Myeloid folliculin balances mTOR activation to maintain innate immunity homeostasis

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Myeloid folliculin balances mTOR activation to maintain innate immunity homeostasis

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Introduction

The mTOR complex 1 (mTORC1) is a central kinase complex that senses ambient nutrient status to determine cell behavior via phosphorylation of various downstream substrates (1). Despite of the important role of mTORC1, its detailed function in innate immunity has yet to be determined.

In most cells, mTORC1 activity is highly sensitive to nutrient status, in particular the presence of amino acids (2–4). A lysosome-associated machinery, comprising v-ATPase, Ragulator complex, and Rag GTPases, is essential for amino acid sensing and subsequent mTORC1 activation (5). When the intracellular amino acid level is sufficient, Rag GTPases directly bind and recruit mTORC1 to the lysosome surface, where Rheb activates mTORC1 (6). Rag GTPases comprise heterodimers of RagA or B, with RagC or D (2). Activity of Rag GTPases is tightly regulated through guanyl nucleotide status of each Rag GTPase component. RagA/B is active while loaded with GTP , while RagC/D is active while loaded with GDP (2). GEFs and GAPs for each Rag GTPase modulate their activity (7, 8). Recently the protein folliculin (FLCN) was identified as a GAP for RagC/D, suggesting that it plays a role in amino acid sensing (9, 10).

FLCN was originally identified as a tumor suppressor gene, mutated in Birt-Hogg-Dube syndrome (11). Loss-of-function mutations of FLCN lead to significant mitochondrial biogenesis and lysosome biogenesis in many cell types (12), although how this transcriptional program was activated remained unclear until recently. Our group recently showed in adipocytes that the transcription factor TFE3 is a dominant downstream mediator of FLCN action (13). TFE3 is a member of the TFE/MiTF bHLH transcription factor family, which includes TFEB, a master regulator of lysosome biogenesis (14). FLCN sequesters TFE3 and possibly other TFE/MiTF members in the cytoplasm upon ambient amino acid availability. Mechanistically FLCN acts as a GAP to RagC/D, promoting its active GDP-bound form (9, 10), which in turn stimulates mTORC1 to phosphorylate a 14-3-3-binding motif of TFE3, thereby leading to its cytoplasmic retention (13, 15, 16). Interestingly, however, while loss of FLCN abrogates TFE3 phosphorylation, loss of FLCN does not affect phosphorylation status of other canonical mTORC1 sub-
strates (e.g., S6K1), indicating that FLCN modulates mTORC1 substrate specificity. In fact, in numerous contexts FLCN deletion induces higher canonical mTORC1 activity, leading to hyperproliferation and tumorigenesis (17–20). The mechanism by which canonical mTORC1 signaling is activated in the absence of FLCN is not known.

In this study, we investigate the role of FLCN in myeloid cells, reasoning that specific activation of mitochondrial and lysosomal biogenesis would affect both proliferation and immune activation of these cells. We show that LPS suppresses FLCN expression, leading to hypersensitive inflammatory responses in cell culture and in vivo, and that these responses are mediated via TFE3. Moreover, we identify a TFE3-mediated feedback loop that explains canonical mTORC1 activation when FLCN is deleted. This study thus identifies a FLCN-TFE3 axis that connects immune responses and canonical and noncanonical mTORC1 activities in myeloid cells.

Results
FLCN deletion in myeloid/macrophage cell lineage leads to constitutive activation of TFE3. To test the role of FLCN in myeloid cells/macrophages, a lentiviral CRISPR-Cas9 system was used to generate genome-edited RAW 264.7 mouse macrophage cells with endogenous FLCN KO (Figure 1A). In the absence of FLCN, the phosphorylation of TFE3 at Ser320 was abolished (Figure 1A), as seen in other cell types (13). Consistent with absence of Ser320 phosphorylation, which is required for cytoplasmic retention of TFE3, TFE3 localized to the nuclei in FLCN-KO cells (Figure 1B), and target genes encoding for lysosome proteins were induced (Figure 1C). To test if similar observations would be made in primary cells, rather than immortalized cells, we cultured bone marrow–derived macrophages (BMDMs) from FLCNlox/lox Lyz2 Cre mice (FLCN myeloid KO mice) versus littermate controls. KO efficiency of FLCN, confirmed by qPCR, was nearly 90%, and gene expression of GPNMB, a canonical TFE3 target gene (21), was markedly increased in FLCN-KO BMDMs (Figure 1D). Immunofluorescence staining of endogenous TFE3 showed strong nuclear localization following FLCN deletion (Figure 1E), and lentiviral ectopic expression of FLCN rescued the cytoplasmic retention of TFE3 (Figure 1F). Lysosomal genes, including Lamp1, Ctsz, Ctsd, Mcoln1, Tpp1, and Clcn7, known to be regulated by TFE3 (22), were induced by 1.5-fold to 3.5-fold in FLCN-deleted BMDMs compared with WT control cells, and lysosomal biogenesis was strongly induced, as shown by immunofluorescence staining of LAMP2 (Figure 1, G and H). Collectively, these data thus show that FLCN promotes cytoplasmic retention of TFE3 in macrophages, inhibiting the ability of TFE3 to activate its target lysosomal genes.

