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# Lanosterol modulates TLR4 mediated innate immune responses in macrophages

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## SUMMARY

Macrophages perform critical functions in both innate immunity and in cholesterol metabolism. Here we report that activation of toll-like receptor 4 (TLR-4) in macrophages causes lanosterol, the first sterol intermediate in the cholesterol biosynthetic pathway, to accumulate. This effect is due to type I interferon (IFN)-dependent histone deacetylase 1 (HDAC-1) transcriptional repression of lanosterol-14α-demethylase, the gene product of *Cyp51A1.* Lanosterol accumulation in macrophages, either due to treatment with ketoconazole or induced conditional disruption of

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**AUTHOR CONTRIBUTIONS**

EA, CF-H and YS conceived and designed the study. EA, MF-F, AC-D, WT, JM-M, GWC and YS performed experiments and analyzed data. JSP, GC, MAL, DW, CF-H, and YS assisted with experimental design and data interpretation. EA and YS wrote the manuscript, which was commented on by all authors.

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*Cyp51A1* in mouse MΦs *in vitro,* decreases IFNβ-mediated STAT1-STAT2 activation and IFNβ-stimulated gene expression. These effects translate into increased survival to endotoxemic shock by reducing cytokine secretion. Additionally, lanosterol accumulation increases membrane fluidity and ROS production thus potentiating phagocytosis and the ability to kill bacteria. This improves resistance of mice to *Listeria monocytogenes* infection by increasing bacterial clearance in the spleen and liver. Overall our data indicate that lanosterol is an endogenous selective regulator of macrophage immunity.

**Keywords**

Lanosterol; innate immunity; TLR-4; macrophage; Cyp51A1

## INTRODUCTION

Macrophages (MΦs) are effector cells of innate immunity that phagocytose bacteria and secrete both pro-inflammatory and antimicrobial mediators. They are able to sense a wide variety of inflammatory and immune stimuli and respond by adapting their gene expression profile to provide protection against microbial infections and maintain tissue homeostasis (Mosser and Edwards, 2008). In response to TLR4 activation, MΦs activate molecular mechanisms that both positively and negatively regulate inflammatory responses (Iyer et al., 2010; Medzhitov and Horng, 2009). TLR4 signaling induces immediate/early gene expression through activation of transcription factors such as NFκB, AP-1and IRF3 (Glass and Natoli, 2016; Medzhitov and Horng, 2009). Mediators induced by these, such as type I interferons (IFNs) can then induce secondary response genes (e.g. IFN-stimulated gene products) (Ivashkiv and Donlin, 2014) that both support innate immunity and set the stage for adaptive immunity. TLR4 signaling also results in downregulation of a broad program of gene expression, although molecular mechanisms responsible for this are less well characterized.

The crosstalk between innate immune responses and cholesterol homeostasis is instrumental for proper MΦ function (Castrillo et al., 2003; Im et al., 2011). Indeed, both Sterol-Responsive-Element-Binding-Proteins (SREBPs), which activate cholesterol synthesis and uptake (Jeon and Osborne, 2012) and Liver-X-Receptors (LXRs), which control of cholesterol efflux (Hong and Tontonoz, 2014), participate in regulating several immune functions (Castrillo et al., 2003; Im et al., 2011; Spann et al., 2012; York et al., 2015). Moreover, recent observations indicate that post-cholesterol oxysterols, including 25-hydroxycholesterol (25-HC), participate in the immune activation of MΦs in response to different inflammatory stimulus (e.g. TLR4 or type I IFN) therefore emerging as important regulators of immune functions elicited by MΦs(Bauman et al., 2009; Blanc et al., 2011; Liu et al., 2013; Reboldi et al., 2014; Shibata et al., 2013). Moreover, type I IFN-induced responses produce perturbations in the intracellular homeostasis of cholesterol, which in turn can regulate these processes (York et al., 2015). Although downregulation of cholesterol biosynthetic enzymes and the concomitant accumulation of sterol intermediates has been reported in response to TLR4 agonists in a MΦ cell line (Dennis et al., 2010) the role of these intermediates in regulating host responses to pathogens has not been elucidated.

Intriguingly, while in lymphoid cells, several precursors of cholesterol biosynthesis are essential for their development and differentiation (Hu et al., 2015; Santori et al., 2015), it remains unclear how sterol intermediates of the cholesterol biosynthesis influence MΦs physiology.

In the present study we show that transcription of lanosterol-14α-demethylase (Cyp51A1) is down-regulated in TLR4-activated MΦs due to a secondary repressive response that is dependent on type I IFN production and on the activation of histone deacetylases. Consequently, CYP51A1 protein levels were reduced, and lanosterol, first sterol of cholesterol biosynthesis, accumulated intracellularly. Strikingly, both pharmacological accumulation of lanosterol through administration of ketoconazole (KT), a competitive inhibitor of CYP51A1, or inducible conditional deletion of *Cyp51A1* in MΦs increased the survival of mice subjected to endotoxemic shock, which was associated with diminished pro-inflammatory cytokine secretion. Mechanistically, LPS/IFNβ-triggered STAT1-STAT2 activation in MΦs that accumulated lanosterol was attenuated, resulting in reduced expression of IFN type I-mediated cytokines. Additionally, we found that lanosterol accumulation increased membrane fluidity and ROS production, thus potentiating phagocytosis and the ability to kill bacteria. As such, mice treated with KT exhibited a survival advantage to *Listeria monocytogenes* infection and increased bacteria clearance in spleen and liver.

Our data indicate that innate immune transcriptional downregulation of CYP51A1 induces lanosterol accumulation in MΦs promoting antimicrobial activity and favoring anti-inflammatory response in MΦs. We further identify lanosterol as an endogenous mediator of innate immune responses of MΦs.

## RESULTS

### CYP51A1 downregulation in LPS/IFNγ-treated macrophages promotes the accumulation of lanosterol

To better understand the role of non-immune related genes on innate immune responses, we performed an unbiased whole genome expression analysis on LPS/IFNγ-activated bone marrow derived MΦs (BMDM) (Figure 1A). Ingenuity pathway analysis on significantly downregulated genes (Figure S1A) revealed that the expression of key enzymes of the cholesterol synthesis, such as the lanosterol-utilizing enzymes lanosterol-14α-demethylase (*Cyp51A1*) and 24-dehydrocholesterol reductase (*Dhcr24*) was reduced in LPS/IFNγ-treated MΦs (Figure 1B) and in agreement with a previous report (Dennis et al., 2010). This effect was translated to protein levels, which decreased over time after LPS/IFNγ stimulation (Figure 1C). CYP51A1 catalyzes the de-methylation of the 14α-methyl group from lanosterol, an obligatory step of cholesterol synthesis, while DHCR24 converts all the sterols from the Bloch to the Kandutsch-Russell pathway and desmosterol into cholesterol (Sharpe and Brown, 2013) (Figure S1B). Importantly, the downregulation of CYP51A1 and DHCR24 was also observed in human peripheral blood monocyte-derived MΦs (hMΦs), suggesting that this process is also relevant in humans (Figure 1D).

Classical activation of MΦs is mediated by a combined effect of type II IFNs (IFNγ) and LPS (Schroder et al., 2004). The effect of the LPS + IFNγ on CYP51A1 or DHCR24 protein expression did not differ to that observed with LPS alone, while the stimulation with IFNγ alone did not produce any effect on the expression of these enzymes (Figure S2A&B). Therefore, co-stimulation with IFNγ is not necessary to promote the downregulation of CYP51A1 or DHCR24 and indicates an IFNγ-independent mechanism is responsible for the changes in the expression of these enzymes. Thereafter, experiments were performed in the absence IFNγ.

