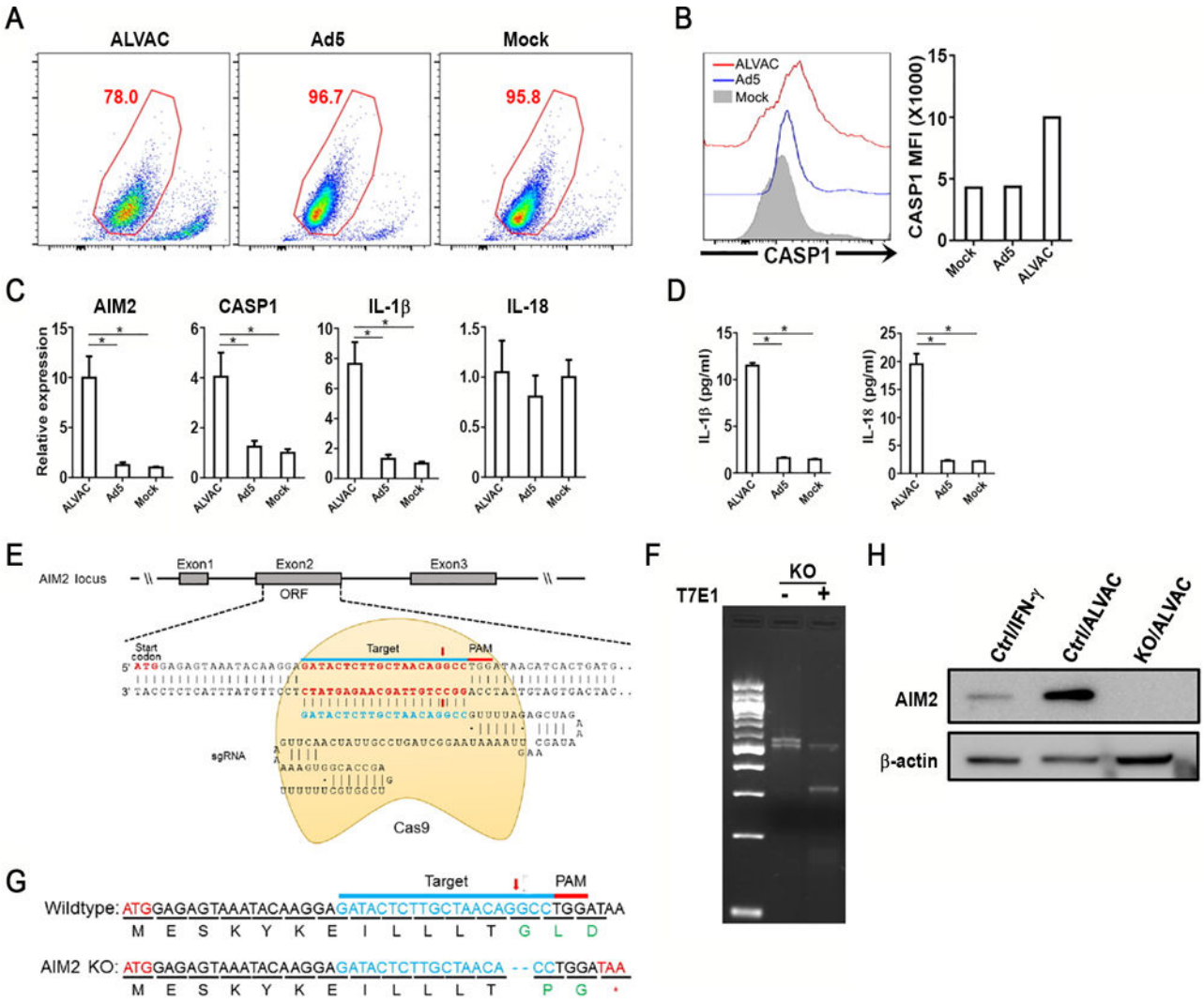

Figure 3. Generation of AIM2 KO THP-1 cells using CRISPR/Cas9

(A–D) ALVAC also induces inflammasome activation and pyroptosis in human THP-1 cells. THP-1 cells were infected with ALVAC or Ad5 or left un-infected for two days. Cell viability (A), caspase-1 activity (B), and production of mature IL-1β and IL-18 (D) were measured as described above. Expression of genes in AIM2 inflammasome pathway (*AIM2, CASP1, IL-1β and IL-18*) were measured at 24 hours post-infection by real-time PCR (C). (E–H) Generation of AIM2 KO THP-1 cells using CRISPR/Cas9. (E) Schematic illustration of CRISPR/Cas9-meidated AIM2 knockout; guide RNA was designed to target exon 2 of *AIM2* locus. Confirmation of successful and efficient ablation of AIM2 in edited THP-1 cells by sequencing (F) and T7-endonuclease 1 assay (G). Ablation of AIM2 protein in KO THP-1 cells was confirmed by western blot (H). Following treatment with IFN-γ (75 ng/ml) or ALVAC for 24 hours, KO-control THP-1 and AIM2 KO THP-1 cells were collected for western blot analysis.

