AMPK hyperactivity maintains the survival of vasculogenic T cells in patients with Takayasu's arteritis

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Abstract

Objective

Takayasu's arteritis (TAK) is a progressive autoimmune vasculitis that mainly affects the aorta and its major branches. While recent studies have identified proinflammatory T cells, including Th1 and Th17 cells, as the dominant infiltrates in the arterial adventitia, mechanisms underpinning the maintenance of such vasculogenic T cells remain obscure.

Methods

75 patients with TAK and 30 age-matched healthy controls were enrolled in this study. CD4 T cells from TAK patients were activated with anti-CD3/CD28 beads to mimic vasculogenic T cells. The survival of T cells was detected by quantifying Annexin-V$^+$7-AAD$^+$ fractions. Expression and activity of AMP-activated protein kinase (AMPK) were determined using phosflow cytometry and immunoblots. Specific inhibitors and shRNA were applied to block the function of AMPK and Notch1, while erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were used to reflect the disease activity of TAK patients.

Results

T cells from TAK patients undergo spontaneous differentiation into vasculogenic proinflammatory T cells with prolonged survival capacity. Mechanistic explorations uncover AMPK hyperactivity in such T cells from TAK patients, promoting mitochondrial metabolism and their survival. Such AMPK hyperactivity results from the robust Notch1 activity in TAK T cells. Accordingly, T cell-intrinsic phosphor-AMPK reflects the disease activity in clinical TAK patients.

Conclusion

AMPK hyperactivity is essential for maintaining the vasculogenic proinflammatory T cells in TAK patients, serving as a promising therapeutic target for TAK management.

Key words

Takayasu's arteritis, AMP-activated protein kinase

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Received on September 27, 2022; accepted in revised form on November 16, 2022.

Funding: this work was supported by a grant from the Natural Science Foundation of Jilin Province, China (YDZJ202101ZYTS116).

Competing interests: none declared.

Introduction

Takayasu's arteritis (TAK) is a progressive autoimmune vasculitis that mainly affects the aorta and the major branches, with increasing morbidity and mortality (1-5). This disease is more common in females and can be seen in all ethnicities (1, 3-5). While imaging tools such as CTA, MRA and contrast-enhanced ultrasonography are used for disease diagnosis and inflammation assessment of vascular layers, diagnoses are still frequently delayed, and the underlying pathogenesis of TAK remains largely undefined, resulting in limited treatment options in clinical practice (3-6).

In patients with TAK, adventitia of affected arteries is frequently infiltrated with immune cells, which drive vascular inflammation and subsequent organ damage (3, 4, 7). Specifically, proinflammatory CD4 T cells, including T helper type 1 (Th1) and Th17 cells, are the dominant vasculogenic infiltrates in the vascular layers of TAK patients, assigning IFN-γ and IL-17 as critical cytokines involved in disease pathogenesis (3, 4, 7, 8). Thus, mechanistic studies for the differentiation and maintenance of such vasculogenic Th1 and Th17 cells are relevant for advancing our understanding of TAK pathogeneses and subsequent therapeutic explorations. Our recent studies have identified Notch1 hyperactivity and the mechanistic target of rapamycin complex 1 (mTORC1) hyperactivity as the critical molecular basis for the maldifferentiation of proinflammatory T cells in TAK patients (3). Notch1 drives the expressions and lysosomal localisation of mTORC1 in T cells, resulting in mTORC1 hyperactivity for the maldifferentiation of Th1 and Th17 cells in patients with TAK (3). Targeting Notch1 and mTORC1 by specific inhibitory chemicals and genetic manipulations is effective in abrogating the development of vasculitis in humanised chimeras, which represent human TAK (3, 4). However, how vasculogenic T cells were maintained in TAK patients remains unknown.

