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Criticality of plasma membrane lipids reflects activation state of macrophage cells

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Signaling is of particular importance in immune cells, and upstream in the signalling pathway many membrane receptors are functional only as complexes, co-locating with particular lipid species. Work over the last 15 years has shown that plasma membrane lipid composition is close to a critical point of phase separation, with evidence that cells adapt their composition in ways that alter the proximity to this thermodynamic point. Macrophage cells are a key component of the innate immune system, responsive to infections, regulating the local state of inflammation. We investigate changes in the plasma membrane's proximity to the critical point, as a response to stimulation by various pro- and anti-inflammatory agents. Pro-inflammatory (IFN- γ , Kdo-LipidA, LPS) perturbations induce an increase in the transition temperature of the GMPVs; anti-inflammatory IL4 has the opposite effect. These changes recapitulate complex plasma membrane composition changes, and are consistent with lipid criticality playing a master regulatory role: being closer to critical conditions increases membrane protein activity.

1. Introduction

Macrophages are extremely versatile cells of the innate immune system able to activate and adapt their functionality depending on the specific milieu [1]. Following phagocytosis of material resulting from trauma, or pathogens, or detection of specific functional molecules, macrophages can change their gene regulatory state and polarize into activated states, where for example they produce immune effector molecules such as cytokines for intercellular communication [2–4]. The responses manifested as a consequence of different stimulation have been classified in two broad activation states, based on both genetic expression profiling and phenotypic behavior: M1, or classically activated, macrophages have an enhanced bactericidal and tumoricidal capacity and produce high levels of pro-inflammatory cytokines, while M2 macrophages produce low levels of cytokines and have a wound-healing capacity by contributing to the production of collagen and extracellular matrix [1,3,5]. The stimuli that promote M1 macrophage activation are mainly IFN- γ , LPS and Granulocyte-macrophage colony-stimulating factor (GM-CSF). IFN- γ is a cytokine mainly produced by natural killer (NK) and T helper 1 (Th1) cells; signaling from the IFN- γ receptor (IFNGR) controls the regulation of specific genes related to the production of cytokine receptors, cell activation markers and adhesion molecules [1]. Lipopolysaccharides (LPS) are a class of molecules of the outer membrane of gram-negative bacteria, these molecules are recognized by the TLR4 receptor [6,7]. TLR4 activation triggers the downstream production of pro-inflammatory cytokines such as TNF- α and presentation of antigens [8]. In contrast, macrophages polarize into M2 mainly in response to IL4 and IL13 stimuli. IL4 is produced by T helper 2 (Th2) cells, basophils, and mast cells in response to a tissue injury and in presence of some fungi and parasites [3]. M2 cells are sensitive to infections, their production of pro-inflammatory cytokines is minimal, and their phagocytic activity is low [1,3].

In the transduction of signals a fundamental regulatory role is thought to be played by the plasma membrane composition [9]. There are many examples of specific protein-lipid affinity, but also strong evidence of more general mechanisms such as the propensity of lipid mixtures to form cholesterol rich domains, or domains of a preferred thickness, which then imply a preferred partitioning of certain trans membrane proteins [10–12]. Any mechanism that modifies local recruitment of membrane proteins, in the context of an assembly step such as dimerization necessary for function, can therefore directly be a regulator of receptor activity. This generalises a well known theme in membrane biochemistry, that proteins with lipid raft affinity have a higher chance to interact [13]. The key structures in this study of macrophages, the TLR4 receptor and its co-receptor CD14, are both known to have raft affinity: CD14 is found in lipid rafts both before and after LPS activation, while TLR4 receptors are initially found in non-raft regions and then translocate to rafts after the activation [14]. It has also been shown that the use of lipid raft inhibitors reduces significantly the production of cytokines related to LPS activation [15]. Moreover, lauric fatty acid seems to be responsible for the recruitment and dimerization of TLR4 into lipid rafts [16]. All together these facts strongly hint that plasma membrane composition, and in particular the propensity to form lipid rafts or domains, are fundamental regulators of protein interaction; we explore this theme with respect to activation of macrophages and the activity of TLR4 receptors.

Various authors have put forward the idea that the lipid raft phenomenology is linked to the propensity for the lipidic component of the membrane to undergo liquid-liquid phase separation [12], as was observed in plasma membrane extracts [17]. Vesicles extracted from the plasma membrane of cells have the same characteristics of certain ternary lipid mixtures, of particular interest the spontaneous appearance of transient lipid domains which is a universal property of systems in vicinity of a critical point [17,18]. From a biological point of view, being poised close to a critical point could be advantageous to accelerate a whole set of membrane biochemistry, since the cell would require much less energy to create lipid heterogeneity. Modulating the lipid composition is thus a mechanism for global regulation of activity on the membrane [12]. Giant plasma membrane vesicles (GPMVs) allow to study the properties of the

