



Effect of PTEN inactivating germline mutations on innate immune cell function and thyroid cancer-induced macrophages in patients with PTEN hamartoma tumor syndrome

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Abstract

PTEN hamartoma tumor syndrome (PHTS) is caused by inactivating germline PTEN mutations with subsequent activation of Akt-mTOR signaling, leading to an increased risk of developing thyroid carcinoma (TC). Activation of Akt-mTOR signaling is essential for innate immune cell activation and reprogramming of TC-induced macrophages. Here, we aim to assess the effect of PTEN mutations on innate immune cell function in PHTS patients, especially in the context of TC, using a unique ex vivo model. Monocyte-derived cytokine responses were assessed in 29 PHTS patients and 29 controls. To assess the functional profile of TC-induced-macrophages, a co-culture model with two TC cell lines was performed. Rapamycin, a lactate transport blocker and metformin were used as modulators of the Akt-mTOR pathway and cell metabolism. Monocytes from PHTS patients showed increased production of IL-6, TNF- α , IL-8 and MCP-1, and higher lactate production. After co-culture with TC cell lines, TC-induced macrophages showed significantly increased production of cytokines in both patients and controls, especially after co-culture with a PTEN-deficient TC cell line; these effects were abolished after use of rapamycin or a lactate transport blocker. Metformin blocked the production of anti-inflammatory cytokines. In conclusion, innate immune cells from PHTS patients have increased lactate production and a more proinflammatory phenotype, especially after co-culture with PTEN-deficient TC. Metformin promotes a proinflammatory phenotype by blocking anti-inflammatory cytokine response, whereas rapamycin reduces production of proinflammatory cytokines. This indicates that PHTS patients may benefit from treatment with mTOR blocking agents to limit the inflammatory response in the tumor microenvironment.

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Introduction

PTEN hamartoma tumor syndrome (PHTS), previously called Cowden syndrome, is a hereditary tumor syndrome leading to increased susceptibility for benign and malignant tumors, including non-medullary thyroid cancer (TC). PHTS is caused by inactivating germline mutations in the tumor suppressor gene Phosphatase and tensin homolog (PTEN), located on chromosome 10 [1, 2]. PTEN is a negative regulator of phosphatidylinositide 3-kinases (PI3K)- protein kinase B (Akt)- mammalian target of rapamycin (mTOR) signaling, an important cancer promoting pathway [3], with inactivation of PTEN resulting in overactivation of this pathway. The PI3K-Akt-mTOR pathway is not only important for cancer promotion, but it has also been shown to be a key regulator of immune cell activation, specifically in lymphocytes [4, 5]. Indeed, several reports described PHTS patients developing auto-

immune conditions, peripheral lymphoid hyperplasia, and displaying changes in the number of T- and B-cells [6–8].

Furthermore, there is increasing evidence suggesting that PTEN inactivation and subsequent overactivation of Akt-mTOR has a pivotal role in innate immune cell function. Xie *et al.* showed that specific inhibition of PI3K, leading to reduced mTOR activation, resulted in suppressed proinflammatory cytokine production [9], suggesting that PI3K-Akt-mTOR pathway activation mediates proinflammatory cytokine secretion. The data from our group have shown that activation of the PI3K-Akt-mTOR pathway leads towards a metabolic shift in myeloid-derived cells, resulting in increased aerobic glycolysis. This increased glycolysis provides energy and metabolic substrates needed for innate immune cell activation and increased (proinflammatory) cytokine production [10, 11]. Our group has also shown that both β -glucan, the main cell wall constituent of *C. albicans*, and the Bacillus Calmette-Guérin vaccin (BCG) induce training in innate immune cells *in vitro* and *in vivo*, via activation of the Akt-mTOR-HIF-1 α pathway [10–13].

Although these results would suggest that the altered PTEN function in PHTS patients results in altered innate immune cell function, this has not yet been investigated. This becomes particularly relevant considering that tumor-infiltrating innate immune cells, specifically tumor-associated macrophages (TAMs), play a key role in carcinogenesis [14]. We have previously shown that the Akt-mTOR pathway is highly activated in TC-induced macrophages, resulting in increased aerobic glycolysis, leading to long-term epigenetic and functional reprogramming of TC-induced macrophages towards a more proinflammatory phenotype [15]. Since PHTS patients have inactivating PTEN mutations affecting Akt-mTOR activation, it is possible that the functional phenotype of TC-induced macrophages in PHTS patients might be altered, e.g. more proinflammatory, which in turn could affect tumor development and progression.

Therefore, in the present study we aim to assess the effect of heterozygous, germline PTEN mutations on innate immune cell function in PHTS patients, with a special focus on innate immune cell function in the context of TC. To achieve this, we have first assessed the monocyte-derived cytokine responses after stimulation of peripheral blood mononuclear cells (PBMC) of PHTS patients and controls with Toll-like receptor (TLR) ligands. Furthermore, since the Akt-mTOR pathway plays a major role in trained innate immunity and this pathway is possibly affected in PHTS patients because of inactivation of PTEN, we have assessed whether *ex vivo* training of monocytes was altered in PHTS patients. Since PHTS patients have an increased risk to develop TC, we assessed whether PHTS patients have an altered functional profile of TC-induced-macrophages in a

co-culture model with different TC cell lines (both PTEN-deficient and PTEN-sufficient). We focused on TC because recent literature suggests that TAMs may play an important role in the pathogenesis of these tumors, particularly since poorly differentiated or undifferentiated TCs are highly infiltrated with TAMs and the abundance of these infiltrates correlates with the prognosis [16, 17]. Finally, we performed pharmacological perturbation experiments using modulators of the Akt-mTOR pathway and cell metabolism to investigate the mechanism in this model.

Results

Baseline characteristics of PHTS patients

In total, 29 PHTS patients and 29 controls were included in this study. Mean age of PHTS patients was 38.4 ± 12.4 years compared to 27.2 ± 6.6 years ($p = 0.0007$) in controls. Twenty-eight patients had a confirmed PTEN mutation, one patient had all clinical characteristics of PHTS and a PTEN mutation was proven in first degree relatives. Seven pairs of family members were included in the PHTS patient cohort. Of the PHTS patients, five were previously diagnosed with cancer (three breast cancer, one thyroid cancer) and two were diagnosed with asthmatic bronchitis; no other autoimmune or infectious diseases were reported. Five patients used medication at the time of inclusion that did not interfere with immune function.

PHTS patients have an increased fraction of monocytes compared to controls

Whole blood leukocyte counts were only available for four PHTS patients and four controls. Mean total leukocyte cell counts were comparable between PHTS patients and controls. Interestingly, PHTS patients had a significant higher percentage of monocytes of $9.4 \pm 0.79\%$, compared to $6.6 \pm 0.56\%$ in controls ($p = 0.029$). After PBMC isolation, PHTS patients had significant lower fraction of lymphocytes ($75.8 \pm 7.3\%$) $p = 0.0083$, and higher fractions of monocytes $21.6 \pm 5.8\%$ ($p = 0.019$) and neutrophils $1.8 \pm 2.6\%$ ($p = 0.012$) compared to controls with $80.7 \pm 5.7\%$, $17.8 \pm 5.5\%$ and $0.7 \pm 0.7\%$, respectively.

