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## Activation of the NLRP3 inflammasome by vault nanoparticles expressing a chlamydial epitope

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### Abstract

The full potential of vaccines relies on development of effective delivery systems and adjuvants and is critical for development of successful vaccine candidates. We have shown that recombinant vaults engineered to encapsulate microbial epitopes are highly stable structures and are an ideal vaccine vehicle for epitope delivery which does not require the inclusion of an adjuvant. We studied the ability of vaults which were engineered for use as a vaccine containing an immunogenic epitope of *C. trachomatis*, polymorphic membrane protein G (PmpG), to be internalized into human monocytes and behave as a “natural adjuvant”. We here show that incubation of monocytes with the PmpG-1-vaults activates caspase-1 and stimulates IL-1 $\beta$  secretion through a process requiring the NLRP3 inflammasome and that cathepsin B and Syk are involved in the inflammasome activation. We also observed that the PmpG-1-vaults are internalized through a pathway that is transiently acidic and leads to destabilization of lysosomes. In addition, immunization of mice with PmpG-1-vaults induced PmpG-1 responsive CD4<sup>+</sup> cells upon re-stimulation with PmpG peptide *in vitro*, suggesting that vault vaccines can be engineered for specific adaptive immune responses. We conclude that PmpG-1-vault vaccines can stimulate NLRP3 inflammasomes and induce PmpG-specific T cell responses.

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## Keywords

Vaults; inflammasomes; *Chlamydia*; lysosomes

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## 1. INTRODUCTION

*Chlamydia trachomatis* is the most prevalent bacterial sexually transmitted disease (STD) in the United States. Chlamydial infections in women can cause pelvic inflammatory disease (PID) and result in infertility, ectopic pregnancy, and chronic pelvic pain [1–3]. Most *Chlamydia* infections are asymptomatic, increasing the risk of transmission of *Chlamydia* to unsuspecting females and result in PID [4–6]. Identification of protective responses is a key component of vaccine development. Intensive studies have been done in order to dissect immunity towards to resolution of primary chlamydial infection, and immunity to reinfection in mouse genital infection model. CD4<sup>+</sup> T cells play major role in resolving primary genital infection [7], particularly IFN- $\gamma$  secreting CD4<sup>+</sup> T cells (Th1 cells) [8], with or without CD8<sup>+</sup> T cells or antibody [9, 10]. CD4<sup>+</sup> T cells and/or antibody are also essential for resistance to reinfection. However, CD8<sup>+</sup> T cells appear to be unnecessary against reinfection [10]. Development of a protective vaccine for prevention of *Chlamydia* PID is challenging due to difficulties in identifying and delivering relevant T cell antigens and developing a safe adjuvant that does not produce excessive inflammatory responses which can diminish the likelihood of public acceptance [11–13].

The full potential of vaccines relies on development of effective delivery systems and adjuvants and is critical for development of successful vaccine candidates. Vaults are large cytoplasmic ribonucleoprotein (RNP) particles consisting of three proteins and a small untranslated RNA [14, 15]. Their function within cells has not been identified but reports have suggested their involvement with multidrug resistance, cell signaling and innate immunity [16–24]. *In vitro* expression of MVP in insect cell can form hollow vault-like particles identical to native vaults [25]. An MVP interaction domain (INT) associates non-covalently with MVP binding site and can be used to internally package other proteins of interests. We have shown that vaults can be engineered *in vitro* as a vaccine which effectively delivers antigen for generation of a protective immune response. However, we and others [26–28] also discovered that recombinant vaults can interact with host immune cells and display self-adjuvanting properties, distinguishing them from other vaccine preparations. Moreover, we reported that vaults engineered to contain a recombinant *Chlamydia* protein (MOMP-vault vaccine) induced strong protective anti-chlamydial immune responses without eliciting excessive inflammation as measured by TNF- $\alpha$  production [29]. We hypothesized that vaults vaccines act as “smart adjuvants” and can be engineered to produce a tailored immune response against specific antigens by housing proteins in the central cavity of the recombinant vault that is hollow and large enough to accommodate multiple copies of foreign epitopes [26, 29]. Our data further suggested that the vault vaccine induced inflammasomes, an innate immune response that could possibly account for the self-adjuvanting property of vault-vaccines upon phagocytosis.

Inflammasomes serve as the first line of immune defense against inducers of cellular stress [30]. Following detection of stress inducers such as infection, inflammasomes promote

maturation and secretion of IL-1 $\beta$  [31]. The inflammasome containing the Nod-like receptor (NLR) family member, NLRP3, is one of the best studied inflammasomes and can be activated by a wide range of stimuli, including membrane-damaging toxins, pathogen associated molecular patterns (PAMPs), and danger associated molecular patterns (DAMPs) [32–35]. The NLRP3 inflammasome can also be stimulated by large particles such as monosodium urate (MSU) crystals, silica, nanoparticles, and the adjuvant, alum, which can lead to lysosomal damage after engulfment by phagocytes and the release of lysosomal proteases such as cathepsin B [36–38]. When these stimuli are detected, NLRP3 interacts with the adaptor, ASC (Apoptosis-associated speck-like protein containing a CARD), which in turn recruits the protease, pro-caspase-1. When pro-caspase-1 is assembled into the inflammasome, it becomes auto-activated and cleaved into a 20 kD fragment and induces caspase-1-dependent maturation and secretion of proinflammatory cytokines such as IL-1 $\beta$  [35, 39–44]. Upon activation of the NLRP3 inflammasome, the mature IL-1 $\beta$  is secreted out of the cell. In many cells such as monocytes and macrophages, the activated 20 kD form of caspase-1 is also secreted.

In this report, we have used a different chlamydial protein, PmpG-1, and convincingly show that PmpG-1-vault vaccines induce NLRP3 inflammasome activation that differs from other particulate induces following phagocytosis *in vitro*. PmpG-1-vault vaccines also induce a T cell response against a PmpG-1 peptide demonstrating that vault-vaccines can be engineered for a tailored immune response.

## 2. MATERIALS AND METHODS

### 2.1 Assembly of PmpG-1-vaults vaults

Recombinant baculoviruses were generated using the Bac-to-Bac protocol (Invitrogen, Carlsbad, CA). The 17 amino acid coding region of PmpG-1 (ASPIYVDPAAAGGQPPA) was fused to the N-terminus of the INT domain derived from VPARP (amino acids 1563–1724) by PCR using the following primers: PmpG-1-INT Forward BamHI-5'/GGGATCCATGGCAAGCCCAATTTATGTCGACCCAGCAGCAGCAGGTGG TCAACCACCAGCATGCACACAACACTGGCAGGA-3' and INT Reverse XhoI-5'-GCTCGAGTTAGCCTTGACTGTAATGGAGGA-3' using INT in pET28 as the template. The resultant PCR product containing the fused PmpG-1-mINT was purified on a Qiagen column (Qiagen, Germantown, MD), digested with BamHI and XhoI, gel purified, and ligated to pFASTBAC to form PmpG-1-mINT pFASTBAC. Construction of cp-MVP-z in pFASTBAC has been described previously [25]. All primers used in this study were purchased from Invitrogen (Carlsbad, CA). Sf9 cells were infected with cp-MVP-z, and PmpG-1-INT recombinant baculoviruses at a multiplicity of incubation (MOI) of 0.01 for approximately 72 h and then pelleted and stored at –80°C until needed. PmpG-1-INT and cp-MVP-z pellets were lysed on ice in buffer A [50 mM Tris-HCl (pH 7.4), 75 mM NaCl, and 0.5 mM MgCl<sub>2</sub>] with 1% Triton X-100, 1 mM dithiothreitol, 0.5 mM chymostatin, 5  $\mu$ M leupeptin, 5  $\mu$ M pepstatin) (Sigma, St. Louis, MO). Lysates containing PmpG-1-vaults were mixed with lysates containing PmpG-1-INT and incubated on ice for 30 min to allow the INT fusion proteins to package inside of vaults. As a control, another lysate of cp-MVP-z pellets was prepared without PmpG-1-INT. Recombinant vaults were purified as previously

described and resuspended in sterile RPMI media [25, 45, 46]. The protein concentration was determined using the BCA assay (Pierce) and sample integrity was analyzed by negative stain electron microscopy and SDS-PAGE with Coomassie staining.

