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Loss of AMPK exacerbates experimental autoimmune encephalomyelitis disease severity

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ABSTRACT

AMP-activated protein kinase (AMPK) is an energy sensing metabolic switch in mammalian cells. Here, we report our novel finding that AMPK is lost in all immune cells of experimental autoimmune encephalomyelitis (EAE), an inflammatory disease of Central Nervous System (CNS). AMPKα1 is predominantly expressed in T cells and antigen presenting cells (APCs), which are primarily involved in EAE disease progression. AMPK is lost at protein level in spleen macrophages, total T cells and their subsets (CD4, CD8 and regulatory T cells) isolated from EAE afflicted animals compared to control, without affecting its mRNA levels suggesting that the loss of AMPK protein is the result of posttranscriptional modification. To examine its pathological relevance in inflammatory disease, EAE was induced in wild type (+/+) and AMPKα1 null mice (−/−) using MOG_{35-55} peptide. AMPK$\alpha1^{-/-}$ mice exhibited severe EAE disease with profound infiltration of mononuclear cells compared to wild type mice however, AMPKα2 is not involved in enhancing the severity of the disease. Spleen cells isolated from AMPK$\alpha1^{-/-}$ immunized mice exhibited a significant induction in the production of IFNγ. Our study identifies AMPK as a down regulated target during disease in all immune cells and possibly restoring AMPK may serve as a novel therapeutic target in autoimmune diseases like multiple sclerosis (MS).

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Introduction

AMP-activated protein kinase (AMPK) is a phylogenetically conserved intracellular energy sensor which plays a central role in the regulation of glucose and lipid metabolism [1]. It is a heterotrimeric complex enzyme comprising of a catalytic (α1 or α2), a regulatory (β1 or β2), and an AMP-binding regulatory (γ1, γ2, or γ3) subunits [1]. AMPK gets activated by alterations in the AMP:ATP ratio in response to energetic stress and requires phosphorylation of Thr^{172} in the activation loop of the α catalytic subunit [2]. Once activated, AMPK induces ATP-generating catabolic pathways including glucose and fatty acid oxidation, while inhibiting ATP-consuming anabolic pathways including cholesterol, fatty acid, and triacylglycerol synthesis [1,3,2]. Three upstream kinases have been identified as activators of AMPK, the tumor suppressor LKB1, calcium/calmodulin-dependent protein kinase (CaMKK) and TGF-beta-activated kinase-1 (TAK1) [4–6].

MS is an inflammatory autoimmune demyelinating disease of CNS, in part, mediated by myelin-specific CD4 T cells [7]. Different classes of immunomodulatory drugs with distinct mechanisms of action have been approved for MS treatment [8–10]. However, current MS medications are either partially effective with significant side effects or less effective for long term treatment [11,12]. Therefore, identification of novel targets for developing a new class of drugs is essential, which can be used for MS therapy alone or in combination with existing drugs.

In the present work, we have investigated the status of AMPK in immune cells during EAE disease and its possible role in disease pathology.

Materials and methods

Animals. Female 6- to 8-wk-old C57BL/6 and SJL mice were obtained from National Cancer Institute (NCI), and were housed in

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pathogen free conditions. The generation of AMPKα1(−/−) and α2(−/−) mice has been described previously [13–15]. AMPKα1(−/−)(background of C57B6/129), α2(−/−) mice (background of C57B6) and their respective wild type (WT) mice were maintained on a 12:12-h light–dark cycle and received standard rodent chow and water *ad libitum*. Genotyping was performed by PCR using DNA from a tail-piece as described before [13–15]. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina, Charleston. Paralyzed mice were afforded facile access to food and water.

Peptide, reagents and cell culture. Myelin proteolipid protein peptide ($PLP_{139-151}$) (HSLGKWLGHPDKF) and myelin oligodendrocytes protein peptide (MOG_{35-55}) (MEVGWYRSPFSRVVHLYRNGK) were purchased from Peptide International Inc. Louisville, KY USA. The anti-pAMPKα, pACC and AMPKα antibodies were purchased from Cell Signaling. The anti-AMPKα1, β1/2 and -γ1 and -γ2 Abs were purchased from Epitomics (Burlingame, CA).