LPS but not CpG suppresses FLCN, which mediates TFE3 nuclear localization. TFE3 and TFEB translocate to nuclei upon LPS stimulation (23), but how LPS mediates TFE3 nuclear localization is not known. We treated BMDMs with LPS, as well as CpG, mimicking bacterial DNA stimulation, for 24 hours. Interestingly, LPS induced a time-dependent suppression of FLCN in BMDMs, resulting in a 90% loss of FLCN at the 24-hour time point, while CpG treatment showed no effects on FLCN expression (Figure 2A). Treatment with a number of additional TLR agonists, including Poly I:C (TLR3), Pam3CSK4 (TLR1/2), and R848 (TLR7/8), demonstrated that LPS (TLR4) has the most profound effect on FLCN expression (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.126939DS1), implying a largely specific effect of TLR4 activation. Interestingly, LPS stimulation did not alter the mRNA expression of FLCN, indicating that the regulation of FLCN occurs at the protein level (Supplemental Figure 1B). Moreover, treatment with the proteasome inhibitor MG132 only partially rescued LPS-induced FLCN protein suppression (Supplemental Figure 1C), indicating some, but a relatively minor, role for the proteasome in regulating FLCN protein stability.

Consistent with the above findings, LPS, but not CpG treatment, resulted in nearly 85% nuclear translocation of TFE3, despite, in both cases, the adoption of a spread out morphology, with membrane projections indicative of activated status (Figure 2B). TFE3 Ser320 phosphorylation was dramatically reduced in LPS-treated cells (Figure 2C, top), the timing of which coincided with TFE3 being found in nuclear fractions (Figure 2C, bottom), while CpG, if anything, had the opposite effects. Treatment with the mTOR inhibitor Torin-1 mimicked the effect of LPS (Figure 2C). Finally, and importantly, ectopic expression of doxycycline-inducible FLCN-HA in BMDMs exposed to LPS completely prevented translocation of TFE3 to the nucleus, even after 24 hours of stimulation (Figure 2D). Taken together, these data demonstrate that LPS promotes nuclear translocation of TFE3 via suppression of FLCN/ mTOR-mediated phosphorylation of TFE3.
TFE3 promotes canonical mTORC1 activity through a transcriptional RagD feedback loop. FLCN is a GAP for RagC/D, promoting its activated GDP-bound state, and RagC/D is required for mTORC1 activity (3, 9). We therefore assessed if the phosphorylation status of canonical mTORC1 substrates is also blunted by FLCN deletion, as is TFE3. Surprisingly, however, p-S6 and p-mTOR levels were increased, rather than decreased, in BMDMs lacking FLCN (Figure 3A, left lanes). Moreover, neither activation of canonical mTOR by LPS nor CpG was affected by FLCN deletion in BMDMs (Figure 3A, middle and right lanes). Importantly, the increase in canonical mTOR was reversed by concomitant deletion of TFE3 (DKO, Figure 3A), indicating the presence of a TFE-mediated feedback loop. To test whether activated TFE3 is sufficient to activate this feedback loop, we ectopically expressed a TFE3 mutant unable to be phosphorylated by mTORC1 (S320A). As expected, TFE3 S320A was constitutively localized in nuclei while WT TFE3 was mostly cytoplasmic (Figure 3B, left). Strikingly, TFE3 S320A was sufficient to strongly induce mTORC1 activity, as evidenced by increased phosphorylation of mTOR (Ser2448) and S6 ribosomal protein (Ser240/244) (Figure 3B, right).
We next interrogated the mechanism by which TFE3 activates the canonical mTORC1 pathway in BMDMs. MiTF/TFE transcription factors have been reported to transcribe RagD to facilitate mTORC1 activity (24). Of all Rag GTPases, RagD was the most strongly induced in BMDMs lacking FLCN, and the induction required the presence of TFE3 (Figure 3C). Moreover, LPS stimulation induced a selective 13-fold increase in RagD mRNA expression, while CpG stimulation showed no effects (Figure 3D). To test the epistatic relationship between FLCN and RagD, we knocked down RagD via siRNA in WT versus FLCN-KO BMDMs. As shown in Figure 3E, siRagD entirely reversed the activation of canonical mTORC1 signaling induced by FLCN loss. Collectively, these data therefore indicate that deletion of FLCN in macrophages selectively prevents mTORC1-mediated suppression of TFE3 and that activation of this TFE3 branch directly leads to a RagD feedback loop that in fact promotes canonical activity of mTOR (Figure 3F).

