

Title: Local amplifiers of IL-4R α -mediated macrophage activation promote repair in lung and liver

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Abstract:

The type 2 immune response controls helminth infection and maintains tissue homeostasis but can lead to allergy and fibrosis if not adequately regulated. We have discovered local tissue-specific amplifiers of type-2 mediated-macrophage activation. In the lung, surfactant protein A (SP-A) enhanced IL-4-dependent macrophage proliferation and activation, accelerating parasite clearance and reducing pulmonary injury following infection with a lung-migrating helminth. In the peritoneal cavity and liver, C1q enhancement of type 2 macrophage activation was required for liver repair following bacterial infection, but resulted in fibrosis following peritoneal dialysis. IL-4 drives production of these structurally related defense collagens, SP-A and C1q, and the expression of their receptor, myosin 18A. These findings reveal the existence within different tissues of an amplification system needed for local type 2 responses.

One Sentence Summary: Specific signals within the tissues are required to enhance IL-4R α mediated macrophage activation and proliferation during infection and injury.

Main Text:

The type 2 cytokines IL-4 and IL-13, which signal through IL-4R α , trigger a specialized macrophage phenotype (M(IL-4)) (1) that promotes control of helminth infection (2) and tissue repair (3, 4). M(IL-4)s also contribute to pathology associated with type 2 immunity, including allergy, asthma, and fibrosis (4). However, little is known about tissue-specific factors that might promote both beneficial and detrimental actions of M(IL-4)s.

In the lung, alveolar macrophages (aM ϕ s), together with the respiratory epithelium, are covered by pulmonary surfactant, a lipid-protein network in which surfactant protein A (SP-A) constitutes the major protein component (5). SP-A is a versatile recognition protein (5) that is a member of a group of secreted soluble defense collagens that include the first component of the complement system (C1q), collectins (e.g., SP-A, SP-D, mannan-binding lectin), ficolins, and

adiponectin (6). Because of its abundance and known role in immune defense (5), we asked whether SP-A was involved in the local regulation of M(IL-4) effector function in the lung.

M(IL-4)s have a critical role in lung repair following infection with the lung-migrating nematode *Nippostrongylus brasiliensis* (3). We therefore infected WT, IL-4R α -deficient and SP-A-deficient mice with *N. brasiliensis* infective larvae. Larvae migrate to the lung where they mature for ~2 days, and reach the small intestine by 3 days post infection (p.i.). The type 2 response peaks 6-7 days after inoculation. We observed an up-regulation of SP-A protein (**Fig. 1A**) and mRNA (**Fig. S1A**) in lungs of *N. brasiliensis* infected C57BL/6 mice at 6 days p.i, which was dependent on IL-4R α . Consistent with a role for SP-A during type 2 immunity to nematode infection, SP-A-deficient mice had greater adult worm burdens (**Fig. 1B**) and egg output (**Fig. S1B**), and significantly impaired lung repair processes (**Fig. 1C, D**) than wild type (WT) mice. The failure to heal was associated with a failure to up-regulate tissue-repair related gene *Coll1a1* (Collagen, type I, alpha 1) (**Fig. 1E**) and increased expression of *Mmp12*, an extracellular matrix-degrading enzyme (**Fig. 1F**). Greater lung damage in SP-A-deficient mice was indicated by increased numbers of red blood cells and neutrophils in bronchoalveolar lavage (BAL) at 6 days p.i (**Fig. 1G, H**). The absence of SP-A resulted in reduced expression of the M(IL-4) proteins RELM α (**Fig. 1I**), Ym1, and arginase (**Fig. S1C,D**) in aM ϕ s. Secretion of RELM α and Ym1 protein into the alveolar fluid (**Fig. S1E**) was also reduced in SP-A-deficient mice compared with WT mice.

Consistent with the known ability of IL-4 to cause macrophage proliferation during helminth infection (7), aM ϕ s from WT mice exhibited significant proliferation (**Fig. 1J, K**) and increased aM ϕ s numbers (**Fig. S1F**) 6 days following *N. brasiliensis* infection. However, SP-A-deficient mice failed to exhibit significantly enhanced aM ϕ proliferation (**Fig. 1J, K**) resulting in fewer total macrophage numbers relative to WT mice (**Fig. S1F**). There was no evidence that differences were caused by a failure of SP-A-deficient mice to mount appropriate ILC2, T_H2 (**Fig. S1G & H**) or local type 2 cytokine responses (**Fig. S1I**). Importantly, aM ϕ s from uninfected SP-A-deficient mice were normal in number, phenotype and ability to respond to IL-4 *ex vivo* (**Fig. S2A-D**).

To ascertain if defects in SP-A-deficient mice were caused by defective IL-4R α responsiveness in vivo, WT and SP-A-deficient mice were injected intra-peritoneally (ip) with IL-4 complex (IL-4c). IL-4c delivery increased the amount of SP-A protein in BAL (**Fig. 1L**) in WT mice and induced M(IL-4) markers and proliferation in aM ϕ s isolated from BAL (**Fig. 1M, N** and **S3A-C**) of WT but not SP-A-deficient mice, reflected by diminished secretion of RELM α and Ym1 to the alveolar fluid in SP-A-deficient mice (**Fig. S3D**). IL-4c delivery decreased IL-4R α expression in both WT and SP-A-deficient aM ϕ s (**Fig. S3E**).

Enhancement of M(IL-4)s may underlie the previously reported contribution of SP-A to tissue integrity in other models of acute lung injury (8, 9). The pro-type 2 effects we report here contrast with reports that associate SP-A with protection in asthma (10). However, in addition to promoting M(IL-4) and proliferation of macrophages, the anti-inflammatory properties of SP-A (5, 6, 8, 9, 11) may suppress the strong inflammatory responses that are responsible for more severe asthma. Our data are supported by the finding that SP-D-deficient mice, which lack SP-A (12), also have reduced M(IL-4) responsiveness (13).

To determine whether SP-A acts directly on aM ϕ s, we first tested the ability of adherence-purified macrophages from the alveolar and peritoneal spaces to proliferate in vitro in response to 1 μ g/ml of IL-4; aM ϕ exhibited significant proliferation, but peritoneal macrophages (pM ϕ) failed to proliferate despite expressing M(IL-4) activation markers (**Fig. S4A**). We then tested the ability of SP-A to enhance IL-4 treatment and included C1q as a control because it is a defense collagen structurally homologous to SP-A (5, 6). We found that SP-A, but not C1q, significantly boosted IL-4-mediated aM ϕ proliferation and M(IL-4) markers (**Fig. 2A**). IL-4R α -deficient aM ϕ showed no proliferation or activation when stimulated with IL-4 (1 μ g/ml) in the absence or presence of SP-A (**Fig. S4B**). Notably, SP-A significantly enhanced proliferation and activation induced by IL-4 in both human (**Fig. S4C**) and rat aM ϕ s (**Fig. S4D**).

To our surprise C1q, but not SP-A, significantly increased IL-4-mediated proliferation and M(IL-4) marker expression in pM ϕ (**Fig. 2A**). To verify these findings in vivo, IL-4c was delivered to C1qa-deficient mice. Consistent with the in vitro studies, mice lacking C1q exhibited reduced IL-4-dependent activation and proliferation in pM ϕ but not aM ϕ (**Fig. 2B**). Similar to SP-A in

the lung (**Fig. 1L**), C1q levels increased in the peritoneal fluid after IL-4c delivery (**Fig. S5A**), indicating that IL-4 drives production of a local signal to amplify its effect on tissue macrophages. Importantly, the pM ϕ phenotype, number, and ability to respond to IL-4 *ex vivo* are normal in C1qa-deficient mice (**Fig. S5 B-F**). Thus, SP-A and C1q were induced by IL-4 in the lung and peritoneal cavity, respectively, where they acted to enhance proliferation and M(IL-4) activation in a tissue-specific manner.

To determine which receptor mediates SP-A effects on IL-4-stimulated aM ϕ s, we inhibited known receptors for SP-A (5). We observed that the blockade of Myosin18A (Myo18A, *aka* SP-R210), but not signal inhibitory regulatory protein α (SIRP α) or calreticulin (*aka* cC1qR), abrogated SP-A-mediated enhancement of IL-4-induced arginase activity in rat aM ϕ s (**Fig. S6A**). Blockade or RNA silencing of Myo18A consistently abolished SP-A-mediated enhancement of IL-4-induced proliferation and activation of mouse (**Fig. 2C**), human (**Fig. S6B**) and rat (**Fig. S6C**) aM ϕ s. Myo18A is an unconventional myosin that does not operate as a traditional molecular motor, having both intracellular and cell-surface locations (14), and was recently defined as CD245 (15). Immune activation results in Myo18A localization on the cell surface, where it binds to the collagen-like domain of SP-A (6, 16) and we confirmed that an intact collagen-like domain is required to enhance IL-4-mediated type 2 responses (**Fig. S6D**).

Because C1q is structurally homologous to SP-A in its supra-trimeric assembly and collagen tail (5, 6), we addressed whether Myo18A was also responsible for the actions of C1q. Indeed, blockade of Myo18A prevented C1q enhancement of IL-4 driven activation and proliferation of pM ϕ (**Fig. 2C**). *In vitro*, IL-4 promoted Myo18A localization on the cell surface of both aM ϕ and pM ϕ (**Fig. S7A, B**), which was maximal 24 hours after stimulation. Cell surface expression of Myo18A was similarly observed *in vivo* following IL-4c delivery (**Fig. S7 C, D**), independent of the presence or absence of SP-A or C1q. We confirmed the role of Myo18A *in vivo* by intranasal or intraperitoneal delivery of anti-Myo18A antibody. Receptor blockade significantly reduced IL-4-induced proliferation and M(IL-4) activation of aM ϕ and pM ϕ (**Fig. 2D**), as well as secretion of RELM α and Ym1 to the alveolar and peritoneal fluid (**Fig. S7E, F**). Thus, Myo18A receptor blockade in the lung or peritoneal cavity phenocopied SP-A or C1qa deficiency, respectively. Together these data indicate that Myo18A is a common receptor or co-receptor for

defense collagens present on aMφs and pMφs, which determines macrophage capacity to respond to IL-4 and whose cell surface expression is itself induced by IL-4. Myo18A lacks a transmembrane domain (14), and thus must act in concert with transmembrane co-receptors for signal transduction that likely impart tissue-specificity.

The relevance of C1q as a local factor to enhance type 2 responses is supported by the fact that unlike most other complement components, the majority of C1q is produced by myeloid cells in peripheral tissues (17). To determine the physiological relevance of C1q in this context, we evaluated its role in a murine model of peritoneal fibrosis (18). Peritoneal fibrosis is a frequent and serious consequence of peritoneal dialysis (19) associated with alternatively activated macrophages in both humans and mice (18-20). We administered Dianeal-PD4, a clinically-used lactate-based dialysate, every other day for 28 days to WT, C1qa⁻, and IL-4Rα-deficient mice. In WT, but not in C1qa-deficient mice, Dianeal-PD4 treatment provoked the induction of C1q (**Fig. 3A**) and morphologic changes in tissue sections of the parietal peritoneum, showing significant enlargement of the submesothelial zone caused by collagen deposition (**Fig. 3B, C**). Dianeal-PD4 treatment induced markers of fibrosis including collagen mRNAs (*Colla1* and *Col3a1*) (**Fig. 3D** and **Fig. S8A**), alpha-smooth muscle actin (*Acta2*) (**Fig. 3E**), and vascular endothelial growth factor (*Vegf*) (**Fig. S8B**). Significant upregulation of these markers was not observed in C1qa-deficient mice. Conversely, *Mmp12* mRNA was up-regulated in C1qa-deficient mice relative to WT mice (**Fig. S8C**) consistent with monocyte infiltration (**Fig. S8D**) (21), and an anti-inflammatory role for C1q (22). Dianeal-PD4 treatment also induced intracellular expression and protein secretion of the M(IL-4) markers RELMα, Ym1, and Arg (**Fig. 3F-H**) and moderate proliferation of pMφs (**Fig. 3I**) in WT but not C1qa-deficient mice. Despite the clear induction of M(IL-4) markers by PD4 delivery and their dependence on C1q, responses of IL-4Rα-deficient mice were equivalent to WT mice (**Fig. 3A-I** and **Fig. S8 A-D**). Although initially surprising, Dianeal-PD4 is a lactate-based solution, and lactate can induce M(IL-4) markers by acting downstream of IL-4Rα through stabilization of HIF1α protein (23). Indeed, we observed that the induction of proliferation and M(IL-4) markers following Dianeal-PD4 delivery was essentially absent in mice lacking HIF1α in macrophages (**Fig. S9**). Together, our data indicate that C1q significantly amplifies peritoneal fibrosis by promoting a type 2 macrophage phenotype driven by lactate and dependent on HIF1α. These results are consistent

with human studies in which C1q is strongly associated with increased fibrosis of skeletal muscle (24).

Critically, we needed to ascertain whether C1q functioned as a type 2 amplifier in tissues beyond the peritoneal cavity and settings that are dependent on IL-4R α . We thus assessed Myo18A expression on the cell surface of resident macrophages from mice treated with or without IL-4c (**Fig. 4A**). Consistent with our functional data, Myo18A was expressed in macrophages from the lung and peritoneal cavity, as well as in liver, spleen and adipose tissue, and significantly increased by IL-4 exposure. In contrast, there was minimal Myo18A on pleural cavity macrophages, which explained our failure to identify a role for C1q in the pleural cavity following IL-4c delivery (**Fig. S10**). As predicted by the Myo18 expression data, we found that macrophages from the liver of C1qa-deficient mice had significantly lower levels of proliferation and M(IL-4) activation compared with WT mice following IL-4c delivery (**Fig. 4B**). Importantly, we observed C1q upregulation in the liver of IL-4c treated mice (**Fig. 4C**). Of relevance, the number and phenotype of liver macrophages are normal in C1qa-deficient mice (**Fig. S11**). Antibody blockade of Myo18A following IL-4 treatment of isolated liver macrophages verified that C1q generated its effects through Myo18A (**Fig. S12**). We thus sought a model in which M(IL-4)s in the liver played a substantive role.

Infection of the liver by the Gram-positive bacteria *Listeria monocytogenes* causes necroptotic death of resident liver macrophages (Kupffer cells) followed by recruitment of monocytes, which control *L. monocytogenes* infection (25) and repopulate the liver macrophage population (26). Following an initial type 1 response, the type 2 response begins at 3 days p.i. with IL-4-mediated activation and proliferation of liver macrophages acting to repair infection damage (26). To ascertain the relevance of C1q and confirm the role of IL-4R α in this process, we infected WT, C1qa- and IL-4R α -deficient mice with *L. monocytogenes* and performed sample analysis 3.5 days later. Infection resulted in IL-4R α -dependent up-regulation of C1q mRNA in liver (**Fig. 4C**) while enhanced expression of RELM α and Ym1 by liver macrophages (**Fig. 4D** and **Fig. S13A**) and macrophage proliferation (**Fig. 4E** and **Fig. S13B**) were dependent on both IL-4R α and C1q. Consistent with the requirement for basophil-derived IL-4 in *L. monocytogenes*-induced liver macrophage proliferation (26), we observed increases in IL-4 and IL-13 cytokines

in liver homogenates (**Fig. S13C**). C1q and IL-4R α deficiency were associated with increased liver injury as assessed by liver transaminases in blood (**Fig. 4F**) and a failure to up-regulate tissue-repair related genes (*Acta2* and *Colla1*) (**Fig. 4G**). Notably, at 3.5 days p.i., liver bacterial burden was higher in WT than in C1qa- or IL-4R α -deficient mice (**Fig. 4H**). Gene-deficient mice had increased numbers of recruited monocytes (**Fig. 4I**) and higher iNOS expression in monocytes (CD11b⁺ Ly6C⁺) and liver macrophages (CD11b⁺ F4/80⁺) (**Fig. S13 D, E**) but pro-inflammatory cytokines in liver homogenates were comparable (**Fig. S13F**). These data suggest that C1q, through its ability to orchestrate IL-4R α -dependent type-2-mediated responses, decreases the bactericidal capacity of monocyte-derived macrophages, but also limits liver injury and promotes the return to homeostasis (**Fig. 4D-I** and **Fig. S13**).

