J Microbiol Immunol Infect.
2008;41:78-87

Original Article

Frequency and phenotypic analysis of $CD4^+CD25^+$ regulatory T cells in children with juvenile idiopathic arthritis

Chih-Ming Wei[1,2], Jyh-Hong Lee[1], Li-Chieh Wang[1], Yao-Hsu Yang[1], Luan-Yin Chang[1], Bor-Luen Chiang[1,3]

[1]Department of Pediatrics, National Taiwan University Hospital, Taipei;
[2]Huai-Te Li Pediatric Clinic, Taipei County; and [3]Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan

Received: November 14, 2006 Revised: March 10, 2007 Accepted: April 26, 2007

Background and Purpose: The aim of this study was to investigate the frequency of $CD4^+CD25^+$ regulatory T cells and their phenotypic expression in peripheral blood of children with active and non-active juvenile idiopathic arthritis (JIA) and healthy controls, to determine if their frequency or phenotypic expression is involved in the immunoregulation of this disease.

Methods: From October 2004 to October 2005, 55 JIA patients and 55 age- and gender-matched healthy controls were enrolled in the study at National Taiwan University Hospital. Flow cytometry was used to determine the frequency of $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ in $CD4^+$ T cells and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) expression on $CD4^+CD25^+$ by tricolor staining. Basic profiles, medication history, clinical symptoms and laboratory data were obtained by chart review and outpatient department interviews.

Results: There was no significant difference in expression of $CD4^+CD25^+$ T cells between patients with inactive JIA and normal controls (13.74 ± 3.25% vs 12.85 ± 3.68%, $p>0.05$). The expression of $CD4^+CD25^{hi}$ T cells was significantly lower in inactive JIA patients than in normal controls (1.89 ± 1.01% vs 2.76 ± 1.28%, $p<0.01$). The expression of CTLA-4 on $CD4^+CD25^+$ T cells was also significantly lower in inactive JIA patients compared with controls (4.37 ± 2.02% vs 6.33 ± 2.57%, $p<0.001$).

Conclusion: We speculate that a decreased frequency of $CD4^+CD25^{hi}$ regulatory T cells and lower level of CTLA-4 expression on $CD4^+CD25^+$ regulatory T cells might play a role in the immunoregulation of JIA.

Key words: Arthritis, juvenile rheumatoid; CD4-positive T-lymphocytes; Cytotoxic T lymphocyte antigen 4; Immunophenotyping; T-lymphocyte subsets

Introduction

The establishment of a full repertoire of pathogen-specific lymphocytes to protect the host and the function of the lymphocytes to recognize self-antigens sometimes concur. Some of these potentially autoreactive T cells capable of recognizing tissue-specific antigens escape negative selection in the thymus and are released into the periphery. They can be cloned from lymphocytes in the lymph nodes of mice and human circulation, they are exquisitely regulated, and their activation can result in autoimmune diseases [1]. 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 responses. The failure of this regulatory network can result in autoimmune disease [2,3].

$CD4^+CD25^+$ regulatory T (Treg) cells play a key role in the regulation of immune responses [4]. Treg cells, which are also part of the normal human immune repertoire, control auto-aggressive T and B cells in humans [5,6]. They can block both the activation and the effector function of autoreactive T cells that escape other mechanisms of tolerance in human peripheral blood [5-7]. Some recent studies have identified a subset of $CD4^+CD25^+$ Treg cells that exhibit strong in vitro regulatory ability in a similar way to murine $CD4^+CD25^+$

Corresponding author: Dr. Bor-Luen Chiang, Department of Pediatrics, National Taiwan University Hospital, Fl. 7, No. 7 Chung-Shan South Road, Taipei 100, Taiwan.
E-mail: gicmbor@ha.mc.ntu.edu.tw

Treg cells [8,9]. This $CD4^+CD25^{hi}$ T cell subset in humans comprises about 2% of circulating $CD4^+$ T cells and inhibits the proliferation and secretion of cytokines [10]. It is not known, however, whether the frequency of this subset is lower in humans with autoimmune diseases.

Co-stimulatory or accessory molecules on $CD4^+CD25^+$ Treg cells may be related to Treg cell-induced suppression of immunity. These molecules are potent modulators of Treg cell development, homeostasis, and suppressor function [11]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), is constitutively expressed on naïve Treg cells and is suspected of playing a critical role in Treg cell function [12]. CTLA-4-deficient mice are known to develop fatal lymphoproliferative disease and immune dysregulation of multiple organs [13], and CTLA-4 has been shown to block the ability of Treg cells to suppress autoimmunity in an in vivo model of colitis [14].

Juvenile idiopathic arthritis (JIA), one of the most common rheumatic diseases in children, is defined as swelling and tenderness occurring in one or more joints in children under the age of 16 years with arthritis, and lasting longer than 6 weeks [15]. It is a heterogenous group of conditions of unknown etiology, each of which has specific clinical features and prognostic implications [16]. The course and outcome of JIA can vary greatly, ranging from full recovery to lifelong symptoms and significant disability [17,18]. The chronic nature of the inflammatory process in JIA suggests a disturbance in immune regulation, which may be a result of an excessive inflammatory response co-occurring with a deficiency in control mechanisms.

This study compared the frequency of $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ Treg cells and CTLA-4 expression on Treg cells in peripheral blood of patients with active and inactive JIA and healthy controls. We also investigated whether differences in frequencies of these markers correlate with the different clinical courses by subdividing the study group into oligoarticular JIA, polyarticular JIA and systemic JIA.