We next determined if the effect of LPS on the downregulation of these enzymes is a physiological response that also occurs *in vivo*. Thus, MΦs were elicited via intraperitoneal injection of thyoglicollate. After 3 days mice were injected with LPS and 24h later MΦs were isolated and the protein levels of CYP51A1 and DHCR24 were analyzed. Thioglycollate-recruited MΦs of mice injected with LPS showed reduced expression of CYP51A1 and DHCR24 than their vehicle (PBS) injected counterparts (Figure 1E).

We then evaluated whether the reduced levels of CYP51A1 and DHCR24 were associated with alterations in the *de novo* synthesis of cholesterol. Previous studies in RAW 264.7 cells treated with the TLR4 ligand Kdo2-Lipid A (KLA) reported a decrease in *Cyp51A1* and *Dhcr24* expression and lanosterol accumulation over 24h of stimulation (Andreyev et al., 2010; Dennis et al., 2010). However, cholesterol content analyzed by mass spectrometry was increased. This latter effect was attributed to increased lipoprotein uptake from the cholesterol-containing media. We first tested whether the uptake of exogenous cholesterol through lipoproteins present in the culture media was involved in the downregulation of these enzymes. As shown in Figure 1F, incubation of MΦs in media containing lipoprotein deficient serum (LPDS) did not alter the TLR4-mediated downregulation of CYP51A1 or DHCR24 at either mRNA or protein levels (Figure 1F & Figure S2C) and indicating that the effect is not due to a negative cholesterol-mediated feedback regulation, but mediated by the inflammatory stimulus *per se*. We next analyzed the incorporation of radioactive acetate into non-saponifiable lipids after TLR4 stimulation. As shown in Figure 1G, LPS treatment produced a slight reduction of [1-2-$^{14}$C]-acetate incorporation into non-saponifiable lipids (corresponding mainly to cholesterol, 7-dehydrocholesterol, dehydrodesmosterol and desmosterol) and to a significant increase of radioactivity incorporation into lanosterol (Figure 1G). General reduction of the flux of carbon into sterols is also supported by the accumulation of non-sterol isoprenoids in LPS treated MΦs (Figure 1G). Mass spectrometry analysis confirmed the accumulation of lanosterol (~4-fold increase) while total cellular cholesterol content was not significantly altered after 24h of stimulation (Figure 1H). Since dehydrolanosterol, the product of DHCR24-mediated conversion of lanosterol, was not significantly affected upon TLR4 stimulation (Figure 1H), we concluded that the accumulation of lanosterol after TLR4 stimulation is mainly a consequence of the LPS-mediated downregulation of CYP51A1 expression.

## CYP51A1 is transcriptionally downregulated independently of newly synthesized 25-HC and of SREBP activation

To elucidate the mechanism responsible for in the downregulation of CYP51A1 we first tested whether TLR-4 activation induces *Cyp51A1* mRNA instability by inhibiting transcription with actinomycin D. *Cyp51A1* mRNA levels in LPS-treated MΦs were similar to those observed in presence of actinomycin D independently of the stimulation (Figure 2A). mRNA transcription requires promoter recruitment of RNA polymerase 2 (Pol2) followed by phosphorylation at Ser5 at the CT domain of Pol2 (Pol2S5) for transcriptional initiation and subsequently at Ser2 on actively elongating Pol2 (Pol2S2) (Phatnani and Greenleaf, 2006). Pol2 and Pol2S5 chromatin immunoprecipitation (ChIP) from LPDS-cultured BMDM showed that Pol2 and Pol2S5 occupancy on the promoter of *Cyp51A1* is decreased upon LPS treatment which was accompanied by reduction of Pol2S2 recruitment (Figure 2B & Figure S2D). ChIP-sequencing analysis of LPS-stimulated BMDMs also showed the decreased of Pol2 occupancy on the *Cyp51A1* promoter (Figure S2E).

In MΦs, both type I or II IFNs stimulate the expression of cholesterol 25-hydroxylase (*Ch25h*) (Blanc et al., 2013; Liu et al., 2013; Park and Scott, 2010; Reboldi et al., 2014; Shibata et al., 2013), the enzyme responsible for the conversion of cholesterol to 25-HC (Lund et al., 1998) which inhibits cholesterol synthesis via SREBP inactivation (Adams et al., 2004). Therefore, we examined if LPS-mediated accumulation of this oxysterol could be responsible for the observed downregulation of CYP51A1 expression. To do so, we analyzed CYP51A1 expression over time after LPS stimulation in BMDMs isolated from wild-type (WT) and *Ch25h*$^{-/-}$ mice. As expected, LPS induced the expression of *Ch25h* in WT BMDMs (Figure 2C, right panel) while downregulating CYP51A1 over time (Figure 2C left & 2D). *Ch25h* mRNA and 25-HC were undetectable in *Ch25h*$^{-/-}$ BMDMs (Figures 2C right & 2E right). LPS-mediated decrease of CYP51A1 was comparable in WT or *Ch25h*$^{-/-}$ BMDMs (Figure 2C left & 2D) and leading to the accumulation of lanosterol (Figure 2E left). Thus, 25-HC does not play a major role in the early LPS-mediated transcriptional downregulation of Cyp51A1.

SREBP2 activity is tightly regulated by cellular sterol levels, and when intracellular cholesterol levels are decreased, it promotes the transcriptional activation of genes responsible for cholesterol synthesis and uptake (Jeon and Osborne, 2012). We next assessed the contribution of SREBPs in the TLR4-mediated downregulation of CYP51A1 in hMΦs after testing SREBP2 antibody on human hepatic cells (Figure S2F). As expected, hMΦs cultured under conditions of cholesterol deprivation showed the expected increase in SREPB2 processing when compared to cells incubated in media containing FBS with lipoproteins (Figure 2F). In this scenario, the increase in SREBP2 processing correlated with the expected increase in CYP51A1 protein expression (Figure 2F). When hMΦs were stimulated with LPS in cholesterol-free media we did not observe a decrease in the mature form of SREBP2 (Figure 2F) that could account for the decrease of mRNA and protein levels of CYP51A1. To further explore the role of SREBP2 we knocked down its expression in BMDMs using siRNA (Figure S2G). Protein levels of CYP51A1 were decreased upon either LPS stimulation or *Srebp2* silencing (Figure 2G). Interestingly, LPS stimulation in *Srebp2*-silencing conditions caused a further decrease in the protein levels of CYP51A1

when compared to LPS stimulation in non-silencing (NS) control conditions or to *Srebp2* silencing in the absence of LPS stimulation (Figure 2G). We then blocked SREBP processing with a serine protease inhibitor that is selective for SREBP site 1 protease (S1P), PF-429242 (Hawkins et al., 2008). As expected, PF429243 treatment efficiently inhibited the simvastatin-induced cleavage of SREBP2 (Figure 2F). In non-LPS stimulated conditions, PF-429242 treated MΦs showed the expected gradual decrease of Cyp51A1 expression, but stimulation with LPS decreased CYP51A1 protein levels regardless of the presence of PF429242 (Figure 2H). To summarize, inhibiting SREBP2 expression or its activation does not alter LPS-mediated CYP51A1 downregulation. On the other hand, when SREBP activation was induced by inhibition of cholesterol biosynthesis via incubation in the presence of either simvastatin or zaragozic acid (Figure S1B) the expression of CYP51A1 was induced (Figure 2I). In these conditions, treatment with LPS stimulation was still able to promote the downregulation of CYP51A1 independently of previous SREBP activation (Figure 2I). Therefore, induction of SREBP activation does not prevent LPS-mediated downregulation of CYP51A1. Cumulatively, SREBP does not activation is not directly involved in the early TLR4-mediated repression of CYP51A1.

