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Cochlin produced by follicular dendritic cells promotes anti-bacterial innate immunity

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Running title

Role of cochlin in innate immunity

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Abstract

Cochlin, an extracellular matrix protein, shares homologies with the Factor C, a serine protease in horseshoe crab *limulus* critical for anti-bacterial responses. Mutations in the *Coch* gene are responsible for the DFNA9 syndrome, a disorder characterized by neurodegeneration of inner ear that leads to hearing loss and vestibular impairments. The physiological function of cochlin, however, is unknown. Here, we report that cochlin is specifically expressed by the follicular dendritic cells, and selectively localized in the fine extracellular network of conduits in the spleen and lymph nodes. During inflammation, cochlin is cleaved by aggrecanases and secreted into blood circulation. In models of lung infection with *Pseudomonas aeruginosa* and *Staphylococcus aureus*, *coch*^{-/-} mice show reduced survival linked to defects in local cytokine production, recruitment of immune effector cells and bacterial clearance. By the production of cochlin, the FDCs may have a previously unknown function in innate immune response in defense against bacteria.

Keywords

Cochlin, LCCL domain, conduit, follicular dendritic cells, aggrecanase, *Pseudomonas aeruginosa*, *Staphylococcus aureus*.

Introduction

Cochlin, encoded by the gene *coch*, is a highly abundant extracellular matrix protein in the cochlea and vestibule of the inner ear (Robertson et al., 1994; Robertson et al., 1997). Missense mutations and in-frame deletions in the *coch* gene are etiologically linked to the autosomal dominant DFNA9 syndrome, a disorder characterized by late onset nonsyndromic hearing loss and vestibular dysfunctions with associated neurosensory degeneration in the inner ear (Manolis et al., 1996). While the neurodegeneration associated with DFNA9 is believed to be caused by a gain-of-function by the mutated cochlin, the normal physiological function of cochlin remains unknown. The N-terminus of cochlin contains a LCCL domain also present in Factor C, a serine protease of the horseshoe crab *Limulus* involved in activating the coagulation cascade in response to LPS (Iwanaga et al., 1992). The expression of cochlin is highly selective; it is abundantly expressed in the inner ear and also found in the spleen (Robertson et al., 1997). To understand the physiological function of cochlin in the spleen, we investigated the splenic cell type that produces cochlin, the regulation of its production and its possible role in immunity.

Follicular dendritic cells, a type of stromal cells of the secondary lymphoid organs, are recognized as key organizers of B cell follicles and central to the development of germinal centers (GCs) where cooperation of multiple cell lineages leads to the formation of isotype-switched, high-affinity immunoglobulin and the establishment of humoral immune memory (Allen and Cyster, 2008). FDCs function by presenting native antigen in the form of immune complexes to B cells, supporting both T-dependent and -independent B cell activation, and suppressing apoptosis in B cells with high affinity

antigen-binding receptors resulting from successful somatic hypermutation processes. In the secondary lymphoid organs, FDCs are tightly associated with a fine tubular extracellular matrix network – named conduits – used as an adhesion substrate for cell’s crawling movement and involved in distributing small molecules through the splenic white pulp and lymph nodes, respectively (Bajenoff et al., 2006; Gretz et al., 2000; Nolte et al., 2003; Sixt et al., 2005). FDCs may secrete cytokines such as CXCL13 into the conduits, and capture small antigens from the circulation (Rozenendaal et al., 2009). While innate immune factors are known to promote the FDC’s accessory functions with regards to mediate B cell activation, FDCs are not yet known to have any effect in directly modulating innate immunity (El Shikh et al., 2007; Garin et al., 2010; Suzuki et al., 2010).

The homology of cochlin to the *Limulus* Factor C protein led us to postulate that cochlin may participate in host resistance to infection. In the present study, we investigated the role for cochlin in immunity. We demonstrate that cochlin is produced specifically by the FDCs in the secondary lymphoid organs and exclusively present in the fine extracellular network of conduits. However, cochlin deficiency has no effect on acquired immunity. On the other hand, cochlin LCCL domain is released into the blood after proteolytic cleavage in response to LPS and bacterial infection. Finally, we provide evidence that cochlin-deficiency leads to a defective innate responses and an increased sensitivity to *P. aeruginosa* infection. Our study demonstrates cochlin as the first immune modulator released from conduits to act at the systemic level, revealing a novel biological function of conduits and a novel role and mechanism of the FDCs in regulating innate immunity.

Results

Cochlin is expressed and secreted by follicular dendritic cells

We investigated the expression of cochlin using a highly specific monoclonal antibody (Yao et al., 2010), and found that cochlin is expressed in spleens and lymph nodes (Figure 1A, S1A). However, since both protein and mRNA of cochlin are not detectable in isolated splenocytes, which contain mostly B and T cells (Figure S1B), we considered the possibility that cochlin is expressed by stromal cells in the spleens. Using *in situ* immunocytochemistry, we found that cochlin is present in the fine network-like structure located in the B cell zone in the white pulp of spleens and in the center of the B cell follicles in lymph nodes (Figure 1B, S1C). Since this localization corresponds to that of the follicular dendritic cells (FDCs), we examined the co-immunostaining of CR2.1, a marker for FDC, and cochlin. Indeed, cochlin is localized in the close proximity of the FDCs (Figure 1C). Cultured primary WT, but not coch^{-/-}, FDCs express and secrete cochlin *in vitro* (Figure 1D, E). We thus conclude that FDCs express and secrete cochlin in the spleen and the lymph nodes.

Cochlin accumulates in the lumen of the conduits

Since cochlin is an extracellular protein and distributed in a fine network-like structure in the spleen and lymph nodes (Figure 1), we considered the possibility that cochlin might be associated with the conduits, an extracellular matrix network involved in distributing chemokines and small blood and lymph-borne molecules through the splenic white pulp and lymph nodes respectively (Gretz et al., 2000; Nolte et al., 2003; Roozendaal et al., 2009; Sixt et al., 2005). We investigated whether cochlin co-localizes with known

markers for conduits such as collagen, ER-TR7, and perlecan in the spleens. We found that cochlin colocalizes with the subset of conduits present in the B cell zone, but not in the periarteriolar lymphocyte sheath (PALS) (Figure 2A). While most of the conduit constituents are synthesized by enwrapping fibroblastic reticular cells (FRC), only conduits in the B cell zone are associated with FDCs in addition to FRCs (Gretz et al., 2000; Lokmic et al., 2008; Roozendaal et al., 2009; Sixt et al., 2005). The specific association of cochlin with the conduits in the B cell zone further supports that cochlin is produced by the FDCs.

Conduits are formed by a central collagen bundle core surrounded by a microfibrillar layer which is enwrapped in a basement membrane. High resolution fluorescence microscopic analysis of splenic samples co-immunostained with anti-cochlin and antibodies for laminin-1 and perlecan, markers for the basement membrane, or with antibody for ER-TR7, a marker for the microfibrillar layer, demonstrate that cochlin is wrapped by the basement membrane and by the microfibrillar layer. Indeed, fluorescence microscopy analysis suggests that cochlin colocalizes with the central collagen core (Figure 2B). Using electron microscopic analysis, we found that cochlin colocalizes with the central collagen bundles and with fibrillar collagen in the conduits (Figure 2C). We thus concluded that cochlin is synthesized by the FDCs and secreted in the lumen of the FDCs-associated conduits.

We next assessed the function of cochlin by analyzing the FDC-associated conduits in the spleen and lymph nodes of coch^{-/-} mice. Cochlin deficiency did not modify the FDC-associated conduit's structure as seen in fluorescent and electronic microscopy (Figure S2A-C). In the spleen or the lymph nodes, immune cells crawl on the

surface of the conduits to circulate (Bajenoff et al., 2006), and cochlin deficiency did not impair B cell, FDCs or T cell localization in the spleen or the lymph nodes of coch^{-/-} mice (Figure S2A-B, and data not shown). Conduits also allow for the flow of low molecular weight molecules ($MW \leq 70$ kDa) such as cytokines and small antigens (Gretz et al., 2000; Nolte et al., 2003; Roozendaal et al., 2009) in their lumen. Cochlin deficiency did not modify either the flow of small molecules nor the molecular weight cut-off of the components circulating through the conduits, as observed by following the flow of 10, 70 and 500 kDa fluorescent dextran in coch^{-/-} spleens (Figure S2D-F). FDCs adhere to the surface of the conduits and capture circulating small antigens before transferring them to the B cells, or alternatively B cells directly sample small antigens circulating in the conduits (Roozendaal et al., 2009). However, cochlin deficiency *in vivo* did not impair the close association of FDCs with the conduits (Figure S3A). Thus, we conclude that cochlin deficiency has no effect on the adherence of FDCs to the conduits.

We next tested whether cochlin was involved in the transfer of small antigens from the conduits to the immune cells, but cochlin deficiency did not alter the ability of FDCs or B cells to capture small antigens at their surfaces in the lymph nodes (Figure S3B-C). In addition, coch^{-/-} mice did not show any defects in the immunoglobulin response to the small antigens TEL (turkey egg lysozyme, 12-14 kDa) trafficking through the conduits lumen, nor to the large antigen PE (phycoerytherin, 240 kDa) excluded from the conduits, as anticipated, and used here as a control (Figure S3D) (Roozendaal et al., 2009). Furthermore, coch^{-/-} mice showed no defect in germinal center formation (Figure S3E) or antibody affinity maturation during primary and secondary responses (Figure S3F-G). Taken together, we conclude that although cochlin is specifically associated with

the conduits in the B cell zone, cochlin deficiency has no detectable effect on adaptive immune response in our experimental conditions.

Cochlin cleavage products are released in the blood during inflammation

As the conduit lumens connect with the blood circulation (Gretz et al., 2000), we next tested whether cochlin is released into the blood stream using western-blot analysis of the serum for detection. In basal conditions, two weak immunoreactive bands reacting with anti-cochlin antibody were present in the blood, which were absent in the sera from coch^{-/-} mice (Figure 3A). We named them p8 and p18 according to their respective apparent molecular weights. Since the *Limulus* Factor C which contains a homologous LCCL domain is secreted into the hemolymph in response to LPS (Iwanaga et al., 1992), we next tested if cochlin p8 and p18 are released into the blood during LPS response. We found that the levels of cochlin p8 and p18 in sera greatly increased shortly after intraperitoneal (i.p.) injection of LPS in WT but not coch^{-/-} mice (Figure 3A). In accordance with cleavage followed by secretion, full-length cochlin is significantly reduced in the spleen starting 4 h after LPS injection, and p18 peaks transiently in the spleen from 2 h to 4 h following LPS injection before gradually decreasing but being maintained at a detectable level. Altogether these data suggest that full-length cochlin is processed into the p8/p18 forms in the spleen prior to their release in the blood.

The levels of TNF- α , one of the first cytokines detectable in the blood, peaked at 1 h after LPS injection (Figure S4A). We observed that TNF- α injection is sufficient to induce the release of the cochlin p8/p18 forms into the blood (Figure 3B). This suggests that cochlin p18/8 release in the blood is mediated downstream of TNF- α release

following LPS injection. Thus, increased levels of cochlin p8 and p18 in the blood are not restricted to LPS stimulation *per se* but may be a common phenomenon under various inflammatory conditions involving TNF- α .

The cochlin p8/p18 forms correspond to the LCCL domain.

Since the anti-cochlin antibody used for the WB analysis recognizes the N-terminal part of cochlin (Yao et al., 2010), which contains a LCCL domain, the p8 and p18 are most likely derived from the LCCL domain. Consistent with this possibility, we have previously shown that cochlin p8/p18 forms are detectable in conditioned media of 293T cells transfected with a cochlin expression vector (Yao et al., 2010). To further characterize the identity of the cochlin p8/p18, we treated conditioned media of 293T cells expressing cochlin with N-glycosidase F, since cochlin is known to be glycosylated (Kommareddi et al., 2007; Robertson et al., 2003). We observed that following N-glycosidase F treatment, the cochlin p18 disappeared while the p8 level correspondingly increased (Figure 3C), suggesting that cochlin p18 is the glycosylated form of p8.

We then determined the cleavage site of full-length cochlin by Edman degradation of the purified C-terminal FLAG-tagged cleavage product corresponding to that of p18 (Figure 3D); “ATGRAV” was found to be its N-terminal residues. As this sequence corresponds to a.a. 135-140 of full-length cochlin, we concluded that cochlin is cleaved between E134 and A135. Cochlin p8/p18 fragments are recognized by the monoclonal antibody 9A10D2 raised against the N-terminal cochlin antigen corresponding to a.a. 7-227 (Yao et al., 2010). As a.a. 1-25 correspond to the signal peptide, we hypothesized that the p8/p18 N-terminal fragment may correspond to the a.a.

26-134. We compared the migration profile in a SDS-PAGE gel of p8/p18 in conditioned media of 293T cells expressing full-length cochlin, and in conditioned media of 293T cells expressing cochlin a.a. 1-134 following PNGase F treatment. The expression from the vector coding for cochlin a.a. 1-134 resulted in bands of the same apparent molecular weights as p8/p18. Taken together, we concluded that p8 corresponds to cochlin N-terminal fragment a.a. 26-134, which is then glycosylated to form p18 (Figure 3E).

We next investigated the release of the C-terminal domain using the polyclonal antibody recognizing epitopes predominantly in this domain. The C-terminal domain remained undetectable in the serum even after LPS injection (Figure S4B), and IF staining using the same antibody showed no decrease in cochlin intensity in the spleen following LPS injection (Figure S4C-D). We concluded that, contrary to the LCCL domain, the C-terminal domain of cochlin remains in the spleen following cochlin cleavage.

To determine the levels of LCCL domains in sera under control or LPS stimulated conditions, we next used purified coch26-134-HA (hereafter named LCCL-HA) of known concentration as a standard. We found that the levels of p8/p18 under basal condition are too low to be precisely determined while 8 h after LPS injection the concentration of p8/p18 reach $\sim 0.4 \mu\text{g/ml}$ (Figure S4E-F).

Cochlin is processed by aggrecanase-1 and -2

To explore the mechanism that mediates the cleavage of full-length cochlin, we incubated 293T cells expressing cochlin with chemical inhibitors of different proteases. We found that in the presence of MMP inhibitor III, a broad spectrum metalloprotease inhibitor, no

p8/p18 fragments were detected in the culture medium of 293T cells expressing cochlin (Figure 4A). Since cochlin is cleaved between E134-A135, we next investigated whether two glutamyl endopeptidases in the metalloprotease family known to cleave E-A bonds, namely aggrecanase-1 and -2, were involved in processing cochlin (Nagase and Kashiwagi, 2003). Compound 800, a specific inhibitor of aggrecanases (patent US2007/0043066) inhibits the release of p8/p18 fragments in the culture medium of 293T cells expressing cochlin (Figure 4B). In addition, we observed that aggrecanase-1 and -2 physically interact with cochlin as shown by co-immunoprecipitation assay in 293T cells (Figure 4C) and that purified aggrecanase-1 or -2 can directly cleave full-length cochlin to the cochlin p18 fragment in an *in vitro* cleavage assay (Figure 4D).

To investigate the mechanism that leads to the processing and secretion of cochlin following LPS or TNF- α stimulation, we assessed aggrecanase expression in the spleen following LPS or TNF- α injection. The *ADAMTS-4* (A Desintegrin And Metalloproteinase with ThromboSpondin motifs) gene encoding aggrecanase-1 is not expressed in the spleen in basal conditions but its transcription is greatly activated as soon as 1 h after LPS injection (Figure 4E). *ADAMTS-4* mRNA was also induced following TNF- α injection (Figure 4F). On the other hand, *ADAMTS-5* gene encoding aggrecanase-2 is expressed in the spleen under basal conditions. However, the expression of *ADAMTS-5* not only is not activated following LPS or TNF- α injection but actually decreases transiently following LPS injection (Figure 4E-F). Consistently, the expression of *ADAMTS-4* mRNA in cultured FDCs was induced by LPS whereas that of *ADAMTS-5* mRNA in FDCs was constitutive (Figure 4G).

At the protein level, splenic aggrecanase-1 was induced 2 h after LPS injection, corresponding exactly to the kinetics of the observed cochlin cleavage in the spleen and the secretion of cochlin p18/p8 into the blood (Figure 4H, 3A). Consistent with the processing of cochlin by aggrecanases, the levels of p18/p8 induced in the serum by LPS were reduced by the injection of compound 800 (Figure 4I). We next tested the interaction of endogenous cochlin with aggrecanase-1 *in vivo*. Aggrecanase-1 co-immunoprecipitates with cochlin in spleen lysates from WT mice injected with LPS (Figure 4J). Neither aggrecanase-1 nor cochlin bands were detectable in the anti-cochlin immunoprecipitation from the splenic lysate of a coch^{-/-} mouse treated in the same conditions, demonstrating the specificity of the assay. Together with the *in vitro* data, our results strongly indicate that aggrecanase-1 cleaves cochlin *in vivo* during LPS and TNF- α responses.

Cochlin-deficiency leads to defects in anti-bacterial innate immunity

To characterize the role of cochlin in anti-bacterial innate immunity, we first examined the role of cochlin in LPS responses, based on the homology of its LCCL domain with the evolutionarily ancient related Factor C protein from the horseshoe crab *Limulus*. Factor C is critical in sensing LPS and in the consequent activation of the hemolymph coagulation cascade as well as in complement deposition onto the surface of bacteria (Ariki et al., 2008; Iwanaga et al., 1992; Koshiba et al., 2007). However, we did not detect any interaction between the cochlin LCCL domain and LPS (data not shown). Coch^{-/-} macrophages showed no defect in the response to LPS *in vitro*, nor did the addition of recombinant cochlin LCCL domain have any impact on this response (Figure

S5A-B). Moreover, *coch*^{-/-} mice showed normal coagulation and cytokine responses to LPS injection *in vivo* (Figure S5C-D), and their viability to LPS challenge was not modified as compared to that of WT mice (Figure S5E). We thus concluded that cochlin is not involved in the control of the LPS response.

We next tested whether cochlin took part in the detection of bacteria by pattern recognition receptors (PRRs) independently of LPS recognition by TLR4-MD2. *In vitro*, *coch*^{-/-} macrophages respond normally to peptidoglycans (PGN) which activate TLR2 and Nod-1 and -2 receptors, and the addition of recombinant cochlin LCCL domain did not impact this response (Figure S5A). We next investigated the role of cochlin in the detection of intact bacteria using *P. aeruginosa* (strain PA14) as a model. Since *coch*^{-/-} macrophages, in the presence or absence of recombinant cochlin LCCL domain, showed no difference in cytokine responses to the infection by PA14 *in vitro* (Figure S5F), we concluded that cochlin is not involved in the mechanism of bacterial detection *per se*.

We next reasoned that while the molecular mechanisms used by cochlin and *Limulus* Factor C might have diverged, their function might nevertheless be conserved and we investigated the role of cochlin in response to bacteria *in vivo*. We examined how cochlin might impact infection caused by *P. aeruginosa*, a gram-negative bacteria usually not pathogenic in healthy individuals but which constitutes a major cause of morbidity for cystic fibrosis patients with chronic lower respiratory tract infection, as well as acute opportunistic pneumonias in immunocompromised individuals (Sadikot et al., 2005). Innate immune responses that recruit neutrophils to the lungs are the primary means by which mammals resist *P. aeruginosa* infections (Koh et al., 2009). Similar to LPS injection, intranasal (i.n.) infection with *P. aeruginosa* (strain PA14) triggered the release

of cochlin p8/p18 into mouse sera (Figure 5A). More importantly, 24 h after i.n. infection of PA14, coch^{-/-} mice showed significantly higher bacterial loads in the lungs, blood and spleens compared to WT mice (Figure 5B). This defect in controlling infection with *P. aeruginosa* was associated with higher levels of cytokines such as IL-6 and IL-18 in the serum at 24 h (Figure 5C), and decreased survival of coch^{-/-} mice (Figure 5D). Similarly, coch^{-/-} mice showed higher bacterial loads in the lungs, blood and spleens as compared to WT mice 24 h following i.n. infection with the gram-positive bacteria *Staphylococcus aureus* (LAC strain) (Figure 5E), demonstrating that cochlin anti-bacterial function is not restricted to one specific bacteria strain, and definitively excluding a LPS-dependent mechanism. Since cochlin LCCL had direct effect neither on the *in vitro* viability and growth of PA14 and LAC nor on biofilm formation (Figure S5G-J), we concluded that cochlin is unlikely to be a bactericidal effector and hypothesized that it may regulate innate immune response. Indeed, analysis of the broncho-alveolar lavage of infected lungs showed that coch^{-/-} mice had a defect in the local secretion of IL-6, TNF- α , IL-1 β and in the macrophage and neutrophil chemoattractants MCP-1 and KC at 8 h post-infection with PA14 (Figure 5F), followed by a defective recruitment of monocytes/small macrophages and neutrophils at 12 h post-infection (Figure 5G). Differentiated alveolar macrophage counts were similar in BAL of WT and coch^{-/-} mice at this time point (data not shown). Since cochlin was not expressed at the mRNA level in lung under basal condition nor following infection with PA14 (Figure 5H) but cochlin LCCL protein accumulated in the lung following infection with PA14 (Figure 5I), we hypothesized that cochlin cleavage by aggrecanases and the subsequent release of cochlin LCCL into the blood was necessary for cochlin to control bacteria loads in the lung. Indeed inhibition of

aggrecanases by i.v. injection of compound 800 greatly increased lung bacteria loads, demonstrating that aggrecanase activity participate in the control of bacteria growth (Figure 5J). More importantly, the effect of compound 800 was significantly attenuated in *coch*^{-/-} mice compared to that of WT mice as shown by the statistical significance of the interaction in the two-way ANOVA. We thus conclude that cochlin cleavage accounts for a significant part of the anti-bacterial effect by aggrecanases, although aggrecanases also control bacteria growth through additional cochlin-independent mechanisms.

Taken together, we conclude that cochlin, specifically expressed by the follicular dendritic cells and selectively localized in the fine extracellular network of conduits in the spleen and lymph nodes, is an important modulator of innate immune response. During infection/inflammation, cochlin is cleaved by aggrecanases and secreted into blood circulation and regulates local cytokine production, recruitment of immune effector cells and bacterial clearance (Figure 6). We propose that by regulating the production of cochlin, the FDCs may have a previously unknown function in innate immune response in defense against bacteria.

Discussion

Our study describes cochlin as a novel modulator of innate immunity produced by the FDCs, the known critical modulators of humoral immunity promoting activation and maturation of B cells within follicles (Allen and Cyster, 2008). FDCs are tightly associated with conduits, a fine extracellular network in spleen and lymph nodes that is involved in small antigens and cytokines transport. While other conduit-associated cytokines have been shown to regulate local cell migration within the lymphoid organ, our study demonstrates cochlin as the first immune modulator locally deposited by the FDCs into the conduits and released upon inflammation into the systemic circulation. Our study supports the notion that the conduit is not only a channel mediating passive diffusion of small molecules, but also a storage structure for ready-to-be-released immune regulators to play active roles in modulating immunity. Moreover, our study suggests a novel role for the FDCs in modulating innate immunity.

We identified the FDCs as the producer of cochlin in the secondary lymphoid organs. FDCs play a critical role in germinal center formation and B cell activation, affinity maturation and differentiation (Allen and Cyster, 2008). Coch^{-/-} mice showed no defects in germinal center formation, immunoglobulin responses or antibody affinity maturation, suggesting that cochlin in the FDCs may serve no function with regards to B cell activation. A previous study implicated cochlin in cell-adhesion, most likely through its two C-terminus von Willebrand factor A-like (vWFA) domains (Bhattacharya et al., 2005). However, cochlin deficiency does not modify FDC localization around the conduits or their ability to capture antigen flowing in the conduit's lumen, suggesting that cochlin does not play a critical role in mediating adhesion of FDCs. Although cochlin is

associated with structures mediating fluid flow such as the trabecular meshwork in the eyes, the conduits in the secondary lymphoid organs, as well as in the inner ear (Bhattacharya et al., 2005; Nolte et al., 2003; Robertson et al., 2006; Roozendaal et al., 2009), cochlin-deficiency causes no impairment in the flow inside the conduit, suggesting that cochlin does not play a critical structural role in supporting the integrity of the fine tubular structure. As abnormal accumulation of cochlin in the inner ear and the eyes has been proposed to interfere with fluid flow and contribute to pathological conditions (Bhattacharya et al., 2005), a possible future study may be to identify any condition where abnormal accumulation of cochlin in the conduits may interfere with the flow which might provide further insights for the physiological function of the conduits in the secondary lymphoid organs.

