Supplementary Information for
Electrostatic influence on IL-1 transport through the GSDMD pore

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Materials and Methods

Structure and Electrostatics Analysis. To simulate cargo transport through the GSDMD pore, we used the cryo-EM structure of GSDMD (PDB: 6VFE), the crystal structure of IL-1β (PDB: 811B), and a molecular model of pro-IL-1β generated using the I-TASSER server based on the crystal structure of IL-1β. Models of charge-mutant GSDMD (GSDMD AP1 and AP2 mutants) and pro-IL-1β (pro-IL-1β AP1 and AP2' mutants) were generated using in silico mutagenesis using the MOLARIS-XG (version 9.15) simulation package (1). All structural representations were generated using the PyMOL Molecular Graphics System. Electrostatic potential surfaces shown in Fig 1 were calculated using the Adaptive Poisson-Bolzmann Solver (APBS) plugin in PyMOL.

Setup of Cargo Transport Model. To reduce computational cost, we used coarse-grained models of the wildtype or charge-mutant GSDMD pore and IL-1β cargoes. The coarse-grained representations were constructed using MOLARIS-XG (version 9.15) (1) with main chains treated explicitly and side chains described by a united-atom model. Coarse-grained modeling in MOLARIS-XG has been improved with a focus on the description of protein electrostatics (2, 3). To account for neutralization of basic lipid-binding residues in GSDMD pore by acidic lipids in the membrane, we immersed the pore into a 320 × 320 × 80 Å³ membrane grid with grid particles separated by 5 Å spacing. For each lipid-binding residue in GSDMD, its nearest membrane particle was assigned a partial charge (−γQ) to mimic acidic lipids, where γ is the lipid-binding degree and Q is the charge of the lipid-binding residue.

We first modelled cargo transport under a simplistic scenario where the cargo trajectory follows the central axis of the pore (Z-axis) (Fig. 1C). In this case, we positioned the center-of-mass of the GSDMD pore at the origin. The cargo started from 20 nm above the origin (intracellular, hereafter -20 nm) to 20 nm below the origin (extracellular, hereafter 20 nm) and moved with 0.5 Å spacing. We also simulated the transport of pro-IL-1β and mature IL-1β through the three-dimensional GSDMD pore conduit (Fig. 1D). The accessible space for the cargo in this case is a cylinder covering the pore conduit in x-y directions with a height of 40 nm in the Z-direction (from -20 nm to 20 nm). Similarly, a spacing of 0.5 Å is adopted in each direction.

Calculation of Free Energy. After the coarse-graining setup of a cargo transport system, we performed an energy minimization run followed by a relaxation run until the system was fully relaxed, using the MOLARIS-XG package (1). To reliably evaluate the charges of ionized residues, a Monte Carlo proton transfer (MCPT) (2) was adopted for all ionizable residues. The MCPT uses a Monte Carlo procedure to perform proton transfer between charged groups, update their charge configurations, and optimize charge distributions. After that, both the pore and cargo were treated as rigid body. The whole GSDMD-membrane system was then used to calculate free energy during cargo transport. Free energies expressed in kcal/mol were calculated after the MCPT as ΔG = 332Σi,j Qijrijefffij explained in the main text (4). The parameter α in the electrostatic screening factor fij = exp(−rijα √V) is initially parameterized to reproduce the folding free energy of the coarse-grained protein which corresponds to an effective Debye length of 2.3 nm. It turns out to work well in the system studied here.

For Scenario 1, each cargo was randomly rotated 10 times. For each orientation, cargo with fixed orientation was used to calculate free energy values during its transport. The averaged results over different cargo orientations were reported. For Scenario 2, a randomly chosen cargo orientation was used to demonstrate the result is consistent with Scenario 1.