## CYP51A1 downregulation in macrophages is caused by a type I IFN response and is mediated by HDAC1 activation

LPS-mediated TLR4 signaling elicits two parallel signaling pathways: the MyD88 pathway, which triggers NF-κB and AP-1 activation, TLR-stimulated genes (TSGs) and related inflammatory cytokine production, and the TRIF pathway, which activates the interferon regulatory factor-3 (IRF3) transcription factor that mediates the subsequent up-regulation of genes encoding type I IFNs and co-stimulatory molecules (Takeda and Akira, 2004). Secreted type I IFNs signal through their heterodimeric receptors, IFNAR1 and IFNAR2, to form a heterotrimeric transcription factor formed by STAT1, STAT2 and IRF9, promoting an autocrine loop that induces IFN-stimulated genes (ISGs) (Ivashkiv and Donlin, 2014). We thus tested if the stimulation of BMDMs with type I IFNs (e.g. IFNβ, Figure S3A positive control of treatment) reduced the expression of CYP51A1. As shown in Figure 3A, both mRNA and protein levels of CYP51A1 were reduced and lanosterol levels were increased in response to IFNβ stimulation (Figure 3B). In order to better characterize the signaling pathway accounting for CYP51A1 downregulation, we tested the effect LPS or IFNβ on Cyp51A1 expression in BMDMs isolated from WT, *myD88*$^{-/-}$ or *ifnar1*$^{-/-}$ mice. The downregulation of CYP51A1 at mRNA and protein levels was observed in response to LPS or IFNβ in both WT and *myD88*$^{-/-}$ BMDMs (Figure 3C & D). However, neither LPS nor IFNβ reduced CYP51A1 expression in *ifnar1*$^{-/-}$ BMDMs (Figure 3C & D). These results are consistent with RNAseq data of *ifnar1*$^{-/-}$ BMDMs treated with LPS over time where *Cyp51A1* expression was not downregulated as opposed to WT BMDMs (Figure S3B). In agreement with those data, lanosterol accumulation after LPS or IFNβ stimulation is observed in WT or *myD88*$^{-/-}$ BMDMs but prevented in *Ifnar1*$^{-/-}$ BMDMs (Figure 3E). These data indicates that autocrine secretion of type I IFNs by TLR-4 activation promotes the downregulation of CYP51A1 after LPS stimulation and the type I IFNs, but not type II IFNs (Figure S2A), is the underlying stimulus responsible for the LPS-mediated downregulation of Cyp51A1 expression.

To further understand the basis of LPS-mediated *Cyp51A1* transcriptional downregulation, we investigated the chromatin landscape of the *Cyp51A1* locus. ChIP-sequencing analysis of the Cyp51A1 locus of LPS-stimulated BMDMs (Ostuni et al., 2013), showed a diminish occupancy of transcription factor PU.1 (master regulator and pioneer transcription factor of the myeloid lineage), a marked decrease in the active enhancer markers histone 4 acetylation (H4ac) and of histone 3 lysine 4 mono-methylation (H3K4me1), as well as a reduction in the reduced active promoter marker H3K4me3 (Figure 3F). Taken together, these data suggest that LPS alters enhancer and promoter maintenance and represses the *Cyp51A1* locus.

The observed decrease in histone acetylation in the *Cyp51A1* locus after LPS treatment led us to investigate whether histone deacetylases (HDACs) were participating in the downregulation of CYP51A1 expression. As shown in Figure 3G, pre-treatment with a pan-HDAC inhibitor, prevented either LPS or IFNβ mediated downregulation of CYP51A1 expression. More specifically, while inhibition of HDAC1 produced similar effect to that observed with the pan-HDAC inhibitor (Figure 3G and Figure S3C), the HDAC3-specific and/or HDAC4-specific inhibitors did not reduce the LPS or IFNβ-mediated downregulation of CYP51A1 (Figure 3G). Taken together our data indicate that upon LPS or IFNβ stimulation the *Cyp51A1* locus is repressed through a mechanism that involves HDAC1-mediated deacetylation.

## Lanosterol accumulation in mice improves survival to endotoxemic shock via reduced STAT1/STAT2-mediated expression of pro-inflammatory cytokines

To determine if there is a functional role of lanosterol in TLR4-induced immune responses in MΦs, we first investigated the effect of its accumulation on the expression of several pro-inflammatory cytokines. To this end, we exploited the CYP51A1 inhibitor ketoconazole (KT), which blocks the demethylation of lanosterol thus promoting its accumulation(Iglesias and Gibbons, 1989). This azole drug is commonly used as an antimycotic agent, as it inhibits the conversion of lanosterol to ergosterol (Van Den Bossche et al., 1979). As expected, treatment of BMDMs with KT resulted in increased lanosterol content (Figure S4A). Interestingly, we found that, in response to LPS, KT-treated MΦs showed reduced expression of several inflammatory mediators of TLR4 and/or IFNβ activation in MΦs (Thomas et al., 2006) such as of *IL-6*, *Tnfa*, *Ccl2*, *Infβ*, *Cxcl9*, *Mx1* or *Mx2* (Figure 4A). KT, as well as other azoles, has been described to have pleotropic anti-inflammatory effects in different cell types(Kanda and Watanabe, 2006; Tsuji et al., 2012). Thus, to avoid potential unspecific effects of KT not related with the accumulation of lanosterol, we silenced the expression *Cyp51A1* in MΦs (Figure S4B). Importantly, and similar to KT-treated MΦs, LPS-induced expression of pro-inflammatory mediators was diminished in BMDMs silenced for *Cyp51A1* (Figure S4C).

We then investigated the mechanism behind the diminished expression of pro-inflammatory mediators in response to TLR4 activation. KT treatment of BMDMs did not produce a significant difference in the activation of IKKα/β, IκBα or TBK in response to LPS stimulation (Figure S4D). Thus, it appears that the alteration in TLR-4 mediated responses caused by lanosterol accumulation does not affect either MyD88 or TRIF signaling, but instead acts upon the autocrine/paracrine loop after IFN-β induction. LPS strongly induce

IFNβ expression through the TRIF pathway and IFNβ signaling in turn triggers STAT1-STAT2 phosphorylation and activation(Ivashkiv and Donlin, 2014). Interestingly, in KT-treated BMDMs phosphorylation of STAT1 and STAT2 in response to IFNβ or LPS stimulation was considerably reduced when compared to vehicle treated MΦs (Figure 4B). Similar effects on the phosphorylation of STAT1 and STAT2 in response IFNβ or LPS were obtained when *Cyp51A1* was knocked-down by siRNA (Figure S4E).

We then analyzed the effect of lanosterol accumulation in endotoxin shock *in vivo*. We injected KT treated mice with a lethal dose of LPS and monitored their survival. KT treated mice had a survival advantage over control DMSO injected mice (Figure 4C). Furthermore, plasma concentrations of IL-6 or CCL2 were reduced compared to control injected mice (Figure 4D). Although it is well established that LPS-induced lethality is caused by additional factors other than overproduction of cytokines, these data show the importance of lanosterol in preventing death from endotoxemia.

To further explore how CYP51A1 downregulation might participate in the regulation LPS-mediated responses in MΦs, we generated a transgenic mouse expressing a tamoxifen (TMX)-inducible Mer-iCre fusion protein driven by the *Csf1r* promoter (Qian et al., 2011), crossed with *Cyp51A1*$^{fl/fl}$ mice (Keber et al., 2011) to ablate *Cyp51A1* postnatally in the monocyte/MΦ compartment (i.e. *Cyp51A1*$^{fl/fl}$;*Csf1r-Mer-iCre-Mer* referred as Cyp51A1iMΦKO) and thus avoiding possible developmental effects of early embryonic conditional deletion (Gomez Perdiguero et al., 2015). 4-hydroxyTMX (OH-TMX)- induced ablation of *Cyp51A1* in cultured BMDMs obtained from Cyp51A1iMΦKO mice (Figure S4F) had increased lanosterol levels compared to BMDMs from *Cyp51A1*$^{fl/fl}$ control mice (Figure 4E). We then evaluated the effect on the expression of pro-inflammatory mediators in OH-TMX- *vs.* ethanol-treated Cyp51A1iMΦKO BMDMs. In line with our previous results, in response to LPS stimulation, OH-TMX-treated Cyp51A1iMΦKO BMDMs showed reduced expression of *IL-6*, *Tnfa*, *Ccl2*, *Infβ*, *Cxcl9*, *Mx1* or *Mx2* when compared to ethanol-treated (Figure 4F). Furthermore, STAT1-STAT2 phosphorylation was also attenuated in response to IFNβ or LPS (Figure 4G). Importantly, treatment with OH-TMX of control *Cyp51A1*$^{fl/fl}$ BMDMs did not affect expression of inflammatory genes, CYP51A1 protein levels nor STAT1-STAT2 activation in response to inflammatory stimulation (Figure S4 G&H). Consistently, when challenged with a lethal dose of LPS, TMX-treated Cyp51A1iMΦKO mice were more resistant to LPS-induced lethality than TMX treated control *Cyp51A1* $^{fl/fl}$ mice (Figure 4G) and plasma levels of IFNβ and CCL2 were reduced when compared to that of control mice (Figure 4I).

