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碩士論文

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Master Thesis

探討在輔助型T細胞中酪胺酸磷酸化之c-Maf對於介白素四與二十一啟動子的結合機轉

Recruitment of tyrosine-phosphorylated c-Maf to *Il4* and *Il21* promoters in helper T cells

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夏日的陽光走廊，那裡是我進入免疫所的起點。兩年過去了，有時候，拿著那張免疫所的學生證，還會想到兩年前口試時驚慌的模樣。免疫所這條狂奔過上百次的長廊，終於要說再見了。兩年又幾個月的日子最要感謝的是繆希椿老師，老師為我開啟了免疫學的大門，帶領著我在這複雜的領域摸索，學習。在實驗上，不論我有多少的問題，多荒謬的想法，老師都會為我解答。也會在我有所突破的時候，給我讚美和鼓勵。老師也給了我很多和其他實驗室交流學習的機會，包括到日本筑波大學的暑期進修等等。除了研究之外，老師也像家人一樣的關心我有沒有照顧好自己，由衷的感謝能加入老師的研究室。我也要感謝我的口試委員，伍安怡老師以及顧家綺老師，兩位老師在我的論文以及研究方向上面，給了很多寶貴的建議，指出了許多我不足的部分，也提供了我很多改進的方法，讓我在學術上，能更紮實的一步一步的前進。也感謝伍老師，在我準備申請學校和面試時，給了很多的建議，還有顧老師讓我能夠有機會成為課堂助教，學習了很多不曾接觸的事務。也感謝免疫所其他的老師們，在 seminar 還有課堂上及生活上的關心與教導，感謝老師們，我才能夠完成這篇論文。

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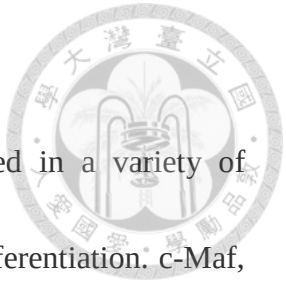
中文摘要

Maf 家族的蛋白質，參與了細胞當中各種不同的生物反應與機制，包括水晶體的發育，免疫細胞的分化以及腫瘤的形成。c-Maf 是 large Maf 家族成員的一員，在免疫系統中扮演著多樣且十分重要的角色。c-Maf 被發現可以在第二型輔助性 T 細胞中轉錄活化介白素四的表現，也可在第十七型輔助性 T 細胞中轉錄活化介白素二十一的表現。在我們之前的研究當中發現，c-Maf 在第 21,92,131 號酪胺酸的位置可以被磷酸化。而且在這三個位置的磷酸化有助於 c-Maf 結合到介白素四的啟動子，也可促進第二型輔助性 T 細胞分泌介白素四。在本篇當中，為了進一步了解 c-Maf 在第十七型輔助性 T 細胞中，對介白素二十一的調控。我們首先確認了 c-Maf 可以在第十七型輔助性 T 細胞中被磷酸化。接著我們將野生型 c-Maf(WT c-Maf)和磷酸化位置被突變的 c-Maf(Y3F c-MAf)過度表現在第十七型輔助性 T 細胞。發現介白素二十一的表現在 Y3F c-Maf 當中有下降的趨勢。我們也同時使用染色質免疫沉澱分析，發現相較於 WT c-Maf，Y3F c-Maf 結合到介白素二十一的啟動子的能力較差。總結來說，我們發現 c-Maf 的磷酸化會影響 c-Maf 結合到啟動子的能力，進而也影響到對介白素轉錄活化的能力。



Abstract

Maf (Musculoaponeurotic fibrosarcoma) proteins are involved in a variety of biological processes such as oncogenesis, lens development and differentiation. c-Maf, one of the large Maf proteins, plays important roles in immune system. c-Maf is the specific transcription factor of the IL-4 and IL-21 genes in type 2 helper T cell (Th2) and type 17 helper T cell (Th17), respectively. In our previous study, we found that c-Maf undergoes phosphorylation, a post-translational modification, in Th2 cells. The Tyrosine 21, 92, and 131 residues are identified as sites for tyrosine phosphorylation in c-Maf. We previously reported that the tyrosine-phosphorylation of c-Maf enhances its transactivity on IL-4 gene expression in Th2 cells. To understand the regulation of IL-21 cytokine gene expression by tyrosine-phosphorylation of c-Maf in Th17 cells, we showed c-Maf is subjected to tyrosine-phosphorylation in Th17 cells. We introduced WT c-Maf or c-Maf mutant carrying deficiency in tyrosine-phosphorylation (Y3F) into primary murine Th17 cells via retrovirus transduction and measured the gene expression of IL-21 with quantitive PCR and ELISA assay. We demonstrated that IL-21 production is reduced by c-Maf Y3F. Importantly, We performed chromatin immunoprecipitation to analyzed the binding ability of WT c-Maf or c-Maf Y3F to IL-21 promoter and found that c-Maf Y3F binds relatively weakly to IL-21 promoter compared to WT c-Maf.

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Chapter I Introduction

1.1 Helper T cell differentiation

$CD4^+$ T lymphocytes are very important in adaptive immune system. They were first classified into Th1 and Th2 cells by Mossman and Coffman in 1986 (Mosmann et al., 1986; Mosmann and Coffman, 1989). Furthermore, IL-17 producing Th17 cells (Harrington et al., 2005; Park et al., 2005), the follicular helper T cells (Tfh) (Schaerli. et al., 2000) and regulatory T cells (Treg) (Sakaguchi et al., 1995) were classified and studied in recent years. $CD4^+$ T cells, by producing cytokine, help host against invading microorganism, mediate antibody formation, recruit monocyte and neutrophil, regulate antiviral response and autoimmunity (Zhu and Paul, 2008).

Naïve $CD4^+$ T cell differentiation is involved signals from other cells and the microenvironment. T cell receptor and CD28 co-receptor can provide the activation and survival signal for naïve $CD4^+$ T cells, and the differentiation decision is governed by the cytokines around the cells (Smith-Garvin et al., 2009). The subtypes of $CD4^+$ T cells are defined by the signature cytokines they secret. Th1 cells induced by IL-12 and IFN-γ can express T-bet, a Th1 specific transcription factor, and produce IFN-γ via signal transducer and activator of transcription 4 (STAT4) signaling, and inhibit Th2 lineage. On the contrary, Th2 cells primed by IL-4 express GATA3, a Th2 specific transcription factor, and produce IL-4, IL-5 and IL-13 via STAT6 signaling. Th17 cells promoted by IL-6 and TGF-β produce IL-17 via STAT3 and IL-21 signaling (Yang et

al., 2005; Zhou et al., 2009). Th cells are involved in different adaptive immune responses. Th1 cells are involved in cellular immunity and the clearance of intracellular pathogens. While Th2 cells can response helminthes infection and also help the regulation of B cell class-switching to IgE. Th17 cells are important in the clearance of extracellular bacteria and are involved in autoimmune disease, such as Experimental autoimmune encephalomyelitis (EAE) in mice (Zhou et al., 2009; Zhu et al., 2010).

Th2 cells mediated the humoral immune response by producing various cytokines, including IL-4, IL-5, IL-13. (Ho and Glimcher, 2002). IL-4 is a multifunction cytokine in the regulation of immune response. IL-4 receptor is a receptor complex consisting of IL-4Rα and common gamma chain. The binding of IL-4 to it receptor lead to a series of phosphorylation signaling cascade, and regulate the cell growth, proliferation and differentiation.(Renate et al., 1996) IL-4 can induce B cell class switch, and differentiation to plasma cell. (Graham et al., 1990; Luzina et al., 2012)

Th17 cells play an important role in the clearance of extracellular pathogens during infections and also involved in autoimmunity. IL-6 and TGF-beta are essential for the Th17 differentiation. IL-6 is a pro-inflammatory cytokine which induced c-Maf expression; whereas TGF-beta is thought to be an anti-inflammatory cytokine and inhibit c-Maf expression (Hiramatsu et al., 2010). Under the stimulation of IL-6 stimulation, Th17 major transcription factor RORγt will be up regulated. RORγt can

transactivate IL-17 and IL-23R expression. IL-6 can induce c-Maf expression, which then transactivates IL-21 production (Korn et al., 2009). The IL-21 signaling enhances IL-23R expression on cell surface and expands the Th17 differentiation. In the late phase of Th17 differentiation, IL-23 plays a critical role in expanding and maintaining the Th17 cell population and enhances IL-17 production. IL-17 can recruit monocytes and neutrophils to the site of inflammation and functions in host defense against extracellular parasite (Jin and Dong, 2013; Korn et al., 2009; Wei et al., 2007).