Calculation of Cargo Release Rate. We performed kinetic simulations to quantify the cargo release rate under two scenarios – cargo trajectory overlapping with the pore central axis (Scenario 1, Fig. 1C) and cargo trajectory covering a three-dimensional space that represents the pore conduit (Scenario 2, Fig. 1D). The master equation is shown in the main text. For Scenario 1, in each step, a Monte Carlo move of one spacing (0.5 Å) was proposed with equal probability to move forward or backward, and the Metropolis algorithm (5) was employed to accept the move with probability paccept = min[1, exp(−(ΔGnew − ΔGold)/kBT)], where T is set to room temperature (298K), k_B is the Boltzmann constant, ΔGnew and ΔGold are free energies of the system at the new and old positions, respectively. Each system under Scenario 1 was simulated using 10 random cargo orientations, 10,000 trajectories, and 1,000,000 maximum Monte Carlo moves. However, for Scenario 2, the cargo trajectory is not limited to the central axis of the pore and can cover the entire accessible space of the pore conduit, which is a more realistic scenario. Therefore, we performed kinetic simulations to quantify the cargo release rate only for Scenario 2.
Carlo steps per trajectory, and averaged results were reported. The cargo release rate is quantified as the reverse of MFPT (6), whose unit is the number of Monte Carlo steps. For scenario 2, the cargo was proposed move to one of the six directions \((\pm x, \pm y, \pm z)\) for 0.5 Å for each Monte Carlo step with equal probability. Similarly, the Metropolis algorithm was employed to accept the move. Each system under Scenario 2 was tested with a total of 1,000 simulated trajectories and 1,000,000 maximum Monte Carlo steps per trajectory. For both Scenarios, the reflecting boundary condition and the adsorbing boundary condition were adopted at the starting point and the ending point of pore, respectively. The code for Scenario 2 is available on Github; simplifying it to Scenario 1 is straightforward.

**Molecular Cloning.** The full-length human GSDMD coding sequence was cloned into the pDB.His.MBP vector between Ndel and Sall restriction sites. The His6-MBP tag was attached at the N-terminus of GSDMD and followed by a linker cleavable by tobacco etch virus protease (TEV). Residues 259 through 275 at the flexible loop between GSDMD-NT and GSDMD-CT were replaced the cleavage site sequence of human rhinovirus 3C protease (3C) (LEVLFQ/GP). This construct is functionally equivalent of WT GSDMD given that the inserted 3C cleavage site is C-terminal to the pore-forming GSDMD-NT fragment. We therefore use GSDMD to refer to this construct for simplicity. The coding sequence for full-length murine pro-IL-1β were inserted into a modified pET28a bacterial expression vector immediately following the N-terminal His6-SUMO tag between Ndel and NotI restriction sites. The sequence coding the pro-domain of pro-IL-1β was then truncated to generate a vector expressing His6-SUMO-tagged mature IL-1β. All plasmid sequences were verified before use.

**Protein Purification.** The plasmid encoding GSDMD was transformed into *E. coli* BL21 (DE3) competent cells. Successful transformants were selected on Luria broth (LB) agar plates containing 50 μg/mL kanamycin, and colonies were grown in LB medium at 37 °C under vigorous shaking. At an OD_{600} of around 0.6, the cultures were cooled to 18 °C and 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. After overnight incubation, *E. coli* cells were harvested by centrifugation at 3,500 rpm for 30 min, washed with PBS, frozen with liquid nitrogen, and stored at -80 °C. Frozen cell pellets were fully thawed and resuspended in Buffer A (50 mM HEPES at pH 7.0, 150 mM NaCl) supplemented with 5 mM imidazole and sonicated for cell lysis. MBP-tagged GSDMD was enriched by affinity using Ni-NTA beads (Qiagen) and eluted off the bead by Buffer A supplemented with 500 mM imidazole. The eluate was further purified by gel filtration using the Superdex 200 (10/300) column (GE Healthcare Life Sciences). Plasmids encoding IL-1β, pro-form or mature form, were transformed into *E. coli* BL21 (DE3) RipL competent cells for expression in LB medium supplemented with 50 μg/mL kanamycin. Protein expression was induced with 0.2 mM IPTG when OD600 reached 0.8 and the culture was then incubated at 18 °C overnight with shaking. The *E. coli* cells were then pelleted by centrifugation at 3,500 rpm for 30 min and resuspended in Buffer A for sonication. After cell lysates were clarified with centrifugation, Ni-NTA beads (Qiagen) were added to the supernatant to bind His$_6$-SUMO-tagged proteins for enrichment. The His$_6$-SUMO tag was cleaved off on Ni-NTA column using His$_6$-tagged ULP1 protease overnight at 4 °C. Flowthrough containing a tag-free IL-1 family protein was then concentrated and further purified by gel filtration using the Superdex 75 (10/300) column (GE Healthcare Life Sciences) equilibrated with Buffer A. Only fractions under the chromatography peaks were collected for experiments to maximize protein quality, and SDS-PAGE was performed to confirm protein purity.