Monocytes from PHTS patients have increased cytokine production

In order to investigate cytokine production capacity, PBMCs isolated from PHTS patients and controls were stimulated with microbial ligands in standard culture medium containing high glucose concentrations (11 mM) or

medium containing physiological concentrations of glucose (5 mM). During stimulation of PBMCs in standard culture medium, LPS induced significant higher levels of Interleukin 1 receptor antagonist (IL-1Ra) ($p = 0.035$) (Fig. 1b). In contrast, there were no significant differences in proinflammatory cytokine production (e.g., IL-6, tumor necrosis factor α (TNF- α), IL-1 β , IL-8 and Monocyte chemoattractant protein 1 (MCP-1)), and IL-10 production between the PBMCs from PHTS patients and controls upon stimulation with LPS or *C. albicans* (Fig. 1).

Interestingly, however, in physiological-glucose conditions, naive monocytes (e.g. co-cultured with RPMI medium only, see Fig. 1A-3 and Methods section) from PHTS patients showed increased production of IL-6 ($p = 0.0464$), TNF- α ($p = 0.0304$), IL-8 ($p = 0.0006$) and MCP-1 ($p = 0.0304$) upon TLR-4 stimulation compared to naive monocytes from controls (Fig. 2). TLR-2 stimulation resulted in a significant higher IL-1Ra production in naive monocytes from PHTS patients compared to controls ($p = 0.0262$), while the production of the other cytokines and chemokines did not change (Supplementary Figure S1). Importantly, unstimulated naive monocytes from PHTS patients already released more IL-8 compared to controls ($p = 0.0172$) but similar levels of IL-1Ra; Unstimulated release of other cytokines was below detection limit (Supplementary Figure S2).

Similar trained immunity with β -glucan and BCG in monocytes from PHTS patients and controls

In vitro training with β -glucan resulted in significantly higher IL-6 and TNF- α production after LPS restimulation by monocytes from both PHTS patients ($p = 0.0063$ and $p = 0.0277$) and controls ($p = 0.0273$ and $p = 0.0235$), respectively (Fig. 1c), but did not differ between PHTS and controls. Monocytes from PHTS patients showed significantly higher TNF- α production after in vitro training with BCG ($p = 0.0371$), and the same trend was visible for IL-6 production (albeit the difference was not statistically significant), while BCG training in controls did not reach statistical significance.

TC-induced macrophages from PHTS patients and controls display a proinflammatory phenotype

Co-culture of monocytes from controls and PHTS-patients with the TPC-1 cell line, followed by subsequent stimulation with LPS, resulted in significantly higher IL-6 ($p = 0.0005$ and $p = 0.0034$), IL-8 ($p = 0.0005$ and $p = 0.0005$), MCP-1 ($p = 0.0020$ and $p = 0.0005$), IL-10 ($p = 0.0020$ and $p = 0.0049$) and IL-1Ra ($p = 0.0005$ and $p = 0.0342$) production in TC-induced macrophages compared to naive

macrophages (Fig. 2). TNF- α production after co-culture with the TPC-1 cell line was only significantly higher in cells from controls ($p = 0.0024$). No significant differences were observed between PHTS patients and controls in cytokine production after co-culture with TPC-1.

Co-culture of monocytes with the PTEN-deficient FTC-133 cell line and subsequent stimulation with LPS resulted in significantly higher production of all cytokines in TC-induced macrophages compared to naive macrophages from both PHTS patients and controls, except for IL-10, which was significantly higher only in controls ($p = 0.0322$). Again, no differences between PHTS patients and controls were observed. The magnitude of increase in LPS-stimulated production of proinflammatory cytokine TNF- α was significantly higher when the monocytes of PHTS patients and controls were co-cultured with the PTEN-deficient TC cell line (FTC-133-induced macrophages), compared to cells that were co-cultured with the TPC-1 cell line with normal PTEN activity (TPC-1-induced macrophages) (Fig. 2). In contrast, the increase in the production of both anti-inflammatory cytokines IL-10 and IL-1Ra was very low and comparable between TPC-1-induced macrophages and FTC-133-induced macrophages (Fig. 2).

Restimulation with TLR-2 ligand Pam3Cys of TC-induced macrophages resulted in increased cytokine production after co-culture with TC-cell lines as well, especially after co-culture with FTC-133 (Supplementary Figure S1).

Unstimulated TPC-1-induced macrophages produced significantly more IL-1Ra compared to the naive monocytes in both controls and PHTS patients (Supplementary Figure S2). Compared with naive monocytes, the unstimulated FTC-133-induced macrophages produced significantly more IL-8 in both controls and PHTS patients and significantly more IL1Ra in PHTS patients. (Supplementary Figure S2). Again, no significant differences between PHTS patients and controls were found.

Blocking of lactate transport inhibits IL-6, MCP-1 and IL-10 production in TC-induced macrophages

Lactate is hypothesized to be an important mediator in the functional reprogramming of TC-induced macrophages and it is an important modulator of immune responses [15, 18]. PBMC's lactate production was significantly higher in PHTS patients compared to controls, especially in the unstimulated (RPMI control) condition ($p = 0.0210$) (Fig. 1b). These results suggest that in PBMCs from PHTS patients the Akt-mTOR pathway is upregulated, resulting in increased aerobic glycolysis and lactate production. Therefore, we wanted to investigate the effect of blocking lactate transport on monocyte cell function in our co-culture model.