M(IL-4)s have recently emerged as important players in homeostatic processes (27), but IL-4R α -dependent pathways are amplified during helminth infection with uncontrolled amplification leading to fibrosis (4, 28, 29). Our data show that IL-4 drives production of local specific factors (SP-A and C1q) and expression of their receptor (Myo18A) on the macrophage surface for full M(IL-4) activation and proliferation (**Fig S14**). These findings reveal the existence within distinct tissues of an amplification system needed for type 2 function. SP-A and C1q are typically produced by alveolar epithelial type II and myeloid cells, respectively, indicating that several different cell types must respond to IL-4 for signal amplification.

The study raises a number of critical questions. What are the Myo18A co-receptors that mediate tissue-specificity? What factors negatively regulate or stop the positive M(IL-4) loop? What are the intracellular signaling pathways regulated by Myo18A and its co-receptors? Critically, SP-A, C1q, and Myo18A are highly conserved across mammalian species, and we have shown the ability of SP-A and Myo18A to enhance human alveolar M(IL-4) proliferation.

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Author Contributions

C.M.M. designed and performed research, analyzed and interpreted data and wrote the manuscript; L.H.J-J., B.G-F, T.E.S., J.A.K., N.L. and E.R. performed research, contributed to experimental design and manuscript preparation; R.G-P. performed research; D.A.F., A.A., C.S. and Z.C. contributed tools, provided expertise and edited the manuscript; D.M.Z. contributed to project funding, provided expertise and edited the manuscript; C.C. and J.E.A funded and designed the research, organized the project and analyses, and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing financial interests

The authors declare no conflict of interest

SUPPLEMENTARY MATERIALS

Materials and Methods

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FIGURE LEGENDS

Fig. 1. Higher worm burden, greater nematode-induced lung damage and reduced IL-4-induced proliferation and activation in mice lacking SP-A. Samples were assessed 6 days after *N. brasiliensis* (Nb) infection. **(A)** SP-A protein expression in lung tissue of WT and IL-4R $\alpha^{-/-}$ mice. **(B)** Adult larvae in the small intestine. **(C)** Lung damage, quantified by ‘mean linear intercept’ from micrographs of H&E stained lung sections, and **(D)** microscopy of H&E stained lung sections (scale bars, 500 μ m) in WT and SP-A $^{-/-}$ mice. **(E)** Amplification of *Colla1*- and **(F)** *Mmp12*-encoding mRNA in lung tissue. Number of **(G)** red blood cells and **(H)** neutrophils isolated in BAL. Expression of **(I)** RELM α by aM ϕ from BAL. **(J)** BrdU incorporation and **(K)** Ki67 expression by aM ϕ from BAL. Data are representative from two independent experiments (mean \pm SEM; naïve: 3 mice, Nb: 6 mice). **(L-N)** WT and SP-A $^{-/-}$ mice treated with 5 μ g IL-4c (i.p.) at days 0 and 2 and analyzed at day 4. **(L)** Relative SP-A levels in BAL (representative western blot shown) of WT mice treated with IL-4c or PBS. **(M)** RELM α expression and **(N)** BrdU incorporation in aM ϕ . Data pooled from three independent experiments (means \pm SEM) (PBS: 9 mice, IL-4c: 11 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with the untreated/uninfected group. $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$ and $^{\circ\circ\circ}p < 0.001$ when WT vs. SP-A $^{-/-}$ groups are compared.

Fig 2. SP-A and C1q act through Myo18A to enhance IL-4-induced proliferation and activation of alveolar and peritoneal macrophages, respectively. **(A)** Murine macrophages were treated with IL-4 in the presence or absence of SP-A or C1q. BrdU incorporation and Ym1 secretion are shown. **(B)** For aM ϕ s (red), 5 μ g IL-4c was delivered ip at days 0 and 2, and BAL cells analyzed at day 4. For pM ϕ s (blue), 1 μ g IL-4c was delivered ip at day 0, and peritoneal cells analyzed at day 1: BrdU incorporation and RELM α expression are shown. **(C)** Murine macrophages were treated with anti-Myo18A or rabbit IgG plus either IL-4+SP-A (aM ϕ) or IL-4+C1q (pM ϕ). BrdU incorporation and Ym1 secretion are shown. **(D)** Concurrently with IL-4c delivery, some WT mice were treated intra-nasally or ip with either anti-Myo18A or rabbit IgG antibody. BrdU incorporation and RELM α expression are shown in aM ϕ s and pM ϕ s. All statistical analysis was performed by ANOVA followed by the Bonferroni multiple-comparison

test. **(A and C)** Results are presented as means (\pm SEM) from three different cell cultures with at least three biological replicates. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, when compared with untreated cells; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$, when SP-A+IL-4- or C1q+IL4-treated are compared with IL-4-treated; $##p < 0.01$, and $###p < 0.001$, the effect of anti-Myo18A antibody on cells treated with SP-A+IL-4 or C1q+IL4. **(B and D)** Data were pooled from three independent experiments (means \pm SEM) (PBS: 6 mice, other groups: 9 mice). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, when compared with PBS treated mice; $^{\circ}p < 0.05$, when WT vs. C1qa^{-/-} mice treated with IL-4c are compared (B); $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$ when anti-Myo18A vs rabbit IgG treatment is compared in IL-4c-treated mice (D).

Fig. 3. C1q enhances peritoneal fibrosis induced by a lactate dialysate. WT, C1qa^{-/-} or IL-4Rα^{-/-} mice were either untreated (C) or injected ip with Dianeal PD-4 every other day for 28 days. Samples were analyzed a day after the last delivery. **(A)** Total amount of C1q in the peritoneal washes was determined by ELISA. **(B)** Quantification of the thickness of the submesothelial compact zone from **(C)** microscopy of Masson's trichrome stained parietal peritoneum slices (scale bars, 0.1 mm). Amplification of **(D)** *Colla1*- and **(E)** *Acta2* -encoding mRNA in peritoneal tissue. Expression of **(F)** RELMα, **(G)** Ym1, **(H)** Arg and **(I)** Ki67 by pMφs. Results are representative from two independent experiments (means ± SEM) (untreated: 3 mice, PD4: 6 mice). ANOVA followed by the Bonferroni multiple-comparison test or Student's *t*-test (A) was used. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 when compared with control group; °*p* < 0.05, °°*p* < 0.01, and °°°*p* < 0.001 when WT vs. C1qa^{-/-} mice treated with Dianeal PD-4 are compared.

Fig. 4. C1q is required for appropriate macrophage activation in the liver during *Listeria monocytogenes* infection. **(A, B, C)** WT or C1qa^{-/-} mice received 1 μg IL-4c (ip) at day 0, and samples were analyzed at day 1. **(A)** Myo18A expression on the surface of resident macrophages (identified as described in the methods) from the indicated tissues. **(B)** BrdU incorporation and RELMα expression of liver macrophages. **(C)** (upper panel) IL-4-induced amplification of *C1q*-encoding mRNA in the liver. **(C-I)** WT, C1qa^{-/-}, or IL-4Rα^{-/-} mice were left uninfected or received intravenous infection with 10⁴ *L. monocytogenes* (Lm) c.f.u., and samples were assessed at 3.5 days p.i. **(C)** (lower panel) *L. monocytogenes*-induced amplification of *C1q*-encoding mRNA. **(D)** Expression of RELMα by liver macrophages. **(E)** BrdU incorporation by liver macrophages. **(F)** Quantification of ALT and AST in serum. **(G)** Amplification of *Acta2*- and *Colla1*-encoding mRNA in the liver. **(H)** Liver bacterial load. **(I)** Number of monocytes in liver single cell suspensions. Data are representative from two independent experiments (mean ± SEM; naïve: 4 mice, *Lm*: 5 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. **p* < 0.05, and ****p* < 0.001, when compared with the uninfected group; °*p* < 0.05, °°*p* < 0.01, and °°°*p* < 0.001 when WT vs. C1qa^{-/-} or IL-4Rα^{-/-} infected groups are compared.

Figure 1:

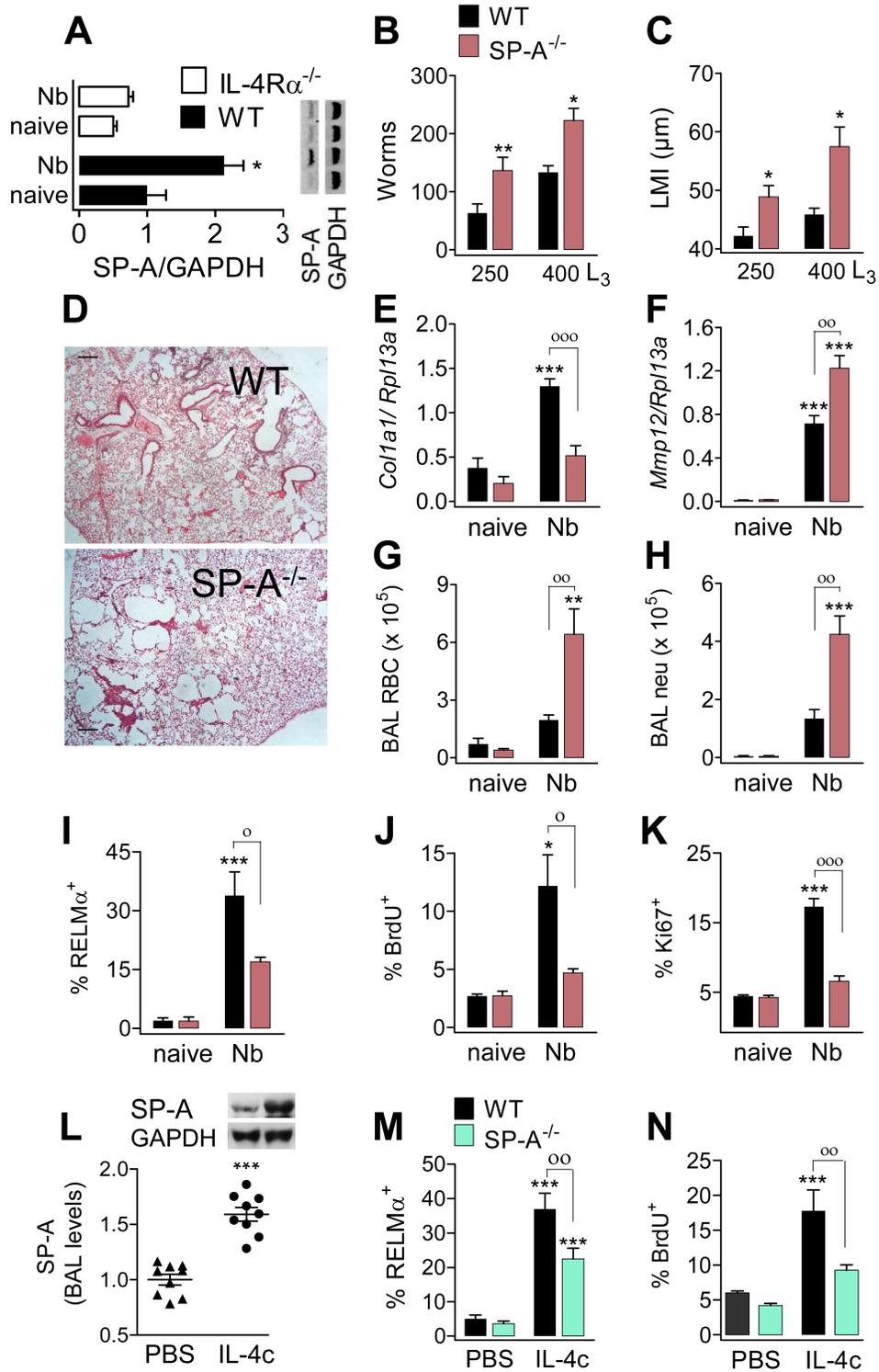


Figure 2:

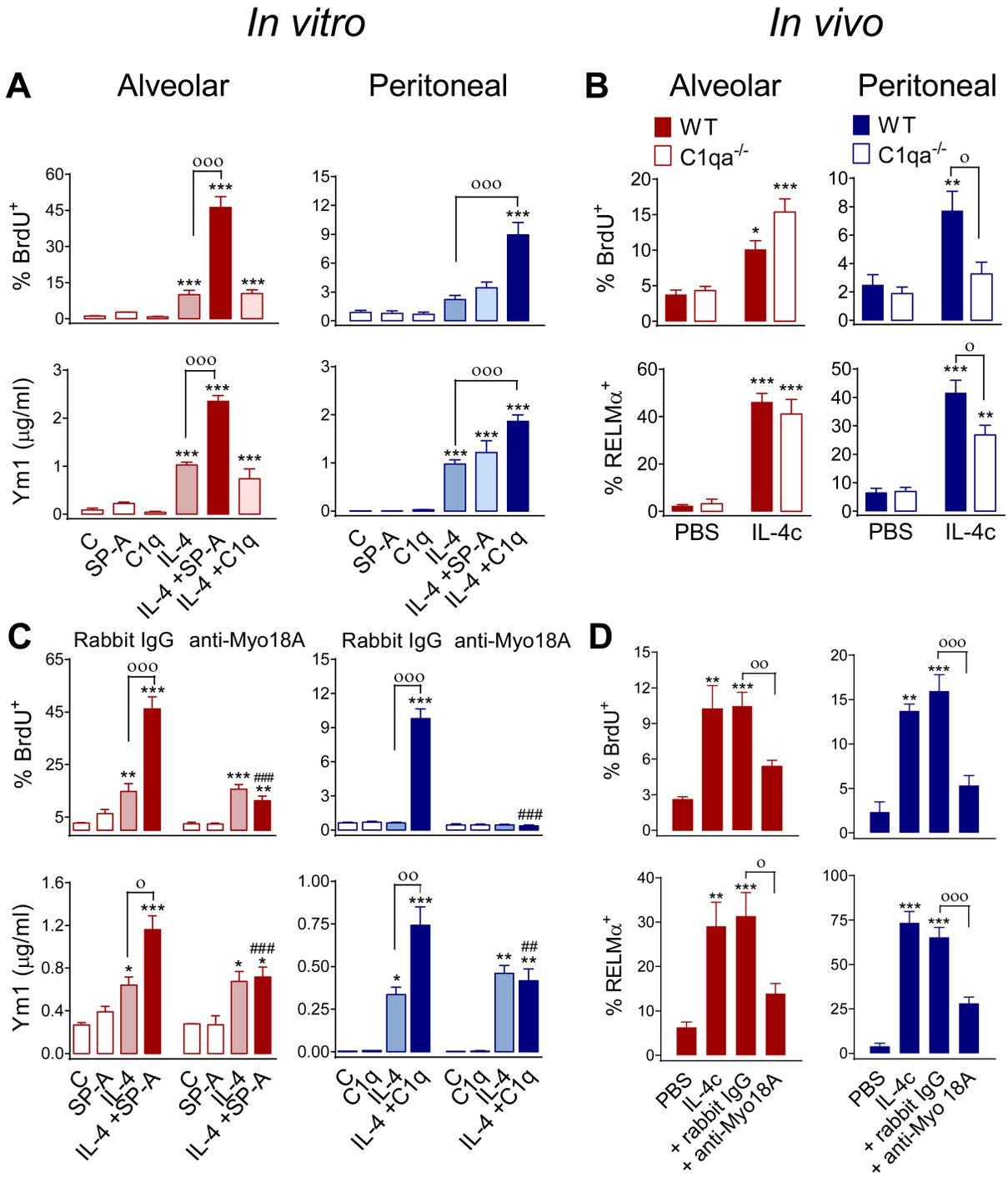


Figure 3:

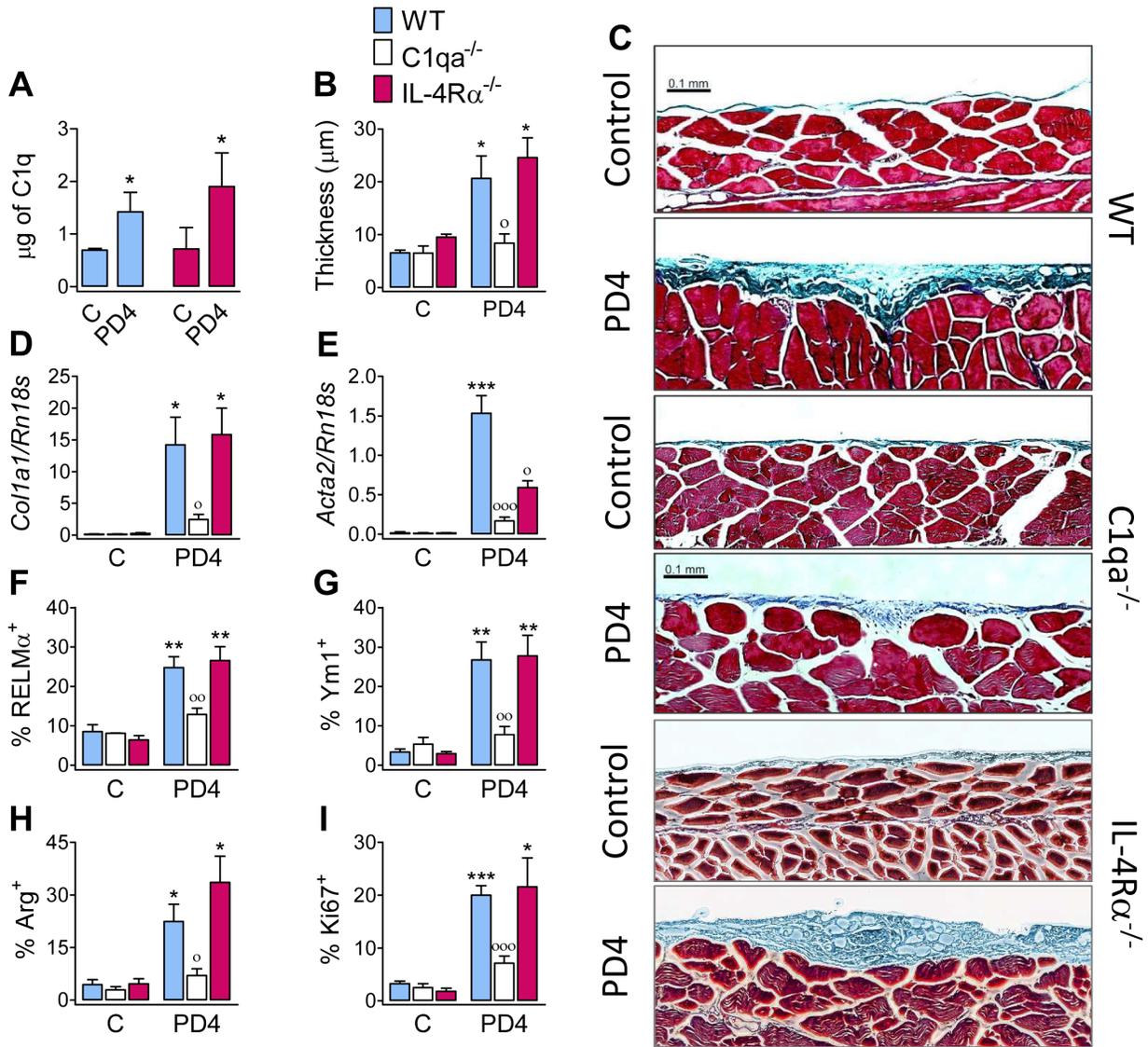
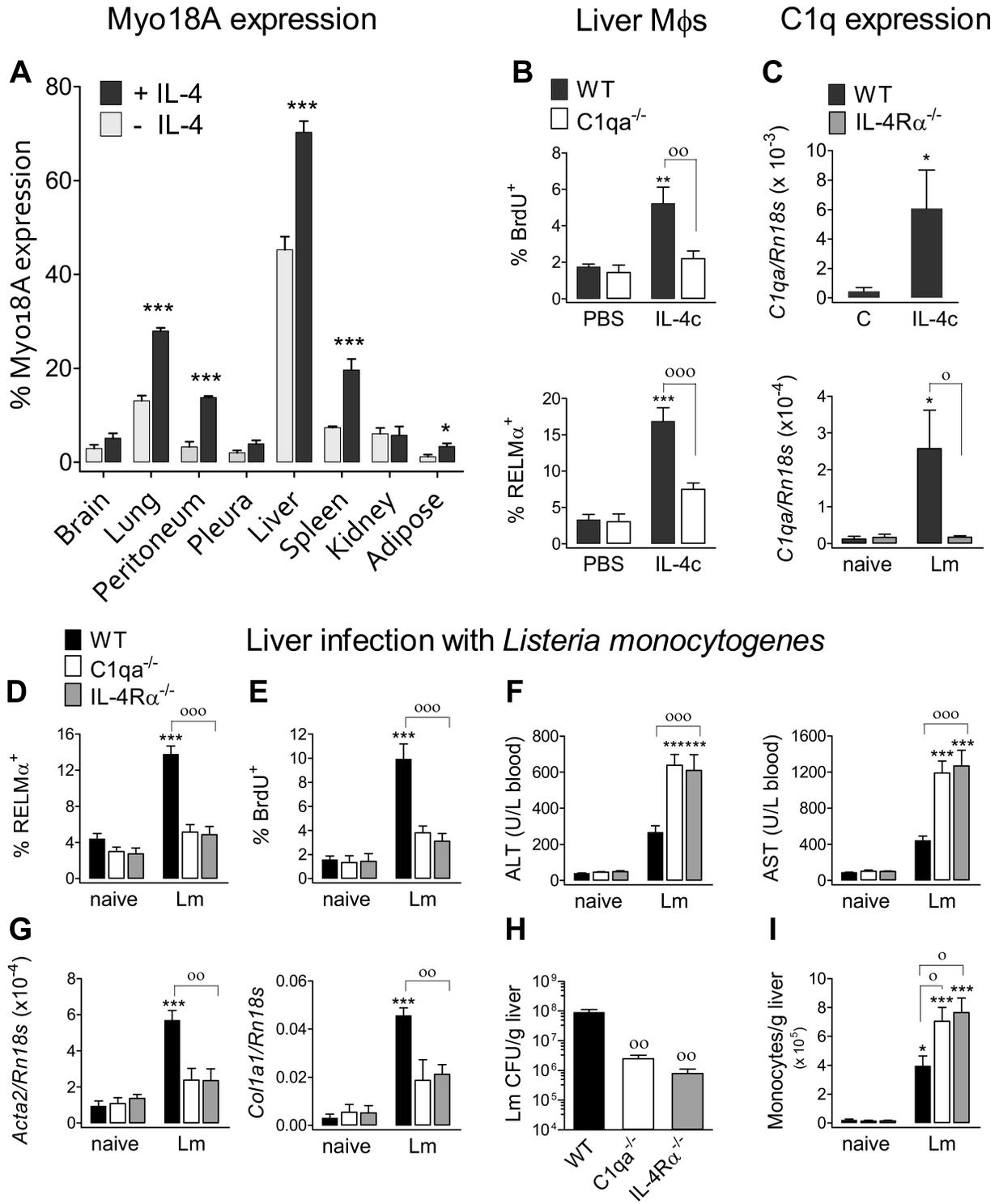


Figure 4:





Supplementary Materials for

Local amplifiers of IL-4R α -mediated macrophage activation promote repair in lung and liver

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Figs. S1- S14

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Materials & Methods

Proteins

Surfactant protein A was isolated from BAL of patients with alveolar proteinosis using a sequential butanol and octylglucoside extraction (11, 30-33). The purity of SP-A was checked by one-dimensional SDS-PAGE in 12 % acrylamide under reducing conditions and mass spectrometry. The oligomerization state of SP-A was assessed by electrophoresis under non-denaturing conditions (31-33), electron microscopy (33), and analytical ultracentrifugation as reported elsewhere (32). SP-A consisted of supratrimeric oligomers of at least 18 subunits. Each subunit had a relative molecular mass (Mr) 36 kDa. Recombinant human SP-A1 (SP-A1^{hyp}) was expressed in insect cells and purified from the medium by mannose affinity chromatography (31, 32). The stability of SP-A1^{hyp} collagen domain was assessed by circular dichroism (31, 32) and differential scanning calorimetry (32). The oligomerization state of SP-A1^{hyp} was evaluated by electrophoresis under non-denaturing conditions (31, 32) and analytical ultracentrifugation (32). SP-A1^{hyp} consists of trimers and hexamers. The endotoxin content of native or recombinant human SP-A was < 0.1 endotoxin units /mg of SP-A as determined by Limulus amoebocyte lysate assay (GenScript, Piscataway, New Jersey). Levels of mouse SP-A from BAL, pleural exudate, or lung tissue were detected by western-blot analysis as reported elsewhere (11, 30-33). Native human C1q was obtained from Abcam (Cambridge, UK). C1q consists of octadecameres with a Mw 410 kDa.

Experimental animals

C57BL/6 WT and gene-targeted mice were used in this study. SP-A (*Sftpa1*^{-/-}) (34), C1q (*C1qa*^{-/-}) (35), IL-4Rα (*Il4ra*^{-/-}) (36) and myeloid-specific HIF-1α (*Hif1a*^{fllox/fllox}; *LysMcre*^{+/-}) (37) deficient mice and WT mice were bred and maintained at the University of Edinburgh in specific-pathogen free conditions. Sex-matched mice were 6-8-weeks old at the start of the experiment, and all mice were housed in individually ventilated cages. *Sftpa1*^{-/-} and *Sftpa1*^{+/+} littermates were genotyped using specific primers (**Supplementary Table 1**). Mice were not randomized in cages, but each cage was randomly assigned to a treatment group. Investigators were not blinded to mouse identity during necropsy; however, the analysis of adult worms, eggs in faeces, lung and peritoneal pathology were performed in a blinded fashion.

Experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and the Spanish guidelines for experimental animals. All researchers were accredited for animal handling and experimentation by the UK and Spanish government Home Office. Dispensation to carry out animal research at The University of Edinburgh was approved by the University of Edinburgh Animal Welfare and Ethical Review Body and granted by the UK government Home Office; as such all research was carried under the project licenses PPL60/4104, PPL70/8548 and PPL70/8470. Sample size was calculated on the basis of the number of animals needed for detection of macrophage proliferation in WT mice, based on published experiments (38-40). Data was not excluded under any circumstances.

Sprague Dawley rats (~350 g) were purchased from Harlan (Indianapolis, IN). All animal experiments were fully compliant with the regulations set by the local ethical committee. Animals were treated according to the Directive 2010/63/EU of The European Parliament and the Spanish act RD53/2013 of 8th February 2013 on protection of animals used for experimentation and other scientific purposes.

***Nippostrongylus brasiliensis* infection**

Mouse-adapted *N. brasiliensis* was maintained by serial passage through C57BL/6 mice, as described previously (41). Mice were infected subcutaneously with 250 or 400 *N. brasiliensis* third-stage larvae. Analysis of samples was performed at day 6 post-infection. Egg output was analyzed in faeces and adult worm burden was determined by removing the small intestine and exposing the lumen by dissection. For macrophage proliferation analysis, mice were injected with 100 µl of 10 mg/ml BrdU in Dulbecco's phosphate buffered saline 3h before experimental end-point. The lungs and pleural cavities were washed to obtain the bronchoalveolar lavage (BAL) and pleural exudate. Subsequently, the right lung was perfused and fixed for histology. Alternatively, one section of the left lung was stored for mRNA quantification; another section was homogenized to obtain single cell suspensions for flow cytometry analysis, and a third section was stored for SP-A quantification. In this case, lung tissue was homogenized in HBSS containing protease inhibitor cocktail (Sigma). SP-A was detected in both lung homogenates and BAL by western blot analysis using an anti-mouse SP-A (GeneTex, Inc, Irvine, CA). SP-A levels were normalized by BAL

volume or GAPDH. Only samples on which the same BAL volume was recovered were used for SP-A quantification.

BAL cells were obtained by washing the lung with Dulbecco's phosphate buffered saline containing 0.5% BSA (m/v). Cells from pleural or peritoneal exudate were obtained by washing either pleural or peritoneal cavity with RPMI 1640 containing 2 mM L-glutamine, 200 U/ml penicillin, 100 µg/ml streptomycin. Single cell suspensions of thoracic lymph node tissue were re-stimulated *ex vivo* with *N. brasiliensis* excretory secretory antigen (42) (1 µg/ml) or anti-CD3 (1 µg/ml), and cell supernatants were analysed by ELISA 72 h later.

***Listeria monocytogenes* infection**

Listeria monocytogenes infections were performed as previously described (26). Briefly, frozen stocks of *L. monocytogenes* (Lm10403s) were thawed and then diluted in fresh Brain-Heart-Infusion (BHI) medium to reach mid-log growth phase. Mice were intravenously injected via the lateral tail vein with 1×10^4 *L. monocytogenes* c.f.u. suspended in 200 µl of PBS. Analysis of samples was performed at day 3.5 post-infection. The livers were perfused *in situ* with phosphate-buffered saline (PBS) before collection. For macrophage activation and proliferation analysis, mice were injected with 100 µl of 10 mg/ml BrdU in Dulbecco's phosphate buffered saline 3h before experimental end-point. One section of the liver was homogenized to obtain single cell suspensions for flow cytometry analysis, a second section of the liver was stored for mRNA quantification; a third section was stored for cytokine quantification by ELISA; and a fourth section was homogenized to analyze bacterial burden. To analyze cytokine secretion by ELISA, the liver tissue was weighed before homogenization in Hank's balanced salt solution (HBSS) containing protease inhibitor cocktail. To quantify bacterial burden, serial dilutions of cell suspensions in PBS were plated on BHI agar plates. After 24 hr of incubation at 37°C, colony-forming units were counted. To analyze liver damage, blood was collected from the inferior vena cava and alanine transaminase (ALT) and aspartate transaminase (AST) levels were determined in the serum.

