Magnetic Phagosome Motion in J774A.1 Macrophages: Influence of Cytoskeletal Drugs

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ABSTRACT The role of the different cytoskeletal structures like microfilaments (MF), microtubuli (MT), and intermediate filaments (IF) in phagosome motion is unclear. These cytoskeletal units play an important role in macrophage function (migration, phagocytosis, phagosome transport). We investigated ferromagnetic phagosome motions by cell magnetometry. J774A.1 macrophages were incubated with 1.3-μm spherical magnetite particles for 24 h, after which more than 90% of the particles had been phagocytized. Phagosome motions can be caused either by the cell itself (relaxation) or by applying magnetic twisting forces, yielding cell stiffness and viscoelastic properties of the cytoskeleton. Apparent viscosity of the cytoplasm was non-Newtonian and showed a shear-rate-dependent power law behavior. Elastically stored energy does not force the magnetic phagosomes back to their initial orientation: 57% of the twisting shear was not recoverable. Cytoskeletal drugs, like Cytochalasin D (CyD, 2 - 4 μM), Colchicine (CoL, 10 μM), or Acrylamide (AcL, 40 mM) were added in order to disturb the different cytoskeletal structures. AcL disintegrates IF, but affected neither stochastic (relaxation) nor directed phagosome motions. CyD disrupts MF, resulting in a retarded stochastic phagosome motion (relative decay 0.53 $\pm$ 0.01 after 5 min versus 0.34 $\pm$ 0.01 in control), whereas phagosome twisting shows only a small response with a 9% increase of stiffness and a small reduction of recoverable strain. CoL depolymerizes the MT, inducing a moderately accelerated relaxation (relative decay 0.28 $\pm$ 0.01 after 5 min) and a 10% increase of cell stiffness, where the pure viscous shear is increased and the viscoelastic recoil is inhibited by 40%. Combining the two drugs conserves both effects. After disintegrating either MF or MT, phagosome motion and cytoskeletal stiffness reflect the behavior of either MT or MF, respectively. The results verify that the dominant phagosome transport mechanism is MF-associated. MT depolymerization by CoL induces an activation of the F-actin synthesis, which may induce an accelerated relaxation and an increase of stiffness. Cell mechanical properties are not modulated by MF depolymerization, whereas MT depolymerization causes a loss of viscous resistance and a loss of cell elasticity. The mean energy for stochastic phagosome transport is $5*10^{-18}$ Joules and corresponds to a force of 7 pN on a single 1.3-μm phagosome.

INTRODUCTION

The cytoskeleton mediates several basic cell functions: chemotaxis, migration, phagocytosis, phagosome-lysosome fusion, and intracellular signaling (Valerius et al., 1982; Ding et al., 1993; Stossel, 1993; Hwang and Ding, 1995; Rivero et al., 1996; Janmey, 1998). The target cells for defense in the lungs are alveolar macrophages (AM). After deposition of foreign matter like particles, bacteria, fungi, or viruses, AM can recognize the location of the particles and migrate to the site in order to phagocytize the particles. This requires many pathways of signal transduction and intracellular cytoskeletal reorganizations. The field of cytomagnetometry offers a novel tool to monitor such cytoskeletal processes in vitro and in vivo. Ferromagnetic microparticles act as inert tracers for cell functions. They are phagocytized rapidly and form ferromagnetic phagosomes, which are aligned in a strong magnetic field pulse. The phagosomes are transported within cells along the cytoskeleton (phagosome-lysosome fusion) and therefore require a coupling to cytoskeletal filaments. Stochastic phagosome rotations are caused by the random motion of the magnetic phagosomes, resulting in a disorientation of the aligned particles and a decay of the remanent magnetic cell field (RMF). This process is called relaxation. A directed particle twist can be caused in a weak external magnetic field, where a magnetic torque acts to the dipole particles. The resistance to this shear yields a direct measure of the cytoplasmic stiffness and the integrity of the cytoskeleton.

It is not yet clear which of the substructures of the cytoskeleton (MF, MT, and IF) are involved in intracellular phagosome transport and which motor proteins drive the phagosomes along the cytoskeleton. In vitro studies suggest that MT provide the network for the transport of intracellular vesicles and organelles (Dabora et al., 1988; Sheetz, 1996; Blocker et al., 1997), caused by a microtubular motor from the kinesin and dynein family (Sheetz et al., 1986; Schroer et al., 1989; Hirokawa, 1996). But the process of particle phagocytosis is mediated by MF; Cytochalasin D inhibits phagocytosis, whereas Colchicine does not (Newman et al., 1990; Yamaya et al., 1995). This would require a translocation of phagosomes from the microfilamentous to the microtubular system. Earlier studies of magnetic phagosome transport suggested a role for MF, because phagosome motion was impaired by cytochalasin D (Valberg and Feldman, 1987; Valberg and Butler, 1990). Additionally, it was

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shown that organelles and coated latex beads could also be transported along MF by myosin motors (Sheetz and Spudich, 1983; Pollard and Ostap, 1996).

Therefore, cytomagnetometric studies were performed on J774A.1 macrophages under the influence of different cytoskeletal drugs. Cytochalasin D (CyD) was used to disrupt the MF, colchicine (CoL) was used to disrupt the MT, and acrylamide (AcL) was used to disrupt the IF. These studies shall help to discriminate between the role of the different cytoskeletal filaments in stochastic phagosome transport and in cytoskeletal tension in macrophages. Besides the questions about intracellular phagosome transport and the cytoskeletal integrity, this study may help us to understand dysfunctions of alveolar macrophages in the human lungs. Cellular dysfunctions associated with the cytoskeleton can cause a retarded phagocytosis (McCulloch and Knowles, 1993) and an impaired phagosome-lysosome fusion (Horwitz, 1983), which may result in a retarded cellular killing and a retarded alveolar clearance.

METHODS

Cytomagnetometric device

The device for measuring in vitro cell probes was designed for in vivo human studies of alveolar clearance and for macrophage cell functions like phagocytosis, phagosome transport, and cytoskeletal integrity (Stahlhofen and Möller, 1993; Möller et al., 1997) and was called magnetopneumography. The adaptation of this method to in vitro studies with cultivated macrophages was called cytomagnetometry. Ferromagnetic microparticles, which had been phagocytized by macrophages, were magnetized and aligned by discharging a capacitor (1 mF, 1000 V) into a copper coil (12 cm mean diameter), producing a magnetic field pulse of 500 mT for 6 ms. After the magnetizing current has decayed, the magnetized particles produce a weak RMF of about 30 pT. The probe is moved under a superconducting magnetic field sensor where the weak magnetic field is detected. This sensor is a second-order gradiometer system and consists of 4 circular loops (10 cm diameter), 2 of which are coupled in opposite current direction and thereby cancel out the interference from external noisy fields. The magnetic flux induced in these loops is transferred to a superconducting quantum interference device (SQUID). To improve sensitivity, the entire system is enclosed in a magnetically shielded room. Both shielding methods, the gradiometer system and the shielded room, allow the detection of very weak magnetic fields ($\approx$1 pT). During the measurement the cells were kept in an incubator at a constant temperature of 37°C.