Our fluorescent and electron microscopy data consistently show that cochlin colocalizes with collagen-forming fibrillar and bundle structures in the conduits. A similar association with type II fibrillar collagen has previously been shown in the inner ear (Mizuta et al., 2008). Indeed, type I, II and IV collagens bind to the second vWFA domain but not the LCCL-domain of cochlin (Nagy et al., 2008). Our data reveal that the LCCL domain is released in the blood following cochlin cleavage between its LCCL domain and its C-terminal domain containing the two vWFA domains. While cochlin C-terminal domains (named p40 and p45) are found in the spleen (Rodriguez et al., 2004), they remained undetectable in the blood in both basal and inflammatory conditions. Thus, our working model is that cochlin binds to conduit collagen through its vWFA domains, and following cochlin cleavage by aggrecanases, the LCCL domain is released into the blood stream while the C-terminal domain remained bound to collagen in the conduits.

Multiple cochlin isoforms have been described that result from both alternative splicing and post-translational modifications, and their expression patterns show tissue specificity (Bhattacharya et al., 2005; Ikezono et al., 2001; Kommareddi et al., 2007; Mizuta et al., 2008; Robertson et al., 2001). Our study reports p8/p18 as a new circulating form of cochlin, highly reminiscent of the N-terminal p16 fragment named cochlin tomoprotein (CTP) recently identified in the ear perilymph, but absent from inner ear tissue (Ikezono et al., 2004). Our data open new perspectives in the identification of CTP, which may be identical to p8/p18, and in its mechanism of secretion that may be dependent on aggrecanases. In addition, our finding that p18 is a glycosylated product of p8 is consistent with the residue N102 being a consensus site for N-linked glycosylation.

Our observation that cochlin cleavage site [E-AtgRavsTA] was highly similar to the established aggrecanase-1 consensus cleavage site [E-(AFVLMY)-X(0,1)-(RK)-X(2,3)-(ST)-(VYIFWMLA)] lead us to identify cochlin as a new substrate for aggrecanase-1 and -2. These glutamyl-endopeptidases from the ADAMTS metalloprotease family play a key role in arthritis pathogenesis by cleaving the cartilage matrix proteoglycan aggrecan (Lin and Liu, 2010). The other substrates of the ADAMTS family include several components of the extracellular matrix such as COMP (cartilage oligomeris protein), biglycan, TIMP-4, matrillin-2 and -3 (Hills et al., 2007). We found that aggrecanase-2, but not aggrecanase-1, is constitutively expressed in the spleen suggesting that the low basal circulating level of cochlin p8/p18 in the blood may result of aggrecanase-2 activity. Aggrecanase-1 expression is highly induced in the spleen following LPS or TNF- α injection with kinetics matching exactly these of cochlin cleavage. This observation is consistent with multiple reports claiming that aggrecanase-1

expression is up-regulated by inflammatory cytokines (Bondeson et al., 2006; Cross et al., 2006; Song et al., 2007; Tortorella et al., 2001; Yamanishi et al., 2002).

Our discovery that cochlin LCCL domain is a blood circulating protein with increased level during inflammation, led us to investigate a systemic role of the LCCL domain in the innate immune response. LCCL is an autonomous folding domain consisting of a central α helix wrapped by two β sheets named after the first three proteins identified to contain this domain (*Limulus* factor C, Cochlin and *Lgl-1* (late gestation lung)) and later found in various secreted proteins with modular structures (Liepinsh et al., 2001). The horseshoe crab *Limulus* coagulation factor C is a serine protease secreted in the hemolymph, activated upon LPS binding and is critical in anti-bacterial immunity. In particular, factor C mediates the degranulation of the amoebocytes, the initiation of a protease cascade resulting in coagulation, and the activation of the complement system (Ariki et al., 2008; Iwanaga et al., 1992; Koshiba et al., 2007). The contribution of the LCCL domain in the function of factor C remains unknown, and in particular LCCL does not bind LPS (Koshiba et al., 2007; Tan et al., 2000). A role in anti-bacterial innate immunity has also been suggested for the mammalian protein *Igl-1* (also named CRISPLD2 for cysteine-rich secretory protein LCCL domain containing 2) expressed in various organs and notably detected in the serum. *Igl-1* binds to LPS and sequesters it away from the PBMCs surface (Wang et al., 2009). Cochlin LCCL sequence and secretion pattern homologies with *Limulus* factor C and *Igl-1* led us to hypothesize a conservation of their functions. Indeed, we discovered that cochlin is critical in innate immunity using models of *P. aeruginosa* and *S. aureus* i.n. infection. However, contrary to factor C or *Igl-1*, cochlin effector function is

independent of LPS, as cochlin LCCL domain does not bind to LPS (data not shown and (Liepinsh et al., 2001)), and impacts LPS response neither *in vitro* nor *in vivo*. In addition, the low affinity of the *P. aeruginosa* LPS for TLR4 and the absence of phenotype of the TLR4^{-/-} mice in regards to *P. aeruginosa* infection predict that the TLR4-mediated LPS response is accessory in the immunity against this pathogen, and modulation of this pathway is not expected to impact the outcome of the infection (Feuillet et al., 2006; Ramphal et al., 2005). Finally cochlin role in controlling the gram-positive bacteria *S. aureus* definitely demonstrates a LPS-independent mechanism.

Our study shows a defect of coch^{-/-} mice in local cytokine and chemoattractant production in the lung and in the subsequent recruitment of immune cells leading to an impaired control of the bacteria and a consequent reduced viability following infection. These data confirm previous studies describing that the recruitment of neutrophils and macrophages to the lung is absolutely critical for the outcome of *P. aeruginosa* infection and that blunted early local inflammatory response in the lung leads to later higher bacteria load, uncontrolled systemic cytokine secretion and reduced survival (Horino et al., 2009; Koh et al., 2009). Taken together, our study demonstrates an unexpected function of FDC in modulating innate immunity and a new role for secondary lymphoid organ and particularly local lymph nodes in the amplification of the innate immune response by the release of modulators such as cochlin.

Figure legends

Figure 1. Cochlin is produced and secreted by FDCs in the spleen and the lymph nodes.

A. Spleen and lymph node lysates from WT and coch^{-/-} mice were analyzed by WB.

B.C. Lymph node and spleen sections from WT mice were co-stained for cochlin and B cell (B220) (objective lens 10X) (B) or FDCs (CR2.1) (objective lens 20 X) (C).

D.E. Primary FDCs were isolated from WT and coch^{-/-} mice. Lysates from *in vitro* cultured FDCs were analyzed by WB (D). Culture media were subjected to anti-cochlin IP and analyzed by WB (E). Spleen lysates from WT and coch^{-/-} mice, as well as lysates from 293T cells expressing cochlin were used as controls.

Figure 2. Cochlin concentrates in the lumen of conduits.

A.B. Spleen sections from WT mice were co-stained for cochlin and the conduits markers collagen, ER-TR7, perlecan and laminin-1. Objective lens 10X (A). Objective lens 100X, 3D reconstructions were performed using deconvolved Z-series (B).

C. Spleen fine sections were stained for cochlin (gold beads, black arrowheads; fc, fibrillar collagen, cb, collagen bundles).

Figure 3. Cochlin LCCL domain is released into the blood following proteolytic cleavage upon inflammation.

A.B. WT and coch^{-/-} mice were injected i.p. with LPS (5 mg/kg) (A), TNF- α (15 μ g, 8 h) (B) or PBS as control.

C. The p8 fragment is the deglycosylation product of p18. Serum free culture medium from 293T cells expressing cochlin was treated by PNGase F for 1 h (-, untreated; mock, 1 h w/o PNGase F).

D. The C-term fragment corresponds to a.a. 135-552. Anti-FLAG IPs from the culture medium of 293T cells expressing cochlin-FLAG.

E. The p8/p18 fragments correspond to a.a. 26-134. Serum-free culture media of 293T cells expressing the full length or the N-terminal domain (a.a. 1-134) of cochlin treated with PNGase F as indicated.

Figure 4. Cochlin is cleaved by the metalloproteases aggrecanase-1 and -2.

A-B. Aggrecanase inhibitors block cochlin cleavage. 293T cells expressing cochlin were cultured in serum free medium with the indicated concentrations of MMP inhibitor III or compound 800 for 24 h.

C. Cochlin interacts with aggrecanase-1 and -2. Anti-FLAG IP from lysates of 293T cells expressing the indicated constructs (*, non specific band).

D. Aggrecanase-1 and -2 cleave secreted full length cochlin. Purified full length cochlin was treated with aggrecanase-1 (left panel) or aggrecanase-2 (right panel).

E-F. *ADAMTS-4* gene expression is up-regulated in mice spleen upon LPS or TNF- α injection. Spleens from mice injected i.p. with LPS (5 mg/kg) (E), mTNF- α (5 μ g or 15 μ g, 8h) (F) or PBS (as control) were analyzed by RT-PCR for the expression of *ADAMTS-4* and *ADAMTS-5*.

G. Aggrecanase-1 and -2 are expressed by FDCs. *In vitro* FDC cultures were treated with LPS (100 ng/ml, 6h or overnight (O/N)). Aggrecanase-1 and -2 expressions were analyzed by RT-PCR.

H. Aggrecanase-1 protein level is up-regulated in the spleen upon LPS injection with a kinetics similar to cochlin cleavage. Spleens from coch^{+/-} and ^{-/-} mice injected i.p. with LPS (5 mg/kg) were analyzed by WB.

I. Aggrecanase-1 and -2 specific inhibitor C8OO inhibits LPS-induced p18 release into the blood. WT and coch^{-/-} mice were injected intraperitoneally with LPS (1 mg/kg) and C8OO (100 mg/kg) or vehicle only (1.3% DMSO+1.3% solutol) for 8 h. n=2 mice per group.

J. Aggrecanase-1 interacts with cochlin *in vivo* upon LPS injection. Spleen lysates from WT and coch^{-/-} mice injected i.p. with LPS (5 mg/kg, 4 h) were subjected to anti-cochlin IP.

Figure 5. Cochlin promotes anti-bacterial immunity against *P. aeruginosa*.

A. Release of cochlin p8/p18 into the serum during infection with PA14. Sera from WT and coch^{-/-} mice i.n. infected with PA14 for 10 h were analyzed by WB. Sera from mice injected i.p. with LPS (5 mg/kg, 8 h) were used as positive controls.

B. Increased CFU number in lung and bacterial spread in coch^{-/-} mice. Coch^{-/-} and WT mice were i.n. infected with PA14 for 24 h. n=8 per group; *, p<0.01; **, p<0.001); data are representative of 4 independent experiments.

C. Enhanced systemic cytokine burst in coch^{-/-} mice. Coch^{-/-} and WT mice were i.n. infected with PA14 for 24 h. IL-6 and IL-18 concentrations in sera were measured by ELISA. Uninfected mice were used as negative controls (NC). n=8 per group; *, p<0.01.

D. Reduced survival of infected coch^{-/-} mice. Coch^{-/-} and WT mice were i.n. infected with PA14. n=8 per group; Gehan-Breslow-Wilcoxon test p<0.05; data are representative of 2 independent experiments.

E. Increased CFU number in lung and bacterial spread in coch^{-/-} mice following *S. aureus* i.n. infection. Coch^{-/-} and WT mice were i.n. infected with LAC for 24 h. n=8-9 per group; *, p<0.01; **p<0.001; data are representative of 2 independent experiments.

F. Decreased cytokine levels in the BAL fluid of coch^{-/-} mice. Coch^{-/-} and WT mice were i.n. infected with PA14 for 8 h. Cytokines concentrations in the BAL fluid were determined by ELISA. n=7 per group; *, p<0.05.

G. Decreased lung recruitment of PMNs in coch^{-/-} mice. Coch^{-/-} and WT mice were i.n. infected with PA14 for 12 h. Cell numbers and composition in the BAL was analyzed by flow cytometry. Neutrophils and monocyte/small macrophages are defined as CD11c⁻/CD11b^{high}/GR-1⁺ and CD11c⁻/CD11b^{mild}/GR-1⁻ respectively. n=7 per group; *, p<0.05.

H.I. Cochlin is not produced but LCCL accumulates in the lung following *P. aeruginosa* i.n. infection. Coch^{-/-} and WT mice were infected with PA14 for 8 h. Lung were analyzed for cochlin mRNA expression by RT-PCR (H) and cochlin LCCL protein accumulation by WB (I). *, unspecific band.

J. Reduced effect of aggrecanase inhibition on CFU number in the lung of coch^{-/-} mice. Coch^{-/-} and WT mice were i.v. injected with C800 (100 mg/kg) or vehicle only (-) and i.n. infected with PA14 for 24 h. n=5 per group; *, p<0.01; **, p<0.001; ns, non

significant. The effect of cochlin deficiency on the increase in CFU following C800 injection was assessed by a two-way ANOVA using “genotype”, “C800 treatment” and the interaction between “genotype” and “C800 treatment” as fixed factors. “genotype”: $F_{1,18}=11.68$, $p=0.0035$; “C800”: $F_{1,18}=55.07$, $p<0.001$; “genotype*C800”: $F_{1,18}=5.57$, $p=0.0313$.

Figure 6. Working model.

Cochlin, produced and secreted by the follicular dendritic cells into the lumen of the conduits of the spleen and the lymph nodes, is processed by the aggrecanases whose expression are increased under inflammatory conditions. Circulating LCCL domain of cochlin modulates innate immune response at the site of infection by promoting immune cell recruitment and protection against bacterial infection.

Materials and Methods

Expression vectors

Cochlin expression vectors have already been described (Yao et al., 2010) *ADAMTS-4* and *ADAMTS-5* genes were cloned from total cDNA isolated from C57B6 mouse spleen using the primers adamts4-f 5'-CATTTTGGTGCCGCAGATG-3', adamts4-r 5'-CGGGACAGTGAGGTTATTTCC-3', adamts5-f 5'-CACTATGCGGCTCGAGTG-3', adamts5-r 5'-CAGGCTAACATTTCTTCAGCAGAC-3' in pcDNA3.

Reagents

Compound 800 (patent US2007/0043066) was custom synthesized by Shanghai ChemPartner Co., Ltd.

Antibodies

The monoclonal anti-cochlin 9A10D2 Ab raised against a.a. 7-227 of cochlin has been described (Yao et al., 2010) (WB analysis). Rabbit polyclonal antibody P13 was raised against cochlin a.a.7-227 (histology staining and immunoprecipitation). The rat monoclonal AD4/4D2E10 anti-aggrecanase-1 antibody was raised against full-length murine aggrecanase-1.

FDC isolation

FDCs were isolated from LN as described (Sukumar et al., 2006) and cultured in FDCs media (DMEM, FCS 10%, Hepes 20 mM, glutamine 2 mM, gentamicin 50 µg/ml, non-essential amino acid 1X, β-mercaptoethanol 4x10⁻⁴ %).

***In vitro* cleavage assay**

The C-term FLAG-tagged aggrecanase-1 and -2 were purified by IP from culture media of 293T cells transfected with the murine ADAMTS-4 (aggr-1) or ADAMTS-5 (aggr-2) constructs. The full length C-term FLAG-tagged cochlin was similarly purified by IP from culture media of transfected 293T cells in the presence of MMP inhibitor III (50 µM). Proteins were eluted by FLAG peptide, and dialyzed in cleavage buffer (Tris HCl pH 7.5 50 mM, NaCl 100 mM, CaCl₂ 10 mM, NaN₃ 0.02%, NP-40 0.02%). Cochlin and aggrecanase-1 or -2 were incubated in cleavage buffer at 37 °C, and reactions were stopped by Laemmli buffer.

Bacteria infection

Gender-matched 6- to 9-week old coch^{-/-} and C57B6 control mice were sedated with ketamine hydrochloride (65 mg/kg) and xylazine (13 mg/kg), and infected i.n. with PA14 (1-3x10⁷ CFU) or LAC (1-3x10⁸ CFU). For CFU enumeration, lungs, blood, and spleens were harvested and homogenized in LB, and dilutions plated on LB-agar plates.

Broncho-alveolar lavages (BAL) were collected in 2 ml of PBS and spun at 1500 rpm for 5 min.

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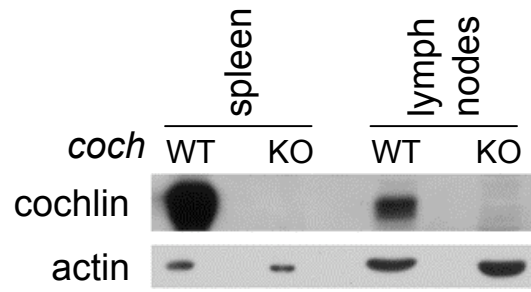
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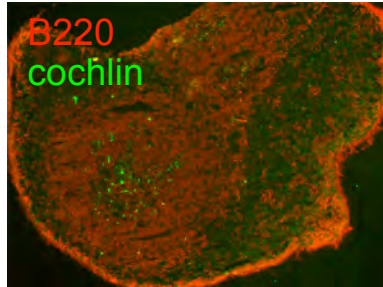
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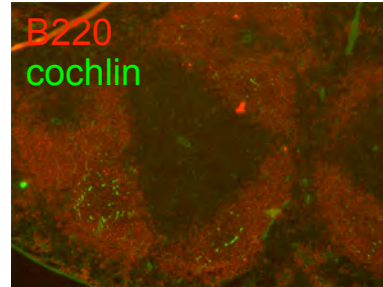
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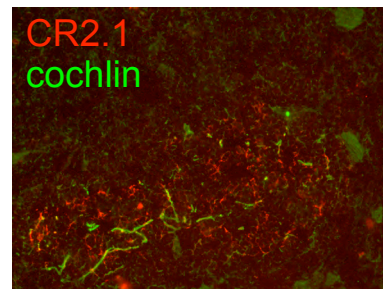
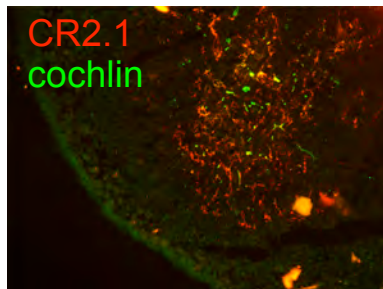
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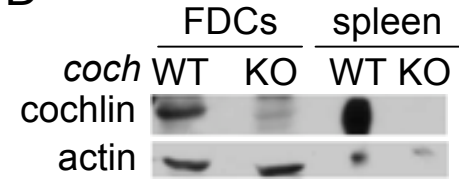
C Spleen



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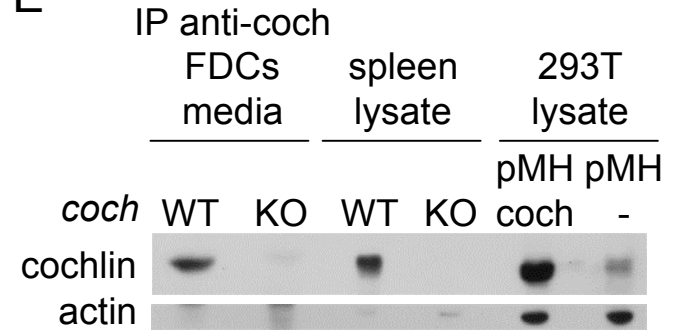


Figure 1

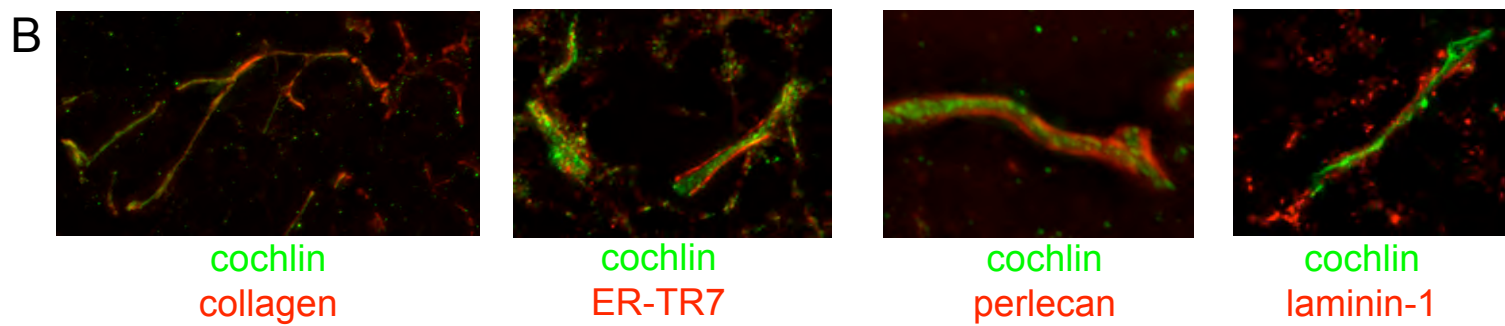
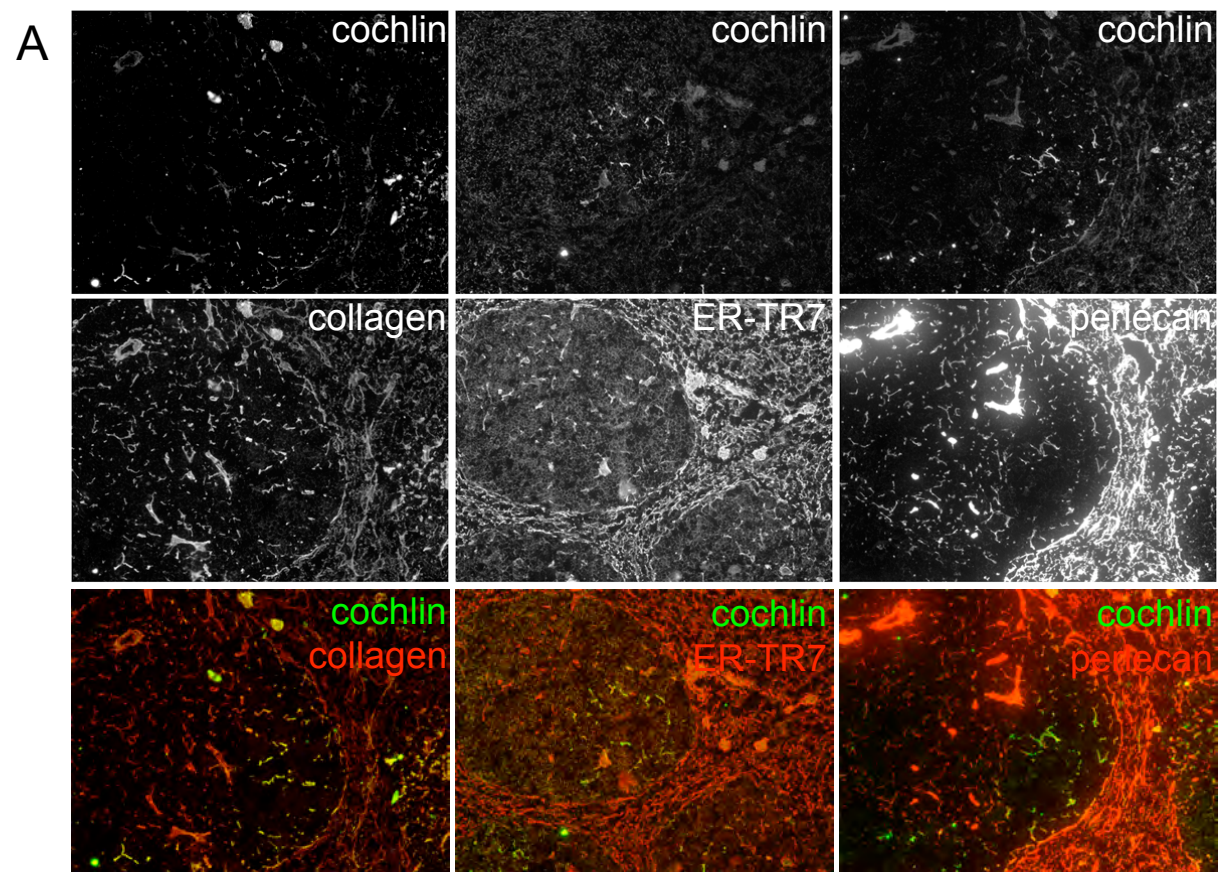