The PmpG-1 was cloned in frame with the INT (interaction domain amino acids 1563–1724 of VPARP ref) protein by PCR ligation, resulting in a ~20 kD fusion protein. Addition of this fusion protein to vaults results in packaging inside the particle [47]. An IgG binding domain (the Z domain) was engineered to the C-terminus of MVP to enhance immunity [29] and a cysteine-rich peptide was added to the N-terminus of MVP to enhance particle stability [47]. These vaults are referred to as cp-MVP-z and following packaging of the PmpG-1-INT fusion protein they are designated cp-MVP-z/ PmpG-1-INT (and abbreviated PmpG-1-vaults).

## 2.2 Cell culture and inhibitor treatment

THP-1 cells were grown in RPMI 1640 (Sigma-Aldrich) with 10% FBS (Invitrogen) and 10 µg/ml gentamicin. A total of  $1 \times 10^6$  cells per well in a 6-well plate were differentiated with 500 nM PMA for 3 hrs. Differentiated THP-1 cells were washed with 1XPBS 3 times and incubated for 24 hrs at 37°C with 5% CO<sub>2</sub>. Z-WEHD (100 nM) and CA-074 Me (10 µM) were used 1.5 hrs before treatment with vaults. Syk-inhibitor (10 µM) was used 30 minutes prior to addition of vaults. PmpG-1-vaults (250 nM) were incubated with cells, and after 6 hrs post-incubation, we collected the supernatant from the treated cells.

## 2.3 Gene product depletion by RNA interference

THP-1 stably expressing shRNA against NLRP3, ASC, Syk and caspase-1 were obtained by transducing THP-1 cells with lentiviral particles containing shRNAs. The sequences 5'-CCGGGCGTTAGAAACACTTCAGAACTCGAGTTCTTGAAGTGTTCCTAACGCTTTTG-3' for human NLRP3 (Sigma; catalog number NM\_004895), 5'-CCGGCGGAAGCTCTTCAGTTTCACACTCGAGTGTGAACTGAAGATCCGTTT TTG-3' for human ASC (Sigma; catalog number NM\_013248), 5'-CCGGGCAGGCCATCATCAGTCAGAACTCGAGTTCTGACTGATGATGGCCTGCTT TTT-3' for human spleen tyrosine kinase (Syk) (Sigma; catalog number NM-003177), and five sequences for caspase-1 (Sigma; catalog number NM-001223): 5'-CCGGGAAGAGTTTGAGGATGATGCTCTCGAGAGCATCATCCTCAAACCTTTCT TTTT-3', 5'-CCGGTGTATGAATGTCTGCTGGGCACTCGAGTGCCAGCAGACATTCATACAT TTTT3', 5'-CCGGCACACGTCTTGCTCTCATTATCTCGAGATAATGAGAGCAAGACGTGTGT TTTT3', 5'-CCGGCTACAACCTCAATGCAATCTTTCTCGAGAAAGATTGCATTGAGTTGTAGT TTTT3', 5'-CCGGCCAGATATACTACAACCTCAATCTCGAGATTGAGTTGTAGTATATCTGGT TTTT-3' were used separately to silence gene expression following the manufacturer's instructions. Nontarget shRNA control cells were also generated using an irrelevant sequence (Sigma; catalog number SHC002 V). Cells ( $3 \times 10^5$ ) were plated at 35% confluency 24 h prior to transduction and then the corresponding lentiviral transduction

particles were added at an moi of 3 overnight. Fresh media were added the next day, and transduced cells were selected by addition of media containing 2 µg/ml puromycin (Sigma). The knockdown (KD) efficiency was tested by qPCR. mRNA was isolated from cells after indicated treatments or incubations using the Qiagen RNeasy Kit (Qiagen, Valencia, VA) following the manufacturer's instruction.

#### 2.4 IL-1β & TNF-α ELISA assay

Supernatant from vaults-treated cells was collected after 6 hrs post-incubation and stored at -80°C until ready for use in the assay. Measurement of IL-1β was carried out using human IL-1β ELISA kit (eBioscience, San Diego, CA), following manufacturer's instructions.

#### 2.5 Western blotting

Supernatants from vaults-treated cells were collected and precipitated with TCA. Samples were lysed using 1× RIPA Lysis Buffer (Millipore) with 1× protease inhibitor cocktail (Biovision) and loaded onto a 12% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Millipore). For detection of the active caspase-1 subunit (p20), the blot was probed with 1 mg/ml rabbit anti-human caspase-1 antibody (Millipore), and then incubated again with conjugated 1:10000 dilution of anti-rabbit IgG horseradish peroxidase (Millipore). To detect mature IL-1β, the blot was probed with IL-1β antibody (Cell Signaling) at a 1:1000 dilution, and then incubated again with 1:10000 dilution of anti-mouse secondary antibody (Santa Cruz Biotechnology). Western blotting detection reagents (Amersham Biosciences) were used following manufacturer's instructions and chemiluminescence was detected using a gel doc system (Bio-Rad).

#### 2.6 Fluorescence-activated cell sorting (FACS)

THP-1 cells ( $2 \times 10^6$ /well) were plated in 6-well plates and primed for 3 hr with 0.5 µM Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO). Recombinant PmpG-1-vaults were dual-labeled with the fluorescent dyes FITC and TRITC by primary amine reaction following manufacturer's instructions (Pierce, Thermo Scientific, Rockford, IL). Unconjugated dye was removed by filtration on a PD-10 column (GE Healthcare, Piscataway, NJ). Primed THP-1 cells were incubated in duplicate with FITC-TRITC dual-labeled vaults for 6, 18, 24 or 48 h. Half of the treatments were incubated with bafilomycin (Sigma-Aldrich, St. Louis, MO), an ATPase inhibitor, for 30 min to neutralize all subcellular compartments. Cells were collected by trypsinization, washed and immediately analyzed by flow cytometry using a BD FACSCalibur (BD Biosciences, Franklin Lakes, New Jersey) and data was analyzed using Flowjo software (Tree Star, Inc., Ashland, OR). A total of  $10^5$  cells were analyzed.