Spherical ferrimagnetic iron oxide particles (magnetite, Fe_3O_4) were prepared according to the method described in Möller et al. (1990). The particles were collected on filters and resuspended in ethanol. After fractionating the particles for the individual cell probes, ethanol was removed by centrifugation, and the particles were resuspended in culture medium containing 5% fetal calf serum. The particles were not further coated, in order not to activate specific cell surface ligands for phagocytosis, like the Fc receptor. Phagocytosis mediated by the Fc receptor can induce several defense reactions, like the oxidative burst (the release of reactive oxygen radicals) and the release of the second messenger Ca^{2+}, which can interfere with the polymerization of actin and tubulin and can influence the stiffness of the cytoskeleton (Kobzik et al., 1993). We suggest that the nonspecific scavenger receptor mediates phagocytosis of the magnetite particles (Kobzik, 1995).

In vitro cell probe

J774A.1 macrophages originate from a BALB/c/National Institutes of Health mouse (Ralph and Nakoinz, 1975) and were obtained from the German Collection of Animal Cell Cultures (Tumorbank, DKFZ Heidelberg, Germany). Cells were grown in RPMI 1640 medium (Sigma GmbH, Deisenhofen, Germany), supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin, and 0.3 g/L-glutamine in $NaHCO_3$ and Hepes (25 mM) buffer. Cells grew with a doubling time of 2 days and were divided every 4 days. In all experiments 1.3-μm spherical ferrimagnetic iron oxide particles were used. For cytomagnetometric measurement, cells and particles were incubated in 30-mm diameter cell culture petri dishes (Nunc GmbH, Wiesbaden, Germany). Ten-microgram particles ($\approx$2 million) were dispersed in culture medium and incubated in the dish for 2 h before the cells were added in order to disperse the particles homogeneously over the surface area of the petri dish. One million J774A.1 macrophages were added to the probe and incubated at 37°C in 5% CO_2 for 24 h in order to allow a complete phagocytosis of the particles. After 24 h, light microscopic observations revealed no particles outside of the macrophages. In the mean, two particles/macrophage should appear, but around one-third of the macrophages had not phagocytized any particle, and some cells had more than two and, in extreme cases, up to ten particles phagocytized, which shows an inhomogeneity of phagocytic activity. This may induce some error in estimating the rheological properties. However, the response to the drugs should be influenced in a minor way.

Cytoskeletal drugs

The different cytoskeletal filaments can be influenced with different drugs. All drugs were purchased from Sigma. Cytochalasin D (2–4 μM) was used to depolymerize MF, colchicine (10 μM) was used to depolymerize MT, and acrylamide (4 mM) was used to disrupt the IF (Wang et al., 1993). The cells were incubated together with the drugs for at least 15 min in the incubator. Then measurements of relaxation, particle twist, and E_r lasted between 10 and 15 min. Incubation with CoL for longer duration resulted in a time dependence of relaxation and particle twist and is shown separately. Incubation with either CyD or AcL for longer duration (up to 1 h) did not further influence the results and is therefore not shown.

Detection of intracellular ATP

ATP was detected by releasing intracellular ATP into solution containing luciferin and luciferase (FL-ASP kit, Sigma). The reaction with ATP emits light, which was detected by a bioluminometer (Biolumant LB 9500, Fa. Berthold, Wildbad, Germany). The ATP measurements were calibrated by the addition of an ATP reference concentration. Together with the number of viable cells in the probe (detected by a CASY cell counter, Reutlingen, Germany) the mean ATP content per cell could be estimated.

Principles of modeling phagosome motions in macrophages

The motion of vesicles and phagosomes happens perpetually within living cells and is part of the intracellular transport system. Phagocytosis reflects a special feature of this system, where foreign matter is ingested. Phagosomes are thereby formed that enclose the foreign substance. Phagosomes fuse with lysosomes, which contain reactive substances for killing and dissolution. The magnetic iron oxide test particles resist this dissolution for long times and monitor the intracellular phagosome motions. Fig. 1 summarizes the principles of phagosome motions, which can be followed in cytomagnetometry. A stochastic phagosome motion (rotation), called relaxation, is caused by the stochastic transport of the phagosomes along the

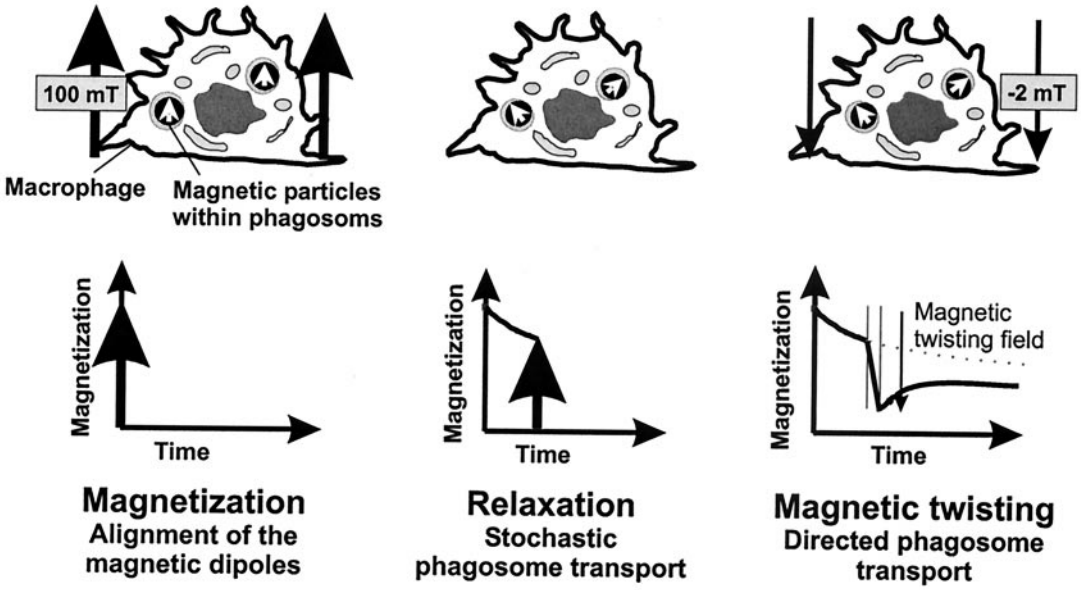


FIGURE 1 Schematic views of magnetic particles within macrophages during the three stages of the investigation showing the direction and strength of the magnetic field, the orientation of the dipoles formed by the magnetized particles, and the decay of the measured remanent magnetic cell field (RMF) over time.

cytoskeletal filaments. Relaxation describes the decay of the magnetic field and originates from the randomization of the ensemble of magnetic particles, which had been aligned by a magnetic field pulse. We assume that the phagosomes are coupled to the cytoskeletal filaments by motor proteins (like myosin, kinesin, or dynein) and that the hydrolization of ATP provides the energy to move the phagosomes and to induce rotational random kicks to the phagosome (Fig. 2 *a*). If this intracellular randomization energy E_r behaves like thermal energy kT, we have the case of rotational Brownian motion, which implies an exponential relaxation in a Newtonian viscosity (Nemoto, 1982). Additionally, we can apply a magnetic twisting force f to the particles. It was shown that MF are viscoelastic (Zaner and Valberg, 1989) and magnetic particle twisting in macrophages suggested elasticity of the cytoskeleton, too (Möller et al., 1997). Therefore, we proposed a model where viscoelasticity is included in modeling relaxation and particle twisting (Nemoto and Möller, 2000). This model describes phagosome motions in a rheological environment called a Voigt-Maxwell body, which consists of a series system of a viscoelastic body (a viscosity in parallel with an elasticity) with a pure viscosity, and which shall be

