

1 **The conical shape of DIM lipids promotes *Mycobacterium***
2 ***tuberculosis* infection of macrophages**

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23

24 **Abstract**

25 Phthiocerol dimycocerosate (DIM) is a major virulence factor of the pathogen
26 *Mycobacterium tuberculosis* (*Mtb*). While this lipid promotes the entry of *Mtb* into
27 macrophages, which occurs via phagocytosis, its molecular mechanism of action is
28 unknown. Here, we combined biophysical, cell biology, and modelling approaches to
29 reveal the molecular mechanism of DIM action on macrophage membranes leading to
30 the first step of *Mtb* infection. MALDI-TOF mass spectrometry showed that DIM
31 molecules are transferred from the *Mtb* envelope to macrophage membranes during
32 infection. Multi-scale molecular modeling and ³¹P-NMR experiments revealed that DIM
33 adopts a conical shape in membranes and aggregate in the stalks formed between
34 two opposing lipid bilayers. Infection of macrophages pre-treated with lipids of various
35 shapes uncovered a general role for conical lipids in promoting phagocytosis. Taken
36 together, these results reveal how the molecular shape of a mycobacterial lipid can
37 modulate the biological function of macrophages.

38

39 INTRODUCTION

40 Phthiocerol dimycocerosate (DIM/PDIM) are highly hydrophobic lipids
41 containing two multiple-methyl-branched fatty acid chains (**Fig. 1-a**). These lipids are
42 mostly found in the cell wall of pathogenic mycobacteria and are particularly abundant
43 in *Mycobacterium tuberculosis* (*Mtb*)¹, the causative agent of tuberculosis. They
44 constitute one of the main *Mtb* virulence factors². Indeed, *Mtb* strains lacking DIM are
45 drastically attenuated³ and are more likely to be killed by the early pulmonary innate
46 immune response⁴, when the bacteria encounter macrophages. Recent work has
47 revealed that DIM modulate macrophage metabolism⁵ and immune functions^{6,7}. In
48 particular, DIM increase the ability of *Mtb* to infect macrophages by modulating
49 phagocytosis⁸, a fundamental immune process involving membrane remodeling.
50 However, how DIM intervene in these cellular processes remains poorly understood.

51 *Mtb* synthesizes a large variety of lipid virulence factors, most of which are
52 amphipathic glycolipids. These glycolipids act through their saccharide domains as
53 potential ligands for membrane receptors on macrophages to induce *Mtb* phagocytosis
54 ⁹. Lacking a saccharide moiety, DIM cannot engage in such interactions. In contrast,
55 the molecular mechanism involving DIM may be related to a global effect on the
56 physical properties of the host cell membrane, such as its fluidity and organization⁸.
57 Modifying such properties can be a successful strategy for bacteria to modulate
58 eukaryotic cell functions. Several types of pathogenic mycobacteria apply this strategy
59 to influence the fate of their host cells. For example, *M. ulcerans* produces the lipid-
60 like endotoxin mycolactone which interacts with host membranes and disturbs their
61 lipid organization¹⁰. In addition, pathogenic mycobacteria use lipoarabinomannan to
62 enter neutrophils and prevent phagolysosome formation¹¹.

63 The biophysical properties of DIM in biological membranes not yet been
64 characterized at the molecular level. In particular, it is unclear if such a complex and
65 large lipid can be incorporated in a simple phospholipid bilayer, and what shape DIM
66 must adopt in such a membrane. The shape of lipid molecules, determined by
67 structural properties¹² like their head group size, acyl chain lengths and degrees of acyl
68 chain unsaturation, can drastically affect the structure and organization of biological
69 membranes^{13,14}. Studying how the molecular shape of lipids may disorganize lipid
70 bilayers and how this can be related to biological function is still a challenge¹⁵. It
71 requires linking the structure of molecules and their biophysical actions at the
72 nanoscale to macroscopic consequences on the cell functions. To achieve this for DIM,

73 we developed a multidisciplinary approach combining multiscale Molecular Dynamics
 74 (MD) simulations, solid-state NMR and cell biology experiments. This revealed how
 75 the molecular shape of DIM can affect macrophage membranes to promote
 76 phagocytosis.
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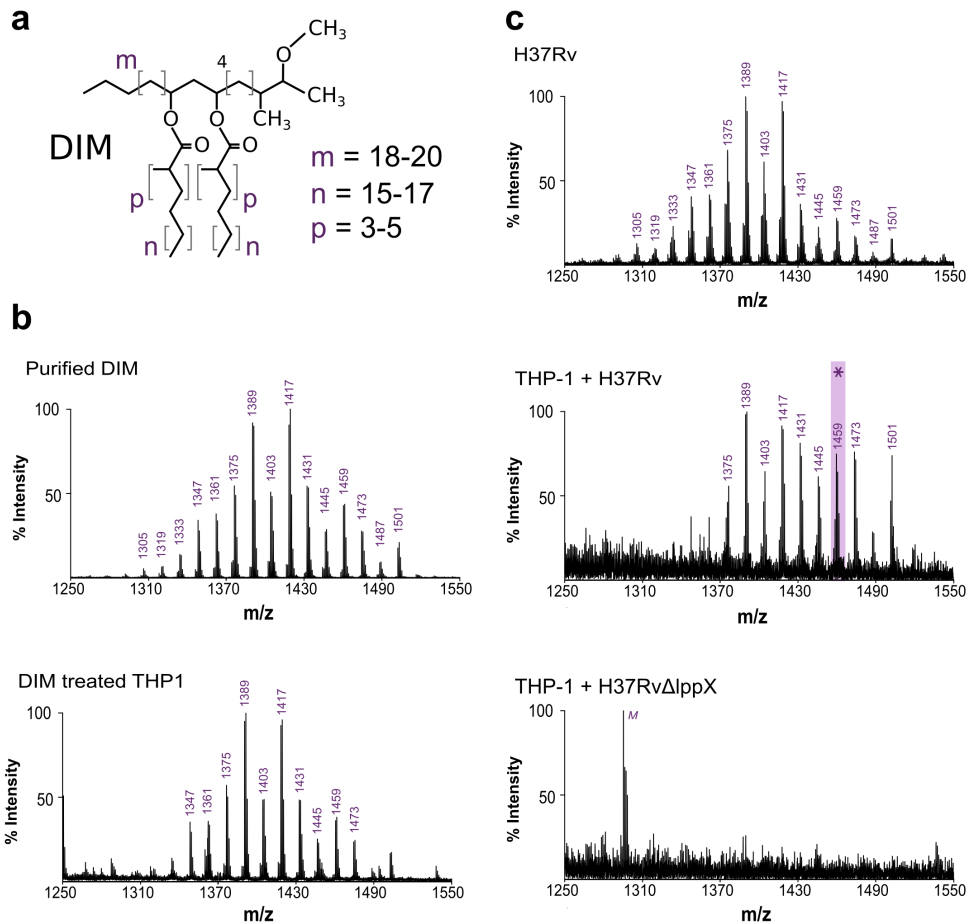


Figure 1: DIM are transferred from the bacterial envelope to macrophage membranes. (a) Structure of the DIM family of lipids, where m denotes the range of carbon atoms on the phthiocerol moiety, and n and p on the mycocerosate moieties. (b) MALDI-TOF mass spectra of purified DIM and of the membrane fraction of macrophages treated with DIM. (c) MALDI-TOF mass spectra of WT Mtb (HR37v) and of the membrane fraction of macrophages infected by H37Rv or by the H37RvΔlppX mutant. M: low intensity peak corresponding to the detection of the matrix molecule in the DIM region of interest. The star symbol highlights the mass of the DIM molecule chosen for the modelling, with m=18, n=17, and p=4.

78

79 RESULTS

80

81 DIM are transferred to host cell membranes during macrophage infection

82 First, we used MALDI-TOF mass spectrometry to assess whether DIM added
 83 to host cells is incorporated into their membranes. Human macrophage (THP-1) cells

84 were treated with purified DIM, and the mass spectrum of the extracted lipids was
85 compared with the spectrum of purified DIM. The structure of DIM consists of a long
86 chain of phthiocerol (3-methoxy, 4-methyl, 9,11-dihydroxy glycol) esterified with two
87 mycocerosic acids (long-chain multiple-methyl-branched fatty acids) (**Fig. 1-a**). In
88 agreement with the MycoMass database¹⁶, the purified DIM mass spectrum is
89 characterized by a cluster of pseudomolecular ions $[M + Na]^+$ between $m/z = 1305$ and
90 $m/z = 1501$, in increments of $m/z = 14$ (**Fig. 1-b**) reflecting the variability of chain
91 lengths and methylations of the molecule. We observed that the spectrum of the
92 extracted lipids from DIM-treated THP-1 cells showed a very similar series of peaks to
93 that of purified DIM (**Fig. 1-b**). These peaks were absent in the spectrum of lipid
94 extracts from untreated cells (**Fig. S1-a**). Hence, exogenously delivered DIM can be
95 inserted into macrophage membranes and were detectable by MALDI-TOF mass
96 spectrometry.

97 We next investigated if DIM could be transferred from the *Mtb* envelope to
98 macrophage membranes during infection. To test this, we infected THP-1
99 macrophages with the WT *Mtb* strain H37Rv for 2 h at a multiplicity of infection (MOI)
100 of 15:1. At 40 h post-infection, the membrane fraction of the infected macrophages
101 showed a mass spectrum similar to the lipid signature of DIM isolated from the H37Rv
102 inoculum (**Fig. 1c**). We noticed a distinct shift towards longer DIM chain lengths
103 consistent with the reported increase in molecular mass of DIM during *Mtb* infection¹⁷.
104 The residual bacterial contamination of the macrophage membrane fractions was less
105 than 1500 cfu, well below the threshold for detection of DIM extracted directly from
106 bacteria (between 10^5 and 10^7 cfu, see **Fig. S1b**). Our data therefore strongly support
107 the model that DIM is transferred from *Mtb* to the membranes of infected macrophages.

