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Elasticity and adhesion of resting and lipopolysaccharide-stimulated macrophages

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Abstract **Colloidal Force Microscopy was employed to study the viscoelastic and adhesive properties of macrophages upon stimulation with lipopolysaccharide (LPS). Force vs. distance measurements were performed. The adhesion of LPS-stimulated cells (separation force = 37 ± 3 nN) was almost twice as high as that of resting macrophages (16 ± 1 nN). Upon retraction pulling of membrane tethers was observed. Tether lengths and forces at which rupture take place did not depend on stimulation. The reduced Young's modulus *K*, a measure of cytoskeleton elasticity, was three times lower than that of the control. The data show that LPS has profound effects on cytomechanical and adhesion properties of macrophages.**

Keywords: Macrophage; Lipopolysaccharide; Elasticity; Adhesion; Colloidal force microscopy

1. Introduction

Lipopolysaccharide (LPS), a major outer membrane component of Gram-negative bacteria, plays an important role in the pathogenesis of a Gram-negative infection [1]. The primary target cells of LPS in mammalian species are professional phagocytes including monocytes/macrophages [2–5]. Interaction of LPS with these cells induces multiple biological responses such as secretion of cytokines and an increased expression of adhesion molecules [6–8]. Furthermore formation of lamellipodia and an extensive cell spreading is observed [9].

While these changes of cytomechanical properties and activities are well understood on a qualitative level, little is known in quantitative terms. Colloidal force microscopy (CFM) was applied to directly measure adhesive forces and the visco-elastic behaviour of the membrane and the cytoskeleton system.

CFM is a variation of force microscopy [10–12]. Instead of a nanodimensional tip a colloidal particle of micrometer dimensions glued to the cantilever of the atomic force device is used as the sensing interface. The advantage of this particular technique is that force–distance curves can be directly converted into interaction energies taking advantage of the known and well-defined topology of the colloidal particles. In view of the comparatively large area of the test colloid being in contact with the cell surface the technique provides an average value of the interaction rather than specific interaction events such as binding with receptors or the like. When the particle is pressed against a cell it complies and allows the cantilever to move further down. From the recorded force–distance profile mechanical parameters of the cell can be derived.

Here, we show that adhesion forces and cytoskeleton elasticity differed remarkably between stimulated and unstimulated cells indicating that the LPS signal has been transduced into defined mechanical changes. To our knowledge this is the first quantification of cytomechanical changes of macrophages upon stimulation with LPS.

2. Materials and methods

2.1. Macrophages

Human peripheral blood mononuclear cells from healthy donors were obtained by centrifugation over a Ficoll-Isopaque (Pharmacia, Freiburg, Germany) density gradient. After repeated washings in phosphate buffered saline containing 0.3 mM EDTA, the monocytes were isolated by counterflow elutriation using the JE-6B elutriation system (Beckman Instruments, Palo Alto, CA, USA) as described previously [13].

The purity of the cell preparation was >90% as assessed by morphological screening and immunofluorescence staining with a monoclonal antibody against CD14 (BL-M/G14, DiaMak, Leipzig, Germany). To obtain macrophages monocytes (1.2×10^6/3 ml) were seeded onto 13 mm diameter sterile coverslips (CELLocate®, Eppendorf, Hamburg, Germany) in 35 mm diameter cell culture dishes and left in culture medium at 37 °C and 5% CO_2 for 5–7 days. The culture medium contained: RPMI 1640 (Sigma–Aldrich, Taufkirchen, Germany) supplemented with 2% inactivated human serum of a donor with the bloodgroup AB, 80 U/ml penicillin (Seromed® Biochrom KG), 80 μg/ml streptomycin (Seromed® Biochrom KG), 4 mM glutamine (Seromed® Biochrom KG), 48 μM β-mercaptoethanol (Sigma–Aldrich), 1 mM sodium pyruvate (Seromed® Biochrome AG), 1 mg/ml sodium bicarbonate (SERVA, Heidelberg, Germany), 0.4% vitamin solution (Gibco, Eggenstein, Germany) and 1% non-essential amino-acid mixture (Seromed® Biochrom KG).

