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Lysosomal phospholipase A_2: A novel player in host immunity to *Mycobacterium tuberculosis*

Bianca E. Schneider[1,2], Jochen Behrends[1], Kristine Hagens[1], Nadine Harmel[1], James A. Shayman[3] and Ulrich E. Schaible[1,2,4]

[1] Department of Molecular Infection Biology, Cellular Microbiology, Research Center Borstel, Borstel, Germany
[2] Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK
[3] Department of Internal Medicine, Nephrology Division, University of Michigan, Ann Arbor, MI, USA
[4] German Centre for Infection Research, TTU-TB, Borstel, Germany

Phospholipases catalyze the cleavage of membrane phospholipids into smaller bioactive molecules. The lysosomal phospholipase A_2 ($LPLA_2$) is specifically expressed in macrophages. $LPLA_2$ gene deletion in mice causes lysosomal phospholipid accumulation in tissue macrophages leading to phospholipidosis. This phenotype becomes most prominent in alveolar macrophages where $LPLA_2$ contributes to surfactant phospholipid degradation. High expression of $LPLA_2$ in alveolar macrophages prompted us to investigate its role in host immunity against the respiratory pathogen *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Here we report that adaptive immune responses to *M. tuberculosis* were impaired in $LPLA_2$ deficient mice. Upon aerosol infection with *M. tuberculosis*, $LPLA_2$ deficient mice showed enhanced mycobacterial counts but less lung immunopathology and pulmonary inflammatory responses. Compromised T-cell priming in the lymph nodes was associated with impaired pulmonary T-cell recruitment and activation. Together with reduced Th1 type cytokine production, these results indicate that $LPLA_2$ is indispensable for the induction of adaptive T-cell immunity to *M. tuberculosis*. Taken together, we identified an unexpected and novel function of a lysosomal phospholipid-degrading enzyme.

Keywords: Immunity · Macrophages · Phospholipase · T cells · Tuberculosis

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Introduction

Phospholipases form a ubiquitous class of enzymes that cleave membrane phospholipids into smaller bioactive molecules [1]. Enzymes of the phospholipase A_2 (PLA_2) family play multiple roles in lung infection and inflammation [2]. Apart from being involved in the generation of bioactive lipids such as eicosanoids, which are involved in inflammation by regulating cytokine and chemokine responses, direct bactericidal activity due to hydrolysis of bacterial-membrane phospholipids has also been shown for secretory PLA_2 ($sPLA_2$) [2, 3]. Despite the essential function of lysosomes for membrane degradation, which is a primary function of phagocytes, only a few responsible enzymes were identified so far. The lysosomal phospholipase A_2 ($LPLA_2$),

Correspondence: Prof. Ulrich E. Schaible
e-mail: uschaible@fz-borstel.de

newly designated group XV PLA A_2, is specifically expressed in macrophages with predominance in alveolar macrophages where it is of specific relevance for surfactant phospholipid degradation [4]. $LPLA_2$ is a broad specificity PLA_2 that is characterized by the presence of both PLA_2 and transacylase activity [5]. Among the main substrates of the $LPLA_2$ are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which account for 84 and 2% of the surfactant lipid in mice, respectively [4]. $LPLA_2$ knockout mice develop a lysosomal storage disease, that is, phospholipidosis, which becomes obvious by phospholipid accumulation in lysosomes of alveolar macrophages and peritoneal macrophages [6]. By 1 year of age, $LPLA_2$ KO mice develop increased lung surfactant phospholipid levels and splenomegaly. The $LPLA_2$ KO phenotype demonstrates an essential function of this enzyme in phospholipid turnover and membrane homeostasis.

The causative agent of tuberculosis, *Mycobacterium tuberculosis*, establishes infection in the lung by aerosol transmission from infected people. With 1.7 million deaths and 8 million new cases annually, tuberculosis continues to have a major impact on public health worldwide (WHO). After entering the lung in small aerosol droplets, *M. tuberculosis* is taken up by resident alveolar macrophages, which provide the initial niche for the pathogen's survival and replication. Infected alveolar macrophages together with interstitial DCs enter the lung parenchyma where they initiate a local inflammatory reaction [7]. At the same time, DCs and probably other APCs transport *M. tuberculosis* to the draining lymph node, where antigen-specific $CD4^+$ and $CD8^+$ T cells are primed [8, 9].

The predominance of $LPLA_2$ in alveolar macrophages together with this cell's function in immunity and as initial habitat for *M. tuberculosis* prompted us to ask whether $LPLA_2$ contributes to host responses against this intracellular pathogen. Upon *M. tuberculosis* aerosol infection, we observed abolished T-cell priming in mediastinal lymph nodes (mLNs) of $LPLA_2$ KO mice, which was associated with recruitment and activation of pulmonary T cells and impaired proinflammatory mediator production compared with WT mice. Antigen-specific $CD4^+$ T cells from infected lungs of $LPLA_2$ deficient mice failed to secrete IFN-γ. Consequently, Th1 cytokine responses were strongly reduced, and mycobacterial tissue loads significantly increased in $LPLA_2$ KO when compared with WT mice. Our findings reveal an essential and novel role of $LPLA_2$ in bridging innate and adaptive immune functions during *M. tuberculosis* infection.

Results

Control of *M. tuberculosis* infection is impaired in $LPLA_2$ deficient mice

After entering the airways, *M. tuberculosis* is taken up by resident alveolar macrophages, which provide the first line of defence but also a niche for its survival and replication. $LPLA_2$ is highly expressed in alveolar macrophages [4], which prompted us to analyze the outcome of and host responses to *M. tuberculosis* infection in $LPLA_2$ KO mice. Mycobacterial loads were significantly increased in lungs of $LPLA_2$ KO mice when compared with those from C57BL/6 WT mice (Fig. 1A), indicating a much better growth and survival of *M. tuberculosis* in the absence of $LPLA_2$. Significantly higher bacterial loads 6 months after infection showed that this phenotype was not transient but sustained over time (Fig. 1A). The difference in lung CFU between $LPLA_2$ and WT mice became even more pronounced after high-dose infection (600 CFU per lung), recovering >1 log higher bacterial loads from $LPLA_2$ deficient than from WT lungs 48 days after infection (Fig. 1B). In line with CFU data, higher loads of tubercle bacilli were detected by acid-fast staining in $LPLA_2$ KO lung sections when compared with WT lungs (Fig. 1C and D).

Survival time of $LPLA_2$ KO mice was shortened upon high-dose *M. tuberculosis* infection compared with that of WT controls but was only observed very late in the chronic phase of infection and statistically not significant (Fig. 1E).

