Janus-faced liposomes enhance antimicrobial innate immune response in *Mycobacterium tuberculosis* infection

Emanuela Greco a,1, Gianluca Quintilianni a,1, Marilina B. Santucci b, Annalucia Serafino a, Anna Rita Ciccgaglione c, Cinzia Marcantonio a, Massimiliano Papì d, Giuseppe Maulucci a, Giovanni Delegúe e, Angelo Martino f, Daniela Colletti g, Loredana Sarmati h, Massimo Andreoni i, Alfonso Altiere i, Mario Alma b, Nadia Caccuri a, Diana Di Liberto a, Marco De Spiriti a, Nigel D. Savage a, Roberto Nisini a, Francesco Dieli a, Tom H. Ottenhoff a, and Maurizio Fraziano a,2

Departments of “Biologic” and “Clinical Infectious Diseases, University of Rome “Tor Vergata,” 00133 Rome, Italy; bDepartment of Infectious, Parasitic, and Immunemediated Diseases, Istituto Superiore di Sanità, 00161 Rome, Italy; Italy; Institutes of “Physics” and “Microbiology, Catholic University of Sacred Heart, 00168 Rome, Italy; dDepartment of Epidemiology and Preclinical Research, National Institute of Infectious Diseases “Lazzaro Spallanzani,” 00149 Rome, Italy; eUnit of Tisiology and Bronchopneumology, S. Camillo-Forlanini Hospital, 00151 Rome, Italy; fDepartment of Bispathology and Medical and Forensics Biotechnologies, University of Palermo, 90135 Palermo, Italy; and gDepartment of Infectious Diseases, Leiden University Medical Center, 2333 ZA, Leiden, The Netherlands

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We have generated unique asymmetric liposomes with phosphatidylserine (PS) distributed at the outer membrane surface to resemble apoptotic bodies and phosphatidic acid (PA) at the inner layer as a strategy to enhance innate antimycobacterial activity in phagocytes while limiting the inflammatory response. Results show that these apoptotic body-like liposomes carrying PA (ABL/PA) (i) are more efficiently internalized by human macrophages than by nonprofessional phagocytes, (ii) induce cytosolic Ca2+ influx, (iii) promote Ca2+-dependent maturation of phagolysosomes containing *Mycobacterium tuberculosis* (MTB), (iv) induce Ca2+-dependent reactive oxygen species (ROS) production, (v) inhibit intracellular mycobacterial growth in differentiated THP-1 cells as well as in type-1 and -2 human macrophages, and (vi) down-regulate tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-1β, IL-18, and IL-23 and up-regulate transforming growth factor (TGF)-β, IL-6 (17). These results suggest that apoptotic body-like liposomes may be a potential therapy to control tuberculosis (TB) and other mycobacterial infections by mimicking key components of the cell death process.

Host phospholipids play a critical role in the activation of the antimicrobial innate immune response (1). In particular, phospholipase D (PLD) activation is necessary for intracellular killing of pathogens induced by natural ligands, such as ATP (2, 3), and by microbial ligands, such as Cpg oligodeoxynucleotides (4). Interestingly, *Mycobacterium tuberculosis* (MTB), unlike the nonpathogenic *Mycobacterium smegmatis*, inhibits PLD activation during phagocytosis, a process that is associated with intracellular survival of the pathogen (4). PLD catalyzes the hydrolysis of the membrane phospholipid, phosphatidylcholine, to generate the metabolically active phosphatidic acid (PA), PA is an important second messenger involved in multiple physiological functions, including (i) the assembly and activation of NADPH oxidase, (ii) the regulation of cytoskeleton organization, and (iii) the modulation of the vesicular trafficking and membrane fission/fusion events, responsible for phagocytosis and phagolysosome maturation (1, 5). MTB has been also reported to block phagolysosome maturation by inhibiting host sphingosine kinase (6) and phosphatidylinositol 3-kinase activity (7), both involved in different steps of phagolysosome biogenesis. A report by Anes et al. showed that different bioactive lipids, like arachidonic acid (AA), ceramide (Cer), sphingosine (Sph), sphingomyelin (SM), phosphatidylinositol (PI), and sphingosine 1-phosphate (SIP), promoted phagolysosome maturation and intracellular mycobacterial killing in murine macrophages (8).

In the same context, we previously demonstrated that lysophospholipids, such as SIP and lysosphatidic acid (LPA), (i) promote in vitro PLD-dependent phagolysosome maturation and PLD-dependent intracellular killing of MTB in human macrophages (9) and type II alveolar epithelial cell line A549 (10), (ii) induce ex vivo intracellular killing of endogenous mycobacteria in bronchoalveolar lavage cells isolated from patients affected by tuberculosis (TB) (11, 12), and (iii) reduce in vivo pulmonary mycobacterial burden and histopathology in murine models of TB (9, 13).