108 To verify whether DIM exposure at the surface of *Mtb* is required for their
109 transfer to macrophage membranes, we infected macrophages with a mutant strain
110 (H37Rv Δ lppX) lacking LppX, a lipoprotein required for the translocation of DIM to the
111 outer membrane of *Mtb*¹⁸. After infection, we did not observe the typical mass spectrum
112 of DIM in the membrane fraction of H37Rv Δ lppX-infected cells (**Fig. 1c**). We verified
113 that the WT and mutant strains produced similar amounts of DIM (**Fig. S1b**).

114 Taken together, these results demonstrate that DIM molecules are indeed
115 transferred to the membranes of macrophages during infection, provided they are
116 exposed at the surface of *M. tuberculosis*.

117

118 DIM accommodate into a bilayer membrane by adopting a conical shape

119 Given their long aliphatic chains and their overall hydrophobic properties, we
120 sought to understand how DIM might physically be accommodated in a bilayer
121 membrane. We used a multi-scale modelling approach to gain insight into the
122 conformation of such a complex lipid embedded in a simple phospholipid bilayer.

123 During macrophage infection, *Mtb* produces DIM of higher molecular weight
124 than under non-infectious conditions¹⁷ (**Fig. 1-c**). We therefore modelled the structure
125 of a DIM molecule with a molecular mass of 1459 Da (star symbol in **Fig. 1-c**), *i.e.*
126 having chain lengths and number of methylations corresponding to $m=18$, $n=17$, and
127 $p=4$ (**Fig. 1-a**). 800 ns of atomistic Molecular Dynamics (MD) simulations of a single
128 DIM molecule in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid
129 bilayer revealed that DIM is deeply embedded in the membrane and may transit
130 between the two opposing leaflets (**Fig. 2-a**). DIM oxygen atoms preferentially
131 remained in the proximity of the POPC ester bonds while the acyl chains stretched into
132 the membrane hydrophobic core. The very long acyl chains (containing up to 27 carbon
133 atoms) prevented confinement of the DIM molecule to one single leaflet. Instead, DIM
134 seemed to be accommodated within the phospholipid bilayer by extending these
135 chains in the inter-leaflets space (see density profile in **Fig. 2a**).

136 To further explore the dynamics of the DIM molecule within the membrane, we
137 designed a Coarse-Grained (CG) model (**Fig. 2b**) based on the MARTINI force field
138 (see Methods). This force field is well adapted to model a large variety of lipids and
139 their actions on membranes and proteins^{19,20}. CG modelling of a single DIM molecule
140 in a POPC bilayer confirmed that DIM extended its long acyl chains in between the two
141 leaflets as seen in the atomistic simulation (**Fig. 2b, c**). Using CG modelling we were
142 able to extend the simulation to longer time scales to see multiple DIM translocations
143 from one leaflet to the other (**Fig. 2c**). We then increased the number of DIM molecules
144 up to a molar DIM-to-POPC ratio of about 7% (**Fig. S4**). At low concentrations (1%,
145 2%, and ~4%), DIM molecules diffused freely inside the bilayer, while at 7% they
146 started to strongly interact with each other and form aggregates in between the two
147 leaflets. This behavior is also observed, both experimentally and computationally, for
148 molecules with similar structural features, like triglycerides^{21,22}.

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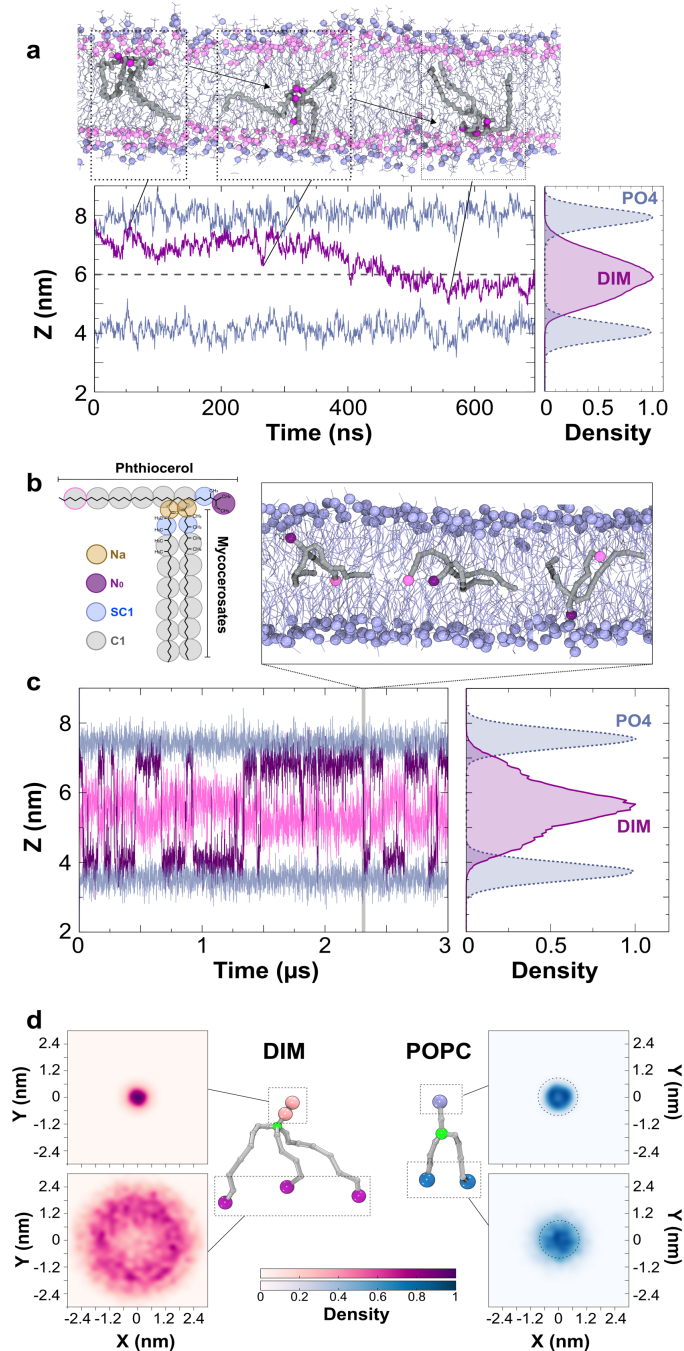


Figure 2: Position and shape of a single DIM molecule in a POPC bilayer. (a) upper inset: atomistic simulation of a DIM molecule (in gray licorice) showing its passage from one leaflet of the POPC bilayer to the opposite one. DIM oxygen atoms are represented in purple, POPC phosphorous atoms are displayed in light blue and POPC oxygen atoms in pink. Bottom left, in purple, evolution of the z-position for the center of mass of DIM's oxygen atoms during the course of the atomistic simulation. In light blue, averaged z-position of the phosphorous atoms of the POPC molecules. Bottom right, densities of the positions of the DIM lipid and POPC phosphate groups revealing the embedded DIM position in the bilayer. (b) Coarse-grained model of the DIM molecule (see Methods for details). (c) Evolution of N0 and the last C1 particles on the phthiocerol moieties (in purple and pink, respectively) during the course of the CG simulation. This plot shows a C1 particle confined around the interleaflet space while the N0 particle stayed in the proximity of the POPC oxygen atoms. Upper inset, DIM (in grey) transit from one leaflet to the other during CG simulation. Right inset, densities of the DIM lipid and POPC phosphate groups. (d) 2D density projections of each extremity for DIM and POPC molecules in the x-y membrane plane (CG simulation, see also Fig. S5 displaying densities for atomistic simulations) highlighting the conical (resp. cylindrical) shape of DIM (resp. POPC) lipid. Particles depicted in green were used for molecule centering.

153 We next sought to understand how the position of the DIM acyl chains in the
154 inter-leaflet space affected its overall shape. To do so, we projected the positions of
155 the lipid extremities onto the 2D membrane plane (**Fig. 2d**). When centering the
156 molecule on the junction of the chains, this revealed very large movements of the three
157 acyl chain extremities, while the most polar end of the phthiocerol chain remained
158 largely static. For POPC, a similar projection displayed a completely different behavior,
159 with comparable densities for both the headgroup and the hydrophobic acyl chain
160 extremities (**Fig. 2d**). Comparable results were obtained from atomistic simulations
161 (**Fig. S5**). These results can be related to the effective shape of each molecule: while
162 it is known that POPC has a cylindrical shape, consistent with our simulations, which
163 is suitable to form planar lipid membranes, our results indicated that DIM molecules
164 adopt a strongly conical shape in a lipid bilayer.

165

166 **DIM drive the formation of non-bilayer membrane structures**

167 This conical molecular shape of DIM may have important consequences for the
168 organization of DIM-containing membranes. Indeed, conical lipids are known to
169 destabilize the lamellar membrane phase (L_{α}) and favor the appearance of a non-
170 bilayer inverted-hexagonal phase (H_{II})²³. The transition from an L_{α} -phase to an H_{II} -
171 phase can be studied using ³¹P-NMR spectroscopy by monitoring the NMR spectra at
172 increasing temperature (see Methods). We have employed Magic Angle Spinning
173 (MAS) NMR spectroscopy for enhanced sensitivity. A mixture of the phospholipids 1,2-
174 dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1-stearoyl-2-oleoyl-*sn*-
175 glycero-3-phosphocholine (SOPC)²⁴ has been used for a range of lipids to study their
176 propensity to induce non-bilayer phases^{25,26}. To study the influence of DIM, we used
177 lipid membranes made of a 3:1 (mol/mol) mixture of DOPE and SOPC (see Method).
178 With this lipid composition, the membranes remained in the L_{α} phase for temperatures
179 up to 322 K (**Fig. 3a,c**). However, incorporating 5% of DIM into the lipid mixture
180 destabilized the L_{α} phase, and induced a transition from a L_{α} phase at low temperature
181 (284 K, **Fig. 3a**) to an H_{II} phase configuration at high temperature (310 K and 322 K,
182 **Fig. 3a**) as evidenced by the ³¹P NMR spectra. Thus, DIM destabilize the L_{α} phase in
183 our model membranes and promote the transition to the H_{II} phase.