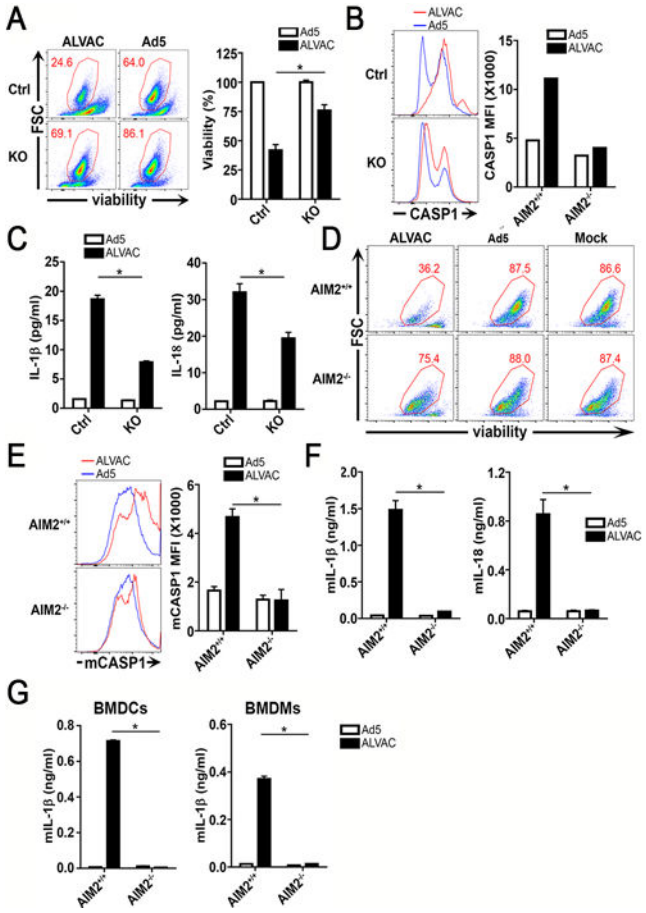

Figure 4. AIM2 is functionally required for ALVAC-induced inflammasome activation

(A–C) AIM2 KO or control (Ctrl) THP-1 cells were infected with ALVAC and Ad5 for two days. Cell viability (A), caspase-1 activity (B) and production of mature IL-1β and IL-18 (C) were measured as described above. (D–F) BMDCs from wild-type ($AIM2^{+/+}$) and AIM2 KO ($AIM2^{-/-}$) mice on the C57BL/6J background were infected with ALVAC, Ad5 or un-infected for two days. Cell viability (D), caspase-1 activity (E) and production of mouse mature IL-1β and IL-18 (F) were measured as described above. (G) BMDCs and BMDMs from wild-type ($AIM2^{+/+}$) and AIM2 KO ($AIM2^{-/-}$) mice on the C57BL/6 background were infected with ALVAC, Ad5 or un-infected for two days. Mouse mature IL-1β production were measured as described above. *, $p < 0.05$.