Figure 2

C

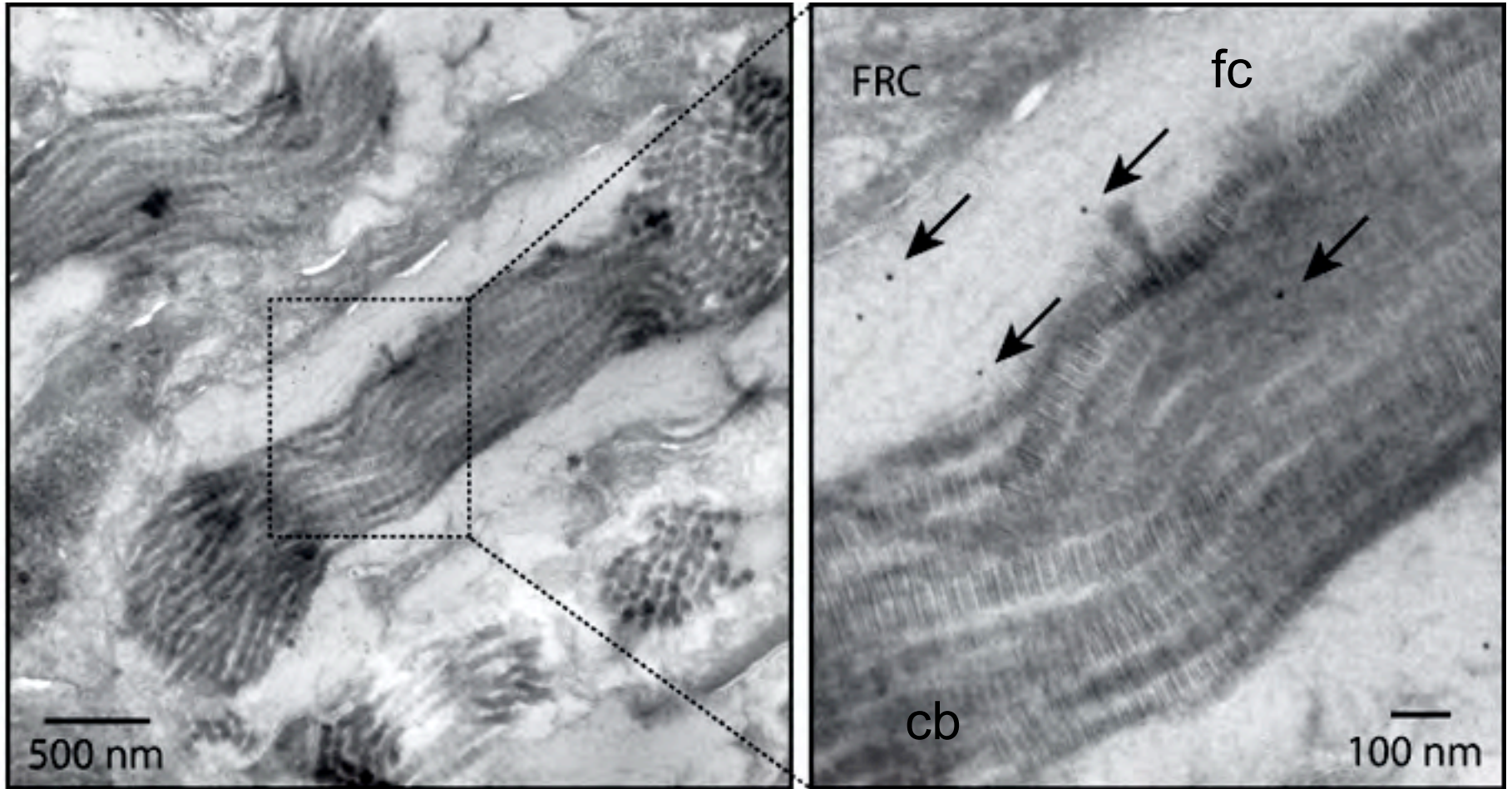
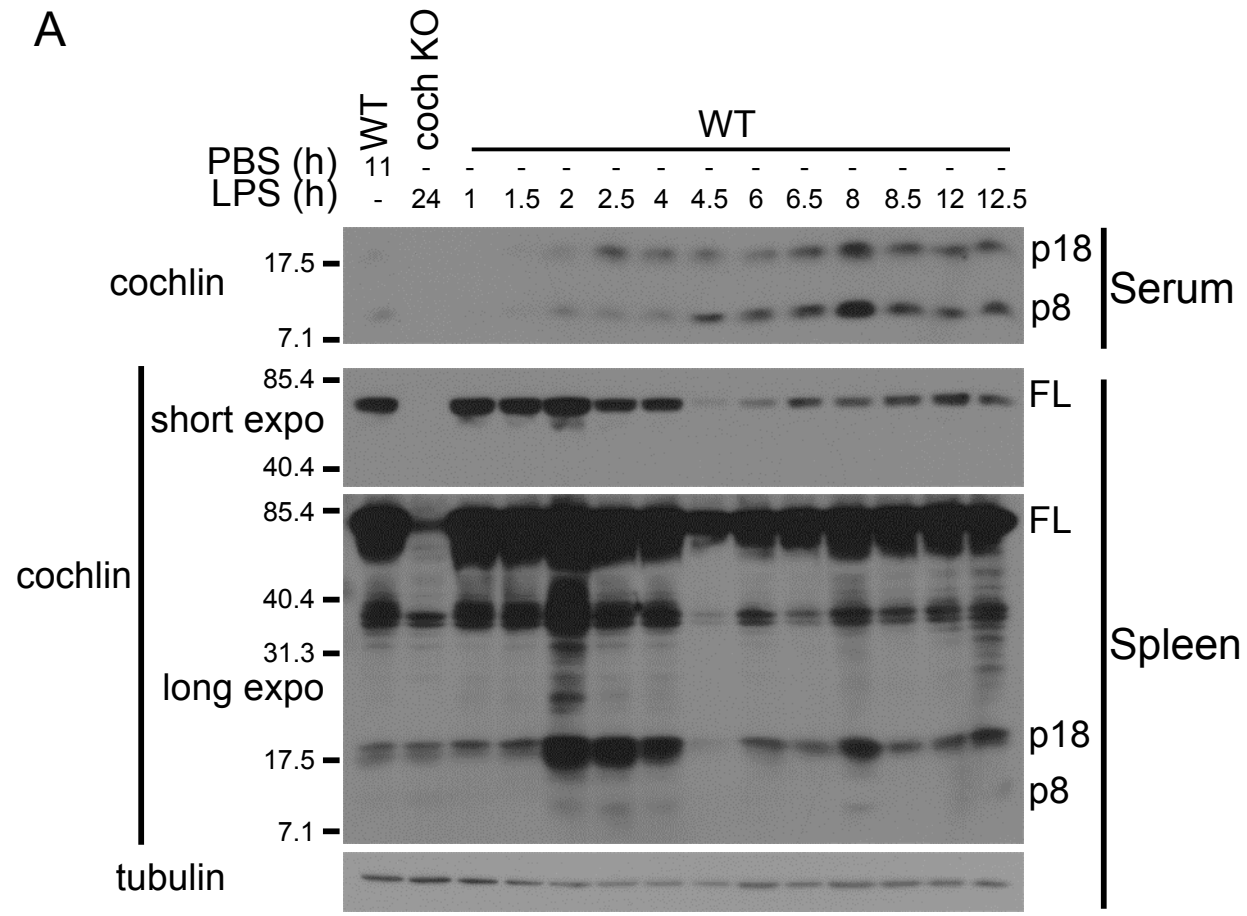


Figure 2, continued

A



B

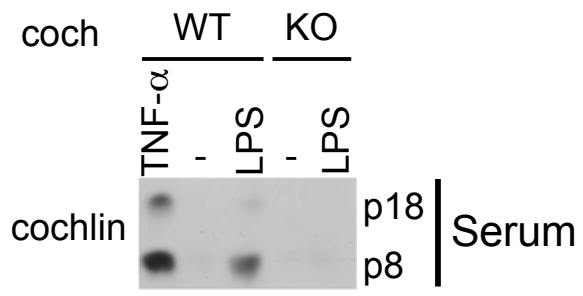


Figure 3

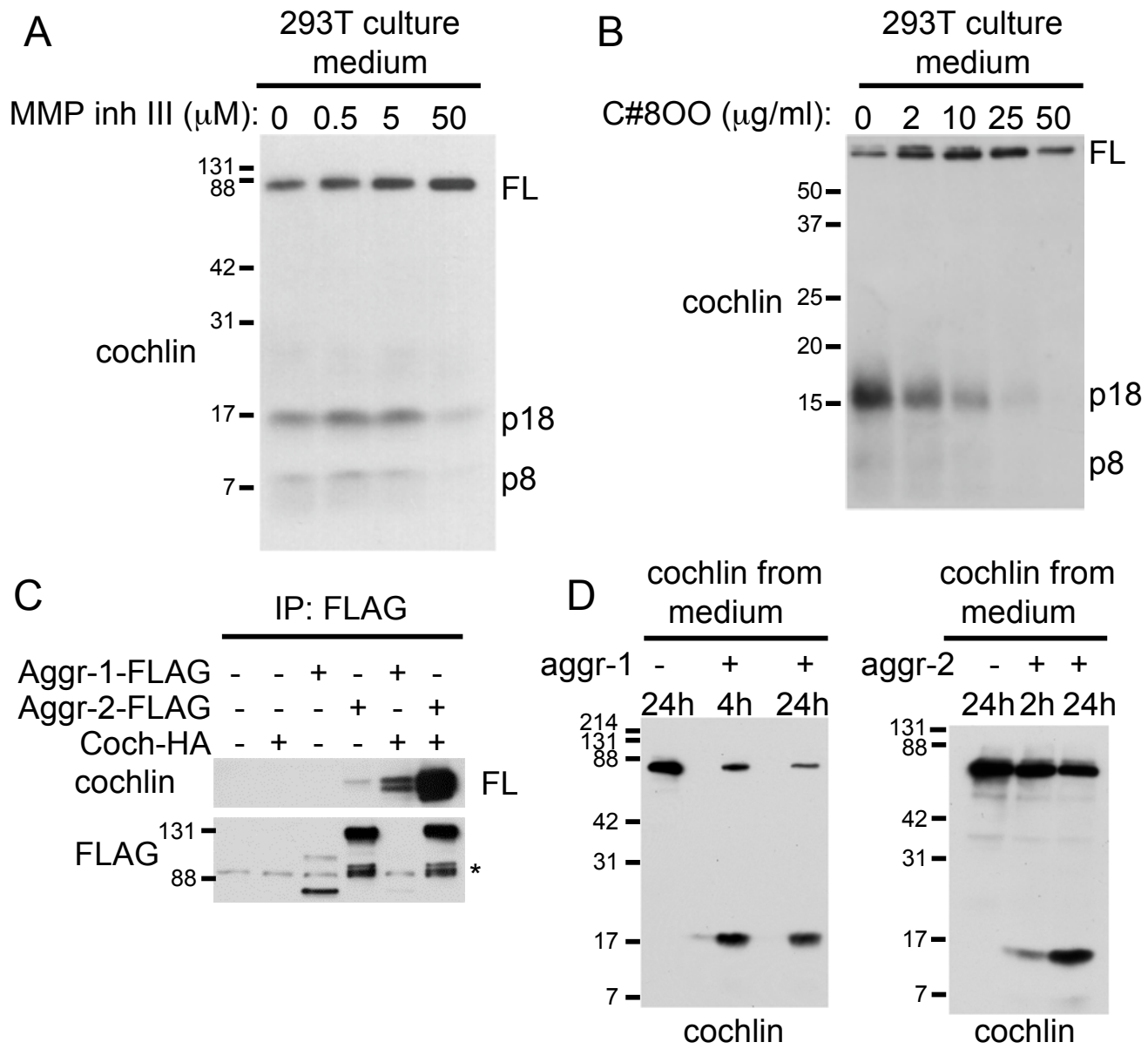


Figure 4

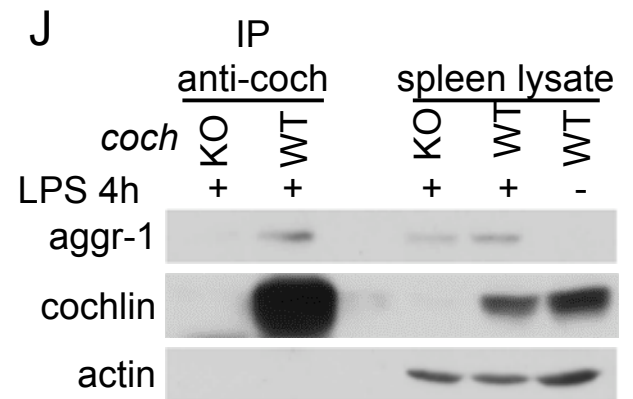
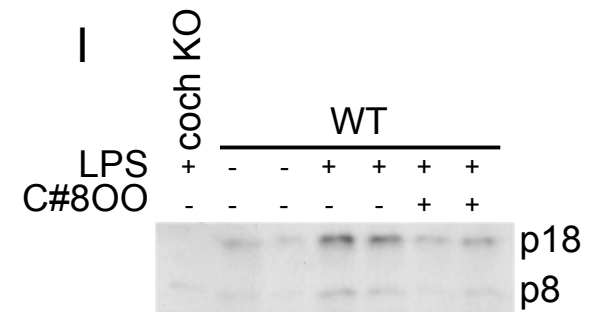
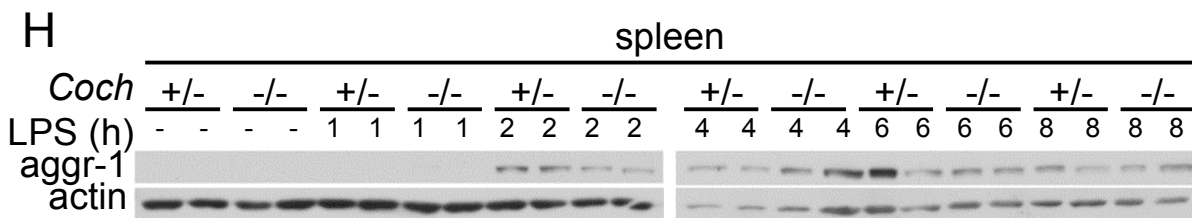
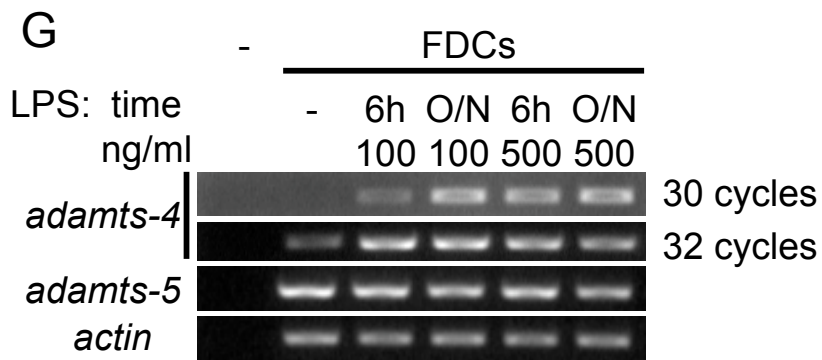
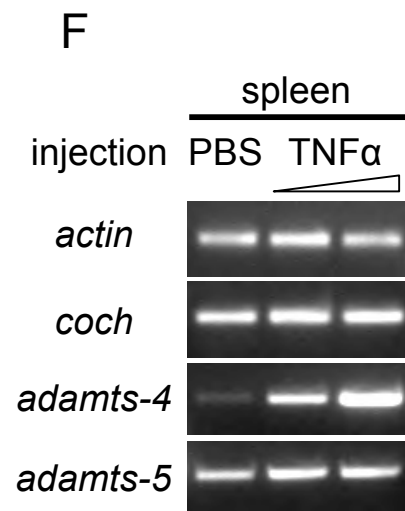
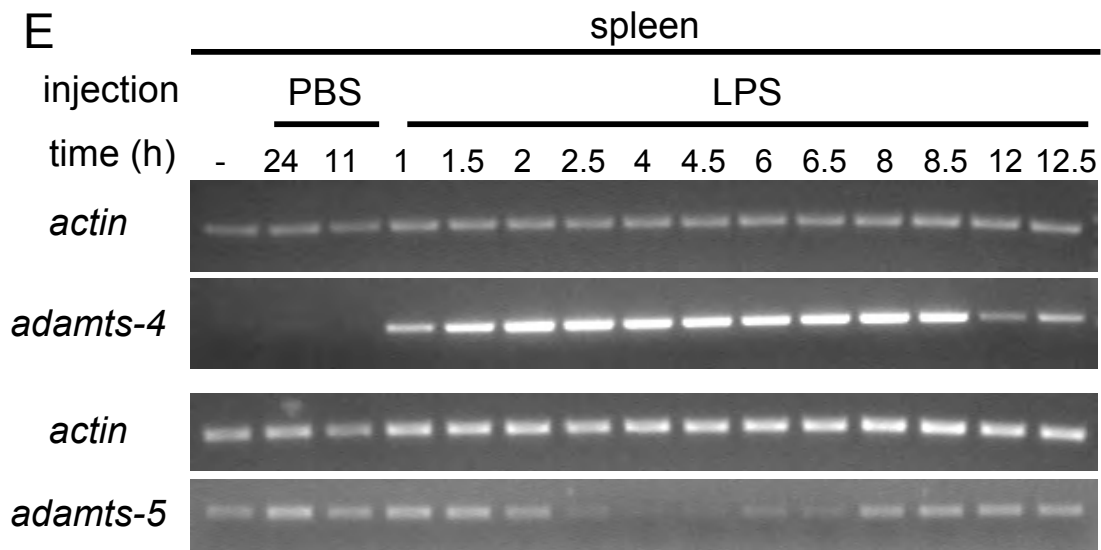


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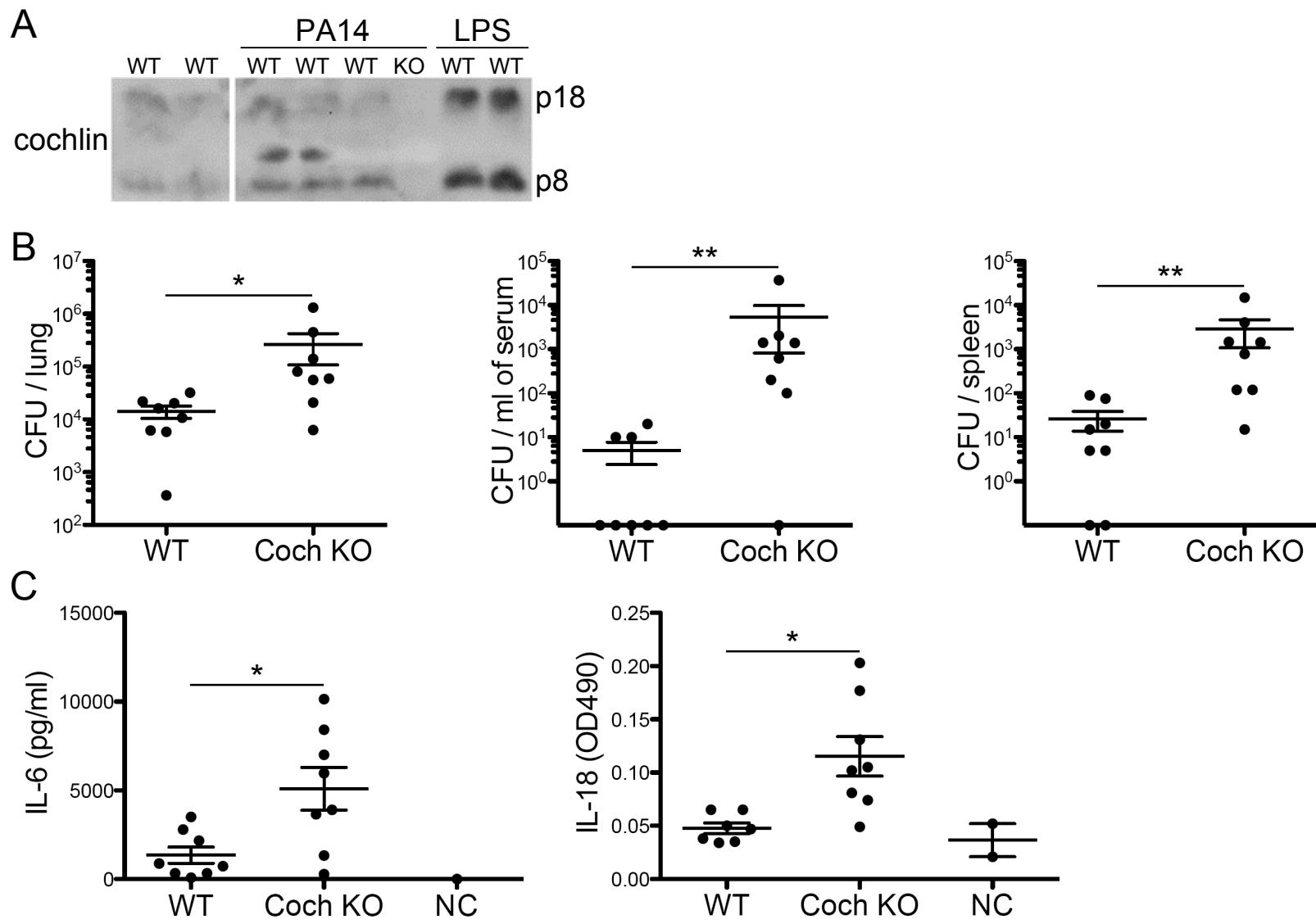


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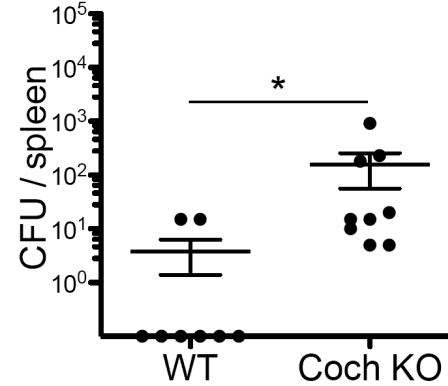
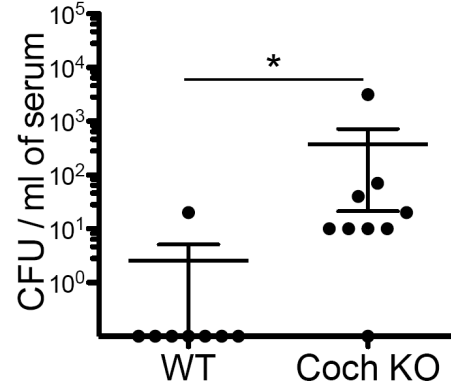
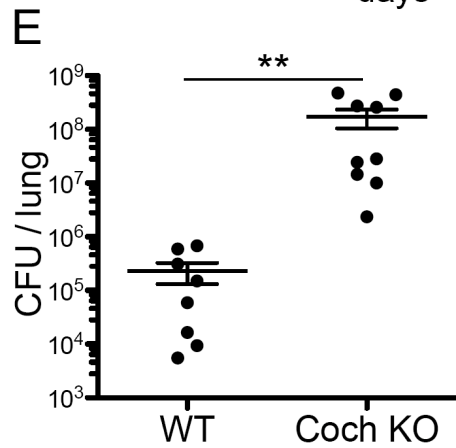
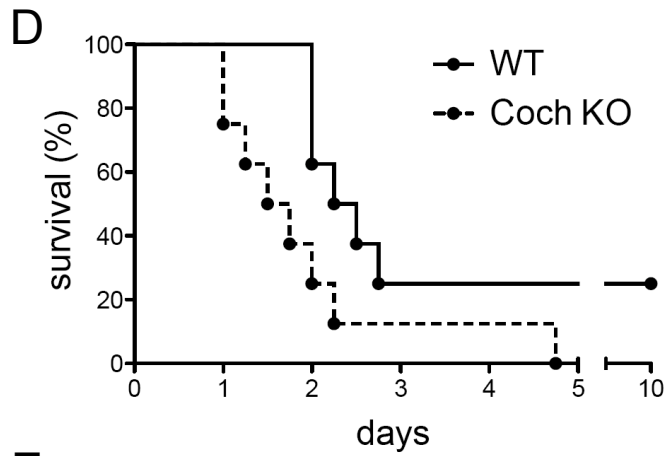