Peritoneal fibrosis model

Peritoneal fibrosis was induced by continuous administration of Dianeal PD-4 (Baxter, Deerfield, Illinois), as described previously (18) with slight modifications.

Mice received a total of 5 or 14 injections of 500 μ l (~200 ml/kg) of Dianeal PD-4 (ip) on alternate days. Animals were sacrificed a day after the last delivery. The right section of the parietal layer of the peritoneum was collected for histology analysis. The left section of the peritoneum was stored in RNAlater for subsequent analysis. Peritoneal macrophages were isolated from the peritoneal cavity as described below. Note: Although timing of macrophage activation and proliferation in this model varied slightly between males and females all animals developed peritoneal fibrosis after 14 injections.

Histology

The right lung lobes were perfused and fixed with 10% neutral buffered formalin, incubated overnight and transferred to 70% ethanol. Lungs were paraffin-embedded, sectioned and stained with H&E. Linear means intercept (Lmi) method was used to quantify emphysema like damage (43). To calculate Lmi, 20 random non-overlapping fields (magnification x200) per H&E stained lung sample were taken. Six horizontal lines were drawn across each image (ImageJ 1.44) and the total number of alveolar wall intercepts counted per line. The length of each line was then divided by the number of intercepts to give the Lmi value. Images that included large bronchi and vessels were avoided and analysis was performed in a blinded and randomised fashion. With respect to histological analysis of the peritoneum tissue, the right parietal layer of the peritoneum was fixed with 10% neutral buffered formalin, incubated overnight and transferred to 70 % ethanol. Subsequently, peritonea were paraffin-embedded, sectioned and stained for Masson's trichrome. The thickness of the submesothelial compact zone was measured using ImageJ and this value was used to score the extent of peritoneal fibrosis. Five independent (\times 200) images were taken to examine the overall section. For each image, six horizontal lines were randomly drawn across the submesothelial compact zone to measure its thickness and the average value of each sample was used for analysis.

IL-4 complex delivery and *in vivo* blocking of Myo18A

IL-4 was delivered as a 2:1 molar ratio of recombinant mouse IL-4 (Peprotech) and anti-IL-4 mAb (clone 11B11; BioXcell,) (38, 39). For pulmonary macrophage analysis, mice were injected ip with IL-4 complex (IL-4c) containing 5 μ g of IL-4 and 25 μ g of 11B11, or PBS vehicle control on days 0 and 2. Simultaneously, 50 μ g of

anti-Myo18A neutralizing antibody (provided by Dr Zissis C. Chronos (16)) or Rabbit IgG (R&D Systems) were delivered intra-nasally on days 2 and 3, and samples were collected on day 4. For studies of peritoneal macrophages, liver macrophages, and other tissue resident macrophage populations, mice received a single ip injection of 1 µg of IL-4 and 5 µg of 11B11, or PBS vehicle control. Anti-Myo18A neutralizing antibody (16) or Rabbit IgG (100 µg) was delivered 2 hours before IL-4c injection and samples were collected 24 hours later. Mice received a pulse of BrdU 3 h before experimental end-point. Bronchoalveolar, pleural and peritoneal cavity lavages as well as lung and liver tissue collection were performed as described above.

RNA extraction and quantitative real-time PCR

A section of the left lung, liver or parietal peritoneum was stored in RNAlater (Ambion, Carlsbad, CA). Tissue was homogenised in Trizol (Invitrogen) with a TissueLyser (Qiagen, Hilden, Germany). Similarly, human alveolar macrophages were collected with Trizol, and RNA was prepared according to manufacturer's instructions. Reverse transcription was performed using 1 µg of total RNA, 50 U Tetro reverse transcriptase (Bioline, London, UK), 40 mM dNTPs (Promega, Fitchburg, WI), 0.5 µg Oligo dT15 (Roche, Basel, Switzerland), and RNasin inhibitor (Promega). Transcript levels of genes of interest were measured by real-time PCR with the Lightcycler 480 II system (Roche) using SYBR Green I Master kit and specific primers (**Supplementary Table 2**) as previously described (43). Transcript levels of C1qa mRNA were measured using Taqman Master kit and the primers Mm00432142 (Applied Biosystems). PCR amplification was analyzed using 2nd derivative maximum algorithm (LightCycler 480 Sw 1.5, Roche) and the expression of the gene of interest was normalized to a housekeeping gene *Rn18s*, *Rpl13a*, or *GAPDH*. In the case of human alveolar macrophages, cells from at least 8 humans were used for all groups.

Flow Cytometry

Single cell suspensions from left lung, liver and other tissues were prepared by digesting with 80 U/ml DNase (Life Technologies, Carlsbad, California) combined with 0.2 U/ml liberase TL (Roche) in HBSS at 37°C for 30 minutes. In some cases, liberase was combined with collagenase B (0.2 mg/ml) and D (0.4 mg/ml) (Roche). Tissue was homogenized by forcing through a 70 µM cell strainer. Cells from tissue

homogenates, BAL, pleural exudate, and peritoneal cavity exudate were treated with red blood cell lysis buffer (Sigma) and counted using an automated cellometer T4 (Peqlab). Cells were incubated with Fc block (CD16/CD32 and mouse serum) (BD Biosciences, Franklin Lakes, NJ) and stained with fluorescent conjugated antibodies to CD19 (clone 6D5; Biolegend, Cambridge, UK), Siglec F (clone E50-2440; BD Biosciences), Ly6G (clone 1A8; Biolegend), CD3 (clone 17A2; Biolegend), CD45.2 (clone 104; Biolegend), Ly6C (clone HK1.4; Biolegend), CD11c (clone N418; Biolegend), CD11b (clone M1/70; Biolegend), F4/80 (clone BM8, eBiosciences, Hatfield, UK), I-A/I-E (MHCII) (clone M5/114.15.; eBiosciences), CD102 (clone 3C4 (MIC2/4); Biolegend), GATA6 (ref. D61E4; Cell Signaling, Danvers, MA), TER119 (clone TER-119; Biolegend), CD4 (clone RM4-5; Biolegend), FcεR1 (clone MAR-1; Biolegend), NK-1.1 (clone PK136; Biolegend), CD90.2 (clone 30-H12; Biolegend), ICOS (clone C398.4A; Biolegend) and unconjugated anti-Myo18A antibody (16) (5 µg/ml) or isotype control followed by secondary reagents (Invitrogen). The following surface markers identified alveolar macrophages: lineage⁻ (CD19, Ly6G, CD3 and TER119), Ly6C⁻, CD45.2⁺, CD11c⁺ and SiglecF⁺. Peritoneal, pleural, and liver macrophages as well as other tissue resident macrophages were identified as lineage⁻ (CD19, SiglecF, Ly6G and CD3), CD11c⁻, Ly6C⁻, CD45.2⁺, CD11b⁺ and F4/80⁺ or ^{high} (38). ILC2 cells were identified as lineage⁻ (CD3, CD11b, CD11c, FcεR1, CD19, and NK1.1) CD90.2⁺ ICOS⁺ CD45.2⁺ cells. T helper cells were identified as CD45.2⁺, CD3⁺ and CD4⁺. Neutrophils were identified as CD45.2⁺, CD11b⁺ and Ly6G⁺. Monocytes were identified as CD45.2⁺, CD11b⁺ and Ly6C⁺.

Following surface staining, cells were fixed with 2% paraformaldehyde in Dulbecco's phosphate buffered saline for 20 min at room temperature, permeabilized with Perm wash (BD Biosciences), and then stained with anti-RELMα, biotinylated anti-Ym1, APC-conjugated anti-Arg-1 (R&D Systems, Minneapolis, MN), or isotype control followed by secondary reagents (Invitrogen). For detection of Ki67 and measurement of BrdU incorporation, cells were stained for surface markers, then fixed and permeabilized using FoxP3 staining buffer set (eBioscience), and subsequently stained with Ki67 set (clone BV56, BD Biosciences) or anti-BrdU (clone BU20a, Biolegend) for 30 min at room temperature. Cells were incubated first with or without DNase for 30 mins at 37°C before staining with anti-BrdU antibody. For intracellular cytokine staining, lung single cell suspensions were re-stimulated ex vivo in complete RPMI1640 media (supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml

penicillin, 100 µg/ml streptomycin) containing 1 µg/ml ionomycin, 500 ng/ml PMA, and 10 µg/ml Brefeldin A for 4 hours at 37°C. Following surface staining and fixing, cells were intracellularly stained with anti-IL-4 (clone 11B11, Biolegend), IL-13 (clone eBio13A; eBiosciences), IL-5 (clone TRFK5; Biolegend), and relevant isotypes. Expression of RELM α , Ym1, Arg-1, Ki67, IL-4, IL-13, and IL-5 was determined relative to isotype control staining. Incorporation of BrdU was determined relative to staining of non-DNase treated cells. Live/Dead (Life Technologies) was used to exclude dead cells from analysis. Samples were analyzed by flow cytometry using Becton-Dickinson FACS LSR II and FlowJo software.

ELISAs

Quantification of RELM α (PeproTech, Rocky Hill, NJ) and Ym1 (R&D Systems) were performed by ELISA in BAL, pleural and peritoneal exudates, as well as in cell supernatants of *ex vivo* cultures of peritoneal or alveolar macrophages. IL-4, IL-5, IL-13, TNF- α , and IFN- γ (BioLegend and eBioscience) were measured in supernatants of *ex vivo* re-stimulated thoracic lymph node cells and liver homogenates. C1q quantification was performed by ELISA (Source BioScience, Nottingham, UK) in peritoneal cavity exudates. ELISA assays were performed following manufacturer instructions.

Isolation and culture of primary alveolar, peritoneal, and liver macrophages

Alveolar macrophages were obtained from BAL of mice and rats, and from human lung biopsies obtained from patients that were submitted to a lobectomy. An informed consent was obtained from all donors. The review board and the ethics committee of the Sabadell Hospital approved this study, which was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. BAL was performed with Dulbecco's phosphate buffered saline with or without 0.2 mM EDTA and 0.5% BSA (m/v). Macrophages were purified by adherence for at least 90 min at 37°C, and 5% CO₂ as previously reported (44). Adherent cells were 94.0 \pm 1.1 % viable (trypan blue exclusion test). Flow cytometry analysis determined that 90 \pm 1 % of adherent cells isolated from BAL were CD11c and SiglecF positive. To estimate the purity of isolated human macrophages, cells were cytopun in a CytoSpin 3 Cytocentrifuge (Shandon Scientific, Waltham, MA), and the cytopun preparations were stained either with anti-CD68 or Diff-Quick kit (Diagnostics Grifols, Barcelona,

Spain) following the manufacturer's protocol. $95 \pm 1.5 \%$ ($n=6$) of adherent cells were macrophages.

Peritoneal macrophages were obtained from mice by washing the peritoneal cavity with RPMI 1640 containing 2 mM L-glutamine, 200U/ml penicillin, 100 μ g/ml streptomycin. For thioglycollate-induced macrophages, mice were injected with 0.4 ml of 4% Brewer's thioglycollate (Sigma) for 48–96 h before peritoneal cells were harvested. Cells were separated from the lavage fluid by centrifugation (250 x g, 5 min), resuspended in RPMI 1640 medium (5% heat-inactivated FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, supplemented with glutamine 2 mM), and purified by adherence. Viability of adherent cells was assessed by trypan blue exclusion test. Flow cytometry analysis determined that $90 \pm 1 \%$ of adherent cell isolated from the peritoneal exudate were F4/80 and CD11b positive.

Liver macrophages were isolated from mouse livers as reported previously (45) with some modifications. Before collection of the tissue, mice were culled and bled by cutting the inferior vena cava. Subsequently the liver was perfused in situ with PBS. The liver was then excised and kept in HBSS. Subsequently, the organ was minced to small pieces and digested with collagenase B (0.2 mg/ml), collagenase D (0.4 mg/ml) (Roche) and 80 U/ml DNase (Life Technologies, Carlsbad, California) in HBSS at 37°C for 30 minutes. Digested tissue was filtered through a cell strainer (70 μ m) to prepare a single cell suspension. The filtrate was centrifuged twice at 300xg (4°C) for 5 min to wash out the residual enzymatic solution. The pellet was resuspended and centrifuged at 50xg (4°C) for 3 min to separate non-parenchymal from parenchymal cells. The pellet containing parenchymal cells was discarded. The supernatant was centrifuged again at 300xg for 5 min. The cell pellet was seeded on 96-well culture plates in complete culture medium [RPMI 1640 medium (10% heat-inactivated FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, supplemented with glutamine 2 mM)]. Following incubation for 2 h in a humidified atmosphere of 95% air with 5% carbon dioxide (CO₂) at 37°C, the cells were gently washed with fresh culture medium. Viability of adherent cells was assessed by trypan blue exclusion test. Flow cytometry analysis determined that $70 \pm 5 \%$ of adherent cells isolated from the liver homogenates were F4/80 and CD11b positive.

In vitro stimulation of macrophages

Macrophages were pre-cultured for 24 h in RPMI 1640 medium with 5% FBS. Subsequently, cells were treated with IL-4 (0.5-1 µg/ml) (ImmunoTools, Berlin, Germany) and/or SP-A (25, 50 and 100 µg/ml) or native human C1q (10 and 100 µg/ml). The following blocking antibodies were added 2 hours before stimulation: 10 µg/ml of anti-Myo18A (16), 10-50 µg/ml anti-SIRPα (eBioscience), and 10-50 µg/ml anti-calreticulin (Thermo Scientific). Under these conditions cell viability was higher than 97%. Macrophage cultures were plated in triplicate wells and each series of experiments was repeated at least three times.

Cell proliferation assays

For 5-ethynyl-2'-deoxyuridine (EdU)/BrdU incorporation analysis, cells were treated with IL-4, SP-A, C1q and combinations thereof for 24 hours. Then, cells were exposed to 10 µM EdU/BrdU for another 24 hours. For confocal microscopy analysis of EdU incorporation, cells were fixed with 2% formaldehyde for 15 minutes at room temperature and permeabilized with 0.2% saponin in PBS. EdU was detected with Alexa Fluor 647-azide using Click-iT EdU assay kit (Life Technologies). Sequential double immunostaining was performed with a monoclonal antibody to CD11c (AbD Serotec, Kidlington, UK), and immune complexes were visualized with FITC-conjugated secondary antibody (Invitrogen). Micrographs were taken with a Leica TCS SP2 Confocal System. Flow cytometry analysis of BrdU and Ki67 expression was performed as described above.

Arginase activity assay

Arginase activity was measured as previously reported (46). Briefly, rat alveolar macrophages were lysed with 50 µl of 50 mM Tris-HCl pH 7.5, Triton X-100 0.1 %, 1 mM benzamidine, 200 µg/ml aprotinin, and 200 µg/ml leupeptin. After 30 min shaking at 4°C, arginase was activated with 50 µl of 10 mM MnCl₂ and 50 mM Tris-HCl, pH 7.5, for 10 min at 55° C. L-arginine hydrolysis was measured by incubating the cell lysate with 25 µl of 0.5 M L-arginine (Sigma) (pH 9.7) at 37°C for 1 h. The reaction was stopped by addition of 200 µl H₂SO₄ /H₃PO₄ /H₂O (1:3:7 v/v). The produced urea was quantified at 570 nm after addition of 25 µl of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 99°C for 45 min. Urea production was normalized to cell number for each treatment by

quantifying cells with the WST-1 reagent (Roche), following manufacturer's instructions. One unit of arginase activity is defined as the amount of enzyme that catalyses the formation of 1 μ mol urea per min.

siRNA-mediated gene-silencing effects *in vitro*

After isolation, primary alveolar macrophages were resuspended in Amaxa® mouse macrophage nucleofector solution (Lonza) and nucleofected with 100 nM siRNA using a nucleofector 2b device (Lonza). Experiments were conducted using two Stealth siRNAs directed against rat Myo18A (RSS322720 and RSS322721) (Applied Biosystems, Carlsbad, California). Medium GC Stealth siRNA was used as control (12935300) (Applied Biosystems). Myo18A expression was detected by Western blot analysis with an anti-Myo18A antibody (16). After 48 hours post nucleofection, Myo18A expression was reduced $72 \pm 4\%$ for RSS322720 and $71 \pm 5\%$ for RSS322721 compared to control. At this time-point, cells were stimulated.

