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25-hydroxycholesterol suppresses interleukin-1-driven inflammation downstream of type I interferon

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Abstract

Type-I interferon (IFN) protects against viruses yet it also has a poorly understood suppressive influence on inflammation. Here we report that activated mouse macrophages lacking the IFN-stimulated gene, *cholesterol 25-hydroxylase* (*Ch25h*), and that are unable to produce the oxysterol 25-hydroxycholesterol (25-HC) overproduce inflammatory interleukin 1 (IL-1) family cytokines. 25-HC acts by antagonizing sterol response element-binding protein (SREBP) processing to reduce *Il1b* transcription and to broadly repress IL1-activating inflammasomes. In accord with these dual actions of 25-HC, Ch25h-deficient mice exhibit increased sensitivity to septic shock, exacerbated experimental autoimmune encephalomyelitis, and a stronger ability to repress bacterial growth. These findings identify an oxysterol, 25-HC, as a critical mediator in the negative-feedback pathway of IFN signaling on IL1-family cytokine production and inflammasome activity.

As well as having potent antiviral activity, type I interferon (IFN) has a suppressive influence on immunity, an action that helps prevent uncontrolled inflammation and that underlies its utility in treatment of certain autoimmune diseases such as multiple sclerosis (1-4). This suppressive action also contributes to the increased propensity for bacterial infection following viral infection (1, 2). A central facet of the IFN-mediated suppressive effect is down-regulation of inflammasome activity and interleukin-1 (IL1 production (3, 5). However, which of the several hundred IFN-stimulated genes are responsible for these effects are poorly defined. The IFN-stimulated gene *cholesterol 25-hydroxylase* (*Ch25h)* (6) was recently shown to have broad anti-viral activity (7, 8), but whether this gene has an anti-inflammatory role is unknown.

Ch25h is an enzyme that hydroxylates cholesterol at the 25 position to generate 25-hydroxycholesterol (25-HC) (9). Ch25h is strongly induced in myeloid cells by Toll like receptor (TLR) ligands in a type I IFN-dependent manner (10). Recently we and others identified a role for a downstream product of Ch25h, 7,25-dihydroxycholesterol (7α,25-

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HC), as a ligand for EBI2 (GPR183) (11, 12). During studies comparing Ch25h- and EBI2-deficient mice, we found that *Ch25h*$^{-/-}$ mice had increased frequencies of IL17A^{+} T cells in spleen and lymph nodes (Fig. 1A). The frequencies of IFNγ-producing and regulatory T cells were unaffected (fig. S1A). Ch25h-deficient mice also had increased neutrophil counts (Fig. 1B), a phenotype seen in other strains with elevated IL17A^{+} cells (13, 14). *Gpr183*$^{-/-}$ mice were not affected in these parameters (fig. S1B). These data indicated that Ch25h was acting via an EBI2-independent pathway to regulate IL17A^{+} T cell and neutrophil numbers, two cell populations that often promote inflammation.

Because Ch25h can be expressed at high levels in activated macrophages (7, 8, 15-17), we asked whether the IL17A-polarizing activity of lipopolysaccharide (LPS)-stimulated macrophage cultures was altered by Ch25h-deficiency. Culture supernatants from Ch25h-deficient macrophages induced more IL17A^{+} T cells than supernatants from control macrophages (Fig. 1C and fig. S1C, D). T helper 1 (T_H1) and T_H2 cell differentiation were unaffected (Fig. 1C and fig. S1C). As expected, LPS-stimulation of bone marrow-derived macrophages (BMDMs) caused *Ch25h* induction in an IFN-α receptor (IFNαR)-dependent manner (fig. S1E) and this was associated with increased 25-HC in culture supernatants (fig. S1F). However, when 25-HC was added to T cell cultures at this concentration (100nM) it was without effect (fig. S1G). These data suggested that the *Ch25h*$^{-/-}$ and control macrophage culture supernatants were differing in some other constituent that affected Th17 cell differentiation.

