LRH-1 mediates anti-inflammatory and antifungal phenotype of IL-13-activated macrophages through the PPARγ ligand synthesis

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Liver receptor homologue-1 (LRH-1) is a nuclear receptor involved in the repression of inflammatory processes in the hepatointestinal tract. Here we report that LRH-1 is expressed in macrophages and induced by the Th2 cytokine IL-13 via a mechanism involving STAT6. We show that loss-of-function of LRH-1 in macrophages impedes IL-13-induced macrophage polarization due to impaired generation of 15-HETE PPARγ ligands. The incapacity to generate 15-HETE metabolites is at least partially caused by the compromised regulation of CYP1A1 and CYP1B1. Mice with LRH-1-deficient macrophages are, furthermore, highly susceptible to gastrointestinal and systemic Candida albicans infection. Altogether, these results identify LRH-1 as a critical component of the anti-inflammatory and fungicidal response of alternatively activated macrophages that acts upstream from the IL-13-induced 15-HETE/PPARγ axis.

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Macrophages orchestrate innate immune responses by initiating and resolving inflammatory signalling programmes. Emerging evidence indicates that the state of macrophage polarization plays a critical role in the regulation of these inflammatory processes. Two different programmes of macrophage activation, the classical (M1) and the alternative differentiation, classify polarized macrophages with either persistence or resolution of inflammation. M1 macrophages express high levels of opsonic receptors, involved in the production of pro-inflammatory effector molecules such as reactive oxygen and nitrogen intermediates and pro-inflammatory cytokines (interleukin (IL)-1β, tumour-necrosis factor alpha (TNFα), IL-6 and IL-12). These macrophages contribute to inflammation, microbial killing, regulation of cell proliferation and apoptosis. Alternatively activated macrophages are characterized by abundant levels of the anti-inflammatory cytokine IL-10 and non-opsonic receptors, such as C-type lectin receptors and scavenger receptors (CD36), and resolve inflammation by increasing CD36-mediated efferocytosis and secretion of tissue remodelling/repair mediators.

The balance of macrophage differentiation in favour of alternatively activated macrophages can be shifted by the activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) (refs 5,6). PPARγ expression and activity in macrophages is negatively regulated during inflammatory processes. In addition, activated PPARγ transrepresses many inflammation-activated transcription factors, including nuclear factor-kappaB (NF-kB), signal transducers and activators of transcription (STATs), activator protein 1 (AP1) and nuclear factor of activated T-cells (NFAT), resulting in pro-inflammatory mediator inhibition. PPARγ is activated by endogenous ligands derived from the metabolism of arachidonic acid (AA). Among these ligands, 15-deoxy-A12,14-prostaglandin J2 (15d-PGJ2), metabolized through the COX1/COX2 cyclooxygenases, and the 12- and 15-hydroxyeicosatetraenoic acids (HETEs), metabolized through 5 and 12/15 lipoxigenases, are essential for PPARγ endogenous activation. In addition to cyclooxygenases and lipoxigenases, cytochrome P450 (CYP) enzymes are also considered to be critical for the metabolism of AA in epoxy (EETs) and in hydroxy (HETEs) derivatives. Within the CYP family, the CYP1 family is mainly involved in the generation of 12- and 15-HETEs through CYP1A1 and CYP1B1.

Liver receptor homologue-1 (LRH-1, NR5A2) is a nuclear receptor highly expressed in the intestine, liver, pancreas and ovary. Although LRH-1 has been recognized as an orphan receptor, phospholipids, including the phosphatidyl inositol second messengers, and more recently the 12C-fatty acyl-containing phospholipid, dilauroyl phosphatidylcholine (DLPC), have been described to bind the ligand-binding pocket and to act as LRH-1 agonists. LRH-1 plays important roles in embryonic development, cholesterol and bile acid homeostasis, and promotes hepatic glucose sensing through its sequence and we evaluated the mutated NR5A2 promoter activity in wild-type macrophages (Fig. 1g,h), chemical inhibition of STAT6 by AG490 (Fig. 1g) or genetic deletion of STAT6 (Fig. 1h), consistent with these observations, IL-13 failed to increase NR5a2 mRNA and protein levels in macrophages deficient for STAT6 (Fig. 1f) suggesting that STAT6 mediates the transcriptional regulation of LRH-1. We then performed transient transfection assays in primary macrophages to assess the effect of IL-13 and STAT6 on NR5a2 promoter activity. While 4 h of IL-13 exposure was already sufficient to induce NR5a2 promoter activity in wild-type macrophages (Fig. 1g,h), chemical inhibition of STAT6 by AG490 (Fig. 1g) or genetic deletion of STAT6 (Fig. 1h) attenuated or even abolished this response.

To explore the functionality of this site, we next modified by in vitro mutagenesis its sequence and we evaluated the mutated NR5a2 reporter construct activity on IL-13 exposure (Fig. 1j).
Mutation of the STAT6-RE abolished the activity of the Nr5a2 reporter construct in response to IL-13 in Stat6<sup>+/+</sup> and Stat6<sup>M−/−</sup> macrophages (Fig. 1j). These results demonstrate that STAT6 directly controls the transcription of LRH-1 in response to IL-13.