For FACS analysis of lymphocytes, the spleen was harvested from individual mice, and single cell suspensions were prepared by dissociating the lymphocytes through a 40 µm cell strainer (BD Falcon). Individual cells were washed with 1% PBS followed by red blood cell lysis treatment. Lymphocytes were re-suspended in RPMI 1640 at 4°C until used. For intracellular cytokine staining, lymphocytes isolated from spleen were incubated in RPMI 1640 in the presence of PmpG-1<sub>303-311</sub> peptide for 6–8 hrs. Brefeldin A (Sigma) was added 4 hrs before the end of culture. Cells were directly stained with fluorochrome-labeled

antibodies against CD3 (clone 145–2C11) or CD4 (clone GK1.5). After washing, the cells were incubated with Cytofix/Cytoperm (BD Biosciences) for 1 h and stained with fluorochrome-conjugated anti-IFN- $\gamma$  (clone XMG1.2), washed again, re-suspended in Cell Fix solution, and analyzed on a SORP BD LSR II (Beckman Dickinson, Franklin Lakes, NJ). FACS data were analyzed by Flowjo (Tree Star, Oregon).

## 2.7 Chlamydiae, immunization and challenge of mice

*Chlamydia muridarum* (MoPn) was grown on confluent McCoy cell monolayers, purified on Renograffin gradients and stored at  $-80^{\circ}\text{C}$  in SPG buffer (sucrose-phosphate-glutamine) as previously described [48]. Female C57BL/6 mice, 5–6 weeks old were housed according to American Association of Accreditation of Laboratory Animal Care guidelines [48]. Mice receiving vaults were anesthetized with a mixture of 10% ketamine plus 10% xylazine and immunized i.n. with 100  $\mu\text{g}$  PmpG-1-vaults in 20  $\mu\text{l}$  saline for a total of 3 times every two weeks. Mice were hormonally synchronized by subcutaneous injection with 2.5 mg of medroxyprogesterone acetate (Depo Provera, Upjohn, Kalamazoo, MI) in 100  $\mu\text{l}$  saline 7 days prior to a vaginal challenge with  $1.5 \times 10^5$  IFU of *C. muridarum* and infection was monitored by measuring infection forming units (IFU) from cervical-vaginal swabs (Dacroswab Type 1, Spectrum Labs, Rancho Dominguez, CA) as previously described [48].

## 2.8 Colocalization studies

The following antibodies were used for immunofluorescence at the indicated dilutions: anti-early endosome antigen 1 (EEA1, G-4; 1:100; Santa Cruz Biotechnology, Dallas, TX), anti-lysosomal-associated membrane protein1 (LAMP1, clone H4A3; 1:100; Biolegend, San Diego, CA), anti-microtubule-associated protein 1 light chain 3 (LC3, clone 166AT1234; 1:100; Abgent, San Diego, CA), and AF488-goat anti-mouse immunoglobulin G (IgG; 1:400; Invitrogen, Carlsbad, CA). For colocalization studies, THP-1 cells ( $1.5 \times 10^5$ ) were seeded onto 18 mm glass coverslips and incubated at  $37^{\circ}\text{C}$  (with 5%  $\text{CO}_2$ ) for 72 hrs in the presence of 10 ng/ml PMA. Purified PmpG-1-vaults were labeled with DyLight 650 according to the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, IL). Coverslips containing primed THP-1 cells were incubated with 30  $\mu\text{g}$  of DyLight 650-labeled PmpG-1-vaults for 15 min, 30 min, and 1 h. Cells were fixed in 3.7% paraformaldehyde in  $1 \times$  PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM  $\text{MgCl}_2$ ) for 15 minutes at room temperature. Cells were washed 3 times in  $1 \times$  PHEM buffer before permeabilization for 10 minutes in 0.25% Triton X-100 in  $1 \times$  PHEM buffer. Following permeabilization, the cells were washed 3 times in  $1 \times$  PHEM buffer prior to incubation in blocking solution (10% normal goat serum in  $1 \times$  PHEM buffer) for 1 h at room temperature. Cells were further incubated with the appropriate primary antibody diluted in blocking solution for 1 h at room temperature, rinsed 3 times in  $1 \times$  PHEM buffer and further incubated for 1 h in secondary antibody prepared in blocking solution. Following staining with the secondary antibody, the cells were washed 3 times with  $1 \times$  PHEM buffer and mounted in VectaShield Hard Mount with DAPI (Vector Labs, Inc., Burlingame, CA) and visualized using a Yokogawa CSU-22 spinning disc confocal scanner and a Hamamatsu C9100–13 EMCCD camera mounted on a Zeiss Axiovert 200m stand. The images were captured using Slidebook 5 software (Intelligent Imaging Innovations, Inc., Denver, CO). The optimal conditions including the number of vault particles used for each experiment

were determined empirically. Images were acquired with a 100× oil immersion objective and were processed using ImageJ (<http://rsb.info.nih.gov/ij/>). In addition, 10 images were used to determine colocalization by applying the Pearson's correlation coefficient located in the JACoP Plugin module.

### 3. RESULTS

#### 3.1 PmpG-1-vault-vaccines stimulate secretion of IL-1 $\beta$ and activated caspase-1 from monocytes

Treatment of THP-1 monocytes with PMA (phorbol-12-myristate-13-acetate) stimulates their differentiation into macrophages. PMA-primed THP-1 cells also synthesize pro-IL-1 $\beta$ , making them good models for studying inflammasome activation. To evaluate whether PmpG-1-vault-vaccines could affect inflammasome activation, we measured IL-1 $\beta$  secretion from PMA-primed THP-1 cells incubated with recombinant vaults containing the chlamydial epitope, PmpG. A significantly higher level of IL-1 $\beta$  was detected in the supernatants after 6 hrs of incubation with the PmpG-1-vaults "vaults" than from cells without vaults (Figure 1A). The empty vaults (without any epitope) were also tested but do not induce an immune response (data not shown) [29], and therefore were not tested further here. To determine whether the IL-1 $\beta$  secretion is dependent on caspase-1 activation, we incubated the cells with a caspase-1 inhibitor, zWEHDfmk [49]. This inhibitor also blocks caspase-4 and caspase-5, which could potentially modulate inflammasome activity [50]. When cells are pre-treated with the caspase inhibitor before adding the vaults, a dramatic decrease in IL-1 $\beta$  secretion and processing was observed (Figure 1A). ELISA of secreted (activated) caspase-1 and Western blot analysis confirmed that the inhibitor also blocked caspase-1 activation (Figure 1C), as expected.

#### 3.2 Incubation of cells with PmpG-1-vaults activates the NLRP3 Inflammasome

The NLRP3 inflammasome can be activated by a broad range of stimuli, including nanoparticles and crystals [51]. We therefore examined whether PmpG-1-vaults may induce IL-1 $\beta$  secretion and caspase-1 activation through the NLRP3 inflammasome. We focused on several representative NLRP3 components such as the adaptor ASC, the NLR family member NLRP3, the protease caspase-1, and the mediators Syk and cathepsin B. To test whether these components may play a role in vault-induced IL-1 $\beta$  secretion, we applied inhibitors of each component and also depleted some components by RNA interference.

When CA-074 Me, an inhibitor of cathepsin B, was incubated with cells 1.5 hrs before incubation with the PmpG-1-vaults, there was a large inhibition of IL-1 $\beta$  secretion (Figure 1A). The inhibitor alone had no effect on IL-1 $\beta$  secretion (data not shown). Similarly, pre-incubation with a Syk inhibitor for 30 minutes significantly decreased PmpG-1-vault-induced IL-1 $\beta$  secretion (Figure 1A). These results suggest that both Syk recruitment and lysosomal destabilization are involved in vault-induced inflammasome activation.