Phospholipids may also play a critical role in cell-to-cell signaling. In this context, the exposure of phosphatidylserine (PS) (14, 15), on the outer leaflet of plasma membrane represents one of the most striking and consistent changes of apoptotic cells. Exposure of PS plays a central role in the recognition and phagocytosis of apoptotic bodies by macrophages (16). The functional consequence of a PS-dependent recognition and ingestion of apoptotic cells by macrophages is the release of antiinflammatory cytokines and the inhibition of the production of proinflammatory cytokines (17). These features have highlighted the possibility of using apoptotic cells to manipulate the immune response for therapeutic gain to reduce the immunopathology (18).

The purpose of the present study was to evaluate the possibility of recovering or strengthening antibacterial innate immune responses by providing PA, involved in phagolysosome biogenesis, using liposomes as a vehicle. In this context, the possibility of


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engineering liposomes characterized by the expression of different phospholipids at the outer and inner membrane surface has been described (19) and offers a technological platform to asymmetrically distribute bioactive phospholipids involved in different cell functions. On these grounds, we have generated unique asymmetric apoptotic body-like liposomes (ABL), with PS distributed at the outer membrane surface to resemble apoptotic bodies, thus targeting macrophages while limiting inflammation, and with PA at the inner membrane surface to simultaneously enhance phagolysosome biogenesis-related processes. These double-faced liposomes were tested for their potential enhancing effect on innate immunity functions and bacterial killing.

Results

Biophysical Characterization of Liposomes. To check the asymmetric distribution of phospholipids in liposome preparations, we first stained liposomes with Cy5-Annexin V and monitored fluorescence emission distribution on the outer surface of liposome membrane by flow cytometry analysis. The results show the presence of Annexin V binding sites on the liposome surface in ABL/PA, visualized by increased fluorescence emission after Cy5-Annexin V staining (Fig. S1A). To demonstrate the presence of PS within liposomes, ABL carrying 1-myristoyl-2-(12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]lauroyl)-sn-glycero-3-phosphate (NBD-PA) (a fluorescent PA analog) were analyzed by confocal microscopy. The results, shown in Fig. S1B, reveal the presence of PA inside the liposome membrane. The level of asymmetry of phospholipids is further confirmed when NBD-PA is incorporated either inside the liposome membrane (PS/NBD-PA) or at the outer liposome surface (NBD-PA/PS) (Fig. S1C). In this first case, on addition of the quencher, the signal decreases ~9% but it drops ~65% when NBD-PA is incorporated at the outer leaflet. These results demonstrate that ~90% of NBD-PA is confined at the inner liposome surface when it is produced as PS/NBD-PA and that ~65% of NBD-PA is distributed at the outer surface when it is produced as NBD-PA/PS, according to a natural tendency exerted by PA to distribute within the liposome surface (20).

The liposome’s size has been tested by using dynamic light scattering. The mean hydrodynamic radius is reported for all of the preparations investigated [ABL/phosphatidylcholine (PC), PC/PC, PC/PA, and ABL/PA] in Fig. S1D. All of the samples show a hydrodynamic radius of ~1 μm with the exception of the ABL/PC sample whose radius is 2.5 μm, which could reflect the increased size due to the presence of PS and PC that modifies the thermodynamic equilibrium by shifting the mean radius toward larger values (21).

Internalization of ABL/PA and Inhibition of Proinflammatory Responses. The ability of ABL carrying NBD-PA (PS/NBD-PA) to be phagocytosed by Phorbol 12-Myristate 13-Acetate (PMA) differentiated THP-1 (dTHP-1) cells was analyzed by confocal microscopy and compared with control liposomes expressing PC at the outer leaflet of the liposome surface (PC/NBD-PA) (Fig. 1). As expected the expression of PS at the outer liposome surface (PS/NBD-PA) induces liposome internalization within macrophages (Fig. 1A), which is significantly enhanced, in comparison with PC/NBD-PA liposome preparations (Fig. 1B). In agreement, the frequency of ABL/PA carrying NBD-PA for 90 min and phagocytosis was analyzed by confocal fluorescence microscopy. (B) Summary of the mean percentage ± SD of dTHP-1 cells with at least one liposome internalized over total cells by counting ≥100 dTHP-1 cells per sample. Three different experiments were assessed. *P = 0.002 by Student’s t test. (C) Comparative analysis of internalization of ABL carrying NBD-PA in A549 cells, dTHP-1 cells, monocyte-derived macrophages (MDM), type 1 macrophages (M1), and type 2 macrophages (M2). Results are expressed as mean ± SD of three independent determinations (for A549 and dTHP-1 cells) and three different donors (for MDM, M1, and M2 cells). (D) dTHP-1 cells were stimulated with the same number of ABL/PA for 18 h and then analyzed by real-time PCR for detection of cytokine mRNA levels. Values of fold induction were the means ± SD of three independent cellular experiments, each performed by pooling of mRNA from at least three biological replicates derived by cells seeded in different plates.