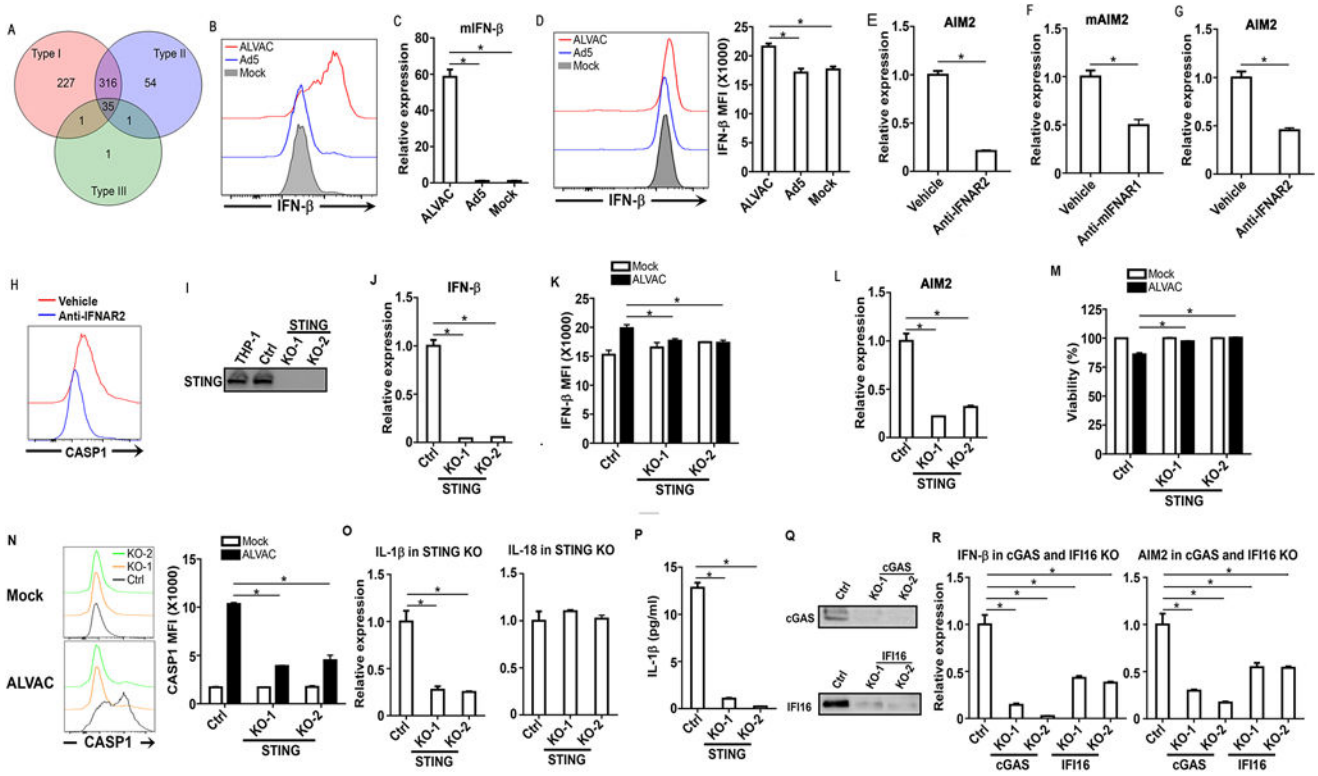

Figure 5. cGAS/IFI16-STING-Type I IFN pathway is required for ALVAC-induced AIM2 priming and inflammasome activation

(A) Identification of IFN-inducible genes among the genes up-regulated by ALVAC. 622 up-regulated genes (FC>2) was analyzed in the online Interferome database. (B–D) The ALVAC-induced IFN-β up-regulation. MDDCs, BMDCs and THP-1 cells were infected with ALVAC and Ad5 or left un-infected for 24 hours. Human IFN-β production in MDDCs (B) and THP-1 (D) or mouse IFN-β expression (C) were measured by flow cytometry or real-time PCR. (E–G) The role of type I IFNs in ALVAC-induced AIM2 up-regulation. MDDCs, BMDCs and THP-1 cells were infected with ALVAC in presence of IFNAR2 (human) or mIFNAR1 (mouse) antibodies for 24 hours. AIM2 expression in MDDCs (E), BMDCs (F) and THP-1 (G) were measured by real-time PCR. (H) Role of type I IFNs in ALVAC-induced inflammasome activation. THP-1 cells were infected with ALVAC in the presence of IFNAR2 antibody for 48 hours. Caspase-1 activity was measured as described above. (I and Q) Confirmation of STING, cGAS and IFI16 knockout (KO-1 and KO-2) in two gene-edited THP-1 cells by Western blot. (J–L) The role of STING in ALVAC-induced IFN-β and AIM2 expression. STING knockout (KO-1 and KO-2) or control (Ctrl) THP-1 cells were infected with ALVAC for 24 hours. IFN-β expression (J) and production (K) as well as AIM2 expression (L) were measured by real-time PCR or flow cytometry. (M, N and P) STING knockout (KO-1 and KO-2) or control (Ctrl) THP-1 cells were infected with ALVAC for 2 days. Cell viability (L), caspase-1 activity (M) and production of mature IL-1β (P) were measured as described above. (O and R) KO cells (STING, cGAS and IFI16) or control (Ctrl) THP-1 cells were infected with ALVAC for 1 days. The expression of IL-1β, IL-18 (O), IFN-β and AIM2 (R) were measured as described above. *, $p < 0.05$.

