Critical role of phospholipase A\textsubscript{2} group IID in age-related susceptibility to severe acute respiratory syndrome–CoV infection

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Oxidative stress and chronic low-grade inflammation in the lungs are associated with aging and may contribute to age-related immune dysfunction. To maintain lung homeostasis, chronic inflammation is countered by enhanced expression of proresolving/antiinflammatory factors. Here, we show that age-dependent increases of one such factor in the lungs, a phospholipase A\textsubscript{2} (PLA\textsubscript{2}) group IID (PLA\textsubscript{2}G2D) with antiinflammatory properties, contributed to worse outcomes in mice infected with severe acute respiratory syndrome–coronavirus (SARS-CoV). Strikingly, infection of mice lacking PLA\textsubscript{2}G2D expression (Pla2g2d\textsuperscript{-/-}) converted a uniformly lethal infection to a nonlethal one (>80% survival), subsequent to development of enhanced respiratory DC migration to the draining lymph nodes, augmented antivirus T cell responses, and diminished lung damage. We also observed similar effects in influenza A virus–infected middle-aged Pla2g2d\textsuperscript{-/-} mice. Furthermore, oxidative stress, probably via lipid peroxidation, was found to induce PLA\textsubscript{2}G2D expression in mice and in human monocyte–derived macrophages. Thus, our results suggest that directed inhibition of a single inducible phospholipase, PLA\textsubscript{2}G2D, in the lungs of older patients with severe respiratory infections is potentially an attractive therapeutic intervention to restore immune function.

Aging is associated with a decline in immune function, generally termed immunosenescence (Caruso et al., 2009; Cannizzo et al., 2011), which in turn is often linked to age-related increases in oxidative stress (De La Fuente and Miquel, 2009). Oxidative stress associated with aging has been attributed to the initiation and maintenance of chronic low-grade inflammation (“inflammaging”; Cannizzo et al., 2011). A consequence of immunosenescence is that many viral infections are more severe in aged individuals (Chen and Subbarao, 2007; Assiri et al., 2010; Frieman et al., 2012). For example, 6–10-wk-old C57BL/6 mice are highly resistant to SARS-CoV and remain asymptomatic even in the absence of interferon signaling (Frieman et al., 2010), whereas as mice age from
6 wk to 22 mo they become progressively more susceptible to the infection (Zhao et al., 2011).

Mice that succumb to SARS-CoV often develop poor T cell responses (Zhao et al., 2009; Channappanavar et al., 2014). T cell priming to SARS-CoV requires migration of respiratory DCs (rDCs) from the lungs to the mediastinal LNs (MLN). Primed T cells then migrate back to the lungs. We previously showed that rDC migration was greatly decreased in uninfected and SARS-CoV–infected aged compared with young mice. Concomitant with this decreased rDC migratory capacity was an increase in prostaglandin D2 (PGD2) in the lungs (Zhao et al., 2011). PGD2, an eicosanoid with both proinflammatory and antiinflammatory/proresolving properties is a downstream metabolite of arachidonic acid (AA; Samuelson et al., 1987; Rossi et al., 2000; Straus et al., 2000; Serhan, 2014). PGD2 binds to two different G-protein–coupled receptors: DP1 expressed on macrophages and DCs (antiinflammatory) and DP2 expressed on Th2 cells (proinflammatory). PGD2 signaling through the DP1 receptor on respiratory DCs (rDCs) and alveolar macrophages (AMs) in the naive and virus-infected lung, results in, among other effects, decreased CCR7 expression (Hammad et al., 2007; Zhao et al., 2011). This, in turn, results in decreased rDC migration from the lungs to the MLN and poor T cell responses.

To determine the factors behind age-related increases in PGD2 levels in the lungs, we initially focused on gene expression in CD11c+ cells (AM and rDC) since these cells are key modulators of the immune response in the lungs. We used middle aged (10–13 mo) mice, since these mice, like middle-aged SARS patients, are much more susceptible to the infection than young (6–8 wk) mice, but have not yet developed many of the features of immunosenescence observed in aged (>18 mo animals) and are easier to obtain. Analysis of several genes involved in AA metabolism and oxidative stress in the lungs revealed, most strikingly, that levels of a phospholipase, PLA2G2D, were significantly higher in CD11c+ cells in the lungs of middle-aged compared with young mice (confirmed in Fig. 1 A) and contributed to poor outcomes after SARS-CoV infection (Zhao et al., 2011). Therefore, we next focused on genes involved in the AA pathway, including several phospholipases, cyclooxygenases, and synthases, which could modulate PGD2 levels in the lungs. Of all the genes in the AA cascade, only levels of secreted phospholipase A2 group IIId (PLA2G2D) were significantly higher in CD11c+ cells in the lungs of middle-aged compared with young mice (using two-fold differences as a cutoff; Fig. 1, C and D). We confirmed these results by quantitative real-time PCR (qPCR) analyses of CD11c+ cell RNA by separating CD11c+ and CD11c− cells from middle-aged mice as measured by mean fluorescent intensity (MFI; Fig. 2 B). In addition, middle-aged mice harbored a higher frequency of CD11c+ cells expressing PLA2G2D compared with young mice (Fig. 2 C).

Together, these results indicate that PLA2G2D levels are highly increased in the lungs of middle-aged mice, and that CD11c+ cells are the major source for its production. However, regulatory T cells (T reg cells; CD4+FoxP3+) have been implicated as a source for PLA2G2D in previous studies (von Allmen et al., 2009) and lungs of uninfected middle-aged mice harbor higher frequency and number of T reg cells compared with young mice (Fig. 2 D). To address whether T reg cells are an additional source for PLA2G2D, we depleted CD4+ T cells, which includes T reg cells, from middle-aged mice and analyzed lungs for expression of PLA2G2D. We observed no changes in the mRNA expression of PLA2G2D after CD4 T cell depletion (Fig. 2 E), suggesting that CD4+ T cells, including T reg cells, are not a major source of PLA2G2D in the lungs.

Because CD11c+ cells comprise only a minority of the total cells in the lungs, expression of PLA2G2D by these cells could make a relatively small contribution to its total production. However, this was not the case because levels of PLA2G2D were significantly higher in unfractionated lung mRNA from middle-aged compared with young mice. Notably, PLA2G2D levels were even higher in samples obtained from aged (22 mo) mice (Fig. 3 A). Assessment of other genes in the AA cascade
generally confirmed the microarray results shown in Fig. 1 C and showed that there were no differences in the levels of expression of the cyclooxygenase enzymes COX₁ and COX₂ or other PLA₂s (PLA₂G2E, PLA₂G3, PLA₂G4, PLA₂G5, and PLA₂G10) as mice aged (Fig. 3, B and C). One exception is that there were significantly higher mRNA levels of hematopoietic PGD₂ synthase (HPGDS), which is important for PGD₂ production and could contribute to elevated PGD₂ levels in the lungs of middle-aged compared with young mice (Fig. 3 C; Murata et al., 2013).