Cumulatively, these data indicate that lanosterol accumulation through enzymatic inhibition or inducible genetic ablation of *Cyp51A1* reduces LPS/IFNβ triggered STAT1-STAT2 activation in MΦs, which results in reduced expression of interferon type I-mediated cytokines and increased survival to endotoxemic shock.

## Lanosterol improves phagocytosis by increasing membrane fluidity, bacteria clearance and confers survival advantage to *Listeria monocytogenes* infection

Besides their important role in immunomodulation through the secretion of cytokines, MΦ phagocytic activity is key to their microbicidal function (Mosser and Edwards, 2008). KT-

treated MΦs exhibited improved phagocytosis of pHrodo-conjugated *E.coli* bacterial particles (Figure 5A). KT might also inhibit CYP3A4 (Svecova et al., 2008), therefore to exclude off-targets effects of we used mifepristone, a non-azole CYP3A4 inhibitor and did not observe any significant effect on pHrodo particle uptake (Figure S5A). Importantly, when *Cyp51A1* was silenced improved phagocytosis was also observed (Figure S5B).

Plasma membrane composition affects lateral mobility of lipids and membrane-associated proteins, as well as phagocytosis. Previous reports suggest that lanosterol, due to its less planar structure compared to cholesterol, increases membrane fluidity (Miao et al., 2002) therefore favoring phagocytosis (Berlin and Fera, 1977). Incubation of MΦs with LPS or KT produced an increase in membrane fluidity as indicated by the decrease in generalized polarization assessed by the shift of laurdan emission spectrum (Figure 5B) and consistent with the accumulation of lanosterol observed in these conditions. Although additional mechanisms might be in play, accumulation of lanosterol treatment (Figure S5C) is sufficient to cause an increase in membrane fluidity (Figure S5D). Interestingly, upon KT treatment MΦs displayed improved ability to kill bacteria (Figure 5C), which was associated with increased reactive oxygen species (ROS) production (Figure 5D). This effect was also observed in MΦs isolated from Cyp51A1iMΦKO mice (Figure S5E).

We then assessed the role of lanosterol accumulation in a model of bacterial infection *in vivo*. After assessing that KT did not affect the growth of Listeria (Figure S6F), we treated mice with KT and tested their survival upon infection. KT treated mice were resistant to death following Listeria infection (Figure 5E). This effect was explained by significantly lower bacteria burden in spleen and liver (Figure 5F). MΦs also play a major role in the early innate defense against Listeria. Increased production of I IFNβ increases susceptibility to Listeria (Solodova et al., 2011) while *ifnar1*$^{-/-}$ mice are resistant (Auerbuch et al., 2004). Consistently, we found that plasma levels of IFNβ were significantly reduced in KT treated mice (Figure 5G).

Our results indicate that innate immune transcriptional downregulation of *Cyp51A1* induces lanosterol accumulation in MΦs promoting antimicrobial activity and favoring negative feedback of type I IFN mediated responses.

## DISCUSSION

In the present study, we identified lanosterol as an endogenous modulator of MΦ innate immune responses, expanding the connection between the roles of MΦs in host defense and in cholesterol metabolism (Castrillo et al., 2003; Im et al., 2011). The key findings of the present study are that host responses to pathogens mediated by TLR4, through production of type I IFN reprogram lipid metabolism by genetically downregulating the cholesterol biosynthetic pathway, specifically affecting *Cyp51A1* expression and resulting in the accumulation of lanosterol. In particular, we show that lanosterol reduces the capacity of MΦs to secrete inflammatory cytokines, but enhances their phagocytic activity. Consequently, lanosterol accumulation reduces mortality to endotoxemia while increasing protection from infection by Listeria.

Previous studies have shown that MΦ respond to cholesterol loading by accumulating desmosterol which suppresses inflammatory cytokine secretion (Spann et al., 2012). Conversely, in response to viral infections, which is integral to the protective immune response requiring a type I IFN, reduction of cholesterol biosynthesis has been is linked to activation of innate immunity (York et al., 2015), while geranylgeraniol, a non-sterol intermediate of the mevalonate pathway, reduces the antiviral effects of IFNs (Blanc et al., 2011). Here we provide evidence that accumulation of the first sterol of the cholesterol biosynthetic pathway, lanosterol, is a physiological response to TLR4 activation through a mechanism that requires the repression of the *Cyp51A1* gene and that is dependent on type I IFN production. In agreement with previous studies, we observed decreased expression of several enzymes of the synthesis of cholesterol after TLR4 stimulation of MΦs (Dennis et al., 2010), a phenomenon that is also observed upon type I IFN activation (Blanc et al., 2011), and this leads to a slight reduction of flux through the cholesterol biosynthetic pathway (Dennis et al., 2010; York et al., 2015) and the accumulation of lanosterol (Dennis et al., 2010). Lanosterol is the substrate of both lanosterol-14α-demethylase (CYP51A1) in the Bloch pathway and DHCR24 in the Kandutsch-Russell pathway. The expression of both genes is reduced after TLR4 activation *in vitro* in hMΦs and, both *in vitro* and *in vivo* in murine MΦs. In most tissues DHCR24 preferentially reduces more distal sterols (Mitsche et al., 2015) together with the fact that lanosterol is the least preferred substrate for DHCR24(Bae and Paik, 1997), indicates that the accumulation of lanosterol observed upon TLR4 stimulation is primarily a consequence of a transcriptional downregulation of *Cyp51A1*.

In agreement with previous reports, we show that in addition to the downregulation of *Cyp51A1*, TLR4 agonists and type I IFN stimulate the expression *Ch25h* and production of 25-HC in MΦs (Blanc et al., 2013; Dennis et al., 2010; Liu et al., 2013; Lund et al., 1998; Reboldi et al., 2014; Shibata et al., 2013), which is a well recognized type I IFN effector response (Blanc et al., 2013; Blanc et al., 2011; Reboldi et al., 2014) and a negative regulator cholesterol biosynthesis enzymes through the repression of SREBP processing *in vitro* (Jeon and Osborne, 2012; Stromstedt et al., 1996). 25-HC mediated modulation of the type I IFN inflammatory responses of BMDMs have been linked to 25-HC-mediated decreased nuclear localization and expression of SREBP2 (Blanc et al., 2011). In line with this finding, RNA-seq analysis on LPS-treated BMDMs obtained from *Ch25h*$^{-/-}$ showed elevated expression of SREBP targets when compared to LPS-treated WT BMDMs (Reboldi et al., 2014). However, *Ch25h*$^{-/-}$ mice exhibit intact cholesterol metabolism (Diczfalusy, 2013; Russell, 2003). In the absence of endogenous 25-HC synthesis we found that the kinetics of the downregulation of CYP51A1 in response to LPS stimulation are indistinguishable from the one observed in WT MΦs and lanosterol levels, in response to LPS stimulation, are increased in both in *Ch25h*$^{-/-}$ or WT BMDMs. These observations suggest that newly synthesized 25-HC is not responsible for the early transcriptional downregulation of *Cyp51A1* expression and subsequent lanosterol accumulation. Notably, TLR4 activation Polymerase 2 occupancy in the Cyp51A1 promoter was observed at 1h after LPS stimulation, which is consistent with a 25-HC-independent mechanism.