Methods

Patients and control subjects

We enrolled 55 newly-diagnosed JIA patients treated at the National Taiwan University Hospital between 1990 and 2004 into this study. Each patient was diagnosed as having JIA in our hospital, received regular oral medication, and was followed up in our outpatient department for at least 1 year. The diagnosis and classification of JIA were based on the revised classification criteria for JIA [19], which categorizes oligoarticular JIA into two types: persistent oligoarticular type (per-OA JIA) and extended oligoarticular type (ext-OA JIA). We also enrolled 55 healthy age- and gender-matched controls.

We excluded patients and healthy controls who had acute asthma or other acute allergic diseases, including allergic rhinitis, atopic dermatitis, and urticaria, as well as other inflammatory or infectious conditions in the 2 weeks leading up to study enrollment to prevent the effect of other inflammatory diseases. We also excluded patients receiving etanercept therapy [20].

To analyze the dynamic changes of the above markers, we classified the JIA patients into inactive and active groups. The inactive group had forty three patients, and the active group twenty four. Twelve of the patients were categorized as sometimes inactive or sometimes active, depending on stage of disease at each sampling.

Active JIA (acute exacerbation stage) was defined based on having a clinical history of active arthritis symptoms or diagnosis by pediatric rheumatologists based on joint swelling or pain. In addition to joint swelling, the patients should have at least one of the other symptoms, including tenderness, erythema, heat, and limited range of motion of the involved joints. Inactive JIA (chronic stable stage) was defined by the absence of active arthritis symptoms and signs, although there might be mild elevation in erythrocyte sedimentation rate and complement level (C3, C4) due to the chronicity of inflammation.

Medical records were retrospectively reviewed from the follow-up period until the latest date of blood sampling. The JIA subtype, clinical course, and medications used in the last 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 as the time period between the age at onset and the age at blood sampling or clinical remission. Clinical remission was defined as the absence of active arthritis and systemic features for at least 6 consecutive months without any medication, including non-steroidal anti-inflammatory drugs, steroids, and disease-modifying antirheumatic drugs. The demographic characteristics of the subjects, their disease status, and current medication are summarized in Table 1.

Table 1. Demographic and clinical characterstics and current medication use of patients and controls

Variable	Controls (n = 55) No. (%)	Inactive JIA (n = 43) No. (%)	Active JIA (n = 24) No. (%)
Gender (F/M) [ratio]	20/35 (0.57)	16/27 (0.59)	9/15 (0.6)
Age (years; mean ± SD) [range]	12.35 ± 5.24 (5-25)	13.67 ± 5.50 (5-25)	12.06 ± 5.67 (5-25)
Age at onset (years; mean ± SD) [range]	NA	8.86 ± 3.95 (2-15)	8.05 ± 2.98 (2-14)
Subtype			
Persistent oligoarticular JIA	NA	16 (37.2)	9 (37.5)
Extended oligoarticular JIA	NA	10 (23.3)	3 (12.5)
Polyarticular JIA	NA	11 (25.6)	6 (25.0)
Systemic JIA	NA	6 (14.0)	6 (25.0)
Human leukocyte antigen B27	NA	13 (30.2)	5 (20.8)
Rheumatoid factor	NA	4 (9.3)	2 (8.3)
CRP (μg/L; mean ± SD) [range]	NA	26 ± 23 (0-78)	313 ± 298[a] (81-1047)
Prednisolone	NA	12 (27.9)	9 (37.5)
Azathioprine	NA	22 (51.2)	11 (45.8)
Sulfasalazine	NA	19 (44.2)	15 (62.5)
Methotrexate	NA	3 (7.0)	4 (16.7)
Cyclosporin	NA	3 (7.0)	1 (4.2)
NSAID	NA	37 (86.0)	21 (87.5)
Disease duration (years; mean ± SD) [range]	NA	4.54 ± 3.32 (1-14)	3.95 ± 4.26 (1-14)

Abbreviations: JIA = juvenile idiopathic arthritis; F = female; M = male; SD = standard deviation; CRP = C-reactive protein; NSAID = non-steroidal anti-inflammatory drugs; NA = not applicable

[a] $p<0.05$ between active and inactive disease.

Monoclonal antibodies

Cell staining was performed using mouse anti-human monoclonal antibodies (mAbs) fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinine chlorophyll protein (Per-CP) conjugate. The following mAbs were used: CD25 (immunoglobulin G [IgG]1, FITC, clone 2A3); CD4 (IgG1, Per-CP, clone SK3); and CTLA-4 (IgG2a kappa, PE, clone BNI3). The peripheral blood was stained with FITC-conjugated anti-CD25 (FL1), PE-conjugated anti-CTLA-4 (FL2) and Per-CP-conjugated anti-CD4 (FL3). To avoid non-specific Fc receptor staining, we used appropriate isotype controls of mouse anti-human mAbs: IgG1 (FITC, clone X40) for CD25 and IgG2a kappa (PE, clone G155-178) for CTLA-4 (CD152). All mAbs were purchased from Becton Dickinson Immunocytometry System (BD Biosciences, San Jose, CA, USA).

Sample preparation and flow cytometry analysis

Fresh blood was obtained by venipuncture and flow cytometry experiment was performed within 8 h of sampling to maintain viable cells and ensure accurate flow cytometry data. Three-color fluorochrome-conjugated monoclonal antibody sets were used to stain T cell surface markers. All antibodies were used at concentrations titrated for optimal staining according to manufacturer's instructions. Briefly, an aliquot of whole blood was stained with the conjugated mAb or appropriate isotype control mAb and incubated for 30 min in the dark at 4°C. Erythrocytes were lysed with FAC™ lysing solution (BD Biosciences), mixed thoroughly, and incubated for 15 min in the dark at 4°C. Samples were centrifuged at 2000 rpm for 10 min.