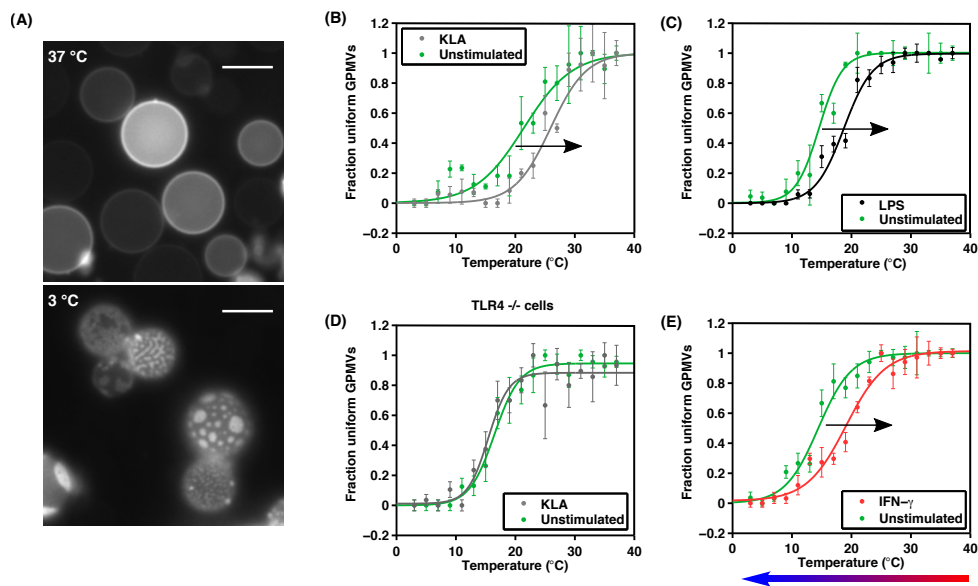


Figure 1. The plasma membrane of macrophage cells is close to critical composition and changes its transition temperature in response to signaling molecules. (A): Fluorescence microscope image of GPMVs at 37 and 3°C. Scalebar 5 μ m. (B-E): Fraction of GPMVs showing just one phase over the total of vesicles observed in function of the temperature. The data show a sigmoidal trend and are fitted with a hyperbolic tangent from which are extracted the transition temperature at mid height and the width of the transition. We compare the sample obtained from cells treated with KdoLipidA (B), LPS (C), IFN- γ (E), for 12h compared to a non-treated control condition prepared in parallel. All these “pro-inflammatory” treatments shift the transition temperature towards higher temperatures. The colored arrow at the bottom indicates the direction of the temperature variation imposed on the GPMV samples during the imaging process. (D): The knock-out TLR^{-/-} cells do not vary transition temperature when stimulated with KdoLipidA (in contrast to panel (A)), remaining the same to the unstimulated controls.

1 membrane lipids as isolated systems [19,20]. These vesicles are thought to maintain the protein
 2 and lipid diversity of the mother membrane [20,21], and at low temperatures the lipids can phase
 3 separate laterally into micron sized domains [17,22,23].

4 GPMVs as systems to study the transition temperature of the plasma membrane have
 5 shown systematic dependency on growth, temperature [24] and cell cycle [25], and on the
 6 epithelial-mesenchymal transition in cancer cells [26], and indeed in both situations the transition
 7 temperature of GPMVs recapitulates broad systematic composition changes that move the cell
 8 composition closer or farther from the critical point. In literature there are previous studies on
 9 the effect on lipid composition of macrophage activation [27,28], but these are bulk assays and
 10 report on the changes in a huge number of lipid species, making it difficult to interpret the
 11 results in simple terms. The work presented here shows that these complex changes in lipidomics,
 12 as reported in literature [27,28], may have a simple interpretation, in terms of their effect on
 13 the membrane phase separation. Investigating the effects of different kinds of macrophage
 14 cell stimulants (LPS, KLA, IFN- γ , IL4), known to differentiate macrophages into two different
 15 activation states, we show opposite changes with respect to the proximity of the critical point in
 16 the two cell types, consistent with biological function.

17 2. Materials and methods

1 (a) Cell Culture

2 The immortalized BMDM cell lines were obtained from Dr. Eicke Latz (Institute of Innate
 3 Immunity at the University of Bonn, Bonn, Germany), and Dr. Kate Fitzgerald and Dr. Douglas
 4 T. Golenbock (University of Massachusetts Medical School, MA, USA). C57BL6 TLR4^{-/-} mice
 5 were obtained from Dr. S. Akira (Osaka University, Osaka, Japan) [29]. iBMDM and TLR4^{-/-}
 6 iBMDM were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, MO,
 7 USA) supplemented with 10% (v/v) heat-inactivated Hyclone fetal calf serum (FCS; Thermo
 8 Scientific, UT, USA), 2mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin and streptomycin
 9 (Sigma-Aldrich), and 20mM HEPES (Sigma-Aldrich). Cells are cultured for at least two days and
 10 brought to confluence in a single 175 cm² flask. From confluence, cells are plated in separate
 11 dishes. To test the effect of stimulants on the melting temperature an equal number of cells
 12 are plated for each condition; we use a density of about 6-7·10³ cells/mm². After 12 hours
 13 the culture medium is changed with (or without for the control condition) the addition of
 14 stimulating agents. Then, after the stimulation time, we start the GPMVs production protocol.