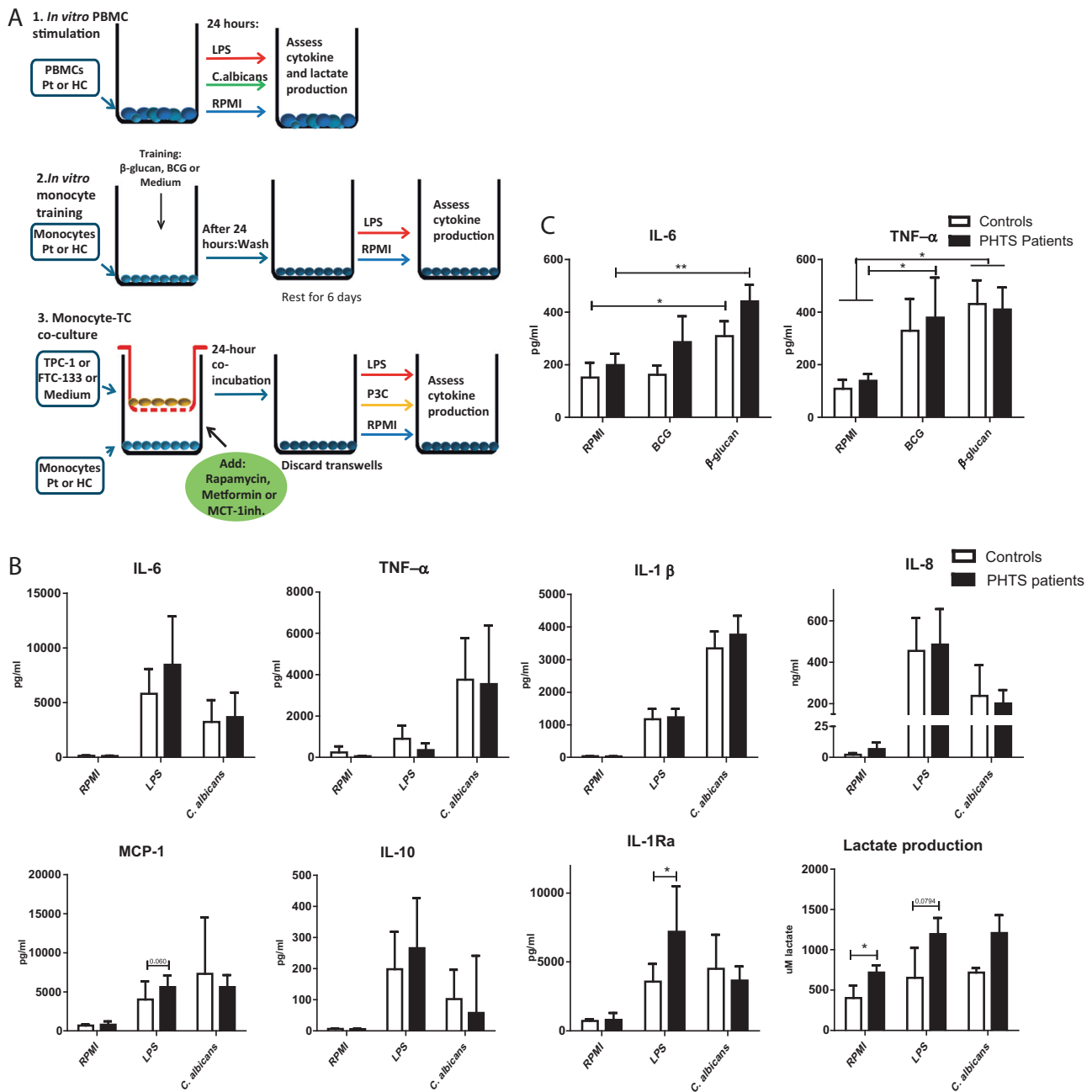


Fig. 1 Experimental setup, PBMCs cytokine production and ex vivo monocyte training. **a** Experimental set up. We performed three types of experiments. 1. PBMCs from PHTS patients ($n = 12$) and controls ($n = 12$) were stimulated for 24 h in normal culture medium (11 mM glucose) to assess monocyte-derived cytokine production and PBMCs lactate production. 2. Ex vivo monocyte training experiments with monocytes from PHTS patients and controls, using BCG and β-glucan as training agents and restimulation with LPS as secondary stimulus to assess cytokine production. 3. *in vitro* Monocyte-TC co-culture in low-glucose-containing medium (5 mM), using monocytes from PHTS patients and controls and co-cultured them for 24 h with TC-cell lines TPC-1 (RET/PTC rearrangement) and FTC-133 (PTEN-deficient) to

create TC-induced macrophages or with medium to maintain naive monocytes; Then we restimulated the naive monocytes and TC-induced macrophages with LPS or P3C and assessed cytokine production. In these experiments we also used Rapamycin, or lactate transport inhibitor (MCT-1 inhibitor) to assess the mechanism. **b** PBMCs cytokine production after 24 h of stimulation with indicated ligands (12 PHTS patients and 12 controls) in normal culture medium (11 mM glucose); **c** Cytokine production after training with indicated compounds and restimulation with LPS (10 PHTS patients and 11 controls). Data shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.001$. PBMCs peripheral blood mononuclear cells, BCG Bacillus calmette-guérin, TC thyroid cancer, P3C Pam3Cys;

Treatment with MCT-1 lactate transporter blocker resulted in decreased levels of IL-6, MCP-1 and IL-10,

without significant effect on TNF-α, IL-8 or IL-1Ra production after co-culture with both TC cell lines and

