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Phosphoenolpyruvate Regulates the JunB-Dependent Pathogenic Th17 Transcriptional Program

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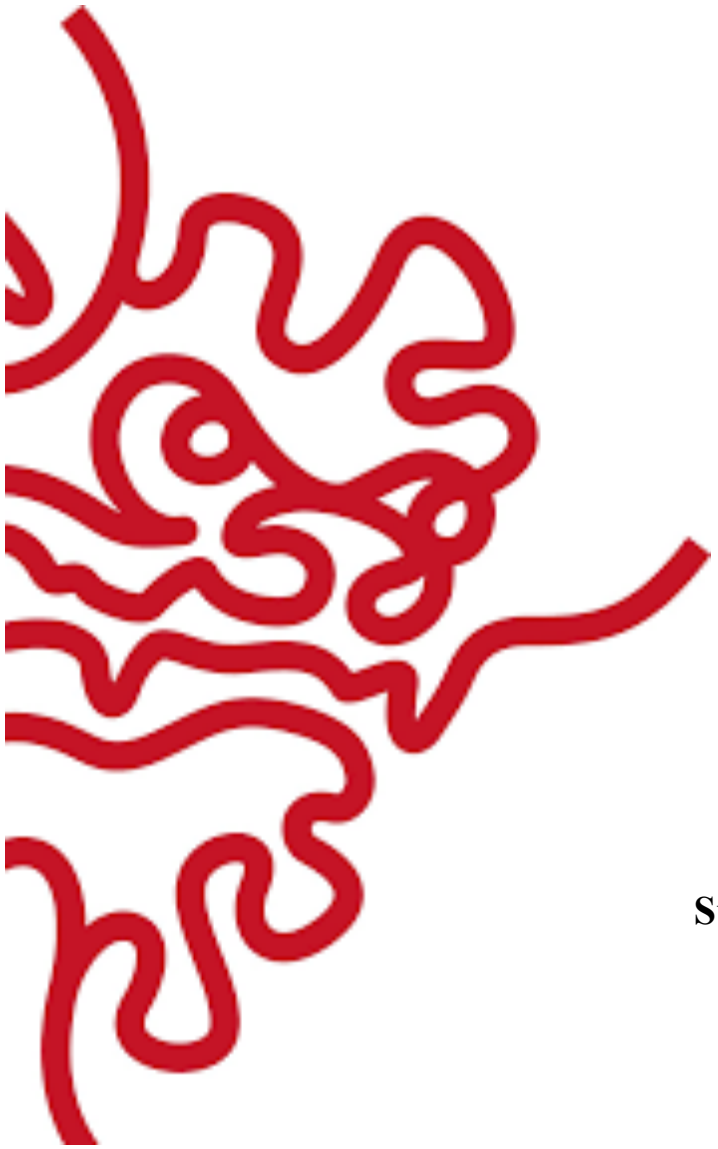

by

Tsung-Yen Huang

Supervisor: Hiroki Ishikawa

February 21, 2023

Declaration of Original and Sole Authorship

I, Tsung-Yen Huang, declare that this thesis entitled “Phosphoenolpyruvate regulates the JunB-dependent pathogenic Th17 transcriptional program” and the data presented in it are original and my own work.

I confirm that:

- No part of this work has previously been submitted for a degree at this or any other university.
- References to the work of others have been clearly acknowledged. Quotations from the work of others have been clearly indicated, and attributed to them.
- In cases where others have contributed to part of this work, such contribution has been clearly acknowledged and distinguished from my own work.
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Abstract

Aerobic glycolysis, a metabolic pathway essential for effector T cell survival and proliferation, regulates the differentiation of autoimmune T helper (Th)17 cells, but the mechanism underlying this regulation is largely unknown. Here, we identify a glycolytic intermediate metabolite, phosphoenolpyruvate (PEP), as a negative regulator of Th17 differentiation. PEP supplementation or inhibition of downstream glycolytic enzymes in differentiating Th17 cells increases intracellular PEP levels and inhibits the expression of Th17 signature molecules, such as IL-17A. However, PEP supplementation does not significantly affect metabolic reprogramming, cell proliferation, and survival of differentiating Th17 cells. Mechanistically, PEP regulates the JunB-dependent pathogenic Th17 transcriptional program by inhibiting the DNA-binding activity of the JunB/BATF/IRF4 complex. Furthermore, daily administration of PEP to mice inhibits the generation of Th17 cells and ameliorates Th17-dependent autoimmune encephalomyelitis. These data demonstrate that PEP links aerobic glycolysis to the JunB-dependent pathogenic Th17 transcriptional program, suggesting the therapeutic potential of PEP for autoimmune diseases.

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Abbreviations

2-DG	2-deoxy-D-glucose
2-HG	2-hydroxyglutarate
2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
α-KG	α-ketoglutarate
AA	Amino acid
ACC1	acetyl-CoA carboxylase 1
acetyl-CoA	acetyl coenzyme A
AhR	Aryl hydrocarbon receptor
AICE	AP-1-IRF4 composite element
AMPK	AMP-activated protein kinase
AP-1	Activator Protein 1
APC	Antigen-presenting cells
ATAC-seq	Assay for transposase-accessible chromatin using sequencing
ATP	Adenosine triphosphate
BATF	Basic leucine zipper ATF-like transcription factor
BCL-2	B-cell lymphoma2
BCL-6	B-cell lymphoma 6
C/EBP	CCAAT-enhancer-binding proteins
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
ChIP	Chromatin immunoprecipitation
CIA	Collagen-induced arthritis
CIP	calf intestinal phosphatase
CNS	Central nervous system
CPT	Carnitine palmitoyl transferase
CTLA 4	Cytotoxic T lymphocyte-associated protein 4
CXCL	Chemokine C-X-C motif ligand 1
CXCR	C-X-C chemokine receptor type
DEG	Differentially expressed genes
EAE	Experimental autoimmune encephalomyelitis
ECAR	Extracellular acidification rates
EOMES	Eomesodermin
F1,6BP	fructose 1,6 bisphosphate
F6P	fructose-6-phosphate
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FOXP3	Forkhead box P3

G3P	Glyceraldehyde 3-phosphate
G6P	Glucose 6-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA-binding protein 3
GLS	Glutaminase
GLUT	Glucose transporter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOT1	Glutamate oxaloacetate transaminase 1
GPI	Glucose-6-phosphate isomerase
HA	Heptelidic acid
HA-tag	Human influenza hemagglutinin-tag
HIF1a	Hypoxia-inducible factor 1-alpha
HK	Hexokinase
HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
iTreg cell	inducible regulatory T cell
LAG-3	Lymphocyte-activation gene 3
LDHA	Lactate dehydrogenase A
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MUFA	Monounsaturated fatty acid
NAD	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T-cells
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
npTh17	Non-pathogenic Th17
OCR	Oxygen consumption rates
OVA	Ovalbumin
OXA	Oxalate
OXPHOS	Oxidative phosphorylation
PCK1	Phosphoenolpyruvate carboxykinase 1
PD-1	Programmed cell death protein 1
PEP	Phosphoenolpyruvate
PGAM	Phosphoglycerate mutase
PI3K	Phosphoinositide 3-kinase

PKM	Pyruvate kinase muscle isozyme
PPP	Pentose phosphate pathway
pTh17	Pathogenic Th17
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
ROR	Retinoid-related orphan receptor
ROS	Reactive oxygen species
RPTOR	Regulatory Associated Protein Of MTOR Complex 1
RT-qPCR	Quantitative reverse transcription PCR
RUNX1	Runt-related transcription factor 1
SAM	S-adenosyl methionine
SERCA	Sarco/endoplasmic reticulum Ca2+ ATPase
SFA	Saturated fatty acid
SFB	Segmented filamentous bacteria
SGK	Serum glucocorticoid kinase
SLC	Solute carrier
SLE	Systemic lupus erythematosus
SOCS3	Suppressor of cytokine signaling 3
SREBP	Sterol regulatory element-binding protein
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
Tbx21	T-Box Transcription Factor 21
TCA	Tricarboxylic acid
TCR	T-Cell Receptor
Tfh cell	Follicular T helper cell
TGF	Tumor growth factor
Th cell	T helper cell
TNF	Tumor necrosis factor
TPI1	Triosephosphate Isomerase 1
Tr1 cell	T regulatory type 1 cell
TRAF6	TNF receptor associated factor 6
Treg cell	Regulatory T helper cell
TRIM	Tripartite motif containing
TSC2	Tuberous sclerosis complex 2

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Chapter 1: Introduction

1.1. An overview of the adaptive immune system

The mammalian immune system comprises innate and adaptive immunity, which collaborate to protect the body from pathogen infection. Innate immunity is the first line of defense against pathogen invasion. It includes the anatomical barriers of skin and mucous membranes, the complement system, and several types of leukocytes including mast cells, dendritic cells, macrophages, granulocytes, natural killer (NK) cells, and innate lymphoid cells [1, 2]. Innate immunity quickly responds to invading pathogens by recognizing molecules common to a wide variety of pathogens, called pathogen-associated molecular patterns (PAMPs). Innate immunity is also responsible for the induction of adaptive immunity, immune responses tailored to pathogen-specific antigens.

Adaptive immunity plays an essential role in pathogen clearance from the body when innate immunity cannot eliminate them [1]. Although activation of adaptive immunity requires several days, it provides an effective defense by identifying, eliminating, and remembering invading pathogens and toxic substances. Adaptive immunity is mediated by two types of lymphocytes: B (bursal or bone marrow-derived) cells and T (thymus-derived) cells. B cells mediate humoral immunity by producing antibodies, while T cells differentiate into functionally distinct subsets and participate in various immune responses.

There are two well-defined subpopulations of T cells: CD8 T cells and CD4 T cells, so named because they express surface glycoproteins CD8 and CD4, respectively. Effector CD8 T cells are known as cytotoxic T cells because they kill cells infected with intracellular pathogens and cancer cells. On the other hand, CD4 T cells, which can be categorized into various subsets, play essential roles in the control of other immune cells involved in adaptive immune responses. The functions of different CD4 T cell subtypes are further discussed in the next sections.

1.2. CD4 T Cell subsets

CD4 T cells orchestrate adaptive immune responses by releasing small proteins called cytokines, which modulate a variety of cell behavior. Cytokines can be divided into proinflammatory and anti-inflammatory cytokines according to their functions. Proinflammatory cytokines, including IFN-γ, IL-1β, and IL-6, enhance inflammatory response by promoting proliferation, phagocytosis, and free radical production of innate immune cells. These cytokines are also necessary for the maturation and differentiation of B cells and T cells. In contrast, anti-inflammatory cytokines, such as IL-10 and TGF-β, suppress inflammatory responses through various mechanisms, such as inhibiting the secretion of proinflammatory cytokines in antigen-presenting cells (APC) and promoting T cell exhaustion [1,3-6].

CD4 T cells can be divided into several different T helper (Th) subsets or T regulatory (T reg) cells based on the expression of cytokines or transcription factors characteristic of each subset [3-6]. Th cells, also known as conventional CD4 T cells, help various immune cells including B cells and CD8 T cells in immune responses. In contrast, Treg cells suppress immune responses and thus play an essential role in resolving inflammatory responses and preventing autoimmunity.

Th subsets and Treg cells are derived from naïve CD4 T cells. Naïve CD4 T cells are kept in a metabolically inactive state and incapable of regulating immune response until stimulated with specific antigen [7]. Naive CD4 T cells must receive three essential stimuli for their activation

and differentiation to effector subsets: (1) the interaction between their T cell receptors (TCRs) and MHC II-antigen complexes on antigen-presenting cells (APCs), (2) the costimulatory signal provided by the interaction between CD28 and CD80/CD86, (3) cytokines secreted by APCs or other immune cells. When naïve CD4 T cells are activated by specific antigens presented by APCs with costimulatory signals, they rapidly increase cell size and proliferation rate and differentiate into specific Th or Treg subtypes according to environmental cytokines [8]. Cytokines activate signal transducers and activators of transcription (STAT) family proteins, which in turn induce expression of lineage-specifying transcription factors [9] that drive expression of genes required for differentiation and maturation of each CD4 T cell subsets [9-11].

1.3. Differentiation and functions of Th subsets and Treg cells

In this section, I will briefly discuss the differentiation and functions of major CD4 T cell subsets other than Th17 cells, and the details of Th17 cell differentiation and function, the main topic of this paper, will be discussed in the next chapter. Th subsets and Treg cells discussed in this thesis are shown in Fig.1.1.

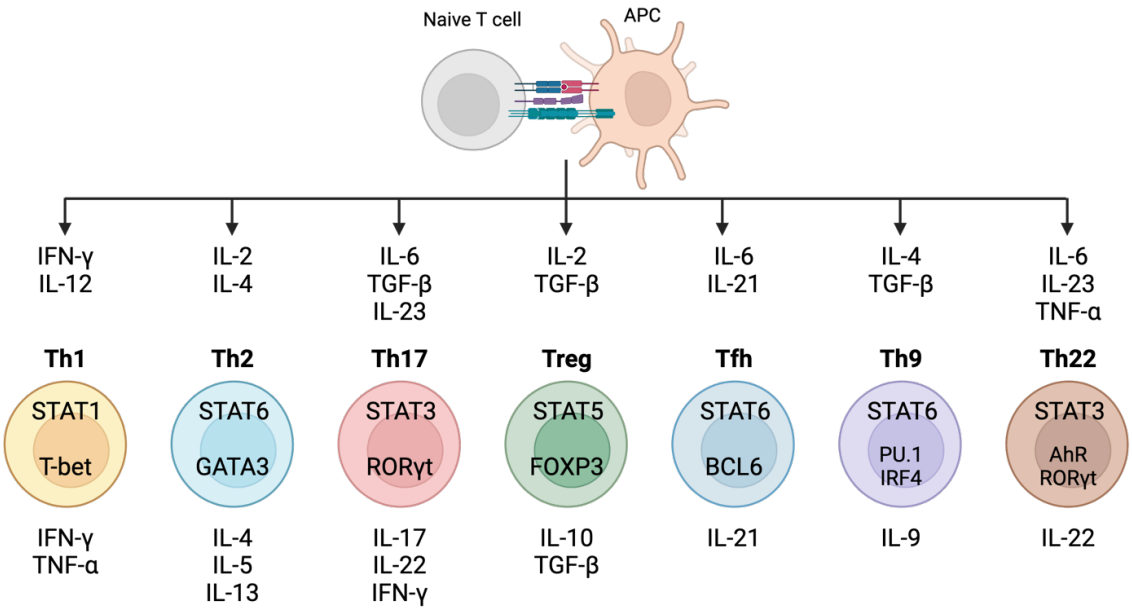


Fig. 1.1. The differentiation and function of CD4 T cell subsets
After receiving antigen stimulation, naïve CD4 T cells differentiates into various effector Th subsets according to the environmental cytokines. The cytokines and signaling pathways required for the differentiation of the major CD4 T cell subsets, and their signature cytokines and transcription factors, are shown in the figure.

Th1 cells are preferentially generated during intracellular bacteria or protozoa infections[11]. In response to these pathogens, APCs induce the expression of IL-2, IL-12, and IFN-γ. These cytokines activate STAT1 and STAT4 and lead to the expression of the Th1-lineage defining transcription factor, T-bet, in naïve T cells stimulated with specific antigens [11-14]. Th1 cells facilitate cell-mediated immune responses mainly by secreting proinflammatory cytokines IFN-

γ and TNF-α. IFN-γ promotes differentiation of M1 macrophages, which secret proinflammatory cytokines and produce nitric oxide and reactive oxygen intermediates to mediate antimicrobial response [15-17]. IFN-γ and TNF-α also facilitate immune responses by recruiting CD8 T cells and NK cells to infected or tumor tissues [18,19].

Th2 cells are mainly generated against threats of helminths, extracellular bacteria, and toxins [11]. Th2 differentiation is promoted by IL-4 signal, which activates STAT6, thereby inducing expression of the Th2-lineage defining transcription factor, GATA3 [11,20-22]. The signature cytokines of Th2 cells, including IL-4, IL-5 and IL-13, play important roles in promoting the proliferation of B cells and the production of IgE [23,24], which can trigger the degranulation of mast cells to release inflammatory mediators including histamine [25]. IL-4 and IL-13 also promote the differentiation of M2 macrophages, which produce extracellular matrix components essential for wound healing [17,26,27]. Th2 cells also secrete IL-5, which promotes the maturation of eosinophils to control helminth infection [28]. However, overactivation of Th2 cells causes mastocytosis, allergy and allergic asthma [20,28,29].

Tfh cells are critical for B cell responses and the maintenance of germinal centers (GCs), critical microstructures for maturation of plasma cells and memory B cells in the secondary lymphoid tissue [30]. IL-21, a cytokine produced by Tfh cells, supports the proliferation of differentiating B cells [30]. CD40-ligand (CD40-L) expressed on Tfh cells is a costimulatory molecule interacting with CD40 on differentiating B cells, which promotes B cell maturation, including somatic hypermutation, class switching, and affinity maturation [3,4,30]. Differentiation of Tfh cells requires IL-6 and IL-21 signal, which induces expression of the Tfh lineage-defining transcription factor, BCL-6, through STAT3 activation [31-33]. BCL-6 promotes the expression of Tfh signature molecules, including IL-21, IL-21 receptor (IL-21R), C-X-C chemokine receptor type 5 (CXCR5), and programmed cell death protein 1 (PD-1) [30,31].

In contrast to Th subsets, Treg cells suppress immune responses. Induced Treg (iTreg) differentiation is dependent on IL-2 and TGF-β, which activate STAT5 and induce expression of the Treg lineage-defining transcription factor, Foxp3 [34,35]. Treg cells express anti-inflammatory cytokines, IL-10 and TGF-β, to suppress the activities of innate immune cells and conventional CD4 T cells[36]. CTLA-4, a co-inhibitory molecule, on Treg cells inhibits dendritic cell activation by interacting with CD80 and CD86 on the dendritic cells. Furthermore, dendritic cell maturation is inhibited by Treg cells through lymphocyte activation gene 3 (LAG-3), another co-inhibitory molecule. In addition, Treg cells not only suppress T-cell proliferation by depleting IL-2 in the microenvironment, but also induce T-cell apoptosis by secreting perforins and granzymes. Furthermore, PD-1 ligands (PD-L1) on Treg cells interact with PD-1 on B cells and exhausted T cells, thereby inhibiting their responses [34-39].

Th9, Th22, and T regulatory type 1 (Tr1) cells can be also considered distinct CD4 T cell subsets, but their roles in immune responses are still not fully understood. Th9 cells, characterized by expression of IL-9, IL-10, and IL-21, are responsible for anti-helminth and anti-tumor immune responses and might also be involved in allergy, autoimmune diseases, and tumorigenesis [40-43].Th9 differentiation relies on IL-4 and TGF-β, which activate STAT6 and induce the lineage-specifying transcription factors, PU.1 and IRF4 [41-43]. Th22 cells, characterized by IL-22 expression, likely play both pathogenic and protective roles in autoimmune disorders. Th22 differentiation requires IL-6, IL-23, and TNF-α signal, which induces expression of transcription factors, aryl hydrocarbon receptor (AhR) and retinoid-related orphan receptor gamma t (RORγt) [3,43-45]. Tr1 cells can be induced during the resolution of inflammation or infection of *Staphylococcus aureus* in the small intestine [46]. Tr1 cells have

increased expression of Treg-signature molecules including c-MAF, AhR, LAG3, and secret IL-10. IL-27 promotes the differentiation of Tr1 cells by inducing the expression of c-MAF, which in turn promotes the production of IL-10 [46-48].