The differentiation, function, and maintenance of T cells are highly dependent on intracellular energy metabolism (9, 10). AMP-activated protein kinase (AMPK), the guardian of energy metabolism and mitochondrial homeostasis, senses ATP insufficiency and increased AMP/ATP ratios, driving catabolic metabolism and ATP generation (11, 12). Instead, mTOR is activated by amino acids in nutrient-sufficient conditions, promoting anabolic metabolism and ATP consumption (13, 14). Mechanistically, the crosstalk between AMPK and mTOR maintains energy haemostasis through interactions on the lysosome (11, 15, 16). Upon AMPK phosphorylation on the Thr172 of α-subunit, AMPK phosphorylates TSC2 and Raptor to inhibit the activation and stability of mTOR, leading to mTOR inactivation (11, 16). While mTORC1 is crucial for the differentiation of Th1 and Th17 cells, AMPK is critical for the differentiation of regulatory T cells (4, 11, 17). Meanwhile, as a vital energy sensor, whether the activity of AMPK was dysregulated and whether AMPK was involved in the differentiation and maintenance of vasculogenic T cells in TAK patients remain elusive.

In this study, we investigated the potential role of AMPK activity in the differentiation and survival of T cells from TAK patients and pinpointed AMPK hyperactivity as a critical basis for maintaining the vasculogenic Th1 and Th17 cells in those patients. Targeting AMPK could be a promising strategy to impair the survival of vasculogenic T cells for TAK treatment.

Materials and methods

Patients

Seventy-five TAK patients diagnosed with the 1990 ACR Classification Criteria and thirty age-matched healthy individuals were enrolled in this study, and informed consent was obtained from every participant. The clinical features of TAK patients were summarised in Table I. Experiments were performed in accordance with the Helsinki Declaration and approved by the Institutional Review Board of The First Hospital of Jilin University.

T cell isolation and culture

T cell isolation and culture were performed as previously described (3, 4, 18). Specifically, peripheral blood

mononuclear cells (PBMCs) were isolated by gradient centrifugation with Human Lymphocyte Separation Medium (Corning), and T cells were isolated from PBMCs using Human CD4 T Cell Enrichment Kit (StemCell Technologies). The T cell purity was consistently over 90%. T cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Thermo Fisher Scientific) plus Penicillin-Streptomycin-Glutamine (Thermo Fisher Scientific).

Reagents and transfections

The Notch1 inhibitor DAPT, AMPK activator A769662 and AMPK inhibitor Compound C were purchased from Sigma-Aldrich. Human AMPKα shRNA, Notch1 shRNA, and the controls were from Santa Cruz Biotechnology. T cell transfections were performed with Nucleofector kits from Lonza. All reagents were used according to the manufacturer's instructions.

T cell differentiation and survival

T cell differentiation and survival were performed as previously described (3, 4, 18, 19). For the differentiation of proinflammatory T cells, CD4 T cells were activated with anti-CD3/CD28 beads (cell/bead ratio = 2/1, Thermo Fisher Scientific) for four days, followed by analyses of linage-determining transcription factors including T-bet and RORγ using flow cytometry. For the survival of T cells, CD4 T cells were stimulated with anti-CD3/CD28 beads for six days and analysed for Annexin-V$^+$7-AAD$^+$ fractions using Annexin-V Apoptosis Detection Kit with 7-AAD (Stem Cell Technologies).

Flow cytometry

Flow cytometry was performed as previously described (3, 4, 11, 18). Specifically, CD4 T cells were treated with Fix Buffer I (BD Biosciences), Perm Buffer III (BD Biosciences), and stained with antibodies as follows: FITC/APC-Cy7 anti-CD4 (BioLegend, Clone OKT4), PE anti-T-bet (Thermo Fisher Scientific, Clone eBio4B10), APC anti-RORγ (Thermo Fisher Scientific, Clone AFKJS-9), rabbit anti-phospho-AMPK alpha-1,2 (Thr172) antibody (Thermo Fisher Scientific, 44-1150G) plus Alexa Fluor Plus 488 anti-Rabbit IgG (H+L) (Thermo Fisher Scientific, A32731), Alexa Fluor® 594 anti-AMPK alpha-1/2 antibody (Santa Cruz, sc-74461), PE anti-Notch1 (Thermo Fisher Scientific, Cat. 12-5785-82), rabbit anti-activated Notch1 (Abcam, Cat. ab52301) plus Alexa Fluor Plus 488 anti-Rabbit IgG (H+L) (Thermo Fisher Scientific, A32731). Cells were stained for 45 min at 4°C, and flow cytometry was performed on an LSR II flow cytometer (BD). Data were analysed with FlowJo software (Tree Star).