**GSDMD-dependent IL-1β Release from Liposomes.** We adopted previously established protocols to measure the release of IL-1β from GSDMD-permeabilized liposomes (7, 8). Briefly, freshly purified tag-free IL-1β, pro-form and mature together, at a final concentration of around 1 μM each, were loaded into liposomes composed of 25% acidic lipids 1,2-dioleoyl-sn-glycero-3-phospho-l-serine (PS) and 75% 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphocholine (PC). The liposomes were formulated by hydration of dried lipid films (Avanti Polar Lipids) in Buffer A containing the cargoes. Unloaded proteins were removed by pelleting the liposomes at 20,000 g for 15 min in a cold centrifuge and replacing the supernatant with Buffer A. We used a previously determined sub-lytic concentration of purified GSDMD (0.5 μM) to ensure that IL-1β was released through the pores rather than lysed liposomal membranes (7). Resuspended liposomes containing pro-form and mature IL-1β were resuspended in Buffer A. Two negative controls were included in the experiments – no MBP-GSDMD addition, and addition of only MBP-GSDMD but not the activating enzyme (En, which includes TEV and 3C). The experimental group was co-addition of MBP-GSDMD and the enzyme. One aliquot was pelleted before the reaction, and the pellet was washed 3 times and
resuspended to the original volume with Buffer A, as a measure of total IL-1 family proteins loaded into the liposomes. At indicated time points, the liposomes were pelleted, and the supernatants collected. 4x SDS buffer was added to the harvested pellet or supernatants and the samples boiled for 5 min for SDS-PAGE. The resolved proteins were semi-dry transferred to a PVDF membrane (MilliporeSigma) for standard immunoblotting procedures using the TBST buffer (150 mM NaCl, 20 mM Tris-HCl at pH 7.6, 0.1% Tween-20). The primary antibodies used to detect IL-1β was goat polyclonal anti-IL-1β (AF-401-NA, R&D Systems). The incubation conditions were 4 °C overnight and room temperature for 1 h, respectively. The secondary antibody was HRP-conjugated anti-goat IgG used at room temperature for 2 h. The Pierce ECL western blotting substrate (Thermo Fisher Scientific) was used for detection.

**Timescale Estimation for Single Cargo Release from Experimental Data and Theory.** Experimentally, the initial/maximum release rate of mature IL-1β, which we assume does not encounter an energy barrier, was 294%/h, or 0.082%/s, according to Extended Data Fig. 8i in this paper (7). Therefore, on average, a mature IL-1β molecule needs the following amount of time to escape from a 1 µm radius liposome with 2,500 cargo molecules and 750 pores. For the derivation of the number of cargoes and pores per liposome, see below (Tables S3 and S4).

$$T = \frac{1 \text{ s}}{0.082\% \times N_{\text{cargo}}} = \frac{1 \text{ s}}{0.082\% \times 2500} = 0.48 \text{ s}$$