EAE induction, Histology and recall response. AMPK null mice ($\alpha1^{-/-}$ or $\alpha2^{-/-}$) and respective WT mice (6–8 wk old) were immunized on day 0 and 7 by subcutaneous (s.c.) injections in the flank region with total 100 μl of emulsion containing MOG_{35-55} peptide along with killed *Mycobacterium tuberculosis* H37Ra (400 μg). In case of EAE induction in SJL mice, $PLP_{139-151}$ (100 μg/mouse) was used. Each mouse additionally received 200 ng of pertussis toxin (Sigma) by intravenous (i.v.) injection in 300 μl of PBS on day 0 and 7 of immunization and clinical disease was monitored as described earlier [16]. H&E staining of the lumbar region of the spinal cords were performed as described earlier [16]. Myelin MOG_{35-55}-immune spleen cells (2×10^5/100 μl/well) isolated from WT and AMPKα1$^{-/-}$ mice were cultured in the presence of MOG_{35-55} (25 μg/ml). Cell proliferation and the production of cytokines (IFNγ and IL-17) were examined as before [16].

Separation of subpopulation of T cells. Mice were killed at the peak of EAE disease (day 20) and spleens were removed, the single cell suspension was prepared. Red blood cells (RBC) were lysed by 1× pharmalyse and then washed twice with RPMI-1640. Finally, the cells were resuspended in RPMI complete media and counted. Total CD3 T cells were enriched using T cells enrichment columns (R&D Systems) as per manufacturer's instructions. Different subpopulation of T cells (CD4, CD8 and CD4CD25) from control and EAE mice were enriched using MagCellect CD4, CD8 and CD4CD25 regulatory T cells isolation kits, respectively (R&D Systems).

Immunoblot analyses. Cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.5% Nonidet P-40] containing a protease inhibitor cocktail (Sigma) and 50 μg of proteins was used for immunoblot analysis of pAMPKα1, pACC, AMPKα, AMPKα1 and β actin using their specific antibodies as described before [16,17].

AMPK kinase assay. AMPK activity was assayed by immunoprecipitation followed by kinase assay using recombinant ACC protein (Upstate Biotech) as a substrate as described before [16,17].

Nucleotide assay. Adherent, non-adherent and T cells from control and EAE mice were lysed in perchloric acid as described [18]. These extracts were neutralized with 1.5 M KOH/KHCO3 and then separated by high-performance liquid chromatography to measure ATP levels [18].

Statistical analyses. GraphPad Prism software (GraphPad Software Inc.) was utilized throughout for statistical analysis. Kruskal–Wallis test and Student's *t*-test were employed to analyze clinical disease score. Statistics for densitometric values comparison for proliferation and cytokine responses were analyzed with one-way multiple-range ANOVA and Student's *t*-test. A value of $p < 0.05$ and above was considered significant.

Results and discussion

Expression of AMPK subunits and their isoforms

We first examined the expression of different subunits of AMPK and their isoforms in T cells, antigen presenting cells (macrophages, dendritic and endothelial cells) and mouse brain (Suppl. Fig. S1A). The expression of α1, β1 and γ2 was found to be predominant in all the cells examined; however, expression of α2 was detected only in the brain (Suppl. Fig. S1A). Since, EAE disease is primarily mediated by T cells, therefore, we examined its expression in freshly isolated subsets of CD3 T cells (CD4, CD8 and CD4CD25) without any stimulation. The expression of α1, β1, γ1 and γ2 was predominantly expressed in CD4, CD8 and in regulatory (CD4CD25) T cells (Suppl. Fig. S1B). These results were further supported by quantitative PCR using specific primers of AMPK subunits and their isoforms (Suppl. Fig. S1 C).