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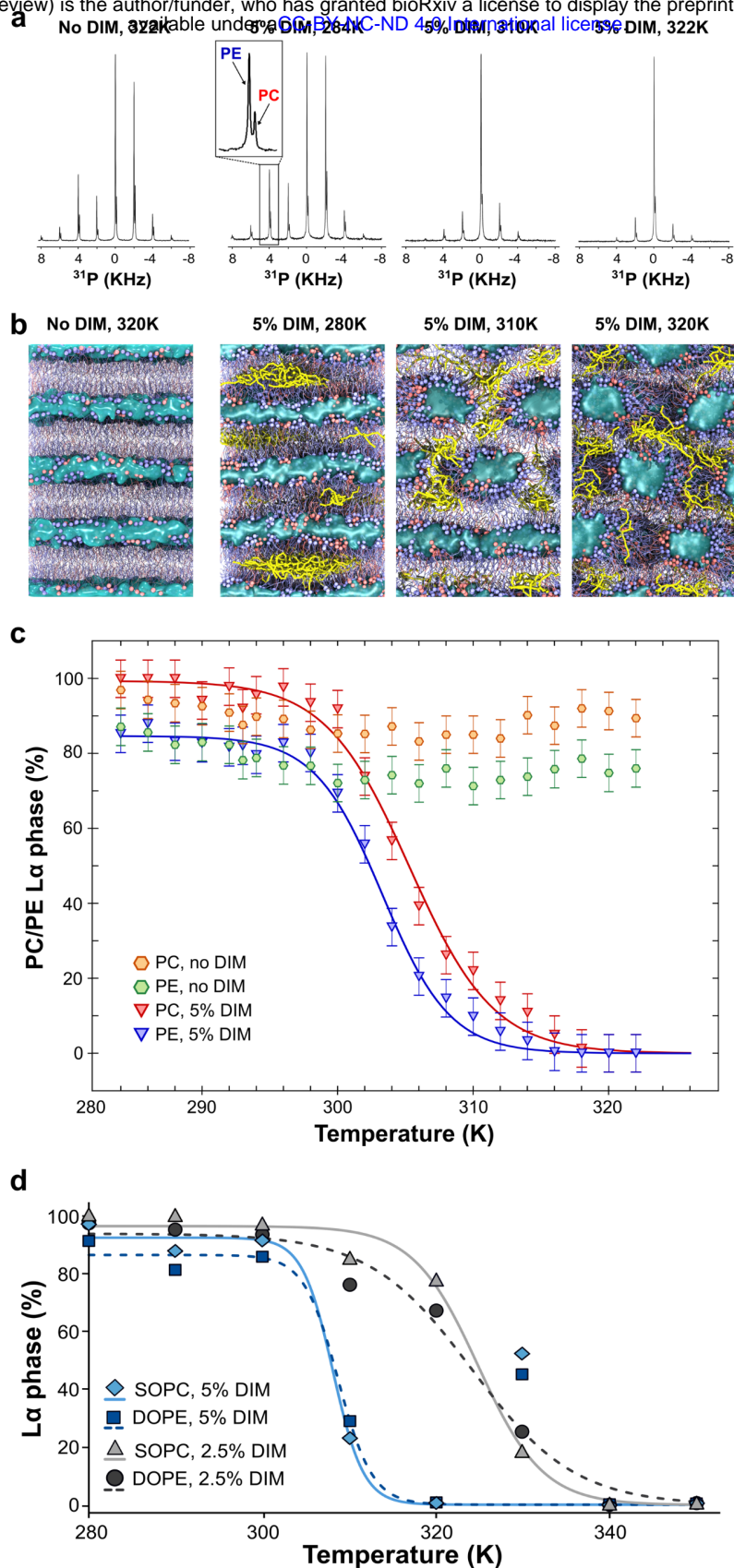


Figure3: DIM induce HII phases. (a) ^{31}P NMR spectra at different temperatures for DOPE/SOPC (3:1, mol/mol) without DIM or containing 5% of DIM. In the MAS ^{31}P NMR spectra, each spinning sideband consists of a DOPE and a SOPC peak. (b) Coarse grained models of the phase transition in DOPE/SOPC (3:1, mol/mol) without DIM or containing 5% of DIM. Increasing the temperature leads to the formation of tubular structures for the DIM-containing systems while the system without DIM stay fully lamellar. Snapshots shown are taken at the end of the 3 μs simulations. SOPC molecules colored in red. DOPE molecules colored in blue. DIM molecules colored in yellow. Water molecules represented as a blue surface. (c) Spectral deconvolution of the ^{31}P NMR spectra giving the percentage of the L α phase as a function of the temperature for DOPE/SOPC (3:1) without DIM (orange and green hexagons) and with addition of 5% DIM (red and blue triangles). In the case of 5% DIM, the phase transition can be approximated by a sigmoid (red and blue lines). (d) SOPC and DOPE phase transitions calculated from CG-MD simulations for 2.5% and 5% of DIM. For the DOPE /SOPC mixture with 5% of DIM, we removed the outlier values at 330K from the curve fitting based on statistical tests (see supplementary material).

187 As a complementary approach to monitor the ability of DIM to induce non-bilayer
188 phases, we performed CG-MD simulations²⁷. We modelled a stack of four lipid bilayers
189 of identical composition (3:1 DOPE/SOPC) as in the ³¹P-NMR experiments, for
190 temperatures ranging from 280 K to 350 K (see Methods for details). Similar to the ³¹P-
191 NMR experiments, the membranes remained in the lamellar phase in the absence of
192 DIM (**Fig. S6**), but a temperature-driven transition occurred when 5% of DIM were
193 added (**Fig. 3b**). These simulations allow a deeper understanding of the molecular
194 process of the H_{II} phase transition in the presence of DIM. At low temperatures,
195 molecules of DIM formed aggregates in the inter-leaflet space (**Fig. 3b**, 280K), as also
196 seen in the POPC bilayer (**Fig. S4**). Increasing the temperature led to the formation of
197 fusion stalks, hourglass-shaped lipid structures formed between neighboring bilayers.
198 DIM aggregated in these stalks to extend their large hydrophobic tails (**Movie S1**). This
199 aggregation stabilized and helped to increase the width of the stalks, eventually leading
200 to the formation of tubular water-filled membrane structures (**Fig. 3b**, 310 K). At still
201 higher temperatures (320 K and higher), DIM molecules diffused freely in the
202 hydrophobic membrane core, thus stabilizing the H_{II} configuration (**Fig. 3b**, 320 K and
203 **Movie S1**).

204 We deconvoluted the ³¹P-NMR spectra recorded between 282 K and 324 K in
205 2 K steps for DOPE/SOPC (3:1) with 5% of DIM, using a set of parameters obtained
206 from reference datasets (see Methods and **Table S2**). The ³¹P chemical shifts of DOPE
207 and SOPC are different. Therefore, each spinning sideband in our spectra consisted
208 of two resolved peaks (**Fig. 3a**) enabling us, in a single spectrum, to independently
209 analyze the percentages of the L_α and H_{II} phases for DOPE and for SOPC. For both
210 lipids, we observed a continuous transition from the L_α to the H_{II} phase described by a
211 sigmoid (**Fig. 3c**, Supplementary Material and **Table S3**). The phase transition
212 midpoint temperatures (T₅₀) were 303.5 K (± 0.4) for DOPE and 305.3 K (± 0.5) for
213 SOPC. Thus, the DOPE lipids were more susceptible to transition to an H_{II} phase than
214 the SOPC lipids, a tendency observed in most of the tested conditions (**Table S3**). This
215 result is in agreement with previous studies showing that conical DOPE prefers the H_{II}
216 phase, in contrast to cylindrical POPC lipids²⁸. In the CG-MD simulations, we evaluated
217 the percentage of L_α phase as a function of temperature from the distribution of lipid
218 tilt angles (see Supplementary material), for SOPC and DOPE (**Fig. 3d**). Here, too, we
219 observed continuous phase transitions that were fitted by a sigmoid with a midpoint

220 transition temperature of ~308 K (**Table S3**), which is in broad agreement with the ³¹P-
221 NMR experiments.

222 CG-MD simulations revealed that the first stage of the mechanism through
223 which DIM drive the L_α-to-H_{II} phase transition involves their aggregation in membrane
224 stalks. Hence, blocking the formation of stalks would be expected to reduce the effect
225 of DIM. To test this, and validate our hypothesis, we replaced a fraction of SOPC with
226 lysophosphatidylcholine (lysoPC), a lipid known to hinder the formation of fusion
227 stalks^{29,30}. ³¹P-NMR experiments on liposomes of DOPE/SOPC/lysoPC (75:25:0),
228 (75:20:5), and (75:15:10), each containing 2.5% of DIM, revealed an increase of the
229 transition midpoint temperature T₅₀ (**Fig. S8**) with increasing percentage of lysoPC,
230 highlighting a diminished effect of DIM when fusion stalk formation is inhibited. We next
231 performed CG-MD simulations to understand the molecular process involved.
232 Consistent with the NMR experiment, including lysoPC in the simulation increased the
233 value of T₅₀ (**Fig. S8a**). From these simulations, we observed that the effect of lysoPC
234 did not involve a direct interaction with DIM, as lysoPC was spread throughout the
235 membranes (**Fig. S8b**). Indeed, ³¹P-NMR experiments on DOPE/SOPC (5:1, mol/mol),
236 which displays an L_α-to-H_{II} transition without DIM, also showed an increase in T₅₀ upon
237 replacing a fraction of SOPC by lysoPC (**Fig. S7**). Thus, limiting the formation of stalks
238 decreased the ability of DIM to effectively drive the formation of non-bilayer membrane
239 structures.