To confirm NLRP3 inflammasome activation by the PmpG-1-vault vaccine, we also depleted ASC and NLRP3 using shRNA method delivered using lentiviral particles. THP-1 cells were treated with a non-target shRNA control, and lentiviral particles to deplete ASC, Syk, caspase-1, and NLRP3 individually. The efficiency of ASC reduction was evaluated by

qPCR (Supplementary Figure S1), which also confirmed specificity of the depletion. When cells were incubated with PmpG-1-vaults, IL-1 $\beta$  secretion decreased dramatically in each depleted cell line, compared to the control group (Figure 1B). These results further strengthen the conclusion that PmpG-1-vaults activate the NLRP3 inflammasome.

We next measured caspase-1 activation in the presence of inhibitors against upstream mediators of the NLRP3 inflammasome. The cathepsin B inhibitor, CA-074 Me, dampened PmpG-1-vault activation by approximately half, suggesting that lysosomal disruption may be involved in this process. The Syk inhibitor also strongly decreased caspase-1 activation (Figure 1A).

The effects of the inhibitors were confirmed by depleting the respective target genes by RNA interference (data not shown). Thus, there was significantly less vault-induced caspase-1 activation when THP-1 cells were depleted of ASC, NLRP3 or Syk. As expected, there was also less caspase-1 activation when the cells were depleted of caspase-1.

The results of processed IL-1 $\beta$  and activated caspase-1 secretion obtained by ELISA (Figure 1) were confirmed by measuring mature IL-1 $\beta$  and activated caspase-1 in the supernatant by Western blot (Figure 2). Incubation of THP-1 cells with vaults stimulated secretion of mature IL-1 $\beta$  in the supernatant, which could be inhibited by pre-incubation with the caspase-1 inhibitor zWEHDfmk (Figure 2A and B). Similarly, activated caspase-1 could be observed in the supernatant of vault-stimulated THP1 cells, and secretion of activated caspase-1 could be inhibited by zWEHDfmk (Figure 2C and D).

To confirm the functional specificity of the shRNA depletion of caspase-1, ASC, NLRP3 or Syk, the THP-1 cell lines were primed with 10  $\mu$ g/ml LPS, and TNF- $\gamma$  secretion was measured. Secretion of this cytokine takes place through an inflammasome-independent pathway, and the results demonstrated that depletion of inflammasome-associated components had no effect (Figure 3). These results showed that depletion of caspase-1, ASC, NLRP3 and Syk by shRNA affected caspase-1 activation and IL-1 secretion, but not cytokine secretion through inflammasome-independent pathways. Taken together, these results imply that the PmpG-1-vault vaccines can activate caspase-1 and stimulate IL-1 $\beta$  secretion through a process involving the NLRP3 inflammasome.

### 3.3 Internalized vaults co-localize with lysosomes

PmpG-1-vaults were labeled with FITC (green) and TRITC (red) (Figure 4A) in order to study their intracellular trafficking by flow cytometry (Figure 4B). The fluorescence of FITC is sensitive to pH, which allowed us to determine whether the particles entered acidic compartments after internalization. One group of cells was treated with bafilomycin (to prevent re-acidification of vesicles) before incubation with PmpG-1-vaults. The results indicated that the majority of PmpG-1-vaults were in acidic compartments after 6 hrs of incubation, but most were at neutral pH after 24 hrs (Figure 4C). These experiments suggest that following phagocytosis, the majority of PmpG-1-vaults are internalized initially into acidic compartments (endolysosomes or phagolysosomes), from which they escape into the cytosol.



This possibility was also addressed by confocal fluorescence microscopy, using DyLight650-labeled PmpG-1-vaults and antibodies against EEA1 (early endosomal marker), Lamp1 (marker of lysosomes), and LC3 (marker of autophagosomes) (Figure 5). The results showed that after 15 min, 30 min and 60 min, approximately 40% of PmpG-1-vaults co-localized extensively with lysosomes (Figure 5), with a significant Pearson's coefficient. This indicates that the majority of PmpG-1-vaults were internalized into lysosomes, which led to lysosomal disruption. These results are consistent with previous results showing that inhibitors of cathepsin B block NLRP3 inflammasome activation in cells incubated with vaults.

### 3.4 Immunization with PmpG-1-vaults induces an immune response *in vivo*

We examined the immune response of mice vaccinated vaginally with the PmpG-1-vault vaccine. Spleen cells were harvested from naïve mice as well as from mice immunized with PmpG-1-vaults three times. Two weeks after the last immunization, all mice were sacrificed and the lymphocytes were isolated from spleens and stimulated *in vitro* overnight. Single-cell suspensions were analyzed by flow cytometry for expression of CD3, CD4, and IFN- $\gamma$ , which are markers for TH1 helper cells, and gated on cells that are specific for MHC-peptide tetramers containing a peptide derived from PmpG-1 (Figure 6). We observed that the cells from immunized mice have a larger percentage of specific TH1 cells within the CD4<sup>+</sup> cell compartment, compared to cells from naïve mice. Taken together, these results show that the immune system can recognize the foreign epitope incorporated into the PmpG-1-vault vaccine which could be used in a subsequent immune response to antigen-expressing *Chlamydia*.

## DISCUSSION

Vaccines that prevent significant infection of the female genital tract are essential to reduce the incidence of PID following *C. trachomatis* infection. We have shown that vaults containing a chlamydial protein (MOMP) markedly reduces infection early after infection suggesting that the self-adjuncting vault vaccine is activating innate immunity while not producing excessive inflammation as measured by TNF- $\alpha$  production [29]. In this study, we characterized this innate immunity as involving inflammasome activation. The results demonstrate that incubation of PMA-primed THP-1 cells with PmpG-1-vaults can activate caspase-1 and stimulate IL-1 $\beta$  secretion through a process requiring the NLRP3 inflammasome. We found that the cathepsin B inhibitor CA-074 Me could partially inhibit this process. Interestingly, when internalized PmpG-1-vaults were visualized in cells, we found that the vaults co-localize at early times with lysosomes. The lysosomal permeabilization assay showed that the PmpG-1-vaults are in acidic compartments at early times, but then transfer to an environment with neutral pH. Once lysosomes are ruptured, they release proteases such as cathepsin B, which have been previously shown to activate the NLRP3 inflammasome.

Syk also modulates vault-mediated inflammasome activation. While the mechanism for this dependence is not yet known, the Syk kinase is known to be recruited into lipid rafts when phagosomes form [52]. It had also been proposed that MVP is involved in intracellular

transport and concentrated in lipid rafts [53]. Considering that vaults are phagocytosed by cells during incubation, we speculate that PmpG-1-vaults might enter the cells through lipid rafts and then interact with Syk kinase and, simultaneously, lysosomes, in order to activate the NLRP3 inflammasome. Alternatively, the PmpG-1-vaults were engineered with a 33 amino acid-peptide called the “Z” domain. This peptide was derived from a staphylococcal binding domain that can bind the Fc portion of IgG at a site distinct from the binding site for the Fc receptor (FcR). It was also previously shown that vaults with a “Z” domain increase binding of mouse IgG [29]. We expected that these vaults would be internalized through the FcR, which also stimulates the Syk pathway [54]. Further studies should elucidate the mechanisms whereby PmpG-1-vaults can stimulate Syk- and cathepsin B-dependent NLRP3 inflammasome activation.