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We next monitored mRNA expression of representative proinflammatory and antiinflammatory cytokines by real-time PCR in dTHP-1 cells, stimulated or not with ABL/PA. The results expressed in Fig. 1D show that after 24 h of liposome treatment, most proinflammatory cytokine mRNAs [interleukin (IL)-12-α, IL-18, and IL-23-α] were down-regulated (~2.60-, ~5.80-, and ~7.60-fold, respectively) in comparison with untreated cells whereas IL-10, IL-27, and IL-6 mRNAs were not altered after treatment (fold inductions between ±2). By contrast, transforming growth factor (TGF)-β mRNA was strongly up-regulated (~4.60-fold induction) after exposure of macrophages with ABL/PA.

ABL/PA Limit Inflammatory Responses and Enhance Intracellular Mycobacterial Killing in the Course of in Vitro M. tuberculosis Infection. To evaluate proinflammatory vs. antiinflammatory properties of ABL/PA in the course of in vitro MTB infection, we measured the production of tumor necrosis factor (TNF)-α, IL-1β, and TGF-β in the supernatant of dTHP-1 cells, infected or not with MTB and in the presence or absence of liposomes, 72 h after infection (Fig. 2). ABL/PA were strongly up-regulated after MTB infection and ABL/PA slightly, although significantly, reduced their release. By contrast, TGF-β production was significantly up-regulated in MTB-infected liposome-treated macrophages in comparison with MTB-
in infected controls; a slight, but not significant, increase in TGF-β was observed in liposome-treated macrophages in comparison with untreated cells.

We next tested the capability of ABL/PA to increase the mycobacterial killing activity of dTHP-1 cells. Results (Fig. 2R) show a significant reduction of intracellular mycobacterial viability after treatment with ABL/PA. A lesser, although still significant, reduction of intracellular mycobacterial growth was also observed after stimulation with liposomes expressing PC outside/PA inside (PC/PA), in agreement with the different abilities of phagocytes to internalize these two types of liposomes (Fig. 1B). Finally, the antimycobacterial effect was specifically induced by PA as no effect was observed after stimulation with ABL/PC. Altogether, these results highlight PA as the component modulating the antimycobacterial function of macrophages.

ABL/PA Promote Ca2+-Dependent Phagolysosome Maturation. Intracellular calcium increase is required for many different signal transduction pathways, including activation of antimycobacterial responses (22, 23). We therefore analyzed cytosolic Ca2+ influx in dTHP-1 cells following stimulation with ABL/PA. Results show an immediate increase of cytosolic Ca2+ after ABL/PA treatment (Fig. 3A), peaking at ~3 min after stimulation (889.5 nM). Cytosolic Ca2+ after ABL/PA stimulation was almost completely inhibited by the presence of ethylene glycol tetraacetic acid (EGTA) and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), used as extracellular and intracellular Ca2+ chelators, respectively (Fig. S3A and B). A progressive reduction in the peak of cytosolic Ca2+ was observed following stimulation with ABL/PC (527.6 nM), PC/PA (122.2 nM), and PC/PC (96.3 nM) control liposomes (Fig. S3C–E). Moreover, the results indicate that the presence of PS on the outer liposomal surface promotes Ca2+ mobilization at 20 min after stimulation whereas the presence of PA within ABL maintained Ca2+ mobilization up to 40 min (Fig. S3F).

