PLD3 and PLD4 are single-stranded acid exonucleases that regulate endosomal nucleic-acid sensing

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The sensing of microbial genetic material by leukocytes often elicits beneficial pro-inflammatory cytokines, but dysregulated responses can cause severe pathogenesis. Genome-wide association studies have linked the gene encoding phospholipase D3 (PLD3) to Alzheimer’s disease and have linked PLD4 to rheumatoid arthritis and systemic sclerosis. PLD3 and PLD4 are endolysosomal proteins whose functions are obscure. Here, PLD4-deficient mice were found to have an inflammatory disease, marked by elevated levels of interferon-γ (IFN-γ) and splenomegaly. These phenotypes were traced to altered responsiveness of PLD4-deficient dendritic cells to ligands of the single-stranded DNA sensor TLR9. Macrophages from PLD3-deficient mice also had exaggerated TLR9 responses. Although PLD4 and PLD3 were presumed to be phospholipases, we found that they are 5’ exonucleases, probably identical to spleen phosphodiesterase, that break down TLR9 ligands. Mice deficient in both PLD3 and PLD4 developed lethal liver inflammation in early life, which indicates that both enzymes are needed to regulate inflammatory cytokine responses via the degradation of nucleic acids.

Evidence is mounting that regulation of the abundance of nucleic acids by nucleases prevents autoimmune and autoinflammatory diseases driven by sensors of nucleic acids. Deficiency in Dnase1 or Dnase1L3 (which encode DNAses) leads to systemic lupus erythematosus in both humans and mice. In mice, Dnase2a deficiency leads to a lethal interferon-mediated disease, while patients deficient in DNASE2a exhibit autoinflammatory anemia, kidney and joint disease. Patients with deficiency in TREX1 develop Aicardi-Goutières syndrome, while Trexl-deficient mice develop a lethal cardiomyopathy inflammatory disease. Prevention of the abnormal accumulation of host nucleic acid in different tissues and cellular compartments is thus performed by an array of enzymes with non-redundant functions.

Proteins of the phospholipase D (PLD) family share a catalytic-site sequence signature (HxxKxxxD, where ‘x’ indicates any amino acid; the ‘HKD motif’) that is present in the cytosolic phospholipases PLD1 and PLD2, but most family members have distinct functions and intracellular localization and reside in subcellular compartments distinct from those in which most other members of the PLD family reside. Studies have linked PLD4 polymorphisms to systemic sclerosis and rheumatoid...
arthritis. Several works have linked PLD3 to Alzheimer’s disease, with some controversy. The functions of PLD3 and PLD4 are unknown. Despite their names, PLD3 and PLD4 have not been demonstrated to have phospholipase activity. Dendritic cells (DCs) and other myeloid cells have high expression of PLD4, and B cells have lower expression of PLD4. PLD3 is structurally similar to PLD4 but has broader expression. We found that PLD3 and PLD4 are 5’ exonucleases with properties strikingly similar to the enzymatic activity described many years ago as ‘spleen acid exonuclease’ or ‘spleen phosphodiesterase’. Mice with mutation of Pld3 and Pld4 had heightened sensitivity to, and diminished turnover of, ligands of TLR9.

Results

PLD4-deficient mice display a TLR9-driven inflammatory syndrome. To assess the effects of PLD4 deficiency, we engineered an allele for the conditional knockout of Pld4 (Pld4fl/fl), from which we generated a homozygous knockout mouse strain (Pld4–/–) and a mouse strain for the conditional knockout of Pld4 (Pld4fl/flCre) (Supplementary Fig. 1a–c). Pld4–/– mice lacked detectable PLD4 protein (Supplementary Fig. 1d) and exhibited a phenotype of chronic activation of the immune system, with splenomegaly (Fig. 1a) and elevated expression of major histocompatibility complex (MHC) class II on the surface of resident peritoneal macrophages (Fig. 1b,c), whereas expression of the costimulatory molecule CD86 was not elevated (Supplementary Fig. 1g). The upregulation of MHC class II expression was driven by IFN-γ produced by innate immune cells, as it occurred in Pld4–/–Rag1–/– mice, which lack B lymphocytes and T lymphocytes (Fig. 1b,c), but not in IFN-γ-deficient Ifng–/–Pld4–/– mice (Fig. 1c). Expression of MHC class II by macrophages was not elevated in Pld4–/–Ly22-Cre mice, in which PLD4 deficiency was limited to macrophages, but was elevated in Pld4fl/flCd11cCre mice, in which PLD4 deficiency was limited to DCs (Fig. 1c); this suggested that PLD4-deficient CD11c+ DCs indirectly promoted the upregulation of MHC class II expression. The plasma of Pld4–/– mice had significantly elevated concentrations of IFN-γ and the chemokine CXCL10, relative to that of Pld4–/– sufficient mice (Fig. 1d,e). Relative to PLD4-sufficient controls, Pld4–/– mice had altered leukocyte subsets, including fewer natural killer (NK) cells, peritoneal B-1 lymphocytes and platelets, and more marginal-zone B cells and blood monocytes (Fig. 1f–j and Supplementary Fig. 1h). However, the number of splenic DCs in Pld4–/– mice was similar to that of PLD4-sufficient mice (Supplementary Fig. 1f,e,f). Consistent with their elevated concentration of IFN-γ, the altered number of B-1 and marginal-zone B cells seen in Pld4–/– mice was partly reversed in Ifng–/–Pld4–/– mice (Fig. 1g and Supplementary Fig. 1i). Moreover, Pld4–/– mice were resistant to the induction of experimental autoimmune encephalomyelitis, in which IFN-γ is protective, but Ifng–/–Pld4–/– mice were not (Supplementary Fig. 2). RNA-sequencing comparison of Pld4–/–Rag1–/– and Rag1–/– splenocytes revealed an ‘interferon signature’ in Pld4–/– mice, as >40 of the 109 genes whose expression was significantly elevated in Pld4–/–Rag1–/– splenocytes (relative to their expression in Rag1–/– splenocytes) are known to be inducible by IFN-γ or type I interferons (Fig. 1k and Supplementary Table 1). Expression of Il12b, Cxcl9 and Il10 was also significantly elevated in Pld4–/–Rag1–/– splenocytes. Of the 33 genes downregulated in Pld4–/–Rag1–/– mice, some are known to be suppressed by interferons, but most are NK cell–specific genes, consistent with the lower number of NK cells in the spleen of Pld4–/– mice and Pld4–/–Rag1–/– mice.

Certain features of the phenotype of Pld4–/– mice resembled the human disease macrophage-activation syndrome, which can be mimicked in mice by repeated challenge with ligands of TLR9. To assess the role of TLR9 in the PLD4-deficient phenotype, we bred Tlr9–/–Pld4–/– mice. Notably, analysis of these mice indicated that all of the abnormalities of the PLD4-deficient mice that we assessed were dependent on TLR9, including splenomegaly, upregulation of MHC class II expression and alterations in the number of monocytes, platelets, NK cells and B-1 cells (Fig. 1c,f–j). In contrast, combined deficiency in both PLD4 and IFN-γ reversed only a subset of the phenotypic features associated with PLD4 deficiency (Fig. 1c,f,g), which suggested that IFN-γ production by Pld4–/– mice was secondary to triggering of TLR9. Histological analysis of Pld4–/–livers revealed a TLR9-dependent increase in the frequency and size of CD68+ macrophages, consistent with mild liver inflammation (Fig. 2). Overall, these results suggested that the main effects of PLD4 deficiency stemmed from enhanced or dysregulated recognition of ssDNA by TLR9 in endolysosomes.