1.2 Overview of Maf family

The Maf (musculoaponeurotic fibrosarcoma) oncoproteins are b-ZIP transcription factors belong to the AP1 (activator protein) superfamily of basic leucine zipper proteins. MAF family is composed of seven members, can further classify into large Maf and small Maf. The small Maf proteins, MafF, MafG and MafK, are essentially composed of a b-Zip domain, but the large Maf proteins, MafA/L-Maf, MafB, Maf/c-MAF and NRL contain an additional amino-terminal transactivation domain. The basic region can directly contact to DNA and the leucine-zipper domain can form a Homo- or Hetero- dimer of the Maf proteins or other AP-1 family proteins (Eychene et al., 2008). As homodimers, Maf proteins can recognize a palindromic DNA sequence, known as Maf response element (MARE) through their bZIP domain. MARE site

composes of phorbol 12-O-tetradecanoate-13-acetate (TPA) responsive element called T-MARE (TGCTGACTCAGCA) or composes of cyclic AMP-responsive element called C-MARE (TGCTGACGTCAGCA). As homodimers, Maf proteins can also recognize and bind to a MARE site or an half MARE site, if they are flanked by 5' AT rich sequence. (Yoshida et al., 2005) Although, Maf proteins have shown to form homo- or hetero- dimers with Maf protein or other AP-1 super family members, small and larger Maf proteins cannot heterodimerize (Kurokawa et al., 2009).

The Maf family also plays an important role during lens development. c-Maf transactivate the γD-crystallin in lens fiber cells. In the c-Maf deficient mice, the lens filer cannot develop, and the structure of the lens fails to complete. In addition, c-Maf also expresses in immune cells and regulates the cytokine production in helper T cells (Kawauchi et al., 1999).

1.3 The function of c-Maf in immune system

c-Maf transactivates IL-4 and IL-21 cytokine production in Th cells. Il-4 promotes the differentiation of the naïve CD4 T cells into Th2 cells. In the presence of TCR-mediated signals, IL-4 signaling activates STAT6, which induces the expression of GATA3. GATA3 can modify the *Il4, Il5* and *Il13* locus to allow the accessibility of other transcription factors, such as c-Maf, that are involved in driving the differentiation

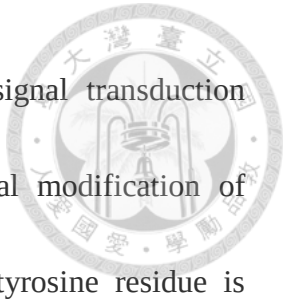
of T cells into Th2 cells (Guo et al., 2004). c-Maf specifically regulate IL-4 production in Th2 cells, but not effect IL-5 and IL-13 production. Also, c-Maf KO $CD4^+$ T cell fails to produce IL-4 (Kim et al., 1999).

c-Maf also regulates IL-21 in both Th17 and Tr1 cells (Pot et al., 2009). IL-21 belongs to the IL-2 cytokine family and has pleiotropic effects on both innate and adaptive immune responses. IL-21 is not only produced by Th17 but also by NKT, Tr1 and Tfh cells (Spolski and Leonard, 2008). IL-21 participates in the differentiation of Th17. It acts in an autocrine manner and enhances the Th17 proliferation. c-Maf directly binds to IL-21 locus and can be induced by ICOS signaling and regulate IL-21 production that in turn regulates the population of Th17 cells and Tfh cells (Bauquet et al., 2009; Pot et al., 2009; Wei et al., 2007).

c-Maf has been shown to regulate IL-10 production during Th17 polarization. IL-10 is a cytokine has anti-inflammatory and suppressive function. IL-10 can regulate the differentiation of B cells, NK cells, helper T cells and Macrophage. c-Maf transactivates the IL-10 production during Th17 polarization (Xu et al., 2009) and also regulate the IL-10 expression in macrophage, especially M2 (Cao et al., 2005).

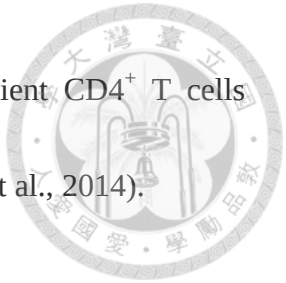
In general, c-Maf plays a critical role in regulating immune system, particularly in Th2, Th17, Tfh and Tr1 cells,

1.4 Post-translational modification by phosphorylation



The tyrosine phosphorylation is important in regulation of signal transduction pathways. Protein phosphorylation is a common post-translational modification of proteins, in which the hydroxyl group of serine, threonine and tyrosine residue is replaced by a phosphoryl group (PO_4^{3-}). In protein phosphorylation, kinase plays an important role in transferring phosphate groups from high-energy donor molecules, such as ATPs to specific substrates. Protein Kinase can be classified into two major groups, tyrosine kinase (TKs), which phosphorylate tyrosine, and serine-threonine kinase, which phosphorylate serine or threonine. Tyrosine kinase can further divided into two groups receptor tyrosine kinase and non-receptor tyrosine Kinase (M. et al., 1991). In immune cells, tyrosine phosphorylation controls various cellular events including cell signaling, cell proliferation, cell cycle regulation and protein trafficking. During signal transduction in immune cells, non-receptor tyrosine kinases, such as Tec family kinase (Itk, Rlk and Tec) have been identified as key components of T-cell-receptor signaling, which can activate downstream mitogen-activated protein kinases (MAPK) signaling cascade. (Schwartzberg et al., 2005) Moreover, TEC kinases serve as important mediators in $CD4^+$ T cells differentiation. T-bet, a Th1 specific transcription factor, has been reported to be phosphorylated by Tec kinase. The phosphorylated T-bet interacts with GATA3, which is essential for Th2 cell differentiation, and interferes with the binding of GATA-3 to IL-4 locus. (Hwang et al., 2005) Itk, another Tec family member,

can also alter the balance between Th17 and T_{reg} cells. *Itk* deficient $CD4^+$ T cells develop higher percentages of $FoxP3^+$ T_{reg} cells (Gomez-Rodriguez et al., 2014).



1.5 Post-translational modification of c-Maf

Members of Large Maf have different function in regulation of biological functions. MafA has been reported to regulate insulin expression (Zhang et al., 2005), while MafB is essential for F4/80 expression in macrophages (Moriguchi et al., 2006). MafA can also undergo phosphorylation by p38 MAP kinase, and the phosphorylation of MafA is a critical for its biological functions. The phosphorylation site in MafA are Thr113, Thr57 and Ser272, and mutations in these residues will reduce the biological activity of MafA (Sii-Felice et al., 2005). MafB undergoes another post-translational modification, SUMOylation. SUMOylation of MafB in Lys32/297 regulate MafB transactivity, Deficiency of MafB SUMOylation increased MafB transactivity and increase macrophage differentiation but not the growth of myeloid progenitor (Tillmanns et al., 2007). In our previous finding, c-Maf undergoes phosphorylation and SUMOylation in Th2 cells. The Tyr21/92/131 residues are the dominant sites of phosphorylation, and phosphorylation of these sites is critical for the recruitment of c-Maf to IL-4 promoter and regulates the transactivity of c-Maf (Lai et al., 2012). On the contrary, SUMOylation of c-Maf in Lys33 residue reduces the recruitment of c-Maf to IL-4 locus

and attenuates c-Maf transactivity (Lin et al., 2010). Furthermore, a recent study also shows that MafB and c-Maf can be phosphorylated by GSK-3, a Ser/Thr kinase, in human MM cell lines. This is the first report showed that c-Maf can be phosphorylated by Ser/Thr kinase (Herath et al., 2014; Rocques et al., 2007).

1.6 Rationale

Previous studies from our lab have shown that post-translational modification of c-Maf is critical for its ability to transactivate downstream *Il4* gene expression. We found that SUMOylation of c-Maf attenuates its IL-4 transcriptional activity (Lin et al., 2010), whereas phosphorylation of c-Maf increases its ability (Lai et al., 2012). We and others also showed that c-Maf can regulate IL-21 in Th17 cells. Here, we focus on the regulation of *Il21* gene expression by c-Maf in Th17 cell. In this study, we showed that c-Maf undergoes tyrosine phosphorylation in both Th2 and Th17 cells. Importantly, the tyrosine phosphorylation of c-Maf increases the binding ability of c-Maf to IL-21 promoter and enhances IL21 production in Th17 cells.