Taken together, these findings support a model whereby *in vivo* administered vault-vaccines are phagocytosed by antigen presenting cells as we have shown *in vitro* using BMDC [47]. Following internalization, we showed in this study, that incubation of monocytes with PmpG-1-vaults can activate caspase-1 and stimulate IL-1 $\beta$  secretion through a process requiring the NLRP3 inflammasome. Inhibitors of the lysosomal protease, cathepsin B, prevented inflammasome activation, implying that lysosomal disruption likely plays a role in caspase-1 activation. This interpretation is consistent with the observation that the PmpG-1-vaults are internalized through a pathway that is transiently acidic and leads to destabilization of lysosomes. PmpG-1-vault interaction within cells are unique from other reported activators of NLRP3 inflammasomes, in that Syk was also shown to be involved in PmpG-1-vault-induced inflammasome activation, which may be due to vault interactions with lipid rafts. Vault vaccines can also be engineered to induce specific adaptive immunity, as we have shown here that immunization of mice with PmpG-1-vaults induces generation of PmpG-1-responsive CD4<sup>+</sup> cells immune cells. Vaults can also be engineered to deliver drugs and promote anti-tumor responses [26, 27, 29]. These studies define vault-vaccines as unique among other vaccines that induce NLRP3 inflammasomes, such as alum, as they are also able to induce specific marked T cell responses against antigens incorporated in the vault body.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

*C. trachomatis*      *Chlamydia trachomatis*

<b>PmpG</b>	Polymorphic membrane protein G
<b>CARD</b>	Caspase recruitment domain
<b>MOMP</b>	Major outer membrane protein
<b>PAMPs</b>	Pathogen associated molecular patterns
<b>DAMPs</b>	Danger associated molecular patterns
<b>NLR</b>	Nod-like receptor
<b>MSU</b>	Monosodium urate
<b>ASC</b>	Apoptosis-associated speck-like protein containing a CARD
<b>Syk</b>	Spleen tyrosine kinase
<b>PMA</b>	Phorbol 12-myristate 13-acetate

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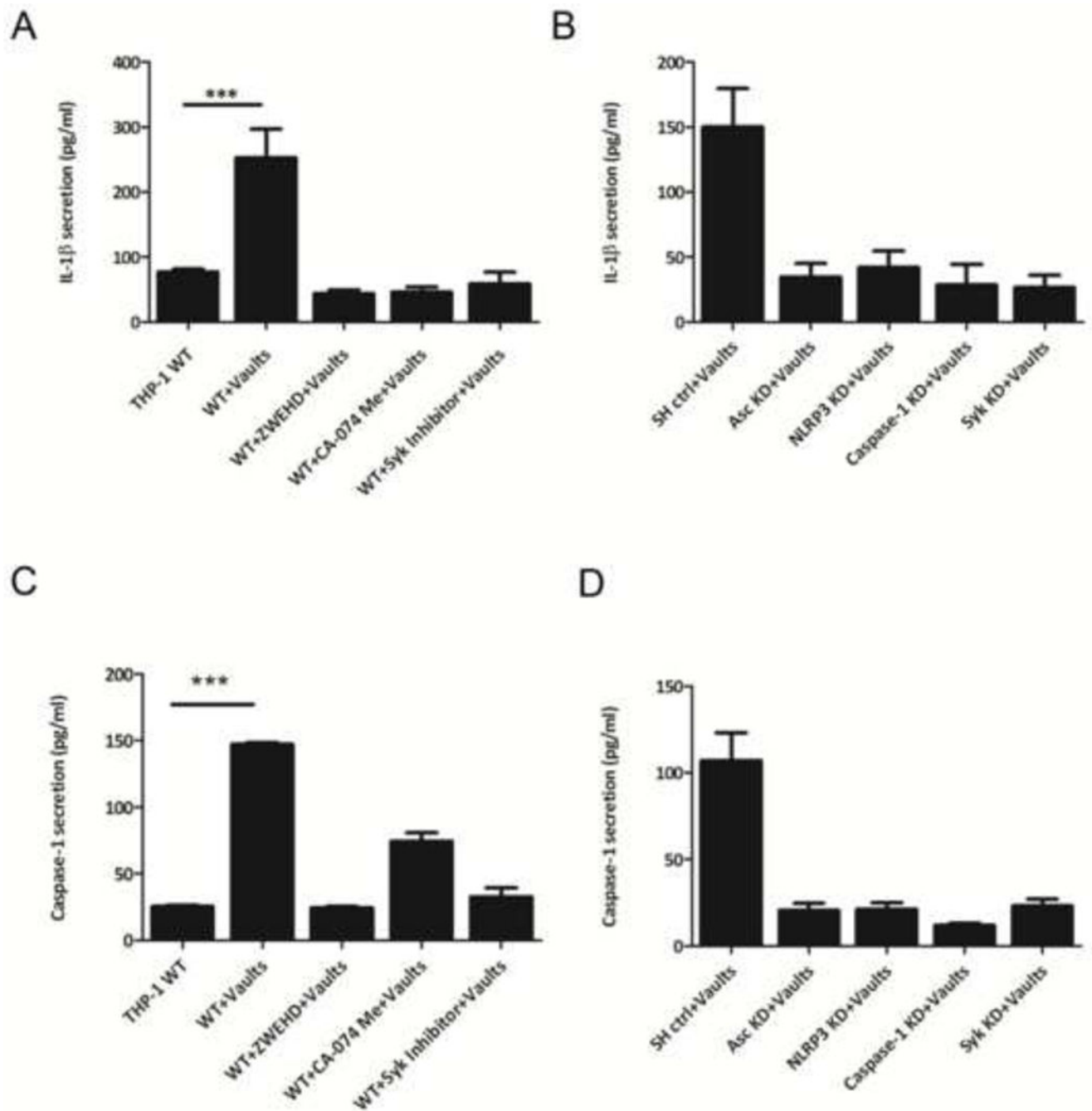
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**HIGHLIGHTS**

- Vault-vaccines activate NLRP3 inflammasomes following phagocytosis
- Syk is necessary for NLRP3 inflammasome activation by vault-vaccines
- Vault-vaccines are internalized into acidic compartments commonly associated with lysosomes
- Vault-vaccines appear in the endocytic pathway following internalization
- Vault-vaccines induce specific T cell responses in vivo following administration without an adjuvant