Because robust expression of PLA₂G2D in lymphoid tissue CD11c⁺ cells was previously reported (Miki et al., 2013), we generally confirmed the microarray results shown in Fig. 1 C and showed that there were no differences in the levels of expression of the cyclooxygenase enzymes COX₁ and COX₂ or other PLA₂s (PLA₂G2E, PLA₂G3, PLA₂G4, PLA₂G5, and PLA₂G10) as mice aged (Fig. 3, B and C). One exception is that there were significantly higher mRNA levels of hematopoietic PGD₂ synthase (HPGDS), which is important for PGD₂ production and could contribute to elevated PGD₂ levels in the lungs of middle-aged compared with young mice (Fig. 3 C; Murata et al., 2013).

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determined whether its levels increased with age in the MLN. As expected, we observed higher expression of PLA2G2D in CD11c\(^+\) compared with CD11c\(^-\) cells in the MLN (Fig. 2 F). However, unlike in the lungs, there were no age-dependent differences in PLA2G2D mRNA expression in MLN CD11c\(^+\) cells between young and middle–aged naive mice. Collectively, these results indicate that PLA2G2D is nearly exclusively expressed in CD11c\(^+\) cells in both lymphoid tissue and lungs and that age-related differences in its expression are confined to the lungs.

**PLA2G2D-dependent alteration in pulmonary lipid profile**

PLA2G2D has been previously described as a proresolving phospholipase acting upstream in the AA-cascade (Miki et al., 2013).

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**Figure 2.** PLA2G2D expression in CD11c cells in the lungs and MLN of middle-aged mice. (A) PLA2G2D mRNA levels in CD11c\(^-\) and CD11c\(^+\) cells from the lungs of young and middle-aged mice. CD11c\(^+\) and CD11c\(^-\) cells were prepared from the lungs of naive young or middle-aged mice using CD11c magnetic beads and analyzed for PLA2G2D mRNA levels using qPCR. Mean ± SEM; two independent experiments, eight mice/group. ***, P < 0.001, unpaired Student’s t test. (B) Representative histograms comparing the mean fluorescent intensity of PLA2G2D expression in CD11c\(^+\) and CD11c\(^-\) cells (gated on CD45\(^+\) cells) of young and middle aged mice (left). Summary data are shown on right. Single-cell suspension from the lungs of naive young and middle-aged mice were stained for PLA2G2D and analyzed by flow cytometry as described in the Materials and methods. Mean ± SEM; three independent experiments, four mice/group. *, P < 0.05; **, P < 0.01; ***, P < 0.001, unpaired Student’s t test. (C) Frequency of CD11c\(^+\) cells (gated on CD45\(^+\)) expressing PLA2G2D in the lungs of naive young and middle-aged mice. The boxed area in the flow cytometry plots indicates cell populations staining positive for PLA2G2D. Mean ± SEM; three independent experiments, four mice/group. *, P < 0.05, unpaired Student’s t test. (D) Frequency and total number of FoxP3\(^+\) CD4\(^+\) T reg cells (gated on CD3\(^+\)CD8\(^-\)). Mean ± SEM; two independent experiments, four mice/group. **, P < 0.01, unpaired Student’s t test. (E) PLA2G2D expression in absence of CD4 T cells. (left) Extent of depletion of CD4 T cells on day 4 after GK1.5 treatment; (right) mRNA expression of PLA2G2D in the lungs of treated (GK1.5) and control groups. CD4 T cells were depleted using mAb GK1.5, delivered i.p. at 0.5 mg/mouse on day 0 and 2. 2 d later, lungs were harvested into TRIzol for quantifying PLA2G2D RNA levels. PBS was administered to control mice. Mean ± SEM; two independent experiments, four mice/group. n.s., not significant, unpaired Student’s t test. (F) CD11c\(^+\) and CD11c\(^-\) cells were harvested from the MLN of naive young and middle-aged mice. PLA2G2D mRNA levels were determined using qPCR. Mean ± SEM; two independent experiments, four mice/group. n.s., not significant; ***, P < 0.001, unpaired Student’s t test.
activity required to release AA was at least partly dependent on PLA2G2D. Levels of 6ketoPGF1α, TXB2, PGF2α, PGD2, and PGE2 were also higher in the lungs of Pla2g2d+/+ compared with Pla2g2d−/− mice (Fig. 4, I and J), suggesting that differences in AA levels resulted in differences in the levels of its downstream metabolites, some of which have antiinflammatory function (PGD2 and PGE2; Vancheri et al., 2004; Kalinski, 2012; Murata et al., 2013). The results also identify PLA2G2D-dependent pools of two other PUFA, DHA, and EPA in the lungs. DHA and EPA are further metabolized largely into proresolving mediators, such as resolvin D1, protectin D1, and resolvin E1 among others (Arita et al., 2005; Rogerio et al., 2012).

