Supporting Information

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SI Materials and Methods

Light Scattering Analysis. Dynamic light scattering (DLS) assessed morphological characteristics of lipid particles. Measurements of various particle samples were carried out with a commercial light-scattering setup (ALV light-scattering spectrometer) consisting of a CGS-5000 rotating arm goniometer, an EMI-9863 photomultiplier tube (PMT), an ALV 5000 multitaug digital correlator, and a Coherent Innova 70 argon ion laser operated at a wavelength of 488 nm at 100 mW. The scattering cell was immersed in a refractive index matching fluid (toluene) maintained at 21 ± 0.1 °C. For each sample dynamic light-scattering data were collected simultaneously and acquired typically for 5 min. The count rate of each acquisition was monitored to exclude runs containing artifacts due to passage through the scattering volume of dust particles.

The DLS technique measures the intensity autocorrelation function $g_2(t) = \langle I(t) I(t + \tau) \rangle / \langle I \rangle ^2$, where $\tau$ is the lag time and brackets represent the ensemble average. $g_2(t)$ can be related to the field autocorrelation function $g_1(t)$ through the Siegert relation $g_2(t) = 1 + g_1(t)$, where $\beta$ is an instrumental constant equal to 1 in our setup. The mathematical form of $g_1(\tau)$ depends on the physical properties of the system investigated. For a monodisperse solution of noninteracting particles a single exponential function with decay time $\tau$ is obtained (1). For a polydisperse sample, $g_1(\tau)$ is no longer a single exponential. In this case the distribution of decay rates on $g_1(\tau)$ can be taken into account by introducing a weighting function

$$ g_1(t) = \int_0^\infty p_1(r) e^{-\Gamma r} dr, \quad [S1] $$

where $p_1(r) dr$ is the intensity-weighted radius distribution function, describing the distribution of the fraction, in the interval $dr$, of the intensity scattered by a particle of hydrodynamic radius $r$. The decay rate is $\Gamma(r) = \frac{k q^2 r^2}{6 \pi \eta}$, where $\eta$ is the water viscosity; $k$ is the Boltzman constant; and $q = \frac{2}{\lambda} \sin \left( \frac{\lambda}{2} \right)$ is the scattering wave vector with $\lambda$ the scattering angle, $n$ the sample’s refraction index, and $\lambda$ the laser wavelength (2, 3). Once $p_1(r)$ is known, the mean hydrodynamic radius ($R$) will be calculated as

$$ R = \int_0^\infty p_1(r) r dr \quad [S2] $$

and the standard deviation (SD) as

$$ SD = \int_0^\infty p_1(r)(r - R) dr. \quad [S3] $$

Asymmetric Distribution of Phospholipids. The distribution of phosphatidylserine (PS) at the outer liposomal surface was initially analyzed by flow cytometry analysis (FACScanibur; Becton Dickinson) after staining with Cy5-Annexin V (BioVision Research Product), used according to the manufacturer’s instructions.

The asymmetric distribution of phospholipids was monitored by a previously described fluorimetric method (4). Briefly, liposomes characterized by the presence of 1-myristoyl-2-[12]-[7-nitro-2-1,3-benzoxadiazol-4-yl]amino]auroyl]-sn-glycero-3-phosphate (NBD-PA) (Avanti Polar Lipids), a fluorescent phosphatic acid (NBD-PA) analog, at the outer or inner surface, were monitored before and after the addition of a quenching solution consisting of 1 M sodium hydroxulite (Na2SO4) (Sigma) in 5 mM 2-[(1,3-diheoxy-2-[hydroxymethyl]propan-2-yl]amino]ethanesulfonic acid (TES), pH 9. Finally, in several experiments the distribution of NBD-PA at the inner liposomal surface was analyzed by confocal laser scanning microscopy, using a Leica TCS-SP5 operating system.

Fluorimetric Analysis of Reactive Oxygen Species (ROS) Generation. ROS generation was analyzed by loading cells with 10 μM of the fluorescent indicator 20,70-dichlorofluorescein diacetate (DCF) (Molecular Probes) for 60 min at 37 °C in the dark. Thereafter, cells were washed twice and stimulated with apoptotic body-like liposomes carrying PA (ABL/PA), ABL/phosphatidylcholine (ABL/PC), PC/PA, or PC/PC for 20, 40, and 60 min. In several experiments, 20 μM 1,2-bis(2-aminophenox)-ethane-N,N,N’,N’-tetraacetic acid acetoxymethyl ester (BAPTA-AM) (Sigma), 100 units/mL polyethylene glycol (PEG)-Catalse or the corresponding amount of PEG, and 3 mM ethylene glycol tetraacetic acid (EGTA) (Calbiochem) were added 30 min, 20 min, and 15 min before liposome addition, respectively.