The finding that Pld4–/– mice had excessive TLR9-driven production of IFN-γ and Il12b mRNA suggested that activation of CD8+ DCs might be dysregulated in these mice. Secretion of IL-12 by CD8+ DCs is known to stimulate IFN-γ production, particularly by NK cells. Sorted PLD4-deficient splenic DCs were found to produce more IL-12p70 and IL-6 in response to two TLR9 ligands, Vacc70V and 2216PS (an A-type CpG oligodeoxynucleotide (ODN)), than did their PLD4-sufficient counterparts; however, the responses of these cells to the B-type CpG ODN 1668PS were similar (Fig. 1l,m). 1668PS is fully modified by phosphorothioate (PS) linkages, 2216PS is partially modified by PS linkages and Vacc70V is unmodified double-stranded DNA (dsDNA) containing CpG motifs (Table 1). In addition, Vacc70V elicited IFN-γ responses in CD8+ DCs through the STING cytoplasmic DNA-sensing pathway, but 1668PS and 2216PS did not, and this did not differ in wild-type cells versus PLD4-deficient cells (Fig. 1n and Supplementary Fig. 3a,b). IFN-γ signals are known to promote IL-12 responses; however, sorted CD8+ DCs isolated from Pld4–/–Ifng–/– mice also showed elevated IL-12 responses to TLR9 stimuli, relative to Ifng–/–CD8+ DCs (Supplementary Fig. 3c). Pld4–/–CD8 CD11b+ splenic DCs also had enhanced IL-6 and IL-12 responses relative to Pld4fl/fl controls, especially to Vacc70V (Supplementary Fig. 3d).
results supported the notion that Pld4−/− CD8+ DCs had enhanced cytokine responses to at least some TLR9 ligands.

**PLD4 and PLD3 are single-stranded 5′ exonuclease.** The results reported above suggested a role for PLD4 in the processing of TLR9 ligands. To test for nuclease activity, we engineered expression of recombinant soluble mouse PLD4 by substituting a secretion signal peptide for the amino-terminal transmembrane and cytoplasmic tail. Using various DNA substrates (Fig. 3a), we then assessed the activity of purified recombinant mouse PLD4. PLD4 degraded a 55-nucleotide (nt) ssDNA (Fig. 3b, lane 2) but not a dsDNA version of the same sequence (Fig. 3b, lane 6). When paired with shorter ssDNAs complementary with the 5′ or 3′ end, PLD4 degraded only a substrate with an unpaired 5′ end (Fig. 3b, lane 4), indicative of 5′-to-3′ ssDNA-exonuclease activity but not 3′-to-5′ ssDNA-exonuclease activity, and demonstrated a lack of endonuclease activity (Fig. 3b, lane 5). PLD4 protein carrying histidine-to-alanine substitutions in both HKD motifs (PLD4-AA) lacked detectable enzymatic activity (Fig. 3b, lanes 7–11). PLD4 cleaved only substrates that lacked phosphorylated 5′ ends and was unaffected by 3′ substitutions in both HKD motifs (PLD4-AA)
phosphorylation (Fig. 3c, lanes 4 and 6). As predicted for an endosomal nuclease, the reaction was most efficient at pH < 6 but was still detectable at pH 7 (Fig. 3d). Parallel experiments with mouse PLD3 revealed a similar profile of substrate specificity and an acidic pH optimum (Fig. 3e–g). We concluded that PLD3 and PLD4 are 5′ exonucleases that are able to cleave ssDNA.

PLD3 and PLD4 appear to be classic spleen phosphodiesterases. The enzymatic properties of PLD4 and PLD3 that we observed were reminiscent of the activity previously described as ‘spleen acid exonuclease’ or ‘spleen phosphodiesterase’ (Enzyme Commission registry number 3.1.16.1, http://www.sbcs.qmul.ac.uk/iubmb/enzyme/EC3/1/16/1.html). We therefore directly compared PLD3 and PLD4 with a commercial preparation of bovine spleen phosphodiesterase (as a positive control) through the use of a diagnostic assay that measured their ability to digest GpA or ApG dinucleotides. Digestion was readily tracked by spectroscopy via the release and loss of adenosine, which is deaminated in a coupled assay.

Mouse and human recombinant soluble PLD3 and PLD4 specifically cleaved the phosphodiester bond between the phosphate and the second nucleotide, which matched the activity described for bovine spleen exonuclease, but PLD4-AA did not (Fig. 3h). Snake venom phosphodiesterase served as a control for possible cleavage at the upstream phosphodiester bond, which leaves 5′ phosphorylated mononucleotides. The results indicated that digestion by PLD3 or PLD4 generated 3′ mononucleotides and nucleosides from short oligonucleotide substrates. The commercial reagent phosphodiesterase II was further purified by size exclusion to diminish complexity of the sample, and the enzymatically active fractions were subjected to analysis by trypsin–liquid chromatography–mass spectrometry (Fig. 3i).
Fig. 3 | Analysis of the nuclease activity of PLD4 and PLD3. a. Nucleic-acid substrates tested, identified by Roman numerals (left margin). b. Denaturing Tris-Borate-EDTA PAGE of the products of incubation (for 2 h at 37 °C in reaction buffer (50 mM MES, pH 5.5, and 100 mM NaCl)) of substrates I–V as in a (above lanes; 2.5 μM; all lacking the 5′ or 3′ phosphate) with soluble recombinant mouse PLD4 or PLD4-AA (100 nM) (above gel); left margin, molecular size, in nt; below, lane numbers. c. PAGE as in b of the products of incubation (conditions as in b) of substrate I lacking phosphate (ssDNA) or with phosphorylation at the 5′ end (5′PO₄) or 3′ end (3′PO₄) (above lanes) with PLD4 or PLD4-AA or neither (left untreated (DNA)) (above gel). d. PAGE as in b of the products of incubation of substrate I with PLD4, in conditions as in b but with buffers adjusted to a pH of 5.0–7.5 (above lanes). e–g. PAGE as in b (e), c (f) or d (g), but with PLD3 or PLD3-AA (10 nM) (above gels) instead of PLD4 or PLD4-AA. In b, the images are cropped between lanes 1 and 2 to eliminate additional undigested control lanes. h. Release of adenosine by the digestion of dinucleotide GpA (left) or ApG (right), as described, by mouse (m) or human (h) PLD3 (3), PLD3-AA (3-AA), PLD4 (4) or PLD4-AA (4-AA) (above lanes; 500 nM) by incubation at 37 °C for 6 h in reaction buffer adjusted to pH 5.0 (PLD4, PLD4-AA) or pH 5.5 (PLD3, PLD3-AA). Data are representative of at least two experiments.
of PS-modified guanine nucleotides known to form unusual secondary structures. Data are from one experiment (mean ± s.d. of three separate cultures)....
Fig. 5 | Stimulatory ability and stability of 2216 and its fragments in the presence or absence of PLD3 or PLD4. **a**, Synthetic substrates tested: lower case indicates PS-linked nucleotides; arrows indicate the ability of DNase II to cut within the palindrome of 2216. **b–e**, IL-12p70 response (b), IFN-α response (c), CD86 response (d) and IL-6 response (e) of various genotypes (key) of CD8α+ DCs (b) or plasmacytoid DCs (c,d) derived from culture in FLT3L (b–d) and gated as CCR9+ B220+ (d), or of thioglycolate-elicited macrophages (e), to ODNs as in a (left margin; 1 μM). Each symbol (b,c,e) represents an individual culture (mean and s.d. of three separate cultures in b, c, e); cells from replicate cultures were pooled for flow cytometry in d, which yielded a single sample per condition. **f**, Electrophoresis of the products of in vitro digestion of the substrates in a (above lanes) without enzyme (left half) or by incubation for 1 h with PLD4 (100 nM; top) or PLD3 (10 nM; bottom) (right half). Data are representative of two experiments with similar results. **g**, TLR9 response of a wild-type HEK-Blue<sup>hTLR9</sup> clone (HEK293), PLD3-deficient HEK-Blue<sup>hTLR9</sup> clone 14 (PLD3 KO 14) or PLD3-deficient HEK-Blue<sup>hTLR9</sup> clone 14 reconstituted with an expression construct for PLD4 (KO + hPLD4) or PLD4-AAA (KO + hPLD4-AAA) (key) to medium alone (Ctrl) or to ODNs as in a (horizontal axis; 1 μM), assessed as activation of an NF-κB reporter and presented as optical density at 630 nm. Each symbol represents an individual culture. Data are representative of at least two experiments with similar results (mean of duplicate cultures). **h**, TBE-urea acrylamide gel electrophoresis (top) of ODNs in lysates of thioglycolate-elicited macrophages from C57BL/6 (WT), PLD3<sup>−/−</sup> or Unc93b<sup>−/−</sup> mice (above lanes) after 3.5 h or 8 h of incubation with medium alone or 3’ biotin--tagged ODN 2216 (2216-Bio) (below gel); bottom, quantification of the intensity of bands in the 5- to 13-nt range (horizontal axis) of lanes 4–9 above (key), presented as the proportion of DNA. Size markers are oligomers of deoxythymidine (left margin, top) and ODNs listed in a (left margin, bottom); asterisks (right margin, top) indicate ODN species increased in abundance in lysates of Plc2γ<sup>−/−</sup> cells. Data are representative of two experiments. **i**, Electrophoresis (top) as in h (in 50 mM histidine buffer for better resolution of small fragments) of 2216-biotin recovered after 6 h of stimulation of wild-type HEK-Blue<sup>hTLR9</sup> cells (WT) or PLD3<sup>−/−</sup> or HEK-Blue<sup>hTLR9</sup> cells (clone 10) transfected with vector only or reconstituted with human PLD4 or PLD4-AAA or mouse PLD3 or PLD3-AAA (above lanes); far left lane, 2216-biotin radiolabeled as a size marker. Bottom, quantification of the intensity of bands in the 5- to 13-nt range (horizontal axis) of lanes 2–7 above (key), presented as in h. Data are from one experiment. *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed t-test).
unphosphorylated 5’ ends and 3’ phosphates, we hypothesized that DNAse II–cleaved 2216PS fragments should be naturally degraded by PLD3 or PLD4. We therefore tested the ability of a series of synthesized fragments of 2216 or 2216PS (Fig. 5a) to stimulate DCs cultured with FLT3L and to stimulate PLD3- or PLD4-deficient thioglycolate-elicited macrophages (Fig. 5b–c). The Plid4−/− DCs and