240 Altogether, the combination of ³¹P-NMR and CG-MD simulations revealed the
241 ability of DIM lipids to perturb membrane organization by promoting a phase transition
242 from the lamellar to the inverted hexagonal phase. The molecular mechanism involves
243 an initial aggregation of DIM lipids in fusion stalks, which then leads to a complete
244 destabilization of the lamellar phase in favor of the inverted hexagonal phase.

245

246 **High potency of DIM to induce non-bilayer phase in comparison to other lipids**

247 We compared the ability (potency) of DIM to induce the H_{II} phase to that of lipids
248 with different structural features using ³¹P-NMR. We first tested the effect of the
249 concentration of DIM on the formation of the H_{II} phase. Figure **4-b** shows that
250 decreasing the DIM concentration to 2.5% and 1% still led to the formation of the full
251 H_{II} phase, albeit at a higher temperature. The increased transition midpoint
252 temperature (**Fig. 4b**) reveals a dose-response relationship, which is also observed in
253 CG-MD simulations (**Fig. 3d**). We then tested the effect of the triglyceride tripalmitin

254 (Fig. 4a), which, like DIM, has three acyl chains. However, incorporation of either 2.5%
 255 or 5% of tripalmitin did not induce a full H_{II} phase transition (Fig. 4b). Thus, the effect
 256 of DIM on non-bilayer structure formation did not seem to be uniquely related to its
 257 three-legged structure.
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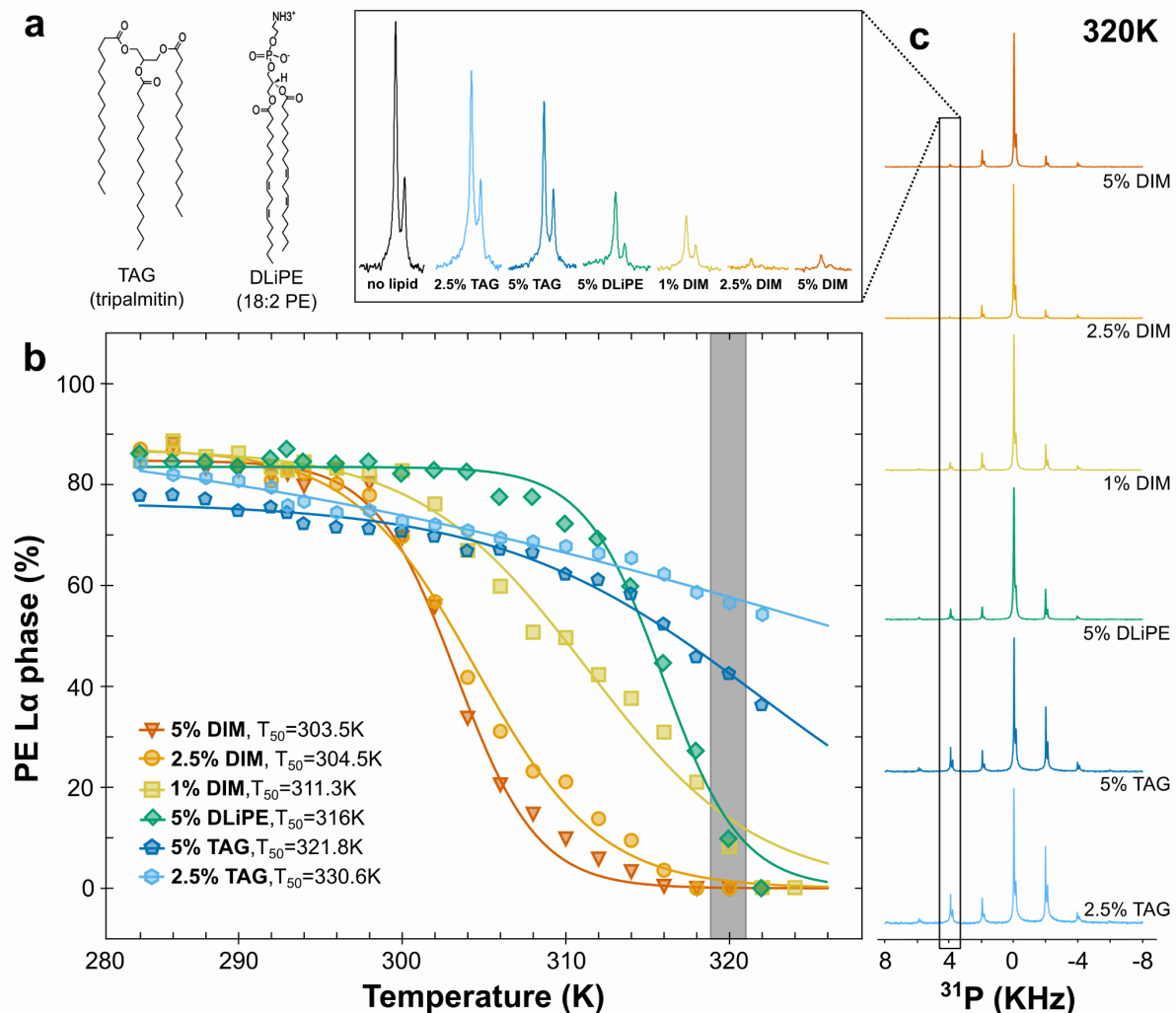


Figure4: Comparison of DIM potency to induce non-bilayer phase with lipids of different shapes. (a) Molecular structures of the triacylglycerol (TAG) tripalmitin and of 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine (DLiPE). **(b)** Evolution of the L_α-to-H_{II} phase transition for the DOPE molecules as a function of temperature for DOPE/SOPC (3:1) containing different concentrations of DIM, DLiPE and TAG (see also Fig. S9 for the respective curves for SOPC molecules). For clarity, error bars are omitted. As seen in Fig. 3, the error was evaluated to ±5%. The gray bar represents the points obtained from the spectra highlighted in c. **(c)** ³¹P NMR spectra for the lipid mixtures containing TAG, DLiPE or DIM at 320 K. The second rotation band (4 kHz), which is strongly related to the evolution of the L_α phase, is magnified in the upper panel. For comparison, the black peak depicts the second rotation band of DOPE/SOPC (3:1) at 320 K.

259

260 We finally analyzed the ability of 1,2-dilinoleoyl-*sn*-glycero-3-
 261 phosphoethanolamine (DLiPE) to induce the L_α-to-H_{II} phase transition and compared
 262 it with DIM. With two double bonds in each acyl chain (Fig. 4a), DLiPE is expected to

263 be a strong enhancer of hexagonal phase formation³¹. With 5% of DLiPE, we observed
264 a full H_{II} phase transition already at 322K (**Fig. 4b,c**) and a transition midpoint
265 temperature for DOPE of 316 K (± 0.4). However, this value is higher than for 5% DIM
266 (303.5K ± 0.4), 2.5% DIM (304.5K ± 0.5) and even 1% DIM (311.3K ± 1.0) (**Table S3**).
267 Thus, DIM molecules are strong inducers of non-bilayer phases, even at low
268 concentrations.

269 Lipid shape can be assessed by studying inverted hexagonal phase in different
270 lipid mixtures³². Here, using the L _{α} -to-H_{II} phase transition temperature as a measure
271 to assess lipid conical shape, we ranked the shape of the different molecules: DIM
272 were strongly conical, DLiPE were less conical, while the tripalmitin were the least
273 conical.

274

275 **Lipid shape modulates the entry of *Mtb* and zymosan particles into macrophages**

276 In our previous experiments, the DIM-deficient mutant H37Rv $\Delta ppsE$ appeared
277 to infect macrophages with a lower efficiency than the WT strain. Coating these DIM-
278 deficient mutant bacteria with DIM restored the WT phenotype while coating mutants
279 with tripalmitin did not have an effect⁸. These results may now be related to their
280 respective conical shapes: strongly conical for DIM and less conical for tripalmitin. To
281 test whether it is specifically the conical shape of DIM that helps *Mtb* to invade
282 macrophages, we evaluated the impact of exogenously added DIM and various other
283 lipids on the capacity of this mutant to invade macrophages in comparison to the WT
284 H37Rv strain (**Fig. 5a**). We confirmed that the DIM-deficient mutant infected a lower
285 percentage of macrophages than the WT strain (**Fig. 5b,c**). Pre-treatment of
286 macrophages with DIM restored the percentage of infected cells to a level comparable
287 to that observed with the WT H37Rv strain in untreated macrophages (**Fig. 5b,c**).
288 Notably, treating macrophages with the conical phospholipid POPE also enhanced the
289 percentage of macrophages infected with the H37Rv $\Delta ppsE$ mutant, whereas treatment
290 with the cylindrical lipid POPC had no significant effect (**Fig. 5c**). These data support
291 the hypothesis that the conical shape of DIM, which induces non-bilayer membrane
292 structures, increases the efficiency of *Mtb* to infect macrophages.

293 To determine whether this effect of DIM on macrophages is restricted to
294 infection by *Mtb*, we examined the effect of DIM and other lipids on the uptake of
295 zymosan (**Fig. 5d**) a fungal polysaccharide frequently used to study non-opsonic

296 phagocytosis³². We found that macrophage pre-incubation with DIM also increased
 297 zymosan uptake by macrophages in comparison to untreated conditions, as did pre-
 298 incubation with POPE but not with POPC (Fig. 5e). These data indicate that DIM and
 299 other conical lipids generally promote phagocytosis by macrophages.