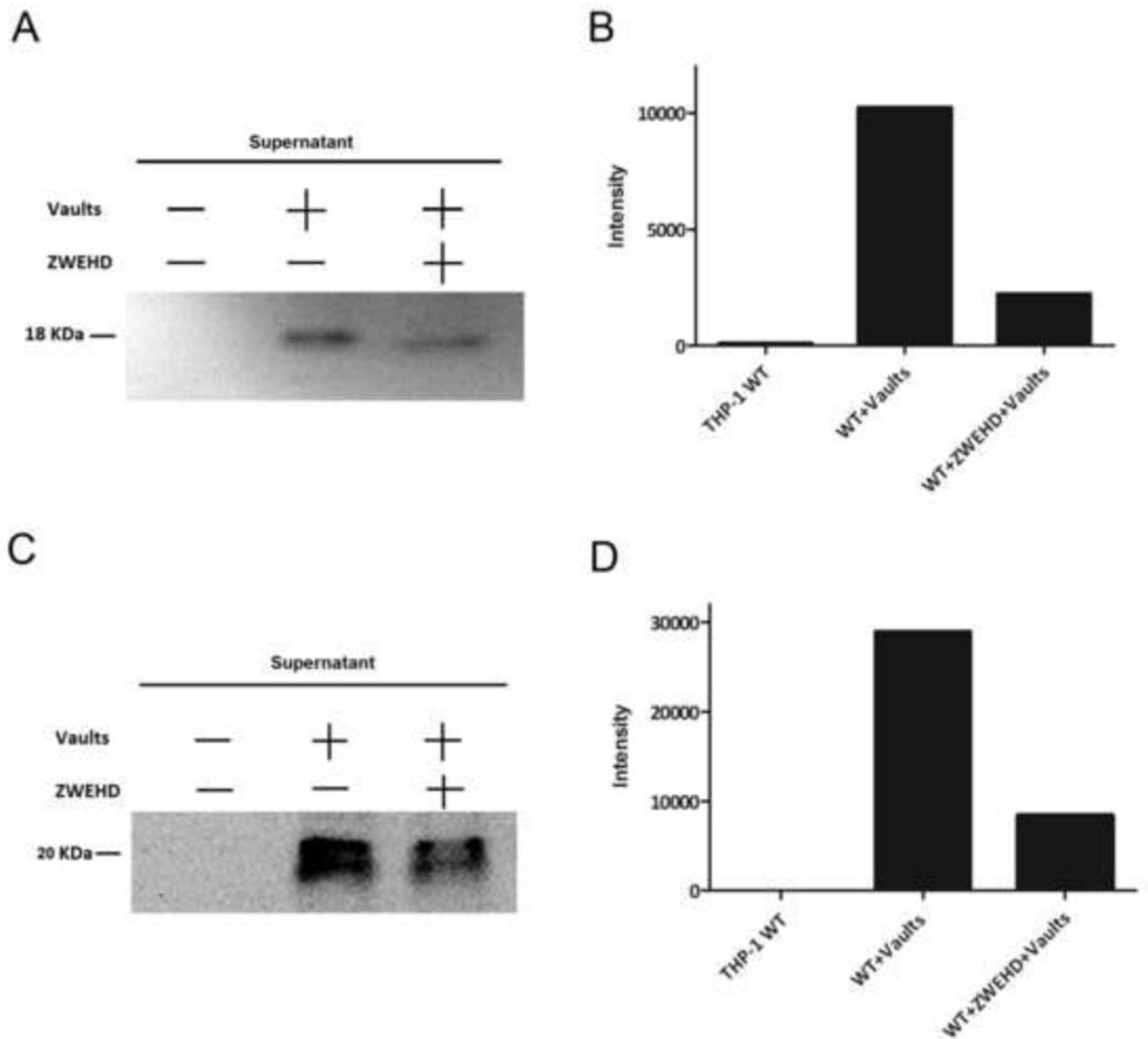


**Figure 1. PmpG-1-vaults activate the NLRP3 inflammasome and induce IL-1 $\beta$  secretion, as measured by an ELISA assay**

THP-1 ( $1 \times 10^6$ ) wild type (WT) cells (A) were incubated in 6-well plates with RPMI 1640 media. Inhibitors of caspase-1 (ZWEHD) or cathepsin B (CA-074) were added individually 1.5 h prior to incubation with PmpG-1-vaults, and the Syk inhibitor was added 0.5 h prior to incubation with PmpG-1-vaults. THP-1 knockdown (KD) cells (B) were incubated with media alone, and 500  $\mu$ g of PmpG-1-vaults were added to each well, except the WT control. Culture supernatants were collected 6 hrs post-incubation and IL-1 $\beta$  was measured by ELISA. IL-1 $\beta$  levels secreted by cells with inhibitors vs cells without inhibitors and by WT

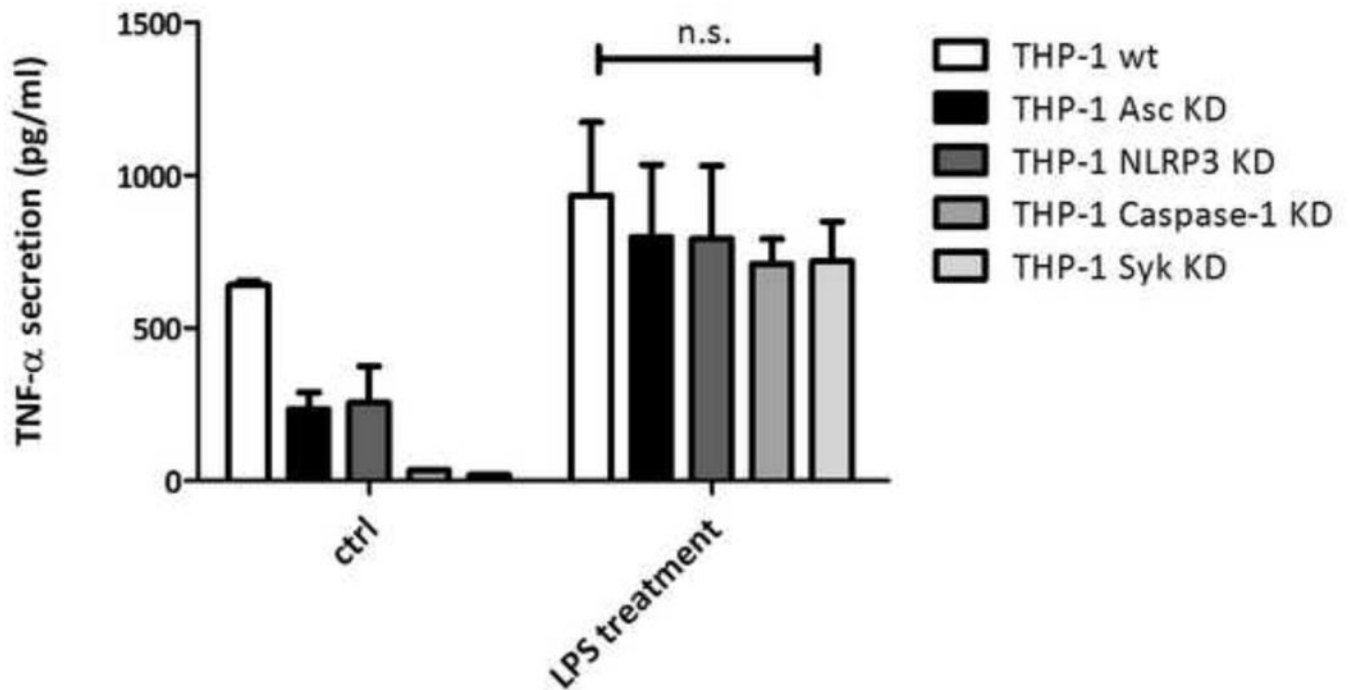


vs KD cells was significantly different ( $p < 0.001$ ). (C) THP-1 ( $1 \times 10^6$ ) WT cells were incubated in 6-well plates. ZWEHD or CA-074 was added individually 1.5 h prior to incubation with PmpG-1-vaults, and the Syk inhibitor was added 0.5 h prior to incubation with PmpG-1-vaults. (D) THP-1 knockdown (KD) cells were incubated with media alone, and 500  $\mu\text{g}$  of PmpG-1-vaults were added to each well, except the WT control. Culture supernatants were collected 6 hrs post-incubation and caspase-1 was measured by ELISA kit. The mean  $\pm$ SD of a representative experiment from six times was analyzed by ANOVA. Caspase-1 levels secreted by cells with inhibitors vs cells without inhibitors and by WT vs KD cells was significantly different ( $p < 0.001$ ). In all cases, cell supernatants were measured in triplicate.