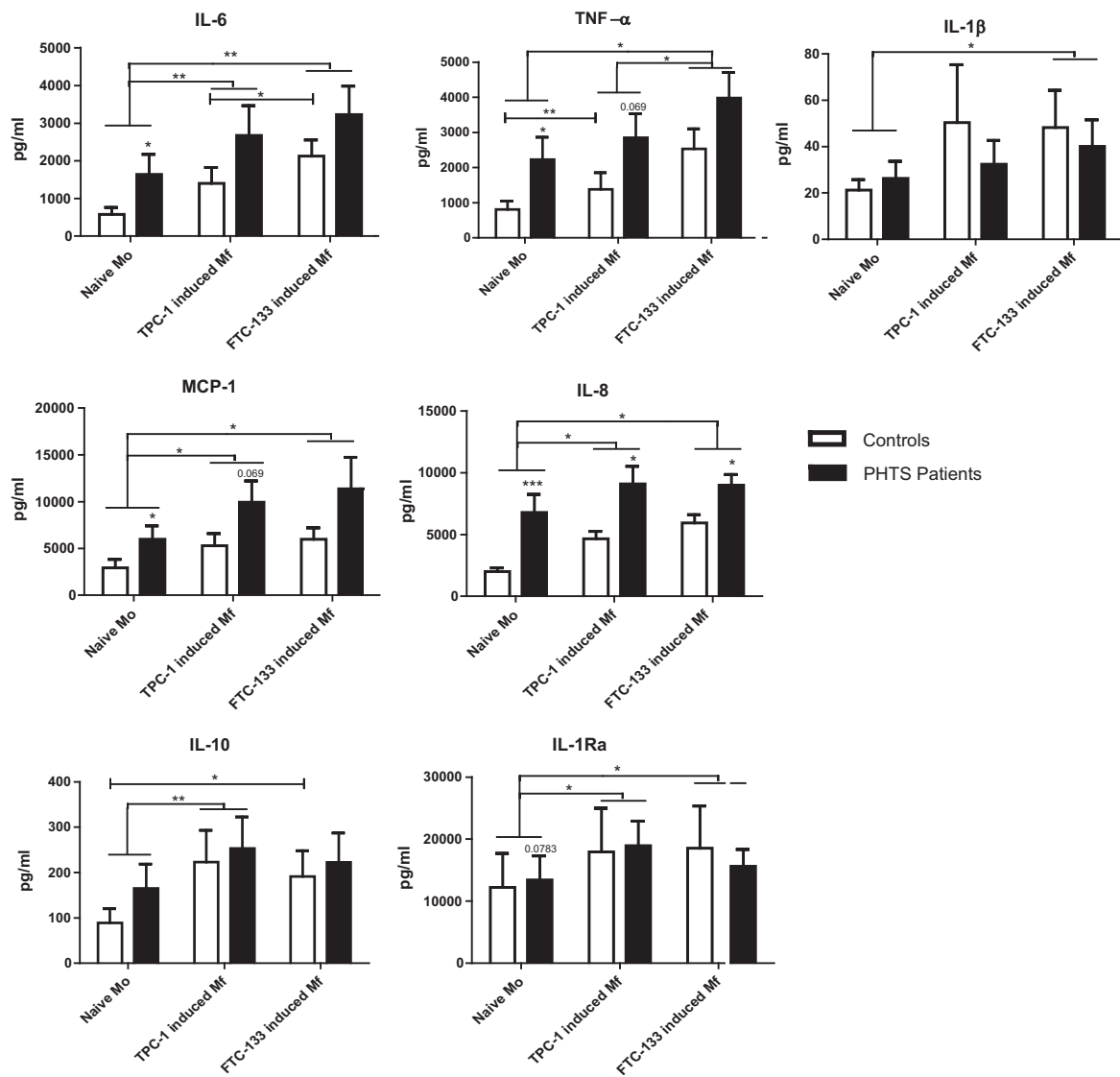


Fig. 2 Cytokine production in naive monocytes and TC-induced macrophages after restimulation with LPS (TLR-4 ligand). Experiments were performed in medium containing 5 mM glucose

(physiological glucose concentration) in a total of 12 PHTS patients and 12 controls. Data shown as Mean \pm SEM. * $p < 0.05$; ** $p < 0.001$

restimulation with a TLR-4 ligand (Figs 3a and 4a) in controls and PHTS patients. The effect of the MCT-1 blocker on cytokine production was similar in magnitude for the controls and the PHTS patients.

In unstimulated TC-induced macrophages from PHTS patients and controls, treatment with the MCT-1 blocker resulted in significant higher levels of IL-8 (Supplementary Figure S3).

Inhibition of the Akt-mTOR pathway inhibits cytokine production in TC-induced macrophages

Blocking of mTOR using rapamycin resulted in a significant decrease in production of proinflammatory cytokines IL-6, TNF- α , IL-8 and MCP-1 (Fig. 3b), as well as a

significant decrease in the anti-inflammatory IL-10 (Fig. 4b). These effects were apparent both with TC-induced macrophages from controls and PHTS patients in all conditions. The effects of rapamycin did not differ in PHTS patients and controls.

Metformin reduced MCP-1, IL-10 and IL-1Ra production in TC-induced macrophages

Metformin is an activator of 5' adenosine monophosphate-activated protein kinase (AMPK), resulting in mTOR inhibition [19, 20], and also directly inhibits oxidative phosphorylation by inhibiting mitochondrial respiratory complex I and ATP synthase [21]. Through these effects, metformin can impact immune cell metabolism and could represent an

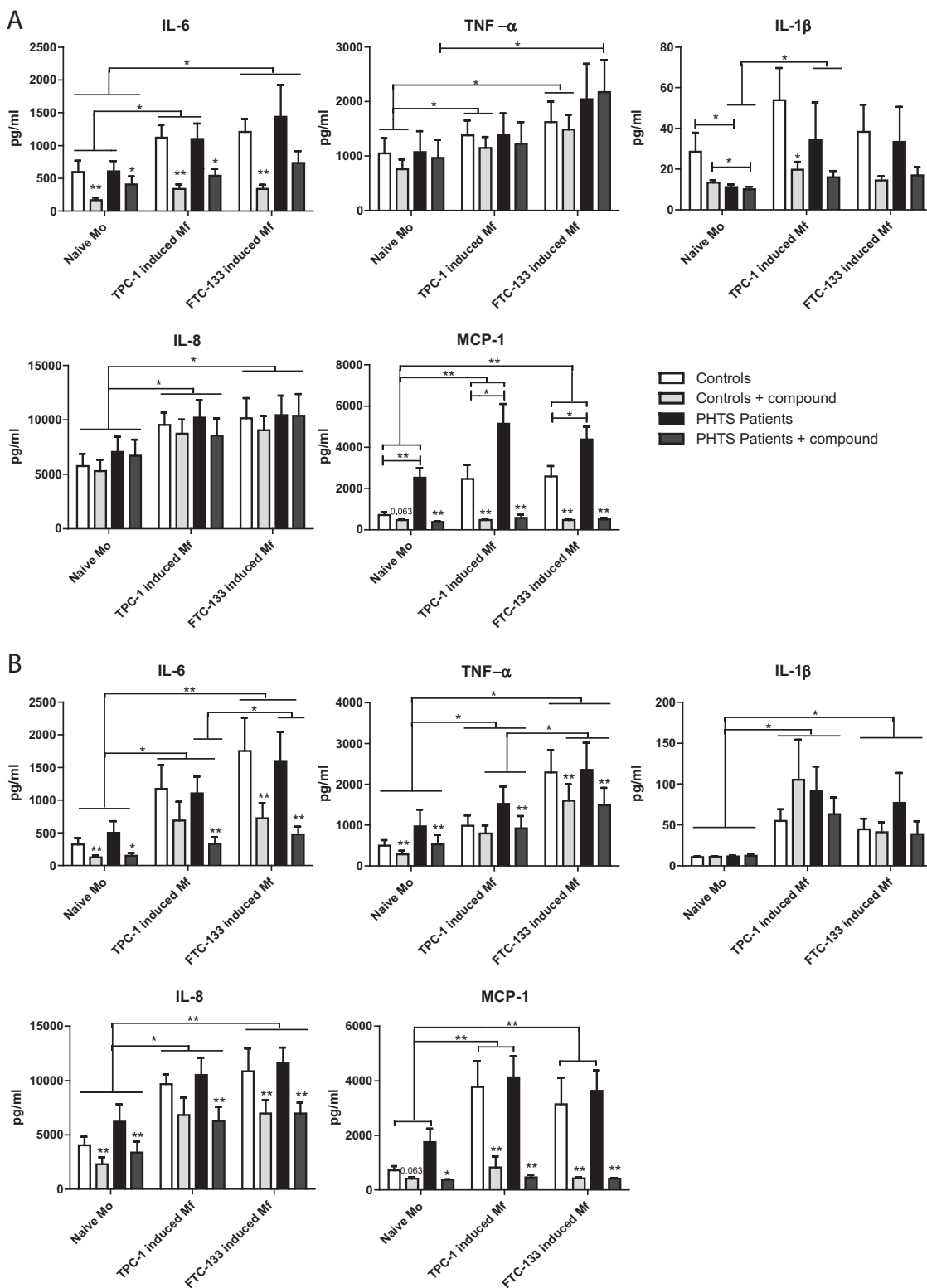


Fig. 3 Pro-inflammatory cytokine production in naive monocytes and TC-induced macrophages after restimulation with LPS in presence of lactate transport inhibitor (a) or rapamycin (b) Experiments where

performed in medium containing 5 mM glucose (physiological glucose concentration) in a total of 9 PHTS patients and 9 controls. Data shown as Mean \pm SEM. * $p < 0.05$; ** $p < 0.001$