1.4. Functions of Th17 cells

Th17 cells are a subset of Th cells defined by the secretion of a proinflammatory cytokine interleukin 17 (IL-17). Pioneering studies using interleukin 23 (IL-23)-deficient mice led to the discovery of Th17 cells [49,50]. These studies demonstrated that IL-23-deficient mice are resistant to the development of experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), and the IL-17-producing CD4 T cells are dramatically reduced in IL-23-deficient mice. In 2005, CD4 T cells expressing IL-17 were identified as a new T helper cell subset that promotes chronic inflammation and autoimmunity in mice [51,52].

The primary function of Th17 cells is to mediate adaptive immune responses against extracellular bacteria and fungi at the mucosal surfaces of the intestine, skin, and lungs. Patients with mutations in genes required for Th17 generation are more susceptible to *Candida albicans* [53-55] and *Staphylococcus aureus* [55-57]infections. Th17 cells regulate anti-pathogen responses mainly through IL-17, which acts on epithelial cells, mesenchymal cells, and neutrophils at the infection site [55,58-60]. Once IL-17 interacts with dimeric IL-17 receptor (IL-17RA and IL-17RC) expressed on non-immune cells such as epithelial cells, it recruits signal transducers ACT1 and TRAF6, thereby activating transcription factors, NF-κB, AP-1 factors, and C/EBP family proteins. These transcription factors then promote the expression of chemokines and proinflammatory cytokines such as CXCL1 and IL-6, which recruits macrophages and neutrophils to the inflammatory sites [59-61]. Additionally, IL-17 can induce the expression of antimicrobial peptides like β-defensins to directly neutralize pathogens [62-64].

Th17 cells also play a crucial role in maintaining the barrier function of colon epithelium[65]. IL-17 induces expression of tight junction proteins, claudins and occludin, in the colon epithelial cell to maintain the epithelial integrity [66-69]. Furthermore, Th17 function is closely associated with the homeostasis of intestinal microbiota. In humans and mice, around 80% of the total plasma cells reside in the intestinal mucosa, where they secret IgA to control the intestinal microbiota [70]. IL-17 maintains levels of mucosal IgA by stimulating the expression of polymeric Ig receptors (pIgR), which is required for transepithelial transportation of IgA from the basolateral membrane to the mucosal layer [71].

Th17 cells not only play a beneficial role in host defense as described above, but also cause various autoimmune disorders [46,72]. Th17 infiltration and IL-17 expression in inflamed tissues are hallmarks of various autoimmune diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, and inflammatory bowel disease (IBD) [4,73,74]. Th17 cells detected in patients or animal models with autoimmune disorders tend to express Th1 signature molecules including T-bet, RUNX1, EOMES, and proinflammatory cytokines, IFN-γ, TNFα and GM-CSF [46,72,75-80]. Th17 cells expressing Th1 signature molecules are also observed in immune responses against extracellular pathogens in mice and humans. Exposure to *Candida albicans* and *Helicobacter hepaticus* promotes the differentiation of human naïve CD4 T cells to Th17 cells expressing IFN-γ [81,82]. The dichotomous roles of Th17 cells in beneficial and harmful immune responses results from their substantial plasticity in response to environmental signals, including cytokines, microbiomes, and nutrients [83-85], which will be discussed in the next section.

1.5. Cytokines and soluble factors that control Th17 differentiation and function

Specific combinations of cytokines can promote *in vitro* differentiation of non-pathogenic or pathogenic Th17 differentiation. For example, naïve CD4 T cells activated in the presence of TGF-β1 and IL-6 differentiate into non-pathogenic Th17 cells, whose gene expression profiles are more similar to those of gut-resident homeostatic Th17 cells than the autoimmune Th17 cells infiltrating the central nervous system (CNS) [86,87]. Adoptive transfer of MOG-specific Th17 cells differentiated *in vitro* by TGF-β1 and IL-6 cannot induce severe EAE [86,88]. However, additional IL-23 stimulation allows TGF-β1/IL-6-induced MOG-specific Th17 cells to cause severe EAE upon adoptive transfer [86]. In contrast, Th17 cells induced by IL-6, IL-1β, and IL-23 in the absence of TGF-β1 exhibit gene expression profiles similar to those of autoimmune Th17 cells infiltrating the CNS [86,87]. MOG-specific Th17 cells induced by IL-6, IL-1β, and IL-23 in the absence of TGF-β1 can also cause severe EAE [86,88]. Thus, TGF-β1 and IL-23 signals are likely associated with non-pathogenic and pathogenic Th17 cells, respectively.

Roles of Th17-inducing cytokines in Th17 generation *in vivo* and autoimmunity have been characterized using knockout mouse models. For example, the deficiency of IL-6 impairs immunization-induced Th17 generation and makes mice resistant to EAE [89]. Similar phenotypes have been observed in mice deficient for IL-1β receptor, IL-1R1 [90], whose expression is promoted by IL-6 signal [91]. These observations indicate that IL-6 and IL-1β signaling are required for Th17 differentiation *in vivo* [89,90]. On the other hand, loss of IL-23 or IL-23R does not affect early Th17 generation in immunization-induced immune responses, but impairs the accumulation of pathogenic Th17 cells expressing IFN-γ and GM-CSF in the late stage of EAE [49,50]. IL-23R expression is induced by IL-6 signal in antigen-primed naïve CD4 T cells and is promoted and maintained by an IL-23 signaling-mediated self-amplification loop [92]. These observations suggest that IL-23 signal is not required for early Th17 differentiation, but is essential for *in vivo* Th17 maturation and maintenance of pathogenic Th17 cells [93], which is consistent with results in *in vitro* Th17 polarization experiments [51,94].

TGF-β1 is known as an anti-inflammatory cytokine required for Treg differentiation, but its role in Th17 differentiation is intriguing. TGF-β1 in cooperation with IL-6 promotes murine Th17 differentiation [86,88,95,96], but its involvement in human Th17 differentiation is still under debate [97-102]. Multiple functions of TGF-β1 signaling likely contribute to non-pathogenic Th17 differentiation. For example, TGF-β1 inhibits Th1 and Th2 programs in Th17 differentiation and promotes activation of STAT3, a critical transcription factor for Th17 differentiation, by inhibiting expression of SOCS3, a negative regulator of STAT3 [103-105]. TGF-β1 also represses Th2 differentiation by inducing the expression of SOX4, a transcriptional factor negatively regulates GATA3 [106]. Furthermore, TGF-β1 signaling induces degradation of SKI, which suppresses Th17 differentiation cooperatively with SMAD4 [107]. Several molecules are likely involved in the anti-inflammatory functions of Th17 cells induced in the presence of TGF-β1. For example, TGF-β1 enhances c-MAF expression, thereby promoting the expression of the anti-inflammatory cytokine, IL-10 [108]. Moreover, TGF-β1-mediated inhibition of growth factor independent-1 (Gfi1) expression increases the expression of ectonucleotidases, CD39 and CD73, which convert ATP into adenosine, an immunosuppressive molecule for T cells and NK cells [109].

Pathogenic Th17 differentiation is promoted by TGF-β3 and inhibited by IL-24. Naïve CD4 T cells activated in the presence of TGF-β3 and IL-6 differentiate into Th17 cells with high expression of *Il23r*, *Tbx21*, and *Csf2*, and MOG-specific Th17 cells polarized with TGF-β3 and IL-6 induce severer EAE compared to TGF-β1/IL-6-induced Th17 cells[86]. On the other hand,

autocrine IL-17A induces *Il24* expression in Th17 cells through activation of NFKB1 and NFKB2, and IL-24 in turn inhibits expression of IL-17F and GM-CSF in Th17 cells in an autocrine manner and inhibits experimental autoimmune uveitis [110,111].

Other environmental factors also affect Th17 differentiation or pathogenicity. An AhR ligand, FICZ, promotes IL-17 and IL-22 expression in Th17 cells and sensitizes mice to EAE development [112]. In addition, salt (NaCl) promotes pathogenic Th17 generation by enhancing IL-23R expression through serum glucocorticoid kinase 1 (SGK1) [84]. Furthermore, serum amyloid A proteins (SAAs), which are produced in the liver in response to acute inflammation, promote the generation of pathogenic Th17 cells [113].

1.6. Transcriptional regulation of Th17 differentiation and function

IL-6-mediated STAT3 signaling is required to initiate Th17 differentiation of TCR-activated naïve CD4 T cells. STAT3 controls the expression of Th17 signature molecules such as RORγt, IL-17A, and IL-17F. IL-6 or IL-23 stimulation promotes the phosphorylation, dimerization, and nuclear translocation of STAT3 [88,114,115]. Activated STAT3 binds to the promoter regions of the target genes, such as *Rorc* (encoding RORgt), *Il17a*, and *Il17f* and promotes their expression [116]. In humans, STAT3 mutations cause increased susceptibility to fungal infection and reduced frequency of Th17 cells [117]. STAT3 recruits an epigenetic regulator Tripartite motif containing 28 (TRIM28) to the STAT3 target gene loci and increases chromatin accessibility [118].

Basic leucine zipper ATF-like transcription factor (BATF), an AP-1 subunit, and interferon regulatory factor 4 (IRF4) are also essential for Th17 differentiation. Mice lacking BATF or IRF4 cannot generate Th17 cells and are resistant to Th17-dependent autoimmunity [119,120]. Expression of BATF and IRF4 are induced by TCR signaling, and IRF4 expression is promoted by IL-1β signaling [91]. BATF and IRF4 cooperatively bind to closed chromatin regions of several Th17-signature gene loci, including *Rorc* and *Il17*, and increase their chromatin accessibility [92,121-123]. This pioneering transcription factor activity is required for the DNA-binding of other Th17-related transcription factors, including STAT3, to their target loci.

RORγt, encoded by *Rorc*, is the lineage-specifying transcription factor of Th17 cells. RORγt-deficient mice lack Th17 cells and are resistant to autoimmunity, while overexpression of RORγt is sufficient to polarize TCR-activated CD4 T cells to Th17 cells [124]. However, RORγt overexpression cannot fully rescue Th17 differentiation in BATF- or IRF4-deficient cells, suggesting that cooperation between these transcription factors is needed for Th17 differentiation [119,120]. RORγt is essential for expressing a small subset of genes upregulated by BATF, IRF4, and STAT3, including Th17 signature genes such as IL-17A, IL-17F, and IL-22 [121,124]. RORγt expression is induced by BATF, IRF4, and STAT3, but is not promoted by RORγt itself [121]. This is in contrast with positive feedback loops for T-bet and GATA3 expression in Th1 and Th2 cells, respectively [125,126], and may partly contribute to the higher plasticity of Th17 cells than Th1 and Th2 cells.

Although the mechanism to switch between pathogenic and nonpathogenic Th17 fates remains unclear, several transcription factors have been proposed to regulate Th17 pathogenicity. JunB has been characterized as a crucial transcription factor for the fate decision of pathogenic Th17 cells [127-129]. During differentiation, JunB is induced by IL-6 in a STAT3-dependent manner. Although JunB is not required for non-pathogenic Th17 differentiation, it is essential for generating IL-23-dependent pathogenic Th17 population, and *Junb* KO mice are resistant to EAE and colitis[127,128]. RBPJ, which is induced downstream of the canonical Notch signaling pathway [130], is likely another modulator of Th17 pathogenicity. RBPJ-deficient Th17 cells

showed elevated IL-10 expression in both nonpathogenic (IL-6 and TGF-β1) and pathogenic (IL-1β, IL-6, and IL-23) Th17 polarizing conditions, and reduced expression of IL-17 in the presence of IL-23. In addition, RBPJ KO mice are resistant to EAE. RBPJ directly binds to the promoter region of *Il23r* to facilitate its expression and suppresses IL-10 expression by inhibiting the c-MAF-mediated transactivation of *Il10*.

1.7. T cell metabolic dynamics

Since the 1960s, it has been observed that T cell maturation, differentiation, and function are closely linked to the regulation of cellular metabolism [131]. In recent years, it has become clear that various metabolic pathways support the survival of different T cell subsets and regulate their differentiation and function [7,132].

Peripheral naïve T cells, which exit from the thymus and circulate throughout the body, are metabolically quiescent. Naïve T cells take up low levels of glucose and rely primarily on mitochondrial oxidative phosphorylation (OXPHOS) to maintain cellular ATP levels [7]. IL-7 is required for naïve T cell homeostasis as it promotes not only the expression of antiapoptotic factor B-cell lymphoma 2 (BCL-2) [229,230], but also the membrane translocation of glucose transporter GLUT1 and glucose uptake through AKT pathway [231]. Maintenance of metabolic quiescence is crucial for the survival of naive T cells, as overactivation of the AKT pathway or deletion of tuberous sclerosis complex 1 (TSC1), a negative regulator of mammalian target of rapamycin (mTOR) signaling, results in metabolic activation and apoptosis [133]. Similarly, the deletion of menin leads to premature TCR activation through mTOR activation and impairs the effector function and memory formation in CD8 T cells [134].

TCR activation reprograms quiescent metabolic states of naïve T cells to active aerobic glycolysis in which glucose is converted into lactate even in the presence of sufficient environmental oxygen for tricarboxylic acid (TCA) cycle and OXPHOS. Aerobic glycolysis connects various metabolic pathways and is crucial for biosynthetic processes required for the growth and proliferation of effector T cells. For example, glycolytic metabolites, glucose 6-phosphate (G6P), glyceraldehyde 3-phosphate (G3P), and 3-phosphoglycerate (3PG), are used as fuel for the pentose–phosphate pathway (PPP), lipid biosynthesis, and serine biosynthesis, respectively. Moreover, the end product of glycolysis, pyruvate, can be utilized for alanine biosynthesis or be translocated into mitochondria and converted to acetyl coenzyme A (acetyl-CoA) to support TCA cycle and OXPHOS [135,136]. In addition, the NAD+ generated from aerobic glycolysis is required not only for the maintenance of glycolysis itself but also for various metabolic pathways including TCA cycle, OXPHOS, and redox metabolism [136]. Thus, aerobic glycolysis may not generate ATP as efficiently as OXPHOS, but it plays an essential role in the generation of biosynthetic precursors that support anabolic demands for clonal expansion and control of the redox state of T cells [136].

Metabolic reprogramming to aerobic glycolysis in activated T cells depends on the phosphoinositide 3-kinase (PI3K)–AKT–mTOR signaling axis [7]. TCR signaling promotes recruitment of the PI3K catalytic subunit p110δ to the immune synapse, activating serine /threonine kinase AKT [137]. AKT then activates mTORC1 through phosphorylating and inhibiting a mTORC1 suppressor, TSC2 [138]. mTORC1 plays an important role in the fate decision of CD4 effector T cells as mTOR deficiency suppresses differentiation of Th1, Th2, and Th17 cells while increasing the generation of Treg cells [139]. mTORC1 promotes aerobic glycolysis and glutaminolysis by activating the expression of transcription factors c-Myc and Hif-1α. c-Myc transactivates the expression of glutamine transporters and enzymes involved in

aerobic glycolysis and glutaminolysis [140], whereas Hif-1α increases the expression of glucose transporters *Glut1* and various glycolytic enzymes including *Hk2*, *Gpi*, *Pkm*, and *Ldha* [141].

Studies on CD8 T cells reveal that, in contrast to effector T cells, memory T cells reduce the activity of mTOR signaling, aerobic glycolysis, and glutaminolysis, and rely primarily on fatty acid oxidation (FAO) and OXPHOS to support their survival and function [142-144]. Treatment of mTOR inhibitors [142,143,145], suppression of glycolysis [146], and glutamine restriction [147] promote memory formation and enhance the anti-tumor activity of CD8 T cells. Consistent with this, activation of the AMPK-dependent mTOR-suppressive pathway by metformin enhances memory T cell generation in response to infection and tumors [143,148]. Carnitine palmitoyl transferase 1a (CPT1a), a mitochondrial long-chain fatty acid transporter, promotes FAO in memory CD8 T cells [144]. Mitochondrial remodeling is another characteristic of memory T cells. In contrast to fragmented mitochondria observed in naïve and effector T cells, mitochondrial biogenesis and mitochondrial fusion are prominent in memory T cells [149]. This likely improves mitochondrial respiration efficiency and protects memory T cells from ROS and mitochondrial DNA damage, contributing to their long-term survival and rapid recall responses upon antigen restimulation [149].

The metabolic reprogramming in the differentiation of Th17 cells is summarized in Fig. 1.2. The detailed description of the role of each metabolic pathway in the differentiation of Th17 and other T cells will be discussed in the following sections.

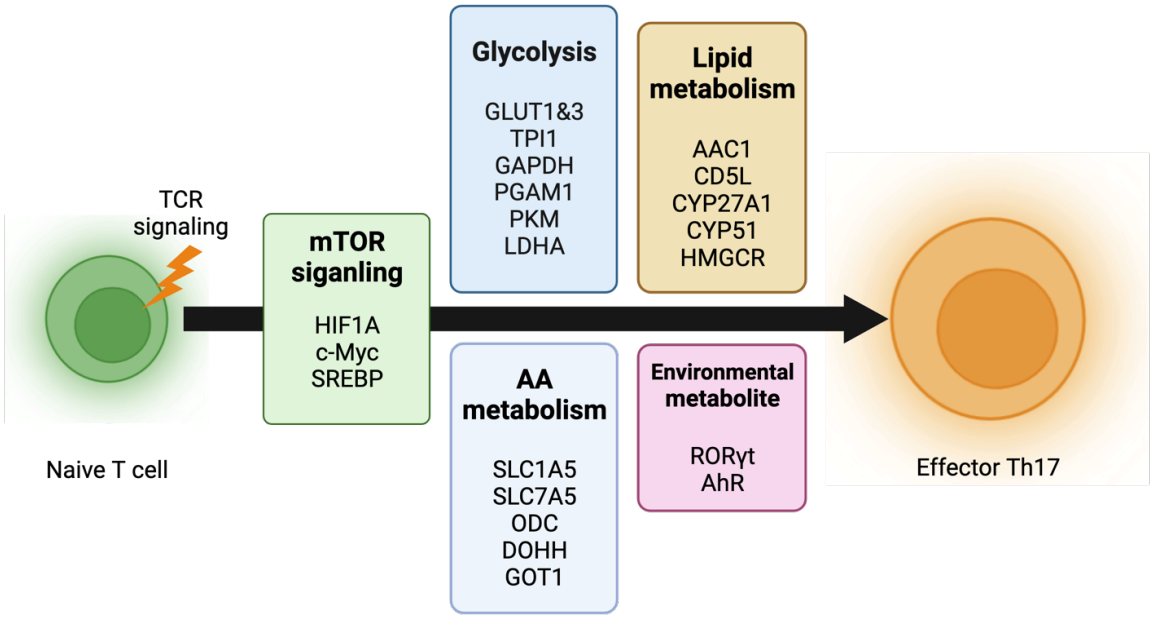


Fig. 1.2. Metabolic reprogramming during the differentiation of Th17 cells
Antigen stimulation activates mTOR signaling. Activation of mTOR signaling releases T cells from metabolic quiescence by promoting the expression of transcriptional factors required to activate the metabolic enzymes and nutrient transporters. Furthermore, nuclear receptors RORγt and AhR control Th17 differentiation by interacting with their agonist metabolites in the environment. Genes involved in each pathway and responsible for modulating Th17 differentiation are listed in the figure.

