行政院國家科學委員會專題研究計畫 成果報告

兒童類風濕關節炎之 CD4+CD25+調節型 T 淋巴球的表面抗原分析

研究成果報告(精簡版)

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Background and Significance

Establishment of a full repertoire of pathogen-specific lymphocytes that can protect the host is coincident with the formation of T cells able to recognize self-antigen. Some of these potentially autoreactive T cells capable of recognizing tissue specific antigens escape negative selection in the thymus and released into the periphery. They can be cloned from lymph nodes of mice and the circulation of human (Ito et al 1993; Ota et al 1990) and they are exquisitely regulated, and their activation can result in autoimmune diseases. To maintain peripheral tolerance, the immune system has developed several mechanisms to suppress or regulate immunity in order to protect the body from sustained harmful immune response (Curotto et al 2002; Shevach et al 2000) and failure of this regulatory network can result in autoimmune disease. One of the key players of immune regulation is the $CD4^+CD25^+$ regulatory T cells (Tregs) (Stephens et al 2000).

Kojima and Prehn discovered that thymectomy in mice in neonatal day 3 (D3T mice model) leads to development of multiorgan autoimmune disease due to loss of a $CD4^+CD25^+$ T cell regulatory population in their peripheral lymphoid tissues (Kojima et al 1981). Subsequently, Sakaguchi et al (Asano et al 1996; Sakaguchi et al 1995) showed that a minor population (approximately 5-10%) of $CD4^+$ T cells was crucial for control of autoreactive T cells in vitro. These $CD4^+$ T cells coexpressed the interleukin-2 (IL-2) receptor α chain (CD25). He demonstrated that the neonatal thymectomized mice lack $CD4^+CD25^+$ T cells due to thymectomy resulted in removal or delay in development of these population, and adoptive transfer of this population into D3T mice or co-transfer with disease-inducing $CD4^+CD25^-$ lymphocytes into atymic nude mice prevented autoimmune disease (Sakaguchi et al 1985). Then a number of animal studies showed that $CD4^+CD25^+$ regulatory T cells (Tregs) can inhibit autoimmune diabetes (Salomon et al 2000; Stephens et al 2000), prevent inflammatory bowel disease (Read et al 2000), prevent the expansion of other T cells in vivo (Annacker et al 2001), and inhibit T cell activation in vitro (Thornton et al 1998). All subsequent in vitro studies by several groups strongly indicate that Tregs play a crucial role in suppression of the immune response (Takahashi et al 1998; Thornton et al 1998)

It is now clear that $CD4^+CD25^+$ regulatory T cells (Tregs) are also part of normal human immune repertoire and it seems likely that the constitutive presence of this regulatory T cell subset also controls autoaggressive T and B cells in humans (Dieckmann et al 2001; Levings et al 2001). Accumulating evidence in human peripheral blood studies suggests that these naturally occurring T cells can actively and dominantly prevent both the activation and the effector function of autoreactive T cells that escape other mechanisms of tolerance (Dieckmann et al 2001; Jonuleit et al 2001; Levings et al 2001; Stephens et al 2001; Taams et al 2002). Therefore, the current challenge is to investigate whether the number or frequency of Tregs are lower in human autoimmune diseases.

In some recent studies report the identification of a subset within the $CD4^+CD25^+$ regulatory T cells in the circulation of normal humans that exhibit strong in vitro regulatory function with characteristics similar to those of murine $CD4^+CD25^+$ regulatory T cells (Stephens et al 2001). This $CD4+CD25^{high}$ T cell subset in human comprise about 2% of circulating $CD4^+$ T cells and they inhibit proliferation and cytokine secretion induced by TCR cross-linking of $CD4^+CD25^-$ responder T cells in a contact-dependent manner . Thus, regulatory $CD4^+$ T cells expressing high levels of CD25 are present in human, providing the opportunity to determine whether alternations of these populations of T cells are involved in induction of human autoimmune disorders.