The supernatant was removed and washed twice with fluorescence-activated cell sorter (FACS) washing buffer (1% phosphate-buffered saline, 2% fetal calf serum, 0.05% sodium azide, 0.5M ethylenediamine tetra-acetic acid) and the cells were re-suspended with FACS washing buffer. Fluorocytometry was performed with the FACS system (BD Biosciences) and all of the data were subsequently analyzed and displayed using Cell QuestPro software (BD Biosciences). Flow cytometric analysis between JIA patients and age-matched controls was performed in case-control paired fashion.

Polymorphonuclear leukocytes (neutrophils), lymphocytes, and monocytes exhibited characteristic light scatter properties that could be identified on a forward versus side light scatter plot with the use of Cell QuestPro data acquisition and analysis software. Lymphocyte populations were located using these parameters, and a live analysis gate (Gate 1 = Region 1, G1 = R1) was set around this population (Fig. 1A and 1B). Large, activated T cells were excluded.

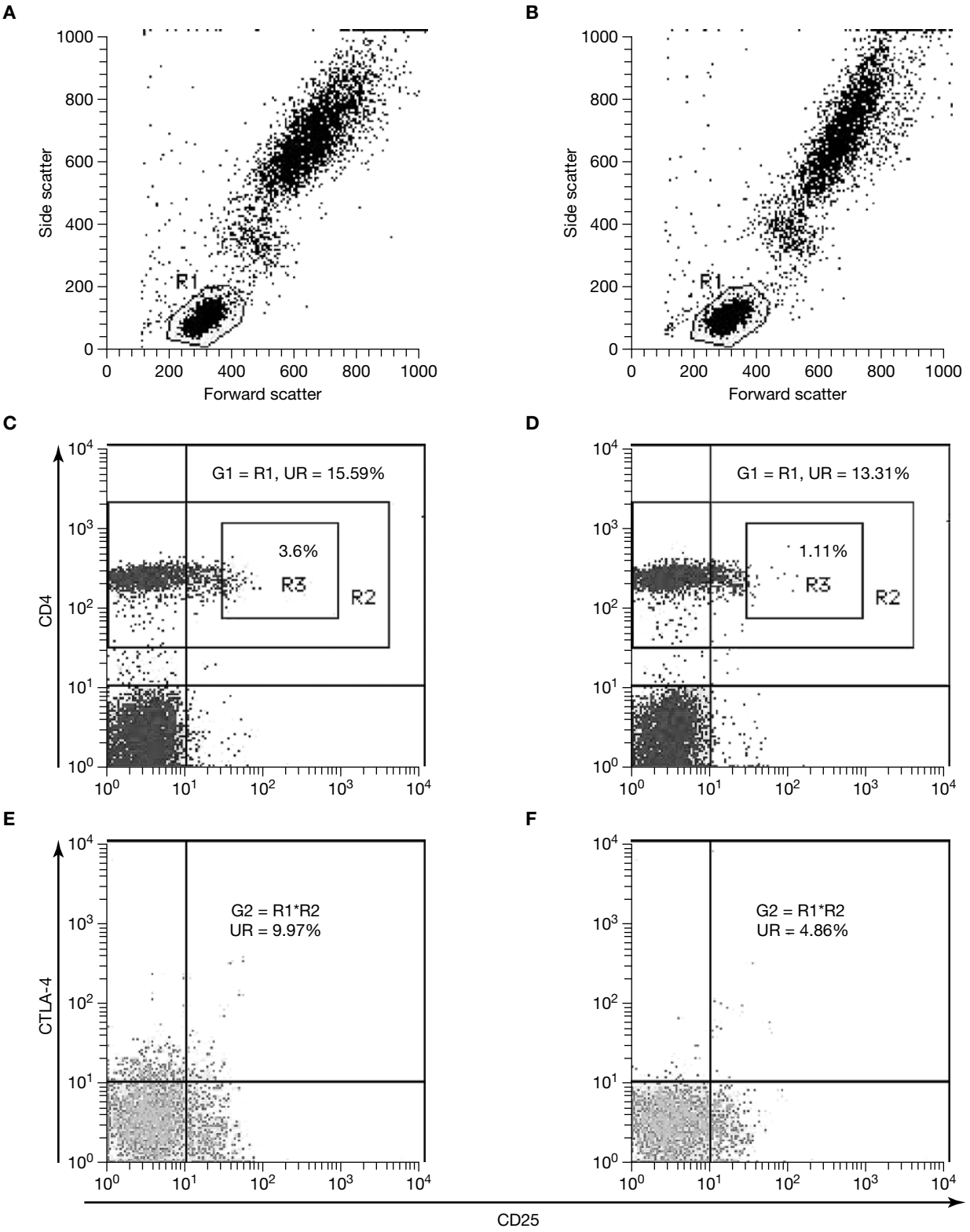

Fig. 1. Comparison of flow cytometric analysis of $CD4^+CD25^+$ or $CD4^+CD25^{hi}$ cell populations and of surface expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on $CD4^+CD25^+$ T cells between controls (A) and inactive juvenile idiopathic arthritis patients (B). Analysis gates show the percentages of $CD25^+$ (C) and $CD25^{hi}$ (D) in $CD4^+$ T cells and percentages of CTLA-4 expression on $CD4^+CD25^+$ T cells (E, F). Isotype controls for each sample were run in conjunction with CD25 staining and with CTLA-4 (immunoglobulin G2a) staining, respectively (data not shown). G1 = gate 1; R1 = region 1; UR = upper limit; R2 = region 2; R3 = region 3.