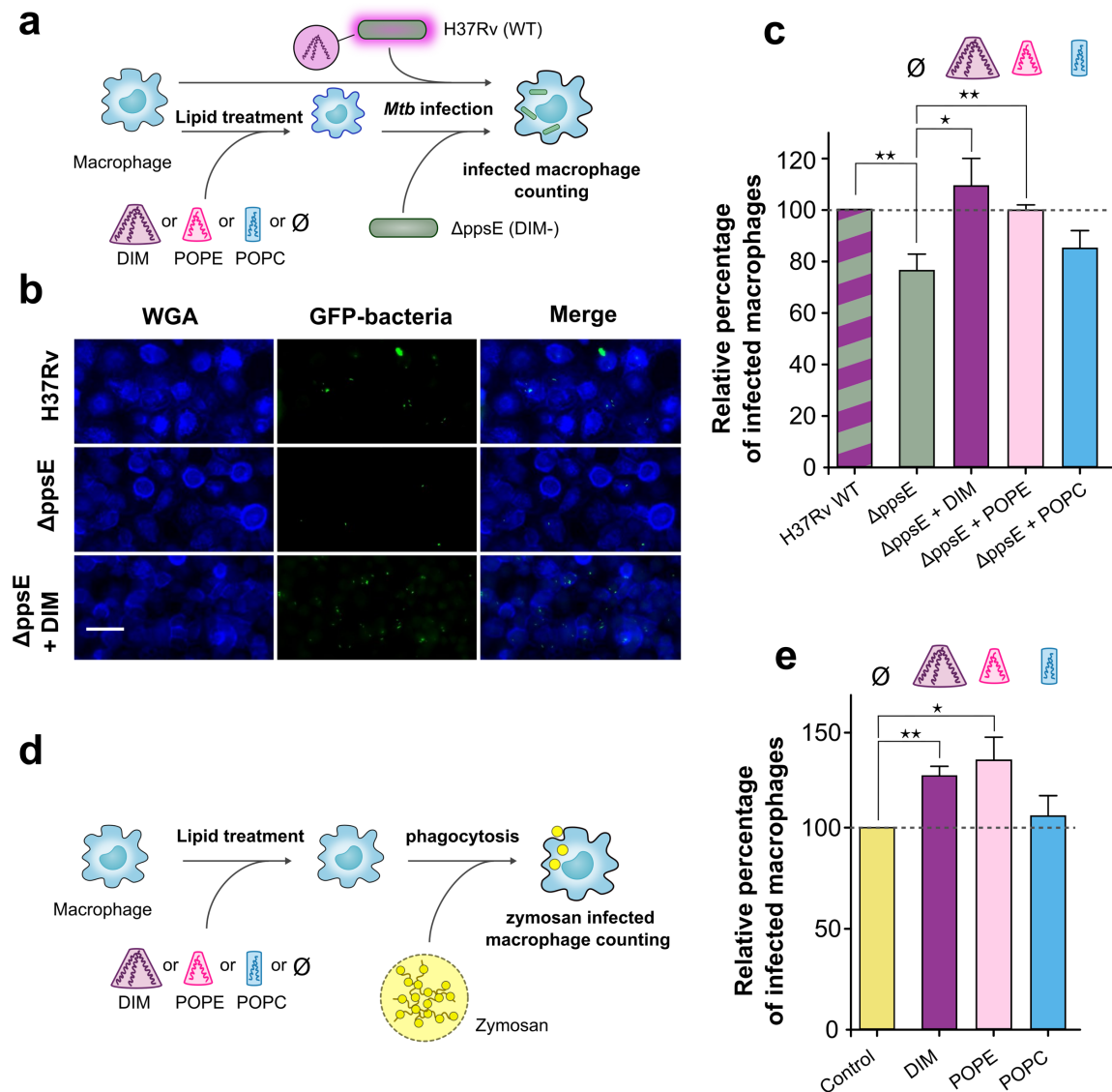


Figure 5: Lipid shapes modulate the entry of the MtbΔppsE mutant and zymosan into macrophages. (a) Macrophages were incubated at 37°C for 1 h with lipid solvent (∅) or 70 μM lipids (DIM, POPC and POPE) and subsequently exposed to GFP-expressing H37Rv WT or ΔppsE (MOI 10:1) for 1 h. Cells were then fixed and processed for the quantification of infected macrophages by fluorescence microscopy. (b) Representative fluorescence microscopy images of untreated or DIM-treated macrophages stained with WGA (membrane marker, blue) and infected with H37Rv WT or ΔppsE (green); scale bar: 30 μm. (c) The histogram represents the percentage of macrophages infected with ΔppsE in lipid-treated and untreated macrophages, expressed with respect to H37Rv WT (100%). (d) Macrophages were incubated with solvent (∅) or lipids, and then put in contact for 1 h with zymosan particles (MOI 30:1). (e) Percentages of macrophages infected with zymosan in untreated cells or cells treated with lipids. Values are expressed with respect to the uptake of zymosan in untreated cells (100%). The values are means + SEM of 8-10 separate experiments. The significance of difference in the percentage of macrophage infection between H37Rv WT and ΔppsE or between untreated and lipid-treated cells were evaluated, *, p < 0.05; **, p < 0.015.

300 DISCUSSION

301 While lipid transfer from *Mtb* to the macrophage membranes during infection
302 had been demonstrated for glycolipids³³, this was never shown for DIM. By using
303 MALDI-TOF mass spectrometry, we established that DIM molecules exposed at the
304 envelope of *Mtb* are indeed transferred to the macrophage membranes during infection
305 (Fig. 1). We envision two mechanisms that could account for this process. DIM may
306 be exchanged by direct contact between the surface of the bacteria and the
307 macrophage membrane at contact sites. Such direct exchange of cholesterol and
308 cholesterol-glycolipids has been observed between *Borrelia burgdorferi* and HeLa
309 cells³⁴. Alternatively, DIM could be transported in the membranes of extracellular
310 vesicles, which are known to be excreted by *Mtb* and other mycobacteria³⁵, followed
311 by fusion of these vesicles with the plasma membrane of the macrophages. Lipid
312 exchange mediated by vesicle fusion was shown for *Borrelia burgdorferi*³⁴ and for
313 *Pseudomonas aeruginosa*³⁶. For the latter process, lipid transfer could be favored by
314 the conical shape, which promotes the fusion of vesicles with the host cell membrane³⁷.
315 Here, by combining ³¹P-NMR with MD simulations, we demonstrated that DIM can
316 adopt such a conical shape and promote the formation of non-bilayer (inverted
317 hexagonal) membrane phases (Fig. 3), structures important for efficient membrane
318 fusion³⁸. Membrane fusion may also be important for sealing of the phagosomal
319 membrane during the ultimate stage of phagocytosis. Notably, our results showed that
320 even DLiPE, a strong enhancer of non-bilayer phases, did not match the strength of
321 DIM in promoting non-bilayer structures (Fig. 4). This may be explained by the fact
322 that DIM lipids preferentially aggregate in transient stalks (Fig. 3b and movie S1) to
323 stabilize them, thereby enhancing non-bilayer phase formation. Based on our
324 modelling, the conical shape of DIM can be related to the accommodation of the very
325 long acyl-chains of DIM to phospholipid bilayers (Fig. 2). This shape may also be
326 adopted by other long acyl chain lipids that are important for *Mtb* infection² and are
327 transferred to the host cell membrane³³ such as trehalose mono- and di-mycolate and
328 the phenolic glycolipids, molecules structurally related to DIM.

329 There is now ample evidence that lipids can modulate membrane protein
330 function^{39,40}. As seen for lipids such as diacylglycerol⁴¹, the conical shape of DIM may
331 modulate membrane protein activity. Accordingly, we find that DIM increase the non-
332 opsonic phagocytosis of zymosan, a process well known to be mediated by a repertoire
333 of membrane receptors, including complement receptor 3 (CR3)³² and the mannose

334 receptor⁴². DIM may act on membrane proteins via different biophysical mechanisms.
335 First, DIM may impose curvature on the host membrane¹², which in turn may modulate
336 integral membrane protein sorting^{43,44} and function⁴⁵. DIM could also trigger
337 reorganization of lipid nano-domains to modulate signaling platforms^{14,46}. Thus, our
338 findings should open new avenues for understanding how *Mtb* subverts other receptors
339 involved in its recognition by the immune system⁴⁷, including Toll-like receptors, NOD-
340 like receptors, and C-type lectin receptors.

341 Modulating the activity of membrane proteins will ultimately modulate cellular
342 functions. Indeed, we showed that DIM promote *Mtb* infection (**Fig. 5**). To confirm the
343 relevance of the conical shape, we showed that conical POPE lipids, but not cylindrical
344 POPC lipids, added to macrophages also restored the infection capacity of a DIM-
345 deficient *Mtb* mutant and improved phagocytosis (**Fig. 5**). Our results also shed new
346 light on previous observations showing that a DIM-deficient *Mtb* mutant coated with
347 tripalmitin lipid was less effective in infecting macrophages than DIM-coated mutants⁸.
348 This can now be understood by the fact that tripalmitin did not promote formation of a
349 non-bilayer phase transition in our model membranes (**Fig. 5**), hence did not display a
350 strong conical shape. To our knowledge, our results demonstrate for the first time that
351 the conical shape of a lipid promotes phagocytosis. The conical shape of DIM and their
352 effect on disorganizing the membrane may also play a role in the induction of
353 phagosomal membrane rupture and cell death^{48,49}. Altogether, this new understanding
354 of how the molecular shape of DIM lipids and their biophysical properties affect
355 biological membranes may help to design host-directed therapeutic strategies to fight
356 Tuberculosis⁵⁰ by preventing the infection of macrophages by *Mtb*.