As MTB is known to reside in immature endosomal compartments sequestered from late endosomes/lysosomes (24–26), the maturation of MTB-containing vacuoles and its dependence on Ca2+ mobilization were investigated in cells stimulated by ABL/PA, in the presence or absence of EGTA, or by PC/PA control liposomes by laser scanning confocal microscopy, using (i) Lysosomal-Associated Membrane Protein (LAMP)-1 and LAMP-3 as markers of lysosomes/late endosomes, (ii) the acido-philic dye LysoTracker Red, (iii) microtubule-associated protein light chain 3 (LC-3) as an autophagy marker, and (iv) DQ-BSA to monitor phagolysosomal protease activity. As expected, green fluorescent phagocytosed mycobacteria resided in LAMP-1 negative compartments (Fig. 3B), consistent with an immature maturation state of the phagosomes. In contrast, the stimulation with ABL/PA, but not with PC/PC control liposomes, induced the expression of LAMP-1 in phagosomes containing MTB, which now appeared yellow, whereas the addition of EGTA almost completely reversed this process. In Fig. 3C, a summary of all percentages of MTB colocalizing in LysoTracker Red, LAMP-1, LAMP-3, LC-3, and DQ-BSA-positive vacuoles over the total intracellular mycobacteria is given (for representative images see Fig. S4A–D). The results show the significant increase in phagolysosome maturation following ABL/PA stimulation and the inhibitory effect exerted by the addition of chloroquine [for LysoTracker Red (LTR) staining] or EGTA (for all markers used), indicating that ABL/PA-induced phagolysosome maturation was Ca2+ dependent. Moreover, the analysis of LC-3, a specific autophagic membrane marker, suggests that this process overlaps, at least in part, with the autophagolysosome pathway (Fig. S4C).

ABL/PA Promote Intracellular Mycobacterial Killing by a Reactive Oxygen Species (ROS)-Dependent and Phagolysosome-Mediated Mechanism. Phagocytes generate ROS by using superoxide-generating NADPH oxidase (NOX) family proteins, which play pivotal roles in host defense against bacterial and fungal pathogens (27). Following stimulation with ABL/PA, a significant increase in ROS production was observed starting with 10 min after stimulation, which remained significantly higher in comparison with that in control liposomes up to 40 min after stimulation (Fig. S5A). This increase was almost completely inhibited by the addition of polyethylene glycol-Catalase (PEG-Cat) (Fig. S5B). Moreover, because Ca2+ mobilization is required for phagolysosome maturation and polymerization of NADPH oxidase occurs on maturing phagosomes (28), we tested the Ca2+ dependence of ROS production. A significant abrogation of ROS production was observed in the presence of the extracellular and intracellular Ca2+ chelator EGTA and BAPTA-AM, respectively (Fig. S6A).

To assess the role of ABL/PA-induced phagolysosome maturation and ROS production in intracellular mycobacterial killing, dTHP-1 cells were infected with MTB, stimulated with liposomes, and then exposed to the lysosomotropic agents chloroquine and NH4Cl, which both increase intralysosomal pH and are considered to be general lysosomal inhibitors. Results show that the antimycobacterial activity increased by ABL/PA was mediated by phagolysosome maturation and acidification because the addition of chloroquine or NH4Cl upon ABL/PA treatment significantly reduced intracellular mycobacterial killing, particularly after 5 d of MTB infection (Fig. S6B). Moreover, to assess the role of ROS in the ABL/PA-induced intracellular mycobacterial killing, MTB-infected cells were also exposed to
PEG-Cat, which converts hydrogen peroxide to water and oxygen and thus reduces ROS activity. The results indicate that PEG-Cat almost completely abolishes ABL/PA-induced intracellular MTB killing.

ABL/PA Induce Intracellular (Myco)Bacterial Killing in Primary Type-1 and Type-2 Macrophages and in Bronchoalveolar Lavage Cells. M1 and M2 macrophages have been previously described as proinflammatory and antiinflammatory macrophages (29, 30), respectively, playing different roles during chronic inflammatory pathologies, such as TB (31). Here, we show that stimulation with ABL/PA determines a significant increase in the killing of intracellular MTB by both types of macrophages (Fig. 4A) in the absence of any macrophage toxicity (Fig. S7 A and B). Thus, the effect of ABL/PA is evident not only in human cell lines, but also in primary human macrophages and different subsets thereof.

On the basis of this finding, we wanted to extend our observations to in vivo-infected human macrophages. To reproduce the effect of ABL/PA on cells from the lungs of patients with TB, cells isolated from bronchoalveolar lavage (BAL) of three patients with active sputum-positive pulmonary TB (patients 1, 2, and 3) were stimulated with ABL/PA and cultured for 3 d, and,