Stimulation	T_m (°C)	T_m err (°C)	σ (K)	σ err (K)	
IL4	12h	12.42	0.98	3.28	0.99
	12h	12.42	0.79	3.19	0.81
	24h	10.46	0.33	3.35	0.35
	24h	14.54	1.06	5.46	1.56
UNST	18.91	0.89	5.45	1.57	
	14.56	0.80	5.75	1.24	
	15.88	0.46	4.39	0.61	
	18.18	0.43	4.17	0.56	
	14.00	0.69	5.20	0.99	
	13.11	0.49	4.90	0.62	
	15.88	0.46	4.39	0.61	
	16.44	0.83	4.28	1.03	
	14.82	0.70	4.23	1.31	
	14.21	1.02	5.31	1.44	
16.95	0.50	6.79	0.85		
16.11	0.60	8.25	1.12		
IFN 12h	20.38	0.99	6.79	1.97	
	19.04	0.87	6.73	1.51	
	18.81	0.45	5.09	0.78	
LPS 12h	20.15	0.20	3.84	0.30	
	18.77	0.89	7.12	1.63	
	18.36	0.61	4.84	1.03	
	15.74	0.93	7.50	1.59	
	21.33	0.41	4.95	0.72	
16.35	0.74	6.27	1.51		
KLA 12h	15.93	0.88	6.35	1.43	
	25.93	0.90	6.02	1.68	
	15.63	1.00	7.60	1.70	
TLR4 ^{-/-} UNST	16.48	0.68	4.22	1.06	
TLR4 ^{-/-} KLA	15.43	0.93	4.84	1.38	

Table 1. Summary of the numerical values of the miscibility temperature and transition width obtained fitting the data with the empirical function $f(T) = A [\tanh((T - T_m)/\sigma) + 1] + C$.

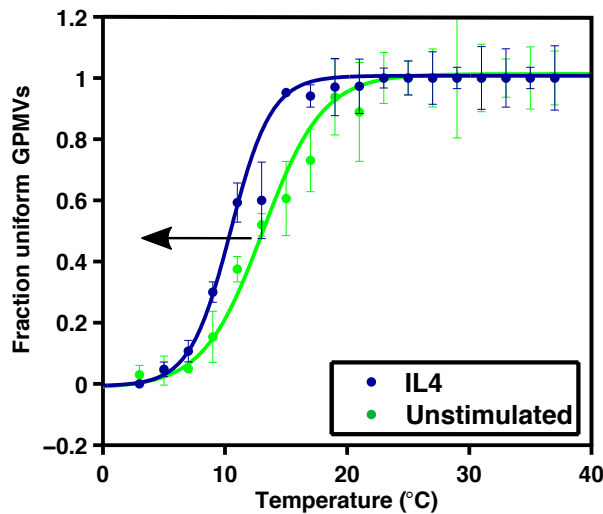


Figure 2. Anti-inflammatory treatment changes the melting temperature in opposite direction compared to pro-inflammatory stimuli, consistent with changes in the composition of the membrane away from the critical point. The data show the fraction of uniform GPMVs as the temperature of the sample is varied. The two curves correspond to 24 hours of IL4 stimulation and to unstimulated conditions. $T_{UNST} = (13.11 \pm 0.49)^{\circ}\text{C}$, $T_{IL4} = (10.46 \pm 0.33)^{\circ}\text{C}$.

- 1 Cell stimulating agents are used at the following concentrations and with the following timings:
- 2 IFN- γ 20 ng/ml (PeproTech) for 12 hours; LPS from *Salmonella* Typhimurium 10 ng/ml (Enzo
- 3 Life Sciences) for 12 hours; Kdo-LipidA 100 ng/ml (KLA, Avanti Polar Lipids) for 12 hours; IL-
- 4 4 20 ng/ml (PeproTech) for 24 hours. These doses were chosen according to previous work on
- 5 M1/M2 macrophage differentiation [30,31] [32].
- 6 To measure the T_m vs cell density dependency, density was measured in two different ways. For
- 7 some experiments, images of the culture were acquired with a low magnification objective and
- 8 the density estimated by counting cells from the image and then dividing their number by the
- 9 field of view area. The same dish was then used to produce GPMVs immediately after. Otherwise
- 10 for each density we had twin dishes, one was used to count the cells with the hemocytometer,
- 11 whilst the other was used to produce GPMVs. To check the effect of stimulation on growth rate,
- 12 an equal number of cells were plated in a multi-well then, for each condition (control, IL4, LPS);
- 13 cells were counted with the hemocytometer after cell adhesion (0h), then stimulated, according
- 14 to previously specified concentrations, and counted after 12h. Cells were initially plated to have
- 15 about $6\text{-}7 \cdot 10^3$ cells/mm² at 0h.