BMDMs lacking FLCN are hypersensitive to LPS and CpG stimuli. LPS-mediated activation of canonical mTORC1 has been implicated in macrophage inflammatory response (25, 26), and TFE3 and TFEB have recently been shown to drive mRNA expression of inflammatory genes in macrophages (23). We therefore reasoned that cells lacking FLCN, in which both of these pathways are activated, may be partially activated, even in absence of inflammatory stimulation, and/or may be hypersensitive to inflammatory signals. As shown in Figure 4A, BMDMs lacking FLCN exhibited morphological signs of spontaneous activation,
including loss of cellular elongation and distinct cell borders, and increased vacuolization. The morphological characteristics of the FLCN-deleted cells resembled those of LPS-stimulated cells (Figure 4A, right). Consistent with these visual observations, quantitative flow cytometry showed that FLCN-deleted BMDMs displayed increased granularity and size (Figure 4B), as well as increased surface expression of CD80 (Figure 4C), reflecting an enhanced baseline activation status compared with that of control cells. Furthermore, real-time qPCR analysis revealed that FLCN deletion strongly decreased the expression of M2 surface marker arginase I (Arg1) and C-type lectin domain family 10 (Clec10a) under IL-4 stimulation (Figure 4D), indicating that FLCN deficiency promotes spontaneous M1-type activation in BMDMs.

We next tested the effects of inflammatory stimulation on FLCN-KO cells. Morphological changes induced by LPS in BMDMs were markedly accentuated in the absence of FLCN (Figure 4B), leading to a synergistic increase in surface CD80 expression and, most strikingly, surface class II MHC expression (Figure 4C). Cytokine and chemokine production is a hallmark of activated macrophages (27). To assess...
the effect of FLCN deletion on secretion of cytokines and chemokines, WT and FLCN-KO BMDMs were stimulated with CpG or LPS for 24 hours. The production and secretion of numerous cytokines, in response to either CpG or LPS stimulation, was enhanced in FLCN-deleted cells compared with WT cells, including key mediators of inflammatory responses, such as granulocyte colony–stimulating factor (G-CSF), TNF-α, IL-6, soluble intercellular adhesion molecule-1 (sICAM-1), and macrophage inflammatory protein 1-α (chemokine [C-C motif] ligand 3 [CCL3]) (Supplemental Figure 2). qPCR studies demonstrated marked induction of numerous cytokine and chemokine genes in FLCN-KO BMDMs under CpG or LPS stimulation, including Tnf-α, Il-6, Il-12, and Ccl2 (Figure 4, E and F). ELISAs confirmed the marked increase in cytokine secretion by BMDMs lacking FLCN (Figure 4G).

These data suggested that FLCN regulates BMDM activation in part via TFE3-mediated regulation of the expression of cytokine genes. To test this notion, BMDMs lacking both FLCN and TFE3 were stimulated with LPS or CpG. As shown in Figure 4G, deletion of TFE3 nearly completely abolished the procytokine secretion effects of FLCN deletion. Together, these data demonstrate that FLCN regulates both baseline and stimulated primary macrophage activation, as well as cytokine production, and that a large part of this regulation occurs via TFE3-mediated regulation of cytokine gene expression.

Loss of FLCN in monocyte lineage leads to expansion of peripheral monocyte progenitors via TFE3. Activation of monocytes in vivo leads to their proliferation and expansion (27). To test the role of myeloid FLCN in vivo, we generated mice lacking FLCN in the myeloid lineage, using Lyz2-Cre drivers (FLCN myeloid KO). The mice did not show any gross phenotype at the age of 2–3 months (data not shown). Myeloid progenitor numbers and mature cell numbers were quantified by flow cytometry in peripheral tissues, including spleen (Figure 5A) and liver (Figure 5B), and in bone marrow (Figure 5C). Strikingly, there was a remarkable expansion of progenitors (GMPs, CMPs, and common monocyte progenitors [cMoPs]) in peripheral tissues but not in bone marrow. Neutrophil and macrophage numbers were also increased in peripheral tissues. Coinciding with myeloid expansion, the spleen weight increased significantly, and liver weight tended to increase in FLCN myeloid KO mice (Figure 5D). Spleen histology assessed by H&E staining revealed disorganized ultrastructure, and macrophage infiltration within the red pulp, in FLCN myeloid KO mice (Figure 5E). Consistent with expansion of myeloid progenitors, peripheral blood monocytes were elevated in FLCN myeloid KO mice versus controls (0.46 ± 0.02 vs. 0.13 ± 0.02, P < 0.01), while other complete blood cell indices showed no differences. Strikingly, all of these observed phenotypes were reversed by codelletion of TFE3 (Figure 5, A–D), indicating that the activation of TFE3 by loss of FLCN mediates the marked expansion of progenitors. Myeloid loss of FLCN thus leads to progenitor expansion and neutrophil/macrophage infiltration in peripheral tissues via activation of TFE3.