**Fig. 6** | Liver inflammation and elevated inflammatory cytokine production in Plid3−/−Plid4−/− mice. **a–c.** Microscopy of hematoxylin and eosin–stained paraffin-embedded sections from Plid3−/−Plid4−/− liver, showing vesicular hepatopathy (steatosis) (a), hemophagocytosis (arrow) (b) and hepatic multinucleate cells (arrow) (c). Scale bars, 50 μm. **d.** Microscopy of hematoxylin and eosin–stained liver sections of littermates of various genotypes (above images) (top row) and CD68 immunofluorescence staining of frozen liver sections derived from different lobes of the same liver as above (bottom). Scale bars, 50 μm. **e.** Concentration of ferritin in serum from 16- to 19-day-old C57BL/6, Plid3−/−Plid4−/−, Plid3−/−Plid4−/+ and Plid3−/−Plid4−/− mice (horizontal axis; n=10, 5, 7, 6 and 12, left to right); dotted horizontal lines indicate assay background. Each symbol represents an individual mouse (error bars, s.d.). P values, two-tailed t-test (*, significant; NS, not significant). **f.** Concentration of ferritin in serum from C57BL/6, Plid3−/−, Plid3−/− and Plid3−/−Plid4−/− mice (horizontal axis; n=4 per group). Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.). P values (in plot), unpaired two-tailed t-test. **g.** IL-6 in supernatants of bone marrow–derived macrophages isolated from 19-day-old C57BL/6, Plid3−/−, Plid3−/− and Plid3−/−Plid4−/− mice (key), assessed after stimulation in vitro with various TLR agonists (horizontal axis). Each symbol represents an individual culture. NS, not significant (P>0.05); *P<0.05, * P<0.01 and ***P<0.001, C57BL/6 versus Plid3−/−Plid4−/− (unpaired two-tailed t-test). Data are representative of two experiments with similar results (mean and s.d. of triplicate cultures).
Pld³⁻ macrophages showed enhanced responses to these shorter ligands, relative to the responses of their PLD3- or PLD4-sufficient counterparts (Fig. 5b–e). When packaged in the transfection reagent Lyovec, the same ODN ligands elicited similar strong responses in DCs, thioglycolate-elicited macrophages still had enhanced responses to the CD45R0+, CD8⁺, CD44⁺, and CD62L⁺ T cells (left half); cells gated as either CD4⁺ T cells or CD8⁺ T cells were further analyzed by staining of CD62L and CD44 (right half). Numbers adjacent to outlined areas indicate percent CD20⁺ IgM⁺ B cells identified in the spleen of mice as in a (key in d), as gated in e. Quantification of CD45R0⁺CD8⁺ T cells (left) or CD8⁺ T cells (right) from the spleen of mice as in c (key in d). Flow cytometry of splenic CD45.2⁺ gated B cell populations from mice as in a (donor genotype, above plot), stained with B220 and IgM. Numbers adjacent to outlined areas indicate percent B220⁺ IgM⁺ B cells. d, Quantification of donor (CD45R0⁺) B220⁺ IgM⁺ B cells identified in the spleen of mice as in a (key, as gated in c. e, Quantification of CD45R0⁺CD8⁺ T cells (left) or CD8⁺ T cells (right) from the spleen of mice as in c (key in d). f, Flow cytometry of splenic CD45R0⁺ cells from mice as in a (donor genotype, right margin), showing gating of CD20⁺, CD4⁺ and CD8⁺ T cells (left half); cells gated as either CD4⁺ T cells or CD8⁺ T cells were further analyzed by staining of CD62L and CD44 (right half). Numbers adjacent to outlined areas indicate percent cells in each. g, Frequency (left) and quantification (right) of CD8⁺ T cells stained as CD44⁺/CD62L⁺ (top row) or CD44⁺/CD62L⁺ (bottom row) in the spleen as in f (key in d, n = 4 (Pld³⁻/Pld4⁺⁻), 4 (Pld³⁻/Pld4⁻⁻) or 3 (Pld³⁻/Pld4⁻⁻)). Data are from one experiment. Each symbol (a.b.d.e.g) represents an individual mouse; bar tops indicate the mean. P values (in plots), two-tailed t-test.