Cytokines that synergize with transforming growth factor-β (TGFβ) to induce Th17 cells include IL-1β, IL-23 and IL-6 (18). Analysis after LPS stimulation revealed that *Il1b* was transiently overproduced in Ch25h-deficient macrophages whereas *Il6* and *Il23* were unaltered (Fig. 2A). LPS stimulated EBI2-deficient BMDMs had wild-type amounts of *Il1b* (fig. S2A). Activated BMDMs lacking *Cyp7b1*, the enzyme that converts 25-HC to 7α,25-HC, also produced normal *Il1b* levels (fig. S2A) and their culture supernatants contained unaltered amounts of 25-HC (fig. S2B), consistent with minimal Cyp7b1 expression in BMDMs (fig. S2C). Flow cytometric analysis showed elevated pro-IL-1β in Ch25h-deficient compared to wild-type macrophages (Fig. 2B). Confirming the presence of secreted IL1β, IL17A induction by Ch25h-deficient macrophage supernatants required T cell IL-1 receptor (IL-1R) expression (fig. S2D). The *Il1b* elevation in *Ch25h*$^{-/-}$ BMDMs occurred without a change in transcript stability and was preceded by induction of precursor mRNA, indicating it was due to increased transcription (fig. 2C).

IL1β secretion occurs after cleavage of pro-IL1β by the inflammasome-activated protease, caspase-1 (19). LPS treatment causes inflammasome priming by up-regulating expression of *Casp-1*, *Nlrp3* and *Asc* (19) and induction of these transcripts occurred normally in Ch25-deficient BMDMs (fig. S2E). Full inflammasome activation depends on exposure to an activating agent such as adenosine triphosphate (ATP) (19). IL-1β was elevated in the supernatant of LPS-stimulated *Ch25h*$^{-/-}$ BMDMs exposed to ATP (Fig. 2D), whereas IL-6 was unaffected (fig. 2D) and IL-23 was too low for ELISA measurement. LPS-stimulated Ch25h-deficient BMDMs also over-produced IL-18, another caspase-1 dependent IL1-family member (19) (fig. 2D). The increased secretion of IL-18 occurred in the absence of any transcript level differences (fig. S2F), which suggested that Ch25h-deficient BMDMs had elevated inflammasome activity.

Type I IFN inhibits NLRP3-containing inflammasomes by an undefined mechanism (2, 4, 5). LPS-primed Ch25h-deficient BMDMs exposed to ATP showed exaggerated caspase-1 activity compared to equivalently treated control macrophages (Fig. 2, F and G) and generated more processed IL1-β (Fig. 2H). A slight elevation in caspase-1 activity was evident in Ch25h$^{-/-}$ macrophages not exposed to ATP (Fig. 2, F and G) consistent with the elevated IL1β bioactivity in these supernatants (Fig. 1C). Increased caspase-1 activity in Ch25h-deficient BMDMs was also observed following treatment with the additional NRLP3-inflammasome activators nigericin and alum, the NLRP4-inflammasome activator flagellin, and the AIM2-inflammasome activator poly (deoxyadenylic-deoxythymidylic) acid [poly(dA:dT)] (Fig. 2I). This broad inflammasome-repressive action of Ch25h suggests that it acts across nucleotide-binding oligomerization domain-like receptors (NLRs) or at a site downstream of the NLRs.

To test whether the Ch25h product was sufficient to regulate *Il1b* expression and caspase-1 activation, LPS -treated BMDMs were incubated with 25-HC. 25-HC suppressed *Il1b* mRNA, protein expression and inflammasome activity in Ch25h-deficient cells, such that the amounts induced were similar to endogenously 25-HC-producing control cells (Fig. 3A and fig. S3A). 25-HC had no significant effect on *ll6* expression (fig. S3A). Mass spectrometry of *Ch25h*$^{-/-}$ BMDMs incubated with 25-HC confirmed their ability to take up the oxysterol (fig. S3B). Incubation with similar concentrations of cholesterol or 7α,25-HC did not cause any change in *Il1b* levels or protein expression (Fig. 3A). Moreover, although transduction of both control and Ch25h-deficient BMDMs with wild-type Ch25h repressed *Il1b* expression and inflammasome activity, transduction with a point mutant that lacks enzymatic function (20) did not (Fig. 3B and S3C).