Having established that both proinflammatory and antiinflammatory/proresolving lipid mediators are up-regulated in the uninfected lungs of middle-aged mice, we next determined whether levels of these mediators were further perturbed in the context of infection. We initially analyzed mice infected with SARS-CoV because middle-aged but not young C57BL/6 mice succumb to infection with this virus, mimicking the human disease (Fig. 5 A; Frieman et al., 2012; Zhao et al., 2012). Because mortality in middle-aged mice begins at 4 d after SARS-CoV infection, we investigated whether levels of lipid mediators, including PGD2, changed at this time. Expression levels of PLA2G2D increased in both young and middle-aged mice after infection (Fig. 5 B). Further, pulmonary lipid profiling on day 4 postinfection (p.i.) revealed significant increases in lung levels of free PUFAs such as AA, EPA, and DHA in middle-aged mice but less so in young mice.
Figure 4. PLA2G2D-dependent alteration of lipid profile in the lungs. Naive young and middle-aged C57BL/6 mice (B–D) and Pla2g2d−/− and Pla2g2d+/+ mice (E–J) were analyzed for pulmonary lipids using ESI-MS as described in Materials and methods. (A) The elution of different endogenous lipids with respect to exogenous standards (SD) is indicated. Retention times and parent m/z to daughter m/z transition are also shown. Levels of (B) free PUFAs, AA, DHA and EPA. Mean ± SEM; three independent experiments, four mice/group. n.s., not significant; *, P < 0.05, unpaired Student’s t test. (C) 6-ketoPGF1α, TXB2, and PGF2α. Mean ± SEM; three independent experiments, four mice/group. n.s., not significant; *, P < 0.05, unpaired Student’s t test. (D) PGE2 and PGD2 in lungs of young and middle-aged C57BL/6 mice are shown. Mean ± SEM; three independent experiments, four mice/group, n.s., not significant; *, P < 0.05, unpaired Student’s t test. (E) Levels of free PUFAs. Mean ± SEM; two independent experiments, four mice/group, unpaired Student’s t test. (F) 6-keto-PGF1α, TXB2, and PGF2α. Mean ± SEM; two independent experiments, four mice/group, unpaired Student’s t test. (G) PGE2 and PGD2 in lungs of young Pla2g2d−/− and Pla2g2d+/+ mice. Mean ± SEM; two independent experiments, four mice/group, unpaired Student’s t test. (H) Levels of...
Levels of lipid mediators with proinflammatory activity, 6-keto-PGF1α, TXB2, and PGF2α, increased in the lungs of young but not middle-aged mice after SARS-CoV infection (Fig. 5 D). In contrast, levels of PGE2 and PGD2, both of which have antiinflammatory activity in the lungs, increased in both young and middle-aged mice after infection (Fig. 5 E).

To determine whether the inducible pool of these lipids is dependent on PLA2G2D, we determined the lung lipid profile in SARS-CoV–infected middle-aged Pla2g2d+/+ and Pla2g2d−/− mice. Whereas levels of free PUFAs, AA, DHA, and EPA increased in the lungs of both groups of mice during infection, Pla2g2d+/+ mice harbored higher levels of all three fatty acids at all times examined (Fig. 5 F), indicating that the inducible pool of each of these fatty acids was at least partly mediated by PLA2G2D. We did not observe an infection-dependent increase in the levels of 6-keto-PGF1α, TXB2, or PGF2α in either group of mice, although levels remained higher in Pla2g2d+/+ mice relative to Pla2g2d−/− mice on day 4 p.i. (Figs. 4 I and 5 G). PGE2 and PGD2 levels increased only in Pla2g2d+/+ mice after infection (Fig. 5 H). These results indicate multifactorial regulation of the inducible AA cascade with basal levels of all lipid mediators were maintained at least partly by PLA2G2D, whereas increases in PGE2 and PGD2 levels observed after SARS-CoV infection in the lungs were largely dependent on PLA2G2D. These data also agree with published studies showing that induction of PLA2G2D generated pools of proresolving but not proinflammatory mediators in the lung and skin in the context of allergic disease (Nagao et al., 2003; Kunikata et al., 2005; Liu et al., 2012; Miki et al., 2013).

PLA2G2D plays a minor role in innate immune functions in the lungs of middle-aged mice

The preferential induction of antiinflammatory lipid mediators in Pla2g2d−/− mice could contribute to less efficient activation of the innate and adaptive immune responses. To address this possibility, we initially analyzed the activation status and function of lung CD11c+ cells harvested from middle-aged and young mice at day 1 after SARS-CoV infection. Cells from middle-aged mice expressed significantly higher levels of maturation markers (CD40, CD80, and MHCIi) and expressed higher levels of TNF in the absence of ex vivo stimulation (basal levels) compared with young mice (Fig. 6, A and B [RP10]). However upon TLR stimulation with LPS or poly I:C, similar frequencies of CD11c+ cells from both young and middle-aged mice produced TNF, although the total number of TNF-producing cells was higher in middle-aged mice (Fig. 6 B, middle and bottom).

To investigate the role of PLA2G2D in this enhanced CD11c+ cell activation in middle-aged mice, we measured CD40, CD80, and MHCIi levels on lung CD11c+ cells harvested from infected middle-aged Pla2g2d+/+ and Pla2g2d−/− mice and also quantified TNF expression after TLR stimulation. PLA2G2D was required for MHCIi but not CD40 or CD80 expression on lung CD11c+ cells in middle-aged mice, and for increased frequency but not number of cells expressing TNF in the absence of any direct ex vivo stimulation with TLR ligands. CD11c+ cells from both Pla2g2d+/+ and Pla2g2d−/− mouse lungs were able to express equivalent amounts of TNF after direct ex vivo stimulation (Fig. 6, C and D). Thus, these results indicate that CD11c+ cells from infected middle-aged Pla2g2d−/− mice were more highly activated in vivo compared with young C57BL/6 mice, but that PLA2G2D has a relatively minor role in age-dependent differences in CD11c+ cell maturation and function.

The effects on TLR-mediated activation of lung CD11c+ cells by Pla2g2d−/− mice returned to normal levels 4 days after infection (Fig. 5 G). PGE2 and PGD2 levels increased only in Pla2g2d+/+ mice after infection (Fig. 5 H). These results show that the major effect of PLA2G2D is to increase levels of antiinflammatory lipid mediators, but changes in expression may also have indirect or direct effects on levels of cytokines and other inflammatory mediators. However, we observed no differences in expression of several proinflammatory molecules or IL-10, a cytokine with antiinflammatory properties, in the lungs of Pla2g2d+/+ and Pla2g2d−/− mice at days 1 or 4 p.i. (Fig. 6 E). Thus, PLA2G2D has only small effects on general innate immune function in middle-aged mice and its absence largely affects only the expression of lipid mediators.