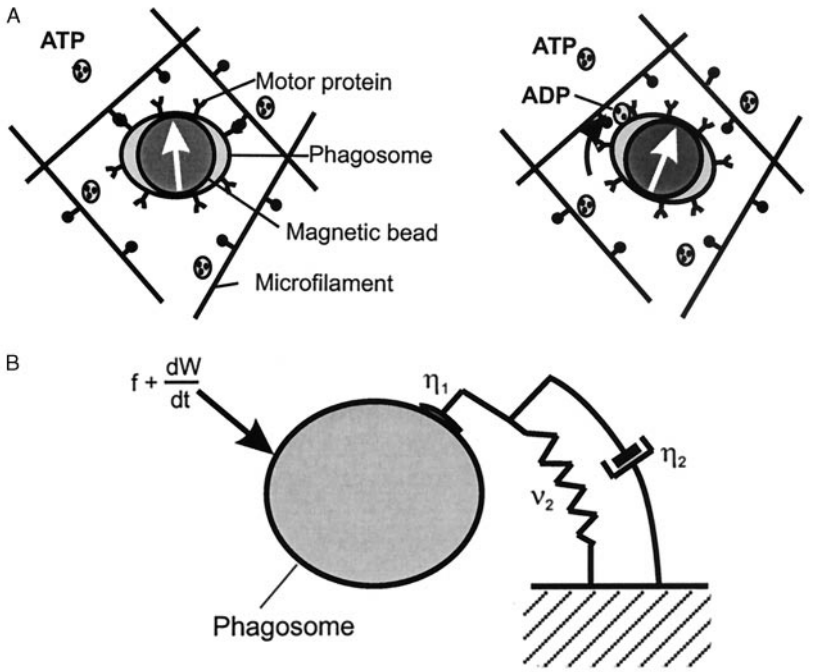


FIGURE 2 (*a*) Molecular model of phagosome transport by dynamic coupling and decoupling of phagosome bound motor proteins with cytoskeletal filaments. Decoupling is achieved by ATP hydrolization. (*b*) Rheological model of phagosome coupling (viscosity η_1) and the viscoelastic properties of the filament. The attachment wall models the extracellular matrix.

summarized in brief. Fig. 2 *b* illustrates the coupling of a phagosome to a cytoskeletal filament, which has viscoelastic properties. We assume a dynamic coupling and decoupling of the phagosome-associated motor proteins with the filament, which is modeled by a viscosity. Relaxation and particle twisting is then described by the differential equations:

$$\frac{d\theta_1(t)}{dt} = \frac{1}{\eta_1}\left(\frac{dW(t)}{dt} + f(t,\theta)\right),$$

$$\frac{d\theta_2(t)}{dt} = -\frac{\nu_2}{\eta_2}(\theta_2(1) - \tilde{\theta}_2) + \frac{1}{\eta_2}\left(\frac{dW(t)}{dt} + f(t,\theta)\right), \quad (1)$$

where $dW(t)/dt$ is the formal differentiation of a Wiener process and describes the Brownian motion and $f(t,\theta)$ describes the external twisting force. $\tilde{\theta}_2$ describes the natural length of the elastic body, which is in the resting state. This differential equation is solved numerically in Nemoto and Möller (2000). The Voigt-Maxwell body is the simplest rheological configuration that can be fitted to all experimental results. The numerical model assumes a continuous and infinite viscoelastic environment, which may not be true for living cells. For the macrophages used in this study with a mean diameter of 12 μm, second-order corrections may be necessary.

Data analysis

Stochastic phagosome motion (relaxation)

After alignment of the magnetic dipoles in a brief magnetic field pulse the decay of the RMF was recorded for 5 min. Relaxation is caused by E_r without external force ($f(t,\theta) = 0$). In this case Eq. 1 can be solved analytically (see Appendix A1). The hydrodynamic relaxation model (Eq. A2) including the Voigt-Maxwell body was fitted to the experimental data by a nonlinear regression algorithm using the Solver software from Microsoft Excel 7.0 and yields a good approximation of all relaxation measurements. Additionally, two robust relaxation parameters were analyzed, being independent of any model (Fig. 3). The normalized RMF after 1 min is b1 = B(1 min)/B_0, which characterizes the initial fast phase of decay, and that after 5 min is b5 = B(5 min)/B_0, which is characteristic of the decay in the slow phase that follows.

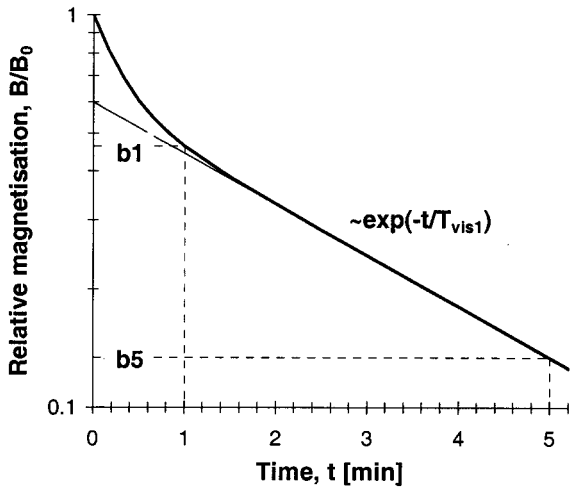


FIGURE 3 Decay of the RMF (relaxation) after particle alignment in a strong magnetic field pulse and estimation of the normalized RMF after 1 min (b1) and after 5 min (b5), respectively.

Randomization energy

The randomizing energy E_r is the source of relaxation. Additionally, it acts against the magnetic aligning force and prevents a complete orientation of the particles, as it will be achieved in primary (strong field) pulse magnetization. The balance between magnetic alignment force and cellular randomization force determines the equilibrium alignment of the particles in the cells and can be described by the so-called Langevin function $L(\alpha)$ (Debye, 1929; Nemoto, 1982):

$$L(\alpha) = \coth\alpha - \frac{1}{\alpha}, \quad \alpha = \frac{mB_M}{E_r} \quad (2)$$

where m is the magnetic dipole moment and B_M is the external twisting field. The experiments were performed in a way that, first, all particles were magnetized and aligned in a strong magnetic field, where all oriented dipoles induce the maximum probe field (B_{max}). After 5 min of relaxation, where the probe field decayed below 50% of the initial value, a weak field of 1 mT strength was applied over 3 min in order to realign the particles. The equilibrium cell field (B_{eq}) is a measure of the achieved dipole alignment. Cellular energy E_r was estimated from a fit with the Langevin model, $B_{eq}(B_M) = B_{max} \cdot L(\alpha)$. This method of estimating randomizing energy is independent of the viscoelastic environment.