25-HC is one of several oxysterol ligands for the nuclear hormone receptors, liver X receptor- α (LXRα, encoded by *Nr1h3*) and LXRβ (*Nρ1η2*)(21, 22), which can negatively regulate *Il1b* expression (23-26). However, whether 25-HC has a physiological role as an LXR ligand remains unclear (27, 28). We did not observe any over-induction of *Il1b* transcripts, intracellular pro-IL-1β or secreted IL-1β following LPS exposure of macrophages generated from LXR-deficient (*Nr1h2*$^{-/-}$ *3*$^{-/-}$*)* compared to control bone marrow (BM) (fig. S3D). Moreover, transduction of LXR-deficient cells with Ch25h led to a similar repression in IL-1β production to that observed with control BMDMs (fig. S3E). These observations suggest that 25-HC acts via an LXR-independent mechanism to regulate *Il1b* transcription.

In vitro, 25-HC can repress sterol response element binding protein (SREBP) processing into active transcription factors, which are required for cholesterol biosynthesis (29, 30) (fig. S3F). However, Ch25h-deficient mice have intact cholesterol metabolism leaving it unclear whether 25-HC has a physiological role as a negative regulator of SREBP processing and, therefore, sterol biosynthesis (9, 28). To further explore how Ch25h might regulate *Il1b* expression and inflammasome activity, we performed RNA sequencing (RNAseq) on LPS-treated BMDMs. This analysis revealed a striking elevation in transcripts for SREBP target genes in *Ch25h*$^{-/-}$ macrophages (Fig. 3C and fig. S3G). It also confirmed the elevation in *Il1b* and showing that *Il18*, *Il6* and tumor necrosis factor (*Tnf)* were not greatly changed (fig. S3H). A panel of IFN-stimulated genes was similarly induced in wild-type and Ch25h-deficient cells compared to their lack of induction in *Ifnar1*$^{-/-}$ BMDMs (fig. S3I). *Nr1h2*$^{-/-}$*3*$^{-/-}$ macrophages showed little alteration in the expression of SREBP target genes (Fig. 3C and fig. S3H). Western blotting confirmed that Ch25h-deficiency was sufficient to deregulate SREBP processing in activated BMDMs (fig. S3J). These data implicate repression of SREBPs as a major pathway of gene regulation by 25-HC in activated macrophages. Sterol biosynthesis is down-regulated in IFN-exposed cells (7)(31) and our findings suggest that Ch25h is a key IFN-induced gene responsible for this repression.

25-HC antagonizes processing of SREBP-1 and -2 by promoting their insulin-induced gene (INSIG)-mediated retention in the endoplasmic reticulum (ER), out of reach of activator proteases in the Golgi (32) (fig. S3F). To test whether 25-HC mediated suppression of *Il1b* could be explained by activation of INSIG, we took advantage of the observation that INSIG overexpression is sufficient to inhibit SREBP processing even without addition of a ligand (32). Indeed, INSIG1 overexpression in Ch25h-deficient BMDMs led to reductions in LPS-stimulated *Il1b* transcripts and pro-IL1-β levels while expression of *Il6* was unaffected (Fig. 3D). In a second approach, we examined macrophages lacking the SREBP-cleavage activating protein (SCAP) that is required for transport of SREBPs from the ER to the Golgi for processing (32) (fig. S3F). SCAP-deficient BMDMs expressed reduced amounts of *Il1b* and underwent reduced caspase-1 activation (Fig. 3E). These data indicate that repression of SREBP-processing contributes to the mechanism of 25-HC mediated downregulation of *Il1b* and inflammasome activity.

To test whether IL1-family cytokine production was regulated by Ch25h *in vivo*, mice were treated with LPS. After a sublethal LPS dose, Ch25h-deficient mice had more IL-1β and IL-18 levels in serum compared to controls (Fig. 4A and fig. S4A). IL-1α, a cytokine that is often co-released with IL-1β (33) was also increased (fig. S4A). Serum IL6 levels were unaffected (fig. S4B) and TNF was too low to measure. Splenic macrophages from the knockout mice showed deregulated expression of several SREBP-target genes (fig. S4C). BM chimera analysis established Ch25h was required in hematopoietic cells for regulation of IL-1β and IL-1α and that both hematopoietic and stromal cell Ch25h regulated IL-18 (fig. S4, D and E). Overexpression of Ch25h in hematopoietic cells led to elevated baseline 25-HC in serum (fig. S4F) and to reduced IL-1β production after LPS treatment (fig. S4G). After exposure to a lethal LPS dose, Ch25h-deficient mice succumbed ~12 hours sooner than controls (Fig. 4B). Although it is recognized that LPS-induced death is influenced by factors besides IL-1 family cytokines, these data establish the important role of this single enzyme in resisting the lethal impact of septic shock (fig. S4H).