Chapter II Materials and Methods

1. Materials

1-1 Buffers

Nuclei Lysis Buffer for ChIP
50 mM Tris-HCl pH 8.1
10 mM EDTA
1 % SDS
Add 1 mM PMSF and protease inhibitor before use.

IP dilution buffer for ChIP
0.01% SDS
1.2 mM EDTA
16.7 M Tris-HCl pH 8.1
167 mM NaCl
Add 1 mM PMSF and protease inhibitor before use

1X Dialysis Buffer for ChIP
2 mM EDTA
50 mM Tris-HCl pH 8.0
0.2% Sarkosyl (only for polyclonal antibodies)

IP Wash Buffer for ChIP
100 mM Tris-HCl 9.0 (8.0 for monoclonal antibodies)
500 mM LiCl
1% NP-40
1% deoxycholic acid

Elution buffer for ChIP
50 mM $NaHCO_3$
1% SDS

PBS (phosphate-buffered saline)
10X Stock solution, 1 liter
80 g NaCl
2 g KCl
11.5 g $Na_2HPO_4 \bullet 7H_2O$
2 g KH_2PO_4

Working solution, pH ~7.3
137 mM NaCl
2.7 mM KCl
4.3 mM $Na_2HPO_4 \bullet 7H_2O$
1.4 mM KH_2PO_4

TAE (Tris/acetate/EDTA) electrophoresis buffer
50X stock solution:
242 g Tris base
57.1 ml glacial acetic acid
37.2 g Na EDTA .$2H_2O$
H2O to 1 liter
Working solution pH~835:
40 mM Tris.acetate
2 mM $Na_2EDTA \bullet 2H_2O$

Separating Gel Buffer
1.5 M Tris pH8.8
0.4% SDS

Stacking Gel Buffer
0.5 M Tris pH6.8
0.4% SDS

10X TBST, 1 Liter Stock
1% Tween-20
80 g NaCl
2 g KCl
30 g Tris-base pH 8.0
Add ddH2O to 1 liter

10X SDS Running Buffer Stock
30.3 g Tris-base
144 g Glycine
10 g SDS

10X Transfer Buffer

29 g Glycine (390mM)

585 g Tris-Base (480mM)

3.7g SDS (0.37%)

20% Methanol is supplied for 1X Transfer Buffer

Resolving Gel Formulation

Separting Gel	8%		10%		12%		15%	
H2O	2.75	ml	2.5	ml	2.25	ml	1.875	ml
40% Bis-acrylamide	1	ml	1.25	ml	1.5	ml	1.875	ml
Separating Gel Buffer	1.25	ml	1.25	ml	1.25	ml	1.25	ml
10% APS	40	µl	40	µl	40	µl	40	µl
TEMED	4	µl	4	µl	4	µl	4	µl

Stacking Gel Formulation

Stacking Gel	4%	
H2O	1.3	ml
40% Bis-acrylamide	0.2	ml
Separating Gel Buffer	0.5	ml
10% APS	30	µl
TEMED	3	µl

RIPA Lysis Buffer

50 mM Tris-HCl, pH 8.0

150 mM sodium chloride,

1.0% Igepal CA-630 (NP-40)

0.5% sodium deoxycholate

0.1% sodium dodecyl sulfate.

5X loading buffer

205mM TrisHCl pH6.8

10% SDS

25% Glycerol

0.025% Bromophenol blue

1.8M β-ME

RBC Lysis Buffer

155 mM NH4Cl
10 mM KHCO3
0.1 mM EDTA
Set pH to 7.2-7.4

1-2 Antibodies and cytokines

Antibodies

Target Gene	React. Spec	Company	Clone/Cat. Number
IFN-γ	Mouse	BioLegend	AN-18/ 517904
IL-4	Mouse	BioLegend	11B11□/ 504115
Flag		Sigma-Aldrich	M2/ F1804
CD3	Mouse	BioLegend	17A2/100223
CD28	Mouse	BioLegend	37.51/102112
Normal mouse IgG	Mouse	Santa Cruz	sc-2025
c-Maf	Rabbit	Santa Cruz	sc-7866
Normal rabbit IgG	Rabbit	Santa Cruz	sc-2027
p-Tyr	Mouse	Santa Cruz	Sc-7020
4G10-HRP		millipore	16-184

Cytokines

Name	Company	Cat. Number
Recombination Human TGF-β-1	PeproTech	100-21C
Recombination Murine IL-6	PeproTech	216-16
Recombination Murine IL-23	Biolegend	589002
Recombination Human IL-2	PeproTech	200-02
Recombination Murine IL-4	Biolegend	504107

1-3 Primers

Name	Sequence
IL-21 promoter F	TGGTGAATGCTGAAAACTGGA
IL-21 promoter R	CTAGGTGTACGTGTGCGTGT
mIL-4 Primer F	CCAGAATAACTGACAATCTGGTGTAAT
mIL-4 Primer R	TTATCAAGAGATGCTAACAATGCAATG
CNS4-F	GCCAGGCAGAGCTACTTAGAAATT
CNS4-R	TGTAGGAGAGAGTGGAACATTTCAA
mIL-4 5'	TAGTTGTCATCCTGCTCTTCTT
mIL-4 3'	GTGTTCTTCGTTGCTGAGAG
mIL-21 5'	TCATCATTGACCTCGTGGCCC
mIL-21 3'	ATGGTACTTCTCCACTTGCAATCCC

2. Experimental procedures

2-1 Mice

Six- to eight-week old Wild type C57BL/6 mice from National Taiwan University College of Medicine Laboratory Animal Center or from national laboratory animal center were used as the source of CD4+ T cells.

2-2 Generation of the reconstituted c-Maf deficient mice

The donor cells for hematopoietic reconstitution were collected from fetal livers of c-Maf deficient fetuses (C57BL/6J-CD45.1) on embryonic 14.5 day and were injected into X-ray irradiated C57BL/6J-CD45.2 mice (7 Gy) by tail vein injection (5 x 106 cells in 200 μl PBS per mouse). After two months of transplantation, the chimerisms of recipient mice were analyzed by flow cytometry. The recipient mice which are reconstituted by transplanted cells showed more than 95% hematopoietic cells from donor cells.

2-3 Purification and differentiation of Th cells

CD4+ T cell were purified from spleen and peripheral lymph nodes of WT CH57BL/6 mice by EasySep CD4 selection kit (STEM CELL CO) according to manufacturer's instruction. On day 0, CD4 T cell were harvested and purified by the

selection kit, Purified T cells ($2x10^6$ cells/ml) were cultured in RPMI complete medium containing 10% FBS, 1X L-glutamine, 1X NEAA, 1X sodium-pyruvate, 1X penicillin/streptomycin, 10 mM HEPES and beta-Mercaptoethanol, and stimulated with plate-bond anti-CD3 (2 μg/ml), soluble anti-CD28 (2 μg/ml) for all cells condition, and additionally with anti-IL-4 (10 μg/ml), IL-12 (1 ng/ml), IL-2 (100 U/ml) for Th1 condition, and anti-IFN-gamma (10 μg/ml), IL-4 (10 ng/ml), IL-2 (200 U/ml) for Th2 condition, and anti-IFN-gamma (10 μg/ml), anti-IL-4 (10 μg/ml), TGF-beta (2.5 ng/ml), IL-6 (20 ng/ml), IL-23 (20 ng/ml) for Th17 condition, For Th2 differentiation, additional IL-2 (200 U/ml) were added in Day2, for Th17 differentiation, additional IL-23 (20 ng/ml), TGF-beta (2.5 ng/ml) , IL-6 (20 ng/ml), were added.

2-4 Retrovirus Preparation from HEK293T cells.

The retroviral plasmids including Gag-pol, Env, and expression vector were co-transfected into HEK 293T cells by Meastrofectin (Omics Bio) transfection reagent according to the manufacturer's instructions. After 48, 72, and 96 hr, the virus-containing medium was collected, and filtered with 0.22 μM filer, then PEG6000 and NaCl were added to the final concentration of 8.5% and 0.3 M respectively. After shaking at 4^oC for 1 hr the virus containing medium was centrifuge at 7,000 X g for 10

min at 4°C, the virus pellet was precipitated and re-suspend in PBS, than stored at -80℃ for future use.