Statistics

Normal distribution of data was determined by visual examination of residuals. Statistical evaluation of different groups was performed either by analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test or by unpaired two-tailed Student's t-test, as indicated. An α level $\leq 5\%$ ($p \leq 0.05$) was considered significant. All statistical calculations were performed using PRISM, (Graphpad, La Jolla, CA).

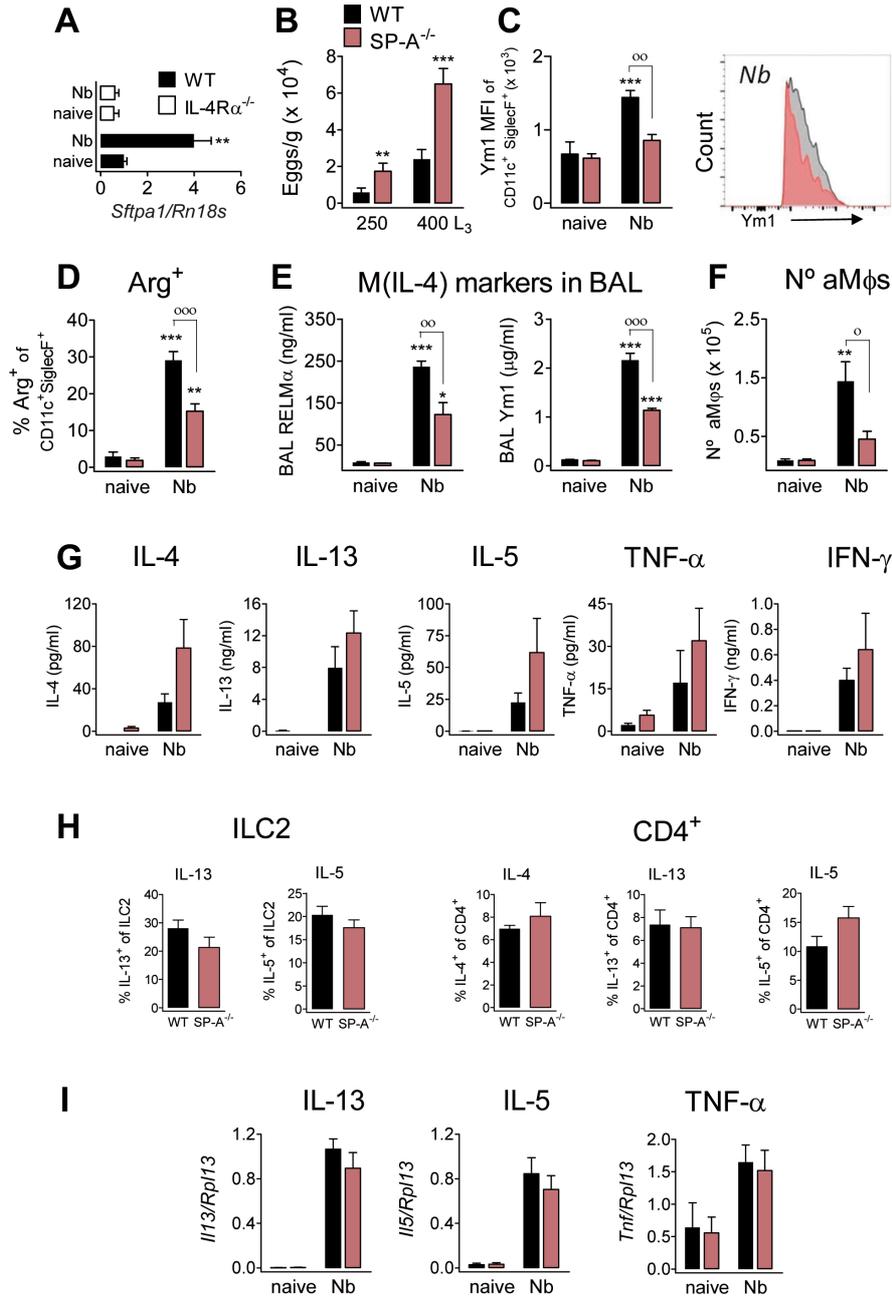


Fig. S1. Further characterization of WT vs. SP-A-deficient mice during *Nippostrongylus brasiliensis* infection. WT or SP-A^{-/-} mice were left uninfected or infected with 250 *N. brasiliensis* L3's, and assessed at day 6 after infection. **(A)** SP-A mRNA expression in lung tissue of WT and IL-4Rα^{-/-} mice. **(B)** Egg output in faeces form WT and SP-A^{-/-} mice. Expression of **(C)** Ym1 and **(D)** Arg by aMφ. **(E)** Analysis of secreted RELMα and Ym1 from BAL by ELISA. **(F)** Number of alveolar macrophages recovered in BAL. **(G)** Levels of IL-4, IL-13, IL-5, TNFα, and IFN-γ in supernatants of thoracic lymph node cells from WT and SP-A^{-/-} mice, cultured with *N. brasiliensis* antigen (1 μg/ml); results are normalized to those obtained for cells cultured with medium alone. **(H)** Expression of IL-13 and IL-5 by ILC2 and IL-4, IL-13 and IL-5 by CD4⁺ cells from single-cell suspensions of lung tissue stimulated *ex vivo* with PMA and ionomycin. **(I)** Amplification of *Il13*, *Il5* and *Tnfr*-encoding mRNA in lung homogenates is also shown. Results are representative from two independent experiments (means ± SEM) (naive: 3 mice, Nb: 6 mice). ANOVA followed by the Bonferroni multiple-comparison test or Student's *t*-test (F) was used. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001, when compared with the uninfected group; °*p* < 0.05, °°*p* < 0.01 and °°°*p* < 0.001 when WT vs. SP-A^{-/-} infected groups are compared.

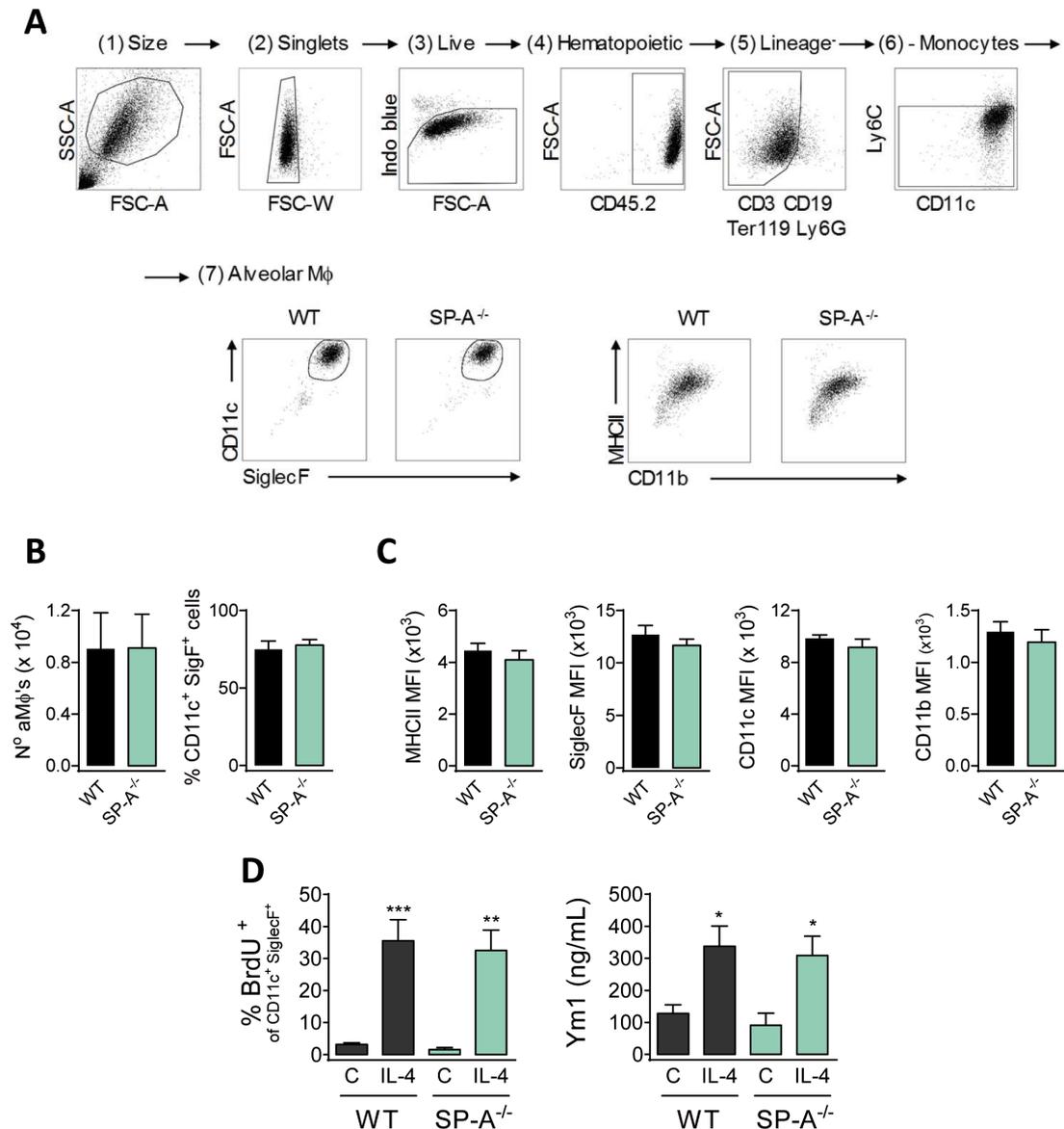


Fig. S2. Characterization of alveolar macrophages from SP-A-deficient mice. (A) Sequential identification of aMφs by flow cytometry. Cells first identified by size (1), and then as singlets (2) and live cells (3). Subsequently, hematopoietic cells were selected by CD45.2 expression (4). Lin⁻ cells (5) were obtained by gating out CD3⁺, CD19⁺, Ly6G⁺, and Ter119⁺ cells (T cells, B cells, neutrophils and red blood cells, respectively). Monocytes were successively excluded by expression of Ly6C (6). Lin⁻ and Ly6C⁻ populations were further subgated on the basis of the expression of CD11c versus SiglecF. aMφ are identified as CD11c^{hi} and SiglecF^{hi} (7); a representative population of aMφ from WT and SP-A-deficient mice is shown. The expression of MHCII and CD11b by aMφ is also shown for both strains. All shown gates are children of the parent gates shown previously. (B) Number and percentage of aMφ recovered in BAL. (C) Expression of MHCII, SiglecF, CD11c and CD11b in aMφ from WT and SP-A-deficient mice. Data are representative from two independent experiments (mean ± SEM; WT: 4 mice, SP-A^{-/-}: 3 mice). Student's *t*-test was used. (D) Purified aMφ from WT and SP-A-deficient mice were treated with or without IL-4 (1 μg/ml) and exposed to BrdU for proliferation analysis. BrdU incorporation and secretion of Ym1 by resident aMφ are shown. The results are presented as means (± SEM) from two different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001, when compared with untreated macrophages.

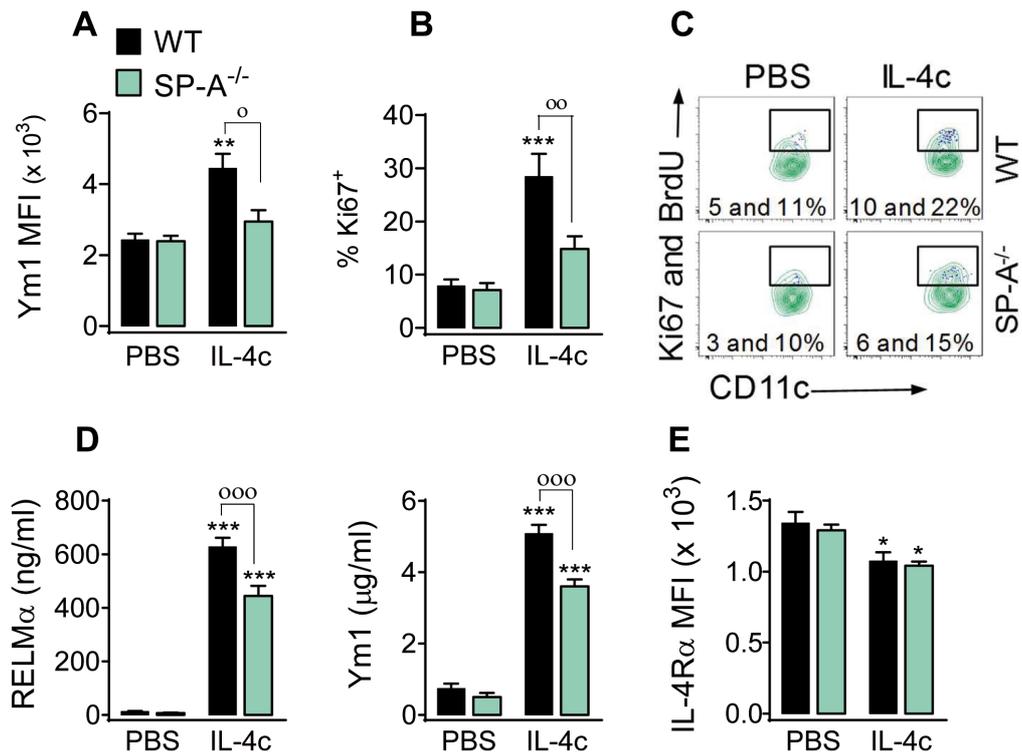


Fig. S3. Alveolar macrophages from SP-A-deficient mice show decreased IL-4-induced proliferation and M(IL-4) activation. WT and SP-A^{-/-} mice were treated with 5 μ g of IL-4c (i.p.) at days 0 and 2; and samples were analyzed at day 4. **(A)** Ym1 and **(B)** Ki67 expression in aM ϕ in response to IL-4c. In **(C)** a representative FACS plot from WT and SP-A^{-/-} mice is shown to demonstrate overlap between BrdU⁺ and Ki67⁺ cells treated with PBS or IL-4c (Ki67 green contour and BrdU⁺ cells blue dots). **(D)** Protein levels of RELM α and Ym1 in BAL measured by ELISA. **(E)** Expression of IL-4R α by aM ϕ from BAL. Data were pooled from three independent experiments (means \pm SEM) (PBS: 9 mice, IL-4c: 11 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with the untreated group. $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$ and $^{\circ\circ\circ}p < 0.001$ when WT vs. SP-A^{-/-} groups are compared.