Fig. 3. ABL/PA promote Ca\(^{2+}\)-mediated phagolysosome maturation in human macrophages. (A) dTHP-1 cells were incubated with 3 \(\mu M\) Fluo-3/AM at 37 °C for 1 h in the dark and were stimulated with ABL/PA or PC/PC control liposomes. After stimulation, fluorescence emission was continuously monitored for 20 min to determine relative alterations in intensity. (B) Confocal microscopy representative images (from three separate experiments) showing the increase of Auramine-stained MTB (green) residing in LAMP-1–positive vacuoles (red) after stimulation with ABL/PA. To distinguish signals deriving from internalized MTB and to avoid mycobacteria adhering on the cell surface, Auramine and LAMP-1 signals were obtained from a 3D reconstruction of images taken throughout 1 \(\mu m\) of thickness inside cells. Cell morphology was visualized by differential interference contrast (DIC) and the merged images of the three signals (Auramine/LAMP-1/DIC) were also shown. (C) Summary of the mean percentage ± SD of MTB colocalizing in acidic (LysoTracker Red-positive), LAMP-1–, LAMP-3–, LC-3–, or DQ-BSA–positive vacuoles after stimulation with ABL/PA or PC/PC control liposomes and the reverse effect exerted by EGTA or by chloroquine, determined by counting ≥40 phagosomes per sample. Three different experiments were assessed. *\(P ≤ 0.001\) in comparison with MTB-infected cells.
finally, the growth of intracellular bacteria was monitored by colony-forming unit (cfu) count. In this setting, MTB infection of macrophages and its effects on macrophage differentiation have already occurred in vivo such that this assay represents a model to measure the possible in vivo efficacy of ABL/PA. Fig. 4B shows that the treatment of BAL cells from patients 1, 2, and 3 with ABL/PA induced a strong reduction of intracellular MTB. Interestingly, when ABL/PA were tested on BAL from a patient with Klebsiella pneumoniae (patient 4), an almost complete eradication of intracellular Klebsiella pneumoniae was observed, suggesting that ABL/PA treatment is not MTB specific, but rather increases the general killing activity of macrophages.

**Therapeutic Application of ABL/PA in M. tuberculosis-Infected Mice Reduces Mycobacterial Load and Inflammatory Response.** To test the possible therapeutic effect of ABL/PA in vivo, MTB-infected mice were treated by intranasal administration of ABL/PA, oral administration of isoniazid (INH), or a combination of both, three times per week for 4 consecutive weeks. At the end of the treatment, mycobacterial load in the lung, spleen, and liver was measured as well as serum levels of TNF-α, IL-1β, IFN-γ, lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate transaminase (AST), and blood urea nitrogen (BUN). Fig. 5 reports that treatment with ABL/PA alone or in combination with INH caused at 6 wk after infection (i.e., after 4 wk of treatment) a 100-fold reduction of pulmonary mycobacterial load (1,100 ± 120 cfu and 2,100 ± 500 cfu, respectively), whereas treatment with INH alone caused only a 2-fold reduction of MTB cfu. Interestingly, opposite results were obtained in the spleen and in the liver where ~10-fold reduction was observed following treatment with INH or INH plus ABL/PA and a slight reduction, which was significant only in the spleen, was shown following treatment with ABL/PA only. To test the specificity of the response, we compared the in vivo effect of ABL/PA with...
control liposomes (ABL/PC, PC/PA, and PC/PC) in combination with INH, because the combined therapy (ABL/PA plus INH) provided the best results in terms of reduction of mycobacterial burden. Addition of control liposomes to INH therapy did not augment the effect of INH both in the lung and in the spleen. On the other hand, when ABL/PA was used in combination with INH, a 100-fold reduction of mycobacterial burden was observed in the lung (3,800 ± 510 cfu), but, as expected, not in the spleen (Fig. S8A).

The therapeutic effect exerted by the treatment with ABL/PA or with ABL/PA plus INH was associated with ~10-fold reduction of TNF-α, IL-1β, and IFN-γ in the serum (Fig. S5B) and with a concomitant reduction of LDH, a nonspecific marker of tissue toxicity, and of ALT and AST as parameters of liver toxicity, in comparison with levels in infected untreated mice (Fig. S8B).

Blood urea nitrogen (as a measure of kidney toxicity) was found unchanged irrespective of the different treatments (Fig. S8B).