Absence of PLA2G2D enhances rDC migration to draining lymph nodes

PGD2 inhibits the migration of airway DCs from lungs to the MLN in middle-aged naive and SARS-CoV–infected mice (Hammad et al., 2003; Zhao et al., 2011) and while levels of PGD2 decreased in Pla2g2d−/− mice, it was not known a priori whether this decrease was sufficient to correct the defect in rDC migration. To determine whether this was the case, we first instilled OVA-FITC intranasally (i.n.) into uninfected middle-aged Pla2g2d+/+ and Pla2g2d−/− mice and analyzing MLN 18 h later for OVA-FITC+ rDCs, indicative of migration from the lungs. We observed that a higher frequency and number of rDCs migrated to the MLN in the absence of PLA2G2D (Fig. 6 F). We next investigated whether rDC migration was augmented in mice infected with SARS-CoV by instilling CFSE i.n. into middle-aged Pla2g2d+/+ and Pla2g2d−/− mice 6 h before infection. 18 h p.i. MLN were harvested and analyzed for the frequency of rDCs that migrated from lungs. SARS-CoV infection of lung CD11c+ cells promotes enhanced migration of rDCs to the MLN, as well as increased accumulation in the draining cervical lymph node (Hammad et al., 2003; Zhao et al., 2011). We observed a higher number of rDCs in the MLN of Pla2g2d−/− infected mice (Fig. 6 F). Migration of DCs from the site of infection to the MLN is mediated by chemokine receptor CCR7 binding to CCL19/21 (Hamilton-Easton and Eichelberger, 1995; Fainaru et al., 2005) and DCs...
Figure 5. PLA₂G₂D-dependent up-regulation of PGD₂ and other lipid mediators after SARS-CoV infection. (A) Survival of young and middle-aged C57BL/6 mice after i.n. challenge with 10⁴ PFU of SARS-CoV (two independent experiments, five mice/group). Naive or infected young and middle-aged C57BL/6 mice (B–E) or middle-aged Pla2g2d⁻/⁻ and Pla2g2d⁺/⁺ mice (F–H) were analyzed for mRNA expression levels of PLA₂G₂D in and lipid expression in the lungs using qPCR and ESI-MS, respectively, as described in Materials and methods. (B) mRNA levels of PLA₂G₂D in the lungs of young and middle-aged mice before (light green) and 4 d p.i. (dark green), expressed relative to Hprt. Mean ± SEM; two independent experiments, four mice/group. *, P < 0.05; ***, P < 0.001, unpaired Student’s t test. Comparison of levels in the lungs of young and middle-aged mice of (C) free PUFAs, AA, DHA, and EPA. Mean ± SEM; two independent experiments, four mice/group. *, P < 0.05; ***, P < 0.001, unpaired Student’s t test. (D) 6ketoPGF₁₀, TXB₂, and PGF₂₀. Mean ± SEM; three independent experiments, four mice/group. *, P < 0.05; **, P < 0.01; ***, P < 0.001, unpaired Student’s t test. (E) PGE₂ and PGD₂ before and 4 d p.i. Mean ± SEM; three independent experiments, four mice/group. *, P < 0.05; **, P < 0.01; ***, P < 0.001, unpaired Student’s t test. (F) Free PUFAs. Mean ± SEM; two independent experiments, five mice/group. n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (unpaired Student’s t test). (G) 6ketoPGF₁₀, TXB₂, and PGF₂₀. Mean ± SEM; two independent experiments, five mice/group. n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (unpaired Student’s t test). (H) PGE₂ and PGD₂ before and 4 d p.i. Mean ± SEM; two independent experiments, five mice/group. n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (unpaired Student’s t test). All lipid concentrations are expressed as nanogram/milligram lung tissue.
Having demonstrated superior migration of rDCs to the MLN in Pla2g2d−/− mice, we next assessed whether this translated into more robust virus-specific CD4 and CD8 T cell responses in infected lungs (Fig. 7). We addressed this possibility by examining the CD4 and CD8 T cell response in SARS-CoV–infected differentiated in the presence of PGD₂ express lower levels of CCR7 (Gosset et al., 2005; Zhao et al., 2011). Consistent with higher expression of PLA₂G₂D and PGD₂ in the lungs, Pla2g2d−/− rDCs expressed higher CCR7 surface levels than Pla2g2d+/+ rDCs, contributing to enhanced rDC migration to the draining lymph nodes in uninfected and SARS-CoV–infected mice (Fig. 6 H).
A higher frequency and number of virus-specific (epitope S525-specific) CD8 T cell and (epitope N110-specific) CD4 T cells (Fig. 7 A) were present in the lungs of Pla2g2d−/− mice compared with Pla2g2d+/+ mice. In addition, we also observed that CD8 T cells from Pla2g2d−/− compared with Pla2g2d+/+ mice produced more IFN-γ on a per cell basis (Fig. 7 B), although there were no differences in the fraction of CD8 T cells that produced both IFN-γ and TNF (Fig. 7 C).

Further, the absence of PLA2G2D augmented CD8 T cell cytolytic activity, measured in an in vivo killing assay (Fig. 7 D). To investigate if there was also a T cell–intrinsic defect in Pla2g2d−/− mice, we performed a set of adoptive transfer experiments, transferring splenocytes from uninfected Pla2g2d+/+ or Pla2g2d−/− mice to CD45 disparate Pla2g2d−/− or Pla2g2d+/+ mice, respectively, before SARS-CoV infection. In each case, the frequency of transferred CD8 T cells that expressed IFN-γ after peptide stimulation was similar to that of endogenous cells, indicating that the defects were T cell extrinsic (Fig. 7 E).

We next assessed whether these enhanced SARS-CoV–specific T cell responses decreased mortality after SARS-CoV infection. To this end, we infected middle-aged Pla2g2d−/− and Pla2g2d+/+ mice with SARS-CoV and monitored survival (Fig. 8 A). More than 80% of Pla2g2d−/− mice survived the infection, whereas all Pla2g2d+/+ mice died. Increased survival reflected enhanced kinetics of viral clearance in Pla2g2d−/− mice (Fig. 8 B), mediated by the more robust T cell response observed in these mice. In addition, decreased pathological changes were observed in the lungs of Pla2g2d−/− compared with Pla2g2d+/+ mice. We detected no differences in the lungs of uninfected mice (Fig. 8 C, naive). Pla2g2d+/+ mice showed much greater perivascular cuffing and cellular infiltration compared with Pla2g2d−/− mice by day 4 p.i. By day 6 p.i., multifocal inflammatory lesions around blood vessels and alveolar edema were significantly greater in Pla2g2d+/+ mice, with few changes observed in Pla2g2d−/− mice.

Enhanced rDC migration to MLN and T cell responses in influenza A virus (IAV)-infected Pla2g2d−/− mice