Immunoblotting

Immunoblotting was performed as previously described (3, 11, 20). An-

Table I. Characteristics of clinical patients.

Parameters	Patients with TAK	Healthy donors
Number of patients	75	30
Age (mean ± SD, years)	41.9 ± 13.6	42.3 ± 11.5
Female	73.3%	70.0%
Disease duration (mean ± SD, months)	9.5 ± 7.3	N/A
Treatment at sampling		
Untreated patients (n, %)	32, 42.7%	N/A
Prednisone (mg/day, mean ± SD)	10.7 ± 6.9	N/A
Immunosuppressants (n, %)	29, 38.7%	N/A
Laboratory		
ESR (mean ± SD, mm/h)	53.87 ± 24.61	N/A
CRP (mean ± SD0, mg/dL)	5.25 ± 2.69	N/A

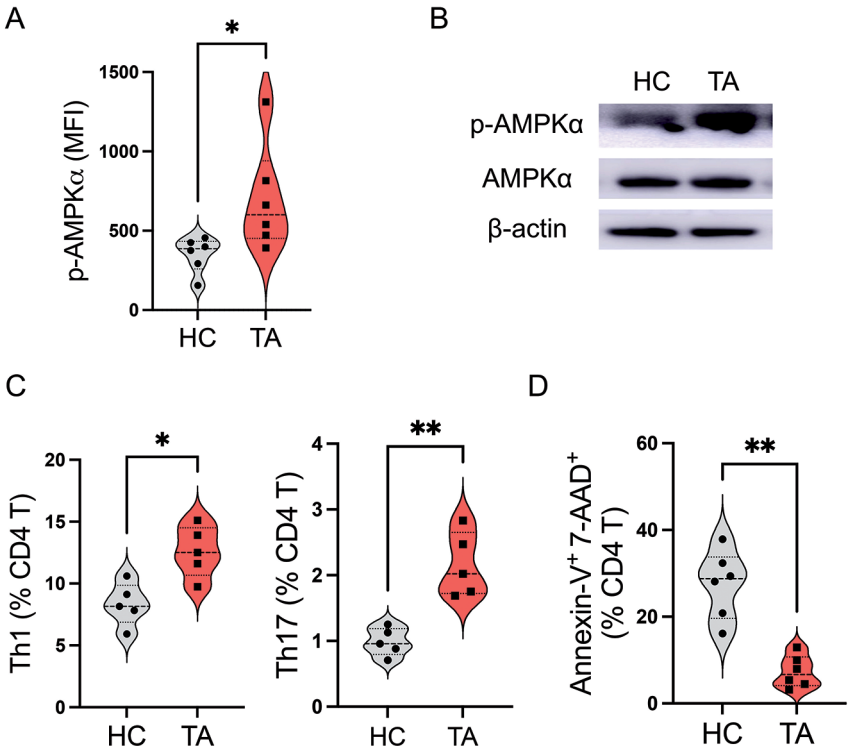

Fig. 1. AMPK hyperactivity and prolonged survival capacity of vasculogenic TAK T cells.
(**A**) CD4 T cells from healthy individuals and TAK patients were activated with anti-CD3/CD28 beads for 3 days and analysed for intracellular phosphor-AMPK using Phosflow cytometry. Collective MFI from 6 healthy-TAK pairs. (**B**) CD4 T cells from healthy and TAK patients were activated with anti-CD3/CD28 beads for 3 days and analysed for phosphor-AMPK using immunoblots. Representative from 4 healthy-TAK individuals. (**C**) CD4 T cells from healthy individuals and TAK patients were activated with anti-CD3/CD28 beads for 4 days and detected for Th1 cells and Th17 cells by quantifying lineage-determining transcription factors T-bet and RORγ. Data from 5 healthy-TA pairs. (D) CD4 T cells from healthy and TAK patients (n=6) were activated with anti-CD3/CD28 beads for 6 days and analysed for survival rate by detecting Annexin-V$^+$7-AAD$^+$ fractions.
*p<0.05, **p<0.01 with unpaired MWW test.

tibodies used were as follows: anti-phosphor-AMPKα (Thr172) antibody (Cell Signaling Technology, Cat. 2531), anti-AMPKα antibody (Cell Signaling Technology, Cat. 2532). β-actin was used as an internal control and detected with anti-β-actin (Santa Cruz Biotechnology, Cat. sc-81178).