$LPLA_2$ deficiency leads to reduced inflammation during *M. tuberculosis* infection

Despite higher numbers of mycobacteria in $LPLA_2$ KO mice, histopathological examination of the *M. tuberculosis* infected lungs revealed less cellular infiltrates affecting smaller tissue areas in these mice when compared with WT ones (Fig. 2A). Consistent with the histological observations, the total immune cell number in the lung after infection was significantly lower in $LPLA_2$ KO mice when compared with WT mice (Fig. 2B). Strikingly, spleens of $LPLA_2$ deficient mice were significantly smaller in size than WT spleens upon *M. tuberculosis* infection, as reflected by lower total cell numbers and organ weight and spleen-to-body weight ratio (Fig. 2B–D). Importantly, $LPLA_2$ deficient spleens contained less mycobacteria than WT ones over the first 6 weeks post aerosol infection with *M. tuberculosis* (Fig. 2E) indicating limited dissemination of *M. tuberculosis* from the pulmonary entry site to peripheral lymphoid organs in $LPLA_2$ KO mice. However, 43 days after infection, *M. tuberculosis* numbers in $LPLA_2$ KO spleens reached those in WT spleens (Fig. 2E), which reveals $LPLA_2$ KO spleens, just like the lungs, as a more favorable environment for mycobacterial growth when compared with WT ones.

Antimicrobial activity of $LPLA_2$ deficient macrophages

Higher mycobacterial loads in lungs of $LPLA_2$ deficient mice prompted us to analyze whether $LPLA_2$ deficiency impairs the antimicrobial effector functions of macrophages. A direct bactericidal effect of $LPLA_2$ could be ruled out by the fact that purified recombinant $LPLA_2$ had only a minor effect on the viability of mycobacteria in vitro, which was tested under different media conditions, that is, Ringer solution and PBS (Fig. 3A and data not

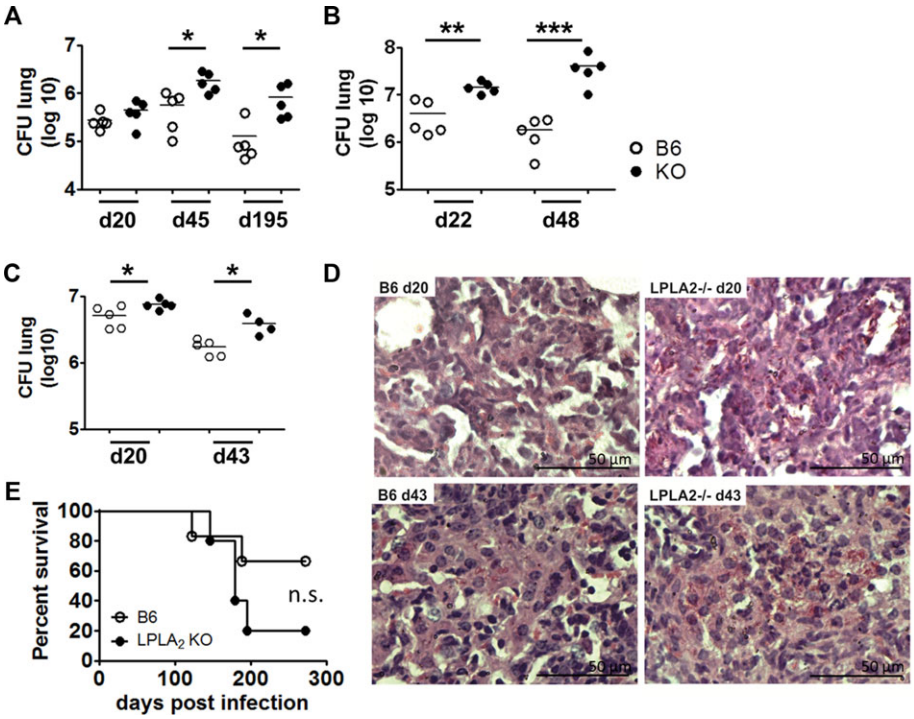

Figure 1. Control of *Mycobacterium tuberculosis* infection in $LPLA_2$ KO mice. $LPLA_2$ KO and WT B6 mice were infected by aerosol with 60 (A), 600 (B and E), or 250 (C and D) *M. tuberculosis* H37Rv per lung. (A–C) Mice were sacrificed after the indicated time points upon aerosol infection and serial dilutions of lung lysates were plated for CFU determination. Symbols and bars represent individual mice and means, respectively. Each graph represents an individual experiment of one (A, B and E) or two (C and D) performed. (D) Acid-fast staining of lung sections 20 and 43 days post-infection (p.i.). Representative images from one mouse out of five analyzed in (C) are shown. (E) Long-term observation upon high-dose *M. tuberculosis* infection. $n = 6$ (WT) and $n = 5$ ($LPLA_2$ KO). Statistical analysis was performed using the Student's *t*-test for bacterial numbers and the log-rank test for survival ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

shown). Moreover, *M. tuberculosis* survived and replicated equally well in both WT and $LPLA_2$ KO macrophages (Fig. 3C). Notably, IFN-γ activation rendered both WT and $LPLA_2$ KO macrophages similarly capable of restricting growth of intracellular *M. tuberculosis* (Fig. 3B) and to produce nitric oxide (NO_2^-) (Fig. 3C). However, we found reduced levels of TNF-α to be secreted into the supernatant by $LPLA_2$ KO peritoneal macrophages 24 h after infection with *M. tuberculosis* (Fig. 3D), suggesting that macrophages are not fully activated in the absence of $LPLA_2$.

Impaired T-cell responses to *M. tuberculosis* in $LPLA_2$ deficient lungs

Reduced cellular infiltrates in $LPLA_2$ KO lungs upon *M. tuberculosis* infection indicated impaired immune cell recruitment to the pulmonary site of infection. This assumption was indeed corroborated by FACS analyses revealing decreased proportions and numbers of $CD11b^{hi}Ly6G^{+}$ infiltrating monocytes in $LPLA_2$ KO compared with WT lungs (Fig. 4A). Moreover, we found significantly reduced