Fig. 7 | Transfer of an inflammatory disease by transplantation of Pld³⁻/Pld4⁺⁻ bone marrow. a, Quantification of platelets (PLT), red blood cells (RBC), reticulocytes (RET) and white blood cells (WBC) in whole blood from B6.SJL CD45.1⁺ host mice at 8 weeks after transfer of CD45.2⁺ bone marrow from Pld³⁻/Pld4⁺⁻, Pld³⁻/Pld4⁻⁻ or Pld³⁻/Pld4⁻⁻ mice (key in d). b, Spleen weight (left) and quantification of splenocytes after erythrocyte lysis (right) in mice as in a (key in d) at 8 weeks after transfer. c, Flow cytometry of splenic CD45.2⁺ gated B cell populations from mice as in a (donor genotype, above plot), stained with B220 and IgM. Numbers adjacent to outlined areas indicate percent B220⁺ IgM⁺ B cells. d, Quantification of donor (CD45.2⁺) B220⁺ IgM⁺ B cells identified in the spleen of mice as in a (key), as gated in c. e, Quantification of CD45.2⁺ T cells (left) or CD8⁺ T cells (right) from the spleen of mice as in c (key in d). f, Flow cytometry of splenic CD45.2⁺ cells from mice as in a (donor genotype, right margin), showing gating of CD20⁺, CD4⁺ and CD8⁺ T cells (left half); cells gated as either CD4⁺ T cells or CD8⁺ T cells were further analyzed by staining of CD62L and CD44 (right half). Numbers adjacent to outlined areas indicate percent cells in each. g, Frequency (left) and quantification (right) of CD8⁺ T cells stained as CD44⁺/CD62L⁺ (top row) or CD44⁺/CD62L⁺ (bottom row) in the spleen as in f (key in d, n = 4 (Pld³⁻/Pld4⁺⁻), 4 (Pld³⁻/Pld4⁻⁻) or 3 (Pld³⁻/Pld4⁻⁻)). Data are from one experiment. Each symbol (a.b.d.e.g) represents an individual mouse; bar tops indicate the mean. P values (in plots), two-tailed t-test.
Enhanced stability of TLR9 ligands in cells lacking PLD3 and PLD4. To assess the degradation of ODNs in vivo, we stimulated wild-type or mutant thioglycollate-elicited macrophages with ODN 2216 carrying a 3′ biotin tag; after 3.5 h or 8 h, we recovered ODNs from cell lysates through the use of streptavidin beads and visualized them by 32P end-labeling. This analysis revealed that the 2216–biotin fragments that demonstrated enhanced stimulatory activity in PLD3+/− macrophages and PLD4+/− DCs were degraded less in lysates of PLD3+/− macrophages than in those of PLD3-sufficient macrophages, which had shorter species composed mainly of the poly(G) 3′ end (<10 nt) (Fig. 5h). The degradation pattern in Unc93b−/− macrophages, which lack signaling via TLR3, TLR7 and TLR9, was identical to that of wild-type macrophages, which indicated that signaling via TLR9 was not required for enzymatic degradation by PLD3. Similar results were obtained for PLD3+/− Hek-Blue<sup>©</sup> cells fed 2216–biotin, in which DNA fragments of 10–15 nt were more abundant than in unmutated Hek-Blue<sup>©</sup> cells, and these DNA fragments were specifically depleted in cells reconstituted with PLD4 or PLD3 but not in those reconstituted with the mutant PLD4-AA or PLD3-AA (Fig. 5i). We concluded that PLD3 and PLD4 had redundant exonuclease function that was able to limit TLR9 responses to CpG ODNs, particularly those lacking PS linkages, by reducing the concentration of ssDNA able to stimulate TLR9, and that these activities were similar in cells of humans and mice.

PLD3 and PLD4 prevent a lethal hepatic autoinflammatory disease. The overlapping enzymatic activities of PLD3 and PLD4 and their co-expression in macrophage subsets prompted us to interbreed PLD3+/− mice and PLD4+/− mice to generate mice deficient in both enzymes. No mice deficient in both genes survived to the age of weaning (Supplementary Fig. 7a). PLD3+/−Pld3+/− mice of each sex were identified, but these mice were undersized relative to the size of their heterozygous littermates, and they died between 12 d of age and 21 d of age with severe liver inflammation (Fig. 6a–d and Supplementary Fig. 7b). The liver of PLD3+/−Pld4+/− mice showed macroscopic white areas of necrosis (Supplementary Fig. 7c), with regions of vesicular steatosis (Fig. 6a), hemophagocytosis (Fig. 6b) and multinucleated hepatocytes (Fig. 6c). Notably, only a single allele of either Pld3 or Pld4 was needed to allow survival and to prevent the substantial liver pathology (Fig. 6d) and Supplementary Table 2). Although CD68<sup>+</sup> myeloid cells were more prevalent in the liver of 16-day-old PLD3−/−Pld4+/− mice than in that of their PLd3+/−Pld4−/− or PLD3−/−Pld4+/− littermates, the liver of PLD3−/−Pld4−/− mice demonstrated extensive infiltration by CD68<sup>+</sup> myeloid cells (Fig. 6d). Serum from PLD3−/−Pld4−/− mice had a higher concentration of ferritin, the cytokines IL-6, IL-10, TNF, IFN-γ, the chemokines CXCL10, MCP3 (CCL7) and MCP1 (CCL2), than that of serum from mice of the same age expressing one or more copies of Pld3 or Pld4, whereas the concentration of IFN-α and IL-10 was not significantly elevated in the PLD3−/−Pld4−/− mice (Fig. 6e,f). We inferred that neonatal mice needed PLD3 and PLD4 to prevent an inflammatory disease consistent with hemophagocytic lymphohistiocytosis.

Analysis of the responses of PLD3−/−Pld4−/− bone marrow–derived macrophages to TLR9 ligands revealed elevated secretion of IL-6 in both enzymes. No mice deficient in both genes survived to the age of weaning (Supplementary Fig. 7a).

PLD3 and PLD4 in hematopoietic cells prevent autoinflammation. To determine whether the immune system itself was the driver of the lethal inflammation in PLD3−/−Pld4−/− mice, we transplanted bone marrow from 19-day-old PLD3−/−Pld4−/−, PLD3+/−Pld4−/− or PLD3+/−Pld4+/− littermate donors into lethally irradiated 6-week-old wild-type CD45.1<sup>+</sup> recipients. At 8 weeks after transfer, blood analysis revealed low platelet counts, anemia, elevated reticulocytes and leukopenia in the recipients that received PLD3−/−Pld4−/− bone marrow but not in recipients given bone marrow from littermates with a single wild-type allele of either Pld3 or Pld4 (Fig. 7a). Despite the overt splenomegaly of recipients of PLD3−/−Pld4−/− bone marrow, the number of splenic leukocytes recovered after erythrocyte lysis was lower in recipients of PLD3−/−Pld4−/− bone marrow than in mice receiving bone marrow expressing either PLD3 or PLD4 (Fig. 7b). Flow cytometry of CD45.2<sup>+</sup> splenocytes revealed a marked reduction in the proportion and number of B cells when both PLd3 and Pld4 were absent, relative to their abundance when either gene was present (Fig. 7c,d). The total number of TCRβ<sup>+</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T cells was also lower in the spleen of recipients of PLD3−/−Pld4−/− bone marrow than in that of mice receiving bone marrow expressing either PLD3 or PLD4 (Fig. 7e); however, the majority of those CD8<sup>+</sup> T cells exhibited an activated phenotype (CD44<sup>+</sup>CD62L<sup>−</sup>), and very few naive CD8<sup>+</sup> T cells (CD44<sup>−</sup>CD62L<sup>+</sup>) were present (Fig. 7f,g). Consistent with the disease observed in PLD3−/−Pld4−/− mice, recipients of PLD3−/−Pld4−/− bone marrow had elevated expression of MHC class II (with CD86 expression similar to that of controls) on peritoneal macrophages, a greater abundance of blood monocytes, and a higher concentration of the cytokines CXCL10, IFN-γ, MCP3, IL-6 in serum compared to recipients of bone marrow in which either PLD3 or PLD4 were present, but IFN-α was not detected in serum (Supplementary Fig. 7d–j). We concluded that both PLD3 and PLD4 limited autoinflammation and that a single wild-type allele of either Pld3 or Pld4 was able to prevent the development of the inflammatory disease transferred via hematopoietic stem cells.