16 (b) GPMVs production

- 17 The procedure for membrane labeling and GPMVs production follows the protocols in [33]
- 18 and [34]. The cells are gently washed twice with PBS, then DiI-C12(3) (Life Technologies) dye
- 19 solution 50 $\mu\text{g/ml}$ in PBS is added and left on ice for 10 minutes to allow incorporation into the
- 20 membrane. Then the cells are washed five times with PBS and twice with GPMV buffer. GPMV
- 21 buffer is formed by 10mM HEPES, 150mM NaCl, 2mM CaCl₂, the pH is adjusted to 7.4 with HCl
- 22 or NaOH. Lastly the vesiculating agent is added and the cells are left in the incubator for 1.5 hours
- 23 at 37°C. 20 μl of vesiculating agent (2mM DTT, 25mM PFA) is used for each ml of GPMV buffer.
- 24 The medium is gently harvested and transferred into a falcon tube. The sample is left at 37°C
- 25 enough to let the blebs deposit on the bottom of the tube: for a volume of 4ml, 24 hours are
- 26 enough for the whole sample to sediment.

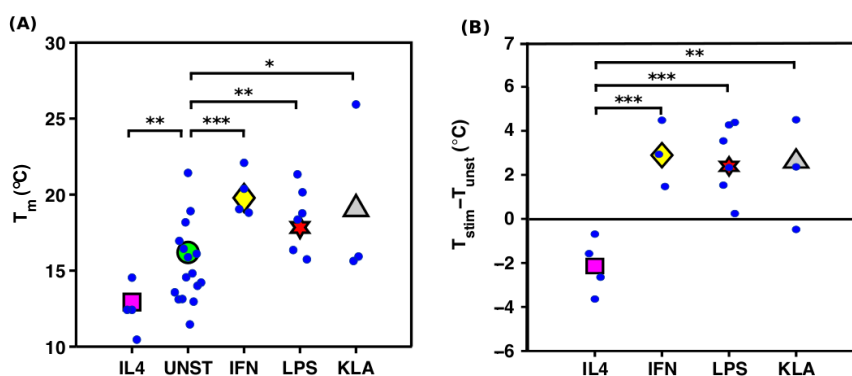


Figure 3. Pro- and anti-inflammatory treatments affect the transition temperature systematically. The scatter in the absolute transition temperature (particularly notable in the unstimulated UNST cells) is reduced significantly comparing with same-day unstimulated controls. (A): Fitted transition temperatures of vesicles produced by macrophage cells treated with IL4, IFN- γ , LPS or Kdo-LipidA. Each small data marker comes from an experiment with between 300 and 600 vesicles. The large markers indicate the average in each distribution, weighted with the errors on T_m . (B): Temperature difference of each stimulation experiment with its control condition. From one-way analysis of variance (ANOVA) we obtained the distributions differences to be statistically significant with * $p < 0.1$, ** $p < 0.05$, *** $p < 0.005$.

1 (c) Isolation of Lipids, and Gel-assisted vesicles formation

2 For the lipids isolation procedure we followed the Bligh and Dyer method [35]: 1 ml of GPMVs
 3 sample is collected and moved to a vial. Then are added 3.75 ml of 1:2 chloroform and methanol
 4 mixture, 1.25 ml of chloroform and 1.25 ml of distilled water. After each step the solution is
 5 vortexed for 1 minute. At this stage the GPMVs burst and the components dissolve in the solution.
 6 The mixture is then centrifuged at 1000 RPM for 5 minutes. This makes the chloroform/methanol
 7 fraction deposit at the bottom of the tube, together with the lipids, while the aqueous and
 8 water soluble component is isolated at the top. Proteins are preferentially located at the interface
 9 between the two phases. The bottom phase is then collected and left under vacuum to let the
 10 solvents evaporate. Finally lipids are re-dissolved in 100 μ l of chloroform.

11 The vesicles are produced through the gel-assisted method as described in [36]: 200 μ l of 5%
 12 (weight/weight) PVA solution is spread on a microscope coverslip with the help of a spincoater
 13 and then left to dry in an oven at 50°C for 30 minutes. Lipids dissolved in 100 μ l of chloroform are
 14 then spread on PVA gel. A chamber is formed with the help of a spacer and a second coverslip
 15 and filled with a solution of sucrose. After 30 minutes the vesicles are collected and diluted in
 16 glucose solution to allow vesicle sedimentation.

17 (d) Imaging

18 The samples are imaged on a Nikon Eclipse Ti-E inverted epifluorescence microscope using a
 19 Nikon PLAN APO 40 \times 0.95 N.A. dry objective and a IIDC Point Grey Research Grasshopper-
 20 3 camera. The perfect focus system (Nikon) maintains the sample in focus even during thermal
 21 shifts. The temperature of the sample is controlled with a home-made computer-controlled Peltier
 22 device. A thermocouple is placed in direct contact with the sample chamber. In each position a
 23 z-stack of 8 images is acquired, spanning across a range similar to the bleb size. The temperature
 24 is decreased across the whole sample with a ramp from 37 to 3°C in steps of 2°C; at each step
 25 the temperature is let to equilibrate for 15 seconds. The abundance of GPMVs produced can vary
 26 from cells prepared in different days, but usually from a dish of 5.5 cm diameter with confluent
 27 cells it is possible to produce blebs for at least 2 experiments. With the quantities described above,
 28 we are able to image up to 100-200 blebs in each field of view.