**LRH-1 is involved in IL-13-induced macrophage activation.** In order to assess the role of LRH-1 in IL-13-induced alternative macrophage differentiation, we generated mice in which the Nr5a2 gene was selectively disrupted in myeloid-derived cells. To generate these animals, mice carrying floxed Lrh-1 alleles were crossed with transgenic mice that express the Cre recombinase under the control of the mouse phagocyte-selective lysozyme promoter<sup>21,26</sup>. Compared with control (Lrh-1<sup>M+/+</sup>) macrophages, LRH-1 mRNA and protein levels were almost undetectable in macrophages derived from the myeloid cell-specific LRH-1-deficient (Lrh-1<sup>M−/−</sup>) mice (Supplementary Fig. 1a–c). Furthermore, the disruption of LRH-1 could not be detected in other LRH-1-expressing tissues, such as the liver and the colon (Supplementary Fig. 1d,e).

We then evaluated the expression of specific markers of classical and alternative activation in untreated or IL-13-treated
LRH-1M+/+ and LRH-1M−/− macrophages during 4 h. Overall, LRH-1M−/− macrophages displayed an upregulation of M1 markers such as Nos2 (encoding the inducible nitric oxide synthase) and the Fcγ-receptors Fcgr3 and Fcgr1 (encoding CD16 and CD64 proteins, respectively), which was mirrored by a downregulation of Chi3l3 (YM1), Mrc1 (MR), Clec7a (Dectin-1), Il1rn (IL-1ra) and Tgb1 (transforming growth factor (TGF)-β1) alternative activation markers (Fig. 2a,b). This was accompanied by an increase in the mRNA and protein levels of the inflammatory cytokines TNFα, IL-1β and IL-6 (encoded by Tnfa, Il1b and Il-6 genes, respectively). I12 pro-inflammatory and I10 anti-inflammatory cytokine mRNA levels remained unchanged in LRH-1M+/+ and LRH-1M−/− macrophages (Fig. 2a). Furthermore, the induction of MR, Dectin-1, CD36, Arg1 (encoding the arginase 1), Chi3l3 and Il1rn expression by IL-13 was strongly diminished in LRH-1M−/− macrophages (Fig. 2a,b). Consistent with reduced alternative activation markers in LRH-1M−/− macrophages, the M1 markers such as Nos2, Ilgam (CD11b), Fcgr3, Fcgr1, Il1b and Il-6 still remained highly expressed (Fig. 2a–c). Consistent with these findings, the induction of alternative activation gene markers observed after 4 h of IL-13 treatment was amplified after 24 h of IL-13 treatment in LRH-1M+/+ macrophages (Supplementary Fig. 2a). Moreover, the decrease in alternative activation markers in LRH-1M−/− macrophages after 4 h of IL-13 treatment was sustained after 24 h of stimulation (Supplementary Fig. 2a). Altogether, these data indicate that LRH-1 is required for repression of pro-inflammatory state and for optimal induction of alternative macrophage activation by IL-13. These findings are consistent with the robust induction of Il10, Tgb1, Il1rn, Mrc1, Clec7a and Cd36 gene expression in LRH-1M+/+ macrophages treated with the LRH-1 agonist DLPC (Supplementary Fig. 2b).

**LRH-1 activates 15-HETE secretion via the control of CYP1As.** The nuclear receptor PPARγ is a key component of the signalling

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**Figure 2 | LRH-1 is involved in IL-13-induced alternative activation of macrophages.** (a) Gene expression analysis of markers of M1 and M2 polarization in peritoneal macrophages from Lrh-1M+/+ and Lrh-1M−/− mice treated with IL-13 for 4 h, determined using RT-PCR. The results were represented in fold induction relative to the untreated Lrh-1M+/+ littermate. (b) Dot-plot representing Dectin-1, CD36 and MR protein expression in macrophages from Lrh-1M+/+ and Lrh-1M−/− mice treated with IL-13 for 24 h. Numbers indicate the % of positive cells. Graphs represent geometric fluorescence quantification for the indicated proteins. (c) Cytokine production of peritoneal macrophages from Lrh-1M+/+ and Lrh-1M−/− mice after IL-13 treatment and C. albicans challenge for 8 h (ratio: 1 macrophage:3 yeasts), quantified by enzyme-linked immunosorbent assay. Results correspond to mean ± s.e.m. of triplicates. Data are representative of three independent experiments. *P<0.05, **P<0.01 compared to the respective untreated control and #P<0.05, ##P<0.01 compared with Lrh-1M+/+ + IL-13. P values were determined using the Bonferroni–Dunnnett method.
pathway triggered by IL-13 and directly controls the expression of markers of alternative activation. To establish whether the increase in alternative activation markers by IL-13 results from direct regulation of PPARγ transcription by LRH-1, we first evaluated Pparg mRNA levels in Lrh-1M+/+ and Lrh-1M−/− macrophages under basal conditions and after IL-13 exposure. The increased Pparg mRNA level by IL-13 in Lrh-1M+/+ macrophages was not affected in Lrh-1M−/− macrophages (Fig. 3a). Moreover, in transient transfection studies, absence of LRH-1 in Lrh-1M−/− macrophages (Fig. 3b) or conversely ectopic expression of LRH-1 in wild-type macrophages (Fig. 3c) did not significantly affect IL-13-mediated PPARγ promoter induction, further indicating that LRH-1 does not regulate the transcription rate of PPARγ. Next, we examined whether LRH-1 was required for PPARγ activation by assessing the impact of IL-13 on a heterologous PPARγ reporter transfected in Lrh-1M+/+ and Lrh-1M−/− macrophages. Remarkably, while in Lrh-1M+/+ macrophages IL-13 significantly induced the PPRE luciferase reporter, no such response could be observed in Lrh-1M−/− macrophages (Fig. 3d). Conversely, co-transfection of the PPRE luciferase reporter with an expression vector for LRH-1 robustly increased PPARγ activation (Fig. 3e), suggesting that LRH-1 induces the activity of PPARγ.