Theoretically, the time for a mature IL-1β to diffuse from the liposome center to any of the 750 pores is the following, based on an established theory for Brownian motion (9) using a diffusion constant (D) estimated for a protein of 30 kD molecular weight in water (Table S3).

$$T = \frac{R_{\text{liposome}}^2}{6D \times \text{Probability of finding a pore}} = \frac{R_{\text{liposome}}^2}{6D \times \frac{\pi \times R_{\text{pore}}^2 \times N_{\text{pore}}}{4 \times \pi \times R_{\text{liposome}}^2}} = \frac{1}{\frac{0.01^2 \times 750}{4 \times 1^2}} = \frac{1}{600} \times 0.01 \times 750 = 0.09 \text{ s}$$
Fig. S1. Illustration of membrane lattice points assigned negative charges. One subunit of the GSDMD pore is shown in cyan and two orientations. The lipid-binding residues in the coarse-grained model are shown as green spheres. Membrane lattice points near the lipid-binding residues and assigned negative charges are shown as black spheres.
Fig. S2. Distribution of cargo along the XY plane of the pore. Profiles of pro-IL-1β (A and C) and mature IL-1β (B and D) are shown. r is the distance from the pore center in the x-y plane in nm; P(r) is the probability of finding a cargo at a specific r. Because the area of XY cross-section circle scales with r, P(r)/r eliminates the geometric effect. The pore center's electrostatics is relatively homogeneous in comparison with the periphery; therefore, P(r) scales linearly with r when r < ~6 nm. The cargo starts to experience a stronger interaction with the pore near the pore periphery (~6 nm < r < 7 nm). With r > 7 nm, the cargo may clash with the pore, hence the steep drop in probability.
Fig. S3. Larger Debye length is necessary for electrostatic filtering. The MFPT was obtained from 3D kinetic simulation for pro- and mature IL-1β with two different Debye lengths. There is no selectivity at the Debye length of 0.78 nm, which cannot explain the experiment.
**Fig. S4.** Interaction free energy values calculated using a Debye length of 2.3 nm for cargos traveling at two off-center positions in the XY plane, x = 3 and 6: the z-direction trajectories are 3 nm and 6 nm away from the central z-axis (x = 0), respectively.
**Fig. S5.** Interaction free energy values calculated using a Debye length of 0.78 nm for cargos traveling at two off-center positions in the in the XY plane. $x = 3$ and $7$: the z-direction trajectories are 3 nm and 7 nm away from the central z-axis ($x = 0$), respectively.
Fig. S6. Schematic picture of cargo release through the GSDMD pore. Cargo release is composed of at least two processes. First, the cargo must find the pore conduit by Brownian motion within the liposome. Second, the cargo needs to go through the pore conduit. Our simulations in this study only consider the second process, which is mainly influenced by electrostatic filtering. We estimated the timescale for the first process and presented back-of-the-envelope calculations in the Supporting Information (see above, Timescale Estimation for Single Cargo Release from Experimental Data and Theory).
Table S1. The MFPT at fixed (x,y) in the XY plane with the Debye length of 2.3 nm

| Cargo          | (x,y) (nm,nm) | Debye length (nm) | MFPT (MC step) |
|----------------|---------------|-------------------|-----------------|
| Pro-IL-1β      | (0,0)         | 2.3               | 9039            |
| Pro-IL-1β      | (3,0)         | 2.3               | 12477           |
| Pro-IL-1β      | (6,0)         | 2.3               | 209588          |
| Mature IL-1β   | (0,0)         | 2.3               | 5944            |
| Mature IL-1β   | (3,0)         | 2.3               | 5908            |
| Mature IL-1β   | (6,0)         | 2.3               | 6683            |
| Cargo     | (x,y) (nm,nm) | Debye length (nm) | MFPT (MC step) |
|-----------|---------------|-------------------|----------------|
| Pro-IL-1β | (3,0)         | 0.78              | 6196           |
| Pro-IL-1β | (6,0)         | 0.78              | 6224           |
| Pro-IL-1β | (7,0)         | 0.78              | 6295           |
| Mature IL-1β | (3,0)     | 0.78              | 6227           |
| Mature IL-1β | (6,0)    | 0.78              | 6167           |
| Mature IL-1β | (7,0)    | 0.78              | 6210           |
Table S3. Given parameters to estimate the timescale for cargo release

