Chronic signaling via the metabolic checkpoint kinase mTORC1 induces macrophage granuloma formation and marks sarcoidosis progression

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The aggregation of hypertrophic macrophages constitutes the basis of all granulomatous diseases, such as tuberculosis or sarcoidosis, and is decisive for disease pathogenesis. However, macrophage-intrinsic pathways driving granuloma initiation and maintenance remain elusive. We found that activation of the metabolic checkpoint kinase mTORC1 in macrophages by deletion of the gene encoding tuberous sclerosis 2 (Tsc2) was sufficient to induce hypertrophy and proliferation, resulting in excessive granuloma formation in vivo. TSC2-deficient macrophages formed mTORC1-dependent granulomatous structures in vitro and showed constitutive proliferation that was mediated by the neo-expression of cyclin-dependent kinase 4 (CDK4). Moreover, mTORC1 promoted metabolic reprogramming via CDK4 toward increased glycolysis while simultaneously inhibiting NF-κB signaling and apoptosis. Inhibition of mTORC1 induced apoptosis and completely resolved granulomas in myeloid TSC2-deficient mice. In human sarcoidosis patients, mTORC1 activation, macrophage proliferation and glycolysis were identified as hallmarks that correlated with clinical disease progression. Collectively, TSC2 maintains macrophage quiescence and prevents mTORC1-dependent granulomatous disease with clinical implications for sarcoidosis.

Granulomas are compact aggregates of mature macrophages with an increased cytoplasmic size whose membranes become interlaced, leading them to be called epithelioid cells1. They are usually formed and maintained in response to the continuous presence of either infectious stimuli, such as bacteria, fungi, protozoa, trematodes and viruses, or in response to non-infectious foreign-body particles. Tuberculosis and chistosomiasis are prime examples of infectious granulomatous diseases, whereas non-infectious granuloma formation is observed in sarcoidosis, Crohn’s disease and primary biliary cirrhosis, as well as in neoplasias2–6. Sarcoidosis is an enigmatic granulomatous disease of unknown etiology that most commonly affects the lung, lymph nodes, skin and liver7. The onset is gradual from an asymptomatic state to a progressive disease that persists in about one-third of patients and can become life threatening8,9. Molecular signals or pathways that control disease progression are largely undefined8,9. When treatment is required, corticosteroids are usually recommended, but they are associated with severe side effects8. As a result of the unknown etiology, there are currently no therapeutic approaches targeting the pathogenetic mechanisms.

Given the fact that granulomas have such a major clinical role in disease pathologies, the morphological properties of granuloma formation have been extensively studied. Generally, the principal and initiating element of a granuloma is the macrophage; for example, alveolar macrophages induce granuloma formation after mycobacterial infection in the lung2,10,11. However, many other cell types are also found later on in mature granulomas, such as neutrophils, dendritic cells, B and T cells, NK cells, and fibroblasts6,11,12. These cells mostly surround the basic macrophage core to form a highly organized granulomatous structure. However, severe combined immunodeficiency (SCID) mice are still able to form granulomas after mycobacteria infection, indicating that adaptive immunity has a non-essential role in granuloma initiation10. Thus, the adaptive immune system contributes more to granuloma organization and protection to limit dissemination of the infection and injury for the host2,13,14. Similarly, although factors such as tumor necrosis factor, interferon-γ, interleukin (IL)-12, IL-10, the chemokine CCL2, heme oxygenase 1 and matrix metalloproteinase 9 regulate granuloma organization and pathogen susceptibility in infectious granulomatous diseases, initial
RESULTS
Myeloid TSC2 deficiency promotes granuloma formation

To define the role of mTORC1 in macrophages, we generated a mouse model in which mTORC1 was constitutively active in myeloid cells by deleting its upstream inhibitor, TSC2 (ref. 17). We crossed Tsc2<sup>fl/fl</sup> mice<sup>25</sup> with transgenic mice expressing Cre under the control of the lysozyme promoter (designated Tsc2<sup>fl/fl</sup>,Lyz2-Cre mice). Conditional myeloid deletion of Tsc2 induced the formation of non-caseating (non-necrotizing) granulomatous aggregates, which were predominantly found in the lung and liver, as well as in the lymph nodes, of 3-month-old Tsc2<sup>fl/fl</sup>,Lyz2-Cre mice that were absent in control mice (Tsc2<sup>fl/fl</sup>) (Fig. 1a and Supplementary Fig. 1a–c). The hypertrophic and epithelioid cell clusters expressed the macrophage marker Mac-<sup>2</sup> and showed strong phosphorylation of S6 (p-S6), a hallmark of mTORC1 activation (Fig. 1b). Although the hypertrophic cells expressed some myeloperoxidase, they could be clearly distinguished from neutrophils (Supplementary Fig. 1d). The phenotype worsened by 6 months and was accompanied by strong inflammatory infiltrates (Fig. 1c). At about 4 months, Tsc2<sup>fl/fl</sup>,Lyz2-Cre mice developed swollen paws and tails as a result of excessive granulomas that were largely composed of hypertrophic Mac-2- and F4/80-positive macrophages (Fig. 1d,e). We also found marked hypertrophy in Tsc2<sup>fl/fl</sup>,Lyz2-Cre peritoneal macrophages (Fig. 1f). Transplantation of Tsc2<sup>fl/fl</sup>,Lyz2-Cre, but not Tsc2<sup>fl/fl</sup>, bone marrow recapitulated granuloma formation.
in the lung and liver, suggesting that hypertrophic macrophage accumulation represents a cell-autonomous phenotype (Fig. 1g).

Alveolar macrophages have a central role in human granulomatous lung lesions. We observed a population of hypertrophic lung cells in Tsc2fl/fl,Lyz2-Cre, but not Tsc2fl/fl, mice (Fig. 1h and Supplementary Fig. 2a). Phenotypic analysis using a previously established protocol revealed that these cells consisted of hypertrophic alveolar macrophages, but not typical interstitial macrophages, infiltrating Lys6C+ monocytes, or other known myeloid or lymphoid immune cells (Supplementary Fig. 2b,c). Functional characterization of the inflamed lung showed enhanced mRNA expression of Lgal3 (encoding Mac-2), Chil3, Ifg1, Il10 and Mrc1, but reduced expression of Nos2 (encoding iNOS), indicating that the macrophages had an enhanced alternative (M2-like) polarization (Fig. 1i). The polarization profile, however, was not strongly maintained in bone-marrow-derived macrophages (BMDMs) in vitro (Supplementary Fig. 2d). Thus, hypertrophic M2-like macrophages progressively accumulated in various tissues of Tsc2fl/fl,Lyz2-Cre mice in a cell-autonomous manner and formed granulomas that disturbed normal tissue homeostasis.