SREBP1 expression is induced in MΦs upon inflammatory stimulation, which both promotes acute inflammatory responses (Im et al., 2011; Reboldi et al., 2014) and

contributes to the resolution of the pro-inflammatory TLR4 signaling(Oishi et al., 2017). However, the reduction of CYP51A1 expression in response to LPS was observed in the absence of SREBP processing or SREBP2 expression, indicating that additional mechanisms must account for the early decreased expression of *Cyp51A1* observed upon inflammatory stimulation. The reported 25-HC-mediated decrease of SREBP2 processing and expression (Blanc et al., 2011) could indeed be responsible for the maintenance of CYP51A1 downregulation or for the modulation of other enzymes of the cholesterol biosynthetic pathway after inflammatory stimulation.

Several studies have highlighted an important role for chromatin remodeling in the control of inflammatory gene expression and MΦ function(Medzhitov and Horng, 2009). In MΦs the myeloid lineage transcription factor PU.1 establishes cell-type specific chromatin architecture and it maintains the accessibility of the genomic cis-regulatory information for constitutive and stimulus-inducible transcriptional regulation (Mancino et al., 2015). PU.1 occupancy of the *Cyp51A1* locus is reduced after LPS stimulation and this is accompanied by a marked decrease in the active enhancer markers H4ac and H3K4me1, as well as in active promoter marker, H3K4me3. Notably, this phenomenon was not observed in other cholesterol homeostasis genes, e.i. LSS, LDLR or Dhcr7 (Ostuni et al., 2013). In both TLR and IFN signaling, different HDACs regulate innate immune responses, and mostly have a repressive role (Aung et al., 2006; Chen et al., 2012). Our data, taken together, show that LPS, acting through type-I IFN-recruited HDAC1 diminished histone acetylation of the *Cyp51A1* locus and reduces Pol2-mediated transcription of *Cyp51A1.*

In response to TLR4 stimulation, activation of MyD88-dependent and MyD88-independent pathways of MΦ results in the release a variety of inflammatory cytokines such as TNF, IL-1 and IL-6 (MyD88-dependent) and IFN-β (MyD88-independent/TRIF-dependent)(Mahieu and Libert, 2007; McNab et al., 2015). Several lines studies have indicated that type I IFNs are important mediators in endotoxemia (Mahieu and Libert, 2007) and in synergy with other cytokines and inflammatory products, can lead to organ damage (Mahieu and Libert, 2007). Thus, to avoid excess inflammation and tissue damage it is important that the production of proinflammatory cytokines is kept under control (Serhan et al., 2007). In line with this, mice ubiquitously deficient for *Ifnβ, Ifnar1, Tyk2 or Stat1*, genes involved in type I IFN signaling, show decreased expression of several proinflammatory cytokines and ISGs in response to LPS and exhibit a better survival to endotoxemia (Dejager et al., 2014; Karaghiosoff et al., 2003; Mahieu and Libert, 2007). One of the most intriguing results of our present work is that accumulation of lanosterol, either via inhibition or knockdown of *Cyp51A1* in MΦs, results in reduced STAT1-STAT2 activation in response to autocrine secretion of type I IFNs after TLR-4 activation. This might, at least in part, contribute to the attenuated expression of *Infβ* and other ISGs including *Ccl2, Cxcl9, Mx1 and Mx2*, as well as *Il6* or *Tnfα* in LPS-mediated autocrine IFNβ regulation in $Ifn\beta^{-/-}$ MΦs (Thomas et al., 2006). Consistently, mice treated with KT and Cyp51A1iMΦKO are less sensitive to LPS-induced mortality and have lower plasma levels of pro-inflammatory cytokines. Thus, type I IFNs, by acting as the underlying stimulus responsible for the LPS-mediated downregulation of CYP51A1, promote lanosterol accumulation, which in turn provides a negative feedback regulation for inflammatory activation.

One of the main physiological roles of MΦs is related to its phagocytic activity. Changes in phagocytosis correlate with changes in membrane composition and fluidity (Avery et al., 1995). Accumulation of lanosterol in MΦs via pharmacological inhibition of Cyp51A1, LPS-mediated Cyp51A1 downregulation or direct lanosterol loading, increased membrane fluidity. As such, we also observed an increased phagocytic activity. Lanosterol structural conformation is less planar compared to cholesterol, therefore increased lanosterol content in membranes increases their fluidity and favors phagocytosis (Miao et al., 2002). Additionally, we observe an increase in ROS production, which explains the enhanced bacterial killing and clearance thus enhancing protection form Listeria infection. We also found that KT-treated mice exhibit decrease secretion of IFNβ which may also account for increased survival to Listeria infection. Indeed, the production of type I IFN is associated with suppression of the innate response and increased susceptibility to Listeria infection (Solodova et al., 2011). In line with these findings, *Ifnra1*$^{-/-}$ are resistant to endotoxemia and to Listeria infection (Auerbuch et al., 2004; Dejager et al., 2014). Thus, lanosterol via decreased activation of STAT1-STAT2 is an essential part of the negative-feedback regulation mechanism regulating cytokine production during inflammatory conditions involving type I IFNs and conferring survival advantage to endotoxemia and resistance to Listeria infection.

KT has been described to have pleotropic anti-inflammatory effects(Kanda and Watanabe, 2006; Tsuji et al., 2012). However, the underlying molecular mechanisms remain poorly understood (Friccius et al., 1992; Hau et al., 2014), and none of these studies have evaluated the contribution KT-mediated inhibition of CYP51A1 and accumulation of lanosterol to the reported anti-inflammatory effects. Our findings suggest that KT, which is currently only used exclusively to treat mycotic infections in immunocompromised patients (Finkel et al., 2009), could be of benefit in additional clinical settings by ameliorating MΦ microbicidal activity or blocking harmful cytokine overproduction.

In summary, our findings indicate that TLR4-mediated transcriptional downregulation of *Cyp51A1* induces lanosterol accumulation in MΦs promoting antimicrobial activity and favoring negative feedback of type I IFN mediated responses. It will therefore be of interest to investigate the role lanosterol in other disease contexts in which inflammation plays a pathogenic role.

## EXPERIMENTAL PROCEDURES

Further details and an outline of resources used in this work can be found in Supplemental Experimental Procedures. Briefly, raw data from microarray experiments were normalized and analyzed by GeneSpring GX software version 11.5 (Agilent Technologies). Data are deposited in NCBI Gene Expression Omnibus (GSE89559). Animal studies were approved by the Institutional Animal Care and Use committee of Yale University School of Medicine. WT C57BL/6 and *Ch25h*$^{-/-}$ 6–12 weeks old mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Ifna1r*$^{-/-}$ and *Myd88*$^{-/-}$ animals were a kind gift from Dr. A. Iwasaki and Dr. D. Goldstein. *Cyp51A1*$^{fl/fl}$~ *Csf1r-Mer-iCre-Mer* (Cyp51A1iMΦKO) mice were generated by crossing B6.129P2-Cyp51tm1Bfro/J (i.e. *Cyp51A1*$^{fl/fl}$) female animals with FVB-Tgm(Csf1r-cre/Esr1*)1Jwp/J (i.e.Csf1r-Mer-iCre-Mer) males.