| Parameter | Value | Unit  | Reference | Note                                                                 |
|-----------|-------|-------|-----------|----------------------------------------------------------------------|
| $D$       | 100   | $\mu$m$^2$/s | [http://book.bionumbers.org/what-are-the-time-scales-for-diffusion-in-cells/](http://book.bionumbers.org/what-are-the-time-scales-for-diffusion-in-cells/) | Protein (~30 kDa) diffusion in water                                   |
| $R_{liposome}$ | 1     | $\mu$m | (10)      | Unextruded liposomes vary from very small (~0.025 $\mu$m) to large (~2.5 $\mu$m) in radii |
| $h$       | 0.5   | nm    | [https://bionumbers.hms.harvard.edu/bionumber.aspx?id=105298](https://bionumbers.hms.harvard.edu/bionumber.aspx?id=105298) | Thickness of lipid bilayer                                             |
| $A_{lipid}$ | 0.5   | nm$^2$ | [https://bionumbers.hms.harvard.edu/bionumber.aspx?id=106993](https://bionumbers.hms.harvard.edu/bionumber.aspx?id=106993) | Surface area per head group of a lipid molecule                       |
| $C_{lipid}$ | 1     | mM    | This study (7) | Lipid (monomer) concentration used experimentally                     |
| $C_{GSDMD}$ | 0.5   | $\mu$M | This study (7) | GSDMD (monomer) concentration used experimentally                     |
| $C_{cargo}$ | 1     | $\mu$M | This study (7) | IL-1 concentration used experimentally                                 |
| $N_{GSDMD}$ | 33    |       | (7)       | Number of GSDMD molecules per pore                                    |
| $R_{pore}$ | 10    | nm    | (7)       | GSDMD pore radius                                                     |
Table S4. Derived parameters to estimate the timescale for cargo release

| Parameter | Value   | Unit     | Derivation                                                                 | Note                                                                 |
|-----------|---------|----------|-----------------------------------------------------------------------------|----------------------------------------------------------------------|
| $N_{\text{lipid}}$ | $5 \times 10^7$ | $\text{lipid}$ | $N_{\text{lipid}} = \frac{N_0}{A_{\text{lipid}}}$ | Number of lipid molecules per liposome (1 $\mu$m radius) |
| $V_{\text{liposome}}$ | 4.19 | $\mu$m$^3$ | $V_{\text{liposome}} = \frac{4}{3} \pi R_{\text{liposome}}^3$ | Volume of liposome (1 $\mu$m radius)                                  |
| $C_{\text{liposome}}$ | $2 \times 10^{-5}$ | $\mu$M | $C_{\text{liposome}} = \frac{C_{\text{lipid}}}{N_{\text{lipid}}}$ | Liposome (polymer) concentration                                    |
| $C_{\text{pore}}$ | 0.015 | $\mu$M | $C_{\text{pore}} = \frac{C_{\text{GSDMD}}}{N_{\text{GSDMD}}}$ | Pore (33-mer) concentration                                       |
| $N_{\text{pore}}$ | 750 |           | $N_{\text{pore}} = \frac{C_{\text{pore}}}{C_{\text{liposome}}}$ | Average number of pores per liposome (1 $\mu$m radius) |
| $N_{\text{cargo}}$ | 2500 |           | $N_{\text{cargo}} = \frac{C_{\text{cargo}} \ast V_{\text{liposome}} \ast \text{Avogadro's number}}{}$ | Average number of cargoes per liposome (1 $\mu$m radius) |
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