Phagosome motion in a weak magnetic field (magnetic twisting)

Application of a weak magnetic field induces an oriented particle twisting and allows investigation of cytoplasmic rheology and mechanical integrity. Particles being suspended in a viscosity η rotate in an external twisting force according to Newton's law:

$$\sigma = \eta\frac{d\theta}{dt}, \quad (3)$$

where $d\theta/dt$ is the shear rate, η is the viscosity, and σ is the applied shear stress. In case of elasticity, the applied stress is proportional to the elastic deformation (strain θ), and we get with the elasticity modulus ν Hooke's law:

$$\sigma = \nu\theta. \quad (4)$$

A summary of ferromagnetic particle twisting in a viscoelastic medium is given in Appendix A2. Additionally, we estimated the cell stiffness as the ratio between mean stress and strain after a constant twisting duration of 10 s. This parameter characterizes the mechanical properties of the cytoskeleton without view of specific viscosity or elasticity and therefore provides an integral view of the cytoskeletal mechanics. A visualization of cytoskeletal elasticity was achieved by applying the twisting field for a short time period of 10 s. Energy being stored during this twisting process is recovered by elastic recoil of the particles. Using a Voigt body as a rheological model of the cytoskeleton, where a viscosity η and the elasticity modulus ν are in parallel (Appendix A3), viscoelastic recoil follows

$$\theta(t) = \Delta\theta(1 - \exp(-t/T_{el})),\ T_{el} = \frac{\eta}{\nu}. \quad (5)$$

The elasticity modulus was estimated from the time path of the elastic recoil. In a Voigt body, elastic recoil returns the dipoles back to their orientation before stress application. Particle twisting in living macrophages does not yield a complete recoil (Fig. 4); most of the strain does not

recover. This required an extension of the Voigt body by an additional viscous element η_1; the whole system is then called a Voigt-Maxwell body. The experiments suggest a two-compartment model of the cytoskeleton. For longer twisting durations the fraction of recoverable strain decreases and disappears after twisting durations longer than 1 min (Möller et al., 1997), which originates from a dissipation of the elastic energy due to stochastic phagosome motions and cytoskeletal rearrangements.

At the beginning of the experiment the particles were aligned in a strong magnetic field pulse and relaxation was followed for 2 min in order to rotate the dipoles a certain angle away from the initial orientation. The weak twisting field (either 0.5 or 1 mT) was applied for 10 s and the elastic recoil was recorded for 2 min, which was sufficient to move back into the normal relaxation behavior. This equilibrium dipole orientation was extrapolated back to the time of switching off the twisting field, using the slow phase of relaxation in order to estimate the fraction of nonrecoverable ($\Delta\theta_1$) and recoverable ($\Delta\theta_2$) strain (see Fig. 4). For each dipole m the system detects the component $m\cos(\theta)$; therefore, the mean orientation angle at each time point was estimated from $\bar{\theta} = \arccos(B(t)/B_0)$. Because of the reverse particle twist, the stress varied only between 80% and 100% of the maximum stress σ during the time of particle twist. Therefore, the equations of motion (Eq. A7) were linearized by neglecting the $\sin(\theta)$ dependency and using a constant mean stress, which was estimated over the total rotational strain $\Delta\theta = \Delta\theta_1 + \Delta\theta_2$. The viscosity η_1 was estimated from the fraction of nonrecoverable strain ($\Delta\theta_1$) during the time period of $\Delta t = 10$ s according to

$$\eta_1 = \frac{\overline{\sigma\sin(\theta - \pi)}}{\Delta\theta_1/\Delta t}. \tag{6a}$$

The parameters of the viscoelastic compartment were estimated from the fraction of recoverable strain ($\Delta\theta_2$) during the twisting period of $\Delta t = 10$ s and the elastic recoil time constant T_{el}:

$$\eta_2 = \frac{\overline{\sigma\sin(\theta - \pi)}}{\Delta\theta_2/\Delta t}\left(1 - \exp\left(-\frac{\Delta t}{T_{el}}\right)\right)\frac{T_{el}}{\Delta t} \tag{6b}$$

and ν_2 from Eq. 5. Because of the short twisting duration of 10 s, E_r has little influence on the estimation of the rheological parameters using the hydrodynamic models. For example, each particle has a remanent magnetic moment of 4.3×10^{-14} Am^2, implying an alignment energy of $m \times B_m = 4.3 \times 10^{-17}$ Joule in a 1-mT twisting field, which is much higher compared to the randomizing energy of some 10^{-18} Joules.

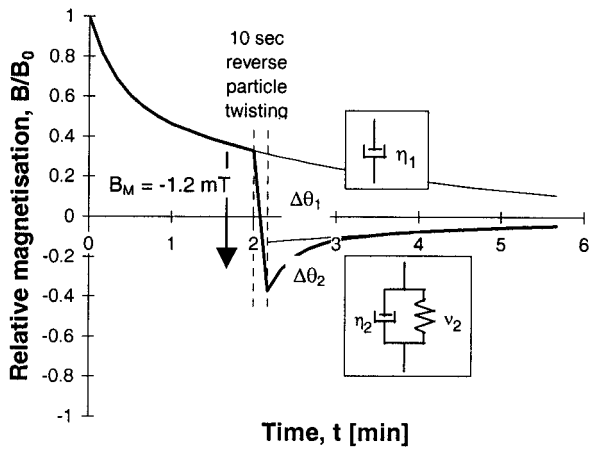


FIGURE 4 Two minutes' relaxation followed by 10 s particle twisting in a reverse weak magnetic field and elastic recoil afterward. The *insets* show the two elements of the Voigt-Maxwell body, which consists of a pure viscous element (η_1) in series with a viscoelastic element (viscosity η_2 and elasticity ν_2).

Every set of drug measurements was performed on at least 5 separate probes together with a separate set of control measurements. All parameters estimated under the set of drugs were normalized by the set of control measurements. A deviation of the normalized parameters from unity denotes an influence of the drug. Using a two-sided Student's t-test, deviations from unity were analyzed for their level of statistical significance. An influence by the appropriate drug was accepted when the level of significance was $P < 0.05$. Pearson's correlation analysis was performed using WINSTAT, Version 1999.2 (Fitch Software, Cambridge, MA).

RESULTS

ATP content per cell under cytoskeletal drugs

Because the free ATP in the cell is an important factor for phagosome transport, the content of free ATP was measured under the influence of the applied cytoskeletal drugs. In J774A.1 macrophages the concentration of free ATP was 3.55 ± 0.39 pg/cell, which is comparable to other cell types. None of the cytoskeletal drugs or combinations of drugs used in this study induced a significant change in the intracellular free ATP. Therefore, we conclude that none of the reactions induced by the drugs is mediated by the intracellular free ATP. All observed effects must be correlated to the structure of the cytoskeleton and the coupling of the phagosomes to the cytoskeleton.

Stochastic phagosome motion (relaxation)

The behavior of relaxation in J774A.1 macrophages under standard conditions (control) and under the cytoskeletal drugs is summarized in Fig. 5, *a* and *b*. Fig. 5 *a* shows the mean normalized relaxation curves of the control probes and after 4 μM CyD, 10 μM CoL, 4 mM AcL, or the combination of CyD and CoL treatment. Additionally, the normalized decay after 1 min and after 5 min (b1 and b5, respectively) is outlined, together with the appropriate standard deviations. Fig. 5 *b* summarizes the normalized response of the relaxation time constants, of the relaxation parameters b1 and b5 and of the randomizing energy E_r under the applied cytoskeletal drugs. According to the hydrodynamic relaxation model, both the mechanical properties of the cytoskeleton and the randomizing energy E_r determine the decay of the RMF. The estimation of the randomization energy E_r results from separate measurements by the Langevin method.