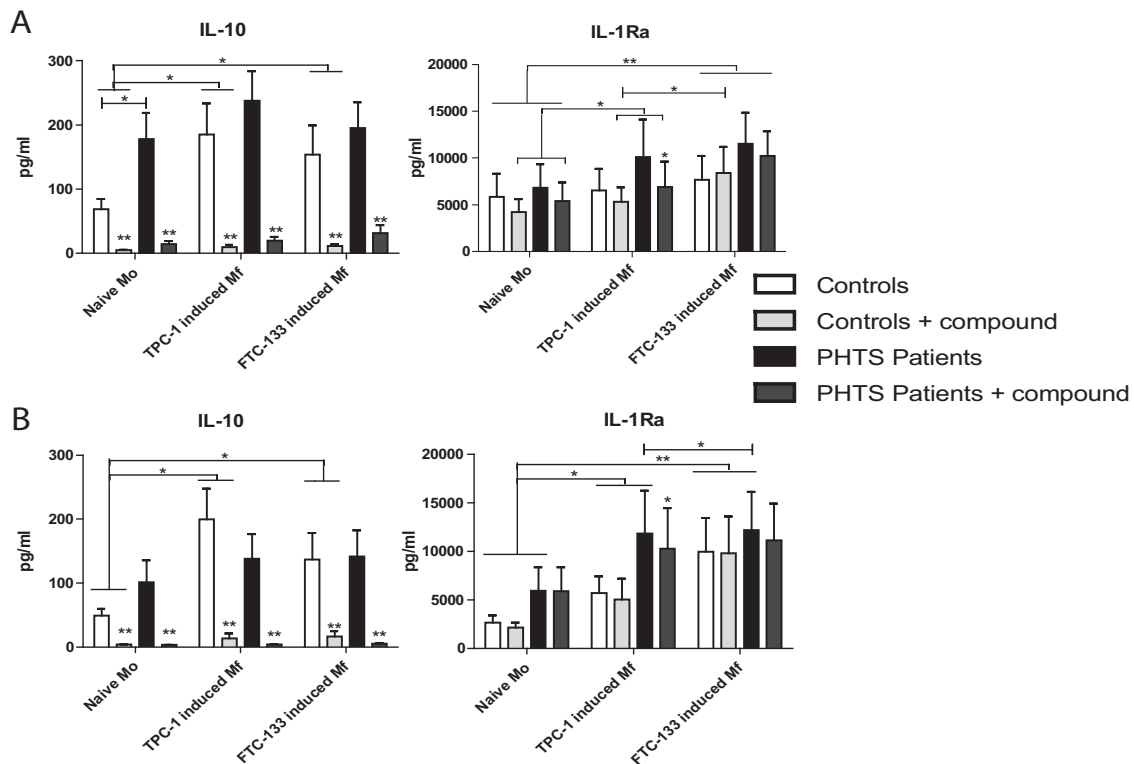


Fig. 4 Anti-inflammatory cytokine production in naive monocytes and TC-induced macrophages after restimulation with LPS in presence of lactate transport inhibitor (a) or Rapamycin (b). Experiments were

performed in medium containing 5 mM glucose (physiological glucose concentration) in a total of 9 PHTS patients and 9 controls. Data shown as Mean \pm SEM. * $p < 0.05$; ** $p < 0.001$

alternative with less toxicity than the treatment with mTOR inhibitors. In addition, metformin is currently being investigated as adjuvant in the treatment of TC. The use of metformin has been associated with inhibition of cell growth in vitro and smaller tumors in animal models, and favorable outcomes in retrospective patient studies [22–24]. Therefore, metformin was added to the co-culture model to assess affects on cytokine production in TC-induced macrophages.

Metformin treatment resulted in similar IL-6, TNF- α , IL-1 β and IL-8 production by naive monocytes and TC-induced macrophages (Fig. 5). Only TPC-1-induced macrophages from controls showed a significant increase in IL-6 production ($p = 0.0273$). In contrast, metformin treatment resulted in significant decreased levels of MCP-1, IL-10 and IL-1Ra in naive monocytes and TC-induced macrophages from both PHTS patients and controls.

Discussion

The PI3K-Akt-mTOR pathway is a major regulator of innate immune responses and patients with germline inactivating mutations in *PTEN* represent a unique model to study the effects of activation of this pathway on the innate

immune system in humans. To our knowledge, this is the first study comprehensively investigating innate immune responses in PHTS patients and the first study focusing on the consequences of PTEN inactivation in the interaction between myeloid cells and tumor cells in humans.

PBMCs from PHTS patients produced significant more lactate compared to controls, indicating that aerobic glycolysis, the main source of lactate production, is increased in the immune cells bearing germline inactivation of PTEN. In physiological, low glucose conditions, naive monocytes from PHTS patients showed increased production of proinflammatory cytokines compared to controls, again suggesting that the Akt-mTOR pathway is upregulated and is not limited by glucose availability. We also showed that proinflammatory cytokine production is highly upregulated in TC-induced macrophages, especially after co-culture with PTEN-deficient TC. Previous results showed that TC-derived factors such as lactate activate the Akt-mTOR pathway in TC-induced macrophages, resulting in a more proinflammatory phenotype [15]. Using a lactate transport inhibitor and rapamycin, we were able to confirm the role of lactate and the Akt-mTOR pathway in promoting a proinflammatory phenotype of TC-induced macrophages. Metformin mainly reduced anti-inflammatory cytokine production. This change in phenotype of TC-induced

