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T 淋巴細胞免疫反應調控基因的辨認與研究(1/3)

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T 淋巴細胞免疫反應調控基因的辨認與研究(1/3)

關鍵詞: T 淋巴細胞,基因調控及免疫反應

中文摘要

初生的 $CD4^+$T 淋巴細胞在 T 細胞受體接受訊號後，會分化為兩個不同的族群：Th1 和 Th2 細胞。成熟的 Th 作用淋巴細胞在免疫系統中扮演協調的角色。Th1 細胞產生干擾素γ、介白素二及淋巴毒素，媒介了延遲性過敏反應和巨嗜細胞的活化。Th1 免疫反應在對抗細胞內病原與媒介器官特異性自體免疫上很重要。相對的，Th2 細胞產生介白素四、五、六、九、十及十三，並媒介 B 細胞產生抗體。Th2 免疫反應在對抗蠕蟲及發炎反應上扮演決定性的角色。

最近的研究顯示 T 淋巴細胞特異的轉錄因子：T-bet、GATA3 與 c-MAF 在 Th1/Th2 的發育上是必需的。T-bet 是 Th1 細胞分化的主要調控者，然而 GATA3 與 c-MAF 則在 Th2 的發育上扮演決定性的角色。其它因子，如細胞激素、多肽媒介者 Eta-1、化學激素 MCP-1、訊息傳導及活化者 4(STAT4)及 STAT6 也會影響 T 細胞的分化。然而許多實驗指出，一些其他未被確定的因子也參與 T 細胞的分化。所以，我們計畫有效率的篩選和辨識這些參與 T 細胞分化的因子(實驗目標一)，並在體內及體外模式中研究他們在 Th1/Th2 的發育與免疫反應中的角色(實驗目標二及三)。

這個計畫的目標是希望能在分子與細胞機轉上，對基因如何調節 T 細胞分化與免疫反應有進一步的了解。此外，我們也希望建立 Th1 或 Th2 免疫系統小鼠模式，以提供對 Th1 疾病(如器官特異性自體免疫)或 Th2 疾病(如氣喘和過敏)更好的了解與控制。

Identification and Study of Genes that Regulate T Lymphocyte Differentiation and Immune Response (1/3)

Keywords： T Lymphocyte, Gene Regulation and Immune Response

Naïve $CD4^+$ T lymphocytes differentiate into two distinct subsets, T helper 1 (Th1) and T helper 2 (Th2), upon T cell receptor engagement. Mature effectors Th lymphocyte plays an orchestrated role in the immune system. Th1 cells produce IFNγ, IL2 and lymphotoxin, and mediate responses of delayed-type hypersensitivity and activation of macrophage. Th1 responses are important for protection against intracellular pathogens and mediate organ-specific autoreactive immune responses. Conversely, Th2 cells produce IL4, IL5, IL6, IL9, IL10 and IL13, and mediate antibody production by B cells. Th2 responses are critical in protection against helminths and responsible for anti-inflammatory reactions.

Recent studies have shown that T lymphocyte specific transcription factors T-bet, GATA3 and c-MAF have essential roles in the Th1/Th2 development. T-bet is the master regulator for the development of the Th1 cell lineage, while GATA3 and c-MAF are crucial for the development of the Th2 cell lineage. Other factors, such as cytokines, polypeptide mediator Eta1, chemokine MCP-1, signal transducer and activator of transcription 4 (STAT4), and STAT6 also influence the T lymphocyte differentiation. However, many studies indicate that some other factors involved in Th differentiation are yet to be identified. Therefore, we intend to effectively screen and identify the factors crucial for T lymphocyte differentiation (Specific Aim 1) and study their roles in Th1/Th2 and immune response *in vitro* and *in vivo* (Specific Aim 2&3).

The goal of this proposal is to provide a better insight of the molecular and cellular mechanisms by which genes regulate T Lymphocyte differentiation and immune response. Furthermore, we hope to establish the Th1/Th2-skewed mouse models for better understanding and controlling of Th1-mediated diseases, such as organ-specific autoimmunity diseases and Th2-mediated diseases, such as asthma and allergy.

Introduction

The cytokines have major roles in Th differentiation. Recent studies demonstrate the cytokines, mainly IFNγ and IL4, influence the differentiation of Th cells by controlling subsets of specific transcription factors, T-bet and GATA3, in a directional and positive feedback manner. Receptors for Th1 or Th2 cytokines also involve in the decision of Th1 or Th2 lineage commitment. For example, the IL12 receptor β chain is the one of the key factors for the Th1-lineage pathway. Other factors, such as cytokines, polypeptide mediator Eta1, chemokine MCP-1, signal transducer and activator of transcription 4 (STAT4), and STAT6 also influence the T lymphocyte differentiation.