Figure 3 | LRH-1 activates CYP1A1- and CYP1B1-dependent 15-HETE production. (a) Pparg mRNA expression in macrophages from Lrh-1M+/+ and Lrh-1M−/− mice treated with IL-13 for 4 h, determined using RT-PCR. The results were represented in fold induction relative to the untreated wild-type littermate. (b) Luciferase activity in macrophages from Lrh-1M+/+ and Lrh-1M−/− mice transfected with PPARγ (PPARγ-luc) promoter construct and treated with IL-13 for 4 h. The results were represented in fold induction relative to the respective control. (c) Luciferase activity in macrophages from C57BL/6 mice co-transfected with PPARγ (PPARγ-luc) promoter construct (in presence of LRH-1) or absence (empty) of LRH-1 (pCMX-LRH-1) and treated with IL-13 for 4 h. The results were represented in fold induction relative to the untreated control (empty). (d) Luciferase activity in macrophages from Lrh-1M+/+ and Lrh-1M−/− mice transfected with a PPRE (PPRE-luc) construct treated with IL-13 for 24 h. The results were represented in fold induction relative to the respective untreated control. (e) Luciferase activity of macrophages from C57BL/6 mice co-transfected with PPRE (PPRE-luc) construct in presence (LRH-1) or absence (empty) of LRH-1 (pCMX-LRH-1), treated with IL-13 for 24 h. The results were represented in fold induction relative to the respective control. (f) Gene expression analysis of arachidonic acid metabolic enzymes in macrophages from Lrh-1M+/+ and Lrh-1M−/− mice treated with IL-13 for 4 h, determined using RT-PCR. The results were represented in fold induction relative to untreated Lrh-1M−/+. (g) Immunoblot analysis of Cyp1b1 and Actin in macrophages from Lrh-1M+/+ and Lrh-1M−/− mice stimulated with IL-13 for 24 h. (h,i) Gene expression analysis of Alox15, Cyp1a1 and Cyp1b1 in macrophages from Stat6−/− and Stat6−/− mice treated with IL-13 (h) and in macrophages from Lrh-1M−/− and Lrh-1M+/+ mice stimulated with DLPC for 4 h (i), determined using RT-PCR. The results were represented in fold induction relative to untreated wild-type littermate. (j) 15-HETE production by macrophages from Lrh-1M−/− and Lrh-1M+/+ mice stimulated with or without IL-13 quantified by enzyme immunoassay (EIA). The results were represented in fold induction relative to untreated Lrh-1M−/+. (k) [3H]AA mobilization in membrane phospholipids of macrophages from Lrh-1M−/− and Lrh-1M+/+ mice stimulated with IL-13 for 2h. (l) 15-HETE production by macrophages from Alox15−/− and Alox15+/+ mice stimulated with IL-13 for 24 h and silenced or not for Cyp1a1 and Cyp1b1 (siRNA Cyp) measured by EIA. The results were represented in fold induction relative to respective untreated control (siRNA C). Results correspond to the mean ± s.e.m. of triplicates. Data are representative of three independent experiments. *P < 0.05, **P < 0.01 compared with the respective untreated control and ***P < 0.05, ****P < 0.01 compared with the corresponding treated or untreated wild-type littermate. P values were determined using Bonferroni-Dunnnett method.
PPARγ is activated by endogenous ligands derived from the metabolism of AA. The COX1/COX2 cyclooxygenases, 5 and 12/15 lipoxygenases, and CYP enzymes are considered to be critical for the conversion of AA to endogenous PPARγ ligands. To identify how LRH-1 may have an impact on PPARγ activation, we next explored whether LRH-1 can coordinate PPARγ ligand availability through the control of the expression of these enzymes. The mRNA levels of Ptg2 (cyclooxygenase 2), Alox5 (5 lipoxygenase) and Hpgds (prostaglandin-D synthase) after IL-13 stimulation were not differentially expressed in Lrh-1M+/+ and Lrh-1M−/− macrophages (Fig. 3i). However, IL-13 robustly induced Alox15 (12/15 lipoxygenase), Cyp1a1 and Cyp1b1 gene expression in Lrh-1M+/+ macrophages, while this induction was blunted in Lrh-1M−/− macrophages. Moreover, Cyp1b1 protein levels were only induced in Lrh-1M+/+ macrophages on IL-13 exposure, but not in Lrh-1M−/− macrophages (Fig. 3g). Unlike Cyp1a1 and Cyp1b1 mRNA levels, which were unresponsive to the IL-13 treatment in Lrh-1M−/− macrophages, Alox15 expression was still moderately induced (Fig. 3i), indicating that Alox15 is only partially controlled by LRH-1.

Consistent with these findings, a strong decrease in Lrh-1M+/+ macrophages on IL-13 exposure, but not in Lrh-1M−/− macrophages (Fig. 3f). Moreover, the simultaneous gene silencing for Cyp1a1 and Cyp1b1 in both Lrh-1M+/+ and Lrh-1M−/− macrophages abolished this induction (Fig. 3i). Altogether, these data indicate that LRH-1 drives the generation of 15-HETE metabolites through its impact on CYP1 gene expression.