1.8. Amino acid metabolism in T cell differentiation and function

Amino acids (AA) influence T cell metabolism through several mechanisms. First, boosted intake of amino acids is required to fully activate T cells. For example, deletion of the neutral amino acid transporter SLC7A5 or the glutamine transporter SLC1A5 impairs the mTORC1 activation, inhibiting the differentiation of Th1 and Th17 cells [150,151]. Second, AA participates in various biosynthetic pathways to support the anabolic demand of proliferating T cells. For example, serine is metabolized in the one-carbon metabolism pathway, generating metabolites required for purine biosynthesis [152]. Mice fed with serine- and glycine-free diets show reduced CD8 effector T cell responses to *Listeria monocytogenes* infection due to insufficient nucleotide biosynthesis [153]. Third, amino acid starvation response (AAR) induced by halofuginone selectively decreases Th17 differentiation and EAE severity by blocking IL-23-mediated STAT3 phosphorylation [154 155].

Several other AA metabolic pathways are involved in the epigenetic regulation of T cell responses. First, methionine metabolism, together with the folate cycle, generates S-adenosyl methionine (SAM) to promote the expression of Th17 signature genes by enhancing the H3K4 methylation [156]. Second, polyamine metabolism governs the fate commitment of CD4 T cell subsets through spermidine production [157,158]. Spermidine is required for chromatin remodeling, histone modifications, and the hypusination of the translation elongation factor eIF5A in T cell differentiation [157]. T cells deficient for *Odc* and *Dohh*, the enzymes responsible for spermidine synthesis and eIF5A hypusination, respectively, cause abnormal upregulation of IFN-γ and IL-17A expression in Th2 and iTreg cells, and mice deficient for these genes develop severe colitis.

Glutaminolysis is indispensable for generating Th1, Th17, and effector CD8 T cells [150,159-162]. In glutaminolysis, glutamine is deaminated by glutaminase (GLS) to glutamate, and glutamate is converted to α-KG by glutamate oxaloacetate transaminase 1 (GOT1). In differentiating Th cells, α-KG is used to replenish metabolites in the TCA cycle or converted to acetyl CoA to fuel lipid synthesis [163]. α-KG can also be converted to 2 hydroxyglutarate (2-HG), which controls Th1/Treg fate decision by inhibiting ten-eleven translocation (TET) methylcytosine dioxygenases, which activates *Foxp3* expression by promoting DNA demethylation at the *Foxp3* locus [162]. The GOT1 inhibitor, aminooxy acetic acid (AOA), reprograms Th17 cells to iTreg cells and ameliorates EAE severity [162].

Glutamine can also contribute to anabolic pathways that regulate T cell proliferation and differentiation, including biosynthesis of glutathione, polyamine, and purine [158,159,161]. Glutamate generated by GLS is required for stabilizing the chromatin landscape in favor of Th17 differentiation, while suppressing the expression of Th1 signature genes [159]. Glutamate is also required to sustain the cellular level of glutathione to quench the reactive oxygen species that impede Th17 differentiation [159].

1.9. Lipid metabolism in T cell differentiation and function

Fatty acid synthesis (FAS) plays an important role in effector T cells. TCR stimulation enhances FAS to generate lipids necessary for rapid cell division while reducing FAO [164]. TCR-activated mTORC1 signaling activates sterol regulatory element-binding proteins (SREBPs), which promote the expression of FAS enzymes such as acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (Fasn) [165]. Loss of SREBP or ACC1 impairs clonal expansion of CD8 T cells during infections [166,167]. Similarly, the loss of ACC1 in CD4 T cells impedes Th17 differentiation and ameliorates EAE, while promoting the differentiation of Treg cells [168]. Mice fed with high-fat diet increase expression of ACC1 in CD4 T cells, which augments the DNA binding of

RORγt to the *Il17a* locus [83]. Various lipid molecules, such as polyunsaturated fatty acids (PUFAs), not only serve as building blocks for membrane biosynthesis, but also regulate the cytoskeleton arrangement and recruitment of membrane proteins at the immunological synapses to modulate TCR signaling [169-173]. In addition, prednisolone, a synthetic glucocorticoid, has been reported to attenuate the glycolytic activity in of CD8 T cells [174].

Treg cells rely on FAO to support the bioenergetic demand during differentiation. TGF-β signaling activates AMPK and promotes FAO in iTreg differentiation [175,176], and Treg cells actively intake fatty acids to sustain FAO [175]. Excess fatty acids in the environment promotes the generation of Treg cells, while inhibiting the development of Th1, Th2, and Th17 cells [175]. In line with this, inhibition of FAS does not affect Treg development [168], while a FAO inhibitor, etomoxir, impedes Treg differentiation [175].

Lipid metabolism is closely associated with Th17 differentiation and pathogenicity. A study on CD5L functions in Th17 cells reveals that cellular lipid composition affects Th17 pathogenicity [65]. CD5L, preferentially expressed in non-pathogenic Th17 cells, increases PUFAs and decreases free cholesterol, saturated fatty acids (SFAs), and monounsaturated fatty acids (MUFAs) in Th17 cells. PUFAs promote nonpathogenic Th17 differentiation by enhancing RORγt-binding to the *Il10* enhancer, whereas SFAs and cholesterol promote pathogenic Th17 differentiation by enhancing RORγt binding to *Il17* and *Il23r* enhancers. In addition, cholesterol synthesis is required for Th17 cell development. Several intermediates of cholesterol synthesis are RORγt agonists to enhance the expression of Th17 signature genes [177-180]. Inhibition of cholesterol synthesis with HMG-CoA reductase inhibitor statin has been shown to inhibit Th17 differentiation and ameliorate EAE progression[181].

1.10. Regulation of CD4 T cell responses by glycolysis-associated molecules

Glycolysis is required for the growth and proliferation of various Th subsets as described in Section 1.7, but glycolysis may also play unique roles in different Th subsets and functional states. In this section, I discuss the roles and functions of glycolysis-related enzymes and metabolites in the regulation of T cell responses.

A glycolytic enzyme, pyruvate kinase m2 (PKM2), promotes Th1 and Th17 responses through various non-glycolytic functions [182,183]. PKM2 is one of the four pyruvate kinases responsible for catalyzing the conversion of PEP to pyruvate to produce ATP, the rate-limiting step in glycolysis. PKM2 tetramers mainly engage in glycolysis, while PKM2 dimers can enter the nucleus and regulate gene expression [184]. In CD4 T cells, dimeric PKM2 promotes the expression of cytokines and transcription factors related to Th1 and Th17 cells and glycolytic enzymes [182]. TEPP-46, a small molecule inducing PKM2 tetramerization and blocking PKM2 nuclear translocation, suppresses T cell activation and cytokine expression of Th1 and Th17 cells *in vitro* and ameliorates EAE [182]. PKM2 likely interacts with STAT3 and controls its activity both *in vitro* [185] and *in vivo* [186].

Other glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase, and lactate dehydrogenase (LDH) also serve non-glycolytic functions that regulate CD4 T cell responses. GAPDH binds to the AU-rich elements of the 3´-untranslated region (UTR) of *Ifng* mRNA and inhibits its translation in Th1 cells under glucose starvation [187]. Enolase, which converts 2-phosphoglycerate (2PG) to PEP, regulates the fate commitment of human iTreg cells by controlling the expression of a splicing variant of FOXP3 [188]. LDHA is required to increase acetyl-CoA to promote histone H3 lysine 9 (H3K9) acetylation at the *Ifng* promoter and enhancer to promote its transcription in TCR-activated Th1 cells [189].

Glycolytic metabolites phosphoenolpyruvate (PEP) and lactate can promote CD4 T cell responses. PEP can bind and inhibit sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), thereby enhancing the activity of nuclear factor of activated T cells (NFAT) [190]. Notably, overexpression of the gluconeogenesis enzyme, PCK1, which generates PEP from oxaloacetate, can increase cellular PEP and promote tumoricidal activities of CD4 T cells under low-glucose conditions [190]. Lactate taken up by the lactate transporter, SLC5A12, promotes IL-17 expression by activating PKM2/STAT3 in human CD4 T cells in chronic inflammatory tissues [191]. Importantly, SLC5A12 expression and lactate-induced metabolic reprogramming are enhanced in CD4 T cells from RA patients, and treatment of the SLC5A12 antibody ameliorates disease severity in the mouse arthritis model [191].

1.11. Metabolic heterogeneity in pathogenic and non-pathogenic Th17 cells

Accumulating evidence demonstrates that Th17 cells have remarkable heterogeneity of cellular metabolism. Glycolytic activity is higher in pathogenic Th17 cells polarized *in vitro* with IL-6, IL-1β, and IL-23 than the nonpathogenic Th17 cells polarized with TGF-β and IL-6, while OXPHOS activity shows the opposite trend [192,193]. Similarly, pathogenic Th17 cells isolated from inflamed CNS or induced by *C. rodentium* infection have elevated glycolysis and amino acid metabolism [193,194]. In contrast, gut-resident homeostatic Th17 cells show lower levels of glycolysis and higher levels of glutaminolysis and OXPHOS than pathogenic Th17 cells [193,194]. These discoveries suggest that pathogenic Th17 cells depend more on glycolysis, while non-pathogenic Th17 cells rely more on mitochondrial respiration. Fig. 1.3. shows the summary of metabolic heterogeneity of pathogenic and non-pathogenic Th17 cells.

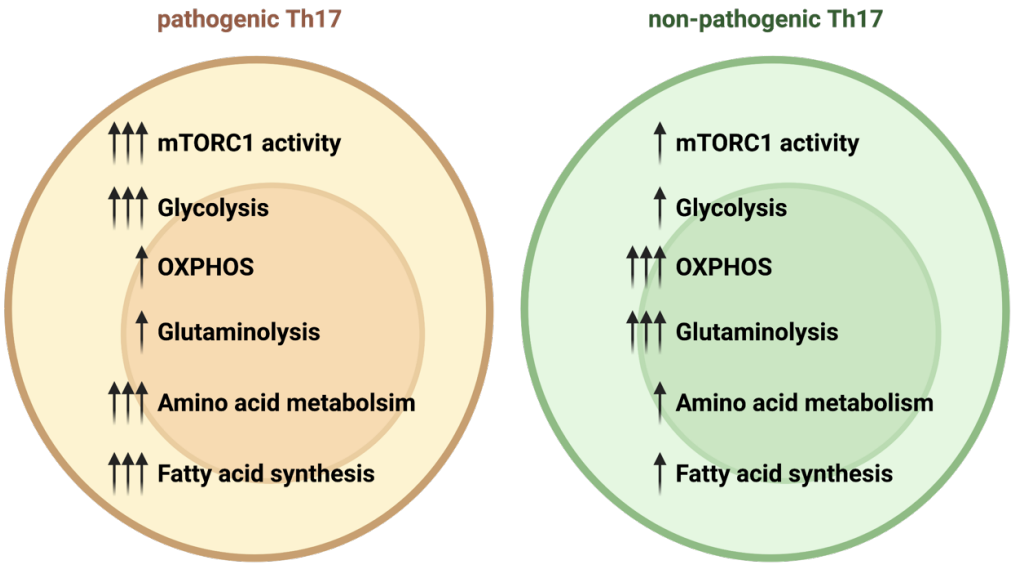


Fig. 1.3. Metabolic heterogeneity in pathogenic and non-pathogenic Th17 cells
Pathogenic Th17 cells gendered *in vitro*[193], induced by C. *rodentium*[194] infection, or EAE[193] induction, are metabolically active with upregulated activities of mTORC1 signaling[195], glycolysis, amino acid metabolism, and fatty acid biosynthesis. In contrast, nonpathogenic Th17 cells, either generated *in vitro*[193] or induced by intestinal commensals[194], are more metabolic quiescent, and depend more on OXPHOS and glutaminolysis.

Therapeutic strategies based on the regulation of glycolysis have been proposed to specifically target pathogenic Th17 cells. Despite their differential metabolic states, glycolysis is indispensable for generating both pathogenic and non-pathogenic Th17 cells, as genetic deletion of *Glut3* [196], *Hif1a* [141], *Tpi1* [197], *Gapdh* [197], *Pgam1* [198], or *Ldha* [197] abolishes Th17 generation. However, loss of the glycolytic enzyme 6-phosphate isomerase (GPI) selectively inhibits the generation of pathogenic Th17 cells in EAE or *Helicobacter hepaticus* infection but not non-pathogenic Th17 cells induced by segmented filamentous bacteria (SFB) [197]. Interestingly, non-pathogenic Th17 cells in the gut can enhance OXPHOS and PPP to compensate for the reduced glycolysis caused by GPI deficiency, but pathogenic Th17 cells in inflamed tissues cannot use this compensatory pathway because of the hypoxic microenvironment [197]. In addition, *Il17a*-Cre/loxP mediated genetic ablation of *Rptor* , a gene involved in mTOC1 signaling, ameliorate EAE progression [195]. *Rptor* deletion suppresses the accumulation of pathogenic $CD27^-$ Th17 cells, but not the $CD27^+$ Th17 cells, which do not produce IFN-γ and express memory T cell-associated markers [195]. Thus, targeting GPI or mTORC1 signaling in mature Th17 cells can be potential strategies for the selective control of pathogenic Th17 cells.

1.12. Glycolysis in Th17 differentiation and IL-17 expression

Although glycolysis is essential for the generation of pathogenic and non-pathogenic Th17 cells as described above, the effect of pharmacological glycolysis inhibitors on Th17 differentiation or IL-17 expression is still controversial. One study reported that 2-deoxy-D-glucose (2-DG), a competitive inhibitor of hexokinase, suppressed Th17 differentiation and decreased the severity of EAE [141], which seems to be consistent with the observations in mice deficient in glycolytic enzymes. However, other studies reported that 2-DG increased *in vitro* Th17 differentiation or IL-17 expression [199] [200]. 2-DG-mediated enhancement of IL-17 expression was also observed in human CD4 T cells [201] and murine CD8 T cells [202]. Furthermore, blockage of a glycolytic enzyme, phosphoglycerate mutase (PGAM), by EGCG also enhanced IL-17A expression in IL-6/TGF-β-induced non-pathogenic Th17 cells and pathogenicity in passive EAE transfer experiments [203].

Several mechanisms by which 2-DG promotes Th17 differentiation have been discussed. First, ER stress caused by 2-DG may be responsible for enhanced Th17 differentiation as ER stress inhibitors inhibited IL17 expression augmented by 2-DG treatment [199]. Second, since IL-2 signal inhibits Th17 differentiation, downregulation of IL-2Rα expression by 2-DG may enhance Th17 differentiation [200]. However, mannose supplementation rescued IL-2Rα expression but did not decrease IL-17A expression, suggesting that 2-DG promotes IL-17A expression independently of the regulation of IL-2R α expression.

1.13. Research Motivation

Glycolysis is essential for the clonal expansion and differentiation of all conventional CD4 Th cells, but the roles of individual glycolytic steps in Th17 differentiation are not fully understood. The role of glycolysis in Th17 differentiation is controversial. Although it is demonstrated that inhibition of glycolysis by 2-DG protected mice from experimental autoimmune encephalomyelitis (EAE) [141], emerging pieces of evidence have shown that glycolysis can play as a negative regulator of Th17 differentiation, as several recent studies report that glycolytic inhibition by 2-DG or other inhibitors promotes Th17 differentiation [199,200]. These paradoxical results suggest that glycolysis may serve as both positive and negative regulators of Th17 differentiation in a context-dependent manner, and there might be uncharacterized glycolysis-associated negative regulators of Th17 differentiation.

In this study, I identified a glycolytic intermediate metabolite, phosphoenolpyruvate (PEP), as a negative regulator of pathogenic Th17 generation. I found that PEP inhibits the generation of Th17 cells *in vitro* and *in vivo*, and administration of PEP to mice ameliorates Th17-dependent autoimmune encephalomyelitis. Mechanistically, PEP regulates the JunB-dependent pathogenic Th17 transcriptional program by inhibiting the DNA-binding activity of JunB, BATF, and IRF4. These findings shed light on glycolysis-dependent negative regulation of pathogenic Th17 differentiation, which might be a novel therapeutic target for autoimmune diseases.

Chapter 2. Materials and Method

2.1. Mice

C57BL/6 mice were obtained from Clea (Tokyo, Japan), and OT-II and B6SJL mice were from the Jackson Laboratory (ME, USA). All mice were maintained under specific pathogen-free conditions. Gender-matched 6-12-week-old mice were utilized for experiments. All animal experimental protocols were approved by the Animal Care and Use Committee at Okinawa Institute of Science and Technology Graduate University.

2.2. Antibodies

The following antibodies were used for flow cytometry analysis and fluorescence-activated cell sorting, with 1:200 dilution: anti-IFN-γ (XMG1.2; Biolegend), anti-IFN-γ (MP6-XT22; Biolegend), anti-IL4 (11B11; Biolegend), anti-IL13 (eBio13A; eBioscience), anti-IL10 (JES5-16E3; Biolegend), anti-IL17A (TC11-18H10.1; Biolegend), anti-RORγt (B2D; eBioscience), anti-FOXP3 (150D; Biolegend), anti-T-bet (4B10; Biolegend), anti-GATA3 (16E10A23; Biolegend), anti-CD4 (GK1.5; Biolegend), anti-CD62L (MEL-14; Biolegend), anti-CD25 (PC61; Biolegend), anti-CD44 (IM7; Biolegend), anti-IL2 (JES6-5H4; Biolegend), anti-CD3 (17A2; Biolegend), anti-CD45.1 (A20; Biolegend), and anti-CD45.2 (104; Biolegend). Antibodies for western blotting were as below: anti-BATF (WW8; Santa Cruz, USA), anti-IRF4 (4964; CST, USA), anti-JUND (329; Santa Cruz), anti-JunB (C11; Santa Cruz), anti-STAT3 (79D7; CST), anti-phospho STAT3 (Tyr705) (D3A7; CST), anti-HIF1α (28b; Santa Cruz), anti-β Actin (6D1; MBL, Japan), anti-PKM2 (D78A4, CST); anti-Histone H3 (D2B12; CST), anti-β tubulin (PM054; MBL), anti-Flag (M2; Sigma-Aldrich), anti-HA tag (Medical & Biological Laboratories), anti-mouse IgG, HRP-linked (7076; CST), and anti-rabbit IgG, HRP-linked (7074; CST).

2.3. *in vitro* CD4 T-cell differentiation

Naïve murine CD4 T cells were isolated from spleens using MojoSort mouse CD4 naive T cell isolation kit (Biolegend) for most of *in vitro* T cell culture experiments. For RNA sequencing, ATAC sequencing, and ChIP PCR, CD4 T cells were first enriched with MACS magnetic cell sorting system with anti-CD4 microbeads (Miltenyi), and then naïve CD4 T cells ($CD4CD25^{-}CD62L^{hi}CD44^{lo}$) were sorted by FACS AriaII or AriaIII (BD). Isolated naïve CD4 T cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) supplemented with 10% FBS, 1X streptomycin-penicillin (Sigma-Aldrich), β-mercaptoethanol (55 μM; Invitrogen, USA), and anti-CD28 antibody (1 μg/mL; 37.51, Biolegend) in 24-well ($2x10^5$ cells / well) or 48-well ($1x10^5$ cells / well) plates coated with anti-CD3ε antibody (5 μg/mL; 145-2C11, Biolegend). The medium was further supplemented with IL-2 (20 ng/mL, Biolegend), IL-12 (20 ng/mL; Biolegend), and anti-IL-4 (1 μg/mL; 11B11, Biolegend) for Th1; IL-2 (20 ng/mL), IL-4 (100 ng/mL; Biolegend), and anti-IFN-γ (1 μg/mL; R4-6A2, Biolegend) for Th2; IL-6 (20 ng/mL; Biolegend) and TGF-β1 (3 ng/mL; Miltenyi) for npTh17; IL-6 (20 ng/mL), IL-1β (20 ng/mL; Biolegend) and IL-23 (40 ng/mL; Biolegend) for pTh17; TGF-β1 (15 ng/mL), IL-2 (20 ng/mL), anti-IL-4 (1 μg/mL), and anti-IFN-γ (1 μg/mL) for iTreg differentiation. In several experiments, additional inhibitors or metabolites were added to the culture medium. Glycolytic metabolites were dissolved in water or PBS first, adjusted the pH value to 7.3, then added to the culture medium to reach the desired concentration. For analysis of cytokine expression, cells were harvested at indicated time points, re-stimulated with phorbol 12- myristate 13-acetate

(PMA; 50 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Sigma-Aldrich), and brefeldin A (5 μg/mL; Biolegend) for 4 hours. Cells were then fixed with 4% paraformaldehyde, permeabilized in permeabilization/wash buffer (421002, Biolegend), and stained with antibodies against cytokines. For analysis of expression of transcription factors, Foxp3 staining buffer set (00-5253-00, eBioscience) was used according to the manufacturer's instructions.