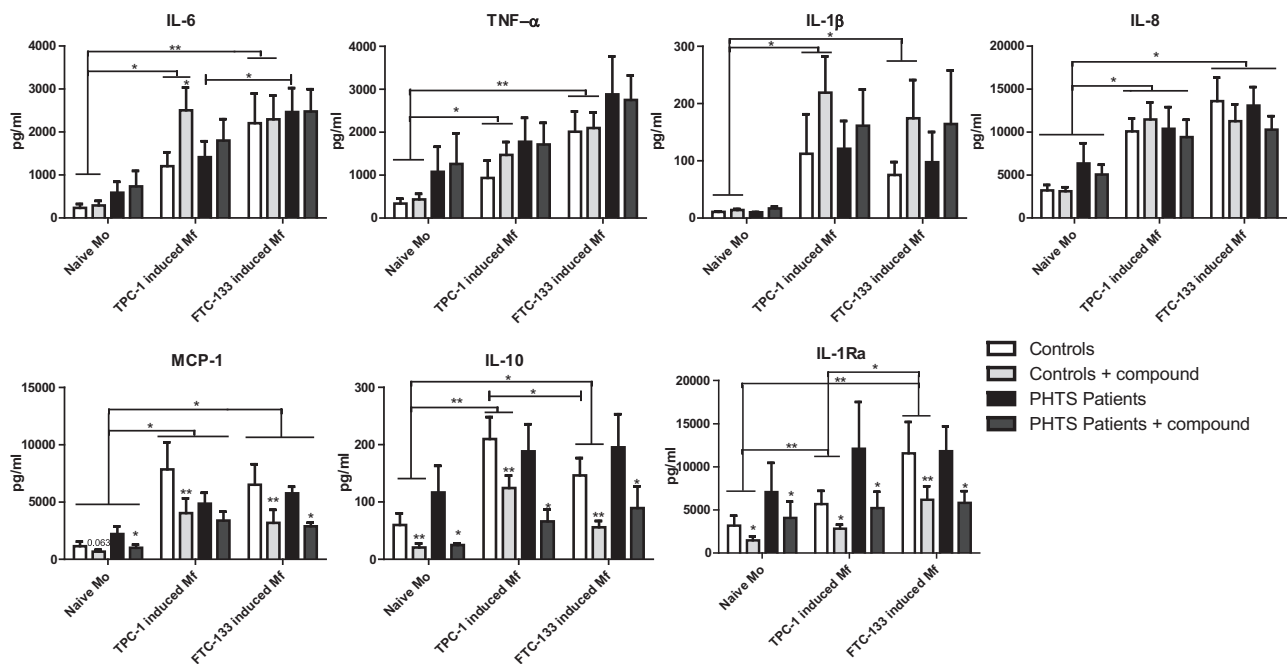


Fig. 5 Cytokine production in naive monocytes and TC-induced macrophages after restimulation with LPS in presence of metformin. Experiments were performed in medium containing 5 mM glucose

(physiological glucose concentration) in a total of 6 PHTS patients and 9 controls. Data shown as Mean \pm SEM. * $p < 0.05$; ** $p < 0.001$

macrophages could affect the potential treatment efficacy of metformin in TC. These results are summarized in Fig. 6.

Unstimulated PBMCs from PHTS patients produced significant higher levels of lactate compared to controls, indicating an increased aerobic glycolysis in these cells, most likely as a result of the activation of the Akt-mTOR pathways. As previously shown, the shift in glucose metabolism from oxidative phosphorylation to aerobic glycolysis provides rapid energy and biological substrates needed for innate immune cell activation [10]. When myeloid cells from patients with PHTS were cultured in a medium with physiological glucose concentration, replicating the *in vivo* conditions, they produced significantly higher levels of IL-6, TNF- α , IL-8 and MCP-1 upon LPS stimulation compared to controls. There is evidence in cancer cells that PTEN acts as regulator of Glucose Transporter 1 (GLUT 1) expression, thereby limiting glucose consumption [25, 26]. GLUT1 is also expressed on monocytes and its expression is upregulated in response to LPS [27]. Since our PHTS patients have a loss-of-function mutation in PTEN, it is possible that GLUT1 expression is upregulated in monocytes from PHTS patients, especially in low-glucose conditions. Glucose availability is therefore not a limiting factor, and activation of Akt-mTOR can thus increase aerobic glycolysis further.

The observation that patients with PHTS have a larger fraction of monocytes compared to controls is interesting. One possible explanation could be the fact that the PHTS patients were older than the controls. In a previous large cohort of volunteers, an association of older age with

reduced lymphoid cell levels and increased myeloid cell levels was shown [28]. However, PTEN deficiency has also been associated with significantly lower numbers of common lymphoid progenitor cells in the bone marrow of transgenic mice, whereas the common myeloid progenitor populations remained unchanged and numbers of subsets of myeloid cells were increased [29]. More recent reports also provide evidence that glycolysis and GLUT1 expression play an important role in myelopoiesis. Hematopoietic GLUT-1 deficiency results in reduced hematopoietic stem and progenitor cell (HSPC) proliferation and myelopoiesis in an ApoE^{-/-} mouse model [30]. In the context of trained immunity, β -glucan was shown to increase hematopoietic progenitor expansion and myelopoiesis in the bone marrow of mice, by enhancing glycolysis. This increase in glycolysis was also associated with protection against myelo-suppression and DNA damage resulting from hematopoietic stress [31]. Considering these recent findings, our results showing increased aerobic glycolysis in cells from PHTS patients and our hypothesis that PHTS patients might have increased GLUT1 expression due to loss of PTEN function could therefore also explain the observed increase number of monocytes in our PHTS patients.

Regarding the altered lymphocyte counts, Amer *et al.* described decreased T cell percentage in two PHTS patients [6], and other earlier reports have discussed altered T- and B- lymphocyte cell numbers in PHTS patients as well [32–34]. Recently, Chen *et al.*, also showed that although numbers of regulatory T cells were reduced in PHTS

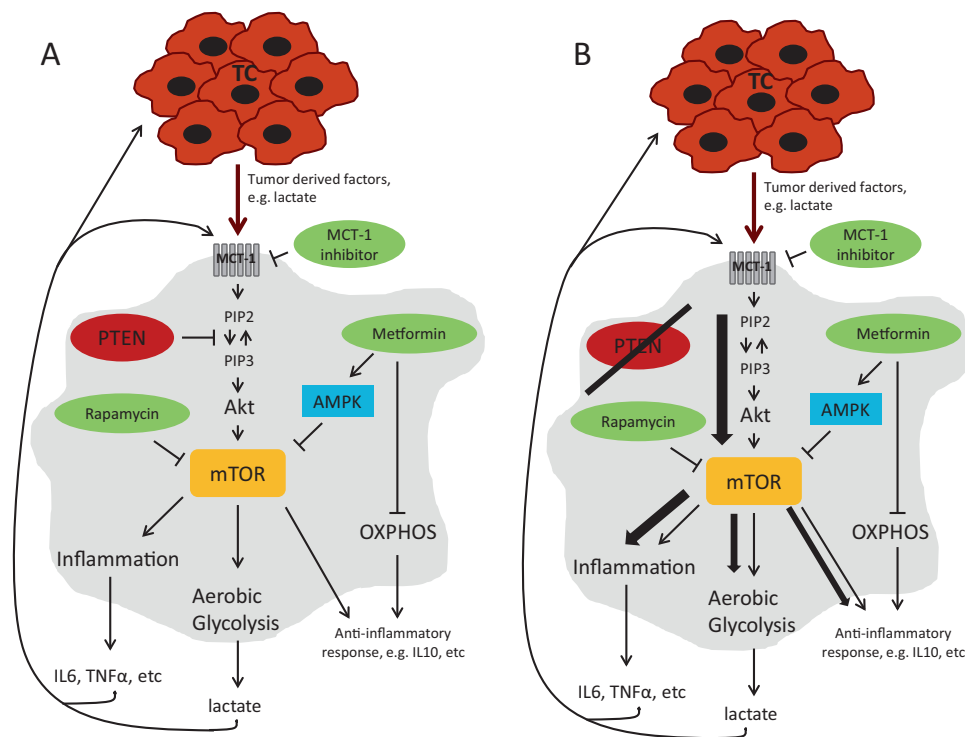


Fig. 6 The role of PTEN and the Akt-mTOR pathway in TC-induced macrophages. **a** Soluble tumor-derived factors such as lactate activate the Akt-mTOR pathway in tumor-associated macrophages, resulting in increased aerobic glycolysis and inflammation, leading to production of lactate and proinflammatory cytokines such as IL-6 and TNF- α . In turn, these factors can influence the tumor (paracrine signaling) and monocyte itself (autocrine signaling). PTEN is an important negative regulator of the Akt-mTOR pathway, preventing overactivation of the Akt-mTOR pathway. **b** When PTEN function is lost, the Akt-mTOR