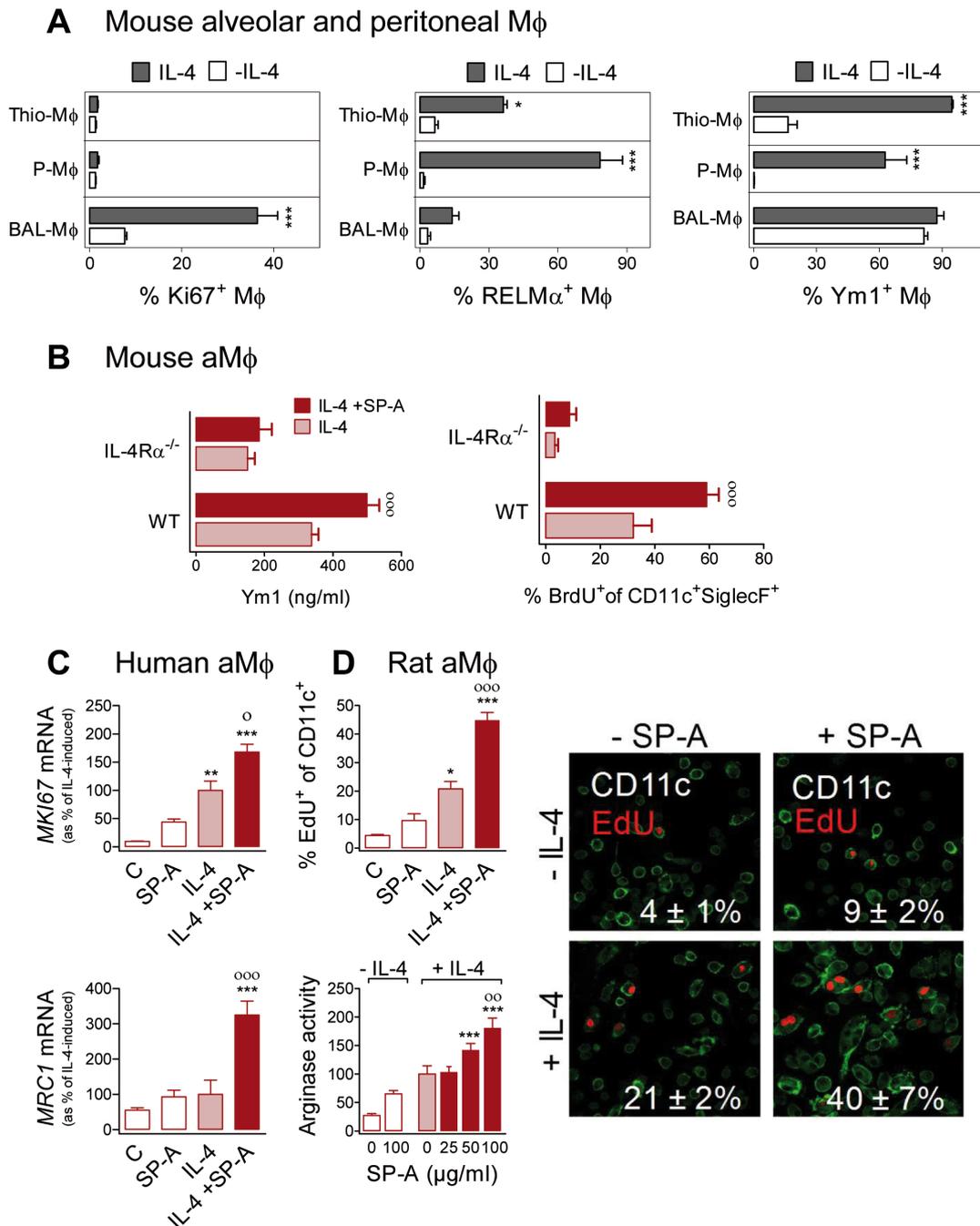


Fig. S4. Proliferation and activation of macrophages isolated from mice, humans, and rats. (A) Purified alveolar (BAL-M ϕ), peritoneal [resident (P-M ϕ)] and thioglycollate-recruited (Thio-M ϕ) from mice were treated with or without IL-4 (0.5-1 μ g/ml). Ki67, RELM α , and Ym1 expression was assessed by FACS. Note: cultured aM ϕ are Ym1⁺ regardless of IL-4 treatment. Purified aM ϕ from mice (B), humans (C), or rats (D) were treated with or without IL-4 (0.5-1 μ g/ml) in the presence or absence of SP-A. (B) BrdU incorporation and secretion of Ym1 by cultured aM ϕ from WT and IL-4R α ^{-/-} mice in the presence and absence of SP-A. (C) *MKI67* and *MRC1* mRNA expression by human aM ϕ as measured by qRT-PCR. (D) EdU incorporation analyzed by flow cytometry and arginase activity of rat aM ϕ . A representative confocal micrograph of rat aM ϕ immuno-stained with CD11c-FITC and EdU-Alexa Fluor 647 is shown. Numbers below indicate percentage \pm SEM of EdU⁺ cells from three independent experiments. The results are presented as means (\pm SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. * p < 0.05, ** p < 0.01, and *** p < 0.001, when compared with untreated macrophages; $^{\circ}p$ < 0.05, $^{\circ\circ}p$ < 0.01, and $^{\circ\circ\circ}p$ < 0.001, when SP-A+IL-4-treated macrophages were compared with IL-4-treated macrophages.

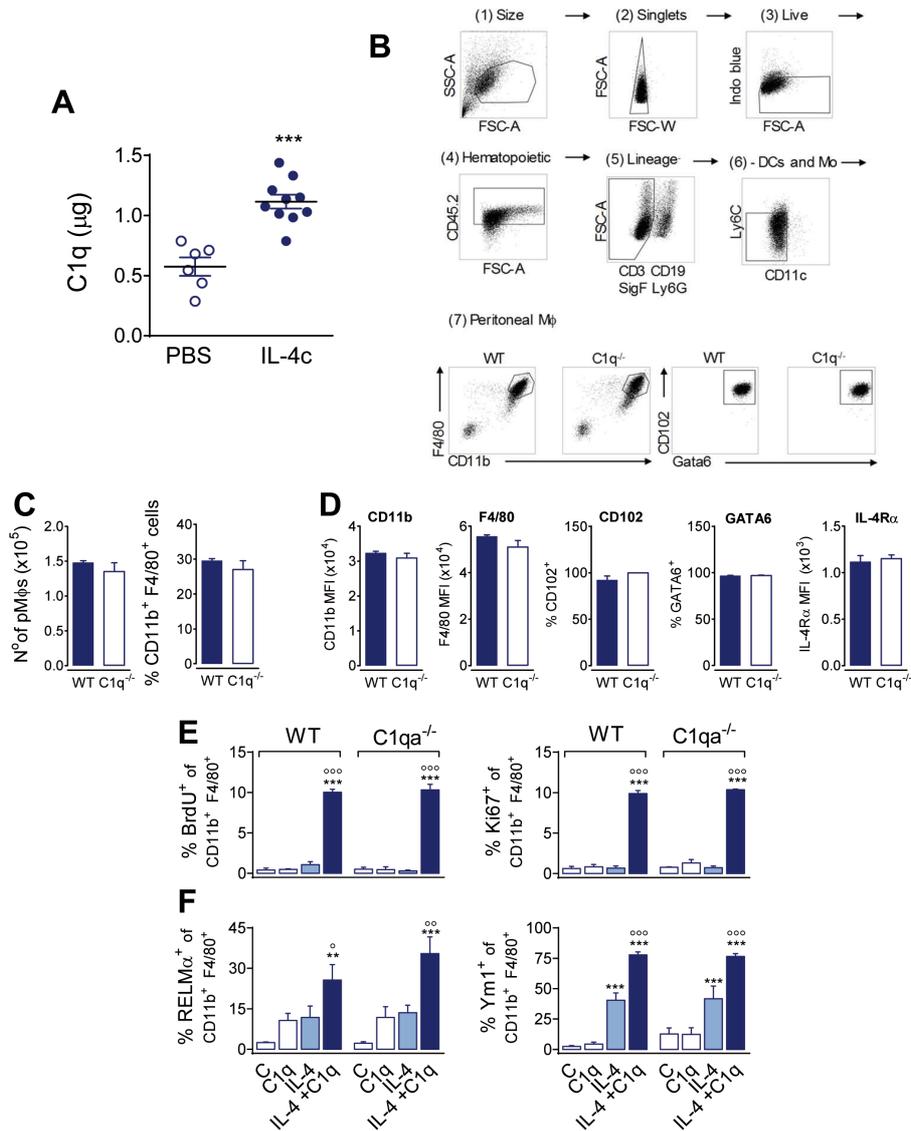


Fig. S5. C1q protein levels in the peritoneal cavity and characterization pMφ of from C1q-deficient mice. (A) Following IL-4c delivery, C1q protein was measured by ELISA in WT mice. (B) Sequential identification of pMφ by flow cytometry. Cells first identified by size (1), and then as singlets (2) and live cells (3). Subsequently, hematopoietic cells were selected by CD45.2 expression (4). Lin⁻ cells (5) were obtained by gating out CD3⁺, CD19⁺, Ly6G⁺ and SiglecF⁺ cells (T cells, B cells, neutrophils and eosinophils, respectively). Monocytes and dendritic cells were successively excluded by expression of Ly6C and CD11c (6). Lin⁻, CD11c⁻ and Ly6C⁻ populations were further subgated on the basis of the expression of CD11b versus F4/80. pMφ are CD11b^{hi} and F4/80^{hi} (7); a representative population of pMφs from WT and C1q-deficient mice is shown. The expression of CD102 and Gata6 by pMφs is also shown for both strains. All shown gates are children of the parent gates shown previously. (C) Number and percentage of pMφ recovered in the peritoneal washes. (D) Expression of CD11b, F4/80, CD102, Gata6, and IL-4Rα in pMφ from WT and C1q-deficient mice. Data are representative from two independent experiments (mean ± SEM; WT: 4 mice, C1q^{-/-}: 4 mice). In C and D, Student's *t*-test was used. In E and F, purified pMφ from WT and C1q-deficient mice were treated with or without IL-4 (1 μg/ml or 10 ng/ml) in the presence or absence of C1q, and exposed to BrdU. (E) Proliferation (BrdU incorporation and Ki67 expression) in response to 1 μg/ml of IL-4. (F) M(IL-4) activation (RELMα and Ym1 expression) of pMφs in response to 10 ng/ml of IL-4. The data shown are means ± SEM of two different pMφ cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. ***p* < 0.01, and ****p* < 0.001, when compared with untreated cells (C); °*p* < 0.05, °°*p* < 0.01, and °°°*p* < 0.001, when C1q+IL-4-treated macrophages were compared with IL-4-treated macrophages.

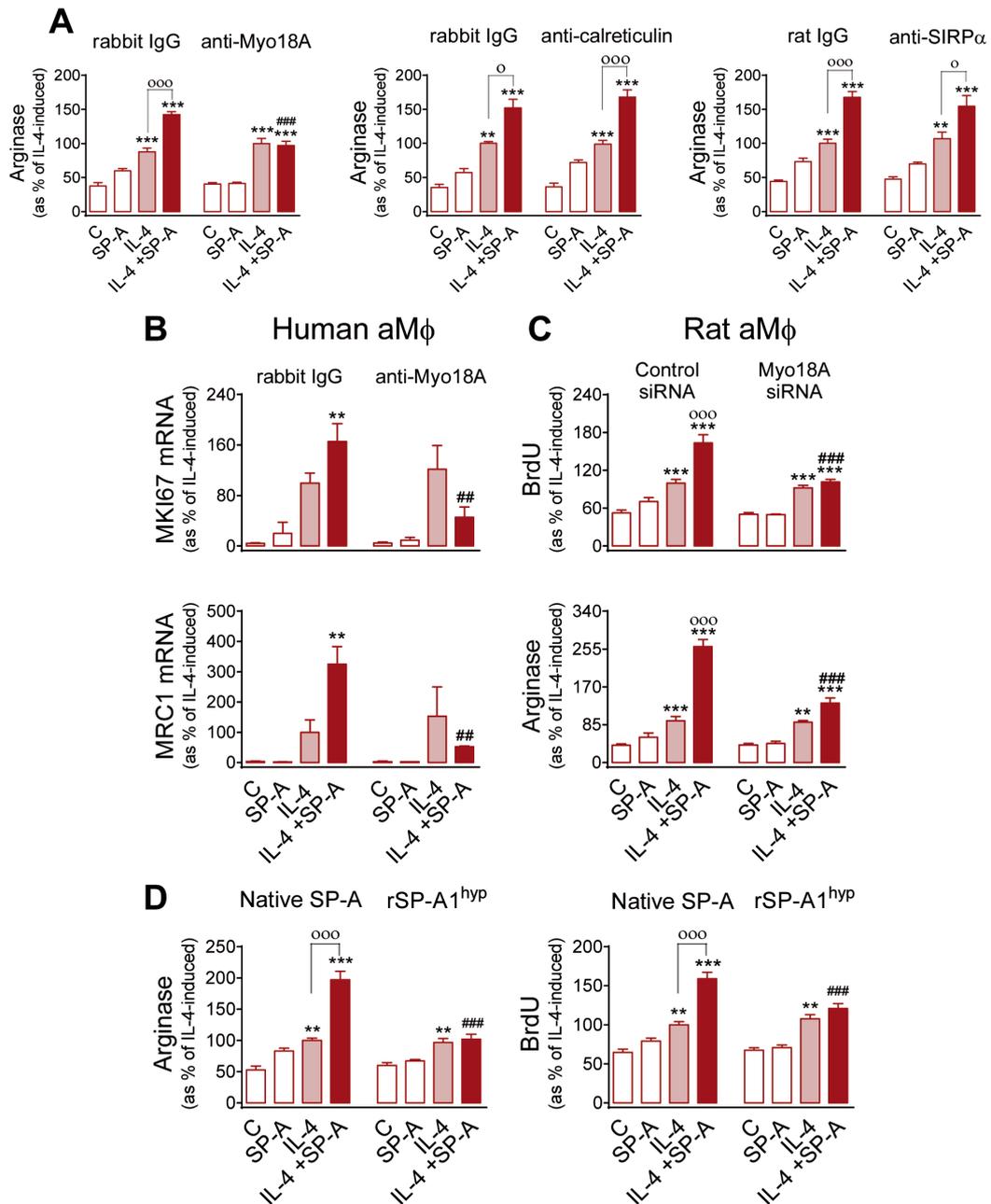


Fig. S6. (A-C) Myo18A mediates SP-A's effects on aM ϕ s isolated from humans and rats. (A) Purified rat aM ϕ were treated with either anti-Myo18A (10 μ g/ml), anti-calreticulin (10-50 μ g/ml), anti-SIRP α (10-50 μ g/ml), or an isotype control for two hours. Next, cells were IL-4-stimulated with or without native SP-A. **(B)** mRNA expression of *MKI67* and *MRC1* by human aM ϕ treated with anti-Myo18A antibody. **(C)** BrdU incorporation and arginase activity in rat aM ϕ that were nucleofected with Myo18A (RSS322720) or control siRNA. Similar results were found using RSS322721 Myo18A siRNA. **(D) The collagen-like domain of SP-A is required for functional interaction with Myo18A on aM ϕ .** Purified rat aM ϕ were IL-4-stimulated with or without either native human SP-A or recombinant human SP-A1 expressed in insect cells (SP-A1^{hyp}). Recombinant human SP-A1 expressed in insect cells lacks the prolyl hydroxylation, resulting in improper folding of the collagen domain (31). The data shown are means \pm SEM of three different alveolar or pM ϕ cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. ** p < 0.01, and *** p < 0.001, when compared with untreated cells (C); $^{\circ}$ p < 0.05, and $^{\circ\circ}$ p < 0.001, when SP-A+IL-4-treated macrophages were compared with IL-4-treated macrophages; ### p < 0.01, and ### p < 0.001, when either the effect of Myo18A siRNA vs. control siRNA or anti-Myo18A vs. rabbit IgG are compared or when the effects of native SP-A vs. SP-A1^{hyp} on macrophage activation and proliferation are compared.