Discussion

We have presented evidence that the endolysosomal proteins PLD3 and PLD4 trimmed the 5′ end of single-stranded ODNs and thus degraded them and limited their ability to stimulate TLR9. Consistent with that conclusion, PLD3 and PLD4 are most active at an acidic pH (5–5.5) and are localized to endolysosomes<sup>15,17</sup>. Notably, neither enzyme seems to have phospholipase activity<sup>25,38</sup>.

Human genome-wide association studies have linked polymorphisms of PLD3 and PLD4 to inflammatory diseases. In animals, spontaneous PLD4-null mutations have been linked to skin disease and runting. A mouse mutant identified on the BALB/c background strain this (‘thin hair with small size’) seems to have abnormalities distinct from those of PLD4 deficiency on the C57BL/6 background that we have investigated here (http://www.informatics.jax.org/reference/J:171492). A spontaneous PLD4-null mutation occurred in a herd of cattle, whereby homozygous calves were runts with skin lesions, respiratory- and digestive-tract inflammation and mild anemia<sup>39</sup>. It will be of interest to determine if these phenotypes are also connected to activation of TLR9.

The similarities in the enzymatic activities of PLD3 and those of PLD4 and their partly overlapping expression patterns would suggest that they have redundant functions in key cell types. The RNA-profiling data indicated that PLD4 had a narrow tissue distribution, with high levels in DCs and myeloid cells, whereas PLD3 had a broad distribution and seemed to digest artificial substrates with greater efficiency than did PLD4. PLD3 enters the lysosome by an unusual sorting and ubiquitination pathway regulated by the ESCRT complex<sup>30</sup>, and other PLD3-deficient mice reportedly show no altered phenotype except for effects on the morphology of the lysosomal system<sup>39</sup>. The phenotype of our Pld3−/− mice also seemed more benign than that of Pld4−/− mice. This observation might be
explained by the high levels of PLD4 normally present in cells with high expression of TLR9. Although Pld4−/− mice had a relatively mild form of macrophage-activation syndrome, the residual PLD3 in myeloid cells probably provided protection from more-severe disease, as a single allele of either Pld3 or Pld4 protects mice against lethal inflammation early in life.

Nucleic-acid sensors are endowed with a restricted fine specificity and affinity for ligands. Responses are limited by the quantity of ligands available and their intracellular localization. PLD3 and PLD4 provide DNAase activity directed against ssDNA in endolysosomes; it is probably no coincidence that they reside in the same compartment as active TLR9. Notably, PLD3 and PLD4 required DNA substrates with a non-phosphorylated 5′ end and the absence of secondary structure. Nucleic acids with such features are relatively rare in nature but are generated efficiently in endolysosomes by DNAase II, which cleaves dsDNA down to ssDNA fragments of ~10 nt carrying 3′ phosphate and 5′ hydroxyl groups. DNAase II is also needed to process DNA hairpins, such as A-type CpG ligands, for recognition by TLR9. Published work has indicated that TLR9 has two binding sites: one that binds ssDNA with a CpG motif and another that binds short ssDNA carrying a 5′ hydroxyl group. Thus, the short stimulatory ligands that TLR9 is predicted to encounter should also be good substrates for PLD3 and PLD4. Endosomal build-up of such short ssDNA in the absence of PLD3 or PLD4 would probably not trigger interferon responses mediated by STING–cGAS, as ssDNA greater than 21 nt in length is required and this might be prevented by the presence of TREX1 in the cytoplasm.

Although mild, the phenotype of Pld4−/− mice is reminiscent of human macrophage-activation syndrome, which is associated with elevated concentrations of IFN-γ, IL-12, CXCL9 and CXCL10, a low number of NK cells and an increase in liver CD68+ cells and can be mimicked in mice by repeated injection of a TLR9 ligand (3′-CpGODN). Notably, all of the features of the phenotype of Pld4−/− mice were absent from mice that also lacked TLR9, consistent with the hypothesis that defective degradation of ligands for TLR9 is central to the phenotype. That was further supported by evidence showing that Pld3−/− macrophages and HEK293-BluehTLR9 cells had exaggerated responses to particular ssDNA ligands of TLR9. Several features of the disease transferred via Pld3−/−Pld4−/− bone marrow into wild-type recipients mimicked dysregulated TLR9 signaling. Mice that receive stem cells expressing a mutant TLR9 that does not require proteolytic activation or traffic normally to endolysosomes succumb to a DC-driven fatal inflammation characterized by profound anemia and a decreased abundance of splenic B cells. The death of Pld3−/−Pld4−/− mice in early life reflects the important role of PLD3 and PLD4 in limiting autoinflammation, and although we predict that TLR9 signaling is required for the disease, this needs to be addressed by further mouse breeding.

Beginning in infancy, the liver of children with primary hemophagocytic lymphohistiocytosis shows several features similar to that of Pld3−/−Pld4−/− mice, including giant-cell hepatitis, steatosis, necrosis and the namesake hemophagocytosis. The high concentrations of ferritin and cytokine in serum and extensive hepatic infiltration by CD68+ cells in Pld3−/−Pld4−/− mice provide further similarities to primary hemophagocytic lymphohistiocytosis, although this disease is usually associated with mutations that result in defective cytotoxic granule contents of, or exocytosis from, NK cells and T cells.

PS linkages are used in synthetic ODNs to hinder their digestion by nucleases. B-type CpG ODNs (such as 1668PS) that are fully modified by PS linkages could drive TLR9-mediated secretion of IL-6 and IL-12 from macrophages and DCs independently of PLD3 or PLD4. However, PS linkages are by no means strictly artificial and are widely present in nature in the DNA of bacteria. The finding that PLD3 and PLD4 digested ODNs carrying PS linkages or strings of guanine residues more slowly than they digested host DNA might favor innate recognition of bacteria or viruses.

We found that PLD3 and PLD4 are 5′ exonucleases with properties strikingly similar to the enzymatic activity described many years ago as ‘spleen acid exonuclease’. The bovine enzyme has an optimum pH of 5.5 and is specific for 5′ single strands; it has also been characterized as a non-processive nuclease that is inhibited by 5′ phosphate and generates 3′-nucleotide monophosphate products. The degradation of ssDNA substrates by PLD3 and PLD4 was similarly non-processive and blocked by 5′ phosphate and generated 3′ nucleotide monophosphates, and the digestion was able to proceed to completion, generating mononucleotides and nucleosides, as does the spleen exonuclease. Although the gene encoding spleen exonuclease has never been cloned, the enzymatic activity of spleen exonuclease has been described in humans, rats, cows and pigs, whose predicted PLD3 proteins are highly conserved (93–96% identical between species). The similarities among bovine, mouse and human PLD3 in enzymatic features and the considerable amino-acid identity suggest that PLD3 might have activity identical to the spleen-phosphodiesterase activity previously described, although PLD4 cannot be excluded as a contributor to this activity, as it is also conserved among these species (>70% identical) and has a very similar enzymatic activity.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41590-018-0179-y.