2-5 Retroviral transduction

Beads-isolated $CD4^+$ T cells were stimulated with plated-bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies with polarizing conditions for 48 hrs. Cells were then infected with GFP-RV Mock, and GFP-RV WT, Y3F c-Maf retrovirus by spin infection. Polybrene (Sigma) were added to the medium at final concentration of 8 μg/ml and centrifuged 2000 RPM for 1 hr at room temperature. After additional 30 to 60 min incubation at 37℃, the virus/polybrene containing medium were removed and replaced by fresh complete RPMI medium with IL-2 200 U/ml for Th2 cells, or IL-23 (20 ng/ml) for Th17 cells. After 48 hours incubation, the transduced cells were sorted by cell sorter according to the GFP expression. GFP^+ cells were re-stimulated at $1x10^6$ cells/ml with plate-bound anti-CD3 antibody (1 μg/ml) for 24 hours. IL-21, IL-17 mRNA level and cytokine production in the supernatant were measured by real time PCR or ELISA. The total cells and sorted cells can also use for ChIP assay.

2-6 ChIP assay

Formaldehyde was added directly to cell culture medium at a final concentration of 1% after PMA (50 ng/ml) and ionomycin (1 mM) stimulation to in vitro skewed retroviral transduced Th2 and Th17 cells. Fixation proceeded at room temperature for 10-15 min on rocking platform shaker. Glycine was added to a final concentration of 0.125M to stop the reaction. The fixed cell were washed with PBS twice then re-suspended in nuclei lysis buffer containing PMSF and protease inhibitor (50 mM Tris-HCl pH 8.1, 10 mM EDTA, and 1% SDS) and incubated on ice for 10 min, then subjected to sonication. The sheared DNA fragments should range in size from 200-600 base pairs. *T*he sheared chromatin extract was then frozen in aliquots at –80 ℃ until required. After sonication, the samples were centrifuged at 15,000 g for 10 min at 4℃ and transfer the supernatant into a new eppendorf. Use 50 μl sample as input, and 200 μl for IP or control. Normal mouse IgG (Santa Cruz) and Monoclonal anti-Flag antibody (Sigma), were add to sample to immunoprecipitate the chromatin and incubated overnight at 4℃. After incubation, 60 μl washed 50% protein G-sepharose beads were added to sample for additional 1-2 hours at 4℃. The sepharose beads were washed four times with IP wash buffer (100 mM Tris pH9.0, 500 mM LiCl, 1% NP-40 and 1% deoxycholic acid), then eluted twice with 150 μl IP elution buffer (100 mM $NaHCO_3$ 1% SDS) by vortex for 15 min each time. Adjusted NaCl to 0.3 M final concentration

with 1 μl RNase A (10mg/ml, Sigma) per sample, then incubated overnight at 65℃. After incubation, 2 μl proteinase K (20mg/ml) were added to each sample, and incubated at least 2 hours at 50℃. After proteinase K digestion, DNA was extracted by phenol/chloroform/isoamyl alchol or Small DNA Fragments Extraction Kit (Geneaid Co.), and then analyzed by real-time PCR analysis.

2-7 Western Blotting

Beads-isolated $CD4^+$ T cells were stimulated with plated-bound anti-CD3 and anti-CD28 antibodies with polarizing conditions for 48 hours, then add additional cytokine for 48 hours. The cells were harvested on day 4 followed by re-stimulation with 50 ng/ml PMA and 1 μM ionomycin for indicted hours. Whole cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 4% nonfat milk or BSA in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 1 hour, the membrane was washed three times with TBST and incubated with antibodies against c-Maf (1:2000 Santa Cruz) or Tubulin (1:5000), at 4°C for 12-16 hours. Membranes were washed three times for 5-10 min and incubated with a 1:5000-1:10000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit light chain antibodies for 1 hour. Blots were washed with TBST three times

and developed at ECL system (Omics Biotechnology) according to the manufacturer's protocols.

2-8 Immunoprecipitation and Immunoblot Analyses

For immunoprecipitation, Th2 and Th17 cells were harvested on day4 followed by re-stimulated with 50 ng/ml PMA and 1 μM ionomycin for 4 hours. The total cell extract was prepared by lysing the cell pellet with RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). After incubation, cell lysate was centrifuged at 15,000 g for 10 min. Cell lysate concentration were detected by PierceTM BCA Protein Assay Kit according to the manufacturer's protocols (Thermo). 500 μl of cell lysate containing 500 μg were precleared with 40μl of protein A-sepharose beads (50% slurry) for 2h. The supernatant was then incubated with 0.8 μg of c-Maf antibody or control antibody at 4°C for 12-16 hours followed by incubation with 30 μl protein A-sepharose beads with gentle rocking. Beads were washed with RIPA lysis buffer for three times and target proteins were eluted with 2x SDS loading dye, boiled at 100°C for 5 min, and analyzed by Western blotting. The blot were detected by anti-c-Maf antibody (Santa Cruz.) or HRP conjugated 4G10

(anti-phosphotyrosine antibody). The signals were detected by ECL system according to the manufacturer's protocols.

2-9 ELISA assay

For the ELISA assay, $CD4^+$ T cells were sorted form WT B6 mice, and skewed to Th17 conditions. 48 hours later, the primary cells were transduced with GFP-RV Mock, or either WT and Y3F c-Maf. After additional 48 hours, retroviral transduced cells were sorted according to the GFP expression, and re-stimulated with plated bond anti-CD3 1 μg/ml for 24 hours. IL-21 and IL-17 production were measured by eBioscience ELISA Ready-SET-Go kit according to manufacturer's instruction.

Chapter III Results

3.1 Endogenous c-Maf undergoes Tyrosine Phosphorylation in both Th2 and Th17 cells.

Previous studies have shown that T cell specific transcription factors can undergo several post-translational modifications, including phosphorylation, SUMOylation or ubiquitination. c-Maf, a major Th2 transcription factor, has been reported undergo SUMOylation and phosphorylation by our research group. We demonstrated that tyrosine phosphorylation deficient c-Maf (Y3F; contains three tyrosine residues mutations, which are Y21F, Y92F and Y131F) has lower transactivity on IL-4 gene in Th2 cells and lower binding ability to IL-4 promoter in Th0 cells. Furthermore, c-Maf induces IL-21 production directly in Th17 cells (Wei et al., 2007) and Tr1 cells (Pot et al., 2009). IL-21 is an autocrine cytokine which is necessary for Th17 differentiation, it is induced by IL-6 and highly express in Th17 cells. Therefore, to further investigate the role of tyrosine-phosphorylation of c-Maf in Th17 cell, and how tyrosine-phosphorylation of c-Maf regulated IL-21 expression. We first examine whether c-Maf can be tyrosine-phosphorylated in primary Th17 cells. $CD4^+$ T cells form WT B6 mice were polarized into Th2 and Th17 cells for 4 days. The endogenous c-Maf and their ability to undergo tyrosine phosphorylation were examined by immunoprecipitation with either anti-c-Maf antibody or a control antibody, and immunoblotted with anti-phosphotyrosine antibody. As Shown in Figure 1,

anti-phosphotyrosine can be detected in the lysates immunoprecipitated with anti-c-Maf, but not in control antibody. This data demonstrated that c-Maf can be tyrosine-phosphorylated in both Th2 and Th17 cells.