1 (e) Software processing

2 A custom Matlab software pipeline has been developed to automatically detect the position and
 3 radius of the GPMVs in the images. It uses the Hough transform to detect circular features. Then
 4 with the help of a graphical user interface the blebs are shown to the user one at the time, the
 5 user can interactively scroll the z-stack and decide if the bleb shows (a) a single phase, (b) phase
 6 coexistence or (c) unclear phenotype. The software randomly picks the vesicle to show, from the
 7 database of all the temperatures, i.e. in this stage the information about the temperature is kept
 8 hidden to the user, so that the decision process (assigning the type a/b/c) is unbiased.

9 3. Results

10 Following established protocols, GPMVs are produced from macrophages using PFA and DTT.
 11 The sample is observed with an optical microscope in a temperature controlled stage. The
 12 temperature is lowered from 37 to 3°C in steps of 2°C. At high temperatures all the vesicles show
 13 a uniform phase. At around 12-22°C phase separation domains start to appear in some GPMVs,
 14 and at low temperatures most of the GPMVs are phase separated (Figure 1 A). Approaching the
 15 transition temperature from below, the contours of the phase separation domains are increasingly
 16 less smooth, and become progressively rough and fragmented until the two phases are completely
 17 mixed (see supplementary Figure S1). Similar image sequences were shown in papers where
 18 other aspects criticality were tested directly [17]. This morphology of domains with temperature
 19 suggests that the GPMVs from macrophages have compositions close to critical. For each
 20 temperature, we calculate the fraction $f(T)$ of GPMVs which show uniform phase or phase
 21 separation. Before producing GPMVs, macrophages are stimulated with one of IFN, LPS or KLA
 22 for 12 hours to induce pro-inflammatory response. The effects of LPS stimulation on IBMDMs
 23 has been tested and characterized in detail of the NF- κ B pathway in our previous study [37],
 24 where cell lines with fluorescent markers were used to measure NF- κ B translocation, and TNF α
 25 promoter activation as a downstream effect. In each data set (Figure 1 B-E) we compare the
 26 stimulated condition with its unstimulated control data set, since we noticed (as has been already
 27 reported in different cell types [24,34]) a significant variability in the transition temperature of
 28 independent repeats; in contrast, the transition temperatures of GPMVs from the same cultures,
 29 even split into separate dishes, are tightly distributed.

30 The transition temperature T_m is obtained by fitting the $f(T)$ data with an empirical sigmoidal
 31 curve:

$$f(T) = A \left[\tanh \left(\frac{T - T_m}{\sigma} \right) + 1 \right] + C, \quad (3.1)$$

32 where T_m and σ are the most interesting parameters to describe the mean and the cell-to-
 33 cell variability (GPMVs originate from individual cells) in the transition temperature of the
 34 population. Error bars are associated with data points by randomly separating the measurements
 35 for a given temperature into three groups, and treating these as independent data sets.

36 Figure 1 B shows the effect of the cell stimulation with KLA for 12 hours. The comparison
 37 with the control condition shows a shift of 4.5°C in the GPMVs transition temperature to higher
 38 temperatures. As expected, LPS and KLA stimulation produce similar effects (see Figure 1 B-
 39 C). Indeed Kdo-LipidA is the active sub-unit of the LPS molecule which is recognized by the
 40 TLR4 trans-membrane receptor [6]. Notice that the comparison between LPS and KLA has to
 41 remain qualitative since there isn't a first-principles way to correlate the doses, except for the
 42 effects on activating cells. Both doses employed here are known to be able to saturate the cell
 43 response, for example in terms of TNF α production [28,37,38]. As a control, repeating the same
 44 experiment of KLA stimulation, this time on TLR4^{-/-} macrophage cells, we obtained compatible
 45 transition trends (Figure 1 D) between the stimulated and unstimulated condition. The absence of
 46 a lipid change in the cells without receptor strongly suggests that the observed phase transition
 47 temperature shift is originated from the metabolic change as a downstream effect, and not by a
 48 direct membrane perturbation by the ligand. We then stimulated cells with IFN- γ , known, like

1 LPS, to have pro-inflammatory effects [1], and obtained the same qualitative effect on the plasma
2 membrane transition temperature (Figure 1 E).

3 Since all the experiments with the "classically activated" conditions were showing a consistent
4 shift in the same direction, we decided to stimulate the cells with interleukin-4 (IL4) which
5 is known to induce a different type of differentiation [3]. Macrophages treated with IL4 have
6 different phenotypes and markers compared to the M1, and a different role in the immune
7 response: they don't produce pro-inflammatory cytokines, but suppress destructive immunity,
8 and are involved for example in wound healing response [3]. IL4 is also known to induce
9 polarization on our same cell line (iBMDM) [39]. The curves in Figure 2 correspond to the
10 control condition and to 24 hours of IL-4 stimulation. Also in this case the stimulation produces
11 a temperature shift, but in contrast to the "classically activated" cells, in this case the T_m shifts
12 towards lower temperatures.