Macrophages that adhered to coverslips were incubated in the presence or absence of LPS (10 μg/ml) for 4 h, at 37 °C, 5% CO_2.

2.2. Colloidal particles

10 μm diameter monodisperse melamine formaldehyde (MF) microparticles labelled with 7-amino-4-methylcoumarin (excitation 360 nm, emission 429 nm) were provided by microparticles GmbH (Berlin, Germany).

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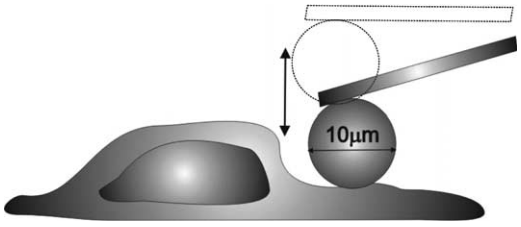
2.3. Confocal laser scanning microscopy (CLSM)

Macrophages were fluorescently labelled with the fluorescent dye MitoTracker® Orange (chloromethyltetramethylrosamine) from Molecular Probes Inc. (Eugene, OR, USA) [14]. The dimension of macrophages in z-direction were measured by means of CLSM (TCS SP II, Leica, Heidelberg, Germany) equipped with a 63× oil objective with a numerical aperture of 1.32.

2.4. Colloidal force microscopy

MF microparticles were attached with epoxy glue (UHU Plus Endfest 300, Uhu, Bühl, Germany) to the free end of a tipless rectangular shaped cantilever (ULTRASHARP, non-contact silicon cantilever CSC12 from Silicon-MDT, Moscow, Russia) using a micromanipulator (Transferman NK2, Eppendorf, Hamburg, Germany). Prior to the measurements the cantilever together with the particle was coated with human serum proteins by dipping the serum solution for 10 min.

Colloidal force measurements were performed by means of the Molecular Force Probe (MFP-1D, Asylum Research, Santa Barbara, USA). The spring constant of each cantilever was determined from its thermal resonance frequency spectra. The sensitivity of the cantilever was determined from reference scans against a glass surface.



Scheme 1. Scheme of colloidal force microscopy to explore cytomechanics of macrophages. A 10 μm MF microparticle coated with serum anchored on a AFM cantilever is moving to and retracting from the cell in a region aside the nucleus.

Scheme 1 shows the principle of the colloidal force measurements. The 10-μm MF microparticle anchored on the cantilever is moved towards a peripheral region of the surface of a macrophage. The cells are labelled with an Orange fluorescent dye and the colloidal sphere is labelled with a UV-fluorescent dye. These two distinguishable fluorescences allow a direct positioning of the colloidal probe on the surface of a macrophage. 80–100 force vs. separation curves were acquired at 500 nm/s pulling rate at each position.

2.5. Elasticity calculation

Approach curves were analysed according to the Chen model, a modification of the Hertz model of elastic indentation [15–17].

Briefly, the Hertz model relates the load F to the indentation δ as $F = KR_i^{1/2}\delta^{3/2}$, with the radius of the indenting colloidal sphere, R_i, and the reduced Young modulus, $K = 4/3[(1-\nu_o^2)/E_o + (1-\nu_i^2)/E_i]^{-1}$, which is a combination of the Young's moduli, E, and the Poisson ratios, ν, of the probed object (subscript o) and the indenter (subscript i), respectively. In the case of large indentations the Hertz model overestimates the K values, the indenter "feels" the hard substrate underneath. Chen's extension corrects for this effect by modifying the K values by a numerically computed function, which itself depends on the Poisson ratio, the cell thickness and the radius of the indenter.

To calculate the indentation from the z displacement proper contact point determination is crucial. The contact point was defined as the displacement at which the force curve initially changed slope taking into account that an accidentally false determination of the contact point would result in irregular K values converging either to zero or infinity.