**Discussion**

Liposomes are vesicles of varying size consisting of a spherical lipid bilayer, which are useful as carriers to deliver pharmacologically active agents or antigens (32), as they can protect their cargo from the environment until controlled release occurs at the target site. Thus, the possibility to asymmetrically distribute bioactive lipids through the liposome membrane provides additional value to liposome-based therapeutic strategies because the cargo of bioactive lipids, used as unique immunomodulators (33), can be preferentially delivered to specific target cells. Here we report a unique study exploring the possibility of using liposomes as carriers of the lipid second messenger PA, known to activate intracellular antimycobacterial signaling (2–5, 9), to promote the antimicrobial response of phagocytes. The preferential targeting of phagocytes is obtained by the expression of PS at the outer leaflet of the liposomal membrane, which makes the liposomes behave as apoptotic bodies. Macrophages are the primary cells initiating granuloma formation and the major cell type in most granulomas (34, 35). Moreover, they both harbor the majority of MTB bacilli and possess effector functions to kill these bacilli. There are a variety of macrophage phenotypes in granulomas with various functions, including antimycobacterial effector mechanisms, pro- and antiinflammatory cytokine production, and secretion of chemokines and proteins associated with tissue remodeling (29, 30). Thus, phagocytes contribute to most aspects of inflammation and control of infection within the granuloma. The results reported here show that the presence of PS at the outer leaflet of the liposomal membrane makes the liposomes phagocytosed more efficiently by human macrophages than by alveolar epithelial cells. Our data also show that phagocytosis of ABL/PA is associated with enhanced ToF-β and reduced proinflammatory cytokine expression, such as IL-12, TNF-α, IL-1β, IL-18, and IL-23. In this context, it has been previously shown that phagocytosis of apoptotic bodies is associated with antiinflammatory responses and resolution of inflammation (18, 36). The antiinflammatory effects of apoptotic bodies have also been demonstrated in vivo. Deliberate instillation of apoptotic cells into sites of local inflammation in the lungs and peritoneal cavity increased production of TGF-β and enhanced resolution of injury (37). Moreover, a decrease in alveolar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia (38), and defective clearance of apoptotic cells in CD44 knockout mice leads to unremitting inflammation following noninfectious lung injury (39). On these grounds, our results support the hypothesis that ABL may have the potential to limit inflammation also in an in vivo setting.

The dynamic interactions between MTB and human macrophages are central in all phases of TB, from initial infection to active disease. A crucial feature in TB is the ability of the tuercle bacilli to escape the microbial activities of macrophages and to persist as intracellular parasites. In this context, MTB was shown to have evolved a number of mechanisms that contribute to its intracellular survival. These include inhibition of (i) PLD-dependent PA generation (4), (ii) Ca2+ signaling (23), and (iii) phagolysosome maturation (40). In particular, PA is involved in the induction of several macrophage antimicrobial activities, such as Ca2+ mobilization and actin polymerization, ROS production, and intracellular trafficking of endocytosed immune complexes to lysosomes (41). Moreover, PA mediates phagolysosome maturation in the course of intracellular mycobacterial killing induced by natural and microbial ligands, such as ATP (2), Sphingosine 1-phosphate (9), LPA (12), and TGF-β.

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both serological proinflammatory cytokines and hematological
parameters of tissue cytotoxicity, so confirming, in an in vivo
experimental model of murine tuberculosis, the double action
of ABL/PA.

Of additional importance and high translational relevance, we
show that ABL/PA stimulation of BAL cells from TB patients as
well as from a patient with K. pneumoniae infection induced a
significant reduction in bacterial growth of the respective en-
dogenous intracellular pathogen. BAL cells are a mixed cell
population and comprise predominantly alveolar macrophages,
T lymphocytes, and neutrophils. In this context, the result
reported herein on BAL cells is relevant because it indicates that
ABL/PA are active in a microenvironment that mirrors that of
reported herein on BAL cells is relevant because it indicates that
the infected lung. ABL/PA caused the activation of Cr³⁺-de-
pendent ROS generation and phagosomal maturation and
these functions were both associated with the reduction/eradi-
cation of intracellular MTB and K. pneumoniae in BAL cells. As
expected from an innate immunity-enhancing compound, the
antimicrobial effect induced by ABL/PA does not seem to dis-
trumiate between intracellular pathogens and thus may repre-
represent a unique strategy to control different microbial pathogens.
In fact, induction of ROS-mediated intracellular killing of K.
pneumoniae has been described following stimulation of alveolar
macrophages with leukotrienes (48).

In conclusion, ABL/PA may be considered as Janus-faced
liposomes, with an external surface exposing PS, resembling
apoptotic bodies and inducing efficient phagocytosis accompa-
nied by the induction of antiinflammatory responses, and with an
inner surface containing PA to enhance the antibacterial func-
tions of infant cells. In many long-lasting infections such as TB,
the activation of an inefficacious immune response may lead to
a chronic inflammation and consequent tissue damage. The
possibility of enhancing innate immunity to treat microbial in-
fec tion by a noninflammatory pathway has been suggested in the
past (49). The existing antibiotic regimens against tuberculosis
last 6 mo, often resulting in patient noncompliance, treatment
failure, infection relapse, and the emergence of drug resistance
(26). In this context, the use of ABL/PA may represent an ex-
emplifying immunotherapeutic strategy to simultaneously reduce
inflammation and strengthen the innate response against
multidrug- or extensively drug-resistant (myco)bacteria.