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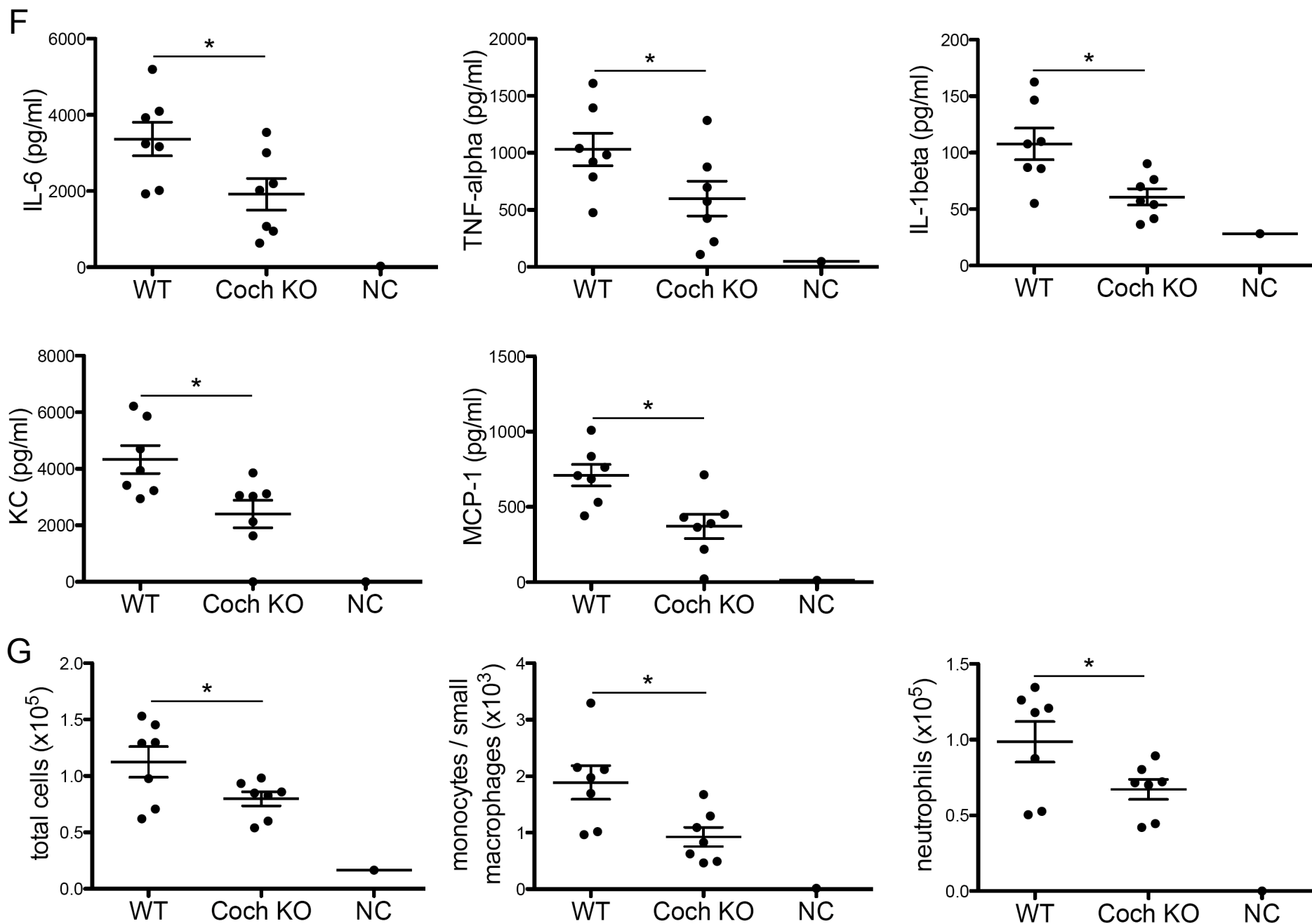


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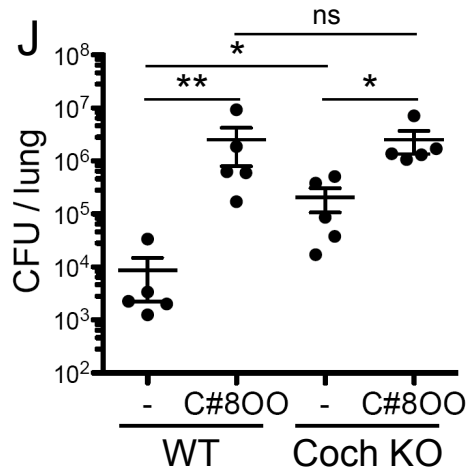
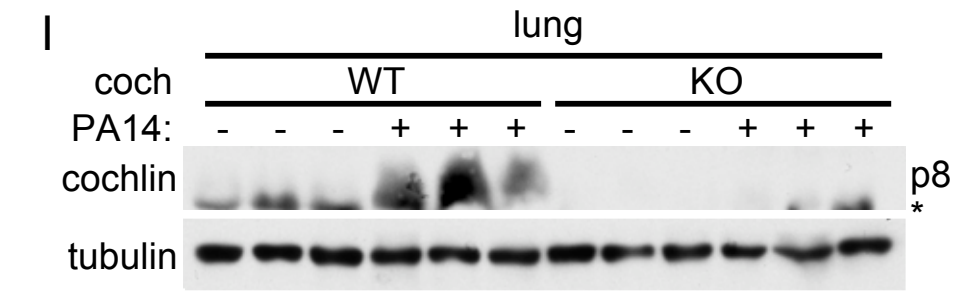
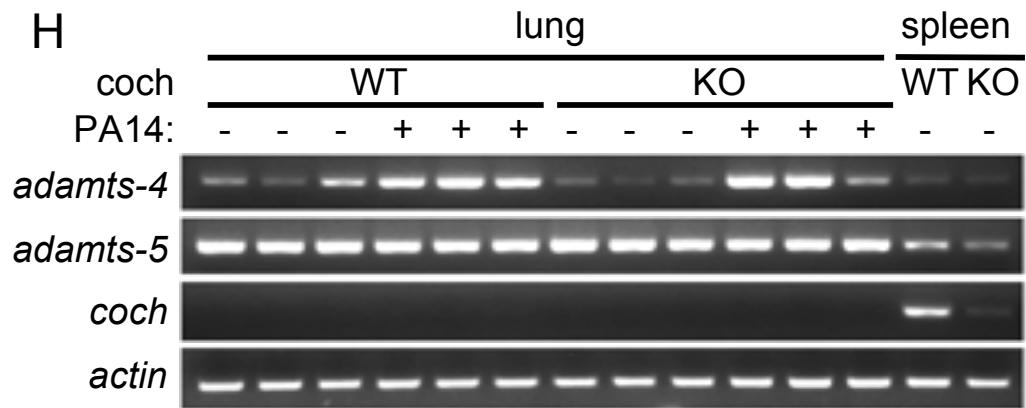


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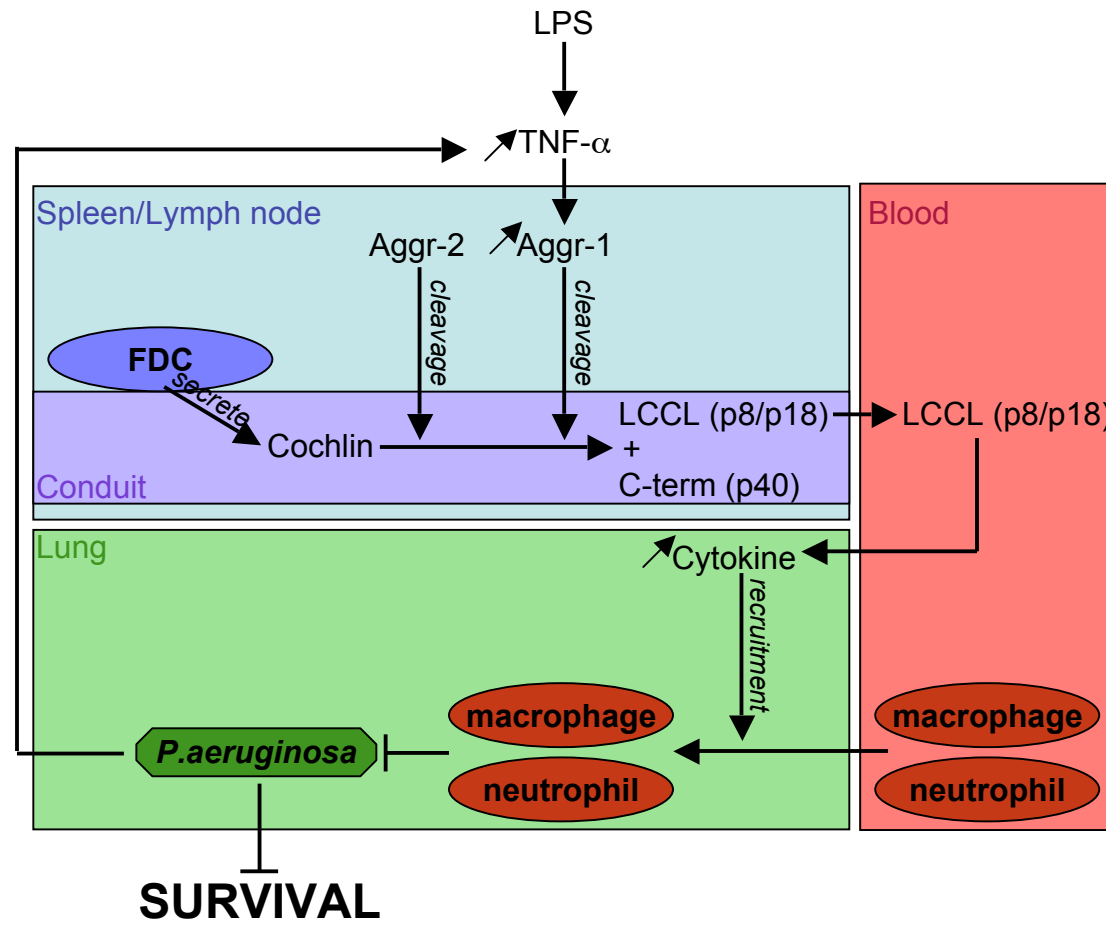


Figure 6

Inventory of supplemental information

- supplemental materials and methods
- supplemental figures S1-S5
- supplemental figure legends

Supplemental Materials and Methods

Mice

Coch^{-/-} mice, kindly provided by Dr Colin L. Stewart (NIH, Frederick, MD) (Rodriguez et al., 2004), were back-crossed for 10 generations with C57B6 mice (Charles River).

Mice were housed at the HCCM Warren Alpert, MCP and IDI animal facilities (Harvard Medical School) under specific pathogen-free conditions. Mice received food and water *ad libitum*. Experiments were performed in accordance with federal and institutional guidelines. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Harvard Medical Area Office for Research Subject Protection.

Cell growth conditions

293T cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) (Gibco) supplemented with 1X penicillin/streptomycin (PS) (Gibco) and 10% Fetal Bovine Serum (FBS) unless otherwise stated.

Reagents

The following reagents were used: LPS from *Escherichia coli* 0111:B4 (Sigma), recombinant TNF- α (Cell Sciences), PNGase F (New England Biolabs), MMP inhibitor III (Calbiochem), complete protease inhibitor cocktail (Roche), protein A/G UltraLink resin (Thermo scientific), anti-FLAG M2 agarose beads (Sigma).

Western-blot analysis

Organs were frozen in liquid nitrogen, ground and lysed in 2X Laemmli sample buffer. Spleen stromal tissue was separated from splenocytes by smashing spleens through a cell strainer. For the lymph node analyses, mixture of various lymph nodes was used (inguinal, axillary, mesenteric, cervical, aortic). Blood was incubated O/N at 4°C and spin at 2 000 rpm for 20 min to isolate serum. 1 µl of mouse serum diluted in 2X Laemmli sample buffer was loaded per well. Samples were analyzed by SDS-PAGE, transferred to PVDF membranes, blocked and probed according to standard procedures.

Histology staining

Lymph nodes and spleens were frozen in OCT. Sections were fixed in -20°C acetone, air dried and blocked (PBS, BSA 1 %, goat serum 10 %, Triton X-100 0.2 %). Sections were incubated with antibodies in blocking buffer and washed 4 times between each step (PBS, BSA 1 %, Triton X-100 0.2 %), before been treated with CuSO₄ 10 mM, ammonium acetate 50 mM pH 5 for 10 min, rinsed in PBS and mounted (PBS 1 X, glycerol 50 %, 0.1 % n-propylgallate). The following antibodies were used: anti-B220-PE, anti-CR2.1-PE, anti-IgD-PE (BD Pharmingen), anti-collagen type 1-biotin (Rockland immunochemical), anti-ER-TR7 (Novus), anti-perlecan-biotin (Thermo Scientific), anti-laminin-1 (R&D systems). Sections were imaged on a Nikon TE2000E Inverted Fluorescence Microscope using MetaMorph software (Molecular Devices). Images acquired with objective lens 100X as Z-series 0.25 mm were deconvolved using AutoQuant X2 software (AutoQuant Imaging, Inc), followed by 3D reconstruction using MetaMorph software.

Electronic microscopy

Spleens were fixed (4 % paraformaldehyde), infiltrated (PBS, sucrose 2.3 M, glycin 0.2M), frozen in liquid nitrogen and sectioned at -120 °C. Sections transferred to formvar-carbon coated copper grids and labeled with anti-cochlin Ab (P13) and protein A-gold (PBS, 1 % BSA) then washed in PBS and double distilled water. Samples were analyzed on a Tecnai G2 Spirit Bio TWIN electron microscope (FEI Company).

RT-PCR

RNAs were purified using Trizol (Invitrogen) followed by the RNA clean-up protocol (Qiagen). Single strand cDNAs were synthesized (Superscript first-strand kit, Invitrogen) and amplified by RT-PCR using the following primers actinF 5'-CACACTGTGCCCATCTACG-3', actinR 5'-CCAGACAGCACTGTGTTGG-3', adamts-4F 5'-GGAGGAGGAGATCGTGTTTCC-3', adamts-4R 5'-GCTGCCATCTTGTCATCTGCTAC-3', adamts-5F 5'-ATCTTTTCGCCATGAGCAGTG-3', adamts-5R 5'-GGTACTCGCCAGTTTTCTTCTTC-3', cochF 5'-AGCGGTTCCCATTCCTGTCAC-3', cochR 5'-GGCTTCCTGGGTACTGCTTTTG-3'.

Immunoprecipitation

Anti-FLAG IP. 293T cells expressing cochlin-HA and aggrecanase-1-FLAG or aggrecanase-2-FLAG were lysed (NP40 1 %, NaCl 150 mM, Tris HCl 500 mM pH 7.6, EDTA 5 mM, PMSF 1 mM, protease inhibitors), and subject to anti-FLAG M2 IPs. After 6 washes in lysis buffer, bound proteins were eluted in 2X Laemmli buffer.

Anti-endogenous cochlin IP. Media from *in vitro* cultured FDCs were collected after 3 days. Ground frozen spleens were homogenized (NP40 1 %, NaCl 150 mM, Tris HCl 500 mM pH 7.6, EDTA 5 mM, PMSF 1 mM, protease inhibitors). Cochlin was immunoprecipitated using the polyclonal anti-cochlin and protein A/G resin. After 6 washes in lysis buffer, proteins were eluted in 2X Laemmli buffer.

Flow cytometry

The BAL cell pellet was stained with anti-CD11b-PE, anti-GR1-APC, anti-CD11c-PECy7 (eBioscience), after the Fc receptors were blocked using anti-CD16/32 (eBioscience). Neutrophils (CD11c⁻/CD11b^{high}/GR-1⁺), monocytes/small macrophages (CD11c⁻/CD11b^{mild}/GR-1⁻) and alveolar macrophages (CD11c^{high}/CD11b^{mild}/GR-1⁻) were analyzed on a FACSCalibur flow cytometer (BD Biosciences) (Gonzalez-Juarrero et al., 2003).

ELISA test

The ELISA test were performed using the following kits: IL1 β , IL-6 and TNF- α ELISA (Quantikine (serum) and DuoSet (cell supernatant, BAL), RnD Systems), IL-18 (MBL), MCP-1 (Biolegend), KC (Raybiotech).

Statistical analysis

The statistical significance of the cytokines concentrations and of the CFU loads in the different organs between coch^{-/-} vs WT mice, or between compound 800 vs vehicle-treated mice were determined by T-test on direct and log-transformed values respectively

(Prism 4, GraphPad). The statistical significance of coch^{-/-} and WT mice survival to PA14 infection was determined by Gehan-Breslow-Wilcoxon test (Prism 4, GraphPad). For the analysis of the effect of cochlin deficiency on C800-induced increase in CFU, bacterial counts were log₁₀ transformed and a two-way ANOVA was conducted. The fixed factors used were “genotype”, “C800 treatment” and the interaction between “genotype” and “C800 treatment”.

- Gonzalez-Juarrero, M., Shim, T.S., Kipnis, A., Junqueira-Kipnis, A.P., and Orme, I.M. (2003). Dynamics of macrophage cell populations during murine pulmonary tuberculosis. *J Immunol* *171*, 3128-3135.
- Rodriguez, C.I., Cheng, J.G., Liu, L., and Stewart, C.L. (2004). Cochlin, a secreted von Willebrand factor type a domain-containing factor, is regulated by leukemia inhibitory factor in the uterus at the time of embryo implantation. *Endocrinology* *145*, 1410-1418.

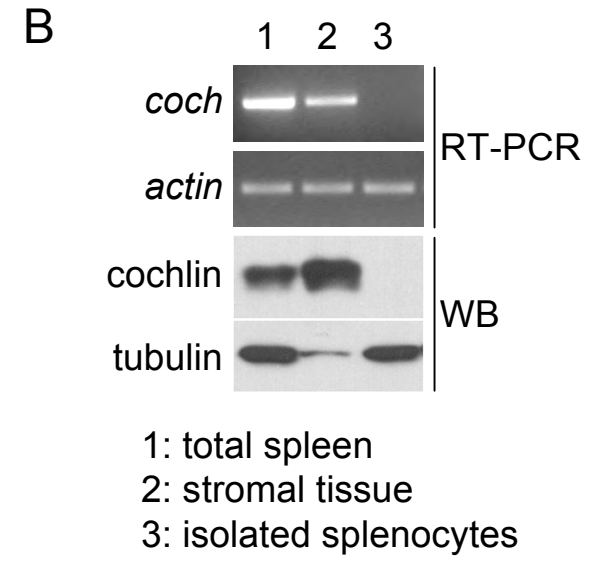
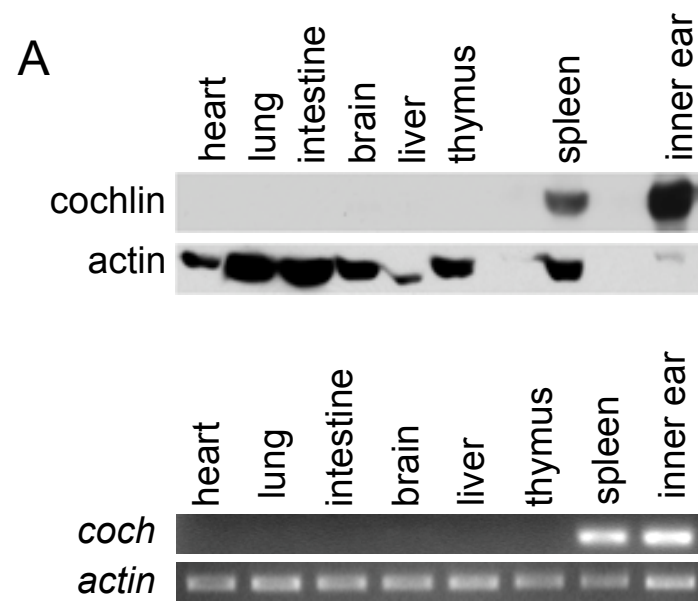