357

358

359

360 **ACKNOWLEDGEMENTS**

361 This work was supported by the CNRS-MITI grants PEPS MPI 2018 and “Modélisation
362 du vivant” 2019, Agence Nationale de la Recherche (grant ANR-16-CE15-0003), the
363 Fondation pour la Recherche Médicale FRM (“Equipe FRM” number
364 DEQ20160334879) and the Centre National de la Recherche Scientifique (CNRS).
365 J.A. was a recipient of a PhD scholarship from the French government. This work was
366 granted access to the HPC resources of CALMIP supercomputing center under the
367 allocation 2019-17036. C.A.D. thanks TRI-Genotoul Imaging facility (Toulouse,
368 France). We acknowledge Life Science Editors for proofreading the manuscript. We

369 thank Olivier Neyrolles, Jérôme Nigou, Laurence Salomé, and Justin Teissié for fruitful
370 discussions and support.

371

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METHODS

506

Antibodies, lipids and reagents

508 The rabbit polyclonal antibody against mycobacteria was produced as previously
509 described⁸. The Rhodamine Red-conjugated goat anti-rabbit secondary antibody and
510 Wheat Germ Agglutinin (WGA), Alexa Fluor[®] 350 conjugate were purchased from
511 Invitrogen. DIM were extracted from *Mycobacterium canetti* as described below. 1-
512 palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0-18:1 PC, POPC), 1-palmitoyl-2-

513 oleoyl-*sn*-glycero-3-phosphoethanolamine (16:0-18:1 PE, POPE), 1-stearoyl-2-oleoyl-
514 *sn*-glycero-3-phosphocholine (18:0-18:1 PC, SOPC), 1,2-dioleoyl-*sn*-glycero-3-
515 phosphoethanolamine (18:1-18:1 PE, DOPE), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-
516 phosphocholine (16:0 lysoPC) and 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine
517 (18:2 PE, DLiPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).
518 Tripalmitin was a generous gift from M. Tropis (Toulouse, France). The other reagents
519 were purchased from Sigma-Aldrich, except when specifically mentioned.

520

521 **Bacterial strains and growth conditions**

522 The strains used in this study included the wild-type (WT) *M. tuberculosis* strain,
523 H37Rv Pasteur (the sequenced strain from Institut Pasteur, Paris) and two distinct
524 H37Rv mutants. The *ppsE* mutant (H37Rv Δ *ppsE*) was constructed in a previous study
525 by insertion/deletion within the polyketide synthase gene *ppsE*⁵¹ required for the
526 synthesis of DIM. The WT strain and the H37Rv Δ *ppsE* mutant were rendered
527 fluorescent by the transfer of plasmid pMV361H *gfp*⁸. The *lppX* mutant (H37Rv Δ *lppX*)
528 was constructed by homologous recombination using the thermosensitive
529 counterselectable plasmid pPR27 as previously described⁵². Briefly, a 2.6kb DNA
530 fragment covering the *lppX* gene was amplified by PCR from H37Rv genomic DNA
531 using primers *lppXA* (5'-GCTCTAGAGTTTAAACGCATTTGAGCAGCCGAG-3') and
532 *lppXB* (5'-GCTCTAGAGTTTAAACGAAGAATACCTGGCCGC-3') and inserted into a
533 cloning vector. The *res- Ω km-res* cassette (Malaga et al., 2003) was inserted at the
534 unique KpnI site within the *lppX* gene to generate the allelic exchange substrate (AES)
535 formed of the *res- Ω km-res* cassette flanked by two arms (of approximately 1kb)
536 specific to *lppX*. This AES was recovered on a PmeI restriction fragment and inserted
537 into the XbaI site of pPR27. The resulting plasmid was transferred into the recipient *M.*
538 *tuberculosis* H37Rv strain and allelic exchange mutants were selected as described
539 previously⁵². Kanamycin and sucrose resistant clones were analyzed by PCR using
540 primers *lppXC* (5'-CAAACGCGTTTCTGGACGG-3'), *lppXD* (5'-
541 GGCAATCCACACGGTCGC-3'), *lppXE* (5'-GAGCATTGAAAGCTCCCACC-3')
542 specific of the *M. tuberculosis* H37Rv genome, and *res1* (5'-
543 GCTCTAGAGCAACCGTCCGAAATATTATAAA-3') and *res2* (5'-
544 GCTCTAGATCTCATAAAAATGTATCCTAAATCAAATATC-3') specific of the *res-*

545 *Ωkm-res* cassette. One clone giving the pattern corresponding to the allelic exchange
546 was retained for analysis and named H37Rv ΔppX (or *PMM76*).

547 All strains were cultured at 37°C in Middlebrook 7H9 liquid medium (BD
548 Difco) containing 10% albumin-dextrose-catalase (ADC) (BD Difco). When required,
549 kanamycin, hygromycin and Tween-80 were added to the medium to a final
550 concentration of 40 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 0.05% (v/v) respectively.

551

552 **Purification of DIM and preparation of lipid solutions**

553 DIM were purified from *M. canetti* as previously described⁵³. Briefly, total mycobacterial
554 lipids were extracted from stationary cultures of *M. canetti*. The bacteria were left
555 successively in $\text{CH}_3\text{OH}/\text{CHCl}_3$ (2:1, vol/vol) for 48 h and in $\text{CH}_3\text{OH}/\text{CHCl}_3$ (1:2, vol/vol)
556 for 24 h. The organic phase was recovered, washed with water and dried. Total lipids
557 were then resuspended in CHCl_3 and the chromatographic separation of DIM was run
558 manually using Sep-Pak Silica Classic Cartridges (55-105 μm particle size; Waters)
559 and an elution gradient of an increasing concentration of diethylether (0-10% (v/v)) in
560 petroleum ether. Fractions containing the isolated compounds were pooled and dried.
561 Stock solutions of purified DIM (40 mg/mL), POPE (20 mg/mL) and POPC (21 mg/mL)
562 were prepared by dissolving the dried lipids in $\text{CH}_3\text{OH}/\text{CHCl}_3$ (2:1, vol/vol). The
563 solutions were then injected in serum-free RPMI 1640 medium (Gibco) at the final
564 concentration of 70 μM (1/400 dilution) and sonicated at 37°C until complete dispersion
565 of the lipids.

566

567 **Macrophage culture**

568 The human promonocytic cell line THP-1 (ECACC 88081201; Salisbury, UK) was
569 cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum
570 (FBS), 2 mM L-Glutamine, 1 mM sodium pyruvate, and 1% MEM non-essential amino
571 acids. For macrophage differentiation, the THP-1 cells were washed and suspended
572 in medium containing 10% FBS. The cells were distributed in a glass petri dish at a
573 density of 3×10^6 cells/petri dish and were differentiated into macrophages with 30 nM
574 phorbol 12-myristate 13-acetate (PMA) for 3 days. Before use, the cells were washed
575 twice with fresh medium.

576 Human blood purchased from the Etablissement Français du Sang in Toulouse
577 (France) was collected from fully anonymous non-tuberculous donors. Human
578 macrophages derived from monocytes (hMDMs) were prepared as previously

579 described⁸. Briefly, monocytes were isolated from peripheral blood mononuclear cells
580 (PBMC) by adhesion on a glass coverslip in 24-well tissue culture plates. Monocytes
581 (5×10^5 cells/well) were differentiated into hMDMs in RPMI 1640 (Gibco),
582 supplemented with 2 mM glutamine (Gibco) and 7% (v/v) heat-inactivated human AB
583 serum for 7 days.

584

585 **Macrophage infection**

586 Single cell suspensions were prepared with exponentially growing strains as previously
587 described⁸. Briefly, the bacteria were grown to mid-exponential growth phase on
588 Middlebrook 7H9 liquid medium supplemented with 10% ADC, and were then pelleted
589 by centrifugation and dispersed in serum-free RPMI 1640 medium using glass beads.
590 The number of bacteria per mL in the suspension was estimated by measurement of
591 the optical density at 600 nm. The bacteria were added to the macrophages at the
592 indicated multiplicity of infection (MOI) and incubated for 1-2 h at 37°C in an
593 atmosphere containing 5% CO₂. Extracellular bacteria or particles were removed by
594 three successive washes with fresh medium.

595

596 **Assay for monitoring DIM transfer to macrophage membranes**

597 For experiments with purified DIM, THP-1 cells were incubated with RPMI 1640
598 medium supplemented with 70 μM DIM at 37 °C and 5% CO₂. After 1 h, the cells were
599 rinsed with fresh medium and detached by incubation with a 0.05% trypsin-EDTA
600 solution (Gibco) for 15 min. The cells were then harvested, centrifuged at 150 x g for
601 10 min and the cell pellet was suspended in RPMI 1640 medium (A).