Statistical analyses

Data were presented as violin plots with all points and analysed using paired or unpaired Mann-Whitney tests. Correlations were performed with Spearman r analyses. Statistics were performed with GraphPad PRISM 9.0 (GraphPad Software), and $p<0.05$ was considered significant.

Results

AMPK hyperactivity in vasculogenic T cells of TAK patients

To explore the potential role of AMPK in the differentiation and survival of vasculogenic T cells in TAK patients, CD4 T cells from TAK patients were stimulated with anti-CD3/CD28 beads to mimic vasculogenic T cells and detected the intracellular levels of phosphor-AMPK. We observed elevated levels of phosphor-AMPK in TAK T cells upon stimulation with anti-CD3/CD28 beads (Fig. 1A-B, Supplementary Fig. S1A). AMPK was not hyperactivated in CD4 T cells from patients with systemic lupus erythematosus (Supplementary Fig. S1B). And, AMPK hyperactivity was also found in CD8 T cells of TAK patients (Supplementary Fig. S1C), assigning AMPK hyperactivity as a feature for vasculogenic TAK T cells.

Further, we confirmed the spontaneous mal-differentiation of Th1 and Th17 cells of TAK T cells. We repeatedly observed higher frequencies of T-bet-expressing Th1 cells and RORγ-expressing Th17 cells upon stimulation with anti-CD3/CD28 beads (Fig. 1C). Meanwhile, we detected the survival of activated T cells by analysing the Annexin-V⁺7-AAD⁺ fractions after six days of stimulation and found a higher survival capacity of vasculogenic TAK T cells (Fig. 1D, Supplementary Fig. S1D). Such an abnormal AMPK activity and survival were not observed in freshly isolated, unstimulated CD4 T cells from TAK patients (Supplementary Fig. S1E), indicating a cell-intrinsic mechanism of TAK T cells upon stimulation. Together, TAK T cells spontaneously differentiate into proinflammatory T cells, with a higher survival rate and AMPK activity.