As the cell thickness used in calculations an average for either macrophage population was obtained from confocal microscopy images (Fig. 1). For both stimulated and control macrophages a value of ~6 μm was found. The Poisson ratio was assumed to be 0.5 following Mahaffy et al. [15]. For verification of the method, polyacrylamide gels of known Young's moduli were prepared according to the protocol of Pelham and Wang [18].

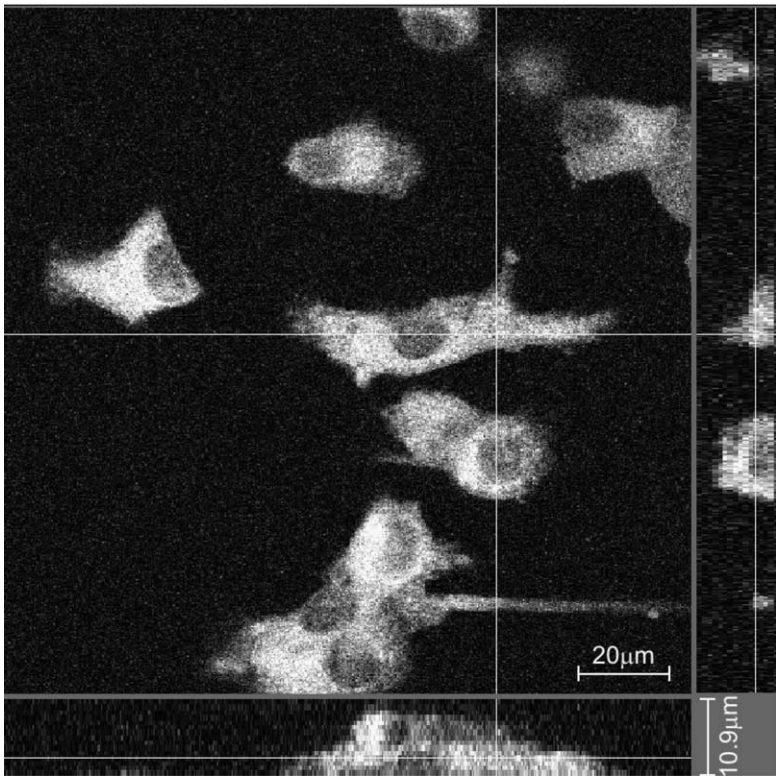


Fig. 1. Confocal fluorescent image of resting macrophages assembled in a 3D frame, taken from a stack of 16 sections $512 \times 512 \times 16$ bit. x–y (centre), y–z (right) and x–z (bottom) views.

3. Results and discussion

Macrophages are known to undergo changes in their adhesion and spreading behaviour upon stimulation with LPS. These changes in cytomechanics, clearly related to the cell functions, can be directly measured by CFM. This technique takes advantage of a colloidal particle glued to the cantilever which is pressed against a cell surface and retracted after contact. Forces as a function of distance can be obtained.

Fig. 2 shows a representative force vs. distance curve measured on a resting cell and Fig. 3 shows the respective traces for a stimulated macrophage. When the spherical particle is moved toward the cell (see the respective upper curve 1 from right to left) the measured force is zero until the sphere touches the cell surface. Long-range electric forces are not observed because of screening in physiological ionic strength. Further movement indents the sphere into the cell leading to a viscoelastic resistance force which gradually increases upon indentation. After approaching the limit of the working range of the device the cantilever is retracted (lower curves from left to right). A hysteresis between approach and retraction is typical. It follows from the viscoelastic nature of the cell. The force curve observes negative values, indicating adhesion before finally separation occurs. The difference between the minimum and the baseline provides the maximum of adhesion from which the adhesion energy can be obtained. The detachment of the colloidal sphere from the cell occurs in a series of smaller steps (see insets in Figs. 2 and 3). These steps represent the rupture of membrane tethers upon retraction. Macromolecular chain pulling, as discussed later, is rather unlikely to be reason for the observed force plateaus.