Immunophenotypic Analysis of Type 1 and Type 2 Macrophages. To get type 1 macrophages (M1) or type 2 macrophages (M2), peripheral blood mononuclear cells were isolated from human buffy coat preparations and monocytes were separated as previously described (5). Monocytes were then suspended in complete medium and incubated for a further 5 d in 24-well plates at the concentration of 10^5 cells/mL in the presence of 100 ng/mL GM-CSF or 20 ng/mL M-CSF to get M1 and M2, respectively. The M1 and M2 phenotype was then confirmed by flow cytometry after staining with FITC anti-CD14 (clone M52B; BD Pharmingen), PE anti-CD16 (clone 3G8; BD Pharmingen), and APC anti-CD163 (clone 215927; R&D Systems) and by ELISA by quantifying TNF-α, IL-6, and IL-10 (all from Thermo Scientific) release in the supernatant.

Cell Viability Assay. Phorbol 12-Myristate 13-Acetate (PMA) differentiated THP-1 cells and 5-d differentiated monocyte-derived macrophages, type 1 and type 2 macrophages, were stimulated with ABL carrying PA. At day 5, cell viability was monitored by Trypan blue exclusion and, at day 4, by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay kit (Molecular Probes). The MTT assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal in metabolically active cells. The formazan is then solubilized, and the concentration determined by optical density at 540 nm. The assay is sensitive with the colorimetric signal proportional to the viable cell number.

Combined Therapeutic Treatment in Mtb-Infected Mice. BALB/c mice (Charles River Laboratories) were kept under specific pathogen-free conditions and used in accordance with institutional guidelines in compliance with national and international law and policies (6). Experiments were performed in specific pathogen-free facilities. Six mice per group (matched for sex and age between 8 and 10 wk) were infected (under light anesthesia) intranasally (i.n.) with 2.5 × 10^7 colony-forming units (cfu) of midlog-phase Mycobacterium tuberculosis (MTB) H37Rv in 0.02 mL of saline. Starting from day 14 after infection and for a further 4 wk, mice received or not 25 mg of isoniazid (INH) (Sigma) per 100 mL of drinking water, in combination or not with 10^5 ABL/PA, or control liposome preparations (ABL/PC, PC/PA, and PC/PC), suspended in 50 μL of phosphate-buffered saline and inoculated intranasally three times per week. At the end of the sixth week, mice were killed and tissue bacillary load of lungs and spleens was quantified by plating serial dilution of...
the lung and spleen homogenates into 7H10 agar, as described previously (6, 7).

**Determination of Serum Transaminases, Lactate Dehydrogenase (LDH), and Blood Urea Nitrogen (BUN).** Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and LDH were measured by the standard photometric method, using an automatic analyzer (type 7350; Hitachi Chemical Diagnostics) and a commercial kit (Sigma-Aldrich) adapted to small-sample volumes, as previously described (6). The normal ranges of ALT, AST, and LDH were obtained by testing sera from 10 mice, age and sex matched with animals used in the experimental groups. BUN levels were measured by impregnating Azostix strips (Bayer) with a drop of fresh blood (8).