3.2 IL-21 production was impaired in Th17 cell transduced with tyrosine phosphorylation deficient c-Maf.

Previous study has shown that c-Maf contains several conserved tyrosine residues. Our previous data demonstrate that Tyr21, Tyr92 and Tyr 131 residues of c-Maf are the dominant phosphorylation sites in Th2 cells, and the IL-4 production in Y3F c-Maf transduced Th2 cells was impaired. We next want to assess whether the Y3F mutant c-Maf can also affect IL-21 production in Th17 cells. To examine the effect of c-Maf phosphorylation on *Il21* gene expression, primary $CD4^+$ T cells from WT B6 mice were activated and skewed under Th17 condition. The cells were transduced with GFP-RV Mock, or with either GFP-RV WT or Y3F c-Maf. After 48 hours, the transduced cells were sorted according to the GFP expression. The *Il21* gene expression level was measured by quantitative real-time PCR. Compare to Mock retroviral transduced cells, WT c-Maf transduced cell showed higher *Il21* gene expression, and Y3F transduced cell showed impaired *Il21* gene expression. (Figure 2, 3, and Figure 4 left panel) However, the *Il17* gene expression is comparable among

Mock, WT or Y3F c-Maf transduced cells (Figure 2, 3, and Figure 4, right panel). Further, In order to eliminate the intrinsic effect of endogenous c-Maf, we use c-Maf deficient $CD4^+$ T cells to repeat the experiment. $CD4^+$ T cells were isolated from c-Maf deficient bone marrow chimeric mice. X-ray irradiated C57BL/6J-CD45.2 mice were reconstituted with fetal liver cells from embryonic 14.5 day of c-Maf deficient mice (C57BL/6J-CD45.1) by tail vein injection (Supplementary Figure). We overexpressed the WT or Y3F c-Maf in c-Maf null Th17 cells. The IL-21 production and gene expression was determined by ELISA and real-time PCR. We found that IL-21 production and gene expression were reduced in Y3F c-Maf transduced c-Maf null Th17 cells, compared to WT c-Maf transduced cells. (Figure 5, upper panel) However, the IL-17 production and gene expression shows no differentiation between Mock, WT or Y3F c-Maf transduced c-Maf KO Th17 cells (Figure 5, lower panel). Collectively, we show that Y3F c-Maf has impaired ability to transactivate IL-21 in both WT and c-Maf KO Th17 cells.

3.3 Tyrosine phosphorylation of c-Maf enhances c-Maf recruitment to IL-4 and IL-21 promoter in Th2 and Th17 cells, respectively.

Previous study has shown that the recruitment of tyrosine phosphorylation deficient c-Maf to IL-4 promoter was attenuated. Here, to further characterize the importance of

tyrosine phosphorylation of c-Maf in primary Th cells. We retrovirally transduced GFP-RV Mock, WT or Y3F c-Maf to primary Th2 or Th17 and performed ChIP assay. We first sorted the retroviral transduced Th2 cells, and analyzed the binding ability of WT or Y3F c-Maf to *Il4* promoter. Y3F c-Maf showed relatively weak binding, 30% reduction, to *Il4* promoter then WT c-Maf did (Figure 6, lower panel). Our findings are consistent with the previous results in Th0 cells. Further, to analyze the recruitment of c-Maf to *Il21* promoter in Th17 cells, we first confirmed whether endogenous c-Maf actually binds to *Il21* locus in Th17 cells. $CD4^+$ T cells were cultured under Th17 condition for 4 days and left un-stimulated or stimulated with P+I for 1 hr, then subjected to ChIP assay. As shown in Figure 7, abundant of c-Maf could bind to *Il21* promoter after P+I stimulation. Next, to address the question of how phosphorylation affects the binding ability of c-Maf in Th17 celld, we retrovirally transduced Th17 cells carrying GFP-RV Mock, WT or Y3F c-Maf and subjected to ChIP assay. While both WT and Y3F c-Maf were recruited to the *Il21* promoter, the recruitment of Y3F-c-Maf to *Il21* promoter was one third reduction compared with WT c-Maf. We use normal mouse IgG as control and the results were normalized by each group's transduction efficiency (Figure. 8). To strengthen our result, we sorted retroviral transduced Th17 cells in day 4 and perform ChIP assay to repeat our findings. The data showed that recruitment of Y3F-c-Maf to *Il21* promoter was from one half to one third reduction

compared with WT c-Maf (Figure. 9). In sum, we demonstrated that the binding ability of c-Maf to *Il21* promoter was impaired in Th17 cells.

Chapter IV Discussion

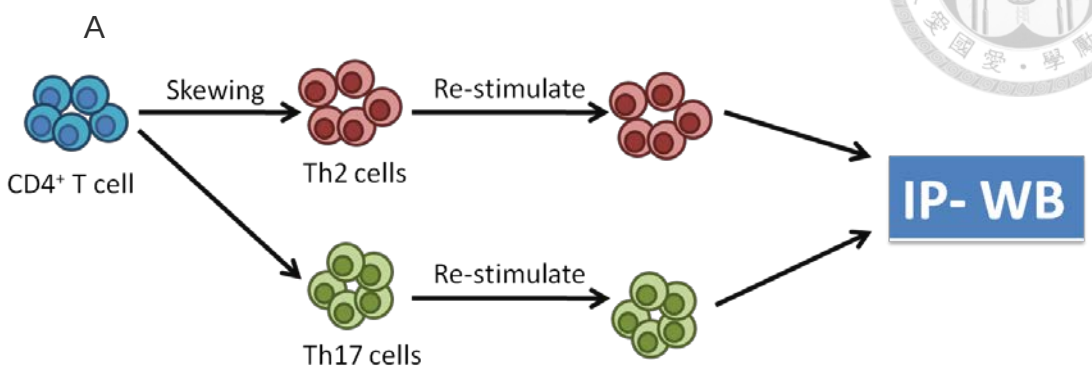
Tyrosine phosphorylation plays a significant role in regulating c-Maf transactivity.

Phosphorylation has shown to be a very important post-translational modification. Phosphorylation can either activate or inhibit protein (Ubersax and Ferrell, 2007). Protein kinase has three major types, tyrosine kinase, serine/threonine kinase or dual specific kinase (Nolen et al., 2004). In Maf family, MafA, a member of Maf family, undergoes phosphorylation in Ser 14 and 65 residues by mitogen-activated protein kinase (MAPK) and is critical for its function (Benkhelifa et al., 2001). Recent study has showed that activation of c-Maf in T cells is dependent on the CARMA1-IKKβ signaling Cascade. It suggested IKKβ might promote the translocation of c-Maf to nucleus. IKKβ, a serine-threonine protein kinase, is involved in the activation of the transcription factor Nuclear Factor kappa B (NF-kappaB). However, there is no direct evidence demonstrating that c-Maf is a direct substrate of IKKβ (Blonska et al., 2013). Previous study from our lab showed that c-Maf undergoes tyrosine phosphorylation in Th2 cells, and the tyrosine phosphorylation of c-Maf enhances the expression of IL-4 gene through increasing the recruitment of c-Maf to Il-4 gene locus (Lai et al., 2012). In this study, we demonstrate that c-Maf undergoes tyrosine phosphorylation in primary Th17 cells. Furthermore, tyrosine phosphorylated c-Maf enhance IL-21 production and increased the binding ability into *Il21* gene locus. Taken together, we believe tyrosine phosphorylation of c-Maf is an important modification of c-Maf and regulates c-Maf

activity in both Th2 and Th17 cells. However, the tyrosine kinase(s) and phosphotase(s) of c-Maf remain to be identified.

Moreover, the modification machinery of c-Maf still need to be further investigated. Identify the kinase(s), phosphotase(s) and the phosphorylation mechanism of c-Maf will help us to understand the regulation of c-Maf tyrosine phosphorylation.

Chapter V Figures


A
Skewing
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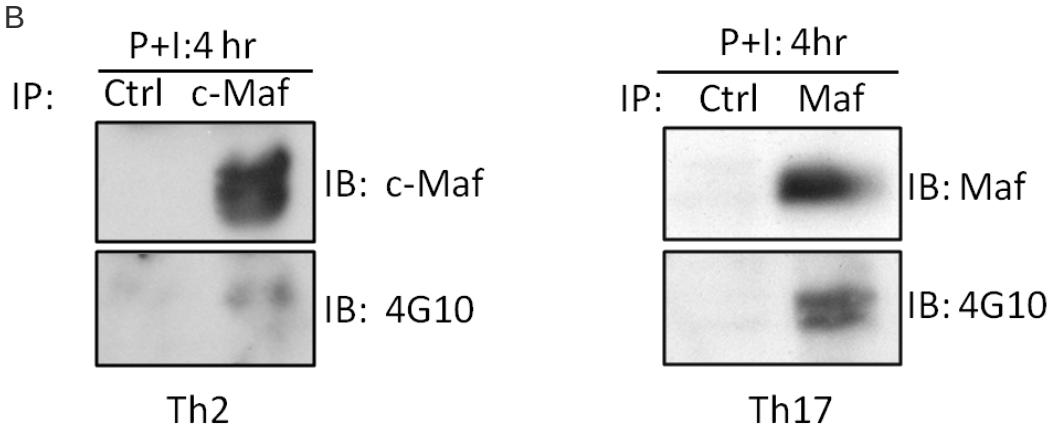
CD4+ T cell
Th2 cells
IP- WB
Re-stimulate
Th17 cells


B
P+I:4 hr
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c-Maf
IB: c-Maf
IB: 4G10
Th2
P+I: 4hr
IP:
Ctrl
Maf
IB: Maf
IB: 4G10
Th17

Figure 1. Endogenous c-Maf undergoes Tyrosine Phosphorylation in both Th2 and Th17 cells.