To determine whether elevated PLA2G2D levels in middle-aged mice affected the host immune response to other respiratory pathogens, we infected Pla2g2d−/− and Pla2g2d+/+ mice with IAV (PR8 strain) and monitored rDC migration, T cell responses and survival. Both the frequency and number of virus-specific T cells in the lungs expressing IFN-γ alone or IFN-γ and TNF. Mean ± SEM; of two independent experiments, five mice/group, unpaired Student’s t test. (D) In vivo cytotoxicity was assessed on day 6 p.i. and the percentage killing was determined as described in Materials and methods. Data show killing of target cells in the lungs of Pla2g2d−/− or Pla2g2d+/+ mice. Mean ± SEM; three independent experiments, five mice/group. *** P < 0.001 (unpaired Student’s t test). (E) Representative flow plots of virus-specific T cell responses in recipient Pla2g2d−/− or Pla2g2d+/+ mice and summary data are shown. Mean ± SEM; two independent experiments with six mice/group. *** P < 0.001 (unpaired Student’s t test).
peroxidation is increased expression of various PLA2s (Brown et al., 2003), raising the possibility that increased oxidative stress in the middle-aged lung is a key factor in PLA2G2D up-regulation. To evaluate whether oxidative stress is increased in the lungs of middle-aged mice, we first measured the levels of malondialdehyde (MDA), a product of lipid peroxidation, and an indicator of oxidative stress (Gutteridge and Halliwell, 1990). Analyses of middle-aged compared with young mice revealed higher lung concentrations of MDA (Imai et al., 2008), indicative of greater pulmonary oxidative stress (Fig. 10 A). Oxidative stress can be countered by treatment with N-acetyl cysteine (NAC) and, as shown in Fig. 10 B, treatment of middle-aged mice with NAC for 10 d reduced MDA levels. Notably, NAC treatment also diminished levels of PLA2G2D mRNA in lung CD11c+ cells, suggesting that augmented chronic oxidative stress mediates elevated PLA2G2D expression

Aging is accompanied by oxidative stress, which triggers lipid peroxidation (Cannizzo et al., 2012). A consequence of lipid peroxidation is increased expression of various PLA2s (Brown et al., 2003), raising the possibility that increased oxidative stress in the middle-aged lung is a key factor in PLA2G2D up-regulation. To evaluate whether oxidative stress is increased in the lungs of middle-aged mice, we first measured the levels of malondialdehyde (MDA), a product of lipid peroxidation, and an indicator of oxidative stress (Gutteridge and Halliwell, 1990). Analyses of middle-aged compared with young mice revealed higher lung concentrations of MDA (Imai et al., 2008), indicative of greater pulmonary oxidative stress (Fig. 10 A).

Oxidative stress can be countered by treatment with N-acetyl cysteine (NAC) and, as shown in Fig. 10 B, treatment of middle-aged mice with NAC for 10 d reduced MDA levels. Notably, NAC treatment also diminished levels of PLA2G2D mRNA in lung CD11c+ cells, suggesting that augmented

Figure 8. Pla2g2d−/− mice are protected from SARS-CoV infection. Middle-aged Pla2g2d−/− or Pla2g2d+/+ mice were infected with 10⁴ PFU of SARS-CoV i.n. (A) Mice were monitored daily for survival. Data are representative of two independent experiments, seven mice/group. P < 0.001 (Kaplan-Meier log-rank survival tests). (B) Viral titers in the lungs of Pla2g2d−/− and Pla2g2d+/+ mice at days 1, 4, and 6 p.i. are shown. Mean ± SEM; two independent experiments, seven mice/group. **, P < 0.01 (unpaired Student’s t test). (C) Lung sections from Pla2g2d−/− and Pla2g2d+/+ mice on day 6 p.i. were analyzed for pathological changes. Infiltrating cells (black arrows) and edema (asterisks) are shown. Histological changes were scored for inflammation and edema as described in Materials and methods. Mean ± SEM; two independent experiments, with three to five mice per group. *, P < 0.05; **, P < 0.01. Bars: (10×) 300 µm; (40×) 70 µm.
DISCUSSION

Although many infections are more severe in aged populations, SARS, and possibly MERS show remarkable age-dependent differences in outcomes such that young humans (less than 24 yr) all survived the infection, whereas 15% and 52% of middle-aged and aged individuals, respectively, succumbed (Peiris et al., 2004; Assiri et al., 2013; Saad et al., 2014; Alsolamy, 2015). SARS-CoV–infected mice also show similar steep age-dependent changes in mortality, which we show is due, in large part, to increases in levels of a single secretory PLA2, PLA2G2D, as mice age. A hallmark of aging is lipid peroxidation and subsequent up-regulation of PLA2s (Brown et al., 2003; Sun et al., 2004; Hermann et al., 2014) and our results suggest that, in the lungs, levels of PLA2G2D singularly increase as mice age. PLA2G2D is considered an antiinflammatory/proresolving enzyme, suggesting its up-regulation is actually a measure used in the lung to counter the low-grade chronic inflammation that occurs with aging (Franceschi et al., 2000; Miki et al., 2013). In support of this notion, treatment with low doses of LPS, which induces oxidative stress and low-grade inflammation, resulted in PLA2G2D up-regulation both in CD11c+ cells in the mouse lung (Fig. 10 C) and in human monocyte-derived macrophages (Fig. 10 E). Further, interventions that diminish reactive oxygen species decreased PLA2G2D expression (Fig. 10 B). A recent study demonstrated the proresolving role of PLA2G2D in the setting of contact dermatitis in mice; in its absence, resolution was prolonged (Miki et al., 2013). PLA2G2D would be expected to have similar effects in ameliorating disease in the context of autoimmune disease.

Figure 9. PLA2G2D dampening of the anti-IAV T cell response. Middle-aged Pla2g2d−/− or Pla2g2d+/+ mice were infected with 1020 tissue culture infectious units of IAV (PR8 strain) and analyzed for differences in rDC migration, CD8 T cell response in lungs, and survival. (A) Flow plots and frequency (left bar graph) and number (right bar graph) of airway DCs (CD11c+MHCII+CFSE−) that migrated from the lung to MLN, 18 h after IAV infection. Mean ± SEM; two independent experiments, four mice/group. **, P < 0.01, unpaired Student’s t test. (B) Flow plots and summary data indicate the frequency and numbers of D/P224 tetramer+ CD8 T cells at 10 d.p.i. Mean ± SEM; two independent experiments, four mice/group. *, P < 0.05; **, P < 0.01, unpaired Student’s t test. (C) Percentage survival. Data are representative of two independent experiments, six mice/group. n.s., not significant (Kaplan-Meier log-rank survival tests).
Increased PLA₂G2D expression by lung CD11c⁺ cells results in up-regulation of several eicosanoids, with especially prominent effects on PGD₂ production (Figs. 4 D and 5, E and H). Activation of the PGD₂/DP1 signaling axis on rDCs results in decreased CCR7 expression so that DP1 antagonism results in augmented rDC migration to the draining LN in uninfected and SARS-CoV-infected mice (Hammad et al., 2003; Zhao et al., 2011). Of note, inhibition of DC migration by PGD₂/DP1 signaling is not confined to the lung, because, after skin invasion, Schistoma mansoni releases PGD₂ to inhibit Langerhans cell migration to the draining LN, delaying the initial immune response (Angeli et al., 2001). Similar effects are observed in Pla2g2d⁻/⁻ mice infected with either SARS-CoV or IAV, resulting in enhanced T cell responses (Fig. 7, A, B, and D, and 9 B) and, in the case of SARS-CoV, improved survival (Fig. 8 A). In addition, PGD₂ can also mediate its antiinflammatory effects through binding of 15d-PGJ₂, a dehydration end product of PGD₂, to a nuclear receptor PPARγ (Hilliard et al., 2010).