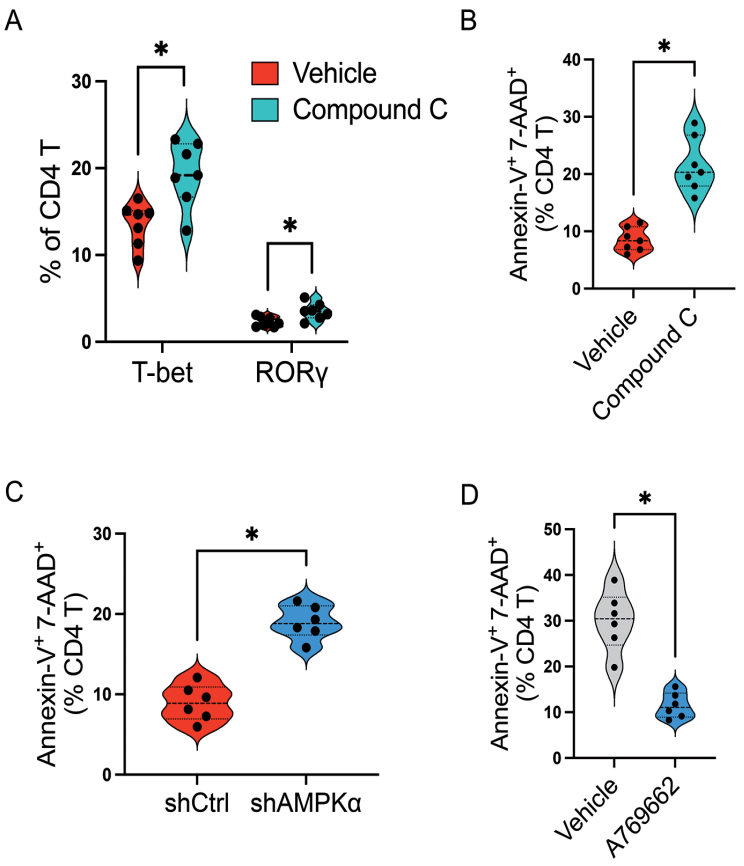

Fig. 2. AMPK promotes the survival rate of vasculogenic TAK T cells.
(**A**) CD4 T cells from TAK patients (n=7) were activated with anti-CD3/CD28 beads in the presence or absence of compound C (10 μM) for 4 days and analysed for the differentiation of T-bet-expressing Th1 cells and RORγ-expressing Th17 cells. (**B**) TAK CD4 T cells from 7 patients were activated with anti-CD3/CD28 beads in the presence or absence of compound C (10 μM) for 6 days and detected for the survival rate. (**C**) CD4 T cells from TAK patients (n=6) were transfected with AMPKα shRNA or the control, activated with anti-CD3/CD28 beads for 6 days, and analysed for the survival rate. (**D**) CD4 T cells from healthy donors (n=6) were activated with anti-CD3/CD28 beads plus A769662 (10 μM) for 6 days.
*$p < 0.05$ with paired MWW test.

AMPK hyperactivity is required for the survival of vasculogenic TAK T cells

To directly detect the function of AMPK in the differentiation and survival of proinflammatory T cells of TAK patients, we isolated CD4 T cells from TAK patients, followed by stimulation with anti-CD3/CD28 beads in the presence or absence of AMPK inhibitor Compound C. Such inhibition of AMPK did not inhibit the differentiation of proinflammatory T cells and actually promoted the frequencies of Th1 and Th17 cells of TAK T cells (Fig. 2A). In contrast, AMPK inhibition fundamentally impaired the survival of vasculogenic TAK T cells, resulting in a higher fraction of Annexin-V⁺7-AAD⁺ T cells (Fig. 2B).

To confirm the function of AMPK in the maintenance of vasculogenic T cells of TAK patients, CD4 T cells from TAK patients were transfected with AMPK shRNA (Supplementary Fig. S2) and stimulated with anti-CD3/CD28 beads. Again, genetic knockdown of AMPK led to the reduced survival rate of TAK T cells, showing elevated frequencies of Annexin-V⁺7-AAD⁺ cells (Fig. 2C). Further, treatment of healthy T cells with AMPK activator A769662 pro-