Although the general character of the force vs. distance curves for control and stimulated cells is similar, there are remarkable quantitative differences in the adhesive force and in cell elasticity while the behaviour of tethers is similar.

Fig. 4 shows the force at maximum adhesion of stimulated and unstimulated macrophages. The mean value of this force for resting macrophages (16 ± 1 nN) was considerably smaller

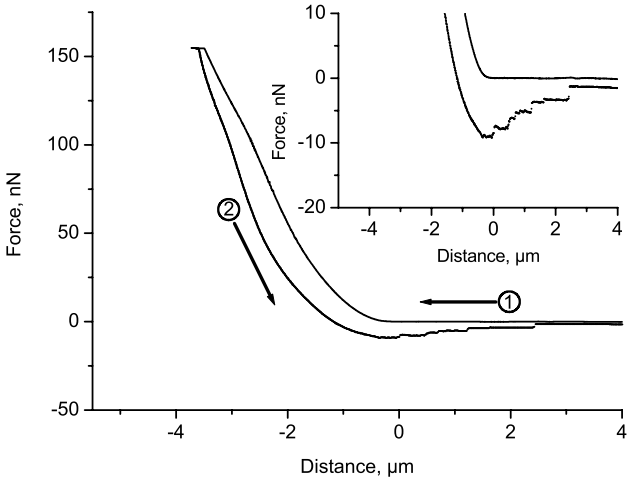


Fig. 2. Example of a force vs. distance curve obtained by CFM showing the interaction of coated MF sphere with a resting macrophage. Curve 1 shows the approach and curve 2 represents the retracting part of the cycle. The inset details the final process of detachment.

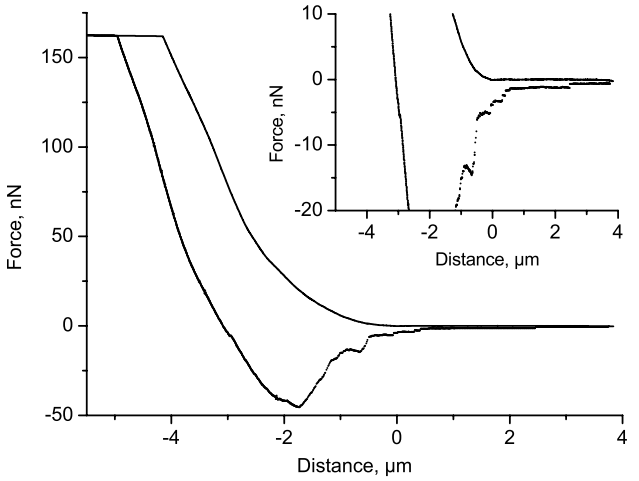


Fig. 3. Example of a force vs. distance curve obtained by CFM showing the interaction of coated MF sphere with an LPS-stimulated macrophage.

than that for stimulated macrophages (37 ± 3 nN). It has to be mentioned that the variation of forces was larger between cells compared with measurements conducted on a single cell at various positions.

The distribution of the force at maximum adhesion for stimulated macrophages is bimodal with centers at 15 ± 2 and 53 ± 2 nN, respectively. This indicates that not all macrophages show the same level of activation. There are even some which probably failed to be stimulated. A time dependence of adhesion over the experimental time of approx. 1 h was not observed. This proves that the cells did neither undergo irreversible changes on multiple loads by the sphere, nor did the adhesion properties of the test particle change in a significant manner.

The multiple rupture steps observed upon retraction were characterised by the distance from the cell surface at which the rupture occurred together with the respective force. This distance was taken in reference to the adhesion maximum and provided the "rupture length". The "rupture force" was