Although we primarily focused on the role of PLA₂G2D in increased PGD₂ production, PLA₂G2D is critical for the synthesis of additional antiinflammatory/proresolving molecules. PUFA-oxygenated metabolites with dedicated proresolving activities, including resolvins, protectins, and maresins (also called specialized proresolving mediators [SPMs]), are derived from ω3 fatty acids such as EPA and DHA (Serhan et al., 2002; Buckley et al., 2014; Serhan, 2014). PLA₂G2D is known to release free PUFAs into a substrate pool that can be further mobilized to produce proresolving lipid mediators such as DHA-derived resolvin D1 and EPA-derived resolvin E1 among many others (Serhan et al., 2002; Miki et al., 2013). Notably, EPA levels were higher in the lungs of uninfected middle-aged compared with young mice (Fig. 4 B), and both EPA and DHA production were higher in uninfected and infected Pla2g2d ⁺/⁺ mice compared with their Pla2g2d⁻/⁻ counterparts (Figs. 4 H and 5 F). However we failed to detect any resolvins or protectins, the downstream metabolites of EPA or DHA in the lungs of mice, possibly reflecting low levels of these molecules in lung tissue. Notably, although EPA is higher in middle-aged compared with young lungs (DHA is nearing to significance; P = 0.12; Fig. 4 B), SPMs are lower in the uninfamed middle-aged mice that were treated with NAC or PBS (control) i.n. for 7 d were separated using magnetic beads and expression of PLA₂G2D was determined by qPCR and lung MDA concentrations were measured by TBARS assay. Mean ± SEM; two independent experiments, four mice/group.

Figure 10. Oxidative stress induces expression of PLA₂G2D. A TBARS assay was used to measure lung MDA concentrations as a measure of oxidative stress in mouse lungs and human MDMs. (A) MDA concentration in the lungs of naive young and middle-aged mice. Mean ± SEM; two independent experiments, four mice/group. **, P < 0.01, unpaired Student’s t test. (B) CD11c⁺ cells from the lungs of naive middle-aged mice that were treated with NAC or PBS (control) i.n. for 10 d, were separated using magnetic beads and expression of PLA₂G2D was determined by qPCR and lung MDA concentrations were measured by TBARS assay. Mean ± SEM; two independent experiments, four mice/group.

![Graphs showing MDA concentrations and PLA₂G2D expression](image-url)
effect of aging on pulmonary CD11c+ cells may reflect constant exposure to airborne antigens, most of which are innocuous. An immune response to these antigens would be undesirable and would contribute to allergic reactions and asthma. In contrast, PLA2G2D in lymph nodes might be expected to have a different role, namely to down-regulate pro-inflammatory responses, and thereby contribute to resolution of the immune response after pathogen control or allergic or autoimmune reactions.

A key question is why SARS-CoV–infected mice are especially affected by age-dependent changes in PLA2G2D expression. One unique feature of SARS is that maximal virus titers in mouse lungs are detected within 24 h after infection (Fett et al., 2013), nearly coincident with the time of maximal migration of rDCs from the lungs to the DLNs. Furthermore, coronavirus replication results in extensive membrane remodeling with the formation of double membrane vesicles and other membranous structures, the sites of viral RNA synthesis and assembly (Novoa et al., 2005; Knoop et al., 2008; Miller and Krijnse-Locker, 2008). This virus–induced reorganization of the host cell membrane causes the accumulation of cone-shaped lipids, specifically lyso-phosphatidylcholine (LPC) or phosphatidylethanolamine (PE; Escribá et al., 1997; Lee and Ahlquist, 2003; Diaz et al., 2010; Lorizate and Kräusslich, 2011; Xu and Nagy, 2015). Because PLA2G2D specifically mobilizes PE to release AA and DHA (Miki et al., 2013), robust replication of SARS-CoV during the first 24 h of infection likely increases the amount of PLA2G2D substrate. Additionally, LPC and PE may form large lipid aggregates; most secreted phospholipases, including PLA2G2D, exhibit a tendency to show increased activity when the substrate is presented in this form (Carman et al., 1995; Gelb et al., 1995). Thus, SARS-CoV infection results in enhanced release of AA into the eicosanoid pathway, which increases the concentration of PGD2 and downstream products of EPA and DHA, further dampening the immune response. Notably, similar processes may occur in patients with MERS-CoV infections, who also exhibit age-dependent increases in susceptibility (Assiri et al., 2013; Saad et al., 2014; Alsalamy, 2015).

In conclusion, it is likely that in the absence of challenge with a pathogen such as SARS-CoV, elevated PLA2G2D expression benefits the host by increasing the level of various immunosuppressive lipid mediators such as PGD2, thereby dampening the response to environmental antigens. However, in the context of an infection, the higher basal levels of PGD2 and other proresolving lipid mediators adversely affect the initiation of a protective innate immune response and ultimately adaptive immune response (Fig. 10 E). Thus, therapeutic interventions that increase the levels of proresolving lipid mediators might have undesirable effects in the context of infection. However, these results also suggest that PLA2G2D would serve as a useful therapeutic target in the lungs of patients after infection with SARS-CoV and perhaps other respiratory viral pathogens, including MERS-CoV. Short-term targeting of a single inducible PLA2 would avoid undesirable effects that might be observed by generalized inhibition of PLA2 function. Such drugs have been developed for some PLA2s (Oslund et al., 2008), although not yet for PLA2G2D, demonstrating the feasibility of this approach.

**MATERIALS AND METHODS**

**Mice, virus, and infection.** 6 wk to 22 mo specific pathogen–free C57BL/6 mice were purchased from Charles River Laboratories or the National Cancer Institute (National Institutes of Health). Pla2g2d−/− and Pla2g2d+/+ mice (H-2b) were generated as described (Miki et al., 2013). After initial development, mice were backcrossed greater than 12 times to C57BL/6NCrSLc mice. Pla2g2d−/− C57BL/6NCrSLc mice were used as controls for all experiments. Mouse-adapted SARS-CoV (MA15) and IAV A/PR8/34 were obtained from Kanta Subbarao (National Institutes of Health) and Kevin Legge (University of Iowa, Iowa City, IA), respectively. For infections, mice were lightly anesthetized using isoflurane and infected i.n. with 1 × 106 PFU SARS-CoV or 108 TCID50 IAV in a 50 µl volume. All animal studies were approved by the University of Iowa Animal Care and Use Committee and meet stipulations of the Guide for the Care and Use of Laboratory Animals.