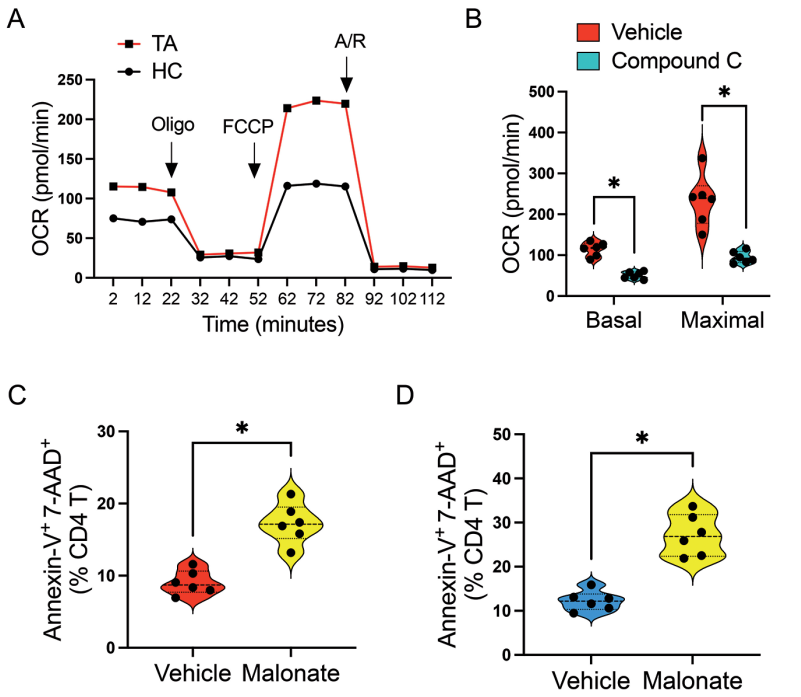

Fig. 3. AMPK drives mitochondrial metabolism for the survival of vasculogenic TAK T cells. **A**: CD4 T cells from healthy and TAK patients (n=5) were activated with anti-CD3/CD28 beads for 3 days and analysed for mitochondrial OCR using the seahorse assay. **B**: CD4 T cells from TAK patients (n=6) were activated with anti-CD3/CD28 beads in the presence or absence of compound C (10 μM) for 3 days. **C**: CD4 T cells from TAK patients (n=6) were activated with anti-CD3/CD28 beads in the presence or absence of malonate (10 mM) for 6 days. **D**: CD4 T cells from healthy individuals (n=6) were activated with anti-CD3/CD28 beads plus A769662 (10 μM) in the presence or absence of malonate (10 mM) for 6 days. *p<0.05 with paired MWW test.

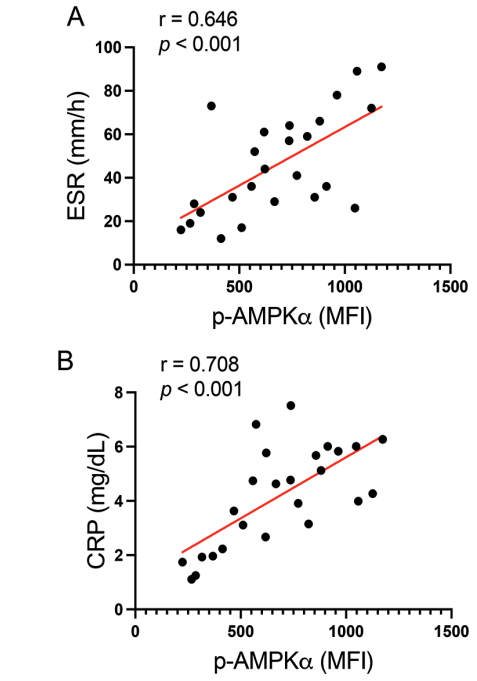

Fig. 5. T cell-intrinsic phosphor-AMPKα reflects disease activity of TAK patients. **A**: The levels of intracellular phosphor-AMPK in circulating CD4 T cells were associated with the ESR levels of TAK patients (n=25). **B**: The levels of intracellular phosphor-AMPK in circulating CD4 T cells were correlated with the CRP levels of TAK patients (n=25). Spearman correlation analyses.

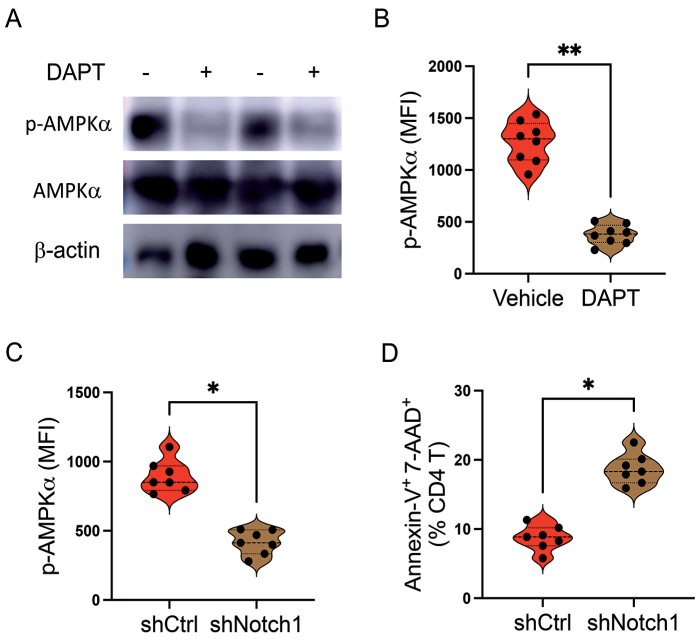

Fig. 4. Notch1 facilitates AMPK activation and the prolonged survival of vasculogenic TAK T cells. (**A-B**) CD4 T cells from TAK patients (n=8) were activated with anti-CD3/CD28 beads with or without DAPT (10 μM) for 3 days and analysed for AMPK activity using immunoblots (**A**) and Phosflow cytometry (**B**). (**C**) CD4 T cells from TAK patients (n=7) were transfected with Notch1 shRNA or the control, and activated with anti-CD3/CD28 beads for 3 days. (**D**) CD4 T cells from TAK patients (n=7) were transfected with Notch1 shRNA or the control, and activated with anti-CD3/CD28 beads for 6 days. *p<0.05, **p<0.01 with paired MWW test.

moted their survival rate (Fig. 2D). In essence, AMPK hyperactivity is essential for the prolonged survival of vasculogenic T cells in TAK patients.