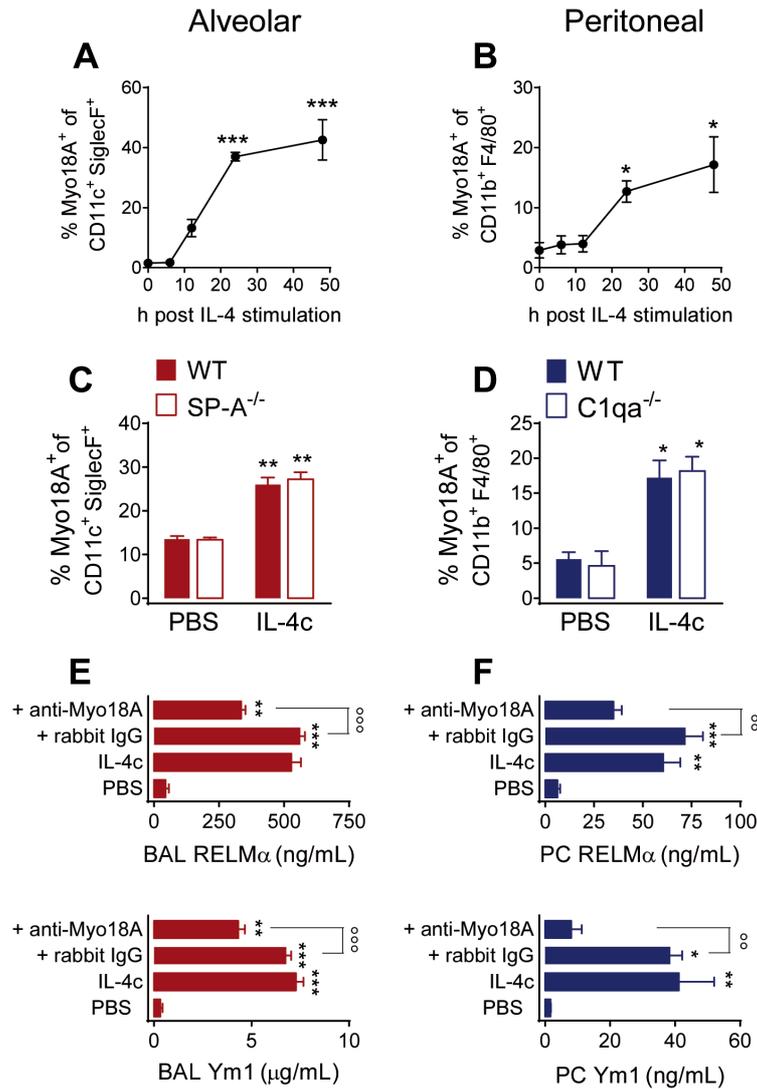


Fig. S7. Myo18A expression in response to IL-4 treatment and further characterization of blocking Myo18A on IL-4-mediated activation of macrophages *in vivo*. Time-dependent expression of Myo18A on the cell surface of alveolar (A) and peritoneal (B) macrophages in response to IL-4 treatment *in vitro*. The data shown are means \pm SEM of three different aM ϕ or pM ϕ cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. * $p < 0.05$, and *** $p < 0.001$, when compared with 0 hours post IL-4 stimulation. (C-D) For aM ϕ s, 5 μ g IL-4c was delivered ip at day 0 and day 2, and BAL cells analyzed at day 4. For pM ϕ s, 1 μ g IL-4c was delivered ip at day 0, and peritoneal cells analyzed at day 1: Myo18A expression on the surface of macrophages from WT, SP-A^{-/-}, and C1qa^{-/-} mice treated with or without IL-4c. (E) For aM ϕ s, mice were treated as in (C-D) and simultaneously, animals were intra-nasally treated with either anti-Myo18A or rabbit IgG at day 2 and 3, and lung samples were analyzed at day 4. (F) Alternatively, for pM ϕ s, mice were also treated as in (C-D) with concomitant delivery of anti-Myo18A or rabbit IgG (ip) 2 hours before IL-4c administration. Concentration of RELM α and Ym1 in BAL (E) and the peritoneal cavity (PC) (F) are shown. Data were pooled from three independent experiments (means \pm SEM) (PBS: 6 mice, other groups: 9 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, when compared with PBS treated mice; $^{\circ}p < 0.01$, and $^{\circ\circ}p < 0.001$ when anti-Myo18A vs rabbit IgG treatment is compared in IL-4c-treated mice.

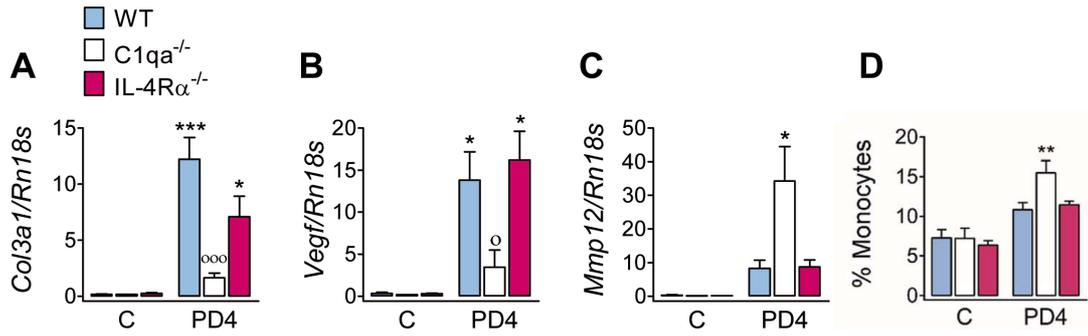


Fig. S8. Further characterization of WT , IL-4Rα and C1q-deficient mice in a model of peritoneal dialysis. WT, C1qa^{-/-} or IL-4Rα^{-/-} mice were either untreated (control, C) or treated with 14 ip injections of Dianeal PD-4 every other day. Samples were analyzed a day after the last delivery. Amplification of (A) *Col3a1*, (B) *Vegf*, and (C) *Mmp12*- encoding mRNA in peritoneal tissue. (D) Percentage of infiltrating monocytes as quantified by FACS. Results are representative from two independent experiments (means ± SEM) (untreated: 3 mice, PD4: 6 mice). ANOVA followed by the Bonferroni multiple-comparison test or Student's t-test (A) was used. *p < 0.05, **p < 0.01, and ***p < 0.001 when compared with control group; °p < 0.05 and °°°p < 0.001 when WT vs. C1qa^{-/-} mice treated with Dianeal PD-4 are compared.

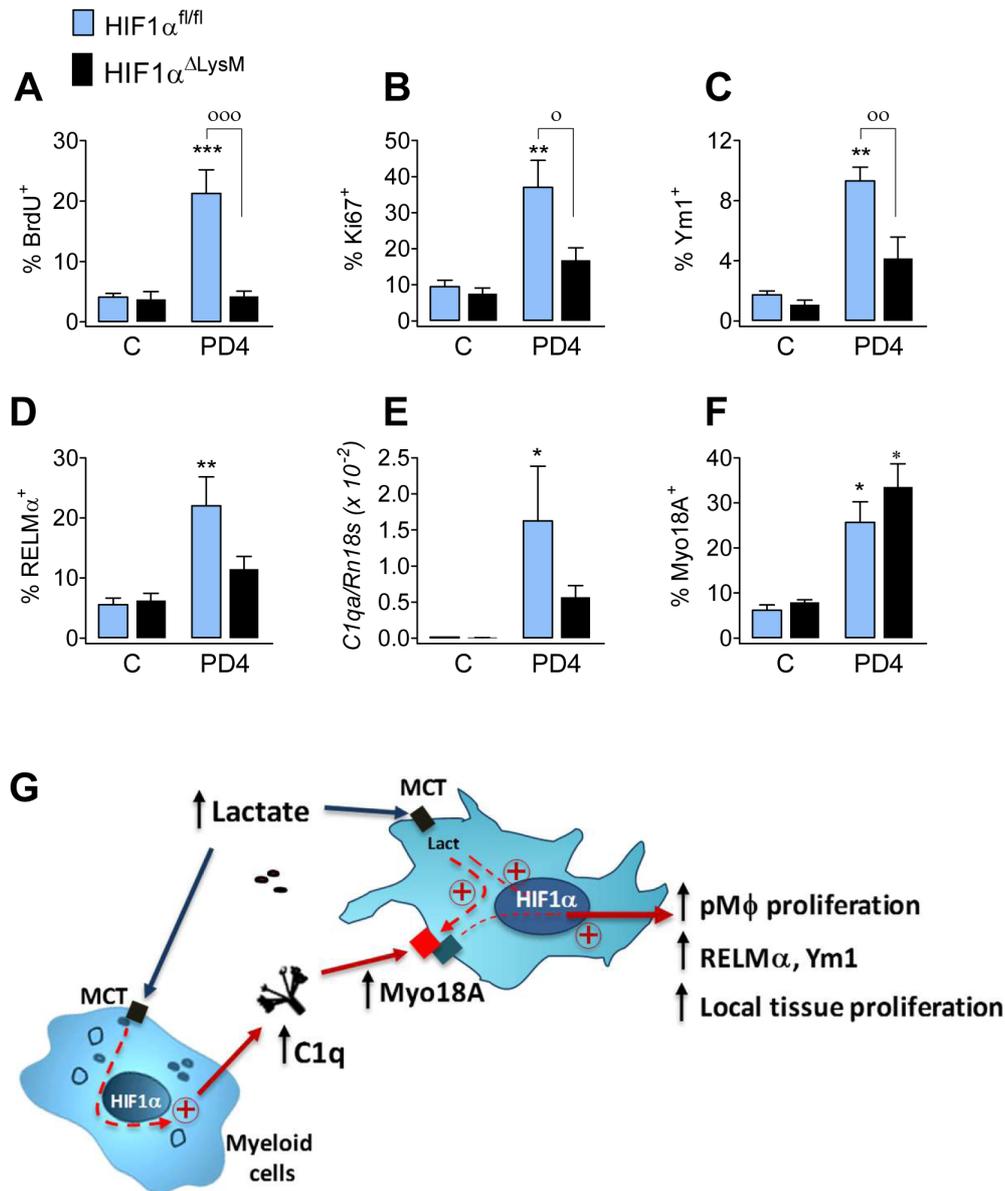


Fig. S9. Lactate-dependent proliferation and type 2 macrophage activation requires stabilization of HIF1 α in a model of peritoneal dialysis. *Hif1a^{flx/flx};LysMcre^{+/-}* mice and littermate WT controls (*Hif1a^{flx/flx};LysMcre^{-/-}*) were either untreated (control, C) or treated with 5 ip injections of Dianeal PD-4 every other day. Samples were analyzed a day after the last delivery. (A) BrdU incorporation in pM ϕ . (B) Ki67 expression. (C) Ym1 and (D) RELM α expression. (E) Amplification of C1qa-encoding mRNA in peritoneal tissue. (F) Myo18A expression on the surface of peritoneal macrophages. Results are representative from two independent experiments (means \pm SEM) (untreated: 3 mice, PD4: 4 mice). ANOVA followed by the Bonferroni multiple-comparison test or Student's t-test (A) was used. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with control group; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$ when WT vs. myeloid specific HIF-1 α -deficient mice treated with Dianeal PD-4 are compared. (G) Schematic of lactate-dependent promotion of a type 2 macrophage phenotype, which is dependent on HIF1 α and C1q in the peritoneal cavity. A lactate-based dialysate increases C1q levels in the peritoneal cavity and expression of myosin 18A on the surface of pM ϕ . Dialysate-induced proliferation of pM ϕ and production of type 2 macrophage markers were blocked in C1q deficiency mice and animals lacking HIF1 α in macrophages, but not in IL-4R α deficient mice. The data suggests that C1q is required to amplify HIF1 α -dependent lactate signaling, which promotes peritoneal fibrosis. (MCT: monocarboxylate transporter for lactate; HIF1 α : hypoxia-inducible factor 1 α)

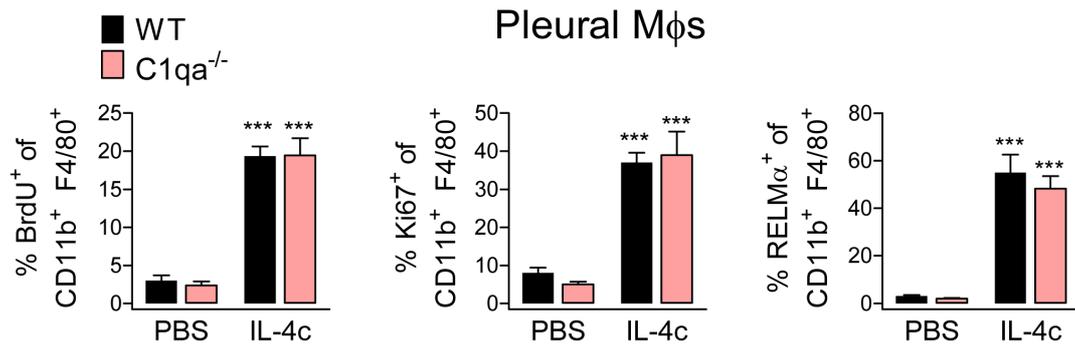


Fig. S10. IL-4 mediated proliferation and activation of pleural cavity macrophages is not enhanced by C1q. WT and C1qa-deficient mice were treated with 1 μ g of IL-4c (ip) at day 0; and samples were analyzed at day 1. Proliferation (BrdU incorporation and Ki67 expression) and M(IL-4) activation (RELM α expression) of resident pM ϕ are shown. Data were pooled from two independent experiments (means \pm SEM) (PBS: 8 mice, IL-4c: 10 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. *** $p < 0.001$ when compared with the untreated group.