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**Author contributions**

A.L.G. participated in many of the studies, identifying anomalies in Pld4- and Pld3-deficient mice and their myeloid cell cytokine responses and in the preparation of Pld3 and Pld4 proteins; D.H. elucidated the enzymatic function of Pld3 and Pld4, carried out all studies in 293 cells, and contributed to other studies; C.H. generated the Pld4−/− and Pld4+/− mutant mice and characterized many features of their phenotypes; A.M. cloned Pld4 cDNA and characterized Pld4 expression in the B cell lineage; V.T. and B.R.L. participated in the analysis of experimental autoimmune encephalomyelitis in Pld4−/− mice; P.D.S., T.R.B. and T.C.T. provided technical support; K.O. provided histological analysis of liver pathology; H.S.C., F.K., P.K., A. Zeitjian, R.L.S., M.B. and S.R.S. were student interns who participated in generating constructs for CRISPR-Cas9 mutagenesis and site-directed mutagenesis of Pld4 and Pld4; A. Zarpellon provided assistance with blood analysis using the IDEX Procyte DX system; B.C. assisted C.H. in the search for potential phospholipase activity of Pld4; E.S.P., M.O.K. and H.H. provided advice and assistance with DC studies (Fig. 11–n) and data in Fig. 4d; J.T. and J.C.d.l.T. provided advice and assistance with RNA virus studies; M.D. assisted with Pld3 reagents and antibodies; L.T. provided advice on and assistance with the expression of Pld4 protein; A.L.G. and D.N. co-directed these studies; and A.L.G., D.H. and D.N. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Methods**

Mice. Targeting of Pld4 was carried out in 129/Sv embryonic stem cells by the TgRI Mouse Genetics Core. The neo+ cassette was removed by breeding to mice expressing Fpr2a in order to generate Pld4+/+ mice. Subsequent breeding to C57/BL6 mice generated Pld4+/− mice, and both Pld4+/+ and Pld4−/− were subsequently backcrossed to C57BL/6 for >20 generations. Pld4−/− mice were genotyped by PCR using the following primers: 32- TTTGCCATACCCCACTACAGCA, #33- GGTGGACGCAAAATCTTAGG, 34- CTCGATCCTCCCAAGAAGACA, which generated product sizes of 300 bp (wild-type), 350 bp (Fpr2a), and 420 bp (altered) for the indicated alleles. The following mouse strains bred with Pld4+/− mice: B16.12975-Rag1−/−/−; B6.12992-Lyz2-tm1(Cref/+); B6.Gc-Tg[Igk-cre-1]1-Reiz/J, B6.12975-Igkflrtv/−/−, C57BL/6J-Tlr9M7Btlr/Mmjax (CpG11), from the Jackson Laboratory. Unc93b1+/+ mice were provided by B. Utterley, University of Texas Southwestern Medical Center. Mutation of Pld3 was carried out by nuclear injection of C57BL/6J embryos with plasmid psCas9(11.1) (1) (gift from Y. Doyama, University of California, Davis) and Adgene (Cambridge, MA) engineered to express the guide sequence GAGCTGCGGTGCTGCCACC targeting Pld3 exon 9, which encodes the first HDK motif. DNA (5 μg/ml) in Tris-HCL pH 7.5, 10 mM EDTA 0.1 mM NaCl 50 mM was microinjected by the TgRI Mouse Genetics Core staff. Pld3−/− mice were genotyped by PCR using P3ExSF-GCACCAGTGCACACAGCAAG and P3ExFR- TACAATGGGAGGAAATGGT, and the PCR product was digested with the restriction enzyme PshA1, as the PshA1 site is only present in the wild-type allele. Experiments were performed as approved by the TgRI IACUC Committee.

DC generation, isolation, and stimulation. DC populations were expanded in vitro from bone marrow precursors using FLT3L as described52. Sorted splenic DCs (50,000 cells per well) were stimulated for either 24 h or 72 h in medium containing GM-CSF (10 ng/ml), mouse IL-4 (10 ng/ml) and rat IL-28A/B (R&D Systems).

Macrophage analysis. Macrophages were elicited in the peritoneal cavity by i.p. injection of 2216PS (1 g/ml, InvivoGen). The following ODNs purchased from IDT or InvivoGen were also used to stimulate macrophages in vitro from bone marrow precursors using FLT3L as described52: 2216PS (1 g/ml), and both 2216PS (0.25 g/ml) and 1668PS (0.25 g/ml). The following ODNs purchased from InvivoGen were also used: 2216PS (1 g/ml), and both 2216PS (0.25 g/ml) and 1668PS (0.25 g/ml). Macrophages were cultured for several days before stimulation. ODN stimuli were added at a concentration of 1 μg/ml. The following ODNs purchased from IDT or InvivoGen were also used: 2216PS (1 μg/ml), 2216PS (1 μg/ml) and 1668PS (0.25 μg/ml) and 1668PS (1 μg/ml). 2216PS (1 μg/ml) and 1668PS (0.25 μg/ml). Macrophages were cultured for several days before stimulation. ODN stimuli were added at a concentration of 1 μg/ml. The following ODNs purchased from IDT or InvivoGen were also used: 2216PS (1 μg/ml), 2216PS (1 μg/ml) and 1668PS (0.25 μg/ml) and 1668PS (1 μg/ml).

**Studies with HEK293 cells.** HEK-Blue2125 cells (InvivoGen) were cultured and stimulated as described in the manufacturer’s instructions. Briefly, HEK-Blue2125 cells were transfected with a plasmid expressing human Pld4, either wild type or with an alanine substitution at position 345, and were cultured for several days before stimulation. ODN stimuli were added at a concentration of 1 μg/ml. The following ODNs purchased from IDT or InvivoGen were also used: 2216PS (1 μg/ml), 2216PS (1 μg/ml) and 1668PS (0.25 μg/ml) and 1668PS (1 μg/ml). 2216PS (1 μg/ml) and 1668PS (0.25 μg/ml). Macrophages were cultured for several days before stimulation. ODN stimuli were added at a concentration of 1 μg/ml. The following ODNs purchased from IDT or InvivoGen were also used: 2216PS (1 μg/ml), 2216PS (1 μg/ml) and 1668PS (0.25 μg/ml) and 1668PS (1 μg/ml).
(B220<sup>+</sup>CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>+</sup>); resident peritoneal B-2 cells (B220<sup>+</sup>CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>+</sup>); resident peritoneal macrophages (size, F4/80<sup>+</sup>CD11b<sup>+</sup>). Antibodies used in this study are identified in Supplementary Table 3. Gating strategies used for DCs, peritoneal macrophages, and bone marrow chimera B cells and T cells are shown in Supplementary Fig. 8.

**Induction and monitoring of experimental autoimmune encephalomyelitis.** Experimental autoimmune encephalomyelitis was induced by immunization with MOG<sub>35–55</sub> peptide (myelin oligodendrocyte glycoprotein amino acids 35–55) emulsified in complete Freund's adjuvant, followed by administration of pertussis toxin as outlined by the manufacturer (Hooke Laboratories), and scores were assigned according to the manufacturer's instructions. Female mice over 8 weeks of age were used. In some cases, C57BL/6 mice congeneric for expression of the marker CD45.1 (B6.CD45.1<sup>+</sup>) were lethally irradiated with two doses of 500 rads and were reconstituted with bone marrow from either Pld4<sup>+/–</sup> mice or Pld4<sup>−/−</sup> mice for 10 weeks before immunization.