Further studies have shown that T lymphocyte specific transcription factors T-bet, GATA3 and c-MAF have essential roles in the Th1/Th2 development. T-bet is the master regulator for the development of the Th1 cell lineage, while GATA3 and c-MAF are crucial for the development of the Th2 cell lineage. ROG, a new member of the POZ family of transcriptional repressors, was initially cloned as a GATA-3 interacting protein. In vitro, ROG can serve as a GATA-3-dependent transcriptional repressor. ROG transcripts can be detected in both Th1 and Th2 cells. Furthermore, over-expression of ROG in Th clones inhibits cytokine production.

However, many studies indicate that other factors involved in Th differentiation are yet to be identified. Therefore, in this proposal, we intend to effectively screen and identify the factors crucial for T lymphocyte differentiation by using the yeast two-hybrid system (Specific Aim 1) and study their roles in Th1/Th2 and immune response *in vitro* and *in vivo* (Specific Aim 2&3).

Specific aim 1. To identify the interacting proteins of T lymphocyte specific transcription factors

T lymphocyte specific transcription factors T-bet, GATA3 and c-MAF have major roles in the Th1/Th2 development. T-bet is the master regulator for the development of the Th1 cell lineage, while GATA3 and c-MAF are crucial for the development of the Th2 cell lineage. ROG is a transcriptional repressor of GATA3. Over-expression of ROG inhibits both Th1 and Th2 cytokines production in Th1 and Th2 clones. How do these factors regulate the differentiation of T cells and immune response? Are any other factors involved to mediate their function? To get a better picture, we plan to conduct a comprehensive screening to identify the interacting protein of these T lymphocyte specific transcription factors by using the yeast two-hybrid system.

Experimental design and methods for specific aim 1.

To identify the interacting proteins of T lymphocyte specific transcription factor c-Maf

The studies described in this aim focus on the isolation of the interacting proteins of c-Maf. c-Maf is crucial for the development of the Th2 cell lineage. I will attempt to clone comprehensively possible interacting partner(s) with the yeast two-hybrid system by using different domains of c-Maf as baits and cDNA libraries from mouse CD4 and CD8 clones as preys.

The Yeast Two-Hybrid Method

The '**preys**' are libraries of plasmid pJG4-5 expressing **cDNAs from (1) Murine CD4 T clone (AE7) and (2) Murine CD8 clone (L3)**.

The '**baits**', different domains of **T cell specific transcription factor c-Maf** was cloned into a plasmid, pGilda. They were transformed into a yeast strain, EGY191 or EGY48. Then, the cDNA library was co-transformed into the bait-transformed EGY191 or EGY48 strain. Those colonies can grow on selection media were streaked. We further performed β-gal colony-lift filter assay, isolated clones from positive yeast clones, identified and purified the prey from *E.coli* transformants, and reconfirmed the assay for the true positive clones. The clones were grouped according insert size and AluI and/or HaeIII restriction patterns. This process have allowed us to exclude sister clones. Then, sequence analysis was performed.

The **alternative approach** for this aim is to identify the interacting proteins by proteomic method. I will employ coimmunoprecipitation (CO-IP) assays with over expression of the bait protein in murine Th1 clone (AE7) and anti-bait antibody generated in our laboratory. The 'CO-IP -complex' will be further separated by electrophoresis, purified by gel extraction and analyzed by micro-peptide-sequencing.

Results:

A.

<u>Construct LexA DB/c-maf (138 bp) plasmid (bait):</u>

The 138 bp c-maf fragment was produced by PCR. We created the EcoR I and Xho I recognition sequence at the two ends of the fragment, respectively. Then the PCR products were cut by EcoR I and Xho I. The bait vector we used here is pGilda, and we digested this vector by EcoR I and Sal I. In the end, we ligated the insert and the vector. The maf-contained vector, pGilda -c-maf(138), is about 6.7 kb (Fig.1).

The bait vector was transformed into EGY191 strain yeast (Trp- Leu- His-).