13 Collecting together all the T_m values from different stimulation experiments (see Figure 3 A)
14 we can see how the IL4 data and the IFN- γ /LPS/KLA data are in two separate temperature
15 ranges, with no data overlapping, while the values from the unstimulated experiments have
16 a much wider range. Statistical analysis confirms the distributions to be significantly different
17 ($p < 0.05$) for almost all of the conditions. Calculating the temperature differences $T_{stim} - T_{unstim}$
18 (i.e. comparing with same day controls), the temperature shifts tighten (Figure 3 B) and show
19 very consistent behaviors: the IL4 data points are all negative, whereas the others are all
20 positive. Similar temperature shifts, of about 2-4 °C, have also been found when comparing
21 melting temperatures of GPMVs from human mesenchymal stem cells (MSCs) differentiated into
22 osteoblasts or adipocytes. Also in this case the plasma membrane lipidic composition is thought
23 to play a key role into tuning lineage specification [40].

24 We then investigated cell density as one of the possible causes for the large variability of T_m
25 in the control condition. The effectiveness of intracellular communication indeed depends on the
26 cell density, and can be conveyed through both mechanical or chemical interaction [41–43]. A
27 set of careful experiments (see supplementary figure S2) shows a linear trend of the miscibility
28 temperature as a function of the cell density, with the higher densities inducing a shift in
29 the T_m in the same direction as the IL4 activated samples. A similar density effect has been
30 observed in similar experimental conditions for other cell types [24]. We also found that LPS
31 stimulation induces differences in the population growth rate, which complicates comparison
32 between controls if one wants the cell density to match. Putting together the cell density assay
33 with the measurement of cell growth, we found that with our cell growth protocol the difference
34 in densities between treated and control conditions can lead to an effect of on only about 0.5°C of
35 the 2°C shifts measured for T_m (see supplementary figure S2 B).

36 The high quality imaging allowed us to investigate also the shape of the phase separation
37 domains appearing in blebs at low temperatures. Some of the domains indeed appear to have an
38 irregular rigid shape similar to a gel phase domain, while others look more rounded like in the
39 situation of liquid-liquid coexistence. With the help of a graphical user interface that shows a 3-4
40 frames time sequence of the vesicle, GPMVs with irregular domains were identified as the ones
41 presenting rigid and not rounded dark regions (see supplementary figure S5). The appearance
42 of gel-looking domains on GPMVs has already been reported [24], but this is the first attempt
43 for a quantification of the phenomenon. Three sets of data in different conditions are shown in
44 Figure 4. In all the cases, in spite of the noise, the fraction of irregular domains over the total
45 of phase separated GPMVs has a clear growth at low temperatures, reaching about 0.4 at 3°C.
46 On the other hand we don't see any significant difference in these trends comparing different
47 stimulation conditions suggesting that they might not play a critical role in the cell activation
48 and differentiation. In the event that these irregular domains could be confirmed as gel domains,
49 this kind of analysis would provide an additional piece of information on the phase diagram of
50 the biological membrane lipid mixture (on which we still have very incomplete knowledge) and
51 might be particularly important in cell biology regulation involving cholesterol [44].

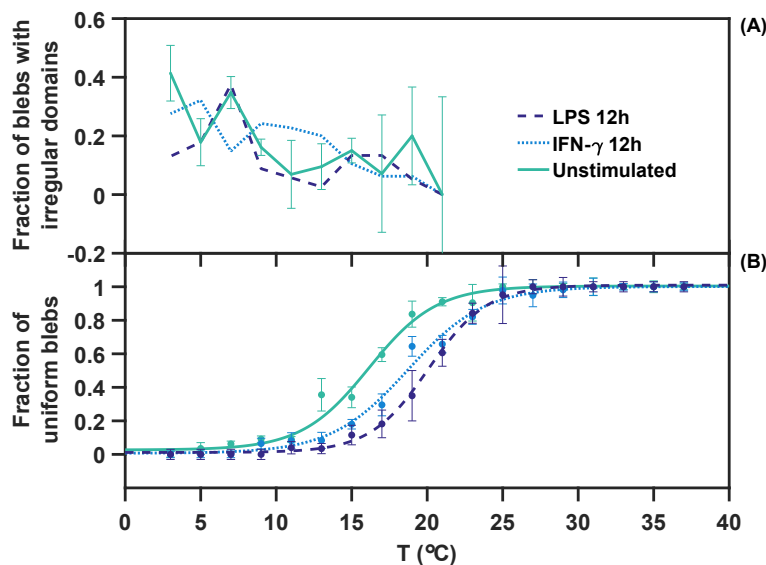


Figure 4. At very low temperatures, irregular shaped domains are observed and attributed to a gel phase. (A) The fraction of GPMVs with irregular domains (over the total of phase separated GPMVs) increases at low temperatures. This fraction grows below T_m , as can be seen comparing in (B) the ‘conventional’ data on liquid-liquid phase separation for the three conditions indicated in the legend. See microscopy images in Supplementary Figure S5.