pathways can become overactivated, resulting in an even higher increase in aerobic glycolysis and inflammation. Blocking the Akt-mTOR signaling, either by inhibiting the lactate transporters (MCT-1) using an MCT-1 inhibitor or blocking of mTOR using rapamycin, results in reduced inflammation. Use of metformin results in activation of AMPK, leading to inhibition of mTOR; metformin use also results in reduced oxidative phosphorylation (OXPHOS), resulting in a stronger reduction of the anti-inflammatory cytokine production (IL-10) as well

patients, the cells were phenotypically normal due to compensatory mechanism [7]. Since our main focus was on innate cell function, we corrected cytokine measurements for these differences in cell fractions to prevent confounding.

In high glucose conditions, PBMCs from PHTS patients showed an increased production of the anti-inflammatory IL-1Ra. IL-1Ra antagonizes IL-1 α and IL-1 β function by binding to the IL-1 receptor and forms a negative feedback loop to limit IL-1 driven inflammation [35]. However, other evidence suggests that higher IL-1Ra concentrations are a marker of inflammation and could be explained by overactivation of the PI3k-Akt pathway. In monocytes, PI3K activation and subsequent Akt activation was shown to be necessary to induce secretion of IL-1Ra in response to LPS, whereas it did not affect IL-1 β mRNA expression or stability [36].

TC-induced macrophages from both PHTS patients and controls showed increased proinflammatory cytokine production, especially after co-culture with the PTEN-deficient cell line FTC-133. The increase in anti-inflammatory cytokines was lower after co-culture with PTEN-deficient TC. These results are in line with our previous data,

demonstrating that TC-derived factors activate the Akt-mTOR pathway, leading towards a more proinflammatory phenotype [15]. We also confirmed the role of mTOR and lactate as important mediators of this effect, by showing that blocking mTOR with rapamycin or blocking the lactate transporter resulted in a significant reduction in cytokine production. In the context of PHTS, the proinflammatory role of lactate on TC-induced macrophages is especially important. In addition to the paracrine effect of tumor-derived lactate on TC-induced macrophages, lactate could also act in an autocrine fashion on TC-induced macrophages because innate immune cells from PHTS patients produce more lactate themselves as well.

Importantly, our results also suggest that co-culture with PTEN-deficient TC, a clinically relevant model for TC in PHTS, induces an even stronger proinflammatory phenotype in TC-induced macrophages compared to the PTEN wild-type cell line TPC-1. PTEN deficiency in TC is associated with more aggressive tumors [37]. Induction of a more proinflammatory tumor microenvironment is known to support tumor progression [38, 39]. Moreover, PTEN-deficient tumors are associated with decreased T cell

infiltration in melanoma [40], indicative of reduced adaptive anti-tumor response. Also, proinflammatory cytokines such as TNF- α , produced by TAMs, have been shown to induce dedifferentiation of tumors, resulting in adaptive immune resistance [41, 42]. While the assessment of these effects were beyond this study, future investigations are warranted to assess the effects of these TC-primed macrophages on the adaptive immune functions, as these might be mechanisms exploited by the PTEN-deficient tumor to become more aggressive in the context of PHTS.

Treatment with metformin, an indirect inhibitor of mTOR through AMPK activation [19, 20] and currently investigated as a potential therapeutic adjuvant in differentiated TC [22, 23, 43], did not affect the production of proinflammatory cytokines of the TC-induced macrophages in our co-culture model, but it reduced the production of anti-inflammatory cytokines IL-10 and IL-1Ra. Metformin has multiple biological effects in addition to modulation of mTOR pathway, including effects on mitochondrial respiration, gene transcription and tumorigenesis itself by inhibition of cell proliferation [38, 39]. Its impact is thus much more complex and difficult to target specifically for the PTEN-mTOR axis, and our results suggest that it is unlikely to represent the most effective approach to modulate this pathway in TAMs.

Activation of Akt-mTOR pathway is an important feature of trained innate immunity, a process of epigenetic innate immune reprogramming that is involved in long-term inflammatory processes in infection, chronic inflammatory disease, and cancer [44]. Moreover, trained immunity has been recently suggested to represent a therapeutic target in cancer [45]. Monocytes from PHTS patients displayed a normal training capacity, without major differences compared to controls. This argues that the increased mTOR pathway due to PTEN defects does not lead to an exacerbation of trained immunity.

One limitation of our study is the relatively small number of patients included in the different experiments and the fact that we were not able to include the whole cohort of patients in all experiments presented here. This is mainly due to the low prevalence of the disease and the limitation in the amount of biological material that we were allowed to collect from each patient. Despite these challenges, we were able to include 29 adult patients with PHTS, which is a very large number of patients from one center and the clinical phenotype of the patients indicate that they are representative for the PHTS population.

In conclusion, this is the first comprehensive assessment of the immunological phenotype of patients with PHTS that showed that innate immune cells from PHTS patients with germline PTEN inactivating mutations have increased lactate production, which results in a more proinflammatory phenotype of monocytes, especially in the context of PTEN-

deficient TC. An important, yet somewhat surprising finding is that metformin promotes a more proinflammatory phenotype in TC-induced macrophages by mainly blocking production of anti-inflammatory cytokines. These results have important translational consequences: they indicate that PHTS patients may benefit from treatment with mTOR blocking agents to limit the inflammatory response in the context of either auto-immune diseases or cancers (such as TC), but also opens the door for understanding the role of this crucial signaling pathway for the modulation of inflammation in other diseases. Further research is warranted to investigate this pathway in PHTS and other pathologies, in order to realize the full therapeutic potential of our findings.

Material and methods

Isolation of PBMCs and monocytes and ex vivo PBMC stimulation experiments

Blood was obtained from patients with PHTS visiting the outpatient clinic and healthy volunteers (controls) after informed consent, in the period between March 2016 and February 2017 (Ethical approval CMO Arnhem-Nijmegen 2010–104). The inclusion of the patients was done unselectively, by inviting consecutive patients visiting our outpatient clinic to participate in the study. The patients were allocated to the different experiments according to the timeline of the study. All experiments were conducted in the Radboud University Medical Center Nijmegen, The Netherlands in accordance with the principles expressed in the Declaration of Helsinki.