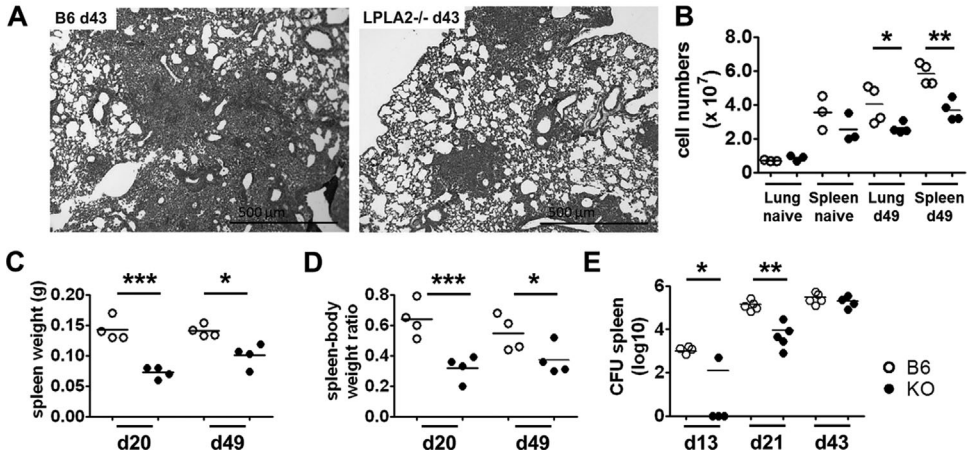

Figure 2. Inflammatory tissue responses in the absence of $LPLA_2$. $LPLA_2$ KO and WT B6 mice were infected by aerosol with 250 (A and E) or 150 (B–D) *Mycobacterium tuberculosis* H37Rv per lung. (A) Representative H&E stains of lung sections of $LPLA_2$ KO or B6 WT mice 43 days p.i. ($n = 5$). (B–D) Total cell numbers, spleen weight, and spleen to body weight ratios were determined from lungs and spleens of naïve or *M. tuberculosis* infected animals. (E) Serial dilutions of spleen lysates were plated for CFU determination. Symbols and bars represent individual mice and means, respectively, and data from one experiment representative of two experiments are shown. Statistical analysis was performed using the Student's *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

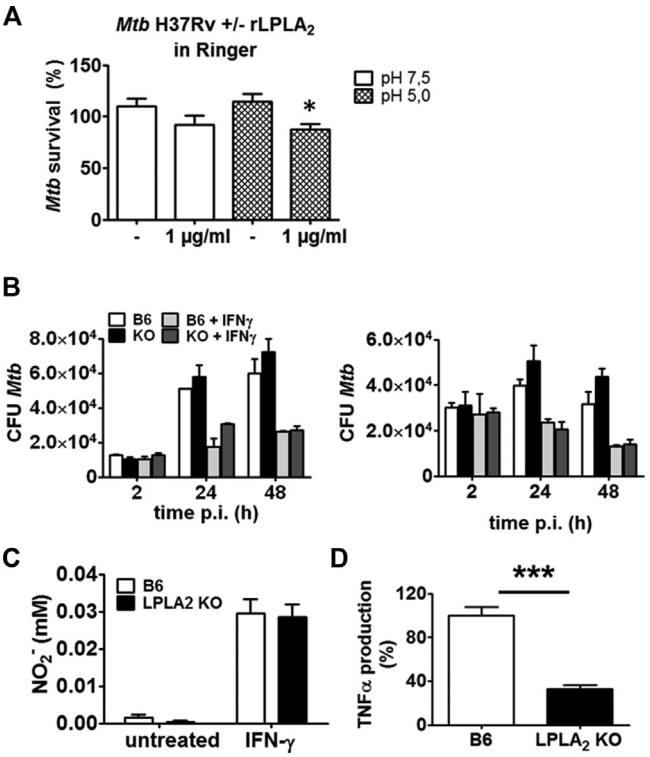

Figure 3. Functional activity of $LPLA_2$ deficient macrophages. (A) *Mycobacterium tuberculosis* was incubated in Ringer solution of pH 5.0 or 7.5, respectively, containing different concentrations of recombinant mouse $LPLA_2$. At the indicated time points, *M. tuberculosis* viability was determined by CFU analysis. Relative survival of *M. tuberculosis* is shown as mean + SEM ($n = 6$) of data pooled from two independent experiments. (B–D) Resting or IFN-γ (2000 U/mL O/N) activated peritoneal macrophages were infected with *M. tuberculosis* H37Rv at a MOI of 1:1. (B) At the indicated time points, serial dilutions were plated onto Middlebrook 7H11 agar plates for CFU determination. Data are shown as mean + SEM from technical replicates ($n = 3$) and are representative of two independent experiments. (C) NO_2^- production was determined 24 h after infection as described in the "Materials and methods" section. Data are shown as mean + SEM representing replicates ($n = 6$) pooled from two independent experiments. (D) TNF-α production by $LPLA_2$ KO peritoneal macrophages relative to WT peritoneal macrophages 24 h after *M. tuberculosis* infection was calculated and pooled from three independent experiments. Statistical analysis was performed using the Student's *t*-test (***$p < 0.001$).

proportions and numbers of both $CD4^+$ and $CD8^+$ T cells in infected $LPLA_2$ KO lungs when compared with WT ones (Fig. 4B). More importantly, recall responses of $CD4^+$ and $CD8^+$ T cells recruited to lungs of $LPLA_2$ KO mice upon *M. tuberculosis* infection were severely impaired as revealed by limited secretion of IFN-γ or TNF-α upon ex vivo restimulation with anti-CD3/CD28 or PPD. Twenty days after infection, WT lungs contained more IFN-γ or TNF-α producing $CD4^+$ and $CD8^+$ T cells in response to anti-CD3/CD28 stimulation ex vivo when compared with $LPLA_2$ KO ones (Fig. 4C–E). Furthermore, a significant proportion of lung-derived $CD4^+$ T cells from WT mice responded to PPD by IFN-γ−production. In contrast, infected $LPLA_2$ KO lungs did not contain PPD-specific IFN-γ−producing T cells (Fig. 4F). Notably, this response was not simply delayed since it was still suppressed 49 days after infection (Fig. 4G). Impairment of pulmonary T-cell responses in $LPLA_2$ deficient mice upon *M. tuberculosis* infection is not due to general incapability to activate T cells as proliferation of ovalbumin specific T cells (OT1, OT2) upon antigen-injection was unaffected by $LPLA_2$ deficiency in infected as well as noninfected mice (Supporting Information Fig. 3, data not shown).

Taken together, $LPLA_2$ deficiency impaired pulmonary T-cell responses upon *M. tuberculosis* infection including recruitment and activation of antigen-specific $CD4^+$ T cells.

Reduced Th1 cytokine responses to *M. tuberculosis* infection in $LPLA_2$ KO mice

In order to identify immune differences in $LPLA_2$ KO versus WT lungs, we analyzed the levels of MCP-1, IFN-γ, TNF-α, IL-10, IL-17, NO, and of the small lipid mediators PGE_2, LTB_4,and LXA_4 upon *M. tuberculosis* infection. No differences were observed regarding tissue levels of these immune mediators between WT and $LPLA_2$ KO mice within the first 2 weeks of infection. However, on day 20 p.i., significantly higher levels of MCP-1 and TNF-α were measured in both lungs and spleens of WT when compared with $LPLA_2$ KO mice (Fig. 5A and B). Although only statistically significant in the spleen, IFN-γ was also reduced in lungs of $LPLA_2$ KO compared with WT. In line with decreased production of the proinflammatory cytokines INF-γ and TNF-α, measurement of NO_2^- in sera of infected mice revealed significantly reduced concentrations of NO_2^- in $LPLA_2$ KO compared with WT sera (Supporting Information Fig. 1A). Levels of IL-10, which counterregulates proinflammatory immune responses, and IL-17A did not differ in the infected lungs, but IL-10 was significantly higher in spleens of $LPLA_2$ KO mice 20 days p.i. (Supporting Information Fig. 1B and C). This difference was only transient and no longer apparent 43 days after *M. tuberculosis* infection. Since nonlysosomal PLA_2 are involved in eicosanoid and proinflammatory lipid mediator production, we also tested the amount of PGE_2, LTB_4, and LXA_4 in infected WT and $LPLA_2$ KO mice. We did not observe differences in the levels of these mediators in lung and mediastinal lymph nodes but an increase in LTB_4 and LXA_4 levels in spleens of $LPLA_2$ KO mice (Supporting Information Fig. 2).