1 The experiments described so far provide evidence that the composition of the plasma
 2 membrane is regulated according to the external milieu, but we still don’t know if the phase
 3 separation phenomenon is lipid driven or if the proteins have any role in the formation of
 4 lipid compartmentalization. To address this, we performed an important and seldom considered
 5 control: comparing the melting temperature of GPMVs with the same sample after a lipid
 6 purification process. A similar experiment has been pioneered by Dietrich *et al.* [45]. The GPMVs
 7 sample was divided in two aliquots, and one of them was dissolved, purified and the vesicles
 8 re-formed through the gel-assisted formation technique, as described in the methods section. The
 9 purified GPMVs produced with this protocol are very few compared with the control sample
 10 moreover it is easier to find them clumped together and are on average smaller. This makes the
 11 recognition of phase separation domains more difficult and prone to errors. Nevertheless, as the
 12 standard GPMVs sample, also the purified GPMVs show phase separation at low temperatures
 13 as well as a very similar phase transition curve (see Supplementary Figure S6 and Supplementary
 14 Figure S7). This means that the phase separation phenomenon on GPMVs is lipid driven and that
 15 the miscibility temperature is mostly unperturbed by membrane proteins. It is worth remarking
 16 here that this experiment has to be interpreted as a qualitative result since we don’t have proof
 17 that the lipid mixture is preserved identical after the purification process, moreover in the
 18 reconstituted vesicles we would have lost any bilayer asymmetry possibly maintained in the
 19 GPMVs.

20 4. Discussion

21 It is well known that the plasma membrane is not just a passive support for activity by membrane
 22 proteins, and here we have developed the theme that the property of lipids to phase segregate
 23 relates to protein interactions [12,46]. GPMVs are an extremely useful system to understand this
 24 aspect of plasma membranes because they maintain the composition of the original membrane,
 25 but they can be studied as an isolated structure and subjected to stringent controls. Our

1 results add to the body of evidence that proximity to the critical point for phase separation
 2 can be a global regulator favoring activity. For the specific case of macrophage cell activation
 3 our results are consistent with previous findings that TLR4 receptor oligomerization takes
 4 place in raft domains [14]. Indeed melting temperatures closer to the physiological temperature
 5 produce bigger and longer lasting fluctuations in the spontaneous domain formation with a
 6 higher chance for protein interaction. Oligomerization of TLR4 would be further promoted as a
 7 positive feedback loop, as well as promotion of oligomerization of any other membrane receptor
 8 that partitions preferentially into lipid domains. These mechanisms would reinforce signaling
 9 cascades and the commitment of macrophages to an activated state.

10 (a) Effect of stimulation on plasma membrane transition temperature

11 We have seen how treatment of macrophages with different stimulating agents affects the melting
 12 temperature of GPMVs. All the stimulants used (IFN- γ , LPS, KLA, and IL4) induced a shift of
 13 few degrees compared to the control condition, meaning that in all the cases the membrane
 14 composition has changed as a consequence of the activation of specific signaling pathways.
 15 Moreover, IFN- γ , LPS and KLA increased the transition temperature (T_m), whereas IL4 had
 16 the opposite effect decreasing T_m . Given that the first three stimulants can be connected to the
 17 activation into the M1 state in macrophages, whereas IL4 is responsible for the differentiation
 18 into the M2 state, this result sheds new light on the importance of plasma membrane composition
 19 in the immune response, and suggests new ways in which lipid composition may be involved in
 20 the regulation of the host defense strategy.

21 From the point of view of the membrane composition, if the melting temperature increases
 22 (coming closer to physiological temperature) it means that spontaneous lipid domains are longer
 23 lived and larger, so that membrane components can partition more strongly; also, the energy
 24 cost to recruit a particular lipid micro-environment around a protein is reduced [46]. It has been
 25 calculated that due to this universal phenomenon, the proximity to critical point, spontaneous
 26 lipid domains exist at sizes of around 22 nm for GPMVs from RBL cells [17]. This argument
 27 considers the dimension of the correlation length ξ at a physiological temperature ($T = 37^\circ\text{C}$), and
 28 experiments that measured T_m , then using the expression $\xi = \xi_o T_m / (T - T_m)$ [18]. This same
 29 argument can now be extended, in light of the temperature shifts presented here and assuming,
 30 as we show from domain morphology, that the composition of GPMVs from macrophages is close
 31 to a critical point. Keeping the same value of ξ_o from [17] (because this is a quantity linked to the
 32 size of the lipid) we can estimate the effect of an increase in T_m from $T_{m\ M1} = 13$ to $T_{m\ M2} =$
 33 20°C (as from figure 3). This results in an increase of the correlation length of the order of 40%
 34 (from $\xi_{M1} = 12$ to $\xi_{M2} = 17$ nm). We expect this to have effect on confinement of proteins and
 35 their local concentration. In the particular system here we can expect this to affect the balance of
 36 dimerization in membrane receptors (TLR4 itself, hence positive feedback) and hence regulate a
 37 variety of signaling pathways that move the macrophage to the committed activate state [47,48].
 38 The arguments on the changing proximity to the critical point are based on the temperature shifts,
 39 which are well defined and consistent over the experiments; the absolute temperatures, however,
 40 may change with cell density and have a day-to-day variability (see supplementary figure S4).