**Virus titration.** Lungs were harvested on the indicated days p.i. Tissue was homogenized in PBS using a manual homogenizer and titered on Vero E6 cells. For plaque assays, cells were fixed with 10% formaldehyde and stained with crystal violet 3 d p.i. Viral titers are expressed as PFU/gram.

**CD11c+ cell magnetic bead separation.** Lung single-cell suspensions were prepared as described. CD11c+ cells were separated using anti-mouse CD11c MicroBeads (Miltenyi Biotech) following the manufacturer’s protocol.

**Microarray analysis.** CD11c+ cells from the lungs of young and middle-aged mice were separated as described above. RNA was purified using a mirVana kit (Life Technologies) according to the manufacturer’s instructions. RNA samples were assessed for purity and quality using an Agilent 2100 Bioanalyzer. RNA for the microarray was processed using a NuGEN WT-Ovation Pico RNA Amplification System together with a NuGEN WT-Ovation Exon Module. Samples were hybridized and loaded onto MouseRef-8 v2.0 Expression BeadChip (Illumina). Arrays were scanned with a HiScan bead array system, and data were collected using GeneChip Operating Software.

**Data from Illumina MouseRef-8 v2.0 Expression BeadChip were normalized and median polished using Robust Multichip Average background correction with log2-adjusted values. After obtaining the log2 expression values for genes, significance testing was performed comparing the two groups (CD11c+ cells from lungs of young and middle-aged mice). False discovery rate was applied to all p-values to correct for multiple testing. Significance of expression differences was assessed using an false discovery rate (FDR) cutoff of 0.05 and a twofold change. Analysis and visualization of data and pathway analysis were performed using PartekGS software and Ingenuity Pathway Analysis software. Complete microarray data have been deposited at the Gene Expression Omnibus under accession no. GSE71868.

**In situ CFSE and OVA–FITC staining.** CFSE and OVA–FITC were purchased from Molecular Probes. CFSE was diluted to a concentration of 8 mM in DMEM, and then administered i.n. (50 µl) 6 h before infection (Legge and Braciale, 2005; Zhao et al., 2011). OVA–FITC was dissolved in 25 mM HEPES, 1 mg/ml Collagenase D (Roche) and 0.1 mg/ml DNase (Roche) for 30 min at 37°C. After pressing through 70 µm nylon filters, lungs homogenates were spun down and treated with red blood cell lysis buffer. Cells were counted using a hemocytometer after dilution in 0.2% Trypan blue. Lymph nodes were dispersed and single-cell suspensions prepared using 70 µm nylon filters and counted.
**Lung histology.** Animals were anesthetized and transcardially perfused with PBS followed by zinc formalin. Lungs were removed, fixed in zinc formalin, and paraffin embedded. Sections were stained with hematoxylin and eosin and examined by light microscopy. Images were acquired using a BX53 microscope (Olympus), DP73 camera (Olympus), and CellSens software (Olympus). All sections were examined at room temperature using 10x or 40x objective lens. Sections were evaluated by a veterinary lung pathologist using postexamination masking techniques (Gibson-Corley et al., 2013). Sections were scored as follows: Inflammation, 1, normal range; 2, focal small patches of perivascular leukocyte aggregates (0–33% of lung); 3, multifocal patches of perivascular leukocyte aggregates (33–66% of lung); 4, widespread patches of perivascular leukocyte aggregates (>67% of lung). Edema: 1, none; 2, minor (0–33% lung fields); 3, (33–66% lung fields); 4, (>67% lung fields).

**Antibodies and flow cytometry.** The following monoclonal antibodies were used: PE or PerCP Cy5.5-conjugated rat anti-mouse CD4 (RM4-5), FITC-conjugated rat anti-mouse CD8 (S3–6–7), FITC or e450-conjugated hamster anti-mouse CD11c (HL3), APC or e480-conjugated rat anti-mouse CD11b, and rat anti-mouse CD16/16/32 (2.4G2) (BioScience); PerCP Cy5.5-conjugated hamster anti-mouse CD3 (145-2C11), PerCP Cy5.5-conjugated rat anti-mouse I-A/I-E (M5/114.15.2), PerCP Cy5.5 or PE-conjugated rat anti-mouse CD8 (30-F11), Alexa Fluor 647 or APC-conjugated rat anti-mouse IFN-γ (XM16.2), and APC-conjugated rat anti-mouse TNF (MP6-XT22; BioLegend); PE or FITC-conjugated hamster anti-mouse CD80 (16–10A1), and FITC-conjugated rat anti-mouse CD40 (1C 10; BD). For PLA2G2D staining, rabbit anti–mouse PLA2G2D antibody (Miki et al., 2013) was used as primary antibody, followed by staining with Alexa Fluor 647-conjugated goat anti-rabbit (Life Technologies).

For surface staining, 1 × 10^6 cells were blocked with 1 µg of anti-CD16/32 antibody and stained with the indicated antibodies at 4°C, except for anti-CCR7 antibody, which was used at 37°C. After washing, cells were fixed using Cytofix Solution (BD). To detect antigen-specific T cells after SARS-CoV infection, 1 × 10^6 cells were cultured in a 96-well round bottom plate in the presence of Brefeldin A and peptides corresponding to a CD8 T cell epitope (S255) or a CD4 T cell epitope (N110; BioSynthesis). To assess the functionality of CD11c+ cells, lung cells were prepared as described above and stimulated with LPS (1 µg/mL), poly I:C (10 µg/ml), or vehicle (RP-10) in the presence of Brefeldin A for 6 h at 37°C. To determine the frequency of CD11c+ cells expressing PLA2G2D, lung cells were incubated for 6 h at 37°C in the presence of Brefeldin A. Cells were then fixed and permeabilized using Cytofix/Cytoperm (BD) and labeled with anti–IFN-γ (T cells), anti-TNF (CD11c+ cells) or anti-PLA2G2D antibody. To detect antigen-specific T cells after IAV infection, lung cells were prepared as described above and PA224-specific CD8 T cells were identified by staining with H2D-P/PA224 tetramer (National Institute of Allergy and Infectious Diseases Tetramer Facility, Atlanta, GA). Flow cytometric data were acquired using a FACSVerse (BD) and were analyzed using FlowJo software (Tree Star).