**Fig. S1.** Biophysical characterization of asymmetric liposomes. (A) Staining with Cy5-labeled Annexin V of apoptotic body-like liposomes carrying phosphatidic acid (ABL/PA), analyzed by flow cytometry. White and red histograms represent the intensity of fluorescence of ABL/PA labeled or not with Annexin V, respectively. (B) Inner distribution of fluorescent PA-NBD analyzed by fluorescence confocal microscopy. (C) Fluorescence intensity of liposomes with phosphatidylserine outside and PA-NBD inside (PS/PA-NBD) and of liposomes with PA-NBD outside and phosphatidylserine inside (PA-NBD/PS) before and after addition of sodium hydrosulfite solution acting as a quencher. (D) Average radius ± SD of the different liposome preparations obtained by dynamic light scattering. (E) Comparative analysis of internalization of ABL/PA-NBD or PC/PA-NBD in dTHP-1 cells upon opsonization of liposomes with either FBS or AB+ human serum. Results are expressed as mean ± SD of three independent determinations.
Fig. S2. Lack of cell toxicity in dTHP-1 cells stimulated with ABL/PA. (A) Cellular viability is monitored after Trypan Blue staining. Values are indicated as the number of living cells after 3 h and 5 d. (B) Cellular toxicity was assessed by MTT assay 3 h and 4 d after liposome stimulation. Absorbance was detected at 540 nm. Results represent the mean ± SD of percentages obtained from three independent experiments and the following four different liposomes were used: (i) phosphatidylserine outside/phosphatidic acid inside (ABL/PA), (ii) phosphatidylserine outside/phosphatidylcholine inside (ABL/PC), (iii) phosphatidylcholine outside/phosphatidic acid inside (PC/PA), and (iv) phosphatidylcholine outside/phosphatidylcholine inside (PC/PC).
Fig. S3. ABL/PA promotes Ca\(^{2+}\) mobilization in dTHP-1 cells. Cells were pretreated with 3 mM EGTA (A) or with 20 μM BAPTA-AM (B) 15 or 30 min prior to ABL/PA stimulation, respectively. After stimulation, fluorescence emission was continuously monitored for 20 min to determine relative alterations in intensity. (C–E) Cells were stimulated or not with the same amount of the following different liposomes: (i) phosphatidylserine outside/phosphatidylcholine inside (ABL/PC) (C), (ii) phosphatidylcholine outside/phosphatidic acid inside (PC/PA) (D), and (iii) phosphatidylcholine outside/phosphatidylcholine inside (PC/PC) (E). (F) Cells were stimulated or not with the same amount of all types of liposomes for 20, 40, or 90 min. The concentration of Ca\(^{2+}\) was determined and data are reported as mean ± SD of triplicate values and are representative of three independent experiments. Differences were evaluated by Student’s t test.
Fig. S4. (Continued)
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Fig. S4. Confocal microscopy representative images (from three separate experiments) showing the increase of GFP-MTB residing in Lysotracker Red (LTR)-positive vacuoles (A) or of Auramine-stained MTB (green) residing in LAMP-3-positive (B), LC3-positive (C), or DQ-BSA–positive (D) vacuoles (red) after stimulation with ABL/PA or PC/PC and the reverse effect exerted by EGTA (A–D) or by chloroquine (A). Cell morphology was visualized by differential interference contrast (DIC) and the merged images of the three signals are also shown.
Fig. S5. Comparison of oxidative response following stimulation with ABL/PA and control liposomes in dTHP-1 cells. dTHP-1 cells were incubated with a solution of DCF (10 μM) at 37 °C for 1 h in the dark and (A) stimulated for 20, 40, and 60 min with the same amount of ABL/PA, ABL/PC, PC/PA, and PC/PC or (B) stimulated with the same amount of ABL/PA, 200 ng/mL PMA, 100 ng/mL LPS, and 100 μM H$_2$O$_2$. One hundred units per milliliter PEG-Cat or the corresponding amount of PEG (as control) was added 20 min before ABL/PA addition to confirm its antioxidative effect. DCF oxidation was determined at 20 min after stimulation by fluorimetric measurement of fluorescence (excitation 488 nm, emission 530 nm). Data are expressed as mean ± SD of triplicate values and are representative of two independent experiments.
Fig. S6. ABL/PA stimulation induces ROS production and ROS-mediated and phagolysosome maturation-dependent intracellular mycobacterial killing. (A) dTHP-1 cells were incubated with a solution of DCF (10 μM) at 37 °C for 1 h in the dark. dTHP-1 cells were stimulated with same amount of ABL/PA for 20, 40, and 60 min. A total of 20 μM BAPTA-AM or 3 mM ethylene glycol tetraacetic acid (EGTA) was added 30 min and 15 min before liposome addition, respectively. DCF oxidation was determined by fluorimetric measurement of fluorescence (excitation 488 nm, emission 530 nm). Results are expressed as mean ± SD of triplicate values and are representative of five different experiments. °P < 0.0001, *P = 0.004, #P = not significant (NS) in comparison with untreated cells, by Student’s t test. (B) dTHP-1 cells were infected with MTB and incubated for 3 and 5 d with ABL/PA plus NH4Cl or chloroquine (Cq) or with ABL/PA plus PEG-Catalase (PEG-Cat). cfu counts are expressed as means ± SD of the triplicate values and are representative of four separate experiments. *P ≤ 0.0001 in comparison with MTB-infected cells, by Student’s t test.

Fig. S7. Lack of cell toxicity in M1 (A) and M2 (B) stimulated with ABL/PA. Liposome preparation was added to the culture for 3 d. Cellular toxicity was assessed by MTT assay 3 h (t0) and 3 d after ABL/PA stimulation. Absorbance was detected at 540 nm. Results represent the mean ± SD of OD of triplicate values coming from one buffy coat and are representative of three different healthy donors.
Fig. S8. Therapeutic role of the combined treatment ABL/PA plus INH in an experimental model of murine tuberculosis. (A) ABL/PA or control liposomes (ABL/PC, PC/PA, and PC/PC) were administered intranasally three times per week, starting from day 14 after intranasal H37Rv infection, for 4 wk in the presence of isoniazid (INH) provided in the drinking water for the same period. Mycobacterial load of the lung and spleen is shown as the mean of cfu ± SD obtained from six mice per group at 6 wk after infection. *P < 0.001 in comparison with MTB-infected control mice. (B) Serum levels of transaminase (ALT and AST), lactate dehydrogenase (LDH), and blood urea nitrogen (BUN) were quantified at 6 wk after infection and expressed as means ± SD of values from six mice per group. **P < 0.05 in comparison with MTB-infected control mice.
Fig. S9. Phenotypic characterization of M1 and M2. (A) Representative histograms showing the membrane expression of CD14, CD16, and CD163 in type 1 and type 2 macrophages. (B) Different patterns of TNF-α, IL-6, and IL-10 release in the supernatants of type 1 and type 2 macrophages. Results are representative of two different donors.