Materials and Methods

Liposome Preparations. Asymmetric liposomes were produced according to
the Pautot et al. method to allow an asymmetric distribution of the outer and
inner phospholipids (19). In particular, to prepare the inner monolayer lipid
suspension, 2.5 mg of phospholipid and 50 μL of anhydrous dodecane
(Sigma) were placed in a 100-μL glass bottle to reach a lipid concentration
of 0.05 mg/mL. The suspension was then sonicated in a bath for 30 min and
left overnight at room temperature. The following day, 250 μL of an
aqueous solution consisting of 100 mM NaCl and 5 mM Tris Buffer, pH 7.4
was added and the solution was stirred with a magnetic stir bar for a further
3 h. To prepare the outer monolayer lipid suspension, 2.5 mg of phospho-
lipid was added to 50 mL of 99:1 dodecane:silicone solution to get a lipid
concentration of 0.05 mg/mL. Thereafter, 2 mL of outer monolayer lipid
suspension was added from 3 mL of either PBS or RPMI 1640 in a 50-mL
plastic tube (Corning). Finally, 100 μL of the inner monolayer lipid suspension
was added over the 2-mL lipid phase and the sample was centrifuged at
710 × g for 10 min. After the centrifugation, liposomes were collected in
the aqueous phase, using a 5-mL syringe with a 16-gauge stainless steel
needle. The liposome suspensions (10 mL) from Avanti Polar Lipids were used for the prepa-
rati on of the inner and outer monolayers: L-α-phosphatidylcholine (PC), L-α-
phosphatidylcholine (PC), L-α-phosphatidic acid (PA), and NBD-PA. Liposomes
were then quantified by flow cytometry (FACS Calibur; Becton Dickinson),
allowing quantification of monodispersed vesicles >0.2 μm in diameter,
whereas their mean radius was analyzed by dynamic light scattering analysis
(SI Materials and Methods).

Bacteria. Pathogenic MTB H37Rv was grown in Middle Brook 719 broth
supplemented with albumin, dextrose, and catalase. Mycobacteria were then
harvested, suspended in sterile PBS, pH 7.2, aliquoted, and stored at −80 °C
until use. Before infection, aliquots were grown on 7H10 plates to titrate the
bacteria after thawing.

Cell Cultures. The human promonocytic THP-1 leukemia cell line, induced
to differentiate by stimulation with PMA, was used as a model of human
macrophages. Cells were grown in complete medium (RPMI 1640 supple-
mented with 10% (vol/vol) FBS, 2 mM l-glutamine, and 5 μg/mL Gentamicin)
supplemented with 1 mM nonessential amino acids and 1 mM sodium py-
ruvate and incubated for 72 h at 37 °C in the presence of 20 ng/mL PMA. In
several experiments, the human lung adenocarcinoma epithelial A549 cell
line (ATCC) was used as a model of human type II alveolar epithelial cells and
cultured as described in ref. 10. Primary type 1 and 2 macrophages were also
used as representative primary phagocytes with distinct functional ac-
tivity (50). To get M1 or M2 macrophages, peripheral blood mononuclear
cells were isolated from human buffy coat preparations and monocytes were
separated, as previously described (51). Monocytes were then suspended in
complete medium and incubated for a further 5 d in 24-well plates at the
concentration of 10⁵ cells/mL in the absence or in the presence of 100 ng/mL
GM-CSF or 20 ng/mL M-CSF (both from R&D Systems) to get MDM, M1 and
M2, respectively. The M1 and M2 phenotype was then confirmed by ELISA by
quantifying TNF-α, IL-6, and IL-10 (all from Thermofisher Scientific) released in
the supernatant and by flow cytometry after staining with anti-CD14, anti-CD16,
and anti-CD163 monoclonal antibodies (Fig. 59 A and B) (50), dTHP-1 cells,
A549 cells, M1, and M2 were then washed and reconstituted in complete
medium, before use in experiments. In all experiments, cells were exposed to
one liposome per cell.

Infection and Evaluation of Intracellular Mycobacterial Growth. Differentiated
THP-1 (5 × 10⁵ cells/well) and M1 and M2 (10⁵ cells/well) were exposed for 3 h to MTB
H37Rv at the multiplicity of infection (MOI) of 1 in 24-well plates. After re-
moval of extracellular bacilli, cells were stimulated with phosphatidylserine
outside/phosphatidic acid inside (ABL/PA), phosphatidylserine outside/phos-
phatidylcholine inside (ABL/PC), phosphatidylcholine outside/ phosphatic acid
inside (PCPA), or phosphatidylcholine outside/phosphatidylcholine inside
(PCPC) and ctf assays were performed at days 3 and 5 postinfection, as
previously described (9). To ascertain whether phagosomal maturation
was responsible for intracellular mycobacterial killing, 10 μM chloroquine or
20 mM NH₄Cl was added to MTB-infected cells together with ABL/PA, as
described in ref. 10. Finally, the role of ROS in intracellular mycobacterial
killing was analyzed by adding 100 units/mL PEG-Catalase.