The gating strategy is used to analyze surface $CD25^+$, $CD25^{hi}$ (R3) expression on $CD4^+$ T cells (Fig. 1C and 1D) and CTLA-4 expression on $CD4^+CD25^+$ T cells (Fig. 1E and 1F). The larger rectangle box (R2 = $CD4^+$ T cells) was the gating area for the analysis of CTLA-4 expression on $CD4^+CD25^+$ T cells. The smaller rectangle box was the gating area for the analysis of $CD25^{hi}$ in $CD4^+$ T cells (Fig. 1C and 1D). Data were acquired from the analysis gate and the percentage of given cell populations expressing fluorescence was determined (Fig 1). Each analysis included measurements from a minimum of 20,000 lymphocytes in R1. $CD25^{hi}$ was defined by sorting gate (fluorescent intensity = 30) by previous in vitro suppression assay studies [10].

Statistical analysis

Data were expressed as the mean ± standard deviation unless otherwise specified. Statistical comparisons of categorical variables between different groups (controls, inactive JIA patients, and active JIA patients) were done using the chi-squared test with Yates' correction or Fisher's exact test. Statistical comparisons of age, age at onset, disease duration, and flow cytometric data among the different groups were performed with the Wilcoxon rank sum test or Kruskal-Wallis test because the sample size was small and data did not have a normal distribution. If statistical significance was found among ≥3 groups with Kruskal-Wallis test, then pairwise comparison was performed using Dunn's multiple comparison test. A p value <0.05 was considered to be significant.

Results

Demographic and clinical characteristics of subjects

This study included of 43 inactive and 24 active JIA patients, 12 of whom changed status over time, and 55 healthy controls. Their demographic characteristics, basic profiles, disease status, and current medications are summarized in Tables 1 and 2. Within the study group, patients with active JIA were not significantly different from those with inactive JIA with regard to gender ratio, age, onset age, subtype, human leukocyte antigen B27, rheumatoid factor, percentage of current medication use, and disease duration (Table 1).

Frequency of $CD4^+CD25^+$ cells in $CD4^+$ T cells

Active JIA patients had a significantly higher percentage of $CD4^+CD25^+$ cells than the healthy controls (15.48 ± 3.74 vs 12.85 ± 3.68, p<0.01). The difference in this measure was not significant between either the inactive JIA patients and healthy controls (13.74 ± 3.25 vs 12.85 ± 3.68, p>0.05) or the active JIA and inactive JIA patients (15.48 ± 3.74 vs 13.74 ± 3.25, p>0.05) [Fig. 2A].

Frequency of $CD4^+CD25^{hi}$ cells in $CD4^+$ T cells

Inactive JIA patients had a significantly lower percentage of $CD4^+CD25^{hi}$ cells than the healthy controls (1.89 ± 1.01% vs 2.76 ± 1.28%, p<0.01), and active JIA patients had a significantly higher percentage of $CD4^+CD25^{hi}$ cells than the inactive JIA patients (2.89 ± 1.56 vs 1.89 ± 1.01, p<0.05) [Fig. 2B].

Table 2. Age, gender, age at onset, human leukocyte antigen B27, rheumatoid factor and current medication use in subtypes of inactive juvenile idiopathic arthritis (JIA) patients

Variable	Persistent oligoarticular JIA (n = 16) No. (%)	Extended oligoarticular JIA (n = 10) No. (%)	Polyarticular JIA (n = 11) No. (%)	Systemic JIA (n = 6) No. (%)
Gender (F/M) [ratio]	5/11 (0.45)	4/6 (0.67)	3/8 (0.38)	4/2 (2)
Age (years; mean ± SD) [range]	12.54 ± 5.58 (5-25)	15.44 ± 4.98 (9-25)	12.86 ± 4.45 (5-19)	15.27 ± 7.83 (7-24)
Age at onset (years; mean ± SD) [range]	8.15 ± 4.04 (2-14)	9.67 ± 3.20 (5-15)	9.26 ± 3.76 (3-14)	8.68 ± 5.69 (2-15)
Human leukocyte antigen B27	8 (50.0)	5 (50.0)	0 (0.0)	0 (0.0)
Rheumatoid factor	2 (12.5)	0 (0.0)	2 (18.2)	0 (0.0)
Prednisolone	4 (25.0)	3 (30.0)	3 (27.3)	2 (33.3)
Azathioprine	9 (56.3)	6 (60.0)	4 (36.4)	3 (50)
Sulfasalazine	9 (56.3)	5 (50.0)	5 (45.5)	0 (0.0)
Methotrexate	0 (0.0)	1 (10.0)	2 (18.2)	0 (0.0)
Cyclosporin	0 (0.0)	0 (0.0)	0 (0.0)	3 (50.0)
NSAID	12 (75.0)	10 (100.0)	10 (90.9)	5 (83.3)
Disease duration (years; mean ± SD) [range]	3.86 ± 2.74 (1-10)	5.77 ± 4.04 (1-14)	3.28 ± 2.40 (1-8)	6.59 ± 4.03 (3-13)

Abbreviations: F = female; M = male; SD = standard deviation; NSAID = non-steroidal anti-inflammatory drugs