2.4. Intracellular PEP Quantification

Naïve CD4 T cells activated under Th17-polarizing conditions at indicated time points were washed twice with PBS, snap-frozen in liquid nitrogen, and then stored at -80^{o}C until further processing. The PEP fluorometric assay was performed with PEP colorimetric/fluorometric assay kits (Sigma-Aldrich) and a SpectraMax M2 96-well reader (Molecular Devices, USA) according to the manufacturer's instructions.

2.5. qRT–PCR

Total RNA was isolated from cells using an RNeasy Plus Mini kit (Qiagen, Germany). cDNA was synthesized with a Revertra Ace qPCR Kit (Toyobo, Japan). PCR was performed with KAPA SYBR fast qPCR kit master mix (Kapa Biosystems) and StepOnePlus Real-Time PCR (Applied Biosystems, USA). Primers used for qPCR are listed in Table 2.1.

2.6. Seahorse assay

Naïve CD4 T cells activated under npTh17- and pTh17-polarizing conditions for 72 h were harvested for measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) with mito stress (Agilent Technologies, USA) and glycolysis stress kits (Agilent Technologies), respectively. Cells were washed twice with PBS, transferred to an analysis plate (2 x 10^5 cells per well) coated with 2% gelatin (Sigma-Aldrich), and incubated at 37^{o}C for 1 h. OCR and ECAR were measured using a Seahorse XFe96 analyzer (Seahorse Bioscience, USA) following the manufacturer's instructions.

2.7. Sample preparation for RNA and ATAC sequencing

Cells activated under npTh17- and pTh17-polarizing conditions for 48 h were harvested, and total RNA was extracted using RNeasy Plus Mini kits (Qiagen). RNA samples were then mixed with ERCC RNA spike-in control mixes (Thermo), and mRNA was isolated with NEBNext poly(A) mRNA magnetic isolation module (E7490; NEB, USA). The sequencing Library was prepared with a Collibri™ Stranded RNA Library Prep Kit for Illumina™ Systems with Human/Mouse/Rat rRNA Depletion Kits (Thermo), according to the manufacturer's instructions. The quality of the cDNA library was checked using Qubit™ dsDNA HS and BR Assay Kits (Thermo) and High Sensitivity DNA Reagents kits (Agilent, USA) with a Qubit 4 fluorometer (Thermo) and a 2100 Bioanalyzer instrument (Agilent), respectively. For ATAC sequencing, naïve CD4 T cells were activated under npTh17-polarizing conditions for 48 h. Then cells were harvested, snap-frozen in liquid nitrogen, and submitted to the OIST Sequencing Section (SQC) for further preparation and sequencing. Both RNA and ATAC sequencing were performed on an Illumina NovaSeq 6000 to generate 150-nucleotide, paired-end reads with a read depth of ≥20 million reads per sample.

2.8. RNA-seq data analysis

Data quality was assessed using FastQC (v.0.11.9) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were further processed to

remove adaptor and low-quality sequences using Trimmomatic1 (v.0.39) software with the options (SLIDINGWINDOW:4:20 LEADING:20 TRAILING:20 MINLEN:35). HISAT23 (v2.2) was utilized to align reads to the GRCm38 reference genome (Mus_musculus.GRCm38.dna.primary_assembly.fa file, downloaded from Ensembl2). We counted the number of reads overlapping the genes in the reference transcriptome annotations (Mus_musculus.GRCm38.98.gtf downloaded from Ensemb4l) with featureCounts from Subread5 (v2.0.1). To detect differentially expressed genes, transcripts with zero expression were first filtered out, and statistical significance was analyzed with the Wald test using DESeq26 (v.1.34.0). Gene set enrichment analysis based on Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed using the clusterProfiler7 R package.

2.9. ATAC-seq data processing

Raw data processing was performed using nfcore/atacseq (v.1.2.1), a bioinformatics analysis pipeline used for ATAC-seq data at the National Genomics Infrastructure at SciLifeLab Stockholm, Sweden. In brief, adapters and low-quality reads were removed with Trim Galore!. Trimmed fastq files were mapped to the GRCm38 mouse reference genome with BWA, and narrow peaks were called with MACS2. The normalized BigWig files, scaled to 1 million mapped reads, were created with BEDTools and bedGraphToBigWig and were uploaded to the UCSC genome browser. Tool versions and full details of the pipeline are available at https://nf-co.re/atacseq.

2.10. Motif analysis

Motif enrichment within 2 kb upstream and downstream of the transcriptional start sites of DEGs in PEP-treated vs. control cells was analyzed using the findMotifs function of Homer (version v4.11). Binding motifs for AP-1 (ATGACTCATC), JunB (RATGASTCAT), BATF (DATGASTCAT), and IRF4 (ACTGAAACCA), and AICE (NAGTTTCABTHTGACTNW) within 10 kb upstream and downstream of transcriptional start sites of DEGs were identified using the mouse mm10 genome with the scanMotifGenomeWide.pl function of Homer v4.11.

2.11. Immunoblot analysis

Cells were lysed with RIPA buffer (Wako, Japan) with a complete protease inhibitor cocktail (Roche, Switzerland). Clear lysates were mixed with 5X sample loading buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% b-mercaptoethanol, and 0.1% bromophenol blue) and subjected to SDS polyacrylamide gel electrophoresis. Blotting was performed with Immobilon P transfer membranes (Millipore) using a Trans-blot electrophoretic transfer system (Bio-Rad). Membranes were blocked with 5% skim milk (Wako) or bovine albumin (Wako) in Tris-buffered saline with 0.1% Tween-20 (Sigma-Aldrich). Then they were hybridized with described antibodies at 4^{o}C overnight, followed by incubation with HRP-conjugated secondary antibodies at room temperature for 2 h. Reactive proteins were detected with Clarity Western ECL (Bio-Rad) or SuperSignal West Femto detection reagents (Thermo) on a Las-3000 imaging system (Fuji film, Japan) or iBright™ CL1500 Imaging System (Thermo).

2.12. Cell transfection

To overexpress mouse BATF and JunB, BATF-HA (C-terminally HA-tagged BATF (gene ID: 53314)) (BATF-HA) and JunB-Flag (C-terminally Flag-tagged JunB (gene ID: 16477)) were amplified from gblock (Integrated DNA Technology) and cloned into pCDNA3.1 (Thermo). HEK293 cells cultured in Dulbecco's modified eagle medium (DMEM) (Thermo) supplemented

with 10% FBS and MEM non-essential amino acids (Thermo) were seeded in 10-cm culture dishes 24 h before transfection with 80% confluency. 5 mg of pcDNA3.1-BATF-HA and pcDNA3.1-JunB-Flag in 250 μl Opti-MEM were mixed with 25 μL of polyethylenimine (1 mg/mL) (Cosmobio, Japan) in 250 μL of Opti-MEM, incubated at room temperature for 30 min, and then added to the cell culture. Cells were harvested after 60 h for co-immunoprecipitation assay.

2.13. Co-immunoprecipitation

HEK293 cells transfected with pcDNA3.1-BATF-HA and pcDNA3.1-JunB-Flag were washed with PBS twice, freeze-thawed by liquid nitrogen twice, and lysed in Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris (pH 7.5) containing complete protease inhibitor cocktail (Roche)) on ice for 30 min (briefly vortexed every 10 min). Then, cellular debris was removed by centrifugation at 13,000 g for 10 min. Lysates (3 mg total protein) were incubated with 3 mg of anti-Flag (M2; Sigma-Aldrich) or anti-mouse IgG (G3A1; CST) antibodies together with or without PEP (50, 200, and 500 mM) on a rotator at 4°C for 16 h, followed by incubation with 15 μL of Dynabeads protein G (Invitrogen) on a rotator at 4°C for another hour. Beads were then washed four times (10 min incubation in each wash) with buffer containing 0.2% Triton X-100, 150 mM NaCl, 20 mM Tris (pH 7.5), and a complete protease inhibitor cocktail (Roche) at 4°C. Immunoprecipitates were eluted by heating the beads in sample buffer (0.05% Bromophenol blue, 2% β-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate in Tris-Cl (pH=6.8)) at 70°C for 15 min.

2.14. Chromatin immunoprecipitation

Cells were harvested at the indicated timepoint, and chromatin immunoprecipitation was performed using SimpleChIP kits (CST) and Dynabeads protein G (Invitrogen) according to the manufacturer's instructions, except for two modifications: (1) the amount of micrococcal nuclease was reduced to 0.05 μL per million cells; (2) chromatin-bound beads were washed with low-salt wash solution 4 times, followed by high-salt wash solution 2 times at 4°C for 5 min. The following antibodies (2 μg per sample) were used for immunoprecipitation: anti-BATF (ww8; Santa Cruz), anti-JunB (C-11; Santa Cruz), anti-IRF4 (4964; CST), anti-mouse IgG (G3A1; CST), and anti-Rabbit IgG (2729; CST).

2.15. Radioisotope labeling of PEP and pull-down assay of JunB with labeled PEP

A published procedure was adopted for labeling PEP with 32P-ATP [204]. Briefly, 600 μCi (from 10 mCi/ml) of γ-^{32}P -ATP were mixed with 800 mM pyruvate and 5 units of rabbit muscle pyruvate kinase (Wako) in a reaction buffer (50 mM Tris (pH7.5), 50 mM KCl, 5 mM MgCl2, and 1 mM DTT) and incubated at 37°C for 1 h. To remove free γ-^{32}P -ATP, the reaction mixture was loaded on a Vivapure Q column (anion-exchange), centrifuged at 500g for 3 min, and then washed twice with reaction buffer. ^{32}P-labeled PEP was eluted stepwise in triethylammonium bicarbonate (TEAB, pH 8.5) buffer (3 mM and 6 mM for the first and second elution, respectively). HEK293 cells overexpressing FLAG-JunB and HA-BATF were lysed as described above. Lysates (1 mg total protein) were incubated with 3 μg of anti-Flag or anti-mouse IgG antibodies on a rotator at 4°C for 16 h, followed by incubation with 15 μL of Dynabeads protein G on a rotator at 4°C for another hour. Beads were then washed four times (10 min incubation in each wash) with buffer containing 0.2% Triton X-100, 150 mM NaCl, 20 mM Tris (pH7.5), and a complete protease inhibitor cocktail at 4°C. Beads were then

resuspended in phosphate buffer (pH 8.0) containing 2 mmol/L DTT, ^{32}P -labeled PEP (4mmol/L) with or without 100 molar-excessive PEP (as a competitor) at room temperature for 1 h. Bead-bound complexes were then washed twice with PBS buffer for 5 min. As an additional control Dynabeads-bound complex/^{32}P-labeled PEP was incubated with 10 unit CIP (Calf intestinal Alkaline phosphatase) in CIP buffer (100mM NaCl, 50mM Tris-HCl, 10 mM MgCl2, 1mM DTT with EDTA-free protease inhibitor cocktail) pH 7.9, and kept at 37º C for 30 mins. Samples were then eluted by heating the beads in 2X sample buffer (80 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, 0.0006% bromophenol blue) at 70ºC for 15 min. Eluted samples were mixed with 5 mL Clear-sol II (Nacalai Tesque), and measurements were taken on a scintillator (Liquid Scintillation Counter; Maker: ALOKA; Model: LSC-6100). Radioactivity was measured as counts per min (CPM) for quantitation and statistical analysis.

2.16. OVA immunization

8-9-week-old, gender-matched B6SJL mice were subcutaneously (s.c.) injected with vehicle (200 µL PBS) or PEP (1g/kg body weight, dissolved in 200 µL PBS, pH adjusted to 7.3), followed 6 h later by intravenous injection of naïve CD4 T cells (1 x 10^6 cells/mouse) isolated from OT-II mice (CD45.1$^+$ CD45.2$^+$) on day 0. One day later (day 1), mice were immunized with $OVA_{323-339}$ (50 µg per mouse; ISQAVHAAHAEINEAGR, GL Biochem, China) emulsified in CFA (200 µL per mouse) supplemented with or without PEP (10 mg/mouse). From day 2 to day 6, mice were s.c. injected with vehicle or PEP as described above. On day 7, mice were euthanized, and cells isolated from inguinal lymph nodes and spleens were analyzed as described above.

2.17. EAE induction

8-week-old, female C57BL/6 mice were s.c. injected with $MOG_{35–55}$ peptides (300 mg per mouse) emulsified in complete Freund's adjuvant (CFA) (200 µL per mouse) containing dead *Mycobacterium tuberculosis* (1 mg per mouse) on day 0. On days 0 and 2, pertussis toxin (400 ng per mouse) was intraperitoneally injected into mice. From day 0 (8 h prior to MOG immunization) until the end of experiments, mice were s.c. injected with vehicle (200 µL PBS) or PEP (1g/kg per mice, dissolved in 200 µL PBS, pH adjusted to 7.3) daily. Disease severity was evaluated on a scale of 1–5 as follows: 1, limp tail; 2, limp tail and weakness of hind legs; 3, limp tail with paralysis of one hind leg; 4, limp tail with paralysis of both hind legs; 5, complete hind and front leg paralysis. Mice with a disease score of 5 were euthanized.

2.18. Metabolite extraction

Control (PBS) or 10mM PEP-treated cells activated under Th17-polarizing conditions for 60 h were collected and washed twice with ice-cold PBS. After cell counting, pellets were frozen at −80°C overnight. Cell pellets were gently washed with ammonium acetate buffer (150 mM) on dry ice to remove PBS. Cells were then resuspended in 100 µL extraction solution (90% methanol, 9.5% water, and 0.5% formic acid), vortexed, and sonicated for 10 min in an ice-cold (2°C) sonication bath. Samples were subsequently centrifuged (14,000g, 4°C, 5 min), and supernatant was collected in a new collection vial. This extraction step was repeated twice. Extracted metabolites (300 µL) were gently mixed with 300 µL water. An equal amount of methyl tert-butyl ether (MTBE, 600 µl) was then added to extracted metabolites, vortexed, and incubated on an orbital shaker at RT for 10 min. For phase separation (ether/aqueous layer), the mixture was centrifuged (14,000g, 4°C, 5 min) and the upper ether layer was removed. This

phase separation step was repeated twice. After removing the ether layer, extracted metabolites in the aqueous layer were vacuum dried and resuspended in 30 µL solution of 95% ultrapure water, 4.5% methanol, and 0.5% formic acid. Samples were incubated in a sonication bath for 2 min, centrifuged (14,000g, 4°C, 10 min), and clear solution from the top was collected carefully in an autosampler vial for MS acquisition. Liquid chromatography-mass spectrometry (LC-MS) data of samples and standard mix (10 µM each; 30 pmole/injection) were acquired in parallel.

2.19. LC-MS acquisition and data analysis of metabolites

Metabolites were chromatographically separated using the Waters M Class Acquity UPLC system coupled with an Orbitrap Q Exactive HF mass spectrometer (ThermoFisher, USA). Chromatography was conducted on a C18 column (UPLC HSS T3 1.8 µm, 1 x 150 mm; Waters, USA) using a 20 min step gradient. Mobile phase consisted of water (A) and LC-grade acetonitrile (B), both containing 0.1% v/v formic acid. The gradient program was 1% B in 0.0-2.1 min, 1-40% B in 2.1–7.0 min, 40% B in 7.0-9.0 min, 40-99% B in 9.0-10.0 min, 99% B in 10.0-13.0 min, and 1% B in 13.1–20.0 min. LC maintained a 50 µL/min flow rate at 40°C (column temperature), and a 3 µL sample (in technical triplicates) was injected from an auto-sampler for each set. In the Orbitrap MS system, mass spectra were sequentially captured in positive and negative modes using an electrospray ionization source. The MS spray voltage was kept at 3.5 kV and 3.0 kV for positive and negative modes, respectively. The S-lens RF level was set at 55, and the capillary temperature at 320°C. The auxiliary gas heater was maintained at 150°C, while sheath gas, auxiliary gas, and sweep gas flow rates were set to 25, 10, and 5 arbitrary units, respectively. Full scan resolution was set to 60,000 at m/z 200, and the AGC target was set to 5×10^5 for a 50 ms maximum injection time. At full scan mode, recorded spectra covered a mass range from 70 to 1000 m/z. Raw MS files were analyzed using Compound Discoverer v3.2 (ThermoFisher, USA) software, and references of expected metabolites were added in the processing workflow. Analysis was performed by adopting general settings, and mass spectral features were analyzed using the "natural product atlas 2020_06" mass list in the search setting under the 'search mass lists' node. Standard metabolite spectra were taken as a reference for sensitivity, mass, and retention time alignment that enabled metabolite discovery. Quantitative peak areas of identified metabolites were normalized to the cell count ratio in control and PEP-treated samples and plotted subsequently.

2.20. Statistical analysis

Unpaired two-tailed Student's t tests and one-way ANOVA followed by Tukey's post-hoc tests were performed with Prism (GraphPad). P values < 0.05 were considered statistically significant.

2.21. Data availability

The RNA-seq and ATAC-seq data that support the finding of this study have been deposited to DDBJ database (DRA014503).

Chapter 3. Results

3.1. Glycolysis regulates IL-17 expression depending on glucose availability

The role of glycolysis in Th17 differentiation is controversial because several studies reported that a glycolysis inhibitor, 2-DG, inhibits IL-17 expression in differentiating Th17 cells, but others showed the opposite results. Given that previous studies evaluated 2-DG effects on Th17 cells polarized in the medium containing different amounts of glucose [141,199], I reasoned that this discrepancy might arise due to differences in cellular glycolytic levels. To assess this possibility, I activated naïve CD4 T cells under non-pathogenic Th17 (npTh17)-polarizing conditions (with TGF-β1 and IL-6) in the presence of different concentrations of glucose and examined the effect of 2-DG treatment (Fig. 3.1A). 2-DG treatment significantly increased IL-17 expression in cells cultured with more than 1 mg/mL glucose, while it decreased IL-17 expression in cells cultured with 0.5 mg/mL glucose. Moreover, Th17 cells polarized in the low-glucose medium (1 g/mL and 0.5 g/mL) had higher IL-17A expression than in the glucose-rich medium (4.5 g/mL) (Fig. 3.1A). This result suggests that the role of glycolysis regulating IL-17 expression depends on glucose availability.