To further disentangle how LRH-1 promotes the production of 15-HETEs in response to IL-13, we assessed 15-HETE production in Alox15-deficient macrophages on Cyp1a1 and Cyp1b1 short interfering RNA (siRNA)-mediated silencing (Fig. 3l and Supplementary Fig. 3b). Interestingly, the increased 15-HETE production by IL-13 was still conserved in Alox15−/− macrophages. Furthermore, the simultaneous gene silencing for Cyp1a1 and Cyp1b1 in both Lrh-1M+/+ and Alox15−/− macrophages abolished this induction (Fig. 3l). Altogether, these data indicate that LRH-1 drives the generation of 15-HETE metabolites through its impact on CYP1 gene expression.

To define whether Cyp1a1 and Cyp1b1 are direct transcriptional targets of LRH-1, transfection assays in Lrh-1M+/+ and Lrh-1M−/− macrophages were performed using a luciferase reporter containing ±1.2 kb of the promoter of the Cyp1a1 and Cyp1b1 genes. IL-13 exposure of Lrh-1M+/+ macrophages...
resulted in an eightfold increase in reporter activity of both Cyp1a1 and Cyp1b1 promoters (Fig. 4a,c). Interestingly, genetic deletion of LRH-1 abolished this response, demonstrating that Cyp1a1 and Cyp1b1 promoters are directly activated by LRH-1.

To identify the critical LRH-1 REs in the Cyp1a1 and Cyp1b1 promoters, we mutagenized the putative RE that were found in silico analysis (Supplementary Fig. 3a), and their response to LRH-1 on IL-13 exposure was compared (Fig. 4a-c). For the Cyp1a1 promoter, mutation of the first LRH-1 RE (site 1) abolished the activity of the reporter construct in response to IL-13, whereas mutation of site 2 was still responsive in Lrh-1M+/+ macrophages (Fig. 4a). Furthermore, whole inhibition of mutated reporter construct activities in Lrh-1M−/− macrophages established that site 1 is the principal site transmitting the effect of LRH-1 on the Cyp1a1 promoter. Thus, this result identified specific recruitment of LRH-1 to site 1, which is most distal to the transcription initiation site in the Cyp1a1 promoter.

For the Cyp1b1 promoter, IL-13 treatment failed to increase the activity of the mutated Cyp1b1 reporter in both Lrh-1M+/+ or Lrh-1M−/− macrophages (Fig. 4c), indicating that LRH-1 binds and activates the Cyp1b1 promoter through a unique sequence between −742 and −728 bp upstream of the transcription initiation site of the gene. Finally, ChIP assays were performed. IL-13 enhanced the recruitment of LRH-1 on both Cyp1a1 and Cyp1b1 sites in Lrh-1M+/+ macrophages, but not in Lrh-1M−/− macrophages (Fig. 4b-d). Altogether, these results demonstrate that LRH-1 directly binds Cyp1a1 and Cyp1b1 promoters and hence controls the transcription of Cyp1a1 and Cyp1b1 genes in response to IL-13.

**IL-13-induced fungicidal properties of macrophages via LRH-1.**

Previous work from our laboratory established the importance of PPARγ in the fungicidal functions of alternatively activated macrophages. On the basis of the current findings suggesting a role for LRH-1 in PPARγ-mediated alternative polarization following IL-13 stimulation, we next investigated whether deletion of LRH-1 in macrophages could have an impact on the outcome of *Candida albicans* infection. The severe systemic infection of mice with *C. albicans* resulted in a significantly lower survival rate of Lrh-1M−/− mice compared with Lrh-1M+/+ mice (P < 0.001; Fig. 6a), supporting a role for LRH-1 in antifungal defence. To further explore the exact function of LRH-1 in the pathophysiology of fungal infection, we evaluated the fungal burden in the intestinal tract and the macrophage microbicidal functions in a murine experimental model of gastrointestinal candidiasis. Lrh-1M−/− mice infected with *C. albicans* had more severe gastrointestinal infection than their wild-type littermates and showed worsened fungal burden in the caecum (Fig. 6b). Remarkably, IL-13, 15-HETE, as well as DLPC, diminished *C. albicans* gastrointestinal colonization in Lrh-1M+/+ mice. However, these effects were lost in Lrh-1M−/− mice treated with IL-13 or DLPC, but not when the PPARγ ligand, 15-HETE, was administered to the animals (Fig. 6b).

To investigate whether LRH-1 in macrophages has any relevant microbicidal phenotype, we evaluated the capacity of Lrh-1M+/+ and Lrh-1M−/− macrophages to kill yeasts in *vitro*. Compared with Lrh-1M+/+ macrophages, Lrh-1M−/− macrophages showed a defect in their ability to kill *C. albicans*, demonstrating the contribution of LRH-1 in macrophage-intrinsic antifungal activity (Fig. 6c). Consistent with our observation, Lrh-1M−/− macrophages were less efficient in engulfing *C. albicans* and producing reactive oxygen species (ROS) after fungal challenge (Fig. 6d,e). Moreover, the defect of Lrh-1M−/− macrophages to exert their antifungal activity was correlated with lower MR and Dectin-1 protein levels after *C. albicans* challenge (Supplementary Fig. 3d). As expected, treatment with IL-13 of Lrh-1M+/+ macrophages increased the killing and the phagocytosis of *C. albicans* and also ROS production in response to *C. albicans*. These inductions were abrogated in Lrh-1M−/− macrophages, underscoring the importance of LRH-1 in these fungicidal functions (Fig. 6c-e).
Interestingly, treatment with 15-HETE increased the fungicidal functions in both \(Lrh-1^{+/+}\) and \(Lrh-1^{-/-}\) macrophages (Fig. 6c–e). Moreover, treatment with IL-13, DLPC and 15-HETE of \(Pparg^{+/-}\) macrophages did not increase the killing of \(C.\) albicans (Fig. 6f), corroborating our findings that PPARγ is downstream from LRH-1 in the signalling pathway triggered by IL-13, leading to macrophage fungicidal activities.