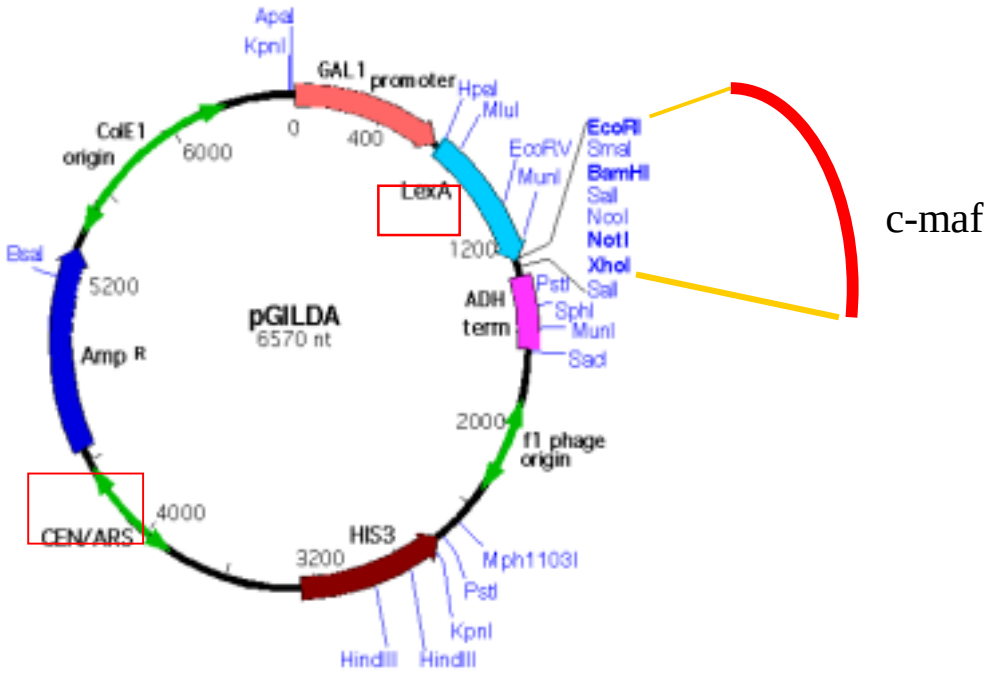

Fig. 1. Map of the plasmid, pGilda -c-maf(138), as a bait.

Yeast transformation:

1. Incubate the yeast in YPAD liquid for 16~18 hours.
2. Dilute the o/n culture in YPAD, let the yeast growth for 3~4 hours.
3. Collect the cell pellt and resuspend in 1 ml TE/LiAc buffer.
4. Mix 10μg carrier DNA and 0.1~1μg plasmid DNA with cell mixture.
5. Add PEG/TE/LiAc buffer.
6. Incubate at 30℃ for 30 min with shaking.
7. Add 70 μl of DMSO, and then mix well gently.
8. Heat shock for 15 min in 42℃, and then chill cells on ice for 5 min.
9. Plate cells on selection plate. Incubate at 30℃ for several days.

Co-transformation

1. Transformation of bait first.
2. Then, the CD4 Th1 cDNA library co-transformed into the bait-transformed EGY191 strain.

Streak transformants that can grow on selection plate:

1. Spread the mating mixture on 150mm-plate, at 200ml per plate.
2. Incubate the plate, colonies appear about 8~21 days.
3. Selection plate contains SD/-4 (-Leu/-Trp/-Ade/-His) and 3-AT.

At present, we got 50 positive colonies from the screening. Reconfirmation and identification of these colonies have preformed (Table 1).

Table 1. The candidates of c-Maf-interacting proteins obtaining by yeast-two-hybrid using c-maf (138bp) as a bait and CD4 AE7 library as a prey

Group	Repeat	Clone	Gene
1	30	1, 4~18, 20~22, 28, 33, 37, 39, 40, 43~46, 49, 53	moues UBC9
2	3	23, 30, 66	snRNP70
3	3	2, 29, 71	18, 28S rRNA
4	2	3, 32	mouse PIAS1
5	1	31	synectin
6	1	65	cytokine like nuclear factor n-pac like protein
7	1	60	actin related protein 2/3 complex subunit 2
8	1	62, 63	cadherin-related neuronal receptor
9	1	82	polyA polymerase member 4
10	1	90	guenine nucleotide binding protein like 2
11	1	36	
12	1	50	mouse colone RP23-392I3, chromosome11
13	1	70	mouse colone RP24-308L17, chromosome 9
14	1	75	mouse colone RP23212N20, chromosome5
15	1	81	mouse colone RP24-571B18
16	1	89	mouse colone RP23-392213, chromosome11

B.