**TSC2 prevents cluster formation and proliferation**

To identify how deletion of TSC2 promotes hypertrophic granuloma formation, we generated BMDMs from Tsc2fl/fl and Tsc2fl/fl,Lyz2-Cre mice. As expected Tsc2fl/fl,Lyz2-Cre BMDMs showed constitutive activation of the mTORC1 pathway, as indicated by phosphorylation of the downstream effector S6 and activation of the translation-inhibition inhibitor 4E-BP1, which can both be blocked by rapamycin (Fig. 2a and Supplementary Fig. 3a). We also found that the activity of mTORC2, as indicated by phosphorylation of Akt serine 473 (p-Akt (S473)), was decreased in these cells (Fig. 2a). In addition, the macrophage growth factor CSF1 and lipopolysaccharide (LPS) induced phosphorylation of TSC2 on Ser939, which was accompanied by mTORC1 activation in wild-type BMDMs (Supplementary Fig. 3b). Expression of the myeloid-specific surface markers F4/80, CD11b, CD115, Gr1 or CD11c was similar between Tsc2fl/fl and Tsc2fl/fl,Lyz2-Cre macrophages, suggesting that the loss of TSC2 does not impair macrophage differentiation (Fig. 2b). However, Tsc2fl/fl,Lyz2-Cre BMDMs formed granulomatous cell clusters after prolonged culture in vitro and had increased granularity and cell size (Fig. 2c,d and Supplementary Fig. 3c,d), reminiscent of the hypertrophic cells observed in vivo. Notably, rapamycin treatment reversed clustering as well as the hypertrophic phenotype of BMDMs and of isolated lung macrophages from Tsc2fl/fl,Lyz2-Cre mice (Fig. 2e and Supplementary Fig. 3e,f). Tsc2fl/fl,Lyz2-Cre dendritic cells or human and mouse fibroblasts, where Tsc2 was knocked down, did not show granulomatous cluster formation, indicating specificity toward the macrophage lineage (Supplementary Fig. 3g,h). We noticed higher numbers of Tsc2fl/fl,Lyz2-Cre BMDM compared with Tsc2fl/fl cells on day 7 of culture, suggestive of enhanced proliferation (Supplementary Fig. 4a). Cell cycle analysis confirmed that CSF1-deprived Tsc2fl/fl,Lyz2-Cre macrophages had enhanced cell cycling, as indicated by higher percentages of cells in the S and G2/M phase. (Fig. 2e and Supplementary Fig. 4b). This proliferative phenotype was also observed in Tsc2fl/fl,Lyz2-Cre peritoneal macrophages in vivo (Fig. 2f). In addition, there were high numbers of Ki67-positive macrophages in the granulomatous lung and skin lesions of Tsc2fl/fl,Lyz2-Cre mice, indicating that macrophage proliferation contributes to the TSC2-dependent granuloma formation (Fig. 2g and Supplementary Fig. 4c). Finally, the macrophage growth factor CSF1 induced much stronger mTORC1-dependent proliferation in Tsc2fl/fl,Lyz2-Cre macrophages than in Tsc2fl/fl cells (Fig. 2h and Supplementary Fig. 4d). These results indicate that deletion of Tsc2 in macrophages is sufficient to spontaneously induce hypertrophy, granulomatous aggregation and hyperproliferation in vitro and in vivo.
TSC2 regulates macrophage quiescence versus inflammation

To decipher the mechanisms that contributed to this phenotype, we performed transcriptome analysis of Tsc2fl/fl and Tsc2fl/fl, Lyz2-Cre macrophages deprived of CSF1. We identified 401 genes that were upregulated in Tsc2fl/fl, Lyz2-Cre macrophages more than twofold, whereas 426 were downregulated in these cells compared with Tsc2fl/fl macrophages (Fig. 3a). Gene set enrichment analysis (GSEA) revealed that E2F transcription factor target genes were the most highly enriched gene set in Tsc2fl/fl, Lyz2-Cre macrophages, suggesting that the TSC2-mTORC1 pathway is a major cell-cycle regulator in macrophages (Fig. 3b,c). We also found that E2F1 expression was slightly higher in Tsc2fl/fl, Lyz2-Cre BMDMs than in controls (Supplementary Fig. 4e). Moreover, a proliferative self-renewal gene signature was strongly enriched in Tsc2fl/fl, Lyz2-Cre cells (Supplementary Fig. 4f).

In contrast, the inflammatory NF-κB response and apoptosis were augmented in Tsc2fl/fl cells (Fig. 3d,e). Analysis of transcription factor binding sites corroborated that genes containing E2F binding sites were highly enriched in Tsc2fl/fl, Lyz2-Cre macrophages, whereas NF-κB binding sites were enriched in Tsc2fl/fl cells (Fig. 3f). Thus, active TSC2 limits E2F activation in macrophages, but promotes NF-κB signaling and apoptosis to balance proliferation, self-renewal and inflammatory properties.