Myeloid FLCN deletion evokes systemic histiocytic inflammation in a TFE3-dependent manner. To determine what tissues would be affected by prolonged activation of monocytes, we allowed FLCN myeloid KO mice to age up to 12 months old. By approximately 8 months of age, FLCN myeloid KO mice displayed mild and patchy alopecia (data not shown). Complete blood cell analysis indicated the development of anemia in these mice, with significantly reduced red blood cell counting, hemoglobin concentration, and low hematocrit (Table 1). In addition, the number of monocytes significantly increased while eosinophils and basophils markedly decreased in the peripheral blood of FLCN myeloid KO mice. By 12 months of age, FLCN myeloid KO mice exhibited marked and diffuse alopecia, extending from the rostral ear margin (including the top of head) to the tail base, with only small patches of hair remaining over the perineum and distal limbs (Figure 6A). Mice were sacrificed at this point, and tissues were dissected, revealing dramatically enlarged spleens and livers in FLCN myeloid KO mice (Figure 6B). Tissue histology, assessed by H&E staining, revealed profound multiorgan infiltration of histiocytes/macrophages...
Figure 5. Loss of FLCN in monocyte lineage leads to expansion of peripheral monocyte progenitors via TFE3. (A–C) Progenitor and mature cell counting by FACS in spleens, livers, and bone marrow isolated from 12-week-old FLCNlox/lox, FLCNlox/lox Lyz2 Cre, and FLCNlox/lox Lyz2 Cre TFE3 KO mice. Values are represented as mean ± SEM from 3–6 animals per group. *P < 0.05, **P < 0.01 vs. FLCNlox/lox, #P < 0.05 vs. FLCNlox/lox Lyz2 Cre, determined by 1-way ANOVA followed by t test (Bonferroni correction). (D) Spleen weight/body weight (percentage) and liver weight/body weight (percentage) were calculated from 6 animals for each genotype at 12 weeks of age. Data are represented as mean ± SEM. **P < 0.01 vs. FLCNlox/lox, *P < 0.05 vs. FLCNlox/lox Lyz2 Cre, determined by 1-way ANOVA followed by t test (Bonferroni correction). (E) H&E staining of spleens from 8-week-old FLCNlox/lox and FLCNlox/lox Lyz2 Cre mice. Scale bar: 200 μm.
in FLCN myeloid KO mice, including spleen, bone marrow, skin, liver, lymph node, and inguinal white adipose tissue (Figure 6C and Supplemental Figure 3). The infiltrating macrophages were large and round, with extensive eosinophilic cytoplasm, hallmarks of activated macrophages. The macrophages in the spleen, liver, and bone marrow frequently contained phagocytosed red blood cells along with yellow-brown pigment (Supplemental Figure 3), likely representing the blood breakdown product hemosiderin. This extensive erythrophagocytosis may explain the observed significant anemia. F4/80 (EMR1) is a heavily glycosylated G protein–coupled receptor and is a well-established marker for mouse macrophages. F4/80 staining indicated profound macrophage infiltration in all analyzed tissues of FLCN myeloid KO mice. In the liver, F4/80 positively stained macrophages formed multifocal aggregates, akin to hepatic granulomas. In the skin, the dermis and subcutaneous fat were expanded by numerous macrophages, displacing hair follicles and likely explaining the alopecia. In the spleen, there was extensive macrophage infiltration within the red pulp and, to a slightly lesser extent, white pulp. Strikingly, once again, all of these gross and histological abnormalities were completely reversed by concomitant genetic deletion of TFE3 (Figure 6), demonstrating that FLCN regulates macrophage/monocyte biology largely via the modulation of TFE3 activity. Collectively, our data demonstrate that activation of myeloid TFE3 by the loss of FLCN induces profound systemic histiocytic inflammation.

**Discussion**

We define here the role of FLCN in myeloid cells as a key modulator of mTORC1 activation, conferring specificity to mTORC1 regulation of lysosomal biogenesis and cytokine production and mediating LPS-induced activation of macrophages.

The role of mTOR in innate immunity and macrophage activation is complex. Numerous studies support the notion that mTOR activity in macrophages suppresses inflammatory responses. For example, treating macrophages with rapamycin increases the secretion of inflammatory cytokines (28). Myeloid disruption of LAMTOR1, an essential component of the amino acid–sensing pathway to mTORC1, results in M1 polarization of macrophages, thus leading to histiocytic inflammation (25, 26). On the other hand, and in seeming contradiction, LPS and other inflammatory activators powerfully activate mTOR signaling, which is required for a full inflammatory response (29, 30). Our work begins to resolve these paradoxes by demonstrating that FLCN confers specificity to the mTOR response. LPS stimulates canonical mTOR signaling to S6K and other substrates while simultaneously suppressing mTOR signaling to TFE3 by virtue of suppressing FLCN (Figure 7). mTORC1 signaling thus has both activating and suppressing effects on macrophage inflammatory responses, and FLCN allows LPS to coordinate activation of the former and suppression of the latter.