The MF disruption drug CyD affects both the relaxation process and the randomization energy E_r. Under >1 μM CyD treatment, relaxation happens significantly more slowly compared to control; the relative decay after 1 min is only 0.75 compared to 0.61 in control probes, and after 5 min it is 0.56 compared to 0.34 (both $P < 0.001$), and the randomization energy E_r was lowered by 20% ($P < 0.01$), which may reduce the driving force for stochastic phagosome motions (relaxation). The higher time constants in the

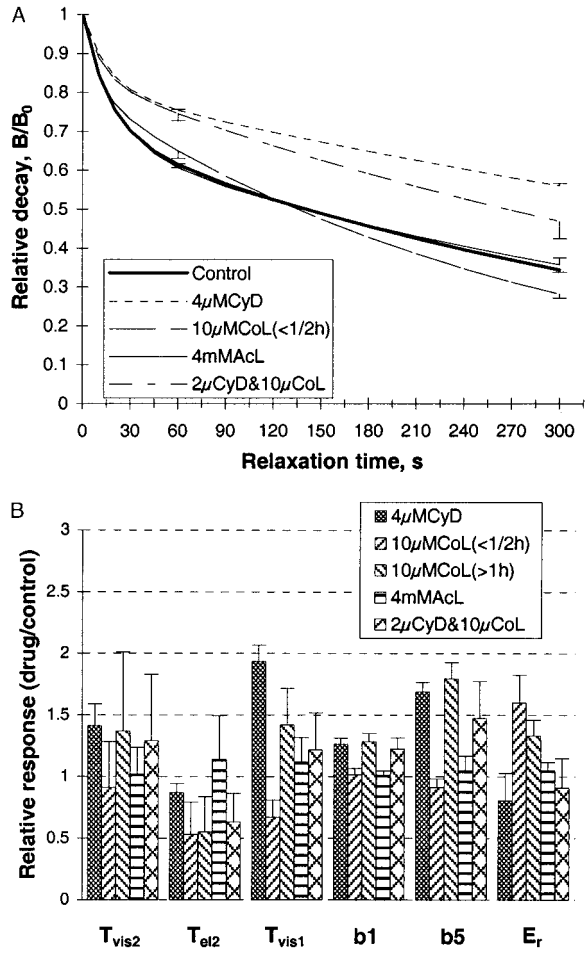


FIGURE 5 (*a*) Stochastic phagosome motion (relaxation) in J774A.1 macrophages without drugs (control) and after 4 μM cytochalasin D (CyD), 10 μM colchicine (CoL), or 4 mM acrylamide (AcL) or combinations of drug treatment. (*b*) Normalized reactions of the relaxation time constants, of relaxation after 1 min (b1) and after 5 min (b5), and of randomization energy E_r after drug treatment.

hydrodynamic relaxation model for both the initial viscoelastic and the slow viscous decay (T_{vis2} and T_{vis1}, respectively) reflect this effect. In the slow exponential phase the decay continues with a rate of 0.11 min^{-1} after 2 μM CyD and 0.07 min^{-1} after 4 μM CyD treatment, compared to 0.14 min^{-1} in control probes. The small decrease of the elastic time constant T_{el2} results from the increase of T_{vis2}, because $T_{el2} = \eta_2/\nu_2$. The inhibition of relaxation by CyD is higher than expected from the 20% reduction of E_r. In summary, CyD significantly inhibits the processes of stochastic phagosome motions, where the main effect results from the pure viscous compartment.

The influence of CoL on phagosome transport is contrary to CyD and depends significantly on the incubation time. There is a dramatic change in relaxation and cytoskeletal stiffness between incubation times of 15 to 30 min and times longer than 1 h. In the short incubation period the randomization energy E_r shows a 60% increase, while the relaxation process is only moderately accelerated. The initial fast relaxation phase (b1) remains unaffected and the slow relaxation phase (b5) shows a moderately faster decay. The time constants of the viscoelastic relaxation model yield clearer results. The slow phase of relaxation is significantly accelerated ($T_{vis1}^{-1} = 0.21$ min^{-1} versus 0.14 min^{-1} in control probes). The elasticity related time constant T_{el2} decreases, resulting from an increase of the elastic modulus ν_2, because η_2 (T_{vis2}) is not influenced. The higher elasticity decreases the fraction of the initial viscoelastic relaxation. For CoL incubation times >1 h, this behavior reverses. Relaxation slows down dramatically ($T_{vis1}^{-1} = 0.10$ min^{-1}) and behaves more slowly compared to 4 μM CyD incubation. But E_r was still 30% increased compared to the control probes.

AcL affects neither the randomization energy E_r nor the relaxation behavior. Combining CyD and CoL (<30 min incubation time) eliminates the effects of the separately added drugs on the randomization energy E_r, which is now in the range of the control probes. But the relaxation behaves significantly more slowly compared to the control probes, in both the fast and slow phases of the decay. The time constants of the viscoelastic relaxation model also reflect this behavior. Both viscosity-related time constants (T_{vis1} and T_{vis2}) increase, resulting in a slower relaxation. The main effect is seen in the decay of the elastic time constant (T_{el2}), which conserves the effect of the drug CoL. In summary, the response of the combination of the two drugs is in the viscoelastic compartment with a CoL-induced increase of the elasticity modulus and an inhibition of the slow phase of relaxation. These results remain unchanged when AcL was added as a third drug.

Magnetic particle twisting

The stiffness (ratio between stress and strain) is a robust parameter for the mechanical behavior of the cytoskeleton because it does not require a rheological model. Therefore, it reports an integral response of the cells to the external stress application and eliminates the effect of the strength of stress. In a pure viscosity or elasticity the stiffness should be independent of stress. Particle twisting in macrophages reveals an increase of stiffness with increasing stress, which is typical for living tissues and is evidence for an intact cytoskeleton. Twisting of phagocytized particles reveals a linear stiffness-to-stress relationship, in vivo and in vitro (Möller et al., 1997). This verifies that twisting of phagocytized particles can act as a probe for the investigation of the mechanical properties and the integrity of living cells.

The influence of the cytoskeletal drugs on the cell stiffness estimated by magnetic phagosome twisting is illustrated in Fig. 6, *a* and *b*. Disrupting the MF by CyD induces a slight increase (9 $\pm$ 5%, $P < 0.05$) in cytoskeletal stiffness. Discriminating between the two rheological compart-