Endotoxemic shock experiments were performed with littermates from breeding *Cyp51A1*$^{fl/fl}$ females with Cyp51A1iMΦKO male mice were used for experiments treated with TMX for 5 days prior LPS injection or; with WT mice treated with KT every other day for 14 days prior LPS. WT mice treated with KT were also used for *Listeria monocytogenes* infection, bacteria burden and survival experiments. Statistical analysis was performed using Student's t test, one-way ANOVA with Bonferroni correction for multiple comparisons or logrank test when appropriate. Normality was checked using the Kolmogorov-Smirnov test. A nonparametric test (Mann-Whitney) was used when data did not pass the normality test. $p \leq 0.05$ was considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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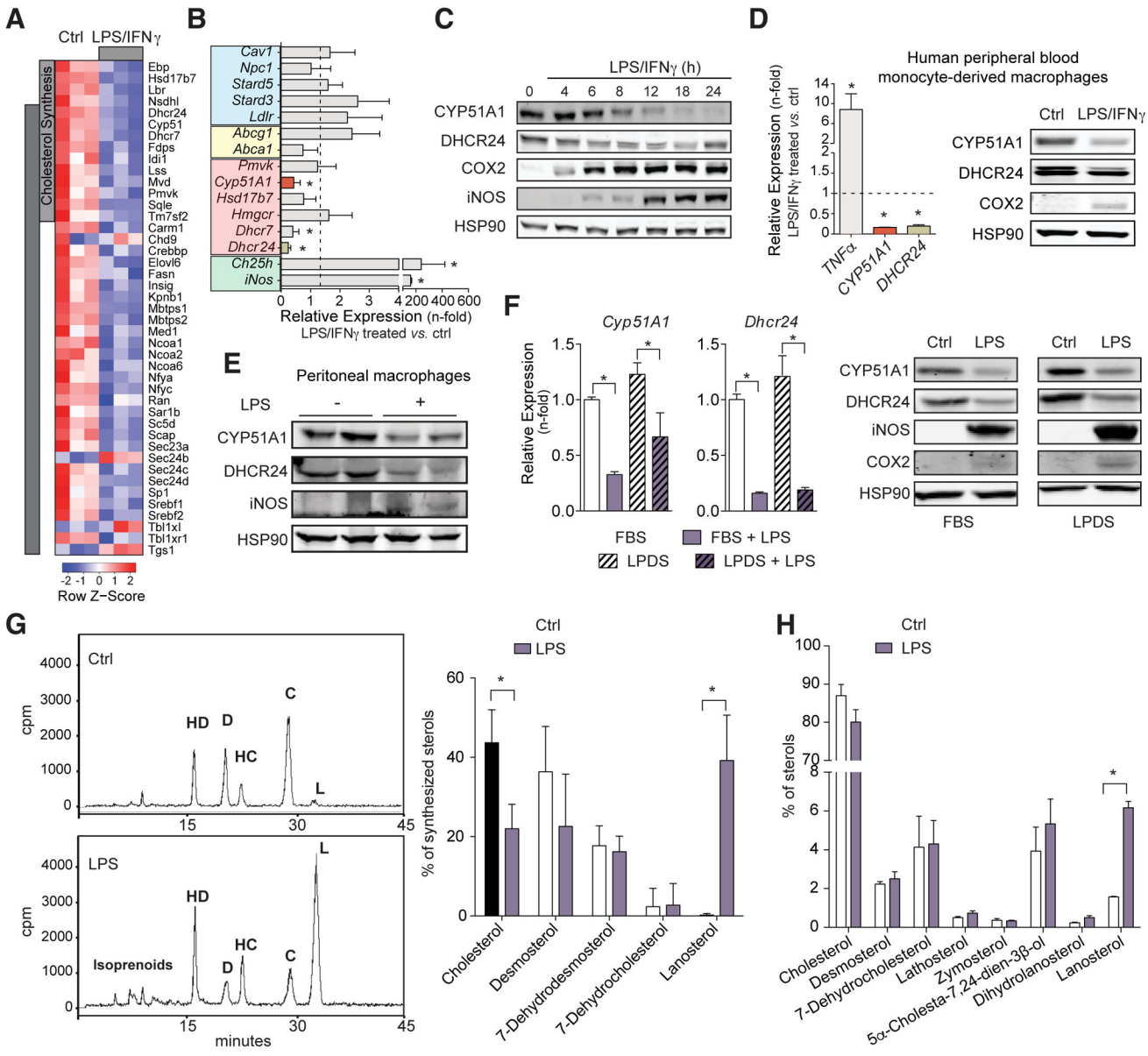


**Figure 1. CYP51A1 downregulation in LPS/IFNγ-treated macrophages promotes the accumulation of lanosterol**

(A) Heat map of differentially expressed cholesterol biosynthesis genes and SREBP2 targets in BMDMs stimulated with LPS (10ng/mL)/IFNγ (20ng/mL) for 8h.

(B) qPCR validation of microarray data. Relative mRNA expression levels of cholesterol trafficking (blue), efflux (yellow), biosynthesis (red), and positive controls of inflammatory stimulation (green), (n=3).

(C) CYP51A1 and DHCR24 protein levels of LPS/IFNγ treated BMDMs for the indicated times (n=3).

(D) Relative mRNA expression (left) and protein levels of CYP51A1 and DHCR24 (right) in hMΦs treated with LPS/IFNγ for 8h (left) or 18h (right), (n=3).

(E) CYP51A1 and DHCR24 protein levels of elicited peritoneal macrophages collected 24h after intraperitoneal injection of LPS (20mg/kg) performed 3 days after initial 3% thioglycollate injection (n=2).
(F) Left: relative mRNA expression, Right: protein levels CYP51A1 and DHCR24 of BMDMs cultured for 24h in regular media containing 20% FBS or in media containing 20% LPDS and then treated with LPS (100ng/mL) for 8h (left) or 18h (Right), (n=3).
(G) Sterol intermediates analysis by HPLC and on-line radioactivity of BMDM treated LPS (100ng/mL) or PBS (Ctrl) for 24h. Left: representative plots of [1–$2^{14}$C]-acetate incorporation into sterols. Right: quantification of total synthesized sterols, expressed as % of total synthesized sterols *vs.* Ctrl, (n=3).
(H) Composition of sterols by GC/MS of BMDM as in G, (% of each sterol specie within total sterol), (n=3).
(C, D, E, F) COX2, iNOS or *TNFα* are positive controls of inflammatory activation. HSP90 is a loading control.
Results are expressed as mean ± SEM. *$p < 0.05$ *vs.* Ctrl unless otherwise indicated.
See also Figure S1.

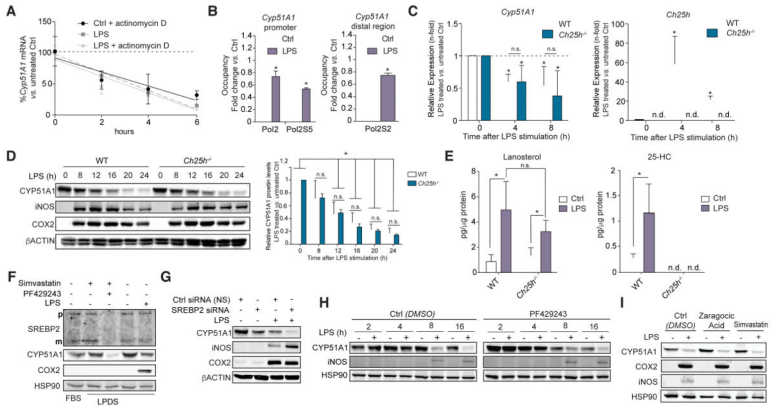

**Figure 2. CYP51A1 is transcriptionally downregulated independently of newly synthesized 25-HC and of SREBP activation**

(A) Cyp51A1 mRNA analysis by qPCR of BMDMs treated with actinomycin-D (30 min) prior LPS (100ng/mL) stimulation for indicated times (n=3).

(B) ChIP analysis with Pol2 and Pol2S5 (left) and PolS2 (right) of BMDMs cultured in media containing 20% LPDS for 24h and treated with LPS for 1h. Quantification of promoter specific bound antibody by qPCR with primers proximal to the TSS of *Cyp51A1*. Data are fold change *vs.* Ctrl (PBS treated) and normalized to input chromatin (n=3).