Figure S1

C

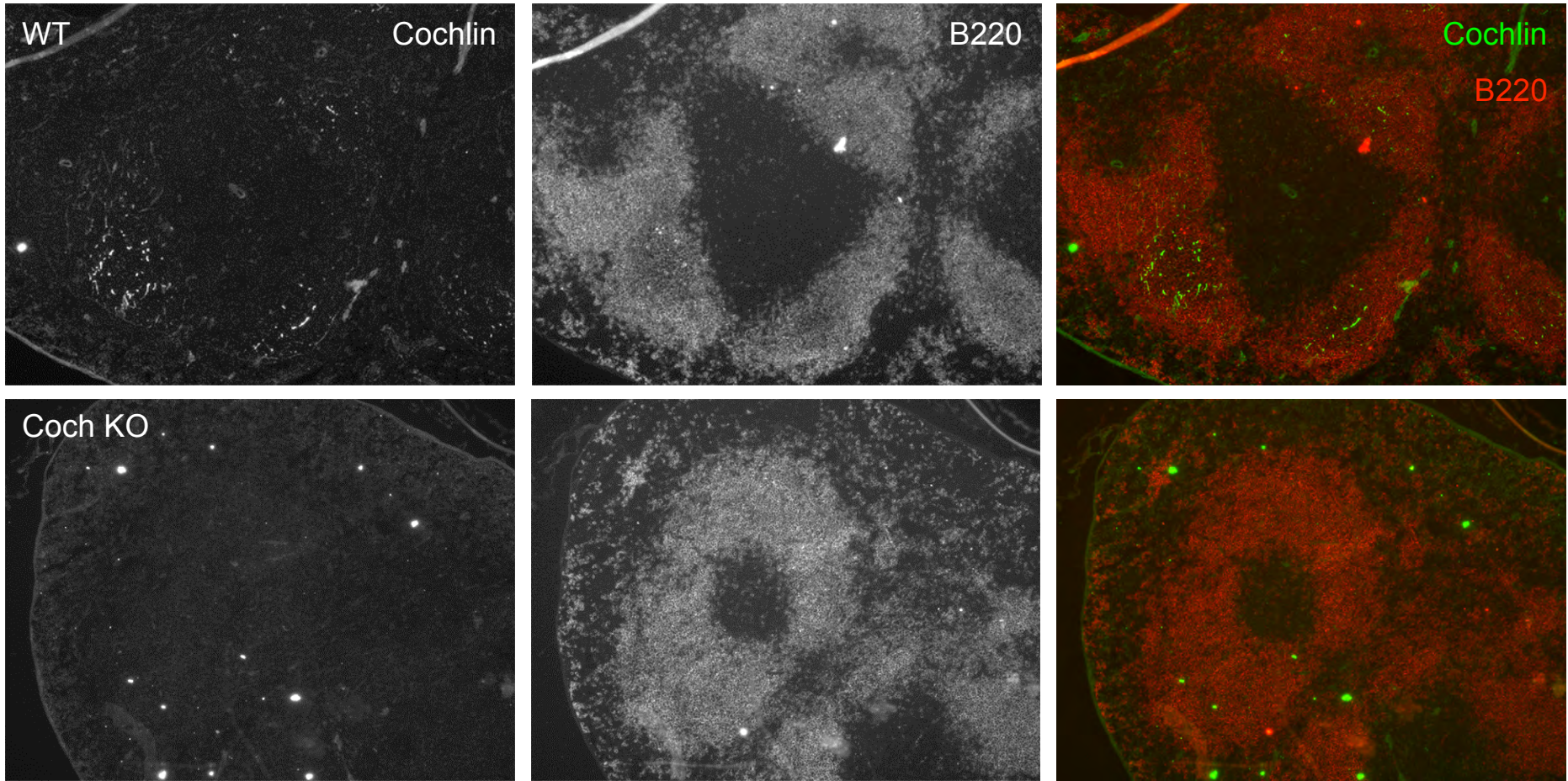


Figure S1, continued

A

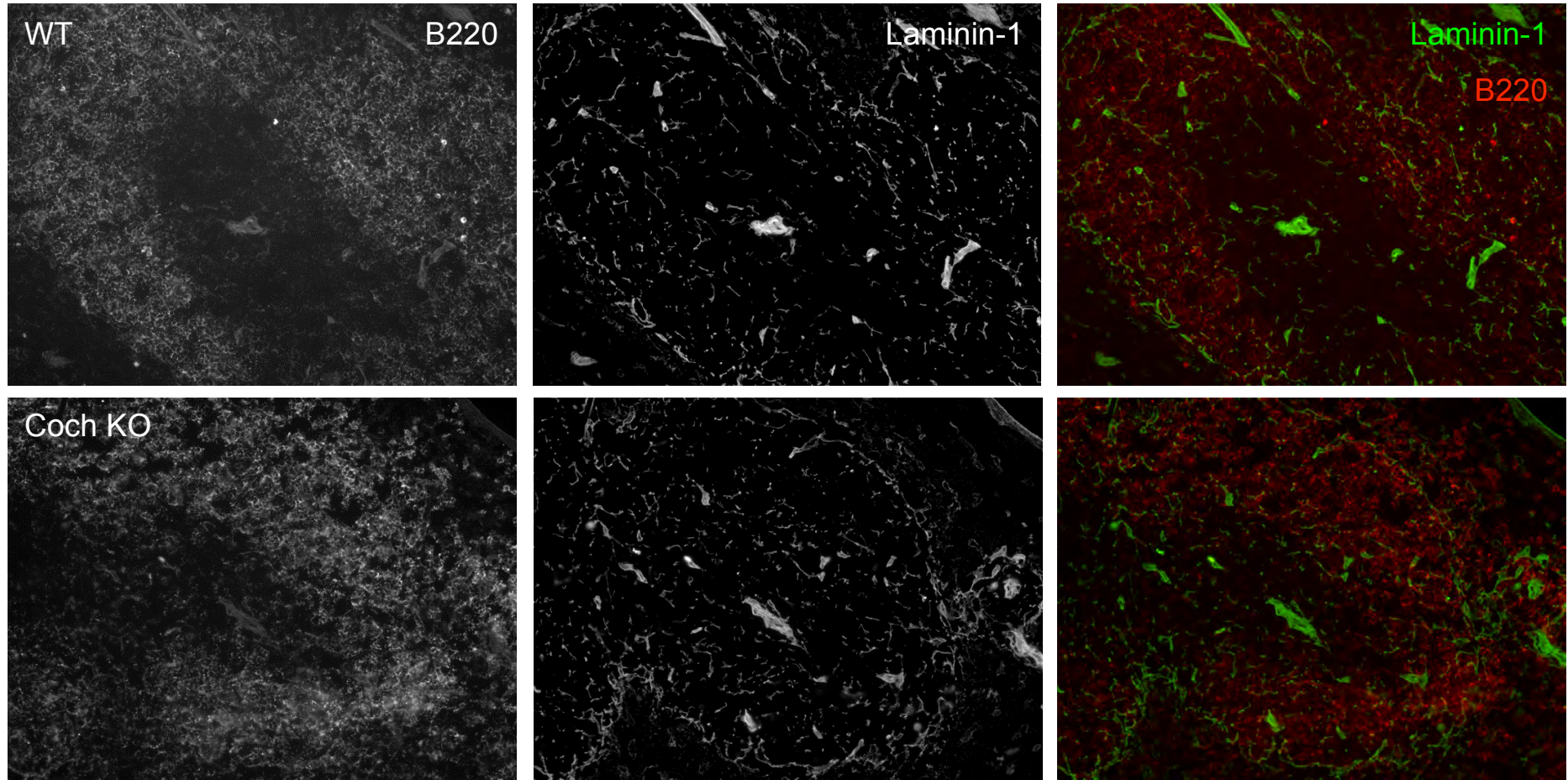


Figure S2

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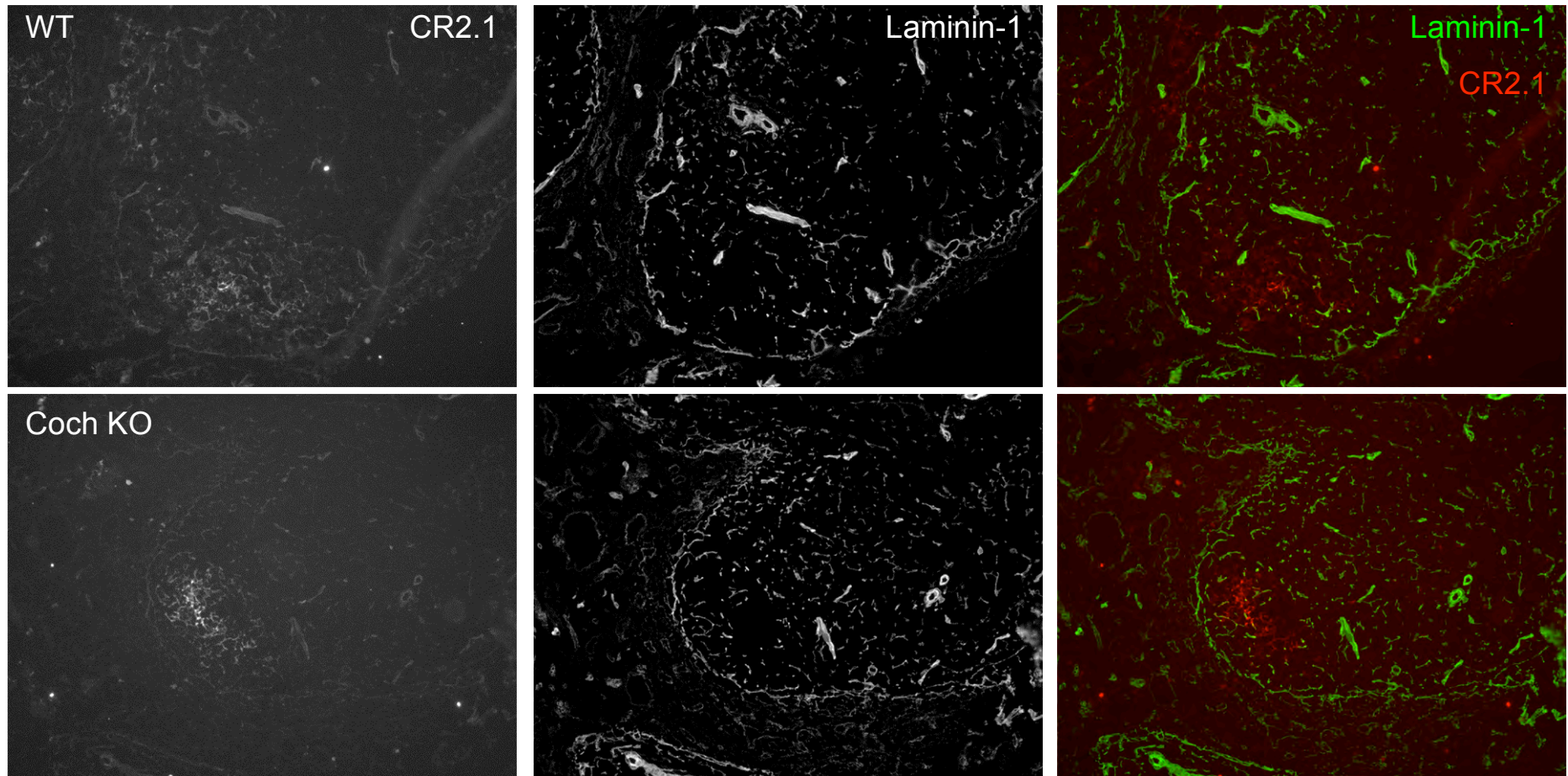


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C

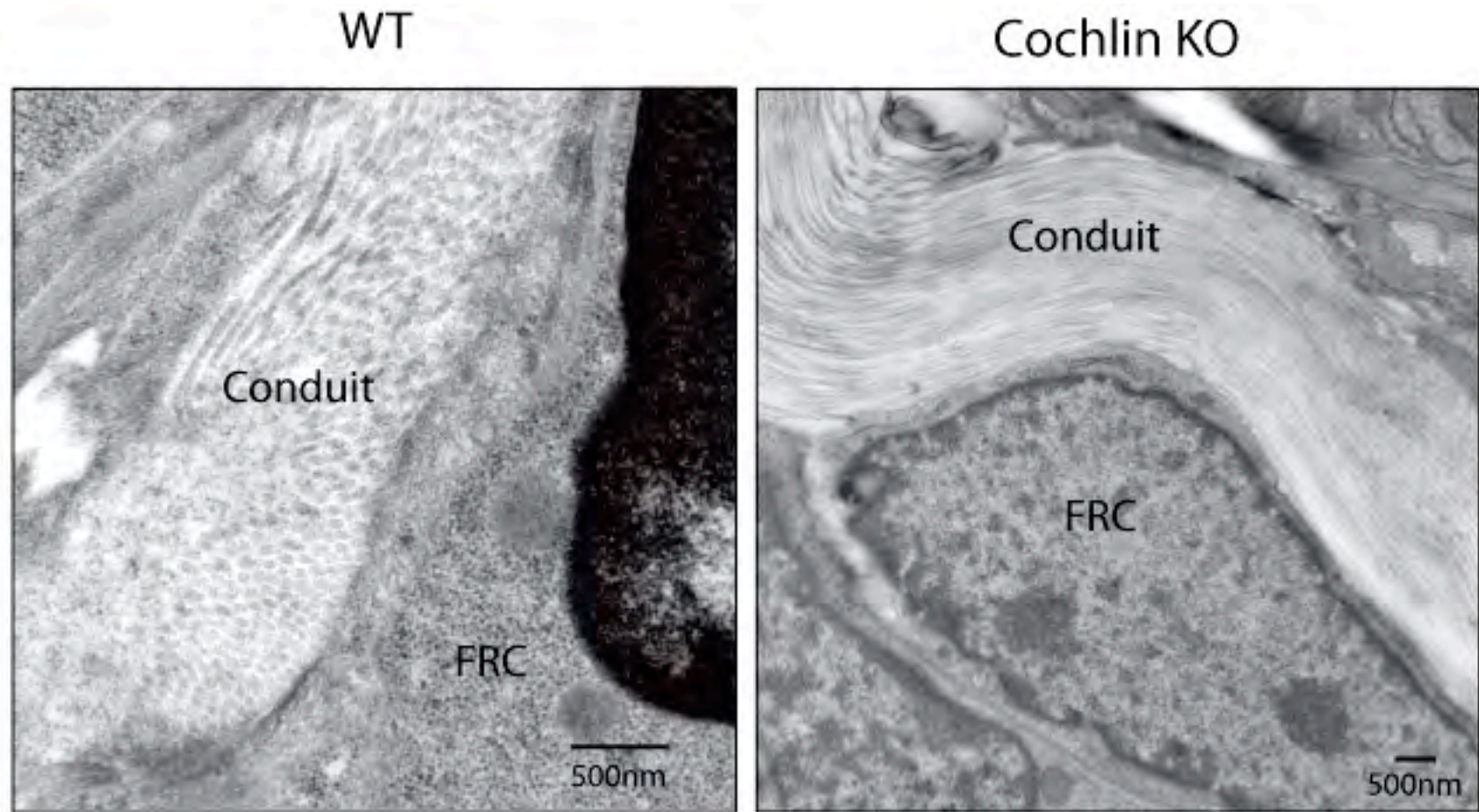


Figure S2, continued

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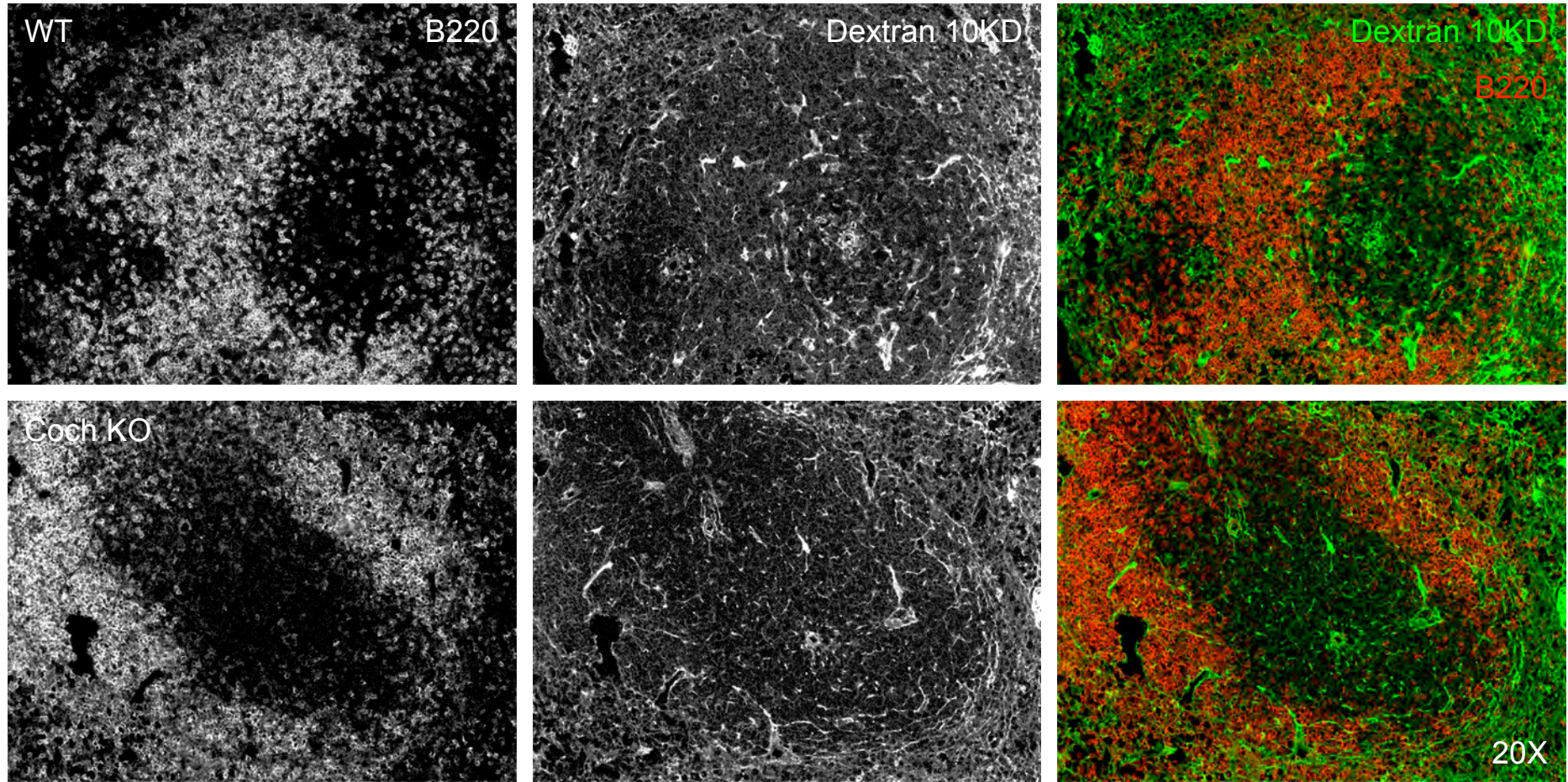


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E

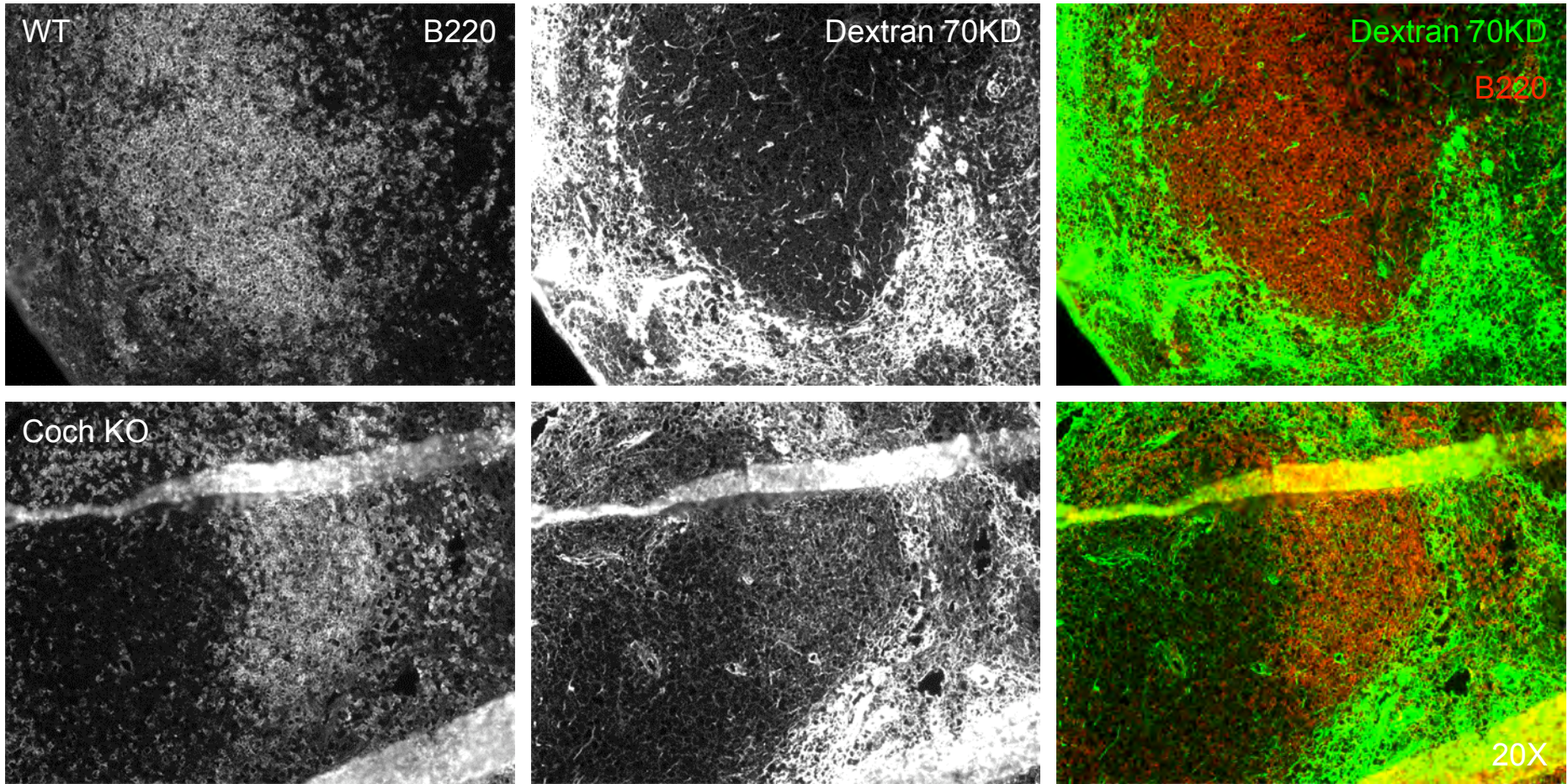


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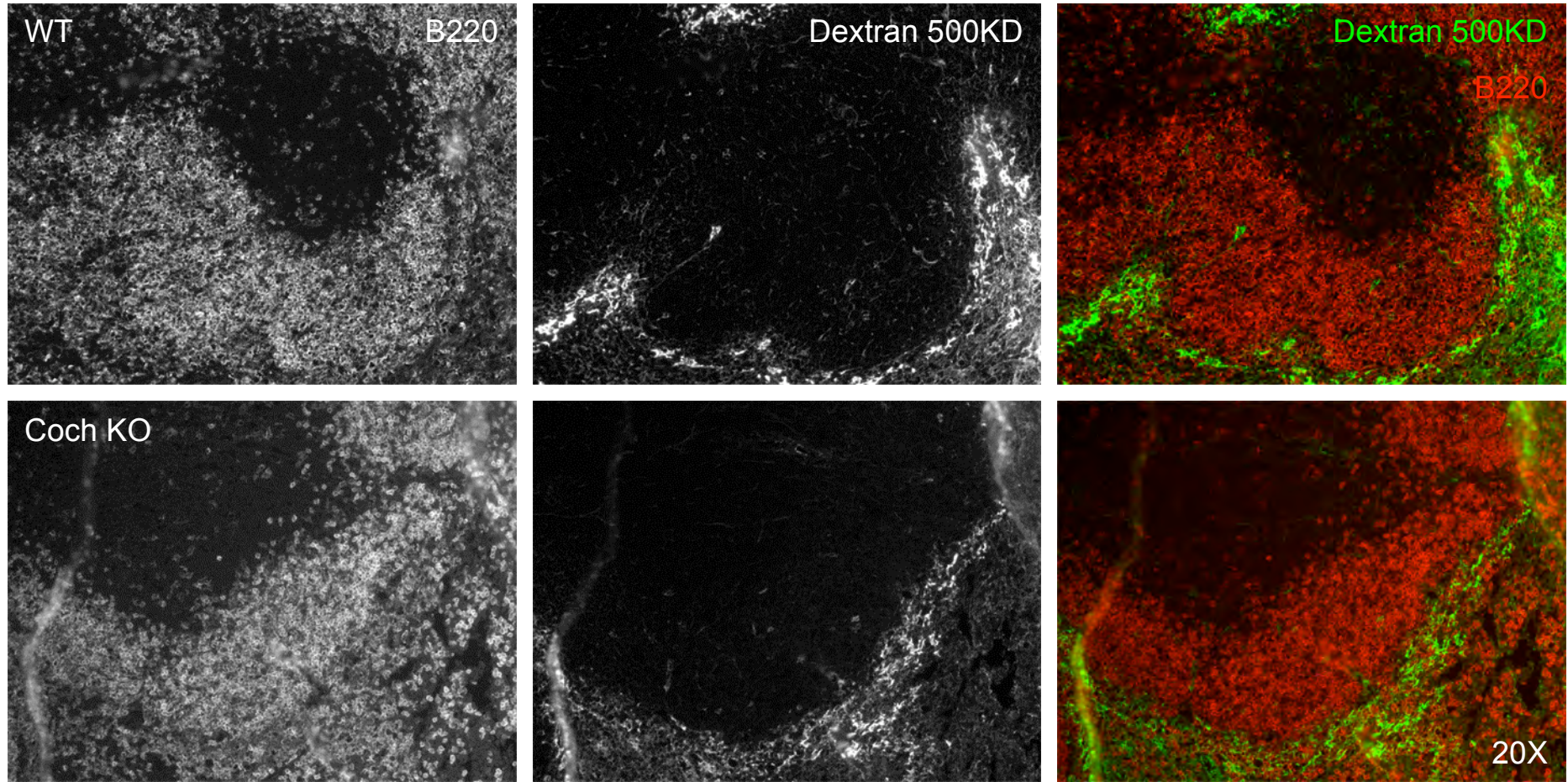