CSF1 induces CDK4 via TSC2-mTORC1 in macrophages

Subsequently, we wanted to investigate the regulation of apoptosis and E2F activation in TSC2-deficient macrophages in more detail. Indeed, we found reduced apoptosis in CSF1-deprived Tsc2fl/fl, Lyz2-Cre macrophages, as shown by a rapamycin-sensitive decrease of cleaved caspase 3 (Fig. 4a). Molecularly, this was associated with enhanced expression of the anti-apoptotic molecules Bcl-2 and survivin and with reduced protein expression of pro-apoptotic PDCD4 (Fig. 4a and Supplementary Fig. 4g). On the other hand, the protein expression of cyclins D1 and A, the phosphorylation of Rb and the degradation of the negative cell-cycle regulator p27Kip1 in CSF1-starved Tsc2fl/fl, Lyz2-Cre BMDMs were indicative of active cell cycling (Supplementary Fig. 5a). This expression pattern was reversed by rapamycin and therefore dependent on mTORC1 (Supplementary Fig. 5a). D type cyclins bind to CDK4, the kinase that drives the G1-to-S transition by activating E2F29. There is evidence that expression of CDK4 is constitutive and not modified by environmental stimuli30. However, we noted enhanced protein expression of CDK4 in Tsc2fl/fl, Lyz2-Cre macrophages that was sensitive to mTORC1 inhibition (Fig. 4b). Even more notable, CSF1 stimulated CDK4 expression in Tsc2fl/fl or CSFBl6/fBMDMs in a dose-dependent manner (Fig. 4c, and Supplementary Fig. 5b-d). However, CSF1 only marginally
Supplementary Fig. 5f

Fig. 4 Inhibition of CDK4 activ-

Tsc2+/+, Lyz2-Cre mice. (a–c) BMDMs were treated with 100 nM rapamycin, solvent control and 10 ng/ml CSF1, as indicated for 18 h. Whole-cell lysates were analyzed by immunoblotting with the indicated antibodies. (d) IHC of CDK4 in lung sections of 3-month-old mice. (e) Left, analysis of CSF1-induced (40 ng/ml) proliferation of BMDMs treated with solvent or the indicated amounts of the CDK4 inhibitor PD-0332991 (n = 4). Right, cell cycle analysis of CSF1-stimulated BMDM treated with solvent or 1 µM PD-0332991 for 18 h (n = 3). (f,g) Tsc2+/+, Lyz2-Cre bone marrow was injected into irradiated wild-type recipient mice. After 10 d, mice were treated daily with PD-0332991 (n = 10) or solvent control (n = 10) daily for 2 months. (f) Lung (left) and liver (right) sections were evaluated by IHC of Mac-2. (g) Area of Mac-2-positive cells compared with total lung area of the treated mice. Two random images per animal were evaluated. (h) BMDMs were treated with 1 µM PD-0332991 or solvent control and stimulated with 10 ng/ml CSF1, as indicated for 18 h. Whole cell lysates were analyzed by immunoblotting with the indicated antibodies. Data are presented as means ± s.e.m. (e) or s.d. (g). *P < 0.01, **P < 0.001 (Student’s t test). Data are representative of three (a–d), one (f,g) or two (h) independent, or cumulative of two (e) experiments. Scale bars represent 40 µm (d) and 100 µm (f).

influenced CDK4 in Tsc2+/+,Lyz2-Cre macrophages, where CDK4 expression was already high (Fig. 4c). CDK4 expression in the hypertrophic macrophages of the Tsc2+/+Lyz2-Cre mice was also enhanced in vivo (Fig. 4d and Supplementary Fig. 5e). Furthermore, mTORC1 inhibition decreased CDK4 expression in human monocyte-derived macrophages (Supplementary Fig. 5f). Inhibition of CDK4 activity with the CDK4/6 inhibitors PD-0332991 or SC-203873 (ref. 31) potently blocked cell cycling in CSF1-stimulated Tsc2+/+ and Tsc2fl/fl,Lyz2-Cre macrophages, but did not induce apoptosis in vitro (Fig. 4e and Supplementary Fig. 5g,h). Accordingly, PD-0332991 treatment of older Tsc2fl/fl,Lyz2-Cre mice that had established granulomas did not result in a marked disappearance of granulomas (data not shown). However, when we initiated granuloma formation by transplanting Tsc2+/+,Lyz2-Cre bone marrow into wild type mice, inhibition of CDK4 with PD-0332991 was able to prevent granuloma induction and formation (Fig. 4f,g). Because inhibition of CDK4 with PD-0332991 blocked proliferation (Fig. 4e), but did not influence CDK4 expression itself (Fig. 4h), we reasoned that CDK4 induction by CSF1 via mTORC1 is causative for cell proliferation. Collectively, these data indicate that the mTORC1 pathway induces the expression of CDK4 following stimulation by CSF1 to drive proliferation and granuloma formation.
TSC2 regulates metabolism via CDK4-dependent glycolysis

Cell proliferation needs to be metabolically supported and mTORC1 has been shown to be a major regulator of cellular metabolism\(^1\). Because we observed the enrichment of genes involved in glycolysis and oxidative phosphorylation in TSC2-deficient macrophages (Fig. 3b), we measured as their indicators the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR), respectively. Basal and LPS-induced ECAR and OCR were both increased in \(Tsc2^{fl/fl}\), Lyz2-Cre BMDMs compared with controls (Fig. 5a and Supplementary Fig. 6a), indicating that glycolysis and mitochondrial respiration were increased under chronic mTORC1 activation in macrophages. Consistent with this, mTORC1-dependent glucose uptake was significantly higher in BMDMs and in peritoneal macrophages from \(Tsc2^{fl/fl}\), Lyz2-Cre mice (Fig. 5b,c). Absolute glucose levels were decreased in \(Tsc2^{fl/fl}\), Lyz2-Cre BMDMs and in the lung of \(Tsc2^{fl/fl}\), Lyz2-Cre animals (Fig. 5d and Supplementary Fig. 6b). Consistent with the higher OCR, constitutive mTORC1 activation increased mitochondrial mass in the cells (Fig. 5e,f), an effect that
was reversed by rapamycin (Fig. 5e). A mitochondrial stress test revealed that Tsc2\(^{fl/fl}\)\_Lyz2-Cre mitochondria had higher mitochondrial spare respiratory capacity (Supplementary Fig. 6c). This high mitochondrial respiration activity was consistent with the reduced citric acid levels found in Tsc2\(^{fl/fl}\)\_Lyz2-Cre BMDMs (Supplementary Fig. 6d). Next, we aimed to determine whether CDK4 contributes to the TSC2-dependent reprogramming of the cellular metabolism. Indeed, the CDK4 inhibitor PD-0332991 significantly inhibited ECAR in Tsc2\(^{fl/fl}\)\_Lyz2-Cre BMDMs (Fig. 5g). Moreover, inhibition of CDK4 reduced CSF1-stimulated ECAR activity in Tsc2\(^{fl/fl}\) BMDMs, but it did not influence glycolysis in CSF1-deprived cells (Fig. 5h), suggesting that CSF1-induced activation of CDK4 via TSC2 contributes to glycolysis in macrophages. We wanted to corroborate these metabolic results in situ by measuring the enzymatic activities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), isocitrate dehydrogenase (IDH) and succinate dehydrogenase (SDH) on frozen tissue sections. We found significantly more p-S6-positive macrophages with high GAPDH activity in the livers of Tsc2\(^{fl/fl}\)\_Lyz2-Cre mice (Supplementary Fig. 6e,f). To identify the cellular functions that are fueled by the enhanced glucose metabolism in Tsc2\(^{fl/fl}\)\_Lyz2-Cre macrophages, we inhibited glycolysis with 2-deoxy-D-glucose (2-DG). 2-DG at doses of only 0.25 mM inhibited proliferation of CSF1-stimulated Tsc2\(^{fl/fl}\)\_Lyz2-Cre and Tsc2\(^{fl/fl}\) BMDMs (Fig. 5i,j). Notably, 2-DG induced cleavage of caspase-3 in CSF1-deprived Tsc2\(^{fl/fl}\) and in Tsc2\(^{fl/fl}\)\_Lyz2-Cre BMDMs, but was unable to do so in CSF1-stimulated macrophages (Fig. 5m). These results indicate that deletion of TSC2 rewrites the metabolism of macrophages toward increased CDK4-dependent glycolysis and mitochondrial respiration that is critical for proliferation, but also inhibits apoptosis under growth-factor-deprived conditions.