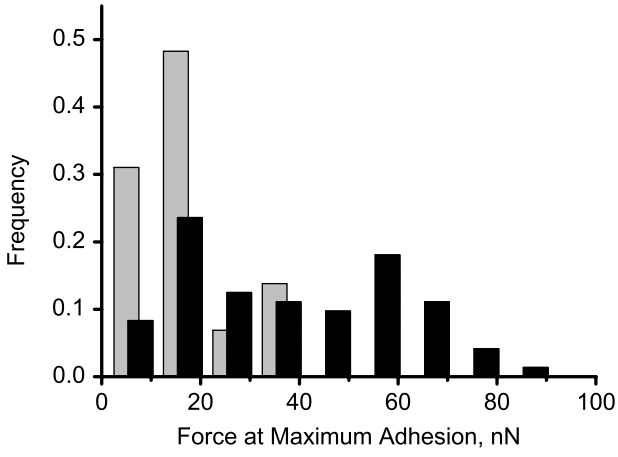


Fig. 4. Histogram showing the distribution of the force at the adhesion maximum for LPS-stimulated, filled columns, and control macrophages, light gray columns.

defined as the height of the force step. Although rupture or detachment of the tether took place at this particular force it represents also the tether pulling force as it is evident from the plateau character of the force steps. Fig. 5 shows the correlation between the "rupture force" and the "rupture length" both for resting and stimulated macrophages as a dot plot. The distributions are clearly coincident. The value of rupture length for resting (2.9 ± 1.8 μm) (±S.D., $N = 479$) is similar to the corresponding value for stimulated macrophages (3.3 ± 1.5 μm) (±S.D., $N = 452$). The presence of terraces of constant force (cf Fig. 3) indicates that these events cannot be related to the stretching of proteins in which case the force would increase hyperbolically with separation until rupture takes place [19]. It was thus concluded that the terraces represent the pulling of membrane tethers. The latter can be pulled almost without increasing resistance until the reservoir of membrane material is depleted.

The reduced Young's modulus, K, was calculated from the approach curves according to the modified Hertz model [15]. Gel composed of 3% polyacrylamide/0.3% bisacrylamide were employed to verify the method. We calculated a Young's modulus of 1490 ± 90 Pa for the gel which is in good agreement with a shear modulus of 5500 dyn/cm^2 ($E = 1460$ Pa) reported by Flanagan et al. [20]. The elastic behaviour of the probing sphere itself was derived from test measurement against glass. The test sphere was soft, with $K \sim 0.2$ MPa, at their peripheral region over a depth of approximately 50 nm. With increasing load the K value of the sphere increased strongly, toward its final value of $K \sim 1$ MPa at a depth of 100 nm. This softness of the peripheral region of the test MF colloid was attributed to the fuzzy character of the surface of the sphere. It has to be mentioned that the true value of the elasticity of MF particles would be of the order of $K \sim 14$–18 GPa corresponding to a Young's modulus of 8–10 GPa for MF [21]. Naturally, this is beyond the range of the cantilever sensitivity. This value is 10^5–10^7 times the elasticity of cells and can thus be neglected in subsequent calculations of cell elasticity [22,23].

The calculated reduced Young's modulus, K, of macrophages generally increased with increasing indentation indicating either a hardening of the cytoskeleton of the cell upon applied pressure and deformation or the presence of less compliant organelles located underneath the testing sphere. Small and gradual increases of the K modulus can be attributed to the effect of strain-induced hardening of the cytoskeleton [24,25] whereas a larger increase, sometimes a fivefold increase was observed, was supposed to be caused by the presence of underlying organelles.

For purpose of comparison the K values at a fixed indentation of 500 nm were calculated both for resting and stimulated macrophages. At this moderate degree of indentation the influence of underlying organelles is not yet pronounced. Hence, it can be assumed that the calculated elasticities reflect largely the properties of the cytoskeleton and the membrane. Fig. 6 shows the distribution of the calculated reduced Young's moduli. It is rather obvious that stimulated macrophages became considerably "softer". On average a threefold increase in softness upon stimulation was found.