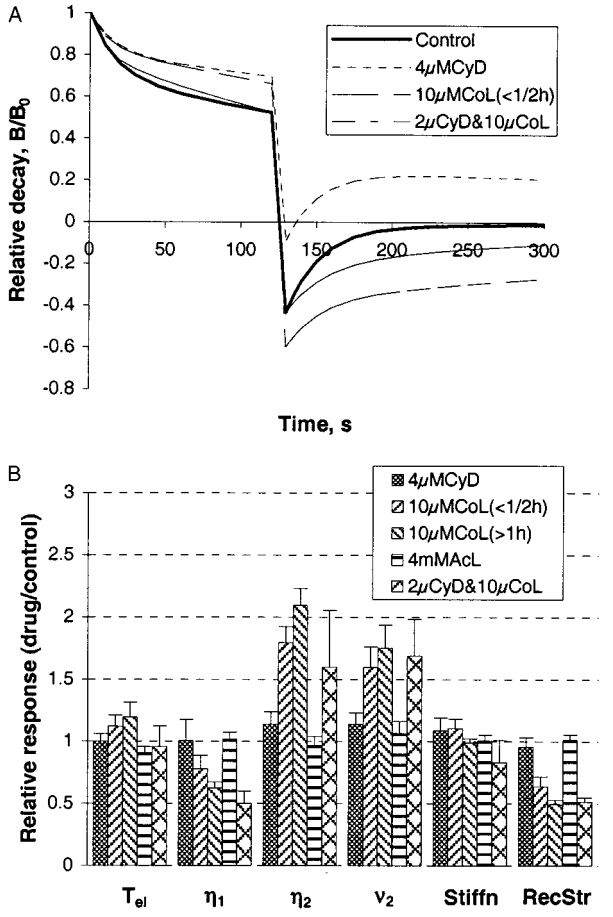


FIGURE 6 (*a*) Magnetic particle twisting and elastic recoil in J774A.1 macrophages without drugs (control) and after 4 μM cytochalasin D (CyD), 10 μM colchicine (CoL), or 4 mM acrylamide (AcL) treatment. (*b*) Normalized reactions after cytoskeletal drug treatment of the time constant of viscoelastic recoil (T_{el}), of the viscoelastic units of the Voigt-Maxwell body (viscosities η_1 and η_2 and elasticity ν_2, see Fig. 4 *inset*), of stiffness (Stiffn), and of the fraction of recoverable strain (RecStr).

ments yields a 14 ± 6% increase in both, the viscosity in the viscoelastic compartment, η_2, and the elasticity modulus, ν_2, whereas the pure viscous compartment, η_1, is not modulated by CyD. The fraction of recoverable strain slightly decreases, from 43% in control probes to 41%. In summary, we got a moderate response of phagosome twisting to CyD with a moderate stiffening of the viscoelastic compartment.

After short-time CoL treatment (<30 min) the stiffness shows a 10 ± 8% increase (n.s.), whereas the pure viscous compartment η_1 shows a 23 ± 11% decrease ($P < 0.01$), and both the viscosity η_2 and elasticity modulus ν_2 of the viscoelastic compartment show a >50% increase ($P < 0.01$) together with a retarded viscoelastic recoil ($P < 0.05$). The higher viscosity and elasticity imply a decrease of the fraction of recoverable strain, from 43% in control probes to 27%. In summary, disrupting MT by CoL induces a slight stiffening of the cytoskeleton with a loss of stiffness in the viscous compartment and a stiffening of the viscoelastic compartment. Long-term CoL treatment (>1 h) further amplifies these effects; the fraction of viscoelastic recoil decreases further to 21%.

AcL has no influence on any of the mechanical parameters of the cytoskeleton. Therefore, the AcL results may show the reproducibility of the control measurements.

The combination of the drugs acting on MF and on MT, CyD and CoL (<30 min), result in a 17 ± 18% loss of cell stiffness (n.s.), whereas the viscosity η_1 shows a 50% decrease and both viscosity η_2 and elasticity modulus ν_2 are increased by more than 50%. The fraction of recoverable strain decreases from 43% in control probes to 22%. In general, the combination of CyD and CoL reflects the response of CoL alone. Compared to the combination of CyD and CoL, there was no further response when AcL was added as a third drug. Table 1 summarizes the main results of the stochastic (b5, E_r) and directed phagosome motion (stiffness) in J774A.1 macrophages under the treatment of the cytoskeletal drug.

DISCUSSION

These investigations verify the role of the different cytoskeletal units in the transport of intracellular phagosomes in macrophages. Several studies suggest an important role for MT in the transport of membranous organelles and phagosomes (Blocker et al., 1997, 1998; reviewed in Hiro-

TABLE 1 Influence of the cytoskeletal drugs cytochalasin D (4 μM, CyD), colchicine (10 μM, CoL) or acrylamide (4 mM, AcL) or combinations of the drugs on cytoskeletal integrity (stiffness) and on stochastic phagosome motion parameters b5 and E_r

Drug	Action on	Drug/control (mean ± SD) Integrity (stiffness)	Stochastic motion (relaxation, b5)	Stochastic motion (energy, E_r)
4 μM CyD	MF	↑ (1.09 ± 0.05)*	↗ (1.7 ± 0.1)**	↓ (0.8 ± 0.2)**
10 μM CoL	MT	↑ (1.1 ± 0.08)*	↘ (0.91 ± 0.06)*	↑ (1.6 ± 0.2)**
4 mM AcL	IF	— (1.0 ± 0.05)	— (1.05 ± 0.12)	— (1.0 ± 0.1)
CyD & CoL	MF & MT	↓ (0.83 ± 0.18)	↗ (1.5 ± 0.3)**	— (0.9 ± 0.2)
All Drugs	MF & MT & IF	↓ (0.91 ± 0.2)	↗ (1.4 ± 0.3)**	— (1.1 ± 0.2)

Mean of the ratio of drug to control ± standard deviation, SD.
— no change, ↗ slower, ↘ faster, ↓ higher, ↑ lower.
* $P < 0.05$, ** $P < 0.01$.

kawa, 1998). Our studies investigate the transport of micron-sized phagosomes from two different views. Relaxation and the measurement of E_r are direct monitors of the coupling dynamics between phagosomes and the cytoskeleton, whereas phagosome twisting views the mechanical properties of the cytoskeletal filaments, which are linked to the phagosomes. After destroying a certain cytoskeletal subunit with a specific drug, the data show the role of the remaining filamentous network in phagosome transport. Immunofluorescent visualization of MF and MT revealed that no filamentous structures remained after CyD and/or CoL drug treatment. None of the effects seen by the cytoskeletal drugs can be associated with the level of ATP in the cells, because it was shown that phagosome transport can be stopped by blocking ATP synthesis (Nemoto et al., 1989).

Intermediate filaments (IF)

Neither stochastic phagosome motion (relaxation) nor directed phagosome motion (magnetic particle twisting) are influenced by AcL, the drug disintegrating IF. Because IF deliver the static part of the cytoskeleton, it is evident that the dynamic behavior of phagosomes is not modulated by AcL.

Microfilaments (MF)

Several studies using cytomagnetometry to investigate the motion of magnetic phagosomes show that CyD slows down the randomization of the oriented dipoles (relaxation), following that phagosomes couple primarily to MF (Valberg and Feldman, 1987; Marugg et al., 1990; Valberg and Butler, 1990). Because CyD disrupts the MF, it was suggested that the transfer of the randomization energy (E_r) to the phagosomes is inhibited. This study supports these suggestions and additionally shows, that the randomization energy E_r is lowered by CyD too and therefore can explain a slower relaxation. But even under high CyD concentrations, the long-term decay proceeds with a mean rate of 0.07 min^{-1}. Because MF-associated transport can be excluded the remaining stochastic phagosome motion reflects MT-associated transport. When comparing both relaxation rates we must conclude that MT-associated phagosome transport is less effective than MF-associated motion with a relaxation rate of 0.14 min^{-1}. Therefore, we conclude that relaxation measurements under the influence of CyD verify that the MF are the primary and most effective filamentous structures for the energy transfer between the cytoskeleton and the phagosomes.