**Granulomas in human sarcoidosis show active mTORC1 signaling**

Our observations in Tsc2\(^{fl/fl}\)\_Lyz2-Cre mice most strongly resemble the histological phenotype of patients with sarcoidosis; for example, alveolar-like epithelioid macrophages that accumulate in the interstitium and express high levels of Lgals3. This prompted us to analyze whether mTORC1 activation is found in biopsies of sarcoidosis patients. We noticed activated mTORC1 signaling in granulomatous lesions in 33% of cases.
of 27 biopsies in an initial screening (Fig. 6a), with the hypertrophic cells also expressing Mac-2 (Fig. 6b). To substantiate these findings, we reanalyzed a published clinical study, which compared active but non-degradable particles and genetic disorders initiate the formation of granulomas. Our results suggest a model in which the inability to modify granuloma formation has not been investigated mechanistically. Moreover, our data indicate that activation of mTORC1 and macrophage proliferation determines the switch from a self-limiting to a progressive form in the human granulomatous disease sarcoidosis.

The induction and maintenance of granulomas remains poorly understood despite their importance for modifying disease pathology. For example, excessive granuloma formation can be detrimental in non-infectious granulomatous disorders such as sarcoidosis or Crohn's disease, but can also favor pathogen growth and survival in tuberculosis and schistosomiasis. Although we understand the morphological sequence of events during granuloma formation, molecular knowledge about signaling pathways that transform macrophages into hypertrophic, epithelioid macrophages that induce granulomas is virtually nonexistent. Unexpectedly, we found that activation of mTORC1 by myeloid-specific deletion of Tsc2 was sufficient to initiate and maintain granulomas by enhancing macrophage proliferation and inhibition of apoptosis in vivo. Notably, activation of mTORC1 already promoted the transformation of BMDMs into hyperproliferative and hypertrophic epithelioid cells that formed granulomatous structures in vitro. These results suggest that granuloma formation is an intrinsic feature of macrophages. Currently, a unifying theory is missing from the explanation of why diverse pathogens, cytokines, non-degradable particles and genetic disorders initiate the formation of granulomas. Our results suggest a model in which the inability to clear a stimulus initiates and maintains mTORC1 activity to induce cell hyperthrophy and granuloma formation. Thus, Tsc2fl/fl Lyz2-Cre mice present a potentially useful tool for studying the molecular principles of granuloma formation in non-infectious and infectious diseases in vivo, and allow the dissection of cell-intrinsic pathways necessary for granuloma initiation from pathogenic signals that modify granuloma organization.

Macrophage proliferation has already been described in sarcoidosis and other human granulomatous diseases. Moreover, macrophages also proliferate in experimental granulomatous models however, the contribution of macrophage proliferation itself to granuloma formation has not been investigated mechanistically. Our data indicate that mTORC1-dependent macrophage proliferation contributes mainly to the establishment of the granulomatous phenotype in Tsc2fl/fl Lyz2-Cre mice. There is a renewed interest in defining the mechanisms of macrophage proliferation in vivo during pathologies such as atherosclerosis or obesity and under steady-state conditions to maintain the macrophage pool in tissues. Our gene expression analysis in BMDMs suggests that cell-cycle progression

**DISCUSSION**

We propose that mTORC1 is a critical rheostat that negatively regulates macrophage quiescence, whereas its constitutive activation is sufficient to initiate granuloma formation in various tissues of mice. Moreover, our data indicate that activation of mTORC1 and macrophage proliferation determines the switch from a self-limiting to a progressive form in the human granulomatous disease sarcoidosis.