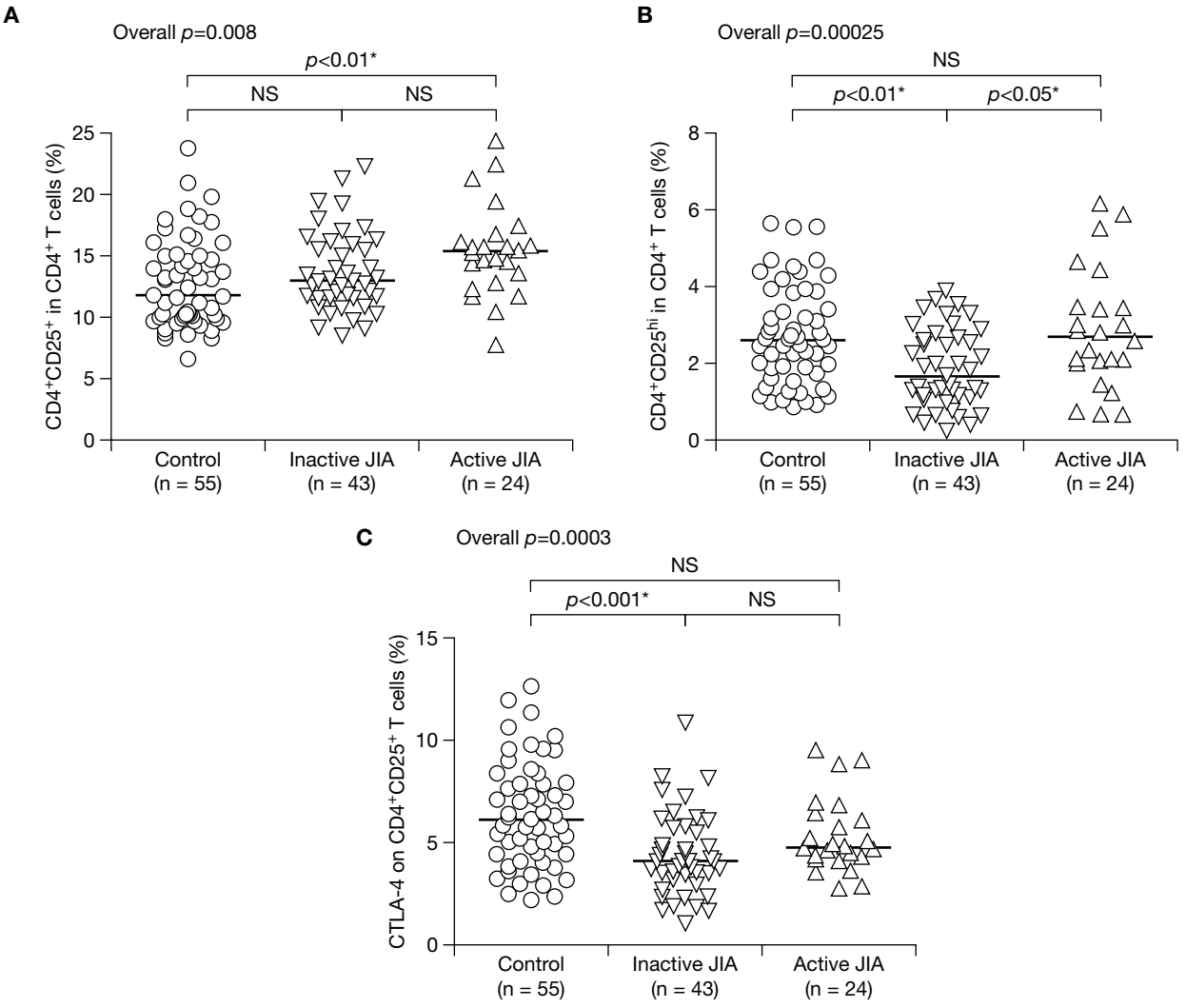

Fig. 2. Comparison of the frequency of (A) $CD4^+CD25^+$ and (B) $CD4^+CD25^{hi}$ regulatory T cells and (C) cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) expression on $CD4^+CD25^+$ regulatory T cells between control subjects, inactive juvenile idiopathic arthritis (JIA) patients and active JIA patients. Dots scatter plot; the horizontal line within the vertical points marks the median [1.7% for patients with inactive JIA vs 2.73% for patients with active JIA and 2.64% for control subjects in (B), respectively]. NS = not significant; *$p<0.05$.

CTLA-4 phenotypic expression on $CD4^+CD25^+$ T cells

The inactive JIA patients had a significantly lower expression of CTLA-4 on Treg cells than the healthy controls (4.37 ± 2.02% vs 6.33 ± 2.57%, $p<0.001$). However, there was no significant difference in this measure between active and inactive JIA patients (5.31 ± 1.81% vs 4.37 ± 2.02%, $p>0.05$) [Fig. 2C].

Frequency of $CD4^+CD25^+/CD4^+CD25^{hi}$ cells in $CD4^+$ T cells and CTLA-4 expression on $CD4^+CD25^+$ T cells

When using the Kruskal-Wallis test to analyze intra-group differences, we found a significant difference only in percentage of $CD4^+CD25^{hi}$ cells (overall $p=0.0004$) [Fig. 3]. The patients with per-OA JIA had a significantly higher percentage of $CD4^+CD25^{hi}$ cells than either those with poly-JIA (2.70 ± 0.70% vs 1.41 ± 1.00%, $p<0.01$) or those with systemic JIA (2.70 ± 0.70% vs 1.03 ± 0.46%, $p<0.01$) [Fig. 3B].