AMPK promotes mitochondrial metabolism for the survival of vasculogenic TAK T cells

As AMPK is well-known for promoting mitochondrial metabolism (21, 22), we detected the mitochondrial oxygen consumption rates (OCR) in activated TAK and healthy T cells to test how AMPK might promote the survival of vasculogenic TAK T cells. We observed higher mitochondrial OCR in vasculogenic TAK T cells, exerting elevated basal OCR and maximal respiratory capacity (Fig. 3A, Supplementary Fig. S3). Such an elevated mitochondrial OCR of vasculogenic TAK T cells could be inhibited by AMPK inhibitor Compound C (Fig. 3B).

To determine whether mitochondrial metabolism was crucial for AMPK-induced maintenance of vasculogenic TAK T cells, CD4 T cells from TAK

patients were stimulated with anti-CD3/CD28 beads in the presence or absence of succinate dehydrogenase inhibitor malonate to block the mitochondrial TCA cycle. Consequently, malonate efficiently abrogated the prolonged survival of vasculogenic TAK T cells, resulting in increased frequencies of Annexin-V^{+}7-AAD^{+} cells (Fig. 3C). In support, malonate also blocked the effect of AMPK activator A769662 on the prolonged survival of healthy T cells (Fig. 3D). Together, AMPK promotes mitochondrial metabolism to maintain the survival of vasculogenic TAK T cells.

Notch1 drives AMPK hyperactivity of vasculogenic TAK T cells

Our recent studies have identified Notch1 in inducing mTOR hyperactivity for the differentiation of vasculogenic T cells in TAK patients (3, 4). While the cross-inhibitory crosstalk between AMPK and mTOR assigns an unlikely function of mTOR in inducing AMPK hyperactivity in vasculogenic TAK T cells, we detected the potential role of Notch1 in instructing AMPK hyperactivity. Thus, CD4 T cells from TAK patients were stimulated with anti-CD3/CD28 beads in the presence or absence of DAPT as a Notch1 inhibitor. We repeatedly observed higher Notch1 expression in TAK T cells (Supplementary Fig. S4A), while DAPT dramatically reduced the level of AMPK phosphorylation in vasculogenic TAK T cells (Fig. 4A-B), indicating a critical role of Notch1 in inducing AMPK hyperactivity.

To confirm the function of Notch1 in AMPK hyperactivity of TAK T cells, those cells were transfected with Notch1 shRNA and stimulated with anti-CD3/CD28 beads. We confirmed that genetic knockdown of Notch1 efficiently reduced Notch1 expression and activity in TAK T cells (Supplementary Fig. S4B), accompanied by a lower AMPK activity and decreased survival rate (Fig. 4C-D). Combing these findings pinpoint Notch1 as a critical molecular basis for AMPK hyperactivity in vasculogenic TAK T cells.

T cell-intrinsic AMPK reflects the disease activity of TAK patients

To explore the clinical relevance of AMPK hyperactivity in vasculogenic T cells from TA patients, levels of intracellular phosphor-AMPK of circulating CD4 T cells were analysed for associations with the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) of TAK patients, which are the most widely performed laboratory investigations to reflect disease activity in clinical practice (23, 24). As shown in Figure 5A-B, levels of T cell-intrinsic phosphor-AMPK were positively correlated with ESR and CRP levels of TAK patients, suggesting that AMPK hyperactivity in T cells predicts active disease in clinical patients.