602 For experiments with DIM in the context of the *M. tuberculosis* cell envelope, THP-1
603 cells were incubated with H37Rv WT or H37Rv ΔLppX (MOI 15:1), washed with fresh
604 RPMI-1640 medium, and further incubated in the presence of serum at 37°C and 5%
605 CO₂. After 40 h, the cells were rinsed with RPMI-1640 medium, detached
606 enzymatically with trypsin and centrifuged at 150 x g for 10 min. The membranes were
607 prepared using a protocol adapted from Rhoades *et al.*³³. The pellet was suspended
608 in 1 mL ice-cold homogenization buffer (1 mM EDTA, 20 mM HEPES, pH 7) containing
609 250 mM sucrose and the cells were disrupted by 25 passages through a 26-gauge
610 needle. Following centrifugation at 3000 x g for 10 min at 4°C in order to sediment
611 nuclei and large cell debris, the supernatant was recovered. This step was repeated
612 twice. The supernatant was layered onto a linear gradient of 30% to 12% sucrose and

613 centrifuged at 2,000 x g for 1 h at 4°C. The upper portion of the gradient containing the
614 membrane fraction was isolated, layered on a discontinuous gradient of 50% to 25%
615 sucrose, and centrifuged at 2,000 x g for 30 min at 4°C. The fraction above the 25%
616 portion containing the membrane fraction was isolated, centrifuged at 110,000 x g for
617 1 h at 4°C and the membrane pellet was taken up in homogenization buffer (B).
618 Total lipids were extracted from the macrophage membranes using the Bligh and Dyer
619 extraction protocol⁵⁴. Briefly, to one volume of cells suspension (A) or membrane
620 fraction (B), 2.5 volumes of CH₃OH and 1.25 volumes of CHCl₃ were added. The
621 mixture was incubated at room temperature for 48 h. Then, 1.25 volumes of CHCl₃
622 followed by 1.25 volumes of CHCl₃ were added. This mixture was left standing for 24 h
623 to separate the organic and aqueous phases. Twenty-four hours later, the organic
624 phase containing the lipids was recovered and dried under a stream of nitrogen. For
625 the membrane fraction samples, the DIM were purified from the total lipid extract by
626 column chromatography using a Florisil column. The Florisil was equilibrated with a
627 solution of petroleum ether/diethyl ether (98:2, v/v). The total lipid extract was dissolved
628 in this solution and DIM were eluted with the same solution.

629

630 **Lipid analysis by MALDI-TOF MS analysis**

631 DIM were analyzed by matrix-assisted laser desorption-ionization time-of-flight
632 (MALDI-TOF) mass spectrometry, as described previously⁵⁵. Lipid residues were
633 dissolved in 20 µL of CHCl₃, deposited on the analysis plate and dried. Then, 0.5 µL
634 of 2,5-dihydroxybenzoic acid (10 mg/mL) dissolved in CHCl₃/CH₃OH (1:1, vol:vol) were
635 deposited on the sample and allowed to crystallize at room temperature. Mass spectra
636 were acquired with a MALDI TOF/TOF 5800 analyzer (Applied Biosystems/AB SCIEX,
637 Framingham, MA, USA) equipped with an Nd:YAG laser (Wavelength 349 nm; pulse
638 rate 400 Hz). The acquisition was carried out in continuous scan mode, in positive
639 mode with a laser intensity of 3500 (arbitrary unit of the software). The final spectrum
640 was obtained by accumulating 10 spectra of 250 laser shots.

641

642 **Phagocytosis assay**

643 Phagocytosis was assessed as described previously⁸. Briefly, human monocyte-
644 derived macrophages (hMDM) cultured on sterile glass coverslips in 24-well culture
645 plates were infected with GFP-expressing bacteria (MOI 10:1) or zymosan (MOI 30:1)
646 for 1 h. When indicated, the hMDM were pre-treated for 1 h with 70 µM lipids or 1/400

647 lipid solvent (CH₃OH/CHCl₃ (2:1, vol/vol) prepared in RPMI as for lipid suspension). At
648 the end of infection, the hMDM were intensively washed and fixed with 4% (w/v) PFA.
649 For mycobacteria, extracellular GFP-bacilli were labelled with rabbit anti-mycobacteria
650 Ab revealed by a Rhodamine Red-conjugated goat anti-rabbit secondary Ab. The cells
651 were then permeabilized with 0.3% Triton X-100 for 5 min and stained for 15 min with
652 WGA conjugated to Alexa Fluor[®] 350 to visualize the hMDM. For zymosan, hMDMs
653 were labelled with TRITC-phalloidin and extracellular zymosan was readily
654 distinguished from internalized particles, which appeared as yellowish grains within a
655 dark phagosome bordered by diffuse red staining. The percentage of cells having
656 ingested at least one bacterium or zymosan particle was determined by fluorescence
657 microscopy using a Leica 43 DM-RB epifluorescence microscope. For each set of
658 conditions, the experiments were performed in duplicate and at least 200 cells were
659 counted per slide. Data are presented as the mean ± standard error of the mean (SEM)
660 of the indicated number of experiments (n). Data were analyzed by the Wilcoxon
661 signed-rank test using GraphPad PRISM (GraphPad Software, GPW5-078069-
662 NBH9780) and p < 0.05 was used as the limit of statistical significance.

663

664 **Formation of multilamellar vesicles for HRMAS NMR experiments**

665 To form multilamellar vesicles (MLV) of well-defined lipid compositions, we mixed
666 appropriate volumes of chloroformic stock solutions of the different lipids in glass
667 tubes. The chloroform was evaporated using a rotating evaporator. We inclined the
668 tubes in the evaporator, resulting in the formation of a thin lipid film over a large area
669 of the tube. The lipids were further dried under vacuum for ~2 h to remove any
670 remaining traces of the solvent. Next, we added sufficient Tris buffer (10 mM Tris,
671 1 mM EDTA, pH 7.4) to the tubes to cover the lipid film. The lipids were left to hydrate
672 at a temperature chosen to favor the formation of the lamellar phase of the liposome
673 membranes (see below for further details). We then vortexed each tube 6 × 30 s. After
674 vortexing, we obtained a cloudy suspension of liposomes. We transferred the liposome
675 suspensions to 1.5 mL centrifuge tubes and centrifuged the tubes for 15 min at
676 16000 g, then stored them at 4°C. Before each NMR experiment, we removed the
677 supernatant and transferred 50 µL of the liposome pellet to a 4 mm MAS rotor, taking
678 care not to warm the liposomes. Before introducing the rotor into the NMR
679 spectrometer, we equilibrated the temperature to ~5°C.

680 In order to be sure to prepare the membranes in the lamellar phase, we adapted the
681 temperature of hydration to the lipid composition. Pure DOPE has a lamellar-to-
682 inverted-hexagonal transition temperature of 10°C. Accordingly, we hydrated the
683 samples of DOPE, DOPE/SOPC (9:1) and DOPE/SOPC (5:1) for 15 min on ice. We
684 vortexed the lipids 6 × 30 s (with cooling on ice for at least 30 s between vortexing
685 periods) in order to prepare and conserve the liposomes in the lamellar phase.
686 DOPE/SOPC (3:1), DOPE/SOPC (1:1) and pure SOPC (which do not form a H_{II} phase
687 at 30°C) were incubated at 30°C in a water bath and vortexed 6 × 30 s with return to
688 the water bath.

689 Incorporating the highly hydrophobic lipid DIM into liposomes requires incubating the
690 lipids at higher temperature, but this would have favored the formation of the inverted-
691 hexagonal phase for some membrane compositions. We therefore adapted our
692 protocol for DIM-containing liposomes and their controls without DIM. For these lipid
693 mixtures, we hydrated the lipids at 37°C on a shaker overnight. We then transferred
694 the tubes to a water bath set at 37°C. The tubes were vortexed for 6 × 30 s, with return
695 to the water bath between vortexing periods. All liposomes with compositions
696 DOPE/SOPC (3:1) +/- DIM, DOPE/SOPC (3:1) +/- TAG and DOPE/SOPC/lysoPC
697 (75:20:5) +/- DIM were prepared according to this protocol. We checked the correct
698 incorporation of DIM in the liposomes using thin-layer chromatography on a few µL of
699 the liposome suspension. We also conducted a separate ¹H-NMR experiment to
700 quantify the incorporation of DIM in liposomes using our protocol (see Supplementary
701 Material and Figures).

702

703 **NMR data acquisition**

704 Phosphorus NMR spectra were acquired on a 500 MHz Bruker Avance spectrometer,
705 in a HRMAS probe, with deuterium lock. The lipid samples (typically 6 mg total lipids
706 in 50 µL of Tris buffer (10 mM Tris, 1 mM EDTA, pH 7.4) were inserted into 4 mm rotors
707 with spherical inserts. The temperature of the sample could be varied between 278 K
708 and 324 K and was controlled to ± 0.1 K with a Bruker variable temperature unit. The
709 temperature was calibrated using the known temperature dependence of methanol
710 chemical shifts. ³¹P chemical shift anisotropies (CSA) were determined from the
711 spinning sideband manifolds at a spinning frequency of 2000 ± 1 Hz. MAS spectra
712 were obtained with a spin-echo sequence ($\pi/2 - \tau - \pi$) where the $\pi/2$ pulse had a length
713 of 5.3 µs (at a power of 107 W), applied at the lipids' isotropic resonance frequency,

714 and the interpulse delay, τ , was 20 μ s. The dwell time was 1 μ s, the acquisition time
715 65 ms, the relaxation delay 1 s and the number of scans 4096. No proton decoupling
716 was applied during acquisition, since it was shown to have no effect on the linewidth
717 at a 2 kHz spinning frequency (i.e. well above the ^1H - ^{31}P dipolar coupling of ~ 500 Hz
718 in fluid lipid bilayers). For every sample, a ^1H NMR spectrum at a spinning frequency
719 of 10 kHz was acquired in order to calibrate the ^1H (using the methylene peak at 1.25
720 ppm with respect to TMS) and ^{31}P chemical shifts (with respect to phosphoric acid at
721 0 ppm, using $\gamma_{\text{P}}/\gamma_{\text{H}} = 0.40480742$). The observed ^{31}P isotropic chemical shifts were
722 -1.00 ± 0.02 ppm for the phosphatidylcholine head group (in SOPC) and -0.27 ± 0.02
723 ppm for the phosphatidylethanolamine head group (in DOPE) and varied only slightly
724 with the lipid compositions explored. Typical linewidths were 40-60 Hz (i.e. ~ 0.25 ppm)
725 so that the PC and PE sideband patterns were well resolved and could be fitted
726 independently.