We also tested the effect of Ch25-deficiency on the course of myelin oligodendrocytes glycoprotein (MOG)-induced EAE, an IL17-driven inflammatory disease (2). In accord with increased IL17A^{+} cell generation and with evidence that IL-1β contributes to central nervous system pathology in this model (4), Ch25h-deficient mice suffered from exacerbated disease compared with controls (Fig. 4C).

Macrophage infection by the bacterium *Listeria monocytogenes* induces type I IFN and Ch25h expression, and bacterial replication in liver and spleen is promoted by type I IFN signaling (1, 2). Ch25h-deficient mice were more resistant to *Listeria* growth than littermate controls (Fig. 4D) and IL-1β, IL-1α and IL-18 were elevated in the serum of infected mice (fig. S5A) and in BMDMs (fig. S5B) and the macrophage supernatants induced greater IL17A^{+} T cell differentiation (fig. S5C). Bacterial growth in BMDMs initially occurred at the same rate and then became more strongly repressed in Ch25h-deficient cells (fig. S5D). Although there is not yet a consensus on the mechanism of IFNαR-mediated augmentation of *Listeria* growth (1, 2), our data indicate that type I IFN-mediated Ch25h induction contributes to host susceptibility to bacterial infection (fig. S5E).

Neutrophil recruitment to the peritoneum in response to the inflammasome activator, alum, is IL-1β-dependent (5). After treatment with alum alone, Ch25h-deficient and wild-type mice showed similar amounts of neutrophil recruitment (Fig. 4E). However, Ch25h-deficient mice suffered an inability to suppress neutrophil recruitment following type I IFN induction by poly(I:C) treatment (Fig. 4E) Similar findings were made in Ch25h-deficient BM chimeras (fig. S5F). Six hours after poly(I:C) treatment wild-type mice showed a 20-fold increase in 25-HC serum concentrations (Fig. 4F). These data indicate that 25-HC functions downstream of type I IFN to repress IL-1β-mediated peritoneal inflammation (fig. S5E).

We identify Ch25h as an essential part of the negative feedback mechanism regulating IL-1 family cytokine production during inflammatory conditions involving type I IFN. 25-HC functions as a repressor of *Il1b* expression and as a broad inhibitor of inflammasome activity. Our findings support a model where 25-HC engagement of INSIG and antagonism of SCAP-dependent SREBP processing reduces *Il1b* expression and inflammasome activity. We speculate that repression of SREBP decreases *Il1b* expression and inflammasome activity by reducing cellular content of key sterols, possibly including cholesterol itself. 25-HC mediated feedback regulation of inflammation requires multiple steps (fig. S3F) and we suggest that this serves as a built-in delay to allow adequate IL1-family cytokine production and inflammasome activation before repression occurs. The anti-inflammatory actions of 25-HC defined in this study reveal a pathway that may contribute to the therapeutic activity of type I IFN in treatment of inflammasome-dependent autoimmune disease (4), the successful use of type I IFN in treatment of some types of cancer (1), and the widespread occurrence of uncontrolled bacterial infection following viral infection (1, 2) (fig. S5E).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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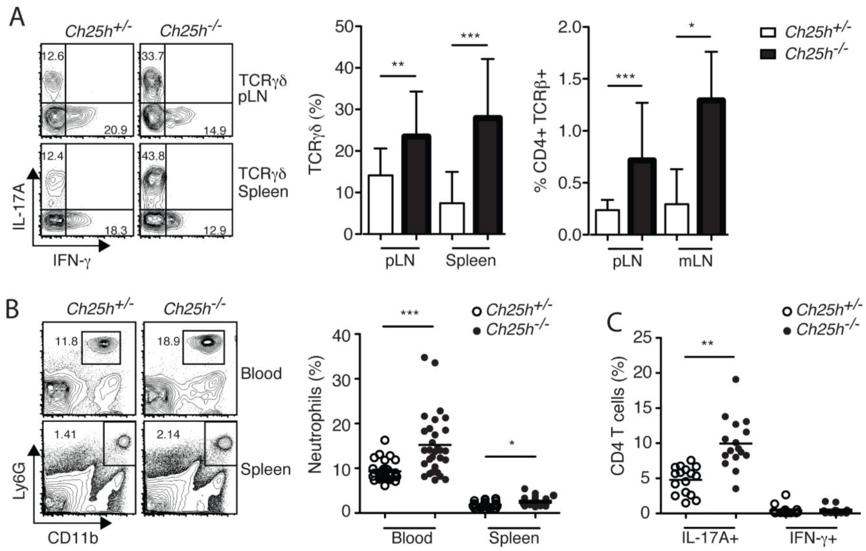