Taken together, these data suggest that $LPLA_2$ deficiency results in diminished Th1 responses during experimental *M. tuberculosis* infection.

$LPLA_2$ deficiency results in impaired T-cell priming in mLNs

To determine whether the reduced T-cell response in the lungs of $LPLA_2$ KO mice was associated with impaired T-cell priming in mLNs, we analyzed the numbers of IFN-γ and TNF-α producing $CD4^+$ and $CD8^+$ T cells by ex vivo restimulation with anti-CD3/CD28. Upon *M. tuberculosis* infection, mLNs from $LPLA_2$ KO mice were smaller in size, as reflected in a significant lower total cell count (Fig. 6A) and significantly reduced $CD4^+$ and $CD8^+$ T-cell numbers compared with mLNs from WT mice (Fig. 6A). On

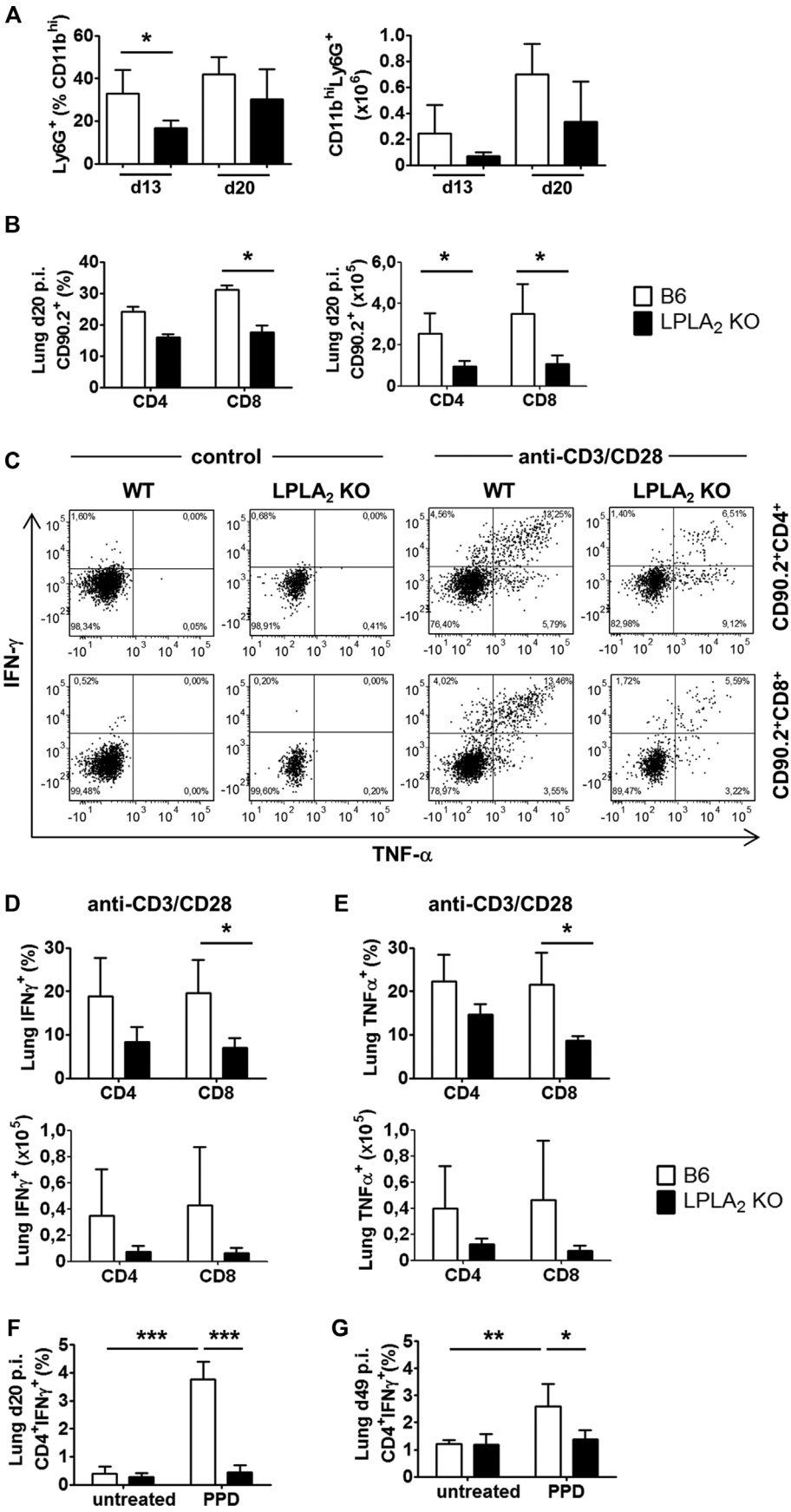

Figure 4. T-cell responses to *Mycobacterium tuberculosis* in lungs of $LPLA_2$ deficient mice. $LPLA_2$ KO and WT B6 mice were infected by aerosol with *M. tuberculosis* H37Rv ((A–E) 250 CFU; (F and G) 150 CFU)). (A) 13 and 20 days p.i. lungs were analyzed for the presence of $CD11b^{hi}Ly6G^+$ cells. (B) 20 days p.i. lungs were analyzed for the presence of $CD4^+$ and $CD8^+$ T cells by flow cytometry. (C–E) 20 days after infection, whole lung lysates were restimulated ex vivo with anti-CD3/CD28 (5 μg/mL, respectively) and analyzed by flow cytometry for the presence of IFN-γ or TNF-α producing $CD4^+$ and $CD8^+$ T cells. (C) Representative dot plots of cytokine-producing $CD4^+$ and $CD8^+$ T cells gated for CD44. (D and E) The frequencies and numbers of gated cytokine-producing $CD4^+$ and $CD8^+$ T cells are shown. (F and G) 20 or 49 days after infection, lung cells were restimulated ex vivo with PPD (5 μg/mL) or left unstimulated and analyzed by flow cytometry for the presence of IFN-γ or TNF-α producing $CD4^+$ T cells gated for CD90.2. (A–G) Data are shown as means + SD ((A–E) $n = 5$; (F and G) $n = 4$) and represent two independent experiments. Statistical analysis was performed by Student's *t*-test ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). For full gating strategies, see Supporting Information Fig. 4.