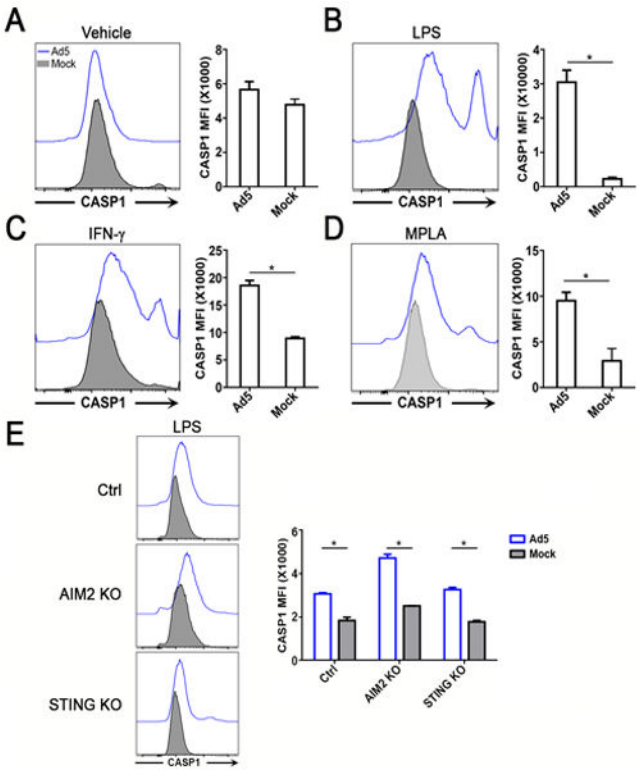

Figure 6. Ad5 vector induces strong inflammasome activation in pre-conditioned THP-1 cells

(A–D) After pre-treatment with LPS (B), IFN-γ (C) or MPLA (D) or left un-treated (A), THP-1 cells were infected with Ad5 vector (MOI=1200) or left un-infected (Mock) for two days. Caspase-1 activity was measured as an indication of inflammasome activation and pyroptosis. (E) After pre-treatment with LPS, AIM2 KO, STING KO and control (Crispr/Cas9 vector only Ctrl) THP-1 cells were infected with Ad5 vector (MOI=1200) or left un-infected (Mock) for two days. Caspase-1 activity was measured as described above. *, $p < 0.05$.

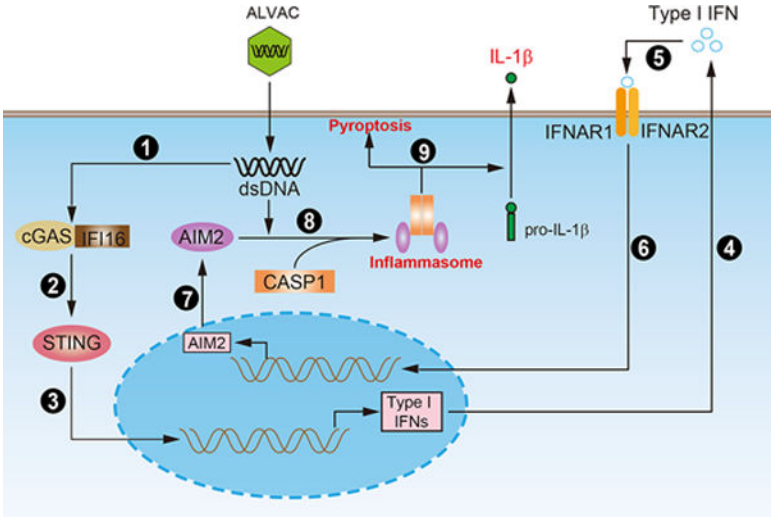

Figure 7. Schematic model for priming and activation of AIM2 inflammasome by ALVAC

After entry into cells, ALVAC genomic DNA is released and recognized by cGAS and IFI16 complex ①, followed by activation of the STING ②. STING activation induces expression (③) and release (④) of type I IFNs (IFN-β). Released type I IFN binds interferon-α/β receptor (IFNAR) ⑤, which induces expression of interferon-inducible genes ⑥, including AIM2 (AIM2 priming) ⑦. Up-regulated AIM2 protein can sense ALVAC genomic DNA and interact with caspase-1 (CASP1) to form activated inflammasomes ⑧. Lastly, activated inflammasomes trigger pyroptosis and production of mature IL-1β and IL-18 ⑨.