Figure S2, continued

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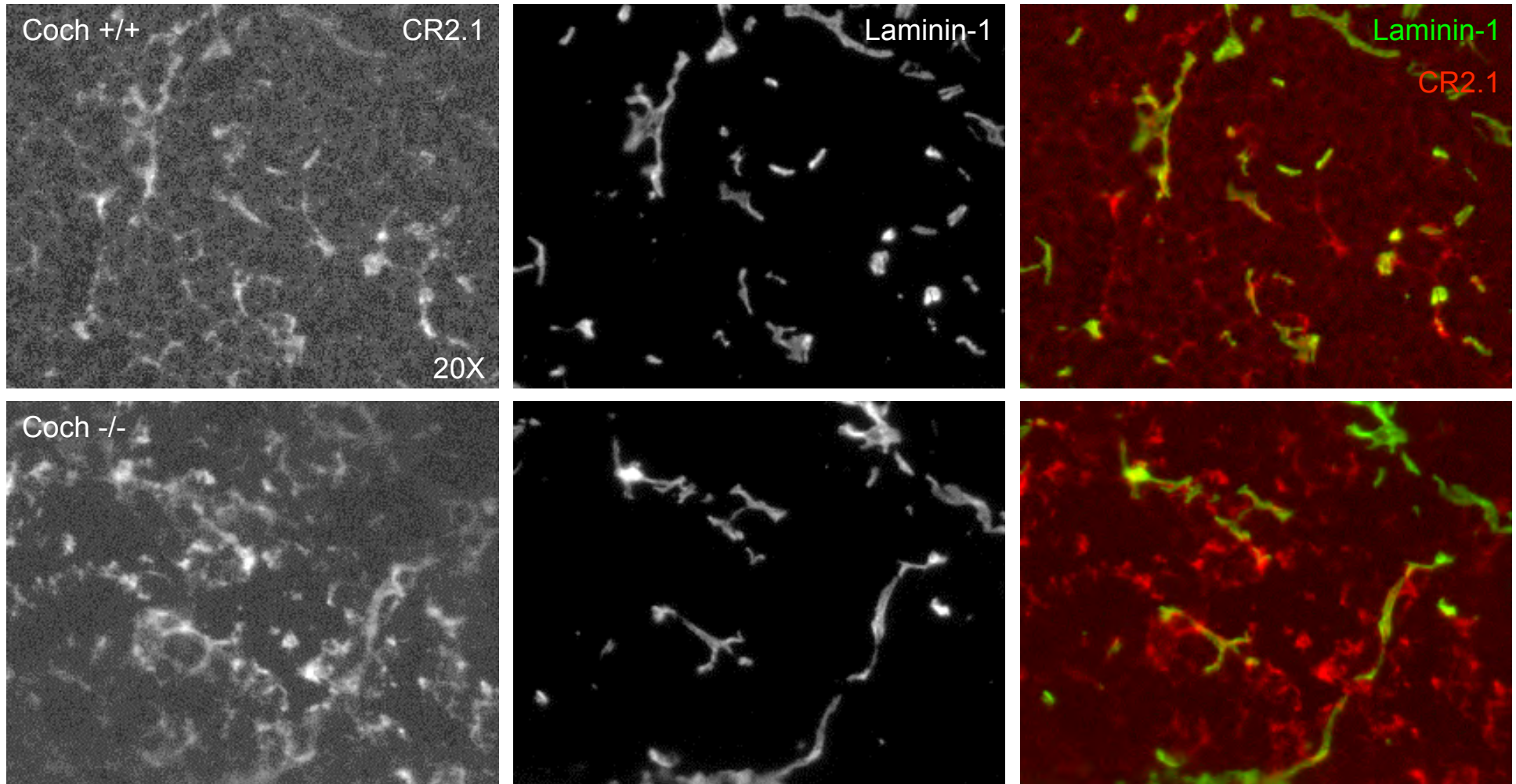


Figure S3, continued

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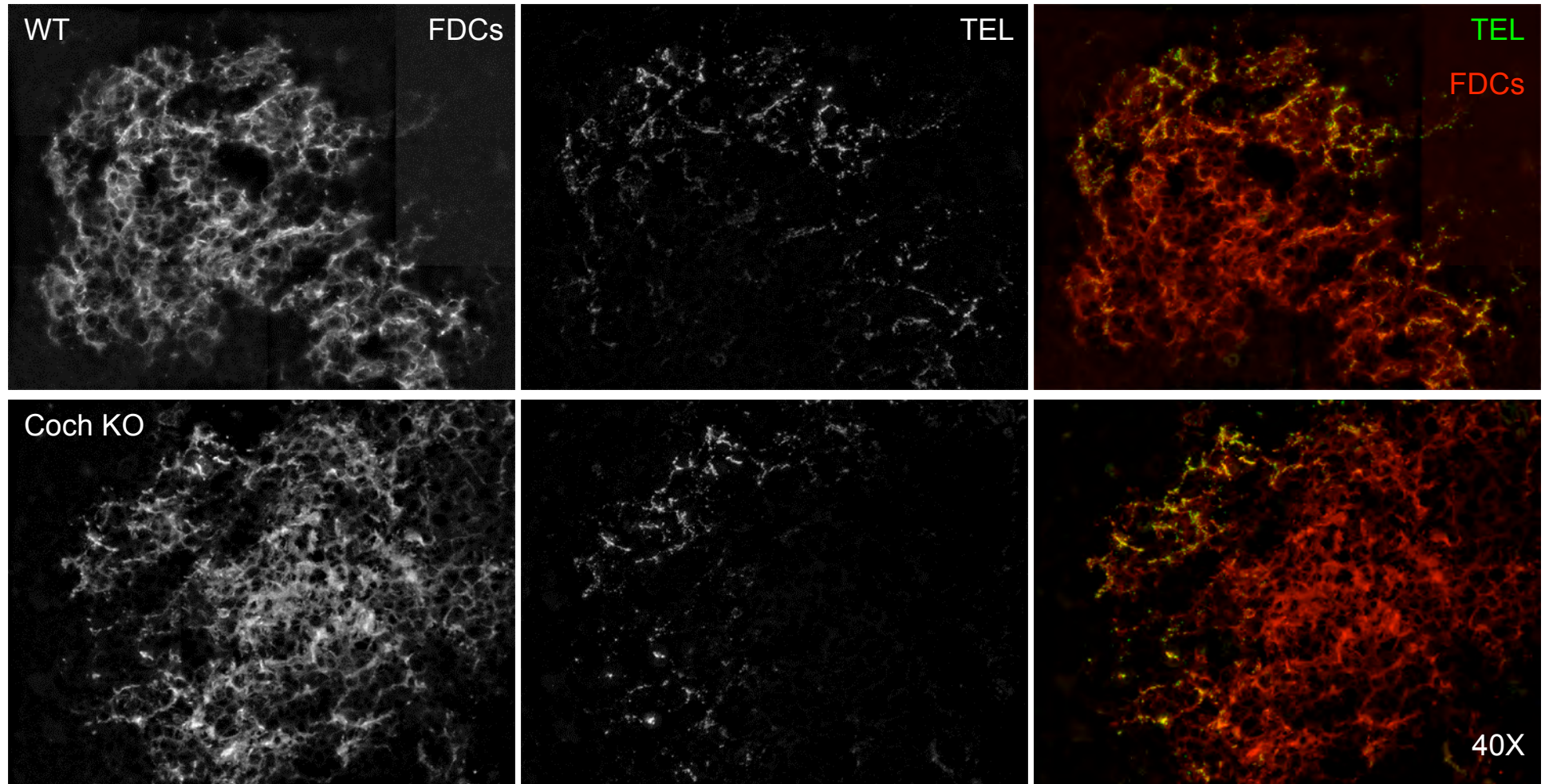


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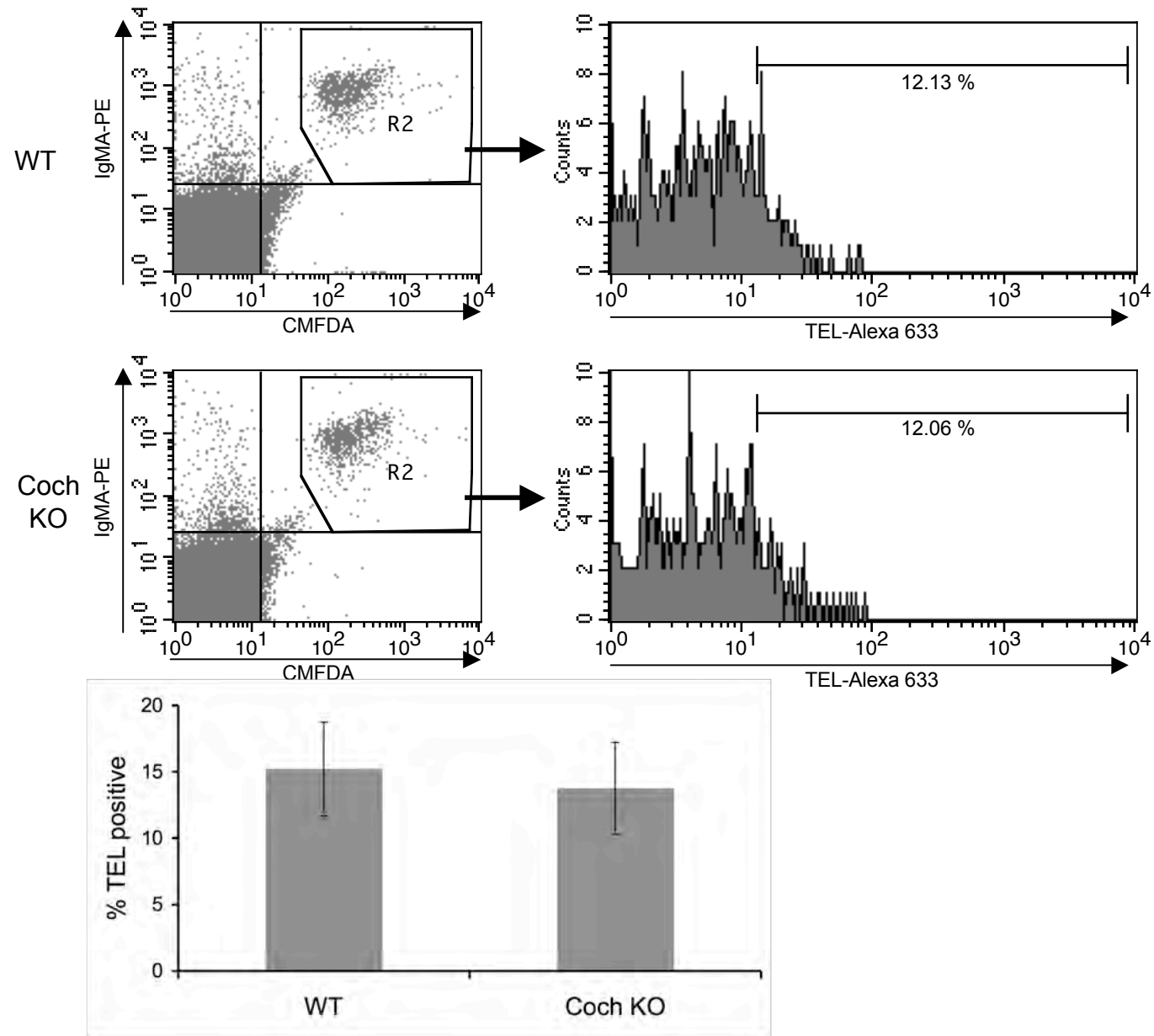


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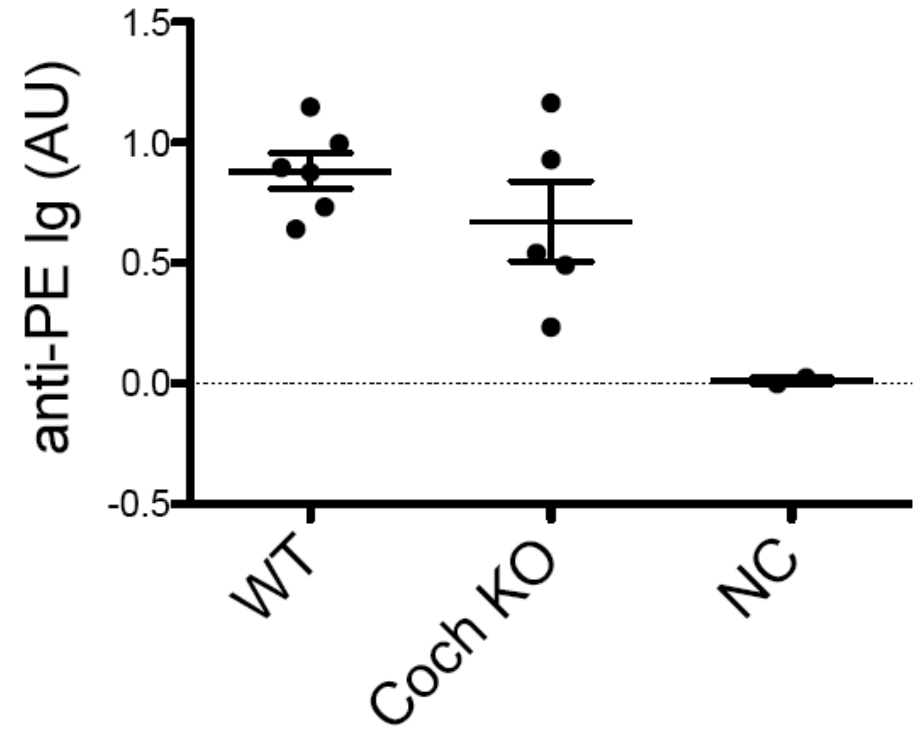
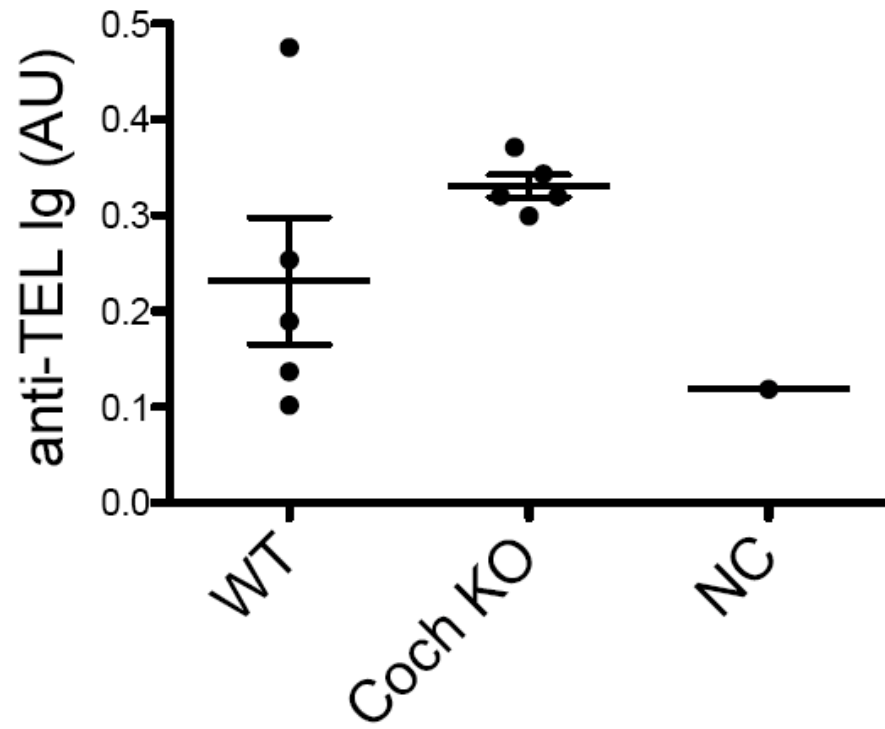


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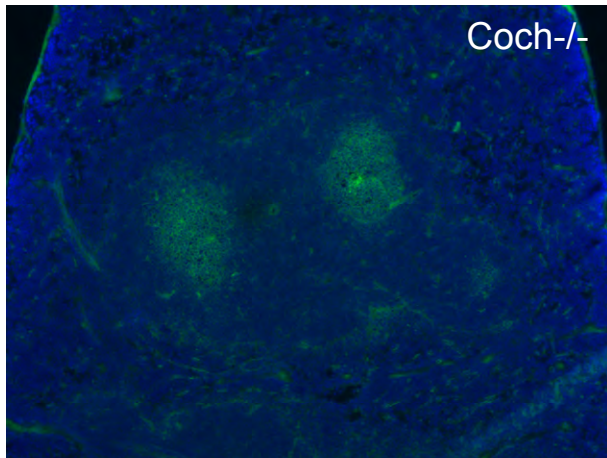
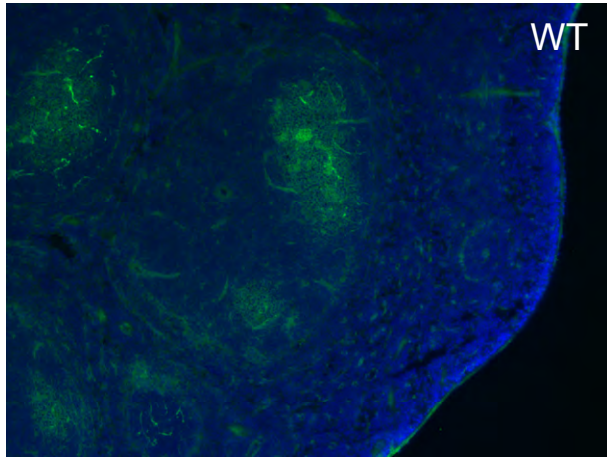


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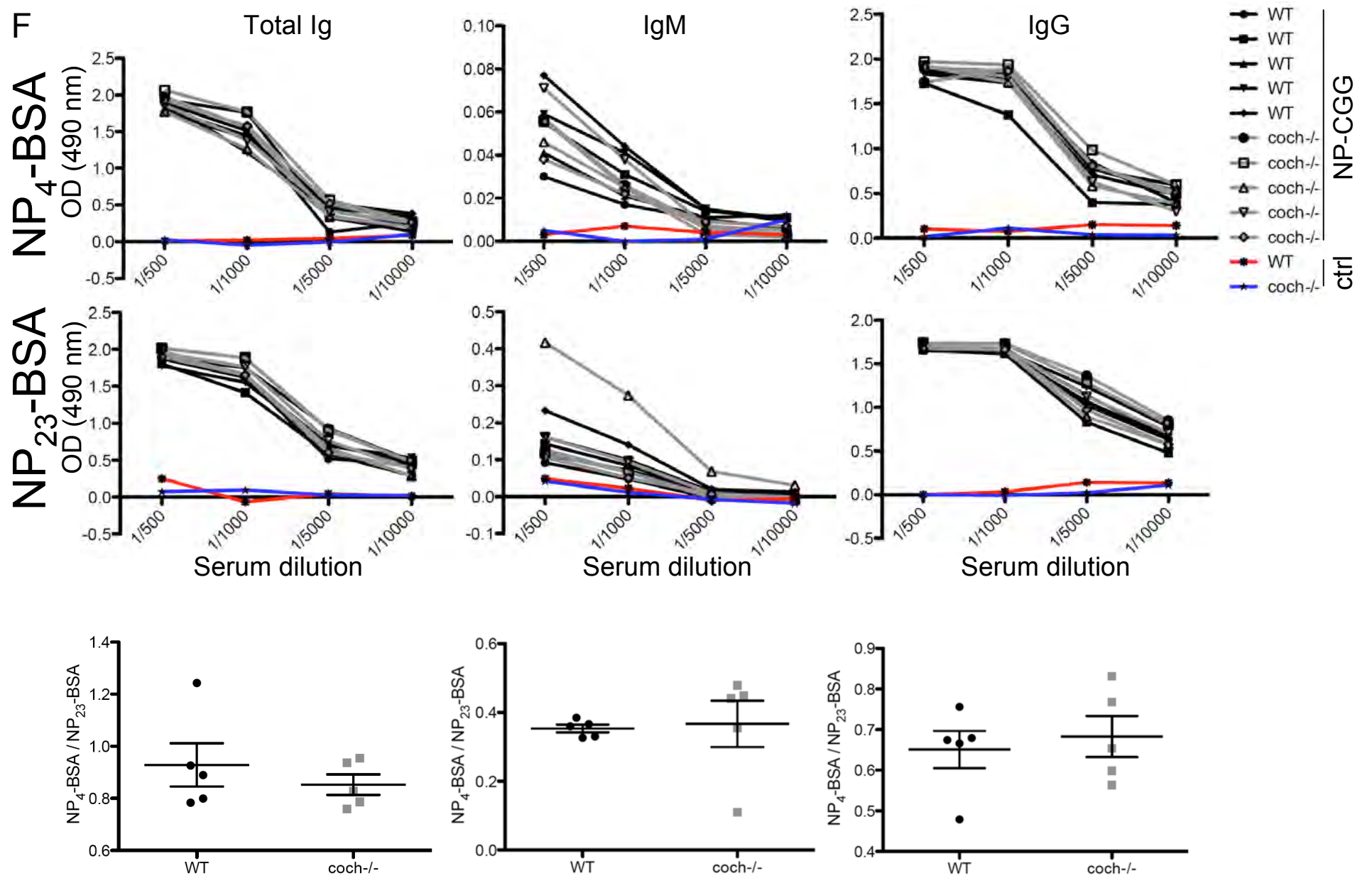


Figure S3, continued

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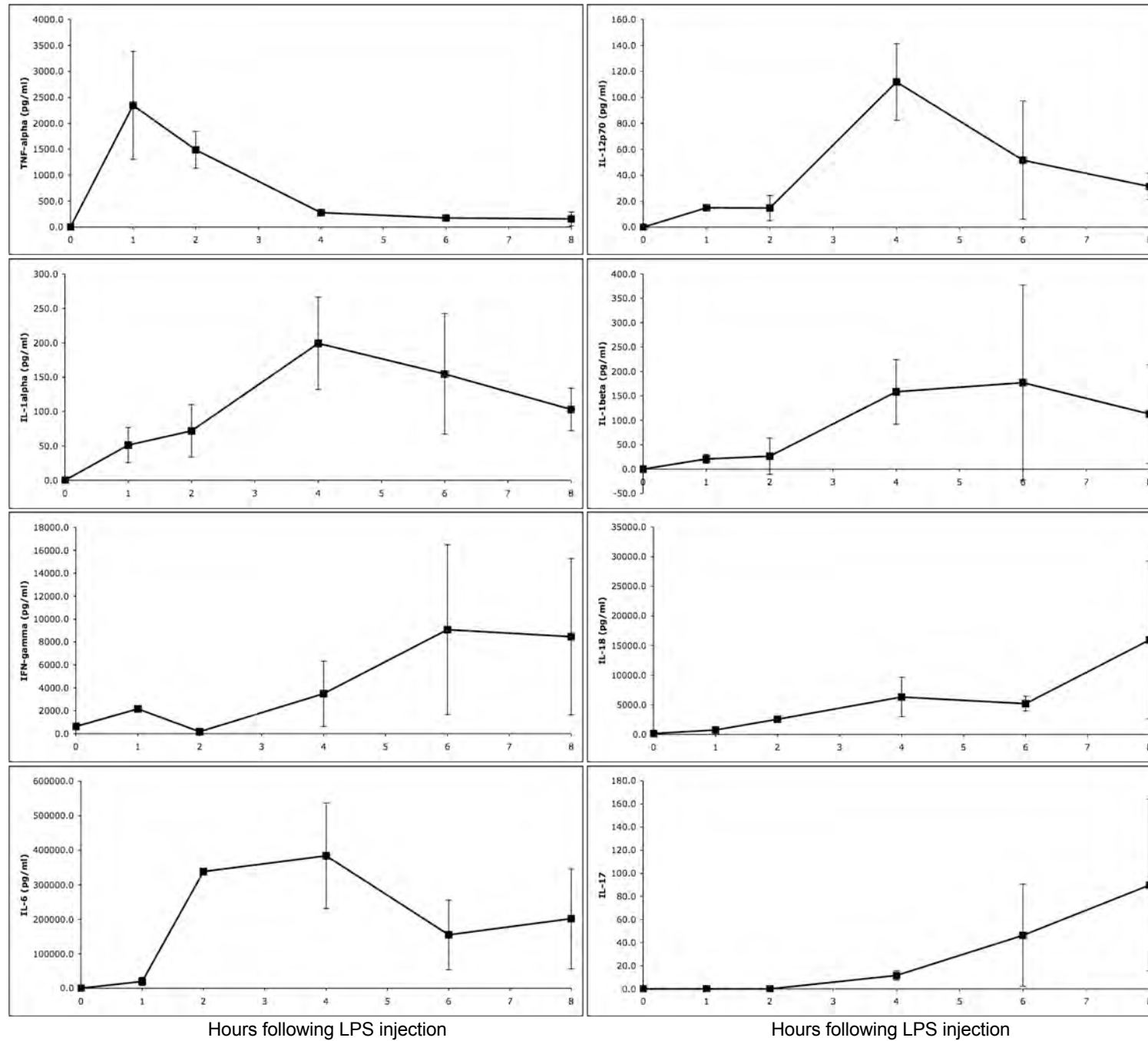