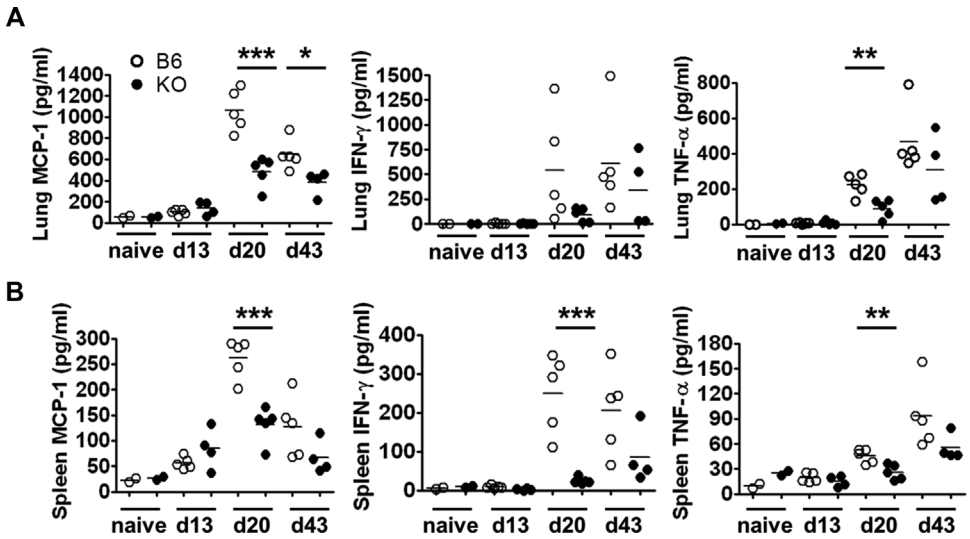

Figure 5. Th1 cytokine responses to *Mycobacterium tuberculosis* infection in $LPLA_2$ KO mice. Cytokine levels were measured in lung (A) and spleen lysates (B) of $LPLA_2$ KO and B6 WT mice on day 13, 20, and 43 post *M. tuberculosis* H37Rv aerosol infection (d1 CFU 250) by CBA as described in the *Materials and methods*. Symbols and bars represent individual mice and means, respectively, and data from one experiment representative of two experiments are shown. Statistical analysis was performed by ANOVA and Tukey's Multiple Comparison test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

day 20 upon *M. tuberculosis* infection, mLNs from $LPLA_2$ KO mice contained significantly lower numbers of $CD4^+$ and $CD8^+$ T cells that responded to anti-CD3/CD28 stimulation with IFN-γ or TNF-α production (Fig. 6B–D) demonstrating defective priming of $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes in the absence of $LPLA_2$.

Impaired TNF-α and CCL5 production in $LPLA_2$ KO mLNs upon *M. tuberculosis* infection

The kinetics of the T-cell response during *M. tuberculosis* infection is dependent on the transport of antigen from the lung to the draining LNs in order to initiate T-cell priming. To ascertain whether impaired T-cell priming was due to defective delivery of *M. tuberculosis* to the mLNs, we analyzed mycobacterial loads in mLNs at different time points over the first 3 weeks of infection. To our surprise, we did not detect differences in mLN CFU at any of the time points analyzed (Fig. 6E). However, when we compared mycobacterial loads in mLNs and lungs, the proportion of *M. tuberculosis,* which was delivered to the mLNs, was decreased in $LPLA_2$ KO mice when compared with WT mice (Fig. 6F and G). Despite this difference, it is unlikely that the reduced transport of mycobacteria to mLNs in $LPLA_2$ KO mice was responsible for impaired T-cell priming since in WT mLNs, the amount of antigen was also sufficient for T-cell priming.

In order to identify mechanisms other than antigen delivery, which could affect T-cell priming in the mLNs, we analyzed the production of a number of relevant chemokines and cytokines. Among those tested, significantly lower amounts of TNF-α and CCL5 were measured in $LPLA_2$ KO mLNs (Fig. 6H and I).

Taken together, production of TNF-α and CCL5 was impaired in $LPLA_2$ KO mLNs upon *M. tuberculosis* infection, whereas transport of *M. tuberculosis* to the mLNs was not affected.

Discussion

Macrophage effector functions are critical for the control of mycobacterial infection and the initiation of an adaptive immune response. $LPLA_2$ is primarily expressed in tissue macrophages such as alveolar and peritoneal ones and contributes to membrane homeostasis and phospholipid turnover including surfactant degradation [6]. $LPLA_2$ deficiency leads to lysosomal accumulation of phospholipids [6], mainly PE and PC, which results in the formation of lamellar inclusion bodies, a hallmark of cellular phospholipidosis. So far, the consequences of $LPLA_2$ deficiency in infection and immunity have not been addressed. Here we show that $LPLA_2$ deficiency impairs establishment of protective pulmonary Th1 immune responses to *M. tuberculosis* in vivo. Impaired adaptive immunity upon *M. tuberculosis* infection was characterized by defective T-cell priming in the draining LNs and deficient $CD4^+$ and $CD8^+$ T-cell recruitment to infected lungs. Moreover, the few recruited pulmonary T cells were unable to produce IFN-γ or TNF-α upon ex vivo restimulation with anti-CD3/CD28. More importantly, antigen-specific pulmonary T cells were not capable of responding to PPD by IFN-γ secretion, suggesting that the pulmonary $LPLA_2$ deficient environment failed to promote activation of mycobacteria-specific $CD4^+$ T cells.

Reduced Th1 immune responses were associated with higher bacterial loads in $LPLA_2$ KO mice. A direct bactericidal effect of the enzyme can most probably be ruled out by the fact that (i) the viability of *M. tuberculosis* was not affected by