FLCN was originally identified as a tumor suppressor gene, mutated in the familial hamartomatous syndrome, Birt-Hogg-Dube syndrome (11, 31). Paradoxically, however, tumors in these patients invariably contain hyperactive mTORC1, rather than suppressed activity as would be expected from the loss of activation of RagC/D, a key mTORC1-activating G protein. Our data not only demonstrate that, in the absence of FLCN, canonical mTORC1 activity is not suppressed, but also explain why mTORC1 activity is in fact increased: the loss of TFE3 phosphorylation leads to its nuclear translocation and dramatic induction of RagD gene expression, thus increasing RagD availability for the mTORC1 complex, leading to stimulation of canonical mTORC1 to S6K and other substrates. This RagD feedback loop likely contributes to neoplastic proliferation in Birt-Hogg-Dube patients, providing a potentially novel target in these patients.

TFE3 and TFEB are increasingly recognized as key regulators of the inflammatory response in macrophages. The pathway is evolutionarily distant, with even the TFE3 and TFEB homolog HLH-30 in *C. elegans* essential for host defense upon bacterial infection (32). In mammals, exposure of macrophages to bacteria, antibody-coated particles, or bacterial antigens, such as LPS promotes nuclear localization of TFE3 and TFEB (23). However, how LPS promotes nuclear localization of TFE3 and TFEB was not clear. Our data demonstrate that the activation of TFE3 is mediated in large part via suppression of FLCN by LPS. TFE3 and TFEB can then engage a large gene network in macrophages, including, most notably, a program of lysosomal biogenesis and a program of cytokines and chemokines (14, 23, 33), both of which are critical for efficient eradication of infection. Certain chronic infections, such as tuberculosis, persist in part by suppressing host myeloid immunity and may benefit from activation of the TFE3/TFEB pathway. Targeting FLCN may thus represent a novel approach to reactivate myeloid function and eradicate infection in such indolent disease.
Importantly, innate inflammatory processes must also be dialed down once infection has resolved or been controlled. Our data demonstrate the consequences of insufficiently downregulated macrophage activation, i.e., marked myeloid expansion and pronounced diffuse histiocytic infiltration. Fortunately, this process appears to be very slow, potentially allowing for short-to-medium-term FLCN-directed therapy. Of note, however, Baba et al. reported that acute loss of FLCN in hematopoietic stem cells leads to bone marrow failure and death in 40 days (20), indicating that any FLCN therapy would have to either be targeted to monocytes or titrated accordingly. Also of note, in the same study, hyper mTORC1 activity was noted in HSCs, for which we provide the likely explanation here: activation of the TFE3-induced RagD feedback loop.

Our epistatic experiments clearly demonstrate that TFE3 is absolutely required for the marked activation of macrophages elicited by suppression of FLCN in cell culture as well as the diffuse histiocytic infiltration observed in vivo with FLCN deletion. Our data thus indicate that TFE3 and TFEB are not redundant in myeloid cells. TFE3 and TFEB have overlapping, though not identical, gene targets, which may explain the necessity of TFE3. Both factors are expressed in myeloid cells, but TFE3 may perhaps be the dominant regulator in these cells. Alternatively, TFE3 and TFEB may form obligate heterodimers, although the existence of such complexes has been questioned (34). Testing if TFEB is also necessary for the effects seen with inhibition of FLCN will be of future interest.

The phenotype of the FLCN myeloid KO mouse recapitulates some of the hallmarks of histiocytic syndromes, including Erdheim-Chester disease (ECD). Much like in patients with ECD, presentation does not occur until a late age. No mutations in FLCN have been noted in ECD, which has been predominantly characterized by somatic activating mutations in the Ras/Erk pathway. However, given the rarity of the disease, a role of FLCN cannot be ruled out by a lack of genetic data at this point in time. Germline mutations would almost certainly be unlikely because systemic mutations in FLCN confer significant evolutionary disadvantages in other tissues. Indeed, FLCN total body KO animals are not viable (35). Nevertheless, epigenetic or other suppression of FLCN may be an important component of these diseases, even in the absence of somatic mutations. The fact that the mTOR inhibitor sirolimus showed efficacy in ECD (36) suggests that activation of this pathway may indeed be relevant, and future studies of ECD genetics might focus on FLCN and TFE3, particularly in the subset of patients without BRAF mutations.

In summary, our work identifies a critical role for FLCN in modulation of the mTORC1 pathway in myeloid cells; demonstrates the existence of specific subbranches of mTORC1 signaling that affect

### Table 1. Complete blood cell analysis of peripheral blood from FLCNlox/lox, FLCNlox/lox Lyz2 Cre, and FLCNlox/lox Lyz2 Cre TFE3-KO mice at 8 months old