(A) The experimental flowchart was shown. **(B)** $CD4^+$ T cells from the spleen and lymph nodes of WT B6 mice were polarized in vitro, with 2 μg/ml of plate bound anti-CD3 and 2 μg/ml of soluble CD28 in the presence of 10 ng/ml of IL-4, 200U/ml of IL-2 and 10 μg/ml of anti-IFN-γ antibody for Th2 cells or 20 ng/ml of IL-6, 20 ng/ml of IL-23, 2.5 ng/ml of TGF-beta and 10 μg/ml of anti-IL-4 and 10 μg/ml of anti-IFN-γ antibody for Th17. The additional cytokine 200 U/ml of IL-2 for Th2 and 20 ng/ml of IL-6, 20 ng/ml of IL-23, 2.5 ng/ml of TGF-beta for Th17 were added on day 2 and the cells were harvested and re-stimulated with PMA and Ionomycin (P+I) on day 4. Cells were lysed with RIPA lysis buffer and then immunoprecipitated with anti-c-Maf antibody. The immunoprecipitate was then probed with anti-c-Maf and anti-phosphotyrosine antibody (4G10).

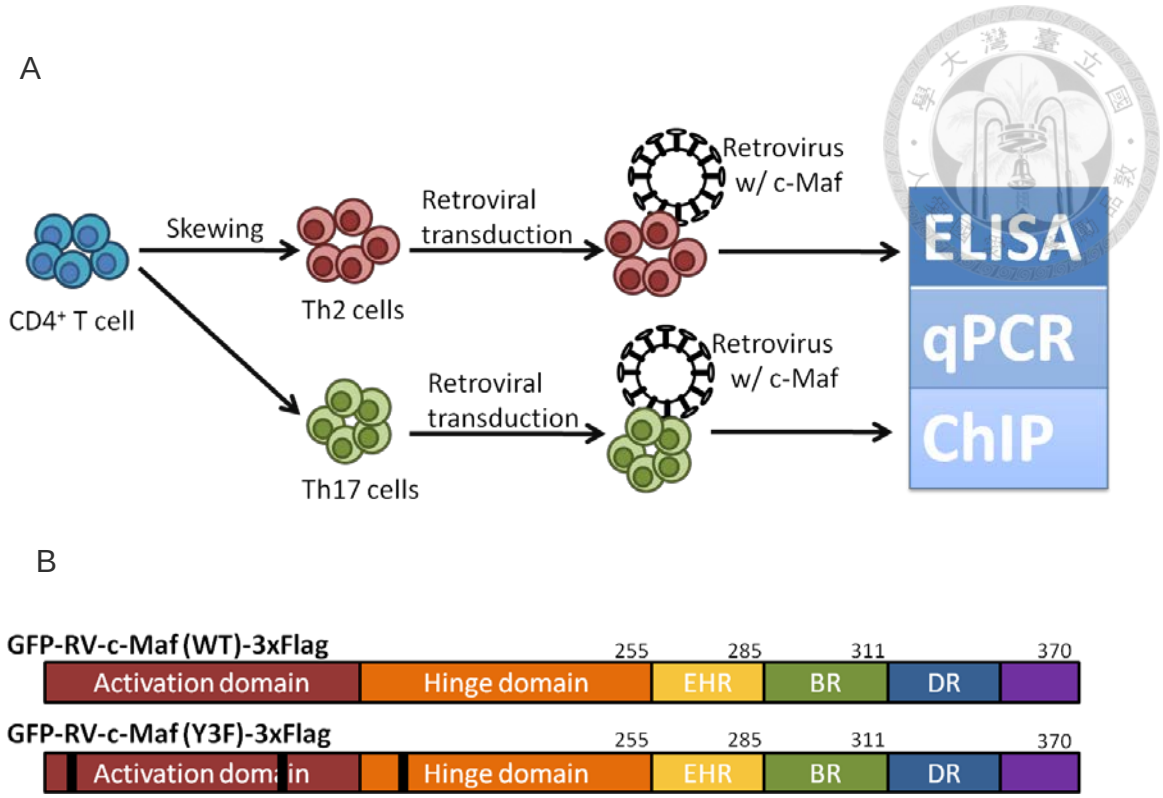


Figure 2. Experimental design and constructs

(A) $CD4^+$ T cells were isolated from the spleen and lymph nodes of WT B6 mice by EasySep CD4 positive selection kit according to manufacturer's instruction. The cells were cultured under Th cells skewing condition. After 48 hours, the cells were transduced with WT and mutants c-Maf. Two days later, the cells can sort with GFP^+ population then re-stimulate with anti-CD3 24 hours for ELISA and qPCR assay, or re-stimulated with P+I then were subjected to ChIP assay. (B) The constructs of GFP RV-c-Maf (WT) 3xFlag and GFP RV-c-Maf (Y3F) 3xFlag are shown. HER: Extended Homology Region. BR: Basic Region. DR: Dimerization Region

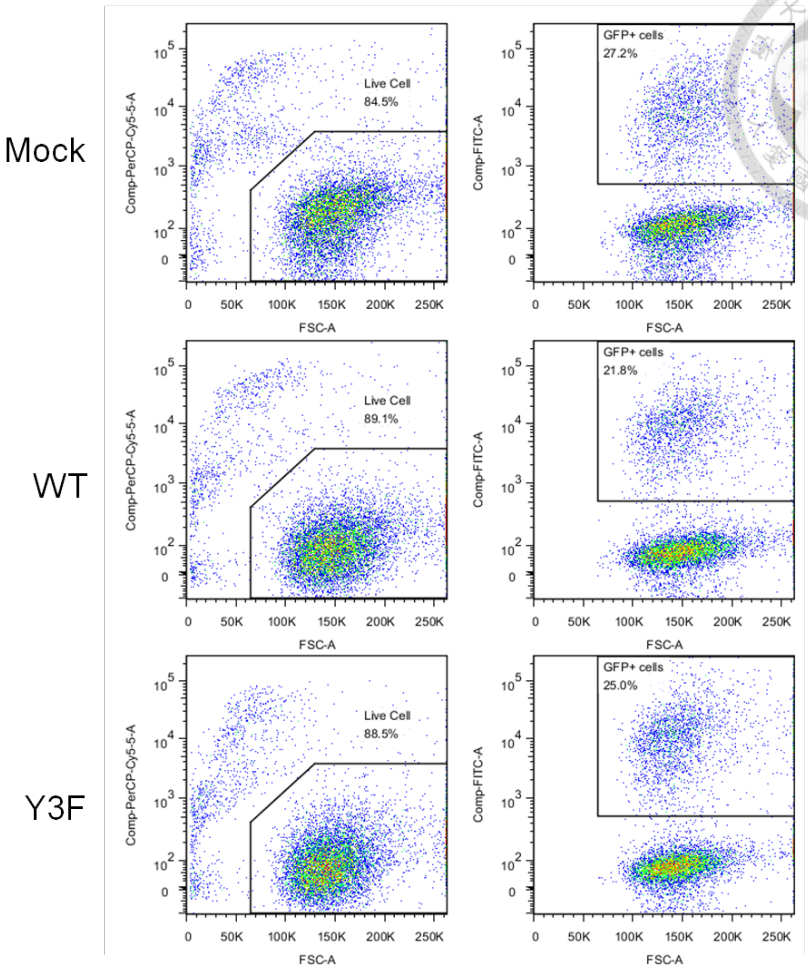


Figure 3. The retroviral transduction efficiency of primary CD4$^+$ T cells was determined by flow cytometry.

The primary CD4$^+$ T cells were transduced with different GFP RV virus, with or without WT or mutant c-Maf. The retroviral transduced cells were analyzed according to GFP ratio as the retroviral transduction efficiency by flow cytomerty at day 4. The cells were subjected to ChIP assay or sorted for ELISA, Real-time and ChIP assays.