Co-stimulatory or accessory signaling in $CD4^+CD25^+$ regulatory T cells has remerged as an important area in our understanding of mechanisms behind Tregs induced suppression. Several methods, particularly gene expression studies, have been instrumental in revealing a plethora of additional molecules expressed on Tregs, such as GITR, 4-1BB(CD137), SLAM(CD15) and CTLA-4(CD152), in the hope of identifying more specific markers on Tregs (Gavin et al 2002; Lechner et al 2001; McHugh et al 2002). Like CD25, most of these receptors are expressed on both Tregs and activated conventional $CD4^+$ T cells. Despite this, it has recently become evident that these molecules are potent modulators of Treg-cell development, homeostasis, and suppressor function.

The TNFR-related protein glucocorticoid-induced TNFR (GITR) is highly constitutive expressed on naïve Treg cells (Ronchetti et al 2002), and has ever been suggested to be a marker for these lymphocytes (McHugh et al 2002). In addition, GITR is expressed at low levels on naïve T cells, and GITR expression is up-regulated upon activation of conventional T cells (Nocentini et al 1997). GITR has been suggested to regulate survival, proliferation, and effector functions of T cell subsets, especially $CD4^+$ effector T cells (Shimizu et al 2002; Tone et al 2003). In particular, GITR has been implicated in inhibiting the function of Tregs that control immune effector cells. Treatment Tregs with GITR ligand (GITR-L) or Ab specific for GITR abrogates the suppressive function of Tregs in vitro. Agonistic mAb specific for GITR breaks peripheral tolerance without eliminating Tregs (Shimizu et al 2002). Taken together, all these studies indicate that GITR plays a crucial role in regulating autoimmune responses.
CTLA-4 (CD152) is a molecule expressed on activated T cells and can deliver a negative signal to down-regulate T cell activation either by delivering a negative signal coincident with TCR stimulation or by competitively inhibiting costimulation by its greater affinity for B7-1 and B7-2 as compared with CD28. In mice model, CTLA-4 deficient mice can develop fatal lymphoproliferative disease and have multiple organ immune pathology (Sharpe et al,1995). CTLA-4 constitutive expressed on naïve Tregs, so a critical role of CTLA-4 in the function of Tregs was proposed. CTLA-4 plays an essential role in function of Tregs was proposed to control mice intestinal inflammation due to anti-CTLA-4 therapy (CTLA-4 blockade) was shown to block the ability of Tregs to suppress autoimmunity in an in vivo model of colitis (Read et al 2000).

JRA is the most common rheumatic disease in children (Cassidy et al 2001) and is defined as children under the age of 16 years with arthritis in one or more joints lasting>6 weeks (Woo et al 1998). It is a heterogenous group of conditions of unknown etiology, each of which has specific clinical features and prognostic implications (Schneider et al 2002). In addition, the course and outcome of JIA can be highly variable, ranging from full recovery, to lifelong symptoms and significant disability (Minden et al 2002; Oen et al 2002). The fact that the inflammatory process in JIA is chronic so we suggest that immune regulation is disturbed. This disturbed regulation may be caused by an excessive inflammatory response together with a deficiency in the mechanisms that control the immune response.

In the present study, we attempted to examine the frequency of $CD4^+CD25^{+,}$ $CD4^+CD25^{hi}$ regulatory T cells and GITR, CTLA-4 phenotypic expression on Tregs in peripheral blood of patients with JIA. Our objective is to compare the above markers in JIA patients and healthy controls. We try to correlate above findings with clinical courses or outcomes and whether differences in this regulatory cell population between the different subtypes of JIA can explain the difference in clinical courses.

In this project, we plan to investigate the frequency and related gene expression of regulatory T cells in JRA patients and normal controls. The information here might shed light on further understanding the molecular mechanisms involved in the regulation of rheumatological diseases.