3.2. Identification of PEP as a negative regulator of IL-17 expression

Since glycolysis is essential for the growth and proliferation of Th17 cells [132,205], reduced expression of IL-17A in 2-DG-treated cells cultured with low levels of glucose is likely due to defective generation of Th17 cells. In contrast, increased expression of IL-17A in 2-DG-treated cells cultured with high levels of glucose suggests that there may be glycolysis-associated negative regulators of Th17 differentiation. To identify such factors, I focused on glycolytic intermediate metabolites. Although several metabolites are known to modulate epigenetic control of gene expression in T cells [7,132], the role of glycolytic intermediate in T cell differentiation is not fully understood. I cultured murine naïve CD4 T cells under npTh17-skewing conditions with different glycolytic metabolites and analyzed IL-17A expression after 60h of polarization. Surprisingly, the supplementation of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), 3-phosphoglycerate (3PG), and phosphoenolpyruvate (PEP) reduced IL-17A expression, without affecting cell viability (Fig. 3.1. B-D). On the other hand, supplementation of fructose 1,6 bisphosphate (F1,6BP) and glycerol-3-phosphate (G3P) induced severe cell death (Fig. 3.1D).

Because PEP reduced IL-17 expression most dramatically, I further investigated the effect of PEP supplementation on Th17 differentiation. To verify whether PEP supplementation can increase intracellular PEP concentrations, I quantified intracellular PEP levels of naïve CD4 T cells activated under npTh17-polarizing conditions with or without PEP supplementation. PEP supplementation significantly increased intracellular PEP levels in 2 h after supplementation (Fig. 3.1E), indicating that intracellular PEP levels in differentiating Th17 cells can be manipulated by increasing environmental PEP.

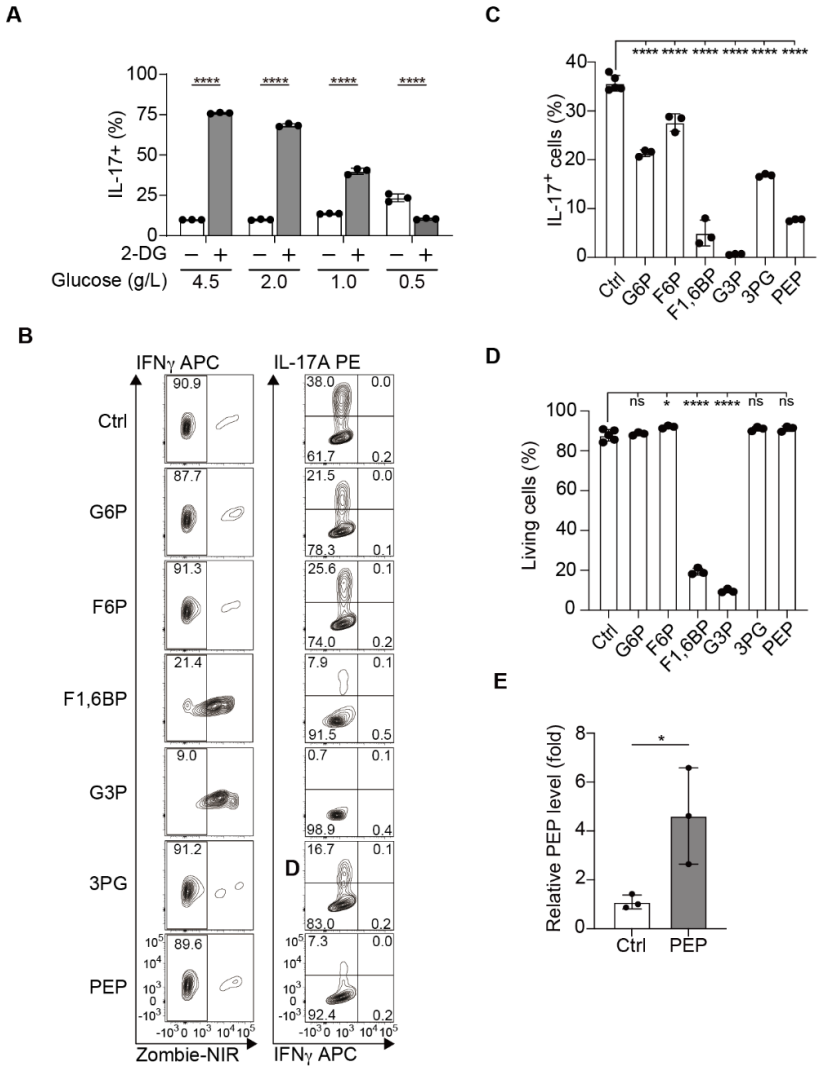


Fig. 3.1. Glycolysis controls IL-17 expression depending on glucose availability in npTh17 cells
(A) Naïve CD4 T cells were activated with CD3/CD28 antibodies under npTh17 conditions (with TGF-β1 and IL-6) in the absence (vehicle control) in the absence or presence of 2-DG (2mM) in RPMI-1640 media containing the indicated concentrations of glucose. IL17-A expression was analyzed by flow cytometry (n = 3). (B-E) Naïve CD4 T cells were activated with CD3/CD28 antibodies under npTh17 conditions in the absence (vehicle control (Ctrl)) or presence of glycolytic intermediate metabolites, G6P (20mM), F6P (10mM), FBP (1mM), G3P (1mM), 3PG (10mM), or PEP (10mM) for 60 h. IL-17A expression (B, C) and cell viability (D) were analyzed by flow cytometry (n = 3). (E) Quantification of intracellular PEP (n = 3). Cells were activated in the absence or presence of PEP (10 mM) for 2 h. The p-value was calculated by a two-tailed unpaired Student's t-test (* $p < 0.05$). (A, C, and D) The p values were calculated by one-way ANOVA with Bonferroni's multiple comparison tests (* $p < 0.05$, **** $p < 0.0001$, ns: not significant). (A-D) Error bars represent mean ± standard deviation (SD). Data are representative of at least two independent experiments.

To further interrogate the relationship between intracellular PEP levels and IL-17A expression, I analyzed the effect of manipulation of PEP levels in npTh17 cells with pharmacological glycolysis inhibitors on IL-17A expression (Fig. 3.2). It has been demonstrated that 2-DG and heptelidic acid (HA) decrease intracellular PEP levels by suppressing the enzymatic activities of hexokinase and glyceraldehyde phosphate dehydrogenase (GAPDH), respectively; while oxalate (OXA) increases PEP levels by inhibiting pyruvate kinase [190]. As previously reported [199], I observed that inhibition of glycolysis with 2-DG dramatically increased IL-17A expression in npTh17 cells (Fig. 3.2A-B). HA also significantly increased IL-17A expression, although not as strongly as 2-DG , while OXA diminished IL-17A expression (Fig. 3.2A-B). Cells treated with these glycolysis inhibitors showed comparable viability to the control cells (Fig. 3.2C). I also found that PEP supplementation suppressed IL-17A expression in 2-DG-treated npTh17 cells (Fig. 3.2D). These results suggest that intracellular PEP adsorbed from the environment or generated by glycolysis can suppress IL-17A expression in Th17 cells independently of its role in glycolysis.

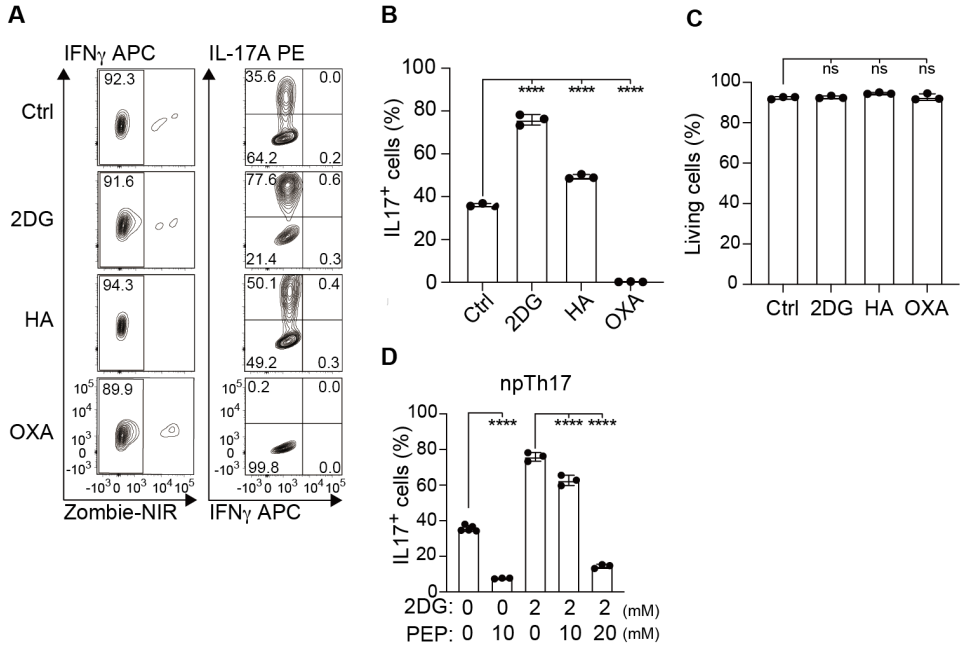


Fig. 3.2. Inhibition of glycolysis enhances IL-17A expression by suppressing PEP generation in Th17 cells.

(A-C) Naïve CD4 T cells were activated with CD3/CD28 antibodies in the absence (vehicle control (Ctrl)) or presence of glycolytic inhibitors under npTh17 conditions (with TGF- β1 and IL-6). IL-17A expression (A, B) and cell viability (C) were analyzed by flow cytometry (n = 3). Cells were polarized in the presence of 2-DG (2mM), HA (1μM), or OXA (2mM) for 60 h. (D) IL-17A expression of cells polarized with the indicated concentration of 2-DG and PEP was analyzed by flow cytometry (n=3). (B, C, and D) The p-values were calculated by one-way ANOVA with Bonferroni's multiple comparison tests (* $p < 0.05$, **** $p < 0.0001$, ns: not significant). Error bars represent mean ± standard deviation (SD). Data are representative of at least two independent experiments.

3.3. PEP treatment inhibits Th17 and Th2 differentiation

Next, I further assessed the effect of PEP supplementation on Th17 differentiation. PEP supplementation decreased IL-17A expression in pathogenic Th17 (pTh17) differentiation induced in the presence of IL-6, IL-1b, and IL-23 as well as npTh17 differentiation in a dose-dependent manner (Fig. 3.3A). Expression of RORγt, the Th17-lineage-specifying transcription factor, was dramatically decreased by PEP supplementation in cells under pTh17-skewing conditions but not under npTh17 conditions (Fig. 3.3B). PEP supplementation decreased mRNA expression of *Il17a* in both npTh17 and pTh17 cells, and *Rorc* expression in pTh17 cells (Fig. 3.3C), indicating that the PEP inhibits IL-17A and RORγt transcription. To examine whether PEP can regulate the IL-17A expression in already differentiated Th17 cells, I polarized naïve CD4 T cells under npTh17 or pTh17 conditions for 84 hours, then treated the cells with PEP or vehicle control (PBS) for 24 hours. PEP supplementation reduced IL-17A expression in already differentiated npTh17 and pTh17 cells, suggesting that PEP can reduce the IL-17 expression in mature Th17 cells (Fig. 3.3D).

I also examined the effect of PEP supplementation on the differentiation of Th1, Th2, and induced T regulatory (iTreg) cells. PEP supplementation significantly reduced IL-4 and IL-13 expression in Th2 cells without affecting GATA3 expression (Fig. 3.3E, F). In Th1 cells, PEP supplementation moderately increased T-bet and IFN-γ expression, while in iTreg cells PEP did not affect FOXP3 expression (Fig. 3.3E, F). These results indicate that PEP supplementation inhibits the differentiation of Th17 and Th2 cells, but not Th1 and iTreg cells.

Ho et al. reported that PEP plays an important role in sustaining TCR-mediated calcium signaling to maintain the effector function of CD4 T cells for anti-tumor defense [190]. Since activation of TCR signaling can enhance production of IL-2 thereby inhibiting IL-17A expression [206], I examined whether PEP supplementation enhances the TCR signaling-mediated IL-2 expression. Interestingly, PEP supplementation decreased IL-2 expression in npTh17 cells (Fig. 3.3F). In addition, the expression of CD25 (IL2RA), a T cell activation marker, was not affected by PEP in npTh17 cells (Fig. 3.4A). I also confirmed that PEP supplementation did not affect the proliferation of both npTh17 and pTh17 cells (Fig. 3.4B). These results suggest that suppression of Th17 differentiation by PEP is not due to the promotion of IL-2 expression or T-cell activation.

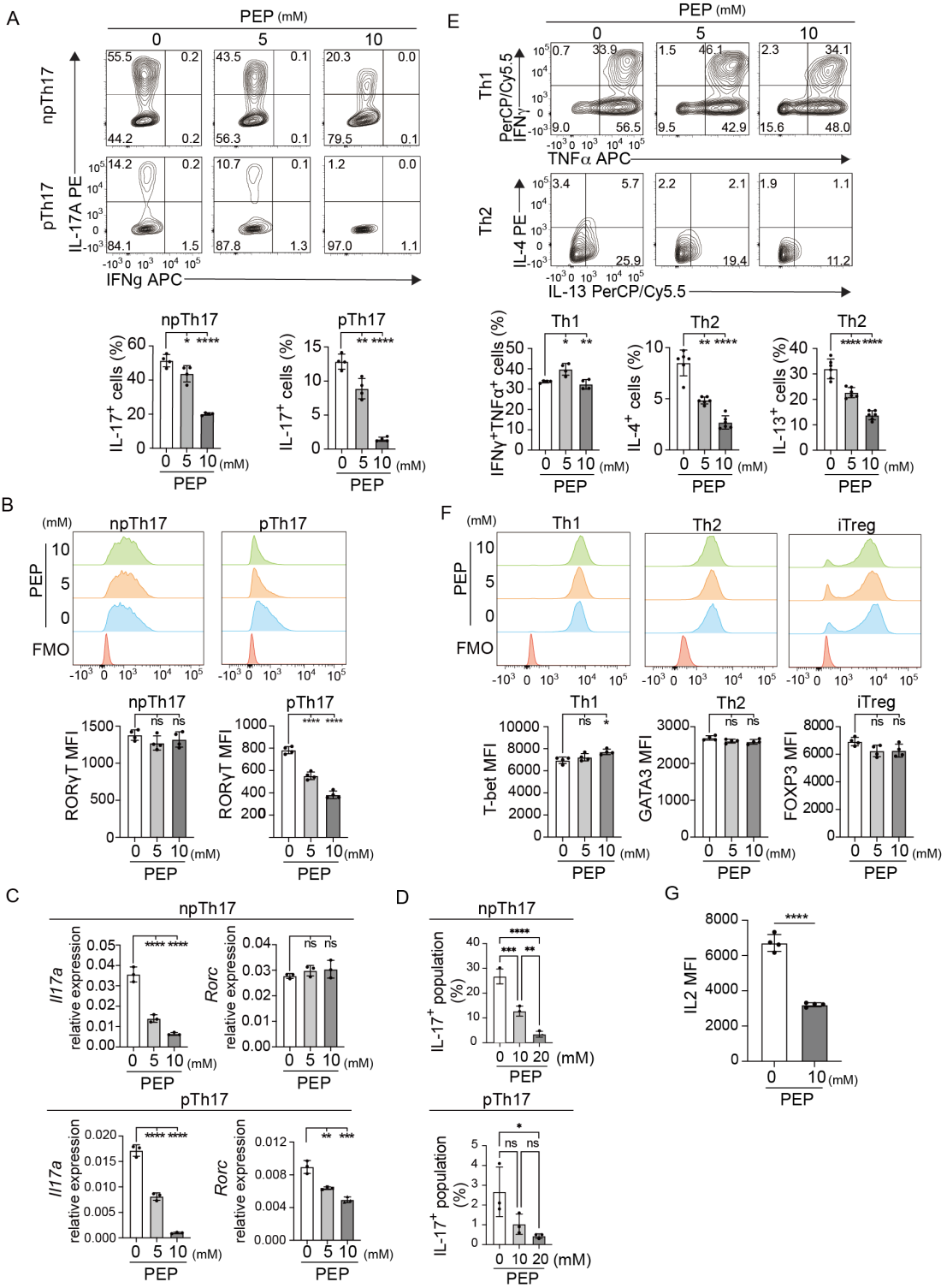

A
PEP (mM)
0
5
10
npTh17
pTh17
IL-17A PE
IFNg APC
55.5
0.2
44.2
0.2
43.5
0.1
56.3
0.1
20.3
0.0
79.5
0.1
14.2
0.2
84.1
1.5
10.7
0.1
87.8
1.3
1.2
0.0
97.0
1.1
npTh17
pTh17
IL-17+ cells (%)
PEP
B
(mM)
PEP
FMO
RORγT MFI
C
Il17a relative expression
Rorc relative expression
D
IL-17+ population (%)
E
Th1
Th2
PerCP/Cy5.5 IFNγ
TNFα APC
IL-4 PE
IL-13 PerCP/Cy5.5
IFNγ+TNFα+ cells (%)
IL-4+ cells (%)
IL-13+ cells (%)
F
iTreg
T-bet MFI
GATA3 MFI
FOXP3 MFI
G
IL2 MFI

Fig. 3.3. PEP supplementation inhibits differentiation of Th17 and Th2 cells
Naïve CD4 T cells were activated with CD3/CD28 antibodies in the absence or presence of PEP (5 or 10 mM) under npTh17 (TGF- β1 and IL-6), pTh17 (IL-6, IL-1β, and IL-23) conditions (A-C, G) or Th1, Th2, or iTreg conditions (E, F). Cells were collected at 48 h (C, G) or 60 h (A, B, D-F) after activation.
(A, B) Flow cytometry analysis of IL-17A and IFN-γ (A) and RORγt (B). Representative plots are shown in the upper panels. Bar graphs showing percentages of cells expressing IL-17A (A) or mean fluorescence intensity (MFI) of RORγt (B) (n = 4).
(C) qPCR analysis of *Il17a* and *Rorc* mRNA expression (n = 3). Relative expression to *Actb* is shown.
(D) Naïve CD4 T cells were activated with CD3/CD28 antibodies under npTh17 (TGF- β1 and IL-6), pTh17 (IL-6, IL-1β, and IL-23) conditions for 84 h and then cultured for another 24 h in the presence of PEP (10 mM and 20mM). Bar graphs showing percentages of cells expressing IL-17A (n=3).
(E, F) Flow cytometry analysis of expression of signature cytokines (E) and transcription factors (F) for Th1, Th2, and iTreg cells. Bar graphs showing percentages of cells expressing each cytokine (E) or MFIs of each transcription factor expression (F) (n = 4).
(G) Flow cytometry analysis of expression of IL-2 (n = 4). The p-value was calculated by a two-tailed unpaired Student's t-test (**** $p < 0.0001$).
(A-F) The p values were calculated by one-way ANOVA with Bonferroni's multiple comparison tests (* $p < 0.05$, **** $p < 0.0001$, ns: not significant). In all panels, error bars indicate mean ± SD. Data are representative of at least two independent experiments.