41 (b) Speculative correlation between membrane composition and receptor 42 activity

43 We suggest here a possible correlation between the role of the cell in the immune defense and
 44 the changes in its membrane composition. One can imagine that these cells, depending on their
 45 activation state, regulate their lipid composition in such a way to tune the proximity to the critical
 46 point, and hence in turn the typical dimension and lifetime of spontaneous lipid domains, in
 47 order to be more or less reactive towards external stimuli. An M1 cell would have bigger and
 48 more long-lasting lipid domains, leading to increased activity of TLR4 receptors, which have raft

1 affinity [14,49–51] (e.g. by increased recruitment to the membrane, and increased dimerization), to
2 induce a faster and stronger inflammatory response with consequent production of inflammatory
3 cytokines. In contrast, in M2 cells the activation of the TLR4 to NF- κ B pathway would be
4 down-regulated through the lipid composition effect. An important element in support of this
5 hypothesis is the reported increased sensitivity to LPS after IFN- γ treatment, both in mice [52]
6 and in macrophages in vitro [53], where a 66% increase of the LPS binding efficiency has been
7 measured. In general activation of TLRs can induce long term changes in the way a cell responds
8 to further stimulation with TLR ligands including sensitization or tolerance [54].

9 (c) Effect of cell density on T_m

10 To investigate the cause of the T_m day-to-day variation, the effect of cell density was tested. The
11 results show that denser populations induce a lower T_m in GPMVs. The same experiment has
12 been very recently performed on rat basophilic leukemia cells (RBL) [24] with the same outcome,
13 the authors suggesting that dense populations could have different physical membrane properties
14 to be able to sense and communicate with touching cells [55].

15 Regarding macrophages, one could relate this result with the shift given by the different
16 kind of stimulations, venturing a picture in which the overcrowded populations have some
17 common behavior with M2 cells. Our hypothesis is that cell density indirectly induces a
18 decrease in T_m , perhaps by triggering the production of cytokines with the same effect of
19 IL4. This idea is supported by a study in which M1/M2-like differentiation was induced by
20 the population density [56]. Moreover BMDMs from high density cultures secrete less pro-
21 inflammatory cytokines, have lower phagocytic ability, and the number of cells showing typical
22 M2 membrane markers like CD11c and Ly-6CLy-6G increases [56]. In this picture, the crowded
23 populations, with no need to further recruit cells and promote additional inflammation against
24 possible infections, diminish their cytokine production, thus acting more like M2 cells.

25 To test the hypothesis of the interaction through cytokines, we performed an experiment
26 where the medium was periodically changed every 2 hours. The "washed" sample shows a
27 higher T_m compared to the control, where cytokines would be accumulating in the medium,
28 see Supplementary Figure S3. This is compatible with a scenario in which the control condition is
29 affected by an accumulation of M2-inducing cytokines such as IL4.

30 Even though the density has been proven to be an important factor in the day-to-day
31 variability in the T_m of unstimulated macrophages, this is not enough to fully explain the
32 variability between independent repeats, indeed just keeping the cells in separate cultures is
33 enough to produce some variability (Supplementary Figure S4).

34 5. Conclusions

35 The biological question addressed here concerns macrophage cells, which we conditioned via
36 pro- and anti-inflammatory stimuli, before extracting GPMV and measuring their phase transition
37 temperatures. From the morphology of domains, it is clear that phase separation happens in the
38 proximity of a critical point (second order transition). Considering all the transition temperatures
39 together, we get a very consistent picture: transition temperatures following IL4, as opposed
40 to IFN- γ /LPS/KLA treatment, form two non-overlapping intervals (respectively at 10-15°C
41 and 15-25°C). The absolute temperature changes induced by stimulation are always around
42 2°C compared to control. We have described a physical mechanism that can underpin this
43 correlation between the immune response role of macrophage cells and the lipid composition
44 of their plasma membranes, where signaling activation initiates, as part of an amplification of
45 response towards cell differentiation (to an activated, inflamed state). Moreover, for the first
46 time, albeit in preliminary fashion, we carried out experiments on vesicles reconstructed from
47 purely the lipid fraction of GPMV from the plasma membrane of macrophages. We observed their
48 phase behaviour, comparing it to the GPMV. The reconstructed vesicles show phase separation
49 apparently the same as the GPMV. The fact that separation is unaffected by the extraction of

1 proteins means the lipids are undisputed key regulators of phase separation phenomena in the
 2 plasma membrane. Also for the first time, we quantified the fraction of irregular domains on
 3 GPMVs, which are a gel phase. We observed an increase of these at low temperatures. Much
 4 remains to be discovered within the 'critical lipidomics' paradigm, specifically direct experiments
 5 are becoming possible thanks to super resolution approaches [4,11,12], probing membrane protein
 6 copy numbers and states of aggregation and how these are affected by the proximity to lipid
 7 mixture critical points.

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