AMPK activity was down regulated during EAE disease progression

To examine the status of AMPK under inflammatory disease condition, we measured AMPK activity and its phosphorylation in CNS at the peak of EAE disease (day 20). AMPK activity was determined by immunoprecipitation with AMPKα antibodies followed by kinase assay using recombinant Acetyl CoA Carboxylase (ACC) as a substrate. AMPK activity was significantly downregulated at the peak of EAE disease in brain compared to control mice (Fig. 1 A). Similarly, phosphorylation of both AMPK and its bona fide substrate ACC was decreased in lymph nodes under EAE disease (Fig. 1 B). AMPK activity examined by kinase assay in total spleen cells was also found significantly reduced (Suppl. Fig. S2). We further analyzed the expression of AMPK and observed that the protein levels of AMPKα1, -β1/2 and -γ1 subunits were all significantly decreased in spleen cells isolated from EAE except -γ2 (Fig. 1C). This data strongly suggests that AMPK activity gets downregulated during EAE disease in lymphoid and non lymphoid organs. Interestingly, the decreased AMPK activity was due to the loss of AMPKα1 and other subunits (β1/2 and γ1) at protein levels without any changes in their mRNA expression (Data not shown) suggesting that AMPK is regulated at protein translation step or degradation. This also raises the question about the mechanism of regulation of AMPK protein levels during EAE disease. Whether inflammation is a causative effect or an upstream event in loss of AMPK or *vice versa*? Recent studies suggest that AMPK and AMPK-related kinases can be regulated by ubiquitin-dependent proteosome degradation [19,20], which may be one of the mechanisms of its regulation in immune cells during EAE.

To further investigate whether the inhibition of AMPK activity was confined to *in vivo* inflammatory condition or it could be mimicked *in vitro,* a macrophage cell line (Raw 267.4) was used and the inflammatory responses were stimulated with LPS/IFNγ (1 μg/50 ng/ml) for 18 h followed by examination of phosphorylation of AMPK and ACC. The stimulus with LPS/IFNγ downregulated AMPK activity as documented by decreased phosphorylation of AMPKα and ACC (Suppl. Fig. S3Ai). To mimic EAE disease, we generated Th1 conditioned media from *in vitro* stimulated CD4 T cells and macrophage cells were treated with Th1 media (1:20 dilution) for 18 h. Interestingly, phosphorylation of AMPKα and ACC was observed very low compared to untreated cells (Suppl. Fig. S3Aii). Protein levels of AMPK-α1, -β1 and -γ1 subunits were slightly decreased in Raw cells treated with LPS/IFNγ or Th1 media (Suppl. Fig. 3 Ai&ii). Since microglia are resident APC in the CNS and further activates infiltrated T cells during disease, therefore, we examined AMPK in these cells under inflammatory condition. For this, microglial cell line (BV2) was treated with Th1 conditioned media