Figure 1. Elevated frequency of IL17A-producing T cells in *Ch25h*$^{-/-}$ mice and increased IL17A^{+} T cell induction by activated *Ch25h*$^{-/-}$ macrophages

(A) (Left) Representative fluorescence-activated cell sorted (FACS) plots of intracellular staining for IL-17A and IFNγ-in T cell receptor (TCR)γδ+ cells. (Right) Percentage of IL-17A^{+} γδ T cells and IL-17A^{+} CD4^{+} T cells in the indicated organs in *Ch25h*$^{+/-}$ and *Ch25h*$^{-/-}$ mice (n=22 per genotype**,** mean ± SD)**.** (**B**) (Left) Representative FACS plot showing frequency of neutrophils, detected by staining for Ly6G and CD11b, in blood and spleen of *Ch25h*$^{+/-}$ and *Ch25h*$^{-/-}$ mice. (Right) Percentage of neutrophils in blood and spleen for 25 mice of each genotype (mean ± SD)**. (C)** Percent of IL17A^{+} CD4 T cells primed *in vitro* with supernatant of 8-hour LPS-stimulated *Ch25h*$^{+/-}$ or *Ch25h*$^{-/-}$ BMDMs, in the presence of 1 ng/ml of TGFβ, for 4 days. Each point represents cells from an individual mouse and data are pooled from more than 10 experiments. *, P<0.05; **, P<0.01, ***, P<0.005 (unpaired Student's T-test).