To unequivocally establish that the LRH-1/CYP1/HETE axis is involved in macrophage-intrinsic antifungal activity of IL-13, we evaluated the ability of macrophages silenced for Cyp1a1 and Cyp1b1 (Cyp1) to kill \(C.\) albicans. Interestingly, the increase in \(C.\) albicans killing by IL-13 and DLPC was inhibited by the simultaneous gene silencing for Cyp1a1 and Cyp1b1 (Cyp1), but not after 15-HETE stimulation (Fig. 6g). Taken together, these data provide in vivo evidence that LRH-1 is involved in the PPARγ-dependent antifungal functions elicited by IL-13 through CYP1-induced 15-HETE production.

**Discussion**

The nuclear receptor PPARγ is essential for IL-13-induced alternative differentiation of macrophages\(^6\),\(^8\),\(^9\). We have previously demonstrated that IL-13, via the cPLA\(_2\) signalling pathway, induced AA mobilization associated with the nuclear localization of 15d-PGJ\(_2\), an endogenous PPARγ ligand\(^5\). Once activated, PPARγ induces the transcription of Dectin-1, MR and CD36, three genes characteristic of the alternative activation\(^5\),\(^30\),\(^31\). Therefore, the processes leading to PPARγ activation, such as AA release and its subsequent metabolic conversion, could be important aspects of alternative polarization because they are limiting factors for PPARγ ligand synthesis.

AA can be metabolized by the COX1/COX2 cyclooxygenases to PGH2, which in turn is transformed by the PGD synthase into 15d-PGJ2 (refs 32, 33). AA can also be directly metabolized to 12- and HETEs, other endogenous PPARγ ligands, through 12/15 lipoxigenases\(^34\). A third pathway of AA metabolism leading to endogenous PPARγ ligand production is associated with its conversion by the enzymes of the CYP family\(^35\)–\(^37\). The CYP enzymes generate two biological and active classes of eicosanoids, the epoxy (EETs) and hydroxy (HETEs) derivatives\(^10\),\(^11\). The CYP1 family is mainly involved in the formation of mid-chain HETEs, such as 12- and 15-HETEs, through CYP1A1 and CYP1B1 (refs 12, 13).
Here we report that the nuclear receptor LRH-1 is expressed in macrophages and in response to IL-13 directly binds CYP1A1 and CYP1B1 promoters to positively regulate their transcription. Moreover, 15-HETE production following IL-13 stimulation is dependent on the LRH-1/CYP1/15-HETE pathway. Another component in the signalling cascades that drive PPAR activation by IL-13 was highlighted by the fact that macrophages lacking LRH-1 present an increase in pro-inflammatory cytokines and the simultaneous expression of other M1 markers. The involvement of LRH-1 in anti-inflammatory responses was supported by the robust reduction of LRH-1 gene expression in response to Th1 cytokines and conversely by the upregulation by Th2 cytokines. Interestingly, LRH-1 was also induced in human macrophages in response to the Th2 cytokine IL-13 via a mechanism that is most likely also STAT6-dependent, given the presence of several conserved STAT6 REs in the human LRH-1 promoter (Supplementary Fig. 3a). Our findings may further explain why LRH-1 is not sufficient to be compatible with a role as an endogenous PPARγ ligand.

Despite the growing knowledge with regard to the biological function of LRH-1, little is known about how LRH-1 is controlled at the transcriptional level. We identified STAT6 as a transcriptional regulator of LRH-1. This was evidenced by the induction of LRH-1 promoter activity by binding of STAT6 to its RE in the LRH-1 promoter and by the decrease in LRH-1 mRNA and protein levels in macrophages lacking STAT6. On the basis of the established role of STAT6 in PPARγ activation and macrophage polarization, these findings identify LRH-1 as a critical component in the signalling cascades that drive PPARγ-mediated alternative macrophage activation. This is corroborated by the findings that PPARγ activation on IL-13 stimulation is lost in macrophages silenced simultaneously for Cyp1a1 and Cyp1b1 in macrophages abolishes the generation of 15-HETE, provide evidence that its production through the LRH-1/CYP1s axis is crucial in PPARγ activation. This is corroborated by the findings that PPARγ activation on IL-13 stimulation is lost in macrophages lacking LRH-1. Consistent with these observations, treatment of macrophages with the LRH-1 agonist, DLPC, increased the expression of CD36, MR and Dectin-1 PPARγ target genes in wild-type macrophages but not in macrophages lacking PPARγ. Altogether, these results establish that PPARγ activation by IL-13 is dependent on the LRH-1/CYP1/15-HETE pathway. Another endogenous activator to consider in PPARγ activation is 15d-PGJ2. Although we have previously shown that IL-13 generates 15d-PGJ2 production and its nuclear localization in macrophages, the results in this study suggest that it is not sufficient to activate PPARγ. This is supported by previous reports showing that 15d-PGJ2 concentration required to stimulate PPARγ is in the nM range, in contrast to other prostaglandins that are normally active at low nM concentrations. Thus, the levels generated in vivo are not sufficient to be compatible with a role for this metabolite as an endogenous PPARγ ligand.
in agreement with reports showing that LRH-1 controls the expression of anti-inflammatory IL-1ra and the scavenger receptor class B type 1, two markers specific of alternatively activated macrophages 22,23,42.