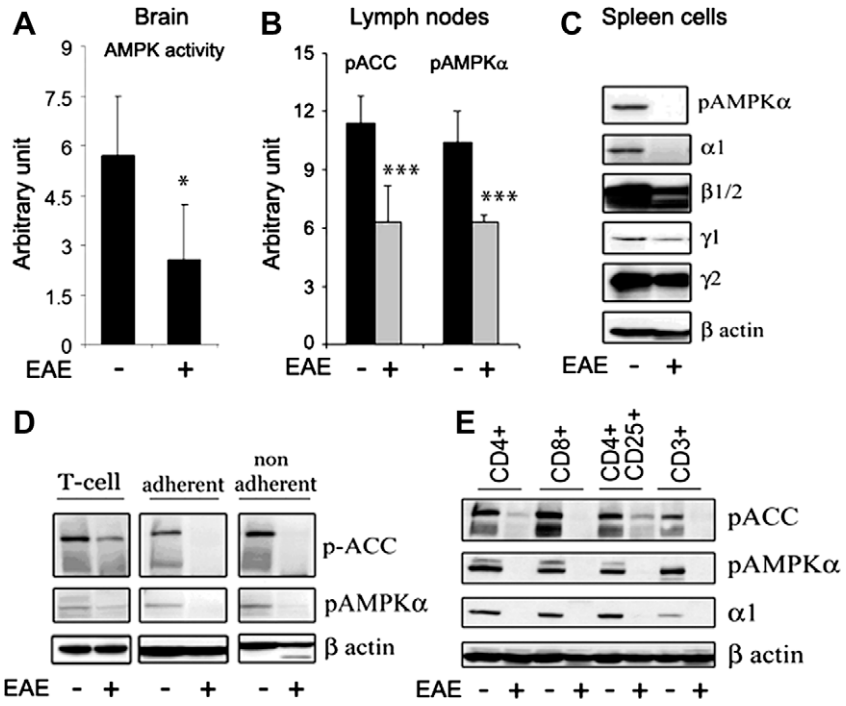

Fig. 1. AMPK was down regulated at the peak of EAE disease. (A) EAE was induced in C57BL/6 mice using MOG_{35-55} peptide and at the peak of disease, mice were killed and AMPK activity was examined in brain homogenate using AMPKα antibody followed by kinase assay as described in methods. $^{*}P < 0.05$ compared to control mice ($n = 3$). (B) Lymph nodes were isolated from control and EAE (SJL) mice at the peak of disease (Day 20) and phosphorylation of ACC and AMPKα was examined by immunoblot and densitometry analysis of pACC and pAMPKα were normalized with β actin. $^{***}P < 0.001$ compared to control mice ($n = 3$). (C) Immunoblot of various proteins including pAMPKα, -α1, -β1/2, -γ1, -γ2 and β actin were performed in the lysate isolated from spleen cells from control and EAE mice (C57B6) as described above (A). Blots are representation of three independent experiments. (D) Spleen cells were isolated from the control and EAE diseased mice (B6) at peak of disease (day 20) and T cells, adherent and non-adherent cells were fractioned as described in method. Phosphorylation of ACC and AMPKα was examined using immunoblot analysis. (E) Subpopulations of CD3 T cells (CD4, CD8 and CD4CD25) were isolated from control and EAE mice (B6) and processed for analysis of pACC, pAMPKα, α1 and β actin by immunoblot using their specific antibodies as described in methods.

at different dilutions (from 5 to 100) for 24 h. The phosphorylation of AMPK and ACC was decreased with the increase of Th1 conditioned media (Suppl. Fig. S3B). Protein levels of AMPK-α1, -β1 and -γ1 subunits were not changed in BV2 treated with Th1 conditioned media. These data strongly suggest that AMPK activity is down regulated under *in vitro* and *in vivo* inflammatory disease conditions.

AMPK was down regulated in sub-fractions of spleen cells during EAE disease

Spleen cells are comprised of mainly T cells, B cells, macrophages and dendritic cells. Since we observed the downregulation of AMPK in total spleen cells during EAE disease progression, we further examined its activity in subpopulations of spleen cells. For this, we separated spleen cells isolated from control and EAE mice in three different fractions: (1) adherent cells (mainly macrophages); (2) enriched CD3 positive T cells; and (3) non-adherent cells (total lymphocytes). We observed that phosphorylation of ACC and AMPKα was reduced in all fractionated cells isolated from total spleen cells including CD3 T cell, adherent and non-adherent cells as evident from immunoblot analysis of pACC and pAMPKα (Fig. 1D). We further examined the phosphorylation of ACC and AMPKα in total T cells (CD3) and its subsets (CD4, CD8 and CD4CD25) from control and EAE diseased mice. As depicted in Fig. 1E, we observed the inhibition of phosphorylation of ACC and AMPKα in total T cells (CD3) and their subsets, which was found due to the loss of AMPKα1 protein levels in EAE disease compared to control mice. These results indicate that reduced AMPK activity is a general phenomenon in immune cells during EAE disease process. Our findings are in contrast to others where they have shown the activation of AMPK by antigen receptor and Ca2 + in T lymphocytes mediated by CaMKK [21]. The difference in the outcome of their and our study may be due to the nature of stimulation as they have used PMA/ionomycin or TCR engagement by CD3 ligation (*in vitro* studies), whereas we have examined the AMPK in immune cells *in vivo* under disease condition without any *in vitro* stimulation. Our results have direct relevance to the inflammatory autoimmune disease like EAE/MS and may be implicated to other inflammatory diseases.

A well characterized mechanism of AMPK activity modulation is the alteration of cellular energy levels reflected by an altered AMP:ATP ratio [1]. Therefore, we investigated the energy status in immune cells under disease by measuring the intracellular ATP and AMP levels in total spleen cells, spleen macrophage and CD3 T cells isolated from control and EAE mice. Higher levels of ATP was detected in total spleen cells (10.3 vs. 6.9 nmol/10^6 cells, $P < 0.001$) and in spleen macrophage cells (10.8 vs. 5.46 nmol/10^6 cells, $P < 0.001$), however, no change was found in ATP levels in T cells (7.7 vs. 8.6 nmol/10^6 cells, $P < 0.089$) isolated from EAE mice compared to the control (Suppl. Fig. S4). We were unable to detect AMP levels in total spleen cells, CD3 and spleen macrophage cells suggesting these cells exhibit a normal energy status in EAE mice.