Discussion

In this study, we compared the frequency of $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ Treg cells and CTLA-4 expression on Treg cells in the peripheral blood of patients with active and inactive JIA and healthy controls. We found no significant difference in frequency of $CD4^+CD25^+$ T cells

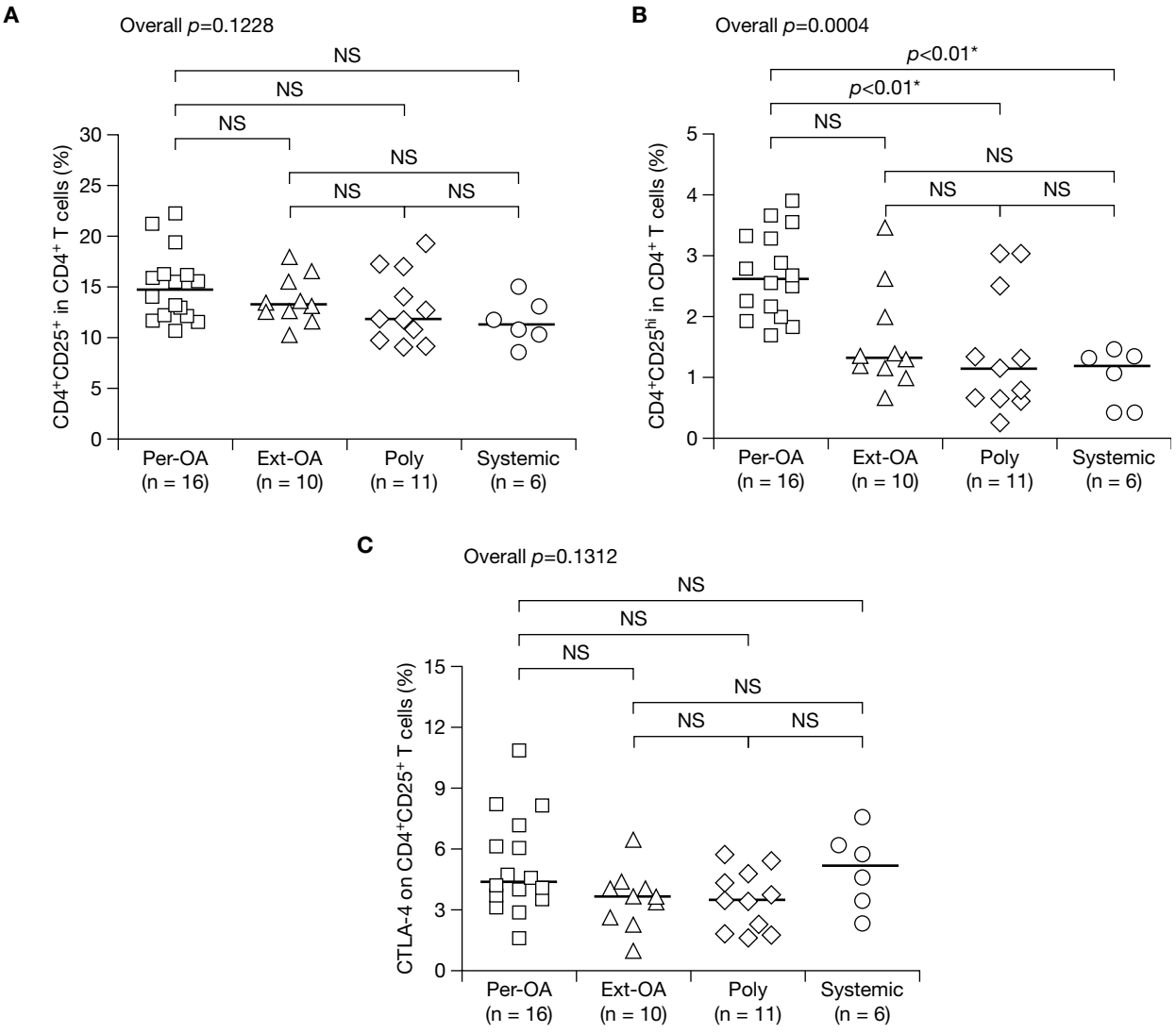

Fig. 3. Comparison of the frequency of (A) $CD4^+CD25^+$ and (B) $CD4^+CD25^{hi}$ regulatory T cells and (C) cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) expression on $CD4^+CD25^+$ regulatory T cells between different types of inactive juvenile idiopathic arthritis (JIA) patients. Dots scatter plot; the horizontal line within the vertical points marks the median [2.63% for patients with persistent oligoarticular type (per-OA) JIA vs 1.35% for patients with extended oligoarticular type (ext-OA) JIA vs 1.17% for patients with polyarticular (poly) JIA and 1.21% for patients with systemic JIA in (B), respectively]. NS = not significant; *$p<0.05$.

between patients with inactive JIA and normal controls, a significantly lower frequency of $CD4^+CD25^{hi}$ T cells in inactive JIA patients compared to normal controls, and a significantly lower frequency of CTLA-4 expression on $CD4^+CD25^+$ T cells in inactive JIA patients compared to controls. Our intragroup study found the only significant difference to be in frequency of $CD4^+CD25^{hi}$ cells, with patients with per-OA JIA having a significantly higher percentage of these cells than those with polyarticular JIA or systemic JIA.

Kojima and Prehn discovered that due to the loss of a $CD4^+CD25^+$ Treg cell population in peripheral lymphoid tissues, thymectomy in 3-day-old mice led to the development of multiorgan autoimmune disease [21]. Sakaguchi and colleagues showed that 5-10% of $CD4^+$ T cells, which co-expressed the interleukin-2 receptor alpha chain (CD25), were needed to control autoreactive T cells in vitro [22-24]. Animal studies have found that $CD4^+CD25^+$ Treg cells are able to inhibit autoimmune diabetes [25], prevent inflammatory bowel disease [14], prevent the expansion of other T cells in vivo [26], and inhibit T cell activation in vitro [27]. Although Treg cells are thought to play an important role in the pathogenesis of autoimmune diseases

in animal models, their role in human autoimmune disease has not yet been thoroughly demonstrated. Although a lymphocyte population with identical phenotypic and functional properties has recently been identified in the human thymus and peripheral blood [9,10], many questions about whether and how Treg cells can actively regulate autoimmune diseases in humans still need to be answered.