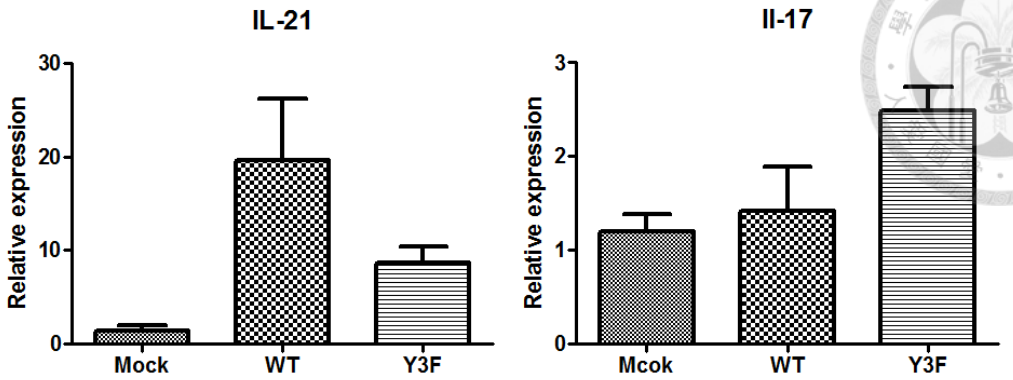


Figure 4. Mutation of c-Maf in Phosphorylation site (Y3F) suppresses *Il21* expression, but not *Il17*, in WT Th17 cells

Retroviral transduced Th17 cells were sorted by GFP expression after retroviral transduction for 48 hours. *Il17a* and *Il21* mRNA level was determined by real-time PCR after addition anti-CD3 stimulation for 24 hours, and the relative expression was normalized to the β-actin.

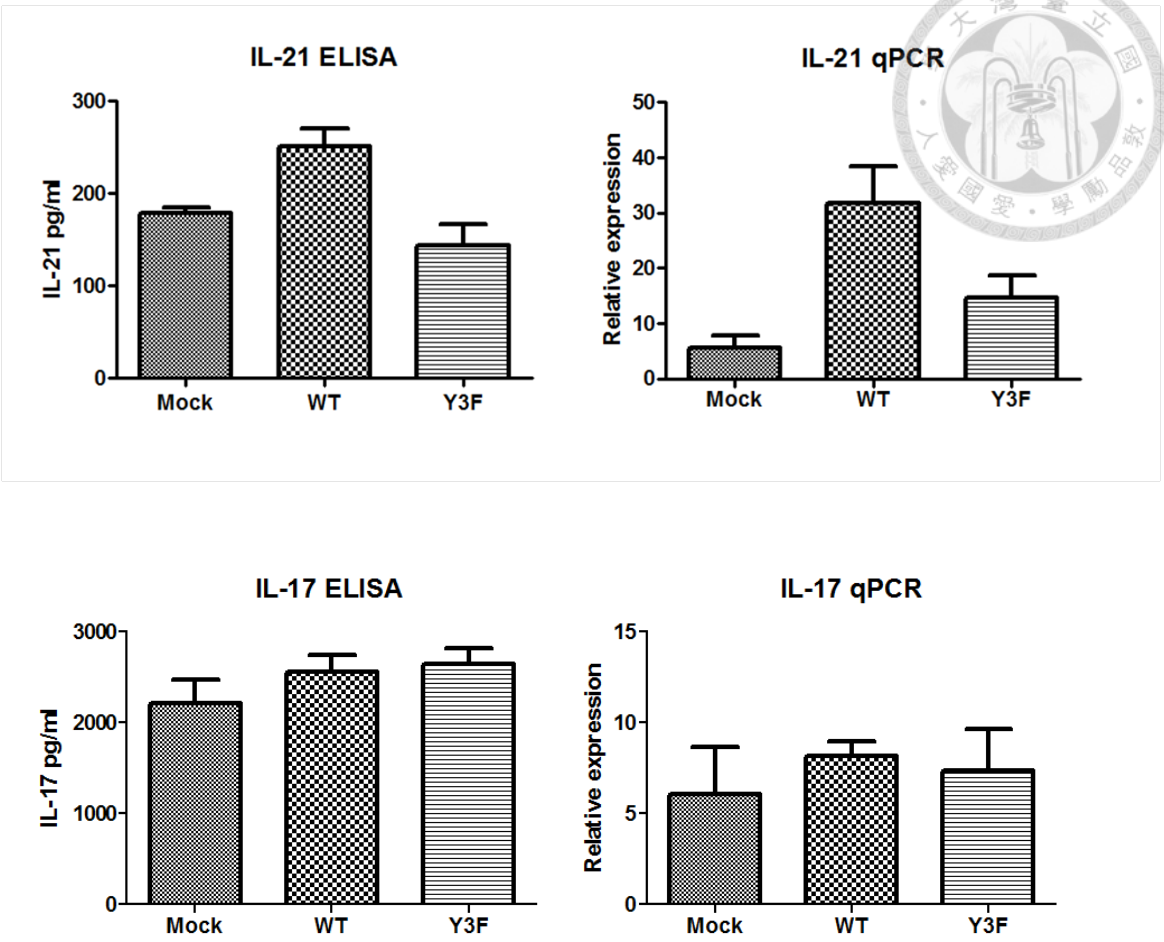
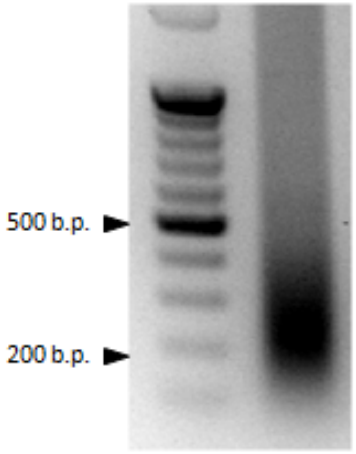


Figure 5. Mutation of c-Maf in Phosphorylation site (Y3F) suppresses IL-21 production, but not IL-17 in c-Maf KO Th17 cells

Retroviral transduced c-Maf KO Th17 cells were sorted by GFP expression after retroviral transduction for 48 hours. IL-21 and IL-17 production of these cells were determined by ELISA assay after addition anti-CD3 stimulation for 24 hours. *Il17a* and *Il21* mRNA level was determined by real-time PCR and the relative expression was normalized to the β-actin.

A



B

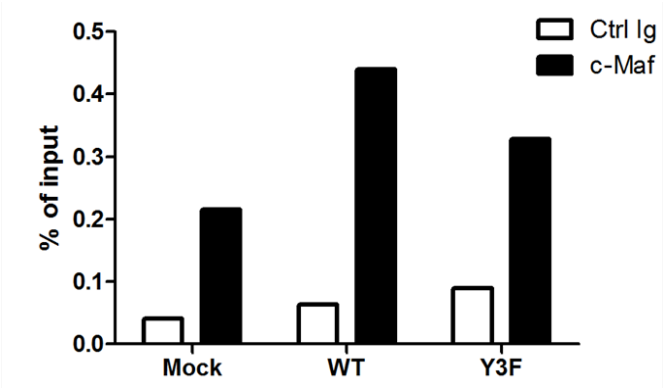


Figure 6. The recruitment of WT and Y3F mutant of c-Maf to IL-4 promoter in WT Th2 cells.

(A) To check the sonication condition, the sheared chromatin was reverse cross-linked and the DNA was extracted by Small DNA Fragments Extraction Kit (Geneaid.Co) according to manufacturer's instruction. (B) Retroviral transduced Th2 cells were sorted according to the GFP expression and re-stimulated with PMA/ionomycin (P+I) for 1 hour. Then the cells were fixed with 1% formaldehyde, chromatins were sheared by sonication and immunoprecipited with control or anti-Flag antibody. The precipitated DNA was analyzed by quantitative PCR using primer against IL-4 promoter. The relative binding levels was presented as percent of input in histogram. The result was calculated by Ct value as follow:

$$\%\ \text{of Input} = \frac{2^{-(\text{anti-c-Maf DNA Ct value})}}{2^{-(\text{control Input DNA Ct value})}} \times 100\%$$