<table>
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<th>FLCNlox/lox</th>
<th>FLCNlox/lox Lyz2 Cre</th>
<th>FLCNlox/lox Lyz2 Cre TFE3 KO</th>
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<td>RBC (×10^6/μl)</td>
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<td>Hemoglobin (g/dl)</td>
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<td>MCH (pg)</td>
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<td>MCHC (g/dl)</td>
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<td>RDW (%)</td>
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<td>WBC (×10^3/μl)</td>
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<td>Neutrophils (×10^3/μl)</td>
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<td>Lymphocytes (×10^3/μl)</td>
<td>7.34 ± 0.66</td>
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<td>Monocytes (×10^3/μl)</td>
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<td>Eosinophils (×10^3/μl)</td>
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<td>Basophils (×10^3/μl)</td>
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<td>Platelets (×10^3/μl)</td>
<td>1030.19 ± 45.4</td>
<td>855.94 ± 32.96</td>
<td>981.86 ± 64.15</td>
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Values are presented as mean ± SEM. One-way ANOVA was performed, followed by a t test (Bonferroni correction). n = 8 per group. *P < 0.01 vs. FLCNlox/lox; †P < 0.05, ‡P < 0.01 vs. FLCNlox/lox Lyz2 Cre. RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; WBC, white blood cells.
Figure 6. Myeloid FLCN inactivation leads to systemic histiocytic inflammation in mice, which is largely rescued by TFE3 deletion. (A) Gross images of FLCN<sup>lox/lox</sup>, FLCN<sup>lox/lox</sup> Lyz2 Cre, and FLCN<sup>lox/lox</sup> Lyz2 Cre TFE3 KO mice at 8 and 12 months old. (B) Gross images of spleens and livers from 12-month-old mice of different genotypes. Data are represented as mean ± SEM from 9 animals for each genotype. **P < 0.01 vs. FLCN<sup>lox/lox</sup>, ##P < 0.05 vs. FLCN<sup>lox/lox</sup> Lyz2 Cre, determined by 1-way ANOVA followed by t test (Bonferroni correction). (C) H&E staining of spleens, bone marrow, skin, livers, lymph nodes, and inguinal white adipose tissue (iWAT) from 12-month-old mice of different genotypes. Hemophagocytosis was indicated by black arrow. Black scale bar: 100 μm. White scale bar (lymph node): 50 μm. (D) F4/80 staining (green) and DAPI (blue) in the spleens, livers, and skin from 12-month-old mice of different genotypes. Scale bar: 200 μm.
inflammatory signaling differently; provides a mechanism by which LPS promotes TFE3 nuclear translocation and lysosome biogenesis; and illustrates the effects of chronic low-grade macrophage activation in vivo. The work thus elucidates fundamental aspects of myeloid biology.

Methods

Animals. FLCN-floxed animals (17) were crossed with Lyz2-Cre mice (from Jackson Laboratory) to obtain FLCN myeloid-specific KO animals. TFE3-deficient animals (37) were bred to FLCN myeloid KO mice to obtain FLCN TFE3 double-KO animals. All animal experiments were littermate controlled and were performed on C57B6 and FVB or 129 mixed strains. Mice were housed in 12-hour light and dark cycles and fed with standard rodent diets or special diets if specified.

Antibodies. Phospho-TFE3 (Ser320) antibody was a gift and has been described (23). Other antibodies used were as follows: TFE3 (MilliporeSigma, HPA023881), FLCN (Abcam, ab124885), LAMP2 (Abcam, ab13524), HA tag (Cell Signaling Technology, 2367), β-actin (Cell Signaling Technology, 4970), phospho-p70S6K (Thr389) (Cell Signaling Technology, 9234), total p70S6K (Cell Signaling Technology, 2708), phospho-S6 ribosomal protein (Ser240/244) (Cell Signaling Technology, 2215), total S6 ribosomal protein (Cell Signaling Technology, 2217), phospho-mTOR (Ser2448) (Cell Signaling Technology, 5536), mTOR (Cell Signaling Technology, 2983), and RagD (Abcam, ab187679).

Complete blood cell analysis. The blood samples were collected with lavender top collection tubes containing EDTA, sent to the Pennsylvania Veterinary Laboratory, and analyzed using a hematology analyzer at times up to 24 hours after blood collection.

Isolation and processing of organs. Bone marrow cells were removed from the leg bones of mice by flushing the bone marrow cavity with cold PBS and filtering the cells over a 70-μm Falcon cell strainer. Spleens were

Figure 7. Model of how LPS specifically modulates different arms of mTOR signaling. LPS activates canonical mTORC1/S6K signaling, likely in large part via suppression of the TSC complex, while suppression of FLCN by LPS specifically suppresses noncanonical mTORC1/TFE3 signaling. Subsequent nuclear translocation of TFE3 activates expression of the lysosome biogenesis program, of cytokine transcription, and of RagD. Expression of RagD forms a feedback loop, leading to hyperactivation of canonical (non-FLCN requiring) mTOR activity.
removed and weighed for analysis of splenomegaly. Splenocytes were harvested from whole spleens after digestion in DNase I (Roche) and collagenase (Roche) at 37°C for 30 minutes. Spleen digests were filtered over a 70-μm strainer. Liver leukocytes were harvested by filtering the bulk liver through a 70-μm strainer. After centrifugation, the liver cell pellet was resuspended in 30% Percoll and layered over 70% Percoll prior to being spun at room temperature at 1000 g for 20 minutes. The liver leukocytes were harvested at the Percoll interface. After the tissue and blood cells were harvested, red blood cell lysis was performed using ACK lysis buffer from Lonza prior to cell counting. Cells were counted on a Countess Automated Cell Counter from Thermo Fisher Scientific to calculate the number of cells harvested from each organ. The cells were then placed in culture or subjected to cell surface marker immunofluorescence staining for FACS analysis.