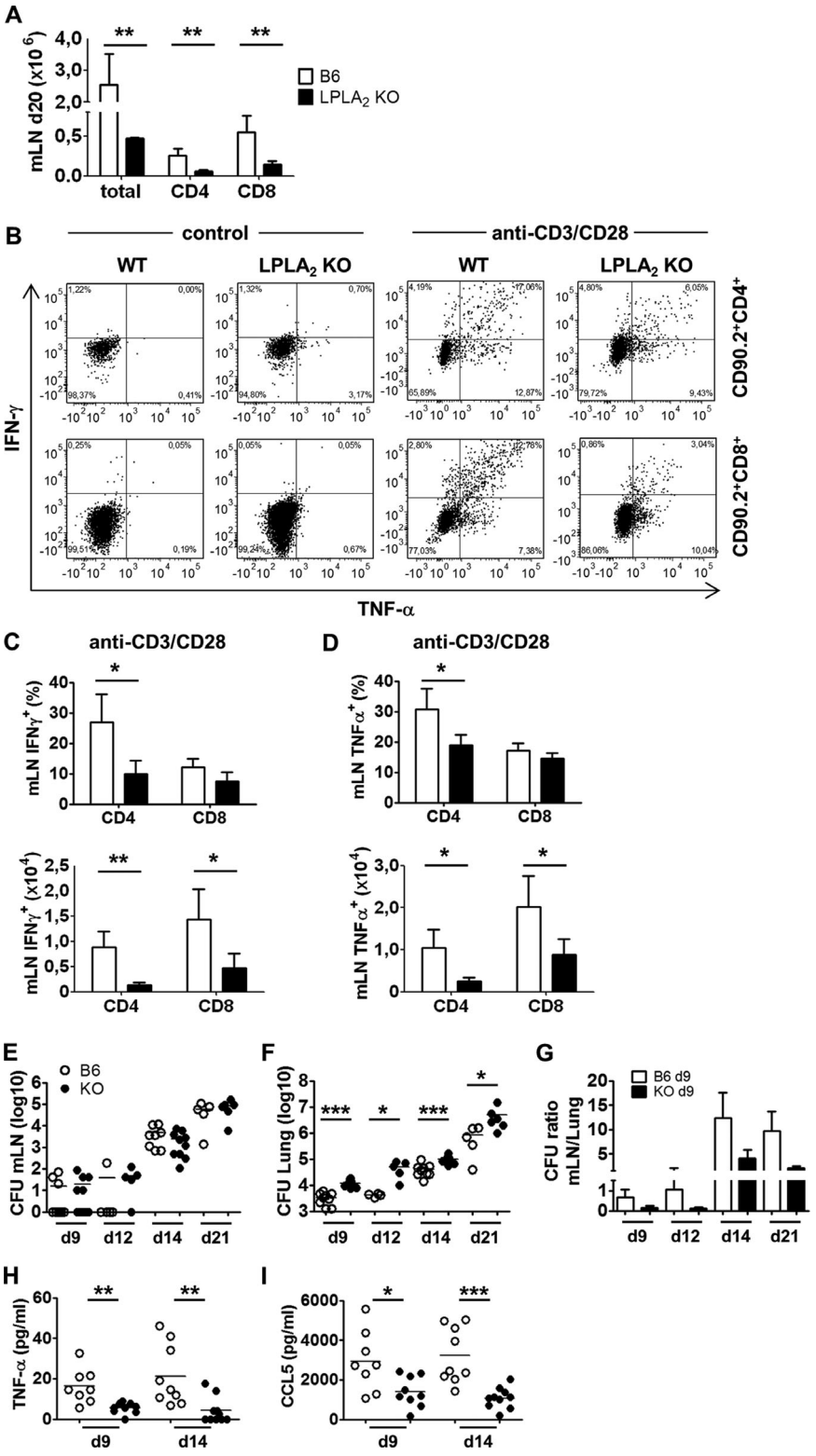

Figure 6. T-cell priming and cytokine production in mediastinal lymph nodes. $LPLA_2$ KO and WT B6 mice were infected by aerosol with *Mycobacterium tuberculosis* H37Rv (d1 CFU 250). (A) 20 days p.i. mediastinal lymphnodes (mLNs) were analyzed for absolute cell numbers and the presence of $CD4^+$ and $CD8^+$ T cells by flow cytometry. (B–D) MLN lysates were prepared on day 20 p.i., restimulated ex vivo with anti-CD3/CD28 (5 μg/mL, respectively), and analyzed by flow cytometry for the presence of IFN-γ or TNF-α producing $CD4^+$ and $CD8^+$ T cells. (B) Representative dot plots of cytokine-producing $CD4^+$ and $CD8^+$ T cells gated for CD44 from one mouse out of five analyzed are shown. (C and D) The frequencies and numbers of gated cytokine-producing $CD4^+$ and $CD8^+$ T cells are shown as means + SD ($n = 5$). (A–D) Data are shown as means + SD and represent one experiment with five mice per group. Statistical analysis was performed by Student's *t*-test ($^*p < 0.05$; $^{**}p < 0.01$). (E and F) Mice were sacrificed after the indicated time points upon aerosol infection and serial dilutions of mLN and lung lysates were plated for CFU determination. Symbols and bars represent individual mice and means, respectively, and data pooled from two independent experiments are shown. (G) The ratio of *M. tuberculosis* CFU in mLNs relative to the lungs is given as means + SD ($n = 5$–8). (H and I) Levels of TNF-α and CCL5 were determined in mLN lysates by CBA as described in the "Materials and methods" section. Symbols and bars represent individual mice and means, respectively, and represent one experiment with eight–ten mice per group. Statistical analysis was performed by Student's *t*-test ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). For full gating strategies, see Supporting Information Figure 4.

in vitro treatment with recombinant $LPLA_2$, (ii) pathogenic *M. tuberculosis* survived and replicated equally well in both WT and $LPLA_2$ KO macrophages, and (iii) tissue macrophages, when preactivated with IFN-γ, restricted mycobacterial growth equally well in the presence or absence of $LPLA_2$. These findings indicate that in vivo $LPLA_2$ KO macrophages are insufficiently activated or macrophages recruitment is limited in the absence of $LPLA_2$, or both. In fact, we observed lower numbers of $CD11b^{hi}Ly6G^+$ inflammatory monocytes infiltrating the lung tissue of $LPLA_2$ KO mice upon *M. tuberculosis* infection than that

of WT mice. The failure to recruit inflammatory monocytes to the site of infection most likely affects control of *M. tuberculosis* and thereby promotes bacterial growth as observed in the absence of $LPLA_2$.

Another reason for the failure of $LPLA_2$ KO mice to control *M. tuberculosis* is most probably reduced production of the proinflammatory cytokine TNF-α. Macrophages and T cells are the primary TNF-α sources in tuberculosis. For both, we have shown impaired TNF-α production in the absence of $LPLA_2$. IL-10, a cytokine counterregulating inflammatory Th1 responses, was only enhanced in $LPLA_2$ KO mouse spleens at day 20 p.i. but otherwise comparable between KO and WT mice and is therefore not likely to dampen proinflammatory immune responses in the absence of $LPLA_2$ during the whole course of infection. We can also rule out a regulatory role for IL-17A that was not affected by lack of $LPLA_2$. The same applies for PGE_2 synthesis, a bioactive lipid mediator known to inhibit macrophages functions on T-cell activation [10–13]. To date, no role for $LPLA_2$ has been observed in arachidonic acid release and prostanoid formation and according to our own data PGE_2, LTB_4, and LXA_4 production is not impaired in *M. tuberculosis* infected mice in the absence of $LPLA_2$.