研究方法

Patients and control subjects:

We plan to collect JIA patients newly diagnosed and treated at the National Taiwan University Hospital enrolled in this study. They all diagnosed definitely JIA in our hospital, received regular oral medication use and in our OPD follow-up at least 1 years. The diagnosis and classification of JIA were based on revision of the classification criteria for juvenile idiopathic arthritis. Then subtypes in oligoarticular JIA are included persistant oligoarticular type (per-OA JIA) (affects no more than four joints throughout the disease course) and extended oligoarticular type (ext-OA JIA) (affects a cumulative total of five joints or more after the first 6 months of disease).

Healthy controls matched in age and sex were included. Because CD25, GITR and CTLA-4 are all activation markers in $CD4^+$ T cells, elevated in many conditions and their frequencies are dynamic changes. To avoid other factors affect our data, we defined the excluding criteria.

Excluding criteria: patient who had acute asthma attack or other acute allergic disease exacerbation like allergic rhinitis, atopic dermatitis, urtiaria, other inflammatory or infection conditions (ex: fever or upper respiratory tract infection) in recent 2 weeks to avoid dynamic phenotypic changes due to activation induced increased CD25, GITR and CTLA-4 expression on $CD4^+$ T cells

To analyze the dynamic changes of above markers, we classified the different JIA patients into two groups. Active or inactive stages was classified by the following criteria:

Active JIA (acute exacerbation stage) was defined by clinical active arthritis symptoms by patients or parents, signs by pediatric rheumatology physicians (joints with swelling not due to deformity and at least one of the following: tenderness, erythema, heatness, limited range of motion in the peripheral joint, **or** pain with limitation of range of motion not due to deformity in the hip joint **or** intermittent spiking fever>7 days without other defined etiology in systemic JIA) **combined with** elevated acute-phase reactant (C-reactive protein=CRP>0.8mg/dl). **Inactive JIA (chronic stable stage)** was defined by no active arthritis symptoms and signs and normal CRP level (<0.8mg/dl) although their erythrocyte sedimentation rate (ESR) and complement level (C3, C4) are often mild elevated due to their chronic inflammation status. The **exclusion criteria** are the same as control subjects and added patients received enbrel therapy (**21**). And our definition of "active joint" is like the previous journal article which was different from the American College of Rheumatology criteria (**Enb 1**). The study was approved by the Institutional Review Board at National Taiwan University Hospital. Informed consent was obtained from parents or from patients directly when they were older than 12 years old.

Medical records will be retrospectively reviewed of the follow-up period till the latest date of blood sampling and the definition of JIA subtypes, clinical courses and current medication use in recent 1 month were all recorded. **Age at onset** was defined as the age of the first physical symptoms or sign consistent with the diagnosis of JIA. **Disease duration** was defined the years between age at onset and age at blood sampling or age at clinical remission. **Clinical remission** was defined as the absence of active arthritis and systemic features for at least 6 consecutive months without use any

medication included non-steroid anti-inflammatory drugs (NSAID), steroid and disease modifying anti-rheumatic drugs (DMARD). The demographic characteristics of all subjects, their disease status, and current medication are shown in Table 1.

Monoclinal antibodies

Cell staining was performed using mouse anti-human mAbs fluorescein isothiocyante (FITC), phycoerythrin (PE) and peridinine chlorophyll protein (Per-CP) conjugate. The following mAbs were used: CD25 (IgG1, FITC, clone 2A3); CD4 (IgG1, Per-CP, clone SK3); GITR (IgG1, PE, clone 11046); and CTLA-4 (IgG2a κ, PE, clone BNI3). We also select appropriate isotype controls of mouse anti-human mAbs: IgG1 (FITC, clone X40) for CD25, IgG1 (PE, clone X40) for GITR and IgG2aκ (PE, clone G155-178) for CD152 to avoid non-specific staining. All mAbs were purchased from Becton-Dickinson Immunocytometry System (BD, San Jose, CA), while PE-conjugated anti-hGITR/TNFRSF 18 IgG1 antibody was purchased from R&D Systems Inc (Minneapolis, MN).