PBMCs were isolated from blood by density gradient centrifugation using Ficoll-plaque (GE Healthcare, Diegem Belgium). PBMCs were washed three times in cold Phosphate Buffered Saline (PBS, Braun Melsungen, Germany) and resuspended in RPMI 1640 Dutch modification culture medium (Life Technologies, Carlsbad, California, USA) supplemented with gentamycin 50 μ g/ml, pyruvate 1 mM, glutamax 2 mM, and counted using a Coulter particle counter (Beckman Coulter Inc., CA, USA). Composition of PBMCs, e.g., the percentage of monocytes and lymphocytes, was assessed on the Sysmex XN-450 automated hematology analyzer (Sysmex Europe GmbH, Germany).

PBMCs were either stimulated for 24 h with RPMI, 10 ng/ml LPS (*E. coli* strain O55:B5, Sigma Chemical Co, St. Louis, MO) or heat-killed *Candida albicans* (1×10^6 /ml, *C. albicans* ATCC MYA-3573 (UC 820)). A total of twelve PHTS patients and twelve controls provided blood to assess innate immune cell function in 24 h PBMC stimulation experiments.

For the in vitro trained innate immunity model, monocytes were isolated within the PBMC fraction using Percoll

isolation as described previously [46]. Briefly, 20×10^6 PBMCs were layered on top of a hyperosmotic Percoll solution (48.5% Percoll (Sigma-Aldrich, St. Louis, MO, USA), 41.5% sterile H₂O, 0.16 M filter-sterilized NaCl) and centrifuged for 15 min at $580 \times g$. The interphase layer was isolated, and the cells were washed with cold PBS. After resuspension in RPMI culture medium, Percoll-isolated monocytes were allowed to adhere to polystyrene flat-bottom plates (Corning, NY, USA) for 1 h at 37 °C; the cells were washed with warm PBS to obtain maximal purity.

Ex vivo trained innate immunity model in human monocytes

The ex vivo experimental model of trained innate immunity in human primary monocytes has been described previously [46]. Briefly, Percoll-isolated monocytes (100,000 cells/well) were added to flat-bottom 96-well plates and left to adhere for 1 h. After washing with warm PBS, monocytes were incubated with RPMI alone (medium control) or *C. albicans* cell wall component β -glucan 1 μ g/ml (kindly provided by Prof. D. Williams, University of East Tennessee) or 1 μ g/ml bacillus Calmette-Guérin (BCG, InterVax Ltd, Ontario, Canada) for 24 h. Cells were washed once with warm PBS and incubated for 6 days in culture medium with 10% serum. Culture medium was changed on day 3. Cells were restimulated with RPMI or 10 ng/ml LPS (*E. coli* strain O55:B5, Sigma Chemical Co, St. Louis, MO). After 24 h, supernatants were collected and stored at -20 °C until cytokine measurement. Ten PHTS patients and eleven controls were included in the ex vivo monocyte training experiments.

Co-culture model of TC cell lines and human monocytes

The TC cell line in-vitro experiments were performed using TPC-1 (papillary, RET/PTC rearrangement and FTC133 (follicular, PTEN-deficient) cell lines [47]. The cancer cell lines were resuscitated and grown in culture medium RPMI 1640 Dutch modification (Life Technologies, Carlsbad, California, USA) supplemented with gentamycin 50 μ g/ml, pyruvate 1 mM, glutamax 2 mM and 10% Fetal Calf Serum (Gibco, Life Technologies). A trans-well system with ThinCert™ cell culture inserts on a 24-well plate (Greiner Bio-One GmbH, Austria) was used for co-culture of TC cells and human monocytes. A total of 1.0×10^5 cells of each TC cell line in 250 μ l culture medium were added to the upper compartment of the trans-well system. Upper compartments with medium only were used as controls. The cells were incubated for 24 h at 37 °C, 5% CO₂. Next, the cells were washed with PBS and fresh co-culture medium containing a physiological concentration of glucose, was

added: RPMI (Life Technologies, Carlsbad, California, USA), supplemented with glucose 5 mM, pyruvate 1 mM, gentamicin 50 μ g/ml and HEPES 10 mM (Life Technologies, Carlsbad, California, USA). The in vitro co-culture experiments were performed in medium with physiological concentration of glucose to replicate the in vivo intratumoral situation.

PBMCs were isolated as described above and resuspended in the co-culture medium. A total of 1.0×10^6 PBMCs in 500 μ l were added to the lower compartment of the trans-well system. After 1 h of incubation, the non-adherent cells comprising mainly lymphocytes were discarded, and adherent monocytes were incubated with TC cell lines or medium alone for 24 h at 37 °C in co-culture medium.

Next, the cell culture inserts were discarded and the adherent monocytes, that we henceforth refer to as “naïve” monocytes or TC-induced macrophages respectively, were stimulated for 24 h with RPMI, 10 ng/ml LPS (*E. coli* strain O55:B5, Sigma Chemical Co, St. Louis, MO), as substitute for endogenous TLR4 ligand signaling, or 10 μ g/ml Pam3Cys (EMC Microcollection, Germany) as a substitute for endogenous TLR2 ligand signaling. Supernatant was collected and stored at -20 °C until further analysis.

To study the effects of co-culture with TC cell lines on monocyte function, we included twelve PHTS patients and controls for the effect of TLR-4 stimulation, and seven PHTS patients and controls for the effect of TLR-2 stimulation.

In some experiments the mTOR inhibitor rapamycin (100 nM, Bio Connect, Huissen, The Netherlands), lactate transport inhibitor α -cyano-4-hydroxycinnamic acid (1 mM, Sigma) or metformin (1,1-Dimethylbiguanide hydrochloride, 3 mM Sigma) were added to the medium during co-culture of TC cell lines and monocytes.

Nine controls and nine PHTS patients were included to study the effect of rapamycin and the lactate transport inhibitor in the co-culture model; nine controls and six PHTS patients were included to study the effect of metformin.

Cytokine, chemokine and lactate production

IL-6, IL-8, IL-10 (Sanquin, Amsterdam, Netherlands), TNF- α , IL-1 β , MCP-1 and IL-1 receptor antagonist (IL-1Ra) (R&D, the Netherlands), concentrations in the culture supernatant were measured by commercial ELISA kits according to the instruction of the manufacturer. Lactate was measured by a Lactate Fluorometric Assay Kit (Biovision, CA, USA). All cytokine data was corrected for by PBMC cell count fractions.

Statistical analysis

All analyses were performed in Graphpad prism 5 (CA, USA). Differences in cytokine and lactate production between patients and controls were analyzed using Mann–Whitney test. To analyze within group differences (e.g., differences in cytokine production after co-culture with each cell line), we used a Friedman test and post-hoc Wilcoxon matched-pairs signed rank test. Overall, all statistical test were performed two-sided. $*P < 0.05$, $**P < 0.01$. The data are shown as means \pm SEM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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