**Cellular immunophenotyping.** Fluorescently labeled cells were analyzed on a BD LSRII or Miltenyi MacsQuant. FACS data were analyzed using FlowJo software, which was used to create all flow cytometry plots. All populations were gated on forward and side scatter to limit inclusion of dead cells, debris, and doubles. Live cells were identified by excluding cells staining positive for Live-Dead reagents obtained from Thermo Fisher Scientific. Inflammatory monocytes were identified as Ly6G^−Ly6C^−Cr2^−^ or Ly6G^+^ Ly6C^+^CD115^+^ cells. Myeloid progenitors were identified as CMPs (Lin^−^cKit^+^CD105^−^CD16/32^−^CD115^+^), GMPs (Lin^−^cKit^+^CD105^−^CD16/32^−^CD115^+^), and MDPs (Lin^−^cKit^+^CD105^−^CD115^−^Ly6C^+^), or cMoPs (Lin^−^cKitCD105^−^CD115^−^Ly6C^+^). The lineage panel included antibodies against B220, CD4, CD5, CD8a, CD11b, CD11c, CD90.2, DX5, Ly6G, NK1.1, and Ter119. The following fluorescently labeled antibodies were used: PE anti-mouse B220 antibody (clone RA3-6B2) (Biolegend, 103207), PE anti-mouse CD4 antibody (clone RM4-5) (BD Biosciences, 553049), PE anti-mouse CD5 antibody (clone 53-7.3) (BD Biosciences, 553023), PE anti-mouse CD8α antibody (clone 53-6.7) (Biolegend, 100708), APC/Cy7 anti-mouse CD11b antibody (clone M170) (BD Biosciences, 557657), PE anti-mouse CD11b antibody (clone M170) (eBiosciences, 12-0112-82), PE anti-mouse CD11c antibody (clone HL3) (BD Biosciences, 557401), APC/Cy7 anti-mouse CD16/32 antibody (clone 93) (Biolegend, 101328), PE anti-mouse CD49b antibody (clone DX5) (Biolegend, 108908), PE anti-mouse CD90.2 antibody (clone 53.2.1) (BD Biosciences, 553006), PerCP/Cy5.5 anti-mouse CD105 antibody (clone M17/18) (Biolegend, 120416), APC anti-mouse CD115 antibody (clone AFS98) (eBiosciences, 17-1152-82), PE/Cy7 anti-mouse CD115 antibody (clone AFS98) (eBiosciences, 25-1152-82), APC anti-mouse Ccr2 antibody (clone 475301) (R&D Systems, FAB5538A-100), PE anti-mouse Ccr2 antibody (clone 475301) (R&D Systems, FAB5538A-100), APC anti-mouse c-Kit/CD117 antibody (clone 2B8) (BD Biosciences, 553356), PE anti-mouse GR-1 antibody (clone RB6-8C5) (Biolegend, 108408), PE anti-mouse IL-12 (p40/ p70) antibody (clone C15.6) (BD Biosciences, 554479), PacBlue anti-mouse Ly6C antibody (clone HK1.4) (Biolegend, 128014), FITC anti-mouse Ly6G antibody (clone 1A8) (BD Biosciences, 561105), PE anti-mouse Ly6G antibody (clone 1A8) (BD Biosciences, 551461), PE anti-mouse NK1.1 antibody (clone PK136) (Biolegend, 108708), PE/Cy7 anti-mouse pSTAT1 antibody (clone A15158B) (Biolegend, 686408), PE anti-mouse Sca-1 antibody (clone D7) (Biolegend, 108108), and PE anti-mouse Ter119 antibody (clone TER-119) (Biolegend, 116208).

**Cell culture.** For preparation of BMDMs, mice were killed by cervical dislocation, and the femur and tibia of the hind legs were dissected and bone marrow cavities were flushed with 5 ml cold, sterile PBS. After lysing red blood cells, the bone marrow cells were washed, resuspended, and differentiated into BMDMs in DMEM with 10% FBS, 10% L929 conditioned medium, 100 μg/mL streptomycin, and 100 U/ml penicillin. Six days after initial BMDMs cell culture, the purity of F4/80^+^ cells was >90%, as determined by flow cytometry. L-929 cells (ATCC, CCL-1) were grown in DMEM with GlutaMAX and sodium pyruvate (Gibco, 10569-044) supplemented with 1% fetal bovine serum. RAW cells and BMDM cells were stimulated with vehicle (PBS), 10 μg/ml CpG 1826 or 100 ng/ml LPS from Escherichia coli 0111:B4 (MilliporeSigma, L4391). The class B CpG1826 oligonucleotide was synthesized by Integrated DNA Technologies. Cell culture supernatants were collected for cytokine detection by BD OptEIA ELISA sets (BD Biosciences) following the manufacturer’s instructions.