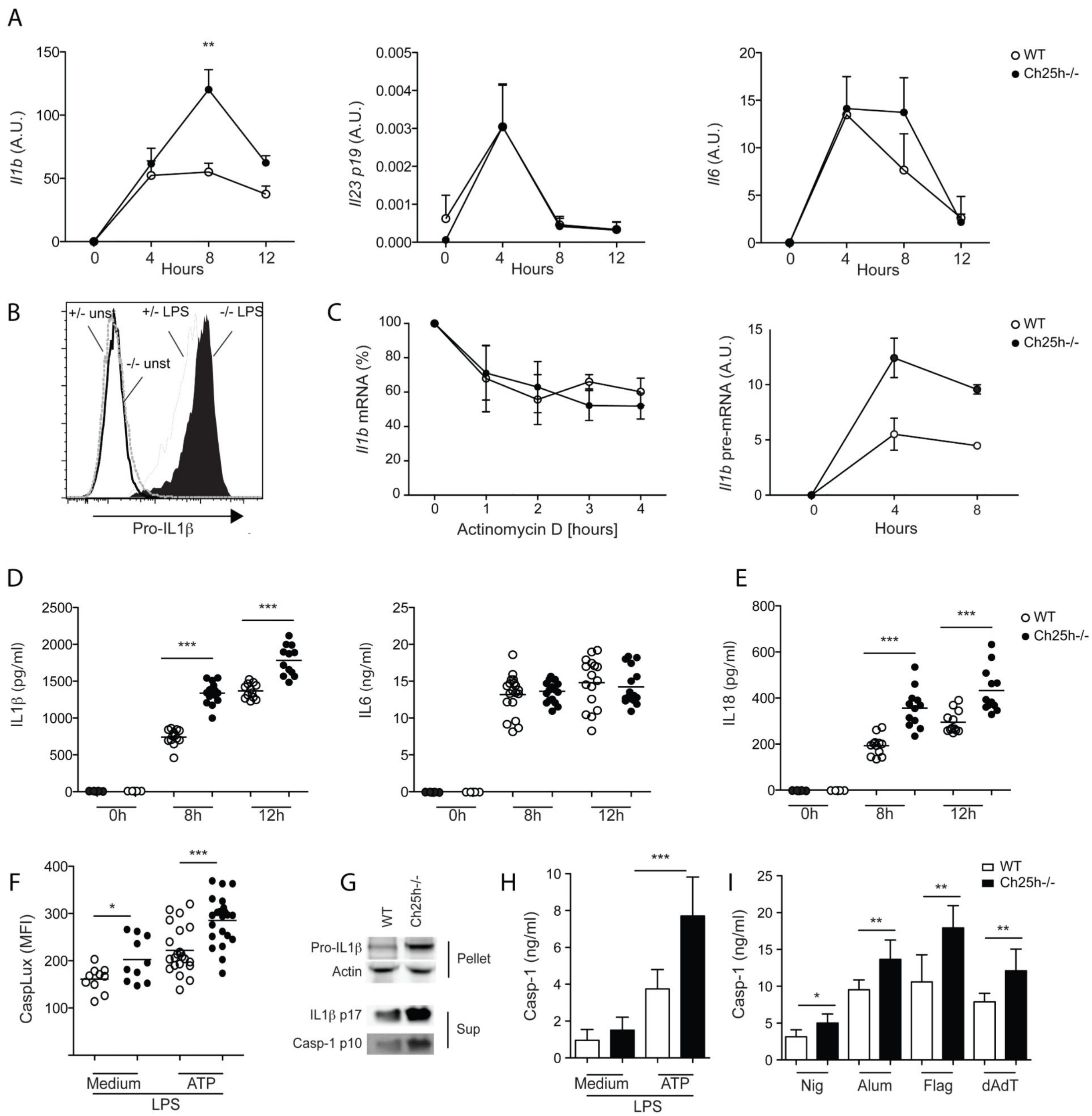

Figure 2. LPS-activated *Ch25h*$^{-/-}$ macrophages overproduce IL-1β and show increased inflammasome activity

(A) Time course quantitative real-time fluorescence polymerase chain reaction (qPCR) analysis of *lllb, Il23 and Il6* expression in BMDMs stimulated with LPS. Data are standardized by comparison to *Hprt* and A.U. indicates arbitrary unit (mean ± SD from six independent experiments). **(B)** Pro-IL-1β intracellular level (arbitrary units) in BMDMs stimulated with LPS for 8 hours. Histogram plot is representative of data from more than 20 mice of each type. (**C)** (Left) Time course qPCR analysis of *ll1b* mRNA expression after treatment with actinomycin D for the indicated time, starting 4 hours after LPS stimulation. (Right) Time course qPCR analysis of intron-containing (newly transcribed) pre-*ll1b* mRNA

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expression in BMDMs after stimulation with LPS. Both panels show means ± SD from three independent experiments. **(D)** (Left) IL-1β secretion by BMDMs stimulated with LPS for 8 or 12 hours and incubated with 5 mM ATP for 45 min. (Right) IL-6 secretion by BMDMs stimulated with LPS for 8 or 12 hours. **(E)** IL-18 secretion by BMDMs stimulated with LPS for 8 or 12 hours and with ATP for 45 min. **(F)** Intracellular level of activated caspase-1 in BMDMs stimulated with LPS for 8 hours and ATP for 45 min. Each point in (D), (E), and (F) represents data for an independent BMDM culture, and data are pooled from five experiments. **(G)** Caspase-1 secretion by BMDMs stimulated with LPS for 8 hours and with ATP for 45min. MFI,mean fluorescence intensity. **(H)** Immunoblotting on cell pellet (pellet) and supernatant (sup) for pro-IL-1β and mature IL-1β (p17) and for activated caspase-1 (p10) in BMDMs stimulated with LPS for 8 hours and with ATP for 45 min. **(I)** Caspase-1 secretion by BMDMs stimulated with LPS for 8 hours, after culture with nigericin, alum, flagellin, or poly(dA:dT). Bars in (G) and (I) show means (±SD) of three experiments.