Quantification of Cytokines by Real-Time PCR and ELISA. RNA extraction
and real-time PCR were performed as previously described (52). Briefly, total RNA
was extracted from 2 × 10⁵ cells, using RNeasy kits (Qiagen) as described by
the manufacturer, and quantified by optical density. One hundred nanograms
of total RNA was reverse transcribed using the high-capacity cDNA
Archive Kit (Applied Biosystems) and random hexamer primers in an ABL
Promo 200 Sequence Detector System (Applied Biosystems), using the fol-
lowing thermal profile: 25 °C for 10 min, 42 °C for 1 h, and 95 °C for 5 min.
PCR reactions were performed in triplicate using TaqMan chemistry with
primer and probe sets from the Assay-on-Demand list (Applied Biosystems).
Each gene profile was compared with the standard curve of the reference
and calculation of the slope of log(ng RNA) vs. ΔCt was always <0.1. Fold
induction was then calculated by ΔΔCt method (53), using the 18s mRNA
level to normalize values and the mRNA level of basal condition (unstimu-
lated dTHP-1) as a calibrator. Values of fold induction were the means ± SD
of three independent cellular experiments.

The levels of IL-1β, TNF-α, and TGF-β in the supernatant of dTHP-1 cells
infected or not with MTB at the MOI of 1 and stimulated or not with ABL/PA
were assessed by commercially available kits [human TNF-α ELISA kit and
human IL-1β ELISA kit (Thermo Scientific) and DRG TGF-β ELISA (DRG
International)] and used according to the manufacturer’s instructions.

Fluorimetric Analysis. The efficiency of liposome internalization by different
cell types (A549, dTHP-1, MDM, M1, and M2) was analyzed by comparing
the fluorescence (λex = 488 nm, λem = 525 nm) of cells before and after 90 min exposure to ABL
carrying NBD-PA. In several experiments, the possible contribution of serum
obstruction in liposome internalization was investigated by exposing cells to
ABL/PA-NBD or PC/PA-NBD liposomes in the presence of complete medium
supplemented with 10% FBS or 10% AB human serum. Percentage of the
liposome internalized was calculated according to the following formula:
[fluorescence arbitrary units (FAU) of cells exposed to liposome carrying

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Finally, intracellular Ca\(^{2+}\) was measured after labeling cells with the 3+\(\mu\)M fluorescent intracellular Ca\(^{2+}\) indicator Fluo-3/AM (Molecular Probes), as described in ref. 10, followed by incubation at 37 °C with the different liposome preparations used at the ratio of one liposome per cell. The concentration of Ca\(^{2+}\) was determined from fluorescence ratios, as previously described (54).

Fluorescence emission was monitored by the use of a Perkin-Elmer LS50B luminescence spectrometer.

**Confocal Microscopy Analysis**

The degree of maturation of MTB-containing endosomes was assessed after 18 h stimulation of dTHP-1 cells with ABL/PA or PCPC, in presence or absence of 3 nM EGTA or 10 nM chloroquine, by analyzing the colocalization of bacilli with lysosomes after staining the mycobacteria with auramine and the lysosomes with (i) the abdophilic dye Lysotracker Red (Molecular Probes) (49), (ii) Alexa Fluor 647 anti-LAMP-1 monoclonal antibody (IgG1, clone MX-09.12.5, Santa Cruz Biotechnology), (iii) Alexa Fluor 647 anti-LAMP-1 monoclonal antibody (IgG2a, clone 25), Transduction Laboratories, Becton Dickinson, or (iv) anti-LC3 purified rabbit polyclonal antibody (clone name RB7481, Abgent). Briefly, cells were washed with PBS, fixed by 10 min incubation with 4% paraformaldehyde at 4 °C, and permeabilized with 0.2% Triton X-100 for 1 h in PBS comprising 10 \(\mu\)M rabbit IgG for 1 h (Molecular Probes). In several experiments, the cells were stained with secondary antibody Alexa Fluor 488-conjugated anti-rabbit IgG for 1 h (Molecular Probes). Several experiments were performed with various combinations. In some experiments, the cells were incubated with 10 \(\mu\)M Alexa Fluor 488-conjugated anti-LAMP-1 antibody (IgG2b, clone 25; Molecular Probes) (55) and followed by 2 h incubation with a polyclonal antibody (clone name RB7481; Abgent). Briefer, cells were washed with PBS, fixed and processed for fluorescent intracellular Ca\(^{2+}\) test.

**Statistical Analysis.** Statistical analysis was carried out by the Graphpad Prism 3.0 software package. Comparison between groups was done using Student’s t test. P < 0.05 was considered statistically significant.

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