CyD influences magnetic phagosome twisting in a minor way; none of the twisting parameters is significantly modulated. Because twisting reveals a static view of the phagosome to cytoskeleton coupling, we suggest that because of the more frequently MF-phagosome coupling-decoupling and the higher turnover of MF, particle twisting primarily reflects the mechanical properties of the (quasi-static) MT. We conclude that MF play a dominant role in phagosome transport mechanisms and are of secondary relevance in magnetic particle twisting.

Microtubuli (MT)

Administration of 10 μM CoL for $<$30 min induces a slightly accelerated decay and does not inhibit the phagosome transport mechanisms. The decrease of the elasticity-related time constant (T_{el2}) results from an increase of the elasticity modulus, which is also verified in the twisting experiments, and modulates a decrease of the fraction of initial fast (viscoelastic) decay. The pure viscous (long-term) decay is accelerated compared to the control probes and proceeds with a rate of 0.21 min^{-1}. Additionally cellular randomization energy E_r shows an increase under 10 μM CoL. Mechanical measurements of neutrophils under 10 to 100 μM CoL treatment suggest that this accelerated relaxation can result from an activation of the synthesis of F-actin (Tsai et al., 1998), which further activates the MF transport efficiency. These data again verify that the energy transfer to the phagosomes primarily requires an intact MF network, as was shown by CyD incubation, the contribution of MT is of secondary relevance.

The magnetic twisting experiments yield a moderate increase in cytoskeletal stiffness after CoL treatment. MT depolymerization significantly reduces the resistance in the viscous compartment with an increase of the fraction of nonrecoverable strain. The strong decrease of the fraction of recoverable strain results from an increase of the viscosity η_2 and elasticity modulus ν_2 and a retarded viscoelastic recoil. The decrease of the viscosity η_1 correlates with the higher rate of decay in the slow phase of relaxation. The loss of resistance in the viscous compartment after MT depolymerization suggests a strong correlation of this compartment with MT. The remaining stiffness should primarily reflect the mechanical properties of MF. In vitro studies of the mechanical properties of actin suspensions show primarily viscoelastic properties (Zaner and Valberg, 1989; Janmey et al., 1994). Because MT depolymerization does not increase the viscoelastic compartment, we suggest that this might result from an interlink of the two filamentous structures and a modification of the equilibrium cytoskeletal tension (Langford, 1995).

Longer incubation times of CoL significantly modulate the relaxation behavior. The acceleration of phagosome transport after short-time CoL incubation reverses and becomes further inhibited compared to CyD treatment, whereas the mechanical response to CoL is further amplified by the longer incubation time. We suggest that the impairment of relaxation by long CoL incubation times is an early indication of cytotoxicity because it was shown that

CoL could induce apoptosis (Takano et al., 1993; Bonfoco et al., 1995), which may modulate cellular and cytoskeletal functions. Therefore, the results under long CoL treatment should be interpreted as pathophysiological cell functions. In summary, MT play a secondary role in phagosome transport, but appear to be dominant structures in the static mechanical structure of the cell.

MF and MT depletion (combination of drugs)

Under CyD and CoL the effect of the single added drugs on E_r disappears, which demonstrates that energy may be present for phagosome transport. But the impaired relaxation shows that the transport machinery is not working properly because the filamentous structures (roads) are not present. Under the combination of drugs we get a profile of results as was obtained for CyD in relaxation measurements with an impairment of phagosome transport, and for CoL in twisting experiments with a stiffening of the viscoelastic compartment. The effects of the cytodrugs on cell stiffness get lost and we get a small decrease of cell stiffness, which verifies the depolymerization of the cytoskeleton.

Stochastic phagosome motion (relaxation) and magnetic phagosome twisting yield different responses to the applied cytoskeletal drugs and only partially correlate. The viscoelastic time constants T_{el2} in the relaxation behavior correlates with the viscosity η_2 and the elasticity modulus ν_2 in magnetic particle twisting (coefficient of correlation, c.c. = 0.78, $P < 0.05$) and also with the fraction of viscoelastic recoil (c.c. = 0.65, $P < 0.05$). This verifies in part the viscoelastic relaxation model. But the slow-phase relaxation time constant T_{vis1} has no correlation with any of the mechanical parameters, which appears from the CyD response with an impairment of relaxation without affecting phagosome twisting. Only after CoL treatment the faster relaxation correlates with the loss of viscosity in the pure viscous compartment. Therefore we conclude that η_1 may not be a Newtonian viscosity and describes the coupling characteristics between phagosomes and the cytoskeletal filaments.

Mechanical measurements on passive neutrophils by micropipette aspiration reveal comparable responses to cytoskeletal drugs (Tsai et al., 1994, 1998). Studies using magnetic twisting cytometry (Wang et al., 1993; Wang, 1998) to investigate the mechanical properties of endothelial and smooth muscle cells report a significant decrease of cytoskeletal stiffness after treatment with the above drugs, which could not be verified in this study. In Wang's studies ferromagnetic particles were coated by an RGD peptide for an attachment to the integrin ligands on the outer cell membrane. The integrin receptors primarily interlink with the MF. The particles are statically coupled to the cytoskeleton and do not undergo active transport phenomena. It appears that the coupling of phagosomes to the cytoskeleton is more complex, and we have to suggest the role of active transport phenomena.

Driving force of the phagosomes

The measurement of the randomization energy E_r allows us to estimate the mean force and energy that act on the surface of the phagosomes in order to transport them along the MF and MT. E_r counterbalances the magnetic twisting torque mB_M. This magnetic torque corresponds to a mean force F_M acting on the surface of the particle at a distance of the radius r of the sphere: $E_r = rF_M$. An energy of $\approx 5 \times 10^{-18}$ Joule implies a force of ≈ 7 pN (7×10^{-12} N), when the magnetic phagosome behaves with a radius of 0.7 μm. This is in agreement with measurements of the force generation during the motion of vacuoles in amoebae (Ashkin et al., 1990) and during translatory motion of magnetic phagosomes in J774A.1 macrophages (Bausch et al., 1999). The high viscosity of the cytoplasm (>50 Pa · s) requires a high driving force in order to mediate the intracellular transport.

CONCLUSIONS

In living cells, phagosome motion is a complex behavior in which both MF and MT, together with their motor proteins, are involved. The depletion of specific cytoskeletal structures by cytoskeletal drugs verifies the role of each of the filaments in relaxation and twisting measurements. This study shows that in J774A.1 macrophages, stochastic phagosome transport requires an intact MF system, which agrees with the essential function of MF in phagocytosis and phagosome-lysosome fusion, whereas magnetic twisting experiments evaluate a dominant role of MT in the static mechanical properties of the cell. The proposed model can understand all results of phagosome motion and magnetic phagosome twisting. This model can provide an understanding on a molecular basis with an interaction between motor proteins and the filamentous network. Phagosome motion and phagocytosis by alveolar macrophages can be investigated in vivo in humans by magnetopneumographic methods (Stahlhofen and Möller, 1993). This requires the inhalation and deposition of about 1 mg of ferrimagnetic microparticles. The size of these particles and the inhalation maneuver can be adapted for a preferred deposition in the alveolar region. Additionally, the clearance capacity of the lungs can be measured and correlated with the above cell functions. This allows correlations between dysfunctions of the cellular defense system in the human lungs and cytoskeletal dysfunctions of alveolar macrophages in specific lung diseases.