727 Every liposome sample was equilibrated overnight at 277 K before NMR
728 measurements. We then measured its ^{31}P MAS spectrum at temperatures from 284 K
729 to 324 K, in 2 K increments every 100 min (25 min equilibration plus 75 min acquisition
730 time for each temperature). For lipid mixtures which did not undergo a lamellar to
731 inverse-hexagonal phase transition (i.e. with a small proportion of DOPE) the spectra
732 were shown to be fully reversible when going down from 324 K to 284 K. However,
733 once the H_{II} phase had formed, it did not revert to the lamellar phase, at least after one
734 day of equilibration at 284 K. Hence, all measurements were done for increasing
735 temperatures, starting from the lowest temperature, taking care of maintaining the
736 liposomes at low temperature during their preparation and before NMR
737 measurements. The actual insertion yield of DIM in the lipid bilayers was determined
738 by ^1H -NMR (see Supplementary material).

739

740 **Spectral deconvolution**

741 We analyzed each ^{31}P spinning sideband manifold using the solid line shape analysis
742 tool (SOLA) available in Topspin 3.5. CSA parameters were calculated using the
743 Haeberlin convention for the anisotropy values $\Delta\delta = \delta_{\parallel} - \delta_{\perp}$ as commonly done in the
744 membrane literature and as explained before⁵⁶. We estimated the uncertainty of $\Delta\delta$
745 values to be ± 0.2 ppm, based on several measurements and fitting performed on
746 independent samples. We first determined the CSA parameters of lipids organized in

747 a single phase (e.g. SOPC for the lamellar L_{α} phase and DOPE for the H_{II} phase), at
748 several temperatures between 284 K and 324 K. The CSA parameters for each lipid
749 and each phase varied slightly and linearly with temperature, due to increased motion
750 at higher temperatures: from 49.6 ± 0.2 ppm (293 K) to 45.0 ± 0.2 ppm (333 K) for
751 SOPC in the L_{α} phase; from -22.2 ± 0.2 ppm (278 K) to -20.1 ± 0.2 ppm (333 K) for
752 DOPE in the H_{II} phase. The following linear regressions were obtained: $\Delta\delta = -0.0944$
753 $T + 76.599$ for SOPC in the L_{α} phase, $\Delta\delta = -0.0409 T - 33.627$ for DOPE in the H_{II}
754 phase, where T is the temperature in Kelvin; these regressions were used to calculate
755 CSA values at intermediate temperatures. Since the lipids in the H_{II} phase experience
756 additional motional averaging due to lateral diffusion around the aqueous channels,
757 their CSA is obtained by averaging two components $\delta_{//}$ and δ_{\perp} , divided by two, for
758 reasons discussed by Cullis and De Kruijff⁵⁷. The CSA values in the L_{α} and H_{II} phases
759 have opposite signs. Hence, the CSA value of SOPC in the H_{II} phase was taken to be
760 -0.5 times its value in the L_{α} phase, while the CSA value of DOPE in the L_{α} phase was
761 2 times its value in the H_{II} phase. Knowing the temperature-dependent CSA
762 parameters for both lipids in both phases allowed a drastic reduction of the number of
763 parameters to be fitted when analyzing lipid mixtures in which the two phases coexist.
764 After optimizing the peak positions and linewidths, the SOLA fitting algorithm only has
765 to search for the L_{α} - H_{II} proportions that best reproduce the experimental spectra. The
766 intensity of the spinning sideband at +4 kHz is a good reporter of the proportion of L_{α}
767 phase, since this peak is almost absent from the typical H_{II} phase spinning sideband
768 manifold⁵⁸ (see Fig. 4c). This fitting protocol was very robust and prevented the
769 artefacts and instabilities arising from fitting too many parameters. We should stress
770 that our procedure is based on the assumption that the spectra consist of a linear
771 combination of the spectra of the two lipids in the L_{α} and H_{II} phases, possessing CSA
772 parameters identical to their values in the pure phases at the same temperature. This
773 simplifying assumption was sufficient to characterize the lipid phase transitions,
774 although we cannot exclude that the actual lipid behavior is more complex.

775

776 **Quantum mechanics**

777 To calculate partial charges for DIM lipid, a molecular dynamics simulation was first
778 performed for 40 ns in the NPT ensemble. Lipid14⁵⁹ and general AMBER force field
779 (Gaff)⁶⁰ were used in order to describe bonded and non-bonded terms within the

780 AMBER program. From this trajectory, 30 structures were extracted based on the
781 clustering method (*kclust* from the MMTSB Toolkit, <http://www.mmtsb.org>, with the
782 radius of 2.5 Å). These structures were fully optimized at the HF/6-31G* quantum
783 chemical method using the Gaussian 09 suite of programs (<http://gaussian.com/>).
784 Partial charges were then obtained using RESP method⁶¹ (RESP-A1) implemented in
785 the RED tools⁶². For each structure, partial charges were obtained by charge fitting
786 to the electrostatic potential at points selected according to the Merz-Singh-Kollman
787 scheme^{63,64} as proposed by the RESP-A1 method. Before charge calculations, a
788 reorientation procedure was applied in order to maintain one ester (O=C-O) group in
789 the same orientation for all 30 structures. Partial charges for the head groups of the
790 DIM model are displayed in Figure S2.

791

792

793 **MD simulations**

794 The atomistic MD simulations were performed with the Amber16 software⁶⁵
795 (<http://ambermd.org>). The system contained a membrane of 300 POPC molecules and
796 was solvated with TIP3P water model using the CHARMM-GUI server^{66,67}. The DIM
797 molecule was positioned in the POPC bilayer with the polar core in the proximity of the
798 POPC oxygen atoms (see Fig. 2a). In order to avoid steric clashes of the DIM molecule
799 with POPC, the system was initially minimized by executing 1500 iterations of the
800 steepest descent (SD) algorithm, followed by 1500 iterations of the conjugate gradient
801 (CG) algorithm, with weakly restrained solute ($k = 10 \text{ kcal/mol/Å}^2$). Next, a short 100
802 ps MD run was performed on weakly restrained solute with temperature varying linearly
803 from 0 to 303 K. The temperature control was achieved using the Langevin dynamics
804 with the collision frequency parameter γ equal to 1.0 ps^{-1} . The integration step used in
805 this run was 1 fs. Throughout the calculations a cutoff of 10 Å was used for electrostatic
806 interactions. The MD simulation continued with the equilibration of the system,
807 consisting of 10 consecutive MD steps of 500 ps each, at constant temperature of 303
808 K with no restraints, with the integration step of 2 fs. The production run was then
809 launched for 800 ns with constant pressure of 1 bar. The Langevin dynamics was used
810 to control the temperature, with $\gamma = 1.0 \text{ ps}^{-1}$, while the pressure was controlled by the
811 anisotropic Berendsen barostat with the pressure relaxation time $\tau_p = 1 \text{ ps}$. Bonds
812 involving hydrogen were constrained with the SHAKE algorithm.

813 Coarse-grain simulations were performed using GROMACS 2016⁶⁸ with the MARTINI
814 force field 2.2^{69,70}. We have designed the CG model of DIM by following the original
815 MARTINI parametrization strategy⁷¹. We approximated 3-4 heavy atoms constituting
816 the long hydrophobic acyl chains by one C1 particle. Small methyl branched parts were
817 approximated by a small particle SC1. The slightly polar extremity of the phthiocerol
818 chain was approximated by a N₀ particle while the glycerol ester moieties were
819 represented by an intermediate hydrophobicity particle Na as usually done for other
820 phospholipids (see Fig. 2b). Force constants and equilibrium values for bonds and
821 angles were extracted from the atomistic simulation (see Fig. S3). All the systems were
822 first gradually equilibrated as proposed in the CHARMM-GUI MARTINI Maker
823 protocol⁷²). Coulomb interactions were treated using the reaction-field potential and
824 Lennard–Jones interactions were treated using shifted potentials with a cut-off radius
825 of 1.1 nm. For systems with a pure SOPC bilayer or DIM molecules embedded in
826 POPC bilayer, pressure was maintained at 1 bar using the Parrinello-Rahman
827 algorithm⁷³ with a semi-isotropic pressure control. The temperature was kept at 310 K
828 using the v-rescale algorithm⁷⁴. For pure DOPE and SOPC-DOPE systems undergoing
829 lamellar to hexagonal transitions, we used the protocol described by S.J. Marrink and
830 A. E. Mark²⁷. We stacked 4 membranes of SOPC-DOPE mixture at a 3:1 ratio with a
831 hydration level of approximately 2-3 water CG-particles per lipid (equivalent to c.a. 9-
832 10 atomistic water molecules) in order to see the phase transition in a reasonable
833 amount of time as detailed in S.J. Marrink and A. E. Mark paper²⁷. A Berendsen
834 thermostat in combination with a Berendsen barostat⁷⁵ were used. A fully anisotropic
835 coupling pressure was applied with a reference pressure of 1 bar. Systems were
836 equilibrated at 280K and then 8 different simulations were launched with temperatures
837 ranging from 280K to 350K with an increment of 10K. In all CG systems, a time step
838 of 20 fs was used. See Table S1 for a summary of the simulations. Parameters for CG
839 and atomistic representations of DIM lipids will be available on the MARTINI website:
840 cgmartini.nl. Density profiles were performed using the Gromacs tool density
841 (<http://manual.gromacs.org/documentation/2016/onlinehelp/gmx-density.html>). MD
842 simulations figures were performed using VMD⁷⁶. Scripts used to analyze MD
843 simulations will be available at: <https://github.com/MChavent>

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847 **ADDITIONAL REFERENCES**

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