In addition to the key role of LRH-1 in the acquisition of alternative activation of macrophages, this study also provides mechanistic insight into the hierarchy between STAT6, LRH-1 and PPARγ to achieve this phenotype. Our findings showing that loss of induction of alternative activation markers in Stat6−/− macrophages can be restored by exogenous 15-HETE support the notion that STAT6 is required for macrophage-alternative activation through PPARγ-dependent mechanism. Moreover, the use of Pparg−/− macrophages provides evidence for the existence of distinct mechanisms in the transcriptional regulation of genes characteristics of alternative activation. Our results demonstrate that the transcriptional regulation of Arginase 1, Fizz 1 and YM1 involves directly STAT6 and that Dectin-1, MR and CD36 are regulated indirectly by STAT6 through the LRH-1/PPARγ axis. These observations are not only consistent with the requirement of STAT6 to induce the majority of PPARγ target genes 43 but also with the identification of PPARγ as a positive regulator of alternative activation 6.

Consistent with the involvement of the LRH-1/PPARγ pathway in inducing MR and Dectin-1 expression during IL-13-mediated alternative activation, loss of LRH-1 and PPARγ in macrophages also severely compromised their capacity to kill, to engulf C. albicans and to produce ROS. This is in line with the fact that LRH-1 is upstream from PPARγ in the signalling pathway leading to the induction of MR and Dectin-1, two C-type lectin receptors strongly involved in the antifungal functions of macrophages against C. albicans 8,27,31. LRH-1 deficiency in myeloid cells also rendered the mice highly susceptible to gastrointestinal and systemic C. albicans infection, highlighting LRH-1 of myeloid lineage as a key effector of host fungicidal functions. Although we have not characterized the role of neutrophils in this infectious context, our in vitro and in vivo results identify LRH-1 as a nuclear receptor indispensable for alternative activation of macrophages and for its associated antifungal functions.

In conclusion, we have shown that loss of LRH-1 in macrophages prevents IL-13-induced alternative activation of macrophages, demonstrating the pivotal role of LRH-1 in the differentiation of macrophages towards an anti-inflammatory and antifungal phenotype. In response to IL-13, LRH-1 expression is increased in macrophages through STAT6 and controls the expression of CYP1A1 and CYP1B1 enzymes, which catalyses the generation of 15-HETE PPARγ ligand. Altogether, these results establish that the alternative polarization of macrophages by IL-13 is dependent on the STAT6/LRH-1/Cyps/15-HETE/PPARγ axis (Fig. 7). Finally, deletion of LRH-1 in myeloid cells renders mice susceptible to gastrointestinal and systemic C. albicans infection, highlighting LRH-1 as a critical factor for antifungal functions. Synthetic agonists of LRH-1 activity may, hence, constitute promising compounds for the treatment of anti-infectious and anti-inflammatory diseases.

**Methods**

**Mice.** Male mice aged 10–12 weeks on C57BL/6 background were used for in vitro and in vivo experiments. Mice were bred and handled by following protocols approved by the Conseil Scientifique du Centre de Formation et de Recherche Étiquetage Expérimental Mérico Chirurgical and the ethics board of the Midi-Pyrénées ethic committee for animal experimentation (Experimenter permission number 31–067, approval no. BE155503). All cages were changed twice weekly, and all manipulations of the animals were carried out in a laminar blow hood under aseptic conditions. The photoperiod was adjusted to 12-h light and 12-h dark. C57BL/6 mice were purchased from Janvier (France) and Stat6−/− mice and Alox15−/− mice were purchased from Jackson Laboratories. Pparg−/− mice deleted for Pparg specifically in macrophages have been described earlier 30,31. Nest2α (encoding LRH-1) macrophage specific knockout mice (referred as Lrh-1−/−/− mice) were obtained by crossing mice carrying floxed Lrh-1 alleles with transgenic mice expressing the Cre recombinase under the control of the mouse phagocyte-selective lysozyme promoter 21,22. For Lrh-1−/−/− and Pparg−/−/− mice, the corresponding floxed littersmates were used as controls throughout all the experiments. Corresponding littersmates were used as controls for Stat6−/− and Alox15−/−/− mice.

For the in vivo experiments, a gastrointestinal infection with the C. albicans strain was established by gavage with 50 × 106 C. albicans per mouse (n = 10 per group). Mice were treated or not intraperitoneally (i.p.) with IL-13 (Clinisciences), DLPC (Sigma) or 15-HETE (Cayman). For IL-13 treatment, injections of 4 μg per mouse were performed 1 day before and 3 days after the infection with C. albicans (two injections). For DLPC (300 μg per 10 g of mouse) and 15-HETE (28 μg per 10 g of mouse), i.p. injections were realized 1 day before the day of the infection with C. albicans and then every 2 days (five injections). Control groups received saline solution only with DMSO. After 6 days of infection, the ceca were removed aseptically for the experiments.

For C. albicans systemic infection, yeasts were administered i.p. (100 × 106 yeasts per mouse). Survival studies were conducted using 32 mice per group and were repeated twice.