The human regulatory or suppressive functions of Treg cells have been observed to occur only when the cells express high levels of CD25, which are distinct from the $CD25^+$ T cells [10]. Recently, FoxP3 has been identified as a specific marker of Treg cells, distinguishing them from recently activated, non-Treg cells [28,29]. One study of JIA reported the mean expression of FoxP3 mRNA to be significantly higher in $CD4^+CD25^{bright}$ than in $CD4^+CD25^+$ T cells, and its expression seemed to correlate well with the clinical subtypes and clinical courses of JIA [30]. Hence, the main subsets involved in the regulatory function of Treg cells are in the $CD25^{hi}$ T cells. Our data on the frequency of $CD4^+CD25^+$, $CD4^+CD25^{hi}$ cells in peripheral blood between inactive JIA patients and healthy controls were consistent with the above study, causing us to wonder whether or not this reduced frequency of $CD4^+CD25^{hi}$ cells contributes to the development of JIA.

In some studies of the frequency of $CD4^+CD25^+$, $CD4^+CD25^{hi}$ cells in peripheral blood, adult systemic lupus erythematosus patients had significantly lower levels of Treg cells than normal controls [31], and type 1 diabetes mellitus patients had significantly lower levels of Treg cells than normal controls [32], although one study of peripheral blood in adults with rheumatoid arthritis showed significantly higher levels of Treg cells in $CD4^+$ T cells than in normal controls [33]. Many previous studies detecting and isolating Treg cells in humans have been complicated by the fact that CD25 is a marker of recent T cell activation. However, none of the above studies classified patients into acute exacerbation (active) or chronic stable (inactive) stages and compared the difference. Our study classified JIA patients into inactive and active groups to avoid acute inflammation-induced increases in CD25 expression when comparing inactive JIA patients and healthy controls. Therefore, our finding that the frequency of $CD4^+CD25^+$ T cells was significantly higher in patients with active JIA than healthy controls may only indicate an activation-induced increase in CD25 expression.

The presence and enrichment of $CD4^+CD25^{bright}$ T cells has been found in the synovial fluid of human joints with rheumatoid arthritis when compared to their peripheral blood, and that these T cells were able to suppress reactive T cells, leading the investigators to speculate that the enriched functional $CD4^+CD25^{bright}$ Treg cells in inflamed joints exerted an immunosuppressive effect locally, and influenced disease activity and progression [33]. In a similar study of patients with JIA, $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ Treg cells expressed more FoxP3 mRNA in synovial fluid than peripheral blood, and they were found to regulate reactive T cells more actively in in vitro co-culture experiments [30]. Since the joint is the main target organ in JIA, Treg cell populations in synovial fluid can represent an active inflammation, suggesting the existence of a negative feedback system in which Treg cells are generated and triggered in the ongoing inflammation. Our data revealed that the greater frequency of $CD4^+CD25^{hi}$ cells we found in active JIA compared to inactive JIA patients may be the result of a similar compensatory mechanism.

JIA is not a homogenous disease, but consists of various subtypes with striking differences in both severity and outcome [19]. A much less favorable prognosis in JIA, often involving non-remitting destructive and disabling arthritis, requires aggressive immunotherapy. Therefore, we compared the frequencies of these measures in patients with per-OA JIA, a subtype with a mild self-limiting, remitting course of disease, with patients with poly-JIA and systemic JIA, subtypes with less favorable, non-self-remitting courses involving joint erosion or systemic involvement. The only significant difference this intragroup comparison found was in the frequency of $CD4^+CD25^{hi}$ in $CD4^+$ T cells. Patients with per-OA JIA had a higher frequency of $CD4^+CD25^{hi}$ T cells than other subtypes, a finding that was similar to that of another study [30]. Therefore, the $CD25^{hi}$ subsets in Treg cells might play a major role in immunoregulation.

In mouse models, CTLA-4 blockade can reverse and abolish the suppressive function of Treg cells [12,14] and block CTLA-4 in vivo. This anti-CTLA-4 treatment can result in the abrogation of any protection offered by co-transfer of the $CD4^+CD25^+$ population in mice models of the autoimmunity of diabetes and intestinal inflammation [14,25]. Recently, it has been shown that the expression level of CTLA-4 in human $CD4^+CD25^+$ Treg cells clones correlates with their suppressive capacity [34,35]. Another recent

report has also proposed an indirect mechanism by which CD28 and CTLA-4 expression are involved in the function of Treg cells [36]. Although CTLA-4 expresses itself and functions on the cell surface, some studies directly analyzing human blood did not find $CD4^+CD25^{hi}$ T cells to express CTLA-4 there [10]. A similar finding could be discovered in mice, in which CTLA-4 is constitutively expressed, mainly in cytoplasm of murine $CD4^+CD25^+$ Treg cells, but is rarely detected on the cell surface [14]. However, due to the longer kinetics of suppression, the peak levels of CTLA-4 expression can be induced on T cells by 2-3 days post-activation [37]. Our patients had chronic inflammation for at least 1 year, making it very likely that their T cells would be found with CTLA-4 and its subpopulations, including Treg cells. Because the kinetics of cell surface CTLA-4 parallel intracellular CTLA-4 [38], CTLA-4 expression on the surface of T cells can also be used as a measure of intracellular CTLA-4. The expression levels of CTLA-4 are lower on the Treg cells of inactive JIA patients than on the Treg cells of healthy controls, and the difference in CTLA-4 expression may play a role in the immunodysregulation of JIA.