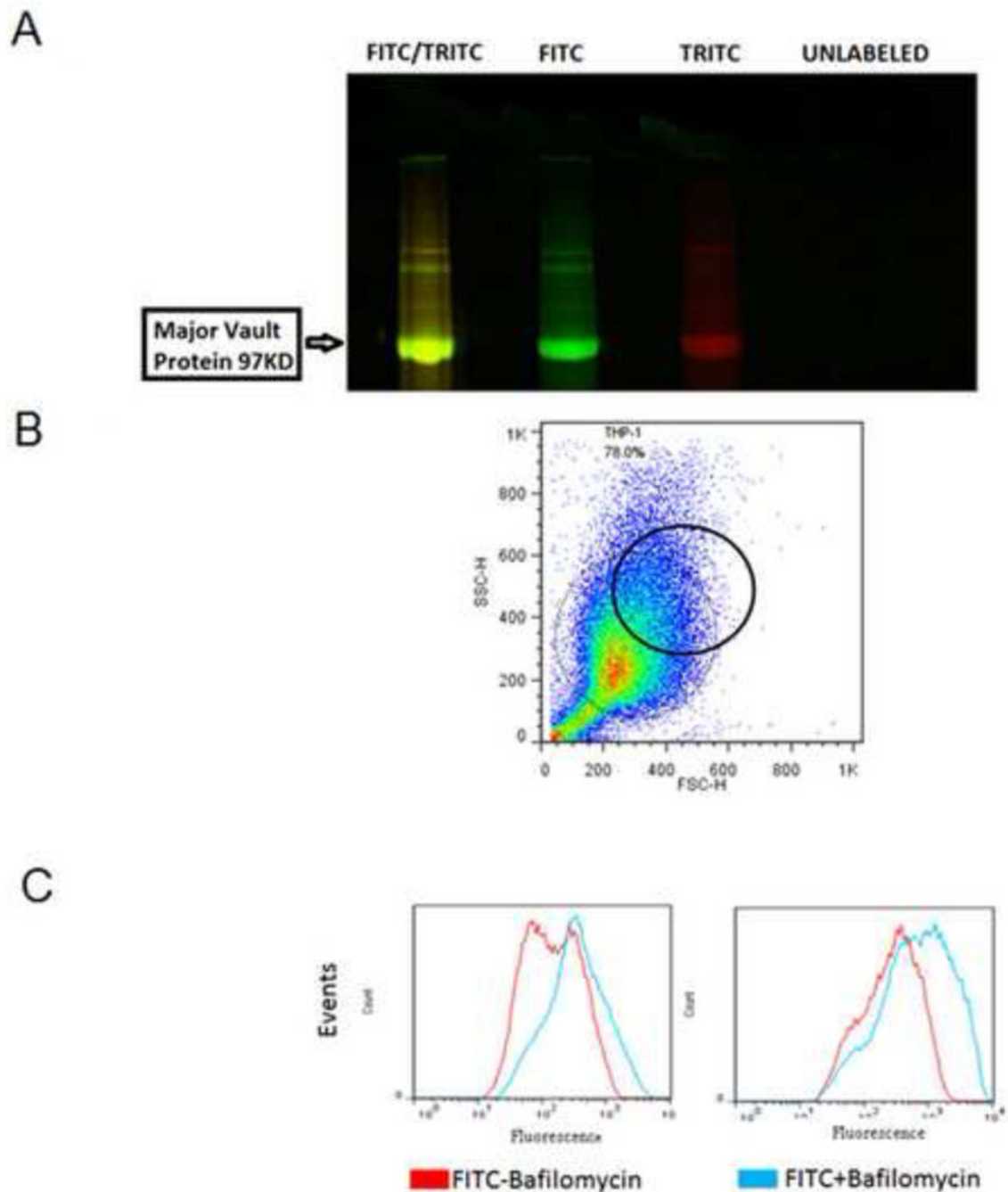


**Figure 2. PmpG-1-vaults activate the NLRP3 inflammasome and caspase-1, as measured by Western blot**

THP-1 ( $1 \times 10^6$ ) wild type (WT) cells (A) were incubated in 6-well plates with RPMI 1640 media. ZWEHD was added 1.5 h prior to incubation with PmpG-1-vaults. THP-1 knockdown (KD) cells (B) were incubated with media alone, and 500  $\mu$ g of PmpG-1-vaults were added to each well, except the WT control. Culture supernatants were collected 6 hrs post-incubation and IL-1 $\beta$  or caspase-1 were detected by Western blot. (A) Western blot of the supernatant probed for IL-1 $\beta$ . (B) Histogram showing the intensity of the bands in the Western blots. (C) Western blot of the supernatant probed caspase-1. (D) Histogram showing the intensity of the bands in the Western blots.