**PCR and primers.** Total RNA was extracted from CD11c+ and CD11c– lung cells or whole lungs of naive or SARS-CoV-infected mice at the specified time p.i. using TRIzol reagent (Invitrogen). 2 µg of RNA were used for cDNA synthesis. 2 µl of cDNA were added to 23 µl of PCR cocktail containing 2XSYBR Green Master Mix (ABI) and 0.2 µM of forward and reverse primer. Amplification was performed in an ABI Prism 7700 thermocycler. Cycle threshold (Franceschi et al., 2000) values were normalized to those of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). Cycle threshold (Ct) values were normalized to those of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) by the following equation: ΔCt = Ct(gene of interest) − Ct(HPRT). All results are shown as a ratio to HPRT calculated as 2^(-ΔΔCt).

**LC/MS.** Lungs were harvested into 2 ml methanol and homogenized using a tissue grinder. 200 µl were transferred to a second tube and diluted to a final volume of 3 ml of 60% methanol (vol/vol). After incubation for 30 min at 37°C, 100 µl (0.1 mg/µl) of internal standard cocktail was added. Samples were diluted to a final concentration of 10% methanol (vol/vol) with water. For AA, PGD2, PGE2a, PGF2a, TXB2, and 6-keto-PGF1α, internal standards were AA-d8, PGE2-d4, PGE2e-d4, PGF2ox-d4, TXB2-d4, and 6-keto-PGF1α-d4, respectively, all purchased from Cayman Chemical and added at the aforementioned concentration. For DHA and EPA, LTβR-d4 was used as the internal standard. Samples were centrifuged at 3,000 rpm for 10 min and the supernatant was subjected to solid phase extraction using a Strata-X 33 mm Polymeric Reversed Phase columns (60 mg/3 ml). Columns were purchased from Phenomenex, activated with 3 ml methanol, and washed with 3 ml water. Samples were eluted with 1 ml methanol, vacuum dried, and resuspended in 100 µl of mobile phase A. The analyses of PUFAs and their metabolites were performed using a Waters Acuity triple quadrupole mass spectrometer coupled with liquid chromatography (Acquity H class UPLC). Samples were then applied to an Acquity UPLC BEH C18 column (130 Å, 1.7 µm, 2.1 mm × 100 mm) and fractionated using a step gradient with mobile phase A (63:37:0.02% water/acetonitrile/formic acid) and mobile phase B (1.1 acetonitrile/isopropanol) at a flow rate of 300 µl/min for most analytes. For DHA and EPA, the mobile phase A was 65:35:0.01% methanol/water/acetic acid and mobile phase B was 100% methanol with 0.1% acetic acid. Columns were run at a flow rate of 250 µl/min. Primary standards were analyzed in parallel with the samples to enable quantification of the endogenous lipids. Detection of each lipid was performed by multiple reaction monitoring.

**Adaptive transfer of bulk splenocytes.** Naive CD45.1 (Pla2g2d−/−) or CD45.2 (Pla2g2d−/+) splenocytes were adaptively transferred by intravenous inoculation into CD45.2 (Pla2g2d−/−) or CD45.1 (Pla2g2d−/+) mice, respectively, at 50 × 10^6 cells/mouse. 1 d after transfer, mice were infected with SARS-CoV i.n. Lungs were harvested 6 dpi and analyzed for virus-specific T cells as described in the preceding sections.

**In vivo cytotoxicity assay.** This assay was performed as described previously (Barber et al., 2003). In brief, splenocytes from CD45.1 mice (Pla2g2d+/−, WT) were stained with high (1 µM, CFSEhi) or low (100 nM, CFSElo) concentrations of CFSE. CFSEhi cells were then pulsed with S255 (10 µM) at 37°C for 1 h. 5 × 10^5 cells from each group (CFSEhi and CFSElo) were mixed together (10^6 cells total) and transferred i.n. into mice CD45.2 Pla2g2d−/− and Pla2g2d−/− mice at 6 d p.i. Lungs were harvested 12 h later and analyzed for numbers of CFSEhi and CFSElo CD45.1 cells. Percentage killing was calculated using the formula: 100 − [(% peptide pulsed in infected/% peptide pulsed in uninfected) × 100] (Barber et al., 2003).

**LPS treatment.** Young mice (6–8 wk) were treated i.n. with LPS (E. coli O111:B4) at 0.1 µg/mouse in 50 µl for 7 d.

**Antioxidant treatment.** Middle-aged mice (10–13 mo) were treated with N-Acetyl-L-Cysteine (Sigma-Aldrich; 100 mg/kg, i.p.) for 7 d.

**TBARS assay.** Pulmonary oxidative stress was measured using a TBARS assay kit according to the manufacturer’s protocol (Cayman Chemicals). Data are expressed as the concentration of MDA (Imai et al., 2008) in infected lungs.

**Human monocyte-derived macrophages.** Human peripheral blood mononuclear cells were purchased from leukocyte reduction columns were obtained from anonymous volunteers that had consented to blood donation at the DeGowin Blood Center at the University of Iowa. Consent forms were approved by the University of Iowa’s Institutional Review Board. To obtain monocytes, PBMCs were cultured in tissue culture plates at a seeding density of 1 × 10^6 cells/ml in RP-10 media (RPMI-1640 medium [Invitrogen] with 10% FBS [Atlanta Biologicals] and 2 mM l-glutamine supplemented with 5 mg/ml M-CSF [eBioscience] at 37°C with 5% CO2. After 4 d, the plates were washed with Hank’s balanced salt solution devoid of divalent cations (Invitrogen) to remove nonadherent cells. Adherent cells (predominantly MDMs)
were then trypsinized and pelleted. Pelleted MDMs were resupended at $1 \times 10^6$ cells/ml in RPMI supplemented with M-CSF (5 ng/ml) and incubated at 37°C overnight. MDMs were subsequently seeded at a density of 5 x $10^3$/ml and incubated for 10 h at 37°C and 5% CO2. MDMs were then treated with 0.1 nM LPS (E. coli O111:B4; Sigma-Aldrich) and M-CSF (1 ng/ml) daily for 10 d.

**Statistics.** A Student’s t test was used to analyze differences in mean values between groups except in the case of the microarray data. All results are expressed as mean ± SEM. P-values of <0.05 were considered statistically significant.

**Online supplemental material.** Fig. S1 is a schematic representation of the effects of PLA2G2D in lungs of mice during SARS-CoV infection. Table S1 shows a list of genes in pulmonary CD11c+ cells expressed differentially in young and middle-aged mice. Table S2 shows qPCR primer sequences used in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20150632/DC1.

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