APPENDIX

A1: Model for relaxation

A first step to model relaxation is to describe the stochastic particle twisting as rotational Brownian motion process. In this model, thermal energy kT induces random rotational pulses on the particles that cause a stochastic twisting of the magnetic dipole particles. These rotational move-

ments happen within a viscous environment (viscosity η). We apply this model to the intracellular rotation of particle containing phagosomes in macrophages, where kT is replaced by the cellular energy E_r. Eq. 1 describes this behavior for one single viscosity and without external force ($f = 0$). When using spherical particles, the decay follows an exponential function according to Nemoto (1982):

$$\frac{B(t)}{B_0} = e^{-t/\tau_r} \quad \tau_r = \frac{\kappa V \eta}{2E_r}, \tag{A1}$$

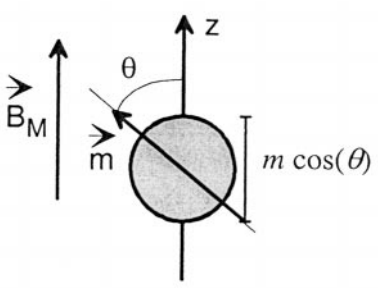
where V is the volume of the particles, η is the viscosity of the cytoplasm, and κ is the rotational shape factor ($\kappa = 6$ for spheres). An exponential relaxation could be verified using monodisperse spherical magnetic particles, which were suspended in a Newtonian viscosity (Möller et al., 1992). In vivo and in vitro investigations of relaxation with spherical particles in macrophages yield deviations from a single exponential decay. An initially fast decaying phase (over the first 30 s) was followed by a slower exponential phase lasting up to 20 min. Because particle twisting reveals an elastic recoil behavior and a large fraction of nonrecoverable strain, stochastic phagosome motion was modeled in a Voigt-Maxwell body (Eq. 1). A detailed description of this model is shown in Nemoto and Möller (2000). After pulse magnetization the stochastic motion of the magnetic dipoles can be analytically predicted as:

$$\frac{B(t)}{B_0} = \exp\left(-\frac{t}{T_{vis1}}\right) \times \exp\left(-\left(\frac{T_{el2}}{T_{vis2}} + \frac{T_{el2}}{\sqrt{T_{vis1} \cdot T_{vis2}}}\right) \cdot \left(1 - \exp\left(-\frac{t}{T_{el2}}\right)\right)\right),$$

$$T_{vis1} = \frac{\kappa V \eta_1}{2E_r}, \quad T_{vis2} = \frac{\kappa V \eta_2}{2E_r}, \quad T_{el2} = \frac{\eta_2}{\nu_2}, \tag{A2}$$

T_{vis1} describes the slow phase of decay, T_{vis2} the initial decay, and T_{el2} modulates the fraction of initial fast decay. The rheological bodies are illustrated in Fig. 3. These models assume a continuum of the viscoelastic surrounding and may require corrections for the finite cell. A comparison between the particle (phagosome) diameter of 1.3 μm and the cell diameter of 12 μm may require second-order corrections.

A2: Particle twisting in a magnetic field (secondary magnetization)



Secondary magnetization describes the twist of the dipole particles in a weak magnetic field. A magnetic torque $N_{mag} = mB_M \sin\theta$ acts on a remanent dipole m in an external magnetic field B_M that twists the particle until the dipole is aligned with the magnetizing field. θ is the angle between B_M and m. The viscosity η of the surrounding fluid resists this twist and produces a hydrodynamic retarding torque $N_{hyd} = -\kappa V \eta \cdot d\theta/dt$; $d\theta/dt$ is the angular velocity of rotation (shear rate); V is the volume and κ is the shape factor of the particle. The balance of both torques describes the dipole particle rotation:

$$\frac{d\theta}{dt} = -\frac{m \cdot B_M}{\kappa \cdot V \cdot \eta} \sin\theta \tag{A3}$$

with the solution

$$\tan\frac{\theta(t)}{2} = \tan\frac{\theta_0}{2} e^{-t/\tau}, \qquad \tau = \frac{\kappa \cdot \eta}{M_T \cdot B_M}, \tag{A4}$$

$M_T = m/V$ is the remanent magnetization of the particles, and θ_0 is the initial orientation of the dipole m. Only the component of magnetic moment parallel to the magnetizing field, $m \cdot \cos\theta(\theta_0, t)$, is detected. From Eq. A4, $\cos\theta(\theta_0, t)$ can be expressed as:

$$\frac{B(t)}{B_{max}} = \cos\theta(\theta_0, t) = \frac{1 - \tan^2\frac{\theta_0}{2}\exp\left(\frac{-2t}{\tau}\right)}{1 + \tan^2\frac{\theta_0}{2}\exp\left(\frac{-2t}{\tau}\right)}. \tag{A5}$$

The time constant τ (Eq. A4) shows that particle twisting is independent of particle size. This method of viscosity measurement was calibrated with magnetite spheres, suspended in a highly viscous Newtonian fluid (Möller et al., 1992). Equations A3-A5 hold only for Newtonian viscosities, where the viscosity is independent of the shear rate. Including elastic properties makes the system non-Newtonian. Nevertheless, secondary magnetization measurements can be analyzed with Eq. A3, yielding a shear rate dependent apparent viscosity. Because the relative RMF is a measure of $\cos\theta(t)$, the orientation angle $\theta(t) = \arccos(B(t)/B_{max})$ and the shear rate $\Delta\theta/\Delta t$ can be predicted. Then Eq. A3 is used to estimate the apparent viscosity as:

$$\eta(\dot{\theta}) = -\sigma\frac{\sin(\theta - \pi)}{d\theta/dt}, \qquad \sigma = \frac{M_T \cdot B_M}{\kappa}. \tag{A6}$$

A3: Viscoelastic recoil

The relaxation and particle twisting measurements have shown that the viscosity of the cytoskeleton behaves non-Newtonian and has elastic properties. The simplest viscoelastic body is a Voigt body, where a viscosity η is in parallel with an elastic element with elasticity ν. The differential equation for this simple viscoelastic system follows from Eq. 1, when neglecting dissipative processes ($dW/dt = 0$):

$$\frac{d\theta}{dt} = -\left(\frac{\sigma}{\eta}\sin\theta - \frac{\nu}{\eta}(\theta - \theta_0)\right), \tag{A7}$$

θ_0 is the orientation before particle twist. During particle rotation in a viscoelastic Voigt body, elastic energy is stored, which drives elastic recoil after removing B_M. Eq. A7 predicts elastic recoil behavior ($\sigma = 0$) as:

$$\theta(t) = \Delta\theta\left(1 - \exp\left(-\frac{\nu}{\eta}t\right)\right). \tag{A8}$$

For a known viscosity η, the elasticity modulus ν can be estimated from the time path of the elastic recoil process. In a Voigt body, elastic recoil returns the dipoles to their orientation before stress application, independent of applied stress. The experimental results show this behavior only for weak twisting fields (Möller et al., 1997). Particle twisting with higher stress does not yield complete recoil; some nonrecoverable strain remains, which might indicate permanent deformations in the cytoplasm. Therefore, the Voigt body was extended by an additional viscous element; the whole system is then called a Voigt-Maxwell body.

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