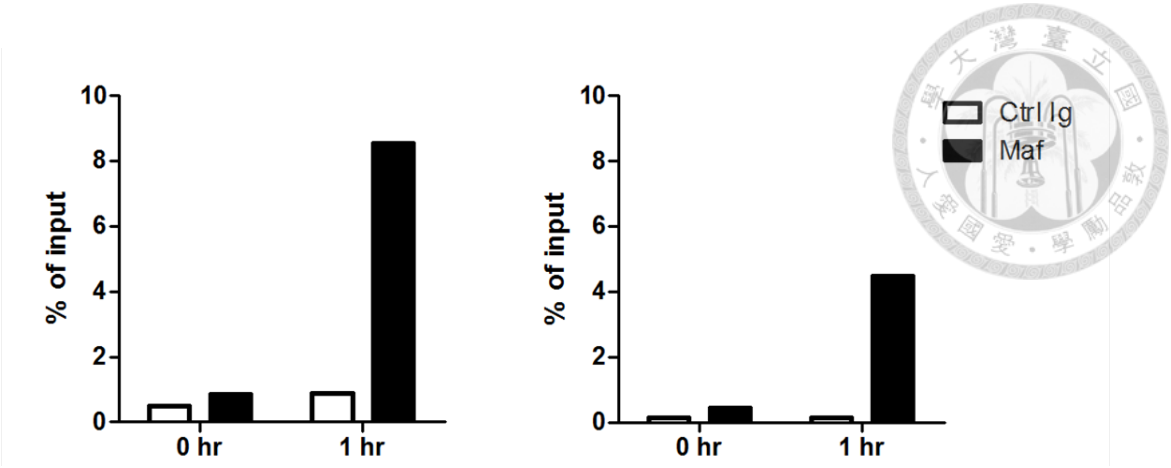


Figure 7. The recruitments of endogenous c-Maf to IL-21 promoter in Th17 cells

Th17 cells were harvested and re-stimulated with or without P+I for indicated periods of time on day 4. The cells were fixed with 1% formaldehyde, chromatin were sheared by sonication and immunoprecipited with control or anti-c-Maf antibody. The precipitated DNA was analyzed by quantitative PCR using primer against IL-21 promoter. The relative binding levels was presented as percent of input in histogram, and the results were calculated with Ct value as showed before. Two independent experiments are shown.

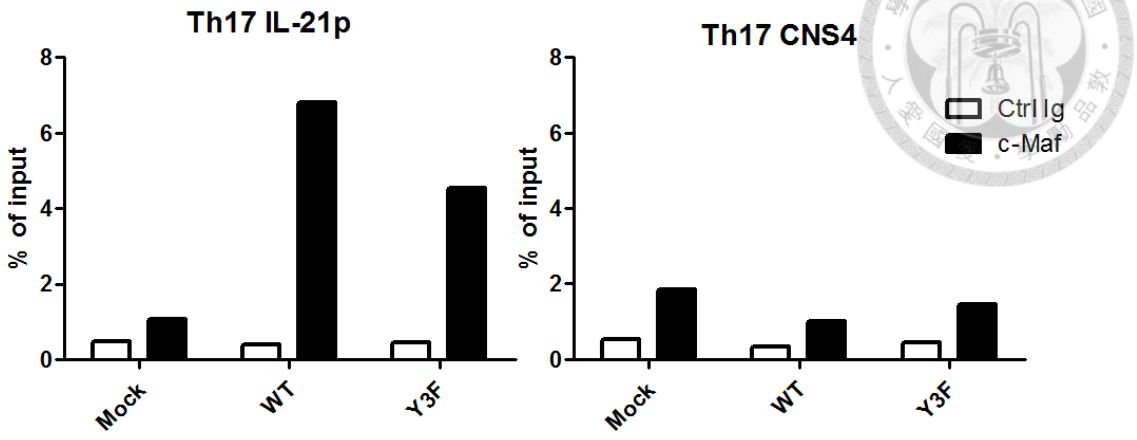


Figure 8. The recruitment of WT and Y3F mutant of c-MAf to IL-21 promoter in WT Th17 cells.

Retroviral transduced Th17 cells were stimulated with PMA/ionomycin for 1 hour. The cells were fixed with 1% formaldehyde, chromatin were sheared by sonication and immunoprecipited with control or anti-Flag antibody. The precipitated DNA was analyzed by quantitative PCR using primer against IL-21 promoter or CNS4 region as negative control (Hiramatsu et al., 2010). The relative binding level was presented as percent of input in histogram. The results were calculated with Ct value and normalized with transduction efficiency, then calculated as showed before.

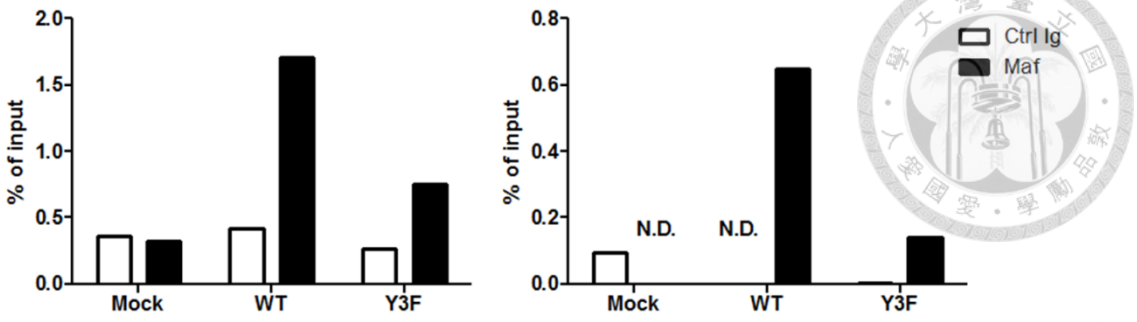
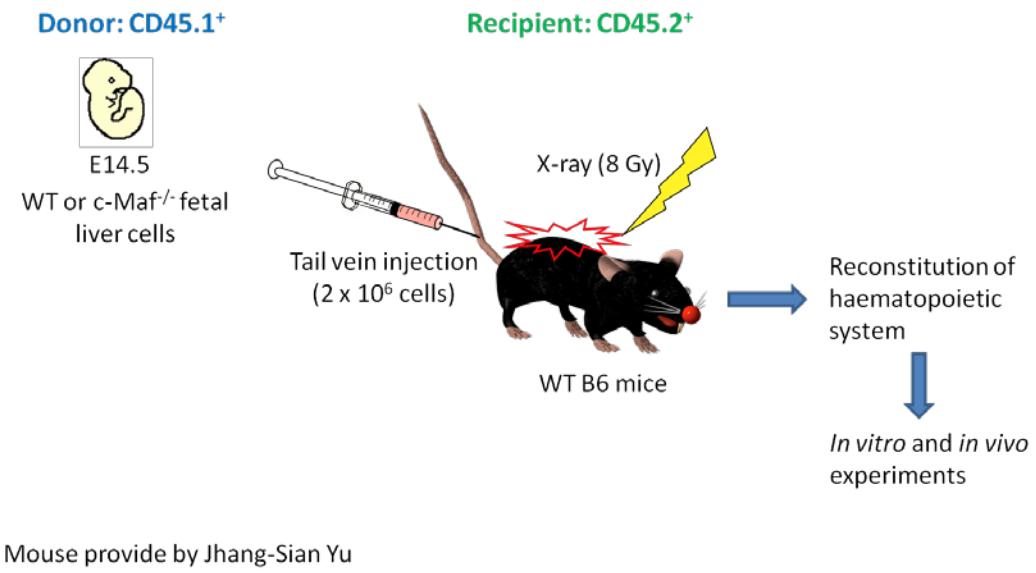


Figure 9. The recruitment of WT and Y3F mutant of c-Maf to IL-21 promoter in WT Th17 cells.

Retroviral transduced Th17 cells were sorted according to the GFP expression and re-stimulated with P+I for 1 hour. Then the cells ($1x10^7$) were fixed with 1% formaldehyde, chromatin were sheared by sonication and immunoprecipited with control or anti-Flag antibody. The precipitated DNA was analyzed by quantitative PCR using primer against IL-21 promoter. The relative binding levels was presented as percent of input in histogram. The results were calculated with Ct value as showed before. Each data represent cells pooled from 5 to 7 times independent retroviral transduced Th17 cells. Two independent experiments are shown.

Supplementary



Supplementary Figure. c-Maf KO bone marrow chimeric mice.

The donor cells for hematopoietic reconstitution were collected from fetal livers of c-Maf deficiency fetuses (C57BL/6J-CD45.1) on embryonic 14.5 day and were injected into X-ray irradiated C57BL/6J-CD45.2 mice (7 Gy) by tail vein injection (5 x 10^6 cells in 200 μl PBS per mouse). After two months of transplantation, the chimerisms of recipient mice were analyzed by flow cytometry. The recipient mice which are reconstituted by transplanted cells showed more than 95% hematopoietic cells from donor cells.

Chapter VI References

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