Sample preparation and flow cytometric analysis

Fresh blood were obtained by venipunture and perform sample preparation in 8 hours to get more viable cells and precise flow data. Three-color fluorochrome-conjugated monoclonal antibody sets were used stain T cell surface marker. All antibodies were used at concentrations titrated for optimal staining by manufacturer's instructions. Briefly, an aliquot of whole blood were stained with the conjugated mAb or appropriate isotype control mAb then incubated for 30 minutes in the dark at 4°C temperature. For lysing whole blood after immunofluoresence staining, erythrocytes were lysed with FAC^{TM} lysing solution (BD, San Jose, CA), mixed thoroughly, incubated for 15 minutes in the dark at 4°C temperature. Centrifugation with 2000 rpm for 10 min was performed, removed supernatant then washed with FACS washing buffer (1% phosphate-buffered saline, 2% fetal calf serum, 0.05% sodium azide, 0.5M EDTA) 2 mililitres twice and resuspend cells with FACS washing buffer. Fluorocytometry was performed with the FACS Calibur system (BD, San Jose, CA). All fluorocytometric data were subsequently analysed and displayed with Cell QuestPro software (BD, San Jose, CA).

Determination of regulatory T cells-related genes with real-time PCR

To further investigate the expressed level of Foxp-3, GITR and CTLA-4, IL-10 and TGF-b genes, mRNA and cDNA will be isolated from the CD4+ T cells of JIA patients and normal controls. All the DNA samples will be subjected to analysis of gene levels with the method of real-time PCR. The primers is similar to the data of our previous study (Lee et al, in press).

Statistical analysis

Data were expressed as the mean+-SD unless otherwise specified.
Statistical comparisons of categorical variables between different groups (control, inactive and active JIA patients) were done using the Chi-square test with Yate's correction or Fisher's exact test. Statistical comparisons of age, onset age, disease duration and flow cytometric data between different groups were performed with the non-parametric Wilcoxon Mann-Whitney U test. And statistical comparisons of flow cytometric data between different subtypes of JIA subjects were performed with the Wilcoxon Mann-Whitney U test and Kruskal-Wallis (rank-sum test) with Bonferroni correction for unpaired variables to compare differences between groups with non-Gaussian dustribution. A probability (p) value of <0.05 was considered to indicate a statistically significant difference.

結果

Frequency of CD4+CD25+(Treg/Treg ^{hi}) cells in CD4+ T cells and GITR/CTLA-4 phenotypic expression on Treg cells in inactive JIA patients and healthy controls

A total fifty-five healthy control and forty-six JRA patients were included in this study. Their basic profiles are summarized in **Table 1 and Table 2.** The demographic characteristics of all subjects are shown in Table 1 and Table 2 shows the clinical characteristics of the included patients. Since the regulatory function of Treg cells preferentially reside within the CD4+CD25 ^{hi} (Treg ^{hi}) population (A21), we analyzed PB samples for relative numbers of both population**.** The number of Treg and Treg ^{hi} cells are expressed as a percentage of all CD4+ T cells and the GITR/CTLA-4 are expressed as a percentage on Treg cells **(Table 1)**

Our results reveal a significantly lower percentage of Treg ^{hi} in JIA patients (mean+-SD, 1.92+-0.97%) when compared with healthy controls (mean+-SD, 2.76+-1.82%, **p<0.001).** But the difference in Treg cells percentage between these groups are non-significant, **p=0.509).** On the phenotypic expression of Treg cells, we find that the percentage of GITR expression on Treg cells are higher in JIA patients (mean+-SD, 14.82+-5.19%) when compared with healthy controls (mean+-SD, 11.97+-6.07%, **p=0.014<0.05, Fig. 2C)**. And CTLA-4 expression on Treg cells are lower in JIA patients (mean+-SD, 4.69+-2.28%) when compared with healthy controls (mean+-SD, 6.33+-2.57%, **p=0.001<0.05)**