Impaired degradation of lipid membranes may affect presentation of both bacterial lipid and protein antigens. Only recently, Paduraru and colleagues described a role for $LPLA_2$ in iNKT cell-mediated CD1d recognition [14]. In other murine lysosomal storage disease models, the numbers of CD1d-restricted iNKT cells are often diminished, irrespective of the glycolipid species accumulated or the glycolipid catalytic/synthetic pathway genes affected [15]. In our hands, however, $LPLA_2$ deficient APCs were not defective in their ability to stimulate both CD1d-dependent autoreactive as well as αGalCer reactive NKT-cell clones (data not shown). In contrast to NKT cells, which have limited immune function in murine tuberculosis, conventional Th1 $CD4^+$ T cells are essential for controlling *M. tuberculosis* in mice [16, 17]. Our data demonstrate that impaired recruitment of $CD4^+$ T cells and Th1 responses render $LPLA_2$ KO mice more susceptible to infection by *M. tuberculosis*. Importantly, recruitment and activation of $CD8^+$ T cells are also hampered in the absence of $LPLA_2$. It should, however, be noted that T-cell activation was not generally impaired as ovalbumin specific $CD4^+$ and $CD8^+$ T cells proliferated normally in WT and $LPLA_2$ KO lungs upon adoptive transfer and subsequent antigen injection.

Failed recruitment of immune cells is most probably due to defective production of chemokines at the site of infection. In fact, levels of MCP-1 (CCL2), which is chemotactic for macrophages, DCs, NK cells, and T cells, were significantly lower in lungs and spleens of *M. tuberculosis* infected $LPLA_2$ KO mice than WT mice. Hence, decreased production of MCP-1 could at least in part explain reduced inflammation and subsequent impaired recruitment of IFN-γ producing T cells in infected $LPLA_2$ KO lungs. Mice lacking CCL2 display a similar phenotype regarding decreased pulmonary recruitment of macrophages upon aerosol infection with *M. tuberculosis*. However, these mice show similar numbers of activated T cells and only a transient reduction in the number of IFN-γ secreting T cells [18]. Accordingly, these mice only show a transient increase in mycobacterial loads. In contrast, mycobacterial loads in the lungs of $LPLA_2$ KO mice still exceed that in lungs of WT mice by 1 log after several months of infection. Furthermore, dissemination of *M. tuberculosis* to the spleen is not affected in CCL2 KO mice. Therefore, decreased levels of MCP-1 alone cannot account for the observed phenotype of *M. tuberculosis* infected $LPLA_2$ KO mice. Production of TNF-α was also affected by $LPLA_2$ deficiency. TNF-α does not only participate in macrophages activation but promotes recruitment of immune cells to the site of infection by upregulation of endothelial adhesion molecules [19] and chemokine production [20, 21]. Hence, insufficient production of TNF-α might further limit production of chemokines involved in the recruitment of leukocytes to the site of infection. In line with this, we found decreased levels of the chemokines KC and MIP-1β early in *M. tuberculosis* in lungs of $LPLA_2$ KO mice (data not shown).

M. tuberculosis dissemination is a prerequisite for priming of adaptive immunity [22]. Our data suggest that $LPLA_2$ deficiency impairs early dissemination of mycobacteria to the spleen but not to the lung draining mLNs. However, while the absence of $LPLA_2$ did not affect mycobacterial loads in the mLNs, it significantly reduced the production of TNF-α and CCL5, both of which contribute to early protection against *M. tuberculosis* infection [23, 24]. As activated macrophages are the main TNF-α producers, diminished production of this cytokine indicates that $LPLA_2$ deficient macrophages are not fully activated in vivo. Although DCs are regarded the predominant APC population responsible for T-cell priming in the draining LN during *M. tuberculosis* infection [8], defective macrophages activation can still limit activation and maintenance of T-cell responses. Like TNF-α, CCL5 is produced by a wide variety of cells including macrophages in response to *M. tuberculosis* infection [25]. In addition to its chemotactic property attracting a number of immune cells including T cells and macrophages, CCL5 directly contributes to T-cell activation [26–28]. Thus, significantly abated CCL5 production in mLNs is likely to preclude T-cell priming and proliferation in $LPLA_2$ KO mice.

The fact that $LPLA_2$ KO mice, despite having higher bacterial loads, succumb to infection earlier than WT mice but only at very late time points, even upon a high initial dose of *M. tuberculosis*, indicates that the limited T-cell activation observed is still sufficient to keep the infection at bay for long time. It is, however, important to note that inflammatory responses were less pronounced in tissues of $LPLA_2$ KO in comparison to WT mice, resulting in milder pathological sequelae, which is beneficial for the host as long as immune control restricts mycobacterial replication to some extend at the same time [29].

In conclusion, our study demonstrates that $LPLA_2$ is involved in protective host immunity to tuberculosis. Deficiency of $LPLA_2$ compromises macrophages functions in vivo leading to both impaired innate and adaptive immunity to *M. tuberculosis*. These results identify an unexpected and novel functional principle of this lysosomal phospholipid-degrading enzyme.

Materials and methods

Bacterial strains and culture

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.5% glycerol and Tween 80, and OADC (Oleic acid, Albumin, Dextrose, Catalase) enrichment medium (BD). Bacterial cultures were harvested and aliquots were frozen at −80°C until later use. Viable cell counts in thawed aliquots were determined by plating serial dilutions onto Middlebrook 7H11 agar plates followed by incubation at 37°C for 4 weeks.

Mice, infection, and colony forming units

C57BL/6 and $LPLA_2$ KO mice were bred and housed under specific pathogen-free (SPF) conditions at the Biological Service Facility of the London School of Hygiene and Tropical Medicine (London, UK) or the Research Center Borstel (Germany). $LPLA_2$ KO mice were generated by targeted disruption of the *lpla2* gene in a clean C57BL/6 N background as described [6]. In any given experiment, mice were matched for age and sex and used at the age of 12 weeks and older, because at that age, the phospholipidosis phenotype of $LPLA_2$ KO mice becomes more evident [6]. For infection experiments, mice were maintained under specific barrier conditions in BSL 3 facilities. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 or German Animal Protection Law and were approved by the London School of Hygiene and Tropical Medicine Ethical Review Committee or the Ethics Committee for Animal Experiments of the Ministry for Agriculture, Environment, and Rural Areas of the State of Schleswig-Holstein, Germany (Kommission für Tierversuche/Ethik-Kommission des Landes Schleswig-Holstein) under the license 102–8/09 ("Die Bedeutung der lysosomalen Phospholipase A2 (LPLA2) für die Immunantwort gegen den Tuberkuloseerreger in der Maus"/"The role of lysosomal phospholipase A2 (LPLA2) for immune response to the causative agent of tuberculosis in the mouse model").

For infection of experimental animals, *M. tuberculosis* stocks were diluted in sterile distilled water/1% v/v Tween-80/1% w/v albumin. Infection was performed via the respiratory route by using an aerosol chamber (Glas-Col, Terre-Haute, IN, USA). Animals were exposed for 60 min to an aerosol generated by nebulising the prepared *M. tuberculosis* suspension. The inoculum size was verified by sacrificing mice 24 h after infection and determining the bacterial load in the lungs of infected mice. For each experiment, the exact day 1 CFU is indicated in the figure legend.