**CD68<sup>+</sup> liver stains.** Livers from age- and sex-matched mice were frozen in optimal cutting temperature (O.C.T., Sakura). Then 7-µm sections were cut, fixed with acetone for 10 min and stained with anti-CD68 Alexa Fluor 647 (FA-11, BioLegend). After washing in PBS, nuclei were stained with Hoechst 33342 for 5 min before mounting in Prolong Diamond Antifade mounting medium (Invitrogen). Sections were imaged using a Keyence BZ-7100 fluorescence microscope. The frequency and area of CD68<sup>+</sup> cells were determined using Fiji software<sup>54</sup>. In brief, color images were split into channels and a threshold set for each channel. The 'analyze particles' tool was used to estimate number of nuclei in image (Hoechst blue channel). The 'analyze particle' tool was then applied to the CD68<sup>+</sup> channel (Red channel). The frequency was then calculated. The 'measure' tool was used to calculate the average area of the particles above the threshold in the CD68<sup>+</sup> channel.

**Mass spectrometry.** Phosphodiesterase II (Worthington Biochemical, Cat#: LS003602, LOT #: 36016915) was fractionated on a Superdex 200 column in PBS, and two adjacent fractions with peak enzymatic activity were pooled. The solution was reduced (200 mM DTT), alkylated (200 mM iodoacetamide) and cut at cutting temperature (O.C.T., Sakura). Then 7-µm sections were cut, fixed with acetone for 10 min and stained with anti-CD68 Alexa Fluor 647 (FA-11, BioLegend). After washing in PBS, nuclei were stained with Hoechst 33342 for 5 min before mounting in Prolong Diamond Antifade mounting medium (Invitrogen). Sections were imaged using a Keyence BZ-7100 fluorescence microscope. The frequency and area of CD68<sup>+</sup> cells was determined using Fiji software<sup>54</sup>. In brief, color images were split into channels and a threshold set for each channel. The ‘analyze particles’ tool was used to estimate number of nuclei in image (Hoechst blue channel). The ‘analyze particle’ tool was then applied to the CD68<sup>+</sup> channel (Red channel). The frequency was then calculated. The ‘measure’ tool was used to calculate the average area of the particles above the threshold in the CD68<sup>+</sup> channel.

**RNA-expression analysis and sequencing.** The RNA-expression data in Fig. 4c were from the BioGPS MOE430 Gene Atlas Dataset<sup>44</sup>. For the sequencing analysis in Fig. 1k, total RNA was isolated from splenocytes using a kit (RNA easy, Qiagen). RNA was prepared into RNA-seq libraries using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina following the manufacturer’s recommended protocol. The libraries were then amplified by PCR for 15 cycles using barcoded PCR primers and were purified and size selected using AMPure XP Beads before loading onto an Illumina NextSeq500 for 75-base single-read sequencing. A total of 16.3–19.6 million ‘passed filter’ reads were obtained for each of the six samples. Reads were compared to the mouse genome build mm10 and were quantitated using the program Salmon. The DC RNA-seq data reported in Fig. 4d were generated as follows. DC subsets were purified from spleens of 10–14 C57BL/6 mice per sample as described<sup>45</sup>. In brief, cell sorting was performed on a BD Influx machine. RNA was extracted using an RNeasy Mini Kit (Qiagen), and residual genomic DNA removed using RNase-free DNase (Qiagen). Sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA kit, and sequencing was carried out by Micromon using High-Output SBS on the Illumina NextSeq 500. RNA-seq data analysis was performed in Degust<sup>56</sup> (http://degust.erc.monash.edu; version 3.1.0) by the Monash Bioinformatics platform personnel, D.R Powell and A. Baroughare.

**Statistical analysis.** Analysis of differential gene expression was carried out using the program Edge. Other data comparisons used the program Prism (Graph Pad), with correction for multiple comparisons. When two groups were compared, an unpaired, two-tailed t-test was used. Actual P values are provided in Supplementary Table 4.

**Data availability.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

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- [ ] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- [ ] Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- [ ] Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Flow sorting data was collected using BD Diva software. Flow cytometry data was collected LSRII flow cytometer. Serum cytokine analysis using FlowCytomix beads was acquired by LSRII flow cytometer. Cytokine ELISA data was collected using Softmax Pro.

Data analysis

Flow cytometry data was analyzed using FloJo software. RNA-seq data was analyzed using either Salmon, (Figure 1k and Supplementary Table 1, or Degust (http://degust.erc.monash.edu/ 790 Version: 3.1.0) for Figure 4d. CD68+ macrophages were enumerated from images using FIJI (Image version 2.0.0-rc-65/1.5u). Serum cytokine analysis using FlowCytomix beads was acquired by LSRII flow cytometer and mean fluorescence values generated using FlowJo. Mean fluorescence values were converted to pg/ml using standards and calculations using Prism standard curve interpolation.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
In determining the number of mice to be used in these experiments, we referred to “Statistical aspects of planning and design of immunological experiments”, Elton, R.A. and McBride, W.H. In The Handbook of Experimental Immunology 4th edition, 1986, Vol 4, Chapter 131, pp131.1-131.6, Blackwell, Oxford. To provide a p<0.05 with 80% power in comparing two means with similar sample sizes and normal distributions, the formula used is:

\[ n = \frac{2(\text{S.D.})^2}{d^2} \]

where n, S.D., and d are the sample size, standard deviation of measurements and the differences in the experimental and control means. The referenced text notes that, for added stringency, 20 would be replaced by 30. Because in measurements of relevant parameters of leukocyte function the standard deviation approximates the mean, in order to detect a two-fold difference between experimental groups a sample size of 5-7 mice is needed per group. Usually, additional redundancy was added, such as independent experiments. We also carried out independent comparisons of Pld4+/- vs Pld4+/- littermates, Pld3+/+- vs Pld3+/- littermates, and separate analysis of cohorts of Pld4+/fl vs age matched Pld4+/-, or Tlr9-/- vs Tlr9-/- Pld4+/- mice.

**Data exclusions**
No data were excluded from any analysis with the exception of a single animal in Fig 7. One animal (out of a total of 4) receiving Pld3-/-Pld4-/- BM was excluded because it had only 10%CD45.2+ cells in the bone marrow and spleen, indicating incomplete bone marrow reconstitution with donor bone marrow.

**Replication**
Figure 1. Spleen weights were determined from age and sex matched animals over four experiments and pooled into one figure. MHC II expression levels were determined once for some genotypes (Pld4fl/fl LysMCre, Pld4fl/fl CD11cCre, RAG-/-, PLD4-/-, RAG -/- PLD4+/+), twice for (IFNg-/- and IFNg-/- PLD4--/-) and four times for PLD4fl/fl and PLD4-/- which were combined into one figure. Blood monocytes, IFNg and CXCL10 levels from blood were determined from age and sex matched animals with two different age ranges. Each point is derived from an individual animal. RNA was isolated from three different RAG1/- (WT) or Pld4-/- RAG1/- (KO) spleens and analyzed for gene expression. Splenic DCs were sorted and stimulated in triplicate cultures. These experiments were performed 4 times with similar results.

Figure 2. Analysis of CD68+ macrophages in frozen liver sections was performed on a total of 3 Pld4fl/fl, 4 Pld4-/-, 4Tlr9-/- and 4Tlr9-/-Pld4-/- mice. Figure 3. DNA or RNA oligonucleotides were digested with PLD4 or PLD3 proteins at least 4 times with similar results. Digestion of dinucleotide substrates with recombinant PLD3 or PLD4 and commercial phosphodiesterases was performed twice.

Figure 4. Peritoneal macrophages from Pld3-/- and control mice were plated in triplicate and each experiment performed at least 3 times. Spleen weight, NK cell proportions, and MHCI levels of macrophages of Pld3-/- and control mice was performed twice with similar results, with a single experiment shown in Fig S1l-n.