(C) Relative mRNA expression of *Cyp51A1* (left) or *Ch25h* (right) of WT of *Ch25h*$^{-/-}$BMDMs incubated with LPS for the indicated times (n= 4).

(D) Left: CYP51A1 protein levels of LPS treated BMDMs for the indicated times. Right: quantification of CYP51A1 protein levels normalized by βACTIN (n= 4)

(E) Quantification of Lanosterol (left) or 25-HC (right) by GC/MS normalized protein content of WT of *Ch25h*$^{-/-}$ BMDMs incubated with LPS for 24h, (n=3).

(F) CYP51A1 protein levels and precursor (p) and mature (m) forms of SREBP-2 of BMDMs incubated in regular media containing 20% FBS or 20% LPDS as indicated, treated with Simvastatin (5μM) for 12h, PF429243 (10μM) for 12h or LPS (100g/mL) for 4h, respectively (n=4).

(G) CYP51A1 protein levels of BMDMs transfected with 30nM of non silencing (NS) control siRNA or *Srebp2* siRNA and treated or not with LPS, as indicated, for 8h (n=3).

(H) CYP51A1 protein levels of BMDMs treated with PF429243 (10μM) or vehicle (DMSO) Ctrl stimulated with LPS for the indicated times.

(I) CYP51A1 protein levels of BMDM incubated with DMSO (Ctrl), zaragozic acid (10μM) or simvastatin (5μM) for 2h prior LPS stimulation for 8h (n=3).

(D, F, G, H, I) COX2 and iNOS are positive controls of activation. βACTIN or HSP90 are loading controls.

Results are expressed as mean ± SEM. *p < 0.05 *vs.* Ctrl unless otherwise indicated. Not significant (n.s.). Not detectable (n.d.)

See also Figure S1 and S2.

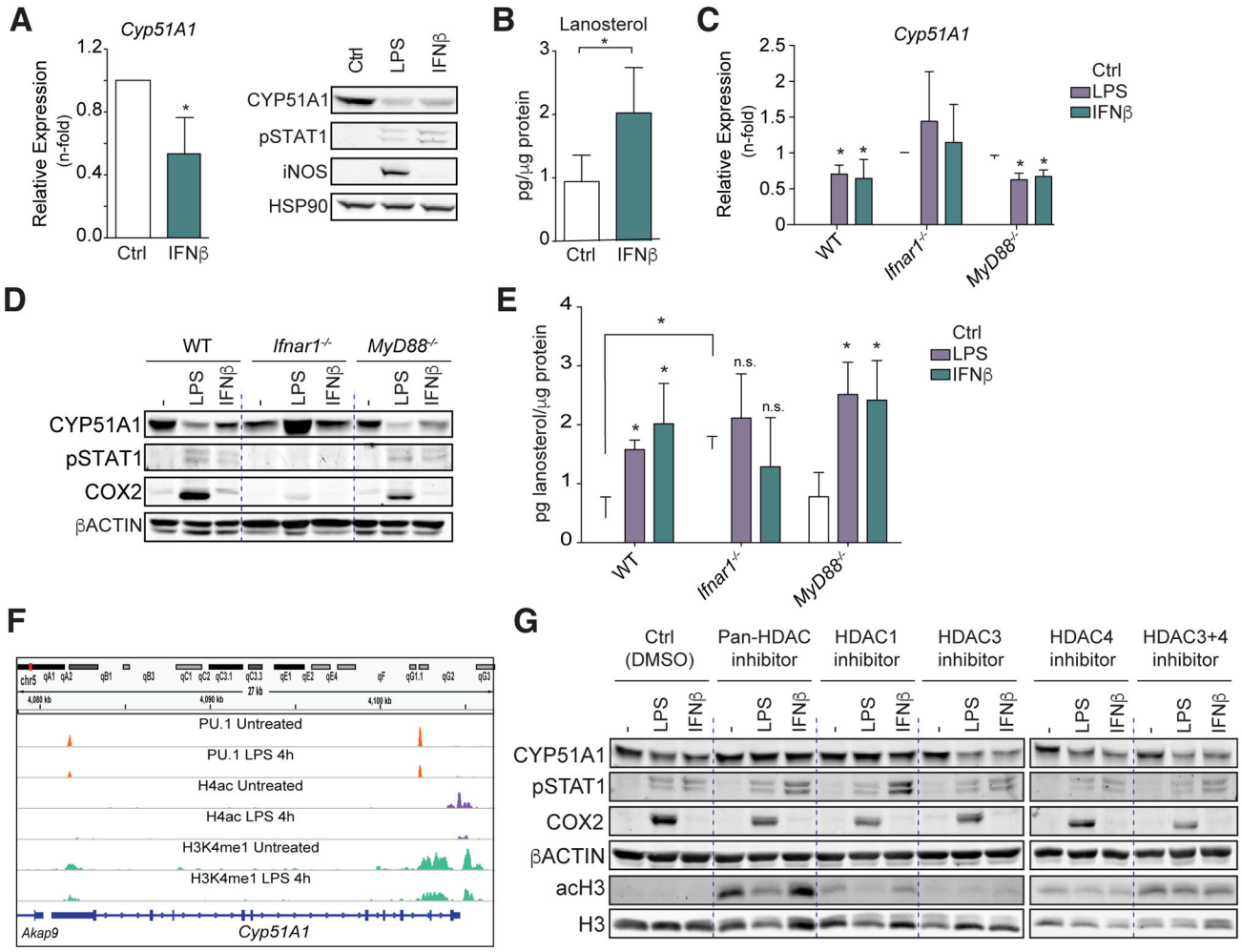


**Figure 3. CYP51A1 downregulation in macrophages is caused by a type I IFN response and is mediated by HDAC1 activation**

(A) Relative mRNA expression of *Cyp51A1* (left) and CYP51A1 protein levels (right) of BMDMs stimulated with IFNβ(1000U/mL) for 4h (left) or with LPS (100ng/mL) or IFNβ for 12h (right), (n=3).

(B) Quantification of lanosterol by GC/MS normalized protein content of BMDMs incubated with IFNβ for 24h, (n=3).

(C–D) Relative mRNA expression (C) or protein levels (D) of CYP51A1 of WT, *Ifnar1*$^{-/-}$ or *myD88*$^{-/-}$ BMDMs stimulated with LPS or INFβ for 4h (C) or 12h (D), (n=3).

(E) Quantification of Lanosterol by GC/MS normalized protein content of BMDMs WT, *Ifnar1*$^{-/-}$ or *myD88*$^{-/-}$ BMDMs as in (B).

(F) ChIP sequencing analysis of PU.1, H4ac, H3K4me1 pull-down in BMDM treated with LPS for 4hours or control. Pioneer transcription factor of the myeloid lineage (PU.1), active enhancer markers histone 4 acetylation (H4ac), histone 3 lysine 4 mono-methylation (H3K4me1), active promoter marker H3K4me3. (Ostuni et al., 2013).

(G) CYP51A1 protein levels of BMDMs treated with different HDAC inhibitors. Pan-HDAC: panabinostat (50nM), HDAC1: CAY10398 (10μM), HDAC3: RGFP966 (5μM),

HDAC4: tasquinimod (10mM) for 1h prior LPS or IFNβ stimulation for 12h (n=3). Dashed blue lines are for treatment group separation and do not indicate cropped blots. H3 and AcH3 are controls for HDAC inhibitor action.

(A, D, G) pSTAT1, iNOS, COX2 are controls of activation of inflammatory activation, respectively. HSP90 or βACTIN are loading controls.

Results are expressed as mean ± SEM. *$p < 0.05$ *vs.* Ctrl unless otherwise indicated.

See also Figure S3.