**Plasmids, transfection, and virus vector production.** FLCN-HA or TFE3 cDNA was transferred to pLenti CMV Puro DEST (a gift from Eric Campeau, Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts, USA) (Addgene, plasmid 17452). Amino acid sequences surrounding S320 were as follows (the serine residue or substituted alanine residue is under-
lined): TFE3 WT (TPAITVSNCPAELPNIK) and TFE3 S320A (TPAITVSNACPAELPNIK). Lenti transfer plasmid psPAX2 (a gift from Didier Trono, School of Life Sciences, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) (Addgene, plasmid 12260) and pMD2.G (a gift from Didier Trono) (Addgene, plasmid 12259) were cotransfected to HEK293T cells by Fugene HD (Promega). Seventy-two hours later, supernatant was harvested and cell debris was removed by a low-protein-binding 0.45-μm syringe filter. Virus particles containing supernatant were stored at −80°C until use. For lenti or retroviral infection, the spinfection method was used. Cells were plated on 6-well multiwell plates, and 2 ml virus containing supernatant and 2 ml fresh medium were added per well. Polybrene was added to medium with a final concentration of 8 µg/ml. A 6-well plate was centrifuged at 1000 g for 1.5 hour (spinfection), and then the medium was replaced with fresh medium. Drug selection with the appropriate marker was started 24–48 hours after the spinfection.

Gene editing by the CRISPR/Cas9 system. The protocol distributed by the Zhang laboratory at Massachusetts Institute of Technology was used. lentiCRISPR version 2 was a gift from Feng Zhang (Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA) (Addgene, plasmid 52961). Guide RNAs (gRNAs) specific to genes of interests were designed by using a web-based algorithm from the Zhang laboratory (Optimized CRISPR Design; http://crispr.mit.edu). Briefly, gRNA was designed as close to the start codon as possible, and the top 1 or 2 candidates were picked up and validated by Western blot. The gRNA sequence was as follows (the pam sequence is excluded): mouse nontarget control (5′-ATTGTTCGACCGTCTACGGG-3′), mouse flcn (5′-GAGCCCTTGGTGAGTCCATA-3′). Cells were infected with lentivirus encoding for CAS9/gRNA, followed by puromycin selection, and used as populations.

Western blot analysis. Tissue or cell samples were lysed by RIPA buffer containing phosphatase inhibitor (PhosSTOP, Roche) and proteinase inhibitor (Complete miniproteinase inhibitor cocktail, Roche). Samples were sonicated followed by centrifugation to remove insoluble debris or lipid. Protein concentration was quantified by the BCA protein assay kit (Thermo Fisher Scientific), and the same amount of protein (10–20 µg) was loaded to 4%–20% gradient Tris-glycine polyacrylamide gel (Bio-Rad) and electrophoresed (SDS-PAGE). After the SDS-PAGE, protein samples were transferred to PVDF membrane (MilliporeSigma). Membranes were blocked with 5% skim milk for 1 hour and then incubated with the respective primary antibodies (see Antibodies above). Appropriate HRP-conjugated secondary antibodies were selected according to the host species of primary antibodies, and images were taken by using a digital imager (GE Healthcare Life Sciences, ImageQuant LAS 4000).

Gene expression analysis. mRNA expression was quantified by quantitative reverse transcription–PCR. Total RNA was extracted by using the TurboCapture mRNA kit (Qiagen) for cells. qPCR was performed by using the SYBR Green method (iQ SYBR Green super mix), and values were normalized to 36B4, Hprt, and Gapdh expression by the ΔΔCt method. Primer sequences are provided in Supplemental Table 1.

Histology analysis and immunofluorescence staining of cells. Isolated tissues were fixed in 3.7% formaldehyde for 24–48 hours followed by dehydration steps. Tissues were embedded in paraffin and sectioned, and then H&E staining was performed. Stained sections were examined under light microscope, and images were captured. Cells were plated on the glass slips coated with gelatin. Cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 0.3% Triton X-100/PBS (PBST). Blocking and primary antibody incubation were performed in PBST containing 5% BSA overnight followed by secondary antibody incubation. Images of the cells were taken via confocal microscopy.

Statistics. Values are presented as mean ± SEM. Student’s t test (2-tailed unpaired t test) was performed for single comparison. For the comparisons of the groups more than 3, 1-way ANOVAs were performed, followed by a 2-tailed t test (Bonferroni correction). P < 0.05 was considered significant.

Study approval. All mouse experiments were performed according to procedures approved by the University of Pennsylvania Institute for Animal Care and Use Committees.

Author contributions
JL, SW, and LKW designed and conducted the experiments, analyzed/interpreted the data, and wrote the manuscript. CB helped perform critical experiments and provided intellectual input for the experiments. EMB provided intellectual input for the study design, interpreted the data, and wrote the manuscript. ZA designed the study, interpreted the data, and wrote the manuscript.
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