Frequency of CD4+CD25+(Treg/Treg ^{hi}) cells in CD4+ T cells and GITR/CTLA-4 phenotypic expression on Treg cells in different subtypes of inactive JIA patients

We use one-way ANOVA test (post-hoc: LSD methods) to analyze these 4 subtypes of JIA patients. Our results reveal a significantly lower percentage of Treg in systemic JIA patients (mean+-SD, 11.12+-2.18%) when compared with persistant oilgo-articular (per-OA) type JIA patients (mean+-SD, 14.69+-3.26%, **p=0.023<0.05).** And a significantly higher percentage of Treg ^{hi} in per-OA type JIA patients (mean+-SD, 2.66+-0.66%) when compared with extended oligo-articular (ext-OA) type (mean+-SD, 1.68+-0.82%, p=0.003<0.05), polyarticular type (mean+-SD, 1.46+-0.97%, p<0.001) and systemic type JIA patients (mean+-SD, 1.19+-0.7%, **p<0.001, respectively).**

On the phenotypic expression of Treg cells, we find that the percentage of GITR expression on Treg cells are higher in systemic type JIA (mean+-SD, 18.5+-7.73%) when compared with per-OA JIA patients (mean+-SD, 13.18+-3.74%, **p=0.03<0.05).** And CTLA-4 expression on Treg cells are higher in per-OA JIA (mean+-SD, 5.66+-2.51%) when compared with ext-OA JIA (mean+-SD, 3.77+-1.48%, p=0.027<0.05) and polyarticular type JIA patients (mean+-SD, 3.63+-1.44%, **p=0.015<0.05, respectively).**

Frequency of CD4+CD25+(Treg/Treg ^{hi}) cells in CD4+ T cells and GITR/CTLA-4 phenotypic expression on Treg cells in inactive persistant oligoarticular form of JIA patients and healthy controls

We also compare frequency of Treg/Treg ^{hi} cells in CD4+ T cells and phenotypic expression on Treg cells in inactive Per-OA form of JIA patients and healthy controls due to Per-OA JIA is a rare form self-limited, self remission autoimmune disease in human. And we want to see the role of regulatory T cells in their relative benign clinical course. Our results reveal no significant difference between frequency of Treg cells in

healthy controls (mean+-SD, 12.85+-3.68%) and per-OA JIA (mean+-SD, 14.69+-3.26%, **p=0.069.** There are also no significant difference between healthy controls and per-OA JIA patients in frequency of Treg ^{hi} (mean+-SD, 2.76+-1.28% versus 2.66+-0.66%, **p=0.677, Fig. 3F)**, in GITR expression on Treg cells (mean+-SD, 11.97+-6.07% versus 13.18+-3.74%, **p=0.441, Fig. 3G)**, and CTLA-4 expression on Treg cells (mean+-SD, 6.33+-2.57% versus 5.66+-2.51%, **p=0.351, Fig. 3H)**.

Frequency of CD4+CD25+(Treg/Treg ^{hi}) cells in CD4+ T cells and GITR/CTLA-4 phenotypic expression on Treg cells in all form of inactive JIA patients with or without subarticular joint erosion

Because a much less favorable prognosis in JIA often involves s nonremitting destructive and disabling arthritis, so we analyze the frequency of Treg/Treg ^{hi} cells and phenotypic expression on Treg cells in all forms of JIA patients with or without subarticular joint erosion. We find that frequency of Treg ^{hi} are lower in joint erosion groups compared with no joint erosion groups (mean+-SD, 1.65+-1.19% versus 1.99+-0.9%) . And CTLA-4 expression on Treg cells are borderline significant in joint erosion groups compared with no joint erosion groups (mean+-SD, 3.85+-1.34% versus 4.92+-2.44%, p=0.079)