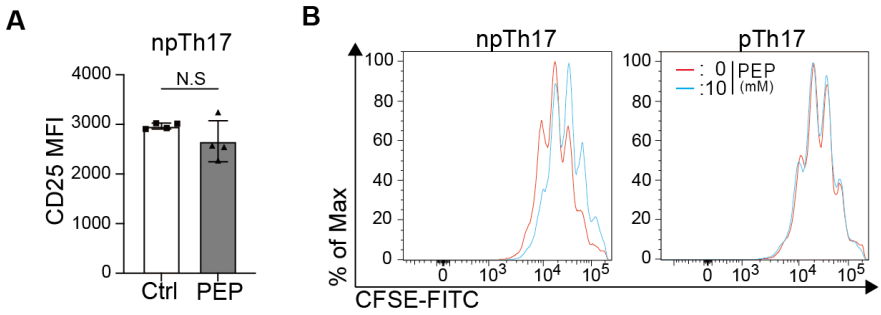


Fig. 3.4. PEP supplementation does not affect CD25 expression and proliferation of Th17 cells
Naïve CD4 T cells were activated with CD3/CD28 antibodies in the absence (Ctrl) or presence of PEP(10 mM) under npTh17 (with TGF-β1 and IL-6) or pTh17 (with IL-6, IL-1β, and IL-23) conditions for 48 h.
(A) CD25 expression in control or PEP-treated cells was analyzed by flow cytometry. The bar graph shows CD25 MFI. The p-value was calculated by a two-tailed unpaired Student' s t-test (ns: not significant). Error bars represent mean ± SD. Data are representative of at least two experiments.
(B) CFSE dilution in control or PEP-treated cells was analyzed by flow cytometry. Data are representative of two independent experiments.

3.4. PEP supplementation does not affect Th17 metabolism

The conversion of PEP to pyruvate by pyruvate kinase is one of the two enzymatic reactions that generate ATP in glycolysis, and pyruvate links glycolysis to mitochondrial TCA cycle. Therefore, PEP supplementation might affect not only glycolysis but also mitochondrial activity. To investigate this possibility, I treated differentiating Th17 cells with PEP and monitored their glycolytic and mitochondrial activities with a Seahorse analyzer (Fig. 3.5A-D). The analysis showed that neither the extracellular acidification rates (ECAR) (Fig. 3.5A-B) nor the oxygen consumption rates (OCR) were influenced by PEP supplementation (Fig. 3.5C-D). In addition, PEP supplementation did not alter the reactive oxygen species (ROS) levels in differentiating Th17 cells (Fig. 3.5E). These data suggest that PEP supplementation does not affect levels of glycolysis and oxidative phosphorylation in Th17 cells.

PKM2, one isoform of the pyruvate kinase, is required for the differentiation of Th1 and Th17 cells by translocating into the nuclei to promote lineage-specific transcriptional programs [182]. To test whether the PEP supplementation affects the balance of Pkm2 cytoplasmic/nuclear distribution, I examined the PKM2 expression in the cytoplasmic and nuclear fractions in differentiating npTh17 cells with PEP treatment. The result demonstrated that PEP supplementation did not alter the PKM2 expression or nuclear translocation (Fig. 3.5F).

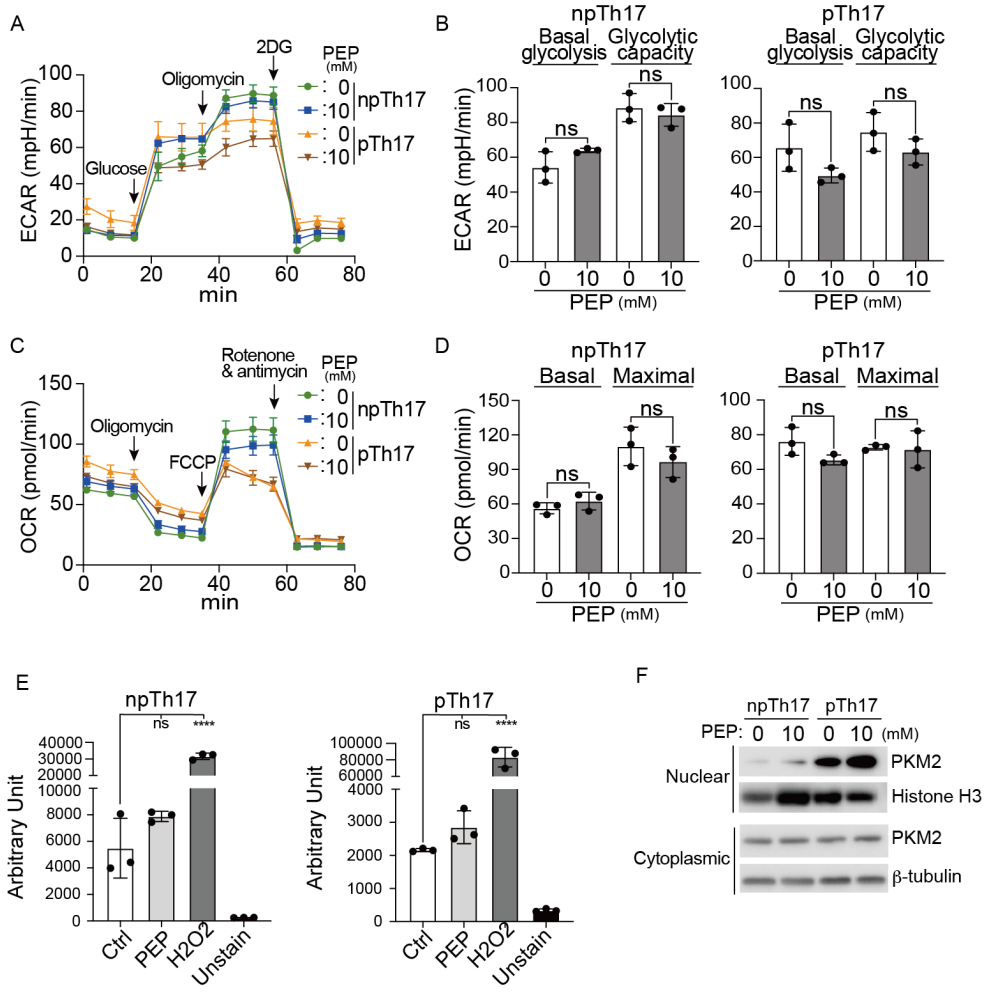


Fig. 3.5. PEP supplementation does not affect cellular metabolism and PKM2 activation
(A-F) Naïve CD4 T cells were activated with CD3/CD28 antibodies in the absence (Ctrl) or presence of PEP (10 mM) under npTh17 or pTh17 conditions. Cells were collected at 48 h (F) or 72 h (A-E) after activation.
(A, B) ECAR over time (A), ECAR after glucose addition (basal glycolysis) and after oligomycin addition (maximal glycolytic capacity) (B) were analyzed (n=3).
(C, D) OCR over time (C), basal OCR, and maximal OCR (D) were analyzed (n=3).
(E) Naïve CD4 T cells were activated with CD3/CD28 antibodies in the absence (Ctrl) or presence of PEP (10 mM) under npTh17 or pTh17 conditions for 48 h. ROS production was analyzed by staining with DCFDA / H2DCFDA Cellular ROS Assay Kit. Cells treated with 0.03% H2O2 were included as positive controls.
(F) Immunoblot analysis of PKM2 in nuclear and cytoplasmic fractions. Nuclear histone H3 and cytoplasmic b-tubulin were also detected as fraction markers. (B, D, E) The p-values were calculated by one-way ANOVA with Bonferroni's multiple comparison tests (**** $p < 0.0001$, ns: not significant). Error bars indicate mean ± SD. In all panels, data are representative of at least two independent experiments.

3.5. PEP regulates Th17 transcriptional program

To further dissect the effect of PEP supplementation on Th17 differentiation, I sought to examine the global alteration of transcriptome induced by PEP in Th17 cells. I performed RNA-sequencing analysis of npTh17 and pTh17 cells differentiated in the presence or absence of PEP supplementation for 48 h. PCA analysis showed that PEP-treated cells have distinct expression profiles compared to the control cells, and the influence of PEP supplementation on the transcriptome was more significant in pTh17 cells than in npTh17 cells (Fig. 3.6A). There were 300 differentially expressed genes (DEGs; 199 upregulated genes and 101 downregulated genes) between control and PEP-treated cells under npTh17-polarizing conditions, and 552 DEGs (241 upregulated genes and 311 downregulated genes) under pTh17-polarizing conditions (Fig. 3.6B). Gene set enrichment analysis revealed that PEP supplementation downregulated pathways related to chemokine/cytokine signaling and inflammatory responses (Fig. 3.6C). Notably, no gene ontology (GO) related to cellular metabolism was identified, which is consistent with our observation that glycolysis and oxidative phosphorylation in Th17 cells were not affected by PEP supplementation (Fig. 3.5A-D).

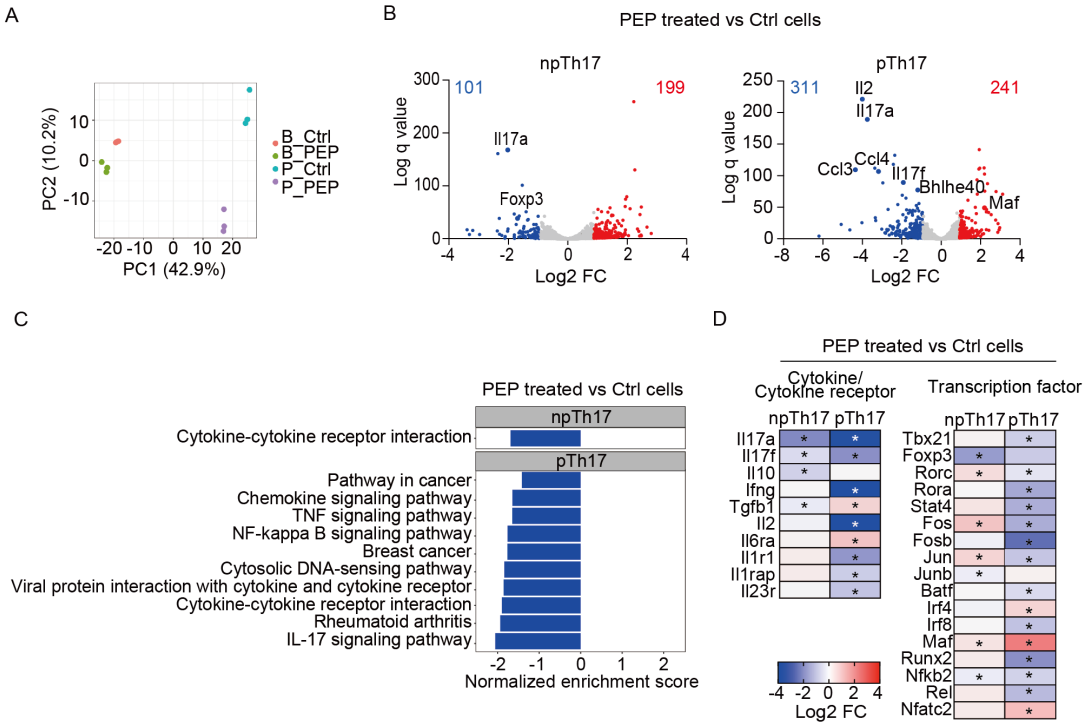
Next, I analyzed the expression of genes related to T cell biology (cytokines, cytokine receptors, and transcription factors) and found that PEP supplementation reduced the expression of *Il17a* and *Il17f* in both npTh17 and pTh17 cells, *Il10* in npTh17 cells, and *Ifng* in pTh17 cells (Fig. 3.6D). Interestingly, PEP supplementation reduced the expression of many cytokine receptors and transcription factors in pTh17 cells but not in npTh17 cells. These included cytokine receptors involved in the development of pathogenic Th17 cells (*Il1r1*, *Il1rap*, and *Il23r*), transcription factors involved in T cell differentiation or function (*Rorc*, *Rora*, *Fos*, *Jun*, *Batf*, *Irf8*, *Nfkb2*, *Rel*, *Tbx21* and *Stat4*). In addition, PEP supplementation enhanced expression of *Maf*, an AP-1 family protein that suppresses the proinflammatory function of Th17 cells, in pTh17 cells. These results suggest that PEP may primarily target transcriptional regulatory mechanisms specific to pathogenic Th17 cells, although it regulates IL-17A expression in both pathogenic and non-pathogenic Th17 cells.

To understand the mechanism by which PEP regulates the Th17 transcriptional program, I sought to identify transcription factors that regulate the expression of PEP-target genes including *Il17a*. I searched for motifs enriched within ±2 kb from the transcriptional start sites of DEGs (300 and 552 in npTh17 and pTh17, respectively) using the findMotifs function of Homer. Interestingly, the binding motifs for several members of AP-1 family proteins and interferon regulatory factors (IRF) were identified in both npTh17 and pTh17 cells (Fig. 3.6E).

It is known that the members of AP-1 and IRF family proteins, including JunB, BATF, and IRF4, are essential for initiating and maintaining the transcriptional program for Th17 differentiation [119-121]. To further evaluate the involvement of AP-1family proteins and IRF4 in the regulation of DEGs expression in PEP-treated cells, we searched the binding motifs of AP-1 (ATGACTCATC), JunB (RATGASTCAT), BATF (DATGASTCAT), and IRF4 (ACTGAAACCA), and AICE (NAGTTTCABTHTGACTNW) motifs within ±10 kb from the transcriptional start sites of the DEGs. The result showed that around 80% of the DEGs contain the binding motifs for JunB, BATF, and IRF4, as well as AICE motifs (Figure. 3.6F)

Among the AP-1 transcriptional family members, JunB has been reported to play a pivotal role in Th17 pathogenicity [127-129]. Given the observation that PEP supplementation inhibited more Th17-related gene expression in pTh17 cells than npTh17 cells, I hypothesized that JunB is involved in the PEP-mediated suppression of Th17 differentiation. By comparing the DEGs and the JunB-regulated genes reported in our previous study [128], I found that 80 npTh17 DEGs (26.7%) and 167 pTh17 DEGs (30.2%) overlapped with the JunB-regulated genes, respectively

(Fig.3.6G). There are 32 JunB-regulated genes affected by PEP in both npTh17 and pTh17 cells, which are mainly under the functional categories of cytokines and chemokines (*Il17a, Ccl4, Lta*), inflammatory response (*Crabp2, Abca1, Lgals3, Serpinb1a, Zfp608*), and differentiation regulation of lymphocyte (*Selp, Il1ra, Nr4a2, Nr4a3, Bcl6*) (Table. 3.1).



E

npTh17

Rank	Motif	Logo	q-value	% of Targets
1	IRF1		0.0390	26.64

pTh17

Rank	Motif	Logo	q-value	% of Targets
1	IRF3		0.0001	47.71
2	ISRE		0.0020	13.12
3	IRF1		0.0036	24.25
4	Fra1		0.0083	55.27
5	Fra2		0.0083	49.70
6	AP-1		0.0083	66.20

F

	npTh17		pTh17	
Motif	#DEGs Containing motif	%	#DEGs Containing motif	%
AP-1	255	85	477	86
JunB	243	81	452	82
BATF	246	82	465	84
IRF4	231	77	429	78
bZIP-IRF(AICE)	235	78	459	83

G

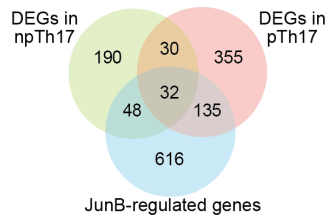


Fig. 3.6. PEP regulates JunB-dependent transcriptional program in Th17 cells
Naïve CD4 T cells activated with CD3/CD28 antibodies in the absence (Ctrl) or presence of PEP (10 mM) under npTh17 or pTh17 conditions for 48 h were analyzed by RNA-sequencing (n=3).
(A) Principal component analysis of RNA-seq data.
(B) Volcano plots showing differentially expressed genes (DEGs) in PEP-treated vs control cells (log2 fold change (FC) > 1, $p < 0.05$). Genes upregulated and downregulated by PEP treatment are shown in red and blue, respectively.
(C) Gene set enrichment analysis of PEP-treated vs control cells under npTh17 and pTh17 conditions.
(D) Heat map showing expression of cytokines, cytokine receptors, and transcription factors affected by PEP treatment under npTh17 and/or pTh17 conditions. Statistical significance was analyzed with the Wald test using DESeq26 (* $p < 0.05$, ** $p < 0.01$).
(E) Motif enrichment within ±2 kbp of the transcriptional start sites (TSS) of DEGs in PEP-treated vs. control cells was analyzed.
(F) Percentages of genes containing AP-1 or AICE motifs (within ±10 kbp of the TSS) among DEGs in PEP-treated vs control cells.
(G) Venn diagram showing the overlap between DEGs in PEP-treated vs control cells and JunB-regulated genes (log2 FC > 0.8, $p < 0.05$ in JunB KO vs control Th17 cells (GSE86499)).

BATF and IRF4 are responsible for epigenetic remodeling in Th17 differentiation [119,120]. To evaluate the influence of PEP supplementation on chromatin accessibility in differentiating Th17 cells, I performed an ATAC-seq analysis of npTh17 cells polarized with or without PEP supplementation for 48 h. I found that PEP supplementation only affected chromatin accessibility of a limited number of gene loci (Table. 3.2) that did not include DEGs in PEP-treated cells, such as *Il17a*, (Fig. 3.7). Overall, these results suggest that PEP regulates the Th17 transcriptional program mediated by AP-1 family proteins, and JunB might be a major target.

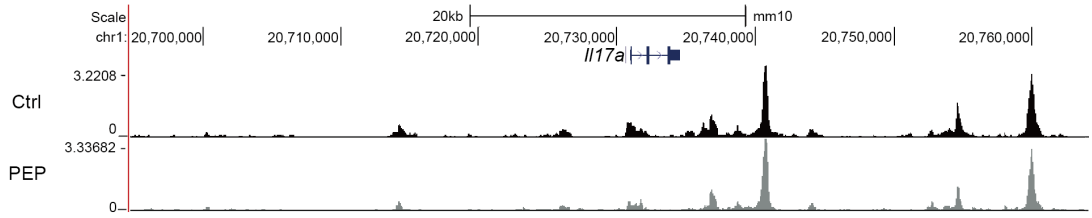


Fig. 3.7. PEP supplementation does not affect chromatin accessibility at the Il17a locus.
Naïve CD4 T cells were activated with CD3/CD28 antibodies in the absence (Ctrl) or presence of PEP (10 mM) under npTh17 (with TGF-β and IL-6) or pTh17 (with IL-6, IL-1β, and IL-23) conditions for 48 h and analyzed by ATAC-seq. The results for the Il17a locus are shown by UCSC Genome Browser (UCSC Genomics Institute).

3.6. PEP regulates AP-1 functions

The observation that PEP affected the expression of genes regulated by JunB prompted me to evaluate JunB expression and activity in PEP-treated Th17 cells. First, I examined the expression of JunB and other AP-1 family members in npTh17 and pTh17 cells by western blotting. PEP supplementation moderately reduced the expression of BATF and JunB and slightly enhanced the expression of IRF4 in pTh17 cells but not in npTh17 cells. On the other hand, expression of other transcription factors involved in Th17 differentiation, including JUND, HIF1α, STAT3, and phosphorylated STAT3, were not affected by PEP supplementation (Fig. 3.8A, B).

Next, I examined whether PEP supplementation controls their function by affecting their DNA-binding ability. It has been reported that JunB/BATF dimer interacts with IRF4 to transactivate IL-17A expression through binding to the AICE motifs located near *Il17a* coding regions [127,128]. I monitored the binding of BATF, IRF4, and JunB to four major binding sites located at the *Il17a* locus by ChIP PCR in npTh17 cells at 36 h after differentiation (Fig. 3.8C). PEP supplementation suppressed the binding of BATF, IRF4, and JunB to the 3' intergenic and promoter regions at the *Il17a* locus (Fig. 3.8D). Interestingly, PEP seems to inhibit the DNA-binding of these transcription factors in a locus-specific manner, as I did not observe significant changes in DNA-binding of BATF, IRF4, and JunB at the *Irf8* locus, another JunB-regulated gene [127], in PEP-treated cells (Fig. 3.8D). These results suggest that PEP impedes the DNA-binding ability of BATF, IRF4, and JunB at specific loci, including *Il17a*, in Th17 cells.