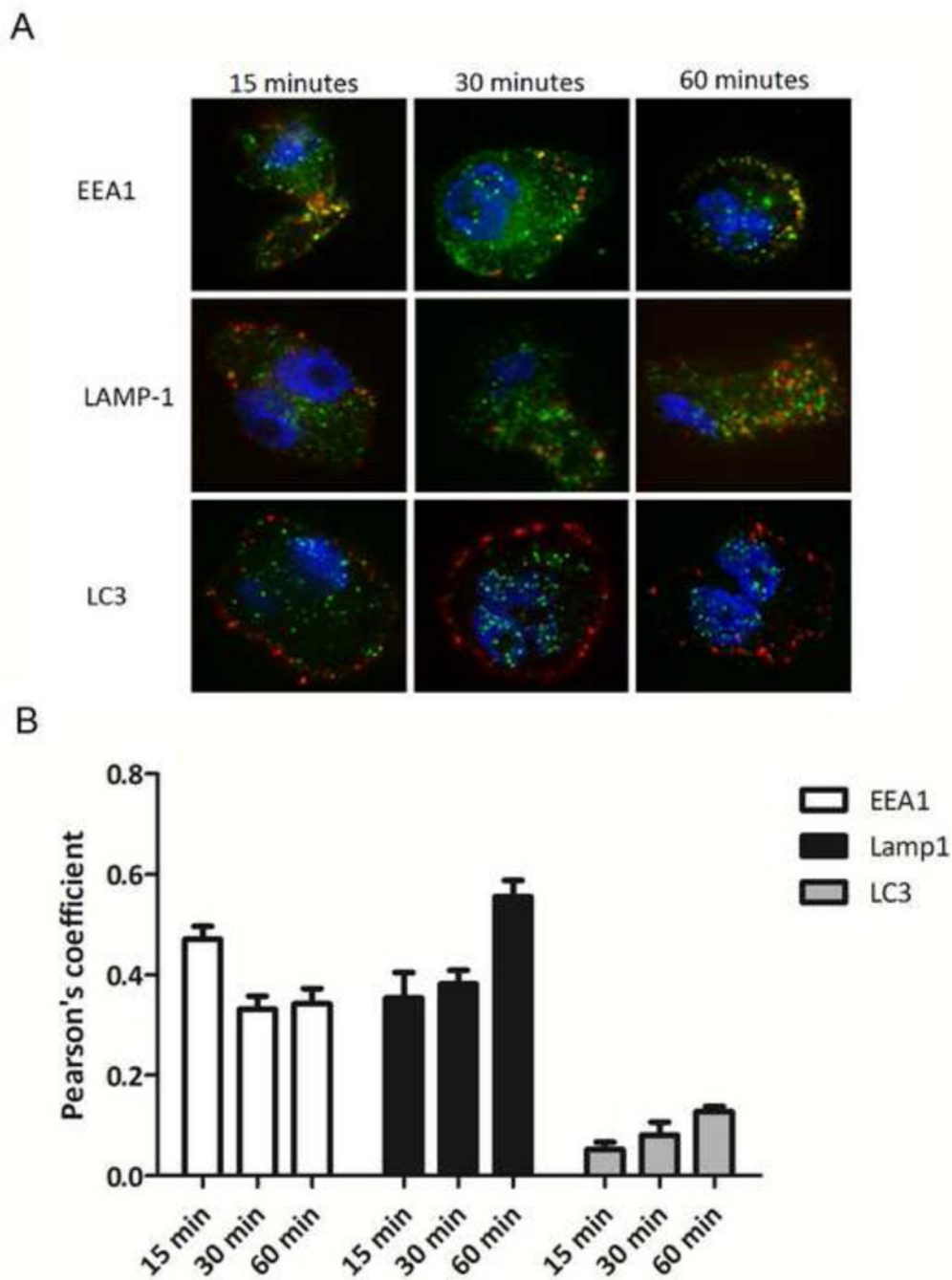


**Figure 3. TNF- $\alpha$  levels were not affected by depletion of inflammasome-related genes**  
 THP-1( $1 \times 10^6$ ) knockdown (KD) cells were incubated in 6-well plates with RPMI 1640 media. LPS (100 ng/ml) was added as stimulator. Culture supernatants were collected 24 hrs post-incubation, and TNF- $\alpha$  was measured by ELISA. TNF- $\alpha$  levels from WT cells were compared to KD cells stimulated by LPS. The values for the WT and KD cells were not statistically significant:  $p < 0.5$  for WT vs caspase-1 KD, and  $p < 0.5$  for WT vs Syk KD cells.

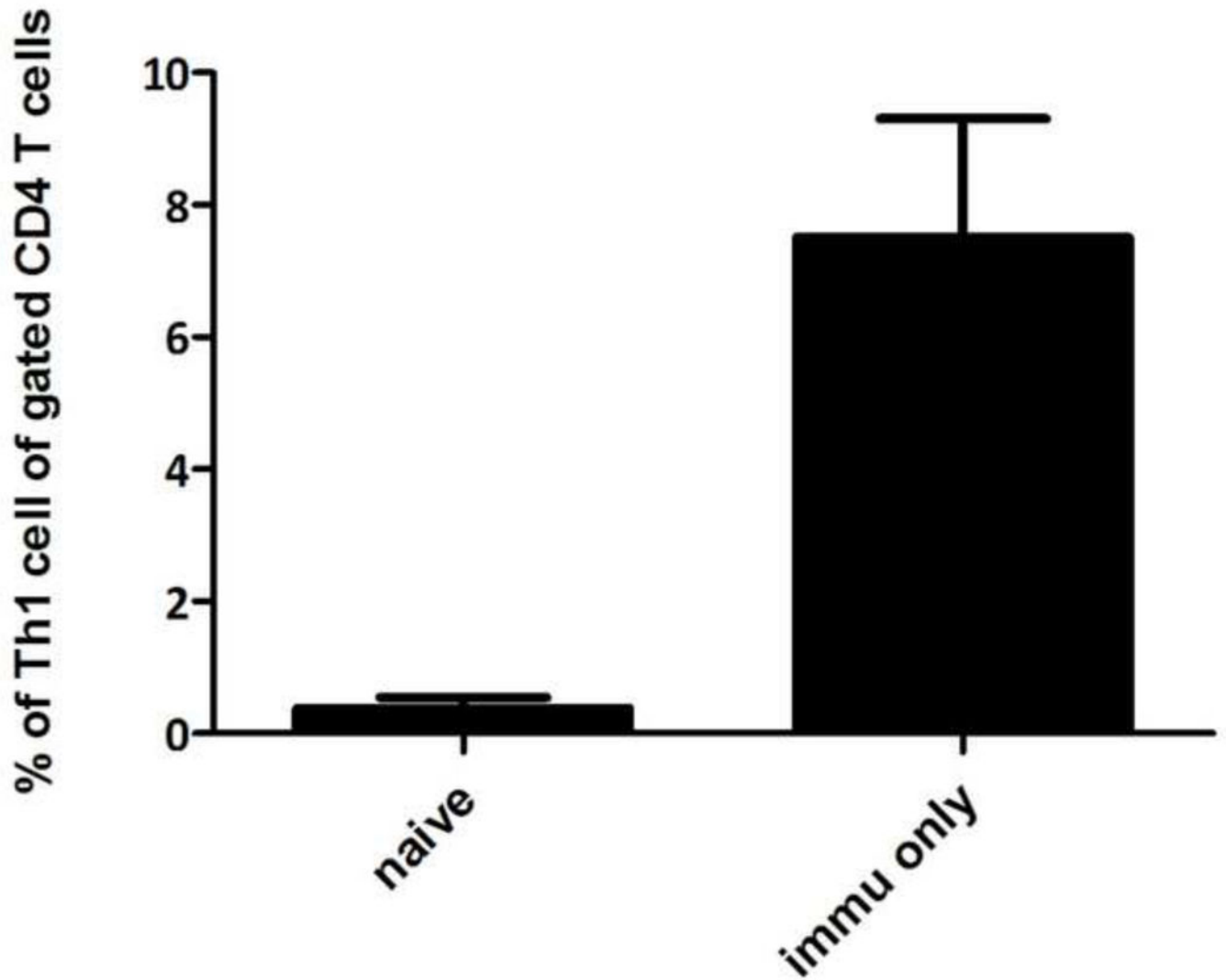


**Figure 4. PmpG-1-vaults are internalized into an acidic compartment**

(A) PmpG-1-vaults dual-labeled with FITC and TRITC fluorophores and incubated with PMA-activated THP-1 cells. (B) Gating scheme (black circle) showing the dot plot of PMA-activated THP-1 cells after PmpG-1-vaults incubation. FITC can only fluorescence when inside acidified chambers and fluorescence can be modified with bafilomycin which prevents re-acidification of vesicles while TRITC constitutively fluoresces. (C) Overlay histogram of FITC-labeled vault fluorescence +/- bafilomycin after 6 hrs and 24 hrs post vault exposure.



**Figure 5. Uptake of PmpG-1-vaults and colocalization within the endocytic pathway**  
 (A) THP-1 cells were grown on 18 mm glass cover slips and treated with 30  $\mu$ g of DyLight 650 labeled PmpG-1-vaults for 15, 30, and 60 min and imaged by confocal microscopy. For immunofluorescence staining, THP-1 cells were reacted with either anti-EEA1 mouse mAb, anti-Lamp1 mouse mAb, or anti-LC3 mouse mAb followed by Alexa Fluor 488-conjugated goat anti-mouse to identify endocytic compartments. (B) Colocalization of PmpG-1-vaults within each compartment was determined by calculation of the Pearson's correlation coefficient of the red and green channels using ImageJ.



**Figure 6. PmpG-1-vaults immunization induces a cellular immune response *in vivo***  
Spleen cells were harvested from naïve mice as well as mice immunized with PmpG-1-vaults containing a total of 15 µg PmpG-1 peptide, 7 days after challenge. Bars indicate percentage of CD3<sup>+</sup>CD4<sup>+</sup>IFNγ<sup>+</sup> (Th1) cells out of CD4<sup>+</sup> cells following *in vitro* stimulation with PmpG-peptide (mean % ± SEM). n=4, p<0.001 by Student's t-test.