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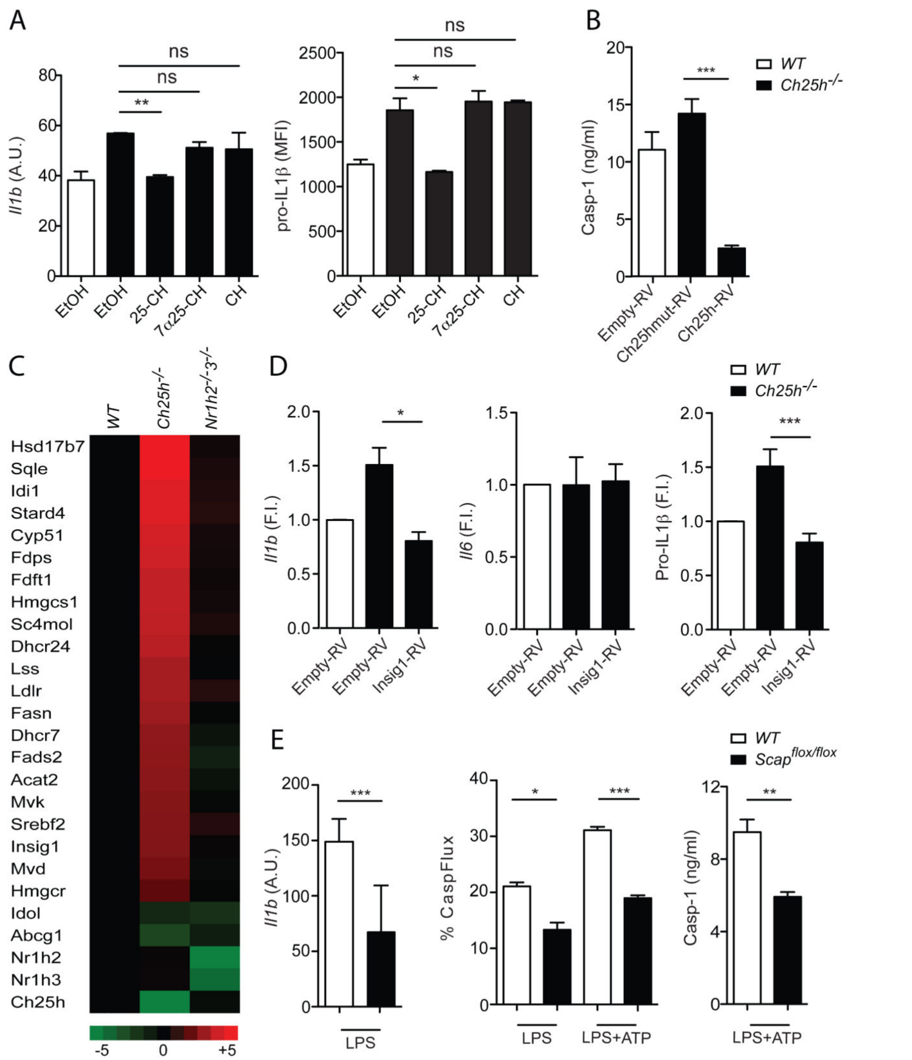

Figure 3. 25-HC represses IL1β expression and inflammasome activation and Ch25h-deficient macrophages show over-expression of SREBP-target genes

(A) *Il1b* expression and pro-IL1β intracellular level in wild-type and *Ch25*$^{-/-}$ BMDMs stimulated with LPS for 8 hours in the presence of 100nM 25-HC, 7α,25-HC or cholesterol (CH) or with carrier (mean ± SD from three independent experiments) EtOH, ethanol. **(B)**, Secreted caspase-1 in BMDMs retrovirally (RV) transduced with empty vector, vector encoding Ch25h or mutated Ch25h and then stimulated with LPS for 8 hours and ATP for 45 min (mean ± SD from three independent experiments**). (C)** Heat map of differentially expressed genes after RNAseq of wild-type, *Ch25h*$^{-/-}$ and *Nr1h2*$^{-/-}$*3*$^{-/-}$ BMDMs stimulated with LPS for 8 hours (determined based on log2 fold change). **(D)** Effect of *Insig1* overexpression on *Il1b, Il6* and pro-IL1β levels in 8 hour LPS-stimulated BMDMs (mean ± SD from three independent experiments)**. (E)**ll1β expression, intracellular level of activated caspase-1 and secreted caspase-1 in wild-type and *Scap*$^{flox/flox}$ BMDMs retrovirally transduced with ER-Cre, treated for 2 days with 4-hydroxytamoxifen and then stimulated with LPS for 8 hours (mean ± SD from three independent experiments**).** *, P<0.05; **, P<0.01, ***, P<0.005 (unpaired Student's T-test).