JunB and BATF form heterodimers to regulate the expression of target genes [207,208]. To test whether PEP supplementation disturbs the formation of JunB/BATF dimers, I added PEP into lysates of HEK293 cells overexpressed HA-tagged BATF and Flag-tagged JunB and performed co-immunoprecipitation analysis. PEP addition did not affect the coimmunoprecipitation of JunB with BATF, suggesting that PEP supplementation does not influence the JunB/BATF dimerization in Th17 cells (Fig. 3.8E).

PEP has been shown to control protein functions as an allosteric regulator or phosphate donor [185,209-211]. Therefore, I assessed whether PEP interacts with JunB by a biochemical assay using isotope-labeled PEP. I incubated purified FLAG-tagged JunB protein with ^{32}P-labeled PEP (4mM), with or without non-labeled PEP (100mM) or calf intestinal phosphatase (CIP). Measurement of radioactivity reveals that labeled PEP was pulled down with Flag-JunB (Fig. 3.8F). Furthermore, the level of labeled PEP pulled down with JunB was reduced by the coincubation with unlabeled competitor PEP but not with CIP treatment. These results suggest that PEP may control the DNA-binding ability of JunB by binding to JunB or JunB-interacting proteins, but not as a phosphate donor.

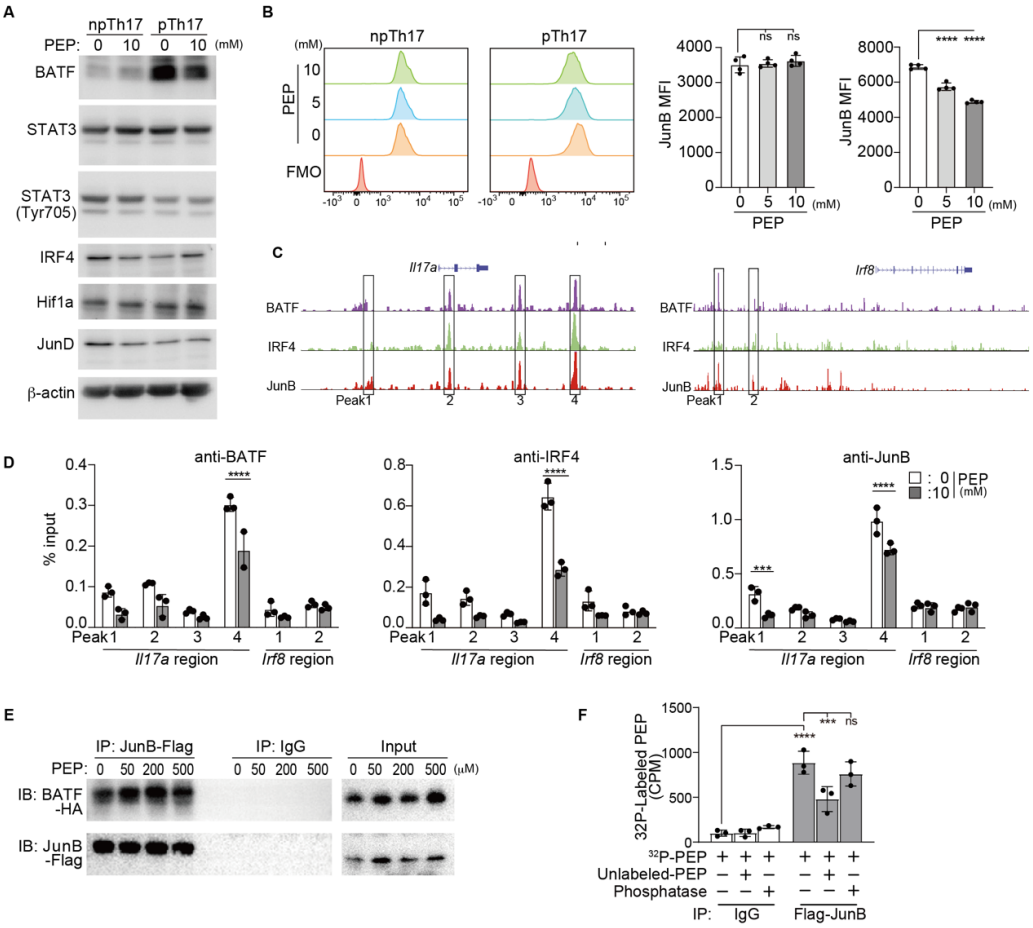


Fig. 3.8. PEP inhibits DNA binding of JunB, BATF, and IRF4 at the *Il17a* locus

(A, B) Naïve CD4 T cells were activated in the absence or presence of PEP (5 or 10 mM) under npTh17 or pTh17 conditions for 60 h. (A) Expression of Th17-related transcription factors were analyzed by immunoblot. (B) Expression of JunB was analyzed by flow cytometry. (C) ChIP-seq peaks for JunB, BATF, and IRF4 were detected at *Il17a* and *Irf8* loci. ChIP-seq data were from GSE86535. Schematic diagrams at the tops of panels indicate transcription start sites (arrows) and exons (filled boxes) of each gene. Open boxes represent regions detected by ChIP-PCR in D. (D) ChIP-PCR analysis showing DNA-binding of JunB, BATF, and IRF4 at the loci of *Il-17a* and *Irf8*. Cells were collected at 36 h after activation (n=3). (E) Immunoblot analysis (IB) of the immunoprecipitation (IP) of FLAG-tagged JunB together with HA-tagged BATF expressed in HEK293 cells. IP was performed in the absence or presence of PEP. (F) Analysis of interaction between JunB and radiolabeled PEP. Radiolabeled PEP (4mM final concentration) was mixed with FLAG-JunB on anti-FLAG beads pull-down from FLAG-JunB overexpressed HEK293 cells. In the control group, either unlabeled PEP (100mM final concentration) or calf intestinal alkaline phosphatase (10 units) was added. The radioactivity of eluted immune precipitates was measured by a scintillator (ALOKA; Model: LSC-6100). (B, D, and F) The p-values were calculated by one-way ANOVA with Bonferroni's multiple comparison tests (*** $p < 0.001$, **** $p < 0.0001$, ns: not significant). Error bars indicate mean ± SD. Data are representative of at least two independent experiments.

3.7. PEP administration inhibits *in vivo* Th17 differentiation and EAE

Because PEP supplementation significantly suppressed IL-17A expression *in vitro*, I next evaluated the effect of PEP treatment on Th17 differentiation *in vivo* using murine models. I transferred ovalbumin (OVA)-specific OT-II naïve CD4 T cells into congenic recipients on day 0, followed by immunization with OVA peptides emulsified in complete Freund's adjuvant (CFA) with or without PEP (0.5g / kg) on day 1. Daily subcutaneous injection of PEP (1g/kg) or vehicle was performed until day7, then the cells in the inguinal lymph nodes and spleens were analyzed by flow cytometry. PEP treatment reduced IL-17A and RORγt expression in the transferred OT-II T cells in the lymph nodes, while expression of the IFN-γ and FOXP3 was not affected (Fig. 3.9A, B). PEP treatment also reduced IL-17A expression in the OT-II T cells in the spleens (Fig. 3.9C, D).

I next sought to determine whether PEP treatment can inhibit experimental autoimmune encephalomyelitis (EAE), a Th17-driven autoimmune disease model of multiple sclerosis. Mice were immunized with MOG peptides emulsified in CFA on day 0, followed by pertussis toxin injection on day 0 and day 2 for EAE induction. PEP (1g/kg) or vehicle was subcutaneously injected daily from day 0, and the body weight and clinical score of animals were recorded daily. The result demonstrated that EAE severity was significantly ameliorated in the PEP-receiving group (Fig. 3.9E). These results indicate that PEP administration inhibits *in vivo* Th17 generation and Th17-mediated EAE.

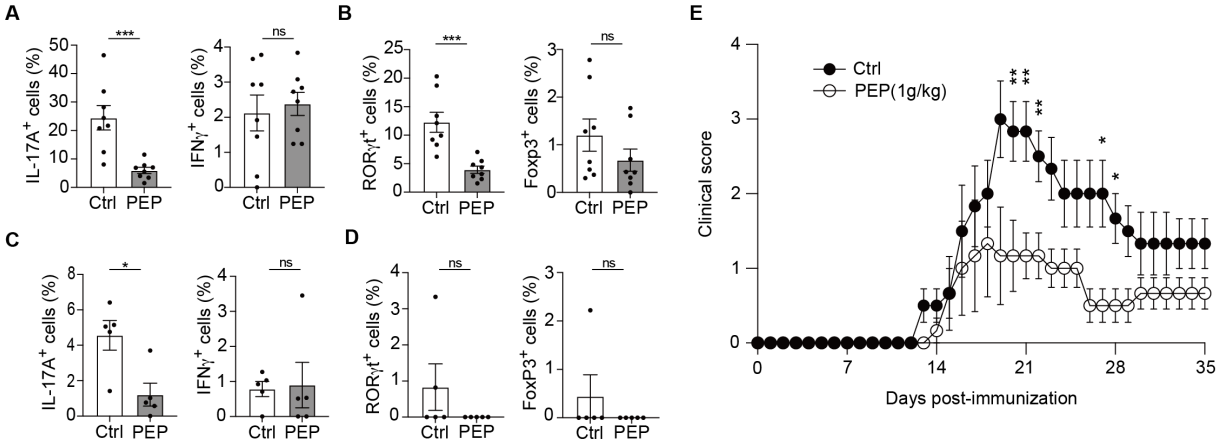


Fig. 3.9. PEP inhibits Th17 differentiation *in vivo* and ameliorates EAE

(A-D) Flow cytometry analysis of expression of IL-17A and IFN-y (A) or RORγt and Foxp3 (B) in OT-II T cells. Congenic recipient mice were adoptively transferred with OT-II T cells on day 0, followed by immunization of OVA emulsified in CFA on day 1. Mice were daily injected s.c. with vehicle (Ctrl) or PEP (1 g/kg). On day 7, cells were isolated from the lymph nodes (A,B) or spleens (C, D) and analyzed. Error bars indicate the mean ± standard error of the mean (SEM) (n = 8 from two independent experiments). The p-values were calculated by two-tailed unpaired Student's t-tests (*** $p < 0.001$, ns: not significant). (E) Disease scores in EAE mice treated with vehicle or PEP (1 g/kg). Mice were daily injected s.c. with vehicle (Ctrl) or PEP (1 g/kg). Error bars indicate the mean ± SEM. * $p < 0.05$. *p*-values were calculated by two-way ANOVA with Šidák test. Data is representative of two independent experiments.

Chapter 4. Discussion, Limitation of the Research, and Outlook

4.1. Discussion

In this study, I identified PEP as an immunoregulatory metabolite that inhibits Th17 differentiation. PEP supplement selectively inhibits the differentiation of Th2 and Th17 cells, but not Th1 and Treg cells. In Th17 cells, suppressing glycolysis by glucose limitation or glycolytic inhibitors boosts IL-17A expression, whereas PEP supplementation or blockage of PKM activity decreases IL-17A expression. Mechanistically, accumulated cellular PEP suppresses the JunB-dependent Th17 transcriptional program and the transcription of *Il17a* by impeding the binding of JunB, BATF, and IRF4 to the 3' intergenic and promoter regions. *In vitro* binding assay reveals that PEP can interact with JunB, suggesting that PEP might control JunB activity by allosteric regulation. Notably, PEP treatment ameliorates EAE progression in mice. Overall, these results indicate that PEP modulates the JunB-dependent Th17 transcriptional program.

The discovery of the immunoregulatory function of PEP provides new insight into how glycolysis modulates Th17 differentiation. My data revealed that treatment with glycolytic inhibitors 2-DG or HA, which downregulates cellular PEP levels [190], enhances IL-17A expression, while OXA, which increases cellular PEP levels [190], inhibits IL-17A expression in Th17 cells. Moreover, treatment with glycolytic intermediates G6P, F6P, 3PG, or PEP suppresses IL-17 expression. These results suggest that glycolysis can play an inhibitory role in Th17 differentiation. Nonetheless, the role of glycolysis in Th17 differentiation is still controversial. It has been known that glycolysis is essential for Th17 development, based on the effects of knockout of glycolytic enzyme genes on Th17 generation [141,212,213], but recent studies have reported that inhibition of glycolysis by 2-DG can upregulate IL-17 expression in Th17 cells [199,200]. This discrepancy might be due to the differences in environmental nutrition levels and cellular glycolytic status, as the culture medium and Th17-polarizing method varied from study to study.

How glucose availability under different physiological conditions modulates T-cell metabolism remains largely unknown. In physiological conditions, glucose availability varies from tissue to tissue. While blood and lymphoid organs are considered nutrition-rich, glucose can be scarce in inflammation sites or tissues due to the competition of nutrients from proliferating immune cells, other somatic cells, or microbes. Since T cells travel among different tissues during differentiation, metabolic reprogramming might be necessary for modulating the proliferation and function of T cells residing in different tissues. A recent study reports that intestine-resident CD8 memory T cells show different dependency on environmental glucose, fatty acids, and glutamine compared to their circulating counterparts [232]. Therefore, the fine-tuning of glycolytic activity might be required to maximize the differentiation efficiency of T cells. My discovery that low-glucose culture condition enhances Th17 differentiation (Fig. 3.1A) suggest that Th17 cells might evolve to survive in a glucose-scarce environment, as they usually reside in the mucosal surface of intestine, skin and lung. Taken together, my discovery suggests that while glycolysis is critical for Th17 differentiation and clonal expansion, it also provides PEP-mediated rheostat control to avoid Th17 pathogenicity.

I found that a fixed concentration of 2-DG (2 mM) could either promote or decrease IL-17A expression under high or low glucose culture conditions, respectively. This is consistent with previous reports that 2-DG treatment increases IL-17A expression in a dose-dependent manner but decreases IL-17A expression when the concentration exceeds the threshold [199,200]. These observations suggest that a certain level of glycolysis is necessary for IL-17A expression but overactivation of glycolysis inhibits IL-17A expression. I speculate that the overactivation of glycolysis accumulates PEP, thereby inhibiting IL-17A expression. Because pathogenic Th17 cells generated *in vitro* or *in vivo* are more glycolytic than non-pathogenic Th17 cells [193,194], PEP-dependent negative regulation of IL-17A expression may be more prominent in pathogenic Th17 cells than the non-pathogenic Th17 subsets. This might be one explanation why PEP supplement induced a more obvious impact on the expression of cytokines and transcription factors in the pathogenic Th17 cells than the non-pathogenic ones (Fig. 3.3A-D, Fig. 3.6A-E). In addition to this heterogeneity in the glycolytic capacity of Th17 cells, changes in environmental glucose levels also affect the Th17 response, as observed that high glucose intake exacerbates the development of Th17-dependent EAE [214]. How changes in environmental glucose levels affect PEP-dependent negative regulation of Th17 responses in different tissues is an interesting question to be further explored.

This study revealed that PEP could modulate AP-1-dependent Th transcriptional program. Like *Junb* knockout[127-129], PEP treatment reduces *Il17a* expression in both non-pathogenic and pathogenic Th17 cells, inhibiting *Rorc* and *Il23r* expression specifically in pathogenic Th17 cells, reducing binding of BATF and IRF4 at the *Il17a* locus, and ameliorates EAE. Furthermore, PEP treatment suppresses IL-4 and IL-13 expression in *in vitro* polarized Th2 cells, which is similar to the *Junb* knockout[215,216]. On the other hand, JunB partners, BATF and IRF4, play a critical role in promoting the chromatin accessibility of T cell lineage-associated loci[121-123], and IRF4 is an essential player in T cells' metabolic transition and clonal expansion after TCR stimulation [217,218]. However, although PEP inhibits the recruitment of BATF and IRF4 to *Il17a* 3'-UTR, neither the chromatin accessibility nor the cellular metabolism is significantly affected by PEP in Th17 cells. Taken together, these results suggest that PEP plausibly regulates Th17 and Th2 differentiation by targeting JunB.

Despite the similarities mentioned above, PEP treatment does not affect the JunB-dependent cell survival promotion and the expression of several JunB target genes in Th2 and Th17 differentiation. In Th2 differentiation, loss of JunB causes apoptosis and significantly reduces the number of viable cells [216], whereas PEP treatment increases the number of viable Th2 cells. (Fig. 4.1). On the other hand, in Th17 differentiation, loss of JunB, but not PEP treatment, upregulates the expression of T-bet, IFN-γ, FOXP3, and IRF8[127,128]. Consistent with these observations, the PEP-mediated inhibitory effect on JunB DNA-binding activity seems to be locus-specific. As shown by ChIP-PCR results, PEP treatment inhibits the binding of BATF, IRF4, and JunB at the *Il17a* locus but not the *Irf8* locus (Fig. 3.8D). This locus-specific effect of PEP-mediated control of JunB, BATF, and IRF4 binding activity may account for the impact of PEP supplementation on the expression of a specific subset of JunB target genes. The mechanism by which PEP regulates JunB DNA binding activity in a locus-specific manner is an interesting question to be addressed in the future.

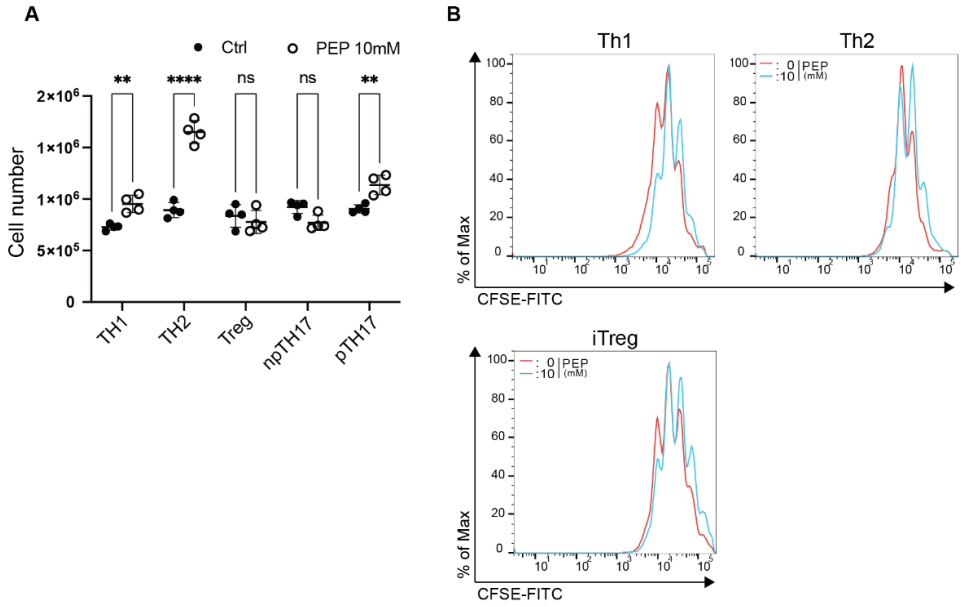


Fig. 4.1. The effect of PEP supplement on the proliferation of CD4 Th cells
Naïve CD4$^+$T cells were activated with CD3/CD28 antibodies in the absence (Ctrl) or presence of PEP(10 mM) under Th1, Th2, or iTreg conditions for 48 h. The harvest cells were used for cell number counting and CFSE staining.
(A) Cell number of live CD4$^+$Th cells in the presence or absence of PEP during polarization. The number of live cells was counted by a Guava Muse Cell Analyzer. The p values were calculated by two-way ANOVA with Šidák test. (** $p < 0.01$, **** $p < 0.0001$, ns: not significant). Error bars indicate mean ± SD. Data are representative of two experiments.
(B) CFSE dilution in control or PEP-treated cells was analyzed by flow cytometry. Data are representative of two independent experiments. CFSE staining result of npTh17 and pTh17 is demonstrated in Fig. 3.4B.