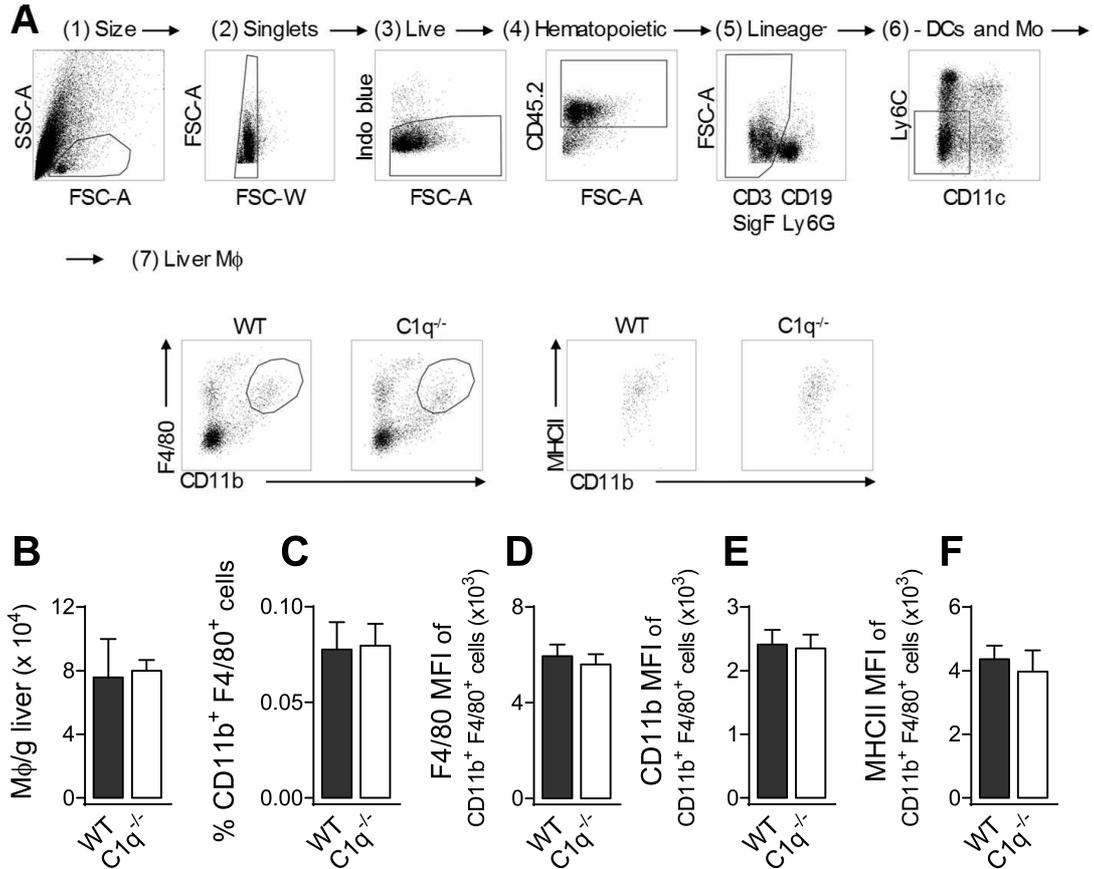


Fig. S11. Characterization of liver macrophages from C1q-deficient mice. (A) Sequential identification of liver macrophages by flow cytometry. Cells were first identified by size (1), and then as singlets (2) and live cells (3). Subsequently, hematopoietic cells were selected by CD45.2 expression (4). Lin⁻ cells (5) were obtained by gating out CD3⁺, CD19⁺, Ly6G⁺ and SiglecF⁺ cells (T cells, B cells, neutrophils and eosinophils, respectively). Monocytes and dendritic cells were successively excluded by expression of Ly6C and CD11c (6). Lin⁻, CD11c⁻ and Ly6C⁻ populations were further subgated on the basis of the expression of CD11b versus F4/80. CD11b⁺ and F4/80⁺ cells are liver macrophages (7); a representative population of liver macrophages from WT and C1q-deficient mice is shown. The expression of MHCII and CD11b by liver macrophages is also shown for both mice strains. All shown gates are children of the parent gates shown previously. (B) Number and (C) percentage of liver macrophages from tissue homogenates. Expression of (D) F4/80, (E) CD11b, (F) MHCII, and (G) IL-4Rα in liver macrophages from WT and C1q-deficient mice. Data are representative of two independent experiments (mean ± SEM; WT: 4 mice, C1q^{-/-}: 4 mice). Student's *t*-test was used.

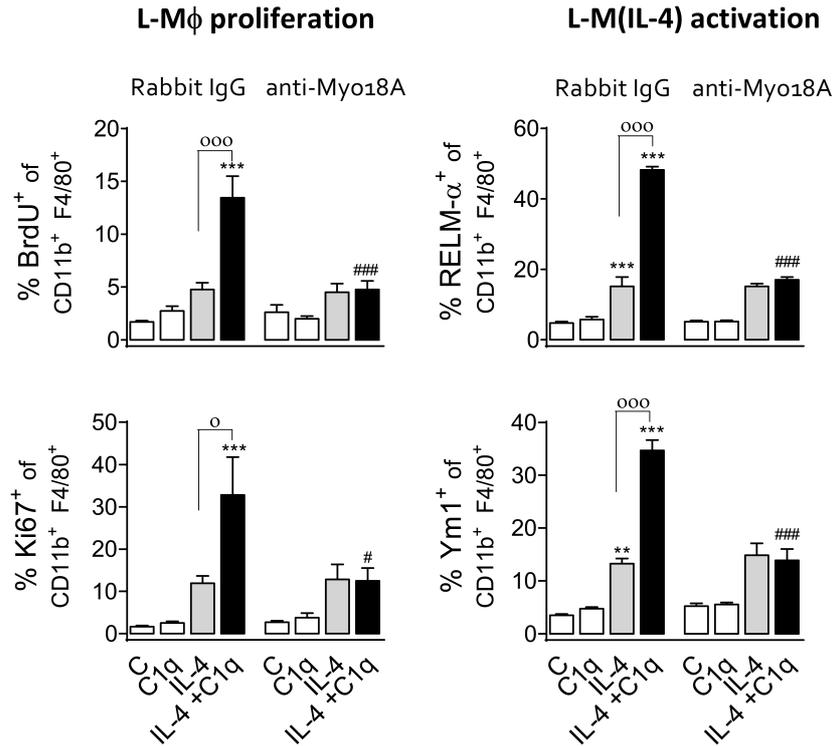


Fig. S12. Myo18A mediates C1q enhancement of IL-4-induced effects on liver macrophages. Purified mouse liver macrophages were treated with anti-Myo18A or rabbit IgG for one hour. Next, cells were IL-4-stimulated with or without C1q. Proliferation (BrdU incorporation and Ki67 expression) and M(IL-4) activation (RELM α and Ym1 expression) of liver macrophages are shown. The results are presented as means (\pm SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. ** $p < 0.01$, and *** $p < 0.001$, when compared with untreated cells; ° $p < 0.05$ and °° $p < 0.01$, when C1q+IL4-treated macrophages were compared with IL-4-treated macrophages; # $p < 0.05$, and ### $p < 0.001$, when the effect of anti-Myo18A antibody is compared in cells treated with C1q+IL4.

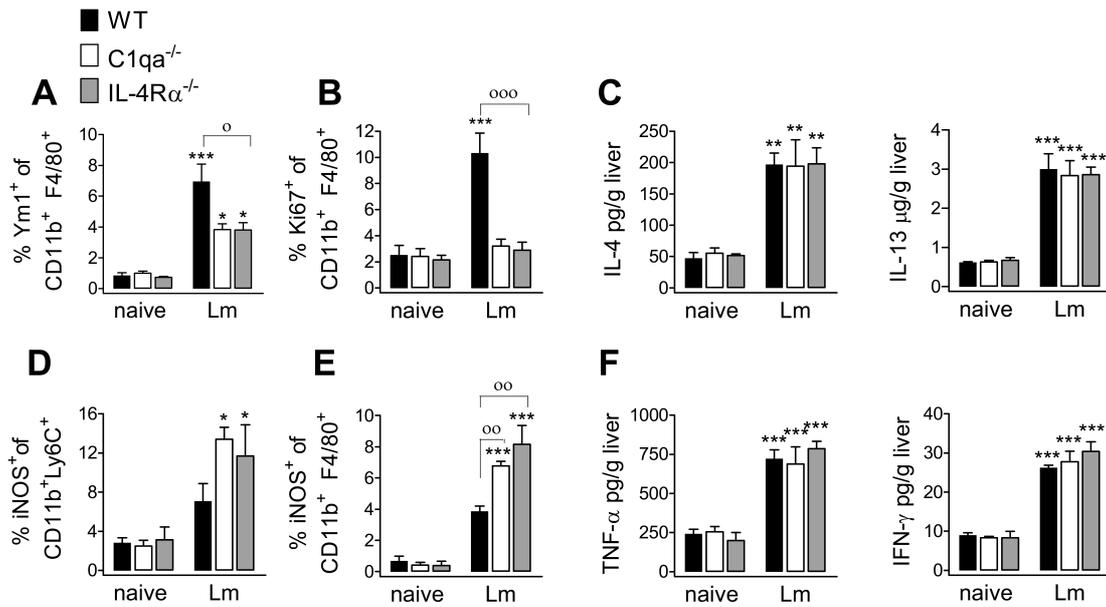


Fig. S13. Further characterization of WT vs. C1qa-deficient mice during *Listeria monocytogenes* infection. WT, C1qa^{-/-}, or IL-4Rα^{-/-} mice were left uninfected or received intravenous infection with 10⁴ *L. monocytogenes* c.f.u. and samples were assessed at day 3.5 after infection. **(A)** Expression of Ym1 by liver macrophages. **(B)** Ki67 expression by liver macrophages. **(C)** Levels of IL-4 and IL-13 in the supernatants of liver homogenates from WT, C1qa^{-/-}, and IL-4Rα^{-/-} mice. **(D-E)** iNOS expression by liver monocytes and macrophages. **(C)** Levels of TNFα and IFN-γ in the supernatants of liver homogenates from WT, C1qa^{-/-}, and IL-4Rα^{-/-} mice. Data are representative from two independent experiments (mean ± SEM; naïve: 4 mice, Lm: 4-6 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. *p < 0.05, **p < 0.01 and ***p < 0.001, when compared with the uninfected group; °p < 0.05, °°p < 0.01, and °°°p < 0.001 when WT vs. C1qa^{-/-} or IL-4Rα^{-/-} infected groups are compared.

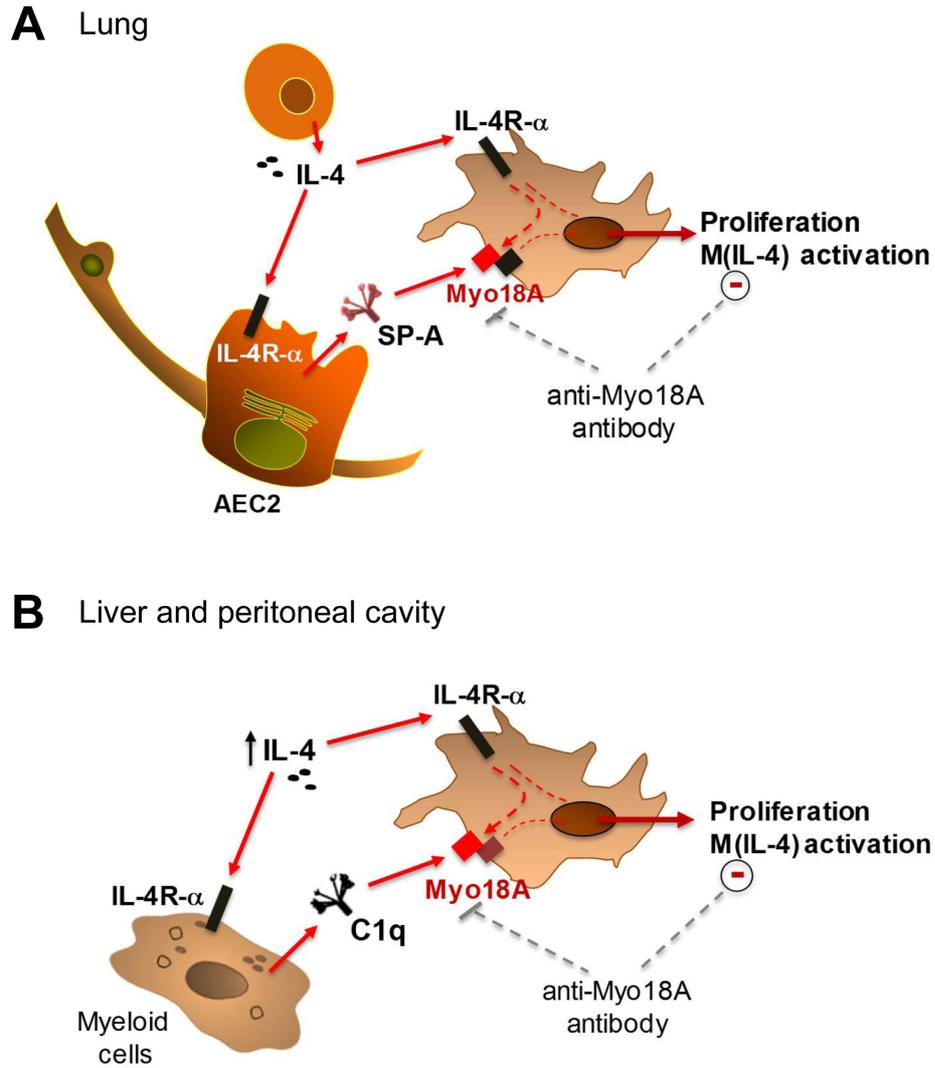


Fig. S14. Schematic of direct and indirect effects of IL-4. Rather than IL-4 (or IL-13) signaling to macrophages alone, IL-4 drives the production of a second signal for full M(IL-4) activation and proliferation. **(A)** IL-4 increases the production of SP-A by alveolar epithelial type II cells (AEC2) and the expression of myosin 18A on the surface of aM ϕ . SP-A deficiency or receptor blockade abrogated IL-4-induced proliferation of aM ϕ and reduced M(IL-4) activation. **(B)** In the liver and the peritoneal cavity, IL-4 increases the production of C1q and the expression of myosin 18A receptor on the surface of liver and peritoneal macrophages. C1q deficiency or receptor blockade impedes full M(IL-4) activation and proliferation.

Supplementary Table 1. Genotyping of the *Sftpa1*^{-/-} and *Sftpa1*^{+/+} littermates

SP-A-deficient mice genotyping	
Primer	Sequence
<i>Sftpa1</i> F neo	GTGGGGTGGGATTAGATAAATGC
<i>Sftpa1</i> 1743-1766	GCATTAGACGACAGAACTCCAGCC
<i>Sftpa1</i> R 1981-1957	TACTGAGAGATGTGTGCTTGGTGAG

Supplementary Table 2. Primer sequences used for quantitative RT-PCR of genes of interest.

Gene	Primer	Sequence
<i>Sftpa1</i>	For	CTGGAGAACATGGAGACAAGG
	Rev	AAGCTCCTCATCCAGGTAAGC
<i>Col3a1</i>	For	AAGGGTGAAGTCGGTGCTC
	Rev	TCCAGCTCCACCTCTAGCA
<i>Colla1</i>	For	TCTGGTCTCCAGGGTCCTC
	Rev	GTCTTTGCCAGGAGAACCAG
<i>Acta2</i>	For	CCAACCGGGAGAAAATGAC
	Rev	CAGTTGTACGTCCAGAGGCATA
<i>Vegf</i>	For	ACTCGGATGCCGACACGGGA
	Rev	CCTGGCCTTGCTTGCTCCCC
<i>Mmp12</i>	For	CAATTGGAATATGACCCCCTGT
	Rev	AGCAAGCACCCCTTCACTACAT
<i>Rpl13a</i>	For	CATGAGGTCGGGTGGAAGTA
	Rev	GCCTGTTCCGTAACCTCAA
<i>Rn18s</i>	For	GTAACCCGTTGAACCCCAT
	Rev	CCATCCAATCGGTAGTAGCG
<i>MKI67</i>	For	TCGACCCTACAGAGTGCTCA
	Rev	GTGGGGAGCAGAGGTTCTTC
<i>MRC1</i>	For	CAGATGCCCGGAGTCAGATC
	Rev	TTTATCCACAGCCACGTCCC
<i>GAPDH</i>	For	GATCATGAGCAATGCCTCCT
	Rev	TGTGGTCATGAGTCGTTCCA

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