The induction and maintenance of granulomas depends on constitutive mTORC1 activation. After a treatment period of 3 weeks with the mTORC1 inhibitor everolimus, the granulomas completely resolved in 8-week-old and 20-week-old Tsc2fl/fl Lyz2-Cre mice and we observed a normal lung architecture, whereas granulomas were well visible in placebo-treated Tsc2fl/fl Lyz2-Cre mice (Fig. 7a, b). Moreover, the swelling of the extremities also disappeared following 2 weeks of mTORC1 inhibition in the old mice in vivo (Fig. 7c, d). Mechanistically, the hypertrophic macrophages downregulated S6 phosphorylation and underwent rapid apoptosis by 2 d after everolimus treatment (Fig. 7e). These results led us to conclude that the development and maintenance of granulomas initiated by hypertrophic macrophages in Tsc2fl/fl Lyz2-Cre mice depends on the constitutive activation of mTORC1 and its inhibition restores normal tissue homeostasis in these mice.
and E2F transcription factors are the main targets of TSC2-mTORC1 in macrophages, a finding that was unexpected despite the knowledge of the role of mTORC1 in cell proliferation in other cells \(^{31,42}\). An important finding from our study is that CDK4 expression was induced by the growth factor CSF1 via TSC2-mTORC1 to promote macrophage proliferation. It is currently believed that CDKs remain largely constant throughout the cell cycle \(^{30}\); however, we found that the cell cycle was not only controlled by the amount of cyclins, but also by the expression of a CDK. It can be speculated that the presence of adequate amounts of CDKs such as CDK4 is rate limiting in addition to sufficient amounts of cyclins. Consistent with this suggestion, transgenic expression of CDK4 in the epidermis is sufficient to drive cell-cycle progression as well as hypertrophy \(^{33}\), similar to our findings in Tsc2\(^{fl/fl}\)-Lyz2-Cre mice. Moreover, alveolar macrophages, which possess a high self-renewal proliferative capacity, also express high levels of CDK4. Although we found enhanced proliferation, we also observed the upregulation of survival genes and decreased apoptosis in BMDMs in vitro and in Tsc2\(^{fl/fl}\)-Lyz2-Cre mice in vivo. This contributed to the maintenance of the granulomas, as inhibition of mTORC1 for 2 d readily induced apoptosis in the macrophages in vivo and completely resolved the granulomas. Thus, TSC2 and mTORC1 maintain a balance between proliferation and apoptosis that is important for macrophage tissue homeostasis.

Transitions between quiescent and activated or proliferating states require the reprogramming of the cellular metabolism to support these functional changes \(^{44,45}\). We observed a prominent remodeling of the cellular metabolism toward increased glycolysis and oxidative phosphorylation in Tsc2\(^{fl/fl}\)-Lyz2-Cre macrophages by enhancing GAPDH, IDH and SDH activities that supported macrophage proliferation. Limiting the glycolytic flux radically suppressed cell cycling and proliferation, corroborating the important role of glucose as a carbon source for macrophages \(^{45}\). Our findings describe a previously unknown role for mTORC1 in the regulation of the cellular metabolism \(^{21}\) and indicate that CDK4 contributes to mTORC1-dependent glycolysis to fuel proliferation.

Sarcoidosis onset is gradual, from an asymptomatic state to a progressive disease that can become life threatening \(^{9}\). Notably, a genome-wide association study identified 12q13.3-q14.1 as a risk locus for Mycobacterium tuberculosis. Cell 159, 1497–1509 (2014). Here, we report that CDK4 is highly expressed in Tsc2\(^{fl/fl}\)-Cre mice. We observed a prominent remodeling of the cellular metabolism toward increased glycolysis and oxidative phosphorylation in Tsc2\(^{fl/fl}\)-Lyz2-Cre macrophages by enhancing GAPDH, IDH and SDH activities that supported macrophage proliferation. Limiting the glycolytic flux radically suppressed cell cycling and proliferation, corroborating the important role of glucose as a carbon source for macrophages \(^{45}\). Our findings describe a previously unknown role for mTORC1 in the regulation of the cellular metabolism \(^{21}\) and indicate that CDK4 contributes to mTORC1-dependent glycolysis to fuel proliferation. Sarcoidosis onset is gradual, from an asymptomatic state to a progressive disease that can become life threatening \(^{9}\). Notably, a genome-wide association study identified 12q13.3-q14.1 as a risk locus for Mycobacterium tuberculosis. Cell 159, 1497–1509 (2014).

**METHODS**

Methods, including guidelines for data availability and any associated access codes and references, are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

The study was conceived by M.L. and T.W. The research was carried out by M.L., H.T.T.P., K.K., T.S., A.M., F.D., B.S., M.R., B.K., N.S., P.K. and T.W. Resources were provided by S.B., V.S., M. Mikula, M. Müller, W.W., A.H., M.H., M.J.G. and T.W. T.W. wrote the original draft of the manuscript. All of the authors reviewed and edited the manuscript. The study was supervised by T.W.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Bone marrow transplantation. 8- and 20-week-old Tsc2fl/fl, Lyz2-Cre mice were gavaged with 5 mg/kg body weight everolimus or provided placebo daily for three weeks. Short time treatment lasted for 2 d, whereas half the dose everolimus or placebo was administered in the morning, the other half in the evening. Everolimus and placebo were dissolved in mouse drinking water. Everolimus (as 2% microemulsion) and placebo were provided by Novartis.

Bone marrow transplantation. 4-week-old female C57BL/6;129 mice were lethally irradiated with 9.5 Gy using a XYLON Maxishot (XYLON International GmbH). The next day they received 104 mononuclear bone marrow cells from male Tsc2fl/fl or Tsc2fl/fl, Lyz2-Cre mice into the tail vein. Phenotypic analysis was performed 3 months after bone marrow transplantation.

Treatment with everolimus. Cells were cultured at 37 °C in a humidified CO2 incubator. Bone marrow was isolated from femur, tibia and humerus and differentiated for 6 d on petri dishes (for the whole bone marrow, six plates were used containing each 10–12 ml) in 10–20% L929 supplemented media containing 10% FBS (Gibco), 2 mM l-glutamine (Lonza), 100 U/ml penicillin, 100 g/ml streptomycin (Sigma), 50 µg/ml β-mercaptoethanol (Gibco). 3 d later floating cells were removed and adherent cells were split 1:2 in fresh L929 supplemented media. Differentiated BMDM (96% of the cells were positive for F4/80 and CD11b) were seeded in 10% FBS (Gibco), 2 mM l-glutamine (Lonza), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 50 µg/ml β-mercaptoethanol (Gibco). 3 d later floating cells were removed and adherent cells were split 1:2 in fresh L929 supplemented media. Differentiated BMDM (96% of the cells were positive for F4/80 and CD11b) were seeded in 10% FBS (Gibco), 2 mM l-glutamine (Lonza), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 50 µg/ml β-mercaptoethanol (Gibco) supplemented with 20-µg/ml CSF2. 2 d later half the medium was replaced and the next day floating cells were removed carefully and fresh medium was added. Differentiated DCs (floating cell portion; 92% of the cells were positive for CD11c and CD11b) were re-seeded in CSF2 supplemented media. Human PBMCS were isolated using standard picoll gradients, floating cells were removed and monocytes were cultured for 7 d in RPMI 1640 (Lonza) containing 10% FBS (Gibco), 100 µg/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma). Inhibitors or stimuli were used in following concentrations, except otherwise indicated: Rapamycin 100 nM (Calbiochem), Torin 250 nM (Tocris), Palbociclib (PD-0332991) HCl 1 µM (Selleckchem), 2-Bromo-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione 0.5-1 µM (Santa Cruz), murine CSF1 10 ng/ml (Peptech), human CSF1 20 ng/ml (Peptech), IL-4 10 ng/ml (Peptech), LPS (E. coli 0111:B4) 100 ng/ml (Sigma), IFN-γ 20 ng/ml (Peptech), murine CSF2 20 ng/ml (Peptech).