Figure 5 FLT3L DC cultures and thioglycolate elicited macrophages of each genotype were plated in triplicates and stimulated with various 2216 subfragments. These experiments were performed at least 4 times with similar results (DC and macrophages) and 3 times with HEK293 PLD3 deficient cells. (h) Recovery and identification of 2216 fragments from macrophage lysates by electrophoresis was performed twice. The similar recovery experiment (i) using HEK293 PLD3 deficient line with or without reconstitution with PLD3 or PLD4 proteins was performed once. Figure 6. Six Pld3+/-Pld4-/- animals were identified and a summary table outlining liver pathology for these animals is given in Supplementary Table 2.

Sera from a total of 12 Pld3-/-Pld4-/- animals was tested for cytokines and compared to 10 C57BL/6, and ranges of 5-7 each littermate genotype controls. Bone marrow macrophages from control or Pld3-/-Pld4-/- were plated in triplicate and stimulated with various TLR ligands. This experiment was repeated three times with similar results. A total of four individual sera from Pld3-/-Pld4-/- animals were compared with controls for ferritin levels in a single elisa. Figure 7. Blood, spleen data from individual recipients 8 weeks after receiving bone marrow from Pld3-/-Pld4-/- mice or littermate controls. This experiment was done once.

**Randomization**
Both male and female animals of either Pld4fl/fl, Pld4-/-, C57BL/6, Tlr9-/-Pld4-/-, Tlr9-/-, Pld3-/- and Pld3-/-Pld4-/- genotypes were used in experiments. They were always age and sex matched with each other in each experiment. In some experiments, Pld3+/-Pld4+/-, Pld3-/-Pld4+/-, or Pld3+/Pld4-/- genotypes (littermates) were used as controls for Pld3-/-Pld4-/- animals (not always sex matched).

**Blinding**
Genotypes were blinded in the liver histology pathology analysis summarized in Supplementary Table 2. Genotypes were not blinded in the other experiments because the quantitative data obtained were not subject to observer bias.
# Reporting for specific materials, systems and methods

## Materials & experimental systems

| n/a  | Involved in the study |
|------|-----------------------|
| □    | Unique biological materials |
| x    | Antibodies |
| x    | Eukaryotic cell lines |
| x    | Palaeontology |
| x    | Animals and other organisms |
| x    | Human research participants |

## Methods

| n/a  | Involved in the study |
|------|-----------------------|
| x    | ChIP-seq |
| x    | Flow cytometry |
| x    | MRI-based neuroimaging |

## Unique biological materials

### Policy information about availability of materials

**Obtaining unique materials**

Unique materials in this manuscript include Pld4 deficient mouse lines and anti Pld4 monoclonal antibodies, Pld3 deficient mice and HEK293hTLR9 Pld3 deficient cells. These will be made available to the scientific community upon request.

## Antibodies

### Antibodies used

A table listing the clones and catalog numbers of monoclonal antibodies used has been provided in Supplementary Data.

### Validation

Pld4 mAb staining was confirmed for specificity by staining cells from Pld4-/- compared to Pld4 WT mice.

## Eukaryotic cell lines

### Policy information about cell lines

**Cell line source(s)**

The eukaryotic cell line 293EBNA was used to generate recombinant soluble PLD3 and PLD4 in this study. This cell line was purchased from Invitrogen. HEK293 Blue hTLR9 cells were purchased from Invivogen.

### Authentication

The 293EBNA cell line was grown in media containing 0.5mg/ml G418. The HEK293 Blue hTLR9 cell line responded to 2006 PS TLR9 stimuli by the production of SEAP as described by Invivogen. No genetic authentication was performed. PLD3 deficient clones generated from parental HEK293 Blue hTLR9 cell line were confirmed by sequencing of PCR products derived from genomic DNA.

### Mycoplasma contamination

The 293 EBNA cell line and the HEK293 Blue hTLR9 cell line have not been tested for mycoplasma.

### Commonly misidentified lines

(See [ILAC register](#))

The 293 EBNA cell line is a derivative of HEK293 cells listed in the commonly misidentified cell line database. Growth in G418 should maintain the EBNA episome to promote EBNA-1 mediated gene replication. This cell line was used to produce recombinant proteins that were later purified, so the genetic origin of the cell line should have little influence on the characteristics of the purified protein used. HEK293 Blue hTLR9 cell line was used to generate PLD3 deficient human cells that could be tested for TLR9 responses. The genetic origin of the cell line should have little influence on the interpretation of the results, as all sub-lines were derived from original parent line purchased from Invivogen.

## Animals and other organisms

### Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

The following laboratory animal strains were used. Pld4-/- and Pld4fl/fl animals were generated in a 129/Ev strain ES cell and backcrossed to C57BL/6J. Pl3-/- mice were generated on the C57BL/6J background. Tlr9 deficient and Unc93b3d/3d mice were generated by ENU mutagenesis on the C57BL/6 background. The following mouse strains bred with Pld4-/- mice including B6.129S7-Rag1tm1Mom/J, B6.129P2-Lys2tm1(cre)Ifo/J, B6.Cg-Tg(Itgax-cre)1-1Reiz/J, B6.129S7-Ifngtm1Ts/J, C57BL/6JTLR9M7Btlr/Mmjax (CpG11) were from the Jackson Laboratory. Unc93b3d/3d mice were obtained from Dr Bruce Beutler. Both male and female mice of ages ranging from 1.5 month to 8 months were used. Pld3-/-Pld4-/- mice or their littermates were generated by interbreeding the Pld3-/- and Pld4-/- lines and were studied between 12 and 21 days of age.

**Wild animals**

No wild animals were used.

**Field-collected samples**

No field-collected samples were used.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Peritoneal macrophages were isolated by flushing the peritoneal cavity with 5 mls of Advanced DMEM media. Tissue suspensions were generated by mashing between frosted glass slides. Bone marrow was released from tibia and femurs by a mortar and pestle crushing method. Red blood cells were lysed with ammonium chloride (0.83%) and cells filtered to generate single cell suspensions. Isolated cells were washed with FACS Buffer (1xPBS with 1%BSA, 10mM HEPES pH8, 1mM EDTA) prior to incubation with 1ug 2.4G2 antibody (Fc block) on ice for 15 minutes before staining.

Instrument
Data collection was performed using BD LSRII analyzers.

Software
Flow cytometry data was acquired on BD LSRII machines using BD Diva software. Data analysis was performed using Flow Jo (Treestar) software or FCS Express 6.

Cell population abundance
DC cell subsets were sorted on a BD FACS Aria machine. Post sort samples were analyzed on the same FACS Aria machine indicating greater than 98% purity for sorted subsets.

Gating strategy
Samples were gated to exclude doublets based on FSC-A vs FSC-W. Plasmacytoid DCs were sorted for CD45RA+CD11c+. CD8+ cDCs were sorted based on being CD45RA-CD11c+,CD8a+,SIRPa-. Peritoneal macrophages were gated to exclude doublets, then based on high FSC, and high levels of F4/80 stain. Peritoneal B1 cells were gated to exclude doublets, then gated on small leukocytes by FSC. B1 cells were determined by B220 lo CD19 hi (IgM+) vs B2 cells which were B220hiCD19hi (IgM+). Bone marrow reconstituted recipients were initially gated on FSCA-vs FSC-W to exclude doublets, followed by size for lymphocyte gates. Only CD45.2+ cells were included for further analysis of B cells (B220+IgM+) or T cells (TCRb+). T cells were further characterized by CD4 or CD8 staining, and CD44 and CD62L staining. These gates are outlined in Fig. S8.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.