Figure S4

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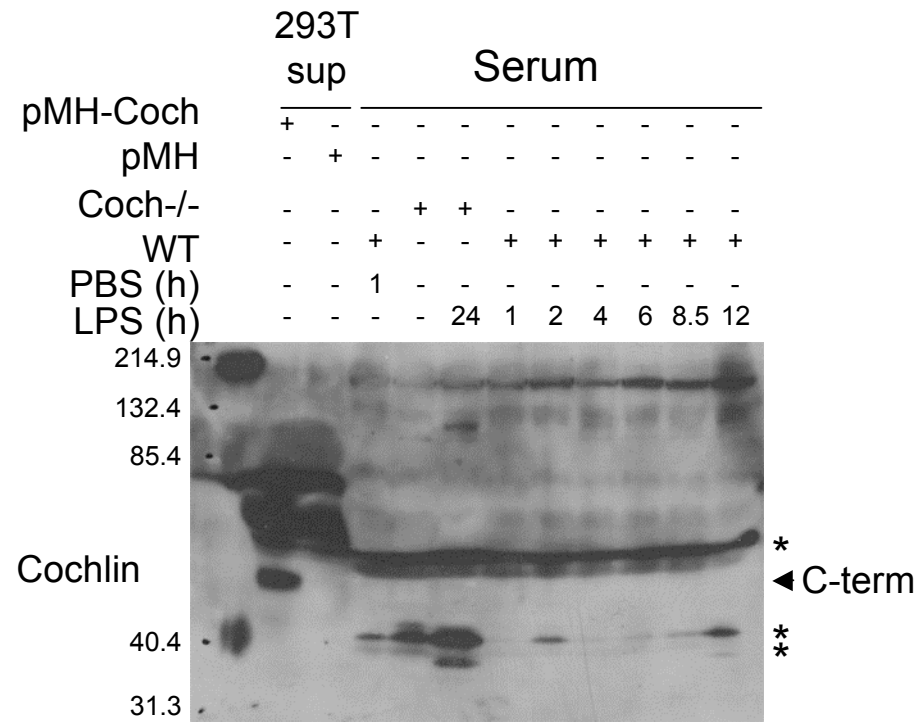


Figure S4, continued

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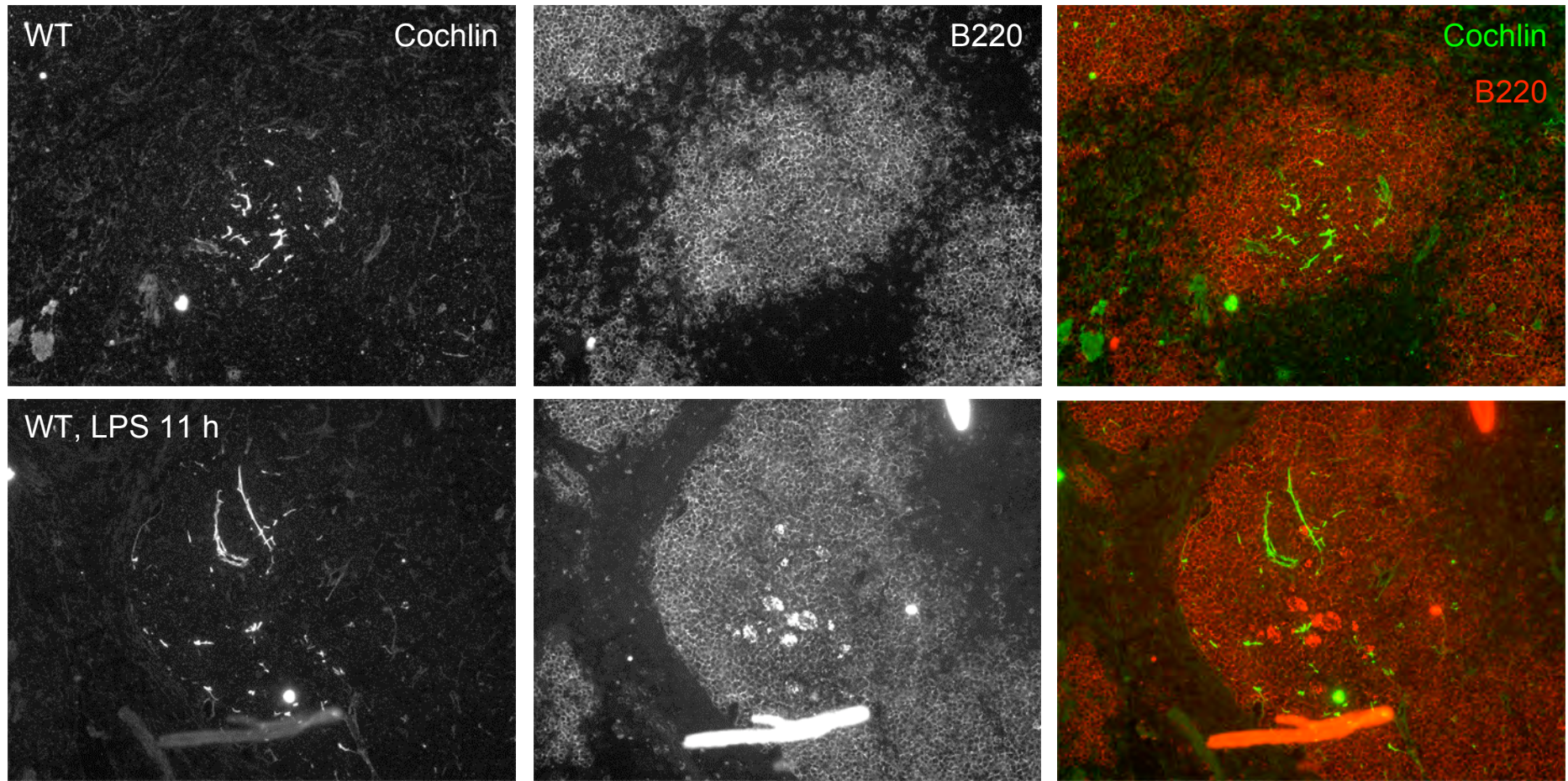


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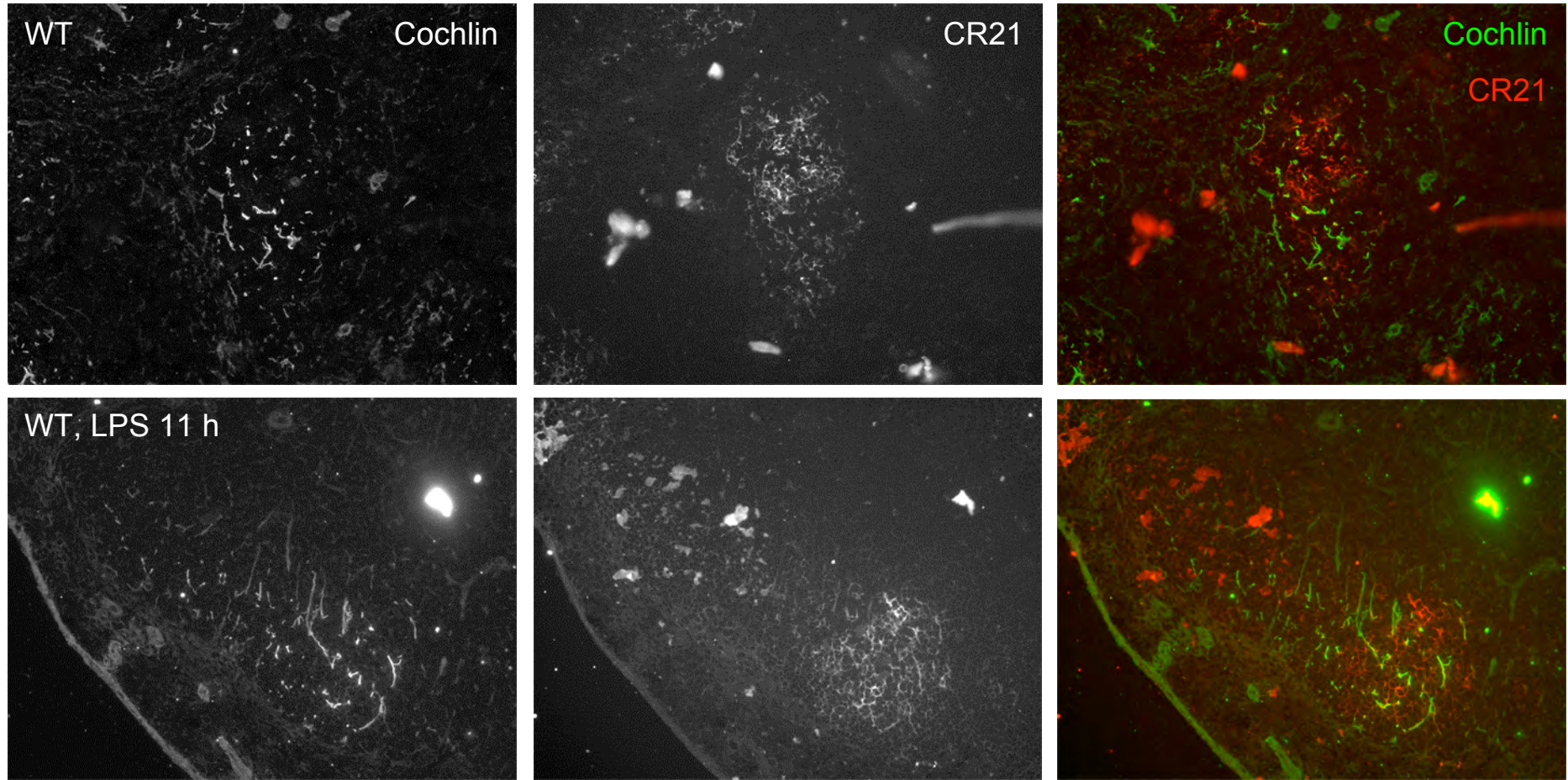


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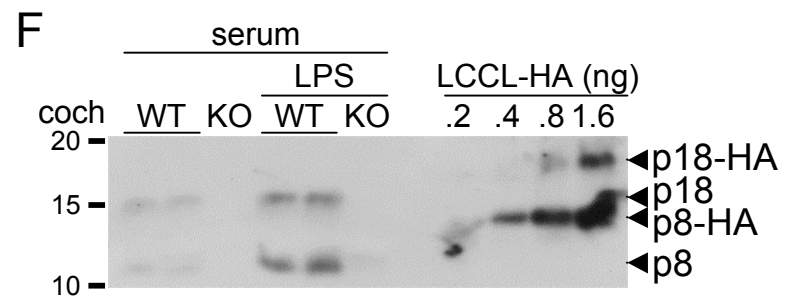
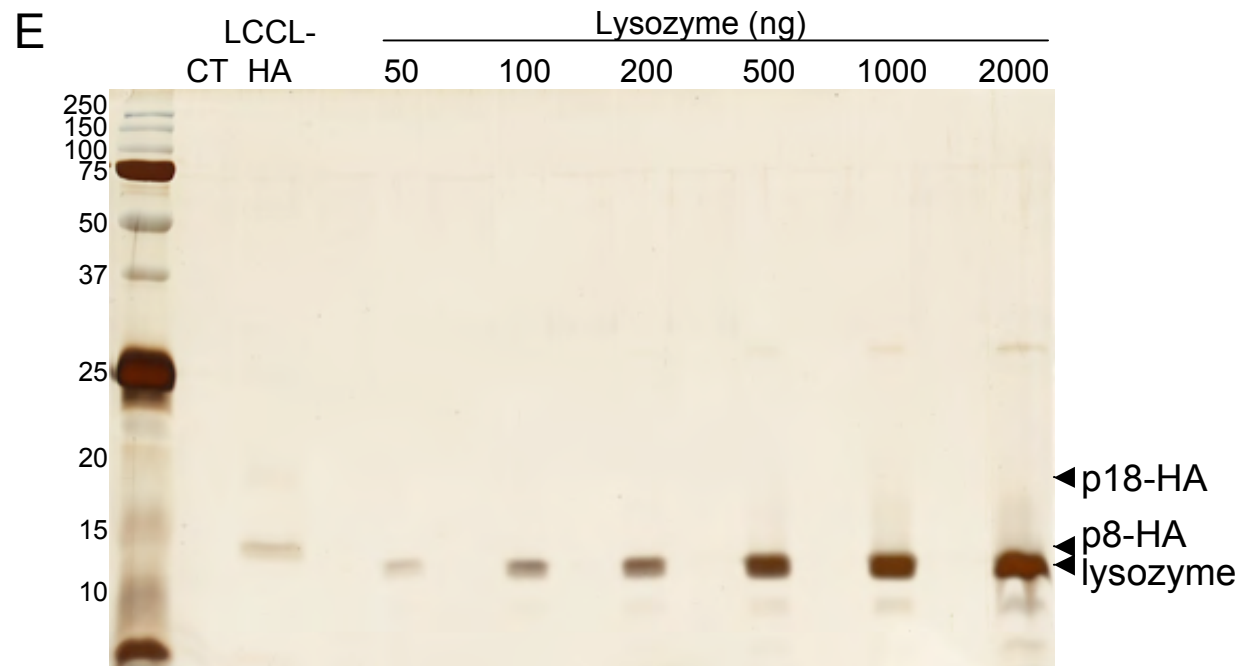


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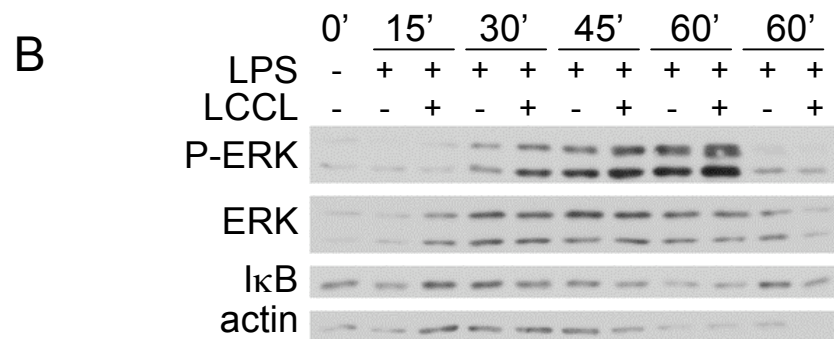
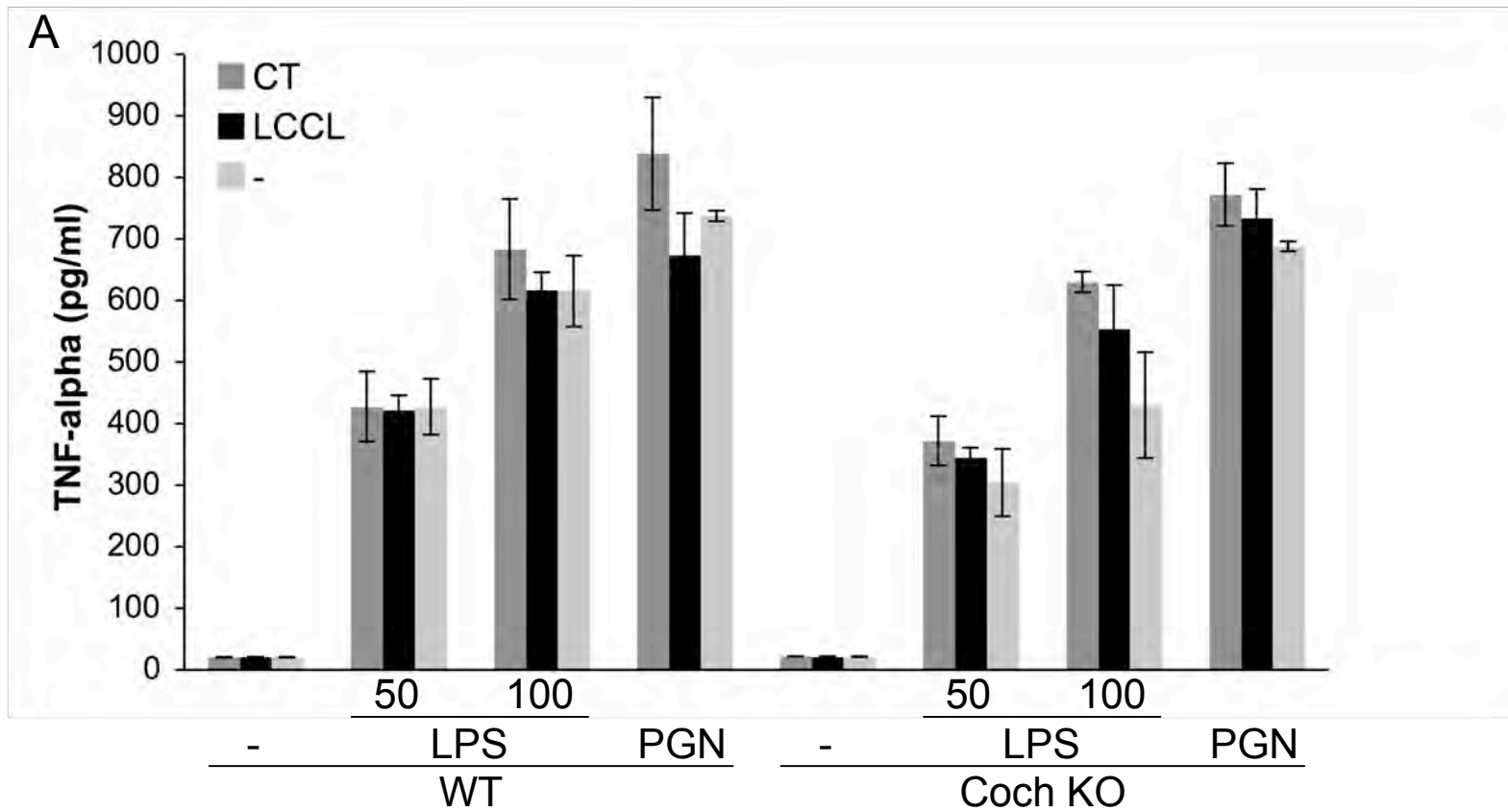


Figure S5

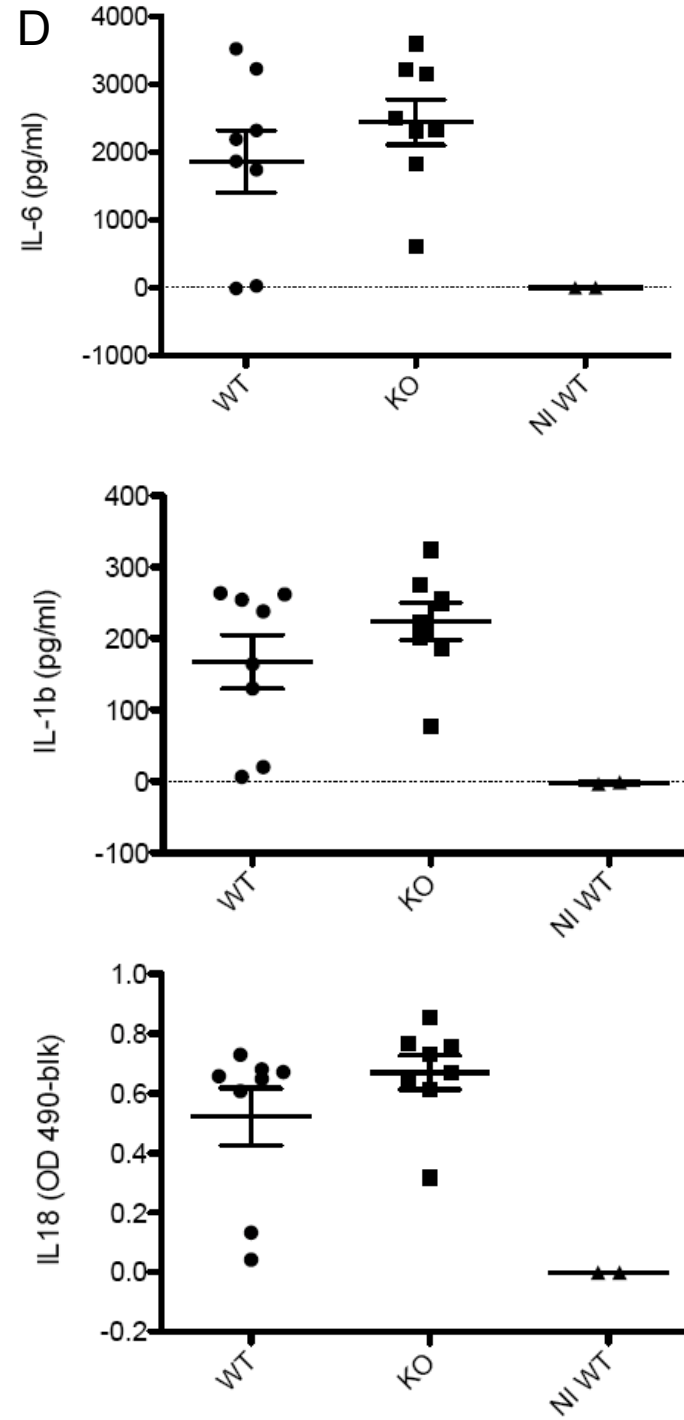
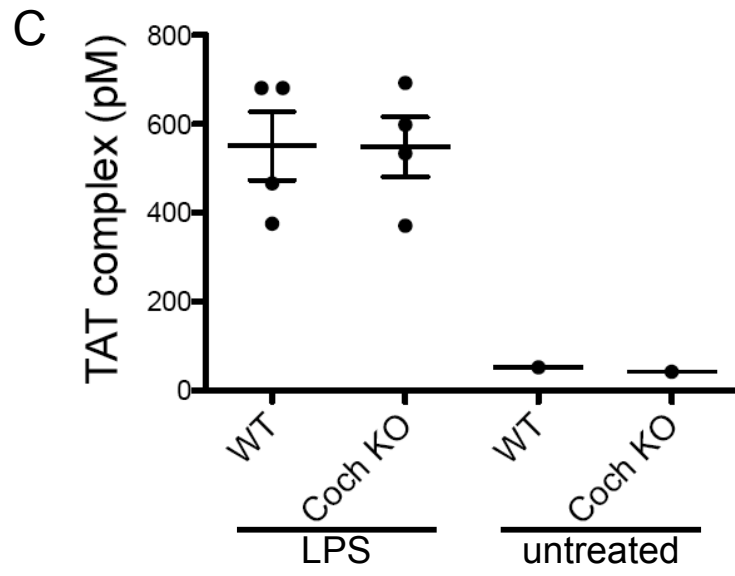
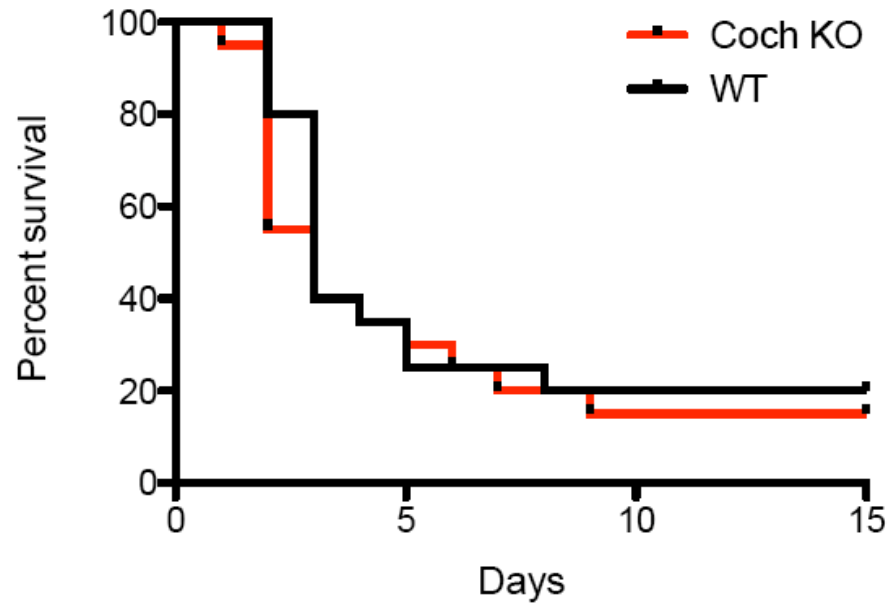