siRNA in fibroblasts. CCD1079Sk were obtained from ATCC (CRL-2097); IMR-90 were bought from ATCC (CCL-186); both cell lines were STR profiled. All cell lines were tested negative for mycoplasma contamination. Primary human foreskin CCD1079Sk and lung IMR-90 fibroblasts, as well as an immobilized mouse embryonic fibroblast cell line were maintained on tissue culture-treated plates in Dulbecco’s Modified Eagle Medium (DMEM) at 4.5 g/l glucose (Invitrogen) supplemented with 10% FCS (Sigma) and 2 mM L-Glutamine.

siRNA transfection of primary and immortalized fibroblasts was performed using Lipofectamine RNAiMAX (Invitrogen) as described previously50. Pooled siRNAs specifically targeting human TSC2 or mouse Tsc2 (Dharmacon, ONTARGETplus SMART pool reagents) were delivered to the cells at a final concentration of 50 nM. A pool of four non-targeting siRNAs was used as a control for non-sequence-specific effects. Non-treated cells were co-analyzed to monitor non-specific effects due to the transfection procedure. 72 h after the knockdown the MEFs were analyzed under the microscope and harvested for immunoblotting, whereas CCD1079Sk and IMR-90 were reseded for 60 h, serum-starved for 12 h before analysis.

Proliferation assay. For CellTiter-Blue analysis, 1.8 × 105 cells were seeded in 96-well plates. 4 h later they were inhibited as indicated for 60 min and thereafter stimulated with 40 ng/ml CSF1. At the indicated time points CellTiter-Blue (Promega) was added 1:11 and cells were further incubated for 2 h at 37 °C. Fluorescence intensity was measured at a 530-nm excitation wavelength and a 590-nm emission wavelength using a Synergy HT Photometer (Biotek Instruments). A replicate plate was used for each time point.

Flow cytometry. BMDMs were harvested and suspended in FACS buffer (PBS with 2% FBS and 2 mM EDTA). Spleens were forced through a 70-µm cell strainer and red blood cells were lysed with ACK lysis buffer (NH4Cl 0.15 M, KHCO3 10 mM, EDTA 0.1 mM). Perfused lungs were chopped in small pieces and digested at 37 °C for 1 h in RPMI 1640 (Lonza) containing 11.5% HBSS (Gibco) 4.4% FBS (Gibco), 1 mg/ml Collagenase Type I (Gibco) and 50 U/ml DNase I (Invitrogen). Tissue was forced through a 70-µm cell strainer, thereafter red blood cells were lysed and the flow-through of a 40-µm cell strainer was collected in FACS buffer. For surface marker staining cell suspensions were blocked with TruStain FCx (BioLegend) for 20 min and stained light protected for 25 min on ice with the indicated antibodies. Pellets were washed and suspended in fresh FACS buffer prior analysis. Antibodies from BioLegend were F4/80 (BM8) 1:100, CD11b (M1/70) 1:100–200, CD11c (N418) 1:100, Gr-1 (RB-6-8C5) 1:100, CD64 (X54-5/7.1) 1:50, CD4 (RM4-5) 1:100, CD8α (53-5.7) 1:100 and I-Å (AF6-120.1) 1:100, CD11b (M1/70) 1:100–200, CD11c (N418) 1:100, CD45.2 (104) 1:100, Mac-2 (M3/38) 1:100, CD124 (M16/69) 1:50, CD4 (RM4-5) 1:100, CD8α (53-5.7) 1:100 and I-Å (AF6-120.1) 1:100. CD11b (M1/70) 1:100–200, CD11c (N418) 1:100, CD45.2 (104) 1:100, 1:100, 1:100 CDSe (eBio500A2) and CD11f (AF589) 1:100 were obtained from eBioscience and Ly-6C (AL-21) 1:200, CD19 (ID3) 1:100 and Siglec-F (E50-5/7.1) 1:100 were purchased from BD. Data were obtained on a FACS Calibur instrument or FACS Canto II (both BD Biosciences) and processed using FlowJo (Tree Star Inc., Ashland, OR). Gating for myeloid populations in the lung was performed as previously described27. T-cells were defined as CD45.2+CD3e+ and positive for CD4 or CD8 respectively, B-cells were defined as CD45.2+CD19-1+Ab1.

Cell cycle analysis. For cell cycle analysis, 150 µl of cell suspension was added dropwise (while vortexing) to 750 µl 85% ethanol (to obtain a final concentration of 70% ethanol for fixation) and frozen at −20 °C for at least one night. Peritoneal lavage cells, after erythrocyte lysis, were stained with CD11b+ fixation. The pellet was suspended in 300 µl PI-staining solution (50 µg/ml propidium iodide, 250 µg/ml RNase A, 400 nM trisodium citrate dehydrate, 0.1% TritonX-100), incubated for light protected for 20 min on ice and resuspended in PBS. Cells were analyzed on a FACS Calibur (BD Biosciences).