Bacterial load in lung, mLNs, and spleen was evaluated at different time points after aerosol infection by mechanical disruption of the organs in 0.05% v/v Tween 20 in PBS containing a proteinase inhibitor cocktail (Roche Diagnostics) prepared according to the manufacturer's instructions. Tenfold serial dilutions of organ homogenates in sterile water/1% v/v Tween 80/1% w/v albumin were plated onto Middlebrook 7H11 agar plates and incubated at 37°C. Colonies were counted after 4 weeks of culture.

Macrophage isolation and infection

To collect resident peritoneal macrophages, peritoneal cavities of naive mice were lavaged with cold PBS (calcium and magnesium free) containing 0.5 mM EDTA. Peritoneal macrophages were suspended in RPMI 1640 medium containing 10% fetal calf serum, 5% Glutamine, 1% HEPES, and 50 mM β-mercaptoethanol and plated into 96-well plates at 2×10^5 cells per well for CFU analysis. Nonadherent cells were removed by washing with PBS.

To determine the intracellular survival of *M. tuberculosis* H37Rv in WT and $LPLA_2$-deficient macrophages, resting or IFN-γ (2000 U/mL O/N) activated peritoneal macrophages were infected with *M. tuberculosis* H37Rv at a MOI of 1:1 for 2 h. Extracellular bacteria were removed by washing with PBS, and cells were further incubated or lysed with 0.5% v/v Triton X-100 in H_2O at the indicated times. Serial dilutions were plated onto Middlebrook 7H11 agar plates followed by incubation at 37°C.

In vitro activity of recombinant $LPLA_2$ on *M. tuberculosis*

M. tuberculosis was incubated in Ringer solution of pH 5.0 or 7.5, respectively, containing 1 μg/mL recombinant mouse $LPLA_2$ [30]. After 30 and 120 min, *M. tuberculosis* viability was determined by plating serial dilutions onto Middlebrook 7H11 agar plates followed by incubation at 37°C for 4 weeks.

Multiplex cytokine assays

The concentrations of CCL5, MCP-1, IFN-γ, TNF-α, and IL-10 were determined in mLNs, lung, or spleen homogenates from uninfected and infected mice by cytometric bead array (BD Biosciences) according to the manufacturer's protocol. For detection of IL-17A, the enhanced sensitivity beads (BD) were used, which can detect cytokine concentrations down to 0.274 pg/mL.

Nitric oxide assay

Nitric oxide was determined in sera from infected mice or in cell culture supernatants from infected macrophages as NO_2^- following reduction of NO_3, using the Griess-reaction described previously [31].

Small lipid mediator detection

The concentration of PGE_2, LTB_4, or LXA_4 was determined in mLNs, lung and spleen homogenates from WT and $LPLA_2$ KO mice by The Parameter PGE_2 or LTB_4 Immunoassays (R&D Systems), or the Lipoxin A_4 BioAssay ELISA Kit (United States Biological), respectively, according to the manufacturer's protocols.

Histology

Organs from *M. tuberculosis*-infected mice were fixed with 4% w/v PFA in PBS for 24 h and embedded in paraffin. Sections (3 μm) were rehydrated by running through xylenes, alcohols of decreasing concentrations, and finally water. Sections were stained with hematoxylin and eosin (H&E). Mycobacteria in lungs were visualized by acid-fast staining (TB Stain Kit K, BD Biosciences) according to manufacturer's protocol.

Cell isolation from lung, spleen, and mLNs

Lungs were removed, sectioned, and incubated in PBS containing liberase (1.5 mg/mL; Worthington Biochemical) and DNAse I (250 μg/mL; Worthington Biochemical) at 37°C for 90 min. Digested lung tissue was broken into single-cell suspension by subsequent passage through a 100 μm pore size nylon cell strainer. MLNs and spleens were also passed through a 100 μm pore size nylon cell strainer to obtain single-cell suspensions. Remaining erythrocytes were lysed and cells were resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, 1% Hepes (v/v), β-mercaptoethanol (50 μM), and 10% (v/v) heat-inactivated fetal calf serum (complete RPMI 1640 medium).

Flow cytometry

In order to analyze surface expression of CD90.2, CD4, CD8, CD11b, and Ly6G, single-cell suspensions were incubated with fluorescently labelled antibodies (BD Biosciences) for 30 min at 4°C and subsequently analyzed by flow cytometry on a FacsCantoII® flow cytometer (BD Biosciences) equipped with a 405 nm, 488 nm, and 633 nm laser and the FCSExpress software (DeNovo™ Software).

Ex vivo T-cell restimulation

Lung or mLN cells (1×10^6) was stimulated for 5 h with a purified protein derivative (PPD) of *M. tuberculosis* (5 μg/mL) or anti-CD3 and anti-CD28 (5 μg/mL, respectively) in the presence of GolgiPlug (BD Biosciences, contains brefeldin A). Subsequently, cells were stained with anti-CD90.2, anti-CD44, anti-CD4, and anti-CD8 antibodies (BioLegend) for 20 min at 4°C. After washing, cells were incubated for 20 min with Cytofix/Cytoperm (BD Biosciences). Cells were washed with Perm/Wash buffer (BD Biosciences), and stained with anti-IFN-γ (XMG1.2) and anti-TNF-α (MP6-XT22) antibodies for 45 min at 4°C. Cells were analyzed using a BD FACSCanto flow cytometer (BD Biosciences) and FCSExpress software.

Adoptive transfer of ovalbumin specific T cells

$CD4^+$ or $CD8^+$ T cells were isolated from spleens of OT2 or OT1 mice, respectively, by negative selection via magnetic activated cell sorting and labelled with CFSE (Invitrogen) at a final concentration of 5 μM. CFSE-labelled T cells (1×10^7 OT1 and 1×10^7 OT2 cells) were adoptively transferred into B6 or $LPLA_2$ mice, which had been infected with *M. tuberculosis* 20 days before. One day after adoptive transfer, 250 μg Ovalbumin in PBS or PBS alone were injected intraperitoneally. After 3 days, lungs were harvested and single-cell suspensions were stained for CD90.2, CD4, and CD8 and analyzed for proliferation of $CFSE^+$ T cells using a BD FACSCanto flow cytometer (BD Biosciences) and FCSExpress software.

Statistical analysis

The unpaired Student's *t*-test was employed to compare bacterial titres, cell numbers, organ weight, T-cell frequencies and cytokine levels, and the log-rank test to compare the survival of mice after *M. tuberculosis* infection.

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Abbreviation: **$LPLA_2$**: Lysosomal phospholipase A_2

Full correspondence: Prof. Ulrich E. Schaible, Research Center Borstel, Priority Area Infections, Parkallee 1, 23845 Borstel, Germany
Fax: +49-4537-188-2091
e-mail: uschaible@fz-borstel.de

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