In conclusion, a decreased frequency of $CD4^+CD25^{hi}$ in $CD4^+$ cells and a decreased expression of CTLA-4 on Treg cells may be related to the immunodysregulation of JIA. In different stages of JIA, we speculate that changes in phenotypes such as $CD25^{hi}$ on Treg cells, which lead to enhanced capacity to suppress autoimmunity, seem to be the mechanisms by which Treg cells regulate local or systemic inflammation. These suggested explanations should be interpreted with great caution, however, because the number of patients with active JIA in our study was limited and because the expression of CD25 may also be increased after T cell activation. The frequency of $CD4^+CD25^{hi}$ T cells may regulate the clinical course. $CD4^+CD25^{hi}$ seems to play more of a role in the limiting and even reversal of established autoimmune pathology of per-OA JIA than in other subtypes of JIA. However, despite the highly significant difference in frequency of $CD4^+CD25^{hi}$ between per-OA and other subtypes of JIA, its frequency cannot be used to predict whether oligoarticular JIA will progress to a less favorable outcome or persist as a per-OA stage. A definite conclusion in this regard is limited by our study's small number of patients with systemic JIA and limited follow-up periods. We also did not perform functional assays of Treg cells in any of the above studies. The role of autoreactive T cells in the pathogenesis of JIA is unknown. While $CD25^{hi}$ and CTLA-4 expression on Treg cells may be associated with the immunoregulation of JIA, there are many possible mechanisms needing further investigation.

References

1. Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL, Hafler DA. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. Nature. 1990;346:183-7.
2. Curotto de Lafaille MA, Lafaille JJ. CD4(+) regulatory T cells in autoimmunity and allergy. Curr Opin Immunol. 2002;14:771-8.
3. Shevach EM. Regulatory T cells in autoimmunity. Annu Rev Immunol. 2000;18:423-49.
4. Stephens LA, Mason D. CD25 is a marker for CD4+ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both CD25+ and CD25- subpopulations. J Immunol. 2000;165:3105-10.
5. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J Exp Med. 2001;193:1303-10.
6. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med. 2001;193:1295-302.
7. Taams LS, Vukmanovic-Stejic M, Smith J, Dunne PJ, Fletcher JM, Plunkett FJ, et al. Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. Eur J Immunol. 2002;32:1621-30.
8. Stephens LA, Mottet C, Mason D, Powrie F. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. Eur J Immunol. 2001; 31:1247-54.
9. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med. 2001;193:1285-94.
10. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol. 2001;167:1245-53.
11. Ndhlovu LC, Takeda I, Sugamura K, Ishii N. Expanding role of T-cell costimulators in regulatory T-cell function: recent advances in accessory molecules expressed on both regulatory and nonregulatory T cells. Crit Rev Immunol. 2004;24:251-66.
12. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25+CD4+ regulatory T cells constitutively expressing

cytotoxic T lymphocyte-associated antigen 4. J Exp Med. 2000;192:303-10.
13. Tivol EA, Borriello F, Scwetitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3:541-7.
14. Read S, Malmström V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med. 2000;192:295-302.
15. Woo P, Wedderburn LR. Juvenile chronic arthritis. Lancet. 1998;351:969-73.
16. Schneider R, Passo MH. Juvenile rheumatoid arthritis. Rheum Dis Clin North Am. 2002;28:503-30.
17. Minden K, Niewerth M, Listing J, Biedermann T, Bollow M, Schöntube M, et al. Long-term outcome in patients with juvenile idiopathic arthritis. Arthritis Rheum. 2002;46: 2392-401.
18. Oen K, Malleson PN, Cabral DA, Rosenberg AM, Petty RE, Cheang M. Disease course and outcome of juvenile rheumatoid arthritis in a multicenter cohort. J Rheumatol. 2002;29:1989-99.
19. Petty RE, Southwood TR, Baum J, Bhettay E, Glass DN, Manners P, et al. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1997. J Rheumatol. 1998;25:1991-4.
20. Ehrenstein MR, Evans JG., Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med. 2004;200:277-85.
21. Kojima A, Prehn RT. Genetic susceptibility to post-thymectomy autoimmune diseases in mice. Immunogenetics. 1981;14:15-27.
22. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155:1151-64.
23. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T-cell subpopulation. J Exp Med. 1996;184: 387-96.
24. Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. J Exp Med. 1985;161:72-87.
25. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity. 2000;12: 431-40.
26. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. J Immunol. 2001;166:3008-18.
27. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med. 1998; 188:287-96.
28. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299:1057-61.
29. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003;4:330-6.
30. de Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J Immunol. 2004;172:6435-43.
31. Liu MF, Wang CR, Fung LL, Wu CR. Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. Scand J Immunol. 2004;59: 198-202.
32. Kukreja A, Cost G, Marker J, Zhang C, Sun Z, Lin-Su K, et al. Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest. 2002;109:131-40.
33. van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum. 2004;50:2775-85.
34. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004;22:531-62.
35. Bluestone JA. Is CTLA-4 a master switch for peripheral T cell tolerance? J Immunol. 1997;158:1989-93.
36. von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat Immunol. 2005;6:338-44.
37. Greenfield EA, Nguyen KA, Kuchroo VK. CD28/B7 costimulation: a review. Crit Rev Immunol. 1998;18:389-418.
38. Alegre ML, Noel PJ, Eisfelder BJ, Chuang E, Clark MR, Reiner SL, et al. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. J Immunol. 1996; 157:4762-70.