Alternatively, 1 µM EdU (Invitrogen, Click-iT EdU Flow Cytometry Assay) was added to the culture medium and cells were replaced in the incubator. Two hours later the cells were washed with 1% BSA-PBS and harvested. The pellets where resuspended in cold (~20 °C) MeOH and incubated for 10 min at ~20 °C, whereafter MeOH was discarded. Cells were washed and 125 µl Click-iT reaction cocktail was added to each sample. After 30 min (at 20 °C and light protected) the pellets were washed again with 1% BSA-PBS and resuspended in 125 µl RNaseA/T7-AAD (8 µg/ml and 400 µg/ml respectively). After a further light protected 30 min incubation at 20 °C the supernatant was discarded and resuspended in FACS buffer. Cells were analyzed on a FACS Calibur (BD Bioscience).
Shock-frozen lung tissue was homogenized in 3× BMDM (n=Cells were seeded in XF24 plates in 10% Mφ. Cells were seeded overnight in eight-well nature immunology System. Relative expression was normalized to TRI Reagent in a Precellys homogenizer. BMDM were washed and suspended mRNA expression analysis. Determination of metabolites in lung and BMDM.

Mitochondrial staining. For staining of mitochondria, 60 nM of MitoTracker Green FM (Life Technologies) was added to the cell culture medium for 30 min, thereafter cells were harvested and analyzed by flow cytometry. Cells were analyzed on a FACS Calibur (BD Biosciences).

Western Blot. Cells were washed with ice cold PBS, scraped in PBS and the pellet was dissolved in lysis buffer (20 mM Hepes pH 7.9, 0.4 M NaCl, 25% (v/v) Glycerin, 1 mM EDTA, 0.5 mM Na3VO4, 0.5 mM DTT, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche) and 4 μg/ml aprotinin, 4 μg/ml leupeptin, 0.6 μg/ml benzamidinchlorid, 20 μg/ml tryps in inhibitor and 2 mM PMSF (all from Sigma). Protein concentration was measured and equal amounts of denaturated lysate were resolved on 7.5–12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 4% low-fat milk for 1 h at 20 °C and incubated with primary antibodies at 4 °C overnight. HRP-conjugated secondary antibodies (Bethyl) were used: 1:10,000 for p-Akt (Ser473) (Cell Signaling Technology), 1:500 for p-Akt (Thr308) (Cell Signaling Technology), 1:1,000 for p-S6 (S240/244) (Cell Signaling Technology) and 1:1,000 for p-4E-BP1 (Thr37/46) (Cell Signaling Technology). The membranes were washed and incubated with 1:5000 HRP-conjugated anti-rabbit secondary antibodies (BioRad). Blots were visualized using ECL substrate (Thermo Scientific).

Immunofluorescence microscopy. Cells were seeded overnight in eight-well PCA Chamber slides (Sarstedt). Cells were washed, fixed for 10 min with 4% paraformaldehyde at 20 °C and rinsed with PBS. Excess PFA was quenched for 15 min with 100 mM glycine whereafter slides were incubated for 30 min in PBS. Cell were then permeabilized for 7 min with 0.5% Triton X-100 and p-21 (M-20) (1:500, p27Kip1 (C-19) 1:1,000, cyclin A (C-19) 1:500, CDK4 (C-22) 1:1,000, CDK6 (H-96) 1:500, and E2F1 (K95) 1:500 were from Santa Cruz.

Western Blot. Cells were washed in PBS to stop the reaction. Tissue sections were further used for antibody stainings after blocking for 30 min with 3% BSA and 0.01% Triton X-100. Sections were then either stained with directly labeled antibodies or no antibodies to control for background staining. Next, slides were washed and stained with DAPI and mounted in Mowiol 4-88. We used the following directly conjugated antibodies: Mac-2 (M3/38) Alexa Fluor 647 (BioLegend) and p-S6 (54D2) (Cell Signaling Technology) and enzyme reactions were carried out for 15 min as previously reported.14 Slides were washed in PBS to remove the antibody stainings. Tissue sections were incubated in GAPDH, LDH, IDH or SDH activity assay medium and enzyme reactions were carried out for 15 min in 4.5 g/l glucose-supplemented medium as described previously.20 TissueFAXS acquisition was performed using a Zeiss Observer Z1 microscope equipped with a stage for 12-slides, a Hamamatsu Photonics Orca flash 4.0 Camera and a X-Cite Series 120PQ Laser (TissueGnostics). All images were acquired using the TissueFAXS 4.0 Slides software by TissueGnostics and then analyzed by TissueQuest 4.0.1.0128 software (TissueGnostics). The analysis of DAPI was set to the default settings and cell detection parameters were set to a max exterior radius of 7 μm. The raw data, containing all relative intensities for each event (cell), was exported to Excel.

Glucose uptake. For 2-NBDG uptake, BMDM were harvested, suspended in PBS and incubated with 100 μM 2-NBDG (Life Technologies) for 10 min at 37 °C. Peritoneal lavage, after erythrocyte lysis, was stained with F4/80 before 2-NBDG addition. Cells were washed and analyzed on a FACS Calibur (BD Biosciences).

Microarray analysis. BMDM (n=4 per group) were seeded for 18 h in 10% Mφ media. Quigen RNeasy Plus Micro Kit was used to isolate total RNA according to manufactures instructions. Total RNA was subjected to microarray analysis on Affymetrix GeneChip Mouse Gene 2.0 ST arrays. Normalization of the data was done with the Affymetrix Expression Console. Analysis was performed with the Affymetrix Transcriptome Analysis Console (TAC) v3.0. We considered genes to be differentially regulated between Tsc2fl/fl and Tsc2fl/fl, Lyz2-Cre BMDM that were more than twofold differently expressed with an ANOVA significance value of smaller than 0.05. Microarray data have been deposited under GEO accession number GSM77075.

Gene set enrichment analysis (GSEA). For the identification of enriched gene signatures, we used the gene set enrichment analysis (GSEA) tool available from the Broad Institute website. GSEA was performed by comparison of normalized gene-expression data obtained from Tsc2fl/fl and Tsc2fl/fl, Lyz2-Cre BMDM (n=4/group). We used 1,000 gene-set permutations for testing of significance. We screened the collection of signatures under the categories H (hallmark gene sets) and C3.TFT (transcription factor targets). In addition, we used the KEGG–APOPTOSIS and self-renewal set (“WONG_EMBRYONIC_STEM_CELL_CORE”) from the Broad Institute website.

Extracellular flux analysis. Cells were seeded in XF24 plates in 10% Mφ media. The next day, cells were washed and ECAR and OCR were measured in 4.5 g/l glucose-supplemented medium in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Results were normalized to the actual cell count after measurements.