**Human macrophages.** Monocytes were obtained from healthy blood donors (Etablissement Français du Sang, EFS Toulouse). Written informed consents were obtained from the donors under EFS contract no. 21/PVNT/TOU/UPS04/2010–0025. Following articles L1243-4 and R1243-61 of the French Public Health Code, the contract was approved by the French Ministry of Science and Technology (agreement no. AC 2009-921). Human peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by a density gradient centrifugation method on Lymphoprep (Abcys). Monocytes were isolated by adherence to plastic for 2 h in SFM ( Gibco) at 37 °C, 5% CO2. The macrophages were obtained after 3 days of culture only in SFM medium.

**Preparation of mouse resident peritoneal macrophages.** After being killed, resident peritoneal cells were harvested by washing the peritoneal cavity with 5 ml of sterile NaCl 0.9%. Collected cells were centrifuged at 1,500 rpm for 10 min and the cell pellet was suspended in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with glutamine (Invitrogen), penicillin, streptomycin (Invitrogen) and 5% heat-inactivated fetal calf serum. Cells were allowed to adhere for 2 h at 37 °C and 5% CO2. Nonadherent cells were then removed by washing with PBS.

**Reverse transcription and real-time PCR.** After washing, adherent macrophages were immediately stimulated with IFNγ (40 U/ml−1, Clinisciences), IL-6 (50 ng ml−1, Clinisciences), LPS (1 ng ml−1, Sigma), IL-4 (30 ng ml−1, Millenyi Biotech), IL-13 (50 ng ml−1, Clinisciences), IL-10 (50 ng ml−1, Clinisciences), 15-HETE (1 μM, Cayman) or DLPC (50 μM, Sigma) for 4 or 24 h. In indicated experiments, adherent macrophages were pre-incubated or not with a Jak-2/STAT6 inhibitor, AG490 (1 nM, Tebu-Bio).
The mRNA preparation was made using the EZ-10 Spin Column Total RNA Miniprep Super Kit (Bio Basic) using the manufacturer’s protocol. Synthesis of cDNA was performed according to the manufacturer’s recommendations (Thermo electron). RT-qPCR was performed on a LightCycler 480 system using LightCycler SYBR Green I Master (Roche Diagnostics). The primers (Eurogentec) were designed with the software Primer 3. Actb (Actin) mRNA was used as the invariant control. Serial dilutions of pooled cDNA were used as external standards in each run for the quantification. Primer sequences are listed in Supplementary Table 1.

In situ hybridization. In situ hybridization was performed with digoxigenin-labelled mRNA probe (Plasmid pBSKK Lrh-1) as previously described44. Briefly, this manual nonradioactive method allows to detect specific complementary mRNA sequences at the cellular level using digoxigenin-labelled probes in a five-step procedure: hybridization of the probe to pretreated tissues at 65°C; post-hybridization stringent washes; blocking steps to prepare for the immunodetection; primary antibody anti-DIG-AP incubation; and colorimetric enzymatic detection. The detection step lasts for 2–3 days.

Western blot analysis. Nuclear protein extracts were prepared, and lysates were subjected to western blotting as described previously45. Briefly, nuclear protein lysates were extracted following standard procedures. Protein extracts were separated using SDS-PAGE. After protein transfer, membranes were incubated overnight at 4°C with either a rabbit anti-Lrh-1 (ref. 21; 1/1,000), a rabbit polyclonal anti-Tbp (Abcam, ab63766, 1/500), a rabbit anti-Cy3pIb1 (Santa Cruz, sc-133490, 1/200) or a Actin (Santa Cruz, sc-1615, 1/1,000) and then for 1 h at room temperature with a peroxidase conjugated secondary antibody. Membranes were washed, and proteins were visualized with the SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific). Images have been cropped for presentation. Full-size images are presented in Supplementary Fig. 4.

Transfection experiments. Macrophages were pre-incubated or not with AG490 (1 mM) and then incubated with 1 µg of DNA per well of the indicated plasmids (pGL3 promoter LHR1-Luciferase, pCMX-LHR1, pPFRE luciferase, pGL3 promoter pCMX-GFP-luciferase or plasmid pGL4.12 promoter Cpyp1b1-luciferase) with JetPei (Polyplus transfection) for 8 h according to the manufacturer’s instructions. Then, the cells were stimulated or not with IL-13 (50 ng ml⁻¹) for 18 h. Supernatant was removed, lucerase substrate was added and luminescence was measured with the Envision lucimeter (Perkin Elmer). For siRNA experiments, mouse Cpyp1a1 and Cpyp1b1 and control siRNA were purchased from OriGene. Macrophages were incubated with 20 µM of control siRNA or Cpyp1a1 and Cpyp1b1 siRNA and with Lipofectamine 2000 (Invitrogen) for 18 h according to the manufacturer’s instructions. Cells were then stimulated with IL-13, DLPC or 15-HETE for 18 h.

ChIP. ChIP analysis was performed as described previously with minor adaptations46,47. Briefly, the liver and colon from Lrh-1 and luminescence was measured with the Envision luminometer (Perkin Elmer). The detection step lasts for 2–3 days. Post-hybridization stringent washes; blocking steps to prepare for the immunodetection; primary antibody anti-DIG-AP incubation; and colorimetric enzymatic detection. The detection step lasts for 2–3 days.