The PEP quantification results demonstrate that the intracellular PEP level of differentiating Th17 cells can be increased by either exposure to PEP for 2- 60 hours (Fig. 3.1.E and Fig. 4.2A), or 3PG, an upstream glycolytic intermediate of PEP, for 2 hours (Fig. 4.2B), suggesting that PEP and 3PG can permeabilize into differentiating Th17 cells *in vitro*. The transportation mechanism of PEP across the mammalian cell membrane is still not fully understood. It is reported that inhibition of the activity of the anion transporters impedes the transportation of PEP in erythrocytes [233,234], suggesting that the transportation of PEP is controlled by the anion transporter such as SLC4 family transporters [235] or organic anion transporters [236]. However, the dosages of PEP utilized in my supplementation experiments are much higher than the detected amount of PEP in the human serum (10-100 μM), suggesting that PEP might not be able to regulate the function of Th17 cells under normal physiological conditions. Nonetheless, PEP supplementation is an ideal approach to study the effects of alteration of intracellular PEP level on Th17 differentiation, and to evaluate its therapeutic potential on autoimmune diseases.

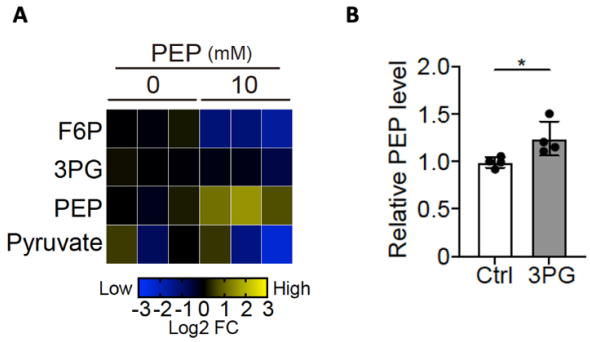


Fig. 4.2. The effect of PEP supplement on the proliferation of CD4 Th cells
(A) Heat map showing levels of glycolytic metabolites measured by LC-MS (n=3 biological replicates). Naïve $CD4^{+}$T cells were activated with CD3/CD28 antibodies in the absence or presence of PEP(10 mM) under npTh17 condition for 60 hrs. Cells were then harvested, and the intracellular level of indicated metabolites was analyzed by LC-MS.
(B) Quantification of intracellular PEP (n = 4). Cells were activated in the absence or presence of 3PG (10 mM) for 2 h. The p-value was calculated by a two-tailed unpaired Student's t-test (* $p < 0.05$). Data are representative of two experiments.

My biochemical assay using radioisotope-labeled PEP showed that PEP could directly interact with JunB and/or JunB-interacting proteins. I also observed that PEP inhibits the DNA-binding of the JunB/BATF complex but not the dimerization of JunB and BATF. These data suggest that PEP interacts with and modulates the activity of the JunB complex to bind to target DNA. The mechanism underlying this remains unknown, but I speculate that interaction with PEP may change the conformation of JunB or JunB-interacting proteins or the composition of JunB complex. Notably, the amino acid sequences of the C-terminal DNA binding domain and basic leucine zipper domain are highly conserved among Jun family members [219,220], implying the possibility that PEP may regulate the DNA-binding ability of other Jun family proteins. This can be addressed by proteomic analysis of the impact of PEP supplement on JunB interactome, identification of the PEP-binding site(s) in JunB, and functional assay with JunB mutated in PEP-binding sites.

4.2. Limitations of the research

Several challenges remain for further insight into the immunoregulatory role of PEP and the therapeutic potential of PEP for Th17-dependent autoimmune diseases. First, the role of endogenous PEP in T cell differentiation is not yet fully clarified, although my data on the effects of glycolytic inhibitors suggest that endogenous PEP inhibits Th17 differentiation. Since inhibition of glycolytic enzymes impedes all downstream glycolytic events, I could not distinguish the effect of changes in PEP levels from that of other events regulated by glycolytic inhibitors and their role in T cell differentiation. To address this issue, another approach targeting the PEP immunoregulatory function, such as the interaction between PEP and JunB, would be helpful in future research. Second, my data suggest that inhibition of EAE by PEP administration is likely due to decreased Th17 generation, but it cannot rule out the involvement of PEP regulation of other immune cells. The role of PEP in other immune cells, such as macrophages and Treg cells, in which AP-1 plays a critical role, needs to be elucidated in the future. Third, the mechanism by which PEP regulates Th17 differentiation needs further clarification. The mechanism of action of PEP in inhibiting the DNA binding of JunB, BATF, and IRF4 is not yet clear. In addition, PEP likely regulates other transcription regulatory mechanisms in Th17 cells based on my RNA-seq data. Furthermore, although no significant effects of PEP supplementation on glycolysis and OXPHOS were observed, the effects on the Th17 metabolome remain to be determined. Fourth, the role of PEP in human Th17 cells should be addressed in the future to further evaluate the therapeutic potential of PEP against autoimmune diseases.

4. 3. Conclusion and Outlook

This study demonstrates that PEP suppresses Th17 differentiation and Th17-dependent autoimmune diseases. PEP supplement significantly decreases the differentiation of Th2 and Th17 cells, but not Th1 and Treg cells. PEP directly binds to JunB and impedes the binding of JunB, BATF, and IRF4 to the *Il17a* locus, thus hindering the expression of IL-17A through allosteric control (Fig. 4.2). Importantly, PEP treatment ameliorates EAE progression in mice. Taken together, these results indicate that PEP is an immunoregulatory metabolite that regulates the Th17 transcriptional program and the therapeutic potential of PEP against autoimmune diseases.

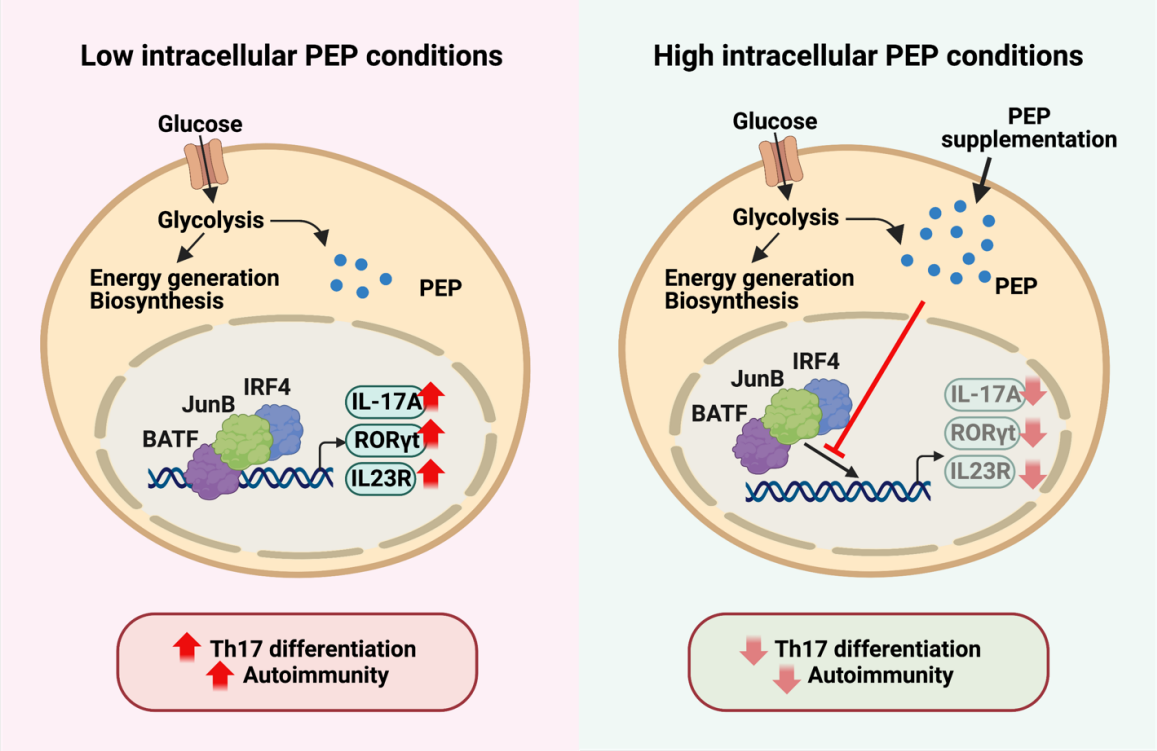


Fig. 4.3. Figure Summary: Phosphoenolpyruvate regulates the JunB-dependent pathogenic Th17 transcriptional program

In Th17 cells, accumulated cellular PEP impedes the binding of AP-1 family proteins and their transactivation partner BATF, JunB, and IRF4 to the *Il17a* locus and other Th17 signature genes, thus inhibiting the differentiation and pathogenic features of Th17 cells.

PEP treatment may provide advantages over the current therapies for autoimmune diseases, such as multiple sclerosis (MS). The current drugs for MS include inhibitors of glycolysis or mitochondrial activities that target lymphocyte activation, signaling cascades of proinflammatory cytokines, or restraining T cell proliferation[221-224]. Because of their low target specificity, these therapies often weaken the whole immune system or prevent the growth of other somatic cells, causing severe side effects and increased susceptibility to infections [221-223,225]. In this respect, PEP might cause fewer side effects because my data suggest that PEP can specifically suppress the proinflammatory function of Th17 cells without hindering the proliferation and function of other $CD4^+$ T cells. With chemical modification to improve PEP stability and/or sophisticated cell type-specific delivery vehicles such as nanoparticles[226-228], PEP and its chemical derivatives could be novel therapeutic agents against autoimmune disorders.

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Appendices

Table 2.1. List of PCR primers

	Gene	Sequences
qPCR	Actb_F	CATTGCTGACAGGATGCAGAAGG
	Actb_R	TGCTGGAAGGTGGACAGTGAGG
	Il17a_F	CAGACTACCTCAACCGTTCCAC
	Il17a_R	TCCAGCTTTCCCTCCGCATTGA
	Rorc_F	AGCGCACCAACCTCTTTTCAC
	Rorc_R	ATGAAGCCTGAAAGCCGCTTG
ChIP-qPCR	Il17a_Promoter_F	ACCAGTGAGGAACCCACCTA
	Il17a_Promoter_R	CCTGAAAATCGAGTCAAGCAG
	Il17a_Intron_F	GGTAGGTTTTCTGCAGTCCT
	Il17a_Intron_R	GCTGAATGACCCCGATTTTC
	Il17a_3'UTR1_F	TGCCAACAGGTCAGTTTCAA
	Il17a_3'UTR1_R	CCATTCAGAAGGATCCCTGA
	Il17a_3'UTR2_F	GGCTTGTCCCTCACATACCT
	Il17a_3'UTR2_R	ATATGGGCATGAGCAAAGTG

Table 3.1. List of shared DEGs and JunB-regulated genes (related to Fig. 3.6G)

npTH17		pTH17				All
Il17a	Chst11	Il17a	Mcoln2	Izumo1r	Cd80	Il17a
Il18r1	Arhgef3	Il17f	Penk	Thy1	Gap43	Il24
Il18rap	Lgals3	Neurl3	Ccne2	Dmxl2	Cd96	Selp
Bcl2	Cx3cr1	Sema4c	Atp6v0d2	Parp16	Nfkbiz	Ifi203
Serpinb5	Ccr8	Npas2	Cd72	Rora	Nxpe3	Irf6
Cd55	Ccr5	Il1r1	Nr4a3	Aqp9	Mx1	Gad2
Il24	Tgtp1	Serpine2	Abca1	Ccrl2	Myo1f	Il1rn
Prg4	Tgtp2	Fam124b	Gm5152	Ccr4	Ltb	Nr4a2
Sell	Ccl3	St8sia4	Il6	Plek	Lta	Car5b
Selp	Ccl4	Il24	Rasl11b	Rel	Ddr1	Cd5l
Slamf7	Serpinb1a	Lad1	Kit	Tgtp2	Runx2	Crabp2
Ifi203	S1pr3	Ptgs2	Cxcl3	Il3	Ppic	Csf1
Atf3	Il9	Tnfsf4	Areg	Acsl6	Zfp608	Gbp7
Irf6	Glrx	Selp	Cxcl10	Tm4sf5	Iigp1	Atp6v0d2
Gata3	Ston2	Ifi203	Anxa3	Aldoc	Slc15a3	Nr4a3
Plxdc2	Evl	Irf6	Serpine1	Nos2	Nmrk1	Abca1
Gad2	Rai14	Itih5	Peg10	Slfn2	Il33	Cxcl10
Il1rn	Arc	Gad2	Gimap7	Ccl3		Srgap3
Eng	Ly6c1	Il1rn	Tnip3	Ccl4		Cyp2s1
Stom	Ly6g	Slc2a6	Mgll	Dusp14		Chst11
Nr4a2	Apol7e	Ak1	Eif4e3	Gngt2		Lgals3
Sdcbp2	Lrrk2	Fmnl2	Srgap3	Mpp2		Tgtp2
Procr	Bcl6	Nr4a2	Apold1	Fam20a		Ccl3
Cd40	Lta	Ermn	Fosb	Zfp750		Ccl4
Foxp3	Zfp608	Ifih1	Cyp2s1	Akr1c18		Serpinb1a
Ar	Iigp1	Dhrs9	Plekhf1	Idi2		Il9
Tspan6	Cd74	Fam171b	Nkg7	Serpinb1a		Ston2
Car5b	Ms4a6c	Pla2g4f	Arrdc4	Serpinb6b		Ly6c1
Cd5l	Ms4a6b	Sord	Irf7	Serpinb9		Bcl6
Crabp2	Gfra1	Tgm2	Perp	Cd83		Lta
Csf1		Sulf2	Marcks	Il9		Zfp608
Gstm1		Dgkk	Cd24a	Ctla2a		Iigp1
F3		Maoa	Slc29a3	Plk2		
Gbp7		L1cam	Adamts14	Hdac9		
Atp6v0d2		Maged1	Prf1	Arhgap5		
Nr4a3		Cysltr1	Gadd45b	Egln3		
Abca1		Car5b	Chst11	Fos		
Gsap		Gpm6b	Lum	Ston2		
Cxcl10		Tlr7	Ifng	Kcnk10		
Msi1		Zbtb10	Tbc1d30	Ifi27		
Vax2		Il2	Il23a	Crip2		
Srgap3		Fgf2	Ikzf4	Itgb8		
Klrd1		Cd5l	Ddx60	Capsl		
St8sia1		Crabp2	Neto2	Osr2		
Cyp2s1		Lingo4	Lgals3	Ly6c1		
Gvin1		Ecm1	Tgm1	Tnfrsf13c		
Nupr1		Ppm1j	Cysltr2	Ccdc184		
Lsp1		Csf1	Tnfsf11	Wnt1		
Themis		Sgms2	Casp4	Rtp4		
Zfp365		Gbp7	Gpr83	Bcl6		

Table 3.2. List of ATAC candidates

Geneid	Chr	Start	End	Length	Log2 FC	Annotation		Gene Name	Gene Type
Interval_5210	10	22142311	22142570	260	-1.7	intron	6169	E030030I06Rik	protein_coding
Interval_14092	12	74803945	74804497	553	-1.7	Intergenic	373111	Kcnh5	protein_coding
Interval_372	1	37286376	37287281	906	-1.6	Intergenic	12868	1700074A21Rik	TEC
Interval_41773	4	3089526	3090982	1457	-1.6	Intergenic	14338	Vmn1r-ps2	unprocessed_pseudogene
Interval_60907	9	3015992	3016356	365	-1.5	intron	520	Gm10720	protein_coding
Interval_64032	9	110907235	110908159	925	-1.5	Intergenic	9164	Gm590	protein_coding
Interval_60909	9	3029501	3029868	368	-1.4	promoter-TSS	2162	Gm10717	protein_coding
Interval_41326	3	138874374	138875197	824	-1.4	intron	-45624	Gm16060	processed_pseudogene
Interval_29322	18	3004844	3005509	666	-1.3	Intergenic	22705	Vmn1r-ps151	unprocessed_pseudogene
Interval_7317	10	100542837	100543874	1038	-1.3	exon	45903	4930430F08Rik	protein_coding
Interval_38522	3	8244715	8246306	1592	-1.3	Intergenic	31179	Gm7103	processed_pseudogene
Interval_42986	4	63935297	63936155	859	-1.3	Intergenic	-39627	Rpl17-ps4	processed_pseudogene
Interval_29435	18	6404830	6406526	1697	-1.2	Intergenic	-34018	Gm6291	processed_pseudogene
Interval_31948	19	21106153	21106920	768	-1.2	Intergenic	-152335	Tmc1	protein_coding
Interval_62362	9	61243534	61244008	475	-1.2	Intergenic	127856	Gm10655	lincRNA
Interval_64609	9	124256379	124257987	1609	-1.2	Intergenic	54716	2010315B03Rik	protein_coding
Interval_7733	10	121315540	121316716	1177	-1.2	Intergenic	-4939	Tbc1d30	protein_coding
Interval_50234	6	31169197	31170096	900	-1.2	intron	-22228	Gm37728	TEC
Interval_50151	6	29747936	29748930	995	-1.2	intron	-2328	Gm13717	processed_pseudogene
Interval_59443	8	86888002	86888409	408	-1.1	Intergenic	2518	2010110E17Rik	lincRNA
Interval_36516	2	128031842	128032864	1023	1.0	intron	-57571	Gm23101	snRNA
Interval_31917	19	17359374	17360010	637	1.0	Intergenic	-3025	Gcnt1	protein_coding
Interval_33433	2	11540366	11540912	547	1.0	intron	-5945	Gm37975	TEC
Interval_51621	6	88817038	88817314	277	1.0	intron	24759	Abtb1	protein_coding
Interval_11549	11	99424185	99424832	648	1.0	exon	-2250	Krt12	protein_coding
Interval_49418	5	142901522	142902780	1259	1.0	TTS	1503	Actb	protein_coding
Interval_46404	5	33265315	33266601	1287	1.0	TTS	9023	Ctbp1	protein_coding
Interval_33237	2	4729809	4730550	742	1.0	intron	12349	Bend7	protein_coding
Interval_20345	14	66157436	66158236	801	1.1	intron	16876	Chrna2	protein_coding
Interval_52138	6	112861449	112862382	934	1.1	intron	-32457	Srgap3	protein_coding
Interval_57793	7	145088623	145090048	1426	1.1	Intergenic	-151139	Ccnd1	protein_coding
Interval_26681	17	12759436	12759887	452	1.1	intron	-8250	Igf2r	protein_coding
Interval_63651	9	103335992	103337070	1079	1.1	exon	-7405	Topbp1	protein_coding
Interval_140	1	20774059	20776725	2667	1.1	Intergenic	4162	Il17f	protein_coding
Interval_34369	2	34143848	34144615	768	1.1	Intergenic	-32788	Gm38389	lincRNA
Interval_62715	9	69025925	69026486	562	1.1	intron	190865	AC127262.1	pseudogene
Interval_49417	5	142898924	142900025	1102	1.1	Intergenic	4179	Actb	protein_coding
Interval_58912	8	70823749	70825107	1359	1.2	Intergenic	15292	Arrdc2	protein_coding
Interval_46140	5	24759289	24760185	897	1.2	Intergenic	-8413	Crygn	protein_coding
Interval_13252	12	21226275	21227862	1588	1.3	intron	20254	Itgb1bp1	processed_transcript