AMPKα1 null mice (−/−) exhibited exacerbated EAE disease severity compared with WT littermates

Since we observed loss of AMPKα1 at protein level in all immune cells, next we examined the effect of AMPKα$1^{-/-}$ gene disruption on the development of EAE disease using AMPKα1 null mice. EAE was induced in AMPKα$1^{-/-}$ and WT mice following active immunization with MOG_{35-55}. As shown in Fig. 2 and in Table

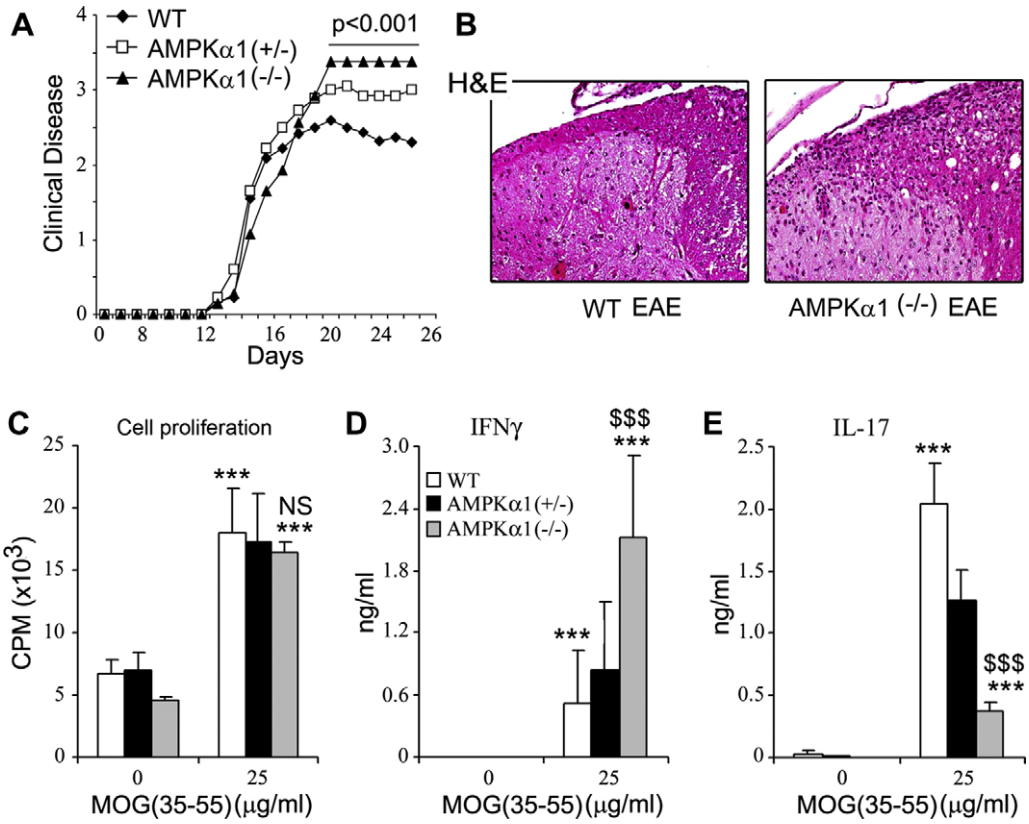

Fig. 2. AMPKα1$^{-/-}$ mice exhibited severe EAE disease compared to WT. (A) Active EAE was induced in AMPKα1$^{-/-}$ mice and WT littermate (C57B6/129) with immunization of 100 μg of peptides MOG_{35-55} on day 0 and 7. The clinical symptoms were scored every day in a blinded manner. Data points are presented as the mean ± SEM. $P < 0.001$ refers to wild type EAE mice (two-way ANOVA). Numbers of mice used in the study are summarized in Table 1. (B) Spinal cords (lumbar regions) were harvested at the peak day of the disease from AMPKα1$^{-/-}$ and WT mice. Tissues were fixed (10% buffered formalin) and embedded in paraffin, and the paraffin sections were stained with H & E and visualized at 10× to determine infiltration. (C) At the peak of EAE disease, spleens cells from AMPKα1$^{-/-}$, AMPKα1$^{+/-}$ and WT mice were isolated and stimulated *ex vivo* with MOG_{35-55} (25 μg/ml) peptide for 48 h and than [3H]-thymidine was added to the cells and incubated for additional 18 h and counted for cell proliferation. (D and E) The culture supernatants of spleen cells were collected at 72 h and analyzed for inflammatory IFNγ and IL17 by OptEIA system. $^{***}p < 0.001$ compared with MOG_{35-55} untreated spleen cells; $^{\$\$\$}p < 0.001$, NS, not significant from control MOG_{35-55} treated spleen cells.