Construct LexA DB/c-maf (hinge domain) plasmid (bait):

The 440 bp c-maf fragment (encode hinge domain of c-Maf) was produced by PCR. We created the EcoR I and Xho I recognition sequence at the two ends of the fragment, respectively. Then the PCR products were cut by EcoR I and Xho I. The bait vector we used here is pGilda, and we digested this vector by EcoR I and Sal I. In the end, we ligated the insert and the vector. The maf-contained vector, pGilda -c-maf(440), is about 7 kb.

The bait vector was transformed into EGY48 strain yeast (Trp- Leu- His-).

Yeast transformation:

10. Incubate the yeast in YPAD liquid for 16~18 hours.
11. Dilute the o/n culture in YPAD, let the yeast growth for 3~4 hours.
12. Collect the cell pellt and resuspend in 1 ml TE/LiAc buffer.
13. Mix 10μg carrier DNA and 0.1~1μg plasmid DNA with cell mixture.
14. Add PEG/TE/LiAc buffer.
15. Incubate at 30℃ for 30 min with shaking.
16. Add 70 μl of DMSO, and then mix well gently.
17. Heat shock for 15 min in 42℃, and then chill cells on ice for 5 min.
18. Plate cells on selection plate. Incubate at 30℃ for several days.

Co-transformation

3. Transformation of bait first.
4. Then, the CD8 L3 cDNA library co-transformed into the bait-transformed EGY48 strain.

Streak transformants that can grow on selection plate:

4. Spread the mating mixture on 150mm-plate, at 200ml per plate.
5. Incubate the plate, colonies appear about 8~21 days.
6. Selection plate contains SD/-4 (-Leu/-Trp/-Ade/-His) and 3-AT.

At present, we got 40 positive colonies from the screening. Reconfirmation and identification of these colonies have preformed (Table 2).

Table 2. The candidates of c-Maf-interacting proteins obtaining by yeast-two-hybrid using c-Maf (hinge domain) as a bait and CD8 L3 library as a prey

Group	Repeat	Clone	Gene
A	5	17,20,21,35,37	Mus musculus RIKEN cDNA 2010311D03 gene (2010311D03Rik), mRNA
B	5	1,3,18,28,39	Mus musculus integrin beta 7, mRNA
C	3	6,25,42	Mus musculus adrenodoxin mRNA, complete cds;Mus musculus ferredoxin 1 (Fdx1), mRNA
D	3	22,24,30	Mouse protein tyrosine phosphatase (70zpep) mRNA, complete cds
E	3	10,36,41	Mus musculus endothelial cell-specific molecule 1 (Esm1), mRNA
F	2	14,15	Mus musculus golgi phosphoprotein 3-like, mRNA
G	3	7,8,19	Mus musculus heat shock protein 8 (Hspa8), mRNA

H	1	4,	Mus musculus cleavage and polyadenylation specific factor 2 (Cpsf2)
I	1	12,	Mus musculus S-adenosylhomocysteine hydrolase (Ahcy), mRNA
J	1	16,	Mus musculus replication protein A1(Rpa1), mRNA
K	1	23,	Mus musculus topoisomerase (DNA) II beta binding protein, mRNA
L	1	26,	Mus musculus protein kinase, cAMP dependent regulatory, type I, alpha
M	1	27,	Mus musculus polymerase (RNA) II (DNA directed) polypeptide J
N	1	33,	Mus musculus similar to Speer1-ps1 protein
O	1	38,	Mus musculus proteasome (prosome, macropain) subunit, beta type 7
P	1	40,	Mus musculus spectrin alpha 2, mRNA
Q	1	31,	Mus musculus 0 day neonate head cDNA, RIKEN full-length enriched library, clone:4833426G12 product:hypothetical protein,full insert sequence Homo sapiens chromosome 9 open reading frame 82, mRNA (cDNA clone MGC:88643 IMAGE:5431506), complete cds
R	1	9,	Mus musculus kidney CCL-142 RAG cDNA；Mus musculus prosaposin (Psap), mRNA
S	1	32,	Mus musculus CH-rich interacting match of PLAG1 mRNA, complete cds Mus musculus ring finger and CHY zinc finger domain protein containing 1
T	1	5,	Mus musculus ring finger and CHY zinc finger domain containing 1
U	1	29,	Mus musculus BAC clone RP23-257F7 from 7, complete sequence
V	1	2,	Mus musculus NADH dehydrogenase 6, mitochondrial, mRNA
W	1	11,	Mus musculus RIKEN cDNA 2010012C16 gene (2010012C16Rik), mRNA

Conclusion:

We have effectively screened and identified the factors which interacting with Th2 specific transcription factor c-Maf. Therefore, we study aggressively on whether these proteins mediating the expression of cytokine gene and their roles on T lymphocyte differentiation.

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