Table 1. Demographic, frequency and phenotypic expression on Treg cells of patients and control subjects

	Control	Inactive	Active	
Case No.	55	46		
Sex F/M (ratio)	20/35 (0.57)	16/30 (0.53)		
Age ^{A} (range) (year)	12.4+-5.2 (5-25)	13.2+-5.2 (5-25)		
CRP ^{A} (range) (mg/dl)	N.A.			
Predonine ^{A,B}(case no., %)	N.A.			
Imuran ^{A,B} (case no., %)	N.A.			
Sulfasalazine ^{A,B} (case no., %)	N.A.			
MTX ^{A,B} (case no., %)	N.A.			
CsA ^{A,B} (case., %)	N.A.			
Treg % ^{A}	12.85+-3.68	13.31+-3.31		
Treg ^{hi} % ^{A}	2.76+-1.82*	1.92+-0.97		
GITR % ^{A}	11.97+-6.07*	14.82+-5.19		
CTLA-4 % ^{A}	6.33+-2.57*	4.69+-2.28		

N.A.: not applicable
F/M: Female/Male
A: mean+-standard deviation
B: daily dose in mg
* P<0.05 compared between 2 groups

	Control	Inactive JIA
Case No.	55	46
Sex F/M (ratio)	20/35 (0.57)	16/30 (0.53)
Age ^{A} (range) (year)	12.4+-5.2 (5-25)	13.2+-5.2 (5-25)
Treg % ^{A}	12.85+-3.68	13.31+-3.31
Treg ^{hi} % ^{A}	2.76+-1.82*	1.92+-0.97
GITR % ^{A}	11.97+-6.07*	14.82+-5.19
CTLA-4 % ^{A}	6.33+-2.57*	4.69+-2.28

N.A.: not applicable
F/M: Female/Male

A: mean+-standard deviation
* P<0.05 compared between 2 groups

Table 2. Profiles of age, sex, subtypes, age at onset, HLA-B27, RF, joint erosion, number of drugs use in JIA patients

Subtype	Persistant Oligoarticular form	Extended Oligoarticular form	Polyarticular form	Systemic form	
Number (%)	17 (37%)	11 (23.9%)	12 (26.1%)	6 (13%)	
Sex F/M (Ratio)	5/12 (0.42)	4/7 (0.57)	3/9 (0.33)	2/4 (0.5)	
Age [A] (range) (year)	11.5+-4.6 (6-21)	15.2+-4.8 (9-25)	13.1+-4.3 (5-19)	14.8+-8.3 (7-24)	
Age at onset [A] (range) (year)	8.2+-3.9 (2-14)	9.7+-3 (5-15)	9.2+-3.7 (3-15)	7.9+-6.3 (2-15)	
Disease duration [A] (range) (year)					
Predonine [A,B](case no., %)					
Imuran [A,B] (case no., %)					
Sulfasalazine [A,B] (case no., %)					
MTX [A,B] (case no., %)					
CsA [A,B] (case no., %)					
HLA-B27 (+/-) (ratio)	7/10 (0.7)	5/6 (0.83)	0/12 (0)*	0/6 (0)*	
RF (+/-) (ratio)	2/15 (0.13)	0/11 (0)*	3/9 (0.33)	0/6 (0)*	
AH (+/-) (ratio)	0/17 (0)	0/11 (0)	0/12 (0)	4/2 (2)	
Joint erosion (+/-) (ratio)	2/15 (0.13)	4/7 (0.57)	3/9 (0.33)	1/5 (0.2)	
Number of immunosuppressant drugs use [A] (range)	1.9+-1.3 (0-4)	2.8+-1 (2-5)	2.3+-1.1 (1-5)	4+-0.9 (3-5)	

F/M: Female/Male
RF: Rheumatoid factor
AH: Autoimmune hemolytic anemia
+/- : Positive/Negative
A: mean+-standard deviation
* P<0.05 compared between 2 groups

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