Table 1
EAE in AMPKα1 WT, heterozygote and in homozygous littermates.

	Number of mice	Incidence	% Incidence	Mean maximum score at peak	Peak day
WT	14	14/14	100	2.59 ± 0.35	20
AMPKα1 (+/−)	13	13/13	100	3.05 ± 0.37**	21
AMPKα1 (−/−)	7	7/7	100	3.37 ± 0.10***	20

Active EAE was induced in AMPKα1 WT, heterozygote and in homozygous mice with MOG_{35-55} peptide. Total number of mice used in study, incidence, clinical disease score and peak day are reported in this table. Mean maximum score are given at the peak of disease. $^{***}P < 0.001$, $^{**}P < 0.01$ as compare with WT EAE (Student *t*-test).

1, the severity of disease was enhanced in AMPKα1$^{-/-}$ mice (3.37 ± 0.1; $p < 0.001$) compared with their WT littermates (2.59 ± 0.35) without any change in the onset of the disease (Table 1). The development of severe paralytic symptoms was apparent in AMPKα1$^{-/-}$ by day 16 with the maximal disease severity score of 3.37 being observed till end of the study (day 26). Although all mice in both groups succumbed to the disease, there was no mortality associated with the acute attack. The hallmark of EAE disease is the infiltration of inflammatory cells into the CNS, leading to tissue damage [22]. Therefore, we examined the status of infiltration of inflammatory cells into the CNS of WT and AMPKα1$^{-/-}$ mice. As shown in figure 2B, AMPKα1$^{-/-}$ EAE mice showed profound infiltration of immune cells in the CNS compare to WT mice. We also examined EAE disease induction in AMPKα2$^{-/-}$ mice and did not observe any significant change in EAE clinical score in AMPKα2$^{-/-}$ mice compare with WT mice (Supp. Fig. S5), suggesting that α2 isoform does not play role in EAE pathology. Induction of EAE disease predominantly involved the participation of APCs and T cells to induce inflammatory cascade, which express mainly α1. Our novel observations, the loss of AMPKα1 during EAE disease and exacerbation of EAE clinical symptom in AMPKα1$^{-/-}$ null mice suggesting a critical role of AMPK in the regulation of inflammatory disease progression.

To confirm that AMPKα1$^{-/-}$ mice were sensitized to MOG_{35-55} peptide, we measured the Ag-induced T cell proliferation *ex vivo*. As shown in Fig. 2C, *in vitro* culture of spleen cells from WT and AMPKα1$^{-/-}$ mice showed an antigen-dependent proliferation with no significant change suggesting that the severity of disease in AMPKα1$^{-/-}$ mice is not due to the higher proliferation of MOG-specific T cell responses. Further, in response to Ag stimulation, spleen cells from WT and AMPKα1$^{-/-}$ mice induced production of IFNγ and IL17 (Fig. 2 *D&E*), however, AMPKα1$^{-/-}$ cells produced higher amount of IFNγ with lesser IL17 compare with WT cells. Spleen cells isolated from AMPKα1$^{-/-}$ mice displayed normal cell proliferation and pro-inflammatory cytokines production suggesting that loss of AMPK did not affect the normal cellular functions

which is consistent with a recent report [23]. However, loss of AMPKα1 enhances the production of IFNγ with lesser production of IL17 compare with WT mice required further detail study to establish the role of AMPKα1 in modulation of pro-inflammatory cytokines under inflammatory environment. Loss of AMPKα1 expression in all immune cells and the higher production of pro-inflammatory cytokines during EAE disease indicate an inverse correlation. But, to establish this relationship, enzymatic active AMPKα1 has to be restored in immune cells and examine if levels of cytokines and EAE disease course can be modulated.

Altogether, our study identified for the first time that energy sensor is lost during disease in all immune cells and its genetically deficient mice exhibits an increased severity of EAE disease suggesting its critical role in inflammatory disease progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.106.

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