Flow cytometry. The analysis was performed on nonadherent macrophages harvested by washing the peritoneal cavity with 5 ml of sterile NaCl 0.9%. Collected cells were centrifuged at 10,000 g for 10 min and the pellet was suspended in PBS medium supplemented with 1% fetal calf serum (FCS). Surface expression of CD11c and CD11b was detected, respectively, using fluorescein isothiocyanate (FITC)-Conc1-1 monoclonal antibody (mAb; Serotec MCA2289F, 1/100) or PE-CD3 MAb (Santa Cruz, sc-13572, 1/100) and was compared with an irrelevant isotype control. To evaluate the mannose receptor (MR) surface expression, we have used MR-specific ligand conjugated with FITC (Sigma A7790, 1 mg ml⁻¹). All stainings were performed on PBS—1% FCS medium. A population of 10,000 cells was analysed for each data point. All analyses were carried out in a Becton Dickinson FACS Explorer and the CellQuestPro software.

ELISA Cytokine titration and EIA quantification of 15-HETE. Peritoneal macrophages were stimulated with IL-13 for 18 h and challenged with non-ozonized C. albicans at a yeast-to-macrophase ratio of 3:1 for 8 h. The production of TNF-α, IL-1β and TGF-β in the cell supernatants was determined with a commercially available OptiELISA kit (BD Biosciences) according to the manufacturer’s instructions.

For 15-HETE quantification, the macrophages were stimulated with IL-13 for 18 h and 15-HETE were measured using EIA as recommended by the manufacturer’s protocol (15(5)-HETE EIA kit, Cayman).

AA mobilization. Peritoneal macrophages were prelabelled with [³H]AA (1 μCi) and then treated with C. albicans at a yeast-to-macrophage ratio of 3:1 for 18 h. The prelabelled macrophages were then treated with 15-HETE (50 ng ml⁻¹) for 1 h. The cellular lipids were extracted twice with hexane/isopropanol (3:2, v/v) and the [³H]AA content in membrane phospholipids was quantified by measurement of the radioactivity by beta liquid scintillation counting, as described with minor adaptations48.

C. albicans strain. The strain of C. albicans used throughout these experiments was isolated from a blood culture of a Toulouse-Rangueil Hospital patient5. Fluorescent C. albicans was prepared by adding C. albicans to FITC (Sigma) dissolved in sodium carbonate buffer (pH 9.5) at room temperature for 3 h and washed by centrifugation three times in sodium carbonate buffer before storage in aliquots of water at 4°C.

Phagocytosis assay and ROS quantification. For analysis of phagocytosis of C. albicans, cultured macrophages were pretreated or not with IL-13, 15-HETE or DLPC for 18 h and then challenged with six FITC-labelled yeasts per macrophage. Phagocytosis was initiated at 37°C with 5% CO₂ and stopped after 1 h by washing the macrophages with ice-cold PBS. The number of C. albicans engulfed by macrophages was determined with fluorescence quantification using the Envision (Perkin Elmer) fluorimetry-based approach.

The oxygen-dependent respiratory burst of macrophages (ROS production) was measured by chemiluminescence in the presence of 5-amin-2,3-dihydro-1,4-phthalazinedione (luminol) using a commercially available (55°C) controlled chemiluminescent luminometer (Wallac 1420 Victor2). The generation of chemiluminescence was monitored continuously for 1 h after incubation of the cells with luminol (66 μM) and pretreatment with IL-13, 15-HETE or DLPC for 18 h and challenge with C. albicans (yeast-to-macrophage ratio: 3:1). Statistical analysis was performed using the area under the curve expressed in counts × seconds.

Killing assay. The killing assay was performed as previously described49. Cells were allowed to interact for 30 min at 37°C with C. albicans (at a ratio of 0.3 yeast per macrophage) and unbound yeasts were removed by four washes with medium. Macrophages were then incubated at 37°C for 4 h. Control plates were kept at 4°C to provide a measure of live C. albicans in the wells. After incubation, the medium was removed and cells were lysed by incubation for 5 min at 25°C with water at a pH of 5.0. And the number of live yeast was determined by plating on Sabouraud plates and incubation overnight at 37°C.

Quantification of C. albicans in the caecum. Cell lysis and DNA extraction. After mouse infection, cecum was aseptically removed and then crushed using lysing matrix tubes (MP Biomedicals). Tissue sample homogenate (250 μl) was resuspended in 200 μl of lysis buffer for 2 h at 65°C and DNA was then extracted with isopropanol and eluted with an elution buffer (High Pure PCR Template preparation kit, Roche Diagnostics).

Light cycle-based PCR assay. The Light Cycler PCR and detection system (Roche Diagnostics) was used for amplification and online quantification. PCR analysis was performed as described previously37. Serially diluted samples of genomic fungal DNA obtained from C. albicans cultures (40 × 10⁶ cells) were used as external standards in each run. Calibration curves were plotted on the logarithm of the concentration of template DNA to evaluate the number of yeast cells present in each tissue homogenate.
Statistical analysis. For each experiment, the data were subjected to one-way analysis of variance followed by the means multiple comparison method of Bonferroni–Dunnnett. For survival study, statistical significance was determined by a log-rank test. P < 0.05 was considered as the level of statistical significance.

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Author contributions

A.C., K.S., B.P. and L.L. designed the study, analysed the data and wrote the manuscript. L.L. and A.H. performed and analysed the experiments. S.S., C.M., B.C., M.A.E., E.M., J.B. and A.V. generated tools and/or helped with specific experiments.

Additional information

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