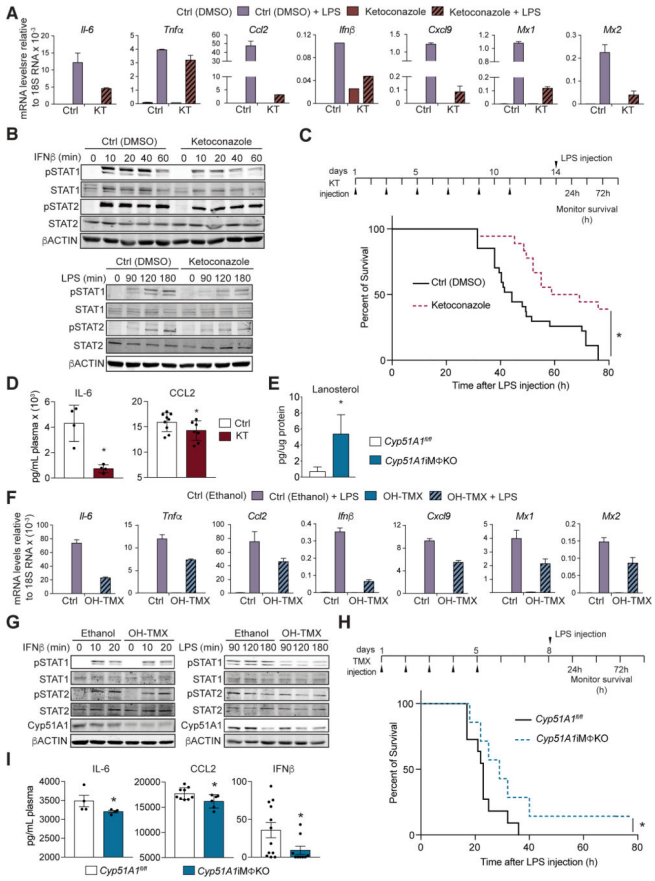

**Figure 4. Lanosterol decreases inflammatory cytokine secretion and improves survival to endotoxemic shock**

(A) qPCR analysis of mRNA levels of indicated genes of BMDMs treated with KT (10μM) or DMSO 12h prior of LPS (100ng/ml) for 4h.

(B) pSTAT1/STAT1 and pSTAT2/STAT2 protein levels of BMDMs treated with KT 12h prior to IFNβ (1000U/mL) (upper panels) or LPS (lower panels) for the indicated times (n=3).

(C) Survival of WT mice treated with (25mg/kg) of KT as in indicated and subjected to a lethal dose of LPS (60 mg/kg). KT-treated mice (n=18), DMSO Ctrl treated mice (n=25).

(D) Plasma levels IL6 or CCL2 by ELISA 3h after LPS injection (60mg/kg) of mice injected with KT as in (C). Each dot represents the mean of triplicate sample of individual animal.

(E) Quantification of Lanosterol by GC/MS normalized protein content of BMDMs isolated from *Cyp51A1*$^{fl/fl}$ or *Cyp51A1iMFΦKO* 3 days after of TMX Cre-mediated induction as described in experimental procedures (n=3 per group).

(F) qPCR analysis of mRNA levels of indicated genes of *Cyp51A1iMFΦKO* BMDMs and treated at day 5 of differentiation with OH-TMX (10μg/mL) or Ethanol for 2 days and then stimulated with LPS 100ng/mL for 4h.

(G) pSTAT1/STAT1 and pSTAT2/STAT2 protein levels of *Cyp51A1iMFΦKO* BMDMs treated with OH-TMX or Ethanol as in (F) prior to IFNβ (upper panels) or LPS (lower panels) for the indicated times (n=3). CYP51A1 protein levels are show as control of TMX induced deletion.

(H) Survival of *Cyp51A1*$^{fl/fl}$ or *Cyp51A1iMFΦKO* treated as indicated and subjected to a lethal dose LPS (60 mg/kg). *Cyp51A1*$^{fl/fl}$ (n=11), *Cyp51A1iMFΦKO* (n=6).
(I) Plasma levels IL6 or CCL2 determined by ELISA 3h after LPS injection (60mg/kg) of mice of *Cyp51A1*$^{fl/fl}$ or *Cyp51A1iMFΦKO* mice injected with TMX as indicated in (H). Each dot represents the mean of triplicate sample of individual animal.
(A, G) βACTIN is a loading control.
(A, F) Data are mean of duplicate samples ± SD of one representative experiment out of four with similar results.
(C, H) Kaplan–Meier survival curves compared by log-rank test. *$p < 0.05$ (C) or *$p < 0.1$ (H).
(D, E, I) Results are expressed as mean ± SEM. *$p < 0.05$ *vs.* Ctrl.
See also Figure S4.

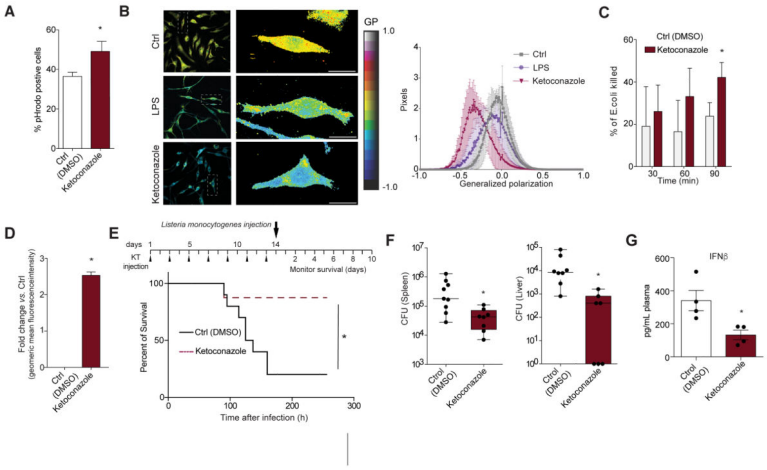


**Figure 5. Lanosterol improves phagocytosis and bacteria clearance and confers survival advantage to *Listeria monocytogenes* infection**

(A) FACS analysis of uptake of opsonized *E.coli* pHrodo particles in CD11b+/Ly-6G- cells from thioglycollate elicited peritoneal cells from KT or DMSO treated mice as in Figure 4C. Data are % of pHrodo positive cells (n=3).

(B) Representative micrographs of BMDMs treated with DMSO vehicle control, LPS 100ng/mL plus DMSO or KT (10μM) for 12h, stained with laurdan (left) and analyzed as described in experimental procedures. Higher GP value indicates that membranes are more ordered and less dynamic or fluid (right). The GP value of each pixel was used to generate a pseudocolor GP image (left). Representative experiment out of 3 with similar results.

(C) Bacteria killing assay of tdTomato-*E.coli* in cells obtained as in (A) and analyzed by FACS. Data % of killed bacteria in CD11b+/Ly-6G- cells (n=3).

(D) ROS determination with CellRox by FACS in BMDMs treated with KT (10μM) for 12h. Data (geometric mean fluorescent intensity) expressed as fold change *vs.* DMSO Ctrl (n=3, by triplicate).

(E) Survival of WT mice treated 25mg/kg of KT as in indicated and infected by retro-orbital injection with $1\times10^5$ Listeria particles. KT-treated mice (n=10), DMSO treated Ctrl mice (n=15). Kaplan–Meier survival curves compared by log-rank test.

(G) Bacteria Burden in spleen and liver 48h post-infection with $1.5\times10^4$ Listeria particles. CFU were determined from spleen and liver. Each dot represents data obtained from individual animals.

(H) Plasma levels of IFNβ by ELISA of mice treated with KT as indicated in (E) and then infected with $1.5\times10^4$ Listeria particles for 48h. Each dot represents the mean of triplicate sample of individual animal.

Results are expressed as mean ± SEM. *$p < 0.05$ *vs.* Ctrl.

See also Figure S5.