The following mean elasticities, K, were calculated: 0.51 ± 0.31 kPa (S.D., $N = 31$) for stimulated macrophages, and 1.5 ± 1.1 kPa (S.D., $N = 32$) for resting macrophages. These values correspond to Young's moduli of 0.29 and 0.83 kPa, respectively. The values of the resting macrophages are well in the range of Young's moduli reported in the literature. Rotsch et al. [26] measured the elasticity of rat liver macrophages by AFM in the so-called Force Mapping Mode. They reported a Young's modulus of 2 kPa. However they used a cantilever tip the radius of which is smaller by three orders of magnitude. Therefore the applied pressure is in spite of the smaller indentation higher by one order of magnitude. Eventually this leads to locally occurring strain hardening.

Considering the LPS-induced reduction of elasticity it is worth to mention that Wojcikiewicz et al. [27] have found a similar softening for leukocytes stimulated by phorbol myristate acetate (PMA). This threefold softening of the macrophages following stimulation can be attributed to changes of the cytoskeleton structure. Actin filaments, a major component of the cytoskeleton, form relatively weak gels with a shear modulus of 500 Pa [25] corresponding to a Young's modulus of 1.3 kPa, which is fairly close to the measured macrophage elasticities.

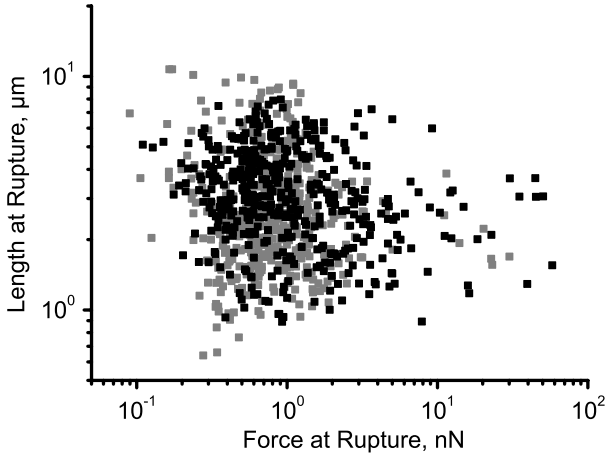


Fig. 5. Detailed analysis of the process of detachment of the probing sphere from the cell surface. The dots represent the correlation between "rupture force" and "rupture length". Solid squares – stimulated macrophages. Light gray squares – control.

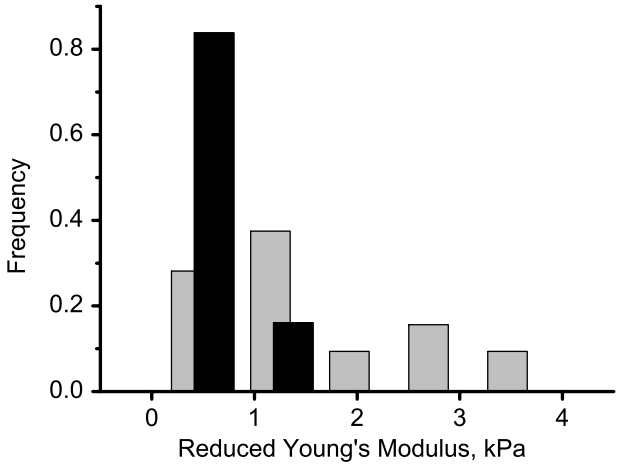


Fig. 6. Histogram showing the distribution of the reduced Young's modulus, a measure of elasticity, for stimulated, filled columns, and control macrophages, light gray columns.

It is worth to mention that the stimulation did not only result in a decreased elasticity but was also accompanied by a more viscous behaviour. This can be concluded from the force vs. separation curves. The sphere sinks deeper into the stimulated than into the resting cell at a comparable force, since a broader shift between the approach and the retraction curves was observed. Furthermore, the spheres detach from stimulated cells at a position several hundred nm deeper than the contact point, suggesting that the original cell shape cannot be recovered as quick as the indenter is pulled away under the experimental conditions.

4. Conclusion

When stimulated with LPS macrophages became noticeably softer. In parallel the adhesion increased remarkably. These cytomechanical changes reflect an underlying transformation of cell physiology related to increased expression of adhesion molecules and changes of the cytoskeleton mechanics related to the activation of cell spreading.

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