Metabolic imaging of enzymatic activities. This method allows the characterization of particular cells types and the simultaneous determination of their intracellular enzyme activities in structurally intact tissue (A. Haschemi, personal communication). Briefly, tissues were harvested, cut in 10-mm2 pieces, snap-frozen in O.C.T. (Tissue-Tek, Sakura) and stored at −80 °C. Tissue sections were incubated in GAPDH, LDH, IDH or SDH activity assay medium and enzyme reactions were carried out for 15 min as previously reported.14 Slides were washed in PBS to remove the antibody stainings. Tissue sections were then either stained with directly labeled antibodies or no antibodies to control for background staining. Next, slides were washed and stained with DAPI and mounted in Mowiol 4-88. We used the following directly conjugated antibodies: Mac-2 (M3/38) Alexa Fluor 647 (Biologend) and p-S6 (5240/244) Alexa 594 (Cell Signaling Technology). TissueFAXS acquisition was performed using a Zeiss Observer Z1 microscope equipped with a stage for 12-slides, a Hamamatsu Photonics Orca flash 4.0 Camera and a X-Cite Series 120PQ Laser (TissueGnostics). All images were acquired using the TissueFAXS 4.0 Slides software by TissueGnostics and then analyzed by TissueQuest 4.0.1.0128 software (TissueGnostics). The analysis of DAPI was set to the default settings and cell detection parameters were set to a max exterior radius of 7 μm. The raw data, containing all relative intensities for each event (cell), was exported to Excel.

Determination of metabolites in lung and BMDM. 1 ml methanol/chloroform/water (M:C:W = 2.5:1:0.5) was added to frozen BMDM or lung tissue in a 2 ml Preccells lysis kit tube. The samples were homogenized with 1 mm ceramic beads in a Preccells 24 homogenizer for 15 s at 5,000 rpm twice and centrifuged for 5 min at 14,000g and 4 °C before the supernatant was transferred to an Eppendorf tube. The pellet was washed with 100 μl Mφ media by short vortexing and centrifuging it again before combining the wash supernatant with the first supernatant. Samples were dried in in a ScanSpeed MiniVac Beta at 35 °C, 1,100 rpm and 0.0001 mbar and subsequently frozen at −80 °C. To induce a phase separation, samples were desolvated in 500 μl Mφ media and 200 μl milliQ H2O, vortexed for 3 s and centrifuged at 14,000g for 5 min at 4 °C. The polar upper phase was transferred to a new Eppendorf tube and dried in the speedvac at 30 °C, 650 rpm and 0.0001 mbar. The polar fractions were incubated with Methoxaminhydrochloride (30 °C / 90 min) and
subsequently with MSTFA (Methyl-N-(trimethylsilyl)-trifluoroacetamide) (37 °C/30 min) to derivatize polar functional groups of the analytes before they were analyzed via GC(EI)MS. Areas of derivates belonging to one standard substance (confirmed by standard analysis and matching the spectra to the Golm Metabolome Database) were summed up and linear regressions were used to quantify the analytes in the samples.

**Human sarcoidosis samples.** Pathological specimens of lung or mediastinal lymph nodes obtained from 27 patients with proven sarcoidosis were retrieved from the archives of the Department of Pathology, Medical University Vienna. The project was authorized by the Ethical Committee of the Medical University of Vienna (Ref. Nr. 194/2016) and was carried out in compliance with Austrian legislation. Diagnosis was based on radiological, clinical and histopathological criteria. Tissues were fixed in 4% formaldehyde and embedded in paraffin.

For the reanalysis of a previous study, we downloaded the corresponding raw data from the NCBI GEO homepage (accession number GSE19976) and analyzed it with the Affymetrix Expression Console, the Affymetrix TAC v3.0, and the GSEA tool. The unsupervised cluster analysis of the microarray data of Tsc2^fl/fl and Tsc2^fl/fl, Lys2^Cre BMDM was performed with the genes that were more than 1.5-fold differently expressed in progressive relative to self-limiting sarcoidosis patients with an ANOVA significance value of smaller than 0.05.

**Histology.** Mouse tissues were washed, fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned. Feet tissue was additionally decalcified with 15% EDTA pH 7.2. Primary antibodies used for immunohistochemical stainings were CDK4 (C-22) from Santa Cruz, p-S6 S240/244 (rabbit polyclonal) and cleaved caspase 3 (5A1E) were purchased from CST. Ki-67 (H-300) was from Santa Cruz or from Abcam (ab66155), Mac-2 (M3/38) was obtained from Cederlane and MPO was from Dako. Biotinylated horse anti-mouse IgG or biotinylated horse anti-rabbit IgG were from Vector Laboratories. Novocastra streptavidin-HRP (Leica) and AEC-high sensitivity substrate chromogen (Dako) were used for detection of primary antibodies. Alternatively VECTASTAIN Elite ABC Kit and DAB (Vector Laboratories) were used. For immunofluorescent staining of paraffin slides, primary antibodies used were p-S6 S240/244 from CST, Mac-2 (M3/38) either from Cederlane or directly labeled from BioLegend and F4/80 (Cl:A3-1) from Serotec. Species-matched secondary antibodies (A488 and A546) were purchased from Invitrogen. Some images were quantitatively analyzed with ImageJ. Percentage of positive staining was determined by calculating the ratio between stained area and total tissue area within a predefined threshold. All pixels in the image with values below the threshold were counted.

**Statistical analysis.** Data were analyzed with GraphPad Prism (Version 6) or with Microsoft Excel. Data sets were compared with an unpaired two-tailed t-test and differences were considered significant if \( P < 0.05 \). In this explorative study no statistical methods were used to predetermine sample size. Group sample size was chosen using records of variance in previous experiments. Mice or samples were randomly assigned to experimental groups or processing orders. The relationship of mTORC1 activation and proliferation in human sarcoidosis samples was evaluated in a blinded fashion and investigated using fisher’s exact test.

**Data availability.** Further information regarding data and reagents used is available by request to the corresponding author. Gene Expression Omnibus Microarray data have been deposited under accession code GSE77075.

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