Discussion

In patients with TAK, vasculogenic T cells including Th1 and Th17 cells are the dominant infiltrates in vascular layers, causing vasculitis and organ damage (3, 4, 7). Due to Notch1-promoted mTORC1 expression and the subsequent lysosomal localisation, mTORC1 hyperactivity drives the mal-differentiation of Th1 and Th17 cells in TAK patients (3, 4). Herein, we extend previous studies by showing that AMPK hyperactivity is a feature of TAK T cells, resulting in the prolonged survival of vasculogenic TAK T cells. Consequently, AMPK would be a promising target for controlling the maintenance of vasculogenic T cells in TAK patients.

AMPK is a critical energy sensor that senses increased AMP/ATP ratios, leading to lysosomal translocation and subsequent phosphorylation by LKB1 (12, 25). Upon phosphorylation at Thr-172, AMPK is activated and drives mitochondrial biogenesis and fusion, resulting in robust mitochondrial metabolism for ATP generation (21). However, recent studies have assigned a crucial role to AMPK in T cell biology (26, 27). Of interest, a great deal of data have suggested that AMPK activity is required for the differentiation of regulatory T cells and the formation of memory T cells (26-29). Meanwhile, AMPK interacts with mTOR, inhibiting mTORC1 stability and activity and thus blocking the differentiation of pro-inframammary Th1 and Th17 cells (11). In support, AMPK inhibition by Compound C promoted the mal-differentiation of Th1 and Th17 cells from TAK T cells, which might be related to the lack of AMPK-mediated mTOR inhibition. Meanwhile, we demonstrate that AMPK is essential for the survival of vasculogenic TAK T cells, which is a crucial mechanism underlying TAK pathogenesis. Blockade of AMPK fundamentally reduces vasculogenic TAK T cells' survival rate, which might benefit TAK patients. Since phosphor-AMPK levels in circulating T cells closely reflect TAK patients' disease activity, targeting T cell-intrinsic AMPK could be a promising therapeutic strategy for TAK treatment. Similarly, AMPK also promotes the survival of T cells in patients with T-cell acute lymphoblastic leukaemia (30).

Notch1 is a highly conserved molecule critically involved in cell biology, including differentiation and survival (31, 32). In the present study, we identify Notch1 as the molecular basis for AMPK hyperactivity in vasculogenic TAK T cells. In patients with TA, Notch1 not only drives mTORC1 activation for differentiation of proinflammatory T cells but also promotes AMPK activation to maintain those vasculogenic T cells. Accordingly, targeting Notch1 is effective in inhibiting vasculitis in humanised chimeras that represent human TAK (3). In support of our findings, Notch1 is reported to induce AMPK activation in T-cell acute lymphoblastic leukaemia cells by increasing the energy burden (30). Mechanistically, Notch1 has been implicated in regulating LKB1, which is a critical enzyme for AMPK phosphorylation on lysosomes (33, 34). Thus, Notch1 might promote the function of LKB1 in facilitating AMPK activation in TAK T cells.

Herein, we provide evidence for demonstrating an essential function of AMPK hyperactivity in maintaining the survival of vasculogenic TAK T cells. Such T cells are from clinical patients and healthy individuals, assigning current findings to closely mimic the realistic conditions. However, we would like to admit some limitations of this study. One is that the patient cohort is relatively small, and such findings could be strengthened with a larger patient cohort. Another is that the precise

molecular mechanism for how Notch1 drives AMPK phosphorylation remains unclear. As Notch1 and AMPK are both relevant molecules for metabolic haemostasis (35-37), further studies might uncover novel therapeutic targets for TAK management. Also, TAK patients exert a specific Tfh cell signature in circulating and aorta-infiltrating CD4 T cells (38), while the potential role of AMPK in such T cell lineages remains inclusive. In addition, biological agents that are used in TAK treatment are capable of remedying certain vascular lesions (39), suggesting specific treatment such as steroids and methotrexate could influence the results obtained. Herein, although we observed that TAK T cells showed higher p-AMPK and untreated TAK T cells tended to exert higher p-AMPK than those under concomitant treatments (Supplementary Fig. S5), the possible effect of the specific treatment still requires successive studies.

In summary, AMPK is highly activated in vasculogenic T cells of TAK patients and is critically involved in maintaining the survival of such T cells. Mechanistically, Notch1 drives the AMPK activation in vasculogenic T cells from TAK patients, assigning Notch1 and AMPK as promising targets for controlling vasculitis in clinical patients.

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