Figure S5, continued

E

LPS 7 mg/kg



LPS 5 mg/kg

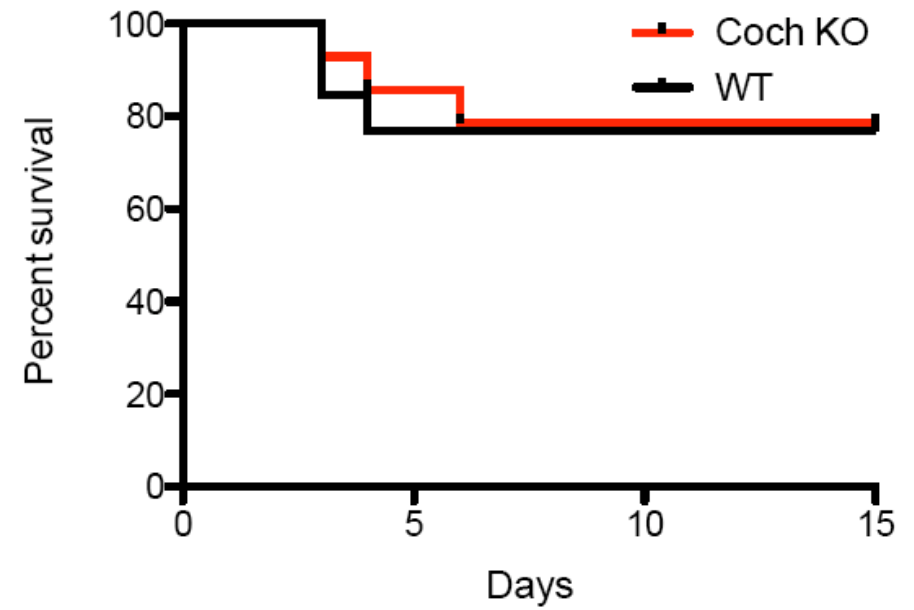


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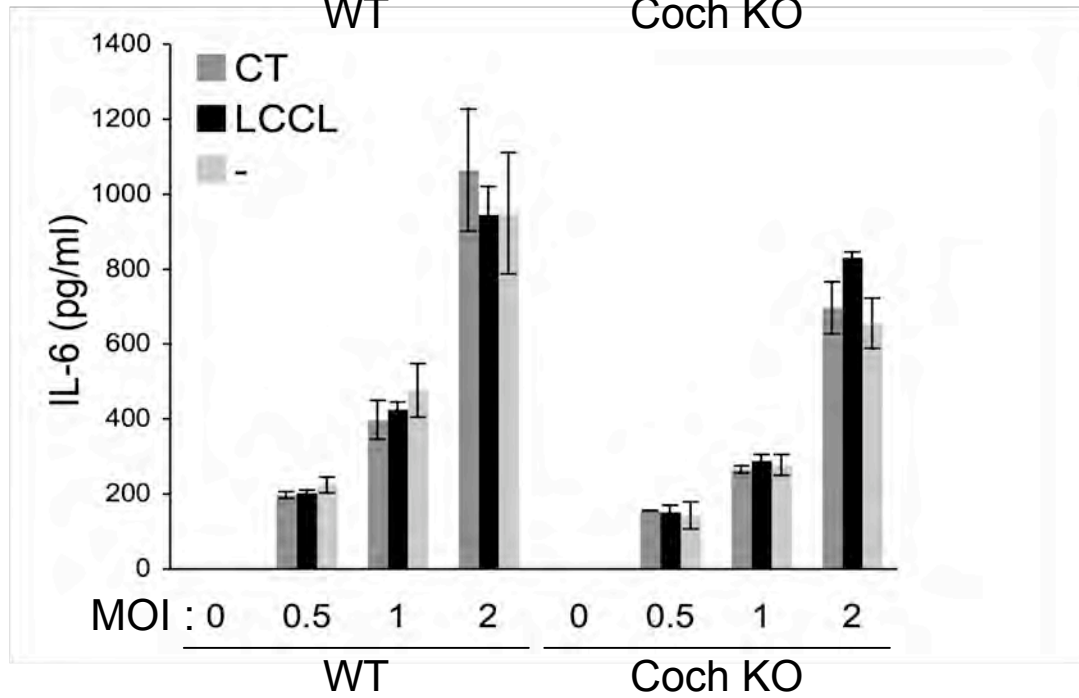
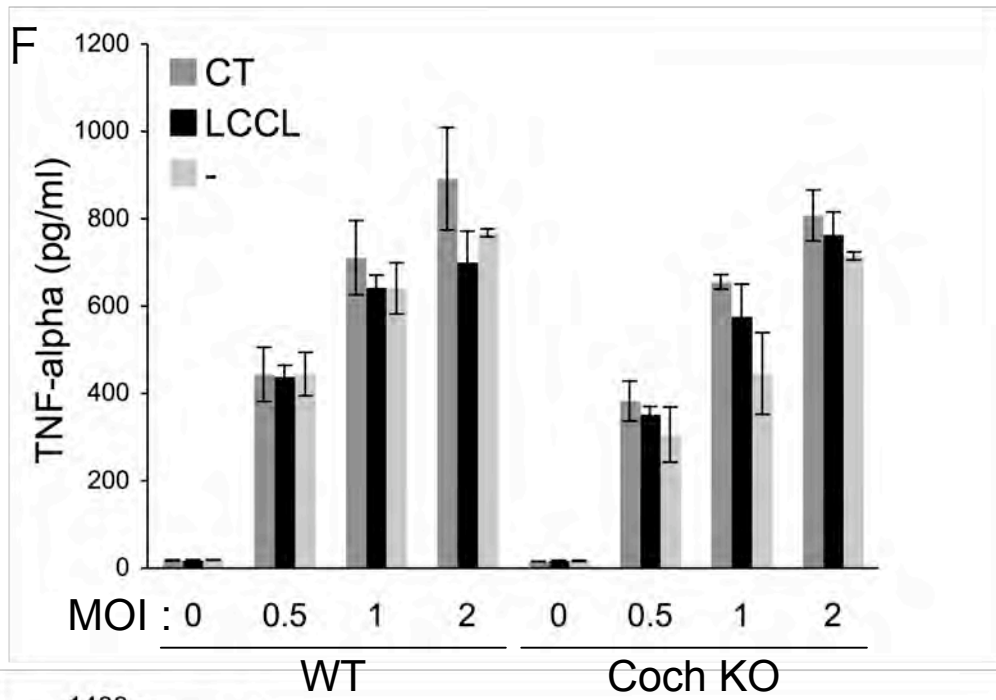
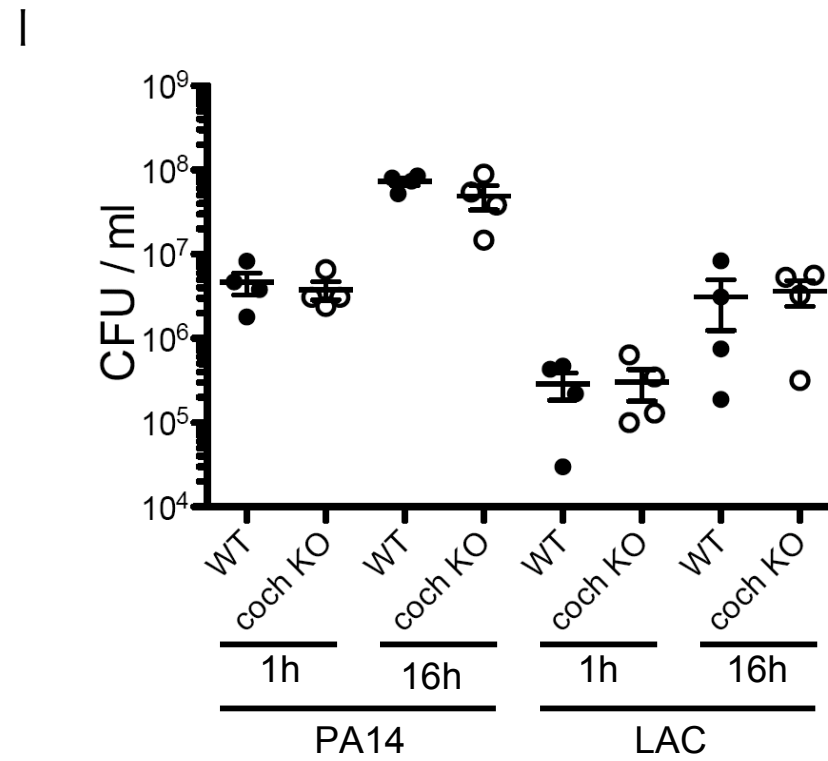
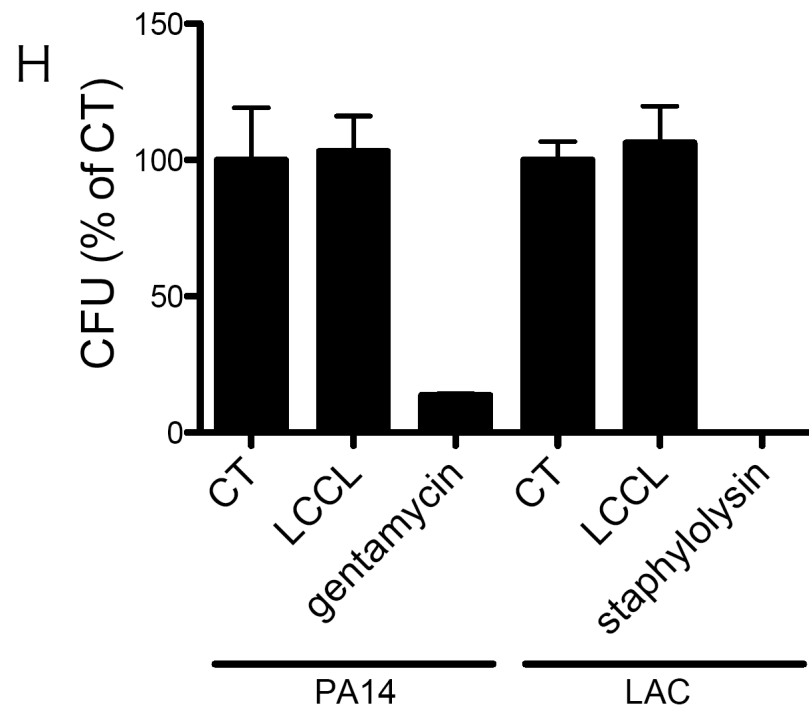
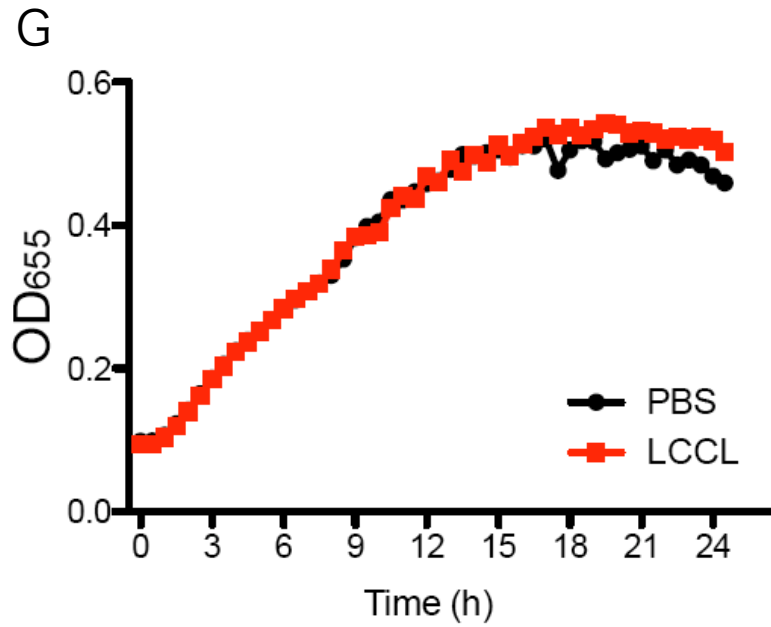


Figure S5, continued



J

LCCL-HA (μg/ml)	0	0.25	0.5	1
PA14	1.02±0.09	0.99±0.02	0.93±0.06	1.00±0.10
SH1000	1.53±0.09	1.65±0.05	1.63±0.05	1.86±0.17

Figure S5, continued

Supplemental Figure legends

Figure S1. Cochlin is expressed by splenic stromal tissue but not by isolated splenocytes.

A. Cochlin expression in different organs was analyzed by WB and RT-PCR.

B. Total spleen (lane 1), spleen stromal tissue (lane 2) and isolated splenocytes (lane 3) were analyzed by RT-PCR and WB for cochlin expression.

C. Specificity of the anti-cochlin P13 polyclonal antibody in immunohistochemistry.

Spleen sections from WT and coch^{-/-} mice were co-stained for cochlin (P13) and B cell (B220). Objective lens 10X.

Figure S2. Cochlin-deficiency does not impair conduit function in adaptive immunity

A-B. FDCs-associated conduits distribution is not impaired by cochlin deficiency. A, spleen sections from WT and coch^{-/-} mice were co-stained for B cell (B220) and laminin-1 (Sigma, L9393). Objective lens 20X. B, spleen sections from WT and coch^{-/-} mice were co-stained for FDCs (CR2.1) and laminin-1 (Sigma, L9393). Objective lens 20X.

C. The structures of the FDCs-associated conduits are not impaired by cochlin deficiency.

Fine sections of spleen from WT and coch^{-/-} mice were embedded in plastic and processed for electronic microscopy. Conduits in the spleen of coch^{-/-} mice show a structure similar to the conduits of WT mice with collagen bundles surrounded by FRCs.

D-F. Small molecules flow, and large molecule exclusion, in the lumen of the conduits are not impaired by cochlin deficiency. WT and coch^{-/-} mice were intravenously injected with 10 KD (D), 70 KD (E) or 500 KD (F) fluorescein-dextran (Invitrogen, D1820, D1822, D7136). Mice were sacrificed 5 minutes later and their spleens were fixed in

paraformaldehyde 4% for 1 h. Spleen sections (20 μm) were co-stained for B cells (B220). Objective lens 20X.

Figure S3. Cochlin-deficiency does not impair humoral adaptive response to small antigens.

A. FDCs are associated with conduits in the absence of cochlin. Spleen sections from WT and coch^{-/-} mice were co-stained for FDCs (CR2.1) and laminin-1 (Sigma, L9393). Objective lens 20X.

B. Small antigen deposition on FDCs is not impaired by cochlin deficiency. WT and coch^{-/-} mice were passively immunized by intraperitoneal injection of anti-lysozyme (200 μl), and FDCs were stained in vivo by intraperitoneal injection of 8C12-Alex 568 (2 μl). 24 h later, mice were injected subcutaneously with 1 μg of TEL-Alexa 488 in the flank and in the base tail on each sides (2 μg total per mouse). 8 h later, mice were sacrificed and inguinal lymph nodes were flash frozen in OCT, sectioned, and observed by fluorescence microscopy. Objective lens 40X. Images were acquired as Z-series 0.25 μm , deconvolved using AutoQuant 2X, and subjected to 3D reconstruction.

C. Small antigen acquisition by B cells is not impaired by cochlin deficiency. B cells purified from spleen of MD4 mice and stained with CMFDA were intravenously transferred to WT and coch^{-/-} mice (13×10^6 cells/mouse). 24 h later, 5 ng TEL-Alexa633 was injected subcutaneously in the footpad of the mice. 1 h later mice were sacrificed, and cells from popliteal lymph nodes were stained with anti-IgMA-PE and anti-B220-PerCP and analyzed by Flow cytometry on FACS Calibur (BD). The percentages of TEL-

positive cells were assessed on the gated IgMA+CMFDA-double positive cells (1 representative lymph node is presented for each group, n=8 per group).

D. Antibody responses to small and large antigens are not impaired in coch^{-/-} mice.

50 µg of TEL or PE (in a total volume of 10 µl) were injected subcutaneously in both footpads at day d1 and d21. Blood was collected at d28 and sera were analyzed for total anti-TEL and anti-PE Igs by ELISA. (n=5-6 for each group, representative results of 2 independent experiments are shown).

E. GC formation is not impaired in coch^{-/-} mice. Mice were immunized intraperitoneally with SRBC (80x10⁶ cells/mouse) for 7 days. Germinal center were stained with biotin-PNA (Sigma-Aldrich) and FITC-streptavidin (Molecular Probes) on spleen sections.

Objective lens 10X.

F. Ig primary response, class switching and affinity maturation are not impaired in coch^{-/-} mice. Mice were immunized IP with NP-CGG (0.3 mg/mouse in PBS, biosearch technologies) (n=5 per group). Sera were collected 12 days later and different dilutions were analyzed by ELISA in wells coated with NP₄-BSA and NP₂₃-BSA (biosearch technologies). Ratio of the OD read in the wells coated with NP₄-BSA over coated with NP₂₃-BSA (NP₄-BSA / NP₂₃-BSA) in a non saturating condition (total Ig serum dilution 1/1000, IgM serum dilution 1/500, IgG serum dilution 1/5000) for each mouse is shown. n=5 mice per group, data are representative of 2 independent experiments.

G. Ig secondary response, class switching and affinity maturation are not impaired in coch^{-/-} mice. Mice were immunized IP with NP-CGG (n=5 per group) at day 0 (0.3 mg/mouse) and day 30 (0.05 mg/mouse). Sera were collected at day 30 and day 40 (30d+10d) and different dilutions were analyzed by ELISA in wells coated with NP₄-

BSA and NP₂₃-BSA. Ratio of the OD read in the wells coated with NP₄-BSA over coated with NP₂₃-BSA (NP₄-BSA / NP₂₃-BSA) in a non saturating condition (total Ig serum dilution 1/3000, IgM serum dilution 1/1000, IgG serum dilution 1/3000) for each mouse is shown. n=4 mice per group, data are representative of 2 independent experiments.

Figure S4. Cochlin LCCL is released into the blood upon inflammation.

A. Kinetics of cytokines secretion in the sera following LPS injection. C57B6 mice were intraperitoneally injected with LPS (5 mg/kg) for the indicated time (h following LPS injection). At each time point, mice were sacrificed and cytokines level in the blood was assessed (Pierce Biotechnology). n=2 for each time point.

B. Cochlin C-terminal domain is undetectable in serum. WT and coch^{-/-} mice were injected IP with LPS (5 mg/kg) or PBS as control for the indicated times. Sera were analyzed by WB using the anti-cochlin P13 polyclonal antibody recognizing mostly epitopes on the C-terminal domain. Supernatant of 293T cells overexpressing cochlin-HA is used as a positive control. *, unspecific bands.

C-D. Cochlin C-terminal localization remained stable following LPS injection. WT and coch^{-/-} mice were injected IP with LPS (5 mg/kg) for 11 h. Spleen sections were co-stained for cochlin (P13) and B cells (B220) (C), or FDCs (CR2.1) (D). Objective lens 20X.

E-F. Quantification of cochlin LCCL domain in mice serum. E, HA-LCCL was purified from serum-free supernatant of 293T cells transfected with an expression vector coding for cochlin aa 1-132 fused with a C-terminal HA tag (pMH-coch1-132). Cell supernatant was subject to immunoprecipitation using anti-Flag M2 agarose beads O/N at 4°C. Beads

were washed 5 times in PBS. LCCL-HA was eluted with HA peptide (I2149, Sigma) (100 µg/ml in PBS) 2 h at 4°C and dialyzed in PBS using 3.5 K MWCO Slide-A-Lyzer dialysis cassettes (Pierce Net). Control preparation (CT) was obtained by the same procedure using 293T cells transfected with the empty pMH vector. The concentration of the purified LCCL-HA was assessed by the co-migration of 20 µl of the preparation with a ladder of known quantities of lysozyme and silver-staining of the gel. The concentration of the purified LCCL-HA was evaluated as 4 µg/ml. F, the concentrations of LCCL in the serum of mice, in basal condition and following LPS injection, were assessed by the co-migration of 1 µl of serum with a ladder of different dilutions of LCCL-HA, followed by an anti-cochlin (PY13) WB. We evaluated the LCCL concentration in serum as to be 0.2 µg/ml in basal condition and 0.4 µg/ml following LPS intraperitoneal injection (5 mg/kg, 8 h).

Figure S5. Cochlin has no effect on LPS or PGN response by macrophages and *P. aeruginosa* growth *in vitro*.

A. Inflammatory peritoneal macrophages from WT or coch^{-/-} mice were treated with LPS (50 or 100 ng/ml) or PGN (20 µg/ml) for 4 h the absence of serum and in the presence of LCCL-HA preparation (0.4 µg/ml, 10%), control preparation (CT, 10%) or media only (-). TNF-α secretion in macrophage supernatant was measured by ELISA.

B. Inflammatory peritoneal macrophages from WT mice were treated with LPS (100 ng/ml) for the indicated time (in min) in the absence of serum and in the presence of LCCL-HA preparation (0.4 µg/ml, 10%), control preparation (CT, 10%).

Phosphorylation of ERK and degradation of IκB was assessed by WB.

C-D. Cochlin deficiency does not impaired LPS-induced thrombin-anti-thrombin (TAT) complexes formation, or cytokine response *in vivo*. C, LPS (7 mg/kg of body weight) were intraperitoneally injected to C57B6 (WT) and *coch*^{-/-} mice. Mice were sacrificed 6 h later and 200 µl of NaCO₃ 3.2% solution was injected intracardiacally – to prevent artifactual activation of thrombin – 20 seconds before 600 µl of blood was collected by the same syringe. Blood was immediately centrifuged at 2000 g for 20 min at 4°C. TAT concentration in the serum was assessed by ELISA (Enzyme Research Laboratories) according to the manufacturer's instructions. D, mice were intraperitoneally injected with LPS (6 mg/kg). 10 h later, TNF- α , IL-1 β and IL-18 level in the serum were measured by ELISA.

E. Survival of *coch*^{-/-} mice to LPS-induced septic shock is not modified.

7 or 5 mg of LPS per kg of body weight were injected IP in *coch*^{-/-} and C57B6 littermate mice and their survival were followed during 15 days. (7 mg/kg: n=19 WT, n=20 *coch*^{-/-}; 5 mg/kg: n=14 WT, n=13 *coch*^{-/-}, all males 8-10 weeks old).

F. Cochlin is not involved in the direct recognition of PA14 by macrophages.

Inflammatory peritoneal macrophages from WT and *coch*^{-/-} mice were infected by log-phase growing PA14 bacteria in the absence of serum and in the presence of LCCL-HA preparation (0.4 µg/ml, 10%), control preparation (CT, 10%) or media only (-).

Gentamycin (50 µg/ml) was added after 30 min. TNF- α and IL-6 concentration in the media 4 h and 6 h post-infection respectively was measured by ELISA. Macrophages do not express cochlin and *Coch*^{-/-} macrophages were used as target cells devoid of any potential contamination with endogenous cochlin from mouse peritonea.

G-I. Cochlin LCCL does not have direct bactericidal activity. G, growth curve of PA14 in LB as followed by OD₆₅₅ over 24 h at 37°C in the presence of purified LCCL-HA (0.4 µg/ml, 10%), or PBS (10%). H, Mid-log growing phase PA14 and LAC were resuspended in PBS (10⁷ CFU/ml) and treated with purified LCCL-HA (0.8 µg/ml, 10%), control preparation (CT, 10%), gentamycin (0.4 µg/ml) or staphylolysin (0.4 µg/ml) at 37°C for 1 h. Numbers of live bacteria following treatment were then assessed by plating on LB plates. The presented results are averages of triplicate and are representative of 2 independent experiments. I. Mid-log growing phase PA14 and LAC were resuspended (10⁷ - 10⁶ CFU/ml respectively) in sera from WT or coch^{-/-} mice previously challenged with LPS (5 mg/kg, 8 h, 4 mice per group) and incubated for 1 h at 37°C or 16 h at 25°C. Numbers of live bacteria following treatment were then assessed by plating on LB plates.

J. Cochlin LCCL does not inhibit biofilm formation. Bacteria from Tryptic Soy Broth (TSB) cultures were resuspended in fresh TSB to a density of OD⁶⁰⁰=0.1 and diluted 1:100. 200µL of this suspension was transferred to wells of 96-polystyrene wells (Costar) and incubated 24 h at 37°C in the presence of the indicated amount of purified LCCL-HA. After incubation, the wells were washed with sterile water three times, air-dried, stained with crystal violet (0.4% in ethanol 95%) (Sigma) 15 minutes at room temperature, and washed three times with sterile water. The dye was dissolved in 30% acetic glacial and absorption was measured at 600nm