Table 1

Primer sequence for validation of viral vector infection

Viral vector	Gene	Sequence
Ad5	Hexon	F: GCCGCAGTGGTCTTACATGCACATC R: CAGCACGCCGCGGATGTCAAAGT
ALVAC		F: ATTGCGCGATGTAGATAAATGTTACAAAC R: GCATCAAAGAGTATAGCTTCATACCCTG

Table 2

Primer sequence for quantification of gene expression

Species	Gene	Genbank ID	Sequence
Homo sapiens	GAPDH	NM_001256799.2	F: CAATGACCCCTTCATTGACC R: GACAAGCTTCCCGTTCTCAG
	Caspase 1	NM_033292.3	F: CCACAATGGGCTCTGTTTTT R: CATCTGGCTGCTCAAATGAA
	IL-1β	NM_000576.2	F: AAGGCGGCCAGGATATAACT R: CCCTAGGGATTGAGTCCACA
	IL-18	NM_001562.3	F: TTGTCTCCCAGTGCATTTTG R: GAAGCGATCTGGAAGGTCTG
	IFNB1	NM_002176.3	F: TGCTCTGGCACAACAGGTAG R: CAGGAGAGCAATTTGGAGGA
	AIM2	NM_004833.1	F: TAGCGCCTCACGTGTGTTAG R: TTGAAGCGTGTTGATCTTCG
	POP3	NM_001320010.1	F: GATTTAAAACGCTGGCATGG R: TGCTCTTCCCGAAGACATTT
Mus musculus	mGAPDH	NM_008084.3	F: GGGTGTGAACCACGAGAAAT R: CCTTCCACAATGCCAAAGTT
	mCaspase 1	NM_009807.2	F: CACAGCTCTGGAGATGGTGA R: GGTCCCACATATTCCCTCCT
	mIL-1β	NM_008361.4	F: GAGTGTGGATCCCAAGCAAT R: TACCAGTTGGGGAACTCTGC
	mIL-18	NM_008360.1	F: ACTTTGGCCGACTTCACTGT R: GGGTTCACTGGCACTTTGAT
	mIFNB1	NM_010510.1	F: CCCTATGGAGATGACGGAGA R: ACCCAGTGCTGGAGAAATTG
	mAIM2	NM_001013779.2	F: AACGCAGATGGAAGTTGCTT R: GCCAGACCTCAAGTCTCCTG
	mP202	NM_011940.2	F: GGCAATGTCCAACCGTAACT R: CACTGGAAGGGGTCTGATGT

Table 3

Gene-specific guide sequences used for gene editing

Name	Target exon	Sequence (PAM is underlined)
AIM2 KO	2	GATACTCTTGCTAACAGGCCTGG
Caspase-1 KO-1	4	GGGTTGTCCTGCACTGCCTGAGG
Caspase-1 KO-2	2	AAGCTGTTTATCCGTTCCATGGG
STING KO-1	3	GCAAGCATCCAAGTGAAGGGCGG
STING KO-2	2	GTTAAACGGGGTCTGCAGCCTGG
cGAS KO-1	1	CGGCCCCCATTCTCGTACGGAGG
cGAS KO-1	1	AGACTCGGTGGGATCCATCGGGG
IFI16 KO-1	4	GACATTTGGCCACTGTTTTCGGG
IFI16 KO-2	2	TGCCCAAACCAGCATCACCTCGG

Table 4

Primer sequence for validation of gene editing

Target gene	Name	Experiment	Sequence
AIM2	AIM2-V1	T7E1 Sequencing	F: AATGTGCACTGGGGGTCATA R: TCCTACTCAAATCTCTTTTACTACTGC
Caspase-1	Caspase1-V1	T7E1 Sequencing	F: AGATCAGGATTGTCCTCCTCAG R: CTTTCACCCCACTCTATCCTTG
Caspase-1	Caspase1-V2	T7E1 Sequencing	F: CAAAGCTCGGGTCTTATCCA R: AGGGGGACATTTCACTGATG
STING	STING-V1	T7E1	F: TGTTGCTGCTGTCCATCTATTT R: AACCCACTGTTCCAGGACATTA
STING	STING-V2	T7E1	F: GAATGGTCATGGATTTCTTGGT R: TGCAGTGAGTCACCTGGAGT
cGAS	cGAS-V1	T7E1	F: CTCCACGAAGCCAAGACCT R: CTCATAGTAGCTCCCGGTGTTC
cGAS	cGAS-V2	T7E1	F: TCCTGAAACGGATTCTTCTTTC R: GCTCTTTTTCTGCCGGGAT
IFI16	IFI16-V1	T7E1	F: TCCCTCTCTATCCTGACTCCAG R: TCCATTTCTGGGGTCTCATATT
IFI16	IFI16-V2	T7E1	F: CCATTTTCTCTTGTAGGCTCAC R: AAGAGTTTCAGCCAGGTCTTCA