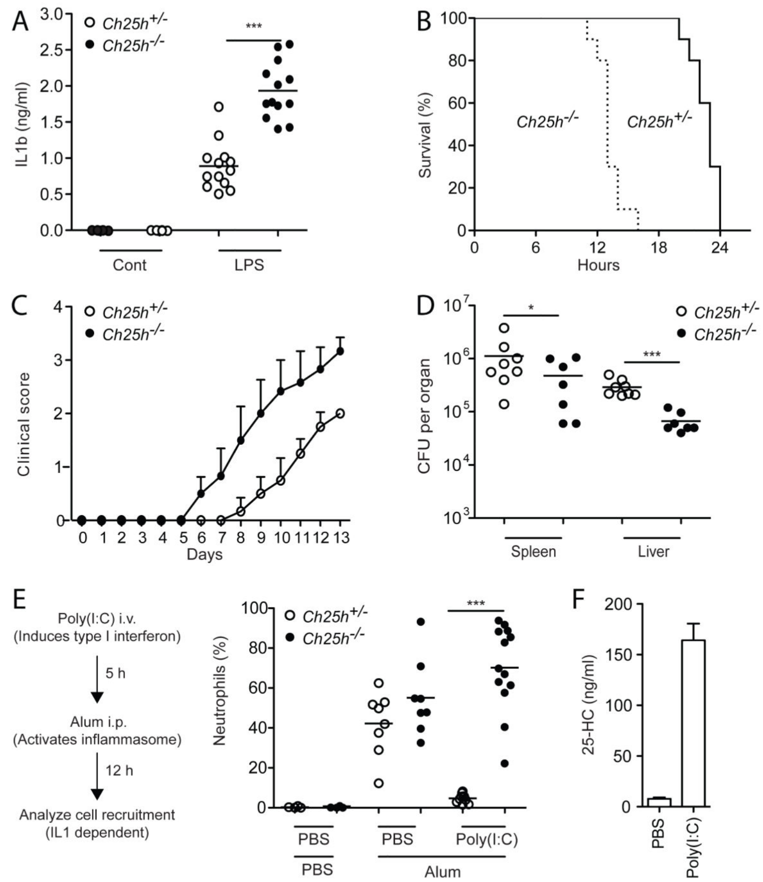

Figure 4. Exaggerated IL-1 family cytokine production, septic shock and EAE and improved anti-bacterial response in Ch25h-deficient mice

(A) Serum IL-1β in *Ch25h*$^{+/-}$ and *Ch25h*$^{-/-}$ mice 12 hours after injection of 20 mg/kg LPS. Each point represents an individual mouse and data are pooled from three experiments**. (B)** Survival of mice (n=10 per genotype) injected with 50 mg/kg LPS (P<0.0001 by Gehan-Breslow-Wilcoxon test). **(C)** EAE severity in *Ch25h*$^{+-}$ and *Ch25h*$^{-/-}$ mice immunized with $MOG_{35\text{-}55}$ in CFA plus PTX (n=12 per genotype, mean ± SD). **(D)** Bacterial growth in spleen and liver of mice infected with 5×10^5 *L. monocytogenes* for 3 days. **(E)** Frequency of neutrophils in the peritoneal cavity of mice challenged with PBS or Poly(I:C) followed by alum. Treatments were as indicated in diagram on left. Each point represents cells from an individual mouse and data are pooled from at least three experiments**. (F)** 25-HC concentration in serum of PBS or poly(I:C) injected mice (mean ± SD from three independent experiments)**.** *, P<0.05; **, P<0.01, ***, P<0.005 (unpaired Student's T-test).